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DISSERTATION

ECOLOGICAL GENOMICS OF THE OPPORTUNISTIC FUNGUS *TRICHODERMA*

ausgeführt zum Zwecke der Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

unter der Leitung von

Doz. Dr. Irina S. Druzhinina

Institut 166

Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften,

eingereicht an der Technischen Universität Wien,

Fakultät für Technische Chemie

von

Mag.rer.nat. Lea Atanasova

Matrikelnummer 0504094

Pantzergasse 6, 1190 Wien

Wien, am

Nonici
Mami in Tatu
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"It is not the strongest or the most intelligent
who will survive but those who can best manage change."
— Charles Darwin

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KURZFASSUNG

Trichoderma (Hypocreales, Ascomycota) ist eine Gattung der filamentösen Pilze, die durch ihr hohes opportunistisches Potenzial und ihre Anpassungsfähigkeit an verschiedene ökologische Bedingungen in zahlreichen natürlichen und künstlichen Habitaten verbreitet ist.

Die Fruchtkörper der *Trichoderma* spp. (Teleomorph; geschlechtliche Form) wurden hauptsächlich auf Basidiomyceten gefunden. Viele *Trichoderma* Anamorphe (asexuelles Entwicklungsstadium) haben die Fähigkeit biotrophe Interaktionen (z.B. Parasitismus) mit anderen Pilzen einzugehen. Aufgrund dieser Eigenschaft sind *Trichoderma* spp. für Forschung und Industrie von großer Bedeutung und werden zur Bekämpfung von phytopathogenen Pilzen als Biofungizide eingesetzt. *Trichoderma* Mykoparasitimus steht daher im Fokus zahlreicher wissenschaftlicher Studien, was dazu führte, dass mittlerweile sieben Genome (*T. asperellum*, *T. atroviride*, *T. citrinoviride*, *T. harzianum* sensu stricto, *T. longibrachiatum*, *T. reesei* und *T. virens*) sequenziert und für die Öffentlichkeit freigegeben wurden.

In der vorliegenden Dissertation wird die Ökologie der Gattung *Trichoderma* anhand von genomischen, transcriptomischen und ökophysiologischen Studien diskutiert. Die komparative Transkriptomik zeigte unterschiedliche Mykoparasitismus-Strategien der *T. atroviride*, *T. reesei* und *T. virens*. Die phylogenomische Studie der drei genannten Spezies zeigte, dass Mykoparasitismus eine Ureigenschaft dieser Gattung ist. Dies wurde auch in der Fallstudie über die evolutionär bedingten Unterschiede des mykoparasitischen Potenzials der eng verwandten, jedoch genetisch getrennten Spezies *T. reesei* und *T. parareesei*, unterstrichen. Die Studie des Polyketid-Synthase Gens *pks4* in *T. reesei* brachte hervor, dass dieses Gen für die Pigmentbildung verantwortlich ist und auch Einfluss auf andere wichtige ökophysiologische Funktionen, inklusive Mykoparasitismus dieser Spezies hat.

Diese Dissertation bietet erste Einblicke in den Mykoparasitismus von *Trichoderma* aus genomischer und ökophysiologischer Perspektive.

SUMMARY

Trichoderma (Hypocreales, Ascomycota) is a genus of common filamentous fungi that display a remarkable range of lifestyles. They have been isolated from numeral natural and artificial habitats demonstrating their high opportunistic potential and adaptability to variety of ecological conditions. Fruiting bodies of *Trichoderma* spp. are mainly found associated with basidiomycetes and many of *Trichoderma* anamorphs can form biotrophic associations (in a broad sense parasites) with different fungi. This property is widely used by researchers and industry for the antagonization and eventual killing of fungal pathogens of plants (biological control or biocontrol). The exploitation of the mycotrophic properties of *Trichoderma* in biotechnology and agriculture made the genus well studied and brought it in focus of numerous *-omic* studies including the seven complete genomes (*T. asperellum, T. atroviride, T. citrinoviride, T. harzianum* sensu stricto, *T. longibrachiatum, T. reesei* and *T. virens*) recently released for public access.

This doctoral thesis focuses on the ecology of the genus *Trichoderma***,** discussing it by our ecophysiological, genomic and transcriptomic studies. The comparative transcriptomic analysis of *T. atroviride, T. reesei* and *T. virens* revealed different strategies of *Trichoderma* mycoparasitism. The phylogenomic study of the three mentioned species showed that mycoparasitism is an innate property of the genus. This was further underlined by the case study on the versatility of antagonistic potentials in closely evolutionary related but genetically delimited species *T. reesei* and *T. parareesei*. The study of a polyketide synthase gene (*pks4*) in *T. reesei* revealed the involvement of a pigment producing *pks* in such ecophysiological functions as stress resistance, defense against competitive microorganisms and in mycoparasitism.

This thesis provides the first insight into ecological genomics of *Trichoderma* through the genome-wide integrated analysis of mechanism, evolution and ecophysiology.

NOMENCLATURE AND CURRENT TAXONOMIC ASSUMPTIONS

The main subject of this thesis is the pleomorphic genus of filamentous fungi *Trichoderma*. The sexual (teleomorphic) stage was known by the generic name *Hypocrea* (Hypocreales, Ascomycota), whereas the asexual (anamorphic or mitosporic) stage is called *Trichoderma*. The majority of the genetic diversity of the genus is represented by its sexual forms[1,2,3] and some species are isolated equally frequently as both anamorphs and teleomorphs. Several common species have lost their ability to reproduce sexually and have become clonal species (or agamospecies)[4,5,6,7]. The nomenclature of these fungi is complicated because of their pleomorphism. In this regard the ICBN (International Code of Botanical Nomenclature) which was adopted at the IBC (International Botanical Congress) in Melbourne in July 2011 for fungi suggested that from 1 January 2013 only one official name is allowed for each pleomorphic fungus. However, the ISTH as a subcommission of the International Commission on the Taxonomy of Fungi (ICTF IUMS) is expected to recommend on the names in *Hypocrea* and *Trichoderma*. The use generally follows the principle of priority of publication, but a new passage in Art. 57.2 says that an anamorph-typified name (e.g. ***Trichoderma***) that has priority must not be taken up until retention of the teleomorph-typified name (e.g. ***Hypocrea***) has been considered by the General Committee and rejected. Another new Art. 14.n says that lists of preferred names may be submitted to the General Committee, which will refer them to the Nomenclature Committee for Fungi and committees of experts for examination. After approval, these names will then become permanent, i.e. treated as conserved.

This thesis coincided with the transitional phase in fungal nomenclature what resulted in some taxonomic inconsistiencies throughout the work. Thus, in the most of the published manuscripts the first mentioning of a fungal species is provided in accordance with the previously valid requirement of the Article 59 of the ICBN implying the teleomorph priority rule. Materials that have been prepared after January 1st 2013 comply with the new rule. The generic group is termed *Trichoderma*.

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[1] Jaklitsch WM (2009) European species of *Hypocrea* Part I. The green-spored species. Stud Mycol., 63:1-91.
[2] Jaklitsch WM (2011) European species of *Hypocrea* part II: species with hyaline ascospores. Fungal Divers, 48(1):1-250.
[3] Druzhinina IS, Seidl-Seiboth V, Herrera-Estrella A, Horwitz BA, Kenerley CM, Monte E, Mukherjee PK, Zeilinger S, Grigoriev IV, Kubicek CP (2011) Review: *Trichoderma*: the genomics of opportunistic success. Nat Rev Microbiol, 9(10):749-59. Erratum in: Nat Rev Microbiol, 9(12):896
[4] Druzhinina IS, Komoń-Zelazowska M, Kredics L, Hatvani L, Antal Z, Belayneh T, Kubicek CP (2008) Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. Microbiology, 154(Pt 11):3447-59.
[5] Druzhinina IS, Kubicek CP, Komoń-Zelazowska M, Mulaw TB, Bissett J (2010) The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. BMC Evol Biol, 10:94.
[6] Druzhinina IS, Komoń-Zelazowska M, Atanasova L, Seidl V, Kubicek CP (2010) Evolution and ecophysiology of the industrial producer *Hypocrea jecorina* (Anamorph *Trichoderma reesei*) and a new sympatric agamospecies related to it. PLoS One, 5(2):e9191.
[7] Samuels GJ, Ismaiel A, Bon MC, De Respinis S, Petrini O (2010) *Trichoderma asperellum* sensu lato consists of two cryptic species. Mycologia, 102:944–966.

ABBREVIATIONS AND WEB RESOURCES

BCA Bayesian Concordance Analysis

cDNA complementary deoxyribonucleic acid

DNA deoxyribonucleic acid

DOE The United States Department of Energy (DOE): http://energy.gov/

ENTREZ NCBI Entrez cross-database search: www.ncbi.nlm.nih.gov/Entrez/

FunCat Functional Catalogue for systematic classification of proteins from whole genomes: http://mips.helmholtz-muenchen.de/proj/funcatDB/search_main_frame.html

GeneDoc: sequence editing software http://www.nrbsc.org/gfx/genedoc/

GO database of Gene Ontology terms: http://www.geneontology.org/

IBC International Botanical Congress

ICBN International Code of Botanical Nomenclature: http://www.iapt-taxon.org/nomen/main.php

ICTF International Commission on the Taxonomy of Fungi: http://www.fungaltaxonomy.org/

ISTH International Subcommission on *Trichoderma* and *Hypocrea:* http://www.isth.info/

JGI DOE Joint Genome Institute: http://www.jgi.doe.gov/

KEGG Kyoto Encyclopedia of Genes and Genomes: http://www.genome.jp/kegg/

KOG EuKaryotic Orthologous Groups (KOG), a eukaryote-specific version of the Clusters of Orthologous Groups (COG) tool for identifying ortholog and paralog proteins

LA Lea Atanasova, the author of the thesis

MCMC Markov chain Monte Carlo

MycoCosm the fungal genomic resource tool of JGI: http://genome.jgi-psf.org/programs/fungi/index.jsf

NCBI National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/

NRPS Non-Ribosomal Peptide Synthetase

Pfam database of protein families that includes their annotations: http://pfam.sanger.ac.uk/

PKS polyketide synthase

qPCR real-time polymerase chain reaction

RNA Ribonucleic acid

rpb2 RNA polymerase II subunit gene

s.l. sensu lato

s.s. sensu stricto

tef1 translation elongation factor 1α

VOCs volatile organic compounds

WSCs water soluble compounds

Interactive key, images, descriptions, distributions and nomenclature of the genus *Trichoderma* by Samuels et al.: http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm

JGI *Trichoderma* genome platforms

T. asperellum: http://genome.jgi.doe.gov/Trias1/Trias1.home.html

T. atroviride: http://genome.jgi.doe.gov/Triat2/Triat2.home.html

T. citrinoviride: http://genome.jgi.doe.gov/Trici1/Trici1.home.html

T. harzianum: http://genome.jgi.doe.gov/Triha1/Triha1.home.html

T. longibrachiatum: http://genome.jgi.doe.gov/Trilo1/Trilo1.home.html

T. reesei: http://genome.jgi.doe.gov/Trire2/Trire2.home.html

T. reesei RUT C-30: http://genome.jgi.doe.gov/TrireRUTC30_1/TrireRUTC30_1.home.html

T. virens: http://genome.jgi.doe.gov/TriviGv29_8_2/TriviGv29_8_2.home.html

Transcriptomic database

The microarray data and protocols related to this study are available at the GEO web site http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE23438.

THESIS OVERVIEW

This is a cumulative thesis that consists of (i) an introducing review of the literature on ecology of *Trichoderma* as revealed by DNA barcoding tools, (ii) three chapters containing published experimental results or materials prepared for submission and relevant comments, (iii) an outlook, and (iv) two appendices containing manuscripts with materials presented in the thesis and manuscripts of the author that were not included in the main part of the thesis respectively. The introduction of the thesis has been submitted as an invited review for the book chapter *Ecophysiology of* Trichoderma *in genomic perspective*[8]. The first authorship publications presented in the Chapters 1 and 2 have been published in peer-reviewed scientific journals, the publication of Chapter 3 "*Polyketide synthase gene* pks4 *of* Trichoderma reesei *provides pigmentation and defense against biotic and abiotic stress*" has been submitted to *Eukaryotic Cell*.

[8] **Atanasova L** (2013) Ecophysiology of *Trichoderma* in genomic perspective. In: Gupta VK, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina IS and Tuohy M (eds.) Biotechnology and Biology of *Trichoderma*. Elsevier BV, Amsterdam, The Netherlands, invited review submitted.

ECOPHYSIOLOGY OF *TRICHODEMA*

Trichoderma in its ecological niche[9]

Members of the filamentous ascomycete genus *Trichoderma* are among the most commonly isolated biotrophic and saprotrophic fungi, frequently found on other fungi, on dead wood and bark, in soil and rhizosphere. *Trichoderma* isolates are also known from marine sponges (Paz et al., 2010; Gal-Hemed et al., 2011), living gramineous and herbaceous plants (Jaklitsch, 2009) and as endophytes (Samuels et al., 2006b; Zhang et al., 2007; Hanada et al., 2008; Mulaw et al., 2010), what demonstrates their ability to occupy various ecological niches. *Trichoderma* diversity of life styles is versatile, and refers to two major nutritional modes- saprotrophy and biotrophy - which are commonly not in disagreement for many of *Trichoderma* species. Most of the species can live in parasitic relationship in which the parasite feeds on a living host organism like other fungi (Figure 1.1). Most of the fruiting bodies of *Trichodema* spp. were found on specific basidiomycetes as well as several of *Trichoderma* spp. can degrade and grow within and on a wide variety of fungi with or without killing them (necrotrophic hyperparasitism and/or mycoparasitism or mycotrophy; Druzhinina et al., 2011). Furthermore, biotrophic interactions were observed also with other organisms such as immunocompromised humans (Kredics et al., 2003; Druzhinina et al., 2008) or nematodes (Casas-Flores et al., 2007) (Figure 1.1). Some species such as *T. reesei,* known by its production of cellulosic and hemicellulosyc enzymes, exhibit saprotrophic nutrition, where the fungus can successfully degrade lignocellulosic compounds by extracellular digestion involved in the processing of dead or decayed organic matter (Figure 1.1). This was observed also *for T. subeffusum, T. luteffusum, T. polysporum, T. phellinicola, T. sulphurea* etc. probably after entire digestion of the host basidiome (Jaklitsch, 2011). Thus, it was hypothesized that the *Trichoderma* ancestral species have first parasitized on wood-rotting fungi and later on explored the wood as an optional ecological niche and have switched to living on the pre-degraded wood rather than the host fungus itself (Rossmann et al., 1999; Druzhinina et al., 2011). However, it was observed that *Trichoderma* spp. can also can establish comensalistic associations in the rhizosphere or in plant tissue (as endophytic associate; Mulaw et al., 2010), or even beneficially stimulate plant growth and evoke plant defense reactions against pathogens (Howel et al., 2003; Benitez et al., 2004; Druzhinina et al., 2011) (Figure 1.1). Furthermore, a species *T. subeffusum* was recently found in association with green algae on decorticated wood (Jaklitsch, 2011). Other mutualistic or comensalistic associations with *Trichoderma* are known from marine sponges (Gal-Hemed et al., 2011), insects (D. Davidson, L. Atanasova and I.S. Druzhinina, unpublished data) and bacteria (L. Atanasova and I.S. Druzhinina, unpublished data), yet their interactions are not yet well understood.

Most of the *Trichoderma* studies focused on the anamorphs of *Trichoderma* from soil (Kullnig et al., 2000; Kubicek et al., 2003; Wuczkowski et al., 2003; Gherbawy et al., 2004; Friedl and Druzhinina, 2012; Zhang et al., 2005; Migheli et al., 2009, Zachow et al., 2009). Thus, the impression that *Trichoderma* is primarily a soil fungus is very commonly distributed in science. The diversity studies showed the dominance opportunistic species such as *T. asperellum, T. asperelloides, T. harzianum* sensu lato*, T. pleuroticola, T. alni, T. hamatum*, *T. atroviride, T. strigosum, T. brevicompactum, T. virens, T. longibrachiatum, T. gamsii, T. citrinoviride, T. koningiopsis, T. spirale, T. koningii* complex etc. Atanasova et al. (2013a) proposed that it is likely that these species obtained the ability to saprotrophic growth in soil due to their great opportunistic potential as suggested based on genomes of *T. atroviride* and *T. virens* (Druzhinina and Kubicek, 2013). Moreover, a consistent co-occurrence of *T. harzianum* sensu lato, *T. spirale*, *T. gamsii*, *T. hamatum* and *T. koningiopsis* in non-rhizosphere soils was noticed in several studies (Hagn et al., 2007; Zachow et al., 2009; Friedl and Druzhinina, 2012). Co-existence of *T. harzianum* sensu stricto and *T.* cf. *harzianum* has been shown by a number of cultivation based studies (see Druzhinina et al. 2010a for references) and was confirmed by metagenomic analyses of soil (Friedl and Druzhinina, 2012). Similar coexistence was also shown for *T. longibrachiatum* and *H. orientalis* isolated from terrestrial (Druzhinina et al., 2008; Kredics et al., 2008) and marine environments (Gal-Hamed et al., 2010).

[9] Ecological niche - the totalilty of the adaptations of an organism to exploit its particular environment.

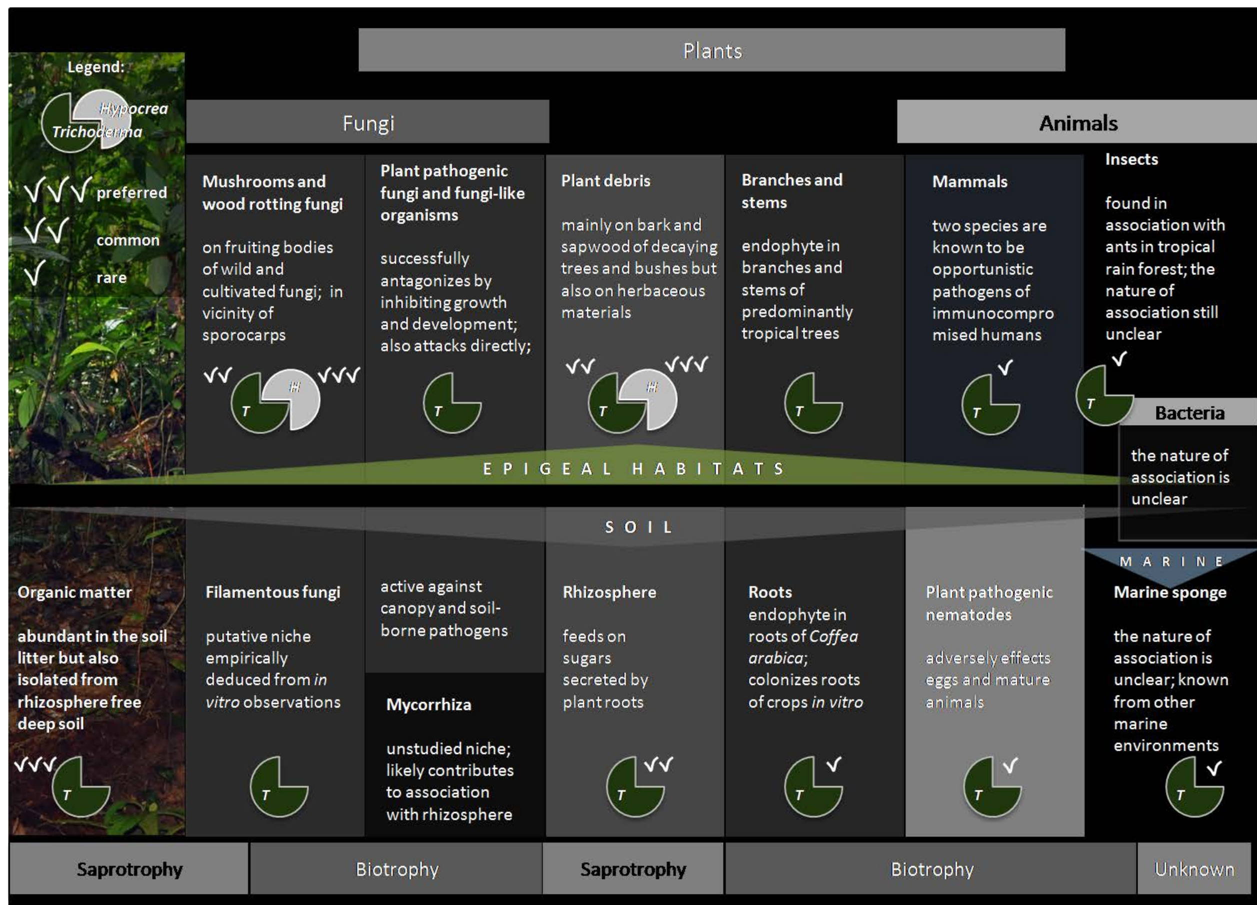


Figure 1.1: Ecological niches of genus *Trichoderma*.

However, the cultivation approaches tend to strongly undergo the isolation bias for rare and slow growing, non-opportunistic species. *In situ* diversity studies of *Trichoderma* in soils (Hagn et al., 2007, Zachow et al., 2009, Meincke et al., 2010, Friedl and Druzhinina, 2012) detected almost exclusively already known species of *Trichoderma*. The latest metagenomic study on *Trichoderma* in soil using a combination of cultivation independent metagenomic methods and *in vitro* modeling of infrageneric interactions showed that there is almost no hidden diversity of this genus in soils, but showed that diversity of *Trichoderma* species different soil layers is not only limited on the highly opportunistic species, but also comprise *T. rossicum*, *T. pachypallidum*, *T. polysporum* and a new species *Trichoderma* cf. sp. nov. MOTU 1A 64 from Section *Longibrachiatum* (Friedl and Druzhinina, 2012). On the other hand the authors showed that the local diversity of *Trichoderma* in soil is biologically determined as not all species which are known to be locally abundant above ground (e.g. most frequent teleomorphic *Trichoderma* species in Central Europe *T. minutisporum* and common *T. viridescens*, *T. viride*, *H. pulvinata*, *T. strictipile* etc. (Jaklitsch et al., 2006; 2009) were not detected (see Friedl and Druzhinina, 2012).

However, the extensive work of Jaklitsch (2009; 2011) on *Trichoderma* in Europe suggested that the biodiversity on and above the litter layers exceed the number of species isolated from soil, and showed that generally *Trichoderma* anamorphs are considerably more common on plant material than the teleomorphs. The majority of *Trichoderma* species (83 %) occurs on wood and bark of trees and shrubs, while only 2 % may be found on gramineous or herbaceous hosts, 2 % on soil and forest debris and 13 % specifically on other fungi (Atanasova et al., 2013a). Although the full diversity of *Trichoderma* species associated with higher plants, basidiomycetes, invertebrates and mammals is not known, most of the taxa have been recovered from dead wood and fruiting bodies of other fungi suggesting that these are the primary ecological niches of this fungus (Druzhinina and Kubicek, 2013).

Mycotrophy of *Trichoderma*

The ability of fungi to feed on other fungi is described as necrotrophic hyperparasitism or mycoparasitism (Druzhinina et al., 2011). Studies of *Trichoderma* teleomorphs in Europe, which included more than 600 specimens from 14 different countries (Jaklitsch 2009; 2011) covering most climatic zones and including 75 species of *Trichoderma* that form teleomorphs, reported that most fruiting bodies of *Hypocrea* spp. are associated with specific basidiomycete fungi; e.g. *H. estonica* and *H. parestonica* strictly growing on *Hymenochaete* spp., *T. fomiticola* growing on *Fomes fomentarius*, and *H. pulvinata* on *Fomitopsis pinicola* and *Piptoporus betulinus* etc. (see Jaklitsch, 2011). Moreover, *Trichoderma* can mostly degrade and grow within the latent fungal structures (sclerotia) of variety of plant pathogenic fungi (see Druzhinina et al., 2011).

Species of *Trichoderma* possess strong mycoparasitic potential and were frequently studied against ascomycetous causative agents of plant diseases *Alternaria alternata*, *Botryotinia fuckeliana* and *Sclerotinia sclerotiorum* (Figure 1.2; see Druzhinina et al., 2011) as well as against basidiomycete *Rhizoctonia solani* (Reithner et al., 2011; Shibu et al., 2012; Yang et al., 2012; Atanasova et al., 2013b). Thus, the exploitation of the mycoparasitic properties of *Trichoderma* in biotechnology and agriculture made the genus well studied and brought it in focus of numerous -*omic* studies including the seven complete genomes (*T. asperellum, T. atroviride, T. citrinoviride, T. harzianum* sensu stricto, *T. longibrachiatum, T. reesei* and *T. virens;* http://www.jgi.doe.gov/). Numerous studies regarding *Trichoderma* spp. mechanisms of action and their commercially valuable purposes (Harman et al., 2004, 2011; Lorito et al., 2010) were released. Most researches in this direction were preformed only with few of *Trichoderma* opportunistic species, such as *T. harzianum* sensu lato species complex, *T. atroviride*, *T. virens*, *T. asperellum* and *T. asperelloides* (Howel et al., 2003; Benitez et al., 2004; Druzhinina et al., 2011; Atanasova et al., 2013b; etc.). Recently, a remarkable genus-wide survey of *Trichoderma* antagonistic potential against ascomycetes *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *Alternaria alternata* as well as against oomycete *Phytophtora* sp. showed that, despite a considerable infraspecific variability, nearly all of 75 molecularly defined *Trichoderma* species are able to reduce the development of tested prey fungi in the average range of 70 % (Druzhinina and Kubicek, 2013). They showed that the mycoparasitism, with few exceptions e.g. *T. polysporum*, is a genus-wide trait, and that the mycoparasitism is a rather strain than species specific (Figure 1.2).

Trichoderma species were found to commonly colonize the substrates on the farms growing edible fungi (*Agaricus bisporus*, *Lentinula edodes*, and *Pleurotus ostreatus*) (Sinden et al., 1953). All over the World in the last three decades severe damages have been caused by *Trichoderma* spp. what led to the extensive studies to identify the green mold disease agents (Muthumeenakshi et al., 1994; Castle et al., 1998; Park et al.; 2004, 2006; Hatvani et al., 2007; Komon-Zelazowska et al., 2007).A survey based on the molecular identification and diagnostic test for *T. aggressivum*, showed that seven species, *T. aggressivum* f. *europaeum*, *T. asperellum*, *T. atroviride*, *T. ghanense*, *T. harzianum* sensu lato, *T. longibrachiatum* and new undescribed phylogenetic species *Trichoderma* sp. DAOM 175924 co-occur in the infected mushroom substrates (Hatvani et al., 2007), but aggressive colonization was claimed to be exclusively due to two *formae speciales* of a new *Trichoderma* species, viz. *T. aggressivum* f. *europaeum* in Europe and *T. aggressivum* f. *aggressivum* in North America (Samuels et al., 2002). The same ITS1 and 2 allele as known for *T.* sp. DAOM 175924 was found on the Hungary oyster mushroom farm (Hatvani et al., 2007) and the study showed that two different, although genetically closely related species, *T. pleurotum* (the correct name *T. pleuroti*, see Atanasova et al., 2013a) and *T. pleuroticola* together with *T. aggressivum* were the causing agents of green mold disease in Hungary, Poland, Romania, Italy, South-Korea and Taiwan (Park et al.; 2004; Hatvani et al., 2007; Komon-Zelazowska et al., 2007). Recently new *Trichoderma* species, *T. mienum*, was isolated from ascospores and dry stromata found on bedlogs for shiitake and oyster mushroom cultivation in Japan (Kim et al., 2012).

Studying *Trichoderma* mycoparasitic response, several genes, enzymes and other effectors were found to be involved among different species and in different stages of their predation (Seidl et al., 2009b; Reithner et al., 2011; Omann et al., 2012; Catalano et al., 2011; Atanasova et al., 2013b). The processes occurring during *Trichoderma* mycoparasitism were recently grouped into four stages (Druzhinina and Kubicek, 2013): 1) waiting for a prey (ambushing); 2)

recognition of the presence of a potential prey (sensing); 3) induction of the biochemical tools to besiege the prey (hunting); and 4) actual attack and eventual killing and feeding on the prey. *Trichoderma* spp. might recognize the other fungi by the excretion of small peptides or other molecules that are released as a response to proteases secreted by the *Trichoderma* spp. (Druzhinina et al., 2011). They may bind to G protein-coupled receptors (such as Gpr1) or nitrogen-sensing receptors on the surface of the *Trichoderma* spp. hyphae, inducing a signaling cascade comprising G proteins and mitogen-activated protein kinases (MAPKs), which may then trigger the activation of still unknown transcription factors which may regulate the genes responsible for secondary metabolite biosynthesis and cell wall lysis of the other fungus (Druzhinina et al., 2011). However, these are only the insights into the opportunistic success of *Trichoderma*, but the mechanisms behind are still not well understood.

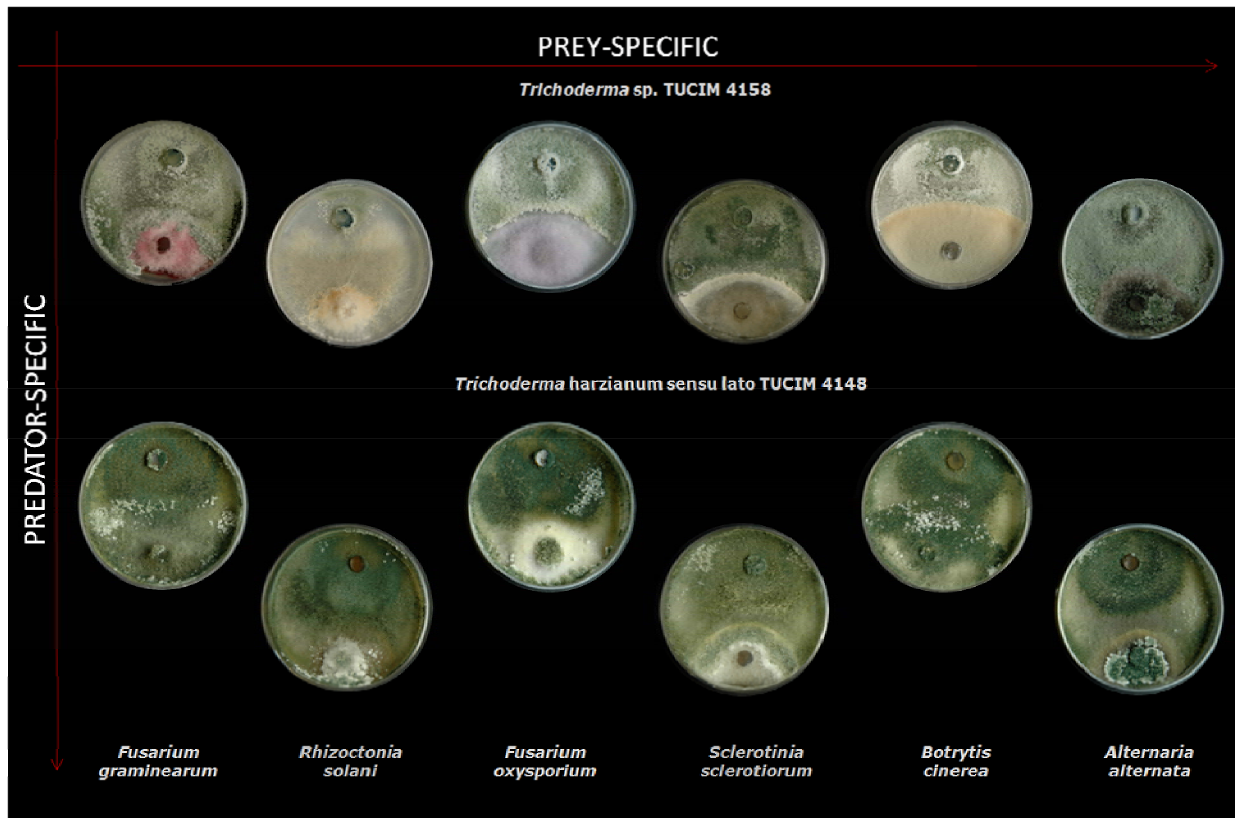


Figure 1.2: *Trichoderma* mycroparasitic poteintial is prey and predator specific. A moderately active strain *T.* sp. TUCIM 4158 compared to strong mycoparasite *T. harzianum* sensu lato TUCIM 4148 in confrontations with six plant pathogenic fungi.

Saprotrophy of *Trichoderma* on dead wood

Most of *Trichoderma* species (83 %) have been reported to occur on dead wood and bark of trees and shrubs (Atanasova et al., 2013a). The fungal degradation pathways of lignocellulosic biomass are described in detail in Kubicek (2013). In the genus *Trichoderma T. reesei* is most prominent industrial producer of cellulases and hemicellulases, enzymes employed in breaking cellulose into simple sugars (glucose and xylose) (Grigoriev et al., 2011). The industrially important strain QM 6a was isolated in 1942 on the Solomon Islands during WW II, and strains used in biotechnology today have been derived from it by the classical mutagenesis based on a series of exposures to radiation with a linear accelerator. The resulting mutants displayed two- to four-fold increase in cellulolytic activity. However, the potential to degrade cellulose is strain specific in *T. reesei* (Druzhinina et al., 2010b) and might be expanded in several strains of *Longibrachiatum* Clade, e.g. in *T. longibrachiatum* and *T. citrinoviride*, whereas the teleomorphs of the latter one were shown to be among very poor producers (Kubicek et al., 1996).

For the degradation of cellulosic materials *T. reesei* can produce at least two cellobiohydrolases (CBHI and CBHII), eight endo-β-1,4-glucanases (EGI to EGIV, EG45, CEL74A , CEL61B and CEL5B) and seven ß-glucosidases (BGLI, BGLII, CEL3B, CEL3C, CELIB, CEL3D, CEL3E) that act synergistically to degrade crystalline cellulose to glucose (Kubicek et al., 2009). The major cellulase genes are coordinately expressed under different inducing carbon source conditions; by cellulose, its derivatives cellobiose and sophorose, lactose, and a monosaccharide L -sorbose, and are subject to carbon catabolite repression (Kubicek et al., 2009; Furukawa et al., 2009). Hemicelluloses, e.g. xylan, galactoglucomannan and xyloglucan, are besides the cellulose the most abundant polysaccharides in plant cell walls (Eriksson et al., 1990; Seiboth et al., 2007). A major part of hemicelluloses consists of simple sugars such as pentoses (D-xylose, L-arabinose) and hexoses (D-galactose, D-mannose or D-glucose). The major components of this xylanolytic system are endoxylanase (1,4-β-D-xylan xylanohydrolase) which hydrolyses the β-1,4 bonds in the main chain generating a mixture of xylo-oligosaccharides, and β-xylosidase (1,4-β-D-xylan xylohydrolase) which cleaves off the terminal xylose units from the non-reducing end of xylo-oligosaccharides and has been reported to be rate limiting in xylan hydrolysis (Gomez et al., 2001). *T. reesei* forms at least three endo-xylanases (XYNI to XYNIII), one ß-xylosidase (BXL1) (Furukawa et al., 2009) and one endo-β-1,4-glucanase which nonspecif cally degrades xylan (Rauscher et al., 2006). Contrary to cellulases, xylanase genes are not co-regulated (Furukawa et al., 2009). Expression of the *xyn1* gene is primarily induced by D-xylose, whereas xylobiose and sophorose induce the *xyn2* (Furukawa et al., 2009) and cellulose, its derivatives and L -sorbose, but not xylan, xylooligosaccharides or D -xylose induce the expression of *xyn3* (Xu et al., 2000). This applies that expression of cellulase and xylanase genes is modulated by a complex network involving several regulatory proteins (Furukawa et al., 2009). Several transcription factors were found to be involved in cellulase and xylanase gene expression in *T. reesei*; three positive transcriptional activators (XYR1, ACE2 and the HAP2/3/5 complex) and two repressors (ACE1 and the carbon catabolite repressor CRE1) (Kubicek et al., 2009). CREI is a Cys2His2 zinc f nger protein that mediates carbon catabolite repression of cellulase and xylanase genes (Strauss et al., 1995; Furukawa et al., 2009). CRE1 and the general transcriptional activator Xyr1 are essential for *xyn1* transcription (Rauscher et al., 2006). XYR1 has been demonstrated to be a general and essential transcription factor controlling expression of the major cellulolytic and xylanolytic genes, regardless of inducer (xylose, xylobiose, sophorose and lactose) and mode of expression (basal, derepression and induction) (Stricker et al., 2006, 2007; Kubicek et al., 2009). ACEII has been shown to be involved in the induction of all major cellulase genes and *xyn2*, where deletion of *ace2* reduced their expression in the presence of cellulose but did not affect induction by sophorose (Aro et al., 2001), whereas ACEI represses expression of all major cellulase genes (*cbh1*, *cbh2*, *egl1* and *egl2*) and the major xylanase genes (*xyn1* and *xyn2*) under inducing conditions with sophorose and cellulose (Saloheimo et al., 2000; Aro et al., 2003) and directly antagonizes Xyr1 function by competing for one of its binding sites (Rauscher et al., 2006).

Recently, Seiboth et al. (2012) have shown that the protein methyltransferase LAE1, known as a master regulator of secondary metabolites in *Aspergillus* spp. (Keller and Bok, 2006; Keller et al., 2007) inf uences cellulase gene transcription in *T. reesei.* The growth of the deletion mutants on cellulose was severely impaired and signif cantly reduced cellulase and xylanase activities were found in the deletion mutant's cultures grown on lactose and xylan respectively. Contrary to non-successfully increased production of all cellulases by increased expression or activity of the cellulase- and hemicellulose specif c transcriptional regulator XYR1 (Stricker et al., 2006; Mach-Aigner et al., 2008), the overexpression of *Lae1* has yielded signif cant improvements in cellulase production in *T. reesei*. A transcriptome analysis of *T. reesei* wild type strain and deletion mutant Δ*lae1* on lactose revealed that in total 6.5 % of all down-regulated genes in Δ*lae1* strain were CAZyme encoding genes, from which the majority represented glycosyl hydrolases (GH) involved in cellulose and hemicellulose degradation (Seiboth et al., 2012). Furthermore, swollenin SWO1, a protein carrying an expansin-like domain that disrupts the crystalline cellulose structure (Saloheimo et al., 2002), CIP1, which contains a signal peptide and a cellulose-binding domain (Foreman et al., 2003; Li et al., 2007), and four xylanases (XYN1 to XYN4) were also down-regulated. The reduction of expression of various glycosidase genes was also ref ected in the decreased ability to grow on several di- or oligosaccharides. Seiboth et al. (2012) implied that the expression of most of the cellulolytic and hemicellulolytic genes is affected by *lae1*. The authors further speculated that LAE1 might play a role in the linkage between asexual sporulation and CAZyme gene transcription in *T. reesei*, like shown in Metz et al. (2011): asexual sporulation triggers massive CAZyme gene expression in *T. reesei* in an inducer-

independent but XYR1-dependent way. The deletion *lae1* mutants in *T. reesei* exhibited absence of conidiation whereas the overexpression strains were hypersporulating. Approximately half of the genes up-regulated during asexual sporulation are identical to a third of the genes down-regulated in Δ*lae1* (Seiboth et al., 2012) and absence of conidial cellulases makes *lae1* deletion mutants unable to germinate on cellulose as a carbon source (Metz et al., 2011). Seiboth et al. (2012) therefore consider it possible that the regulation of sporulation is the prime target of LAE1, and that the effect on cellulase gene expression occurs by a signal for CAZy gene expression created during sporulation.

Trichoderma growth in soil

Species commonly isolated from soils are mostly cosmopolitan and opportunistic *Trichoderma* species known from all over the world (Kubicek et al., 2003; Zachow et al., 2009; Migheli et al., 2009). *S*everal recent studies that were preformed on islands (Migheli et al., 2009; Zachow et al., 2009) are also in agreement with this. Tyrrhenian Island of Sardinia (Migheli et al., 2008), which has become isolated since the early Miocene (7~8 Ma) is considered a hotspot of biodiversity and endemism (Médail and Quézal, 1999; Grill et al., 2006). Among more than 400 strains of *Trichoderma* from 15 soils comprising undisturbed and disturbed environments (forest, shrub lands and undisturbed or extensively grazed grass steppes) Migheli et al. (2009) were able to isolate mostly pan-European and/or pan-global *Trichoderma* species from sections *Trichoderma* and *Pachybasium*, comprising *T. harzianum* s.l., *T. gamsii*, *T. spirale*, *T. velutinum*, *T. hamatum*, *T. koningii*, *T. virens*, *T. tomentosum*, *H. semiorbis*, *T. viridescens*, *T. atroviride*, *T. asperellum*, *T. koningiopsis* and *T. samuelsii* (that time *Trichoderma* sp. Vd2), possessing the genotypes already detected worldwide. Only one isolate represented a new, undescribed species belonging to the *Harzianum* Clade, and a unique allele of *T. hamatum* was additionally found. Similarly, the study of Tenerife island soil (Zachow et al., 2009) revealed similar diversity (*T. harzianum*, *T. spirale*, *T*. cf. *tomentosum*, *T. gamsii*, *T. viridescens*, *T. viride* and *T.* 'viride Ve'). Migheli et al. (2009) suggested that native *Trichoderma* diversity on Sardinia was replaced by extensive invasion of species from Eurasia, Africa and the Pacific Basin, what was supported also by Zachow et al. (2009); the colonization by highly competitive *Trichoderma* species from the continent could explain the extraordinarily high mycoparasitic potential of all *Trichoderma* isolates found on this island against different plant pathogens (Zachow et al., 2009). Furthermore, the diversity reported for the rhizosphere of agricultural soils and crops in South-East Asia (Kubicek et al., 2003) and South America (Hojos-Carvajal et al., 2009) and in Ethiopia (Mulaw et al., 2010) was much higher than those found on both islands. The cultivation-based study of *Trichoderma* species from South-East Asian soils from 19 countries including tropical forest, park, garden and soil near island's seashores revealed nine *Trichoderma* species (Kubicek et al., 2003). Genus *Trichoderma*, as in other studies, dominated with *Trichoderma harzianum* s.l. and *T. virens*, followed by *T. spirale*, *T. koningii*, *T. atroviride*, *T. asperellum*, T. *viride*, *T. hamatum* and *T. ghanense*. Another study of *Trichoderma* diversity from soil and litter in China (Zhang et al., 2005) reported about very similar biodiversity in soil (*T. asperellum*, *T. koningii*, *T. atroviride*, *T. viride*, *T. velutinum*, *T. cerinum*, *T. virens*, *T. harzianum*, *T. sinensis*, *T. citrinoviride*, *T. longibrachiatum*) and two putative new species. Corresponding to fact that most of species the isolated from soil are cosmopolitan as well as strong opportunist a local genetic diversity study of *Trichoderma* in the river Danube national park in Middle Europe (Austria) found *T. harzianum*, *T. rossicum*, *T. cerinum*, *T. hamatum* and *T. atroviride* and *T. koningii* in riparian forest soil (Wuczkowski et al., 2003). Moreover, *T. harzianum* s.l. and an anamorph of *H. orientalis* were even found in a several different strongly alkaline soils from the Northern half of the Nile valley (Egypt) (Gerbawy et al., 2004)

On the other hand a large-scale biogeographic study of neotropic soils performed in Mexico, Guatemala, Panama, Ecuador, Peru, Brazil and Colombia (Hoyos-Carvajal et al., 2009) revealed relatively high diversity of *Trichoderma* species. The most commonly isolated species were *T. asperellum* and *T. harzianum* s.l., but also *T. atroviride*, *T. brevicompactum*, *T. crassum*, *T. erinaceum*, *T. gamsii*, *T. hamatum*, *T. harzianum*, *T. koningiopsis*, *T. longibrachiatum, T. ovalisporum*, *T. pubescens*, *T. rossicum*, *T. spirale*, *T. tomentosum*, *T. virens*, *T. viridescens* and *T. reesei were found.* Additionally, two distinct genotypes of *T. asperellum* with different metabolic profiles and habitat preferences were found, and one of them was later described as *T. asperelloides* (Samuels et al., 2010). Further undescribed species

from this study are still awaiting their formal description. Interestingly, neither *T. koningii* nor *T. viride*, once believed to be common and widely distributed, were isolated from these neotropical soils.
These studies showed that most isolates from soils belong to a few known *Trichoderma* species, which are all very powerful opportunist. Hence, it might be suggested that saprotrophic growth in soil is likely so demanding that only strong environmental opportunistic species can accomplish it.

Rhizosphere competence of *Trichoderma*

Recently, several species have been patented as stimulants of plant growth and immunity (biofertilizers), complementing their role as principal components in commercial biofungicide formulations. Some of these species improve survival of plants in hostile environments and increase plant tolerance to drought and high salinity (Gal-Hamed et al., 2011; M. Marzouk, L. Atanasova and I.S. Druzhinina; J. Zhang, unpublished data). These traits are intimately associated with the profound ability of only some species to grow in the rhizosphere as well as free soil and establish long-term associations *in planta,* however, only ca. 20 % of described *Trichoderma* species have been detected in these ecosystems.

A study from a coffee plant (*Coffea arabica*) rhizosphere in Ethiopia showed remarkable diversity and occurrence of several new or rarely observed genotypes (Mulaw et al., 2010). Infect, a correlation analysis did not detect any other parameter that would influence species richness and distribution, so the authors concluded that the *C. arabica* rhizosphere as ecological niche is the main parameter determining the presence of *Trichoderma*. Similarly, the most dominant species in the vertical soil profiles in Danube riparian forest soils, *T. asperellum,* was reproducibly associated with upper soil horizonts (rhizosphere), but its abundance did not correlate with the carbon and nitrogen content of the soil or pH of the habitat (Friedl and Druzhinina, 2012).

It has been shown that plants respond to the presence of other organisms by activating defense mechanisms, especially against various plant pathogens that trigger their immune defense (Yedidia et al., 1999; Druzhinina et al., 2011). As other non-pathogenic microorganisms, *Trichoderma* spp. trigger induced systemic resistance that results in the accumulation of components of the jasmonate and ethylene signaling pathways in plants (Segarra et al., 2007; Druzhinina et al., 2011). Furthermore, proteomic and transcriptomic studies showed that colonization of plant roots by *Trichoderma* provoke a systemic change in the expression of plant genes regulating stress responses, isoprenoid oxylipins and ethylene biosynthesis, photosynthesis, photorespiration, and carbohydrate metabolism (Vargas et al., 2009; Druzhinina et al., 2011). Several classes of *Trichoderma* molecules, such as xylanases, peptaibols, swollenin and cerato-platanins, induce microbe-associated molecular patterns in plants (Druzhinina et al., 2011). Details on the *Trichoderma* – plant root associations are extensively reviewed in Druzhinina et al. (2011).

Ability to competitively colonize plant roots has often been stressed as an important requirement of *Trichoderma* to act as biocontrol agents (cf. Harman et al., 2004). However, very less is known about the interaction of *Trichoderma* with plant roots *in vivo*, also due to very narrow sampling of species and using of very few opportunistic species as model organisms because they possess a potential as biocontrol agents (Druzhinina et al., 2011). Rhizosphere might provide opportunities for both biotrophy and saprotrophic nutrition on plant roots; *Trichoderma* as saprotroph could degrade highly hydrated polysaccharides such as pectins and hemicellulose which are secreted from the plant roots and tips (mucgel) or might as biotroph interact with plant mycorrhiza (Druzhinina et al., 2011).

Trichoderma versus mycorrhizae

The role of mycorrhiza is very important for the plants, since 92 % of land plants are colonized by mycorrhizal fungi (Druzhinina et al., 2011) and exchange nutrients for minerals. However, it would be remarkably important to know if the interactions between the mycorrhizal fungi and *Trichoderma* are synergistic or the *Trichoderma* species feed on them, since the mycorrhizal fungi present an obvious natural barrier between the plant and *Trichoderma*. Mycorrhiza is

among the poorest studied topic in the research area of *Trichoderma*, as the cultivation and general work with mycorhizae is difficult *in vitro*.

Recently, sequenced genome of ectomycorrhizal fungus *Laccaria bicolor* revealed that during ectomycorrhizae development, *L. bicolor* expresses effector-type small secreted proteins with unknown function, for which it was speculated to have a decisive role in the establishment of the symbiosis, which furthermore induces an increased expression of carbohydrate, oligopeptide and amino acid transporters, suggesting increased fluxes of metabolites at the symbiotic interface (Martin et al., (2008). The unexpected observation that the genome of *L. bicolor* lacks carbohydrate-active enzymes involved in degradation of plant cell walls, but maintains the ability to degrade non-plant cell wall polysaccharides, pointed out the dual saprotrophic and biotrophic lifestyle of the mycorrhizal fungus that enables it to grow within both soil and living plant roots (Martin et al., 2008). A study on *Laccaria bicolor* mycorrhiza formation in the rhizosphere of black spruce in presence of two *Trichoderma* species revealed strong antagonistic effect towards mycorrhizal colonization in both cases, when *Trichoderma* and *L. bicolor* were inoculated together and when establishment of the mycorrhizal fungus in the rhizosphere prior the inoculation of *Trichoderma* was allowed (Summerbell, 1987). Interestingly, one *Trichoderma* strain did not parasitize on *L. bicolor* in agar culture (Summerbell, 1987). In studies where seedlings grown in open pots were used, the same effect of *Trichoderma* was observed towards the formation of mycorrhiza by two *Suillus* spp. (Shemakhanova, 1962), however under certain unusual soil conditions, *Trichoderma* spp. appeared to stimulate the formation of mycorrhiza (Summerbell, 1987). Furthermore, it was also shown that mycorrhization helper bacteria promote ectomycorrhizal establishment of *L. laccata* but inhibit mycorrhiza formation by other fungi (Duponnois et al., 1993). However, *Trichoderma* interactions with bacteria in rhizosphere as well in other habitats are another white spot in *Trichoderma* research.

Trichoderma + bacteria = ?

Close associations with bacteria including endosymbiosis have been recently detected in many fungi (Bianciotto et al., 2003; Compant et al., 2008; Coenye et al., 2001; Lim et al., 2003; Partida-Martinez and Hertweck, 2005). Recent advances in molecular ecology and genomics indicate that the interactions of *Trichoderma* spp. with other organisms such as animals and plants may have evolved as a result of saprotrophy on fungal biomass and various forms of parasitism on other fungi (mycoparasitism), combined with broad environmental opportunism (Druzhinina et al., 2011; see above). Up to date most of *Trichoderma* – bacteria associations are known to either have a beneficial effect on plant disease protection as combined biocontrol agents in agriculture or *Trichoderma* secondary metabolites were studied as effectors of bacterial growth. A study showed that application of *Trichoderma* sp. and several strains of *Pseudomonas* spp. mixture provided greater suppression of the *Gaeumannomyces graminis* var. *tritici* on wheat (Duffy et al., 1996). Further it has been shown that chitinolytic enzymes of *Trichoderma* sp. enhanced the growth of *Enterobacter cloacae* in the presence of chitinous substrates and increased the ability of bacterial cells to bind to hyphae of the fungal pathogens (Lorito et al., 1993). *Trichoderma* spp. were also able to beneficially modify the response of plants to infection by bacteria e.g. *Xanthomonas* spp., *Pseudomonas syringae*, etc. (Woo et al., 2006). Recently, Davidson et al. (2009) performed an experimental study on microbial diversity of artificial and natural nests of *Camponotus* (*Colobopsis*) *cylindricus* (COCY) ants which dominate the lowland dipterocarp rain forest on Borneo. They suggested that the filamentous mycoparasitic fungus *Trichoderma* and bacteria from the nitrogen fixing genus *Burkholderia* are likely associated with nests of these ants. Species of *Campanotus* (*Colbopsis*) genus have been already reported as frequent non-mutualistic ‘weed’ fungi of gardens of leaf cutting ants (see Sen et al., 2009 for the references) present along with *Fusarium*, *Synecephalastrum* and garden-threatening *Escovopsis* fungi. However, no other study has so far reported about such interactions for *Trichoderma*. In the collaboration with D. Davidson we isolated and subcloned a library of *Trichoderma* species including the cellulose producer *T. reesei* which has so far not been isolated as an anamorph from natural habitats. Davidson et al. (2009) proposed a role for *Trichoderma* cellulases in hardening COCY nest cavity walls in natural fallen wood, which are remarkably hard and extremely difficult to crack open, despite extensive decay of external wood surfaces.

As mentioned, Davidson et al. (2009) detected bacteria co-occurring in the ant's nests together with *Trichoderma*. Six COCY spp. were harvested from artificial and natural nests in fallen dead wood and the microbial community of their nests was subsequently assessed using metagenomic approach by universal primers for the bacterial 16S rRNA gene and fungal 18S rRNA gene. All COCY taxa except one nest in the understory and commute extensively over the ground to canopy access trees. Members of bacterial genus *Burkholderia* and fungal genus *Trichoderma* continually appeared in both fiber and carton nests and the latter statistically dominated in the fiber nest, but was not detected in any of unoccupied nests, nor in any of the nests inhabited by ant taxa other than COCY. *Burkholderia* was found in ca. half of COCY spp. nests but was also detected in low amount in unoccupied nests and in nests housing other ant taxa. Davidson et al. (2009) showed that *Burkholderia* and *Trichoderma* co-occurred significantly more than predicted by chance. At the colony level, *Trichoderma* was present in 87.5 % of the occurrences of *Burkholderia*, and *Burkholderia*, in all nests with *Trichoderma* ($P = 0.01$ in Fisher Exact Test in both cases; Davidson et al., 2009). Interestingly, *Trichoderma* also occurred as endophytes in leaves from the canopy and understory and from roots of COCY foraging plants, but was absent from a leaf sample of an adjacent tree of a different species were these species do not forage, yet *Burkholderia* was not detected in any of those samples (Davidson et al., 2009). Together with D. Davidson we further investigate the foraging pattern and the potential collecting of microorganism by these particular ants. Our metagenomic analysis confirmed that *Trichoderma* spp. are present in the buccal pellets of at least one COCY species.

Burkholderia spp. are among the most abundant bacteria in the environment. It is now obvious that the plasticity of their genomes allow them to colonize diverse environmental niches (Holden et al., 2004).Several species of the genus are classified as human, animal or plant pathogens but others may interact with their partner organisms, resulting in various beneficial effects. The comprehensive review on ecological diversity and occurrence of *Burkholderia* spp. in the natural environment has been recently presented by Comopant et al. (2008). For the context of this review the most remarkable are the findings of *Burkholderia* spp. as endosymbionts of both insects (including ants) and fungi (including endophytes, plant pathogens and mycorrhizal agents). Many *Burkholderia* species are intimately associated with rhizosphere and are also found in vegetative and reproductive tissues of plants (Cruz et al., 2001; Vega et al., 2005). Our 16S rRNA sequence analysis and Bayesian phylogenetic analysis provided evidence for the at least eight putative *Burkholderia* taxa, of which at least six 16S rRNA phylotypes suggest hypothetically new species might occur in COCY nests. However, the majority of *Burkholderia* isolates from derived COCY ants are closely related to a common N_2-fixing neotropical soil species *B. tropica*.

Our preliminary results show that several other *Trichoderma* strains that were not isolated from ants nest also harbor *Burkholderia* spp. and that these can alter the carbon utilization and growth rates of *Trichoderma* spp. Up to date no other interactions between *Trichoderma* and bacteria were studied in nature.

Facultative endophytism of *Trichoderma*

Endophytic biotrophy (i.e., mutualistic growth inside a plant tissue) is very common among fungi (Druzhinina et al., 2011), yet very few *Trichoderma* species have been isolated as true endophytes (Holmes et al., 2004; Bailey et al. 2006; 2009; Bae et al., 2009; 2011) and it is not clear if any of *Trichoderma* species are obligate endophytes.

After the study of *Trichoderma* associated with the rhizosphere of *Coffea arabica* (see above) we extended the study to detect endophytes from roots of healthy *C. arabica* (coffee) in Ethiopia (T. Mulaw. I.S. Druzhinina, C.P. Kubicek, L. Atanasova ms in preparation) and detected several of the isolated species new to science to be also present in the rhizosphere e.g. T. *aethiopicum*, as well as few were detected exclusively *in planta*, e.g. *T. flagellatum.* Both species were recently formally described by Samuels et al. (2012), whereas other eight putative new species will be described in the future. Several other *Trichoderma* anamorphs are for now known as obligate endophytes: Zhang et al. (2007) described a rare species, *T. taxi*, from *Taxus mairei* from Jiangxi province, China. Many other true endophytic *Trichoderma* species were found in the association with cacao tree (*Theobroma cacao*) in South America such as *T. theobromicola* from Amazonian Peru and *T. paucisporum* from Ecuador (Samuels et al., 2006b), and *T. martiale* (Hanada et al., 2008) all belonging to *Pachybasium* Clade. Furthermore, it is likely that due to a high opportunistic potential *Trichoderma* species can be facultative endophytic associates of plants. Several *Trichoderma* species were

collected from plants growing in tropical climates e.g. *T. ovalisporum* isolated from *Banisteriopsis caapi* (Holmes et al., 2004), *T. hamatum* (Bae et al., 2009) and *T. caribbaeum* from *Theobroma gileri* (Samuels et al., 2006a) and *T. stilbohypoxyli* from *Theobroma cacao* (Lu and Samuels, 2003). Moreover, *Hypocrea spinulosa* is a unique species that forms fruiting bodies also on various temperate gramineous hosts (e.g. *Chelidonium*, *Plantago*) including those that are partially green (living). The species does not form conidia *in vitro* but abundant fertile stromata were found and thus may be mated (Jaklitsch, 2009). The unique biology of this rare temperate and clearly specialized species suggests that it is also capable of biotrophic growth (endophytic or even parasitic) on herbaceous plants.

Animal nourishment of *Trichoderma*

The most common hosts of the hypocrealean animal pathogens include insect species of Coleoptera, Hemiptera and Lepidoptera, although one species of arthropod pathogen is generally considered to have a narrow host range of one or closely related host species (Sung et al., 2008). These arthropod pathogenic fungi consist of several genera (e.g., *Cordyceps* s.s., *Elaphocordyceps*, *Hypocrella*, *Metacordyceps* and *Ophiocordyceps*) in three families (Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae) (Sung et al., 2008). These multiple fungal lineages are associated with a diversity of arthropods that possibly had a relatively ancient origin as shown by the founding of the oldest fossil evidence of animal parasitism by fungi, *Paleoophiocordyceps coccophagus* parasitized by a fungus similar to asexual states of *Hirsutella* and *Hymenostilbe* of the extant genus *Ophiocordyceps* and order Hypocreales (Sung et al., 2008).

Also *Trichoderma* was found to be able to establish parasitic associations with the animal hosts. It was found as a colonizer of nematode eggs and second-stage juveniles, and it was also reported that it can sometimes penetrate the nematode egg masses (Sharon et al., 2001). Such host–fungus interactions are not abundantly documented and at the molecular level not well understood (Grigoriev et al., 2011). As with mycoparasitism, lytic enzymes, such as chitinases and proteases as well as toxins are important virulence factors in the infection process, but intensive research is necessary to better understand the mechanisms underlying the infection of nematodes by fungi (Druzhinina et al., 2011; Grigoriev et al., 2011).

Trichoderma spp. can cause invasive mycosis in immunocompromised mammals including humans. Such invasions are up to date known only from *H. orientalis,* clonal *T. longibrachiatum*, and possibly *T.* cf. *harzianum* (Guarro et al., 1999; Kredics et al., 2003; Druzhinina et al., 2008; Kantarcioğlu et al., 2009) though there are several species that can grow at 37 °C such as *T. reesei* or *T. citrinoviride*. It has been shown that the lung cell cultures infected with *T. longibrachiatum* stated to rapidly sediment and lose their adhesive properties compared to those infected with *T. reesei*, implying that proteases and/or secondary metabolites from the fungus are acting on these cells (Druzhinina et al., 2011). Clinical *Trichoderma* species were typically isolated from the peritoneal effluent of dialysis patients, infections of immunocompromised transplant recipients, and patients suffering from leukemia, brain abscesses and HIV (Druzhinina et al., 2008). It is still a question whether the ability of *T. longibrachiatum* to attack immunocompromised mammals is a newly gained property of the species since it is a derived lineage on the phylogenetic tree of the whole genus (Kubicek et al., 2011; Atanasova et al., 2013a) or it resembles the ancestral abilities of other hypocreales as shown by Sung et al. (2008).

Most of the famous *Trichoderma* species are environmental opportunists

The use of molecular tools for species identification has raised the number of *Trichoderma* species dramatically and revealed that some *Trichoderma* species are cosmopolitan, but several of them are geographically isolated or occupy very limited ecological niches. The biogeography *Trichoderma* may be deduced from several studies, but it is still impossible to draw conclusions about the whole genus on a world-wide scale, because many regions have not been screened and many more species are expected to be found. However, some diversity studies of *Trichoderma* in limited but widely separated geographical areas (Kullnig et al., 2000; Kubicek et al., 2003; Wuczkowski et al., 2003; Gherbawy et al., 2004; Zhang et al., 2005; Migheli et al., 2008; Zachow et al., 2008; Friedl and Druzhinina, 2012) showed that in

comparison to epigeal diversity of the genus relatively a few species of *Trichoderma* inhabit the temperate soil: *T. asperellum, T. asperelloides, T. pleuroticola, T. alni, T. hamatum, T. atroviride, T. strigosum, T. brevicompactum, T. virens, T. longibrachiatum, T. gamsii, T. citrinoviride, T. koningiopsis, T. spirale, T. koningii* complex. It is notable, that most of the species listed are cosmopolitan and are known from a broad range of habitats.

The core of *Harzianum* Clade is represented by the cosmopolitan *H. lixii –Trichoderma harzianum* sensu lato species complex with a high degree of phylogenetic variation (Druzhinina et al., 2010a). This species complex may be resolved in future and the distribution of each species will require a new evaluation. This Clade contains also species known as green mold disease agents on mushroom farms from all north temperate regions (*T. aggressivum*, *T. pleurotum* and *T. pleuroticola*; Samuels et al., 2002; Park et al., 2006, Komon-Zelazowska et al., 2007). *T. virens* from the sister Virens Clade, is a cosmopolitan, but predominately temperate species.

Except *T. longibrachiatum* and *T. citrinoviride* most of the species of the *Longibrachiatum* Clade have a narrow distribution range such as *T. aethiopicum* (East Africa), *H. andinensis* (high elevations in Venezuela), *T. effusum* (high elevations in India), *T. flagellatum* (Ethiopia), *T. gracile* (Malaysia), *T. konilangbra* (high elevations in East Africa), *T. pseudokoningii* (Australasia) and *T. solani* (Mexico) (Samuels et al., 2012; Druzhinina et al., 2012). Furthermore, *T. reesei*, used for the production of cellulase enzymes and its teleomorph *H. jecorina* as well as its sister species *T. parareesei* (Druzhinina et al., 2010b; Atanasova et al., 2010) are pantropical species.

Section *Trichoderma* comprises 39 species among them cosmopolitan *T. atroviride*, *T. gamsii*, *T. asperellum* and *T. asperelloides*, *T. hamatum*, Koningi species aggregate, rare *T. viride* and endophytic, neotropic *T. theobromicola and T. paucisporum* (see Atanasova et al., 2013a).

Pachybasium Clade was recently subdivided to several unrelated clades (Jaklitsch, 2009; Jaklitsch 2011) and the clade with remaining species around *T. polysporum* collected Australia, Europe, Japan, Korea, New Zealand, North America, is now called pachybasium core group (Jaklitsch, 2011). It contains *T. minutispora* frequently found in middle Europe and North America (eastern U.S.A. and Canada) (Jaklitsch, 2011) and *T. alutaceum* isolated in Europe (Austria, Germany, UK), Japan, and North America, *T. piluliferum* was so far collected only from northern Europe, *T. atlanticum* in middle Europe, rare *T. pachypallidum* in Austria, Czech Republic, Germany and Sweden.

Secondary metabolites of *Trichoderma* for its adaptation to ecological niches

Trichoderma spp. are producers of variety of secondary metabolites - small molecules which also comprise toxins, antibiotica and potential anti-cancer compounds and play important role in signaling, development and interaction with other organisms (Hoffmeister and Keller, 2007; Keller et al., 2005; Osbourn, 2010; Mukherjee et al., 2012a). The recent genome analysis of three *Trichoderma* species has revealed a large number of genes putatively involved in the biosynthesis of secondary metabolites, such as non-ribosomal peptides, polyketides, terpenoids and pyrones (Kubicek et al., 2011; Mukherjee et al., 2012a; 2012b).

The genes for secondary metabolite biosynthesis are often found in clusters (Keller and Hohn, 1997; Mukherjee et al., 2012a). Yet, in vitro cultivation conditions might not induce the expression of most of the secondary metabolites repertoire, thus the putative products often remain unknown (Mukherjee et al., 2012a). High mycoparasitic potential, rhizosphere competence as well as facultative or endophytic plant interactions (see chapters above) render *Trichoderma* species the most widely used biofungicides. Thus, many authors suggested that the involvement of secondary metabolites is crucial for the biocontrol potential of these mycotrophic fungi (Baker et al., 2012; Miao et al., 2012; Mukherjee et al., 2012a; 2012b). This can be supported by the fact that a great diversity of biosynthetic clusters was found in two highly opportunistic and mycotrophic species *T. atroviride* and *T. virens*, compared to a tropical wood decaying *T. reesei* (Kubicek et al., 2011). Most recently, Mukherjee et al. (2012a) proposed that many silent biosynthetic clusters might be activated by competition for resources between *Trichoderma* and its host fungi.

Similar to other ascomycetes, *Trichoderma* spp. produce **nonribosomal peptides**, for example the epipolythiodioxopiperazines (ETPs) and siderophores, as well as a subgroup of peptaibiotics known as peptaibols. The **peptaibols** are a group of small peptides having a high α-aminoisobutyric acid (Aib) content and an amino acid alcohol at the C-terminus, which are exclusively produced by members of the genus *Trichoderma* and closely aligned genera by means of special NRPS enzymes (Kubicek et al., 2007). The genomes of all three sequenced *Trichoderma* spp. contain two genes encoding such peptaibol synthases, one synthesizing short (10-14 aa) and one synthesizing long (18-25 aa) peptaibols. Like all other NRPS enzymes too, the peptaibol synthases consist of a series of modules that act like an assembly line, each incorporating one amino acid into the peptide (Strieker et al., 2010). The core of each module includes adenylation, peptidyl carrier and condensation domains. It is therefore interesting that the larger peptaibol synthase synthesizes only a single peptaibol of 18-25 aa's (Neuhof et al., 2007; Wiest et al., 2002). By gene disruption Mukherjee et al. (2011) and Degenkolb et al. (2012) showed that the smaller synthase synthesizes two small peptaibols (11 and 14 kDa). The obvious three-residue gap between 11- and 14-residue peptaibol families has to be attributed to module skipping (Degenkolb et al., 2012), which could be further kinetically regulated by the chemical structure of the intermediate peptides, thereby giving rise to the multitude of peptides produced by these enzymes in dependence of the available precursor concentrations.

Two other secondary metabolites, whose biosynthesis in *T. virens* is initiated by an NRPS, but which are not formed by *T. atroviride*, are the epipolythiodioxopiperazine-type peptides **gliotoxin and gliovirin** (Patron et al., 2007). The former is exclusively produced by so called "P-strains" of *T. virens*, whereas the latter is only produced by "Q-strains" (Mukherjee et al., 2012b). Gliotoxin has fungistatic action, and is also known from the opportunistic human pathogen *Aspergillus fumigatus* (Eurotiales, Ascomycota) where it has been discussed as a virulence factor (Dagenais and Keller, 2009). Its toxicity is due to the presence of a disulfide bridge in the molecule which can inactivate proteins via reaction with thiol groups and generate reactive oxygen species by redox cycling (Gardiner et al., 2005). Gliovirin has antimicrobial properties particularly against Oomycota (Mukherjee et al., 2012b).

Secondary metabolites may be essential for survival under particular environmental conditions; for example, **siderophores**, which are needed for fungal growth at low iron concentrations. They are mostly produced nonribosomally by large multifunctional peptide synthetases organized into repetitive synthase units, where each of them completes a different single amino acid elongation step (Wilhite et al., 2001). The orthologous NRPS gene clusters involved in siderophore synthesis (*SidD* and *NPS6*) have been identified in *T. atroviride*, *T. reesei*, and *T. virens* (Kubicek et al., 2011; Mukherjee et al., 2012a; 2012b; Lehner et al., 2012). Both orthologous genes, responsible for the synthesis of fusarinine/coprogene type siderophores, were found in latter two species, whereas *T. atroviride* contains only the *SidD* homolog. Furthermore, a putative ornithine-N5-monooxygenase orthologues to *A. nidulans* and *A. fumigatus* SidA, dffA from *A. oryzae*, and *sid1* from *F. graminearum* that catalyze the initial step in siderophore biosynthesis in all intra- and extracellular siderophores were identified in these three *Trichoderma* species (see Lehner et al., 2012).

For the first time screening approach applying liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) was used by Lehner et al. (2012) to screen for iron chelators/siderophores. The study showed a high diversity of secreted siderophores in *Trichoderma*: 18 different siderophores were detected in the culture f ltrates of eight *Trichoderma* species (see Lehner et al., 2012), from which only few has been described so far: ferricrocin, which plays an important role in intracellular iron storage (Eisendle et al., 2006) and is usually described as an intracellular siderophore, was found in *T. harzianum* s.s., *T. atroviride* and *T. virens*, but also to some level in *T. reesei, T. asperellum and T. hamatum*; dimerum acid, that has so far been reported only for *T. virens* (Wilhite et al., 2001), fusigen and coprogen were detected in all species cultures. In total, at least ten putative novel siderophores were found using LC-HRMS(-MS) screening approach and selected criteria for the elucidation of (putative novel) metabolites, relevant to the molecular mechanisms regarding their benef cial use. However, the biosynthesis of ferricrocin and hydroxyferricrocin involves acetylation of N5-hydroxyornithine to N5-acetyl-N5-hydroxyornithine by the *sidL* gene which is not clustered with other siderophore-biosynthetic genes, as well as not regulated by iron availability in *A. fumigatus* (Blatzer et al., 2011). Further, the NPS2 in some *Fusarium* spp. (see Tobiasen et al., 2007), SidC from *A. nidulans* (Eisendle et al., 2006) and Sid2 from *Ustilago maydis* (Yuan et al., 2001), encode NRPS gene responsible for

the production of siderophores ferricrocin and ferrichrome. Orthologues of both genes were found in all three *Trichoderma* species with sequenced genomes (Lehner et al., 2012).

Based on the biosynthesis in *A. fumigatus* (Blatzer et al., 2011) and the finding of the corresponding siderophores and orthologoues genes in *T. atroviride*, *T. virens* and *T. reesei*, Lehner et al. (2012) proposed that the *Trichoderma* spp. equivalently requires f ve genes corresponding to *sidI*, *sidH*, *sidF*, *sidD*, and *sidF* from *A. fumigatus* (Blatzer et al., 2011; for more details see Lehner et al., 2012) for the biosynthesis of fusarinine and coprogen type siderophores, which start with the hydroxylation of ornithine, catalyzed by the SidA monooxygenase. Lehner et al. (2012) proposed that low diversity of genes coding for siderophore synthetases in *Trichoderma* might indicate post-synthetic modif cations as a reason for high compound diversity.

Fungal secondary metabolite production is often linked to environmental factors, e.g. growth substrate might regulate the production of gliotoxin (Park et al., 1991; Mukherjee et al., 2012a) or to physiological traits such as conidiation (Mukherjee et al., 2012a). Regulator methyltransferase LaeA, the ‘velvet’ complex and the illumination co-regulate secondary metabolism and conidiation in fungi (Keller and Bok, 2006; Keller et al., 2007). In *A. nidulans*, VeA physically interacts with VelB and the regulator of secondary metabolism LaeA to form a complex that regulates secondary metabolism and sexual reproduction (Bayram et al., 2008). Deletion of the VeA gene leads to an increase in conidiation in the darkness and reduced biosynthesis of sterigmatocystin (the product of a PKS) and penicillin (the product of a NRPS), while it reduces and delays sexual reproduction (Kato et al., 2003; Kim 2009). In *T. virens*, the deletion of *vel1* impaired production of conidia on solid medium and chlamydospores in rich medium; as well it is shown that *vel1* is also involved in the regulation of the gliotoxin biosynthesis gene *gliP* and other secondary metabolism genes (Mukherjee and Kenerley, 2010).

Polyketides are important secondary metabolites as they possess antimicrobial, anti-cancer and immunosuppressive properties, as well they are involved in organism’s substrate competition and communication (Mukherjee et al., 2012a). 29 out of a total of 47 **PKS-encoding genes** in the three *Trichoderma* spp. fall into an orthologous group (Baker et al., 2012). These authors speculated that *Trichoderma* spp. may comprise only a limited catalogue of PKSs, but the diversity in their respective metabolites may be expanded at the level of ‘accessory enzymes’ that subsequently modify the polyketide products. Indeed, many of the aforementioned cytochrome P450 monooxygenases, FAD-dependent monooxygenases, short chain dehydrogenases/reductases or epimerases are located next to PKS encoding genes. Less is known about the role of these enzymes in *Trichoderma* ecophysiology.

Terpenoids are diverse class of compounds synthesized from five-carbon isopentenyl units. Among the three *Trichoderma* spp. with analyzed genomes *T. virens* genome has an expansion of terpene cyclases, the crucial enzyme for terpenoid synthesis. The fungistatic and anticancer steroidal compound viridin is widely produced by both ‘P’ and ‘Q’ strains of *T. virens* (Howell et al., 1993; Mukherjee et al., 2012a). In addition to terpenoids, several other **volatile metabolites** have been reported to be produced by *T. atroviride* (Stoppacher et al., 2010). *T. brevicompactum* produces trichodermin, a terpenoidtrichothecene type toxin that is highly fungitoxic and phytotoxic, and which is formed by the trichodiene synthase TRI5 (Tijerino et al., 2010). α–pentyl-pyrone, a volatile component with coconut aroma and antifungal activity, is one of the most well studied secondary metabolites from a biocontrol perspective (Cooney et al., 2001; Reithner et al., 2005, 2007). Its formation has been detected in *T. atroviride*, but not in *T. reesei* and *T. virens* (Reino et al., 2008). Its biosynthesis has been suggested to occur via linoleic acid, analogous to jasmonate synthesis in which the necessary hydroxyl group originates from an oxidation by lipoxygenase (Serrano-Carreon et al., 1993).

Furthermore, it is worth to also mention that *T. atroviride* and *T. virens* contain two set of genes encoding **high molecular weight toxins**, which bear high similarity to the Tc-(toxin complex) toxins - approximately 1 MDa protein complexes which are toxic to insect pests - of *Photorhabdus luminescens* (Enterobacteriaceae, Proteobacteria), a bacterium which is mutualistic with entomophagous nematodes and which secretes this toxin into the insect hemocoel upon nematode invasion (Goodrich-Blaire and Clark 2007). In *Yersinia pseudotuberculosis* (Enterobacteriales, Proteobacteria), these toxins were shown to have evolved as virulence factors to mammals (Hares et al., 2008). *T.*

virens contains four paralogs of this gene. The presence of these two potential insecticidal toxin genes in mycoparasitic *Trichoderma* spp. renders it also possible that the two species may be active also against insects.

References[10]

Aro, N., A. Saloheimo, M. Ilmen and M. Penttilä. 2001. ACEII, a novel transcription activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. *J. Biol. Chem*. 276:24309–24314.

Aro, N., A. Saloheimo and M. Penttilä. 2003. ACEI of *Trichoderma reesei* is a repressor of cellulase and xylanase expression. *Appl. Environ. Microbiol*. 69:56–65.

Atanasova, L., W.M. Jaklitsch, M. Komoń-Zelazowska, C.P. Kubicek and I.S. Druzhinina. 2010. Clonal species *Trichoderma parareesei* sp. nov. likely resembles the ancestor of the cellulase producer *Hypocrea jecorina*/*T. reesei*. *Appl. Environ. Microbiol.* 76:7259–7267.

Atanasova, L., I.S. Druzhinina and W.M. Jaklitsch. 2013a. Two hundred *Trichoderma* species recognized based on molecular phylogeny. *In Trichoderma: Biology and Applications*, Eds. by P.K. Mukherjee, Sigh U.S., Horwitz B.A., Schmoll M., Mukherjee M. UK: CABI of Nosworthy Way. Wallingford, Oxon, in press.

Atanasova, L., S. Le Crom, S. Gruber, F. Coulpier, V. Seidl-Seiboth, C.P. Kubicek, I.S. Druzhinina. 2013b. Comparative transcriptomics reveals different strategies of *Trichoderma* mycoparasitism. *BMC Genomics.* 14:121.

Bae, H., R.C. Sicher, M.S. Kim, S.H. Kim, M.D. Strem, R.L. Melnick and B.A. Bailey. 2009. The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*. *J. Exp. Bot*. 60(11):3279-3295.

Bae, H., D.P. Roberts, H.S. Lim, M.D. Strem, S.C. Park, C.M. Ryu, R.L. Melnick and B.A. Bailey. 2011. Endophytic *Trichoderma* isolates from tropical environments delay disease onset and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms. *Mol. Plant. Microbe Interact*. 24(3):336-351.

Bailey, B.A., H. Bae, M.D. Strem, D.P. Roberts, S.E. Thomas, J. Crozier, G.J. Samuels, I.Y. Choi and K.A. Holmes. 2006. Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four Trichoderma species. *Planta*. 224(6):1449-1464.

Bailey, B.A., M.D. Strem and D. Wood. 2009. *Trichoderma* species form endophytic associations within *Theobroma cacao* trichomes. *Mycol. Res*. 113(Pt 12):1365-1376.

Baker, S.E., G. Perrone, N.M. Richardson, A. Gallo and C.P. Kubicek. 2012. Phylogenomic analysis of polyketide synthase-encoding genes in *Trichoderma*. *Microbiology*. 158(Pt 1):147-154.

Bayram, O., S. Krappmann, M. Ni, J.W. Bok, K. Helmstaedt, O. Valerius, S. Braus-Stromeyer, N.J. Kwon, N.P. Keller, J.H. Yu and G.H. Braus. 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science*. 320:1504-1506.

Benítez, T., A.M. Rincón, M.C. Limón and A.C. Codón. 2004. Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol*. 7:249–260.

Bianciotto, V., E. Lumini, P. Bonfante and P. Vandamme. 2003. '*Candidatus Glomeribacter* gigasporarum' gen. nov., sp. nov., an endosymbiont of arbuscular mycorrhizal fungi. *Int. J. Syst. Evo.l Microbiol*. 53:121-124.

Blatzer, M., M. Schrettl, B. Sarg, H.H. Lindner, K. Pfaller and H. Haas. 2011. SidL, an *Aspergillus fumigatus* transacetylase involved in biosynthesis of the siderophores ferricrocin and hydroxyferricrocin. *Appl. Environ. Microbiol*. 77:4959–4966.

Casas-Flores, S.E. and A. Herrera-Estrella. 2007. Antagonism of Plant Parasitic Nematodes by Fungi. *In The Mycota, Environmental and Microbial Relationships* (Vol. 4) 2nd ed. Eds. By C.P. Kubicek and Druzhinina I.S. Germany, USA: Springer. Berlin, Heidelberg, New York, pp. 159–187.

[10] The references in this chapter are formatted according to the requirements of Elsevier BV (Amsterdam, Netherlands) where the review was submitted.

Castle, A., D. Speranzini, N. Rghei, G. Alm, D. Rinker and J. Bissett. 1998. Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. *Appl. Environ. Microbiol*. 64(1):133-7.

Catalano, V., M. Vergara, J.R. Hauzenberger, B. Seiboth, S. Sarrocco, G. Vannacci, C.P. Kubicek and V. Seidl-Seiboth. 2011. Use of a non-homologous end-joining-deficient strain (delta-ku70) of the biocontrol fungus *Trichoderma virens* to investigate the function of the laccase gene lcc1 in sclerotia degradation. *Curr. Genet.* 57(1):13-23.

Compant, S., J. Nowak, T. Coenye, C. Clément and E. Ait Barka. 2008. Diversity and occurrence of *Burkholderia* spp. in the natural environment. *FEMS Microbiol. Rev.* 32(4): 607-26.

Coenye, T., S. Laevens, A. Willems, M. Ohlen, W. Hannant, J.R.W. Govan, M. Gillis et al. 2001. *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples. *Int. J. Syst. Evol. Microbiol.* 51: 1099-1107.

Cooney, J.M., D.R. Lauren and M.E. di Menna. 2001. Impact of competitive fungi on Trichothecene production by *Fusarium graminearum*. *J. Agric. Food. Chem*. 49(1):522-526.

Cruz, L.M., E.M. de Souza, O.B. Weber, J.I. Baldani, J. Döbereiner and F. de Olivera Pedrosa. 2001. 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa* spp.) and pineapple (*Ananas comosus* L. Merril). *Appl. Environ. Microbiol.* 67:2375-2379.

Dagenais, T.R. and N.P. Keller. 2009. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. *Clin Microbiol Rev.* 22:447-465.

Davidson, D.W., N.F. Anderson, S.C. Cook, C.R. Bernau, T.H. Jones, A.S. Kamariah, L.B. Lim, C.M. Chan and D.A. Clark. 2009. An experimental study of microbial nest associates of Borneo’s exploding ants (*Camponotus* [*Colobopsis*] species). J. Hym. Res. 18:341-360.

Degenkolb, T., R. Karimi Aghcheh, R. Dieckmann, T. Neuhof, S.E. Baker, I.S. Druzhinina, C.P. Kubicek, H. Brückner and H. von Döhren. 2012. The production of multiple small peptaibol families by single 14-module Peptide synthetases in *Trichoderma*/*Hypocrea*. *Chem. Biodivers*. 9(3):499-535.

Druzhinina, I.S., M. Komoń-Zelazowska, L. Kredics, L. Hatvani, Z. Antal, T. Belayneh and C.P. Kubicek. 2008. Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. *Microbiology.* 154*:*3447–3459.

Druzhinina, I.S., C.P. Kubicek, M. Komoń-Zelazowska, T.B. Mulaw and J. Bissett. 2010a. The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. *BMC Evol. Biol.* 10:94.

Druzhinina, I.S., M. Komoń-Zelazowska, L. Atanasova, V. Seidl and C.P. Kubicek. 2010b. Evolution and ecophysiology of the industrial producer *Hypocrea jecorina* (Anamorph *Trichoderma reesei*) and a new sympatric agamospecies related to it. *PLoS One.* 5*:*e9191.

Druzhinina, I.S., V. Seidl-Seiboth, A. Herrera-Estrella, B.A. Horwitz, C.M. Kenerley, E. Monte, P.K. Mukherjee, S. Zeilinger, I.V. Grigoriev and C.P. Kubicek. 2011. *Trichoderma*: the genomics of opportunistic success. *Nat. Rev. Microbiol*. 9:749–759.

Druzhinina, I.S, M. Komoń-Zelazowska, A. Ismaiel, W. Jaklitsch, T. Mullaw, G.J. Samuels and C.P. Kubicek. 2012. Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma*. *Fungal. Genet. Biol*. 49(5):358-368.

Druzhinina, I.S. and C.P. Kubicek. 2013. Ecological genomics of *Trichoderma*. *In Ecological genomics of fungi*, Ed. by F. Martin. UK: Wiley-Blackwell. Oxford, in press.

Duffy, B.K., A. Simon and D.M. Weller. 1996. Combination of *Trichoderma koningii* with Fluorescent Pseudomonads of Control of Take-all on Wheat. *Phytophatology*. 86(2):188-194.

Duponnois, R., J. Garbaye, D. Bouchard and J.L. Churin. 1993.The fungus-specificity of mycorrhization helper bacteria (MHBs) used as an alternative to soil fumigation for ectomycorrhizal inoculation of bare-root Douglas-fir planting stocks with *Laccaria laccata*.*Plant and Soil*. 157(2):257-262.

Eisendle, M., M. Schrettl, C. Kragl, D. Müller, P. Illmer and H. Haas. 2006. The intracellular siderophore ferricrocin is involved in iron storage, oxidative stress resistance, germination, and sexual development in *Aspergillus nidulans*. *Eukaryot. Cell*. 5:1596–1603.

Eriksson, K.-E.L., R.A. Blanchette and P. Ander. 1990. *Microbial and Enzymatic Degradation of Wood and Wood Components*. Germany: Springer. Berlin.

Foreman, P.K., D. Brown, L. Dankmeyer, R. Dean, S. Diener, N.S. Dunn-Coleman et al. 2003. Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma* reesei. *J. Biol. Chem*. 278: 31988–31997.

Friedl, M.A. and I.S. Druzhinina. 2012. Taxon-specific metagenomics of *Trichoderma* reveals a narrow community of opportunistic species that regulate each other's development. *Microbiology.* 158:69–83.

Furukawa, T, Y. Shida, N. Kitagami, K. Mori, M. Kato, T. Kobayashi, H. Okada, W. Ogasawara and Y. Morikawa. 2009. Identification of specific binding sites for XYR1, a transcriptional activator of cellulolytic and xylanolytic genes in *Trichoderma reesei*. *Fungal. Genet. Biol.* 46(8):564-74.

Gal-Hemed, I., L. Atanasova, M. Komon-Zelazowska, I.S. Druzhinina, A. Viterbo, and O. Yarden. 2011. Marine isolates of *Trichoderma* spp. as potential halotolerant agents of biological control for arid-zone agriculture. *Appl Environ Microbiol.* 77:5100–5109.

Gardiner, D.M., P. Waring, and B.J. Howlett. 2005. The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis. *Microbiology*. 151:1021-1032.

Gherbawy, Y., I.S. Druzhinina, G.M. Shaban, M. Wuczkowsky, M. Yaser, M.A. El-Naghy, H.-J. Prillinger and Kubicek, C.P. 2004. *Trichoderma* populations from alkaline agricultural soil in the Nile valley, Egypt, consist of only two species. *Mycol. Prog.* 3*:*211–218.

Gómez, M., P. Isorna, M. Rojo and P. Estrada. 2001. Chemical mechanism of beta-xylosidase from *Trichoderma reesei* QM 9414: pH-dependence of kinetic parameters. *Biochimie*. 83(10):961-967.

Goodrich-Blair, H. and D.J. Clarke. 2007. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. *Mol. Microbiol*. 64(2):260-8.

Grigoriev, I.V, D. Cullen, S.B. Goodwin, D. Hibbett, T.W. Jeffries, C.P. Kubicek, C. Kuske, J.K. Magnuson, F. Martin, J.W. Spatafora, A. Tsang and S.E. Baker. 2011. Fueling the future with fungal genomics. *Mycology*. DOI:10.1080/21501203.2011.584577.

Grill, A., P. Casula, R. Lecis, S.B.J. Menken. 2006. *Endemism in Sardinia*. *In*: *Phylogeography of Southern European Refugia*. Eds. By S. Weiss, N. Ferrand. The Netherlands: Springer. Dordrecht, p. 273-296.

Guarro J., M.I. Antolín-Ayala, J. Gené, J. Gutiérrez-Calzada, C. Nieves-Díez, M. Ortoneda. 1999. Fatal case of *Trichoderma harzianum* infection in a renal transplant recipient. *J. Clin. Microbiol*. 37(11):3751-3755.

Munch, and M. Schloter. 2007. A new cultivation independent approach to detect and monitor common *Trichoderma* species in soils. *J. Microbiol. Methods.* 69:86–92.

Hanada, R.E., T. de Jorge Souza, A.W. Pomella, K.P. Hebbar, J.O. Pereira, A. Ismaiel and G.J. Samuels. 2008. *Trichoderma martiale* sp. nov., a new endophyte from sapwood of *Theobroma* cacao with a potential for biological control. *Mycol. Res.* 112:1335–1343.

Hares M.C., S.J. Hinchliffe, P.C. Strong, I. Eleftherianos, A.J. Dowling, R.H. ffrench-Constant and N. Waterfield. 2008. The *Yersinia pseudotuberculosis* and *Yersinia pestis* toxin complex is active against cultured mammalian cells. *Microbiology*. 154(Pt 11):3503-3517.

Harman, G.E., C.R. Howell, A. Viterbo, I. Chet, and M. Lorito. 2004. Trichoderma species - opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol*. 2:43–56.

Harman, G.E. 2011. Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. *New Phytol*. 189:647–649.

Hatvani, L., Z. Antal, L. Manczinger, A. Szekeres, I.S. Druzhinina, C.P. Kubicek, A. Nagy, E. Nagy, C. Vágvölgyi, and L. Kredics. 2007. Green Mold Diseases of *Agaricus* and *Pleurotus* spp. Are Caused by Related but Phylogenetically Different *Trichoderma* Species. *Phytopathology.* 97*:*532–537.

Hoffmeister, D. and N.P. Keller. 2007. Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat. Prod. Rep.* 24:393–416.

Holden, M.T., R.W.Titball, S.J Peacock, A.M. Cerdeño-Tárraga, T. Atkins, L.C. Crossman, T. Pitt et al. 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *PNAS.* 101:14240-14245.

Holmes, K.A., H.-J. Schroers, S.E. Thomas, H.C. Evans and G.J. Samuels. 2004. Taxonomy and biocontrol potential of a new species of *Trichoderma* from the Amazon basin in South America. *Mycol. Prog.* 3:199–210.

Howell, C.R., R. Stipanovic and R. Lumsden. 1993. Antibiotic production by strains of *Gliocladium* virens and its relation to biocontrol of cotton seedling diseases. *Biocontrol. Sci. Technol.* 3:435–441.

Howell, C.R. 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis*. 87:4–10.

Hoyos-Carvajal, L., S. Orduz and J. Bissett. 2009. Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions. *Fungal Genet Biol.* 46:615–631.

Jaklitsch, W.M., G.J. Samuels, S.L. Dodd, B.S. Lu and I.S. Druzhinina. 2006. *Hypocrea rufa*/*Trichoderma viride*: a reassessment, and description of five closely related species with and without warted conidia. *Stud. Mycol*. 56:135–177.

Jaklitsch, W.M. 2009. European species of *Hypocrea* Part I. The green-spored species. *Stud. Mycol*. 63:1–91.

Jaklitsch, W.M. 2011. European species of *Hypocrea* Part II: species with hyaline ascospores. *Fungal. Divers*. 48:1–250.

Kantarcioğlu A.S., T. Celkan, A. Yücel, Y. Mikami, S. Kurugoglu, H. Mitani and K. Altas. 2009. Fatal *Trichoderma harzianum* infection in a leukemic pediatric patient. Med. Mycol. 47(2):207-15.

Kato, N., W. Brooks and A. M. Calvo. 2003. The expression of sterigmatocystin and penicillin genes in *Aspergillus nidulans* is controlled by veA, a gene required for sexual development. *Eukaryot. Cell*. 2:1178-1186.

Keller, N.P. and T.M. Hohn. 1997. Metabolic pathway gene clusters in filamentous fungi. *Fungal. Genet. Biol.* 21: 17–29.

Keller, N.P., G. Turner and J.W. Bennett. 2005. Fungal secondary metabolism – from biochemistry to genomics. *Nat. Rev. Microbiol*. 3:937–947.

Keller, N.P., and J.W. Bok. 2006. Global regulator of secondary metabolite biosynthesis and methods of use. *United States Patent No.* US7,053,204B2.

Keller, N.P., E. Shwab and J.W. Bok. 2007. Method of increasing secondary metabolite production in fungi by chromatin regulation. *United States Patent Application* No.:P06331US.

Kim, H. Y., K.H. Han, M. Lee, M. Oh, H.S. Kim, X. Zhixiong, D.M. Han, K.Y. Jahng, J.H. Kim, and K.S. Chae. 2009. The veA gene is necessary for the negative regulation of the veA expression in *Aspergillus nidulans*. *Curr. Genet*. 55:391-397.

Kim, C.S., T. Shirouzu, A. Nakagiri, K. Sotome, E. Nagasawa and N. Maekawa. 2012. *Trichoderma mienum* sp. nov., isolated from mushroom farms in Japan*. Antonie Van Leeuwenhoek.* 102:629–641.

Komon-Zelazowska, M., J. Bissett, D. Zafari, L. Hatvani, L. Manczinger, S. Woo, M. Lorito, L. Kredics, C.P. Kubicek and I.S. Druzhinina. 2007. Genetically closely related but phenotypically divergent *Trichoderma* species cause green mold disease in oyster mushroom farms worldwide. *Appl. Environ. Microbiol.* 73:7415–7426.

Kredics, L., Z. Antal, I. Dóczi, L. Manczinger, F. Kevei and E. Nagy. 2003. Clinical importance of the genus *Trichoderma*. A review. *Acta. Microbiol. Immunol. Hung*. 50(2-3):105-17.

Kubicek, C.P., U.M. Bölzlbauer, W. Kovacs, R.L. Mach, K. Kuhls, E. Lieckfeldt, T. Börner and G.J. Samuels. 1996. Cellulase formation by species of *Trichoderma* sect. *Longibrachiatum* and of *Hypocrea* spp. with anamorphs referable to *Trichoderma* sect. *Longibrachiatum*. *Fungal Genet Biol.* 20(2):105-14.

Kubicek, C.P., J. Bissett, I. Druzhinina, C. Kullnig-Gradinger and G. Szakacs. 2003. Genetic and metabolic diversity of *Trichoderma*: a case study on South-East Asian isolates. *Fungal. Genet. Biol.* 38:310–319.

Kubicek, C.P., M. Zelazowska-Komon, E. Sandor and I.S. Druzhinina. 2007. Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma*. *Chem. Biodivers.* 4:1068-1082.

Kubicek C.P., M. Mikus, A. Schuster, M. Schmoll and B. Seiboth. 2009. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. *Biotechnol. Biofuels.* 2:19.

Kubicek, C.P., A. Herrera-Estrella, V. Seidl-Seiboth, D.A. Martinez, I.S. Druzhinina, M. Thon, S. Zeilinger, S. Casas-Flores, et al. 2011. Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. *Genome Biol.* 12:R40.

Kubicek, C.P. 2013: Fungi and lignocellulosic biomass. UK: Wiley-Blackwell. Oxford, pp. 29-41.

Kullnig, C.M., G. Szakacs and C.P. Kubicek. 2000. Molecular identification of *Trichoderma* species from Russia, Siberia and the Himalaya. *Mycological Research.* 104:1117–1125.

Lehner, S.M., L. Atanasova, N.K. Neumann, R. Krska, M. Lemmens, I.S. Druzhinina and R. Schuhmacher. 2012. Isotope-assisted screening for iron-containing metabolites reveals high diversity of known and unknown siderophores produced by *Trichoderma* spp. *Appl. Environ. Microbiol*. 79(1):18-31

Li, X.L., S. Spanikova, R.P. de Vries and P. Biely. 2007. Identification of genes encoding microbial glucuronoyl esterases. *FEBS. Lett.* 581:4029–4035.

Lim, Y.W., K.S. Baik, S.K. Han, S.B. Kim and K.S. Bae. 2003. *Burkholderia sordidicola* sp. nov., isolated from the white-rot fungus *Phanerochaete sordida*. *Int. J. Syst. Evol. Microbiol.* 53:1631-1636.

Lorito, M, A. Di Pietro, Hayes, C.K., Woo, S.L. and G.E. Harman. 1993. Antifungal, synergistic interaction between chitinolytic enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*. *Phytopathology*. 83(7):721-728.

Lorito, M., S.L. Woo, G.E. Harman and E. Monte. 2010. Translational research on *Trichoderma*: from 'omics to the field. *Annu. Rev. Phytopathol*. 48:395-417.

Mach-Aigner, A.R., M.E. Pucher, M.G. Steiger, G.E. Bauer, S.J. Preis and R.L. Mach. 2008. Transcriptional regulation of xyr1, encoding the main regulator of the xylanolytic and cellulolytic enzyme system in *Hypocrea jecorina*. *Appl. Environ. Microbiol.* 74:6554–6562.

Martin, F., A. Aerts, D. Ahrén, A. Brun,E.G. Danchin,F. Duchaussoy, J. Gibon J, A. Kohler, E. Lindquist, V. Pereda et al. 2008. The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. *Nature*. 452(7183):88-92.

Meincke, R., N. Weinert, V. Radl, M. Schloter, K. Smalla and G. Berg. 2010. Development of a molecular approach to describe the composition of *Trichoderma* communities. *J. Microbiol. Methods.* 80:63–69.

Médail, F. and P. Quézal. 1999. Biodiversity hotspots in the Mediterranean Basin: Setting global conservation priorities. *Conserv. Biol.* 13(6):1510-1513.

Metz, B., V. Seidl-Seiboth, T. Haarmann, A. Kopchinskiy, P. Lorenz, B. Seiboth and C.P. Kubicek. 2011. Expression of biomass-degrading enzymes is a major event during conidium development in *Trichoderma reesei*. *Eukaryot. Cell*. 10(11):1527-35.

Miao, F.P., X.R. Liang, X.L. Yin, G. Wang and N.Y. Ji. 2012. Absolute configurations of unique harziane diterpenes from *Trichoderma* species. *Org. Lett*. 14(15):3815-3817.

Migheli, Q., V. Balmas, M. Komoñ-Zelazowska, B. Scherm, S. Fiori, A.G. Kopchinskiy, C.P. Kubicek and I.S. Druzhinina. 2009. Soils of a Mediterranean hot spot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European, invasive species of *Hypocrea*/*Trichoderma*. *Environ. Microbiol.* 11:35–46.

Mulaw, T.B., C.P. Kubicek and I.S. Druzhinina. 2010. The Rhizosphere of *Coffea Arabica* in Its Native Highland Forests of Ethiopia Provides a Niche for a Distinguished Diversity of *Trichoderma*. *Diversity.* 2:527–549.

Mukherjee, P.K. and C.M. Kenerley. 2010. Regulation of morphogenesis and biocontrol properties in *Trichoderma virens* by a VELVET protein, Vel1. *Appl. Environ. Microbiol*. 76(7):2345-52.

Mukherjee, P.K., A. Wiest, N. Ruiz, A. Keightley, M.E. Moran-Diez, K. McCluskey, Y.F. Pouchus and C.M. Kenerley. 2011. Two classes of new peptaibols are synthesized by a single non-ribosomal peptide synthetase of *Trichoderma virens*. *J. Biol. Chem.* 286(6):4544-4554.

Mukherjee, P.K., N. Buensanteai, M.E. Moran-Diez, I.S. Druzhinina and C.M. Kenerley. 2012a. Functional analysis of non-ribosomal peptide synthetases (NRPSs) in *Trichoderma virens* reveals a polyketide synthase (PKS)/NRPS hybrid enzyme involved in the induced systemic resistance response in maize. *Microbiology*. 158(Pt 1):155-65.

Mukherjee, P.K., B.A. Horwitz and C.M. Kenerley. 2012b. Secondary metabolism in *Trichoderma*--a genomic perspective. *Microbiology*. 158(Pt 1):35-45.

Muthumeenakshi, S., P.R. Mills, A.E. Brown and D.A. Seaby. 1994. Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in British Isles. *Microbiology.* 140:769–777.

Neuhof, T., R. Dieckmann, I.S. Druzhinina, C.P. Kubicek and H. von Döhren. 2007. Intact-Cell MALDI-TOF mass spectrometry analysis of peptaibol formation by the genus *Trichoderma*: Can molecular phylogenic knowledge predict peptaibol structures? *Microbiology*. 153:3417-3437.

Omann, M.R., S. Lehner, C. Escobar Rodríguez, K. Brunner and S. Zeilinger. 2012. The seventransmembrane receptor Gpr1 governs processes relevant for the antagonistic interaction of Trichoderma atroviride with its host. *Microbiology*. 158(Pt1):107-118.

Osbourn, A. 2010. Secondary metabolic gene clusters: evolutionary toolkits for chemical innovation. *Trends. Genet.* 26:449–457.

Park, M.S., K.S. Bae and S.H. Yu. 2004. Molecular and morphological analysis of *Trichoderma* isolates associated with green mold epidemic of oyster mushroom in Korea. H*ua Zhong Nong Ye Da Xue Xue Bao.* 23:157–164.

Park, S.P., Bae, K.S. and Yu S.H. 2006. Two New Species of *Trichoderma* Associated with Green Mold of Oyster Mushroom Cultivation in Korea. *The Korean Society of Mycology*. 34(3):111-113.

Partida-Martinez, L.P. and C. Hertweck. 2005. Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature*. 437:884-888.

Patron, N.J., R.F. Waller, A.J. Cozijnsen, D.C. Straney, D.M. Gardiner, W.C. Nierman and B.J. Howlett. 2007. Origin and distribution of epipolythiodioxopiperazine (ETP) gene clusters in filamentous ascomycetes. *BMC. Evol. Biol.* 7:174.

Paz, Z., M. Komon-Zelazowska, I.S. Druzhinina, M.M. Aveskamp, A. Shnaiderman, Y. Aluma, S. Carmeli, M. Ilan and O. Yarden. 2010. Diversity and potential antifungal properties of fungi associated with a Mediterranean sponge. *Fungal Diversity.* 42:17–26.

Portnoy, T., A. Margeo, R. Linke, L. Atanasova, E. Fekete, E. Sándor, L. Hartl, L. Karaffa, I.S. Druzhinina, B. Seiboth, S. Le Crom and C.P. Kubicek. 2011. The CRE1 carbon catabolite repressor of the fungus *Trichoderma reesei*: a master regulator of carbon assimilation. *BMC Genomics.* 12:269.

Rauscher, R., E. Würleitner, C. Wacenovsky, N. Aro, A.R. Stricker, S. Zeilinger, C.P. Kubicek, M. Penttil and R.L. Mach. 2006. Transcriptional regulation of *xyn1*, encoding xylanase I, in *Hypocrea jecorina*. *Eukaryot. Cell*. 5(3):447-456.

Reino, J.L., R.F. Guerrero, R. Hernandez-Galan and I.G. Collado. 2008. Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochem. Rev.* 7:89–123.

Reithner, B., K. Brunner, R. Schuhmacher, I. Peissl, V. Seidl, R. Krska and S. Zeilinger. 2005. The G protein alpha subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites. *Fungal Genet Biol.* 42:749-760.

Reithner, B., R. Schuhmacher, N. Stoppacher, M., Pucher, K. Brunner and S. Zeilinger. 2007. Signaling via the *Trichoderma atroviride* mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection. *Fungal. Genet. Biol.* 44:1123–1133.

Reithner, B., E. Ibarra-Laclette, R.L. Mach and A. Herrera-Estrella. 2011. Identification of mycoparasitism-related genes in Trichoderma atroviride. *Appl. Environ. Microbiol.* 77(13):4361-4370.

Rossmann, A.Y., G.J. Samuels, C.T. Rogerson and R. Lowen. 1999. Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). *Stud. Mycol*. 42:1-83.

Saloheimo, A., N. Aro, M. Ilmen and M. Penttilä. 2000. Isolation of the *ace1* gene encoding a Cys(2)-His(2) transcription factor involved in regulation of activity of the cellulase promoter *cbh1* of *Trichoderma reesei*. *J. Biol. Chem.* 275:5817–5825.

Saloheimo, M., J. Kuja-Panula, E. Ylösmäki, M. Ward and M. Penttilä. 2002. Enzymatic properties and intracellular localization of the novel *Trichoderma reesei* betaglucosidase BGLII (cel1A). *Appl. Environ. Microbiol.* 68:4546–4553.

Samuels, G.J., S.L. Dodd, W. Gams, L.A. Castlebury and O. Petrini. 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. *Mycologia.* 94:146–170.

Samuels, G.J., S. Dodd, B.-S. Lu, O. Petrini, H.-J. Schroers and I.S. Druzhinina. 2006a. The *Trichoderma koningii* aggregate species. *Stud. Mycol.* 56:67–133.

Samuels, G.J., C. Suarez, K. Solis, K.A. Holmes, S.E. Thomas, A. Ismaiel and H.C. Evans. 2006b. *Trichoderma theobromicola* and *T. paucisporum*: two new species isolated from cacao in South America. *Mycological Research.* 110:381–392.

Samuels, G.J., A. Ismaiel, M.C. Bon, S. De Respinis and O. Petrini. 2010. *Trichoderma asperellum* sensu lato consists of two cryptic species. *Mycologia.* 102:944–966.

Samuels, G.J., A. Ismaiel, T.B. Mulaw, G. Szakacs, I.S. Druzhinina, C.P. Kubicek and W.M. Jaklitsch. 2012. The *Longibrachiatum* Clade of *Trichoderma*: a revision with new species. *Fungal Diversity.* 55:77–108.

Segarra, G., Casanova E., D. Bellido, M.A. Odena, E. Oliveira and Trillas I. 2007. Proteome, salicylic acid, and jasmonic acid changes in cucumber plants inoculated with *Trichoderma asperellum* strain T34. *Proteomics* 7:3943–3952.

Shemakhanova, N.M. 1962. Mycotrophy of Woody Plants. *Acad. Sci. USSR Inst. Microbiol.* TT 66-51073, 329 pp.

Seiboth, B., C. Gamauf, M. Pail, L. Hartland C.P. Kubicek. 2007. The D-xylose reductase of *Hypocrea jecorina* is the major aldose reductase in pentose and D-galactose catabolism and necessary for β-galactosidase and cellulase induction by lactose. *Mol. Microbiol.* 66(4):890-900.

Seiboth, B., R.A. Karimi, P.A. Phatale, R. Linke, L. Hartl, D.G Sauer, K.M. Smith, S.E Baker, M. Freitag, C.P Kubicek. 2012. The putative protein methyltransferase LAE1 controls cellulase gene expression in *Trichoderma reesei*. *Mol. Microbiol.* 84(6):1150–1164.

Seidl, V., L.F. Song, E.A. Lindquist, S. Gruber, A. Koptchinskiy, S. Zeilinger, M. Schmoll, P. Martinez, J. Sun, I. Grigoriev, A. Herrera-Estrella, S.E. Baker and C.P. Kubicek. 2009. Transcriptomic response of the mycoparasitic fungus *Trichoderma atroviride* to the close presence of a fungal prey. *BMC Genomics.* 10:567.

Serrano-Carreon, L., Y. Hathout, M. Bensoussan and J.M. Belin. 1993. Metabolism of linoleic acid or mevalonate and 6-pentyl-α-pyrone biosynthesis by *Trichoderma* species. *Appl. Environ. Microbiol.* 59:2945–2950.

Sharon, E., M. Bar-Eyal, I. Chet, A. Herrera-Estrella, O. Kleifeld and Y. Spiegel. 2001. Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. *Phytopathology*. 91:687–693.

Shibu, M.A., H.S. Lin, H.H. Yang and K.C. Peng. 2012. *Trichoderma harzianum* ETS 323-mediated resistance in Brassica oleracea var. capitata to *Rhizoctonia solani* involves the novel expression of a glutathione S-transferase and a deoxycytidine deaminase. *J. Agric. Food. Chem.* 60(43):10723-10732.

Sinden, J. and E. Hauser. 1953. Nature and control of three mildew diseases of mushrooms in America. *Mushroom Sci.* 2:177–180.

Slot, J.C. and D.S. Hibbett. 2007. Horizontal transfer of a nitrate assimilation gene cluster and ecological transitions in fungi: a phylogenetic study. *PLoS One*. 2:e1097.

Stoppacher, N., B. Kluger, S. Zeilinger, R. Krska and R. Schuhmacher. 2010. Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma atroviride* by HS-SPME-GC-MS. *J. Microbiol. Methods.* 81:187–193.

Strauss, J., R.L. Mach, S. Zeilinger, G. Stöffler, M. Wolshek, G. Hartler and C.P. Kubicek. 1995. Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. *FEBS. Lett.* 376:103–107.

Stricker, A.R., K. Grosstessner-Hain, E. Würleitner and R.L. Mach. 2006. Xyr1 (xylanase regulator 1) regulates both the hydrolytic enzyme system and the D-xylose metabolism in *Hypocrea jecorina*. *Eukaryot. Cell.* 5:2128–2137.

Stricker, A.R., M.G. Steiger, R.L. Mach. 2007. Xyr1 receives the lactose induction signal and regulates lactose metabolism in *Hypocrea jecorina*. *FEBS. Lett.* 581:3915–3920.

Strieker, M., A. Tanović and M.A. Marahiel. 2010. Nonribosomal peptide synthetases: structures and dynamics. *Curr. Opin. Struct. Biol.* 20:234–240.

Summerbell, R.C. 1987. The inhibitory effect of *Trichoderma* species and other soild microfungi on formation of mycorrhiza by *Laccaria bicolor* in vitro. *New Phytol*. 105:437-448.

Sung, G.H., G.O.Jr. Poinar and J.W. Spatafora. 2008. The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbioses. *Mol. Phylogenet. Evol.* 49(2):495-502.

Tijerino, A., R.E. Cardoza, J. Moraga, M.G. Malmierca, F. Vicente, J. Aleu, I.G. Collado, S. Gutierrez, E. Monte and R. Hermosa. 2011. Overexpression of the trichodiene synthase gene tri5 increases trichodermin production and antimicrobial activity in *Trichoderma brevicompactum*. *Fungal. Genet. Biol.* 48:285–296.

Tobiasen, C., J. Aahman, K.S. Ravnholt, M.J. Bjerrum, M.N. Grell and H. Giese. 2007. Nonribosomal peptide synthetase (NPS) genes in *Fusarium graminearum*, *F. culmorum* and *F. pseudograminearum* and identif cation of NPS2 as the producer offerricrocin. *Curr.Genet*. 51:43–58.

Vargas, W. A., Mandawe, J. C. and C. M. Kenerley. 2009. Plant-derived sucrose is a key element in the symbiotic association between *Trichoderma virens* and maize plants*. Plant Physiol*. 151:792–808.

Vega, F.E., M. Pava-Ripoll, F. Posada and J.S. Buyer. 2005. Endophytic bacteria in *Coffea arabica* L. *J. Basic Microbiol.* 45:371-380.

Wiest, A., D. Grzegorski, B.W. Xu, C. Goulard, S. Rebuffat, D.J. Ebbole, B. Bodo and C. Kenerley. 2002. Identification of peptaibols from Trichoderma virens and cloning of a peptaibol synthetase. *J Biol Chem.* 277:20862-20868.

Wilhite, S.E., R.D. Lumsden and D.C. Straney. 2001. Peptide synthetase gene in *Trichoderma virens*. *Appl. Environ. Microbiol.* 67:5055–5062.

Woo, S.L., F. Scala, M. Ruocco and M. Lorito. 2006. The Molecular Biology of the Interactions Between *Trichoderma* spp., Phytopathogenic Fungi, and Plants. *Phytopathology*. 96(2):181-5.

Wuczkowski, M., I. Druzhinina, Y. Gherbawy, B. Klug, H. Prillinger and C.P. Kubicek. 2003. Species pattern and genetic diversity of *Trichoderma* in a mid-European, primeval floodplain-forest. *Mycological Research.* 158:125–133.

Xu, J., M. Nogawa, H. Okada and Y. Morikawa. 2000. Regulation of *xyn3* gene expression in *Trichoderma reesei* PC-3-7. *Appl. Microbiol. Biotechnol*. 54:370–375.

Yang, C.A., C.H. Cheng, J.W. Lee, C.T. Lo, S.Y. Liu and K.C. Peng. 2012. Monomeric L-amino acid oxidase-induced mitochondrial dysfunction in *Rhizoctonia solani* reveals a novel antagonistic mechanism of *Trichoderma harzianum* ETS 323. *J. Agric. Food. Chem.* 60(10):2464-71.

Yedidia, I.I., Benhamou, N. and I. Chet. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbiol*. 65:1061–1070.

Yuan, W.M., G.D. Gentil, A.D. Budde and S.A. Leong. 2001. Characterization of the *Ustilago maydis sid2* gene, encoding a multidomain peptide synthetase in the ferrichrome biosynthetic gene cluster. *J. Bacteriol.* 183:4040–4051.

Zachow, C., C. Berg, H. Müller, R. Meincke, M. Komon-Zelazowska, I.S. Druzhinina, C.P. Kubicek and Berg, G. (2009) Fungal diversity in the rhizosphere of endemic plant species of Tenerife (Canary Islands): relationship to vegetation zones and environmental factors. *ISME J.* 3:79–92.

Zhang, C.L., I.S. Druzhinina, C.P. Kubicek and T. Xu. 2005. *Trichoderma* biodiversity in China: evidence for a North to South distribution of species in East Asia. *FEMS Microbiol. Lett.* 251:251–257.

Zhang, C.L., S.P. Liu, F.C. Lin, C.P. Kubicek and I.S. Druzhinina. 2007. *Trichoderma* taxi sp. nov., an endophytic fungus from Chinese yew *Taxus mairei*. *FEMS Microbiol. Lett.* 270:90–96.

AIMS OF THE THESIS

Trichoderma is a genus of filamentous fungi with a variety of biotrophic and saprotrophic lifestyles. The ability to parasitize and/or kill other fungi (mycoparasitism) is widely used in agricultures as a protection of plants against plant pathogens (biological control or biocontrol).

The major aim of this thesis was to get inside the ecological genomics of *Trichoderma*, i.e. to reveal molecular mechanisms employed by the fungus in its environment.

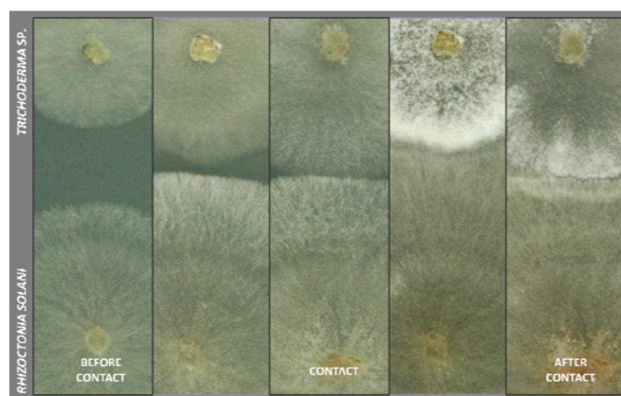
To fulfill this role the following tasks have been formulated:

1. To investigate molecular mechanisms of *Trichoderma* mycoparasitism by comparative transcriptional analysis of cosmopolitan opportunistic species and powerful biocontrol agents *Trichoderma atroviride* and *T. virens* with tropical ecologically restricted species *T. reesei.*

2. To reveal the evolutionary history of *Trichoderma* mycoparasitism by phylogenomic analysis of *T. atroviride*, *T. virens* and *T. reesei.*

3. To reveal the role of polyketide synthase gene *pks4* from *T. reesei* that is putatively responsible for the most characteristic morphological feature of the fungus, its green color of conidia.

1. COMPARATIVE TRANSCRIPTOMICS REVEALS DIFFERENT STRATEGIES OF *TRICHODERMA* MYCOPARASITISM



BMC Genomics

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Comparative transcriptomics reveals different strategies of *Trichoderma* mycoparasitism

Lea Atanasova[1], Stephane Le Crom[2,3,4,5,6], Sabine Gruber[1], Fanny Coulpier[2,3,4], Verena Seidl-Seiboth[1], Christian P Kubicek[1,7] and Irina S Druzhinina[1,7*]

Abstract

Background: *Trichoderma* is a genus of mycotrophic filamentous fungi (teleomorph *Hypocrea*) which possess a bright variety of biotrophic and saprotrophic lifestyles. The ability to parasitize and/or kill other fungi (mycoparasitism) is used in plant protection against soil-borne fungal diseases (biological control, or biocontrol). To investigate mechanisms of mycoparasitism, we compared the transcriptional responses of cosmopolitan opportunistic species and powerful biocontrol agents *Trichoderma atroviride* and *T. virens* with tropical ecologically restricted species *T. reesei* during confrontations with a plant pathogenic fungus *Rhizoctonia solani*.

Results: The three *Trichoderma* spp. exhibited a strikingly different transcriptomic response already before physical contact with alien hyphae. *T. atroviride* expressed an array of genes involved in production of secondary metabolites, GH16 ß-glucanases, various proteases and small secreted cysteine rich proteins. *T. virens*, on the other hand, expressed mainly the genes for biosynthesis of gliotoxin, respective precursors and also glutathione, which is necessary for gliotoxin biosynthesis. In contrast, *T. reesei* increased the expression of genes encoding cellulases and hemicellulases, and of the genes involved in solute transport. The majority of differentially regulated genes were orthologues present in all three species or both in *T. atroviride* and *T. virens*, indicating that the regulation of expression of these genes is different in the three *Trichoderma* spp. The genes expressed in all three fungi exhibited a nonrandom genomic distribution, indicating a possibility for their regulation via chromatin modification.

Conclusion: This genome-wide expression study demonstrates that the initial *Trichoderma* mycotrophy has differentiated into several alternative ecological strategies ranging from parasitism to predation and saprotrophy. It provides first insights into the mechanisms of interactions between *Trichoderma* and other fungi that may be exploited for further development of biofungicides.

Keywords: *Hypocrea*, *T. atroviride*, *T. virens*, *T. reesei*, Mycoparasitism, Gene expression, Biocontrol, Transcriptomics

Background

Mycoparasitism describes the type of biotrophic interactions in which organisms benefit at the expense of the fungi [1]. In a broad sense this property is most proliferated within the fungal family *Hypocreaceae*, and the ability to antagonize, parasitize or even kill other fungi (necrotrophic hyperparasitism, mycotrophy) is particularly common in the genus *Trichoderma* (teleomorph *Hypocrea*, Hypocreales, Dikarya) [1]. The biochemistry and genetics of mycoparasitism has been most thoroughly investigated in only a few species of *Trichoderma* such as *Trichoderma harzianum* sensu lato, *T. atroviride* (teleomorph *Hypocrea atroviridis*), *T. virens* (teleomorph *H. virens*), *T. asperellum* and *T. asperelloides* [1-3] because of their application as agents of biological control of pests (biocontrol) in agriculture. Consequently, several enzymes and other effectors involved in the recognition of a fungus and in the mycoparasitic responses itself have been identified [4-7]. Expressed-sequence-tag (EST) libraries obtained of different *Trichoderma* strains cultivated under various conditions have contributed significantly to the large-scale identification of genes required for the interaction [8-12]. DNA microarrays have been used to

* Correspondence: irina.druzhinina@tuwien.ac.at
[1]Research Area Biotechnology and Microbiology, Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstrasse 1a, A-1060, Vienna, Austria
[7]Austrian Center of Industrial Biotechnology (ACIB), GmBH c/o Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstrasse 1a, A-1060, Vienna, Austria
Full list of author information is available at the end of the article

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study the interaction of *Trichoderma* with plants [13]. So far, however, only two studies used high throughput transcriptomic tools to investigate mechanisms of *Trichoderma* mycoparasitism [5,11].

A comparative analysis of the genome inventory of *T. atroviride, T. virens* and *T. reesei* revealed that mycoparasitism is the innate property of the genus *Trichoderma* [1,4]. It has been suggested that the evolutionary more recent species *T. reesei* adapted to a certain ecological niche and thereby partially lost the strength of its mycoparasitic potential [1,4]. A comparison of genome wide gene expression in vigorous and moderate mycoparasitic *Trichoderma* species may therefore lead to the identification of mechanisms involved in the interaction.

In this study we have applied oligonucleotide tiling microarrays to obtain transcriptional profiles of *T. atroviride* IMI 206040, *T. virens* Gv29-8 and *T. reesei* QM 6a (which were used for genome sequencing) interaction with the plant pathogenic fungus *Rhizoctonia solani* (teleomorph *Thanatephorus,* Basidiomycota). Our data highlight the differences in the mechanisms of these interactions between *Trichoderma* species and emphasize the existence of alternative strategies employed by these fungi.

Results

Confrontation assays and *Trichoderma* mode of action

In order to identify the genes that are involved in *T. atroviride* (*Ta*), *T. virens* (*Tv*) and *T. reesei* (*Tr*) interaction with *R. solani*, we performed dual confrontation assays. *R. solani*, a plant pathogenic fungus was chosen as a model prey fungus because several biocontrol formulations based on *Trichoderma* target various plant diseases caused by this fungus [14] and as it makes severe agricultural damages on a wide range of crops worldwide [15]. The three *Trichoderma* spp. revealed essential differences in their response to confrontation with *R. solani* (Figure 1): *Tr* was at first unable to stop the growth of *R. solani*, and the growth of the latter one was even stimulated for 21% more than when confronted to itself. However, after 10 days at the borderline between the two cultures there was a clear barrage zone that was later consequently overgrown by *Tr* (Figure 1). *Tv* inhibited the growth of *R. solani* by 9%, but it was able to fully overgrow *R. solani* colony and finally killed it. In contrast, at 10th day *Ta* did not stop the growth of *R. solani*, but it straightforwardly overgrew it almost completely (86%, Figure 1). Thus, in terms of this experiment, *Tv* and *Ta* expressed interactions best described as predation (immediate kill and consumption) and parasitism respectively. *Tr* showed neutral reaction with signs of weak mycoparasitism.

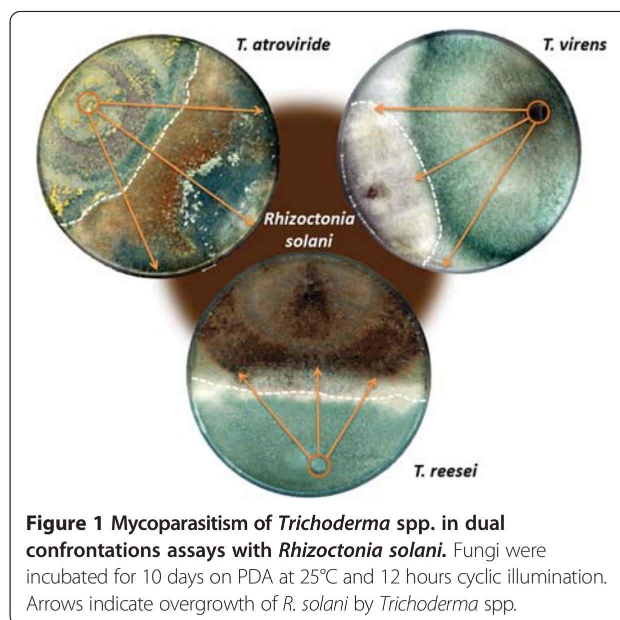

Figure 1 Mycoparasitism of *Trichoderma* spp. in dual confrontations assays with *Rhizoctonia solani*. Fungi were incubated for 10 days on PDA at 25°C and 12 hours cyclic illumination. Arrows indicate overgrowth of *R. solani* by *Trichoderma* spp.

Transcriptional response of *Trichoderma* to the interaction with *R. solani*

The tiling microarray data of *Ta, Tv* and *Tr* were analyzed at three stages of the interaction: (1) before contact with alien hyphae (BC), (2) during the initial contact with the hyphae (C), and (3) when the interaction has been established (after contact; AC) (Figure 2). After data normalization we applied the linear modeling approach and the Bayes statistics implemented in the LIMMA package (http://bioinf.wehi.edu.au/limma/, [16]) to the biological replicates as described in Materials and Methods. In total more than 60% of the genes present in the three genomes were found to be transcribed. Among them 651, 303 and 424 genes were found to be differentially regulated (larger/smaller than $\log_2$ (ratio) = 1.5) in *Ta*, *Tv* and *Tr*, respectively (Table 1; Additional file 1: Tables S1-S3) and thus involved in the interaction. All of up- and down-regulated genes were annotated and categorized as described in the Materials and Methods section. In addition, Functional Catalogue (FunCat, http://mips.helmholtz-muenchen.de/proj/funcatDB/) protein families were assigned to those genes for which a function could be predicted (Table 2). 66, 70 and 62% of the significantly expressed genes of *Ta*, *Tv* and *Tr*, respectively, encoded genes with significant similarity to other fungal proteins (blastp $E < e^{-100}$; Table 1). The remaining genes were annotated as unknown (when orthologues were found in other fungal genera but no function was assigned) or orphan (unique; present only in *Trichoderma* spp.) proteins (see Materials and Methods for definition). The latter comprised only 0.5-0.7% of all transcripts for the three *Trichoderma* species (Table 1). Statistical analysis revealed that the transcriptomic profiles of three

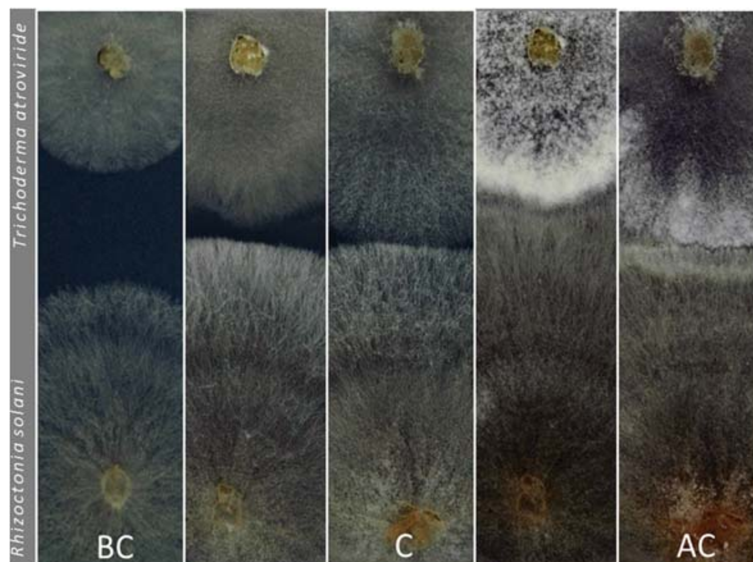

Figure 2 Development of mycoparasitic reaction of *Trichoderma atroviride* against *Rhizoctonia solani*. The mycelium was sampled before contact (**BC**), at the contact (**C**) and after the contact (=overgrowth, **AC**) with R. solani.

investigated species were significantly different (ANOVA, $p < 0.001$). The majority of involved genes in *Tr* and *Tv* (63 and 84% respectively) were down-regulated, whereas the up- and down-regulated genes in *Ta* occurred in approximately equal proportions (52% up- and 48% down-regulated). However, in all species the pattern of up- and down-regulation depended on the confrontation stages (Figure 3).

Interestingly, the majority of significantly up-regulated genes were represented by genes for which orthologues in other *Trichoderma* species are present (Figure 4). In addition, 88 of these genes were shared only between *Tv* and *Ta*. Thus 93.2, 74.4 and 84.8% of all genes that were significantly expressed in either *Tr, Ta* or *Tv*, respectively, were orthologues or at least conserved in *Ta* and *Tv*. In contrast, among those most of genes involved in mycoparasitic response were only found in one species. Only nine orthologuous genes were up-regulated in all three species (Figure 4) when confronted with *R. solani*, and 29 genes were significantly expressed only in the two mycoparasitic species *Ta* and *Tv*; they comprised 16 unknown, three putative MFS transporters, two orphan and two unknown transcriptional activators genes.

The most significant differences were evident at the pre-contact stage (ANOVA, $p < 0.001$): in *Tr,* the majority of differentially regulated genes were up-regulated, whereas in *Tv* and in *Ta* genes in this stage were mostly down-regulated and showed only a small response (Figure 3). At contact with hyphal tips of *R. solani*, *Tr* and *Ta* displayed only a low transcriptomic response, whereas *Tv* showed significant down-regulation of most of its genes (ANOVA, $p < 0.001$). During overgrowth of the prey, expression of most of genes increased in *Ta* and *Tv*, but were down-regulated in *Tr.* However, in general transcriptional response in this stage was significantly different between *Ta* and the other two species (ANOVA, $p < 0.001$).

To confirm the microarray results, quantitative Real-Time-PCR (qPCR) was performed on a subset of the genes from *Tv* and *Ta*. They were chosen from gene families that showed abundant up-regulation during confrontation with *R. solani*, such as GH16 glucanases,

Table 1 Numbers of transcripts that are either up- or down-regulated during the mycoparasitic response of *T. reesei*, *T. atroviride* and *T. virens*

Species	Section	Genes	Transcripts		Proteins					
					With identified function		Unknown		Orphan	
			Total	Up/down (%*)	Total	Up/down (%)	Total	Up/down (%)	Total	Up/down (%)
T. reesei	*Longibrachiatum*	9 143	424	36.6/**63.4**	264	44.7/**55.3**	137	23.4/**76.6**	23	21.7/**78.3**
T. atroviride	*Trichoderma*	11 865	651	**53.1**/46.9	432	**52.1**/47.9	173	**58.4**/41.6	46	43.5/**56.5**
T. virens	*Pachybasium*	12 428	303	15.5/**84.5**	211	19.4/**80.6**	71	8.5/**91.5**	21	0/**100**

*the percentages of up- and down regulated genes are calculated based on the total number of transcripts found within each group of genes; bold fond highlights larger values.

Table 2 Protein groups (FunCat) that exhibited significant up- and/or down-regulation during mycoparasitic interaction in at least one of the three *Trichoderma* spp.*

FunCat category	FunCat number	Annotation	*T. atroviride*				*T. virens*				*T. reesei*			
			11 865 genes				12 428 genes				9 143 genes			
			Total involved	Up	down	Up/ down (%)	Total involved	Up	down	Up/ down (%)	Total involved	Up	down	Up/ down (%)
metabolism	01_05_01_01_09	2-oxoglutarate dependent dioxygenase	*3*	*0*	*3*	0	*1*	*1*	*0*	n.a.	*0*	*0*	*0*	n.a.
	01_04	acid phosphatase	*4*	*4*	*0*	n.a.	*1*	*1*	*0*	n.a.	*1*	*1*	*0*	n.a.
	01_20_05_11	PKS	*5*	*2*	*3*	0.7	*1*	*0*	*1*	0	*4*	*2*	*2*	1
	01_20_35_01_05	flavonol reductase	*4*	*4*	*0*	n.a.	*1*	*0*	*1*	0	*0*	*0*	*0*	
	01_20_35_01_05	isoflavon reductase	*2*	*2*	*0*	n.a.	*0*	*0*	*0*	0	*1*	*0*	*1*	0
	01_20_36	NRPS	*2*	*0*	*2*	0	*4*	*0*	*4*	0	*2*	*0*	*2*	0
	01_25_01	GH glycosyl hydrolase	*51*	*10*	*41*	0.2	*5*	*4*	*1*	4	*36*	*34*	*2*	17
	01_25_03	protease	*15*	*13*	*2*	6.5	*3*	*1*	*2*	0.5	*5*	*2*	*3*	0.7
	01_25_07	lipase/esterase	*6*	*2*	*4*	0.5	*2*	*0*	*2*	0	*3*	*1*	*2*	0.5
	01_25_07	CE carbohydrate esterase	*3*	*1*	*2*	0.5	*0*	*0*	*0*	0	*3*	*3*	*0*	n.a.
transcription	11_02_03_04	C2H2 transcriptional regulator	*5*	*2*	*3*	0.7	*5*	*0*	*5*	0	*9*	*3*	*6*	0.5
	11_02_03_04	transcription factor	*8*	*1*	*7*	0.1	*0*	*0*	*0*	n.a.	*0*	*0*	*0*	n.a.
	11_02_03_04	Zn2Cys6 transcriptional regulator	*11*	*3*	*8*	0.4	*3*	*0*	*3*	0	*5*	*2*	*3*	0.7
protein fate	14_07_04	GCN5-N-acetyltransferase	*6*	*3*	*3*	1	*2*	*1*	*1*	1	*1*	*1*	*0*	n.a.
	14_07_09	SAM-dependent methyltransferase	*4*	*4*	*0*	n.a.	*3*	*0*	*3*	0	*3*	*2*	*1*	2
protein with binding functions	16_01	ankyrin	*2*	*1*	*0*	n.a.	*2*	*0*	*2*	0	*0*	*0*	*0*	n.a.
	16_21	cytochrome P450 subfamily	*8*	*6*	*2*	3	*9*	*0*	*9*	0	*5*	*4*	*1*	4
	16_21	FAD-dependent monooxygenase	*11*	*8*	*3*	2.7	*3*	*2*	*1*	2	*2*	*0*	*2*	0
	16_21_07	GMC oxidoreductase	*2*	*2*	*0*	n.a.	*2*	*0*	*2*	0	*1*	*0*	*1*	0
	16_21_07	short chain dehydrogenase/ reductase	*8*	*5*	*3*	1.7	*11*	*0*	*11*	0	*6*	*0*	*6*	0
cellular transport	20_01_01	oligopeptide transporter	*8*	*3*	*5*	0.6	*1*	*1*	*0*	n.a.	*3*	*1*	*2*	0.5
	20_01_01_01_01	iron permease	*2*	*2*	*0*	n.a.	*0*	*0*	*0*	n.a.	*0*	*0*	*0*	n.a.
	20_01_01_07	inorganic phosphate transporter	*4*	*4*	*0*	n.a.	*1*	*1*	*0*	n.a.	*1*	*1*	*0*	n.a.
	20_01_03	monocarboxylate transporter	*3*	*3*	*0*	n.a.	*0*	*0*	*0*	n.a.	*0*	*0*	*0*	n.a.
	20_01_07	amino acid permease	*5*	*1*	*4*	0.3	*3*	*3*	*0*	n.a.	*4*	*3*	*1*	3
	20_03	MFS (major facilitator superfamily) (12 TMH)	*51*	*15*	*36*	0.4	*7*	*4*	*3*	1.3	*17*	*9*	*8*	1.1
signal transduction mechanism	30_05_02_24	GPCR, G-protein coupled receptor	*6*	*1*	*5*	0.2	*0*	*0*	*0*	n.a.	*2*	*0*	*2*	0
	30_05_02_24	PTH11-type GPCR	*11*	*9*	*2*	4.5	*2*	*0*	*2*	0	*2*	*1*	*1*	1
cell rescue and defense	32_01_05	chaperone/heat shock protein	*0*	*0*	*0*	n.a.	*5*	*5*	*0*	n.a.	*4*	*0*	*4*	0
	32_07	AAA + –type ATPase	*4*	*2*	*2*	1	*8*	*0*	*8*	0	*2*	*1*	*1*	1
	32_07_05	PDR-type multidrug transporter	*3*	*3*	*1*	3	*0*	*0*	*0*	n.a.	*2*	*1*	*1*	1

Table 2 Protein groups (FunCat) that exhibited significant up- and/or down-regulation during mycoparasitic interaction in at least one of the three *Trichoderma* spp.* *(Continued)*

	32_07_05	ABC transporter	*0*	*0*	*0*	n.a.	*2*	*0*	*2*	0	*2*	*0*	*2*	0
	32_07_05	MDR (multidrug resistance-associated protein)	*0*	*0*	*0*	n.a.	*2*	*0*	*2*	0	*1*	*0*	*1*	0
	32_07_07_03	glutathione S-transferase	*3*	*3*	*0*	n.a.	*4*	*0*	*4*	0	*2*	*0*	*2*	0
subcellular localization	70_01	cell wall protein	*5*	*0*	*5*	0	*2*	*0*	*2*	0	*4*	*2*	*2*	1
	70_27	SSCR (small secreted cysteine rich protein)	*8*	*8*	*0*	n.a.	*6*	*0*	*6*	0	*0*	*0*	*0*	n.a.
cell fate	40	HET protein	*2*	*2*	*0*	n.a.	*0*	*0*	*0*	n.a.	*1*	*0*	*1*	0

*ratio values cannot be given when denominator is zero; n.a. means not applicable.

proteases, PKS and SSCPs (see below). Expression of these genes correlated well with the data of the microarray (Additional file 1: Table S4). We therefore conclude that the results of tiling microarray analysis indeed reflect differences in the function of genes involved in mycoparasitism.

The enrichment for the major functional categories (FunCat) was assessed for each category (Table 2) separately. To reveal the common (shared by all three species) and species-specific mechanisms acting at every stage of interaction between *Trichoderma* and *R. solani* we performed the statistical analysis of the respective FunCats. In general, all three species showed significantly different FunCat profiles indicating the presence of alternative interaction strategies (MANOVA; $p < 0.05$). Shifts in gene expression between BC, C and AC stages of the interaction were also strongly species-specific (Figure 5): only a minor portion of genes became down-regulated in C compared to BC and at AC compared to C (ANOVA $< p < 0.001$). However the massive differences were observed in *Ta* when a large group of genes became up-regulated at AC compared to BC and C (Figure 5A).

Statistical analysis of the global comparison of FunCat categories for the three stages for *Tr, Tv* and *Ta*, revealed that most categories broadly scattered both in the up- and down-regulated areas (Table 2). However, some specific features were observed: in *Tr*, genes involved in metabolism and transport were all up-regulated and the latter also in *Tv*; expression of proteins with binding function was increased in *Ta* during the contact phase and proteins involved in self-defense decreased in *Tr* during overgrowth. It is also apparent that during overgrowth regulation of all genes was much more comparable (as indicated by less scattering of values), and only few categories also included up-regulated genes.

Genes up-regulated in interaction with *R. solani*

A comparison of the protein families [Pfam, http://pfam.sanger.ac.uk/] assigned for up-regulated genes in the above analysis revealed very little common responses among the three *Trichoderma* spp., and also between the two opportunistic and strong mycoparasitic species. In fact only a few gene families shared a common trend in all three *Trichoderma* spp. (proteases, heat shock proteins, cytochrome C peroxidase, proline oxidase, ER-bound glutathione-S-transferases, ABC efflux transporters, the pleiotropic drug resistance (PDR) transporters, multidrug resistance MDR-type transporters and nitrilases), indicating that stress response connected with detoxification of potentially hazardous metabolites is a general reaction of *Trichoderma* during antagonism (see the Discussion below).

In addition to the genes named above, all three *Trichoderma* spp. had distinct specific responses. Thus, in addition to expression of the highest number of several protease families (dominated by subtilisin-like and aspartyl proteases), *Ta* increased the transcription of oligopeptide transporters, C-type lectins, small secreted cysteine-rich proteins (SSCPs), PTH11-receptors and ß-glucanases of the GH16 family (Table 2; Additional file 1: Tables S1-S3). In contrast, *Tv* mainly increased the expression of genes for gliotoxin biosynthesis including the genes for its precursors, and genes encoding heat shock proteins. The minor additional responses in *Tv* included increased up-regulation of chaperone proteins, and several dehydrogenases and monooxygenases and significant down-regulation of AAA + –type ATPases and transcriptional regulators. However, it was conspicuous that both strongly opportunistic and mycoparasitic species expressed all these genes already at a stage before hyphal contact with *R. solani,* and were not expressed anymore after the contact with the prey (Figure 5B).

Ta also largely relied on antibiosis: one polyketide synthase (PKS) of the reducing (lovastatin/citrinin) clade I (Triat2:134224; [17]) was expressed at all stages of interaction, and peaked at C. A second PKS gene (Triat2:85006; [17]) from the reducing clade I was expressed only at contact but not at BC and AC. Interestingly, *Ta* also expressed genes encoding two KP4-like killer-toxins that have so far not been described for filamentous fungi. In addition, the up-regulation of genes encoding macrophomate

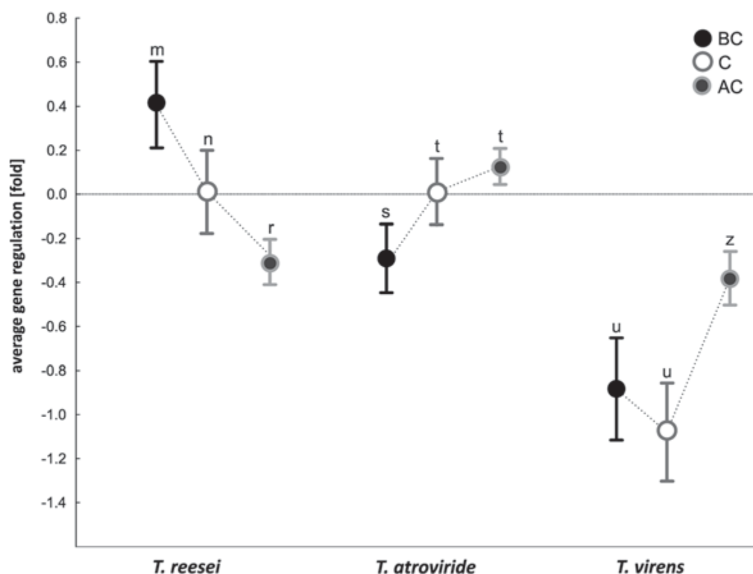

Figure 3 Averaged regulation of all genes (|log$_2$ (ratio)| > 1.5) involved in mycoparasitsm of *T. reesei*, *T. atroviride* and *T. virens* during the three confrontation stages with *R. solani* (BC, C and AC). Vertical bars denote 0.95 confidence intervals. The same letters above the bars indicate the statistical significance (ANOVA) between the stages of each species: for m–n–r $p < 0.005$; s–t $p < 0.02$; u–z $p < 0.001$. The plot shows different trends of gene expression kinetics between the species and does not reflect the gene regulation intensity differences among the species.

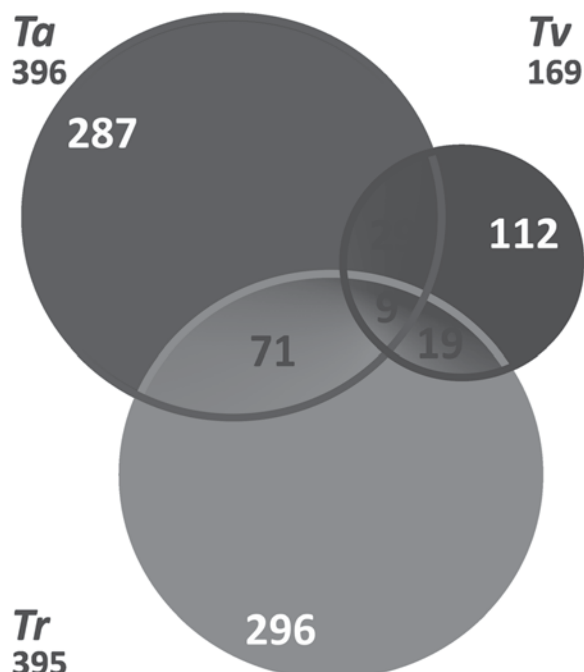

Figure 4 The Venn diagram showing the number of orthologous genes of *T. reesei*, *T. atroviride* and *T. virens* that are involved in mycoparasitic response against *Rhizoctonia solani* (at all stages of confrontation). The total number of orthologous genes per species is given below the respective species name.

synthases, isoflavone reductases and pyoverdin dioxygenases was notable. These genes may be involved in the formation of yet unknown secondary metabolites of *Trichoderma*. Finally, a gene encoding a lipoxygenase (Triat2:33350), which is absent in *Tr* and *Tv,* was up-regulated at C stage. It may be involved in the biosynthesis of γ-pentylpyrone (see details in the Discussion).

In contrast, the transcriptome of *Tr* before contact revealed unique response to the presence of *R. solani*. It showed a massive up-regulation of cellulolytic and hemicellulolytic CAZymes, and ribosomal proteins (Table 2). Furthermore, several transporter proteins (MSF permeases, inorganic phosphate, oligopeptide and amino acid transporters) as well one PKS (Trire2:60118) were up-regulated at this stage. Interestingly, *Tr* also up-regulated subtilisin proteases but none of the aspartyl proteases that were active in *Ta*. At C stage *Tr* significantly reduced the total gene expression, and only genes encoding a multidrug transporter, a stress-responsive protein (RDS1) and another PKS were induced and may indicate defense reaction rather than an attack. The AC stage was characterized by an increased activity of the genes encoding ribosomal proteins, while genes for cellulolytic and hemicellulolytic CAZymes were not expressed anymore.

Genes down-regulated in interaction with *R. solani*

Some gene families were consistently down-regulated in all three species during confrontation with *R. solani*, particularly already at BC stage. These comprised short-chain

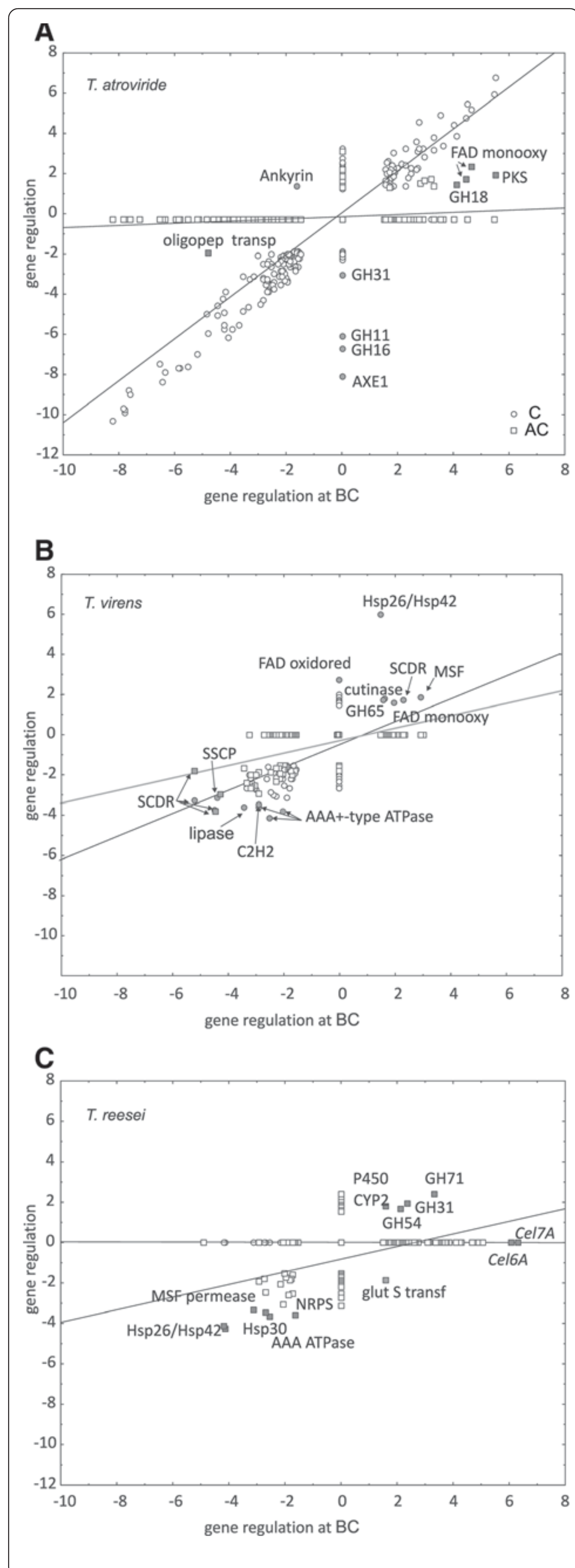

Figure 5 Scatter plots of all regulated genes in each stage for (A) *T. atroviride*, (B) *T. virens* and (C) *T. reesei*, respectively. The $\log_2$ (ratio) regulation for the genes at contact (C, circles) and after the contact (AC, squares) stages are plotted against the genes regulated prior the contact (BC). The genes, for which a stage-specific difference was found, are indicated by their functional names.

dehydrogenases/reductases, transcriptional regulators of the fungal-specific Zn(2)Cys(6) family, non-ribosomal peptide synthases, and AAA + –domain proteases.

The only species-specific response was detected for *Ta,* which showed the opposite expression pattern of genes involved in polysaccharide and lipid hydrolysis and solute uptake to *Tr,* as it strongly down-regulated all these genes already prior the contact (BC).

Genomic distribution of genes involved in mycoparasitism

We also investigated whether the genes expressed during interaction with *R. solani* would be clustered in the genome. Our approach was based on the consideration that under random distribution of transcribed genes, we should (on the average) find them once in batches of 18, 42 and 22 subsequent genes in *Ta*, *Tv* and *Tr*, respectively. These values arise by division of the total number of genes in the *Ta, Tv* and *Tr* (11865, 12518, and 9143, respectively) by the number of genes significantly expressed by them in this study (651, 303 and 424, respectively). To detect significant deviations from these expectations, we manually screened the three genomes for genes that occurred within the vicinity of each other at an at least 3-fold lower value than the above calculated average distributions, as was also done by Seiboth, Aghcheh et al. [18]. We detected that the distribution of about one third of the genes (36.9, 34.7 and 37.2% in *Ta, Tv* and *Tr*, respectively) was not random (Pearson coefficient >0.25) (Table 3). About a third of them were located at the ends of the respective scaffolds implying that they are situated either at the ends of chromosomes or nearby repetitive regions.

Discussion

Although a wide range of *Trichoderma* spp. have been described as mycoparasites [1], detailed studies on the molecular physiology of this trait have been performed mostly with *T. harzianum* CECT 2413 or the *T. atroviride* strains P1 and IMI 206040 [2,3,19]. Based on these studies, it is commonly believed that the expression of cell wall lytic enzymes (particularly chitinases but also ß-glucanases and proteases) and secondary metabolites are the major determinants for success in this process. The present comparison of the two mycoparasites *T. atroviride* and *T. virens* with *T. reesei*, which was unable to efficiently besiege *R. solani*, changes this view in two important aspects: first, the role of induction of

Table 3 Clusters of nonrandomly distributed genes in *T. atroviride, T. virens* and *T. reesei* that are involved in mycoparasitism

Scaffold	*T. atroviride*		*T. virens*		*T. reesei*	
	Gene area*	Density**	Gene area*	Density**	Gene area*	Density**
1	44-47	3/4	583-618	6/36	62-68	3/7
	69-87	7/19	974-990	4/17	129-135	4/7
	1111-1119	4/9			439-452	4/14
	1331-1340	3/10			632-639	3/8
	1717-1726	3/10			734-753	5/20
2	85-93	3/9	3-27	7/25	43-55	4/13
	306-313	3/8	134-148	3/15		
	649-663	6/15	253-274	4/22		
	936-942	3/7	665-695	4/31		
	998-1000	3/3				
	1031-1039	3/9				
	1039-1054	6/16				
3	140-143	4/4	788-792	4/5	4-27	5/24
	189-201	10/13			333-342	3/10
	691-698	3/8			406-411	4/6
	771-773	3/3				
4	37-45	4/9	20-24	4/5	34-45	3/12
	340-349	3/10	242-255	3/14	333-339	3/7
	593-614	6/20	327-346	3/20		
5	12 - 24	7/13	252-262	3/11	209-226	4/14
	97-104	3/8			235-248	4/14
	286-302	5/17			448-463	5/16
	389-412	8/22				
	425-427	3/3				
6	264-268	4/5	2-21	3/20	24-51	6/28
	354-357	3/4			128-139	3/12
	461-483	7/23				
7	178-187	4/10	6-9	3/4	59-72	5/14
	404-408	3/5			286-292	3/7
	484-509	8/26				
	524-532	5/9				
	546-559	4/14				
8	117-129	6/13			276-290	3/15
	155-165	6/11				
	185-189	3/5				
9	173-188	6/16			26-44	4/19
	198-212	4/15				
	489-498	4/10				

Table 3 Clusters of nonrandomly distributed genes in *T. atroviride, T. virens* and *T. reesei* that are involved in mycoparasitism *(Continued)*

10			242-262	3/21	74-83	3/10
					185-187	3/3
					262-272	3/11
					316-344	5/29
11	399-411	5/13				
	462-477	9/16				
12	386-391	3/6			7-11	3/5
	466-468	3/3			160-174	3/15
13	77-85	3/9	277-281	3/5		
	132-139	3/8				
	275-281	3/7				
			219-229	3/11	1-14	3/15
15	20-25	4/6				
	169-174	3/6				
16	55-61	3/7				
17	78-85	3/8			59-86	6/28
	165-175	4/11				
18	45-62	6/18	1-7	5/7		
	135-138	3/4				
19	4 of 12	3/9	107-113	4/7	32-37	3/6
	68-82	6/15				
	141-155	4/15				
20	2-11	5/10			94/100	3/7
21			21-41	3/21	116-120	4/5
			100-109	3/10		
22			101-114	4/15	80-109	7/30
24					29-41	4/13
26			53-63	4/11		
27					40-56	4/17
28			32-51	3/20	68-74	3/7
					98-122	5/25
29					68-79	3/12
					99-113	6/13
32					55-62	3/8
34			3-13	10/11	26-30	3/5
35			32-51	4/20		
45					7-20	3/14
54			1-8	5/8		

* indicates the area on the scaffold in which the cluster was identified, given by the numbers of the first and the last genes on the scaffold. ** the ratio of significantly up- or down-regulated genes to the total number of genes in the cluster.

hydrolytic enzymes and secondary metabolites must be revisited; and second, the two mycoparasites display completely different strategies to antagonize their host/prey.

The latter claim is nicely reflected by the findings that – although the majority of significantly expressed genes in the three *Trichoderma* spp. belong to the gene inventory present in all of them – most of these genes are specific for only in one of the species. This implies that rather the regulation of gene expression than the availability of specific "mycoparasitic" genes is the key for

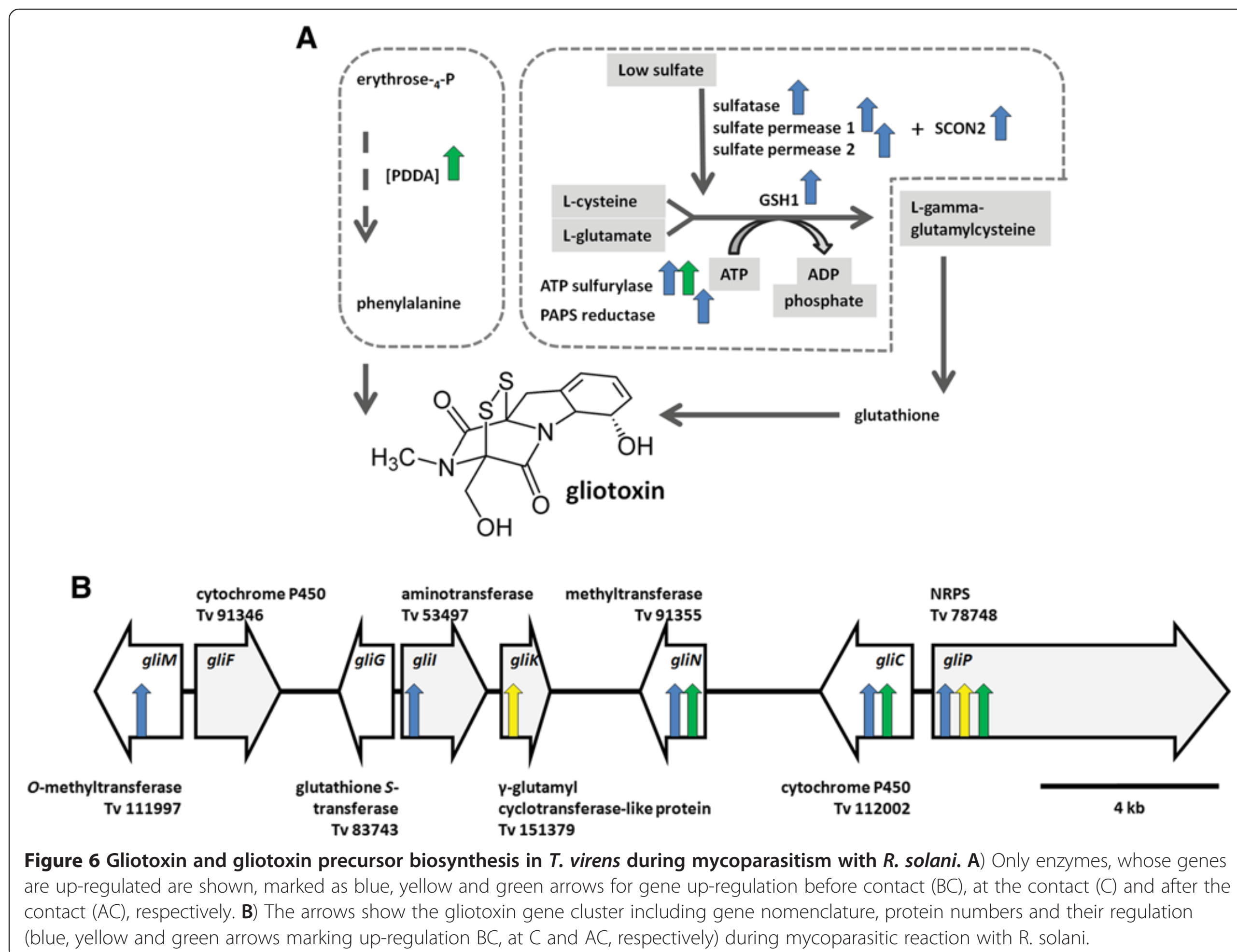

Figure 6 Gliotoxin and gliotoxin precursor biosynthesis in *T. virens* during mycoparasitism with *R. solani*. A) Only enzymes, whose genes are up-regulated are shown, marked as blue, yellow and green arrows for gene up-regulation before contact (BC), at the contact (C) and after the contact (AC), respectively. **B**) The arrows show the gliotoxin gene cluster including gene nomenclature, protein numbers and their regulation (blue, yellow and green arrows marking up-regulation BC, at C and AC, respectively) during mycoparasitic reaction with R. solani.

successful antagonism. The molecular basis for this has not yet been studied: it could be the enhanced number of transcription factors in *Ta* and *Tv* [4] which may have resulted in a more refined transcription pattern of genes related to mycoparasitism. The other reason could be the occurrence of many of these genes in nonsyntenic regions of the genome (at the ends of chromosomes or nearby repetitive regions; *vide supra*), which could have changed the transcriptional environment of these genes.

We have previously reported that *T. atroviride* is able to sense the presence of its host from a distance [20]. This ability to sense the other fungus seems to be a property of all *Trichoderma* species, as all three show a significant and specific expression of a number of genes already before contact. However, the types of genes expressed distinguished *T. atroviride* and *T. virens* from *T. reesei*: while the former two species express a number of genes for which a function in antagonism can be interpreted, *T. reesei* mainly expresses genes for nutrient acquisition. Interestingly, the expression of the same cellulase and hemicellulase genes was strongly down-regulated in *T. atroviride* and highly induced in *T. reesei*, *T. virens* remained unaffected. One interpretation of this observation would be that – upon sensing a potential prey – *T. atroviride* changes gene expression towards an attack, whereas *T. reesei* attempts to compete with the other fungus by faster acquisition of nutrients. Likewise, it is possible that the sensing of a basidiomycete fungus signals the availability of pre-degraded wood to *T. reesei*, in accordance with the model that *T. reesei* became an efficient saprotroph on dead wood by following wood-degrading fungi into their habitat [1,20,21]. This implies that components from the host/prey are able to stimulate this process, and the role of the general cellulase regulator XYR1 [22] therefore warrants examination. The fact that cellulase gene expression came to an immediate stop when *T. reesei* arrived at physical contact with *R. solani* does not contradict this explanation, because cellulases and hemicellulases are secreted into the medium where they have a half-life of 40 and more hours. Thus the cellulases secreted by *T. reesei* before contact may suffice for nutrient acquisition throughout the whole process of interaction observed.

The two strong mycoparasitic species also differed in the tools that they used for combating and killing *R.*

solani. *T. virens* straightforwardly headed for predation by poisoning *R. solani* with gliotoxin. Earlier studies [23-27] have already suggested that gliotoxin formation is the main antifungal principle of a subset of *T. virens* strains, so called "Q-strains" (such as Gv29-8). Our data are consistent with this claim, but it must be noted that they may not apply to "P-strains" as they do not produce gliotoxin [25,26]. In our experiments it was evident from the up-regulation of almost all genes required for gliotoxin biosynthesis, i.e. a two module non-ribosomal peptide synthetase (*gliP*); thioredoxin reductase (*gliT*); O-methyl transferase (*gliM*); a methyl transferase with unknown specificity (*gliN*); glutathione *S*-transferase (*gliG*); cytochrome P450 monooxygenases (*gliC*); amino cyclopropane carboxylate synthase (*gliI*); and a dipeptidase (*gliJ*). This coordinated expression is also supported by the genomic clustering of these genes (Figure 6). However, it was of interest to note that also genes involved in the provision of the precursor of gliotoxin, L-phenylalanine, and of the glutathione required for the formation of the central disulfide bond were induced during one or more of the three stages. Provision of sulfur for cysteine and subsequently glutathione biosynthesis appeared to be an essential requirement already before contact, as indicated by the up-regulation of two sulfate permeases, one sulfatase, the cysteine biosynthesis genes ATP-sulfurylase and PAPS reductase, and of SCON2, an ubiquitin-ligase involved in regulating sulfur metabolism under conditions of low sulfate supply [28]. Most of these genes were up-regulated only before contact, suggesting that *T. virens* could later fulfill the sulfur requirement by feeding on *R. solani*. In contrast, a shortage in L-phenylalanine appears to take place only during overgrowth as illustrated by the increased expression of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, the first enzyme involved in the biosynthesis of aromatic amino acids, and also the regulatory target for this pathway [29].

It is interesting to note that the genes of the gliotoxin gene cluster that are present in *Tr* [23] were not at all expressed during confrontation with *R. solani*, whereas the gliotoxin cluster is absent in *Ta* [23].

T. atroviride, in contrast, appeared to follow a strategy that involved antibiosis as well as the action of hydrolytic enzymes indicating a rather parasitic interaction that does not directly aims to kill the host. With respect to the latter, *T. atroviride* up-regulated almost only ß-1 ,3/1,4-D-glucanases of the GH16 family, of which some also contained a carbohydrate-binding domain of the lectin superfamily (SCOP link 49899). Unfortunately their properties have not been studied in sufficient detail to offer an interpretation why *T. atroviride* overexpressed just this battery of ß-glucanases and not one of the several others that are present in its genome [4].

Proteases, on the other hand, were up-regulated in all three species, although only the aspartyl proteases were consistently up-regulated in the two strong mycoparasites and down-regulated in *T. reesei*. This confirms our earlier conclusion [30] that the proteases play an important role in mycoparasitism.

Up-regulation of genes for secondary metabolite production in *T. atroviride* most conspicuously comprised two PKS genes of the reducing (lovastatin/citrinin-like) clade I [17]. One of them (Triat1:134224) was in fact one of the most up-regulated genes before and at contact with *R. solani*. However, there is indirect evidence for yet more secondary metabolite formation: *T. atroviride* has been reported to produce α–pentyl-pyrone, a volatile component with antifungal activity that is not formed by *T. reesei* and *T. virens* [31]. Its biosynthesis has been suggested to occur via linoleic acid, in analogy to jasmon synthesis by plants in which the necessary hydroxyl group originates from an oxidation by lipoxygenase [32]. A lipoxygenase gene was indeed present in *T. atroviride* (protein ID 33350) but not the two other species, and was significantly up-regulated in *T. atroviride* during contact with *R. solani*. In addition, the up-regulated genes putatively encoding macrophomate synthase, pyoverdin dioxygenase and of isoflavone reductase may be involved in the biosynthesis of so far unknown secondary metabolites. Two PKS (Trire2:60118, Trire2:82208; the first of the same type as the two of *T. atroviride*, and the second belonging to the nonreducing PKS clade I), were also up-regulated in *T. reesei* before and at contact respectively. However, no other genes with potential relationship to secondary metabolism were observed in *T. reesei*. Interestingly, genes encoding nonribosomal peptide synthases (NRPS) – including the two encoding peptaibol synthases, which have been emphasized as important antifungal components from *Trichoderma* [33] – were found to be down-regulated in all three species. Similar findings have been reported recently for *T. atroviride* by Reithner et al. [5].

Another notable finding with *T. atroviride* was the up-regulation of several genes encoding members of GPCR PTH11 class [34], of lectins and of small secreted cysteine rich proteins. PTH11 encoding genes were also among those up-regulated in the mycoparasite *Coniothyrium minitans* during colonization of *Sclerotinia sclerotiorum* [35], thus rendering them candidates for a function in mycoparasitism. Interestingly, a PTH11 receptor of *Botrytis cinerea* is not involved in plant pathogenicity but regulates the expression of a glutathione-S-transferase gene [36]. The lectins also included a gene encoding cyanovirin, a mannose-binding lectin [37]. Lectins have been suggested to be involved in coiling of *Trichoderma* around hyphae of the fungi they attack [38]. SSCPs are abundant in *Trichoderma* [4] and the high expression of several of them in *T. atroviride* suggests a potential role in mycoparasitism. They are involved in the mutualistic behavior of the ectomycorrhizal symbiont of plants

Laccaria bicolor and are active in some plant pathogenic fungi [39].

Only a few gene families shared a common trend: all three *Trichoderma* spp. also displayed an oxidative response in confrontation with *R. solani* (expression of heat shock proteins, cytochrome C peroxidase, proline oxidase, and ER-bound glutathione-S-transferases). Likewise, genes for detoxification processes (ABC efflux transporters, the pleiotropic drug resistance (PDR) transporters and the multidrug resistance MDR-type transporters) were induced. *R. solani* uses radical oxygen species as signaling molecules during sclerotia formation [40], and excretes antifungal components [41], both of which could act in our experiments and may have elicited this response. An ABC-transporter from *T. atroviride* (TAABC2) is involved in biocontrol of *R. solani* [42].

A cyanide hydratase gene with high similarity to corresponding proteins from other ascomycetes was one of the most strongly induced genes in all three *Trichoderma* spp. at all stages of the interaction. The fungal cyanide hydratases form a functionally specialized subset of the nitrilases which catalyze the hydrolysis of cyanide to formamide [43]. One could argue that this nitrilase serves defend against cyanide produced by *R. solani*, but this has not yet been proven so far. On the other hand, bacteria like *Pseudomonas* spp. [44] produce cyanide. Since this gene was expressed by all three *Trichoderma* spp., its expression may be a general response of *Trichoderma* towards the presence of any host, but this clearly needs further investigations.

A significant portion of the genes responding to the presence of *R. solani*, were non-randomly distributed in the genome of all three *Trichoderma* spp. In ascomycetes clustering is primarily known for genes involved in secondary metabolite synthesis [45]. In *T. reesei* the genes encoding cellulases, hemicellulases and other proteins of the CAZome also occur in clusters [46], and we have recently shown that the genes regulated during conidiation also are clustered in the genome [47]. In *Fusarium graminearum* (teleomorph: *Gibberella zeae*) [48] and *Neurospora crassa* (anamorph: *Chrysonilia crassa*) [49], clustering occurs in subtelomeric regions and contains particularly fast evolving genes, such encoding secreted proteins and orphan genes related to ecological success of an organism. In this context, it is reasonable that the genes related to mycoparasitism are clustered in organisms that have specialized on this trait. In *Aspergillus* spp., expression of genes from such clusters is controlled by the putative methyltransferase LaeA [50], a component of the Velvet protein complex [51]. In this regards, we have recently shown that cellulase formation and conidiation in *T. reesei* are in fact regulated by the orthologue LAE1 [18]. Another component of the Velvet protein complex, VEL1 (VeA) controls mycoparasitism in *T. virens* [52]. We were recently able to show that LAE1 indeed is a regulator of mycoparasitism in *T. atroviride* (R.K. Aghcheh, I.S. Druzhinina and C.P. Kubicek, unpublished data).

Conclusions

The present comparison of the two opportunistic and strongly mycoparasitic species *T. atroviride* and *T. virens* with *T. reesei*, which is unable to besiege *R. solani*, reveals different responses of *Trichoderma* spp. to the presence of another fungus. It also demonstrates that there is no common mechanism by which a mycoparasite attacks and kills its host, but that alternative strategies are used. Finally, the observation of a nonrandom distribution of the transcribed genes suggests the possible involvement of epigenetic regulation of mycoparasitism.

Methods

Comparative transcriptomics analysis

For mycoparasitism confrontation assays *T. virens* Gv29-8, *T. atroviride* IMI 206040 and *T. reesei* QM 6a were grown on potato dextrose agar plates (BD Dicfo, Franklin Lakes, NJ, USA), covered with cellophane, at 25°C and 12 hours cyclic illumination and harvested when the mycelia were ca. 5 mm apart, at contact of the mycelia and after *Trichoderma* had overgrown the host fungus *Rhizoctonia solani* by ca. 5 mm. As control, the respective strain of *Trichoderma* was confronted with itself and harvested at contact. Peripheral hyphal zones from each confrontation stage were sampled and shock frozen in liquid nitrogen. Mycelia were ground to a fine powder under liquid nitrogen and total RNA was isolated using the guanidinium thiocyanate method [53]. For cDNA synthesis, RNA was treated with DNase I (Fermentas, Burlington, Canada) and purified with the RNeasy MiniElute Cleanup Kit (Qiagen, Valencia, CA, USA). 5 μg RNA/reaction were reverse transcribed using the SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and a mixture of provided random hexamer primer and oligo(dT) primer.

We designed *T. virens*, *T. atroviride* and *T. reesei* tiling array with 60mer oligonucleotides (oligo length) each 93 bp apart (oligo distance), using the unmasked fasta file of the genomes of *T. atroviride* [http://genome.jgi.doe.gov/Triat2/Triat2.info.html], *T. vir*ens [http://genome.jgi-psf.org/Trive1/Trive1.home.html] and *T. reesei* [http://genome.jgi-psf.org/Trire2/Trire2.home.html]. The design was done with Teolenn [54] using a maximum prefix length match set to 30 for the uniqueness calculation made by genome tools. Complexity is evaluated using the masked genome by counting the number of masked bases for each probe. T_m values are calculated using the nearest neighbor thermodynamic model. No filters were applied after probe parameter

calculations. In order to obtain the probe quality score, the calculated parameters were weighted as follows: 0.4 for T_m, 0.3 for uniqueness, 0.2 for GC content, and 0.1 for complexity. To get final oligonucleotide scores, a weighting of 0.75 was assigned to quality scores, and a weighting of 0.25 was assigned to position scores. Teolenn software designed 415373, 387105 and 359089 final probes respectively for *T. atroviride*, *T. virens*, and *T. reesei*. Oligonucleotides were loaded on Agilent eArray software and 2 × 400 k microarrays were obtained from Agilent. The microarray data and related protocols are available at the GEO web site [www.ncbi.nlm.nih.gov/geo/] under accession number GSE23438. Briefly, the RNAs of two independent biological replicates for each condition (which in turn consisted of pooled RNA preparations from at least three separate cultivations) were reverse-transcribed and labeled with Cy3 or Cy5 dye using the indirect labeling procedure. Dye bias was eliminated using dye switch labeling protocol. We then hybridized 1.5 μg of labeled cDNA with the 2x400k DNA chip (Agilent). The array was read using an Agilent G2505C DNA microarray scanner and the TIFF images extracted with the Agilent Feature Extraction software (version 10.5.1.1) using the 20bit coding ability. Data pre-treatment was applied on each result file to discard flagged spots by Feature Extraction software. The data were normalized without background subtraction by the global Lowess method performed with the Goulphar software [55]. For each experimental condition, the two file results were merged together. For each probe the hybridization ratio was linked to genome annotation coming from the DOE JGI website for a respective genome. The final ratio for each transcript was obtained by averaging the detected hybridization values from all probes located inside the coding sequence on the matching strand. Transcripts with no or only one probe marked as detected were discarded from further analysis. Finally we kept only transcript with a final hybridization ratio greater than $\log_2$ (ratio) = 1.5 or lower than $\log_2$ (ratio) = −1.5. The list of statistically significant differentially expressed genes was obtained by applying the linear modeling approach implemented in lmFit package for Python and the empirical Bayes statistics implemented by eBayes from the limma R package (http://bioinf.wehi.edu.au/limma/; [16]).

Gene identification

The genomes of *T. reesei* QM6a, *T. atroviride* IMI 206040 and *T. virens* Gv29-8 have been annotated [4] and were used as a source for identification of the genes found in this study. A gene was considered to encode a homologue of an already identified protein if it had the Expect value (E) in blastp of $< e^{-100}$ with the next Sordariomycetes member. All unknown and orphan (unique) proteins were subjected to blastp search (May, 2012) to check whether any of them has been identified for another fungus since the *Trichoderma* annotations. Orphan proteins were defined as such that did not occur in any other Pezizomycotina with an E-value $> e^{-20}$. All other proteins, for which no function could be predicted, were termed as unknown.

Statistical data evaluation

Statistical analyses of microarray data were performed using Statistica 6.1 (StatSoft, Inc., Tulsa, OK, USA). For this purpose the cumulative matrix has been assembled (Additional file 1: Table S1-S3). In this matrix every gene was considered as a case and the following grouping variable (predictors) were integrated: protein ID, gene annotation, species, protein family (Pfam), gene regulation (up-regulated/down-regulated), fold gene regulation prior, at and after the contact, and functional category (FunCat). Data were explored by means of descriptive statistics (mean values and standard deviations were calculated for each predictor). Hypotheses were tested using one-way main effects or factorial ANOVA analyses with post-hoc comparisons with Tukey honest significant difference tests (Tukey HSD).

Mycoparasitic potential

The antagonistic potential was evaluated for each species based on dual confrontation plate assay carried out as described for the RNA extraction, except the plate were not covered with cellophane. The evaluation of the plates was carried out after 10 days of incubation (see Figure 1) and the growth of *Trichoderma* against itself was set up as a zero inhibition rate. The growth inhibition of *R. solani* was calculated implying that the distance between the plugs of both fungi is 100%. The inhibition of growth was calculated as a percentage of *Trichoderma* growth subtracted for a correction of the growth against itself. Overgrowth of the prey was calculated similarly using the distance between the *R. solani* plug and the confrontation zone with *Trichoderma* as 100%, and the growth of *Trichoderma* over the *R. solani* mycelium was then calculated as a percentage of overgrowth.

Real Time PCR quantification of transcripts

T. atroviride and *T. virens* were cultivated and harvested as described for the microarrays. DNase treated (DNase I, RNase free; Fermentas) RNA (5 μg) was reverse transcribed with the RevertAid™ First Strand cDNA Kit (Fermentas) according to the manufacturer's protocol with a combination of the provided oligo(dT) and random hexamer primers.

All real-time PCR experiments were performed on a Bio-Rad (Hercules, CA) iCycler IQ. For the reaction the IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was

prepared for 25 μl assays with standard $MgCl_2$ concentration (3 mM) and a final primer concentration of 100 nM each. Primers used are given in Additional file 1: Table S4. The amplification protocol consisted of an initial denaturation step (3 min at 95°C) followed by 40 cycles of denaturation (15 sec at 95°C), annealing (20 sec at 52 and 52.5°C for *T. virens* and *T. atroviride* respectively) and extension (72°C for 10 sec for both species). *Tef1* gene which maintained constant over all conditions (± 20 relative %) was used as the normalizer. Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA (1, 0.1, 10^{-2} and 10^{-3}). Amplification efficiency was then calculated from the given slopes in the IQ5 Optical system Softwarev2.0. The qPCR were performed with the cDNA of 5 pooled biological replicates for each species and condition. Expression ratios were calculated using Livak test model [56] and are given in the Additional file 1: Table S4. Zero values for qPCR indicate the expression levels between $|\log_2 \text{(ratio)}| > 1.5$ as these were not treated as significantly regulated in the microarray analysis.

Additional file

Additional file 1: Table S1. List of *Trichoderma reesei* genes, which expression has been significantly changed in response to mycoparasitism to *R. solani.* **Table S2.** List of *Trichoderma virens* genes, which expression has been significantly changed in response to mycoparasitism to *R. solani.* **Table S3.** List of *Trichoderma atroviride* genes, which expression has been significantly changed in response to mycoparasitism to *R. solani.* **Table S4.** Real Time PCR quantification of selected genes.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LA performed the experiments with *Tr* and *Ta*, carried out qPCR, did the statistical data evaluation, prepared some figures and supplementary materials and participated in ms writing. SLC and FC made microarrays. SG made experiments with *Tv*. VS participated in the design of the study and gene annotation. CPK and ISD designed the study, evaluated the data and wrote the manuscript. ISD prepared some figures and supplementary materials. All authors read and approved the manuscript.

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Author details

[1]Research Area Biotechnology and Microbiology, Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstrasse 1a, A-1060, Vienna, Austria. [2]École normale supérieure, Institut de Biologie de l'ENS, IBENS, Paris F-75005, France. [3]Inserm, U1024, Paris F-75005, France. [4]CNRS, UMR 8197, Paris F-75005, France. [5]UPMC Univ Paris 06, UMR7622, Laboratoire de Biologie du Développement, 9 quai St. Bernard, F-75005, Paris, France. [6]CNRS, UMR7622, Laboratoire de Biologie du Développement, 9 quai St. Bernard, F-75005, Paris, France. [7]Austrian Center of Industrial Biotechnology (ACIB), GmBH c/o Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstrasse 1a, A-1060, Vienna, Austria.

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References

1. Druzhinina IS, Seidl-Seiboth V, Herrera-Estrella A, Horwitz BA, Kenerley CM, Monte E, Mukherjee PK, Zeilinger S, Grigoriev IV, Kubicek CP: *Trichoderma*: the genomics of opportunistic success. *Nat Rev Microbiol* 2011, **9**(10):749–759.
2. Benítez T, Rincón AM, Limón MC, Codón AC: Biocontrol mechanisms of *Trichoderma* strains. *Int Microbiol* 2004, **7**:249–260.
3. Howell CR: Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis* 2003, **87**:4–10.
4. Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, Thon M, Zeilinger S, Casas-Flores S, Horwitz BA, Mukherjee PK, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, Atanasova L, Cervantes-Badillo MG, Challacombe J, Chertkov O, McCluskey K, Coulpier F, Deshpande N, von Döhren H, Ebbole DJ, Esquivel-Naranjo EU, Fekete E, Flipphi M, Glaser F, Gómez-Rodríguez EY, Gruber S, *et al*: Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. *Genome Biol* 2011, **12**(4):R40.
5. Reithner B, Ibarra-Laclette E, Mach RL, Herrera-Estrella A: Identification of mycoparasitism-related genes in *Trichoderma atroviride*. *Appl Environ Microbiol* 2011, **77**(13):4361–4370.
6. Omann MR, Lehner S, Escobar Rodríguez C, Brunner K, Zeilinger S: The seven-transmembrane receptor Gpr1 governs processes relevant for the antagonistic interaction of *Trichoderma atroviride* with its host. *Microbiology* 2012, **158**(Pt 1):107–118.
7. Catalano V, Vergara M, Hauzenberger JR, Seiboth B, Sarrocco S, Vannacci G, Kubicek CP, Seidl-Seiboth V: Use of a non-homologous end-joining -deficient strain (delta-ku70) of the biocontrol fungus *Trichoderma virens* to investigate the function of the laccase gene lcc1 in sclerotia degradation. *Curr Genet* 2011, **57**(1):13–23.
8. Liu PG, Yang Q: Identification of genes with a biocontrol function in *Trichoderma harzianum* mycelium using the expressed sequence tag approach. *Res Microbiol* 2005, **156**(3):416–423.
9. Vizcaíno JA, Redondo J, Suárez MB, Cardoza RE, Hermosa R, González FJ, Rey M, Monte E: Generation, annotation, and analysis of ESTs from four different *Trichoderma* strains grown under conditions related to biocontrol. *Appl Microbiol Biotechnol* 2007, **75**(4):853–862.
10. Suárez MB, Vizcaíno JA, Llobell A, Monte E: Characterization of genes encoding novel peptidases in the biocontrol fungus *Trichoderma harzianum* CECT 2413 using the TrichoEST functional genomics approach. *Curr Genet* 2007, **51**:331–342.
11. Samolski I, de Luis A, Vizcaíno JA, Monte E, Suárez MB: Gene expression analysis of the biocontrol fungus *Trichoderma harzianum* in the presence of tomato plants, chitin, or glucose using a high-density oligonucleotide microarray. *BMC Microbiol* 2009, **9**:217.
12. Liu Z, Yang X, Sun D, Song J, Chen G, Juba O, Yang Q: Expressed sequence tags-based identification of genes in a biocontrol strain *Trichoderma asperellum*. *Mol Biol Rep* 2010, **37**(8):3673–3681.
13. Rubio MB, Hermosa R, Reino JL, Collado IG, Monte E: Thctf1 transcription factor of *Trichoderma harzianum* is involved in 6-pentyl-2H-pyran-2-one production and antifungal activity. *Fungal Genet Biol* 2009, **46**(1):17–27.
14. Ogoshi A: Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kuhn. *Annu Rev Phytopathol* 1987, **25**:125–143.
15. Dean R, VAN Kan JA, Pretorius ZA, Hammond-Kosack KE, DI Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD: The Top 10 fungal pathogens in molecular plant pathology. *Mol Plant Pathol* 2012, **13**(4):414–430.
16. Smyth GK: Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004, **3**:Article 3.
17. Baker SE, Perrone G, Richardson NM, Gallo A, Kubicek CP: Phylogenomic analysis of polyketide synthase-encoding genes in *Trichoderma*. *Microbiology* 2012, **158**(Pt 1):147–154.
18. Seiboth B, Aghcheh RK, Phatale PA, Linke R, Hartl L, Sauer DG, Smith KM, Baker SE, Freitag M, Kubicek CP, *et al*: The putative protein methyltransferase LAE1 controls cellulase gene expression in *Trichoderma reesei*. *Mol Microbiol* 2012, **84**(6):1150–1164.

19. Lorito M, Woo SL, Harman GE, Monte E: Translational research on *Trichoderma*: from 'omics to the field. *Annu Rev Phytopathol* 2010, **48**:395–417.
20. Seidl V, Song LF, Lindquist EA, Gruber S, Koptchinskiy A, Zeilinger S, Schmoll M, Martinez P, Sun J, Grigoriev I, Herrera-Estrella A, Baker SE, Kubicek CP: Transcriptomic response of the mycoparasitic fungus Trichoderma atroviride to the close presence of a fungal prey. *BMC Genomics* 2009, **10**:567.
21. Rossmann AY, Samuels GJ, Rogerson CT, Lowen R: Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). *Stud Mycol* 1999, **42**:1–83.
22. Stricker AR, Grosstessner-Hain K, Würleitner E, Mach RL: Xyr1 (xylanase regulator 1) regulates both the hydrolytic enzyme system and D-xylose metabolism in *Hypocrea jecorina*. *Eukaryot Cell* 2006, **5**(12):2128–2137.
23. Patron NJ, Waller RF, Cozijnsen AJ, Straney DC, Gardiner DM, Nierman WC, Howlett BJ: Origin and distribution of epipolythiodioxopiperazine (ETP) gene clusters in filamentous ascomycetes. *BMC Evol Biol* 2007, **7**:174.
24. Wilhite SE, Lumsden RD, Straney DC: Mutational analysis of gliotoxin production by the biocontrol fungus *Gliocladium virens* in relation to suppression of *Pythium* damping-off. *Phytopathology* 1994, **84**:816–821.
25. Lumsden RD, Locke JC: Biological control of damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* with *Gliocladium virens* in soilless mix. *Phytopathology* 1989, **79**:361–366.
26. Lumsden RD, Locke JC, Adkins ST, Walter JF, Ridout CJ: Isolation and localization of the antibiotic gliotoxin produced by *Gliocladium virens* from alginate prill in soil and soilless media. *Phytopathology* 1992, **82**:230–235.
27. Wilhite SE, Straney DC: Timing of gliotoxin biosynthesis in the fungal biological control agent *Gliocladium virens* (*Trichoderma virens*). *Appl Microbiol Biotechnol* 1996, **45**:513–518.
28. Kumar A, Paietta JV: The sulfur controller-2 negative regulatory gene of *Neurospora crassa* encodes a protein with beta-transducin repeats. *Proc Natl Acad Sci U S A* 1995, **92**(8):3343–3347.
29. Helmstaedt K, Strittmatter A, Lipscomb WN, Braus GH: Evolution of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase-encoding genes in the yeast *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 2005, **102**:9784–9789.
30. Seidl V, Gamauf C, Druzhinina IS, Seiboth B, Hartl L, Kubicek CP: The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. *BMC Genomics* 2008, **11**(9):327.
31. Reino JL, Guerrero RF, Hernández-Galan R, Collado IG: Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochem Rev* 2008, **7**:89–123.
32. Serrano-Carreon L, Hathout Y, Bensoussan M, Belin JM: Metabolism of Linoleic Acid or Mevalonate and 6-Pentyl-α-Pyrone Biosynthesis by *Trichoderma* Species. *Appl Environ Microbiol* 1993, **59**:2945–2950.
33. Mukherjee PK, Horwitz BA, Kenerley CM: Secondary metabolism in *Trichoderma* - a genomic perspective. *Microbiol* 2012, 158:35–45.
34. Kulkarni RD, Thon MR, Pan H, Dean RA: Novel G-protein-coupled receptor-like proteins in the plant pathogenic fungus *Magnaporthe grisea*. *Genome Biol* 2005, **6**(3):R24.
35. Muthumeenakshi S, Sreenivasaprasad S, Rogers CW, Challen MP, Whipps JM: Analysis of cDNA transcripts from *Coniothyrium minitans* reveals a diverse array of genes involved in key processes during sclerotial mycoparasitism. *Fungal Genet Biol* 2007, **44**(12):1262–1284.
36. Gronover CS, Schumacher J, Hantsch P, Tudzynski B: A novel seven-helix transmembrane protein BTP1 of *Botrytis cinerea* controls the expression of GST-encoding genes, but is not essential for pathogenicity. *Mol Plant Pathol* 2005, **6**(3):243–256.
37. Xiong S, Fan J, Kitazato K: The antiviral protein cyanovirin-N: the current state of its production and applications. *Appl Microbiol Biotechnol* 2010, **86**(3):805–812.
38. Neethling D, Nevalainen H: Mycoparasitic species of *Trichoderma* produce lectins. *Can J Microbiol* 1996, **42**(2):141–146.
39. Plett JM, Martin F: Poplar root exudates contain compounds that induce the expression of MiSSP7 in *Laccaria bicolor*. *Plant Signal Behav* 2012, **7**(1):12–15.
40. Papapostolou I, Georgiou CD: Superoxide radical induces sclerotial differentiation in filamentous phytopathogenic fungi: a superoxide dismutase mimetics study. *Microbiol* 2010, **156**:960–966.
41. Aliferis KA, Jabaji S: Metabolite composition and bioactivity of *Rhizoctonia solani* sclerotial exudates. *J Agric Food Chem* 2010, **58**:7604–7615.
42. Ruocco M, Lanzuise S, Vinale F, Marra R, Turrà D, Woo SL, Lorito M: Identification of a new biocontrol gene in *Trichoderma atroviride*: the role of an ABC transporter membrane pump in the interaction with different plant-pathogenic fungi. *Mol Plant Microbe Interact* 2009, **22**:291–301.
43. Basile LJ, Willson RC, Sewell BT, Benedik MJ: Genome mining of cyanide-degrading nitrilases from filamentous fungi. *Appl Microbiol Biotechnol* 2008, **80**:427–435.
44. Michelsen CF, Stougaard P: Hydrogen cyanide synthesis and antifungal activity of the biocontrol strain *Pseudomonas fluorescens* In5 from Greenland is highly dependent on growth medium. *Can J Microbiol* 2011, **58**:381–390.
45. Palmer JM, Keller NP: Secondary metabolism in fungi: does chromosomal location matter? *Curr Opin Microbiol* 2010, **13**:431–436.
46. Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG, Grigoriev IV, Harris P, Jackson M, Kubicek CP, Han CS, Ho I, Larrondo LF, de Leon AL, Magnuson JK, Merino S, Misra M, Nelson B, Putnam N, Robbertse B, Salamov AA, Schmoll M, Terry A, Thayer N, Westerholm-Parvinen A, *et al*: Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nat Biotechnol* 2008, **26**:553–560.
47. Metz B, Seidl-Seiboth V, Haarmann T, Kopchinskiy A, Lorenz P, Seiboth B, Kubicek CP: Expression of biomass-degrading enzymes is a major event during conidium development in *Trichoderma reesei*. *Eukaryot Cell* 2011, **10**(11):1527–1535.
48. Cuomo CA, Güldener U, Xu JR, Trail F, Turgeon BG, Di Pietro A, Walton JD, Ma LJ, Baker SE, Rep M, Adam G, Antoniw J, Baldwin T, Calvo S, Chang YL, Decaprio D, Gale LR, Gnerre S, Goswami RS, Hammond-Kosack K, Harris LJ, Hilburn K, Kennell JC, Kroken S, Magnuson JK, Mannhaupt G, Mauceli E, Mewes HW, Mitterbauer R, Muehlbauer G, *et al*: The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. *Science* 2007, **317**:1400–1402.
49. Kasuga T, Mannhaupt G, Glass NL: Relationship between phylogenetic distribution and genomic features in *Neurospora crassa*. *PLoS One* 2009, **4**:e5286.
50. Strauss J, Reyes-Dominguez Y: Regulation of secondary metabolism by chromatin structure and epigenetic codes. *Fungal Genet Biol* 2011, **48**:62–69.
51. Bayram O, Braus GH: Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiol Rev* 2012, **36**(1):1–24.
52. Mukherjee PK, Kenerley CM: Regulation of morphogenesis and biocontrol properties in *Trichoderma virens* by a VELVET protein, Vel1. *Appl Environ Microbiol* 2010, **76**(7):2345–2352.
53. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979, **18**(24):5294–5299.
54. Jourdren L, Duclos A, Brion C, Portnoy T, Mathis H, Margeot A, Le Crom S: Teolenn: an efficient and customizable workflow to design high-quality probes for microarray experiments. *Nucleic Acids Res* 2010, **38**:e117.
55. Lemoine S, Combes F, Servant N, Le Crom S: Goulphar: Rapid access and expertise for standard two-color microarray normalization methods. *BMC Bioinformatics* 2006, **7**:467.
56. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. *Methods* 2001, **25**(4):402–408.

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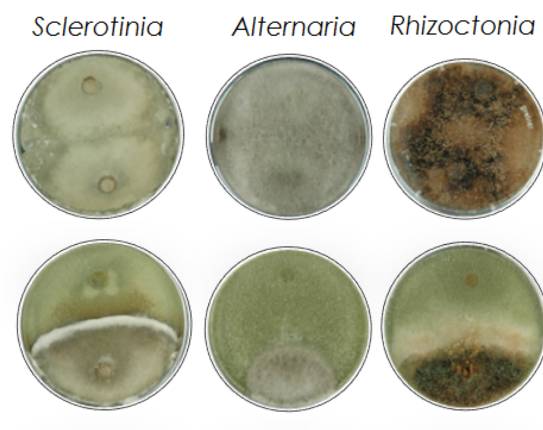
Conclusive remarks and contributions

Lea Atanasova (LA) performed the dual confrontation assays and extracted RNA for *T. reesei* and *T. atroviride*, partially annotated the transcripts, carried out qPCR and its analysis, did statistical data evaluation of transcriptomic analysis, prepared most of the figures and supplementary materials and participated in manuscript writing.

The transcriptomic response in confrontation with the model fungus *Rhizoctonia solani* underlined their different mechanisms of mycoparasitic response in all three species as well as in different stages of mycoparasitic attack. However, the question appeared if the ability to parasitize on other fungi was lost in this species or this property was gained by other so called strong mycoparasite *Trichoderma* spp. later in the evolution. With other words, was the ancestral nutritional mode of *Trichoderma* biotrophic (more exact mycotrophic) or saprotrophic as shown for *T. reesei*?

Nonetheless, little is known about *Trichoderma* mycoparasitism *in vivo*, but what could be studied so far *in vitro* implies that interactions of *Trichoderma* with other fungi are prey/host dependent and additionally the *Trichoderma* antagonistic potential is strain dependent rather than a specific trait of some species. Finally, our knowledge on *Trichoderma* mycoparasitism arrives from the fact that *Trichoderma* teleomorphs are associated with other fungi; from *in vitro* test on mostly plant pathogenic fungi; and from the fact that biocontrol preparations are efficient *in situ*.

2. MYCOPARASITISM IS *TRICHODERMA'S* INHERENT PROPERTY



2.1 Phylogenomics of *Trichoderma* reveals the origin of mycotrophy

Kubicek *et al. Genome Biology* 2011, **12**:R40
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RESEARCH **Open Access**

Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*

Christian P Kubicek[1*], Alfredo Herrera-Estrella[2], Verena Seidl-Seiboth[1], Diego A Martinez[3], Irina S Druzhinina[1], Michael Thon[4], Susanne Zeilinger[1], Sergio Casas-Flores[5], Benjamin A Horwitz[6], Prasun K Mukherjee[7], Mala Mukherjee[6], László Kredics[8], Luis D Alcaraz[2], Andrea Aerts[9], Zsuzsanna Antal[8], Lea Atanasova[1], Mayte G Cervantes-Badillo[5], Jean Challacombe[9], Olga Chertkov[9], Kevin McCluskey[10], Fanny Coulpier[11], Nandan Deshpande[12], Hans von Döhren[13], Daniel J Ebbole[14], Edgardo U Esquivel-Naranjo[2], Erzsébet Fekete[15], Michel Flipphi[16], Fabian Glaser[6], Elida Y Gómez-Rodríguez[5], Sabine Gruber[1], Cliff Han[9], Bernard Henrissat[17], Rosa Hermosa[4], Miguel Hernández-Oñate[2], Levente Karaffa[15], Idit Kosti[6], Stéphane Le Crom[11], Erika Lindquist[9], Susan Lucas[9], Mette Lübeck[18], Peter S Lübeck[18], Antoine Margeot[19], Benjamin Metz[1], Monica Misra[9], Helena Nevalainen[12], Markus Omann[1], Nicolle Packer[12], Giancarlo Perrone[20], Edith E Uresti-Rivera[5], Asaf Salamov[9], Monika Schmoll[1], Bernhard Seiboth[1], Harris Shapiro[9], Serenella Sukno[4], Juan Antonio Tamayo-Ramos[21], Doris Tisch[1], Aric Wiest[10], Heather H Wilkinson[14], Michael Zhang[9], Pedro M Coutinho[17], Charles M Kenerley[14], Enrique Monte[4], Scott E Baker[9,22] and Igor V Grigoriev[9]

Introduction

Mycoparasitism, a lifestyle where one fungus is parasitic on another fungus, has special relevance when the prey is a plant pathogen, providing a strategy for biological control of pests for the management of plant diseases. Today about 60% of the biofungicide market is based on formulations with the common filamentous fungus *Trichoderma* (Verma et al., 2007).

The genome sequences of the two biocontrol species of *Trichoderma* - *T. atroviride* and *T. virens* (Kubicek et al., 2011) showed a remarkable differences to the genome of the industrial cellulase producer *T. reesei* (Martinez et al., 2008) that was reflected in (i) genome size (ii) conservation of gene order, (iii) lack of active mobile elements probably due to repeat-induced point mutation (iv) several gene families expansions in the two above mentioned mycoparasitic species relative to *T. reesei* or other ascomycetes, and are overrepresented in non-syntenic genome regions. Also in contrast to the genomes of other multicellular ascomycetes such as aspergilli (Fedorova et al., 2008; Galagan et al., 2009), *Trichoderma* appear to be have the highest level of synteny of all genomes investigated (96 % for *T. reesei* and still 78/79% for *T. virens* and *T. atroviride* respectively, versus 68 to 75% in aspergilli)., and most of the differences to other ascomycetes occur in the non-syntenic areas. Nevertheless, at a molecular level the three species are as distant from each other as apes from Pices (fishes) or Aves (birds) (Fedorova et al., 2008), suggesting a mechanism maintaining this high genomic synteny. Espagne et al. (2008) proposed that a discrepancy of genome evolution between *P. anserina*, *N. crassa* and the aspergilli and saccharomycotina yeasts is based on the difference between heterothallic and homothallic fungi: in heterothallics the presence of interchromosomal translocation could result in chromosome breakage during meiosis and reduced fertility, whereas homothallism allows translocations to be present in both partners and thus have fewer consequences on fertility. Since *Trichoderma* is heterothallic (Seidl et al., 2009), this explanation is also applicable to it. However, another mechanism, meiotic silencing of unpaired DNA (Shiu et al., 2002) - which has also been proposed for *P. anserina* (Espagne et al., 2008), and which eliminates progeny in crosses involving rearranged chromosomes in one of the partners - may not function in *Trichoderma* because one of the essential genes (SAD2; Borkovich et al., 2004) is missing.

T. reesei - a relatively rare tropical species which is mainly known from its teleomorphic stage *in vivo* - is commonly considered to be a moderate to weak mycoparasite, but the origin of mycoparasitism in the genus remains obscured. Sung et al. (2008) refer to the general fungicolous nature of the Hypocreaceae family to which *Trichoderma* is attributed, however the genus itself is considered to be saprotrophic on plant biomass (Sung et al., 2008). The purpose of this work was to apply the phylogenomic approach to the three *Trichoderma* species with sequenced genomes which have different mycoparasitic potentials and

compare their relations with fungi from related genera using phylogenetic approach, in order to test whether profound mycoparasitism of *T. atroviride* and *T. virens* is an advanced evolutionally feature compared to *T. reesei* or *vice versa* the later species lost the ability to feed on other fungi.

Materials and Methods

One-hundred genes were randomly selected from *T. atroviride*, *T. virens* and *T. reesei*, and *Gibberella zeae* and *Chaetomium globosum* genomes based on their property to fulfill two requirements: they were in synteny in all four genomes, and they were true orthologues (no other gene encoding a protein with amino acid similarity >50% present). After alignment, the concatenated 10,449 amino acids were subjected to Bayesian analysis (Yang et al., 1997) using 1 million MCMC generations. The respective cDNA sequences (31,347 nucleotides) were also concatenated, and Ks/Ka ratios determined using DNASp5 (Librado et al., 2009)

The genus-wide phylogram of *Trichoderma* was constructed by Bayesian analysis of partial exon nucleotide sequences (824 total characters from which 332 were parsimony-informative) of the *rpb2* gene (encoding RNA polymerase B II) from 110 ex-type strains, thereby spanning the major clades of the genus. The tree was obtained after 5 million MCMC generations sampled for every 100 trees, using burnin = 1200 and applying the general time reversible model of nucleotide substitution. The NCBI ENTREZ accession numbers are: 1 [HQ260620]; 3 [DQ08724]; 4 [HM182969]; 5 [HM182984]; 6 [HM182965]; 7 [AF545565]; 8[AF545517]; 16 [FJ442769]; 17 [AY391900]; 18 [FJ179608]; 19 [FJ442715]; 20 [FJ442771]; 21 [AY391945]; 22 [EU498358]; 23 [DQ834463]; 24[FJ442725]; 25 [AF545508]; 26 [AY391919]; 27 [AF545557]; 28 [AF545542]; 29 [FJ442738]; 30[AF545550]; 31 [AY391909]; 32 [AF545516]; 33 [AF545518]; 34 [AF545512]; 35 [AF545510]; 36 [AF545514]; 37 [AY391921]; 38 [AF545513]; 39 [AY391954]; 40 [AY391944]; 41 [AF545534]; 42 [AY391899]; 43 [AY391907]; 44 [AF545511]; 45 [AY391929]; 46 [AF545540]; 47 [AY391958]; 48[AY391924]; 49 [AF545515]; 50 [AY391957]; 51[AF545551]; 52 [AF545522]; 53 [FJ442714]; 54 [AF545509]; 55 [AY391959]; 56 [DQ087239]; 57[AF545553]; 58 [AF545545]; 59 [DQ835518]; 60[DQ835521]; 61 [DQ835462]; 62 [DQ835465]; 63[DQ835522]; 64 [AF545560]; 65 [DQ835517]; 66[DQ345348]; 67 [AF545520]; 68 [DQ835455]; 69 [AF545562]; 70 [AF545563]; 71 [DQ835453]; 72 [FJ179617]; 73 [DQ859031]; 74 [EU341809]; 75 [FJ179614]; 76 [DQ087238]; 77 [AF545564]; 78 [FJ179601]; 79 [FJ179606]; 80 [FJ179612]; 81 [FJ179616]; 82 [EU264004]; 83 [FJ150783]; 84 [FJ150767]; 85 [FJ150786]; 86 [EU883559]; 87 [FJ150785]; 88 [EU248602]; 89 [EU241505]; 90 [FJ442762]; 91 [FJ442741]; 92 [FJ442783]; 93 [EU341805]; 94 [FJ442723]; 95 [FJ442772]; 96 [EU2415023]; 97 [EU341801]; 98 [EU248600]; 99 [EU341808]; 100 [EU3418033]; 101 [EU2485942]; 102 [AF545519]; 103 [EU248603]; 104 [EU248607]; 105 [EU341806]; 106 [DQ086150]; 107 [DQ834460]; 108 [EU711362]; 109 [EU883557]; 110 [FJ150790].

Results

T. reesei, *T. atroviride* and *T. virens* each occupy distanced phylogenetic positions in the genus *Trichoderma*, as shown by a Bayesian *rpb2* tree of 110 *Trichoderma* taxa (Figure 2.1a). In order to determine which of the three species more likely resembles the ancestral state of *Trichoderma*, we performed a Bayesian phylogenetic analysis using a concatenated set of 100 proteins that were encoded by orthologous genes in

syntenic areas in the three *Trichoderma* species and also *G. zeae* and *C. globosum*. The result (Figure 2.1b) showed that *T. atroviride* occurs in a well-supported basal position to *T. virens* and *T. reesei*. These data indicate that *T. atroviride* resembles the more ancient state of *Trichoderma* and that both *T. virens* and *T. reesei* evolved later. The long genetic distance of *T. reesei* further suggests that it was apparently evolving faster than *T. atroviride* and *T. virens* since the time of divergence. To test this assumption, we compared the evolutionary rates of the 100 orthologous and syntenic gene families between the three *Trichoderma* species. The median values of the evolutionary rates (Ks and Ka) of *T. atroviride* - *T. reesei* and *T. virens* - *T. reesei* were all significantly higher (1.77 and 1.47, and 1.33 and 1.19, respectively) than those of *T. atroviride* - *T. virens* (1.13 and 0.96; all P values <0.05 by the two-tailed Wilcoxon rank sum test). This result supports the above suggestion that *T. reesei* has been evolving faster than *T. atroviride* and *T. virens*.

Discussion

Our data suggest that the ancestral state of *Trichoderma* was mycoparasitic. This conclusion was supported by the genus-wide test for the mycoparasitic potential that revealed that all *Trichoderma* species are capable to parasitize on other fungi when several prey/host are tested (Druzhinina and Kubicek, 2013). *T. reesei* appears to have lost 23 % of genes present in *T. atroviride* and maintained in *T. virens*. In our transcriptomic study we have shown that, when sensing the pray, *T. atroviride* and *T. virens* induce the expression of the genes involved in the attack, whereas *T. reesei* attempts to compete with the other fungus by faster acquisition of nutrients (Atanasova et al., 2013; Chapter 1). Thus we conclude that *T. reesei* is a derived species that lost its mycoparasitic potential due to the lost of a great portion of genes by adapting to the saprotrophic way of life. This supports an earlier speculation (Rossmann et al., 1999) that the ancestors of *Trichoderma* were mycoparasites on wood-degrading basidiomycetes and acquired saprotrophic characteristics to follow their prey into their substrate. Indirect evidence for this habitat shift in *T. reesei* was also presented by Slot and Hibbett (2007), who demonstrated that *T. reesei* – after switching to a specialization on a nitrogen-poor habitat (decaying wood) - has acquired a nitrate reductase gene (which was apparently lost earlier somewhere in the Sordariomycetes lineage) by horizontal gene transfer from basidiomycetes. It is also possible that the biotrophic *Trichoderma* ancestor species remained on the decomposed wood after they digested the basidiomycete host and evolved into saprotrophs in order to adapt to a novel ecological niche. This hypothesis is supported by the fact that many *Trichoderma* teleomorphs (e.g. *T. subeffusum, T. luteffusum, T. polysporum, T. phellinicola, T. sulphurea* etc.) associated with basidiomes are often found on medium to strongly decayed wood (see Jaklitsch et al., 2011).

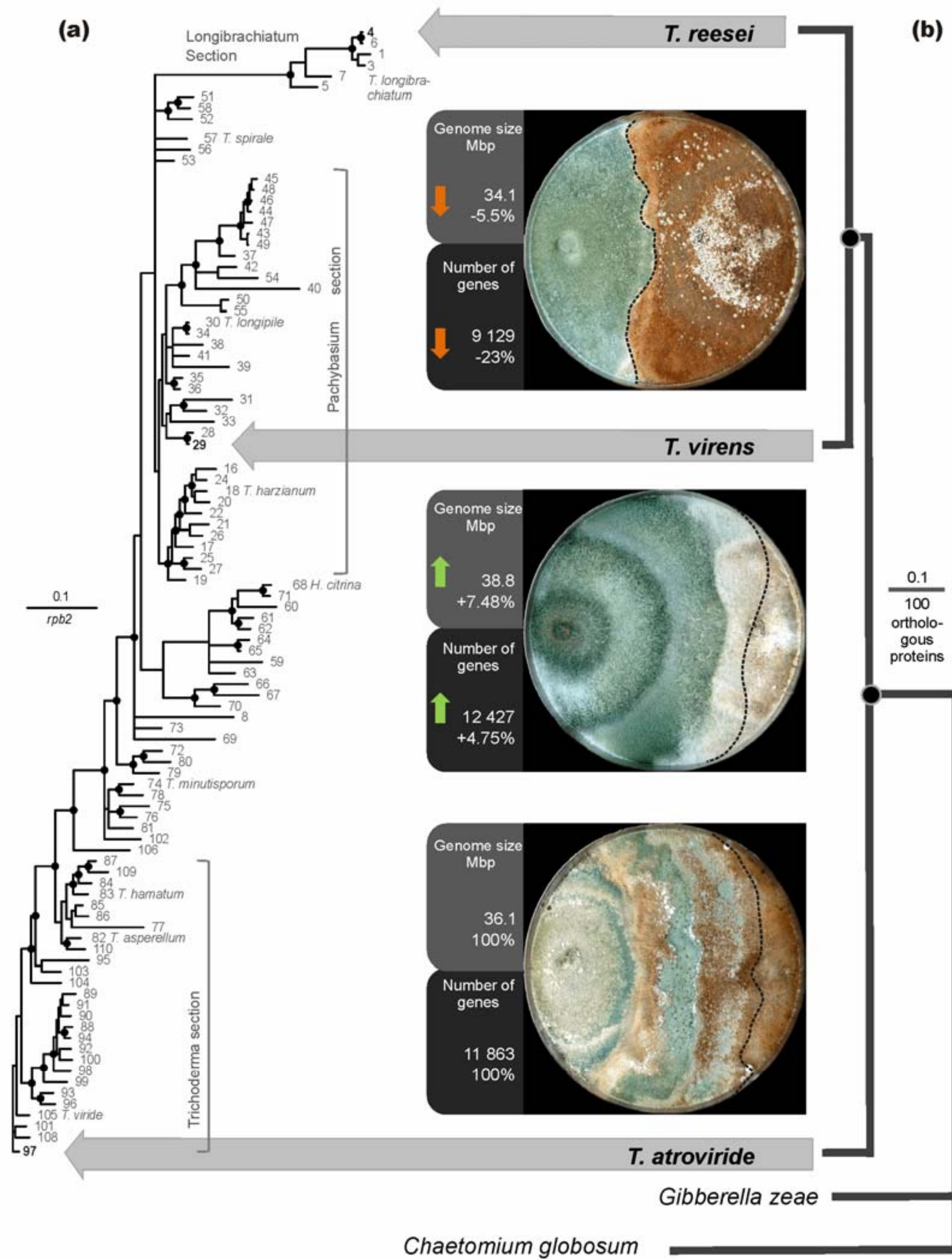


Figure 2.1: Mycoparasitism is an ancient life style of *Trichoderma*. (a) Position of *T. atroviride*, *T. virens* and *T. reesei* within the genus *Trichoderma*. The positions of *T. reesei*, *T. virens* and *T. atroviride* are 4, 29 and 97, respectively - shown in bold), and a few hallmark species are given by their names. For the identities of the other species, see the gene accession numbers (Materials and Methods). (b) Bayesian phylogram based on the analysis of amino acid sequences of 100 orthologous syntenic proteins (MCMC, 1 million generations, 10,449 characters) in *T. reesei*, *T. virens* and *T. atroviride*, *Gibberella zeae* and *Chaetomium globosum*. Circles above nodes indicate 100% posterior probabilities and significant bootstrap coefficients. The numbers in the boxes between (a) and (b) indicate the genome sizes and gene counts and percentage net gain regarding *T. atroviride*. Photoplates show the mycoparasitic reaction after the contact between Trichoderma species and *Rhizoctonia solani*. *Trichoderma* species are always on the left side; dashed lines indicate the position of *Trichoderma* overgrowth of *R. solani*. (From Kubicek et al., 2011)

References

Atanasova L, Druzhinina IS and Jaklitsch WM. (2013) Two hundred *Trichoderma* species recognized based on molecular phylogeny. In *Trichoderma*: Biology and Applications, Eds. Mukherjee PK, Sigh US, Horwitz BA, Schmoll M, Mukherjee M. CABI of Nosworthy Way, UK, Wallingford, Oxon, in press.

Borkovich KA, Alex LA, Yarden O, Freitag M, Turner GE, Read ND, Seiler S, Bell-Pedersen D, Paietta J, Plesofsky N, Plamann M, Goodrich-Tanrikulu M, Schulte U, Mannhaupt G, Nargang FE, Radford A, Selitrennikoff C, Galagan JE, Dunlap JC, Loros JJ, Catcheside D, Inoue H, Aramayo R, Polymenis M, Selker EU, Sachs MS, Marzluf GA, Paulsen I, Davis R, Ebbole DJ, et al. (2004) Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. Microbiol Mol Biol Rev, 68:1-108.

Druzhinina IS, Kubicek CP (2013) Ecological genomics of *Trichoderma*. *In Ecological genomics of fungi*. Ed. by Martin F. Wiley-Blackwell, Oxford, UK, in press.

Espagne E, Lespinet O, Malagnac F, Da Silva C, Jaillon O, Porcel BM, Couloux A, Aury JM, Ségurens B, Poulain J, Anthouard V, Grossetete S, Khalili H, Coppin E, Déquard-Chablat M, Picard M, Contamine V, Arnaise S, Bourdais A, Berteaux-Lecellier V, Gautheret D, de Vries RP, Battaglia E, Coutinho PM, Danchin EG, Henrissat B, Khoury RE, Sainsard-Chanet A, Boivin A, Pinan-Lucarré B, et al. (2008) The genome sequence of the model ascomycete fungus *Podospora anserina*. Genome Biol, 9:R77.

Fedorova ND, Khaldi N, Joardar VS, Maiti R, Amedeo P, Anderson MJ, Crabtree J, Silva JC, Badger JH, Albarraq A, Angiuoli S, Bussey H, Bowyer P, Cotty PJ, Dyer PS, Egan A, Galens K, Fraser-Liggett CM, Haas BJ, Inman JM, Kent R, Lemieux S, Malavazi I, Orvis J, Roemer T, Ronning CM, Sundaram JP, Sutton G, Turner G, Venter JC, et al. (2008) Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. PLoS Genet, 4:e1000046.

Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee SI, Baştürkmen M, Spevak CC, Clutterbuck J, Kapitonov V, Jurka J, Scazzocchio C, Farman M, Butler J, Purcell S, Harris S, Braus GH, Draht O, Busch S, D’Enfert C, Bouchier C, Goldman GH, Bell-Pedersen D, Griffiths-Jones S, Doonan JH, Yu J, Vienken K, Pain A, Freitag M, et al. (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and A. oryzae. Nature, 438:1105-1115.

Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, Thon M, Zeilinger S, Casas-Flores S, Horwitz BA, Mukherjee PK, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, Atanasova L, Cervantes-Badillo MG et al. (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol, 12(4):R40.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25(11):1451-1452.

Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG, Grigoriev IV, Harris P, Jackson M, Kubicek CP, et al. (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat Biotechnol, 26(5):553-60.

Rossmann AY, Samuels GJ, Rogerson CT, Lowen R (1999) Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). Stud Mycol, 42:1-83.

Seidl V, Seibel C, Kubicek CP, Schmoll M (2009) Sexual development in the industrial workhorse Trichoderma reesei. Proc Natl Acad Sci USA, 106:13909-13914.

Shiu PK, Metzenberg RL (2002) Meiotic silencing by unpaired DNA: properties, regulation and suppression. Genetics, 161:1483-1495.

Slot JC, Hibbett DS (2007) Horizontal transfer of a nitrate assimilation gene cluster and ecological transitions in fungi: a phylogenetic study. PLoS One, 2:e1097.

Sung GH, Poinar GO Jr, Spatafora JW (2008) The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbioses. Mol Phylogenet Evol 49(2):495-502.

Verma M, Brar SK, Tyagi RD, Surampalli RY, Valero JR (2007) Antagonistic fungi, *Trichoderma* spp.: panoply of biological control. Biochem Eng J, 37:1-20.

Yang Z, Rannala B (1997) Bayesian phylogenetic inference using DNA sequences: a Markov Chain Monte Carlo Method. Mol Biol Evol, 14:717-724.

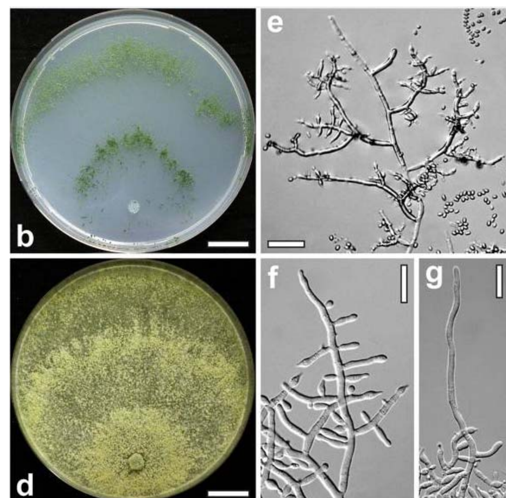
Contributions

Materials presented in this chapter were incorporated in

Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, Thon M, Zeilinger S, Casas-Flores S, Horwitz BA, Mukherjee PK, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, **Atanasova L**, Cervantes-Badillo MG, Challacombe J, Chertkov O, McCluskey K, Coulpier F, Deshpande N, von Döhren H, Ebbole DJ, Esquivel-Naranjo EU, Fekete E, Flipphi M, Glaser F, Gómez-Rodríguez EY, Gruber S, Han C, Henrissat B, Hermosa R, Hernández-Oñate M, Karaffa L, Kosti I, Le Crom S, Lindquist E, Lucas S, Lübeck M, Lübeck PS, Margeot A, Metz B, Misra M, Nevalainen H, Omann M, Packer N, Perrone G, Uresti-Rivera EE, Salamov A, Schmoll M, Seiboth B, Shapiro H, Sukno S, Tamayo-Ramos JA, Tisch D, Wiest A, Wilkinson HH, Zhang M, Coutinho PM, Kenerley CM, Monte E, Baker SE, Grigoriev IV (2011) **Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*.** Genome Biology, 12:R40.

LA retrieved the sequences of 100 orthologous proteins from syntenic areas of *Trichoderma atroviride, T. virens and T. reesei* and also *Giberella zeae* and *Chaetomium globosum,* performed the phylogenetic analysis and contributed to its interpretation.

2.2 Clonal species *Trichoderma parareesei* sp. nov. likely resembles the ancestor of the cellulase producer *Hypocrea jecorina/T. reesei*



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Clonal Species *Trichoderma parareesei* sp. nov. Likely Resembles the Ancestor of the Cellulase Producer *Hypocrea jecorina*/*T. reesei*▽†

Lea Atanasova,[1]‡ Walter M. Jaklitsch,[2]‡ Monika Komoń-Zelazowska,[1]
Christian P. Kubicek,[1] and Irina S. Druzhinina[1]*

Research Area of Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, A-1060 Vienna, Austria,[1] and Faculty Centre of Biodiversity, University of Vienna, Rennweg 14, A-1030 Vienna, Austria[2]

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We have previously reported that the prominent industrial enzyme producer *Trichoderma reesei* (teleomorph *Hypocrea jecorina*; Hypocreales, Ascomycota, Dikarya) has a genetically isolated, sympatric sister species devoid of sexual reproduction and which is constituted by the majority of anamorphic strains previously attributed to *H. jecorina*/*T. reesei*. In this paper we present the formal taxonomic description of this new species, *T. parareesei*, complemented by multivariate phenotype profiling and molecular evolutionary examination. A phylogenetic analysis of relatively conserved loci, such as coding fragments of the RNA polymerase B subunit II (*rpb2*) and GH18 chitinase (*chi18-5*), showed that *T. parareesei* is genetically invariable and likely resembles the ancestor which gave raise to *H. jecorina*. This and the fact that at least one mating type gene of *T. parareesei* has previously been found to be essentially altered compared to the sequence of *H. jecorina*/*T. reesei* indicate that divergence probably occurred due to the impaired functionality of the mating system in the hypothetical ancestor of both species. In contrast, we show that the sexually reproducing and correspondingly more polymorphic *H. jecorina*/*T. reesei* is essentially evolutionarily derived. Phenotype microarray analyses performed at seven temperature regimens support our previous speculations that *T. parareesei* possesses a relatively high opportunistic potential, which probably ensured the survival of this species in ancient and sustainable environment such as tropical forests.

Trichoderma reesei, the anamorph of the pantropical saprotrophic ascomycete *Hypocrea jecorina*, is used in the biotechnological industry for the production of cellulolytic and hemicellulolytic enzymes and recombinant proteins (13, 21). Accordingly, strong interest in this fungus has also recently reemerged in attempts to produce second-generation biofuels to reduce carbon dioxide emissions and the dependence on fossil fuels (oil) (15, 17).

Trichoderma reesei was originally collected on the Solomon Islands during World War II, where it destroyed canvas and other cellulose-containing materials of the U.S. army (18). It is unique among industrial fungi, as *T. reesei* was known only from the single isolate QM 6a for 50 years, and all genetically improved mutant strains used in biotechnology today have been derived from it. Kuhls et al. (14) found that *T. reesei* was indistinguishable from mycelial cultures of the pantropical ascomycete *Hypocrea jecorina*, on the basis of sequences of the internal transcribed spacer of the rRNA gene cluster and randomly amplified polymorphic DNA (RAPD) fingerprinting analysis. Thus, they established the anamorph-teleomorph connection, which is still valid. Other putative anamorphs of *H. jecorina* have more recently been identified as frequent inhabitants of soils in tropical forests of Southeast Asia, South America, and the South Pacific region (1, 7, 12). Druzhinina et al. (4), however, have recently shown that the majority of anamorphic strains are genetically isolated from *H. jecorina*/*T. reesei* and form at least two new phylogenetic species. The more frequent one (*T. parareesei* nom. prov. [4]) was shown to be closely related to and also sympatric with *H. jecorina* but exclusively asexual (clonal or agamospecies). In that work we also provided some details on the ecophysiological characteristics of *H. jecorina* and its sister species. We showed that the extremely efficient cellulase-producing strains are present in all these phylogenetic species and that in general their carbon metabolisms are very similar, although the clonal species are more versatile and efficient in the utilization of their preferred substrata. No details on carbon utilization profiles or temperature-dependent growth rates have been presented. Striking differences between *H. jecorina*/*T. reesei* and *T. parareesei* in conidiation intensity, photosensitivity, and mycoparasitism have been found, all suggesting that the latter species occupies a separate ecological niche and has much stronger opportunistic potential than *H. jecorina* (4).

Here we provide the formal taxonomic description of *Trichoderma parareesei* on the basis of macro- and micromorphologies, carbon utilization profiles at different temperatures, and phylogenetic analysis. We show that this species likely resembles the ancestor of *H. jecorina*/*T. reesei*.

* Corresponding author. Mailing address: Research Area of Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, A-1060 Vienna, Austria. Phone: 4315880117202. Fax: 4315880117299. E-mail: druzhini@mail.zserv.tuwien.ac.at.

‡ These two authors contributed equally to the manuscript and are listed in alphabetical order.

† Supplemental material for this article may be found at http://aem.asm.org/.

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MATERIALS AND METHODS

Material studied. The strains used in this work, their origin, and the NCBI GenBank sequence accession numbers are listed in Table 1. The isolates are stored at −80°C in 50% glycerol in the Collection of Industrial Microorganisms of Vienna University of Technology (TUCIM). For convenience, TUCIM num-

TABLE 1. Strains of *T. parareesei* and *H. jecorina*/*T. reesei* as well as other species from *Trichoderma* section *Longibrachiatum* used in this study[a]

Taxon	C.P.K. strain no.	Other strain no.	Origin	GenBank accession no.	
				chi18-5	*rpb2*
Reesei subclade					
Trichoderma parareesei sp. nov.	717 type	CBS 125925, TUB F-1066	Mexico	HM182987	HM182963
	3426	G.J.S. 07-26	Ghana	HM182991	HM182966
	661	CBS 125862, TUB F-728	Argentina	HM182990	HM182965
	665	TUB F-733	Argentina	HM182992	HM182967
	3420	G.J.S. 04-41	Brazil	HM182989	HM182964
	634	TUB F-730	Sri Lanka	HM182993	HM182968
	3692		Ethiopia	HM182987	HM182962
Hypocrea jecorina	160	G.J.S. 85-236	Celebes, Indonesia	HM183002	HM182977
	282	G.J.S. 97-177	French Guiana	HM182999	HM182974
	1392	G.J.S. 86-401	Puerto Rico	HM183005	HM182980
	1282	G.J.S. 85-249	Celebes, Indonesia	HM182998	HM182973
	158	G.J.S. 85-229	Celebes, Indonesia	HM183003	HM182978
	159	G.J.S. 85-230	Celebes, Indonesia	HM183004	HM182979
	1127	G.J.S. 93-23	New Caledonia	HM183000	HM182975
	1337	G.J.S. 93-22	New Caledonia	HM183001	HM182976
	917	CBS 383.78, QM 6a	Solomon Islands	HM182994	HM182969
	3418	G.J.S. 06-138	Cameroon	HM182997	HM182972
	3419	G.J.S. 06-140	Cameroon	HM182996	HM182971
	155	G.J.S. 86-404	Brazil	HM182995	HM182970
Other species from *Trichoderma* section *Longibrachiatum*					
Trichoderma sp. strain C.P.K. 524	523	TUB F-1034	Taiwan	HM183006	HM182981
	524	TUB F-1038	Taiwan	HM183007	HM182982
Trichoderma sp. strain C.P.K. 1837	1837		Ethiopia	HM183011	HM182986
T. longibrachiatum	1254	CBS 48978	Colombia	DQ087242[b]	EU401511[b]
T. saturnisporum	1266	CBS 33070, ATCC 18903	Georgia, USA	HM183009	HM182984
H. schweinitzii	2002	CBS 121275	Germany, Europe	HM183008	HM182983
T. pseudokoningii	1277	G.J.S. 81-300		HM183010	HM182985

[a] Strains of *T. parareesei* sp. nov., *H. jecorina*, and *Trichoderma* sp. strain C.P.K. 524 were published earlier (1, 4, 12). ATCC, American Type Culture Collection; CBS, Centralbureau voor Schimmelcultures; G.J.S., collection of Gary J. Samuels, USDA, Beltsville, MD; TUB, collection of George Szakacs, TU Budapest, Hungary.
[b] Previously published by Jaklitsch et al. (8).

bers (CPK) are used for the strains throughout the article; in addition, numbers of the original isolators' collections are listed in Table 1.

DNA extraction, PCR amplification, and sequencing. Mycelia were harvested after 2 to 4 days of growth on 3% malt extract agar (MEA; Merck, Germany) at 25°C, and genomic DNA was isolated using a Qiagen (Hilden, Germany) DNeasy plant minikit following the manufacturer's protocol. Amplification of fragments of *chi18-5* (GH18 chitinase CHI18-5, previously *ech42*) and *rpb2* (RNA polymerase subunit II ß) was performed as described previously (9). PCR fragments were purified (PCR purification kit; Qiagen) and sequenced at Eurofins MWG Operon (Ebersberg, Germany).

Phylogenetic analysis. Phylogenetic analysis was done essentially as described previously (4, 9). Briefly, DNA sequences were aligned by use of the Clustal X program (version 1.81) and visually checked in GeneDoc software (version 2.6). The possibility of intragenic recombination, which would prohibit the use of each locus for phylogenetic analysis, was tested by linkage disequilibrium-based statistics implemented in DnaSP software (version 4.50.3) (19). The neutral evolution was tested by Tajima's test, implemented in the same software. The interleaved NEXUS file was formatted using the PAUP* program (version 44.0b10) (22). As the sample size is relatively small (1,670 characters per 26 sequences for the biggest data set), the unconstrained GTR+I+G substitution model was applied to all sequence fragments (Table 2). Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling was performed using the MrBayes program (version 3.0B4) with two simultaneous runs of four incrementally heated chains for 1 million generations. Bayesian posterior probabilities (PPs) were obtained from the 50% majority rule consensus of trees sampled every 100 generations after removal of the first trees using the "burnin" command. The number of discarded generations was determined for each run on the basis of visual analysis of the plot showing the generation versus the log probability of observing the data. PP values lower than 0.95 were not considered significant. Summaries of the model parameters and nucleotide characteristics of the loci used are given in the Table 2.

Phenotype microarrays. Growth rates on different carbon sources were analyzed using a phenotype microarray system for filamentous fungi (Biolog Inc., Hayward, CA), as described by Druzhinina et al. (2) and Friedl et al. (5). Briefly, strains were cultivated on 3% MEA for 5 days. Conidial inocula were prepared by rolling a sterile, wetted cotton swab over sporulating areas of the plates. The conidia were then suspended in sterile Biolog FF inoculating fluid (0.25% Phytagel, 0.03% Tween 40), gently mixed, and adjusted to a transmission of 75% at 590 nm (using a Biolog standard turbidimeter calibrated to the Biolog standard for filamentous fungi). A total of 90 μl of the conidial suspension was dispensed into each of the wells of the Biolog FF microplates (Biolog Inc.), which were incubated at 15, 20, 25, 30, 35, 40, and 45°C in darkness. The optical density (OD) at 750 nm (for detection of mycelial growth [2, 5]) was measured after 18, 24, 42, 48, 66, 72, and 96 h using a microplate reader (Biolog Inc.). Statistical analyses were performed using the Statistica software package (version 6.1; StatSoft Inc., Tulsa, OK).

Growth rate determination on agar plates and morphological observations. The micromorphology of conidiation structures, growth rates, and the comparative morphology of cultures were determined and morphological terms were applied as described by Jaklitsch (10). Growth rates at 15, 25, 30, and 37°C were recorded in a single experiment using 2 strains each of *T. parareesei* and *T. reesei*. In addition, images of cultures grown on 3% MEA for 5 days at 25°C under alternating 12 h of light/12 h of dark were taken. Color was determined as described by Kornerup and Wanscher (11).

Phytotoxicity assays. Phytotoxicity assays were carried out using seeds of garden cress (*Lepidium sativum*) from Austrosaat AG (Vienna, Austria) and strains CBS 125925, CBS 125862, and C.P.K. 665 of *T. parareesei* and strains C.P.K. 917 (QM 6a), C.P.K. 1282, and C.P.K. 3419 of *H. jecorina.* Samples without *Trichoderma* were used as controls. The assay was designed on the basis of a 24-well plate, where each well was inoculated with one seed of *L. sativum*. Surface sterilization of the seeds was performed in 96% ethanol for 15 min, followed by washing of the seeds with sterile double-distilled water, with this step

TABLE 2. Nucleotide properties of phylogenetic markers and MCMC parameters[b]

Parameter	Phylogenetic marker		
	rpb2	*chi18-5*	Concatenated data set
Fragment characterization	Partial exon	Partial exon	Not applicable
No. of characters	852	818	1,670
No. parsimony informative	80	88	168
No. constant	685	680	1,365
Parameters of MCMC analysis			
Mean nucleotide frequency[a] (A/C/G/T)	0.24/0.28/0.28/0.20	0.23/0.33/0.24/0.20	0.24/0.30/026/0.20
Substitution rates[a]			
A↔C	0.12	0.10	0.11
A↔G	0.23	0.23	0.25
A↔T	0.03	0.12	0.06
C↔G	0.08	0.10	0.09
C↔T	0.44	0.37	0.39
G↔T	0.09	0.06	0.08
Alpha[a]	0.18	21.32	0.23
No. of MCMC generations (10^6)/no. of runs	1/2	1/2	2/2
PSRF[a]	1.00	1.00	1
No. of chains/temp (λ)	4/0.2	4/0.2	4/0.2
Sampling frequency	100	100	100
No. of discarded first generations	200	300	300
Total tree length (substitutions/site)	0.58	0.3	0.38

[a] Alpha (shape parameter of the gamma distribution) and potential scale reduction factor (PSRF) values were estimated after GTR MCMC sampling and burning.
[b] Twenty-nine sequences in total were analyzed.

being repeated twice. Individual seeds were introduced into 20 of the 24 wells filled with synthetic low nutrition agar (SNA) medium (for 1 liter, 1 g of KH_2PO_4 and KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$ and KCl, 0.2 g glucose and sucrose, and 20 g of agar-agar; Merck, Darmstadt, Germany), leaving 4 wells for the control of the fungal growth. Pregrown mycelium was introduced to 20 seedlings per strain. The plates were incubated at 25°C under a rhythmic illumination cycle (12 h of light/12 h of dark) for 8 days. Afterwards, the plants were inspected under a stereomicroscope and the lengths of their stems were measured.

MycoBank accession number. The sequence of *Trichoderma parareesei* Jaklitsch, Druzhinina & Atanasova, sp. nov., has been deposited in MycoBank under accession no. MB 515503 (Fig. 1).

RESULTS

Taxonomy. Differt a Trichodermate reesei absentia teleomorphosis, sporulatione multo magis abundante, conidiophoribus sinuosis et conidibus parvioribus, uniformioribus in forma et magnitudine. Phialides in agaro CMD lageniformes vel ampulliformes, 4.5 ad 11 μm longae, 2.5 ad 3.8 μm latae. Conidia viridia, ellipsoidea vel oblonga, glabra, 3.3 ad 6.2 μm longa, 2.5 ad 3.5 μm lata.

Morphology and growth rates of *T. parareesei*. Table 3 presents some characteristics of *T. parareesei*. The morphology and growth rates of *T. parareesei* follow. Optimum growth at 30 to 37°C on all media. On CMD (Sigma corn meal agar plus 10% dextrose) mycelium covering the plate within 3 days at 25 to 37°C. Colony on CMD at 25 to 37°C hyaline, thin; mycelium dense, not zonate, concentrated on the agar surface, of thick, radially arranged primary hyphae and numerous delicate secondary hyphae forming a reticulum. Hyphal width decreasing with increasing temperature. Aerial hyphae scant, short, becoming fertile. Autolytic activity and coilings lacking or inconspicuous. No diffusing pigment produced or agar turning slightly yellowish, 1B3 to 3B3; no distinct odor produced. Chlamydospores at 30 to 37°C after 1 week uncommon, slightly increased in number relative to that at lower temperatures, terminal, mostly in thin, 3- to 4-μm-wide hyphae, less commonly intercalary in wider hyphae, 6 to 22 μm long by 4 to 16 μm wide, length/width ratios of 0.9 to 2.6 ($n = 33$), (sub)globose to pyriform, less commonly fusoid or oblong, smooth, sometimes 2 celled. Conidiation starting after 1 day on simple conidiophores of a straight, flexible main axis often sterile at the tip, and several tree-like side branches, paired, unpaired, or radially emergent from the main axis; appearing as minute white shrubs 0.3 to 0.6 mm in diameter, concentrated in proximal areas and in one to several diffuse, at higher temperatures often narrower, concentric zones. Shrubs loosely disposed and firmly attached to surface hyphae; erect, first with straight to sinuous sterile elongations, becoming green and entirely fertile; terminally 2.5 to 3.5 μm wide, downwards 4.5 to 5 μm. Consecutively, after ca. 3 days, dark green pustules to nearly 2 mm in diameter formed, more or less arranged in concentric zones. Pustules loose, transparent, first white with sterile ends or elongations, turning pale to dark green, maturing from inside, ends becoming fertile; consisting of a loose reticulum bearing several straight or sinuous main axes with highly variable, straight or distinctly sinuous side branches. Conidiophores (all branches) generally narrow, 2.0 to 4.5 μm wide, thick walled, and with thickenings to 5.5 to 6.5 μm when old, flexuous, loosely arranged, usually unpaired, in right angles or inclined upwards, holding solitary phialides in right angles along their length, directly, or terminally on a supporting cell often similar in shape (intercalary phialides) or on few-celled cylindrical branches, sometimes in whorls of 3 on intercalary

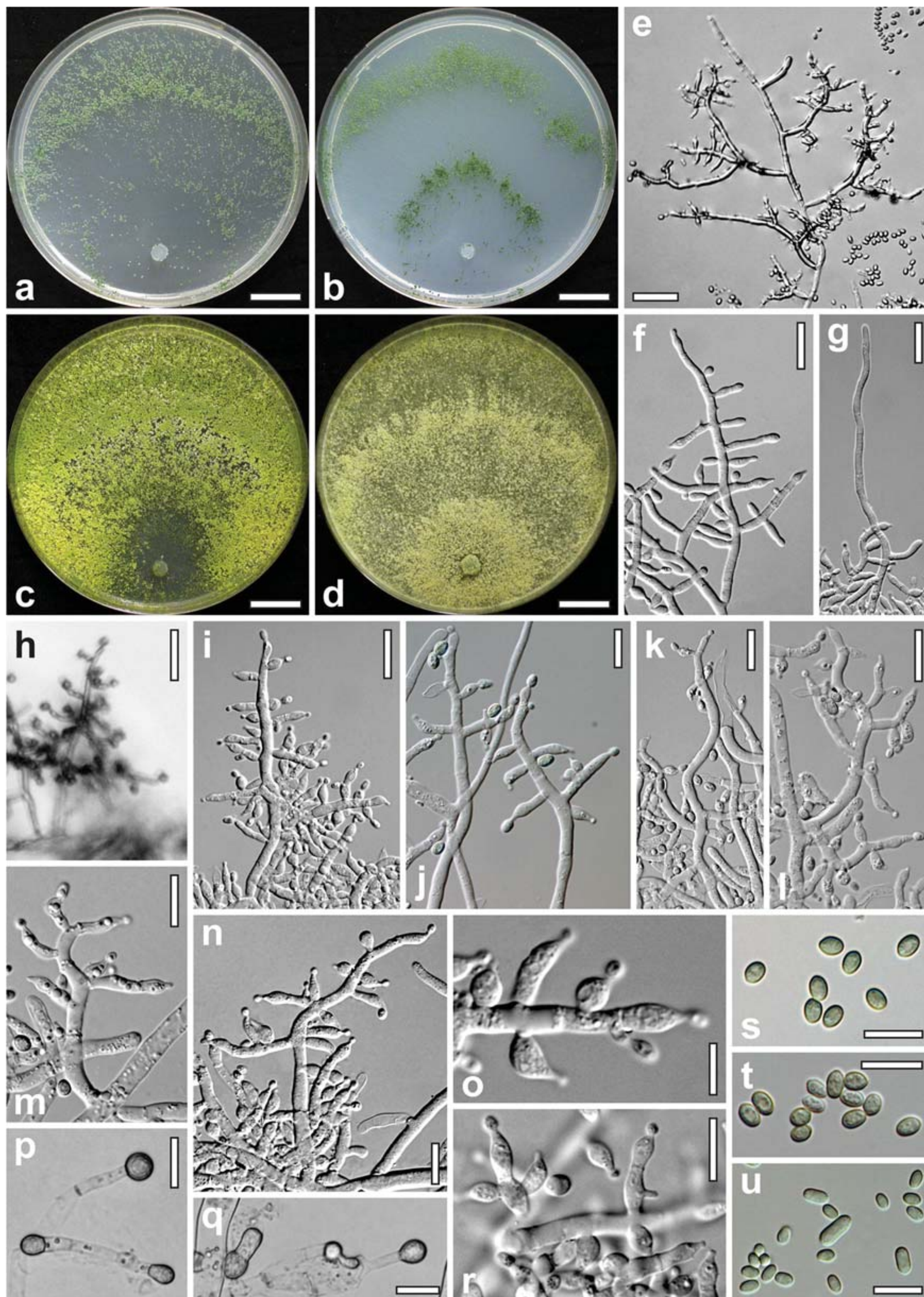

FIG. 1. *Trichoderma parareesei*. (a to c) Cultures after 4 days on CMD (a), SNA (b) and PDA (c); (e) conidiophore without cover glass (9 days); (f) young conidiophore (3 days); (g) young conidiophore with sterile elongation (3 days); (h) conidiophore of a shrub on growth plate (2 days); (i to n) conidiophores after 3 days (i and n) and after 9 days (j to m); (o and r) phialides at 2 days (o) and 8 days (r); (p and q) chlamydospores (7 days); (s and t) conidia (8 days); (e to t) growth on CMD; (a to c, p, and q) growth at 30°C; (e and j to m) growth at 15°C; (f to i, n, o, and r to t) growth at 25°C; (a to c, f, g, i, and n to t) strain C.P.K. 717; (e, h, and j to m) strain C.P.K. 661; (d and u) *Hypocrea jecorina* (*Trichoderma reesei*); (d) culture on PDA after 4 days at 30°C (C.P.K. 917); (u) conidia (C.P.K. 1127, SNA, 25°C, 4 days). Scale bars: 15 mm (a to d), 30 μm (e and h), 15 μm (f, g, i, k, l, p, and q), 10 μm (j, m, n, and r to u), and 5 μm (o).

TABLE 3. Colony phenotype and anamorph characteristic of the species discussed

Parameter	*T. parareesei* sp. nov.	*H. jecorina*/*T. reesei*
Colony type on CMD	Conidia formed abundantly in shrubs and well-defined pustules	Only shrubs; no well-defined pustules formed
Colony radius (mm) at 48 h		
CMD		
15°C	19–21	8–19
25°C	44–47	34–44
30°C	50–56	44–55
37°C	50–53	37–58
PDA		
15°C	15–16	9–16
25°C	44–48	36–49
30°C	53–56	45–59
37°C	57–61	42–59
SNA		
15°C	17–18	9–15
25°C	40–43	32–40
30°C	50–55	40–60
37°C	53–58	32–64
Conidia	Uniformly ellipsoidal; more variable, ellipsoidal to cylindrical at 37°C	Variable, ellipsoidal or oblong with parallel sides
Conidium		
Length (μm)	3.3–6.2 (3.8–4.5)[a]	3.5–9.0 (3.5–6.0)
Width at widest point (μm)	2.5–3.5 (2.8–3.2)	2.2–4.0 (2.5–3.3)
Length/width	1.2–2.0[b] (1.3–1.5)	1.2–2.7[c] (1.3–1.9)
Phialides	Lageniform or ampulliform, often with a cylindrical neck, usually with distinct widening at or above the middle	Lageniform
Phialide		
Length (μm)	4.5–11 (5–8)	5.0–14.5 (6–10)
Width at widest point (μm)	2.5–3.8 (2.7–3.5)	2.2–4.0 (2.5–3.5)
Width at base (μm)	1.4–3.2 (1.7–2.4)	1.3–3.0 (1.7–2.5)
Length/width	1.3–3.6 (1.6–2.7)	1.5–4.6 (2–3.4)

[a] Data in parentheses represent the narrower ranges determined by the mean plus minus standard deviation.
[b] $n = 70$.
[c] $n = 75$.

phialides, but not originating at the same position. Phialides lageniform or ampulliform with often cylindrical neck, straight and symmetric or inequilateral and distinctly curved, with wide or constricted base, usually with distinct widening at or above the middle. Conidia formed in minute, mostly dry heads 10 μm in diameter. Conidia green, uniformly ellipsoidal, smooth, thick walled, eguttulate or with some minute guttules; scar indistinct or pointed; shape and size more variable, ellipsoidal to cylindrical at 37°C. On potato dextrose agar (PDA), mycelium covering the plate within 3 days at 25 to 37°C. Secondary hyphae forming a dense reticulum. Autolytic excretions conspicuous at the colony margin, positively correlated with increasing temperature. Conidiation on PDA and MEA conspicuously abundant, in numerous densely arranged and variably superposed bright yellow-green to dark green pustules (for cultures of *T. parareesei* and *H. jecorina* on 3% MEA, see Fig. S1 in the supplemental material). Diffusing pigment variable, yellow if present. On SNA, mycelium covering the plate within 3 days at 25 to 37°C. Colony similar to CMD; conidiation more abundant, denser, and more regularly arranged in concentric zones.

Holotype. The holotype was isolated from soil of a subtropical rain forest near Iguazu Falls, Argentina, on 4 September 1997, and is deposited as a dry culture (WU 30015; living cultures CBS 125925, C.P.K. 717, TUB F-1066).

Additional isolates examined were CBS 125862 (C.P.K. 661, TUB F-728) from subtropical rain forest, near Iguazu Falls, Argentina, 4 September 1997.

Habitat and distribution. *T. parareesei* has been isolated from soils of subtropical and tropical areas in South America (Brazil, Argentina, Colombia), Central America (Mexico), Africa (Ghana and Ethiopia), and India (4). Hojos-Carvajal et al. (7) noted that the fungus occurs in soil of the African oil palm (*Elaeis guineensis*) in South America.

Anamorph morphology of *Hypocrea jecorina*/*Trichoderma reesei*. To highlight differences in culture and anamorph morphologies, a short description of *H. jecorina*/*T. reesei* cultured in the same experiments is given here (Table 3). On CMD, mycelium covering the plate within 3 to 4 days at 25 to 37°C. Conidiation after 4 to 8 days at 25°C on CMD and SNA concentrated in a distal concentric zone, farinose, green; only shrubs, no well-defined pustules formed. Conidiophores

straight, 2.5 to 5 μm wide, similar to those of *T. parareesei* in shrubs. Larger branches becoming coarsely warted with age. Phialides solitary, lageniform, straight, only rarely slightly curved, sometimes with long cylindrical necks. Conidia variable, ellipsoidal, or oblong with parallel sides, scar indistinct.

Temperature-dependent growth of *T. parareesei* and *H. jecorina* on multiple carbon sources. We have recently reported that *T. parareesei* and *H. jecorina* exhibit qualitatively similar carbon source utilization profiles under standard temperature conditions (25°C) but respond differently to light (4). Moreover, it was noticed that *T. parareesei* showed a more variable utilization profile compared to that of *H. jecorina*. Here we performed phenotype microarrays (PMs) of both species at seven temperatures in darkness in order to get an advanced profile of carbon source utilization by *T. parareesei* in comparison to that of *H. jecorina*. Results show that in general both species have their growth optimum at temperatures between 28 and 35°C. However, *T. parareesei* is able to produce statistically higher biomass in this temperature range compared to that for *H. jecorina* (analysis of variance [ANOVA], $P < 0.05$). Figure 2A shows the temperature-dependent growth rates of both species calculated for the 16 best carbon sources (cluster I) for *H. jecorina*, as estimated by Druzhinina et al. (2). The detailed PM profiles are given in Table S1 in the supplemental material. The most distinct difference between PM profiles of the two species was detected at 35°C. Figure 2B shows examples of faster growth of *T. parareesei* at 35°C on *i*-erythritol, D-mannitol, D-cellobiose, and D-galactose than the rate for *H. jecorina*. An inspection of the growth pattern on *i*-erythritol and D-mannitol can be used in the laboratory to distinguish the two species. In addition to the carbon sources listed above, similar patterns were also observed on gentobiose, D-mannose, D-xylose, D-arabitol, D-trehalose, α-D-glucose, and D-fructose, yet without statistical significance (ANOVA, $P > 0.05$).

Phytotoxicity assays. We have previously reported that *T. parareesei* is considerably different from *H. jecorina* with respect to its ecophysiological adaptation (4). Thus, we found that, contrary to *H. jecorina*, *T. parareesei* is better adapted to growth in light and is more competitive with epigeal fungi than *H. jecorina*. However, all strains of *T. parareesei* have been isolated from soil, suggesting that soil may be its natural habitat. In order to extend our knowledge on the ecology of *T. parareesei*, we have performed a pilot phytotoxicity assay, which tests the influences of *T. parareesei* and *H. jecorina* on the germination of seeds and on the growth of a model plant, *Lepidium sativum*. This plant was chosen for its rapid growth in soil-free cultivations. The results show that both *T. parareesei* and *H. jecorina* are, in fact, able to inhibit growth of *L. sativum* up to 40%, yet the plants were normally developed. Statistically supported differentiation was detected only between each of the investigated fungal species and the control samples (for *T. parareesei*, $P = 0.0009$ [ANOVA] and $n = 59$; for *H. jecorina*, $P = 0.0002$ and $n = 67$), but no statistically significant differences were found between the species. Furthermore, the variation of inhibition among the *T. parareesei* strains was minimal. In contrast, growth of the plants with *H. jecorina* was strain dependent; in particular, strain C.P.K. 3419 inhibited *L. sativum* plants the most conspicuously. Development of the rootlets in the presence of *Trichoderma* was observed to be strain specific, as some strains (C.P.K. 717 and C.P.K. 667 of *T. parareesei* and C.P.K. 3419 of *H. jecorina*) clearly inhibited maturation of *Lepidium* roots.

Evolution of *T. parareesei*. The genealogical concordance phylogenetic species recognition criterion, based on three of the most polymorphic phylogenetic markers applied previously (4), showed that *T. parareesei* constitutes a cryptic phylogenetic species genetically isolated from *H. jecorina*. In order to learn about the evolution of these two species with respect to other related taxa, we used Bayesian phylogenetic analysis of nucleotide sequences of two relatively conserved phylogenetic markers, *rpb2* and *chi18-5*, which allow alignments with other species of *Trichoderma* section *Longibrachiatum* (Fig. 3). The nucleotide properties of these loci and parameters of phylogenetic analyses are given in Table 2. The topology of the phylogram based on the combined data set (Fig. 3A) confirms the result of Samuels et al. (20) and shows that, indeed, *T. longibrachiatum* and species related to it (*H. orientalis* and *Trichoderma* sp. strain C.P.K. 1837 [3]) are the next genetic neighbors to the species studied in this work. Furthermore, it also revealed a monophyletic Reesei subclade combining *T. parareesei*, *Trichoderma* sp. strain C.P.K. 524, and *H. jecorina* (Fig. 3B). The Reesei subclade is characterized by relatively short intercladal genetic distances compared to those present between other species in the section. A detailed inspection of this subclade (Fig. 3B) revealed that *T. parareesei* does not occupy any separate clade but is most closely related to the hypothetical common ancestor of the whole Reesei subclade. This was also confirmed on both individual phylograms for *rpb2* and *chi18-5* (data not shown). Three strains of *T. parareesei* (C.P.K. 3692 [Ethiopia], C.P.K. 634 [Ghana], C.P.K. 3426 [Sri Lanka]) are closest to the ancestral node, while four other strains form a statistically supported subclade derived from it. The phylogram shows that both *H. jecorina* and *Trichoderma* sp. strain C.P.K. 524 derived from the ancestral state, which is contemporarily represented by *T. parareesei*. In contrast to *T. parareesei*, strains of *H. jecorina* show considerable infraspecific polymorphism and occupy the longest branches of the Reesei subclade. These data indicate that *T. parareesei* is evolutionarily older than *H. jecorina* and likely resembles the ancestor of the latter species.

DISCUSSION

In this study we extend our earlier finding (4) of *T. parareesei* as a new phylogenetic species of *Trichoderma* section *Longibrachiatum* to a full taxonomic description. The use of an integrated approach consisting of morphological observation and description, temperature-dependent carbon source utilization profiling, and phylogenetic analysis resulted in a clear differentiation between *T. parareesei* and *T. reesei*, the anamorph of *H. jecorina*, where *T. parareesei* was erroneously ascribed to earlier (1, 7, 12).

Macro- and micromorphological characters. Conidiation in *T. parareesei* is distinctly more abundant than in *H. jecorina* on all media at all temperatures. On richer media such as PDA and MEA, conidiation in *T. parareesei* is conspicuously abundant and is positively correlated with temperatures up to 37°C. Under such conditions, several generations of bright yellow-green pustules form consecutive superposed layers, resulting in a thick, coarsely tubercular colony surface. In *H. jecorina*,

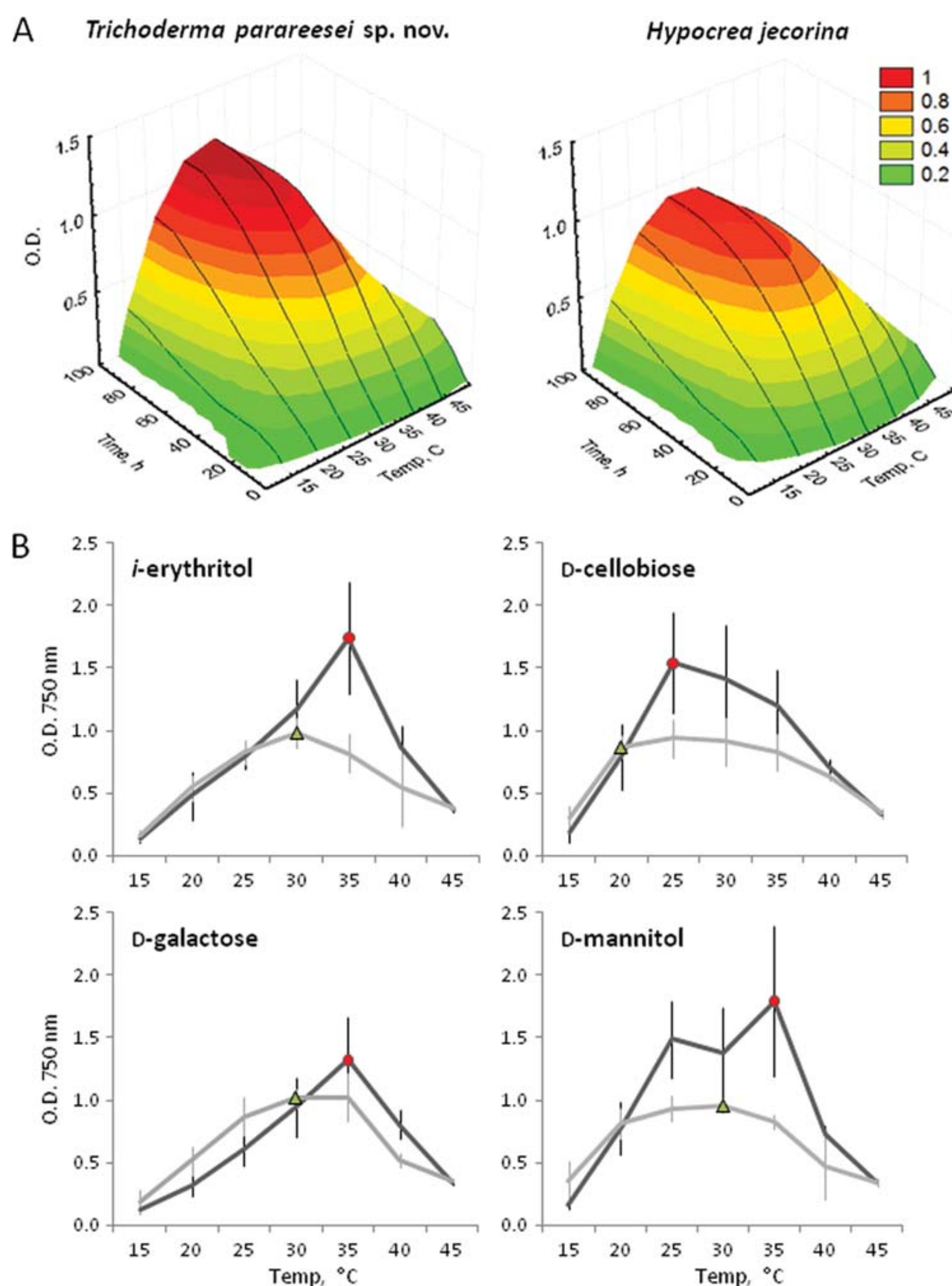

FIG. 2. Temperature-dependent growth of *T. parareesei* and *H. jecorina* on different carbon sources. (A) Temperature-dependent growth rates of both species calculated for the 16 best carbon sources (cluster I, L-arabinose, D-arabitol, D-cellobiose, dextrin, *i*-erythritol, D-fructose, gentobiose, α-D-glucose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-trehalose, D-xylose, γ-aminobutyric acid), as estimated by Druzhinina et al. (2) for *H. jecorina.* The complete carbon source utilization profiles are given in Table S1 in the supplemental material. (B) Temperature-dependent growth of *T. parareesei* and *H. jecorina* on individual carbon sources. Vertical bars correspond to standard deviations, calculated on the basis of the profiles of six and five strains for *T. parareesei* and *H. jecorina*, respectively. Colored labels highlight the differences.

conidiation on PDA is usually yellow and turns green only slowly, while on CMD and SNA, only shrubs and no well-defined pustules are formed. Conidiophores of *H. jecorina* are comparable to those of *T. parareesei* and are formed in shrubs, but they are generally straighter, with slightly longer phialides and conidia that vary in shape and size more conspicuously than those of *T. parareesei*. Phialide length only rarely exceeds 10 μm in *T. parareesei*, while this is common in *H. jecorina*. The formation of a yellow pigment is variable among isolates of both species.

Reasons for genetic isolation and speciation. Our data show that *T. parareesei*, a species that has lost its ability to reproduce sexually, likely closely resembles the anamorphic stage of a common ancestor of all three species detected in the Reesei subclade. We have recently demonstrated that although *T. parareesei* possesses both the *MAT1-1* and *MAT1-2* loci, the

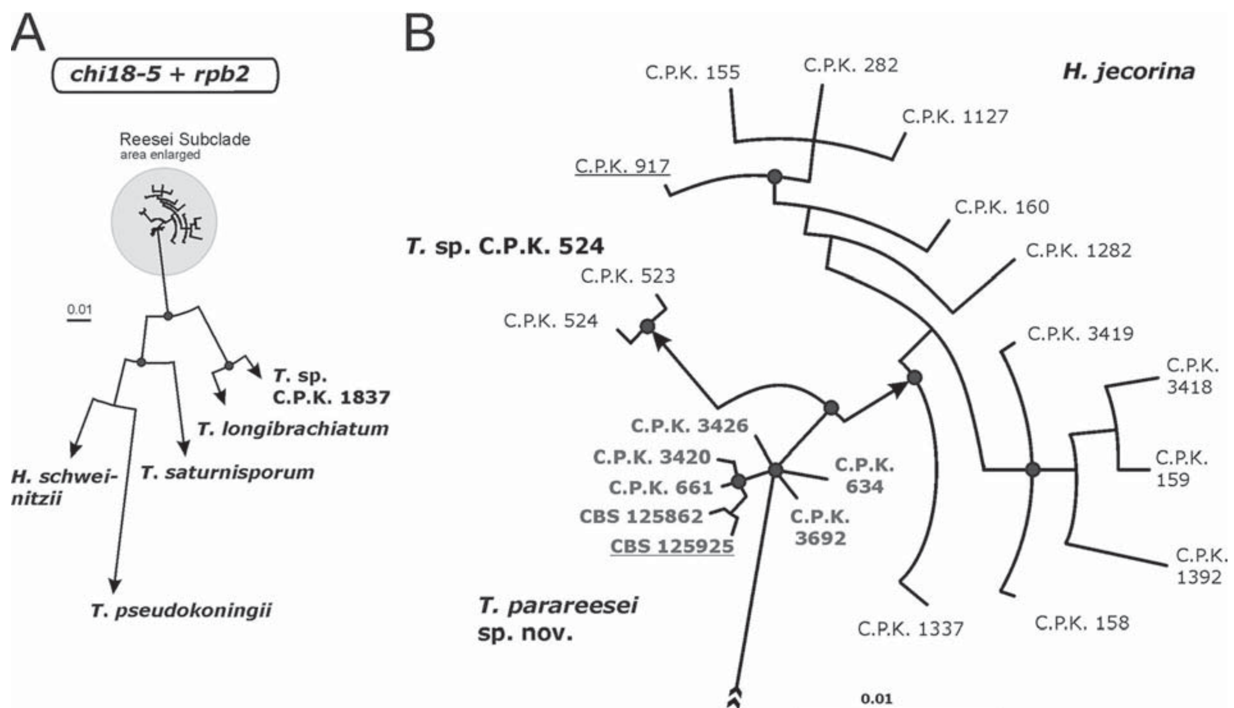

FIG. 3. Bayesian circular phylogram inferred from the concatenated data set of partial exons of *rpb2* and *chi18-5* phylogenetic markers. Symbols at the nodes correspond to PPs of >94%. Arrows point to clades/nodes of phylogenetic species. (A) Phylogenetic position of the Reesei subclade with respect to other species from *Trichoderma* section *Longibrachiatum*; (B) phylogenetic relations between species of the Reesei subclade (enlarged from panel A). The ex type strain of *T. reesei* QM 6a (C.P.K. 917) is underlined.

latter is essentially altered compared to that of *H. jecorina*, where both mating-type loci are functional even *in vitro*. Therefore, we speculate that the inability of sexual reproduction likely originated from mutations in the *MAT1-2* gene (4). Here we used conservative phylogenetic markers (coding fragments of the *chi18-5* and *rpb2* genes) to infer the evolution of the group of species rather than to differentiate *T. parareesei* from the rest. The analyses show that, in fact, *T. parareesei* is the oldest taxon which apparently nearly stopped its evolutionary development and that *H. jecorina* and *Trichoderma* sp. strain C.P.K. 524 arose from it. This is in perfect agreement with our previous results, which showed abundant molecular footprints of sexual recombination for *H. jecorina* and none for *T. parareesei* (4). Thus, we conclude that *T. parareesei* is a relict agamospecies which resembles the ancestor of *H. jecorina* and *Trichoderma* sp. strain C.P.K. 524. In this case, it is interesting to speculate on reasons which led to the survival of *T. parareesei*. The reduction of sexual recombination is reflected in the low level of infraspecific polymorphism of *T. parareesei*, which theoretically makes the species vulnerable to changing environments. We think that the survival of *T. parareesei* was to a large extent possible due to the long-term stability of its habitat, the tropical forest, which is one of the most sustainable and ancient ecosystems on the planet. However, we have also observed that *T. parareesei* has a versatile phenotype in a broad range of temperatures, extensive conidiation, and fast growth rates, which altogether are characteristics in line with strongly opportunistic members of the genus, e.g., *T. asperellum*, *T. longibrachiatum*, and *T. hamatum.* As it is hard to imagine which mechanisms could be exploited by the agamospecies to gain new genetic/phenetic properties (6), we would rather suggest that in *H. jecorina* extensive production of propagules and growth on certain carbon sources was reduced during evolution. This hypothesis is well supported by the observations that *H. jecorina* has an unusual small genome compared to the sizes of the genomes of other ascomycetes and *Trichoderma* species (16; cf. also http://genome.jgi-psf.org/Trive1/Trive1.home.html and http://genome.jgi-psf.org/Triat1/Triat1.home.html), suggesting that its overall phenotype likely reflects some losses in the genome. Druzhinina et al. (4) also showed that *H. jecorina* is photoinhibited to a certain extent, while *T. parareesei* is well adapted to various lighting conditions. Moreover the two species also have differences in their antagonistic potential in dual confrontations with fungi pathogenic for plants, showing that *T. parareesei* is generally more competitive. These considerations call for a comparison of the genomes of *T. parareesei* and *H. jecorina*, because this may provide insights into the original genetic potential of this important industrial species, where speciation occurred by restriction to a narrow habitat and lifestyle.

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REFERENCES

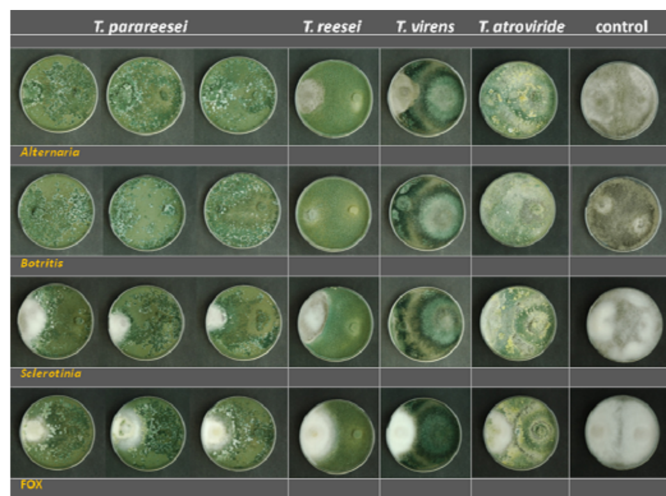
1. **Druzhinina, I. S., A. G. Kopchinskiy, M. Komon, J. Bissett, G. Szakacs, and C. P. Kubicek.** 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet. Biol. **42:**813–828.

2. **Druzhinina, I. S., M. Schmoll, B. Seiboth, and C. P. Kubicek.** 2006. Global carbon utilization profiles of wild-type, mutant, and transformant strains of *Hypocrea jecorina*. Appl. Environ. Microbiol. **72:**2126–2133.
3. **Druzhinina, I. S., M. Komoń-Zelazowska, L. Kredics, L. Hatvani, Z. Antal, T. Belayneh, and C. P. Kubicek.** 2008. Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable to cause invasive mycoses of humans. Microbiology **154:** 3447–3459.
4. **Druzhinina, I. S., M. Komoń-Zelazowska, L. Atanasova, V. Seidl, and C. P. Kubicek.** 2010. Evolution and ecophysiology of the industrial producer *Hypocrea jecorina* (Anamorph *Trichoderma reesei*) and a new sympatric agamospecies related to it. PLoS One **5:**e9191.
5. **Friedl, M. A., M. Schmoll, C. P. Kubicek, and I. S. Druzhinina.** 2008. Photostimulation of *Hypocrea atroviridis* growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress. Microbiology **154:**1229–1241.
6. **Giraud, T., G. Refrégier, M. Le Gac, D. M. de Vienne, and M. E. Hood.** 2008. Speciation in fungi. Fungal Genet. Biol. **45:**791–802.
7. **Hoyos-Carvajal, L., S. Orduz, and J. Bissett.** 2009. Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions. Fungal Genet. Biol. **46:**615–631.
8. **Jaklitsch, W. M., M. Komon, C. P. Kubicek, and I. S. Druzhinina.** 2005. *Hypocrea voglmayrii* sp. nov. from the Austrian Alps represents a new phylogenetic clade in *Hypocrea/Trichoderma*. Mycologia **97:**1365–1378.
9. **Jaklitsch, W. M., C. P. Kubicek, and I. S. Druzhinina.** 2008. Three European species of *Hypocrea* with reddish brown stromata and green ascospores. Mycologia **100:**796–815.
10. **Jaklitsch, W. M.** 2009. European species of *Hypocrea* Part I. The green-spored species. Stud. Mycol. **63:**1–91.
11. **Kornerup, A., and J. H. Wanscher.** 1981. Taschenlexikon der Farben—1440 Farbnuancen und 600 Farbnamen. Muster-Schmidt Verlag, Zürich, Switzerland.
12. **Kubicek, C. P., J. Bissett, C. M. Kullnig-Gradinger, I. S. Druzhinina, and G. Szakacs.** 2003. Genetic and metabolic diversity of Trichoderma: a case study on South-East Asian isolates. Fungal Genet. Biol. **38:**310–317.
13. **Kubicek, C. P., M. Mikus, A. Schuster, M. Schmoll, and B. Seiboth.** 2009. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol. Biofuels **2:**19.
14. **Kuhls, K., E. Lieckfeldt, G. J. Samuels, W. Kovacs, W. Meyer, O. Petrini, W. Gams, T. Börner, and C. P. Kubicek.** 1996. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proc. Natl. Acad. Sci. U. S. A. **93:**7755–7760.
15. **Kumar, R., S. Singh, and O. V. Singh.** 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J. Ind. Microbiol. Biotechnol. **35:**377–391.
16. **Martinez, D., R. M. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S. E. Baker, J. Chapman, O. Chertkov, P. M. Coutinho, D. Cullen, E. G. Danchin, I. V. Grigoriev, P. Harris, M. Jackson, C. P. Kubicek, C. S. Han, I. Ho, L. F. Larrondo, A. L. de Leon, J. K. Magnuson, S. Merino, M. Misra, B. Nelson, N. Putnam, B. Robbertse, A. A. Salamov, M. Schmol, A. Terry, N. Thayer, A. Westerholm-Parvinen, C. L. Schoch, J. Yao, R. Barabote, M. A. Nelson, C. Detter, D. Bruce, C. R. Kuske, G. Xie, P. Richardson, D. S. Rokhsar, S. M. Lucas, E. M. Rubin, N. Dunn-Coleman, M. Ward, and T. S. Brettin.** 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat. Biotechnol. **26:**553–560.
17. **Percival Zhang, Y. H., M. E. Himmel, and J. R. Mielenz.** 2006. Outlook for cellulase improvement: screening and selection strategies. Biotechnol. Adv. **24:**452–481.
18. **Reese, E. T., H. S. Levinsons, and M. Downing.** 1950 Quartermaster culture collection. Farlowia **4:**45–86.
19. **Rozas, J., J. C. Sanchez-DelBarrio, X. Messeguer, and R. Rozas.** 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics **19:**2496–2497.
20. **Samuels, G. J., O. Petrini, K. Kuhls, E. Lieckfeldt, and C. P. Kubicek.** 1998. The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *Longibrachiatum*. Stud. Mycol. **41:**1–54.
21. **Seidl, V., and B. Seiboth.** 2010. *Trichoderma reesei*: genetic strategies to improving strain efficiency. Biofuels **1:**343–354.
22. **Swofford, D. L.** 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer Associates, Sunderland, MA.

Contributions

LA amplified *chi18-5* and *rpb2 loci*, participated in sequence analysis, carried out BIOLOG Phenotype Microarrays and phytotoxicity experiments, performed temperature depending growth rate analyses and statistical analysis, participated in figure design and writing of the manuscript.

2.3 *T. parareesei*, the sibling species of *T. reesei*, is powerful mycoparasite



Introduction

T. reesei QM 6a isolated in 1942, it was a victim of then almost undeveloped taxonomy for *Trichoderma*. Therefore it was first determined to be *T. viride*, and later on recognized as a unique species and named in honor of its detector Elwyn T. Reese *T. reesei* (Simmons, 1977). Bissett (1984) then revised it as being co-specific with *T. longibrachiatum*. Finally, it was recognized to be identical to the pantropical ascomycete *H. jecorina* (Kuhls et al., 1996), which was itself just distinguished as a separate tropical species closely related to *H. schweinitzii* (Samuels et al., 1998). Yet small morphological differences and the inability of these authors to cross it with other *H. jecorina* isolates in the lab led Kuhls et al. (1996) to assume that it is actually a clonally derived asexual form of *H. jecorina*. Our data have clearly rejected this hypothesis: the analysis showed that the original isolate *T. reesei* QM 6a reveals a history of recombination similar to that of the teleomorphic isolates of *H. jecorina*. These findings are also supported by our recent discovery that *T. reesei* QM 6a is a *MAT1-2* idiotype and can indeed be crossed with *MAT1-1* partners of *H. jecorina* (Seidl et al., 2009). *T. reesei* clearly is an isolate identical to *H. jecorina*, and the minor differences in anamorph morphology (Kuhls et al., 1996) and nutrient assimilation (Lieckfeldt et al., 2000) to other (more recent) isolates of *H. jecorina* are likely the result of its maintenance in the laboratory for the last 65 years. Since 1st of January 2013 the name *Trichoderma* applies to all the holomorph species (see Nomenclature and current taxonomic assumptions), thus the name *T. reesei* will be further applied.

Our findings showed that *T. reesei* strains isolated as anamorphs from soil and litter belonged to a cryptic agamospecies, *T. parareesei* [Jaklitsch, Druzhinina & Atanasova] (Atanasova et al., 2010). This sibling anamorphic sister species of *T. reesei* is similarly restricted to the same pantropical altitude around the equator, what represents a rare example of sympatric speciation of a saprotrophic fungus (Giraud et al., 2008). Though not able to reproduce, *T. parareesei* possess both mating loci what suggest the operation of other speciation mechanisms of sympatric speciation rather than the mutation of one *MAT* locus. However, the phylogenetic analyses using conserved loci to infer the evolution of both species revealed *T. parareesei* is the oldest taxon which apparently nearly stopped its evolutionary development and that *T. reesei* arose from it (see Atanasova et al., 2010; Chapter 2.2). Furthermore, we also showed that *T reesei* underwent considerable sexual recombination whereas none was observed for *T. parareesei*.
Generally *T. reesei* is considered to be a weak mycoparasite as well as we previously showed that it has a derived position on phylogenetic tree (Kubicek et al., 2011; Chapter 2.1) thus we were interested in the antagonistic potential of its clonal and ancestral sister species *T. parareesei*.

Material and Methods

To assess the antagonistic potential of anamorphic cultures of *T. reesei* five potential prey fungi have been selected: *Sclerotinia sclerotiorum* C.P.K. 3593 and FOX (*Fusarium oxysporum* species complex, strain C.P.K. 1842) to represent soil and rhizosphere competent pathogens and *F. xylarioides* C.P.K. 3453, *Alternaria alternata* C.P.K. 3594 and *Botrytis cinerea* C.P.K. 3592 to represent epigeal plant pathogens. Potential prey fungi were inoculated as agar blocks of the standard size always 1 cm from the edge of the Petri plate and pre-cultivated on 3% PDA in darkness at 25 °C. Then similar agar blocks with *Trichoderma* cultures were introduced on the opposite side of the plate and cultivated for 10 days. Antagonistic potential was semi-quantified based on both ability to inhibit the growth of a pathogen and ability to overgrow the mycelium of the pathogenic fungus. One of 5 phases for each confrontation was recorded: 0–no inhibition; I - started to inhibit; II - clear signs of inhibition; III -mostly or strongly inhibited; IV - totally inhibited. The ability to overgrow was based on the same scale but recorded using Arabic numbers.

Results

Species of *Trichoderma* are renowned for their mycoparasitic behavior (Harman and Kubicek, 1998). In order to test whether the two species may also differ in their antagonistic abilities, we have selected five plant pathogenic fungi which differ in their primary habitat: *Sclerotinia scleotiorum* and *Fusarium oxysporum* (FOX, *F. oxysporum* species complex) representing soil competent mycobionts; and *F. xylarioides*, *Alternaria alternata* and *Botrytis cinerea* representing pathogens of green plant tissue and thus predominantly epigeal fungi. Figure 2.2 shows the summary of dual confrontation tests assessed after 10 days of co-cultivation on MEA medium. *T. reesei* is able to considerably inhibit growth of *S. scleotiorum*, *A. alternata* and *B. cinerea*. The latter plant pathogen was so completely combated by *T. reesei* C.P.K. 160 and C.P.K. 1127 that its mycelium was overgrown and killed. No antagonism to both *Fusarium* species by *T. reesei* was detected. Strains of *T. parareesei* showed a conserved pattern of mycoparasitic activity with almost no variation among strains, and they also exhibited a superior antagonistic potential against *S. scleotiorum*, *B. cinerea* and particularly *A. alternata*.

		Sc	FOX	Fx	Alt	Bot	antagonistic potential
H. jecorina	C.P.K. 1282	II 2	1	1	II 3	III 1	moderate VII 8
	C.P.K. 1127	II 0	0	2	II 2	IV 3	moderate VIII 7
	C.P.K. 3419	II 1	0	1	2	IV 3	moderate VI 7
	C.P.K. 160	I 0	0	3	II 3	III 3	moderate VI 9
***T. parareesei* nom. prov.**	C.P.K. 665	I 0	1	2	III 3	IV 4	strong VIII 10
	C.P.K. 3426	III 3	0	2	I 3	IV 4	strong VIII 12
	C.P.K. 717	IV 4	1	3	III 3	IV 4	strong XI 15
	C.P.K. 634	IV 3	2	2	III 1	IV 4	strong XI 11
***T.* sp. nov. C.P.K. 524**	C.P.K. 524	III 3	I 2	2	II 1	III 3	strong XI 11

Figure 2.2: Mycoparasitic ability of T. reesei (*H. jecorina*) and *T. parareesei*. Results of dual confrontation tests between *Trichoderma* strains (inoculated on the left side) and the plant pathogenic fungi (inoculated on the right side): Sc – *Sclerotinia scleotiorum*, FOX – *Fusarium oxysporum* complex, Fx – *F. xylarioides*, Alt– *Alternaria alternata*, Bot – *Botrytis cinerea*. Roman numbers indicate the weak (I), moderate (II), strong (III) and very strong (IV) ability of *Trichoderma* to inhibit the growth of the prey fungus. The ability to overgrow the mycelium of prey fungi is given in Arabic numbers on the similar scale. Antagonistic potential is calculated as the mean value for a strain to combat all five pathogens. The dashed lines correspond to the center position between confronted fungi. (From Druzhinina et al., 2010)

Discussion

We tested mycoparasitic potential of closely related species *T. reesei*, *T. parareesei* and *T. sp.* C.P.K. 524 against epigeal and soil born plant pathogens. The position of the *T. sp.* C.P.K. 524 was recently reconsidered in the phylogenetic analysis of the whole Section *Longibrachiatum* and this species was attributed to the *T. parareesei* species clade (Druzhinina et al., 2012).

General observation showed that *B. cinerea* was very weak prey of all tested *Trichoderma* species, though not all of the *T. reesei* strains were able to overgrow this fungus compared to very constant effect of *T. parareesei* strains. Major differences between the species were detected in confrontations with *S. scleotiorum*, *A. alternata* and *Fusarium* spp. where *T. parareesei* was able to antagonize these preys more successfully than *T. reesei*.

The strains of *T. parareesei* showed a conserved pattern of mycoparasitic activity as well as remarkable antagonistic potential against epigeal plant pathogenic fungi. Compared to strong opportunistic *T. virens* and *T. atroviride*, *T. parareesei* successfully inhibited FOX and compared to *T. virens* totally overgrown *A. alternata* (data not shown). However, the significant antagonistic ability similar to highly opportunistic species such as *T. virens* and *T. atroviride* render this species strong mycoparasite against tested plant pathogenic fungi.

Intriguing, *T. parareesei*, a clonal species that is most closely related to the hypothetical common ancestor of *T. reesei*, was revealed to be very strong opportunistic species and *T. reesei* apparently evolved from a common ancestor with mycotrophic nutritional mode and lost the strong ability to parasitize on other fungi during the evolution.

References

Atanasova, L., W.M. Jaklitsch, M. Komoń-Zelazowska, C.P. Kubicek and I.S. Druzhinina (2010) Clonal species *Trichoderma parareesei* sp. nov. likely resembles the ancestor of the cellulase producer *Hypocrea jecorina*/*T. reesei*. *Appl. Environ. Microbiol.* 76:7259–7267.

Bissett J (1984) A revision of the genus *Trichoderma* I. Section *Longibrachiatum* sect. novum. Can J Bot, 62:924–933.

Druzhinina IS, Komoń-Zelazowska M, Ismaiel A, Jaklitsch W, Mullaw T, Samuels GJ, Kubicek CP (2012) Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma*. Fungal Genet Biol, 49(5):358-368.

Giraud T, Refregier G, Le Gac M, de Vienne DM, Hood ME (2008) Speciation in fungi. Fungal Genet Biol, 45:791–802.

Harman GE, Kubicek CP (1998) *Trichoderma* and *Gliocladium* Vol 2. Taylor and Francis, London, pp 393.

Lieckfeldt E, Kullnig CM, Samuels GJ, Kubicek CP (2000) Sexually competent, sucrose- and nitrate-assimilating strains of *Hypocrea jecorina* (*Trichoderma reesei*, Hypocreales) from South American soils. Mycologia, 92:374–380.

Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, Thon M, Zeilinger S, Casas-Flores S, Horwitz BA, Mukherjee PK, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, Atanasova L, Cervantes-Badillo MG et al. (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol, 12(4):R40.

Kuhls K, Lieckfeldt E, Samuels GJ, Kovacs W, Meyer W, et al. (1996) Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proc Natl Acad Sci USA, 93:7755–7760.

Samuels GJ, Petrini O, Kuhls K, Lieckfeldt E, Kubicek CP (1998) *Hypocrea* and *Trichoderma* sect. *Longibrachiatum*. Stud Mycol 41:1–54.

Simmons EG (1977) Classification of some cellulase-producing *Trichoderma* species. Abst Ind Mycol Congress, p 618.

Seidl V, Seibel C, Kubicek CP, Schmoll M (2009) Sexual development in the industrial workhorse Trichoderma reesei. Proc Natl Acad Sci USA, 106:13909-13914.

Contributions

Materials presented in this chapter were incorporated in

Druzhinina IS, Komoń-Zelazowska M, **Atanasova** L, Seidl V, Kubicek CP (2010) **Evolution and Ecophysiology of the Industrial Producer *Hypocrea jecorina* (Anamorph *Trichoderma reesei*) and a New Sympatric Agamospecies Related to It.** PLoS ONE, 5(2):2010.

LA participated in sequence analysis, performed *in vitro* mating between and within *T. reesei* and *T. parareesei*, amplified mating type loci, performed BIOLOG Phenotype Microarrays on BIOLOG FF and ECO plates, did photosensitivity experiments, qualitative and quantitative conidiation tests, dual confrontation assays to asses mycoparasitic potential against soil competent and epigeal plant pathogens and contributed to data anlysis.

2.4 Towards further investigations of *Trichoderma* mycoparasitism

Taxa of Hypocreales exhibit a broad range of nutritional modes and symbioses involving plants, animals and other fungi. Sung et al. (2008) performed ancestral host reconstruction of Hypocreales coupled with phylogenetic dating analyses calibrated with an ancient fungal parasite *Paleoophiocordyceps coccophagus*. Their results showed a plant-based ancestral nutritional mode for Hypocreales, which then ecologically shifted to fungal and plant hosts in Hypocreaceae. In agreement to our result they show that the common Hypocreaceae ancestors functioned as mycoparasites that infect various groups of fungi including mushrooms, bracket fungi, rusts and truffles (Sung et al., 2008). Regarding the plant host, however, most of *Trichoderma* species are found on dead or decomposed wood (especially the teleomorphs). Some of the species indeed can live as facultative and very few as obligate endophytes, but living plant material is not the primary habitat of *Trichoderma* (Atanasova et al., 2013). Thus, further inclusion of *Trichoderma* species in the analysis would even more clearly show that Hypocreaceae arrived from an ancestor which biotrophic mode of nutrition. Anyhow, these facts support the speculation that the ancestors of *Trichoderma* were mycoparasites on wood-degrading basidiomycetes and acquired saprotrophic characteristics to follow their prey into their substrate (Rossmann et al., 1999).

Our comparative study the transcriptional profiles of *T. atroviride* IMI 206040 *T. virens* Gv29-8 and *T. reesei* QM 6a (which genomes were sequenced) in response to the plant pathogenic fungus *R. solani* showed that these three species employ alternative strategies to combat other fungi. The gene regulation of each species is distinct, yet it is necessary to note that this fungus presents one model organism that might not resemble the mechanisms of *Trichoderma* mycoparasitism on other fungi. Thus similar comparative transcriptomic studies using other fungi would be of great importance; for instance plant pathogenic ascomycetes or those species which are found in *Trichoderma* spp. natural habitats e.g. the basidiomes with which teleomorphs of *Trichoderma* spp. are associated or basidiomycetes which grow on dead wood; or even edible fungi from mushroom farms where green mold causes harvest loss. Moreover, *R. solani* is a temperate fungus which might inhabit similar ecological niches as *T. atroviride* and *T. virens*, but not *T. reesei*. The first two species might have evolved to combat this basidiomycete due to their random co-existence in rhizosphere, though this does not necessarily mean that their interaction is of a primary importance for these *Trichoderma* spp. Contrary, *T. reesei* is a tropical saprotroph, which probably does not interact with temperate plant pathogenic fungi in its natural habitat and thus might have not evolved the mechanisms to combat it. Hence, we were interested in the evolution of mycoparasitism in the genus *Trichoderma*. We showed that *T. reesei* is a derived species which successfully adapted to a saprotrophic life style and mycoparasitism is a rather ancestral property of the species. This finding is well concordant to our

results that *T. parareesei*, a sibling species which likely resembles *T. reesei* ancestor and evolved very slowly due to a lack of sexual recombination, is a strong mycoparasite. We are awaiting the genome release of this agamospecies to investigate the genomic traits that might help us to understand the details of *T. reesei* evolution from mycoparasite to a saprotrophic species

References

Atanasova L, Druzhinina IS and Jaklitsch WM (2013) Two hundred *Trichoderma* species recognized based on molecular phylogeny. In *Trichoderma*: Biology and Applications, Eds. Mukherjee PK, Sigh US, Horwitz BA, Schmoll M, Mukherjee M. CABI of Nosworthy Way, UK, Wallingford, Oxon, in press.

Rossmann AY, Samuels GJ, Rogerson CT, Lowen R (1999) Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). Stud Mycol, 42:1-83.

Sung GH, Poinar GO Jr, Spatafora JW (2008) The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbioses. Mol Phylogenet Evol 49(2):495-502.

3. THE POLYKETIDE SYNTHASE GENE *PKS4* OF *TRICHODERMA REESEI* PROVIDES PIGMENTATION AND DEFENSE AGAINST BIOTIC AND ABIOTIC STREES [11]

[11] Materials presented in this chapter have been submitted as:
Atanasova L, Knox BP, Kubicek CP, Baker SE, Druzhinina IS (2013) The polyketide synthase gene *pks4* of *Trichoderma reesei* provides pigmentation and defense against biotic and abiotic strees. ms submitted to Eukariotic Cell.

Abstract

Species of fungal genus *Trichoderma* (teleomorph *Hypocrea*, Ascomycota) are well known after their diverse production of secondary metabolites. Non-ribosomal peptides and polyketides represent a major portion of these products. Recently phylogenomic analysis of polyketide synthase (PKS)-encoding genes in some *Trichoderma* species showed that the majority of PKSs occur as orthologs and that their number in these species is generally low. Recently it was suggested that the PKS encoding gene *pks4* Trire2:82208 from *T. reesei* and their orthologues are most likely responsible for the *Trichoderma* characteristic yellow-green pigment. In this study we show that deletion of this gene in *T. reesei* results in full loss of conidial pigmentation. The ecophysiological characterization of two *pks4* deletion mutants furthermore revealed that its regulation also influences further functions in *T. reesei* lifestyle such as delayed germination rate, altered carbon source utilization, increased UV sensitivity and decreased mycoparasitic defence against other fungi. Furthermore, we show that the *pks4* gene is responsible for the coloration of the perithecia and fertile progeny with white conidia, as well as for the non-melanised parts of stroma and perithecial structures. We also show that regulation of remaining PKS encoding genes present in *T. reesei* genome is influenced by the *pks4* gene.

Introduction

The economically important genus *Trichoderma* (Hypocreales, Ascomycota, Dikarya) is well known for its mycotrophic lifestyle and for the broad range of biotrophic interactions with plants and animals. Moreover it contains several cosmopolitan species characterized by their outstanding environmental opportunism. These properties have given rise to the use of several species in agriculture as biopesticides and biofertilizers while *T. reesei* is applied for production of bioenergy-related enzymes.

The molecular basis of the opportunistic success of *Trichoderma* is not yet well understood. While there is some evidence for a role of some secreted proteins (Druzhinina et al., 2012; Druzhinina and Kubicek, 2013), less is known about a possible role of secondary metabolites. *Trichoderma* spp. are probably best known for their production of peptaibols, which are non-ribosomal peptides with antimicrobial and plant defense-stimulating activities (Mukherjee et al., 2012a). However, less is known about the role of polyketide synthases (PKS) in *Trichoderma* ecophysiology. Genome analysis of several filamentous fungi revealed amount of gene clusters responsible for the secondary metabolite production, yet many of them were not found to be expressed under standard laboratory conditions what makes it difficult to identify the compounds (Brakhage and Schroeckh, 2011; Mukherjee et al., 2012b). *Trichoderma* spp. polyketides are produced by type iterative PKSs, multienzymes consisting of several active sites, capable of catalyzing the fusion of variable numbers of CoA linked acyl monomers, such as acetyl-CoA and malonyl-CoA, into

polymers known as polyketides. Iterative PKSs can be further grouped into non-reducing (NR) and reducing (RD) PKSs according to their domain organization (Kroken et al., 2003). Due to the iterative nature of fungal PKSs, only a single PKS is involved in the biosynthesis of a particular fungal polyketide in most cases (Saha et al., 2012). Recently Baker et al. (2012) have taken a phylogenomic approach to study the PKS repertoire in the genomes of *T. reesei*, *T. atroviride* and *T. virens*. This enabled the putative *in silico* identification of some of them. In total 11 PKS encoding genes were found in *T. reesei* genome, from which two are singlets (i.e. they occur only in *T. reesei*) and nine have orthologues in *T. virens* or/and *T. atroviride* PKSs (Baker et al., 2012). *Pks4*- (Trire2:82208, Triat2:79 and Trive2:77826) in *T. reesei*, *T. atroviride* and *T. virens,* respectively), which encode enzymes of the non-reducing type (clade I), has been shown to have orthologues in other fungi (i.e. *pks* associated with synthesis of aurofusarin in *Fusarium graminearum* (Frandsen et al., 2011; Kim et al., 2005; Malz et al.,2005), bikaverin in *Fusarium fujikuroi* (Linnemannstöns et al., 2002; Wiemann et al., 2009) and DHN melanin in *Aspergillus* spp. (Baker, 2008; Chiang et al., 2011; Jørgensen et al., 2011; Langfelder et al., 1998; Tsai et al., 1998, 2001; Watanabe et al., 1999; 2000). It was therefore hypothesized that PKS4 would likewise be involved in the production of characteristic *Trichoderma* yellow-green pigment (Baker et al., 2012).

Pigment forming PKSs have been shown to have functions beyond providing the color of conidia (Langfelder et al., 1998, Heinekamp et al., 2012, Tsai et al., 1998). In this study we therefore used reverse genetics to examine the functions of *pks4* in *T. reesei*. We will show that a loss of function of *pks4* is indeed responsible for the pigmentation of conidia and fruiting bodies, but also has an impact on mycelia growth rates, conidiation, conidial wall stability, and on the antagonistic abilities of *T. reesei* against other fungi, including formation of inhibitory metabolites. In addition, we will show that deletion of *pks4* significantly affect the expression of other PKS-encoding genes of *T. reesei*. To our knowledge this is the first indication that low-molecular pigments can be involved in defense, mechanical stability and stress resistance in fungi.

Material and methods

Deletion of *pks4* gene in *T. reesei*

The *pks4* was deleted based on the double joined PCR method as described by Yu et al. (2004). Essentially, a typical PCR reaction fused DNA fragments of a 50 flanking sequence, a 30 flanking sequence and a hygromycin B (*hyg*) selection marker. Primers F2 and R3 carried homologous sequence overlapping with the ends of the selection marker. In a first round PCR the amplification of the components using the specific and chimeric primers (see primer list below) was done. In a second PCR round the assembly reaction was carried out without using any specific primers, as the overhanging chimeric extensions act as primers (Yu et al., 2004). In the third PCR round amplification of the final product using nested primers was made (primers F1

and R4). Transformants were randomly picked and examined for double crossover-mediated gene replacement pattern by PCR amplification of the *hyg* locus using a primer pair beyond the flanking regions included in the cassette (primers F1 and R6).

List of primers used for the double-joined PCR transformation of *T. reesei* Qm6a to achieve *pks4* gene deletion:

```
F1    CAATGGCCGAATGTTCTAGC
F2    AGGTACGCATGGAGACAACA
R3    GGAACAAGTTGAGCCAGAGC
F4    GCAATACACGGTGAGAACGA
R5    TACACACGCACTCACGCATA
R6    TGCGGAGGATCGAGACTATT
```

The verification of the *pks4* absence in the genome of *T. reesei* Δ*pks4* was tested by a specific qPCR primers (Table 3.1) with the following amplification protocol; initial denaturation step for 3 min at 95 °C, followed by 40 cycles of denaturation for 15 sec at 95 °C, annealing for 20 sec at 54 °C and extension at 72 °C for 15 sec.

Somatic incompatibility

Somatic compatibility of Δ*pks4* mutants and the parental strain was tested to reveal the genetic identity of the mutants and the influence of the *pks4* knock-out on the recognition of QM 6a genotype. The strains were confronted to each other on 2.4 %PDA (BD Difco, Germany), at 28 °C for seven days in darkness.

Morphological observations

The parental strain QM6a and both deletion mutants were cultivated in darkness and 12h cyclic light on 2.4 % PDA (BD Difco, Germany) at 28 °C for seven days. Spore density was measured quantitatively per cm^2 of developed conidia. For this purpose three 6.2 cm^2 agar fragments were cut from cultures pre-grown at 28 °C for seven days and were rinsed separately in 15 ml of water containing 0.1 % of Tween-80 until visually all conidia were washed out. The OD of the suspension was measured at 540 nm using Biolog turbidimeter calibrated to the Biolog standard for f lamentous fungi. The final concentration value was calculated based on the calibration curve inferred from the serial dilutions of the standard suspension. Furthermore, size of the conidia was assessed by measuring the length of 40 conidia per each strain under the microscope using 400 x magnifications.

Mycelial growth rate and carbon source utilization

Strains growth rates and carbon utilization profile were analyzed using a Phenotype microarray system with Biolog FF microplates for f lamentous fungi (Biolog Inc., Hayward, CA) as described in Druzhinina et al. (2006) and Atanasova et al. (2010). Briefly, the strains were cultivated on 2.4 % PDA for five days in darkness

and conidial inocula were prepared by rolling a sterile, wetted cotton swab over conidiating areas of the plates. The conidia were then suspended in sterile Biolog FF inoculating f uid (0.25 % Phytagel, 0.03 % Tween-40), gently mixed, and adjusted to a transmission of 75 % at 590 nm (using a Biolog standard turbidimeter calibrated to the Biolog standard for f lamentous fungi; 4 x 10^7 spores in 10 ml of phytagel).A total of 90µl of the conidial suspension was dispensed into each of the wells of the Biolog FF microplates (Biolog Inc.) and incubated at 28 °C in darkness and at 12 h cyclic light. The optical density (OD) at 750 nm (for detection of mycelial growth) was measured after 18, 24, 42, 48, 66, 72, 90 and 96 hours using a microplate reader (Biolog Inc.).The growth rate of each strain was assessed by the averaged mycelial density measured on all 95 carbon sources after 0, 24, 48 and 72 hours of incubation in darkness. Statistical analyses were performed using the Statistica software package (version 6.1; StatSoft Inc., Tulsa, OK).

Response to illumination

The three strains were inoculated in the Biolog FF Phenotype microarray plates containing 95 carbon sources and water as described above, incubated in light (20 cm away from Master TLD 15W/840 lamp) and darkness at 28 °C for five days and measured at 750 nm. The growth rates and carbon utilization patterns were compared to those incubated in darkness and the data were statistically analyzed using the Statistica software package (version 6.1; StatSoft Inc., Tulsa, OK).

UV sensitivity

The photosensitivity of the spores lacking the pigment was tested using UV radiation with the HNS 15W OFR UV lamp. The conidia were collected from five days old plates and were filtered through sterile glass wool and the amount of spore suspension containing 4 x 10^7 (75 % turbidity) was estimated using Biolog turbidimeter at 580 nm. The suspensions were then diluted several times to obtain ca. 20 spores per 100 µl which were then plated on four PDA plates per sample (QM 6a and both Δ*pks4* mutants) and four for each sample control. Open plates were then exposed to the UV illumination for five, seven and ten minutes, whereas the control was opened protected from UV light. The plates were then incubated for 48 h at 28 °C and the single spore colonies observed under 10-times magnification were counted and the percentage of germinated spores were normalized by the spore numbers obtained from not exposed plates. Statistical analyses were performed using the Statistica software package (version 6.1; StatSoft Inc., Tulsa, OK).

Spore capacity

Spores of the mutants and the parental strain were transferred to a carbon supporter and were coated with 4 nm Au-Pd layer under vacuum conditions in the high vacuum evaporation unit, type Sputter Coater Quorum Q150T S (Quorum Technologies, Germany). The spores were then observed under a FEI Quanta™

200 Field Emission Scanning Electron Microscope (FEGSEM) using 5kV and high pressure under 25 000 x magnification.

Mating experiments and amplification of *MAT* loci

In vitro mating experiments were carried out on PDA (BD Difco, Germany) medium at room temperature and cycling day light as dual confrontation assays of QM 6a or *pks4* deletion mutants (*MAT1-2*) against CBS 999.97 (*MAT1-1*). Plates were incubated for 14 days, the ascospores were then collected from the mature fruiting bodies and progeny strains were isolated by single ascospore cultivation. Obtained F1 progeny strains were then purified and tested for somatic incompatibility as described above. The strains which expressed somatic incompatibility reaction were further used in this study. Obtained F1 generations of *MAT1-1* and *MAT1-2 pks4* deletion or non-deletion progeny was further cross-mated as described above.

PCR verification of MAT loci and *pks4* inheritance

Mycelia were harvested after 2–4 days of growth on PDA (Difco, Germany) at 28 °C and genomic DNA was isolated using DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer’s protocol. Each progeny strain was screened for mating type by amplifying *mat1-1-3* and *mat1-2-1* loci as described in Druzhinina et al. (2010). Furthermore, all progeny strains were screened for inheritance of *pks4* gene using PCR primers PKS4-2fw (TCATTATACACGGACTTT) and PKS4-1rev (TATAAGCCTGACTGTAGT) at following conditions: 1 min of initial denaturation step at 94 °C followed by 30 cycles of 1 min denaturation at 94°C, 1 min of annealing at 50 °C and 90s of extension at 72 °C. Final extension was carried out at 72 °C for 7 min. The results were verified by real-time PCR (qPCR) primer pair for *pks4* gene listed in Table 3.1 under the conditions described above. The number of cycles was compared to the number of cycles obtained for housekeeping gene *tef1*. The qPCR runs were carried out in iQ5 Bio-Rad qPCR machine under the conditions described below.

Antagonistic potential

Antagonistic ability of *T. reesei* QM 6a and both *pks4* deletion mutants was assessed by dual confrontation tests against *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *Alternaria alternata*. Agar plugs with fresh culture of *Trichoderma* and the plant pathogenic fungus were placed each on opposite poles of a PDA (BD Difco, Germany) plate, 1 cm from the edge. The cultures were incubated at 28 °C for 10 days under the 12h cyclic light. Antagonistic potential was estimated as a percentage reduction of the antagonist growth rate corrected for the growth rate when confronted with itself, so that the growth of antagonist against itself was set up as a zero inhibition rate.

Volatile compounds (VOCs)

The impact of *pks4* gene on VOCs production was tested by "sandwich" culture test. *Trichoderma* strains were first cultivated on the PDA plate for two days at 28 °C and were then sealed together with a plate containing fresh antagonistic fungus on the top. The plates were then further cultivated at the same conditions for four days. The radius of the plant pathogenic fungi was measured every 24h.

Water soluble compounds (WSCs)

WSCs were assessed by growing *Trichoderma* QM 6a and the mutants on the PDA covered by cellophane, which was removed together with *Trichoderma* mycelium after 60 hours. Agar plugs of antagonistic fungi were then put in the middle of the plates and were cultivated for another four days under 12h cycling light and 28 °C. The radius of the fungi was measured every 24h. Additionally the same experiment was performed when the fungus cultivated on PDA was sealed on the top of the growing *T. reesei* under the same conditions as described above.

RNA extraction

Confrontation assays of *T. reesei* QM 6a and both Δ*pks4* strains against themselves and against *Rhizoctonia solani* were carried out on potato dextrose agar plates (BD Dicfo, Germany) covered with cellophane at 28 °C and 12 hours cyclic illumination. Peripheral hyphal zones from each confrontation stage were sampled and were shock frozen in liquid nitrogen. Mycelium of five replicate plates was harvested when the hyphae were ca. 20 mm apart (before the contact) and at contact of the mycelia and was pooled together before the RNA extraction. Mycelia were grinded to a fine powder under liquid nitrogen and total RNA was isolated using the RNeasy extraction Kit (Qiagen, Germany). For cDNA synthesis, DNase treated (DNase I, RNase free; Fermentas, Germany) RNA (5 µg) was reverse transcribed with the RevertAid™ First Strand cDNA Kit (Fermentas, Germany) according to the manufacturer's protocol with a combination of the provided oligo(dT) and random hexamer primers.

Expression analysis

All real-time PCR experiments were performed in an iCycler IQ (Biorad, Germany). For the reaction the IQ SYBR Green Supermix (Bio-Rad, Germany) was prepared for 25 µl assays with standard $MgCl_2$ concentration (3 mM) and a final primer concentration of 100nM each. Primers used are given in Table 3.1. The amplification protocol consisted of an initial denaturation step (3 min at 95°C) followed by 40 cycles of denaturation (15 sec at 95°C), annealing (20 sec at the temperature given in the Table 3.1 for each primers separately) and extension (72 °C for 15 sec). *Tef1* housekeeping gene which maintained constant over all

conditions (± 20 relative %) was used as the normalizer. Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA (0.1, 10^{-2}, 10^{-3} and 10^{-2}). Amplification efficiency was then calculated from the given slopes in the IQ5 Optical system Softwarev2.0. The qPCR were performed with the cDNA of 5 pooled biological replicates for each species and condition separately. Expression ratios were calculated by the Pfaffl test model implemented in the Relative expression software tool (REST) (Pfaffl et al., 2002).

Results

Generation of a *pks4* knock out strain of *T. reesei* QM 6a

The mutants showing an altered phenotype were randomly picked from plates and the deletion of *pks4* was verified by PCR amplification of region outside of the recombination area and only those transformants and qPCR as described in Material and methods. Two candidates, Δ*pks4*-1 and Δ*pks4*-2 were selected for further studies.

Performing Biolog Phenotypic microarray it has been verified that the phenotypes of both candidate strains are very consistent and that the changes observed are in agreement with the previous data from transformation strains (Druzhinina et al., 2006). Furthermore, the genetic identity of the mutants and the influence of the *pks4* knock-out on the recognition of QM 6a genotype were tested by a plate confrontation assay. Both mutants expressed somatic incompatibility reaction (flat zone) and no anastomoses were observed in contact to their parental strain QM 6a, indicating genetic alterations due to transformation. The parental strain recognized the mutants as non-self genotype and thus reacted antagonistic, what eventually led to the partial overgrowth of the mutants. The mutants were well compatible to each other, what suggested that the deletion strains are genetically identical.

Δpks4 mutants are devoid of green conidial pigmentation

The default hypothesis of this work was that PKS4 is involved in pigment formation. This was confirmed: morphological examination on PDA plates revealed that both *pks4* deletion mutants lost the green conidial pigmentation (Figure 3.1a) but the characteristic for *T. reesei* yellow pigmentation of the media remained unchanged. Green conidia coloration did not occur in any of cultivation conditions in light or darkness, and was not recovered by cultivation on any of 95 carbon sources of the Biolog Phenotype Microarrays (Figure 3.1b).

The *Δpks4* strains did not show any statistically significant difference in the conidiation intensity on PDA: after seven days QM 6a and *Δpks4*-1 and *Δpks4*-2 produced in average 9.03, 9.16 and 9.94 x 10^6spores per cm^2, respectively (ANOVA $p > 0.05$; Figure 3.1c). Spore size remained unchanged (ANOVA $p > 0.05$; data not

shown), however, the conidia of the deletion mutants showed less stability against reduced air pressure (Figure 3.1d).

In order to test whether *pks4* is solely responsible for conidial pigment formation, we crossed the Δ*pks4* mutants (which due to their QM 6a background posses *MAT1-2*; Seidl et al. 2009) with the *MAT1-1* strain CBS 999.97. In total 34 pure single spore progeny strains were isolated from mature ascospores. 21, 10 and 3 cultures contained purely white, green and yellowish conidia, respectively. All first generation progeny (F1) strains were PCR screened for the mating types, and revealed equal distribution (15 *MAT1-2* and 18 *MAT1-1*) that was phenotype independent. Specific *pks4* primers were designed (Table 3.1) and used to test F1 for the presence of *pks4* gene. It was thereby found that all the strains with green conidia had inherited the wild-type gene, whereas the yellow and albino-conidia phenotype did not contain *pks4* gene. This proves that the loss of the conidial pigmentation indeed is the direct cause of the *pks4* deletion and that this gene is involved in production of green pigmentation of *T. reesei*.

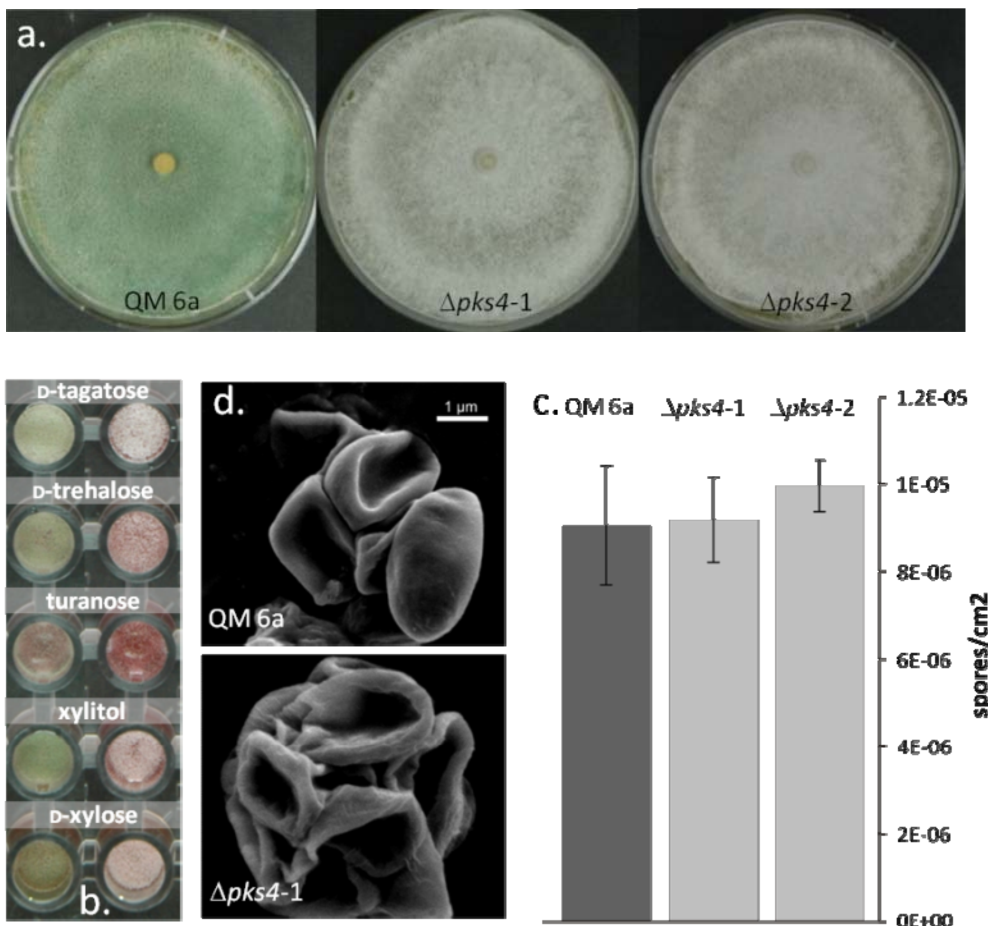


Figure 3.1: Comparative photoplate of QM 6a and the *pks4* deletion mutants' conidiation. a. Morphology and conidial pigmentation of Δ*pks4* and QM 6a cultures. b. Conidiation of QM 6a and Δ*pks4* mutants on D-tagatose, D-trehalose, turanose, xylitol and D-xylose. c. Number of QM 6a and Δ*pks4* mutants' spores measured after seven

days of cultivation PDA. No statistically significant difference in the conidiation intensity was observed (ANOVA p> 0.05). d. Conidial stability observed under a FEI Quanta™ 200 Field Emission Scanning Electron Microscope (FEGSEM) after applied vacuum conditions and high pressure under 25 000 x magnification.

Loss of green pigmentation caused reduced resistance to UV

The lack of green conidia pigmentation was also reflected in an increased sensitivity to UV light: after seven minutes of UV exposure (see M&M for details) 64 % of green spores survived, whereas only 8-20 % of white conidia were able to germinate, both compared to not exposed control (Table 3.2). The prolongation of exposure time for three minutes led to ca. 92 % loss of germination rate of both ∆*pks4* mutants (Table 3.2) while QM 6a germination decreased for only 60 %.

Protein ID*	Name[+]	R or NR	Nr. of amino acids	PKS	Identity [%]	E-value	Accession Nr.	NCBI blastp identification	Reference	forward primer 5' - 3' reverse primer 5' - 3'	Tm
Tr82208	PKS4	NR clade I	2146	yellow-green conidial pigment polyketide synthase	47	0.0	ACJ13039	*alb1* from *A. fumigatus*	Pihet et al., 2009	CCAGCAGACAGATACAAC AACAGTCCAGGCTCATTA	54
Tr81964	PKS8	NR clade I-II	1863	uncharacterized protein	47	0.0	JN257714	*Ada*A from *A. niger* or *VrtA* from *Penicillium aethiopicum* are involved in anthracenone and naphthacenedione production respectively	Li et al., 2012/ Chooi et al., 2010	ATAACACTGGCGTCACAT CAATCAGCACAGCAATCTC	54.7
Tr105804	PKS3	NR clade III	2116	PKS involved in lipid metabolisms	54	0.0	XP_ 003719468	PKS16 polyketide synthase from *Botryotinia fuckeliana*	*Botrytis cinerea* T4 genome consortium	CCGTATCTCTGCTGTATC GTGAACCATCTTGAAGGA	na
Tr73621	singlet -PKS1S	NR clade III	2633	uncharacterized protein	37	0.0	EGE04288	phenolpthiocerol synthesis polyketide synthase ppsB from *Trichophyton equinum*	*Trichophyton equinum* CBS 127.97 genome consortium	AGCATAAGCGGAATACATC AGCCTGAGAAGAGTTGAT	50.2
Tr65172	PKS1	RD clade I: lovastatin/ citrinin diketide	2598	uncharacterized lovastatin/ citrinin - like diketide synthase	35/40	0.0/ 2E-93	BAC20566/ AAP32477	MlcB from *P. citrinum* synthesizes the diketide portion of lovastatin /PKS involved in ochratoxin A from *A. ochraceus*	Abe et al., 2002/ O'Callaghan et al., 2003	AACATCAATCTCAACATC ACACATCGGTATAAGTATA	57
Tr65891	PKS2		2374		37	0.0	ELQ36243	6-methylsalicylic acid synthase from *Magnaporthe oryzae*	Xue et al., 2012	GGACATATTCAACAGGATTCTC GGTGGCAACATCTTCAAG	54
Tr60118	PKS6		2415		46	0.0	AAR90259	uncharacterized polyketide synthase from *Cochliobolus heterostrophus*	Kroken et al., 2003	TCAAGTGGTCTCCTCTATT AATGTGCTGTCTCAATCC	54.7
Tr106272	PKS9		2612		54	0.0	AAR92209	uncharacterized PKS2 from *Gibberella moniliformis*	Kroken et al., 2003	CCGTATCTCTGCTGTATC ATCGTCTGTGATGAAGTG	54.7
Tr73618	singlet - PKS2S		2567		41/40	0.0	BAC20566/ AAD34559	MlcB from *P. citrinum* and LovF from *A. terreus* synthesize the diketide portion of lovastatin and citrinin respectively	Abe et al., 2002/ Hendrickson et al., 1999	TACCATTACACAGACTTG AGCAATCACAACATCATA	50.2
Tr59482	PKS5	RD clade III: t-toxin	2205	uncharacterized t-toxin - like synthase	34	0.0	ABB76806	PKS1 and PKS2 *Cochliobolus heterostrophus* are required for synthesis of T-toxin	Baker et al., 2006	TCTCATTGATGCGTGGTA GCTTGGACTCTCATTCATATC	57
Tr65116	PKS7	RD clade IV: fumonisins	2434	uncharacterized fumonisin-like synthase	40	0.0	ELQ64206	mycocerosic acid synthase from *Magnaporthe oryzae*	Xue et al., 2012	AAGAAGATGTCCGCAACT AAGCACTCATACACAACCT	na

RD - reducing, NR - non reducing PKS; * JGI genome portal http://genome.jgi-psf.org/Trire2/Trire2.home.html; [+] after PKS grouping of Baker et al. (2012)

Table 3.1: *Trichoderma reesei* repertoire of PKS encoding genes, their most related orthologs for which products or functions are identified and the primers and annealing temperatures used in the expression analysis.

Table 3.2: Percentage of germinated spores after the exposure to UV light for seven and ten minutes. Sd annotate standard deviation values.

T. reesei strain	% of germinated spores after UV exposure			
	7 min	sd	**10 min**	sd
QM 6a	63.6	*10.1*	39.4	*6.4*
Δ*pks4*-1	19.7	*6.5*	5.9	*8.4*
Δ*pks4*-2	7.7	*6.5*	7.7	*6.5*

Pks4 is responsible for ascospore discharge and contributes to the development of the brown color of fruiting bodies and stroma

The wild-type morphology of the *T. reesei* teleomorph is conspicuous with perithecial openings appearing as black dots against the light brown background (Figure 3.2a-b; Samuels et al., http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm). Crossing of the two *Δpks4* mutants with the *MAT1-1* tester strain CBS 999.79, as described above, produced stroma with fruiting bodies that were normally pigmented which shot vital and fertile ascospores. Crossings between the albino strains from the collection of F1 described above showed that the pigmentation of both stroma surface and perithecial openings is also altered (Figure 3.2c-e, h-i). Thus, the young stromata appeared to be white with slightly brownish dots of perithecial openings (Figure 3.2c, d). However, the mature and over-mature teleomorphs developed dark brown pigmentation of both stroma surface and perithecial walls and openings (Figure 3.2d, e, h). Importantly, the surface of mature stromata was covered by whitish worm-like structures that originated from perithecial openings (Figure 3.2e, h). Microscopic investigation showed that these structures were entirely composed of mature ascospores as some of them started to germinate (Figure 3.2g). This observation indicates that the mechanism of ascospore discharge is altered in absence of PKS4. Morphology of asci was observed to be normal.

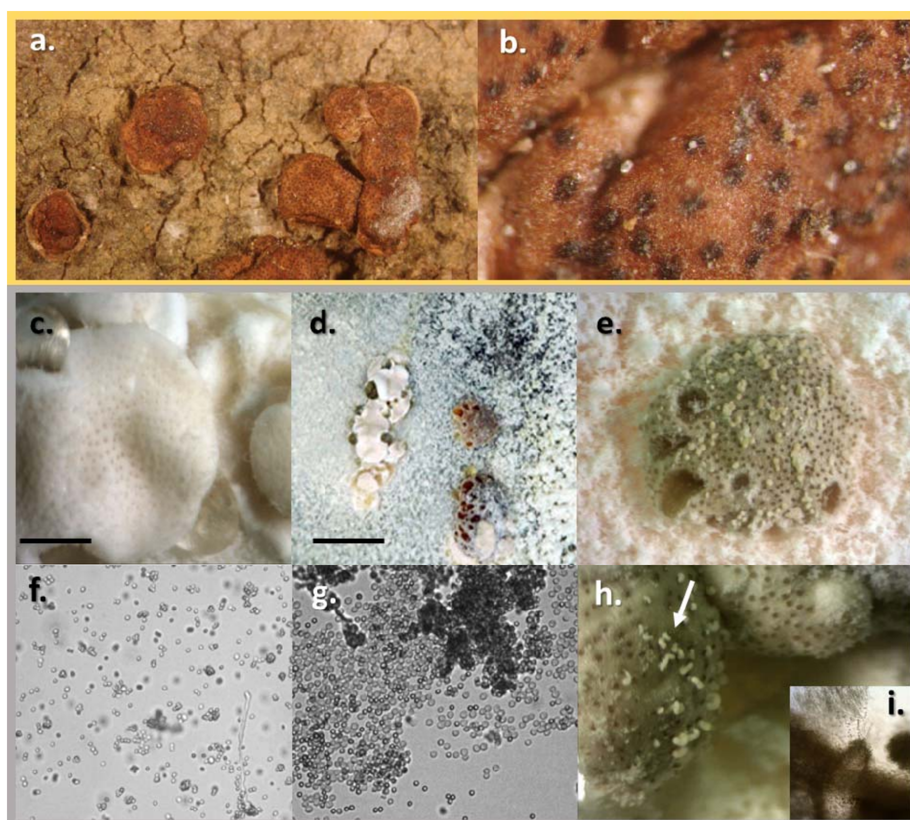


Figure 3.2: Photoplate of *T. reesei* QM 6a and Δ*pks4* mutants' fruiting bodies. a. *T. reesei* fruiting bodies in nature. b. *T. reesei* melanised brown stromata and dark-brown perithecia openings. c. young white stromata and mature, partially melanised brownish fruiting bodies of F1 Δ*pks4* strains mated *in vitro.* The scale annotate 10 mm. *d.* Partially melanised over-mature fruiting bodies of F1 Δ*pks4* strains with dark perithecia openings. The scale annotates 1 mm. e. magnified young white stromata of F1 Δ*pks4* strains with non-melanised openings. f. Conidia of F1 Δ*pks4* strains observed under 200 x magnification. g. Ascospores from the worm-like structures of F1 Δ*pks4* attached to over-mature perithecia indicated by arrows on figure h. i. Pigmented perithecium with over-mature melanised stroma under 200 x magnification.

Loss of pks4 reduces the antifungal potential of *T. reesei*

In dual confrontation assays with several fungi (*Rhizoctonia solani, Sclerotinia sclerotiorum, Botrytis cinerea and Alternaria alternata*) showed that the ability of *T. reesei* QM 6a to inhibit growth of other fungi was reduced in the Δ*pks4* mutants (Figure 3.3a) by 37, 13, 22 and 40 %, respectively. Interestingly, there were no significant differences in ability of QM 6a and the *pks4* mutants to overgrow their opponents, as can be observed for *A. alternata* and *R. solani* on the Figure 3.3a, thus the mycoparasitic ability was not affected. However, the difference was detected in respect to the ability to protect against opponent's metabolites: in confrontations of QM 6a with *A. alternata*, *S. sclerotiorum* and *R. solani* a narrow antibiosis zone was observed likely due to secretion of metabolites toxic for *T. reesei* (Figure 3.3b).This zone was clearly enlarged and appeared with diffused borders in confrontations with the Δ*pks4* mutants suggesting that metabolites secreted by other fungi penetrate colonies of the deletion mutants.

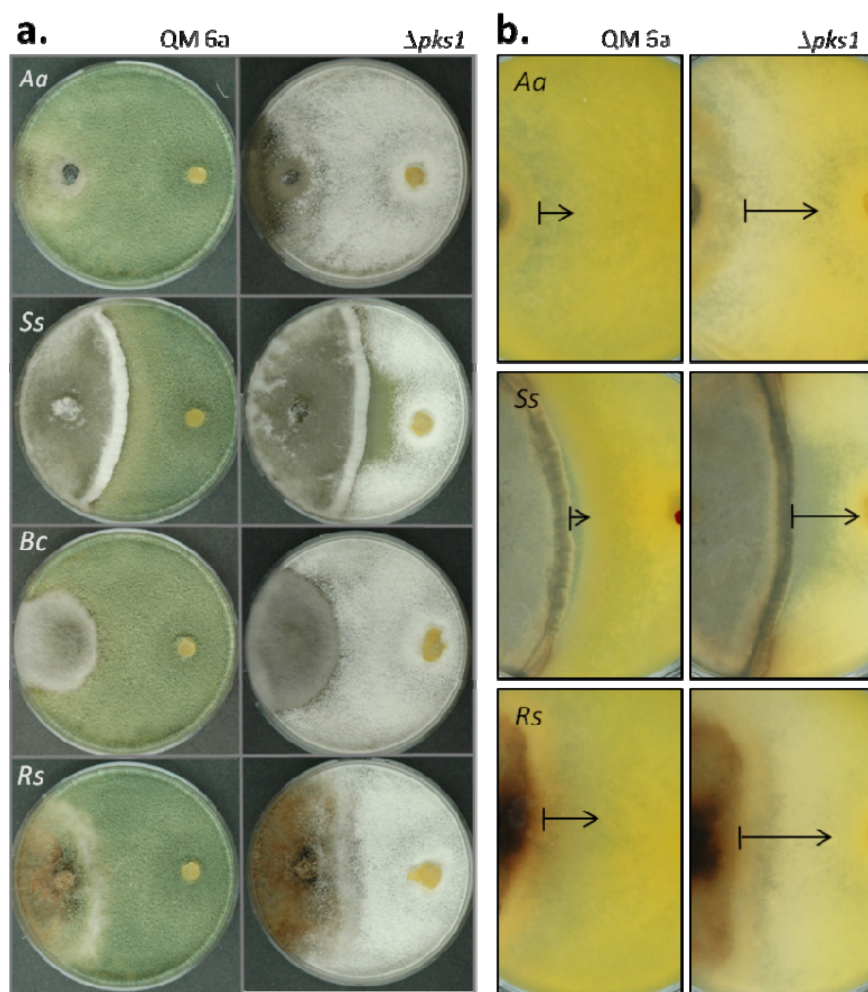


Figure 3.3: Mycoparasitic potential of QM 6a and *pks4-1* deletion mutants. Confrontations panels are shown only for Δ*pks4*-1, both mutants revealed identical mycoparasitic pattern. a. Front sides of confrontation plates with plant pathogenic fungi always on the left side and *Trichoderma* QM 6a and the mutants always on the right. b. Back sides of confrontation plates with plant pathogenic fungi always on the left side and *with T. reesei* QM 6a and the mutants always on the right. Enlarged antibiosis zones for Δ*pks4* strains are indicated by the arrows.

Pks4 affects the production of water soluble and volatile inhibitory compounds

In order to test whether the loss of *pks4* is also reflected in an alteration of water soluble and volatile metabolites produced by *T. reesei*, QM 6a and the two *Δpks4* strains were grown in sealed "sandwich" cultures (see Materials and Methods) with *R. solani, S. sclerotiorum, B. cinerea and A. alternata* so that the test fungi were facing *Trichoderma* cultures on the top. When compared to the effect caused by the both *pks4* deletion mutants with that of QM 6a, the growth of all four tested fungi was strongly reduced by VOCs (Figure 3.4 showing *R. solani* as one example), thus indicating that the *pks4* deletion mutants display an enhanced production of VOCs compared to QM 6a.

In contrast, the formation of fungicidic water soluble compounds (WSCs) by *T. reesei* was reduced in the Δ*pks4* strains (Figure 3.4b). Interestingly, the secretion of WSCs by *T. reesei* QM 6a and the *Δpks4* mutants was inhibited by the presence *R. solani* VOCs (no hyphal contact), and this effect was even enhanced in the *Δpks4* strains (Figure 3.4c).

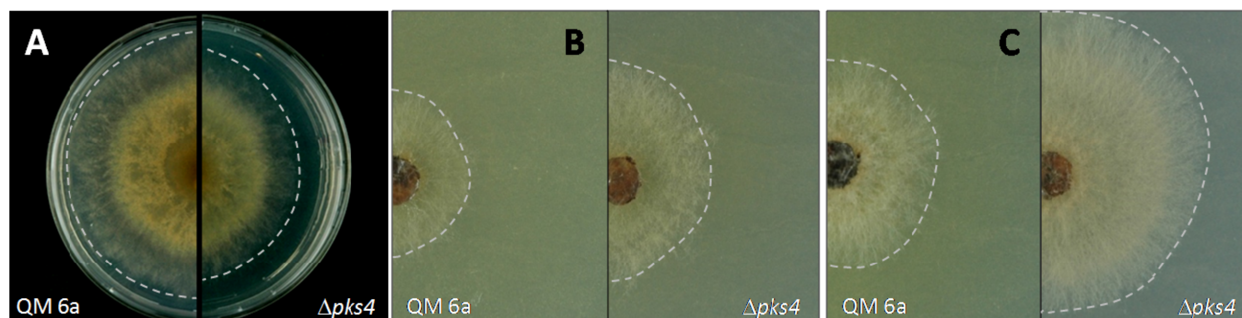


Figure 3.4: Effect of VOCs and WSCs from *pks4* deletion mutant and QM 6a on growth of *R. solani*. A. Reduction of *R. solani* growth by the VOCs secreted by *T. reesei* QM 6a and Δ*pks4*-1 strain after four days incubation. Both mutants consistently reduced the growth of *R. solani*. B. *R. solani* growth on the PDA medium containing WSCs of *T. reesei* and *pks4* deletion mutants. C. Growth of R. solani on the medium with WSCs secreted by the pks4 mutant and QM 6a in the presence of VOCs from *R. solani* during the growth of *T. reesei* strains. Aa, Ss, Bs and Rs mark *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *Rhizoctonia solani*, respectively.

Pks4 is involved in regulation of other PKS genes in confrontation with other fungi

We were also interested whether the loss of PKS4 would impact other PKS-encoding genes in *T. reesei*. To this end, we assessed their expression in QM 6a and the two *Δpks4* mutants when confronted to *R. solani*. Expression of PKS genes was inspected both prior the contact and at contact of the hyphae (Figure 3.6). Two *pks* genes, *pks3* (Trire2:105804) and *pks7* (Trire2:65116), were not detectable in the conditions of our experiments including in the confrontations of strains to itself.

The expression analysis of the remaining eight *pks* genes in *Δpks4* mutants and QM 6a prior and at the contact with the *R. solani* revealed essential change in the regulation patterns depending on the stage of the interaction respectively such as before contact and contact respectively. Before the contact both QM 6a and the mutants up-regulated four *pks* genes from lovastatin/citrinin reducing clade I (*pks1*, *pks2*, *pks6* and *pks9*; for protein ID see Table 3.1). Yet, contrary to the *pks9* (Trire2: 106272) and *pks2* (Trire2: 65891) which expressions were much higher in QM 6a then in the mutants (Figure 3.6a), *pks6* and *pks1* were strongly up-regulated in mutant strains (Figure 3.6a). The remaining two reducing genes *pks5* (Trire2:59482) from the clade of fumonisin – like synthases and the singlet *pks2S* (Trire2:73618) as well as the non-reducing *pks1S* (Trire2:73621) were not influenced in QM 6a prior the contact with *R. solani*, but showed a tendency to up-regulation in both mutants (Figure 3.6a). At contact with *R. solani* the pattern was different: QM 6a down-regulated the majority of its *pks* genes including *pks9* and *pks2* compared to when confronted to itself (Figure 3.6b). These genes were strongly up-regulated before the contact with *R. solani* (Figure 3.6a).

Interestingly, singlet *pks1S* and *pks2S* were differentially down-regulated at the contact of the mutants and *R. solani* (Figure 3.6b), whereas the QM 6a significantly up-regulated these genes at this stage.

The expression of *pks*-genes in the mutants in non-antagonistic condition (confrontation to itself) was deduced by the normalization to values of QM 6a. Most of the eight *pks* genes were down-regulated in the mutants, whereas two singlet genes, *pks2S* that is a member of the lovastatin/citrinin clade and *pks1S* belonging to the non-reducing clade III were up-regulated (Figure 3.5).

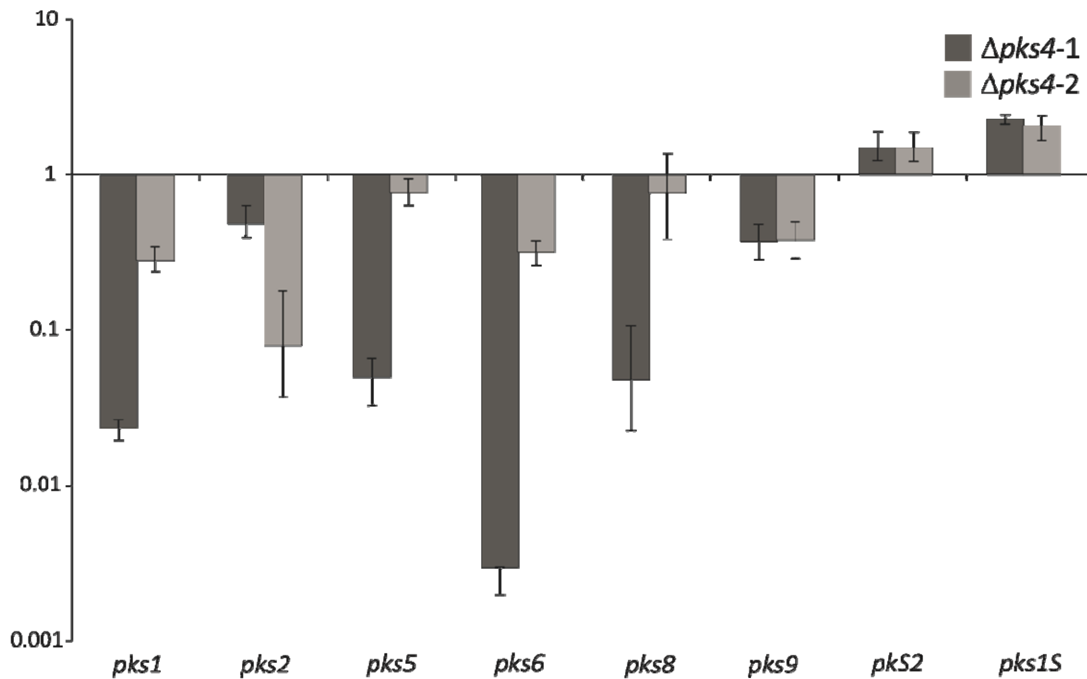


Figure 3.5: Regulation of PKS genes in both Δ*pks4* mutants normalized by the *T. reesei* QM 6a strain. Protein numbers of *pks* genes in the genome: *pks1* (Trire2:65172), *pks2* (Trire2:65891), *pks5* (Trire2:59482), *pks6* (Trire2:60118), *pks8* (Trire2:81964), *pks9* (Trire2:106272), *pks1S* (Trire2:73621) and pks2S (Trire2:73618).

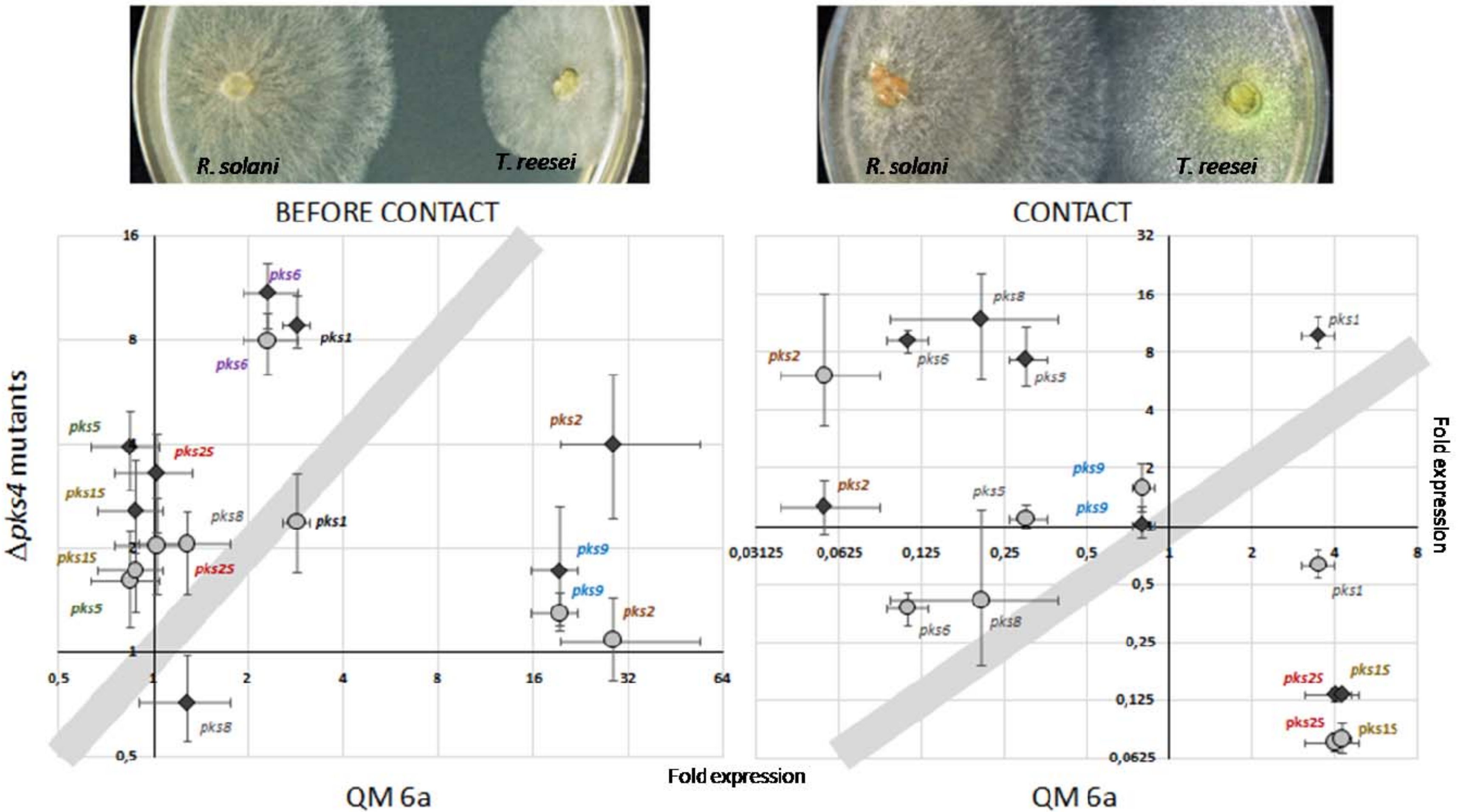


Figure 3.6: Expression rates of *T. reesei* PKS genes. a. Scattered plot of *pks* genes regulation before the contact with *Rhizoctonia solani* in *T. reesei* QM 6a plotted against Δ*pks4* mutants. b. Scattered plot of *pks* genes regulation at the contact with *Rhizoctonia solani* in *T. reesei* QM 6a plotted against Δ*pks4* mutants. Shadowed area determines no differences in regulation of *pks4* deletion mutants and QM 6a. Protein numbers of *pks* genes in the genome: *pks1* (Trire2:65172), *pks2* (Trire2:65891), *pks5* (Trire2:59482), *pks6* (Trire2:60118), *pks8* (Trire2:81964), *pks9* (Trire2:106272), *pks1S* (Trire2:73621) and *pks2S* (Trire2:73618). Those genes marked with color exhibited consistent expression trend between both of the mutants. The figures above the plots show the sampling stage of the *T. reesei* strains at each condition.

Discussion

In this paper, we have functionally characterized the role of *pks4* in *T. reesei*. *pks4* belongs to the non-reducing clade I of fungal *pks*-encoding genes, which includes genes associated with pigment production such as aurofusarin (Frandsen et al., 2011; Kim et al., 2005; Malz et al., 2005) or bikaverin (Linnemannstöns et al., 2002; Wiemann et al., 2009), but also DHN melanin (Baker, 2008; Chiang et al., 2011; Jørgensen et al., 2011; Langfelder et al., 1998; Tsai et al., 1998, 2001; Watanabe et al., 1999; 2000). While the former comprise substances of relatively low molecular weight, melanins - the dark to black pigments, are of high molecular mass that derive from oxidative polymerization of phenolic compounds (Riley, 1997; Heinekamp et al., 2012). The chemical structures of the conidial pigments of *T. reesei* and *Trichoderma* spp. in general have not been elucidated yet, but due to their green and sometimes yellowish color would appear not to be melanins. Yellow pigments e.g. from *A. niger* were shown to be dimeric linear naphtho-y-pyrones (Jørgensen et al. 2011). However, Benitez et al. (1976) preliminarily characterized the conidial pigment of *Trichoderma* sp. (*T. viride* at that time) as a non-indolic melanin-like polyphenol. Consistent with these data, Csiktusnádi Kiss et al. (2000) identified the main pigment fractions of *T. harzianum* as oxidation polymers originating from monomer molecules containing polar substructures and double bonds in the alkyl chain. The dark brown component of the fruiting body and stoma color, which likely represents melanin, is independent on PKS4. In absence of *pks4* the young stroma of *T. reesei* are colorless with slightly darkened openings of perithecia indicating some remained pigmentation. Mature and overmature stromata, however, show some dark brown coloration of the surface and perithecia walls, indicating that melanin is still synthesized but the PKS4 product is different and that it is responsible for the main bright reddish teleomorph color.

The question whether or not the pigments synthesized by PKS4 are melanins is important because our data show that PKS4 is involved in antagonism and defense against other fungi, in mechanical stability of the conidium, and in the discharge of the ascospores. A role in antagonism is also supported by earlier findings that *pks4* is up-regulated during antagonism and mycoparasitic contact of *T. reesei* with *R. solani* (Atanasova et al., 2013). A role in defense, stress resistance, virulence and mechanical stability has so far been shown for melanins but not for the low-molecular weight pigments formed by PKS4 orthologs mentioned above. In addition, some polyketides have been shown to be involved in sexual development (Wolf and Mirocha, 1973; Zimmerman et al., 1995; Graziani et al., 2004; Engh et al., 2007; Nowrousian, 2009), but none of them was a PKS4 ortholog and the mechanism of involvement is still only poorly understood, in most cases also probably related to cell-wall stabilization (Nowrousian, 2009). *T. reesei* PKS4 therefore exhibits a biological function typical for melanin synthesizing PKSs: in human pathogenic fungi, such as *Cryptococcus neoformans* (Nosanchuk et al., 2000; Rosas et al., 2000a,b; Casadevall et al., 2000), *Sporothrix schenckii* (Romero-

Martinez et al., 2000; Morris-Jones et al., 2003), *Paracoccidioides brasiliensis* (Gómez et al., 2001), *Histoplasma capsulatum* (Nosanchuk et al., 2002), and the opportunistic pathogen *A. fumigates* (Heinekamp et al., 2012), melanin is involved in virulence, probably because of resistance against oxidative stress. Also, it contributes to resistance against antifungal drugs in *H. capsulatum* (Hamilton and Holdom, 1999). Melanins have also been demonstrated to play crucial roles in plant pathogenic fungi: in *Magnaporthe grisea*, melanin accumulates between the plasma membrane and the cell wall of an aspersorium creates turgor pressure (Howart and Valent, 1996). In addition, expression of an *A. alternata* melanin biosynthetic PKS in the insect pathogen *Metarhizium anisopliae* exhibited increased virulence (Tseng et al., 2011). In wood decaying Basidiomycota such as *Phellinus weirii*, (Li, 1981) and *Pleurotus ostreatus* (I. Druzhinina, personal communication) melanin is crucially important in reactions of somatic incompatibility when genet borders are marked by thick melanised walls (barrage-reaction) impermeable for competitive fungi, which could also be the reason for the effect of PKS4 on vegetative compatibility in *T. reesei.*

Another interesting consequence of *pks4* loss of function that has not been reported with any other PKS before was its effect on the expression of the other eight *T. reesei pks* genes that has been tested in this study. During normal growth in the absence of a competing fungus, all but two of these eight genes were significantly down-regulated. Since growth of the mutant strains and the wild type occurred at the same rate, these differences cannot be the consequence of different rates of nutrient uptake. In addition, antagonistic interaction with *R. solani* revealed that loss of *pks4* function affected the expression of the other *pks* genes in different ways, some being up-regulated, some down-regulated and some not affected at all. From these data we conclude that PKS4 – or rather the function of its product, e.g. protection and defense against stress – is an important signal for the expression of the other *pks* genes, and that this signal affects *pks* genes in different ways. This is definitely an area that requires further investigations.

Loss of function of *pks4* also led to a decreased synthesis of water soluble inhibitory components by *T. reesei*, and it is tempting to speculate that they would be formed by the affected PKSs. Since their production was tested in the absence of other fungi, they all are down-regulated (cf. Figure 6), and it is not possible to predict which of them is or are the responsible producers. In addition, these components could also be products of other enzymes, such as non-ribosomal peptide synthases of which *T. reesei* has ten (Kubicek et al., 2011).

It was finally interesting to note that deletion of *pks4* increased the production of volatile organic compounds (VOCs) by *T. reesei*. The chemical nature of the VOCs from *T. reesei* have not been identified yet, but their composition from other *Trichoderma* spp. showed that they in majority comprised mainly long aliphatic acids, and their alcohols and esters (Nemcovic et al., 2008; Siddiquee et al., 2012), which are

usually products of fatty acid catabolism (Savage et al., 1996). Their biosynthesis may be favored by the lack of PKS activity which results in an increased access to the cellular pools of acetyl- and malonyl-CoA.

The function of PKSs has so far mainly been investigated with respect to the role of their products in human or plant pathogenesis. Our data show that PKS4 also influences several biological functions in *T. reesei* which are not only related to the interaction with other organisms. Transcriptomic analyses of *T. reesei* have recently shown that many *pks* genes are highest expressed during rapid vegetative growth (Metz et al., 2011; Portnoy et al., 2011; Karimi-Aghcheh et al., 2013), which is not a pattern that would be expected for genes whose function is unrelated to growth. It will thus be worthwhile to perform a deeper investigation of the regulation and role of *pks* genes in fungal physiology.

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References

Abe Y, Suzuki T, Mizvno T, Ono C, Iwamoto K, Hosobuchi M, Yoshikawa H (2002) Molecular cloning and characterization of an ML-236B (compactin) biosynthetic gene cluster in *Penicillium citrinum*. Mol Genet Genomics, 267:636–646.

Atanasova L, Druzhinina IS (2010) Review: Global nutrient profiling by Phenotype MicroArrays: a tool complementing genomic and proteomic studies in conidial fungi. J Zhejiang Univ Sci B, 11(3):151-168.

Atanasova L, Crom S, Gruber S, Coulpier F, Seidl-Seiboth V, Kubicek CP, Druzhinina IS (2013) Comparative transcriptomics reveals different strategies of *Trichoderma* mycoparasitism. BMC Genomics, 14(1):121.

Baker SE (2006) Review: *Aspergillus niger* genomics: past, present and into the future. Med Mycol, 1:S17-21.

Baker SE (2008) *Aspergillus* genomics and DHN-melanin conidial pigmentation. In *Aspergillus* in the Genomic Era. Eds Varga J, Samson RA. Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 73–85.

Baker SE, Perrone G, Richardson NM, Gallo A, Kubicek CP (2012) Phylogenomic analysis of polyketide synthase-encoding genes in *Trichoderma*. Microbiology, 158(Pt 1):147-54.

Brakhage A A and Schroeckh V (2011) Fungal secondary metabolites– strategies to activate silent gene clusters. Fungal Genet Biol, 48:15–22.

Benítez T, Villa TG, GarcíaAcha I (1976) Some chemical and structural features of the conidial wall of *Trichoderma viride*. Can J Microbiol, 22(2):318-21.

Casadevall A, Rosas AL, Nosanchuk JD (2000) Review: Melanin and virulence in *Cryptococcus neoformans*. Curr Opin Microbiol, 3(4):354-358.

Chiang YM., Meyer KM, Praseuth M, Baker SE., Bruno KS, Wang CC (2011) Characterization of a polyketide synthase in *Aspergillus niger* whose product is a precursor for both dihydroxynaphthalene (DHN) melanin and naphtho-c-pyrone. Fungal Genet Biol, 48:430–437.

Chooi YH, Cacho R, Tang Y (2010) Identification of the viridicatumtoxin and griseofulvin gene clusters from *Penicillium aethiopicum*. Chem Biol, 17(5):483-94.

Csiktusnádi Kiss G, Forgács E, Cserháti T, Vizcaino JA. (2000) Colour pigments of *Trichoderma harzianum*. Preliminary investigations with thin-layer chromatography-Fourier transform infrared spectroscopy and high-performance liquid chromatography with diode array and mass spectrometric detection. J Chromatogr A, 896(1-2):61-68.

Druzhinina IS, Schmoll M, Seiboth B, Kubicek CP (2006) Global carbon utilization profiles of wild-type, mutant, and transformant strains of *Hypocrea jecorina*. Appl Environ Microbiol, 72(3):2126-33.

Druzhinina IS, Komoń-Zelazowska M, Atanasova L, Seidl V, Kubicek CP (2010) Evolution and ecophysiology of the industrial producer *Hypocrea jecorina* (Anamorph *Trichoderma* reesei) and a new sympatric agamospecies related to it. PLoS One, 5(2):e9191.

Druzhinina IS, Shelest E, Kubicek CP (2012) Novel traits of *Trichoderma* predicted through the analysis of its secretome. FEMS Microbiol Lett, 337(1):1-9.

Druzhinina IS, Kubicek CP (2013) Ecological genomics of *Trichoderma*. In Ecological genomics of fungi, Ed. Martin F. Wiley-Blackwell, Oxford, UK, in press.

Engh I, Nowrousian M, Kück U (2007) Regulation of melanin biosynthesis via the dihydroxynaphthalene pathway is dependent on sexual development in the ascomycete *Sordaria macrospora*. FEMS Microbiol Lett, 275:62–70.

Frandsen R J, Schütt C, Lund BW, Staerk D, Nielsen J, Olsson S, Giese H (2011) Two novel classes of enzymes are required for the biosynthesis of aurofusarin in *Fusarium graminearum*. J Biol Chem 286:10419–10428.

Gómez BL, Nosanchuk JD, Díez S, Youngchim S, Aisen P, Cano LE, Restrepo A, Casadevall A, Hamilton AJ (2001) Detection of melanin-like pigments in the dimorphic fungal pathogen *Paracoccidioides brasiliensis* in vitro and during infection. Infect Immun, 69:5760–5767.

Graziani S, Vasnier C, Daboussi MJ (2004) Novel polyketide synthase from *Nectria haematococca*. Appl Environ Microbiol, 70:2984–2988.

Hamilton AJ, Holdom MD (1999) Review: Antioxidant systems in the pathogenic fungi of man and their role in virulence. Med Mycol, 37(6):375-89.

Heinekamp T, Thywißen A, Macheleidt J, Keller S, Valiante V, Brakhage AA (2012) *Aspergillus fumigatus* melanins: interference with the host endocytosis pathway and impact on virulence. Front Microbiol, 3:440.

Hendrickson L, Davis CR, Roach C, Nguyen DK, Aldrich T, McAda PC, Reeves CD (1999) Lovastatin biosynthesis in *Aspergillus terreus*: characterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene. Chem Biol, 6(7):429-39.

Howard RJ, Valent B (1996) Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. Annu Rev Microbiol, 50:491-512.

Jørgensen TR, Park J, Arentshorst M, van Welzen AM, Lamers G, Vankuyk PA, Damveld RA, van den Hondel CA, Nielsen KF, Frisvad JC, Ram AF (2011) The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*. Fungal Genet Biol, 48(5):544-553.

Karimi-Aghcheh R, Bok JW,Phatale PA, Smith KM, Baker SE, Lichius A, Omann M, Zeilinger S, Seiboth B, Rhee C, Keller NP, Freitag M, Kubicek CP (2013) Functional analyses of *Trichoderma reesei* LAE1 reveal conserved and contrasting roles of this regulator. G3 3: 369-378

Kim JE, Han KH, Jin J, Kim H, Kim JC, Yun SH, Lee YW (2005) Putative polyketide synthase and laccase genes for biosynthesis of aurofusarin in *Gibberella zeae*. Appl Environ Microbiol, 71:1701–1708.

Kroken S, Glass NL, Taylor JW, Yoder OC, Turgeon BG (2003) Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. Proc Natl Acad Sci USA, 100(26):15670-15675.

Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, Thon M, Zeilinger S, Casas-Flores S, Horwitz BA, Mukherjee PK, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, Atanasova L, Cervantes-Badillo MG, Challacombe J, Chertkov O, et al. (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol, 12(4):R40.

Langfelder K, Jahn B, Gehringer H, Schmidt A., Wanner G, Brakhage AA (1998) Identification of a polyketide synthase gene (*pksP*) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. Med Microbiol Immunol (Berl), 187:79–89.

Li Y, Chooi YH, Sheng Y, Valentine JS, Tang Y (2011) Comparative characterization of fungal anthracenone and naphthacenedione biosynthetic pathways reveals anα-hydroxylation-dependent Claisen-like cyclization catalyzed by a dimanganesethioesterase. J Am Chem Soc, 133(39):15773-15785.

Linnemannstös P, Schulte J, del Mar Prado M, Proctor RH, Avalos J, Tudzynski B (2002) The polyketide synthase gene *pks4* from Gibberella fujikuroi encodes a key enzyme in the biosynthesis of the red pigment bikaverin. Fungal Genet Biol, 37:134–148.

Malz S, Grell MN, Thrane C, Maier FJ, Rosager P, Felk A, Albertsen KS, Salomon S, Bohn L et al. (2005) Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the *Fusarium graminearum* species complex. Fungal Genet Biol, 42:420–433.

Metz B, Seidl-Seiboth V, Haarmann T, Kopchinskiy A, Lorenz P, Seiboth B, Kubicek CP (2011) Expression of Biomass-Degrading Enzymes Is a Major Event during Conidium Development in *Trichoderma reesei*. Eukaryot Cell, 10:1527-1735.

Morris-Jones R, Youngchim S, Gómez BL, Aisen P, Hay RJ, Nosanchuk JD, Casadevall A, Hamilton AJ (2003) Synthesis of melanin-like pigments by *Sporothrix schenckii* in vitro and during mammalian infection. Infect Immun, 71:4026–4033.

Mukherjee PK, Horwitz BA, Kenerley CM (2012a) Secondary metabolism in *Trichoderma*-a genomic perspective. Microbiology, 158(Pt 1):35-45.

Mukherjee PK, Buensanteai N, Moran-Diez ME, Druzhinina IS, Kenerley CM (2012b) Functional analysis of non-ribosomal peptide synthetases (NRPSs) in *Trichoderma virens* reveals a polyketide synthase (PKS)/NRPS hybrid enzyme involved in the induced systemic resistance response in maize. Microbiology, 158(Pt1):155-65.

Nemcovic M, Jakubíková L, Víden I, Farkas V (2008) Induction of conidiation by endogenous volatile compounds in *Trichoderma* spp. FEMS Microbiol Lett, 284(2):231-6.

Nosanchuk JD, Rosas AL, Lee SC, Casadevall A (2000) Melanisation of *Cryptococcus neoformans* in human brain tissue. Lancet, 355:2049–2050.

Nosanchuk JD, Gómez BL, Youngchim S, Díez S, Aisen P, Zancopé-Oliveira RM, Restrepo A, Casadevall A, Hamilton AJ (2002) *Histoplasma capsulatum* synthesizes melanin-like pigments in vitro and during mammalian infection. Infect Immun, 70:5124–5131.

Nowrousian M (2009) A novel polyketide biosynthesis gene cluster is involved in fruiting body morphogenesis in the filamentous fungi *Sordaria macrospora* and *Neurospora crassa*. Curr Genet, 55(2):185-98.

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. NAR, 30:e36.

Pihet M, Vandeputte P, Tronchin G, Renier G, Saulnier P, Georgeault S, Mallet R, Chabasse D, Symoens F, Bouchara JP (2009) Melanin is an essential component for the integrity of the cell wall of Aspergillus fumigatus conidia. BMC Microbiol, 24:9:177.

Portnoy T, Margeot A, Le Crom S, Linke R, Atanasova L, Fekete E, Sándor E, Karaffa L, Druzhinina IS, Seiboth B, Kubicek CP (2011) The CRE1 carbon catabolite repressor of the fungus *Trichoderma reesei*: a master regulator of carbon assimilation. BMC Genomics, 12: 269.

Riley PA (1997) Review: Melanin. Int J Biochem Cell Biol, 29(11):1235-1239.

Romero-Martinez R, Wheeler M, Guerrero-Plata A, Rico G, Torres-Guerrero H (2000) Biosynthesis and functions of melanin in *Sporothrix schenckii*. Infect Immun, 68:3696–3703.

Rosas A. L., Nosanchuk J. D., Feldmesser M., Cox G. M., McDade H. C., Casadevall A (2000a) Synthesis of polymerized melanin by *Cryptococcus neoformans* in infected rodents. Infect Immun 68, 2845–2853.

Rosas AL, Nosanchuk JD, Gómez BL, Edens WA, Henson JM, Casadevall A (2000b) Isolation and serological analyses of fungal melanins. J Immunol Methods, 244(1-2):69-80.

Saha D, Fetzner R, Burkhardt B, Podlech J, Metzler M, Dang H, Lawrence C, Fischer R (2012) Identification of a polyketide synthase required for alternariol (AOH) and alternariol-9-methyl ether (AME) formation in *Alternaria alternata*. PLoS One,(7):e40564.

Samuels GJ, Chaverri P, Farr DF, McCray EB. *Trichoderma* Online, Systematic Mycology and Microbiology Laboratory, ARS, USDA. Retrieved February 26, 2013, from /taxadescriptions/keys/TrichodermaIndex.cfm

Savage TJ, Hristova MK, Croteau R (1996) Evidence for an elongation/Reduction/C1-Elimination Pathway in the Biosynthesis of n-Heptane in Xylem of Jeffrey Pine. Plant Physiol, 111(4):1263-1269.

Seidl V, Seibel C, Kubicek CP, Schmoll M (2009) Sexual development in the industrial workhorse *Trichoderma* reesei. Proc Natl Acad Sci U S A, 106(33):13909-13914.

Siddiquee S, Cheong BE, Taslima K, Kausar H, Hasan MM (2012) Separation andidentification of volatile compounds from liquid cultures of *Trichoderma harzianum* by GC-MS using three different capillary columns. J Chromatogr Sci, 50(4):358-367.

Tsai HF, Chang YC, Washburn RG., Wheeler MH, Kwon-Chung KJ (1998) The developmentally regulated *alb1* gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. J Bacteriol, 180:3031–3038.

Tsai HF, Fujii I, Watanabe A, Wheeler MH, Chang YC, Yasuoka Y, Ebizuka Y, Kwon-Chung KJ (2001) Pentaketide melanin biosynthesis in *Aspergillus fumigatus* requires chain length shortening of a heptaketide precursor. J Biol Chem, 276:29292–29298.

Tseng MN, Chung PC, Tzean SS (2011) Enhancing the stress tolerance and virulence of an entomopathogen by metabolic engineering of dihydroxynaphthalene melanin biosynthesis genes. Appl Environ Microbiol, 77(13):4508-4519.

Watanabe A, Fujii I, Sankawa U, Mayorga ME, Timberlake WE, Ebizuka Y (1999) Re-identification of *Aspergillus nidulans* wA gene to code for a polyketidesynthase of naphthopyrone. Tetrahedron Lett, 40:91–94.

Watanabe A, Fujii I, Tsai H, Chang YC, Kwon-Chung KJ, Ebizuka Y (2000) *Aspergillus fumigatus alb1* encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*. FEMS Microbiol Lett, 192:39–44.

Wiemann P, Willmann A, Straeten M, Kleigrewe K, Beyer M, Humpf HU, Tudzynski B (2009) Biosynthesis of the red pigment bikaverin in *Fusarium fujikuroi*: genes, their function and regulation. Mol Microbiol, 72:931–946.

Wolf JC, Mirocha CJ (1973) Regulation of sexual reproduction in *Gibberella* zeae (*Fusarium roseum* 'Graminearum') by F-2 (Zearalenone). Can J Microbiol, 19:725–734.

Xue M, Yang J, Li Z, Hu S, Yao N, Dean RA, Zhao W, Shen M, Zhang H, Li C, Liu L, Cao L, Xu X, Xing Y, Hsiang T, Zhang Z, Xu JR, Peng YL (2012) Comparative analysis of the genomes of two field isolates of the rice blast fungus *Magnaporthe oryzae*. PLoS Genet, 8(8):e1002869.

Yu JH, Hamari Z, Han KH, Seo JA, Reyes-Domínguez Y, Scazzocchio C (2004) Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet Biol, 41(11):973-81.

Zimmerman WC, Blanchette RA, Burnes TA, Farrell RL (1995) Melanin and perithecial development in *Ophiostoma piliferum*. Mycologia, 87:857–863.

Own contributions

LA performed verification of the *pks4* deletion by qPCR, made complete ecophysiological characterisation, *pks* expression analysis, analysed all the data, contributed to the figures, prepared the draft and with other authors wrote the manuscript.

OUTLOOK

Ecological genomics implies that the studies and analysis of several available genomes can help to understand the common genus-wide traits and detect the unique features of selected species only when the true nature of the organism (i.e. the diversity of is lifestiles and totality of ecophysiological adaptations) has been also investigated.

A high interest in industrially important *Trichoderma reesei*, a producer of large amounts of enzymes employed in breaking cellulose in to simple sugars led to the first sequenced *Trichoderma* species (http://genome.jgi-psf.org/Trire2/Trire2.home.html). The movement toward environmentally friendly agriculture over the past two decades has encouraged the use and facilitated the studies of biocontrol fungi. In recent years the complete genomes of another two *Trichoderma* species, *T. atroviride* and *T. virens* (JGI, http://genome.jgi-psf.org), one of the most studied biocontrol agents of the genus, were sequenced by DOE JGI and became publicly available (http://genome.jgi-psf.org/Trive1/Trive1.home.html and http://genome.jgi-psf.org/Triat1/Triat1.home.html, respectively).
In the year 2012 genomes of four other *Hypocrea/Trichoderma* species have became available by DOE JGI: *T. harzianum* sensu stricto CBS 226.95 (http://genome.jgi-psf.org/Triha1/Triha1.home.html), *T. asperellum* CBS 433.97 (http://genome.jgi.doe.gov/Trias1/Trias1.home.html), *T. longibrachiatum* (http://genome.jgi.doe.gov/Trilo1/Trilo1.home.html) and *T. citrinoviride* (http://genome.jgi.doe.gov/Trici1/Trici1.home.html). The sequencing of these four species, closely related to those already sequenced genomes will allow even more comprehensive molecular-level analysis of the ecological diversity of the genus and it might lead to better understanding of the traits responsible for the loss of sexual reproduction. Furthermore, the availability of *Trichoderma longibrachiatum* genome, the species frequently found as antagonist of nematodes in soil, might allow the identif cation of traits associated with interaction with nematodes. Additionally, sequencing of *T. koningii* by Shanghai Institutes for Biological Sciences (China) and several *T. harzianum* sensu lato genomes has been reported, yet these genomes are not publicly accessible.

Our phylogenomic survey revealed that the mycoparasitic species *T. atroviride* occupies an ancestral position relative to the mycoparasitic and phytostimulating species *T. virens,* while the moderate antagonist of other fungi and a superior producer of cellulases *T. reesei* holds the most derived position on the tree (Chapter 2). This finding indicates that mycoparasitism is the innate property of the genus, which may be either powered by additional features (like in *T. virens*) or reduced (like in *T. reesei*) in a course of evolution and ecological specialization. The latest phylogenetic analysis of the whole genus (see Appendix II-I) is largely in agreement with our postulation. Furthermore, we are preparing an extended phylogenomic

analysis including the newly sequenced agamospecies *T. harzianum* s.s., *T. asperellum* and *T. longibrachiatum*. The putative positions of these species are indicated in Appendix II-I.

Although mycotrophy of *Trichoderma* has been studied for decades, there is still a large gap in our understanding of the molecular events involved in this process. Part of this lack of knowledge is due to the fact that different *Trichoderma* spp. exhibits a strikingly different transcriptomic response to their prey in different stages of mycoparasitic attack as it has been shown in this work (Chapter 1). For example, *T. atroviride* expresses an array of genes putatively involved in secondary metabolism and synthesis of GH16 ß-glucanases, proteases and small secreted cysteine rich proteins; whereas *T. virens*, on the other hand, expresses mainly the genes for gliotoxin biosynthesis and glutathione (which is necessary for gliotoxin biosynthesis) precursors. A comparison of a larger, phylogenetically diverse sample of *Trichoderma* spp. may enable us to sort out the basic physiological events that take place during mycotrophic interaction.

The best studied core of the genus *Trichoderma* contains mycoparasitic soil- or rhizosphere-competent species. The genomes of the opportunistic taxa have been sequenced by the JGI, however, genomes of these species alone do not allow in depth understanding of e.g. *Trichoderma*-plant interactions, interactions with mycorrhiza. Therefore, an increase of *Trichoderma* genomics understanding would be achieved by sequencing the species that to some extent evolved towards association with plants and/or are able to develop in soil including heavily disturbed and arid ecosystems, which may be potentially beneficial for bioenergy grasses. It is important to note that plant growth promotion is a strain specific ability, and may vary among the strains inside of one species. Here we can address the questions of genomic properties of the strains which beneficially influence the plant growth compared to those for which a negative impact on the plants was observed *in vitro*. Furthermore, rhizosphere competent *Trichoderma* species or strains would help us to understand the interaction with plant roots or lead us closer to mycorrhiza – *Trichoderma* interactions. The genomes of cosmopolitan environmental opportunists and are most frequently found in temperate rhizosphere and soil but sometimes have also been observed as facultative epigeal and root endophytes (*T. hamatum*, *T. polysporum*, and some members of the Harzianum clade: *T. cerinum*, *H. alni*, *T.* cf. *harzianum*, *T. pleuroticola*, and *T*. 'afroharzinum' nom. prov. might help us to understand these interactions.

Endophytes occupy very specific ecological niche inside a plant organism. We still do not understand the mechanisms of a putative mutualistic success of obligate *Trichoderma* endophytes, neither what are the benefits for both mutualsits. Moreover, the question emerges what are the genetic properties of cosmopolitan *Trichoderma* species that allow them to switch to endophytic way of life. To answer these questions some of the following putatively obligate endophytic *Trichoderma* species can be sequenced: *T. evansii* (host plant(s): *Lophira alata*, *Cola verticillata* and *Theobroma gileri*; Cameroun, Peru respectively), *T.*

ovalisporum (host plant: *Banisteriopsis caarpi*; Ecuador), *T. theobromicola* (host plant: *Theobroma cacao*; Peru), *T. scalesiae* (host plant: *Scalesia pedunculata* endemic to Galapagos Islands, *T. taxi* (host plant: *Taxus mairei*; China), *T. amazonicum* (host plant: *Hevea* spp.; Peru), *T. caribbaeum* var. *aequatoriale* (*Theobroma* spp.; Tropical America), *T. paucisporum* (*Theobroma cacao*, Ecuador), *T. martiale* (*Theobroma cacao*; Brazil).

For many conidial fungi the facilitated methods for production of DNA-mediated transformation mutants have already been established. Studies using transformation mutant focus on the understanding of specific genes, pathways, or processes, but also on functional genomics studying the impact of genes on the organism's phenotype. Phenotype is the manifested attribute of an organism, combined result of its genes and environment during ontogeny, what became a target for strain improvement in biotechnology (Atanasova and Druzhinina, 2010). In our comparative transcriptomic study of mycoparasitic response of three *Trichoderma* species (Chapter 1) we show that several PKS genes are regulated in different stages of antagonistic attack. In our most recent study (Chapter 3) we show that *T. reesei pks4* gene is indeed involved in defense, stress resistance and mechanical stability of *T. reesei,* but also responsible for green pigmentation characteristic for *Trichoderma* conidia and the pigmentation of both stroma surface and perithecial openings. Additionally there is evidence that the loss of this gene has an impact on the regulation of other *pks* genes in the *T. reesei* genome, but how and why this is achieved still remains unknown. However, this *pks* gene is of high importance for the maintenance of *T. reesei* environmental fitness and its impact will be further studied in the future.

The understanding of *Trichoderma* spp. mechanisms, their life styles and diversity is currently increasing by the sequencing and analyses of their whole genomes. The availability of solid taxonomy within the genus and samples from various habitats and substrates from all over the world combined with *Trichoderma* genome analyses can therefore allow us to explore the genus in the perspective of ecological genomics (Druzhinina and Kubicek, 2013). In this thesis the first link between ecology and genomics of the genus *Trichoderma* was achieved.

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CURRICULUM VITAE WITH COMPLETE LIST OF PUBLICATIONS

Personal information

Name:	**Lea Atanasova**, Mag.rer.nat
Address:	Pantzergasse 6/4, 1190 Vienna, Austria lea.atanasova@gmail.com lea.atanasova@tuwien.ac.at
Date and place of birth:	15.07.1984 in Maribor, Slovenia

Education

Since March 2009	**Vienna University of Technology,** Austria Institute of Chemical Engineering **PhD thesis** (Doz. Dr. Irina Druzhinina). Specialization in Molecular Ecology and Genomics.
October 2006-November 2008	**University of Vienna,** Austria, Biology field of study
November 2008	**Diploma thesis** *Regional dynamics in a Calamagrostis pseudophragmites population system inferred from AFLPs* (Prof. Dr. Joseph Greimler).
October 2007-November 2008	Graduate in the lab of Prof. Dr. Joseph Greimler, Faculty Center of Biodiversity. Specialization in Plant population genetics
September 2005-September 2006	**Student Exchange Program** Erasmus at the University of Vienna, Austria, Faculty for Life science, Biology.
October2003-September 2006	**University of Ljubljana**, Slovenia Biotechnical Faculty, Department of Biology.
1999-2003	Matura at Gimnazija Šentvid school in Ljubljana, Slovenia

Research experience

Since March 2009	**Project Assistant** at Vienna University of Technology Institute of Chemical Engineering

October 2009–December 2009 Research group Microbiology

Project Assistant at Graz University of Technology

Institute of Environmental Biotechnology

Since August 2012 Curator of *TU Collection of Industrially Important Microorganisms* (TUCIM) at Vienna University of Technology
[http://institute.tuwien.ac.at/vt/tucim/DE/]

Grants and Awards

GSA Fungal Meeting Grant, Asilomar 2013: 27th Fungal Genetics Conference; Asilomar, CA, USA, 2013.

FEMS Young Scientist Meeting Grant, EMBO: Comparative Genomics of Eukaryotic Microorganisms; San Feliu de Guixols, Spain, 2011.

Diploma Award of Ecological Society of Germany, Austria and Switzerland (GFÖ); Bayreuth, Germany, 2009.

Austria National Park Research Award of Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management; Vienna, Austria, 2008.

Ad Futura Grant of Slovene human resources development and scholarship fund; Ljubljana, Slovenia, 2006-2008.

Ljubljana student scholarship for special achievements; Ljubljana, Slovenia, 2004-2006.

Teaching

2009S: LU 166.161 **Biotechnology II,** Biodiesel and other valuable products in green microalgae (Lehrgang Diplomstudium Technische Chemie).

2010W, 2011W und 2012W: LU 166.222 LU **Biochemie und Biotechnologie Labor,** Teil Stoffwechselphysiologie (Lehrgang Bachelorstudium Technische Chemie)

2012S and 2013S: LU 166.193 LU **Microbiology**: Nonpathogenic fungi in human life: Molecular ecology and DNA barcode (Lehrgang Masterstudium Technische Chemie)

2011S und 2012S: WU 166.632 **Wahlübungen chemisch: Biochemie, Mikrobiologie und Gentechnik** (Lehrgang Masterstudium Technische Chemie)

Publications

1. Atanasova L, Knox BP, Kubicek CP, Baker SE, Druzhinina IS (2013) **The polyketide synthase gene *pks4* of *Trichoderma reesei* provides pigmentation and defense against biotic and abiotic strees.** *ms submitted*.
2. Atanasova L (2013). **Ecophysiology of *Trichoderma* in genomic perspective**. In: Gupta VK,

Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina IS and Tuohy M (eds.) Biotechnology and Biology of *Trichoderma*. Elsevier BV, Amsterdam, Netherlands, *review submitted*.

3. Atanasova L, Le Crom S, Gruber S, Coulpier F, Seidl-Seiboth V, Kubicek CP, Druzhinina IS (2013) **Comparative transcriptomics reveals different strategies of *Trichoderma* mycoparasitism**. BMC Genomics, 14:121.

4. Atanasova L, Druzhinina IS, Jaklitsch WM (2013) **Two hundred *Trichoderma* species recognized based on molecular phylogeny.** In: Mukherjee PK, Sigh US, Horwitz BA, Schmoll M, Mukherjee M (eds.) *Trichoderma*: Biology and Applications. CABI of Nosworthy Way, Wallingford, Oxon, UK, *in press*.

5. Lehner SM, Atanasova L, Neumann NKN, Krska R, Lemmens M, Druzhinina IS, Schuhmacher R (2012) **Isotope-assisted screening for iron-containing metabolites reveals a high degree of diversity among known and unknown siderophores produced by *Trichoderma* spp**. Appl Environ Microbiol, 79(1):18-31.

6. Gal-Hemed I, Atanasova L, Komon-Zelazowska M, Druzhinina IS, Viterbo A and Yarden O (2011) **Marine isolates of *Trichoderma* as halotolerant agents of biological control for arid-zone agriculture.** Appl. Environ. Microbiol, 77:5100–5109.

7. Portnoy T, Margeot A, Le Crom S, Linke R, Atanasova L, Fekete E, Sándor E, Karaffa L, Druzhinina IS, Seiboth B and Kubicek CP (2011) **The CRE1 carbon catabolite repressor of the fungus *Trichoderma reesei*: a master regulator of carbon assimilation.** BMC Genomics, 12:269.

8. Kubicek CP, Herrera-Estrella A, Seidl V, Le Crom S, Martinez DA, Druzhinina IS, Zeilinger S,Casas-Flores S, Horwitz BA, Mukherjee PK, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, Atanasova L, Cervantes-Badillo MG, Challacombe J, Chertkov O, McCluskey K, Coulpier F, Deshpande N, von Döhren H, Ebbole DJ, Esquivel-Naranjo EU, Fekete E, Flipphi M, Glaser F, Gómez-Rodríguez EY, Gruber S, ***et al.*** (2011) **Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*.** Genome Biology, 12:R40

9. Atanasova L, Jaklitsch WM, Komoń-Zelazowska M, Kubicek CP and Druzhinina IS (2010) **The clonal species *Trichoderma parareesei* sp. nov. likely resembles the ancestor of the cellulase producer *Hypocrea jecorina/T. reesei*.** Appl. Environ. Microbiol, 76(21):7259-67

10. Atanasova L, Druzhinina IS (2010). **Global nutrient profiling by phenotype microarrays: a tool complementing genomic and proteomic studies in conidial fungi.** J Zhejiang Univ. Sci. B, 11(3):2010, review.

11. Druzhinina IS, Komoń-Zelazowska M, Atanasova L, Seidl V, Kubicek CP (2010). **Evolution and Ecophysiology of the Industrial Producer *Hypocrea jecorina* (Anamorph *Trichoderma reesei*) and a New Sympatric Agamospecies Related to It**. PLoS ONE, 5(2):2010.

International Conferences and Symposia

1. Atanasova L, Knox BP, Kubicek CP., Baker SE, Druzhinina IS. ***Trichoderma* reesei polyketide synthase gene *pks4* is necessary for yellow-green pigmentation of conidia and is involved in the establishment of environmental fitness.** Talk. Asilomar 2013: 27th Fungal Genetics Conference; Asilomar, CA, USA, 2013.
2. Atanasova L, Le Crom S, Gruber S, Coulpier F, Seidl-Seiboth V, Kubicek CP, Druzhinina IS. **Functional diversity of *Trichoderma* mycoparasitism.** Poster. ECFG11, Marburg, Germany, 2012.

3. Atanasova L, Le Crom S, Gruber S, Coulpier F, Seidl V, Druzhinina IS, Kubicek CP. **The transcriptional profile of *Trichoderma* mycoparasitism.** Poster. EMBO meeting: Comparative genomics of eukaryotic microorganisms, Sant Feliu de Guixols, Spain 2011.
4. Lopandic K, Bond U, Atanasova L, Druzhinina SI, Sterflinger K. **Molecular characterisation of the yeast interspecies hybrids**. Poster. The 15th International Biodeterioration & Biodegradation Symposium, Vienna, Austria, 2011.
5. Lehner SM, Neumann NKN, Atanasova L, Krska R, Lemmens M, Druzhinina I, Schuhmacher R. **Metabolic profiling of iron-containing metabolites secreted by *Trichoderma* using LC-HR-MS/MS**. Poster. Metabomeeting, Helsinki, Finland, 2011.
6. Marzouk MA, Ali MIA, Yasser MM, Mousa AS, Atanasova L, Komon-Zelazowska M , Kubicek CP and Druzhinina IS. **Molecular ecology of *Trichoderma* in Nile river basin and surrounding ecosystems in Egypt**. Poster. XVI Congress of European Mycologist, Halkidiki, Greece, 2011.
7. Atanasova L., Druzhinina IS. ***Trichoderma* in Its Environment: Ecophysiological Aspects, Aerial Transfer and in Situ DNA Barcoding of Infrageneric Communities**. Talk. IMC9 - The biology of Fungi, Edinburgh, Scotland, UK, 2010.
8. Atanasova L, Davidson DW, Kamariah As, Anderson NF, Druzhinina IS. **Diversity and physiology of *Trichoderma* and *Burkholderia* associated with Borneo's exploding ants.** Poster. IMC9 - The biology of Fungi , Edinburgh, Scotland, UK, 2010.
9. Atanasova L, Jaklitsch WM,Komon-Zelazowska M, Kubicek CP, Druzhinina IS. ***Trichoderma parareesei* sp. nov.: a mycoparasitic agamospecies likely resembles an ancestor of *H. jecorina*/*T. reesei*.** Poster. IOBC Working Group Biological control of fungal and bacterial plant pathogens, Graz, Austria, 2010.
10. Atanasova L, Friedl MA, Bauer H, Puxbaum H, Kubicek CP, Druzhinina IS. **Metagenomic study of an air borne diversity of a mycoparasitic fungus *Trichoderma* (teleomorph *Hypocrea*).** Poster. 3th Joint Conference of German Society for Hygiene and Microbiology, Hannover, Germany, 2010.
11. Gal-Hemed I, Atanasova L, Komon-Zelazowska M, Druzhinina IS, Viterbo A and Yarden O. **Characterization of *Trichoderma* isolates of marine origin and assessment of their potential as biocontrol agents.** Poster. IOBC Working Group Biological control of fungal and bacterial plant pathogens, Graz, Austria, 2010.
12. Tjandrawati Nugroho T, Komon-Zelazowska M, Lea Atanasova, Kubicek CP, DruzhininaIS. **Strategy for the correct identification of Indonesian biocontrol fungi using molecular methods**. Talk. The Gruber-Soedigdo Lecture 2010, University of Groningen, Netherlands, 2010.

Language skills

Slovenian	mother language
German and English	fluent in speaking and writing
Macedonian, Serbian and Croatian	fluent in speaking and reading

Vienna, March 2013

APPENDIX I: CO-AUTHORED MANUSCRIPTS THAT INCLUDE MATERIALS FROM THE THESIS

I) Druzhinina IS, Komoń-Zelazowska M, Atanasova L, Seidl V, Kubicek CP (2010) **Evolution and ecophysiology of the industrial producer *Hypocrea jecorina* (Anamorph *Trichoderma reesei*) and a new sympatric agamospecies related to it.** PLoS One, 5(2):e9191.

II) Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, Thon M, Zeilinger S, Casas-Flores S, Horwitz BA, Mukherjee PK, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, Atanasova L, Cervantes-Badillo MG, Challacombe J, Chertkov O, McCluskey K, Coulpier F, Deshpande N, von Döhren H, Ebbole DJ, Esquivel-Naranjo EU, Fekete E, Flipphi M, Glaser F, Gómez-Rodríguez EY, Gruber S, Han C, Henrissat B, Hermosa R, Hernández-Oñate M, Karaffa L, Kosti I, Le Crom S, Lindquist E, Lucas S, Lübeck M, Lübeck PS, Margeot A, Metz B, Misra M, Nevalainen H, Omann M, Packer N, Perrone G, Uresti-Rivera EE, Salamov A, Schmoll M, Seiboth B, Shapiro H, Sukno S, Tamayo-Ramos JA, Tisch D, Wiest A, Wilkinson HH, Zhang M, Coutinho PM, Kenerley CM, Monte E, Baker SE, Grigoriev IV (2011) **Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*.** Genome Biology, 12:R40.

APPENDIX II: REVIEWS

I) Atanasova L, Druzhinina IS and Jaklitsch WM (2013) **Two hundred *Trichoderma* species recognized based on molecular phylogeny.** In *Trichoderma*: Biology and Applications, Eds. Mukherjee PK, Sigh US, Horwitz BA, Schmoll M, Mukherjee M. CABI of Nosworthy Way, UK, Wallingford, Oxon, *in press*.

II) Atanasova L, Druzhinina IS (2010) Review: **Global nutrient profiling by Phenotype MicroArrays: a tool complementing genomic and proteomic studies in conidial fungi**. J Zhejiang Univ Sci B, 11(3):151-168.

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Evolution and Ecophysiology of the Industrial Producer *Hypocrea jecorina* (Anamorph *Trichoderma reesei*) and a New Sympatric Agamospecies Related to It

Irina S. Druzhinina*, Monika Komoń-Zelazowska, Lea Atanasova, Verena Seidl, Christian P. Kubicek

Research Area of Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria

Abstract

Background: *Trichoderma reesei*, a mitosporic green mould, was recognized during the WW II based on a single isolate from the Solomon Islands and since then used in industry for production of cellulases. It is believed to be an anamorph (asexual stage) of the common pantropical ascomycete *Hypocrea jecorina*.

Methodology/Principal Findings: We combined molecular evolutionary analysis and multiple methods of phenotype profiling in order to reveal the genetic relationship of *T. reesei* to *H. jecorina*. The resulting data show that the isolates which were previously identified as *H. jecorina* by means of morphophysiology and ITS1 and 2 (rRNA gene cluster) barcode in fact comprise several species: i) *H. jecorina/T. reesei* sensu stricto which contains most of the teleomorphs (sexual stages) found on dead wood and the wild-type strain of *T. reesei* QM 6a; ii) *T. parareesei* nom. prov., which contains all strains isolated as anamorphs from soil; iii) and two other hypothetical new species for which only one or two isolates are available. *In silico* tests for recombination and *in vitro* mating experiments revealed a history of sexual reproduction for *H. jecorina* and confirmed clonality for *T. parareesei* nom. prov. Isolates of both species were consistently found worldwide in pantropical climatic zone. Ecophysiological comparison of *H. jecorina* and *T. parareesei* nom. prov. revealed striking differences in carbon source utilization, conidiation intensity, photosensitivity and mycoparasitism, thus suggesting adaptation to different ecological niches with the high opportunistic potential for *T. parareesei* nom. prov.

Conclusions: Our data prove that *T. reesei* belongs to a holomorph *H. jecorina* and displays a history of worldwide gene flow. We also show that its nearest genetic neighbour - *T. parareesei* nom. prov., is a cryptic phylogenetic agamospecies which inhabits the same biogeographic zone. These two species thus provide a so far rare example of sympatric speciation within saprotrophic fungi, with divergent ecophysiological adaptations and reproductive strategies.

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* E-mail: druzhini@mail.zserv.tuwien.ac.at

Introduction

The holomorphic fungal genus *Hypocrea/Trichoderma* (Hypocreales, Ascomycota) contains several hundred species comprising both sexually propagating (*Hypocrea*) as well as apparently mitosporic (*Trichoderma*) taxa without observed ability to sexual reproduction. One species of *Trichoderma* -*T. reesei* - is particularly well known because it is used in the biotechnological industry for the production of cellulolytic and hemicellulolytic enzymes and recombinant proteins [1,2]. This property, and strategies for its further improvement, have recently regained strong interest because of the attempts to produce second generation biofuels to combat carbon dioxide emission and dependence on fossil oil [3,4].

T. reesei was originally isolated as in the Solomon Islands during WW II, where it destroyed canvas and other cellulose-containing material of the US army [5]. This species is unique among industrial fungi, as it was known only from this single wild-type isolate (= QM 6a) for last 50 years, and all mutant strains used in biotechnology today have thus been derived from it. Kuhls et al. [6], however, used molecular characters (internal transcribed spacers 1 sequences of the rRNA gene cluster and RAPD fingerprinting) to conclude that *T. reesei* was indistinguishable from the pantropical ascomycete *Hypocrea jecorina*, and suggested that the latter species is its teleomorph. Yet, although *H. jecorina* is heterothallic [7], the authors were unable to mate *T. reesei* with wild type isolates of *H. jecorina in vitro*. Further, Kuhls et al. [6] noted small phenotypic differences between *T. reesei* QM 6a and wild-type strains of *H. jecorina*. Consequently, the authors suggested that *T. reesei* is a clonal derivate from *H. jecorina* but this argument was not considered in the taxonomic status of the first fungus. Seidl *et al.* [8] recently described that *T. reesei* can indeed be crossed with wild-type isolates of *H. jecorina*, thus rejecting at least one of the above arguments for the clonality of *T. reesei*.

The progress in molecular evolutionary tools and theoretical concepts for species recognition has recently led to the detection of a continuously increasing number of cryptic species within individual morphological or biological fungal taxa [9–13] including *Hypocrea/Trichoderma* [14–16]. Recent world-wide sampling has detected a putative anamorph of *H. jecorina* as being common in soils of South East Asia and particularly in South America [17–19]. The frequent occurence of these strains in soil led us to hypothesize that they may eventually be co-specific with *T. reesei*, and the latter in fact is not being an anamorph of but a cryptic species to *H. jecorina*. The attribution of *T. reesei* to holomorph of *H. jecorina* has so far been only claimed from identical ITS 1 and 2 sequences, but recent studies have shown that sequences from this locus are unable to distinguish between several closely related *Hypocrea/Trichoderma* species [15–17].

Here we will show that *T. reesei* sensu stricto (= QM 6a) indeed belongs to the holomorphic species *H. jecorina*, and exhibits a history of recombination and world-wide gene flow, thus rejecting the hypothesis of being a genetically separated agamospecies derived from *H. jecorina*. In addition, we will also show that strains recently isolated from soils as putative anamorphs of *H. jecorina* in fact form two sibling species and that at least one of them (*T.* parareesei nom. prov.) reproduces asexually, and is the result of sympatric speciation in parallel to *H. jecorina*. Ecophysiological analyses reveal that this sympatric speciation is due to the adaptation to two different habitats.

Results

Sample Design and Genetic Markers

Our sample consisted of 34 strains from teleomorphs and anamorphs (Table 1, already sorted in relation to the results from phylogenetic analysis given below), which were originally identified as *H. jecorina* or *T. reesei* both by morphological analysis as well as ITS1 and 2 based oligonucleotide barcode (*TrichO*Key [17]; online at www.ISTH.info). They covered the whole geographic variability known for these species (South and Central America, Caribbean archipelago, Africa, South Pacific, South East Asia, Macro-and Micronesia and the Indian subcontinent).

A preliminary screening for phylogenetic markers, which were previously used with success in other studies on *Hypocrea/Trichoderma* (such as *tef1, cal1, chi18-5, rpb2* or *act1* [15–17,20]), showed that only the 4th intron of *tef1* and the 2nd and 3rd introns of *cal1* provided sufficient phylogenetic information. Coding regions such as *chi18-5* or *act1* provided insufficient polymorphism (data not shown). We therefore searched for additional genes with long introns and tested their ability to differentiate within *H. jecorina*. One locus fulfilling this requirement is the *las1* gene (fgenesh5_pg.C_scaffold_1000016), which encodes the orthologue of an essential nuclear protein regulating bud formation and morphogenesis in *Saccharomyces cerevisiae* [21]. It is interrupted by four introns, of which the second (307 nts) was selected as the phylogenetic marker (Fig. 1).

Sequencing of *tef1, cal1* and *las1* for the whole strain sample provided 1241 nts, of which 1098 were constant sites and 109 polymorphic characters were parsimony-informative (49 in *tef1*, 38 in *las1* and 22 in *cal1*, respectively). Nucleotide characteristics of the three genes are shown in Table 2.

Molecular Phylogeny of *H. jecorina* Sensu Lato

We used Bayesian phylogenetic analysis of both the concatenated as well as individual gene data sets to test for a phylogenetic structure within *H. jecorina* sensu lato (Fig. 2 and Fig. S1). One single teleomorph isolate from North Sulawesi, Indonesia (C.P.K. 1281 = G.J.S. 85–238 - CBS 638.92), which was described as *H. jecorina* by Kuhls et al. [6] and Samuels et al. [22], consistently formed a single branch separated from all other clades in the *tef1*, *las1* and *cal1* trees (data not shown) indicating that it represents a still undescribed species outside the *H. jecorina* sensu lato clade. Thus, we named it as *H.* sp. nov. G.J.S. 85–238 and excluded it from further analyses. The remaining 33 isolates formed three significantly supported clades in all single gene trees as well as in the combined phylogram, thus fulfilling the criteria of the genealogical concordance phylogenetic species recognition concept [23,24] which recognizes a clade as an evolutionary lineage if its separation is supported by at least two gene trees and not contradicted by the others. One of the obtained clades contained all the teleomorph isolates and also the original isolate of *T. reesei*. As the type strain of *H. jecorina* (specimen No. 989 of the Berkeley and Broom collection from Ceylon at Kew, UK) is not available for molecular analysis, we rely on the attribution of all these strains to the above taxon by Samuels et al. [22] and recognize this clade as a holomorph *H. jecorina/T. reesei* sensu stricto.

The next large clade contained all strains that were isolated from soil as anamorphs (Fig. 2). Since *T. reesei* was not clustered in this clade we call it *T.* sp. nov. 'parareesei nom. prov.' (for simplicity *T. parareesei* nom. prov.) throughout the manuscript, assuming that its formal taxonomic description will soon be published elsewhere. Two isolates (C.P.K. 523 and C.P.K. 524), collected from tree bark in Taiwan formed a separate branch in all of these trees and are therefore recognized as another phylogenetic species *T.* sp. nov. C.P.K. 524, whose formal description will be possible when additional isolates become available.

Geographic Distribution of *H. jecorina* and *T. parareesei* Nom. Prov.

Our sample shows that both *H. jecorina* and *T. parareesei* nom. prov. are cosmopolitan species (Fig. 2), yet restricted to a narrow latitudinal belt around the equator (±20°). They must therefore be considered to be sympatric species, particularly in Central and South America. In order to test, whether the gene sequences would reveal some intraspecific geographic separation within *H. jecorina*, we determined the F_{ST} values for pairwise combinations of strains from different locations (e.g. Caribbean vs. Indopacific, South American vs. Africa etc.). However, the F_{ST} values were within 0.011–0.024 for all combinations (data not shown), thus documenting a high rate of exchange of genetic material over these wide geographic distances, and no evidence for geographic segregation.

In vitro Mating between and within *H. jecorina* and *T. parareesei* Nom. Prov.

The mating type loci of the heterothallic species *H. jecorina* have recently been identified and conditions for successful mating have been established [8]. We thus tested whether the two newly recognized species would contain one of the two mating types of *H. jecorina*. Using primers within conserved regions of the genes *mat1-1-1*, *mat1-1-2*, *mat1-1-3* (indicative for a *MAT1-1* mating type locus, Fig. 3A) and *mat1-2-1* (indicative for a *MAT1-2* locus, Fig. 3A), we in fact identified that all isolates of *T. parareesei* nom. prov. have a *MAT1-1* locus except C.P.K. 661 from northern Argentina, which has a *MAT1-2* locus (Table 1). Both isolates of *T.* sp. C.P.K. 524 also possess a *MAT1-1* locus. RFLP of the complete mating type loci in comparison with *H. jecorina* confirmed the segregation of all three species in three and two different haplotypes of *MAT1-1* and *MAT1-2* respectively (Fig. 3B). Although most of the SNPs of the mating type loci of *T. parareesei* nom. prov. and *T.* sp. C.P.K. 524 were silent mutations, only the amino acid sequence of MAT1-1-2 of *T. parareesei* nom. prov. was

Table 1. Strains of *H. jecorina* sensu lato used in this study.

Taxon	C.P.K. strain Nr	Other strain Nrs	Substratum	Origin	Mating type (*MAT* locus)	GeneBank Nr			
						MAT1-1-2/MAT1-1-3 or MAT1-2	*tef1*	*cal1*	*las1*
Hypocrea jecorina	**160**	G.J.S. 85-236, [6]	arecoid palm	Indonesia, North Sulawesi	*1–2*		GQ354342	GQ354276	GQ354308
	1285	G.J.S. 86-408, [6]	unknown	Brazil, Para	*1–1*		n/a	GQ354295	GQ354329
	1286	G.J.S. 88–6, [6]	unknown	Brazil, Para	*1–1*		GQ354362	GQ354296	GQ354330
	1274	C.T.R. 72–94, [6]	wood	Venezuela	*1–1*		GQ354357	GQ354292	GQ354325
	170	G.J.S. 86–410, [6]	bark	French Guiana	*1–1*		GQ354346	GQ354280	GQ354312
	1273	A.Y.R. 2896, [6]	dead log	French Guiana	*1–2*		GQ354356	GQ354291	GQ354324
	1283	G.J.S. 86–403, [6]	bark of recently dead tree	French Guiana	*1–1*		GQ354360	GQ354294	GQ354328
	282	G.J.S. 97–177, CBS 102271 [38]	fallen twig of *Theobroma*	French Guiana	*1–1*	GQ167152/GQ167152	GQ354347	GQ354281	GQ354313
	283	G.J.S. 97–178, CBS 102270 [38]	fallen twig of *Theobroma*	French Guiana	*1–1*		GQ354348	GQ354282	GQ354314
	1392	G.J.S. 86–401	unknown	Puerto Rico	*1–2*		GQ354368	GQ354302	GQ354336
	1380	G.J.S. 95–82, CBS 498.97	decorticated wood	Puerto Rico	n/a		GQ354364	GQ354298	GQ354332
	1386	G.J.S. 95–2081	bark	Puerto Rico	*1–1*		GQ354365	GQ354299	GQ354333
	1387	G.J.S. 95–2082	bark	Puerto Rico	*1–1*		GQ354366	GQ354300	GQ354334
	1388	G.J.S. 95–123	unknown	Puerto Rico	*1–1*		GQ354367	GQ354301	GQ354335
	1282	G.J.S. 85–249, [6]	log	Indonesia, North Sulawesi	*1–1*		GQ354359	GQ354293	GQ354327
	158	G.J.S. 85–229, [6]	unknown	Indonesia, North Sulawesi	*1–2*		GQ354343	GQ354277	GQ354309
	159	G.J.S. 85–230, [6]	wood	Indonesia, North Sulawesi	*1–1*		GQ354344	GQ354278	GQ354310
	1407	CBS 881.96	unknown	Papua New Guinea	*1–1*		GQ354369	GQ354303	GQ354337
	1127	G.J.S. 93–23, 6]	bark	New Caledonia	*1–2*		GQ354355	GQ354290	GQ354323
	1337	G.J.S. 93–22, [6]	decorticated wood	New Caledonia	*1–2*		GQ354363	GQ354297	GQ354331
	917	CBS 383.78, QM 6a, [6]	cellulose fabrics	Solomon Islands	*1–2*	GQ167145	Z23012	n/a	GQ354321
	3418	G.J.S. 06–138	unknown	Cameroon	n/a		GQ354370	GQ354304	GQ354338
	3419	G.J.S. 06–140	unknown	Cameroon	n/a		GQ354371	GQ354305	GQ354339
	938	G.J.S. 89–7, CBS 836.91, [6]	bark	Brazil	*1–2*		GQ354354	GQ354289	GQ354322
	155	G.J.S. 86–404, [6]	unknown	Brazil, Para	*1–1*		GQ354345	GQ354279	GQ354311
*H.*sp. G.J.S. 85–238	**1281**	G.J.S. 85–238, [6]	wood	Indonesia, North Sulawesi	n/a		GQ354358	n/a	GQ354326
Trichoderma parareesei nom. prov.	3420	G.J.S. 04–41	soil	Brazil	n/a		GQ354372	GQ354306	GQ354340
	3426	G.J.S. 07–26	soil	Ghana	n/a		GQ354373	GQ354307	GQ354341
	661, [17]		soil	Argentina	*1–2*			GQ354286	GQ354318
	665, [17]		soil	Argentina	*1–1*	GQ167143/GQ167148	GQ354352	GQ354287	GQ354319
	717, [17]		soil	Mexico	*1–1*	GQ167144/GQ167149	GQ354353	GQ354288	GQ354320
	634, [17]		soil	Sri Lanka	*1–1*	GQ167142/GQ167147	GQ354351	GQ354285	GQ354317
T. sp. nov. C.P.K. 524	523, [19]		rotting wood	Taiwan	*1–1*		GQ354349	GQ354283	GQ354315
	524, [19]		rotting wood	Taiwan	*1–1*	GQ167141/GQ167146	GQ354350	GQ354284	GQ354316

bold font indicates strains isolated from fruiting bodies (teleomorphs), n/a corresponds to not available
doi:10.1371/journal.pone.0009191.t001

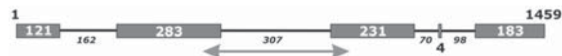

Figure 1. Structure of the *las1* locus in *H. jecorina/T. reesei*. Intron-exon structure of the *las1* locus in *H. jecorina/T. reesei* and position of PCR primers as inferred for *T. reesei* QM 6a.
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identical to that from *H. jecorina*, whereas it was polymorphic in three positions in *T.* sp. C.P.K. 524. The amino acid sequence of the MAT1-1-3 protein of *T. parareesei* nom. prov. differed in two residues from that of *H. jecorina* including one case where the helix breaker P was exchanged by a polar S residue. The most significant difference was observed in MAT1-2-1, which contained a PS insertion (Fig. S2). Thus these data demonstrate that the mating type proteins from *T. parareesei* nom. prov. have undergone alterations which may have affected their functionality.

The availability of two mating types for *T. parareesei* nom. prov. provided us with the possibility to test whether these isolates already developed an infertility barrier to *H. jecorina*, and if they would still be able to mate with each other. Therefore, selected isolates of *H. jecorina* and *T. parareesei* nom. prov. carrying the *MAT1-1* and *MAT1-2* locus respectively, were subjected to pairwise crossing experiments on plates under daylight conditions (indicated by arrows on Fig. 4). Only the heterothallic strain pairs belonging to *H. jecorina* indeed produced either mature fruiting bodies or primordia within 6–12 weeks of incubation, whereas all other combinations did not mate, even after further prolonged incubation time. These data are consistent with the conclusion that – at least in *vitro* - *T. parareesei* nom. prov. is unable to cross with *H. jecorina*, and is also not capable of sexual reproduction with the opposite mating partner of itself.

In silico Analysis of Reproduction Strategies of *H. jecorina* and *T. parareesei* Nom. Prov.

The results of mating experiments, the visual inspection of tree topologies (Fig. 2 and Fig. S1) and eventually the origin of strains (as teleomorph or anamorph, respectively) lead to the assumption that only *H. jecorina* performs sexual reproduction, whereas *T. parareesei* nom. prov. would be a clonal taxon (= agamospecies). In order to investigate this by means of sequence analysis we first used the split decomposition method [25,26] to test for the presence of network relationships in *H. jecorina* and *T. parareesei* nom. prov., using a concatenated dataset of *tef1*, *cal1* and *las1*. This method presents conflicting phylogenetic data, presumably arising from recombination, as an interconnected network of lineages. As shown in Fig. 4, such a network was evident within *H. jecorina* (= the strains isolated as teleomorphs and *T. reesei*) whereas it was absent within *T. parareesei* nom. prov. We especially note that the *ex*-type strain of *T. reesei* was tightly linked to the *H. jecorina* network, thus arguing against its origin as a clonally separated isolate.

Table 2. Nucleotide properties of phylogenetic markers and MCMC parameters.

Parameters		phylogenetic marker			
		tef1	*las1*	*cal1*	concatenated dataset
Fragment characterization		intron	exon/intron	exon/intron	not applicable
Number of sequences		33	33	33	33
Number of characters		509	377	355	1241
	parsimony informative	18	9	7	34
	constant	442	330	326	1098
Parameters of MCMC analysis					
Mean nt frequencies* A/C/G/T		0.21/0.32/0.22/0.25	0.20/0.24/0.34/0.22	0.24/0.28/0.29/0.19	0.22/0.28/0.27/0.23
Substitution rates*	A <-> C	0.02	0.09	0.08	0.09
	A <-> G	0.89	0.22	0.17	0.29
	A <-> T	0.01	0.19	0.16	0.1
	C <-> G	<0.01	0.08	0.06	0.04
	C <-> T	0.03	0.37	0.40	0.31
	G <-> T	0.04	0.06	0.13	0.17
alpha*		0.10	0.10	0.08	0.05
MCMC generations, million/number of runs		1/2	1/2	1/2	2/2
PSRF*		1.00	1.00	1.00	1
Number of chains/Temp (λ)		4/0.2	4/0.2	4/0.2	4/0.2
Sampling frequency		100	100	100	100
Number of discarded first generations		600	400	500	800
Total tree length		3.19	5.79	6.05	0.43

*as estimated after GTR MCMC sampling and burning.
doi:10.1371/journal.pone.0009191.t002

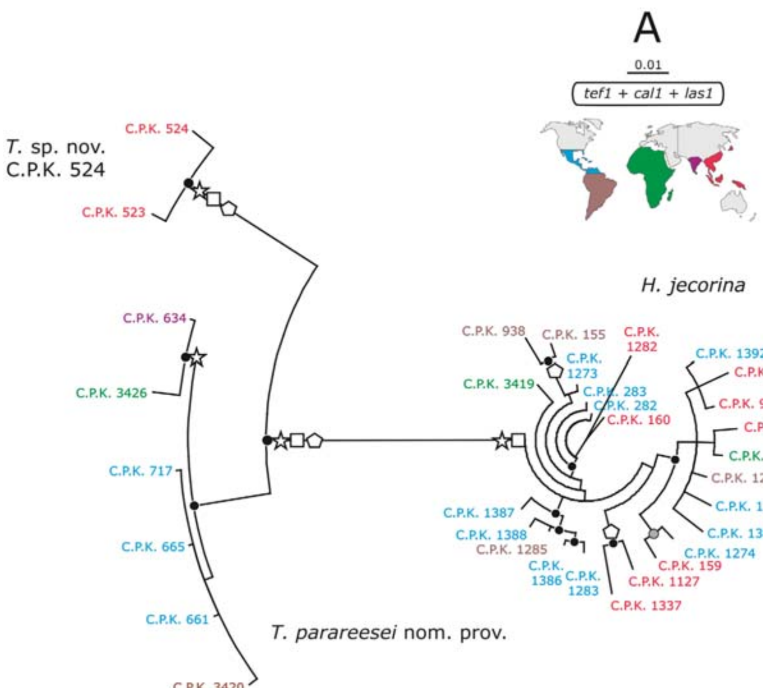


Figure 2. Molecular phypogeny of *H. jecorina* sensu lato. Bayesian circular phylogram inferred from the concatenated dataset of *tef1*, *cal1* and *las1* phylogenetic markers. Symbols at nodes correspond to posterior probabilities (PP) >95%. Filled circles correspond to PP in the concatenated tree, open stars, squares and polygons to PP in *las1*, *cal1* and *tef1* gene trees, respectively. The corresponding phylograms are given in Figure S1. The color code indicates the geographic region from which the isolates were obtained, as explained in the right top inset.
doi:10.1371/journal.pone.0009191.g002

We then used the partition homogeneity test (PHT; [27,28]) to examine the congruence between individual gene trees. This test produces artificial datasets by multiple (10 000) re-sampling and random swapping of observed datasets and subsequent construction of maximum-parsimony trees for every newly sampled 'gene' sequence. For clonally reproducing populations (= no sexual recombination), the sums of the lengths of the gene trees for the observed and re-sampled data should be similar. However, under recombination the sums of the tree lengths should be longer than those for the actual data because of introduction of homoplasy into unlinked sites. This test confirmed our analysis of topologies of single locus trees - the clades containing *T. parareesei* nom. prov. showed congruence of all three loci suggesting the clonality of this species (Fig. 4). The topologies of *H. jecorina* subclades appeared to be not concordant between individual trees, providing the evidence for recombination (Fig. 4).

As another means, we used the index of association (IA) test on a subset of 'clone corrected' data (i.e. individuals with identical alleles of the three loci were excluded so that each haplotype was represented only once; cf. [29]). In this test, complete panmixia (sexual compatibility resulting in recombination) would be indicated by a value of 0 (= the null hypothesis). This value was neither obtained with the complete dataset nor with any of the individual clades (data not shown).

Consistent with the occurrence of teleomorphs *H. jecorina* clade gave a significantly lower value (0.014, $P = 0.006$), whereas strains of *T. parareesei* nom. prov. completely rejected the null hypothesis of recombination (1.060, $P = 0.126$).

Finally we applied the Phi-test, which uses the pairwise homoplasy index (PHI, Φ) to detect refined incompatibility [30]. This method assumes the infinite sites model of evolution in which the detection of incompatibility for a pair of sites indicates recombination. Application of this test to *H. jecorina* did find statistically significant evidence for recombination ($P \ll 0.001$) while it was not the case for *T. parareesei* nom. prov. ($P = 1$). Since the Phi-test is a very robust means which can detect recombination even in the presence of recurrent mutation, we decided to use this method to define the borders of recombining populations. To this end, we first detected a

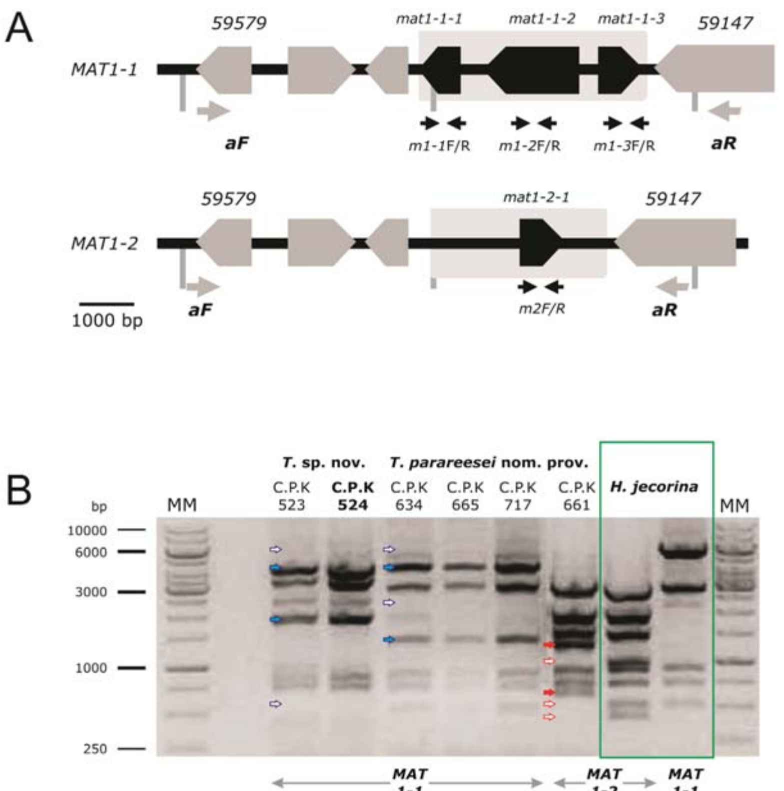


Figure 3. The mating type loci of *H. jecorina*. (A) Schematic presentation of the mating type loci *MAT1-1* and *MAT1-2* and their flanking regions based on the *H. jecorina* data [8]. Primers used to amplify the complete *MAT*-loci are indicated by gray arrows and primers for fragments of the mating type genes (Table 4) by black arrows. Numbers correspond to the respective proteins IDs in the *T. reesei* genome database. (B) Restriction fragment patterns of the mating type loci, amplified with primers aF and aR (Table 4) and digested with *Pst*I. MM, molecular marker (GeneRuler 1 kb ladder, Fermentas). The strains and their respective mating types are indicated as C.P.K. numbers. Small colored arrows show either present (filled) or absent (open) bands in RFLP profiles of C.P.K. strains in respect to the reference strains of *H. jecorina* for *MAT1-1* and *MAT1-2* [8].
doi:10.1371/journal.pone.0009191.g003

non-recombining subsample consisting of six most terminal strains of *H. jecorina* (C.P.K. 160 and 1282 from Indonesia, C.P.K. 155 and 938 from Brazil, C.P.K. 3419 from Cameroon and C.P.K. 1273 from Pacific; $P = 0.34$), and then gradually added strains from two other phylogenetic species until evidence for recombination was detected ($P < 0.05$). No recombination was detected between *T. parareesei* nom. prov. and *H. jecorina*. In contrast, a positive recombination signal ($P = 0.012$) was obtained when both strains of *T.* sp. C.P.K. 524 were tested together with the above listed subset of *H. jecorina*. It suggests that *T. sp.* C.P.K. 524 may also reproduce sexually but the current sample is too small to reveal it.

Thus the four alternative tests suggest that *T. parareesei* nom. prov. is an agamospecies which did not undergo sexual recombination in its recent evolutionary history.

Ecological Specialization of *H. jecorina* and *T. parareesei* Nom. Prov.

The fact that *H. jecorina* and *T. parareesei* nom. prov. evolved in sympatry raises the question about the differences in their ecological niches. Since both fungi are saprotrophs, we tested whether they would differ in their carbon metabolism, response to environmental stimuli or antagonistic ability against other fungi.

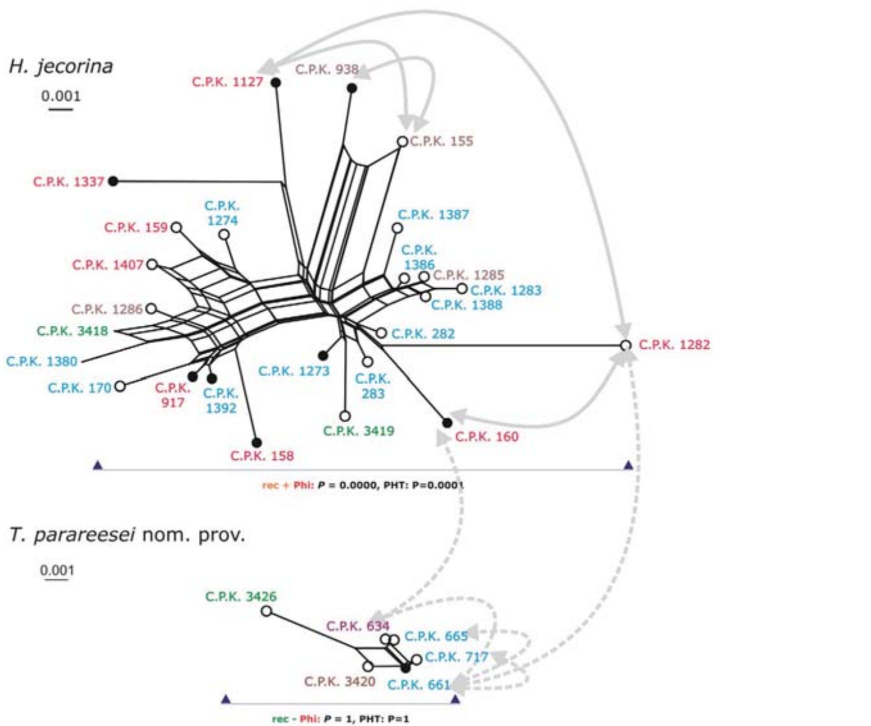
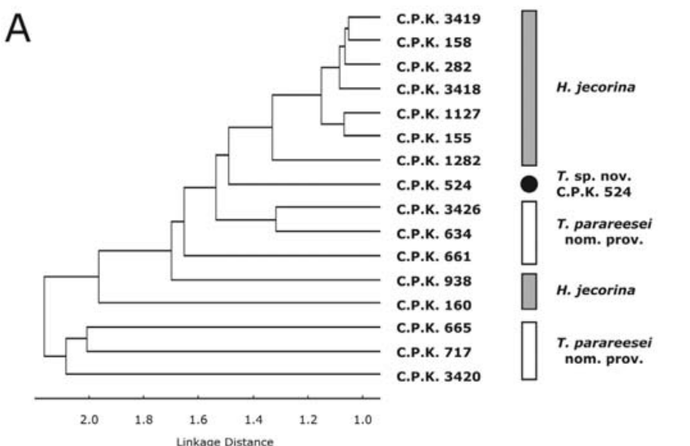


Figure 4. Recombination analysis of *H. jecorina* and *T. parareesei* nom. prov. Reconstruction of possible recombination networks build using the split decomposition method applied to the concatenated dataset (*tef1* + *cal1* + *chi18-5*). Upper shape: *H. jecorina*, low shape: *T. parareesei* nom. prov. Open and filled symbols at OTUs indicate *MAT1-1* and *MAT1-2* mating types respectively. Gaps were treated as missing characters throughout. All networks have been calibrated to fit one scale. The color scheme shows geographic origin of the strain as indicated in Fig. 2. Results from the PHT and Phi tests are shown by arrows and the respective P values, 'rec +' specifying positive recombination result and 'rec -'specifying no recombination detected. PHT indicates the result of partition homogeneity test, Phi corresponds to results of Phi test. Double ended arrow lines show successful (solid line) and failed (dashed line) crossings.
doi:10.1371/journal.pone.0009191.g004

Carbon utilization. With respect to carbon metabolism, *H. jecorina* and the two new species exhibited qualitatively very similar carbon source utilization profiles. The full scale profile will be published elsewhere alone with the formal description of the new species; the list of carbon sources is in Table S1 and in Druzhinina et al. [31]. On a quantitative basis, *T. parareesei* nom. prov. and *T.* sp. C.P.K. 524 generally exhibited faster growth rates on so-called "Cluster I" carbon sources, i.e. the ones which provide fastest growth (such the chitin monomer n-acetyl-β-d-glucosamine or the hemicellulose monomers l-arabinose, d-xylose, d-galactose and corresponding polyols; cf. [31]), and also displayed a broader intraspecific variation (Fig. 5A). In contrast, the majority of *H. jecorina* strains showed a much more conserved quantitative pattern of growth on these carbon sources, yet with a significantly lower growth rate (except C.P.K. 938 and C.P.K. 160). Thus, we conclude that *T. parareesei* nom. prov. has become more versatile and efficient in the utilization of its preferred carbon sources.

Consistent results were obtained when the experiment was repeated with carbon sources typical for soil (EcoPlate™; Biolog Inc., Hayward, CA, USA, see Table S1 for individual carbon sources). Although the growth of both species on EcoPlates was slower than in the experiment above, the results confirm that in general *T. parareesei* nom. prov. is more competent in utilizing these carbon sources (ANOVA, $F(1, 133) = 38.32$, $P = 0.000$).

Since the only known anamorphic strain of *H. jecorina* - *T. reesei* QM 6a is a model fungus for cellulase formation, we tested whether the superiority of *T. parareesei* nom. prov. in carbon assimilation would also be reflected in this trait. We therefore tested 10 randomly chosen strains of *H. jecorina* and five strains of *T. parareesei* nom. prov., and included the cellulase twofold-



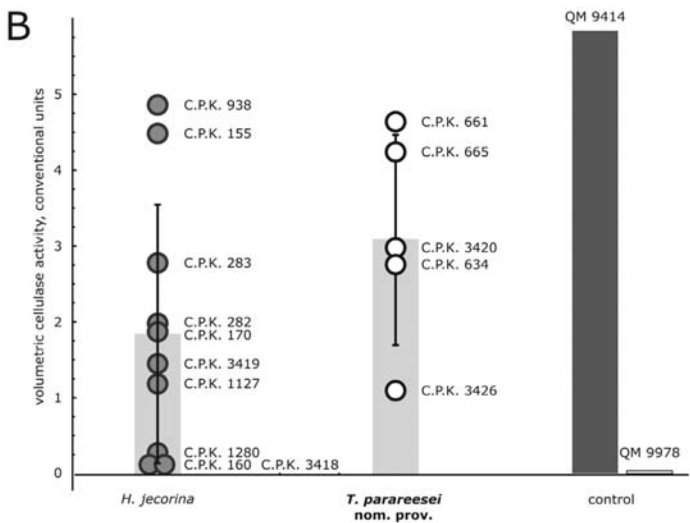


Figure 5. Carbon source utilization by *H. jecorina, T. parareesei* nom. prov. and production of extracellular cellulases. (A) Results of the single linkage cluster analysis (Pearson distance) applied to strains and based on growth on 95 carbon sources and water (Biolog FF MicroPlate ™) inferred from optical density values at 750 nm after 48 hours of incubation (linear growth stage) under ambient illumination conditions. (B) Volumetric cellulase activity of *H. jecorina* and *T. parareesei* nom. prov. Bars correspond to the average values per species and control strains with standard deviations (vertical lines), circles show the values obtained for individual strains. Control corresponds to cellulase overproducing and cellulase negative mutant strains QM 9414 and QM 9978 respectively, both derived from *T. reesei* QM 6a.
doi:10.1371/journal.pone.0009191.g005

overproducing and the cellulase negative mutants QM 9414 and QM 9978 respectively (Fig. 5B). The results show that both species contain extremely efficient cellulase producing strains which gave values close to that of QM 9414. For *H. jecorina* these were two strains isolated from Brazil (C.P.K. 938 and C.P.K. 155); for *T. parareesei* nom. prov. these are also South American C.P.K. 661 and C.P.K. 665 isolated from northern Argentina. Interestingly, three strains of *H. jecorina* showed no cellulase activity, while all strains of *T. parareesei* nom. prov. were efficient producers. Analysis of variance, however, did not detect any statistically significant difference between two species (ANOVA, $P>0.05$). This suggests that the ability to produce cellulases for the degradation of cellulose in the environment has been maintained in both species.

Photosensitivity. Light sensing is an important mechanism in the ecophysiological adaptation of fungi as it is strongly involved in regulation of their reproduction. Seidl et al. [6] reported that light is important for formation of perithecia of H. jecorina. Friedl et al. [32] demonstrated that light plays the role in the conidiation of H. atroviridis. Our recent studies have shown that light influences the mycelial growth of Hypocrea but the effect varies depending on the species: temperate H. atroviridis is strongly stimulated by illumination [33] while mutant strains derived from tropical T. reesei are frequently photoinhibited [34]. Thus, photosensitivity may reflect the ecological niche of the fungus.

Because of the differences in carbon assimilation, we reasoned that the response to light may also be different in *H. jecorina* and *T. parareesei* nom. prov. We thus incubated Biolog FF MicroPlates either in complete darkness or under the conditions of day light (May-June, N 48°) for 72 hours and compared the data (Fig. 6). Indeed, all strains of *H. jecorina* show their best growth rates in darkness, which is basically not changed by exposure to light. We have detected about 10 cases of photoinhibition (on individual carbon sources, see Fig. 6), which were strain- but not species-specific. Growth of only one strain, C.P.K. 160, was considerably stimulated by light on several carbon sources (for example, d-cellobiose, d-mannitol, gentiobiose, d-trehalose, maltotriose and l-fucose). In contrast, the best growth of the majority of *T. parareesei* nom. prov. (all possessing the *MAT1-1*) was under illumination indicating that this species is not capable of normal growth in darkness (Fig. 6). No cases of photoinhibition were detected for *T. parareesei* nom. prov. These data show a striking difference in physiological adaptation of two species and confirm that although they are sympatric they have different ecological niches. The detection of photostimulation in *T. parareesei* nom. prov. is particularly interesting as all its strains were isolated from soil.

Conidiation. *H. jecorina* has a complete holomorphic life cycle and forms both conidia and ascospores, while propagation of *T. parareesei* nom. prov. is dependent on the distribution of its

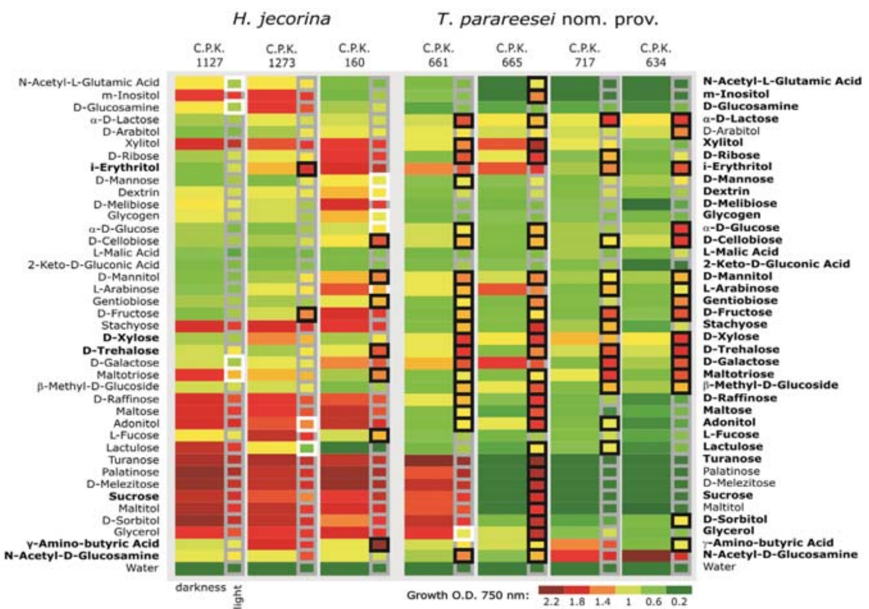


Figure 6. Photosensitivity map of *H. jecorina* and *T. parareesei* nom. prov. Photosensitivity map of *H. jecorina* and *T. parareesei* nom. prov. constructed based on the two way joining cluster analysis. Framed squares show growth under conditions of sun light: white, black and grey frames correspond to photoinhibition, photostimulation and neutral photoresponse respectively. Bold font used for carbon sources indicates those which supported conidiation of *H. jecorina* (left list) and *T. parareesei* nom. prov. (right list) respectively.
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mitospores. A visual inspection of cultures indicated that *T. parareesei* nom. prov. conidiates essentially more intensively than the mycelial forms of *H. jecorina* which leads to the striking difference in culture morphology (data not shown). Since conidiation is carbon source dependent [33], we tested it on 95 carbon sources. The results show that in total *H. jecorina* conidiates only on 7 out of 95 carbon sources (6 of those shown on Fig. 6, see the corresponding legend) while *T. parareesei* nom. prov. forms mitospores on 62 carbon sources (36 of those shown on Fig. 6). A quantification of conidial density per cm^2 of a MEA plate showed that on an average *H. jecorina* formed 3.6 ($\pm$1.78)$\times10^6$ per 10 tested strains, while the six available strains of *T. parareesei* nom. prov. produced an average of 16.05 ($\pm$4.8)$\times10^6$ conidia under the same conditions.

Antagonistic potential against soil competent and epigeal plant pathogens. Species of *Hypocrea/Trichoderma* are renowned for their mycoparasitic behaviour [1]. In order to test whether the two species may also differ in their antagonistic abilities, we have selected five plant pathogenic fungi which differ in their primary habitat: *Sclerotinia sclerotiorum* and *Fusarium oxysporum* (FOX, *F. oxysporum* species complex) representing soil competent mycobionts; and *F. xylarioides*, *Alternaria alternata* and *Botrytis cinerea* representing pathogens of green plant tissue and thus predominantly epigeal fungi. Fig. 7 shows the summary of dual confrontation tests assessed after 10 days of co-cultivation on MEA medium. *H. jecorina* is able to considerably inhibit growth of *S. sclerotiorum*, *A. alternata* and *B. cinerea*. The latter plant pathogen was so completely combated by *H. jecorina* C.P.K. 160 and C.P.K. 1127 that its mycelium was overgrown and killed. No antagonism to both *Fusarium* species by *H. jecorina* was detected. Strains of *T. parareesei* nom. prov. showed a conserved pattern of mycoparasitic activity with almost no variation among strains, and they also exhibited a superior antagonistic potential against *S. sclerotiorum*, *B. cinerea* and particularly *A. alternata*. *T.* sp. C.P.K. 524 behaved similar to *T. parareesei* nom. prov. except that it was the most resistant strain against the isolate of FOX used. We should like to stress that the *in vitro* dual confrontation tests are only an indirect means to assess antagonistic potential of the fungus. However, the significant difference shown between *H. jecorina* and *T. parareesei* nom. prov. suggests that the former is only a moderate mycoparasite with significant variation among isolates, whereas the latter species may exhibit a stronger antagonistic potential against all epigeal fungi tested (Fig. 7).

Discussion

T. reesei QM 6a has been a taxonomic riddle. Originally isolated in 1942, it was a victim of then almost undeveloped taxonomy for *Trichoderma*. Therefore it was first determined to be *T. viride* (because the genus was in that time believed to consist only of this single species [35]), and later on recognized as a unique species and named in honor of its detector Elwyn T. Reese *T. reesei* [36]. Bissett [37] then revised it as being co-specific with *T. longibrachiatum*. Finally, it was recognized to be identical to the pantropical ascomycete *H. jecorina* [6], which was itself just distinguished as a separate tropical species closely related to *H. schweinitzii* [22]. Yet small morphological differences and the inability of these authors to cross it with other *H. jecorina* isolates in the lab led Kuhls et al. [6] to assume that it is actually a clonally derived asexual form of *H. jecorina*.

The present data have clearly rejected this hypothesis: our analysis shows that the original isolate *T. reesei* QM 6a reveals a history of recombination similar to that of the teleomorphic isolates of *H. jecorina*. These findings are also supported by our recent discovery that *T. reesei* QM6a is a *MAT1-2* idiotype and can indeed be crossed with *MAT1-1* partners of *H. jecorina* [8]. Previous failures to obtain crossing is therefore likely due to the use of the incorrect mating type partner or inappropriate experimental conditions. *T. reesei* clearly is an isolate identical to *H. jecorina*, and the minor differences in anamorph morphology [6] and nutrient assimilation [38] to other (more recent) isolates of *H. jecorina* are likely the result of its maintenance in the laboratory for the last 65 years.

A large number of studies have shown that fungal taxa which were defined on the basis of consistent invariant morphological features in fact contain multiple, well differentiated phylogenetic species [9,12–15]. Here we provide a further example for this growing list, the pantropical ascomycete H. jecorina, and show that the 33 strains that were available for this study actually consist of not less than four different species, i.e. H. jecorina, T. parareesei nom. prov., T. sp. nov. C.P.K. 524 and H. sp. nov. G.J.S. 85–238. The formal taxonomic description of T. parareesei nom. prov. will be published elsewhere (L. Atanasova, W.J. Jaklitsch, C.P. Kubicek, and I.S. Druzhinina, manuscript in preparation) but we will refrain from describing the other two phylogenetic taxa based on the small number of strains. Taxonomies with arbitrarily named fungal species (T. parareesei nom. prov. in the present case) have frequently been published [11–14] and enable researchers to communicate effectively before the formal species nomenclature has been established [9].

H. jecorina yielded evidence for sexual recombination even via a large distance and geographic barriers, thus indicating the presence of a very efficient system for transfer of spores, and/or of the fungus itself (e.g. via wood logs, insects etc.). On the other hand, the same tests clearly rejected this possibility for *T. parareesei* nom. prov. Thus, these two species represent a diverged species pair, similar to *H. orientalis* and *T. longibrachiatum* [15].

Our findings show that those strains of *H. jecorina*, which were recently isolated as anamorphs from soil and litter and identified as *H. jecorina* by morphological analysis and molecular barcodes [17] are in fact a cryptic agamospecies, *T. parareesei* nom. prov. It is very intriguing in this regard that this sibling anamorphic sister species of *H. jecorina* is similarly restricted to the same narrow belt of$\pm20°$ altitude around the equator, and otherwise similarly pantropical. It consequently represents a rare example of sympatric speciation (i.e. the evolution of reproductive isolation between co-distributed populations) of a saprotrophic fungus [39].

One of the most obvious reasons for such a divergent speciation resulting in formation of an agamospecies would be a mutation in a gene required for sexual reproduction. In such a scenario *T. parareesei* nom. prov. would have arisen from its ancestor because of a loss of a subpopulation ability to mate. However, our data reject this hypothesis as in such a case all strains of the anamorphic population should have the same mating type. The possession of both *MAT1-1* and *MAT1-2* loci, although unevenly distributed in our (small) sample, suggest the operation of other speciation mechanisms. The other indirect argument against altered mating mechanism as the driving force for speciation comes from the analysis of topologies of phylogenetic trees. If *T. parareesei* nom. prov. would have arisen as an agamospecies by a sudden loss of its ability to sexually recombine, its gene sequences should display lower evolutionary rates compared to *H. jecorina*. In other words the length of the branch leading to *T. parareesei* nom. prov. from the hypothetical ancestor of both species should be then shorter than the one leading to *H. jecorina*. However, the individual trees presented here as well as the analysis of an exon of the gene coding for RNA polymerase subunit B II (C.P. Kubicek, I.S. Druzhinina, unpublished data) revealed similar genetic distances between *H.*

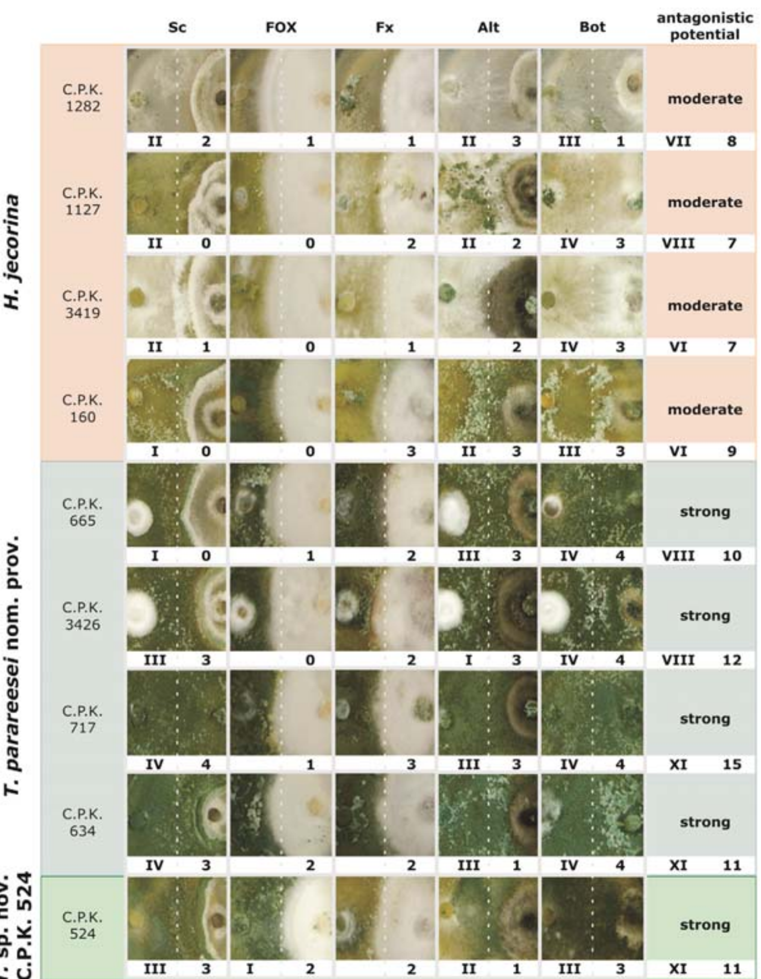

Figure 7. Mycoparasitic ability of *H. jecorina* and *T. parareesei* nom. prov. Results of dual confrontation tests between *Trichoderma* strains (inoculated on the left side) and the plant pathogenic fungi (inoculated on the right side): Sc–*Sclerotinia sclerotiorum*, FOX–*Fusarium oxysporum* complex, Fx–*F. xylarioides*, Alt–*Alternaria alternata*, Bot–*Botrytis cinerea*. Roman numbers indicate the weak (I), moderate (II), strong (III) and very strong (IV) ability of *Trichoderma* to inhibit the growth of the prey fungus. The ability to overgrow the mycelium of prey fungi is given in Arabic numbers on the similar scale. Antagonistic potential is calculated as the mean value for a strain to combat all five pathogens. The dashed lines correspond to the center position between confronted fungi.
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jecorina and *T. parareesei* nom. prov. from their hypothetical ancestor respectively.

The currently most favored explanation for reproductive isolation postulates that hybrid inferiority is caused by antagonistic epistasis between incompatible alleles at interacting loci [40–42]. Theoretical models have shown that sympatric speciation may occur when the same genes control both mating and habitat preference or fitness [43]. Our data would be compatible with this hypothesis: growth and conidiation of *MAT1-1* strains of *T. parareesei* nom. prov.–in contrast to *H. jecorina* - are stimulated by intensive illumination, whereas the *MAT1-2* strain behaves similarly to *H. jecorina*. Evidence for regulation of both mating and carbon source utilization by the blue light has previously been obtained [32–34] demonstrating that sexual reproduction and carbon assimilation may indeed share the same regulatory circuits. The availability of further *MAT1-2* strains of *T. parareesei* nom. prov. will help to differentiate between the effect of mating type loci and ecophysiological divergence of two species.

Sympatric speciation is well known from the evolution of plant and human pathogenic fungi [39], but has not been reported for non-pathogenic saprotrophs like *Hypocrea/Trichoderma*. In this work we applied a bioinformatic sequence analyses and ecophysiological characterization to obtain some insights into the forces thriving speciation in *H. jecorina* and *T. parareesei* nom. prov. We have analyzed the global versatility of their carbon metabolism, response to light, conidiation intensity and mycoparasitic potential (for a summary see Table 3). The differences detected are indeed striking and reveal that *T. parareesei* nom. prov. and *H. jecorina* occupy different ecological niches in the vertical profile of the tropical forest. *T. parareesei* nom. prov. displays all the properties of an environmental opportunist: it shows faster growth on a wider spectrum of carbon sources than *H. jecorina*, and produces a much higher number of propagules on a greater variety of carbon sources. The species is also able to strongly compete with other mycobionts and is mycoparasitic on the epigeal plant pathogenic fungi tested. The latter fact, combined with its profound photostimulation of conidiation, allows us to speculate that *T. parareesei* nom. prov. might occupy an ecological niche connected with photosynthesizing parts of higher plants, i.e. the canopy of the tropical forest.

H. jecorina in turn seems to be a specialized on a narrow habitat, where its surviving strategy mainly relies on the advantages of sexual reproduction. The species has definitely reduced conidiation efficiency (compared for the general mean for the genus, I.S. Druzhinina, personal observations) and is less aggressive against potentially competing fungi. It is remarkable that the quantitative pattern of carbon metabolism of *H. jecorina* is highly conserved (with exception of two fast growing strains, Fig. 5A) which may indicate its nutritional specialization. The fact that its anamorph has only very rarely been found in its natural environment [38] further supports the hypothesis that this species is strongly specialized. In our own work on the assessment of the general *Hypocrea/Trichoderma* biodiversity, we failed to encounter *H. jecorina* in an asexual form in more than 1000 samples, collected worldwide from soil and litter. It is probable that asexual reproduction in *H. jecorina* - like it apparently occurred in the case of the original strain of *T. reesei* and the three strains described in [38] - can be observed only under certain conditions or in certain habitats. Yet the origin of the anamorphic strains G.J.S. 97–177 (CBS 102271) and G.J.S. 97–178 (CBS 102270) both found on dead cacao brooms in Brazil, and G.J.S. 97–38 (CBS 999.97), from soil at a storage lake in French Guiana [38] does not provide a hint towards ecological adaptation of the species. Moreover details of the environment at the US Army camp in Guadalcanal during WW II, where and when *T. reesei* QM 6a was originally isolated, are also not available. Further samplings in tropical regions may eventually disclose the habitat of one of the most prominent producer in biotechnology.

Table 3. Comparative ecophysiology of *H. jecorina* and *T. parareesei* nom. prov.

	H. jecorina	*T. parareesei* nom. prov.	Reference
Known substrata			
teleomorph	wood deris	n. a.	Table 1
anamorph	*unknown*	Soil	Table 1
Phenetic profile			
growth rate	moderate	Fast	
carbon metabolism	invariable	variable	Fig. 5A
cellulase secretion	variable to high	high	Fig. 5B
soil competence	low	moderate	
extracellular yellow pigmentation	strong	weak	Fig. 7
photosensitivity reaction	insensitive to inhibited	stimulated	Fig. 6
photoadaptation to	darkness	illumination	
Conidiation			
density (per cm^2)	$(36\pm17.8)\times10^5$	$(160.5\pm47.7)\times10^5$	n. a.
carbon sources supporting conidiation (out of 95)	7	62	Fig. 6
formation rate	low	High	
Antagonistic potential			
against soil fungi	low to moderate	moderate to strong	Fig. 6
against epigeal fungi	moderate	strong	

n.a. corresponds to not applicable
doi:10.1371/journal.pone.0009191.t003

Methods

Material Studied

The strains, their origin and the sequence accession numbers used in this work are listed in Table 1. The isolates are stored at −80°C in 50% glycerol in the laboratory of Vienna University of Technology (TUW). Strains are grouped according to their identification in the present work. For convenience, TUW-lab codes (C.P.K.) are used for the strains throughout, but other collection numbers are also listed in Table 1.

Molecular Genetic Analysis

DNA extraction, PCR amplification and sequencing. Mycelia were harvested after 2–4 days of growth on MEA at 25°C and genomic DNA was isolated using QIAGEN DNeasy® Plant Mini Kit following the manufacturer's protocol. Amplification of nuclear rRNA gene cluster, containing the ITS1 and 2 and the 5.8S rRNA gene, and of fragments of *tef1* (translation elongation factor 1-α) and *cal1* (calmodulin) was performed as described previously [15]. The *las1* gene (fgenesh5_pg.C_scaffold_1000016), which encodes the orthologue of an essential nuclear protein regulating bud formation and morphogenesis in *S. cerevisiae* [21] was amplified using primers given in Table 4. PCR amplification was carried out in an *i*-cycler (BIO-RAD, USA) for 30 cycles of 94°C for 1 min denaturing, 58°C for 1 min annealing, and 74°C for 50 sec extension. Initial denaturing was at 94°C for 1 min.and the final extension was at 74°C for 7 min. PCR amplification of the mating type loci and the mating type genes was carried out as described [8]. The primers used for *MAT1* loci are listed in Table 4. PCR fragments were purified (PCR purification kit, Qiagen, Hilden, Germany), and sequenced at MWG (Ebersberg, Germany).

Phylogenetic analysis. For the main phylogenetic analysis DNA sequences were aligned with Clustal X 1.81 [44] and then edited using GeneDoc 2.6 [45]. The possibility of intragenic recombination, which would prohibit the use of the respective loci for phylogenetic analysis, was tested by linkage disequilibrium based statistics as implemented in DnaSP 4.50.3 [46]. The neutral evolution of coding fragments (*cal1* and *las1*) was tested by Tajima's test implemented in the same software. The interleaved NEXUS file was formatted using PAUP*4.0b10 [47]. The best nucleotide substitution model for the each locus was determined using jMODELTEST [48]. As Akaike and Bayesian Information criteria (AIC [49] and BIC [50] respectively) selected different nucleotide substitution models for every locus and due to the relatively small size of individual datasets (1242 characters per 34 sequences for the biggest) the unconstrained GTR + I + G substitution model was applied to all sequence fragments (Table 2). Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling was performed using MrBayes v. 3.0B4 with two simultaneous runs of four incrementally heated chains that performed 5 million generations. The length of run (number of generations) for each dataset was determined using AWTY graphical system [51] to check the convergence of MCMC. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of trees sampled every 100 generations after removing the first trees using the "burnin" command. Number of discarded generations was determined for each run based on visual analysis of the plot showing generation versus the log probability of observing the data. PP values lower than 0.95 were not considered significant while values below 0.9 are not shown on the resulting phylograms. Model parameters summaries after MCMC run and burning first samplings as well as nucleotide characteristics of used loci are collected in Table 2.

Detection of recombination. he congruence or incongruence of the three gene genealogies was used to infer recombination between isolates. To this end, three different tests were employed: the incongruence length difference/partition homogeneity test (ILD/PHT) [27,28] using a score of $P<0.05$ to reject the null hypothesis of congruence between loci; the Index of Association (IA) test [29], in which the data were compared to the IAs of artificially recombined datasets; and the Phi-test implemented in SplitsTree [26], which uses the pairwise homoplasy index, PHI (= Φ) statistic, to detect refined incompatibility indicating recombination [30].

In addition we applied split decomposition implemented in the SplitsTree program, version 4.0 [25,26], using pairwise distances under the Kimura 3ST model [52].

Mating type RFLP analysis. The ca. 10 kb large PCR fragments of the complete *MAT*-loci and their flanking regions were digested with *Pst*I (Fermentas, Burlington, Canada). Sequences of *H. jecorina* MAT1-1 and MAT1-2, derived from strain C.P.K. 2189 (CBS 999.97, [6,8,38]) were used as reference strains in the mating experiments.

Table 4. Selected PCR primers used in this study.

Gene or locus	Primer pair	Sequence (5′-3′)
Las1	LAS1 fw	CATCGACGTATGCTGTGAGG
	LAS1 rev	CTTGGCGATGGAGTACATGC
MAT1-1 and *MAT1-2*	aF	CATCGAAGCATCTACCTACTTG
	aR	CGAAGCGAAACACACGAC
mat1-1-1	m1-1F	TCCTCTCAATGCGTTCATGGC
	m1-1R	AGAAGATCATTCTCTGTGTTGGGA
mat1-1-2	m2-1F	CTCGAGAGGGATATACACCAG
	m2-1R	CTTCCTACACGGATGCCAGA
mat1-1-3	m1-3F	ATCTCCATGTCTTTATTCCCGAAG
	m1-3R	CTTGTAGTCGGGATATTTCTCC
mat1-2-1	m2-1F	GCGCACCACGGTATTTCATTG
	m2-1R	ATTTGCGCGGCTTGTATTGG

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Ecophysiological Characterization

Phenotype profiling. The carbon assimilation patterns were investigated using Biolog FF MicroPlate™ and EcoPlate™ (Biolog Inc., Hayward, CA, USA) according to the protocol published recently [31]. The complete lists of carbon sources implemented in both plates are given in Table S1. Briefly, strains were grown on 3% malt extract agar (MEA), and 90 µl of a conidial suspension from them (75±2% transmission at 590 nm) was dispensed into each of the wells of a corresponding Biolog microplate. Inoculated microplates were incubated at 28°C, and optical density (O.D.) determined after 12, 18, 24, 36, 42, 48, 66 and 72 h at 750 nm. Analyses were repeated at least three times for each strain.

In order to estimate the effect of illumination on mycelial growth and conidiation the protocols of Friedl et al. [32,33] were used respectively. Biolog FF MicroPlates were incubated either at natural day light (May-June, N 48°), while plates for darkness experiment were not exposed to any light source in between the measurements. Biolog EcoPlates were incubated in darkness.

Data exploratory statistical analyses were performed using Statistica 6.1 (StatSoft, Inc., Tulsa, OK, USA) data analysis software system.

Antagonistic potential. To assess the antagonistic potential of anamorphic cultures of *H. jecorina* five potential prey fungi have been selected: *Sclerotinia sclerotiorum* C.P.K. 3593 and FOX (*Fusarium oxysporum* species complex, strain C.P.K. 1842) to represent soil and rhizosphere competent pathogens and *F. xylarioides* C.P.K. 3453, *Alternaria alternata* C.P.K. 3594 and *Botrytis cinerea* C.P.K. 3592 to represent epigeal plant pathogens. Potential prey fungi were inoculated as agar blocks of the standard size always 1 cm from the edge of the Petri plate and pre-cultivated on 3% PDA in darkness at 25 C. Then similar agar blocks with *Trichoderma* cultures were introduced on the opposite side of the plate and cultivated for 10 days. Antagonistic potential was semi-quantified based on both ability to inhibit the growth of a pathogen and ability to overgrow the mycelium of the pathogenic fungus. One of 5 phases for each confrontation was recorded: 0– no inhibition; I - started to inhibit; II - clear signs of inhibition; III - mostly or strongly inhibited; IV - totally inhibited. The ability to overgrow was based on the same scale but recorded using Arabic numbers.

Quantitative and qualitative conidiation assessments. Quantitative assessment of conidiation was done by measuring conidia density per cm2 produced on Petri dishes on 3% MEA after 10 days of cultivation under natural light/darkness cycle. For this purpose the 6.2 cm2 fragment of an agar plate was cut and rinsed in 15 ml of water containing 0.1% of Tween-80 until visually all conidia were washed out. The concentration of conidia was estimated based on optical density at 540 nm (Biolog Turbidimeter) and transferred into density values based on the calibration curve inferred from the serial dilutions of the standard suspension. In addition the qualitative conidiation assessment when the ability to produce conidia was estimated in respect to carbon metabolism was done using Biolog FF Microplates. In this case conidiation intensity was estimated according to the nominative scale [32] after 72 and 168 hours of incubation. Values 0 and 1 were assigned to the cases when no aerial mycelium and no conidia were detected respectively. Values above 1 corresponded to different intensities of conidiation from single spores (2) to the full coverage of the microplate well by a thick conidial mat (4).

Cellulase production. Strains were grown in 1-liter Erlenmeyer flasks on a rotary shaker (250 rpm) at 28°C for 72 h in 250 ml of Mandels-Andreotti medium [53] containing 1% (wt/vol) Avicel microcrystalline cellulose as the sole carbon source. Conidia (final concentration, 10^8 per liter) were used as the inoculum. Cellulase activity in the extracellular culture supernatant was measured using 4-nitrophenyl-β-d-lactopyranoside as a substrate dissolved in a 50 mM sodium citrate buffer, pH 5.0. Other conditions for the assay were the same as used for β-glucosidase [54]. One unit (1 U) of enzyme activity is given as the amount of enzyme needed to liberate 1 µmole 4-nitrophenole from the substrate per min. under the conditions of the assay.

Mating Experiments

This was done as described by Seidl et al. [8]. Briefly, the two putative mating partners were placed onto 3% MEA plates 5 cm apart from each other, and incubated at 25°C for 7–10 days in the presence of a natural illumination cycle. In compatibility reactions fruiting bodies were formed at the interaction zone between the two cultures.

Supporting Information

Figure S1 Single loci phylograms. Bayesian circular phylogram inferred from the concatenated dataset of las1 (A), tef1 (B), cal1 (C) phylogenetic markers. Symbols at nodes correspond to posterior probabilities (PP) >95%.
Found at: doi:10.1371/journal.pone.0009191.s001 (1.34 MB TIF)

Figure S2 Amino acid polymorphism of MAT1 sequences. The aa alignments of MAT1-1-2, Mat1-1-3 and MAT1-2 proteins for *H. jecorina*, *T. parareesei* nom. prov. and T. sp. nov. C.P.K. 524 respectively. Arrows indicate polymorphic sites.
Found at: doi:10.1371/journal.pone.0009191.s002 (0.39 MB TIF)

Table S1 Carbon sources of BIOLOG FF microplates.
Found at: doi:10.1371/journal.pone.0009191.s003 (0.10 MB DOC)

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Author Contributions

Conceived and designed the experiments: ID CPK. Performed the experiments: MKZ LA VS. Analyzed the data: ID. Wrote the paper: ID CPK.

References

1. Harman GE, Kubicek CP (1998) *Trichoderma* and *Gliocladium* Vol 2. London: Taylor & Francis. pp 393.
2. Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B (2009) Metabolic engineering strategies for improvement of cellulase production by *Hypocrea jecorina*. Biotechnol Biofuels. In press. .
3. Percival Zhang YH, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 24: 452–481.
4. Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 35: 377–391.
5. Reese ET, Levinsons HS, Downing M (1950) Quartermaster culture collection. Farlowia 4: 45–86.
6. Kuhls K, Lieckfeldt E, Samuels GJ, Kovacs W, Meyer W, et al. (1996) Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proc Natl Acad Sci U S A 93: 7755–7760.
7. Samuels GJ, Petrini O, Manguin S (1994) Morphological and macromolecular characterization of *Hypocrea schweinitzii* and its *Trichoderma* anamorph. Mycologia 86: 421–435.
8. Seidl V, Seibel C, Kubicek CP, Schmoll M (2009) Sexual development in the industrial workhorse *Trichoderma reesei*. Proc Natl Acad Sci U.S.A. In press.
9. Dettman JR, Jacobson DJ, Taylor JW (2006) Multilocus sequence data reveal extensive phylogenetic species diversity within the *Neurospora discreta* complex. Mycologia 98: 436–446.
10. O'Donnell K, Kistler HC, Tacke BK, Casper HH (2000) Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proc Natl Acad Sci USA 97: 7905–7910.
11. O'Donnell K, Ward TJ, Geiser DM, Kistler HC, Aoki T (2004) Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genet Biol 41: 600–623.
12. Rehner SA, Buckley E (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1-a sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia 97: 84–98.
13. Steenkamp ET, Wingfield BD, Desjardins AE, Marasas WFO, Wingfield MJ (2002) Cryptic speciation in *Fusarium subglutinans*. Mycologia 94: 1032–1043.

Appendix I-I

14. Chaverri P, Castlebury LA, Samuels GJ, Geiser DM (2003) Multilocus phylogenetic structure within the *Trichoderma harzianum/Hypocrea lixii* complex. Mol Phylogenet Evol 7: 302–13.
15. Druzhinina IS, Komoń-Zelazowska M, Kredics L, Hatvani L, Antal Z, et al. (2008) Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. Microbiology 154: 3447–3459.
16. Samuels GJ, Dodd SL, Lu BS, Petrini O, Schroers HJ, et al. (2006) The *Trichoderma koningii* morphological species. Stud Mycol 56: 67–135.
17. Druzhinina IS, Kopchinskiy AG, Komon M, Bissett J, Szakacs G, et al. (2005) An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet Biol 42: 813–828.
18. Hoyos-Carvajal L, Orduz S, Bissett J (2009) Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions. Fungal Genet Biol. In press.].
19. Kubicek CP, Bissett J, Kullnig-Gradinger CM, Druzhinina IS, Szakacs G (2003) Genetic and metabolic diversity of *Trichoderma*: a case study on South-East Asian isolates. Fungal Genet Biol 38: 310–317.
20. Komoń-Zelazowska M, Bissett J, Zafari D, Hatvani L, Manczinger L, et al. (2007) Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms. Appl Environ Microbiol 73: 7415–7426.
21. Doseff AI, Arndt KT (1997) LAS1 is an essential nuclear protein involved in cell morphogenesis and cell surface growth. Genetics 147: 857–871.
22. Samuels GJ, Petrini O, Kuhls K, Lieckfeldt E, Kubicek CP (1998) *Hypocrea* and *Trichoderma* sect. *Longibrachiatum*. Stud Mycol 41: 1–54.
23. Dettman JR, Jacobson DJ, Taylor JW (2003) A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. Evolution 57: 2703–2720.
24. Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, et al. (2000) Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31: 21–32.
25. Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23: 254–267.
26. Huson DH (1998) SplitsTree: a program for analyzing and visualizing evolutionary data. Bioinformatics 14: 68–73.
27. Farris JS, Kallersjo M, Kluge AG, Bult C (1995) Constructing a significance test for incongruence. Syst Biol 44: 570–572.
28. Huelsenbeck JP, Bull JJ, Cunningham CW (1996) Combining data in phylogenetic analysis. Trends Ecol Evol 11: 152 157.
29. Maynard Smith J (1992) Analyzing the mosaic structure of genes. J Mol Evol 35: 126–129.
30. Bruen TC, Philippe H, Bryant D (2006) A simple and robust statistical test for detecting the presence of recombination. Genetics 172: 2665–2681.
31. Druzhinina IS, Schmoll M, Seiboth B, Kubicek CP (2006) Global carbon utilization profiles of wild-type, mutant, and transformant strains of *Hypocrea jecorina*. Appl Environ Microbiol 72: 2126–2133.
32. Friedl MA, Kubicek CP, Druzhinina IS (2008) Carbon source dependence of conidiation and its photostimulation in *Hypocrea atroviridis*. Appl Environ Microbiol 74: 245–250.
33. Friedl MA, Schmoll M, Kubicek CP, Druzhinina IS (2008) Photostimulation of *Hypocrea atroviridis* growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress. Microbiology 154: 1229–1241.
34. Schuster A, Kubicek CP, Friedl MA, Druzhinina IS, Schmoll M (2007) The PAS/LOV-domain protein Envoy of the fungus *Hypocrea jecorina* plays several light-dependent and-independent regulatory roles. BMC Genomics 4; 8: 449.
35. Bisby GR (1939) *Trichoderma viride* Pers. ex Fries, and notes on *Hypocrea*. Trans Brit Mycol Soc 23: 149–168.
36. Simmons EG (1977) Classification of some cellulase-producing *Trichoderma* species. Abst Ind Mycol Congress. p 618.
37. Bissett J (1984) A revision of the genus *Trichoderma* I. Section *Longibrachiatum sect. novum*. Can J Bot 62: 924–933.
38. Lieckfeldt E, Kullnig CM, Samuels GJ, Kubicek CP (2000) Sexually competent, sucrose- and nitrate-assimilating strains of *Hypocrea jecorina* (*Trichoderma reesei*, Hypocreales) from South American soils. Mycologia 92: 374–380.
39. Giraud T, Refrégier G, Le Gac M, de Vienne DM, Hood ME (2008) Speciation in fungi. Fungal Genet Biol 45: 791–802.
40. Dettman JR, Sirjusingh C, Kohn LM, Anderson JB (2009) Incipient speciation by divergent adaptation and antagonistic epistasis in yeast. Nature 447: 585–588.
41. Schluter D (2001) Ecology and the origin of species. Trends Ecol Evol 16: 372–380.
42. Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19: 198–207.
43. Rice WR (1987) Speciation via habitat specialization: the evolution of reproductive isolation as a correlated character. Evol Ecol 1: 301–314.
44. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882.
45. Nicholas KB, Nicholas HB Jr (1997) Genedoc: a tool for editing and annotating multiple sequence alignments. Program Available: http://www.psc.edu/biomed/genedoc. Accessed 2009 Aug 21.
46. Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497.
47. Swofford DL (2002) PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10. Sinauer Associates, Sunderland, MA.
48. Posada D (2003) Using MODELTEST and PAUP* to select a model of nucleotide substitution. In: Baxevanis AD, Davison DB, Page RDM, Petsko GA, Stein LD, eds. Current Protocols in Bioinformatics. Hoboken, New Jersey: John Wiley & Sons. pp 6.5.1–6.5.14.
49. Akaike H (1974) A new look at the statistical model identification. IEEE Trans Automat Control 19: 716–723.
50. Schwarz G (1978) Estimating the dimension of a model. The Ann Stat 6: 461–464.
51. Nylander JA, Wilgenbusch JC, Warren DL, Swofford DL (2008) AWTY: A system for graphical exploration of MCMC convergence in Bayesian phylogenetics. Bioinformatics, Program available: http://ceb.csit.fsu.edu/awty. Accessed 2009 Aug 21 25: 581–583.
52. Kuhner MK (2006) LAMARC 2.0: Maximum likelihood and Bayesian estimation of population parameters. Bioinformatics 22: 768–770.
53. Mandels M (1985) Applications of cellulases. Biochem Soc Trans 13: 414–416.
54. Kubicek CP (1981) Release of carboxymethlycellulase and β-glucosidase from cell walls of *Trichoderma reesei*. Eur J Appl Microbiol Biotechnol 13: 226–231.

Genome Biology

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Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*

Christian P Kubicek[1*], Alfredo Herrera-Estrella[2], Verena Seidl-Seiboth[1], Diego A Martinez[3], Irina S Druzhinina[1], Michael Thon[4], Susanne Zeilinger[1], Sergio Casas-Flores[5], Benjamin A Horwitz[6], Prasun K Mukherjee[7], Mala Mukherjee[6], László Kredics[8], Luis D Alcaraz[2], Andrea Aerts[9], Zsuzsanna Antal[8], Lea Atanasova[1], Mayte G Cervantes-Badillo[5], Jean Challacombe[9], Olga Chertkov[9], Kevin McCluskey[10], Fanny Coulpier[11], Nandan Deshpande[12], Hans von Döhren[13], Daniel J Ebbole[14], Edgardo U Esquivel-Naranjo[2], Erzsébet Fekete[15], Michel Flipphi[16], Fabian Glaser[6], Elida Y Gómez-Rodríguez[5], Sabine Gruber[1], Cliff Han[9], Bernard Henrissat[17], Rosa Hermosa[4], Miguel Hernández-Oñate[2], Levente Karaffa[15], Idit Kosti[6], Stéphane Le Crom[11], Erika Lindquist[9], Susan Lucas[9], Mette Lübeck[18], Peter S Lübeck[18], Antoine Margeot[19], Benjamin Metz[1], Monica Misra[9], Helena Nevalainen[12], Markus Omann[1], Nicolle Packer[12], Giancarlo Perrone[20], Edith E Uresti-Rivera[5], Asaf Salamov[9], Monika Schmoll[1], Bernhard Seiboth[1], Harris Shapiro[9], Serenella Sukno[4], Juan Antonio Tamayo-Ramos[21], Doris Tisch[1], Aric Wiest[10], Heather H Wilkinson[14], Michael Zhang[9], Pedro M Coutinho[17], Charles M Kenerley[14], Enrique Monte[4], Scott E Baker[9,22] and Igor V Grigoriev[9]

Abstract

Background: Mycoparasitism, a lifestyle where one fungus is parasitic on another fungus, has special relevance when the prey is a plant pathogen, providing a strategy for biological control of pests for plant protection. Probably, the most studied biocontrol agents are species of the genus *Hypocrea/Trichoderma*.

Results: Here we report an analysis of the genome sequences of the two biocontrol species *Trichoderma atroviride* (teleomorph *Hypocrea atroviridis*) and *Trichoderma virens* (formerly *Gliocladium virens*, teleomorph *Hypocrea virens*), and a comparison with *Trichoderma reesei* (teleomorph *Hypocrea jecorina*). These three *Trichoderma* species display a remarkable conservation of gene order (78 to 96%), and a lack of active mobile elements probably due to repeat-induced point mutation. Several gene families are expanded in the two mycoparasitic species relative to *T. reesei* or other ascomycetes, and are overrepresented in non-syntenic genome regions. A phylogenetic analysis shows that *T. reesei* and *T. virens* are derived relative to *T. atroviride*. The mycoparasitism-specific genes thus arose in a common *Trichoderma* ancestor but were subsequently lost in *T. reesei*.

Conclusions: The data offer a better understanding of mycoparasitism, and thus enforce the development of improved biocontrol strains for efficient and environmentally friendly protection of plants.

* Correspondence: ckubicek@mail.zserv.tuwien.ac.at
[1]Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering Vienna University of Technology, Getreidemarkt 9, 1060 Vienna, Austria
Full list of author information is available at the end of the article

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Background

Mycoparasitism is the phenomenon whereby one fungus is parasitic on another fungus, a lifestyle that can be dated to at least 400 million years ago by fossil evidence [1]. This has special relevance when the prey is a plant pathogen, providing a strategy for biological control of pests for plant protection ('biocontrol'). The movement toward environmentally friendly agricultural practices over the past two decades has thus accelerated research in the use of biocontrol fungi [2]. Probably the most studied biocontrol agents are species of the genus *Hypocrea/Trichoderma*, *Trichoderma atroviride* (*Ta*) and *Trichoderma virens* (*Tv*) - teleomorphs *Hypocrea atroviridis* and *Hypocrea virens*, respectively - being among the best mycoparasitic biocontrol agents used in agriculture [3]. The beneficial effects of *Trichoderma* spp. on plants comprise traits such as the ability to antagonize soil-borne pathogens by a combination of enzymatic lysis, secretion of antibiotics, and competition for space and substrates [4,5]. In addition, it is now known that some *Trichoderma* biocontrol strains also interact intimately with plant roots, colonizing the outer epidermis layers, and acting as opportunistic, avirulent plant symbionts [6].

Science-based improvement of biocontrol agents for agricultural applications requires an understanding of the biological principles of their actions. So far, some of the molecular aspects - such as the regulation and role of cell wall hydrolytic enzymes and antagonistic secondary metabolites - have been studied in *Trichoderma* [3-5]. More comprehensive analyses (for example, by the use of subtractive hybridization techniques, proteomics or EST approaches) have also been performed with different *Trichoderma* species, but the interpretation of the data obtained is complicated by the lack of genome sequence information for the species used (reviewed in [7]).

Recently, the genome of another *Trichoderma*, *Trichoderma reesei* (*Tr*, teleomorph *H. jecorina*), which has a saprotrophic lifestyle and is an industrial producer of plant biomass hydrolyzing enzymes, has been sequenced and analyzed [8]. Here we report the genome sequencing and comparative analysis of two widely used biocontrol species of *Trichoderma*, that is, *Ta* and *Tv*. These two were chosen because they are distantly related to *Tr* [9] and represent well defined phylogenetic species [10,11], in contrast to *Trichoderma harzianum sensu lato*, which is also commonly used in biocontrol but constitutes a complex of several cryptic species [12].

Results

Properties of the *T. atroviride* and *T. virens* genomes

The genomes of *Ta* IMI 206040 and *Tv* Gv29-8 were sequenced using a whole genome shotgun approach to approximately eight-fold coverage and further improved using finishing reactions and gap closing. Their genome sizes were 36.1 (*Ta*) and 38.8 Mbp (*Tv*), and thus larger than the 34 Mbp determined for the genome of *Tr* [8]. Gene modeling, using a combination of homology and *ab initio* methods, yielded 11,865 gene models for *Ta* and 12,428 gene models for *Tv*, respectively (Table 1), both greater than the estimate for *Tr* (9,143). As shown in Figure 1, the vast majority of the genes (7,915) occur in all three *Trichoderma* species. Yet *Tv* and *Ta* contain about 2,756 and 2,510 genes, respectively, that have no true orthologue in any of the other species, whereas *Tr* has only 577 unique genes. *Tv* and *Ta* share 1,273 orthologues that are not present in *Tr*, which could thus be part of the factors that make *Ta* and *Tv* mycoparasites (for analysis, see below).

With respect to other ascomycetes, *Tr, Ta and Tv* share 6,306/7,091, 6,515/7,549, and 6,564/7,733 orthologues with *N. crassa* and *Gibberella zeae*, respectively.

Table 1 Genome assembly and annotation statistics

	T. atroviride	*T. virens*	*T. reesei*
Genome size, Mbp	36.1	38.8	34.1
Coverage	8.26×	8.05×	9.00×
Assembly gaps, Mbp	0.1 (0.16%)	0.2(0.4%)	0.05 (0.1%)
Number of scaffolds	50	135	89
Number of predicted genes	11865	12518	9143
Gene length, bp	1747.06	1710.05	1793,25
Protein length, amino acids	471.54	478.69	492,27
Exons per gene	2,93	2,98	3,06
Exon length, bp	528.17	506.13	507,81
Intron length, bp	104.20	104.95	119,64
Supported by homology, NR	10,219 (92%)	10,915 (94%)	8409 (92%)
Supported by homology, Swissprot	8,367(75%)	8,773 (75%)	6763 (74%)
Has PFAM domain	5,883 (53%)	6,267 (54%)	5096 (56%)

NR, non-redundant database; PFAM, protein families.

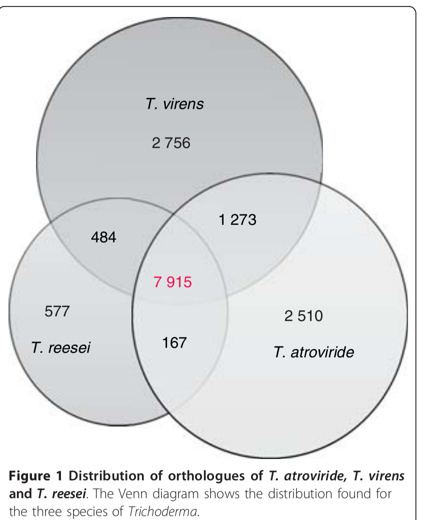

Figure 1 Distribution of orthologues of *T. atroviride, T. virens* and *T. reesei*. The Venn diagram shows the distribution found for the three species of *Trichoderma*.

Thus, approximately a third of the genes in the three *Trichoderma* species are not shared in even the relatively close relative *G. zeae* and are thus unique to *Trichoderma*.

Genome synteny

A comparison of the genomic organization of genes in *Ta, Tv* and *Tr* showed that most genes are in synteny: only 367 (4%) genes of *Tr*, but 2,515 (22%) of genes of *Tv* and 2,690 (21%) genes of *Ta* are located in non-

Table 2 Occurrence of orthologues, paralogues and singletons in the genomes of the three *Trichoderma* spp

Genome	Synteny	Total genes	Orthologs[a]	Non-orthologs	*P*-value[b]
T. atroviride	Syntenic	9,350	7,326	2,024	2.2e-16
	Non-syntenic	2,515	1,265	1,250	
T. virens	Syntenic	9,828	7,326	2,502	2.2e-16
	Non-syntenic	2,690	1,532	1,158	
T. reesei	Syntenic	8,776	7,326	1,450	2.2e-16
	Non-syntenic	367	153	214	

[a]Orthologs that are in all three genomes. [b]Null hypothesis that the proportion of non-orthologs that are syntenic is less than the proportion of non-orthologs that are non-syntenic. *P*-value: null hypothesis that the proportion of paralogs that are syntenic is less than the proportion of paralogs that are non-syntenic.

syntenic regions (identified as a break in synteny by a series of three or more genes (Table 2); a global visual survey can be obtained at the genome websites of the three *Trichoderma* species (see Materials and methods) by clicking 'Synteny' and 'Dot Plot'). As observed for other fungal genomes [13-15], extensive rearrangements have occurred since the separation of these three fungi but with the prevalence of small inversions [16]. The numbers of the synteny blocks increased with their decreased size, compatible with the random breakage model [14] as in aspergilli [15,17]. Sequence identity between syntenic orthologs was 70% (*Tr* versus *Ta*), 78% (*Tr* versus *Tv*), and 74% (*Tv* versus *Ta*), values that are similar to those calculated for aspergilli (for example, *Aspergillus fumigatus* versus *Aspergillus niger* (69%) and versus *Aspergillus nidulans* (68%) and comparable to those between fish and man [17,18].

Transposons

A scan of the genome sequences with the *de novo* repeat finding program 'Piler' [19] - which can detect repetitive elements that are least 400 bp in length, have more than 92% identity and are present in at least three copies - was unsuccessful at detecting repetitive elements. The lack of repetitive elements detected in this analysis is unusual in filamentous fungi and suggests that, like the *Tr* genome [8], but unlike most other filamentous fungi, the *Ta* and *Tv* genomes lack a significant repetitive DNA component.

Because of the paucity of transposable elements (TEs) in the *Trichoderma* genomes, we wondered whether simple sequence repeats and minisatellite sequences may also be rare. To this end, we surveyed the genomes of the *Trichoderma* species using the program Tandem Repeat Finder [20]. We also included the genomes of three additional members of the Sordariomycetes and one of the Eurotiomycetes as reference (Table S1 in Additional file 1). Satellite DNA content varied from as little as 2,371 loci (0.53% of the genome) in *A. nidulans* to 9,893 (1.46% of the genome) in *Neurospora crassa*. Satellite DNA content of the *Trichoderma* genomes ranged from 5,249 (0.94%) in *Ta* to 7,743 (1.54%) in *Tr*. Since these values are within the range that we found in the reference species, we conclude that there is no unusual variation in the satellite DNA content of the *Trichoderma* genomes.

We also scanned the genomes with RepeatMasker and RepeatProteinMask [21] to identify sequences with similarity to known TEs from other organisms. Thereby, sequences with significant similarity to known TEs from other eukaryotes were identified (Table 3). In most cases, the TE families that we detected were fragmented and highly divergent from one another, suggesting that they did not arise from recent transposition events.

Table 3 The major classes of transposable elements found in the *Trichoderma* genomes

	T. atroviridae		*T. reesei*		*T. virens*	
Class	Copy number	Total length (bp)	Copy number	Total length (bp)	Copy number	Total length (bp)
DNA	372	39,899	446	50,448	370	52,358
LTR	533	64,534	559	76,482	541	67,484
Helitrons	40	9,235	45	9,962	34	8,547
LINE	561	65,202	530	54,928	349	59,414
Total[a]		178,870 (0.49%)		191,820 (0.57%)		187,803 (0.48%)

[a]Total in base pairs and percentage of genome of transposable elements found in the genomes. LINE, long interspersed nuclear element; LTR, long terminal repeat.

Based on these results, we conclude that no extant, functional TEs exist in the *Trichoderma* genomes. The presence of ancient, degenerate TE copies suggests that *Trichoderma* species are occasionally subject to infection, or invasion by TEs, but that the TEs are rapidly rendered unable to replicate and rapidly accumulate mutations.

Evidence for the operation of repeat-induced point mutation in *Trichoderma*

The paucity of transposons in *Trichoderma* could be due to repeat-induced point mutation (RIP), a gene silencing mechanism. In *N. crassa* and many other filamentous fungi, RIP preferentially acts on CA dinucleotides, changing them to TA [22]. Thus, in sequences that have been subject to RIP, one should expect to find a decrease in the proportion of CA dinucleotides and its complement dinucleotide TG as well as a corresponding

Table 4 Repeat-induced point mutation ratios for four of the most abundant transposable element families in the three *Trichoderma* species

Sequence	TA/AT ratio	CT+AT/AC+GT ratio	RIP[a]
T. atroviride	0.70	1.35	
LTR Copia	0.42	1.50	
LTR Gypsy	0.97	1.21	
LINE R1	1.86	1.67	
LINE Tad1	0.82	1.32	
T. reesei	0.71	1.28	
LTR Copia	1.04	1.31	
LTR Gypsy	1.01	1.28	
LINE R1	0.99	2.40	
LINE Tad1	0.33	1.30	
T. virens	0.71	1.33	
LTR Copia	0.77	1.48	
LTR Gypsy	0.95	1.16	
LINE R1	0.75	2.14	
LINE Tad1	1.33	0.99	*

[a]The asterisk indicates the family Tad1 from *T. virens* in which the RIP ratios fall within values that are typically associated with RIP. LINE, long interspersed nuclear element; LTR, long terminal repeat; RIP, repeat-induced point mutation; TE, transposable element.

increase in the proportion of TA dinucleotides. The RIP indices TA/AT and (CA + TG)/(AC + GT) developed by Margolin *et al.* [22] can be used to detect sequences that have been subject to RIP. Sequences that have been subjected to RIP are expected to have a high TA/AT ratio and low (CA + TG)/(AC + GT) ratio, with values >0.89 and <1.03, respectively, being indicative of RIP [22,23].

To identify evidence for RIP in the TE sequences, we computed RIP indices for four of the most prevalent TE families in each of the three species (Table 4). Since many of the sequences are very short, we computed the sum of the dinucleotide values within each TE family within each species, and used the sums to compute the RIP ratios. In only one of the 12 families did we find that both RIP indices were within the ranges that are typically used as criteria for RIP. Most of the TE sequences that we identified in the *Trichoderma* genomes are highly degenerate and have likely continued to accumulate mutations after the RIP process has acted on them. We suspect that these mutations have masked the underlying bias in dinucleotide frequencies, making the RIP indices ineffective at identifying the presence of RIP. To overcome this, we also prepared manually curated multiple sequence alignments of the TE families, selecting only sequences that had the highest sequence similarity, and thus should represent the most recent transposon insertion events in the genomes. We were able to prepare curated alignments for all four of the test TE families of *Tr* and *Tv* only for the long terminal repeat element Gypsy and the long interpersed nuclear element R1 in *Ta* (Table S2 in Additional file 1). Among DNA sequences that make up these ten alignments, we detected RIP indices within the parameters that are indicative of RIP in seven alignments. In addition, all seven alignments have high transition/transversion ratios, as is expected in sequences that are subject to RIP.

Finally, screening of the genome sequences of *Tr, Ta* and *Tv* identified orthologues of all genes required for RIP in *N. crassa* (Table 5).

Table 5 Presence of genes in *Trichoderma* known to be required in *N. crassa* for repeat-induced point mutation

N. crassa protein[a]	Accession number[a]	Function[a]	*Trichoderma* orthologue (ID number)		
			T. atroviride	*T. virens*	*T. reesei*
RIP					
RID	XP_959047.1	Putative DMT, essential for RIP and for MIP			
Dim-5	XP_957479.2	Histone 3-K9 HMT essential for RIP; RdRP	152017	55211	515216
Quelling					
QDE-1	XP_959047.1	RdRP, essential for quelling	361	64774	67742
QDE-2	XP_960365.2	Argonaute-like protein, essential for quelling	79413	20883	49832
QDE-3	XP_964030.2	RecQ helicase, essential for quelling	91316	30057	102458
DCL1	XP_961898.1	Dicer-like protein, involved in quelling	20162	20212	69494
DCL2	XP_963538.2	Dicer-like protein, involved in quelling	318	47151	79823
QIP	CAP68960.1	Putative exonuclease protein, involved in quelling	14588	41043	57424
MSUD					
SAD-1	XP_964248.2	RdRP essential for MSUD	465	28428	103470
SAD-2	XP_961084.1	Essential for MSUD	No	No	No

[a]*N. crassa* gene information and abbreviations taken from [36]. DMT, cytosine DNA methyltransferase; HMT, histone methyltransferase; MIP, methylation induced premeotically; MSUD, meiotic silencing of unpaired DNA; RdRP, RNA-dependent RNA-polymerase.

Paralogous gene expansion in *T. atroviride* and *T. virens*

We used Marcov cluster algorithm (MCL) analysis [24] and included ten additional ascomycete genomes present in the Joint Genome Institute (JGI) genome database (including Eurotiomycetes, Sordariomycetes and Dothidiomycetes) to identify paralogous gene families that have become expanded either in all three *Trichoderma* species or only in the two mycoparasitic *Trichoderma* species. Forty-six such families were identified for all three species, of which 26 were expanded only in *Ta* and *Tv*. The largest paralogous expansions in all three *Trichoderma* species have occurred with genes encoding Zn(2)Cys(6) transcription factors, solute transporters of the major facilitator superfamily, short chain alcohol dehydrogenases, S8 peptidases and proteins bearing ankyrin domains (Table 6). The most expanded protein sets, however, were those that were considerably smaller in *Tr* ($P < 0.05$). These included ankyrin proteins with CCHC zinc finger domains, proteins with WD40, heteroincompatibility (HET) and NACHT domains, NAD-dependent epimerases, and sugar transporters.

Genes with possible relevance for mycoparasitism are expanded in *Trichoderma*

Mycoparasitism depends on a combination of events that include lysis of the prey's cell walls [3,4,7]. The necessity to degrade the carbohydrate armor of the prey's hyphae is reflected in an abundance of chitinolytic enzymes (composing most of the CAZy (Carbohydrate-Active enZYmes database) glycoside hydrolase (GH) family GH18 fungal proteins along with more rare endo-β-N-acetylglucosaminidases) and β-1,3-glucanases (families GH17, GH55, GH64, and GH81) in *Trichoderma* relative to other fungi. Family GH18, containing enzymes involved in chitin degradation, is also strongly expanded in *Trichoderma*, but particularly in *Tv* and *Ta*, which contain the highest number of chitinolytic enzymes of all described fungi (Table 7). Chitin is a substantial component of fungal cell walls and chitinases are therefore an integral part of the mycoparasitic attack [3,25]. It is conspicuous that not only was the number of chitinolytic enzymes elevated but that many of these chitinases contain carbohydrate binding domains (CBMs). Mycoparasitic *Trichoderma* species are particularly rich in subgroup B chitinases that contain CBM1 modules, historically described as cellulose binding modules, but binding to chitin has also been demonstrated [26]. *Tv* and *Ta* each have a total of five CBM1-containing GH18 enzymes. Subgroup C chitinases possess CBM18 (chitin-binding) and CBM50 modules (also known as LysM modules; described as peptidoglycan- and chitin-binding modules). Interestingly, CBM50 modules in *Trichoderma* are found not only in chitinases but also frequently as multiple copies in proteins containing a signal peptide, but with no identifiable hydrolase domain. In most cases these genes can be found adjacent to chitinases in the genome.

Together with the expanded presence of chitinases, the number of GH75 chitosanases is also significantly expanded in all three analyzed *Trichoderma* species. As with plant pathogenic fungi [27,28], we have also observed an expansion of plant cell wall degrading enzyme gene families. A full account of all the carbohydrate active enzymes is presented in Tables S3 to S8 in Additional file 1. Additional details about the *Trichoderma* CAZome (the genome-wide inventory of CAZy) are given in Chapter 1 of Additional file 2.

Table 6 Major paralogous gene expansions in Trichoderma

PFAM domain	*T. reesei*	*T. virens*	*T. atroviride*	Other fungi[a]
Unknown protein with ankyrin (PF00023), CCHC zinc finger (PF00098; C-X2-C-X4-H-X4-C) and purine nucleoside phosphorylase domain (01048)	19	***38***	***45***	4
Zn(II)Cys6 transcription factor (00172) cluster 1-5	20	***43***	***42***	5,1
Peptidase S8 subtilisin cluster 1-4	10	***33***	***36***	9,6
Unknown protein with WD40, NACHT and HET domain	13	***38***	***35***	3,4
Short chain alcohol dehydrogenase (PF00106) cluster 1 and 2	20	32	34	4,7
Unknown protein family 1-4	12	***25***	***28***	5
NAD-dependent epimerase (PFAM 01370)	10	***21***	***23***	5,8
Isoflavon reductase, plus PAPA-1 (INO80 complex subunit B), epimerase and Nmr1 domain	9	***18***	***19***	6
Ankyrin domain protein	10	17	19	8
Sugar transporters	11	24	18	10,8
GH18 chitinases	6	***11***	***16***	2
Protein kinase (00069) plus TPR domain	2	***24***	***15***	4,7
Unknown major facilitator subfamily (PF07690) domain	9	15	15	5,5
F-box domain protein	7	10	11	1,7
Ankyrin domain protein with protein kinase domain	6	8	11	2,7
Amidase	4	11	11	2,8
Epoxide hydrolase (PF06441) plus AB hydrolase_1 (PF00561)	5	14	11	3,2
FAD_binding_4, plus HET and berberine bridge enzymes (08031) domain	5	13	11	6,1
FMN oxidoreductases	2	8	10	2,5
Unknown protein with DUF84 (NTPase) and NmrA domain	5	19	10	3,7
Protein with GST_N and GST_C domains	6	12	10	4,6
Class II hydrophobins	6	8	9	1,1
Proteins with LysM binding domains	6	7	9	1,2
Unknown protein family with NmrA domain	2	***11***	***8***	0,2
Pro_CA	5	9	8	1,3
WD40 domain protein	5	11	8	2,2
C2H2 transcription factors	1	***5***	***7***	1,4
GFO_IDH_MocA (01408 and 02894) oxidoreductase	3	9	7	1,5
Protein kinase (00069)	4	6	6	0,7
Nonribosomal peptide synthase	3	4	5	1
SSCP ceratoplatanin-family	3	4	5	1
GH75 chitosanase	3	5	5	1,1
SNF2, DEAD box helicase	3	5	5	1,3
Nitrilase	3	6	5	2,2
GH65 trehalose or maltose phosphorylase (PFAM 03632)	4	4	4	0,8
AAA-family ATPase (PF00004)	4	3	4	1
Pyridoxal phosphate dependent decarboxylase (00282)	2	3	4	1,2
Unknown protein	3	4	4	1,3

[a]Results are from MCL analysis of the three *Trichoderma* species (*Tr, Ta, Tv*) and mean values from ten other ascomycetes whose genomes are present in the JGI database [63]. Eurotiomycetes: *Aspergillus carbonarius, Aspergillus niger*. Sordariomycetes: *Thielavia terrestris, Chaetomium globosum, Cryphonectria parasitica, Neurospora discreta, Neurospora tetrasperma*. Dothidiomycetes: *Mycosphaerella graminicola, Mycosphaerella fijiensis, Cochliobolus heterostrophus*. The number of genes present in the "other fungi" is averaged. Data were selected from a total of 28,919 clusters, average cluster number 5.8 (standard deviation 15.73). PFAM categories printed in bold specify those that are significantly ($P < 0.05$) expanded in all three *Trichoderma* species; numbers in bold and italics specify genes that are significantly more abundant in *Ta* and *Tv* versus *Tr* ($P < 0.05$). GH, glycosyl hydrolase family; GST, glutahionine-S transferase; SSCP, small secreted cystein-rich protein.

Another class of genes of possible relevance to mycoparasitism are those involved in the formation of secondary metabolites (Chapter 2 of Additional file 2). With respect to these, the three *Trichoderma* species contained a varying assortment of non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) (Table 8; see also Tables S9 and S10 in Additional file 1). While *Tr* (10 NRPS, 11 PKS and 2 NRPS/PKS fusion genes [8]) ranked at the lower end when compared to other ascomycetes, *Tv* exhibited the highest

Table 7 Glycosyl hydrolase families involved in chitin/chitosan and β-1,3 glucan hydrolysis that are expanded in mycoparasitic *Trichoderma* species

		Glycosyl hydrolase family						
		Chitin/chitosan[a]		ß-glucan[a]				Total ß-glucan[b]
	Taxonomy	GH18	GH75	GH17	GH55	GH64	GH81	217
Trichoderma atroviride	S	**29**	**5**	5	**8**	**3**	2	18
Trichoderma virens	S	**36**	**5**	4	**10**	**3**	1	18
Trichoderma reesei	S	**20**	**3**	4	**6**	**3**	2	15
Pezizomycota								
Nectria haematococca	S	*28*	2	6	5	2	1	14
Fusarium graminearum	S	19	1	6	3	2	1	12
Neurospora crassa	S	12	1	4	6	2	1	13
Podospora anserina	S	20	1	4	7	1	1	13
Magnaporthe grisea	S	14	1	7	3	1	2	13
Aspergillus nidulans	E	19	2	5	6	0	1	12
Aspergillus niger	E	14	2	5	3	0	1	9
Penicillium chrysogenum	E	9	1	5	3	2	1	11
Tuber melanosporum	P	5	1	4	2	0	*3*	9
Other ascomycetes								
Saccharomyces cerevisiae	SM	2	0	4	0	0	2	6
Schizosaccharomyces pombe	SS	1	0	1	0	0	1	2
Basidiomycota								
Phanerochaete chrysosporium	A	11	0	2	2	0	0	4
Laccaria bicolor	A	10	0	4	2	0	0	6
Postia placenta	A	20	0	4	6	0	0	10

[a]Main substrates for the respective enzyme families. [b]Number of all enzymes that can act on ß-glucan as a substrate. Taxonomy abbreviations: S, Sordariomycetes; E, Eurotiomycetes; P, Pezizomycetes; S, Saccharomycetes; SS, Schizosaccharomycetes; A, Agaricomycetes. The bold numbers indicate glycosyl hydrolase (GH) families that have a statistically significant expansion in *Trichoderma* ($P < 0.05$) or *Ta* and *Tv* (GH18). This support was obtained only when *N. haematococca* and *T. melanosporum* were not included in the analysis of GH18 and GH81, respectively.

number (50) of PKS, NRPS and PKS-NRPS fusion genes, mainly due to the abundance of NRPS genes (28, twice as much as in other fungi). A phylogenetic analysis showed that this was due to recent duplications of genes encoding cyclodipeptide synthases, cyclosporin/enniatin

Table 8 The number of polyketide synthases and non-ribosomal peptide synthetases of *Trichoderma* compared to other fungi

Fungal species	PKS	NRPS	PKS-NRPS NRPS-PKS	Total
Trichoderma virens	**18**	**28**	**4**	**50**
Aspergillus oryzae	26	14	4	44
Aspergillus nidulans	26	13	1	40
Cochliobolus heterostrophus	23	11	2	36
Trichoderma atroviride	**18**	**16**	**1**	**35**
Magnaporthe oryzae	20	6	8	34
Fusarium graminearum	14	19	1	34
Gibberella moniliformis	12	16	3	31
Botryotinia fuckeliana	17	10	2	29
Aspergillus fumigatus	13	13	1	27
Nectria haematococca	12	12	1	25
Trichoderma reesei	**11**	**10**	**2**	**23**
Neurospora crassa	7	3	0	10

synthase-like proteins, and NRPS-hybrid proteins (Figure S1 in Additional file 3). Most of the secondary metabolite gene clusters present in *Tr* were also found in *Tv* and *Ta*, but about half of the genes remaining in the latter two are unique for the respective species, and are localized on non-syntenic islands of the genome (see below). Within the NRPS, all three *Trichoderma* species contained two peptaibol synthases, one for short (10 to 14 amino acids) and one for long (18 to 25 amino acids) peptaibols. The genes encoding long peptaibol synthetase lack introns and produce an mRNA that is 60 to 80 kb long that encodes proteins of approximately 25,000 amino acids, the largest fungal proteins known.

Besides PKS and NRPS, *Ta* and *Tv* have further augmented their antibiotic arsenal with genes for cytolytic peptides such as aegerolysins, pore-forming cytolysins typically present in bacteria, fungi and plants, yeast-like killer toxins and cyanovirins (Chapter 2 of Additional file 2). In addition, we found two high molecular weight toxins in *Ta* and *Tv* that bear high similarity (E-value 0 for 97% coverage) to the Tc ('toxin complex') toxins of *Photorhabdus luminescens*, a bacterium that is mutualistic with entomophagous nematodes [29] (Table S11 in Additional file 1). Apart from *Trichoderma*, they are

also present in *G. zeae* and *Podospora anserina*. Yet there may be several more secondary metabolite genes to be detected: *Trichoderma* species contain expanded arrays of cytochrome P450 CYP4/CYP19/CYP26 subfamilies (Table S12 in Additional file 1), and of soluble epoxide hydrolases that could act on the epoxides produced by the latter (Figure S2 in Additional file 3).

The *Hypocrea*/*Trichoderma* genomes also contain an abundant arsenal of putatively secreted proteins of 300 amino acids or less that contain at least four cysteine residues (small secreted cysteine-rich proteins (SSCPs); Chapter 3 of Additional file 2). They contained both unique and shared sets of SSCPs, with a higher complexity in *Tv* and *Ta* than in *Tr* (Table S13 in Additional file 1).

Genes present in *T. atroviride* and *T. virens* but not in *T. reesei*

As mentioned above, 1,273 orthologous genes were shared between *Ta* and *Tv* but absent from *Tr*. When the encoded proteins were classified according to their PFAM domains, fungal specific Zn(2)Cys(6) transcription factors (PF00172, PF04082) and solute transporters (PF07690, PF00083), all of unknown function, were most abundant (Table S14 in Additional file 1). However, the presence of several PFAM groups of oxidoreductases and monooxygenases, and of enzymes for AMP activation of acids, phosphopatheteine attachment and synthesis of isoquinoline alkaloids was also intriguing. This suggests that *Ta* and *Tv* may contain an as yet undiscovered reservoir of secondary metabolites that may contribute to their success as mycoparasites.

We also annotated the 577 genes that are unique in *T. reesei*: the vast majority of them (465; 80.6%) encoded proteins of unknown function or proteins with no homologues in other fungi. The remaining identified 112 genes exhibited no significant abundance in particular groups, except for four Zn(2)Cys(6) transcription factors, four ankyrins, four HET-domain proteins and three WD40-domain containing proteins.

Evolution of the non-syntenic regions

A search for overrepresentation of PFAM domains and Gene Ontology terms in the non-syntenic regions described above revealed that all retroposon hot spot repeat domains [30] are found in the non-syntenic regions. In most eukaryotes, these regions are located in subtelomeric areas that exhibit a high recombination frequency [31]. In addition, the genes for the protein families in *Tv* and *Ta* that were significantly more abundant compared to *Tr* were enriched in the non-syntenic areas (Table 9). In addition, the number of paralogous genes was significantly increased in the non-syntenic regions. We considered three possible explanations for

Table 9 Number of PFAM domains that are enriched among paralogous genes in non-syntenic areas

	T. reesei	*T. virens*	*T. atroviride*
Zn2Cys6 transcription factors	9	**95**	69
WD40 domains	1	11	14
Sugar transporters	**0**	18	13
Proteases	2	28	23
Cytochrome P450	7	**40**	15
NmrA-domains	2	19	21
Major facilitator superfamily	7	52	60
HET domains	3	26	27
Glycoside hydrolases	3	33	26
FAD-binding proteins	2	28	24
Ankyrins	4	44	37
Alcohol dehydrogenases	**4**	51	71
α/ß-fold hydrolases	2	26	15
ABC transporters	4	14	3
Number of genes	50	485	418
Total gene number in NS areas	92	686	1012

Boxed numbers are those that are significantly ($p < 0.05$) different from the two other species when related to the genome size. PFAM, protein family; NS, non-syntenic; HET, heteroincompatibility.

this: the non-syntenic genes were present in the last common ancestor of all three *Trichoderma* species but were then selectively and independently lost; the non-syntenic areas arose from the core genome by duplication and divergence during evolution of the genus *Trichoderma*; and the non-syntenic genes were acquired by horizontal transfer. To distinguish among these hypotheses for their origin, we compared the sequence characteristics of the genes in the non-syntenic regions to those present in the syntenic regions in *Trichoderma* and genes in other filamentous fungi. We found that the majority (>78%) of the syntenic as well as non-syntenic encoded proteins have their best BLAST hit to other ascomycete fungi, indicating that the non-syntenic regions are also of fungal origin. Also, a high number of proteins encoded in the non-syntenic regions of *Ta* and *Tv* have paralogs in the syntenic region. Finally, codon usage tables and codon adaptation index analysis [32] indicate that the non-syntenic genes exhibit a similar codon usage (Figure S3 in Additional file 3). Taken together, the most parsimonious explanation for the presence of the paralogous genes in *Ta* and *Tv* is that the non-syntenic genes arose by gene duplication within a *Trichoderma* ancestor, followed by gene loss in the three lineages, which was much stronger in *Tr*.

Tr, *Ta* and *Tv* each occupy very diverse phylogenetic positions in the genus *Trichoderma*, as shown by a Bayesian *rpb2* tree of 110 *Trichoderma* taxa (Figure 2). In order to determine which of the three species more likely resembles the ancestral state of *Trichoderma*, we performed a Bayesian phylogenetic analysis [33] using a

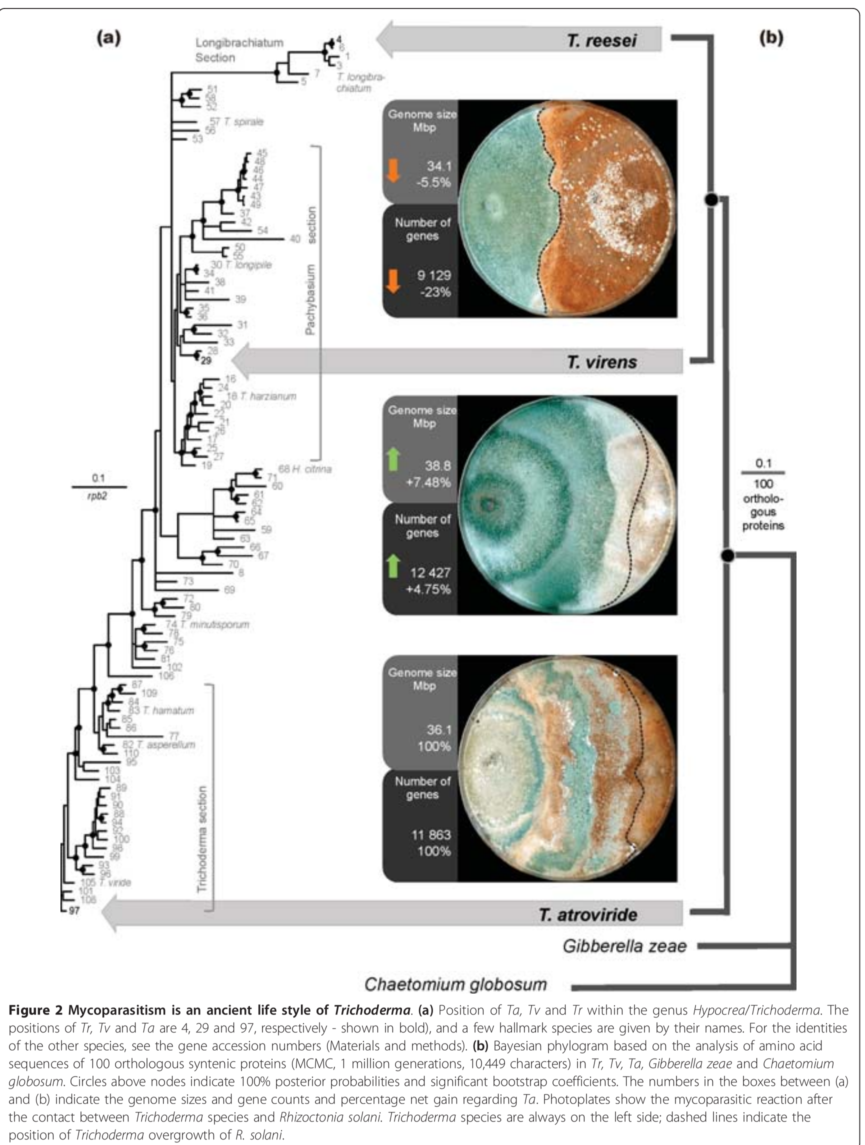

Figure 2 Mycoparasitism is an ancient life style of *Trichoderma*. **(a)** Position of *Ta*, *Tv* and *Tr* within the genus *Hypocrea/Trichoderma*. The positions of *Tr*, *Tv* and *Ta* are 4, 29 and 97, respectively - shown in bold), and a few hallmark species are given by their names. For the identities of the other species, see the gene accession numbers (Materials and methods). **(b)** Bayesian phylogram based on the analysis of amino acid sequences of 100 orthologous syntenic proteins (MCMC, 1 million generations, 10,449 characters) in *Tr*, *Tv*, *Ta*, *Gibberella zeae* and *Chaetomium globosum*. Circles above nodes indicate 100% posterior probabilities and significant bootstrap coefficients. The numbers in the boxes between (a) and (b) indicate the genome sizes and gene counts and percentage net gain regarding *Ta*. Photoplates show the mycoparasitic reaction after the contact between *Trichoderma* species and *Rhizoctonia solani*. *Trichoderma* species are always on the left side; dashed lines indicate the position of *Trichoderma* overgrowth of *R. solani*.

concatenated set of 100 proteins that were encoded by orthologous genes in syntenic areas in the three *Trichoderma* species and also *G. zeae* and *Chaetomium globosum*. The result (Figure 2) shows that *Ta* occurs in a well-supported basal position to *Tv* and *Tr*. These data indicate that *Ta* resembles the more ancient state of *Trichoderma* and that both *Tv* and *Tr* evolved later. The lineage to *Tr* thus appears to have lost a significant number of genes present in *Ta* and maintained in *Tv*. The long genetic distance of *Tr* further suggests that it was apparently evolving faster then *Ta* and *Tv* since the time of divergence.

To test this assumption, we compared the evolutionary rates of the 100 orthologous and syntenic gene families between the three *Trichoderma* species. The median values of the evolutionary rates (K_s and K_a) of *Ta-Tr* and *Tv-Tr* were all significantly higher (1.77 and 1.47, and 1.33 and 1.19, respectively) than those of *Ta-Tv* (1.13 and 0.96; all P values <0.05 by the two-tailed Wilcoxon rank sum test). This result supports the above suggestion that *Tr* has been evolving faster than *Ta* and *Tv*.

Discussion

Comparison of the genomes of two mycoparasitic and one saprotrophic *Trichoderma* species revealed remarkable differences: in contrast to the genomes of other multicellular ascomycetes, such as aspergilli [15,17], those of *Trichoderma* appear to be have the highest level of synteny of all genomes investigated (96% for *Tr* and still 78/79% for *Tv and Ta*, respectively, versus 68 to 75% in aspergilli), and most of the differences between *Ta* and *Tv* versus *Tr* or other ascomycetes occur in the non-syntenic areas. Nevertheless, at a molecular level the three species are as distant from each other as apes from *Pices* (fishes) or *Aves* (birds) [17], suggesting a mechanism maintaining this high genomic synteny. Espagne *et al.* [13] proposed that a discrepancy of genome evolution between *P. anserina, N. crassa* and the aspergilli and saccharomycotina yeasts is based on the difference between heterothallic and homothallic fungi: in heterothallics the presence of interchromosomal translocation could result in chromosome breakage during meiosis and reduced fertility, whereas homothallism allows translocations to be present in both partners and thus have fewer consequences on fertility. Since *Trichoderma* is heterothallic [34], this explanation is also applicable to it. However, another mechanism, meiotic silencing of unpaired DNA [35] - which has also been proposed for *P. anserina* [13], and which eliminates progeny in crosses involving rearranged chromosomes in one of the partners - may not function in *Trichoderma* because one of the essential genes (*SAD2* [36]) is missing.

Our data also suggest that the ancestral state of *Hypocrea*/*Trichoderma* was mycoparasitic. This supports an earlier speculation [37] that the ancestors of *Trichoderma* were mycoparasites on wood-degrading basidiomycetes and acquired saprotrophic characteristics to follow their prey into their substrate. Indirect evidence for this habitat shift in *Tr* was also presented by Slot and Hibbett [38], who demonstrated that *Tr* - after switching to a specialization on a nitrogen-poor habitat (decaying wood) - has acquired a nitrate reductase gene (which was apparently lost earlier somewhere in the Sordariomycetes lineage) by horizontal gene transfer from basidiomycetes.

Furthermore, the three *Trichoderma* species have the lowest number of transposons reported so far. This is unusual for filamentous fungi, as most species contain approximately 10 to 15% repetitive DNA, primarily composed of TEs. A notable exception is *Fusarium graminearum* [27], which, like the *Trichoderma* species, contains less than 1% repetitive DNA [8]. The paucity of repetitive DNA may be attributed to RIP, which has been suggested to occur in *Tr* [8] and for which we have here provided evidence that it also occurs in *Ta* and *Tv*. It is likely that this process also contributes to prevent the accumulation of repetitive elements.

The gene inventory detected in the three *Trichoderma* species reveals new insights into the physiology of this fungal genus: the strong expansion of genes for solute transport, oxidoreduction, and ankyrins (a family of adaptor proteins that mediate the anchoring of ion channels or transporters in the plasma membrane [39]) could render *Trichoderma* more compatible in its habitat (for example, to successfully compete with the other saprotrophs for limiting substrates). In addition, the expansion of WD40 domains acting as hubs in cellular networks [40] could aid in more versatile metabolism or response to stimuli. These features correlate well with a saprotrophic lifestyle that makes use of plant biomass that has been pre-degraded by earlier colonizers. The expansion of HET proteins (proteins involved in vegetative incompatibility specificity) on the other hand suggests that *Trichoderma* species may frequently encounter related yet genetically distinct individuals. In fact, the presence of several different *Trichoderma* species can be detected in a single soil sample [41]. Unfortunately, vegetative incompatibility has not yet been investigated in any *Trichoderma* species, and based on the current data, should be a topic of future research.

Finally, the abundance of SSCPs in *Trichoderma* may be involved in rhizosphere competence: the genome of the ectomycorrhizal basidiomycete *Laccaria bicolor* also encodes a large set of SSCPs, which accumulate in the hyphae that colonize the host root [42].

Gene expansions in *Tv* and *Ta* that do not occur in *Tr* may comprise genes specific for mycoparasitism.

As a prominent example, proteases have expanded in *Ta* and *Tv*, supporting the hypothesis that the degradation of proteins is a major trait of mycoparasites [43]. Likewise, the increase in chitinolytic enzymes and some ß-glucanase-containing GH families is remarkable and illustrates the importance of destruction of the prey's cell wall in this process. With respect to the chitinases, the expansion of those bearing CBM50 modules was particularly remarkable: proteins containing these modules were recently classified into several different groups by de Jonge and Thomma [44]. Proteins that consist solely of CBM50 modules are type-A LysM proteins, and there is evidence for the role of these as virulence factors in plant pathogenic fungi. The high numbers of LysM proteins that are found in *Trichoderma*, however, indicate other/additional roles for these proteins in fungal biology that are not understood yet. Also, the expansion of the GH75 chitosanases was intriguing: chitosan is a partially deacetylated derivative of chitin and, depending on the fungal species and the growth conditions, in mature fungal cell walls chitin is partially deacetylated. It has also been reported that fungi deacetylate chitin as a defense mechanism [45,46]. Chitosan degradation may therefore be a relevant aspect of mycoparasitism and fungal cell wall degradation that has also not been regarded yet. Overall, the carbohydrate-active enzyme machinery present in *Trichoderma* is compatible with saprophytic behavior but, interestingly, the set of enzymes involved in the degradation of 'softer' plant cell wall components, such as pectin, is reduced. A possible plant symbiotic relationship [3] might rely on a mycoparasitic capacity along with a reduced specificity for pectin, minimizing the plant defense reaction.

Although the genes encoding proteins for the synthesis of typical fungal secondary metabolites (PKS, NRPS, PKS-NRPS) are also abundant, they are not significantly more expanded than in some other fungi. An exception is *Tv* and its 28 NRPS genes. However, our genome analysis revealed also a high number of oxidoreductases, cytochrome P450 oxidases, and other enzymes that could be part of as yet unknown pathways for the synthesis of further secondary metabolites. In support of this, several of these genes were found to be clustered in the genome (data not shown), and were more abundant in the two mycoparasitic species *Ta* and *Tv*. Together with the expanded set of oxidoreductases, monooxygenases, and enzymes for AMP activation of acids, phosphopathetheine attachment, and synthesis of isoquinoline alkaloids in *Ta* and *Tv*, these genes may define new secondary metabolite biosynthetic routes.

Conclusions

Our comparative genome analysis of the three *Trichoderma* species now opens new opportunities for the development of improved and research-driven strategies to select and improve *Trichoderma* species as biocontrol agents. The availability of the genome sequences published in this study, as well as of several pathogenic fungi and their potential host plants (for example, [47]) provides a challenging opportunity to develop a deeper understanding of the underlying processes by which *Trichoderma* interacts with plant pathogens in the presence of living plants within their ecosystem.

Materials and methods

Genome sequencing and assembly

The genomes of *T. virens* and *T. atroviride* each were assembled from shotgun reads using the JGI (USA Department of Energy) assembler Jazz (see Table S15 in Additional file 1 for summary of assembly statistics). Each genome was annotated using the JGI Annotation pipeline, which combines several gene prediction, annotation and analysis tools. Genes were predicted using Fgenesh [48], Fgenesh+ [49], and Genewise programs [50]. ESTs from each species (Chapter 4 of Additional file 2) were clustered and either assembled and converted into putative full-length genes directly mapped to genomic sequence or used to extend predicted gene models into full-length genes by adding 5' and/or 3' untranslated regions to the models. From multiple gene models predicted at each locus, a single representative model was chosen based on homology and EST support and used for further analysis. Gene model characteristics and support are summarized in Tables S16 and S17 in Additional file 1.

All predicted gene models were functionally annotated by homology to annotated genes from a NCBI non-redundant set and classified according to Gene Ontology [51], eukaryotic orthologous groups (KOGs) [52], and Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways [53]. See Tables S18 and S19 in Additional file 1 for a summary of the functional annotation. Automatically predicted genes and functions were further refined by user community-wide manual curation efforts using web-based tools at [54,55]. The latest version gene set containing manually curated genes is called GeneCatalog.

Assembly and annotation data for *Tv* and *Ta* are available through JGI Genome Portals homepage at [54,55]. The genome assemblies, predicted gene models, and annotations were deposited at GenBank under project accessions [GenBank: ABDF00000000 and ABDG00000000], respectively. GenBank public release of the data described in this paper should coincide with the manuscript publication date.

Genome similarity analysis and genomic synteny

Orthologous genes, as originally defined, imply a reflection of the history of species. In recent years, many

studies have examined the concordance between orthologous gene trees and species trees in bacteria. With the purpose of identifying all the orthologous gene pairs for the three *Trichoderma* species, a best bidirectional blast hit approach as described elsewhere [56,57] was performed, using the predicted translated gene models for each of the three species as pairwise comparison sets. The areas of relationship known as syntenic regions or syntenic blocks are anchored with orthologs (calculated as mutual best hits or bi-directional best hits) between the two genomes in question, and are built by controlling for the minimum number of genes, minimum density, and maximum gap (genes not from the same genome area) compared with randomized data as described in [56]. While this technique may cause artificial breaks, it highlights regions that are dynamic and picking up a large number of insertions or duplications.

Orthologous and paralogous gene models were identified by first using BLAST to find all pairwise matches between the resulting proteins from the gene models. The pairwise matches from BLAST were then clustered into groups of paralogs using MCL [58]. In parallel we applied orthoMCL [59] to the same pairwise matches to identify the proteins that were orthologous in all of the three genomes. By subtracting all the proteins that were identified as orthologs from the groups of paralogs and unique genes, we were left with only the protein products of gene models that have expanded since the most recent common ancestor (MRCA) of the three *Trichoderma* genomes. We then calculated the *P*-value under the null hypothesis that the number of non-orthologous genes that are non-syntenic is less than the number of non-orthologous genes that are syntenic.

Identification of transposable elements

We scanned the *Trichoderma* genomes with the *de novo* repeat finding program Piler [19]. Next, we searched for sequences with similarity to known repetitive elements from other eukaryotes with the program RepeatMasker [21] using all eukaryotic repetitive elements in the RepBase (version 13.09) database. After masking repetitive sequences that matched the DNA sequence of known repetitive elements, we scanned the masked genome sequences with RepeatProteinMask (a component of the RepeatMasker application). This search located additional degenerate repetitive sequences with similarity to proteins encoded by TEs in the RepBase database.

CAZome identification and analysis

All protein models for *Ta* and *Tv* were compared against the set of libraries of modules derived from CAZy [60,61]. The identified proteins were subjected to manual analysis for correction of the protein models, for full modular annotation and for functional inference against a library of experimentally characterized enzymes. Comparative analysis was made by the enumeration of all modules identified in the three *Trichoderma* species and 14 other published fungal genomes.

Phylogenetic and evolutionary analyses

One-hundred genes were randomly selected from *Ta*, *Tv*, *Tr* and *C. globosum* based on their property to fulfill two requirements: they were in synteny in all four genomes, and they were true orthologues (no other gene encoding a protein with amino acid similarity >50% present). After alignment, the concatenated 10,449 amino acids were subjected to Bayesian analysis [33] using 1 million generations. The respective cDNA sequences (31,347 nucleotides) were also concatenated, and Ks/Ka ratios determined using DNASp5 [62]. The same file was also used to determine the codon adaptation index [32]. In addition, 80 non-syntenic genes were also selected randomly for this purpose.

The species phylogram of *Trichoderma*/*Hypocrea* was constructed by Bayesian analysis of partial exon nucleotide sequences (824 total characters from which 332 were parsimony-informative) of the *rpb2* gene (encoding RNA polymerase B II) from 110 *ex*-type strains, thereby spanning the biodiversity of the whole genus. The tree was obtained after 5 million MCMC generations sampled for every 100 trees, using burnin = 1200 and applying the general time reversible model of nucleotide substitution. The NCBI ENTREZ accession numbers are: 1 [HQ260620]; 3 [DQ08724]; 4 [HM182969]; 5 [HM182984]; 6 [HM182965]; 7 [AF545565]; 8 [AF545517]; 16 [FJ442769]; 17 [AY391900]; 18 [FJ179608]; 19 [FJ442715]; 20 [FJ442771]; 21 [AY391945]; 22 [EU498358]; 23 [DQ834463]; 24 [FJ442725]; 25 [AF545508]; 26 [AY391919]; 27 [AF545557]; 28 [AF545542]; 29 [FJ442738]; 30 [AF545550]; 31 [AY391909]; 32 [AF545516]; 33 [AF545518]; 34 [AF545512]; 35 [AF545510]; 36 [AF545514]; 37 [AY391921]; 38 [AF545513]; 39 [AY391954]; 40 [AY391944]; 41 [AF545534]; 42 [AY391899]; 43 [AY391907]; 44 [AF545511]; 45 [AY391929]; 46 [AF545540]; 47 [AY391958]; 48 [AY391924]; 49 [AF545515]; 50 [AY391957]; 51 [AF545551]; 52 [AF545522]; 53 [FJ442714]; 54 [AF545509]; 55 [AY391959]; 56 [DQ087239]; 57 [AF545553]; 58 [AF545545]; 59 [DQ835518]; 60 [DQ835521]; 61 [DQ835462]; 62 [DQ835465]; 63 [DQ835522]; 64 [AF545560]; 65 [DQ835517]; 66 [DQ345348]; 67 [AF545520]; 68 [DQ835455]; 69 [AF545562]; 70 [AF545563]; 71 [DQ835453]; 72 [FJ179617]; 73 [DQ859031]; 74 [EU341809]; 75 [FJ179614]; 76 [DQ087238]; 77 [AF545564]; 78 [FJ179601]; 79 [FJ179606]; 80 [FJ179612]; 81 [FJ179616]; 82 [EU264004]; 83 [FJ150783]; 84 [FJ150767]; 85

[FJ150786]; 86 [EU883559]; 87 [FJ150785]; 88 [EU248602]; 89 [EU241505]; 90 [FJ442762]; 91 [FJ442741]; 92 [FJ442783]; 93 [EU341805]; 94 [FJ442723]; 95 [FJ442772]; 96 [EU2415023]; 97 [EU341801]; 98 [EU248600]; 99 [EU341808]; 100 [EU3418033]; 101 [EU2485942]; 102 [AF545519]; 103 [EU248603]; 104 [EU248607]; 105 [EU341806]; 106 [DQ086150]; 107 [DQ834460]; 108 [EU711362]; 109 [EU883557]; 110 [FJ150790].

Additional material

Additional file 1: Comparative properties and gene inventory of *T. reesei, T. virens* and *T. atroviride*. This file contains additional information on genomic properties and selected gene families from the three *Trichoderma* species comprising 19 tables. Table S1 summarizes the satellite sequences identified in the *Trichoderma* genomes and four other fungal genomes. Table S2 summarizes manually curated sequence alignments of transposable element families from the *Trichoderma* genomes. Table S3 lists the total number of CAZy families in *Trichoderma* and other fungi. Table S4 lists the glycoside hydrolase (GH) families in *Trichoderma* and other fungi. Table S5 lists the glycosyltransferase (GT) families in *Trichoderma* and other fungi. Table S6 lists the polysaccharide lyase (PL) families in *Trichoderma* and other fungi. Table S7 lists the carbohydrate esterase (CE) families in *Trichoderma* and other fungi. Table S8 lists the carbohydrate-binding module (CBM) families in *Trichoderma* and other fungi. Table S9 lists the NRPS, PKS and NRPS-PKS proteins in *T. atroviride*. Table S10 lists NRPS, PKS and NRPS-PKS proteins in *T. virens*. Table S11 lists the putative insecticidal toxins in *Trichoderma*. Table S12 lists the cytochrome P450 CYP4/CYP19/CYP26 class E proteins in *Trichoderma*. Table S13 lists the small-cysteine rich secreted protein from *Trichoderma* spp. Table S14 lists the most abundant PFAM domains in those genes that are unique to *T. atroviride* and *T. virens* and not present in *T. reesei*. Table S15 surveys the assembly statistics. Table S16 provides gene model support. Table S17 summarizes gene model statistics. Table S18 provides numbers of genes with functional annotation according to KOG, Gene Ontology, and KEGG classifications. Table S19 lists the largest KOG families responsible for metabolism.

Additional file 2: Additional information on selected gene groups of *Trichoderma*, methods used for genome sequencing, and legends for the figures in Additional file 3. Chapter 1: Carbohydrate-Active enzymes (CAZymes). Chapter 2: Aegerolysins and other toxins. Chapter 3: Small secreted cysteine rich proteins (SSCPs). Chapter 4: EST sequencing and analysis. Chapter 5: Legends to figures.

Additional file 3: Figures that illustrate selected aspects of the main text. Figure S1 provides a phylogeny of *Trichoderma* NPRSs. Figure S2 compares the numbers of epoxide hydrolase genes in *Trichoderma* with that in other fungi. Figure S3 compares the codon usage in genes from syntenic and nonsyntenic regions of the genomes of *Trichoderma reesei, T. atroviride* and *T. virens*.

Abbreviations

CAZy: Carbohydrate-Active enZYmes; CBM: carbohydrate binding module; EST: expressed sequence tag; GH: glycosyl hydrolase; HET: heteroincompatibility; KEGG: Kyoto Encyclopedia of Genes and Genomes; KOG: clusters of eukaryotic orthologous groups; NRPS: non-ribosomal peptide synthase; PKS: polyketide synthase; RIP: repeat-induced point mutation; SSCP: small secreted cysteine-rich protein; *Ta*: *Trichoderma atroviride*; TE: transposable element; Tr: *Trichoderma reesei*; Tv: *Trichoderma virens*.

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Author details

[1]Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering Vienna University of Technology, Getreidemarkt 9, 1060 Vienna, Austria. [2]Laboratorio Nacional de Genómica para la Biodiversidad, Cinvestav Campus Guanajuato, Km. 9.6 Libramiento Norte, Carretera Irapuato-León, 36821 Irapuato, Mexico. [3]Broad Institute of MIT and Harvard, 301 Binney St, Cambridge, MA 02142, USA. [4]Centro Hispanoluso de Investigaciones Agrarias (CIALE), Department of Microbiology and Genetics, University of Salamanca, Calle Del Duero, 12, Villamayor 37185, Spain. [5]División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa San José, No. 2055, Colonia Lomas 4a Sección, San Luis Potosí, SLP., 78216, México. [6]Department of Biology, Technion - Israel Institute of Technology, Neve Shaanan Campus, Technion City, Haifa, 32000, Israel. [7]Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India. [8]Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, Szeged, H-6726, Hungary. [9]DOE Joint Genome Institute, 2800 Mitchell Drive, Walnut Creek, CA 94598, USA. [10]School of Biological Sciences, University of Missouri- Kansas City, 5007 Rockhill Road, Kansas City, MO 64110, USA. [11]Institut de Biologie de l'École normale supérieure (IBENS), Institut National de la Santé et de la Recherche Médicale U1024, Centre National de la Recherche Scientifique UMR8197, 46, rue d'Ulm, Paris 75005, France. [12]Chemistry and Biomolecular Sciences, Macquarie University, Research Park Drive Building F7B, North Ryde, Sydney, NSW 2109, Australia. [13]TU Berlin, Institut für Chemie, FG Biochemie und Molekulare Biologie OE2, Franklinstr. 29, 10587 Berlin, Germany. [14]Department of Plant Pathology and Microbiology Building 0444, Nagle Street, Texas A&M University College Station, TX 77843, USA. [15]Department of Biochemical Engineering, Faculty of Science and Technology, University of Debrecen, Egyetem tér 1, Debrecen, H-4010, Hungary. [16]Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Apartado de Correos 73, Burjassot (Valencia) E-46100, Spain. [17]Architecture et Fonction des Macromolécules Biologiques, UMR6098, CNRS, Université de la Méditerranée, Case 932, 163 Avenue de Luminy, 13288 Marseille 13288, France. [18]Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Lautrupvang 15, DK-2750 Ballerup, Denmark. [19]Biotechnology Department, IFP Energies nouvelles, 1-4 avenue de Bois Préau, Rueil-Malmaison, 92852, France. [20]Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Via Amendola 122/O, 70126 Bari, Italy. [21]Wageningen University, Systems and Synthetic Biology, Fungal Systems Biology Group, Dreijenplein 10, 6703 HB Wageningen, The Netherlands. [22]Chemical and Biological Process Development Group, Pacific Northwest National Laboratory, 902 Battelle Boulevard, Richland, WA 99352, USA.

Authors' contributions

CPK, IVG, BH, EM, SEB, CMK, and AHE contributed equally to this work as senior authors. AA, JC, MM, AS, and IVG performed global annotation and analysis, MZ and HS did the assembly, OC and CH finished the assembly, and EL and SL performed the genome and EST sequencing. SEB, AH-E, CMK and CPK designed the study, and coordinated and supervised the analysis; CPK drafted and submitted the paper. All other authors contributed research (annotations and/or analyses). All authors read and approved the final manuscript.

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References

1. Taylor TN, Hass H, Kerp H, Krings M, Hanlin RT: **Perithecial ascomycetes from the 400 million year old Rhynie chert: an example of ancestral polymorphism.** *Mycologia* 2005, **97**:269-285.
2. Vincent C, Goettel MS, Lazarovits G: *Biological Control: A Global Perspective: Case Studies from Around the World* Wallingford, UK: CAB International; 2007.
3. Harman GE, Howell CR, Viterbo A, Chet I, Lorito M: ***Trichoderma* species-opportunistic, avirulent plant symbionts.** *Nat Rev Microbiol* 2004, **2**:43-56.
4. Howell CR: **Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts.** *Plant Disease* 2003, **87**:4-10.
5. Harman GE: **Overview of mechanisms and uses of *Trichoderma* spp.** *Phytopathology* 2006, **96**:190-194.
6. Shoresh M, Harman GE, Mastouri F: **Induced systemic resistance and plant responses to fungal biocontrol agents.** *Annu Rev Phytopathol* 2010, **48**:21-43.
7. Lorito M, Woo SL, Harman GE, Monte E: **Translational research on *Trichoderma*: from 'omics to the field.** *Annu Rev Phytopathol* 2010, **48**:395-417.
8. Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG, Grigoriev IV, Harris P, Jackson M, Kubicek CP, Han CS, Ho I, Larrondo LF, de Leon AL, Magnuson JK, Merino S, Misra M, Nelson B, Putnam N, Robbertse B, Salamov AA, Schmoll M, Terry A, Thayer N, Westerholm-Parvinen A, *et al*: **Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*).** *Nat Biotechnol* 2008, **26**:553-560.
9. Druzhinina IS, Kopchinskiy A, Kubicek CP: **The first one hundred *Trichoderma* species characterized by molecular data.** *Mycoscience* 2006, **47**:55-64.
10. Chaverri P, Samuels GJ, Stewart EL: ***Hypocrea virens* sp. nov., the teleomorph of *Trichoderma virens*.** *Mycologia* 2001, **93**:1113-1124.
11. Dodd SL, Lieckfeldt E, Samuels GJ: ***Hypocrea atroviridis* sp. nov., the teleomorph of *Trichoderma atroviride*.** *Mycologia* 2003, **95**:27-40.
12. Druzhinina IS, Kubicek CP, Komoń-Zelazowska M, Mulaw TB, Bissett J: **The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages.** *BMC Evol Biol* 2010, **10**:94.
13. Espagne E, Lespinet O, Malagnac F, Da Silva C, Jaillon O, Porcel BM, Couloux A, Aury JM, Ségurens B, Poulain J, Anthouard V, Grossetete S, Khalili H, Coppin E, Déquard-Chablat M, Picard M, Contamine V, Arnaise S, Bourdais A, Berteaux-Lecellier V, Gautheret D, de Vries RP, Battaglia E, Coutinho PM, Danchin EG, Henrissat B, Khoury RE, Sainsard-Chanet A, Boivin A, Pinan-Lucarré B, *et al*: **The genome sequence of the model ascomycete fungus *Podospora anserina*.** *Genome Biol* 2008, **9**:R77.
14. Fischer G, Rocha EP, Brunet F, Vergassola M, Dujon B: **Highly variable rates of genome rearrangements between hemiascomycetous yeast lineages.** *PLoS Genet* 2006, **2**:e32.
15. Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee SI, Baştürkmen M, Spevak CC, Clutterbuck J, Kapitonov V, Jurka J, Scazzocchio C, Farman M, Butler J, Purcell S, Harris S, Braus GH, Draht O, Busch S, D'Enfert C, Bouchier C, Goldman GH, Bell-Pedersen D, Griffiths-Jones S, Doonan JH, Yu J, Vienken K, Pain A, Freitag M, *et al*: **Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*.** *Nature* 2005, **438**:1105-1115.
16. Seoighe C, Federspiel N, Jones T, Hansen N, Bivolarovic V, Surzycki R, Tamse R, Komp C, Huizar L, Davis RW, Scherer S, Tait E, Shaw DJ, Harris D, Murphy L, Oliver K, Taylor K, Rajandream MA, Barrell BG, Wolfe KH: **Prevalence of small inversions in yeast gene order evolution.** *Proc Natl Acad Sci USA* 2000, **97**:14433-14437.
17. Fedorova ND, Khaldi N, Joardar VS, Maiti R, Amedeo P, Anderson MJ, Crabtree J, Silva JC, Badger JH, Albarraq A, Angiuoli S, Bussey H, Bowyer P, Cotty PJ, Dyer PS, Egan A, Galens K, Fraser-Liggett CM, Haas BJ, Inman JM, Kent R, Lemieux S, Malavazi I, Orvis J, Roemer T, Ronning CM, Sundaram JP, Sutton G, Turner G, Venter JC, *et al*: **Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*.** *PLoS Genet* 2008, **4**:e1000046.
18. Nadeau J, Taylor B: **Lengths of chromosomal segments conserved since divergence of man and mouse.** *Proc Natl Acad Sci USA* 1984, **81**:814-818.
19. Edgar RC, Myers EW: **PILER: identification and classification of genomic repeats.** *Bioinformatics* 2005, **21(Suppl 1)**:i152-i158.
20. Benson G: **Tandem repeats finder: a program to analyze DNA sequences.** *Nucleic Acids Res* 1999, **27**:573-580.
21. **RepeatMasker Open-3.0.**. [http://www.repeatmasker.org].
22. Margolin BS, Garrett-Engele PW, Stevens JN, Fritz DY, Garrett-Engele C, Metzenberg RL, Selker EU: **A methylated *Neurospora* 5S rRNA pseudogene contains a transposable element inactivated by repeat-induced point mutation.** *Genetics* 1998, **149**:1787-1797.
23. Selker EU, Tountas NA, Cross SH, Margolin BS, Murphy JG, Bird AP, Freitag M: **The methylated component of the *Neurospora crassa* genome.** *Nature* 2003, **422**:893-897.
24. Enright AJ, van Dongen S, Ouzounis CA: **An efficient algorithm for large-scale detection of protein families.** *Nucleic Acids Res* 2002, **30**:1575-1584.
25. Seidl V: **Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions.** *Fungal Biol Rev* 2008, **22**:36-42.
26. Limon MC, Chacón MR, Mejías R, Delgado-Jarana J, Rincón AM, Codón AC, Benítez T: **Increased antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 by addition of a cellulose binding domain.** *Appl Microbiol Biotechnol* 2004, **64**:675-682.
27. Cuomo CA, Güldener U, Xu JR, Trail F, Turgeon BG, Di Pietro A, Walton JD, Ma LJ, Baker SE, Rep M, Adam G, Antoniw J, Baldwin T, Calvo S, Chang YL, Decaprio D, Gale LR, Gnerre S, Goswami RS, Hammond-Kosack K, Harris LJ, Hilburn K, Kennell JC, Kroken S, Magnuson JK, Mannhaupt G, Mauceli E, Mewes HW, Mitterbauer R, Muehlbauer G, *et al*: **The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization.** *Science* 2007, **317**:1400-1402.
28. Dean RA, Talbot NJ, Ebbole DJ, Farman ML, Mitchell TK, Orbach MJ, Thon M, Kulkarni R, Xu JR, Pan H, Read ND, Lee YH, Carbone I, Brown D, Oh YY, Donofrio N, Jeong JS, Soanes DM, Djonovic S, Kolomiets E, Rehmeyer C, Li W, Harding M, Kim S, Lebrun MH, Bohnert H, Coughlan S, Butler J, Calvo S, Ma LJ, *et al*: **The genome sequence of the rice blast fungus *Magnaporthe grisea*.** *Nature* 2005, **434**:980-986.
29. Münch A, Stingl L, Jung K, Heermann R: ***Photorhabdus luminescens* genes induced upon insect infection.** *BMC Genomics* 2008, **9**:229.
30. Wellinger RJ, Sen H: **The DNA structures at the ends of eukaryotic chromosomes.** *Eur J Cancer* 1997, **33**:735-749.
31. Freitas-Junior LH, Bottius E, Pirrit LA, Deitsch KW, Scheidig C, Guinet F, Nehrbass U, Wellems TE, Scherf A: **Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of *P. falciparum*.** *Nature* 2000, **407**:1018-1022.
32. Sharp PM, Li WH: **The codon adaptation index - a measure of directional synonymous codon usage bias, and its potential applications.** *Nucleic Acids Res* 1987, **15**:1281-1295.
33. Yang Z, Rannala B: **Bayesian phylogenetic inference using DNA sequences: a Markov Chain Monte Carlo Method.** *Mol Biol Evol* 1997, **14**:717-724.
34. Seidl V, Seibel C, Kubicek CP, Schmoll M: **Sexual development in the industrial workhorse *Trichoderma reesei*.** *Proc Natl Acad Sci USA* 2009, **106**:13909-13914.
35. Shiu PK, Metzenberg RL: **Meiotic silencing by unpaired DNA: properties, regulation and suppression.** *Genetics* 2002, **161**:1483-1495.
36. Borkovich KA, Alex LA, Yarden O, Freitag M, Turner GE, Read ND, Seiler S, Bell-Pedersen D, Paietta J, Plesofsky N, Plamann M, Goodrich-Tanrikulu M, Schulte U, Mannhaupt G, Nargang FE, Radford A, Selitrennikoff C, Galagan JE, Dunlap JC, Loros JJ, Catcheside D, Inoue H, Aramayo R, Polymenis M, Selker EU, Sachs MS, Marzluf GA, Paulsen I, Davis R, Ebbole DJ, *et al*: **Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism.** *Microbiol Mol Biol Rev* 2004, **68**:1-108.
37. Rossmann AY, Samuels GJ, Rogerson CT, Lowen R: **Genera of *Bionectriaceae, Hypocreaceae* and *Nectriaceae* (*Hypocreales, Ascomycetes*).** *Stud Mycol* 1999, **42**:1-83.
38. Slot JC, Hibbett DS: **Horizontal transfer of a nitrate assimilation gene cluster and ecological transitions in fungi: a phylogenetic study.** *PLoS One* 2007, **2**:e1097.
39. Bennett V, Baines AJ: **Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues.** *Physiol Rev* 2001, **81**:1353-1392.
40. Stirnimann CU, Petsalaki E, Russell RB, Müller CW: **WD40 proteins propel cellular networks.** *Trends Biochem Sci* 2010, **35**:565-574.
41. Migheli Q, Balmas V, Komoň-Zelazowska M, Scherm B, Fiori S, Kopchinskiy AG, Kubicek CP, Druzhinina IS: **Soils of a Mediterranean hot spot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European, invasive species of *Hypocrea/Trichoderma*.** *Environ Microbiol* 2009, **11**:35-46.

42. Martin F, Aerts A, Ahrén D, Brun A, Danchin EG, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V: **The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis.** *Nature* 2008, **452**:88-92.
43. Seidl V, Song L, Lindquist E, Gruber S, Koptchinskiy A, Zeilinger S, Schmoll M, Martínez P, Sun J, Grigoriev I, Herrera-Estrella A, Baker SE, Kubicek CP: **Transcriptomic response of the mycoparasitic fungus *Trichoderma atroviride* to the presence of a fungal prey.** *BMC Genomics* 2009, **10**:567.
44. de Jonge R, Thomma BP: **Fungal LysM effectors: extinguishers of host immunity?** *Trends Microbiol* 2009, **17**:151-154.
45. Baker LG, Specht CA, Donlin MJ, Lodge JK: **Chitosan, the deacetylated form of chitin, is necessary for cell wall integrity in *Cryptococcus neoformans*.** *Eukaryotic Cell* 2007, **6**:855-862.
46. El Gueddari NE, Rauchhaus U, Moerschbacher BM, Deising HB: **Developmentally regulated conversion of surface-exposed chitin to chitosan in cell walls of plant pathogenic fungi.** *New Phytol* 2002, **156**:103-111.
47. **diArk: a resource for eukaryotic genome research.**. [http://www.diark.org/diark/search].
48. Salamov AA, Solovyev VV: ***Ab initio* gene finding in *Drosophila* genomic DNA.** *Genome Res* 2000, **10**:516-522.
49. Birney E, Durbin R: **Using GeneWise in the *Drosophila* annotation experiment.** *Genome Res* 2000, **10**:547-548.
50. Zdobnov EM, Apweiler R: **InterProScan - an integration platform for the signature-recognition methods in InterPro.** *Bioinformatics* 2001, **17**:847-848.
51. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G: **Gene Ontology: tool for the unification of biology The Gene Ontology Consortium.** *Nat Genet* 2000, **25**:25-29.
52. Koonin EV, Fedorova ND, Jackson JD, Jacobs AR, Krylov DM, Makarova KS, Mazumder R, Mekhedov SL, Nikolskaya AN, Rao BS, Rogozin IB, Smirnov S, Sorokin AV, Sverdlov AV, Vasudevan S, Wolf YI, Yin JJ, Natale DA: **A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes.** *Genome Biol* 2004, **5**:R7.
53. Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M: **The KEGG resource for deciphering the genome.** *Nucleic Acids Res* 2004, **32**:D277-D280.
54. ***Trichoderma virens* Gv29-8 v2.0.**. [http://www.jgi.doe.gov/Tvirens].
55. ***Trichoderma atroviride* v2.0[.**. [http://www.jgi.doe.gov/Tatroviride].
56. Castillo-Ramirez S, Gonzalez V: **Factors affecting the concordance between orthologous gene trees and species tree in bacteria.** *BMC Evol Biol* 2008, **8**:300.
57. Moreno-Hagelsieb C, Janga SC: **Operons and the effect of genome redundancy in deciphering functional relationships using phylogenetic profiles.** *Proteins* 2008, **70**:344-352.
58. **MCL - a cluster algorithm for graphs.**. [http://micans.org/mcl/].
59. Li L, Stoeckert CJ Jr, Roos DS: **OrthoMCL: identification of ortholog groups for eukaryotic genomes.** *Genome Res* 2003, **13**:2178-2189.
60. **CAZY - Carbohydrate-Active EnZYmes database.**. [http://www.cazy.org].
61. Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B: **The carbohydrate-active EnZymes database (CAZy): an expert resource for glycogenomics.** *Nucleic Acids Res* 2009, **37**:D233-D238.
62. Librado P, Rozas J: **DnaSP v5: a software for comprehensive analysis of DNA polymorphism data.** *Bioinformatics* 2009, **25**:1451-1452.
63. **JGI genome portal.**. [http://genome.jgi-psf.org].
64. Latgé JP: **The cell wall: a carbohydrate armour for the fungal cell.** *Mol Microbiol* 2007, **66**:279-290.
65. de Groot P, Brandt BW, Horiuchi H, Ram AF, de Koster CG, Klis FM: **Comprehensive genomic analysis of cell wall genes in *Aspergillus nidulans*.** *Fungal Genet Biol* 2009, **46(Suppl 1)**:S72-S81.
66. Lieckfeldt E, Kullnig CM, Samuels GJ, Kubicek CP: **Sexually competent, sucrose- and nitrate-assimilating strains of *Hypocrea jecorina* (*Trichoderma reesei, Hypocreales*) from South American soils.** *Mycologia* 2000, **92**:374-384.
67. Vargas WA, Mandawe JC, Kenerley CM: **Plant-derived sucrose is a key element in the symbiotic association between *Trichoderma virens* and maize plants.** *Plant Physiol* 2009, **151**:792-797.
68. Martens-Uzunova ES, Schaap PJ: **Assessment of the pectin degrading enzyme network of *Aspergillus niger* by functional genomics.** *Fungal Genet Biol* 2009, **46(Suppl 1)**:S170-S179.
69. Berne S, Lah L, Sepcić K: **Aegerolysins: structure, function, and putative biological role.** *Protein Sci* 2009, **18**:694-706.
70. Goodrich-Blair H, Clarke DJ: **Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination.** *Mol Microbiol* 2007, **64**:260-268.
71. Hares MC, Hinchliffe SJ, Strong PC, Eleftherianos I, Dowling AJ, French-Constant RH, Waterfield N: **The *Yersinia pseudotuberculosis* and *Yersinia pestis* toxin complex is active against cultured mammalian cells.** *Microbiology* 2008, **154**:3503-3517.
72. Karasova D, Havlickova H, Sisak F, Rychlik I: **Deletion of *sodCI* and *spvBC* in *Salmonella enterica* serovar *Enteritidis* reduced its virulence to the natural virulence of serovars Agona, Hadar and Infantis for mice but not for chickens early after infection.** *Vet Microbiol* 2009, **139**:304-309.
73. McNulty C, Thompson J, Barrett B, Lord L, Andersen C, Roberts IS: **The cell surface expression of group 2 capsular polysaccharides in *Escherichia coli*: the role of KpsD, RhsA and a multi-protein complex at the pole of the cell.** *Mol Microbiol* 2006, **59**:907-922.
74. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215**:403-410.
75. **SignalP 3.0 Server.**. [http://www.cbs.dtu.dk/services/SignalP/].
76. Brotman Y, Briff E, Viterbo A, Chet I: **Role of swollenin, an expansin-like protein from *Trichoderma*, in plant root colonization.** *Plant Physiol* 2008, **147**:779-789.
77. Max Planck Institute of Developmental Biology: **Bioinformatics toolkit.** [http://toolkit.tuebingen.mpg.de/blastclust].
78. Talbot NJ: **Growing into the air.** *Curr Biol* 1997, **7**:R78-R81.
79. Wösten HA, van Wetter MA, Lugones LG, van der Mei HC, Busscher HJ, Wessels JG: **How a fungus escapes the water to grow into the air.** *Curr Biol* 1999, **9**:85-88.
80. Kubicek CP, Baker S, Gamauf C, Kenerley CM, Druzhinina IS: **Purifying selection and birth-and-death evolution in the class II hydrophobin gene families of the ascomycete *Trichoderma/Hypocrea*.** *BMC Evol Biol* 2008, **8**:4.
81. Viterbo A, Chet I: **TasHyd1, a new hydrophobin gene from the biocontrol agent *Trichoderma asperellum*, is involved in plant root colonization.** *Mol Plant Pathol* 2006, **7**:249-258.
82. Djonovic S, Pozo MJ, Dangott LJ, Howell CR, Kenerley CM: **Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance.** *Mol Plant Microbe Interact* 2006, **19**:838-853.
83. Djonovic S, Vargas WA, Kolomiets MV, Horndeski M, Wiest A, Kenerley CM: **A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize.** *Plant Physiol* 2007, **145**:875-889.
84. Rep M: **Small proteins of plant-pathogenic fungi secreted during host colonization.** *FEMS Microbiol Lett* 2005, **253**:19-27.
85. Mukherjee PK, Hadar R, Pardovitz-Kedmi E, Trushina N, Horwitz BA: **MRSP1, encoding a novel *Trichoderma* secreted protein, is negatively regulated by MAPK.** *Biochem Biophys Res Commun* 2006, **350**:716-722.
86. Armaleo D, Gross SR: **Structural studies on *Neurospora* RNA polymerases and associated proteins.** *J Biol Chem* 1985, **260**:16174-16180.
87. **Vogels 50x salts.**. [http://www.fgsc.net/methods/vogels.html].
88. Jones JDG, Dunsmuir P, Bedbrook J: **High-level expression of introduced chimaeric genes in regenerated transformed plants.** *EMBO J* 1985, **4**:2411-2418.
89. Berrocal-Tito G, Sametz-Baron L, Eichenberg K, Horwitz BA, Herrera-Estrella A: **Rapid blue light regulation of a *Trichoderma harzianum* photolyase gene.** *J Biol Chem* 1999, **274**:14288-14294.
90. Detter JC, Jett JM, Lucas SM, Dalin E, Arellano AR, Wang M, Nelson JR, Chapman J, Lou Y, Rokhsar D, Hawkins TL, Richardson PM: **Isothermal strand displacement amplification applications for high-throughput genomics.** *Genomics* 2002, **80**:691-698.
91. Huang X, Madan A: **CAP3: A DNA Sequence Assembling Program.** *Genome Res* 1999, **9**:868-877.
92. Papadopoulos JS, Agarwala R: **COBALT: constraint-based alignment tool for multiple protein sequences.** *Bioinformatics* 2007, **23**:1073-1079.

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Two hundred *Trichoderma* species recognized based on molecular phylogeny

Lea Atanasova[a], Irina S. Druzhinina[a,*] and Walter M. Jaklitsch[b]

[a] Research Area Biotechnology and Microbiology, Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstrasse 1a, A-1060 Vienna, Austria.

[b] Faculty Centre of Biodiversity, University of Vienna, Rennweg 14, A-1030 Vienna, Austria.

* Corresponding author: Irina S. Druzhinina

Book chapter in press[1]

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Summary

Similar to other fungi, taxonomy of *Trichoderma* has been revolutionized by the application of DNA-based methods. The importance of *Trichoderma* strains as producers of enzymes and secondary metabolites in biotechnology and as biofungicides and biofertilizers in agriculture forced scientists to provide a stable taxonomical framework in the genus. Owing to efficient integration of molecular phylogenetic methods with modern phenetic and ecophysiological assays the number of species in this genus increased rapidly. In this paper we review the contemporary taxonomy of *Trichoderma*, trace its development from the pre-molecular era to the level of phylogenomics and also briefly address ongoing nomenclature debates. We also present species concepts that are applied to the genus and give insight into the species diversity that approached 200 species and show an up-to-date infrageneric structure based on molecular phylogenetic reconstruction.

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Introduction

Taxonomy of *Trichoderma* (Hypocreaceae, Hypocreales, Ascomycota) is relatively young in contrast to the corresponding teleomorph genus *Hypocrea*. The earlier commonly applied morphological identification of *Trichoderma* species was and still is notoriously difficult due to few relatively invariable morphological characters, leading to overlap among species (Samuels, 2006). Thus, the incorrect application of species names to isolates was very common before DNA markers were developed. Samuels (2006) and Druzhinina *et al.* (2006) provided the methodological framework for molecular identification of *Trichoderma* species by means of DNA barcoding and that resulted in the characterization of the first 100 species by molecular data. Since then diversity studies became more meaningful and extensive sampling world-wide (Chaverri *et al.*, 2003a; Hoyos-Carvajal *et al.*, 2009; Jaklitsch, 2009; 2011; Migheli *et al.*, 2009, Gal-Hamed *et al.,* 2010) introduced a new period of *Trichoderma* taxonomy underlain by evolutionary concepts.

Nowadays the availability of solid taxonomy within the genus and samples from various habitats and substrates from all over the world opens the way to ecological genomics of *Trichoderma* when understanding of several available genomes relies on generalizations of genus-wide traits and on detection of unique features of selected species (Druzhinina and Kubicek, 2013). In recent years the complete genomes of three *Trichoderma* species, *T. atroviride, T. virens* and *T. reesei* (JGI, http://genome.jgi-psf.org) were sequenced by DOE JGI and became publicly available (Martinez *et al.*, 2008; Kubicek *et al.*, 2011). Furthermore, three agamospecies (*T. asperellum*, *T. harzianum* sensu stricto and *T. longibrachiatum*) and the holomorphic *H. schweinitzii/T. citrinoviride* were recently subjected to full genome sequencing and are now being released to the public (http://genome.jgi.doe.gov/sordariomycetes/sordariomycetes.info.html).

Trichoderma in its environment

Members of the genus *Trichoderma* are among the most commonly isolated fungi, frequently found on other fungi, on dead wood and bark, in soil and in the rhizosphere of plants. Their antifungal action driven by mechanisms that comprise both enzymatic and antibiotic activities allow them to switch between biotrophic and saprotrophic lifestyles. The current knowledge suggests that all species are fungicolous (Druzhinina *et al.*, 2011; Jaklitsch 2009; Druzhinina & Kubicek, 2013). *Trichoderma* isolates are also known from marine sponges (Paz *et al.*, 2010; Gal-Hemed *et al.*, 2011), living gramineous and herbaceous plants (see *H. spinulosa* in Jaklitsch, 2009) and as endophytes (Samuels *et al.*, 2006b; Zhang *et al.*, 2007; Hanada *et al.*, 2008; Mulaw *et al.*, 2010), demonstrating their adaptability to various ecological niches. *Trichoderma* spp. are furthermore important to mankind as producers of industrial enzymes, biofertilizers and biofungicides (agents of biological control against plant pathogenic fungi), as contaminants of substrates on mushroom farms (Komon-Zelazowska *et al.*, 2007; Park *et al.*, 2006; Hatvani *et al.*, 2007; Druzhinina *et al.*, 2011), but also as opportunistic pathogens of immunocompromised humans (Kredics *et al.*, 2003; Druzhinina *et al.*, 2008). Due to the opportunistic potential of several cosmopolitan *Trichoderma* species they may be also used as indicators of soil health and disturbance (Migheli *et al.*, 2009).

History of morphology-based taxonomy of *Trichoderma*

The genus *Trichoderma* was established by Persoon (1794) based on four species collected in Germany (see Samuels, 2006). Of these taxa only one species, *T. viride* remained in *Trichoderma*. In 1865, the Tulasne brothers for the first time linked *T. viride* to its sexual stage, *Hypocrea rufa*. Until 1969 Bisby's 'one-species concept' (1939) was in place and only few species were added. Accordingly, Joan Dingley (1957), who collected *Hypocrea* specimens in New Zealand and isolated their ascospores into pure culture, characterized ten species, yet recognized all anamorphs as typical of *T. viride*. Therefore it was also believed that all strains of the cellulase producing *T. reesei* that was known since 40s, also belonged to this species. Rifai (1969) published a revolutionary monograph of *Trichoderma*. He recognised nine aggregate species and excluded *Gliocladium virens* from *Trichoderma*. Rifai's work remained the unchallenged authority for *Trichoderma* for 15 years. Between 1972 and 1989, Yoshimichi Doi published several papers describing the life cycles of many species of *Hypocrea*, mostly from Japan and South America, proposing a subdivision of the genus based on stroma anatomy and on anamorph morphology (Doi, 1972), but unfortunately cultures of many of his species have not been preserved (Gams and Bissett, 1998) and herbarium specimens were not available for a long time.

Thus, not many species were included in the genus *Trichoderma* until 1984, when John Bissett (Bissett, 1984; 1991a–c; 1992) started a revision of *Trichoderma*, taking Rifai's work as the basis. Bissett grouped Rifai's aggregate species into the five sections introducing *Longibrachiatum*, *Pachybasium*, *Trichoderma*, and *Hypocreanum* and maintaining *Saturnisporum* previously proposed by Doi *et al.* (1987). However section *Saturnisporum* was later included in *Longibrachiatum*. The remaining section *Longibrachiatum*

and *Hypocreanum* are still valid (Jaklitsch *et al.,* 2011). Bissett (1991a–c) established additional new species within some of these sections, considering morphological characters newly recognised as suitable for species differentiation, expanding the range of morphological variation expressed by anamorphs and as well as adopting some forms previously included in *Gliocladium* (see Druzhinina *et al.*, 2005, Samuels, 2006, Druzhinina *et al.*, 2006 for reviews). He recognized 20 species in his section *Pachybasium*, and five in section *Longibrachiatum* (Bissett; 1984; 1991a–c; 1992; Gams and Bissett, 1998), while the remaining sections were not resolved at that time.

Based on the results obtained with developing molecular methods, Druzhinina and Kubicek (2005) stated that approximately half of the *Trichoderma* spp. that had been identified by morphological analysis and deposited in culture collections were submitted under wrong species names. The unreliability of a purely phenotypic approach in the identification of *Trichoderma* and discrepancies of names established in *Hypocrea* and *Trichoderma* lies in the difficult differentiation of conidiophores (Jaklitsch, 2009), i.e. by homoplasy and/or insufficient variability of characters, which makes morphological species recognition problematic even to *Trichoderma* taxonomists (Kubicek *et al.*, 2008). Thus, most of the studies on the ecology (Danielson and Davey, 1973), enzyme production (Wey *et al.*, 1994; Kovacs *et al.*, 2004), biocontrol (Kullnig *et al.*, 2000), human infection (Gautheret *et al.*, 1995), and secondary metabolite formation (Cutler *et al.*, 1999; Humphris *et al.*, 2002) by *Trichoderma* were based on morphological characterization of species (particularly before 2000), and are thus difficult to interpret. In attempts to overcome limitations of micro-morphological and phenotypic species differentiation researchers proposed several supplementary characters, such as production of secondary metabolites, which revealed a great diversity in this genus (Okuda *et al.*, 1982; Lieckfeldt *et al.*, 1998), physiological assays including isoenzyme profiles that were claimed as an effective taxonomic tool (Leuchtmann *et al.*, 1996; Samuels *et al.*, 1994; Lieckfeldt *et al.*, 1998).

Development of *Trichoderma* taxonomy based on molecular data

Molecular methods based on the characterization of nucleic acid polymorphism provide an almost unlimited number of potential markers[2] for taxonomic studies and reflect phylogenetic relationships between organisms (Lieckfeldt *et al.*, 1998). In the late nineties DNA sequence analysis was introduced and became widely accepted as a standard technique in fungal systematics (Fujimori and Okuda, 1994; Kuhls *et al.*, 1996, 1997; Meyer, 1991; Meyer *et al.*, 1992; Muthumeenakshi *et al.*, 1994; Zimand *et al.*, 1994; Lieckfeldt *et al.*, 1998a; Lieckfeldt *et al.*, 1998; Kindermann *et al.*, 1998, Dodd *et al.*, 2000). This consequently led to more reliable identification of *Trichoderma* species (Druzhinina and Kubicek, 2005; Druzhinina *et al.*; 2006; Samuels, 2006; Jaklitsch 2009) and a rapid increase of species numbers. The first hundred of molecularly characterized *Trichoderma* species were described by the year 2006 (Druzhinina *et al.,* 2006).
The first important finding that arose from the introduction of molecular tools was the demonstration that *Gliocladium* was paraphyletic with *Trichoderma* but that *G. virens* belongs to the latter one (now *T. virens)* (Rehner and Samuels, 1994). Secondly, *Trichoderma* and *Hypocrea* were recognized to be phylogenetically indistinguishable (Rehner and Samuels, 1995), showing that sexual and asexual forms form one holomorphic species.

Molecular data, particularly sequences of the regions coding for ribosomal RNA and fingerprinting techniques, provided first insights into *Trichoderma* taxonomic units (Fujimori and Okuda, 1994; Kuhls *et al.*, 1996, 1997; Muthumeenakshi *et al.*, 1994; Lieckfeldt *et al.*, 1998). Yet, the relationships among the clades and their members could only be resolved using phylogenetic analysis of sequence data. First studies were carried out using the internal transcribed spacer 1 and later the internal transcribed spacer 2 and 5.8S gene of the rRNA gene cluster were included (ITS) (Kindermann *et al.*, 1998; Lieckfeldt *et al.*, 1998a; Dodd *et al.*, 2000). However, it became evident that ITS does not provide sufficient phylogenetic resolution, but was regarded as suitable for barcoding (Druzhinina *et al.,* 2005). The small number of polymorphic sites or, alternatively, frequent but homoplasious substitutions in the sequences does not provide a decent phylogenetic resolution, but such sequences are indicative of clade affiliation for the given species (Druzhinina and Kubicek, 2005). Hence, the intraspecific variability must be known for its correct application in identification. Considering this, ITS1 and 2 sequence hallmarks were successfully combined in the online identification tool *TrichO*Key for the most frequent species (Druzhinina *et al.*, 2005, see the section DNA barcoding as an identification tool for *Trichoderma*). By and by new markers were introduced to enhance the phylogenetic resolution, including the regions encoding 18S and 28S

[2] *Trichoderma* genomes are 30 - 36 MBb and encode 9 000 - 14 000 genes (Mycocosm DOE JGI)

Figure 1: Bayesian phylogram representing the most up-to-date diversity of the genus *Trichoderma*. The tree was inferred from the alignment of 808 nucleotides of the *rpb2* gene for 196 sequences available in NCBI GenBank. Two independent MCMC runs were performed with 10 million generations and sampling frequency after each 100 generation; the first 800 trees have been removed. Nodes supported with posterior probabilities above 0.94 are marked by black cycles. Vertical bars correspond to infrageneric clades recognized in the genus; clades that are monophyletic in this analysis are filled with solid color. Gray shadow highlights an unresolved area on the phylogram. Red arrows label species with whole genomes sequenced. The following ten species are not represented in the tree, because *rpb2* sequences are lacking: *H. albocornea*, *H. cornea*, *H. hunua*, *H. lacuwombatensis*, *T. matsushimae*, *T. neokoningii*, *H. patella*, *H. stellata*, *H. vinosa* and *T. compactum.*

rRNA, varying parts of translation elongation factor 1-alpha (*tef1*), endochitinase (*echi42=chi18-5*) (Lieckfeldt*et al.*, 2000; Kulling-Gradinger *et al.*, 2002), *rpb2* (Chaverri *et al.*, 2003a), actin (*act*) and calmodulin (*cal1*) (Samuels *et al.*, 2006a, Jaklitsch *et al.*, 2008a). They consist of genomic DNA fragment(s) (loci) of either coding or mixed (intron containing) areas, which are used as markers for phylogenetic reconstructions. It means that they are expected to have no or predictable variation within a given species and sequences are available for most or all species of a genus. Numerous studies showed that *tef1* introns provide the most powerful phylogenetic resolution within the genus (see e.g. Samuels *et al.*, 2006a; Jaklitsch *et al.*, 2006a,b; Jaklitsch, 2009, 2011), yet the most informative and consistent results have been obtained using multilocus phylogenetic analyses. The pioneer work on the phylogeny of all described *Trichoderma* species was published by the group of Christian Kubicek in 2002 (Kulling-Gradinger *et al.* 2002) and extended in several subsequent studies frequently treating sections or groups of species that share some essential morphological traits (Chaverri *et al.,* 2003a; Chaverri and Samuels, 2003, 2011; Lu *et al.,* 2004; Druzhinina *et al.*, 2004, 2007, 2008, 2010, 2012; Samuels *et al.*, 2006a; Jaklitsch *et al.*, 2005, 2006a; 2008; Komon-Zelazowska *et al.*, 2007; Jaklitsch, 2009; Atanasova *et al.*, 2010).

Trichoderma longibrachiatum and *T. pseudokoningii* were two of the nine aggregate species that Rifai (1969) had included in the genus (Samuels *et al.*, 2012a) and were later combined into the section *Longibrachiatum* including three other species (Bissett, 1984). Kuhls *et al.* (1996) discovered that another member of this section, *T. reesei*, is the anamorph of *H. jecorina*. The following revision of the section *Longibrachiatum* was published by Samuels *et al.* (1998), recognizing it as a monophyletic group with ten taxa including several teleomorphs. Furthermore, Samuels *et al.* (1998) merged the section *Saturnisporum* with the section *Longibrachiatum*. After 14 years, the phylogeny of this section was revised by Druzhinina *et al.* (2012) and the taxonomic framework with newly detected species, revealing 21 distinct species in total (see the Figure 1 below) among which eight are new (*T. aethiopicum*, *T. capillare*, *T. flagellatum*, *T. gillesii*, *T. gracile*, *T. pinnatum*, *T. saturnisporopsis* and *T. solani*) was formally described by Samuels *et al.* (2012a).

Most species of *Trichoderma* belong to the section *Trichoderma* (Kulling-Gradinger *et al.*, 2002; Druzhinina and Kubicek, 2005). This section is basically in accordance with Bissett's (1991b) concept, but later several other species, particularly of the section *Pachybasium,* were included: *T. hamatum*, *T. pubescens* and *T. strigosum*, (Kindermann *et al.*, 1998; Dodd *et al.*, 2000, Lieckfeldt *et al.*, 2001; Kullnig-Gradinger *et al.*, 2002). Lieckfeldt *et al.* (1998b) described the teleomorph of *T. koningii*, Lieckfeldt *et al.* (1999) added *T. asperellum*, Dodd *et al.* (2002) described *H. neorufa*, Dodd *et al.* (2003) described the teleomorph of *T. atroviride*, Druzhinina *et al.* (2004) added *H. flaviconidia* and Lu and Samuels (2003) *H. stilbohypoxyli*. Several additional species were added in recent years (Samuels *et al.*, 2006a; Hanada *et al.*, 2008; Samuels and Ismaiel, 2009; Samuels *et al.*, 2010). More recently subclades called the *T. koningii* aggregate species group, and the *Viride* and *Viridescens* Clades were recognized within this section and several new species were described (Samuels *et al.*, 2006a; Jaklitsch *et al.*, 2006a). Section *Trichoderma* is a monophyletic group (Chaverri and Samuels, 2003; Samuels *et al.*, 2006a; Jaklitsch *et al.*, 2006a; Jaklitsch *et al.*, 2012) and has not been yet monographed as a whole. Most recently Jaklitsch (2009) included 27 species in this key phylogenetic clade containing the generic type species *H. rufa* / *T. viride.* Later on Jaklitsch *et al.* (2012) also added *H. caerulescens*, *H. hispanica* and *T. samuelsii* reported 43 species in Section *Trichoderma* in total.

Bissett (1991a) based his section *Pachybasium* on *T. hamatum* (formerly *Pachybasium hamatum*) and enlarged it to contain 20 species (Bissett, 1991b), including species like *T. harzianum*, *T. piluliferum*, *T. polysporum* and the anamorph of *H. gelatinosa*. However, it was shown that this section is paraphyletic (Kindermann *et al.*, 1998; Kulling-Gradinger *et al.*, 2002) and subsequently it was subdivided into five clades. *Trichoderma hamatum* and some other species were found to belong to the section *Trichoderma* and all species sharing this morphology and having green ascospores were grouped among several

JN175554 T. cf. parareesei G.J.S. 04-93
HM182965 T. parareesei CBS 125862
HM182981 T. cf. parareesei C.P.K. 524
T. reesei QM6a
JN175547 T. gracile G.J.S. 10-263
T. aethiopicum C.P.K. 1837
JN175513 T. longibrachiatum G.J.S. 07-21
JQ685884 H. orientalis S187
JN175516 T. pinnatum G.J.S. 02-120
JF421253 T. sp. Y.O. 4707
JN258688 T. flagellatum C.P.K. 3334
JN175536 T. pseudokoningii G.J.S. 99-149
JQ513368 T. effusum C.P.K. 254
JN175545 T. citrinoviride G.J.S. TR102
JN175524 T. saturnisporum ATCC 28023
JN175533 T. sp. G.J.S. 09-62
JN175532 T. sp. G.J.S. 01-355
JN175531 H. andinensis G.J.S. 90-140
JN175563 T. ghanense G.J.S. 04-323
JN182313 T. sp. C.P.K. 667
JN182312 T. capillare C.P.K. 2883
JN175546 T. solani G.J.S. 08-81
JN175527 T. gillesii G.J.S. 00-72
JN175528 T. sinense DAOM 230004
JQ513367 T. konilangbra C.P.K. 132
JN175525 T. sp. G.J.S. 99-17
JQ685885 T. saturnisporopsis S19
JN133563 H. novae-zelandiae G.J.S. 81-265
JQ685880 H. britdaniae WU31610
FJ860585 H. sambuci WU29467
FJ860603 T. tremelloides CBS 121140

Longibrachiatum

0.1

FJ860562 T. parepimyces CBS 122769
FJ442770 T. aggressivum G.J.S. 99-29
AY391956 T. tawa G.J.S. 97-174
EU498355 T. alni C.P.K. 3145
HM142372 T. pleuroti CBS 124387
HM142371 T. pleuroticola CBS 124383
HM142366 T. amazonicum IB49
EU498360 T. epimyces CBS 120534
FJ442724 T. cf. harzianum G.J.S. 04-67
FJ442735 H. nigricans G.J.S. 90-254
T. 'afroharzianum' nom. prov. C.P.K. 845
H. lixii G.J.S.96-97
FJ442725 T. inhamatum CBS 273.78
AF545549 T. harzianum CBS 226.95
EU498358 T. brunneoviride CBS 120928
AY391945 T. stramineum G.J.S. 02-84
JN133569 T. velutinum DAOM 230013
AY391918 T. cinnamomeum G.J.S. 97-230
FJ860533 T. dacrymycellum WU 29044
AF545508 H. atrogelatinosa G.J.S. 95-159
AY391902 T. ceraceum G.J.S. 89-136
FJ860532 T. cerinum CBS 120637
AF545557 T. tomentosum DAOM 178713a
AY391900 T. catoptron G.J.S. 02-76
AF545542 H. crassa DAOM 164916
EU341804 T. virens G.J.S. 01-287
AF545511 T. cremeum G.J.S. 91-125
FJ179619 T. sinuosum C.P.K. 1595
AF545540 T. surrotundum G.J.S. 88-73
FJ860601 T. thelephoricola CBS 12118
AY391907 T. chlorosporum PC4
AY391921 H. costaricensis PC21
FJ860534 H. danica CBS 121273
FJ860591 H. spinulosa CBS 311.50
FJ860516 T. aerugineum CBS 120541
AY391899 T. pseudocandidum PC59
FJ179602 T. aureoviride CBS 120536
AY391957 T. thailandicum G.J.S. 97-61
AY391959 H. virescentiflava PC2
FJ860594 T. strictipile C.P.K. 1601
JF461455 T. sp. Y.O. 2407
FJ860542 T. longipile CBS 120953
AF545512 T. cuneisporum G.J.S. 91-93
AF545513 T. phyllostachydis G.J.S. 92-123
AY391954 H. sulawesensis G.J.S. 85-228
FJ860531 T. ceramicum CBS 114576
FJ860566 T. parestonicum C.P.K. 3167
FJ860537 T. estonicum C.P.K. 3149
AY391913 T. chromospermum G.J.S. 94-68
AF545516 T. gelatinosum G.J.S. 88-17
AF545518 T. pseudonigrovirens G.J.S. 99-64
HQ342245 T. stromaticum G.J.S. 97-183
HQ342284 T. lanuginosum G.J.S. 01-176
HQ342282 T. vermipilum PPRI 3559
HQ342279 T. caesareum G.J.S. 01-225
HQ342277 T. medusae G.J.S. 01-171
HQ342281 T. floccosum G.J.S. 01-238
HQ342280 T. ivoriense G.J.S. 01-321
HQ342288 T. rossicum DAOM 230011
HQ342286 T. barbatum G.J.S. 04-308
FJ860587 T. silvae-virgineae CBS 120922
DQ087239 T. helicum DAOM 230021
AF545553 T. spirale DAOM 183974
AF545551 T. oblongisporum DAOM 167085
FJ860548 T. moravicum C.P.K. 2419
AF545546 T. fertile DAOM 167161
JQ621966 T. mienum TUFC 61530
AF545522 H. semiorbis DAOM167636
FJ860539 T. fomiticola C.P.K. 3137

Harzianum

Virens

Chlorospora

Spinulosa

Strictipilosa

Ceramica

Gelatinosa

Stromaticum

Semiorbis

DQ835456 H. americana G.J.S. 94-79
JN133565 H. pulvinata CBS 124357
FJ860574 H. protopulvinata C.P.K. 2434
AF545561 H. citrina CBS 894.85
DQ835462 H. microcitrina G.J.S. 97-248
DQ835454 H. pseudostraminea G.J.S. 90-74
DQ835521 H. decipiens G.J.S. 89-139
FJ860569 H. phellinicola CBS 119283
EU338336 H. victoriensis G.J.S. 99-130
FJ860599 H. sulphurea C.P.K. 1593
FJ860525 H. austriaca CBS 122494
DQ835522 H. subsulphurea M-141
DQ835518 H. eucorticioides G.J.S. 99-61
FJ860578 H. rhododendri CBS 119288
FJ860576 T. psychrophilum C.P.K. 2435
FJ860528 T. calamagrostidis CBS 121133
DQ345347 T. crystalligenum CBS 118980
AF545563 H. megalocitrina BEO 0009
DQ859030 T. taxi ZJUF0869
EU338318 T. brevicompactum CBS 112447
EU338320 T. turrialbense BBA 72294
EU338308 T. arundinaceum G.J.S. 05-184
EU338322 T. protrudens DIS119f
FJ860529 T. margaretense C.P.K. 3127
EU338324 H. rodmanii G.J.S. 91-88
FJ860522 T. auranteffusum C.P.K. 3158
DQ834462
H. alcalifuscescens TFC 181548
DQ834461 H. cinereoflava G.J.S. 92-102
HQ260610 H. peltata G.J.S. 08-207
FJ860567 H. parmastoi CBS 121139
AF545517 T. deliquescens G.J.S. 89-129
AY391926 T. melanomagnum G.J.S. 99-153
FJ860544 T. luteocrystallinum CBS 123828
AF545562 H. avellanea CTR 77-155
FJ860535 T. delicatulum CBS 120631
FJ860596 T. subalpinum C.P.K. 3126
FJ860517 T. albolutescens CBS 119286
FJ179612 H. nybergiana CBS 122496
FJ179618 T. seppoi CBS 122497
FJ179606 T. leucopus CBS 122495
FJ860543 T. luteffusum CBS 120537
FJ860546 T. atlanticum CBS 120632
EU341809 T. minutisporum DAOM 167069
FJ1796002 T. alutaceum CBS 120535
JQ685879 T. pachypallidum CBS 122126
JQ685878 T. polysporum C.P.K. 3131
FJ179614 H. parapilulifera CBS 120921
AF545519 T. piluliferum CBS 814.68
JQ685876 H. folicola CBS 130008
FJ860527 T. bavaricum CBS 120538
FJ860572 T. placentula CBS 121134
FJ179622 T. voglmayrii CBS 117711
FJ860554 T. neorufoides C.P.K. 1904
HQ260621 H. neorufa G.J.S. 96-132
JN715610 H. pezizoides C.P.K. 775
AB646529 T. aeroaquaticum NBRC 108031
FJ007374 T. theobromicola DIS85f
EU883560 T. paucisporum G.J.S. 03-69
EU883557 H. flaviconidia G.J.S. 99-49
EU883558 T. evansii DIS341hi
EU248613 T. pubescens DAOM 166162
EU883556 T. hamatum G.J.S. 98-170
EU248615 T. asperellum G.J.S. 04-105
GU198274 T. yunnanense CBS 121219
JN133560 T. asperelloides G.J.S. 04-187
EU883562 T. lieckfeldtiae G.J.S. 00-14
EU241505 T. intricatum G.J.S. 02-78
FJ860556 H. ochroleuca CBS 119502
FJ442781 T. ovalisporum G.J.S. 04-113
JN715608 T. taiwanense C.P.K. 416
FJ442795 T. koningiopsis G.J.S. 97-273
EU341803 T. dingleyae G.J.S. 99-105
FJ8605412 T. koningii CBS 119500
FJ442783 T. petersenii G.J.S. 04-164
FJ442723 T. caribbaeum G.J.S. 98-43
EU248602 T. dorotheae G.J.S. 99-202
JN715605 H. caerulescens CBS 130012
EU341805 T. stilbohypoxyli G.J.S. 96-32
JN715600 H. hispanica CBS 130540
JN715599 T. samuelsii CBS 130537
FJ860540 T. junci CBS 120926
FJ442703 T. viridescens G.J.S. 94-9
FJ860605 T. valdunense CBS 120923
EU252007 T. scalesiae G.J.S. 03-74
FJ442754 T. atroviride DAOM 222144
EU248597 T. martiale G.J.S. 04-40
JN133561 T. gamsii G.J.S. 04-09
EU711362 T. viride CBS 119325
EU248606 T. strigosum DIS173k
EU248603 T. erinaceum DAOM 230019
JN133566 T. rogersonii G.J.S. 04-157
FJ442772 T. austrokoningii CBS 247.63
FJ860597 T. subeffusum CBS 120929
EU703949 Protocrea pallida TFC 99-209
EU703942 Protocrea farinosa CBS 121554

0.1

1.45

Hypocreanum

Psychrophila

Brevicompactum

Lutea

pachybasium
Core Group

sect. Trichoderma

Appendix I-II

unrelated clades such as e.g. *Ceramica*, *Chlorospora*, *Harzianum*, *Semiorbis*, *Strictipilosa* or *Stromaticum* (Chaverri and Samuels, 2003; Jaklitsch, 2009; Jaklitsch, 2011). The removal of *T. hamatum* determined that Bissett´s sectional name could not be used any more. Lu *et al.* (2004) refined the clade containing the remaining species around *T. polysporum*/ *H. pachybasioides* (Figure 1) and described the teleomorph of *T. minutisporum* and additional new species. This clade was then named *Pachybasioides* Clade, but changed to the *Pachybasium* core group by Jaklitsch (2011), who significantly enlarged it and also included the former genera *Podostroma* and *Podocrea* in this clade (Chamberlain *et al.*, 2004; Jaklitsch *et al.*, 2008c). One species of this section, *H. minutispora*, is the most common species of *Trichoderma* forming a teleomorph in Europe (Jaklitsch, 2011). Most recently Jaklitsch and Voglmayr (2012) added *H. foliicola* to this clade.

Bissett (1991a) erected the section *Hypocreanum* for *Trichoderma lacteum* Bissett (as *Trichoderma lactea*), the newly formally established anamorph of *Hypocrea citrina* (syn. *H. lactea*). Species of this section were later reviewed by Overton *et al.* (2006a,b). They reported that species of *Hypocrea* with anamorphs assignable to *Trichoderma* sect. *Hypocreanum* did not form a monophyletic group. *Hypocrea megalocitrina*, *H. parmastoi*, and *H. alcalifuscescens* do not belong to the major *Hypocreanum* Clade. However, *Hypocrea* spp. with well-defined pseudoparenchymatous stroma tissue, and acremonium- or verticillium-like conidiophores (hypocreanum-like) that produce hyaline conidia variable in size and shape, can be accommodated in a large monophyletic *Hypocreanum* Clade. On the other hand, the simple or reduced hypocreanum-like anamorph occurs in many other clades of *Trichoderma*. Recent phylogenetic analyses of multiple loci revealed 13 species, among others *H. austriaca*, *H. citrina*, *H. decipiens*, *H. phellinicola*, *H. pulvinata*, and *H. sulphurea* that were described in detail (Jaklitsch *et al.*, 2011) and correspond to what Overton *et al.* (2006a,b) proposed as the major *Hypocreanum* Clade (Figure 1).
In addition to Bissett´s sections many additional clades have been established: The largest one containing species with green ascospores is currently named *Harzianum* Clade (Jaklitsch, 2009; Druzhinina *et al.*, 2010a) (Figure 1). The core of this clade is represented by the cosmopolitan *H. lixii* – *T. harzianum* sensu lato species complex (Figure 1) showing a high degree of phylogenetic variation. Species of this complex are most commonly found in soil studies (Kullnig *et al.*, 2000; Kubicek *et al.*, 2003; Wuczkowski *et al.*, 2003; Gherbawy *et al.*, 2004; Migheli *et al.,* 2009; Mulaw *et al.,* 2010; Druzhinina *et al.*, 2005, 2010a) but also above ground (Błaszczyk *et al.*, 2011). Their identity, phylogenetic position and substructure was studied by Chaverri and Samuels (2002, 2003) and Chaverri *et al.* (2003b) and further evaluated by Druzhinina *et al.* (2010a). These studies appreciated a complex speciation process within *H. lixii* - *T. harzianum* species group, which is based on the coexistence and interaction of organisms with different evolutionary histories and on the absence of strict genetic borders between them. Druzhinina *et al.* (2010a) revealed reproductively isolated biological species, evolutionary recent agamospecies and numerous relict lineages with unresolved phylogenetic positions. They confirmed the existence of two genetically isolated anamorphic species, *T. harzianum* sensu stricto and a not yet formally described *T.* sp. 'afroharzianum' (Druzhinina *et al.*, 2010a) that are different from *H. lixii*. A network of recombining strains lacking straight-forward identification is called 'pseudoharzianum matrix' (Druzhinina *et al.*, 2010a) pointing to the complex structure within the group. Since *H. lixii* and *T. harzianum* s. stricto are evidently not the same species, the anamorph-teleomorph relationship *H. lixii*/*T. harzianum* was rejected (Druzhinina *et al.*, 2010a). Moreover, besides *H. lixii* and the above mentioned species, the *Harzianum* Clade currently contains 18 other species (Figure 1), of which some (T. *aggressivum* (Samuels *et al.*, 2002), *T. pleuroti* (established with the grammatically incorrect name *T. pleurotum,* see details below) and *T. pleuroticola* (Park *et al.* , 2006; Komon-Zelazowska *et al.*, 2007)) are known to cause green mold disease in mushroom farms. Jaklitsch *et al.* (2008a) reported that some of the species in this clade such as *H. alni*, which co-occurs with basidiomata of *Macrotyphula contorta*, may be mycoparasites. Also, *T. harzianum* sensu lato is frequently found on other fungi such as e.g. *Phellinus* spp. (Jaklitsch 2009). The current phylogenetic resolution within the *Harzianum* Clade, when compared to other infrageneric groups, illustrates the lack of criteria for species recognition that may be applied for this group (see below).

Furthermore eight smaller clades have been recognized (Figure 1): *Chlorospora* Clade (*H. chlorospora*, *H. costaricensis*, *H. cremea*, *H. sinuosa*, *H. surrotunda*, and *H. thelephoricola*), *Spinulosa* Clade (*H. aeruginea*, *H. danica* and *H. spinulosa*), *Strictipilosa* Clade (*H. cuneispora, H. longipilosa*, and *H. strictipilosa*), *Ceramica* Clade (*H. ceramica*, *H. estonica* and *H. parestonica*), *Semiorbis* Clade (*T. fertile, H. fomiticola*, *H. moravica, T. oblongisporum* and *H. semiorbis*), *Brevicompactum* Clade (*T. arundinaceum*, *H. auranteffusa*, *T. brevicompactum*, *H. margaretensis*, *T. protrudens*, *H. rodmanii* and *T. turrialbense*), *Psychrophila* Clade (*H. calamagrostidis*, *H. crystalligena*, *H. megalocitrina*, *H. psychrophila* and *H. rhododendri*) and *Lutea* Clade (*H. lutea*, *H. luteocrystallina* and *H. melanomagna*) (Chaverri and Samuels, 2003; Degenkolb *et al.*, 2008; Jaklitsch *et al.*, 2009, 2011). Recently a clade containing *T. stromaticum* was considerably enlarged by Samuels *et al.* (2012b).

Species concepts: what is a species in the genus *Trichoderma*?

In mycology the answers to the question "What is a species?" commonly vary, depending on the group of organisms, even within the same fungal family. Concepts are often subjective to a certain degree and are in a constant state of change. The earliest species concepts in fungi relied purely on the morphology of gathered specimens, later such phenotype data were augmented with cultural traits, host specificity, morphology of anamorphs formed *in vitro*, ultra-structure, chemical compounds isolated from the fungus or its physiological traits, and eventually DNA sequences were implemented in taxonomy. Here we give a brief survey of what this means for *Trichoderma*.

Morphological species concept

Phenotype data of *Hypocrea* teleomorphs include size, colour and shape of stromata preferably in the fresh state, colour and other changes caused by drying and by treatment by 3% KOH, perithecium shape and size, anatomy of stromatic tissues, size of asci, colour and size of distal and proximal ascospore cells. Also the colour of the perithecial wall (yellow in *H. neorufa* and *H. neorufoides*, hyaline in other species of the sect. *Trichoderma*), colour change of the perithecial wall by 3% KOH may be useful, e.g. in *H. pachypallida* (peridium orange-red) or to distinguish *H. sinuosa* (peridium hyaline) from *H. thelephoricola* (peridium orange), particularly when the *Steccherinum* host of the latter is not evident (Jaklitsch, 2009, 2011). In addition, the green colour of ostiolar apices in lactic acid is diagnostic for teleomorphs in the section *Longibrachiatum*.
Phenotype data of *Trichoderma* anamorphs include the organisation of conidiation structures (effuse, shrubs or pustules), conidiophore morphology, shape, colour, ornamentation (verrucae, tubercles), size and l/w ratio of conidia, size and shape of phialides, presence of chlamydospores on CMD or SNA. Phenotype data of cultures include growth rates, the optimum temperature of growth on different media, colony appearance, formation of pigments or crystals, odour, zonation of mycelium, aerial hyphae or conidiation, among some other details.

Unculturable herbarium specimens of *Trichoderma* do not exhibit significantly exploitable morphological features for species differentiation and recognition, although they offer much more than e.g. genera like *Colletotrichum*, many Chaetothyriales and Capnodiales, and various endophytes. Also, several of the earliest names are not represented by specimens, are invalid for other reasons or were identified to belong to other genera. Therefore, although 50 epithets were established before Rifai (1969), only *T. viride* and *T. atroviride* P. Karst. (Bissett, 1991c, 1992) remained as valid species in the current concept of *Trichoderma*. Accordingly, the taxonomy of the genus relied on the species epithets established in its teleomorph counterpart *Hypocrea*, where already 286 species and varietal names existed before Dingley (1957).

Hypocrea is a morphologically well-defined genus with 2-celled, disarticulating ascospores in cylindrical asci and perithecia immersed in soft (when fresh) and light-coloured stromata that vary in size, shape and colour. This is only somewhat blurred by species, where the stroma is reduced to a subiculum (e.g. *H. delicatula*) resembling the phylogenetically different genera *Arachnocrea* and *Protocrea* (Jaklitsch *et al.*, 2008b). Some genera that were segregated due to green ascospore colour (*Chromocrea*, *Creopus*), large upright stromata (*Podocrea*, *Podostroma*), or cleistothecial ascomata (*Aphysiostroma*) have been re-united with *Hypocrea* on phylogenetic grounds. Other similar and possibly synonymous genera like *Dialhypocrea* or *Pseudohypocrea* have not been re-assessed recently.

The large number of species established in *Hypocrea* and the morphological conservation, i.e. lack of distinct morphological variations in e.g. ascospores, limits morphological species differentiation and recognition seriously. Some species can easily be identified on its *Hypocrea* teleomorph alone, even in the field, particularly when they are specifically associated with other fungi. As an example, Jaklitsch (2011) showed that it is easily possible to differentiate *H. protopulvinata* from *H. pulvinata* on their common host *Fomitopsis pinicola*. However, in several groups, e.g. sect. *Trichoderma*, it is virtually impossible to identify species using teleomorph morphology with any justifiable confidence, even within a small geographic area.

Doi (1972 and later on) extensively studied the genus and proposed a subdivision based on morphological characters. However, as we know now, organization and morphology of stromatic tissues are plesiomorphic, thus his groupings turned out not to reflect evolutionary relationships.

An important improvement was the study of *Trichoderma* anamorphs formed in ascospore cultures of *Hypocrea*. Doi (1972) already performed meaningful culture studies and later on these were dramatically

expanded by Samuels and collaborators, who, in addition to Bissett (1991b), made the most important contribution to phenotype studies of *Trichoderma*. He introduced essentially useful phenotype parameters like culture description and growth rates to *Trichoderma* taxonomy and used them consistently in many studies (see e.g. Chaverri and Samuels 2003, Samuels *et al.*, 2006a, 2012a; and other references in this paper).

Trichoderma is like anamorph genera in general a so-called form genus, i.e. species of the genus share certain morphological key features. However, as currently conceived in a phylogenetic sense, *Trichoderma* is morphologically highly heterogeneous. Anamorphs in sect. *Hypocreanum* are untypical of *Trichoderma* due to their acremonium- to verticillium-like conidiophores. Such conidiophores are sometimes also found in other clades as "effuse conidiation" alone (e.g. *T. bavaricum* in the *Pachybasium* core group) or in combination with typical *Trichoderma* pustules (Bissett 1991a; Overton *et al.*, 2006a,b; Jaklitsch, 2011). Even more distinct are the anamorphs of *H. lutea* (*T. deliquescens* with strict gliocladium-like conidiophores and before Jaklitsch (2011) classified in the genus *Gliocladium*) and *H. subalpina* (*T. subalpinum* with polypaecilum-like conidiophores and phialides) (Jaklitsch 2011), and the *Stilbella* anamorph of *H. cinereoflava*, which has not yet been combined in *Trichoderma* (Seifert & Samuels 1997). If these forms would have been found alone in nature (in *T. deliquescens* = *Gliocladium viride* this was indeed the case), they would have never been identified as belonging to *Trichoderma*. On the other hand, such forms can easily be differentiated and recognized.

Trichoderma is now a large genus. The high number of species makes it impossible to use phenotype alone to draw conclusions about clear species differentiation, because among closely related species taxonomically exploitable differences in conidiophore morphology, phialides, conidia and growth rates are subtle, particularly in species-rich clades such as sect. *Trichoderma* or the *Harzianum* Clade. Therefore no definition to the morphological species of *Trichoderma* can be formulated. Nevertheless, phenotype data are crucially important, when combined with results of molecular phylogenetics for sound definition and recognition of species. In some cases differences in morphology are used to set up species borders among molecularly recognized groups. For example, without profound phenotype dissimilarities the two species causing the green mold disease of *Pleurotus* - *T. pleuroti* and *T. pleuroticola* would initially be interpreted as a single taxon (Komon-Zelazowska *et al.,* 2007).

Physiological data - Phenotype MicroArrays

Species of the genus *Trichoderma* have the ability to assimilate a great diversity of nutrients, which enables them to colonize versatile ecological niches (Atanasova and Druzhinina, 2010). Testing a large number of fungi on numerous growth media has been attractive to many researchers since a long time. The advanced BIOLOG Phenotype MicroArray technique that provides global analysis of cellular phenotypes using microplates was first published in 1989 by Bochner (1989). He established phenotype characterization based on cell respiration using tetrazolium dye, which is reduced by succinate dehydrogenase, an enzyme active in the citric acid cycle. Reduction of this dye forms a purple colored formazan, which is used to quantify respiration by spectrophotometric measurement of optical density at 490 nm.

The Phenotypic MicroArray technique was applied to *Trichoderma* in numerous studies, which revealed intra- and interspecific variability in *Trichoderma* carbon source utilization (see Atanasova and Druzhinina, 2010 for the review). The development of Biolog FF plates (Biolog, Hayward, CA) designed for filamentous fungi diminished the limitations by inconsistently reduced tetrazolium violet present in GN and GP plates designed for bacteria (Dobranic and Zak, 1999). Kubicek *et al.* (2003) first performed a comparative study of carbon source utilization profiles in Biolog FF plates for *Trichoderma* using 96 South-Asian isolates. The study revealed both species-specific and variable metabolic properties of *Trichoderma* species, showing that this technique cannot be solely used for species identification. However, the method was shown to be useful as a powerful tool that complements molecular phylogenetics. Thus several studies (Kraus *et al.*, 2004; Nagy *et al.*, 2007; Komon-Zelazowska *et al.*, 2007; Druzhinina *et al.*, 2010a; Atanasova *et al.*, 2010; Hoyos-Carvajal *et al.*, 2009) furthermore proved the applicability of this method as supportive in species identification.

A particular advantage of the Phenotype MicroArray techniques becomes evident, when the evolutionary context of two or many genetically closely related species should be resolved. For example, *T. pleuroticola* and *T. pleuroti* form two supported phylogenetic clades that share the putatively closest ancestor. The reliable differentiation of the two species was aided by Phenotype MicroArrays that detected the difference between *T. pleuroticola* and *T. pleuroti* and also separated them from another species causing mushroom green mold disease (*T. aggressivum* on *Agaricus bisporus*) and the large polymorphic neighbor taxon *T. harzianum* sensu lato (Komon-Zelazowska *et al.*, 2007). This application was further developed by Druzhinina *et al.* (2010b), when evolution and ecophysiology of *H. jecorina*/*T. reesei* and related taxa was studied. These authors used Phenotype MicroArrays to show that *T. parareesei*, a clonal sister species of *H. jecorina*/*T. reesei,* is strongly photostimulated, while *H. jecorina*/*T. reesei* is neutral in this respect.

Moreover, carbon utilization profile may be a useful technique for the identification of certain individual taxa (strains and/or species). For example, Nagy *et al.* (2007) used Phenotype MicroArrays to develop a rapid test system that is suitable to screen for chitinase overproducing strains of *T. harzianum* s. stricto.

Hence, Phenotypic MicroArrays can render important in understanding the phenotypic variation at the level of evolving species (Kubicek *et al.*, 2003; Atanasova and Druzhinina, 2010). Furthermore, the intraspecific variability in the spectrum of assimilated carbon sources may well reflect the habitat speciation due to the loss of specific biochemical functions in the process of adaptation to narrow ecological niches (Druzhinina *et al.*, 2010b; Atanasova *et al.*, 2010). On the other hand, Druzhinina *et al.* (2008) studied strains of *T. longibrachiatum* isolated from the lungs of immunocompromised patients and non-clinical strains, did not reveal any specialized subpopulation variability within this species. The reason for this is possibly the ancient pathogenic ability that arose in a common ancestor with *H. orientalis*, of which also clinical strains are known (Druzhinina *et al.*, 2008), joined with the fact that *T. lonigbrachiatum* is a clonal species, i.e. lacking sexual recombination. However, the physiological data might not always reflect the phylogenetic relationships of the taxa, but are recognized to be a valuable tool that contributes to the characterization of species, individual strains, and ecological groups.

Finally, several recent formal species descriptions such as of *T. brevicompactum* (Kraus *et al.*, 2004) and *T. parareesei* (Atanasova *et al.*, 2010), have integrated Phenotypic MicroArrays as a complementary method to make it one of the standard techniques for species recognition (Atanasova and Druzhinina, 2010; Atanasova *et al.*, 2010).

Other physiological methods that have been used recently to aid in the taxonomy of *Trichoderma* are peptaibiomics analysis of the *T. brevicompactum* clade (Degenkolb *et al.*, 2008) and more general polypeptide analyses by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (De Respinis *et al.*, 2010).

Molecular Concepts

Although almost all recent studies are based on concatenated multiloci sequence data (see above but also below) even the 'molecular' species definitions are not strictly homogeneous across the genus. For example, Chaverri *et al.* (2003) studied the intraspecific structure of one of the most common species, *T. harzianum* sensu lato. Although several intraspecific clades have been confirmed by tree topologies of unlinked loci, the authors considered the variability to be insufficient to recognize sibling species. This vision of *T. harzianum* sensu lato was generally confirmed by Druzhinina *et al.* (2010a) who used *in silico* methods to trace sexual recombination in the larger dataset of the same species but revealed only a few genetically isolated putative agamospecies within *T. harzianum* sensu lato. In that study a clade of *T. harzianum* sensu stricto was recognized as a separate taxon distinct from the rest of the strains including the teleomorph *H. lixii* that was formerly attributed to it. A different resolution scheme was applied by Samuels *et al.* (2006a), when the *Koningii* Clade was studied with virtually the same methodology and resulted in the formal description of 12 taxa sharing the same morphology. In that work, genetic polymorphism was weighed differently along the tree. For example, the three terminal subclades on the Large Koningii Branch were determined to be three species (*T. petersenii*, *T. dingleyae* and *T. caribbaeum*), while six highly polymorphic strains on the Small Koningii Branch were combined to *T. austrokoningii* despite genetic distances within this taxon that are larger than those separating some species recognized in the same study (Samuels *et al.*, 2006a). It is obvious that taxonomists consider unequal sets of parameters to distinguish species, being influenced by the number of available specimens (distribution of the species), economic value of strains and biogeography.

Another common practice is to name species based on single specimens and isolates, i.e. with unknown intraspecific polymorphism, ecology and distribution but often clear morphological and/or phylogenetic separation and biogeographic or ecologic specialty. Examples are *T. taiwanense* (Samuels *et al.*, 2006a), *T. neokoningii* or *T. scalesiae* (Jaklitsch *et al.*, 2006a), *H. aeruginea* or *H. danica* (Jaklitsch, 2009), *H. calamagrostidis*, *H. junci*, *H. valdunensis* (Jaklitsch, 2011) or *T. caesareum*, *T. floccosum*, *T. ivoriense* and *T. vermipilum* (Samuels *et al.*, 2012). This indicates a lack of unified criteria to recognize species in this genus.

The most commonly applied concept in *Trichoderma* during the last decade is the Genealogic Concordance Phylogenetic Species Recognition (GCPSR) concept (Taylor *et al.*, 2000; see Druzhinina and Kubicek, 2005; Jaklitsch, 2009). This requires the analysis of several unlinked genetic markers, and implies that if different gene trees are concordant they have the same tree topology due to fixation of formerly polymorphic loci following genetic isolation, and these concordant branches connect species (Taylor *et al.*, 2000). With other words, it requires the analysis of trees of several genes, implying that the phylogenetic position of a true species will be concordant in at least two of them, and not be contradicted in the others (Druzhinina and Kubicek, 2005). Lack of concordance among the gene trees is likely to be due to recombination within a species, and this determines the limits of species (Taylor *et al.*, 2000). The power of GCPSR lies in its comparison of more than one gene genealogies with a requirement that in each of gene genealogy recombination does not take place, and that parts of genes are often used to construct the genealogies (Taylor *et al.*, 2000). Kullnig-Gradinger *et al.* (2002) tried to resolve the phylogeny of *Trichoderma* using ITS1 and ITS2, mitssuDNA, 5th *tef1* intron, a fragment of *chi18-5* (former *ech42*) large exon, however, a rigorous clade concordance was not possible for most of the species because of insufficient phylogenetic resolution by the markers used. Chaverri *et al.* (2003a) analyzed ITS1 and ITS2, 4th *tef1* intron, and short fragments of the actin (*act1*) and calmodulin (*cal1*) exon sequences for *H. lixii/T. harzianum*, which resolved seven concordant phylogenetic lineages but only the 4th intron of *tef1* and rarely *cal1* remained to be used in later analyses.

Taylor *et al.* (2000) proposed to base phylogenetic species concepts on the concordance between five or more gene trees. This requirement is not easily fulfilled in *Trichoderma*. In the past, most researchers made heavy use of ITS1 and/or ITS2 (Kuhls *et al.*, 1997; Kindermann *et al.*, 1998; Lieckfeldt *et al.*, 1998a,b, 2001; Dodd *et al.*, 2000), because this cluster is present in multiple copies in the genome and can thus be easily amplified (Druzhinina and Kubicek, 2005). Furthermore, ITS1 and ITS2 provide only poor phylogenetic resolution due to the high level of homoplasy (Druzhinina *et al.*, 2005) and low polymorphism in some clades, particularly in the sections *Trichoderma* (Samuels *et al.*, 2006a; Jaklitsch *et al.*, 2006) and "*Pachybasium*" (Kullnig-Gradinger *et al.*, 2002; Chaverri *et al.*, 2003a).

Unfortunately, none of the above mentioned markers is alone optimal for phylogenetic resolution of the whole genus, or of large clades such as *Trichoderma*. The 4th intron of *tef1* provides excellent resolution and high clade support for closely related taxa in groups such as the *Harzianum* Clade (*H. lixii*, *T. harzianum*, *T. aggressivum*, *T. tomentosum*, *T. cerinum*, *T. velutinum*, *H. tawa*) or *Viride* Clade (*T. gamsii, T. viride, T. viridescens, H. vinosum*) but it is not alignable for species from different clades. In contrast, the last large (6th) exon of *tef1* contains only limited phylogenetic signals for analysis of diverse clades, and thus resulted in lack of support for almost all basal branches in a combined analysis of the *Pachybasium* Clade sensu Chaverri and colleagues (2003b, 2004), whereas the terminal branches had good support. The same problem was even more apparent with RNA polymerase II subunit 2 (*rpb2*). Additionally, the calmodulin gene (*cal1*) is widely in use since recently, especially in multilocus analyses (Druzhinina *et al.*, 2010a; Druzhinina *et al.*, 2012). Eventually, the optimal combination of genes allowing the application of the GCPSR concept on the whole genus *Trichoderma* has not yet been found. Detailed analysis of various core nucleotide genes available in GenBank for taxonomic purposes suggested that the simultaneous usage of (i) *tef1* large intron and last large exon, (ii) *rpb2* gene, (iii) *chi18-5* last large exon and (iv) ITS1 and 2 as diagnostic regions may lead to the most reliable phylogeny (Druzhinina and Kubicek, 2005). However, new phylogenetic markers would increase the phylogenetic resolution of the genus and recognition of new species. The available whole genome sequences of *Trichoderma* (see details below) will certainly be used as a valuable resource for such genes. For example, genes that are unique in *Trichoderma* but are universally present in all species may give a meaningful phylogenetic resolution. On the other hand a specific search for house-keeping genes with large (> 300 nts) introns may be undertaken. The screening for such markers is currently ongoing in the lab of ISD.

Even when GCPSR is applied successfully, the question of species borders – "whether all subclades and lineages are to be named as different species?" - remain open. Thus, the phylogeny of the *Longibrachiatum* Clade was recently investigated using GCPSR (Druzhinina *et al.*, 2008; Druzhinina *et al.*; 2010b; Atanasova *et al.*, 2010; Druzhinina *et al.*, 2012) where it was shown that some of the taxa comprised clonal species (agamospecies) that reproduce exclusively asexually. It was implied that the loss of sexual reproduction may constitute an important mechanism for speciation in this clade (Druzhinina *et al.*, 2008; Druzhinina *et al.*, 2010b). In the case of truly clonal fungi where no incompatibilities in multilocus data are found, the GCPSR does not always help to differentiate the species (Druzhinina *et al.*, 2012). Therefore, in the latest revision of the *Longibrachiatum* Clade Druzhinina *et al.* (2012) applied a population genetics approach, which can be used to complement species recognition by GCPSR ("4× rule" or "K/θ method" (Birky et al. (2010)). This method bases on the theory that random genetic drift in a single species will produce clades and singlets that all originated from a common ancestor on an average 2 times effective population sizes ago (2Ne generations), and their distance from each other will be less than 2Ne generations. After the speciation a species will be split into two completely separated populations forming two clusters separated by a gap exceeding 2Ne. Thus clusters that are separated by t > 4Ne generations represent the upper 95% confidence limit of the coalescent time, and are characterized by a probability of less than 5 % of those being formed by random genetic drift. The 4x rule therefore supports the cluster as an evolutionary species (Birky *et al.*, 2010). This method rejected the attribution of a number of strains (*T.* sp. G.J.S. 09-62 and *T.* sp. G.J.S. 01-355; see Figure 1) to *H. andinensis* in contrast to GCPSR alone (Druzhinina *et al.*, 2012).

Integrated approach to species recognition in *Trichoderma*

Generally, the more data are available the better, i.e. the higher the confidence in the definition and recognition of a species. Therefore an integrated approach in the decision by which criteria and in what degree two coherent homogeneous groups of specimens or strains differ significantly to reach the conclusion that they represent two different species, is the optimal method. For *Trichoderma* specimen(s) recognition as a new species the ideal set of data consists of molecular data (at least *tef1* introns and *rpb2*), morphology of the teleomorph, morphology of the anamorph, geographic occurrence, substrate preferences and ecophysiological profile (culture morphology, growth rates on various relevant carbon sources and response to abiotic stimuli).
For *Trichoderma* there are various situations prevalent:

1) Ideally both teleomorph and anamorph are known and cultures are available for several isolates. In this setting all necessary information can be provided - and all may be necessary in large and complex clades like e.g. the section *Trichoderma* or the *Harzianum* Clade. The recommended and usually applied work flow here is the initial determination of *tef1*, *rpb2* and one or another additional marker (*chi18-5* or *cal1* will work for most of the groups), followed by a phylogenetic analysis and if this may indicate a new lineage, subsequent recording of phenotype data. If all characters of the teleomorph, anamorph and molecular markers are available, it may easily be possible to identify a single specimen as a new species.
Note, some species could be recognized on the teleomorph alone. This can be true for a small number of species and usually only for limited geographical regions. One example is *H. gelatinosa* in Europe, which can be identified at first sight. However, *H. nigrovirens* in Latin America may look the same, but it should be possible to distinguish it from *H. gelatinosa* by larger ascospores. However, due to the hyper-diversity of the genus formerly undetected species may be masked by a common morphology, therefore molecular data (ideally *tef1*) are needed to reach certainty in identification and in order to determine their position on the generic phylogenetic tree.

2) Rarely only teleomorph that does not yield a culture from ascospores is available. However the specimen is sufficient for DNA amplification of single-copy markers such as *tef1* and *rpb2*. The disadvantage with such species is that there is no culture, which can be studied by other researchers.

3) Commonly, only the anamorph is known, e.g. isolated from soil. In this case the description may be based on ideally more than one isolate and multiloci analysis with *tef1* and ITS1 and 2 as an obligatory marker. Species that is remote enough from all other species by *tef1* phylogenetic analysis are to be characterized based on *rpb2* and sufficiently different phenotype data such as anamorph morphology.

Integrated approaches are standard in *Trichoderma*, but multigene phylogeny is now playing the dominant role.

DNA barcoding as an identification tool for *Trichoderma*

Among 11 gene loci or fragments tested in *Trichoderma*, the most promising ones appear to be the 4th and 5th introns of the gene encoding translation elongation factor 1-alpha (*tef1* or frequently incorrectly named based on the respective protein EF-1α), and the coding portions of endochitinase *chi18-5*, and the RNA polymerase gene (*rpb2*_exon; Liu *et al.* 1999). Given that almost all recent studies used molecular data for the species characterization and that this led to the accumulation of the DNA sequences in the GenBank (http://www.ncbi.nlm.nih.gov/), the researchers are now theoretically able to identify all known species using NCBI search similarity tool blastn (BLAST, Basic Local Alignment tool; http://www.ncbi.nih.gov/BLAST). The evaluation of the results retrieved from the BLAST is however not straight-forward. Before recently the deposition of sequences within NCBI GenBank has not included a

quality control of species identification, and some sequences are still deposited under the name the species was originally obtained and not under the name it has been identified subsequently. On a scientific level, high similarity of sequences does not confirm species identity unless the intraspecific variability of this sequence is also known; and even if it is known that a given species may show, e.g., 1 % nucleotide (nt) variation, this may not apply to the entire sequence, and nts in some positions may be invariable. To offer a convenient method for species identification of the most common species, the oligonucleotide barcode program *TrichO*Key (ITS1 and 2 only), and a sequence similarity search tool (using only verified sequences as database) *Tricho*BLAST (multiloci, combined with *Tricho*MARK, which analyses the quality and suitability of the sequence) were developed (www.isth.info; Druzhinina *et al.*, 2005; Kopchinsky *et al.* 2005). However, with the ongoing identification and description of new *Trichoderma* species, it is now known that ITS1 and 2 are insufficiently polymorphic. Unfortunately, so far no other loci could be detected that allow the development of oligonucleotide barcodes for the whole genus. To eliminate these problems, *Tricho*BLAST incorporated sequences of five independent loci from all genetically characterized *Trichoderma* and *Hypocrea* species (ITS1 and ITS2; two introns and one exon of *tef1*, *rpb2*, and located it in the public domain (www.isth.info). The current version of the ISTH multiloci database includes the most common species (around 100) and is suitable for the identification of almost all strains that are usually isolated from soil. It is important to note that a barcode based on ITS1 and 2 is not always sufficient to identify all species of section *Trichoderma*, because several species have identical ITS (for example, *T. koningii* group). Moreover the fact that *TrichO*Key database has not been updated makes it impossible to identify certain species; e.g. *H. auranteffusa* is identified as *H. gelatinosa* by this tool.

Species numbers, current phylogenetic reconstruction and GenBank

Since the introduction of molecular methods in the taxonomy of *Trichoderma* the number of species has dramatically increased (Samuels, 2006; Druzhinina *et al.*, 2006; Jaklitsch, 2009, 2011). As it has been described above a species is now recognized as a combination of phylogenetic and phenotypic as well as to some extent biogeographic and ecological data (Jaklitsch, 2009). While many species in understudied continents such as South America, equatorial Africa and East Asia still await re-assessment, the genus *Trichoderma* is today exceptionally well documented by gene sequence data that can be retrieved from GenBank, enabling researchers to identify them using online tools described above. The first multiloci phylogeny of *Trichoderma* based on these genes raised the number of species to 47 (Kullnig-Gradinger *et al.*, 2002). In 2005 Druzhinina and Kubicek (2005) reviewed the phylogeny and species number of *Hypocrea/Trichoderma*. At that time 88 taxa were redefined and 14 of those were recognized as holomorphs, while 49 and 25 were exclusively named as *Hypocrea* or *Trichoderma*, respectively. Jaklitsch (2011) already included 135 named species in his phylogenetic tree. Currently, those formally established species characterised by molecular data amount to 194 species, of which 84 are described and named as holomorphs, 48 as *Hypocrea* only and 62 as *Trichoderma* only. Moreover there are at least seven yet unnamed species that have been recognized as genetically isolated taxa by means of multiloci phylogeny (Figure 1) and at least 20 potentially new species proposed based on *tef1* alone (11 by Hoyos-Carvajal *et al.*, 2009 in South America and 8 by Mulaw et al., 2010 in Ethiopia respectively). Thus, the total number of species in the genus *Trichoderma* exceeds 200.
It should however be borne in mind that, after exclusion of synonyms and doubtful names, there are still ca. 200 names of *Hypocrea* teleomorphs that have not been re-assessed, i.e. where no molecular data are available (see http://www.indexfungorum.org/Names/Names.asp).

Figure 1 shows the current phylogenetic tree of *Trichoderma* based on *rpb2* sequences available in the Nucleotide database of NCBI GenBank (http://www.ncbi.nlm.nih.gov/). On this figure names possible *Trichoderma* names were used while in text we refer to *Hypocrea* more often. Fugure 1 contains 183 named species and seven unnamed segregates of the sections that have been recognized as phylogenetic species but not named yet (see section 2.4: Development of *Trichoderma* taxonomy based on molecular data). Although the topology is presented in a different way, the displayed clades are in accordance with those published previously (Chaverri *et al.*, 2004; Druzhinina *et al.*, 2005; Kubicek *et al.*, 2008) and with the most recent genus-wide phylogram of Jaklitsch (2009, 2011), that was based on the analysis of the combined *rpb2* + *tef1* exon datasets. The differences are the following: within the *Harzianum* Clade the Lixii/Harzianum complex is only counted as two species (*T. harzianum* and *H. lixii*), because *T.* 'afroharzianum' has not been formally described and *T. inhamatum* is commonly regarded a synonym of *H. lixii.* However Druzhinina *et al.* (2010) has demonstrated that *T. inhamatum* is a separate species within *T. harzianum* sensu lato. *H. nigricans* requires recollection on *Sasa* in Japan as the isolate labelled with the latter name in Figure 1 originates from Germany and may therefore not represent this species. The *Strictipilosa* Clade is somewhat heterogeneous and contains also *H. phyllostachydis* and *H. sulawesensis*, albeit the latter with a long branch. As addressed above, the clade containing *T. stromaticum* has been considerably enlarged (Samuels *et al.*, 2012b). *Hypocrea albofulva* (accession DQ835524, strain G.J.S. 01-265) may be added to the section *Trichoderma*, although the sequence is currently labelled with *H. ochroleuca* in GenBank, but the geographic distribution of the respective specimen may suggest *H. albofulva*.

The following nine species are not represented in the tree, because *rpb2* sequences are lacking: *H. albocornea*, *H. cornea*, *H. hunua*, *H. lacuwombatensis*, *T. matsushimae*, *T. neokoningii*, *H. patella*, *H. stellata* and *H. vinosa*. Another one is *Trichoderma compactum* (Yu *et al.*, 2007) of the *Harzianum* Clade, where the name is missing in GenBank. Sequences are only available under the strain number, as *Trichoderma* sp. YMF1.01693. *Rpb2* for these species are currently sequenced in the lab of WMJ.

Here we want to point to the responsibility of the authors/submitters to update their submissions with regard to the species name and to possible improvements of their sequences. In addition we want to discuss the situation of sequences available for *Trichoderma* in GenBank.

First, the Taxonomy Browser of GenBank is not an authority for the taxonomy of fungi, for this IndexFungorum (http://www.indexfungorum.org/Names/Names.asp) or MycoBank (http://www.mycobank.org/) has to be consulted in addition to the more recent taxonomic publications that include DNA data. The latter sources are most important, because some data may not be updated in the databases mentioned above, e.g. synonyms of *T. ghanense* have not been implemented in IndexFungorum (see below). Accordingly, it is impossible to determine the number of species of *Trichoderma* based on GenBank accession numbers, but also species identification of this large genus via gene sequences has serious limitations. One reason for this is the shortcomings of the BLAST routine, because it cannot deal optimally with various sequence lengths of *tef1* sequences present in the database. As pointed out above, the *Ticho*MARK tool, placed on ISTH website, was developed to overcome this problem (see Section 2.5.5- DNA barcoding as an identification tool for *Trichoderma*). It is useful to retrieve the most diagnostic regions for such phylogenetic markers as *tef1*, *rpb2* and ITS1 and 2 (Druzhinina & Kopchinskiy, 2006) and to avoid unjustified false positive hits resulted from high scores due to exon - exon similarities in case the reference database is not complete or the query sequence does not contain the diagnostic intron sequences. It is recommended to charge results of sequence similarity search in respect not only to the length of the resulted alignment but also to the position of the hit in the sequence. This is particularly important for the *tef1* gene as over the years different regions of it were sequenced and used for phylogenetic reconstruction. The best sequences are those that contain 4th and 5th introns and a large part of the exon (Kopchinskiy *et al.,* 2005).

Secondly, there are several issues concerning fungal names: As outlined in the nomenclature section below, a general move to the use of *Trichoderma* in favour of *Hypocrea* has not been implemented yet, therefore most species are now filed under *Hypocrea*, in accordance with earlier rules of the ICBN. However, for nine species the combination in *Trichoderma* (*T. atroviride*, *T. aureoviride*, *T. chlorosporum*, *T. cinnamomeum*, *T. cremeum*, *T. piluliferum*, *T. surrotundum*, *T. thailandicum*, *T. thelephoricola*) is present in addition to those in *Hypocrea*, and the respective sequences in GenBank are not named consistently. As an example, 6 sequences can be retrieved from GenBank for *Hypocrea cremea* and 4 for *Trichoderma cremeum*. Then there are also orthographic errors, e.g. Hypocrea caribbea instead of *Hypocrea caribbaea* (*rpb2* accession FJ442723 for strain G.J.S. 98-43) and others.

Some names labels of sequences are taxonomically wrong: *Hypocrea farinosa* (= *Protocrea farinosa*) was erroneously used by Overton *et al.* (2006a) for a species of *Hypocrea*, later established as *H. decipiens* (Jaklitsch *et al.*, 2008b). This has never been updated. Sequences in GenBank under the name *Hypocrea chionea* do apparently not represent that species but belong to *H. caerulescens* or possibly to a phylogenetically close but yet undescribed species (G.J. Samuels, pers. comm.).

Some sequences of *Trichoderma* isolates are deposited under a valid name but they do not belong to the respective species, which can be detected by alignment tests. Such errors require laborious screening and correction.

Other sequences are deposited under a valid name but possibly do not belong to the respective species, due to the lack of taxonomic verification or because they were not collected in the original region. The most recent submission in GenBank of sequences labelled with *Hypocrea pseudogelatinosa* can currently not be assessed taxonomically, because the respective paper has not been published yet. In any case, *tef1* sequences comprising only 208 bp (for references the most diagnostic 4th intron of this gene has ~350 nt

in length) and *rpb2* of only 315 bp of at least 800 nt for the locus used for phylogeny can not be considered as reliable data.

The taxonomy browser displays also names, which additionally contain a strain number that does not belong to the taxon. Names like *Hypocrea* sp. or *Trichoderma* sp. and a strain number constitute the largest number of "names". Most of them originate from various non-taxonomic or non-phylogenetic studies, are only represented by ITS sequences and have thus not been identified, but a considerable fraction comprises known species, where the name has not been updated by the submitters. Names like *Hypocrea* cf. or *Trichoderma* cf. and a species name (e.g. *Hypocrea* cf. *gelatinosa* or *Trichoderma* cf. *stilbohypoxyli*) have not been identified; they may be close to the respective species, but probably not representing that species.

Several species names present in the taxonomy browser are synonyms of other species: *Hypocrea flavovirens* is a synonym of *H. catoptron* (Chaverri and Samuels 2003), but is still used in GenBank for *H. chlorospora*, due to a preliminary identification as *H.* cf. *flavovirens* by Chaverri *et al.* (2003a). This requires an update. Other synonyms: *Hypocrea lactea* (valid name *H. citrina*), *Hypocrea nigricans* (valid name *H. lixii*, but it may be distinct from that species after a thorough re-evaluation), *Trichoderma croceum* (valid name *T. polysporum*/*H. pachybasioides*), *T. fasciculatum* (valid name *T. strictipile*/*H. strictipilosa*), *T. glaucum* (valid name *T. viride*/*H. rufa*), *T. parceramosum* (valid name *T. ghanense*), *T. todica* (valid name *T. ghanense*).

Some names have been updated in the sequence annotations in the meantime, but remain in the list of the taxonomy browser. An essential complication comes from the fact that information about the sequences may only be updated by people listed as authors for the respective submission in NCBI GenBank but not others. Therefore it is advisable to assign also faculty members as sequence authors rather than temporarily employed scholars (PhD students or PosDocs).

Others errors or dubious names: *Hypocrea cordyceps*, as *Podostroma cordyceps*, is represented by ITS and small subunit of the rRNA gene cluster (SSU) sequences, which suggest a species of the section *Trichoderma*. It is unclear whether a contaminant was sequenced. The *tef1* accession AF534577 labelled with *Hypocrea dichromospora* is identical with *H. phyllostachydis* and should be annotated accordingly. The ITS accession JF905628 labelled *Hypocrea fomitopsis* (a species described by Liu *et al.*, 2000 from Yunnan, China on *Fomitopsis pinicola*) clearly belongs to the *T. harzianum- H. lixii* complex. However the Latin description of the taxon indicates that *H. fomitopsis* has hyaline ascospores and hyaline conidia, i.e. it cannot belong to the Harzianum Clade, thus the species cannot be assessed further. Likewise, *Hypocrea muroiana* (taxonomy to be clarified) is represented by ITS, LSU, SSU and *rpb1* sequences, but not by *tef1* or *rpb2*, which would be required for a reliable phylogenetic assessment. It may be identical with another species of the section *Trichoderma*.

Some species names present in GenBank have never been published or the name was changed until publication. They comprise *Trichoderma* esasiaensis, *T*. hebeiensis, *T*. mimkoningii and *T*. virgatum. *T*. pauculosporum was published as *T. paucisporum* (Samuels *et al.*, 2006b).

Last but not least it is important to note that GenBank has recently aggravated submission requirements and classifies deposited sequences as UNVERIFIED, when the submitter does not annotate coding regions. These sequences are unavailable for BLAST analyses. We think that this appears too stringent, because the sequences, even if containing an error, were used as such in phylogenetic analyses where respective protein sequence was not required. Unfortunately such a problem occurred with some of the sequences used in the latest revision of the *Longibrachiatum* Clade.

Taxonomy of domesticated species of *Trichoderma*

The commercial biofungicide Trichoderma harzianum *T22*

The phylogenetically and taxonomically still unresolved *H. lixii* - *T. harzianum* species complex is one of the most commonly sampled fungal groups because it is dominant in the majority of soil ecosystems worldwide and it inhabitates a broad variety of ecological niches (Druzhinina *et al.*, 2010a). The evolutionary success of these fungi may be attributed to the largest known genome anong all sequenced *Trichoderma* species (Mycocosm, DOE JGI) and very complex structures of its populations, e.g. reproductively isolated biological species, sympatric and allopatric phylogenetic species, recently diverged agamospecies and numerous relict lineages with unresolved phylogenetic positions (Druzhinina *et al.*, 2010a). *T. harzianum* sensu lato are effective biocontrol agents for several soil-borne plant pathogens (Harman *et al.*, 2004; Liu *et al.,* 2012; Hasan *et al.,* 2012; L. Espino de Ramer and I.S. Druzhinina, unpublished data) yet recently some researchers also shown that they possess the abilities to enhance systemic resistance to plant diseases and overall plant growth (Harman, 2000; Yedidia *et al.*,2001; Harman *et al.*, 2004, Shoresh and Harman, 2008). As an application in biocontrol of plant diseases the goal was to obtain highly rhizosphere competent strains with substantial ability to compete with spermosphere bacteria (Harman, 2000; Stasz *et al.*, 1988). Two strong biocontrol agents, the rhizosphere competent mutant *T. harzianum* T-95 produced from a strain isolated from a *Rhizoctonia*-suppressive Colombian soil and *T. harzianum* T-12, capable of competing with spermosphere bacteria under iron-limiting conditions were fused in Gary Harman's laboratory (Cornell University, USA) using protoplast fusion (Stasz *et al.*, 1988). The strongly rhizosphere competent, competitive in the spermosphere environment, and broadly effective biocontrol T. *harzianum* strain T22 (1295-22, KRLAG2, or ATCC 20847), was produced for commercial agriculture and is sold as a single strain product for greenhouse, row crop, and turf industries (Harman, 2000). Analysis of *tef1* polymorphism suggests that this hybrid strain is most closely related to *T.* 'afroharzianum' nom. prov. within *T. harzianum* sensu lato complex but it does not belong to *T. harzianum* sensu stricto species (Druzhinina *et al.,* 2010).

Trichoderma asperellum – T. asperelloides *T203*

It was long believed that one of most studied *Trichoderma* biocontrol strains T203 is *T. harzianum* (Yedidia *et al.*, 2000) and later *T. asperellum* T203 (Harman *et al.*, 2004; Viterbo and Chet 2006). Recently, Samuels *et al.* (2010) performed a revision of *T. asperellum* strains and showed that *T. asperellum* sensu lato consist of two criptic species, *T. asperellum* and *T. asperelloides.* According to these results T203 was re-identified as *T. asperelloides*.

Trichoderma reesei – T. parareesei *and closely related species*

T. reesei was originally isolated in 1942 on the Solomon Islands during WW II, whereas its sister species were recognized as independent species only recently (Atanasova et al., 2010; Druzhinina et al., 2010b). *T. reesei* is a biotechnologically highly important taxon used only from the wild-type isolate Qm 6a, so all the mutant strains applied in industry today have thus been derived from it. First it was believed that Qm 6a belongs to *T. viride*, as it was thought that the genus consist only of this single species (Bisby, 1939), but it has been later recognized as a separate species named after its founder Elwyn T. Reese (Druzhinina *et al.*, 2010b). Bissett (1984) thought that *T. reesei* was conspecific with *T. longibrachiatum*, but finally it was recognized to be identical with *H. jecorina* (Kuhls *et al.*, 1996). Because Qm 6a was not able to mate with the wild type *H. jecorina* strains Kuhls *et al.* (1996) assumed that it is actually a clonally derived asexual form of *H. jecorina.* Seidl *et al.* (2009) recently described that *T. reesei* can indeed be crossed with wild-type isolates of *H. jecorina.* Druzhinina *et al.* (2010b) further showed that *H. jecorina/T. reesei* sensu stricto contains most of the teleomorphs (sexual stages) found on dead wood and the wild-type strain of *T. reesei* QM 6a. Studying the phylogenetic relations and ecophysiological characteristics using a large number of strains Druzhinina *et al.* (2010b) and Atanasova *et al.* (2010) showed that the Reesei subclade consist of two phylogenetic species, *T. reesei* and *T. parareesei*, and two putative new species *T.* sp. G.J.S. 04-93 and *T.* sp. C.P.K 524 which were later, by complementing the GCPSR with 4x rule, attributed to *T.* cf. *parareesei* (Druzhinina *et al.*, 2012). Atanasova *et al.* (2010) also showed that *T. parareesei* is more ancient taxon which apparently nearly stopped its evolutionary development likely due to the loss to reproduce sexually and that *T. reesei* arose from it.

Taxonomy of pathogenic *Trichoderma* species

Trichoderma longibrachiatum - H. orientalis *and new related taxa*

Trichoderma longibrachiatum is an opportunistic pathogen of immunocompromised humans that usually represents a common component of *Trichoderma* communities isolated from soil and other environments (Druzhinina *et al.*, 2005; Kubicek *et al.*, 2003; Kullnig *et al.*, 2000; Wuczkowski *et al.*, 2003; Zhang *et al.*, 2005), but it is also known to be a part of the indoor fungal flora (Thrane *et al.*, 2001) and has also frequently been isolated from mushroom farms infected by green mould disease (Hatvani *et al.*, 2007). Abundant isolation of *T. longibrachiatum* was from the archaeological excavation sites at an Iron Age tomb in the Republic of Tatarstan, Russia (F. Alimova & I. S. Druzhinina, unpublished). In clinical studies, *T. longibrachiatum* has been detected in sputum and sinus ethmoidalis of healthy humans (Kredics *et al.*, 2003) and it has been known as the causal agent in the majority of reported *Trichoderma* mycoses (Druzhinina *et al.*, 2008). Recently, Druzhinina *et al.* (2008) performed a multilocus phylogenetic analysis of all available clinical isolates and wild-type strains of the fungus including several cultures of its putative teleomorph *Hypocrea orientalis*. The aim was to test if the opportunistic strains of *T. longibrachiatum* may represent specialized potentially clonal subpopulations within this species. The study revealed that not

only one but two genetically different species *T. longibrachiatum* and *H. orientalis* infect immunocompromised patients, and that clinical isolates are found in all or at least in major gene haplotypes of both species, what rejected the hypothesis that opportunistic *T. longibrachiatum* strains are forming potential subpopulations (Druzhinina *et al.*, 2008). Additionally, Druzhinina *et al.* (2008) also showed that *T. longibrachiatum* and *H. orientalis* are two genetically isolated species, which are reproductively isolated from each other. The analysis of haplotype association, incongruence of tree topologies and the split decomposition method suggested that *H. orientalis* is sexually recombining whereas *T. longibrachiatum* is clonal agamospecies (Druzhinina *et al.*, 2008; Druzhinina *et al.*, 2012). The finding that an opportunistic pathogenic fungus like *H. orientalis* reproduces sexually is uncommon. Nevertheless, both species have been shown to be cosmopolitan sympatric species, they are being isolated from numerous soil samples worldwide, therefore, Druzhinina *et al.* (2008) proposed that together with its appearance as causative agent of invasive mycoses, there is emerging evidence that *T. longibrachiatum* and *H. orientalis* may have a specialized ecological niche(s) which is(are) essentially different from other species of the genus. Druzhinina *et al.* (2012) performed an extended evolutionary analysis of *Longibrachiatum* Clade, where it was shown that there are evidences for sexual recombination among *T. longibrachiatum* and *H. orientalis*, suggesting that speciation in these cases involved loss or gain of sexual reproduction. With the increasing number of isolates, two new species *T. aethiopicum* and *T. pinnatum* (Druzhinina *et al.*, 2012; Samuels *et al.*, 2012a) were recently recognized to be the most closely related species of *T. longibrachiatum* and thus a part of *Longibrachiatum-Orientalis* subclade. No data on their pathogenicity to humans are available so far.

Trichoderma aggressivum, T. pleuroti, T. pleuroticola*, and* T. mienum

For a long time it is known that *Trichoderma* species also colonize the substrates on the farms growing edible fungi (*Agaricus bisporus*, *Lentinula edodes*, and *Pleurotus ostreatus*) and may limit commercial production of these basidiomycetes (Sinden *et al.*, 1953). In the last 30 years *Trichoderma* spp. caused severe damages on the farms with outbrakes all over the World, what evoked extensive research efforts to identify and study these causative agents (Hatvani *et al.*, 2007). First it was believed that this is due to *T. harzianum* (Muthumeenakshi *et al.*, 1994, Castle *et al.*, 1998). Later on a number of *Trichoderma* spp. have been isolated from mushroom compost, but aggressive colonization was claimed to be exclusively due to two *formae speciales* of a new *Trichoderma* species, viz. *T. aggressivum* f. *europaeum* in Europe and *T. aggressivum* f. *aggressivum* in North America (Samuels *et al.*, 2002). However, after the outbreak of *Trichoderma* green mold in Korea (Park *et al.*, 2004) and Itally (Woo *et al.*, 2004) the causative agent appeared to be morphologically distinct from *T. aggressivum* (Park *et al.*; 2004; Woo *et al.*, 2004; Hatvani *et al.*, 2007) and it was proposed that two different new species (*T. koreana* and *T. pleuroti*) are responsible for the epidemics on *P. ostreatus* in Korea, but unfortunately the claim was not documented (Hatvani *et al.*, 2007). Recently, the study of *Agaricus* compost and *Pleurotus* substrate was undertaken to identify the *Trichoderma* green mold agents causing the outbreak on the mushroom farms in Hungary (Hatvani *et al.*, 2007). The survey based on the molecular identification showed that two different, although genetically closely related species, *T. pleuroti* and *T. pleuroticola* together with *T. aggressivum* were the causing agents of green mold disease in Hungary (Park *et al.*; 2004; Hatvani *et al.*, 2007; Komon-Zelazowska *et al.*, 2007). These species have also been detected in Poland, Romania, Italy, South-Korea and Taiwan, thus it was hypostasized that this species are result of sympatric speciation, taking into account that *T. pleuroticola* damages *Pleurotus ostreatus* mainly by competition for nutrients and overgrowing its mycelium, and contrary to *T. pleuroti* and *T. aggressivum*, was found also in environmental samples (Komon-Zelazowska *et al.*, 2007; Kubicek *et al.*, 2008). Additionally, Komon-Zelazowska *et al.* (2007) proposed that a strongly reduced carbon source assimilation profile of *T. pleuroti* might be a reflection of the hypothetic specialization solely on *P. ostreatus.*
Recently the new species *Trichoderma mienum* was isolated from ascospores of dry stromata found on bedlogs for *L. edodes* and *P. ostreatus* cultivation in Japan. Phylogenetic analysis placed the new species in the *Semiorbis* Clade (see Figure 1) most closely to *T. fertile* and *T. oblongisporum* and morphological comparison with other species of the clade showed clear differences in ascospore colour, texture of subperithecial tissue, conidiophore type, formation of chlamydospores, and growth rate (Kim *et al.*, 2012).

Diversity surveys of *Trichoderma*

Trichoderma species were commonly isolated in several surveys (Kullnig *et al.*, 2000; Kubicek *et al.*, 2003; Wuczkowski *et al.*, 2003; Gherbawy *et al.*, 2004; Druzhinina *et al.*, 2005; 2010; 2012; Zhang *et al.*, 2005; Kiyuna *et al.*, 2008; Migheli *et al.*, 2009, Zachow *et al.*, 2008). However, all these studies focused on soil ecosystems, therefore there was still very little known about the distribution of non-soil *Trichoderma*.
Nevertheless, two of these 'soil' studies have been recently performed on islands with an attempt to investigate biogeographically isolated ecosystems and to correlate species occurrence with ecological parameters. A study from the Tyrrhenian island of Sardinia (Migheli *et al.*, 2009), which has become isolated since the early Miocene (~7-8 Ma) and is considered a hotspot of biodiversity and endemism (Médail and Quézal, 1999; Grill *et al.*, 2006); and the Canary island Tenerife (Zachow *et al.*, 2008), which is of relatively recent volcanic origin (2 Ma) and represented by six different vegetation zones characterized by specific abiotic conditions and plant communities. Migheli *et al.* (2009) isolated 482 strains of *Trichoderma* from 15 soils comprising forest, shrub lands and undisturbed or extensively grazed grass steppes. The majority of the isolates was identified as pan-European and/or pan-global *Trichoderma* species from sections *Trichoderma* and "*Pachybasium*", comprising *T. harzianum* sensu lato, *T. asperellum*, *T. atroviride*, *T. gamsii*, *T. hamatum*, *T. koningii*, *T. koningiopsis*, *H. semiorbis*, *T. spirale*, *T. tomentosum*, *T. velutinum*, *T. virens*, *T. viridescens* and *Trichoderma* sp. Vd2 sensu Jaklitsch *et al.* (2006a). Except for one isolate representing an undescribed species from *Harzianum* Clade and one potentially endemic ITS1 allele of *T. hamatum*, all other species exhibited genotypes that were already found in Eurasia or in other continents (Migheli *et al.*, 2009). Furthermore, in the cultivation assays from Tenerife island soils (Zachow *et al.*, 2008) revealed similar species (e.g. *T. gamsii*, *T. harzianum*, *T. spirale*, *T.* cf. *tomentosum*, *T. viride* and *T. viridescens*). The diversity was much lower than those reported for the rhizosphere of agricultural soils and crops in South-East Asia (Kubicek *et al.*, 2003) and South America (Druzhinina *et al.*, 2005; Hoyos-Carvajal *et al.*, 2009) and in Ethiopia (Mulaw *et al.*, 2010). All *Trichoderma* isolates found on this island show an extraordinarily high antagonistic potential towards different groups of plant pathogens (Zachow *et al.*, 2008), supporting the hypothesis of extensive colonization by highly competitive *Trichoderma* species from the continents. However, both studies detected almost exclusively common cosmopolitan and opportunistic *Trichoderma* species, which were already known to science by isolations from many locations from all over the world. In comparison with the whole fungal community, *Trichoderma*-specific communities showed a low diversity and no correlation to plant communities or abiotic factors.

Another large-scale biogeographic study performed in South America (Hoyos-Carvajal *et al.*, 2009) assessing the biodiversity of 183 isolates from Mexico, Guatemala, Panama, Ecuador, Peru, Brazil and Colombia, revealed high diversity of species from neotropical soils, with the highest dominance of *T. asperellum* and *T. harzianum* sensu loto, but also *T. atroviride*, *T. brevicompactum*, *T. crassum*, *T. erinaceum*, *T. gamsii*, *T. hamatum*, *T. harzianum*, *T. koningiopsis*, *T. longibrachiatum, T. ovalisporum*, *T. pubescens*, *T. reesei*, *T. rossicum*, *T. spirale*, *T. tomentosum*, *T. virens* and *T. viridescens*, along with 11 putative new species. *T. asperellum* was the prevalent species and was represented by two distinct genotypes with different metabolic profiles and habitat preferences one of which was later (Samuels *et al.*, 2010) described as *T. asperelloides*. Neither *T. koningii* nor *T. viride*, once believed to be common and widely distributed, were isolated from those neotropical soils.

Furthermore, *Trichoderma* species have also been isolated from marine environments (Paz *et al.*, 2010; Gal-Hemed *et al.*, 2011). Among *Trichoderma* strains isolated from Mediterranean *Psammocinia* sponges, the largest number of isolates belonged to the *T. longibrachiatum* - *H. orientalis* species pair, *T. atroviride* and the *T. harzianum* species complex, which are all known for high mycoparasitic potential (Gal-Hemed *et al.*, 2011). In addition, one isolate of *T. asperelloides* and two putative new species, *Trichoderma* sp. Y.O. 14707 and Y.O. 2407 (see Figure 1), from the *Longibrachiatum* and *Strictipilosa* clades, respectively, have been identified. Moreover, in vitro salinity assays showed that halotolerance is a property specific for a strain rather than for a species and that the majority of the isolates were found to be insensitive to increased salinity, some even demonstrated improved growth in increasingly saline conditions (Gal-Hemed *et al.*, 2011).

Several *Trichoderma* anamorphs were lately isolated as endophytes. Zhang *et al.* (2007) reported about a new species, *T. taxi*, which was isolated as an endophytic fungus of *Taxus mairei* growing at the Guanshan Nature Reserve of Jiangxi province, China. This species is characterized by its white small subglobose conidia and pachybasium-like conidiophores aggregated into compact pustules, and forms a phylogenetically independent branch (Zhang *et al.*, 2007). However, several endophytic species of *Trichoderma* were found in the Amazon basin in species of *Theobroma*, (*T. theobromicola*, *T. martiale*; Samuels *et al.*, 2006b; Hanada *et al.*, 2008) or *Hevea* (*T. amazonicum*; Chaverri *et al.*, 2011), and on Galapagos on *Scalesia* (*T. scalesiae*; Jaklitsch et al., 2006a).

Root endophytic *Trichoderma* were isolated from roots of *Coffea arabica* from Ethiopian coffee-growing areas (T.B. Mulaw, I.S. Druzhinina and L. Atanasova, unpublished data). The production of coffee in this area is affected by tracheomycosis caused by the soil-borne fungus *Gibberella xylarioides. Trichoderma* communities from native and disturbed coffee rhizospheres were recently characterized by high diversity of species, where several of them were new to science (Mulaw *et al.*, 2010). Eight known species were found, among which *T. harzianum* sensu lato and *T. hamatum* were most abundant, followed by *T.*

asperelloides, *T. atroviride, T. gamsii, T. koningiopsis*, *T. longibrachiatum*, and *T. spirale*. The strains of the latter three species were identical to strains known from Europe or South America, while most of the isolates of *T. harzianum* sensu lato were highly similar to strains from Cameroon and Egypt (Mulaw *et al.*, 2010), belonging to the recombining holomorphic strains known as the 'pseudoharzianum matrix' (Druzhinina *et al.*, 2010a). Few isolates were also attributed to the new putative agamospecies *T.* sp. 'afroharzianum' nom. prov. (Druzhinina *et al.*, 2010a), and *T. harzianum* sensu stricto was usually found in a temperate climate. Interestingly, unique genotypes were found for *T. hamatum* and some of *T. atroviride* isolates, and high genetic diversity was detected for *T. spirale*. Importantly also several new species were found: *T. aethiopicum* and *T. flagellatum*, both belonging to the Section *Longibrachiatum* were recently described by Samuels *et al.* (2012a). Other putative new species *T.* sp. C.P.K. 2707 of the *Brevicompactum* Clade, *T.* sp. C.P.K. 1828 related to *T. helicum*, *T.* sp. C.P.K. 2727 closely related to *T. atroviride*, and putative new *Trichoderma* spp. of the *Harzianum* Clade (*T.* sp. C.P.K. 1812, *T.* sp. C.P.K. 1807, *T.* sp. C.P.K. 1833, *T.* sp. C.P.K. 2607, *T.* sp. C.P.K. 2612) are awaiting formal species description.

In a recent diversity study that included isolates from soil, (mushroom) compost and wood (Błaszczyk *et al.*, 2011) the authors identified 110 of 170 isolates to the species level, found 14 species of which the commonest were, in this order: *T. harzianum* sensu lato (ubiquitous and in large excess), *T. aggressivum* (only from mushroom compost), *T. atroviride*, *T. koningii*, *T. viridescens* and *T. citrinoviride*. This result is in agreement with purely soil-derived isolates, although it is not clear which species the residual, non-identified strains may reperesent.

Jaklitsch (2009; 2011) performed an extensive study in Europe that was based on *Hypocrea* teleomorphs. His monograph of European species of *Hypocrea* included more than 620 specimens from 14 different countries and presents the first and very detailed assessment of the diversity of the genus within a larger region covering most European climate zones. He described or re-described the unexpected high number (75) of species that form teleomorphs, while being aware that there are still several more to be thoroughly studied and formally described. His data clearly show that the diversity of *Trichoderma* above soil exceeds the number of species isolated from soil substantially. His results also indicate that the vast majority of species (83 %) occurs on wood and bark of trees and shrubs, while only 2 % may be found on gramineous or herbaceous hosts, 2 % on soil and forest debris and 13 % specifically on other fungi. Of the latter, *H. estonica* and *H. parestonica* occur on *Hymenochaete* spp., *H. fomiticola* on *Fomes fomentarius*, *H. protopulvinata* on *Fomitopsis pinicola*, *H. pulvinata* on *Fomitopsis pinicola* and *Piptoporus betulinus*, *H. sulphurea* on *Exidia* spp., *H. thelephoricola* on *Steccherinum ochraceum*, and *H. phellinicola* on *Phellinus* spp. Many other species overgrow fungi such as *Phellinus* basidiomes (e.g. *T. harzianum* sensu lato), other polypores, corticiaceous basidiomycetes or pyrenomycetes, but the same species also occur on wood and bark without seemingly obvious association with a fungus. However, Jaklitsch (2009) also reported that generally *Trichoderma* anamorphs are considerably more common on plant material than *Hypocrea* teleomorphs and that very large and intense study would be necessary to capture the diversity of *Trichoderma* in Europe above soil in its entirety.

Diversity studies using cultivation-independent methods (metagenomics) usually result in the identification of a high percentage of still unknown fungal phylotypes. In situ diversity of *Trichoderma* has so far been only studied in soils (Hagn et al. 2006, Zachow et al. 2009, Meincke et al. 2010, Friedl and Druzhinina, 2012). These pioneering studies, however, detected almost exclusively already known species of *Trichoderma*, which is in agreement with the discussion above.

Friedl and Druzhinina (2012) found no hidden diversity of *Trichoderma* in primeval undisturbed soils (Austria). Among 411 ITS1 and 2 molecular operational taxonomic units (MOTUs) 407 were safely attributed to 15 existing species or to putatively new taxa that have previously been sampled. A similar outcome was also obtained by Hagn et al. (2007) for arable soil and Meincke et al. (2010) for rhizosphere of *Solanum tuberosum*. These results are in line with published data on the *in situ* diversity of Pezizomycotina in soil: in these studies, *Trichoderma* MOTUs were found only at minor portions compared to other groups of Ascomycota (Buée et al. 2009; Lim et al. 2010).

Trichoderma was considered to be a genus of soil fungi for a long time. This perception was based on abundant isolations from soil samples world-wide. The general strong antifungal activity of *Trichoderma* spp. favors their detection in cultivation-based surveys as they are able to suppress other fungi and thrive on a Petri plate. Qualitative analysis of the diversity revealed in such samples shows the dominance by the same 15 - 20 highly opportunistic species such as *T. alni, T. asperelloides, T. asperellum, T. atroviride, T. brevicompactum, T. citrinoviride, T. gamsii, T. hamatum, T. harzianum* sensu lato, *T. koningii* complex, *T. koningiopsis, T. longibrachiatum, P. pleuroticola, T. spirale, T. strigosum, T. virens* etc. It is likely that these species obtained the ability to saprotrophic growth in soil due to their general outstanding opportunistic potential as suggested based on genomes of *T. atroviride* and *T. virens* (Druzhinina et al. 2011; Kubicek et al. 2011, see below). Consequently, the general belief that *Trichoderma* is a "soil fungus" is not supported.

Trichoderma phylogenomics: which species came first?

The last level of evolutionary resolution is offered by the analysis of the whole genomes. Thus, phylogenetic analysis of 100 orthologous protein sequences available from the three genomes sequenced of *T. reesei* (http://genome.jgi-psf.org/Trire2/Trire2.home.html, Martinez *et al.*, 2008), *T. virens* and *T. atroviride* (http://genome.jgi-psf.org/Trive1/Trive1.home.html and http://genome.jgi-psf.org/Triat1/Triat1.home.html, respectively; Kubicek *et al.*, 2011), representing the three well defined infraspecific groups of the genus (Section *Longibrachiatum*, *Virens* Clade and Section *Trichoderma*, respectively), has revealed that the mycoparasitic species *T. atroviride* occupies an ancestral position relative to the mycoparasitic and phytostimulating species *T. virens,* while the moderate antagonist of other fungi and a superior producer of cellulases and moderate mycoparasite *T. reesei* holds the most derived position in the three (Figure 2). This finding indicates that mycoparasitism is the innate property of the genus, which may be either powered by additional features (like in *T. virens*) or reduced (like in *T. reesei*) in a course of evolution and ecological specialization (Druzhinina *et al.*, 2011). Kubicek *et al.* (2011) complemented their three-species phylogram with an *rpb2*-based tree for 100 species of the genus. That *rpb2* phylogram and the tree based on 100 orthologous protein sequences for genome sequences species are largely in agreement with the postulation that *T. atroviride* represents the oldest state. The same topology is also seen from the phylogram inferred for this chapter (Figure 1).These analyses suggest that *T. virens* and *T. reesei* are evolutionary more derived.

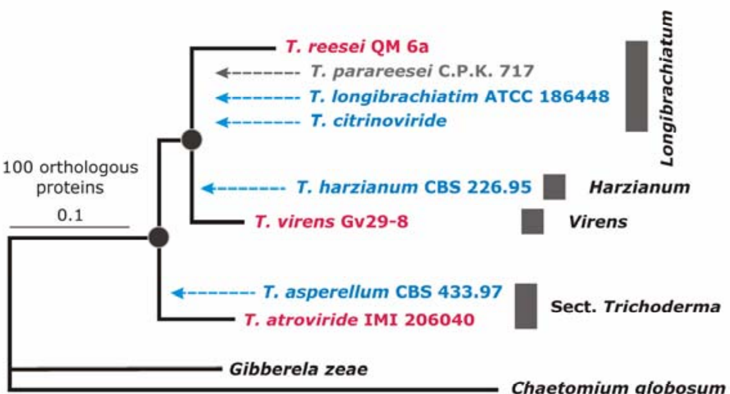

Figure 2: Bayesian phylogram based on the analysis of amino acid sequences of 100 orthologous syntenic proteins (MCMC, 1 million generations, 10,449 characters) in *T. reesei*, *T. virens*, *T. atroviride*, *Gibberella zeae* and *Chaetomium globosum* (see Kubicek et al., 2001 for details). Circles above nodes indicate 100% posterior probabilities and significant bootstrap coefficients. Arrows indicate putative positions of the 5 other *Trichoderma* species which complete genomes sequenced (blue) have been sequenced (grey). Vertical bars correspond to infrageneric clades as on Figure 1

In the year 2012 genomes of four other *Trichoderma* species have been sequenced by DOE JGI and made available for public: *T. harzianum* sensu stricto CBS 226.95 (http://genome.jgi-psf.org/Triha1/Triha1.home.html) and *T. asperellum* CBS 433.97 (http://genome.jgi.doe.gov/Trias1/Trias1.home.html). *T. longibrachiatum* (http://genome.jgi.doe.gov/Trilo1/Trilo1.home.html) ATCC 18648 and *T. citrinoviride* (http://genome.jgi.doe.gov/Trici1/Trici1.home.html). Sequencing of *T. koningii* genome was reported by Shanghai Institutes for Biological Sciences (China) although it is not accessible through web.

The inclusion of the above mentioned species in a phylogram on Figure 2 will certainly refine evolutionary relationships between and within respective clades. However it should not bring major changes on the

genus tree as all novel genomes are closely related to the first three species, which genomes were compared by Kubicek *et al.* (2011).

Hypocrea vs. *Trichoderma* nomenclature

Most Ascomycota have a pleomorphic lifestyle, i.e. they may appear in different forms, the meiotic sexual form or teleomorph and the mitotic asexual form or anamorph. Some genera may even have several synanamorphs or less commonly, synteleomorphs. The holomorph is the whole fungus. Traditionally the different forms received different names, because the anamorph-teleomorph connections were unknown for a long time, even centuries. The name of the teleomorph was used as the valid name of the holomorph. The respective rules were covered by Article 59 of the International Code of Botanical Nomenclature (ICBN). However, several changes were approved in the nomenclature sessions of the 18th International Botanical Congress (IBC) in Melbourne in July 2011. Apart from the name change to the International Code of Nomenclature for algae, fungi, and plants (ICN) the most important changes are the permission of electronic publication from 1 January 2012 in online journals having an ISSN number or books having a ISBN number, the permission to write the descriptive diagnosis of a new species either in English or Latin from 1 January 2012, the mandatory registration of fungal names from 1 January 2012, and the requirement that new fungal taxa can only be described under a single name from 1 January 2013. This latter change is nearly an abolishment of Art. 59 and means also that anamorph and teleomorph names are treated in future as synonyms, and the valid name is determined by the priority of publication.

As outlined above, the genus discussed here manifests itself as teleomorphs known as *Hypocrea* on the generic level, and as anamorphs called *Trichoderma*. The latter name is older than the former, therefore the latter is the candidate of choice for the exclusive use at the generic level. However, a clear priority rule is counteracted by Art. 57.2. In pleomorphic fungi, in cases where, prior to 1 January 2013, both teleomorph-typified and anamorph-typified names were widely used for a taxon, an anamorph-typified name that has priority must not be taken up until retention of the teleomorph-typified name has been considered by the General Committee and rejected. This was added to the Code to guarantee a continuous use of names of economically important fungi. For *Trichoderma* this means that a decision must be taken. For this reason a poll was conducted by the ISTH (http://www.isth.info/), which resulted in a clear preference of *Trichoderma* over *Hypocrea* (70 % and 30 % respectively for 75 votes by November 15, 2012). Accordingly, in future eventually *Trichoderma* may be used for all species of the genus.

What has to be done to achieve that, what are the advantages and the disadvantages or difficulties to reach this goal?

First, one of the favorable facts that is not evident or true for many other genera, is the conspecificity of *Hypocrea rufa*, the type species of *Hypocrea* and *Trichoderma viride*, the type species of *Trichoderma*, which means that the genera are indeed congruent.

However, when we consider other species, there are several problems. One is that several species of *Hypocrea* do not form an anamorph (is this a criterion?), several have an anamorph, but the combination in *Trichoderma* does not yet exist, and last but not least, the priority rule applies also to the names at the species level. For example, the cellulase-producer *Trichoderma reesei* is usually addressed under this name, especially in non-taxonomic context. However, *Hypocrea jecorina* is older. To comply to the rules, a new combination *T. jecorinum* would be the valid name for the species. Similar is the situation in *H. schweinitzii* vs. *T. citrinoviride*, where a new combination *T. schweinitzii* would be required.

As a general solution for such issues a "mass conservation" procedure was implemented in the ICN. An addition to Article 14 runs (not literally): lists of names may be submitted to the General Committee, which will refer them to the Nomenclature Committee for Fungi for examination by subcommittees established by that Committee in consultation with the General Committee and appropriate international bodies. Accepted names on these lists, which become permanent as Appendices once reviewed by the Nomenclature Committee for Fungi and the General Committee, are to be listed with their types together with those competing synonyms (including sanctioned names) against which they are treated as conserved.

Several questions arise in this context: which names should be put on such a list? Those that are deemed to be important with respect to users in industry and agriculture or all names that are "in use", i.e. those where molecular data are available and meaningful? The selection of names poses several problems. It is important to be very cautious in determining the names for conservation. Conserved names cannot be changed except by a tedious procedure that may extend over up to 6 years.

Several questions need to be answered before a name can be selected:

1) Do species pairs having the same epithet in *Hypocrea* and *Trichoderma* indeed represent the same species? There are several homonyms in *Trichoderma*, which do not represent the same species, because the epithets in the other genus were not checked at the time of publication. Examples among others include *T. amazonicum* P. Chaverri & Gazis 2011 vs. *H. amazonica* M.C. Cooke 1887, *T. asperellum* Samuels *et al.* 1999 vs. *H. asperella* Starb. 1899, *H. candida* P. Chaverri & Samuels 2003 vs. *T. candidum* Alb. & Schw. 1805 and *T. candidum* P. Chaverri & Samuels 2003. The latter has already been replaced by *T. pseudocandidum* P. Chaverri, Samuels & Minnis 2009, but is younger than *H. candida* and therefore requires conservation. Also *T. crassum* is not conspecific with *H. crassa* (G.J. Samuels, 2012, pers. comm.). This also shows that it is important to keep in mind that there are still many names in *Hypocrea* and some in *Trichoderma*, before a new species is named, in order to avoid the creation of homonyms, i.e. illegitimate names.

2) Can the anamorph-teleomorph connection of a species be challenged, or: what is the confidence in a link between *Hypocrea* and *Trichoderma* having the same or different epithets?

The highest confidence offer newly established species, where a) the same epithet was published in both generic names at the same time and b) where the *Trichoderma* anamorph was directly derived from ascospores, and c) where the type of the *Trichoderma* name is the (ex-type) isolate from the holotype of the *Hypocrea* (e.g. *H. aeruginea* / *T. aerugineum*, among many others). All other situations imply less confidence in the connection and require scrutinized analyses of types and DNA data.

3) How is the *Hypocrea* name typified / how is the *Trichoderma* name typified?

4) On what grounds was the concept of a species established? How does the phylogenetic structure of the species look like? How is the robustness of the phylogenetic treatment?

To take a short look on problems addressed under 3) and 4) here are two examples (as also addressed above in another context): Samuels *et al.* (2006a) defined the species of the *T. koningii* complex rather narrowly, but *T. austrokoningii* apparently contains different elements, judging on branch lengths of the phylogenetic analysis. However, the types of the *Hypocrea* and *Trichoderma* names are derived from the same material and the names are published at the same time, which means that there is no need to conserve *T. austrokoningii*. On the other hand, Jaklitsch *et al.* (2006a) defined some species more broadly, i.e. with a complex phylogenetic substructure. While the epitype of *T. viride* is derived from the epitype of *H. rufa*, i.e. not posing a problem, the situation is different in *H./T. viridescens*: there are ca. 11 subclades and the neotype of *Eidamia viridescens*, i.e. of *T. viridescens*, is not in the same clade as the holotype of *Hypocrea viridescens*. If the phylogenetic analysis is refined and the subclades are consolidated and formally published as species, then the connection between *T. viridescens* and *H. viridescens* is not valid any more.

5) Is the phylogenetic structure and monophyly of the genus granted? May it be possible in future to remove "basal" or "jumping" species from the genus?

This raises the question whether species like e.g. *Hypocrea alcalifuscescens*, *H. cinereoflava* or *H. avellanea* should be combined in *Trichoderma* or not. This question cannot be answered before additional related taxa are detected or species borders established based on phylogenomic data for more isolates that will be available in near future.

A way how to mitigate the use of the lists planned for conservation in a strict sense, i.e. according to the Code, was published by Gams *et al.* (2012). They suggested to use the terms prioritization vs. suppression instead of conservation vs. rejection. "Among competing names for anamorph and teleomorph of the same taxon (a name pair) one will be given preference, the other is being suppressed; it remains, however, valid and legitimate but just not available. Contrasting with conservation, an act of prioritization need not be fixed for ever, if convincing arguments support a change".

A list of *Trichoderma* names for approval by the Nomenclature Committee for Fungi (NCF) has so far not been produced.

Outlook

As shown above, more and more new species are being described and many more are to be expected. Several continents have not been screened for *Trichoderma* in recent times. Many species have not been re-assessed and particularly many species established by Doi for Japan and South America need re-collection and study, but also many other species described from these regions and also North America in the eighteenth century still await re-examination and sequencing.
Southern Europe including the Canary Islands is currently being intensively studied (W.M. Jaklitsch, unpublished data), collecting both teleomorphs and anamorphs, and many new species are to be expected.

Our attention has been drawn to some recent papers, where minimalistic approaches are applied and accordingly the quality has been decreasing lately to a considerable extent. Often, incomplete sequences of phylogenetic markers are used to draw phylogenetic conclusions from. We therefore strongly advise reviewers and editors of taxonomic and also non-taxonomic journals that are intended to describe new species, to demand from the authors both complete phylogenetic markers (DNA barcodes) for at least *rpb2* and *tef1* sequences for every new species for inspection.We also invite those readers who submit sequences to GenBank under a tentative name to update the name after publication of their paper. This is important for other users of GenBank in order to reach correct identifications.

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References

Atanasova, L. and Druzhinina, I.S. (2010) Review: Global nutrient profiling by Phenotype MicroArrays: a tool complementing genomic and proteomic studies in conidial fungi. *Journal of Zhejiang University. Science B* 11, 151–168.

Atanasova, L., Jaklitsch, W.M., Komoń-Zelazowska, M., Kubicek, C.P. and Druzhinina, I.S. (2010) Clonal species *Trichoderma parareesei* sp. nov. likely resembles the ancestor of the cellulase producer *Hypocrea jecorina*/*T. reesei*. *Applied and Environmental Microbiology* 76, 7259–7267.

Birky Jr., C.W., Adams, J., Gemmel, M. and Perry J. (2010) Using population genetic theory and DNA sequences for species detection and identification in asexual organisms. *PLoS One* 5, e10609.

Bisby, G.R. (1939) *Trichoderma viride* Pers. ex Fries, and notes on *Hypocrea*. *Transactions of the British Mycological Society* 23, 149–168.

Bissett, J. (1984) A revision of the genus *Trichoderma*. I. Section *Longibrachiatum* sect. nov. *Canadian Journal of Botany* 62, 924–931.

Bissett, J. (1991a) A revision of the genus *Trichoderma*. II. Intrageneric classification. *Canadian Journal of Botany* 60, 2357–2372.

Bissett, J. (1991b) A revision of the genus *Trichoderma*. III. Section *Pachybasium*. *Canadian Journal of Botany* 69, 2373–2417.

Bissett, J. (1991c) A revision of the genus *Trichoderma*. IV. Additional notes on section *Longibrachiatum*. *Canadian Journal of Botany* 69, 2418–2420.

Bissett, J. (1992) *Trichoderma atroviride*. *Canadian Journal of Botany* 70, 639–641.

Błaszczyk, L. Popiel, D., Chełkowski, J., Koczyk, G., Samuels, G.J., Sobieralski, K. and Siwulski, M. (2011) Species diversity of *Trichoderma* in Poland. *Journal of Applied Genetics* 52, 233–243.

Bochner, B.R. (1989) Sleuthing out bacterial identities. *Nature* 339(6220), 157–158.

Buée, M., Reich, M., Murat C., Morin, E., Nilsson, R.H., Uroz, S. and Martin, F. (2009). 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist* 184, 449–456.

Castle, A., Speranzini, D., Rghei, N., Alm, G., Rinker, D. and Bissett, J. (1998) Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. *Applied and Environmental Microbiology* 64, 133–137.

Chamberlain, H.L., Rossman, A.Y., Stewart, E.L., Ulvinen, T. and Samuels, G.J. (2004) The stipitate species of *Hypocrea* (Hypocreales, Hypocreaceae) including *Podostroma*. *Karstenia* 44, 1–24.

Chaverri, P. and Samuels, G.J. (2002) *Hypocrea lixii*, the teleomorph of *Trichoderma harzianum*. *Mycological Progress* 1, 283–286.

Chaverri, P. and Samuels, G.J. (2003) *Hypocrea*/*Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae), Species with green ascospores. *Studies in Mycology* 48, 1–116.

Chaverri, P., Castlebury, L.A., Overton, B.E. and Samuels, G.J. (2003a) *Hypocrea*/*Trichoderma,* species with conidiophore elongations and green conidia. *Mycologia* 95, 1100–1140.

Chaverri, P., Castlebury, L.A., Samuels, G.J. and Geiser, D.M. (2003b) Multilocus phylogenetic structure within the *Trichoderma harzianum*/*Hypocrea lixii* complex. *Molecular Phylogenetics and Evolution* 27, 302–313.

Chaverri, P., Candoussau, F. and Samuels, G.J. (2004) *Hypocrea phyllostachydis* and its *Trichoderma* anamorph, a new bambusicolous species from France. *Mycological Progress* 3, 29–36.

Chaverri, P., Gazis, R.O. and Samuels, G.J. (2011) *Trichoderma amazonicum*, a new endophytic species on *Hevea brasiliensis* and *H. guianensis* from the Amazon basin. *Mycologia* 103, 139–151.

Cutler, H.G., Cutler, S.J., Ross, S.A., Sayed, K.E., Dugan, F.M., Bartlett, M.G., Hill, A.A., Hill, R.A. and Parker, S.R. (1999) Koninginin G, a new metabolite from *Trichoderma aureoviride*. *Journal of Natural Products* 62, 137–139.

Danielson, R.M. and Davey, C.B. (1973) The abundance of *Trichoderma* propagules and the distribution of species in forest soils. *Soil Biology & Biochemistry* 5, 485–494.

De Respinis, S., Vogel, G., Benagli, C., Tonolla, M., Petrini, O., Samuels, G.J. (2010) MALDI-TOF MS of *Trichoderma*: a model system for the identification of microfungi. *Mycological Progress* 9, 79–100.

Degenkolb, T., Dieckmann, R., Nielsen, K.F., Gräfenhan, T., Theis, C., Zafari, D., Chaverri, P., Ismaiel, A., Brückner, H., von Döhren, H., Thrane, U., Petrini, O., Samuels, G.J. (2008a) The *Trichoderma brevicompactum* clade: a separate lineage with new species, new peptaibiotics and mycotoxins. *Mycological Progress* 7, 177–219.

Dingley, J.M. (1957) Life history studies in the genus *Hypocrea Fr. Transactions of the Royal Society of New Zealand* 84, 689–693.

Dobranic, J.K. and Zak, J.C. (1999) A microtiter plate procedure for evaluating fungal functional diversity. *Mycologia* 91, 756–765.

Dodd, S.L., Crowhurst, R.N., Rodrigo, A.C., Samuels, G.J., Hill, R.A. and Stewart, A. (2000) Examination of *Trichoderma* phylogenies derived from ribosomal DNA sequence data. *Mycological Research* 104, 23–34.

Dodd, S.L., Lieckfeldt, E., Chaverri, P., Overton, B.E. & Samuels, G.J. (2002) Taxonomy and phylogenetic relationships of two species of *Hypocrea* with *Trichoderma* anamorphs. *Mycological Progress* 1, 409–428.

Dodd, S.L., Lieckfeldt, E. and Samuels, G.J. (2003) *Hypocrea atroviridis* sp. nov., the teleomorph of *Trichoderma atroviride*. *Mycologia* 95, 27–40.

Doi, Y. (1972) Revision of the Hypocreales with cultural observations IV. The genus *Hypocrea* and its allies in Japan (2) Enumeration of the species. *Bulletin of the National Science Museum. Series B, Botany* 15, 649–751.

Druzhinina, I.S., Chaverri, P., Fallah, P., Kubicek, C.P. and Samuels, G.J. (2004) *Hypocrea flaviconidia*, a new species from Costa Rica with yellow conidia. *Studies in Mycology* 50, 401–407.

Druzhinina, I. and Kubicek, C.P. (2005) Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters? *Journal of Zhejiang University. Science B* 6, 100–112.

Druzhinina, I.S., Kopchinskiy, A.G., Komoń, M., Bissett, J., Szakacs, G. and Kubicek, C.P. (2005) An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea. Fungal Genetics and Biology* 42, 813–828.

Druzhinina, I.S. and Kopchinskiy, A.G. (2006) TrichOKEY v. 2-a DNA oligonucliotide BarCode program for the identification of multiple sequences of *Hypocrea* and *Trichoderma*. In: Proceedings of the 8th International Mycological Congress, Cairns, Australia, DVD.

Druzhinina, I.S., Kopchinskiy A.G. and Kubicek C.P. (2006) The first 100 *Trichoderma* species characterized by molecular data. *Mycoscience* 47, 55–64.

Druzhinina, I.S., Komoń-Zelazowska, M., Kredics, L., Hatvani, L., Antal, Z., Belayneh, T. and Kubicek, C.P. (2008) Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. *Microbiology* 154, 3447–3459.

Druzhinina, I.S., Kubicek, C.P., Komoń-Zelazowska, M., Mulaw, T.B. and Bissett, J. (2010a) The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. *BMC Evolutionary Biology* 10, 94.

Druzhinina, I.S., Komoń-Zelazowska, M., Atanasova, L., Seidl, V. and Kubicek, C.P. (2010b) Evolution and ecophysiology of the industrial producer *Hypocrea jecorina* (Anamorph *Trichoderma reesei*) and a new sympatric agamospecies related to it. *PLoS One* 5, e9191.

Druzhinina, I.S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B.A., Kenerley, C.M., Monte, E., Mukherjee, P.K., Zeilinger, S., Grigoriev, I.V. and Kubicek, C.P. (2011) *Trichoderma*: the genomics of opportunistic success. *Nature reviews. Microbiology* 9, 749–759.

Druzhinina, I.S., Komoń-Zelazowska, M., Ismaiel, A., Jaklitsch, W., Mullaw, T., Samuels, G.J. and Kubicek, C.P. (2012) Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma*. *Fungal Genetics and Biology* 49, 358–368.

Druzhinina, I. S. and Kubicek, C.P. (2013) Ecological genomics of *Trichoderma*. In: F. Martinn (ed.) *Ecological genomics of fungi*. Wiley-Blackwell, Oxford, UK, in press.

Finlay, B.J. (2002) Global dispersal of free-living microbial eukaryotic species. *Science* 296(5570), 1061–1063.

Friedl, M.A. and Druzhinina, I.S. (2012) Taxon-specific metagenomics of *Trichoderma* reveals a narrow community of opportunistic species that regulate each other's development. *Microbiology* 158, 69–83.

Fujimori, F. and Okuda, T. (1994) Application of the random amplified polymorphic DNA using the polymerase chain reaction for efficient elimination of duplicate strains in microbial screening. I. Fungi. *The Journal of Antibiotics (Tokyo)* 47, 173–182.

Gal-Hemed, I., Atanasova, L., Komon-Zelazowska, M., Druzhinina, I.S., Viterbo, A. and Yarden, O. (2011) Marine isolates of *Trichoderma* spp. as potential halotolerant agents of biological control for arid-zone agriculture. *Applied and Environmental Microbiology* 77, 5100–5109.

Gams, W. and Bissett, J. (1998) Morphology and identification of *Trichoderma*. In: Kubicek, C.P., Harman, G.E. (eds.) *Trichoderma* and *Gliocladium*. Basic Biology, Taxonomy and Genetics, Taylor & Francis, London, UK, pp. 3–34.

Gams, W., Humber, R.A., Jaklitsch, W., Kirschner, R., Stadler, M. (2012) Minimizing the chaos following the loss of Article 59: Suggestions for a discussion. *Mycotaxon* 119, 495–507.

Gautheret, A., Dromer, F., Bourhis, J.H. and Andremont, A. (1995) *Trichoderma pseudokoningii* as a cause of fatal infection in a bone marrow transplant recipient. *Clinical Infectious Diseases* 20, 1063–1064.

Gherbawy, Y., Druzhinina, I.S., Shaban, G.M., Wuczkowsky, M., Yaser, M., El-Naghy, M.A., Prillinger, H.-J. and Kubicek, C.P. (2004) *Trichoderma* populations from alkaline agricultural soil in the Nile valley, Egypt, consist of only two species. *Mycological Progress* 3, 211–218.

Grill, A., Casula, P., Lecis, R. and Menken, S.B.J. (2006) Endemism in Sardinia. In: Weiss, S., Ferrand, N. (eds.) Phylogeography of Southern European Refugia. Springer, Dordrecht, the Netherlands, pp. 273–296.

Hagn, A., Wallisch, S., Radl, V., Charles Munch, J. and Schloter, M. (2007) A new cultivation independent approach to detect and monitor common *Trichoderma* species in soils. *Journal of Microbiological Methods* 69, 86–92.

Hanada, R.E., de Jorge Souza, T., Pomella, A.W., Hebbar, K.P., Pereira, J.O., Ismaiel, A. and Samuels, G.J. (2008) *Trichoderma martiale* sp. nov., a new endophyte from sapwood of *Theobroma* cacao with a potential for biological control. *Mycological Research* 112, 1335–1343.

Harman, G.E. (2000) Myths and dogmas of biocontrol. Changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Disease* 84, 377–393.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. and Lorito. M. (2004) *Trichoderma* species - opportunistic, avirulent plant symbionts. *Nature reviews. Microbiology* 2, 43–56.

Hasan, M.M., Rahman, S.M., Kim, G.H., Abdallah, E. and Oh, D.H. (2012) Antagonistic potentiality of *Trichoderma harzianum* towards seed-borne fungal pathogens of winter wheat cv. *Protiva* in vitro and in vivo. *Journal of microbiology and biotechnology* 22, 585-591.

Hatvani, L., Antal, Z., Manczinger, L., Szekeres, A., Druzhinina, I.S., Kubicek, C.P., Nagy, A., Nagy, E., Vágvölgyi, C. and Kredics, L. (2007) Green Mold Diseases of *Agaricus* and *Pleurotus* spp. Are Caused by Related but Phylogenetically Different *Trichoderma* Species. *Phytopathology* 97, 532–537.

Hoyos-Carvajal, L., Orduz, S. and Bissett, J. (2009) Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions. *Fungal Genetics and Biology* 46, 615–631.

Humphris, S.N., Bruce, A., Buultjens, E. and Wheatley, R.E. (2002) The effects of volatile microbial secondary metabolites on protein synthesis in Serpula lacrymans. *FEMS Microbiology Letters* 210, 215–219.

Jaklitsch, W.M., Komon, M., Kubicek, C.P. and Druzhinina, I.S. (2005) *Hypocrea voglmayrii* sp. nov. from the Austrian Alps represents a new phylogenetic clade in *Hypocrea/Trichoderma*. *Mycologia* 97, 1365–1378.

Jaklitsch, W.M., Samuels, G.J., Dodd, S.L., Lu, B.S. and Druzhinina, I.S. (2006a) *Hypocrea rufa*/*Trichoderma viride*: a reassessment, and description of five closely related species with and without warted conidia. *Studies in Mycology* 56, 135–177.

Jaklitsch, W.M., Komon, M., Kubicek, C.P. and Druzhinina, I.S. (2006b) *Hypocrea crystalligena* sp. nov., a common European species with a white-spored *Trichoderma* anamorph. *Mycologia* 98, 499–513.

Jaklitsch, W.M., Kubicek, C.P. and Druzhinina, I.S. (2008a) Three European species of *Hypocrea* with reddish brown stromata and green ascospores. *Mycologia* 100, 796–815.

Jaklitsch, W.M., Põldmaa, K. and Samuels, G.J. (2008b) Reconsideration of *Protocrea* (Hypocreales, Hypocreaceae). Mycologia 100, 962–984.

Jaklitsch, W.M., Gruber, S., Voglmayr, H. (2008c) *Hypocrea seppoi*, a new stipitate species from Finland. *Karstenia* 48, 1–11.

Jaklitsch, W.M. (2009) European species of *Hypocrea* Part I. The green-spored species. *Studies in Mycology* 63, 1–91.

Jaklitsch, W.M. (2011) European species of *Hypocrea* Part II: species with hyaline ascospores. *Fungal diversity* 48, 1–250.

Jaklitsch, W.M., Stadler, M. and Voglmayr, H. (2012) Blue pigment in *Hypocrea caerulescens* sp. nov. and two additional new species in sect. *Trichoderma*. *Mycologia* 104, 925–941.

Jaklitsch W.M. and Voglmayr H. (2012) *Hypocrea britdaniae* and *H. foliicola*: two remarkable new European species. Mycologia 105, 1213–1221.

Komon-Zelazowska, M., Bissett, J., Zafari, D., Hatvani, L., Manczinger, L., Woo, S., Lorito, M., Kredics, L., Kubicek, C.P. and Druzhinina, I.S. (2007) Genetically closely related but phenotypically divergent *Trichoderma* species cause green mold disease in oyster mushroom farms worldwide. *Applied and Environmental Microbiology* 73, 7415–7426.

Kim, C.S., Shirouzu, T., Nakagiri, A., Sotome, K., Nagasawa, E. and Maekawa, N. (2012) *Trichoderma mienum* sp. nov., isolated from mushroom farms in Japan. *Antonie Van Leeuwenhoek* 102, 629–641.

Kindermann, J., El-Ayouti, Y., Samuels, G.J. and Kubicek, C.P. (1998) Phylogeny of the genus *Trichoderma* based on sequence analysis of the internal transcribed spacer region 1 of the rDNA cluster. *Fungal Genetics and Biology* 24, 298–309.

Kopchinskiy, A.G., Komon, M., Kubicek, C.P. and Druzhinina I. (2005) TrichoBLAST: A multilocus database for *Trichoderma* and *Hypocrea identifications*. *Mycological Research* 109, 657-660.

Kovacs, K., Szakacs, G., Pusztahelyi, T. and Pandey, A. (2004) Production of chitinolytic enzymes with *Trichoderma longibrachiatum* IMI 92027 in solid substrate fermentation. *Applied Biochemistry and Biotechnology* 118, 189–204.

Kraus, G., Druzhinina, I., Bissett, J., Zafari, D., Prillinger, H.J., Szakacs, G., Zare, R., Gams, W. and Kubicek, C.P. (2004) *Trichoderma brevicompactum* sp. nov. *Mycologia* 96, 1057–1071.

Kredics, L., Antal, Z., Dóczi, I., Manczinger, L., Kevei, F. and Nagy, E. (2003) Clinical importance of the genus *Trichoderma*. A review. *Acta Microbiologica et Immunologica Hungarica* 50, 105–117.

Kubicek, C.P., Bissett, J., Druzhinina, I., Kullnig-Gradinger, C. and Szakacs, G. (2003) Genetic and metabolic diversity of *Trichoderma*: a case study on South-East Asian isolates. *Fungal Genetics and Biology* 38, 310–319.

Kubicek, C.P., Komon-Zelazowska, M. and Druzhinina, I.S. (2008) Fungal genus *Hypocrea*/*Trichoderma*: from barcodes to biodiversity. *Journal of Zhejiang University. Science* 9, 753–763.

Kubicek, C.P., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D.A., Druzhinina, I.S.,Thon, M., Zeilinger, S., Casas-Flores, S., Horwitz, B.A., Mukherjee, P.K., Mukherjee, M.,Kredics, L., Alcaraz, L.D., Aerts, A., Antal, Z., Atanasova, L., Cervantes-Badillo, M.G.,Challacombe, J., Chertkov, O., McCluskey, K., Coulpier, F., Deshpande, N., von Döhren, H.,Ebbole, D.J., Esquivel-Naranjo, E.U., Fekete, E., Flipphi, M., Glaser, F., Gómez-Rodríguez, E.Y., Gruber, S., Han, C., Henrissat, B., Hermosa, R., Hernández-Oñate, M., Karaffa, L., Kosti, I., Le Crom, S., Lindquist, E., Lucas, S., Lübeck, M., Lübeck, P.S., Margeot, A., Metz, B., Misra, M., Nevalainen, H., Omann, M., Packer, N., Perrone, G., Uresti-Rivera, E.E., Salamov, A., Schmoll, M., Seiboth, B., Shapiro, H., Sukno, S., Tamayo-Ramos, J.A., Tisch, D., Wiest, A.,Wilkinson, H.H., Zhang, M., Coutinho, P.M., Kenerley, C.M., Monte, E., Baker, S.E. and Grigoriev, I.V. (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. *Genome biology* 12, R40.

Kuhls, K., Lieckfeldt, E., Samuels, G.J., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Börner, T., and Kubicek, C.P. (1996) Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. *Proceedings of the National Academy of Sciences of the USA* 93, 7755–7760.

Kuhls, K., Lieckfeldt, E., Samuels, G.J., Börner, T., Meyer, W., and Kubicek, C.P. (1997) Revision of *Trichoderma* sect. *Longibrachiatum* including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. *Mycologia* 89, 442–460.

Kullnig, C.M., Szakacs, G. and Kubicek, C.P. (2000) Molecular identification of *Trichoderma* species from Russia, Siberia and the Himalaya. *Mycological Research* 104, 1117–1125.

Kullnig-Gradinger, C.M., Szakacs, G. and Kubicek, C.P. (2002) Phylogeny and evolution of the fungal genus *Trichoderma*—a multigene approach. *Mycological Research* 106, 757–767.

Kyalo, G., Affokpon, A., Coosemans, J. and Coynes, D.L. (2007) Biological control effects of *Pochonia chlamysdosporia* and *Trichoderma* isolates from Benin (West-Africa) on root-knot nematodes. *Communications in Agricultural and Applied Biological Sciences* 72, 219–223.

Leuchtmann, A., Petrini, O., and Samuels, G.J. (1996) Isozyme subgroups in *Trichoderma* section *Longibrachiatum*. *Mycologia* 88, 384–394.

Lieckfeldt, E., Kuhls K. and Muthumeenakshi S. (1998a) Molecular Taxonomy of *Trichoderma* and *Gliocladium* and their Teleomorphs. In: Kubicek, C.P., Harman, G.E. (ed.) *Trichoderma* and *Gliocladium*. Basic Biology, Taxonomy and Genetics, Taylor & Francis, London, UK, pp. 35–56.

Lieckfeldt, E., Samuels, G.J., Börner, T. & Gams W. (1998b) *Trichoderma koningii*: neotypification and *Hypocrea* teleomorph. *Canadian Journal of Botany* 76, 1507–1522.

Lieckfeldt, E., Samuels, G.J., Nirenberg, H.I., Petrini, O. (1999) A morphological and molecular perspective of *Trichoderma viride*: is it one or two species? *Applied and Environmental Microbiology*,65, 2418-2428.

Lieckfeldt, E. and Seifert, K.A. (2000) An evaluation of the use of ITS sequences in the taxonomy of the Hypocreales. *Studies in Mycology* 45, 35–44.

Lieckfeldt, E., Kullnig, C.M., Kubicek, C.P., Samuels, G.J. and Börner, T. (2001) *Trichoderma aureoviride*: phylogenetic position and characterization. *Mycological Research* 105, 313–322.

Lim, Y.W., Kim, B. K., Kim,C., Jung, H.S., Kim, B.S., Lee, J.H. and Chun, J. (2010) Assessment of soil fungal communities using pyrosequencing. *Journal of microbiology (Seoul, Korea)* 48, 284–289.

Liu, P.-G., Doi, Y., Wang, X.-H. and Wang, Q.-B. (2000) The Hypocreaceae of China III. Some fungicolous species of the genus *Hypocrea*. *Mycosystema* 19, 317–327.

Liu, Y.L., Whelen, S. and Hall, B.D. (1999) Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* 16, 1799–1808.

Liu, M., Liu, J. and Wang, W.M. (2012) Isolation and functional analysis of Thmfs1, the first major facilitator superfamily transporter from the biocontrol fungus *Trichoderma harzianum*. *Biotechnology letters* 34,1857-1862.

Lu, B., Samuels, G.J. (2003) *Hypocrea stilbohypoxyli* and its *Trichoderma koningii*-like anamorph: a new species from Puerto Rico on *Stilbohypoxylon moelleri*. *Sydowia* 55, 255–266.

Lu, B., Druzhinina, I.S., Fallah, P., Chaverri, P., Gradinger, C., Kubicek, C.P. and Samuels, G.J. (2004) *Hypocrea*/*Trichoderma* species with pachybasium-like conidiophores: teleomorphs for *T. minutisporum* and *T. polysporum* and their newly discovered relatives. *Mycologia* 96, 310–342.

Martinez, D., Berka, R.M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S.E., Chapman, J., Chertkov, O., Coutinho, P.M., Cullen, D., Danchin, E.G., Grigoriev, I.V., Harris, P., Jackson, M., Kubicek, C.P., Han, C.S., Ho, I., Larrondo, L.F., de Leon, A.L., Magnuson, J.K., Merino, S., Misra, M., Nelson, B., Putnam, N., Robbertse, B., Salamov, A.A., Schmoll, M., Terry, A., Thayer, N.,Westerholm-Parvinen, A., Schoch, C.L., Yao, J., Barabote, R., Nelson, M.A., Detter, C., Bruce, D., Kuske, C.R., Xie, G., Richardson, P., Rokhsar, D.S., Lucas, S.M., Rubin, E.M., Dunn-Coleman, N., Ward, M. and Brettin, T.S. (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*) *Nature Biotechnology* 26, 553–560.

Médail, F. and Quézal, P. (1999) Biodiversity hotspots in the Mediterranean Basin: setting global conservation priorities. *Conservation biology: the Journal of the Society for Conservation Biology* 13, 1510–1513.

Meincke, R., Weinert, N., Radl, V., Schloter, M., Smalla, K. and Berg, G. (2010) Development of a molecular approach to describe the composition of *Trichoderma* communities. *Journal of Microbiological Methods* 80, 63–69.

Migheli, Q., Balmas, V., Komoñ-Zelazowska, M., Scherm, B., Fiori, S., Kopchinskiy, A.G., Kubicek, C.P. and Druzhinina, I.S. (2009) Soils of a Mediterranean hot spot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European, invasive species of *Hypocrea*/*Trichoderma*. *Environmental Microbiology* 11, 35–46.

Minnis, A.M., Samuels, G.J. and Chaverri, P. (2009) Three nomenclatural corrections for species of *Hypocrea*/*Trichoderma*. *Mycotaxon* 109, 245–246.

Mulaw, T.B., Kubicek, C.P. and Druzhinina, I.S. (2010) The Rhizosphere of *Coffea Arabica* in Its Native Highland Forests of Ethiopia Provides a Niche for a Distinguished Diversity of *Trichoderma*. *Diversity* 2, 527–549.

Muthumeenakshi, S., Mills, P.R., Brown, A.E. and Seaby, D.A. (1994) Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in British Isles. *Microbiology* 140, 769–777.

Nagy, V., Seidl, V., Szakacs, G., Komoń-Zelazowska, M., Kubicek, C.P. and Druzhinina, I.S. (2007) Application of DNA bar codes for screening of industrially important fungi: the haplotype of *Trichoderma harzianum* sensu stricto indicates superior chitinase formation. *Applied and Environmental Microbiology* 73, 7048–7058.

Okuda, T., Fujiwara, A. and Fujiwara, M. (1982) Correlation between species of *Trichoderma* and production patterns of isonitrile antibiotics. *Agricultural and Biological Chemistry* 46, 1811–1822.

Overton, B.E., Stewart, E.L., Geiser, D.M. and Jaklitsch, W.M. (2006a) Systematics of *Hypocrea citrina* and allies. *Studies in Mycology* 56, 1–38.

Overton, B.E., Stewart, E.L. and Geiser, D.M. (2006b) Taxonomy and phylogenetic relationships of nine species of *Hypocrea* with anamorphs assignable to Trichoderma section *Hypocreanum*. *Studies in Mycology* 56, 39–65.

Park, M.S., Bae, K.S. and Yu, S.H. (2004) Molecular and morphological analysis of *Trichoderma* isolates associated with green mold epidemic of oyster mushroom in Korea. *Journal of Huazhong Agricultural University* 23, 157–164.

Park, M.S., Bae, K.S., Yu, S.H. (2006) Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. *Mycobiology* 34, 111–113.

Paz, Z., Komon-Zelazowska, M., Druzhinina, I.S., Aveskamp, M.M., Shnaiderman, A., Aluma, Y., Carmeli, S., Ilan, M. and Yarden, O. (2010) Diversity and potential antifungal properties of fungi associated with a Mediterranean sponge. *Fungal Diversity* 42, 17–26.

Persoon, C.H. (1794) Disposita methodica fungorum. *Römer's Neues Magazin für die Botanik* 1, 81–128.

Rehner, S.A., and Samuels, G.J. (1994) Taxonomy and phylogeny of *Gliocladium* analyzed by large subunit rDNA sequences. *Mycological Research* 98, 625–634.

Rehner, S.A. and Samuels, G.J. (1995) Molecular systematics of the Hypocreales, a teleomorph gene phylogeny and the status of their anamorphs. *Canadian Journal of Botany* 73, S816–S823.

Rifai, M.A. (1969) A revision of the genus *Trichoderma*. *Mycological Papers* 116, 1–56.

Samuels, G.J. and Seifert, K.A. (1987). Taxonomic implications of variation among hypocrealean anamorphs. In: J. Sugiyama (ed.) *Pleomorphic fungi: the diversity and its taxonomic implications*. Kodansha Ltd., Tokyo and Elsevier, Amsterdam, pp. 29-56.

Samuels, G.J., Petrini, O. and Manguin, S. (1994) Morphological and macromolecular characterization of *Hypocrea schweinitzii* and its *Trichoderma* anamorph. *Mycologia* 86, 421–435.

Samuels, G.J., Petrini, O., Kuhls, K., Lieckfeldt, E. and Kubicek, C.P. (1998) The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *Longibrachiatum*. *Studies in Mycology* 41, 1–54.

Samuels, G.J., Dodd, S.L., Gams, W., Castlebury, L.A. and Petrini, O. (2002) *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. *Mycologia* 94, 146–170.

Samuels, G.J. (2006) *Trichoderma*: systematics, the sexual state, and ecology. *Phytopathology* 96, 195–206.

Samuels, G.J., Dodd, S., Lu, B.-S., Petrini, O., Schroers, H.-J. and Druzhinina, I.S. (2006a) The *Trichoderma koningii* aggregate species. *Studies in Mycology* 56, 67–133.

Samuels, G.J., Suarez, C., Solis, K., Holmes, K.A., Thomas, S.E., Ismaiel, A. and Evans, H.C. (2006b) *Trichoderma theobromicola* and *T. paucisporum*: two new species isolated from cacao in South America. *Mycological Research* 110, 381–392.

Samuels, G.J. and Ismaiel, A. (2009) *Trichoderma evansii* and *T. lieckfeldtiae*: two new T. *hamatum*-like species. *Mycologia* 101,142-56.

Samuels, G.J., Ismaiel, A., Bon, M.C., De Respinis, S. and Petrini, O. (2010) *Trichoderma asperellum* sensu lato consists of two cryptic species. *Mycologia* 102, 944–966.

Samuels, G.J. and Ismaiel, A. (2011) *Hypocrea peltata*: a mycological Dr Jekyll and Mr Hyde? *Mycologia* 103, 616–630.

Samuels, G.J., Ismaiel, A., Mulaw, T.B., Szakacs, G., Druzhinina, I.S., Kubicek, C.P. and Jaklitsch, W.M. (2012a) The *Longibrachiatum* Clade of *Trichoderma*: a revision with new species. *Fungal Diversity* 55, 77–108.

Samuels, G.J., Ismaiel, A., de Souza, J. and Chaverri, P. (2012b) *Trichoderma stromaticum* and its overseas relatives. *Mycological Progress* 11, 215–254.

Seidl, V., Seibel, C., Kubicek, C.P. and Schmoll, M. (2009) Sexual development in the industrial workhorse *Trichoderma reesei*. *Proceedings of the National Academy of Sciences of the USA* 106, 13909–13914.

Shoresh, M. and Harman, G.E. (2008) The molecular basis of shoot responses of maize seedlings to *Trichoderma harzianum* T22 inoculation of the root: a proteomic approach. *Plant Physiology* 147, 2147–2163.

Sinden, J., and Hauser, E. (1953) Nature and control of three mildew diseases of mushrooms in America. *Mushroom Science.* 2, 177–180.

Stasz, T. E., Harman, G. E., and Weeden, N. F. (1988) Protoplast preparation and fusion in two biocontrol strains of *Trichoderma harzianum*. *Mycologia* 80, 141–150.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S. and Fisher, M.C. (2000) Phylogenetic species recognition and species concepts in Fungi. *Fungal Genetics and Biology* 31, 21–31.

Thrane, U., Poulsen, S.B., Nirenberg, H.I. and Lieckfeldt, E. (2001) Identification of *Trichoderma* strains by image analysis of HPLC chromatograms. *FEMS Microbiology Letters* 203, 249–255.

Viterbo, A. and Chet, I. (2006) TasHyd1, a new hydrophobin gene from the biocontrol agent *Trichoderma asperellum*, is involved in plant root colonization. *Molecular Plant Pathology* 7, 249–258.

Wey, T.T., Hseu, T.H. and Huang, L. (1994) Molecular cloning and sequence analysis of the cellobiohydrolase I gene from *Trichoderma koningii* G-39. *Current Microbiology* 28, 31–39.

Woo, S.L., Di Benedetto, P., Senatore, M., Abadi, K., Gigante, S., Soriente, I., Ferraioli, S., Scala, F. and Lorito, M. (2004) Identification and characterization of *Trichoderma* species aggressive to *Pleurotus* in Italy. Journal of Zhejiang University (Agriculture & Life Sciences) 30, 469–470.

Wuczkowski, M., Druzhinina, I., Gherbawy, Y., Klug, B., Prillinger, H. and Kubicek, C.P. (2003) Species pattern and genetic diversity of *Trichoderma* in a mid-European, primeval floodplain-forest. *Mycological Research* 158, 125–133.

Yedidia, I., Benhamou, N. and Kapulnik, Y. and Chet, I. (2000) Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-203. *Plant Physiology and Biochemistry* 38, 863–873.

Yedidia, I., Srivastva, A.K., Kapulnik, Y. and Chet, I. (2001) Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant Soil* 235, 235–242.

Zachow, C., Berg, C., Müller, H., Meincke, R., Komon-Zelazowska, M., Druzhinina, I.S., Kubicek, C.P. and Berg, G. (2009) Fungal diversity in the rhizosphere of endemic plant species of Tenerife (Canary Islands): relationship to vegetation zones and environmental factors. *The ISME Journal* 3, 79–92.

Zhang, C.L., Druzhinina, I.S., Kubicek, C.P. and Xu, T. (2005) *Trichoderma* biodiversity in China: evidence for a North to South distribution of species in East Asia. *FEMS Microbiology Letters* 251, 251–257.

Zhang, C.L., Liu, S.P., Lin, F.C., Kubicek, C.P. and Druzhinina, I.S. (2007) *Trichoderma* taxi sp. nov., an endophytic fungus from Chinese yew *Taxus mairei*. *FEMS Microbiology Letters* 270, 90–96.

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E-mail: jzus@zju.edu.cn

Review:

Global nutrient profiling by Phenotype MicroArrays: a tool complementing genomic and proteomic studies in conidial fungi*

Lea ATANASOVA, Irina S. DRUZHININA[†‡]

(*Research Area of Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, A-1060 Vienna, Austria*)

[†]E-mail: druzhini@mail.zserv.tuwien.ac.at

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Abstract: Conidial fungi or molds and mildews are widely used in modern biotechnology as producers of antibiotics and other secondary metabolites, industrially important enzymes, chemicals and food. They are also important pathogens of animals including humans and agricultural crops. These various applications and extremely versatile natural phenotypes have led to the constantly growing list of complete genomes which are now available. Functional genomics and proteomics widely exploit the genomic information to study the cell-wide impact of altered genes on the phenotype of an organism and its function. This allows for global analysis of the information flow from DNA to RNA to protein, but it is usually not sufficient for the description of the global phenotype of an organism. More recently, Phenotype MicroArray (PM) technology has been introduced as a tool to characterize the metabolism of a (wild) fungal strain or a mutant. In this article, we review the background of PM applications for fungi and the methodic requirements to obtain reliable results. We also report examples of the versatility of this tool.

Key words: Biolog Phenotype MicroArray, Mitosporic fungi, Carbon metabolism, *Trichoderma*, *Aspergillus*, Biotechnology
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1 Introduction

Conidial fungi (mitosporic Dikarya, also informally called imperfect fungi or Fungi Imperfecti), commonly known as molds and mildews, represent a diverse and economically important biological group of organisms that include animal and plant pathogens, as well as organisms used in biotechnology. Some conidial fungi are responsible for the production of industrially important enzymes, antibiotics, secondary metabolites and foods (e.g., cheeses, sake, and soya sauce), whereas others produce toxins contaminating food products and/or resulting in food spoilage. Conidial fungi are capable of metabolizing a wide variety of nutrients. This characteristic has been exploited extensively to study metabolic pathways and their regulation in model fungi such as *Aspergillus nidulans* and *Neurospora crassa*. Their importance for humankind has consequently led to an increasing number of fungi for which genomic sequences are now available (>130 at http://www.ncbi.nlm.nih.gov/genomeprj as noticed on Dec. 15, 2009). Genome sequencing, however, is only the first step towards the identification and validation of the function of the genes of an organism. To understand the information stored in the genome, one must understand the production of knockout mutants and the consequence for the organism biology, learn the conditions for the expression of the gene, and have knowledge of the stability, compartmentation, and modification of the gene's translation product. To this end, "high-throughput methods", capable of studying all these traits, have and are being developed. For many conidial fungi, facilitated methods for production of knockout mutants have already been established

‡ Corresponding author
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(Sweigard *et al*., 1999; Chaveroche *et al*., 2000; Hamer *et al*., 2001), but new approaches for wide-domain functional characterization of the gene of interest need further elaboration.

In addition to investigation systems, which focus on specific genes, pathways, or processes, functional genomic methods attempt to study the impact of altered genes on the phenotype of the organism, using technologies that provide a cell-wide perspective. The most well known "global" approaches are DNA-microarrays for transcriptomics (Fodor *et al*., 1993; Kahmann and Basse, 2001) and two-dimensional gel electrophoresis (O'Farrell, 1975) for proteomics. These technologies allow for global analysis of the information flow from DNA to RNA to protein. To comprehend, however, how the gene initially is encoded in the genome is ultimately displayed at the cellular level; the phenotype must be considered. Phenotype is the manifested attribute of an organism, the joint product of its genes and their environment during ontogeny. As such, phenotypes are the ultimate goal in strain improvement in biotechnology during the screening of fungi for new processes or products. Therefore, a (semi)high-throughput and comprehensive phenotypic assay would greatly accelerate a functional genomic approach.

Since macronutrients are the major determinants of the fungal phenotype, a comprehensive profiling system should ideally include the maximum number of various nutrient sources in a single assay. This would contain some hundred carbon sources, and fifty to hundred nitrogen sources, sulphur and phosphorous sources, and eventually also include different pH and aeration conditions. Such a maximized system could be prepared in 5–7 microtiter plates with each well containing a standard medium specific for one particular phenotype (e.g., utilization of a sucrose), and allows the quantitative analysis of growth and/or metabolism. Unfortunately, custom synthesis of this type of comprehensive phenotype analysis system is laborious, and probably only feasible with the aid of laboratory robotics. The Phenotype MicroArray™ (PM) system (Bochner *et al*., 2001; Bochner, 1988; 2003) (Biolog Inc., Hayward, CA), however, offers a reasonable compromise and has frequently been used for the analysis of bacteria and yeast mutant strains (http://www.biolog.com/mID_section_13.html for bibliography). Kubicek *et al*. (2003) applied carbon source PMs to soil fungi, thereby detecting species-diagnostic characters in a collection of *Trichoderma* (teleomorph *Hypocrea*) isolates from Southeast Asia. Tanzer *et al*. (2003) demonstrated how global nutrient utilization analysis can be used to elucidate the effects of either genetic alterations or chemical treatments on *Aspergillus nidulans*, *Aspergillus fumigatus*, *Magnaporthe grisea*, and *Mycosphaerella graminicola*. In addition, Druzhinina *et al*. (2006) have applied PMs to investigate phenotypes of various wild-type and mutant strains of *Hypocrea jecorina* (anamorph *Trichoderma reesei*). The results presented in these three studies demonstrate that PMs indeed offer an ideal complement for the phenotypic characterization of gene knockout strains towards understanding genomic and proteomic data.

Here we review the applications of PMs to conidial fungi, and discuss the utility of this system for the high-throughput analysis of global phenotypes and utilization of particular nutrients.

2 Principle and reliability of Phenotype MicroArrays

During the initiative on functional genomics of *Saccharomyces cerevisiae* as a model system, two groups pioneered the testing of a large number of strains against 96 (Ross-Macdonald *et al*., 1999) or 288 (Rieger *et al*., 1997; 1999) phenotypes. In these studies, growth of yeast strains was tested on agar surface. It was problematic because scoring of growth on agar is prone to subjectivity and difficult to scale for high-throughput analysis. Several other researchers have tested their strains by cultivating them on a hundred or more different nutrient media compositions. Bochner (1989) first demonstrated a technique for global analysis of cellular phenotypes using microplates. He advocated the testing of phenotypes using cell respiration as a reporter system. The principle of this assay is the use of a tetrazolium dye (tetrazolium violet), which is reduced by the action of succinate dehydrogenase, enabling the quantification of respiration. Reduction of this dye results in formation of a purple color with a maximum absorbance at 490 nm. The reaction is essentially irreversible; therefore, the resulting dye accumulates in the well over a period of incubation, amplifying the signal and

integrating the amount of respiration over time. Although this assay works well in prokaryotes and yeasts (Bochner *et al*., 2001; Singh, 2009), color formation in imperfect fungi does not always coincide with growth (Fig. 1). Consequently, both Tanzer *et al*. (2003) and Druzhinina *et al*. (2006) quantified growth by mycelial production reading the optical density at 750 nm (OD_{750}).

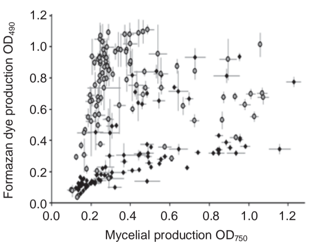

Fig. 1 Correlation between formazan dye production and mycelial density

Formazan dye production was estimated as difference between optical density (OD) values at 490 and 750 nm. Mycelial density directly corresponds to the OD value at 750 nm. Grey circles show the result of the experiment with *Hypocrea jecorina* QM 6a, and black diamonds indicate values from an experiment with *Hypocrea atroviridis* P1 strain. Vertical and horizontal error bars illustrate standard deviation calculated for three independent experiments per each strain

To achieve reproducible OD_{750} measurements for certain fungal species, both the media for inoculum production and inoculum concentration had to be optimized. As an example, for *A. nidulans* and *M. graminicola*, the inoculum densities that resulted in the least variation ranged from 1.25×10^5 to 5×10^5 spores/ml, while for *M. grisea* the optimal inoculum concentration was strictly at 4×10^5 spores/ml (Tanzer *et al*., 2003).

Furthermore, contrary to endpoint assays absorbance data need to be collected over the incubation period to generate complete growth curves for each nutrient source. This is necessary because, for example, different carbon sources result in different growth kinetics (Fig. 2), and assessing growth only at a single time point would eventually be indicative of the early growth phase in one case and the phase of already terminated growth in another.

Fig. 2 Four types of growth patterns revealed for *Hypocrea jecorina* (strain QM 6a) observed on Biolog FF MicroPlate

D-glucose (black diamonds) exemplifies the group of quickly utilised carbon sources, which resulted in maximal biomass values already after 40 h of incubation. D-lactose (open triangles) is typical for carbon sources with medium utilisation rates, which gave maximal mycelium production after 3 d of growth. D-tagatose (open circles) belongs to the group of carbon sources, which provided only slow growth, and still commenced for longer than 72 h. Glucuronamide (black squares) is a compound, which does not support growth of *H. jecorina* QM 6a. Error bars correspond to standard deviation values calculated after three independent experiments

In addition, careful visual monitoring of cultures in test wells over the incubation period is essential: several carbon sources were found to initiate the early sporulation (e.g., 48 h of growth on γ-aminobutyrate for *H. jecorina*), which was reflected by increase in absorbance readings (Druzhinina *et al*., 2006). Careful examination of the time-dependent growth patterns on every nutrient composition as recommended above helps to eliminate this problem.

Finally, a last caveat would be the hyphal morphology. There are a number of carbon sources, which lead to aberrant morphology, and one would expect that thicker or irregularly branched hyphae would yield a different ratio of OD_{750} vs. true biomass. Fortunately, this problem appears to be less important than theoretically predicted: we have tested the growth of *H. jecorina* wild-type strain in submerged culture, on agar surface and in PMs for several carbon sources with a potential morphological effect (e.g., L-sorbose), and generally found significant correlation between them. Therefore, OD_{750} values are equivalent means to measure growth of conidial fungi on PM. Considering all the preconditions discussed

above, the reproducibility of PMs is very high. We have tested 17 wild-type and mutant strains of *H. jecorina* in three independent experiments when turbidity (mycelial production) was measured at several time points (Druzhinina *et al*., 2006). The subsequent statistical analysis did not detect any significant difference between corresponding plates in both cases when values for all carbon sources were averaged or tested individually, although the variable of time was always significant and has revealed three clusters attributed to spore germination, linear hyphal growth, and phase of sporulation and/or growth saturation. In spite of the high reproducibility of PMs, three or more independent tests are required as a precautionary measure against possible air-born contamination of test well(s), which might take place during readings at early growth stages or when the slow growing fungi are investigated.

2.1 Intra- and inter-specific variability in carbon source utilization in conidial fungi

Well studied filamentous fungi genera, e.g., *Aspergillus*, *Neurospora*, *Hypocrea* and *Acremonium*. are known to have a wide range of substrate assimilation (Caddick *et al*., 1994; Tanzer *et al*., 2003; Druzhinina *et al*., 2006; Hoyos-Carvajal *et al*., 2009). For species of genus *Hypocrea*/*Trichoderma*, rapid growth as well as ability to assimilate diverse substrates is a great advantage that allows this genus to colonize many ecological niches in the world. Kubicek *et al*. (2003) compared the inter-specific variability of carbon source utilisation profiles in *Hypocrea*/*Trichoderma*, and confirmed that isolates of most species formed defined clusters, thus proving the applicability of this method in species identification. This approach was then integrated in a description of *T. brevicompactum* sp. nov. by Kraus *et al*. (2004).

PMs were also applied to study the differentiation of two morphologically and ecologically very similar marine species *Dendryphiella arenaria* and *D. salina* (dela Cruz *et al*., 2006), when the qualitative difference in utilization of four carbon sources confirmed the species divergence inferred from genetic data. Moreover, PMs could also differentiate subpopulations within *Dendryphiella* species in relation to their geographic origin (dela Cruz *et al*., 2006).

Nutrient source profiling was also applied by Komon-Zelazowska *et al*. (2007) to determine the evolutionary context of two genetically closely related, but phenotypically very different species (*Trichoderma pleuroticola* and *T. pleurotum*) that cause green mold disease in oyster mushroom (*Pleurotus ostreatus*) farms worldwide. This assay revealed generally impaired growth of *T. pleurotum* on numerous carbon sources according to enhanced assimilation of those by *T. pleuroticola*, which thereby showed very similar metabolic characteristics to its phylogenetically close members in the Harzianum clade of *Hypocrea*/*Trichoderma* (*T. harzianum* and *T. aggressivum*). It was shown that the highest assimilation rates for *T. pleuroticola* occurred on *N*-acetyl-D-glucosamine and quinic acid, which could be useful for differentiation of two causative agents of *Pleurotus* green mold disease. *T. pleuroticola*, on the other hand, can be distinguished from *T. aggressivum* (green mold disease on *Agaricus*) by the inability of the latter to utilize α-ketoglutaric, L-malic, and succinamic acids (Komon-Zelazowska *et al*., 2007). Thus, these kinds of assays are important sources of complementary data for precise species identification and as a starting point for uncovering the ecological niche of a fungus.

Furthermore, as shown in Fig. 3 (biochemical and physiological groups of carbon sources are given in Fig. 1A), PM analyses of different species displayed significant intraspecies diversities (*H. jecorina*) or were conserved (*H. atroviridis*) (Seidl *et al*., 2006; Friedl *et al*., 2008b). It was interesting to see that for *H. jecorina* the level of this variability was in the same range as that obtained by DNA-mediated transformation (Druzhinina *et al*., 2006). It may suggest that the difference between *H. jecorina* isolates is due to the sexual recombination, which is possibly less frequent in *H. atroviridis*.

Yet not every study could successfully confirm species differentiation: we tested the hypothesis that opportunistic strains of *T. longibrachiatum* isolated from the lungs of immunocompromised patients may represent specialized potentially clonal subpopulations within this species, which would allow us to identify specific genetic markers for their diagnosis. In contrast, the results from this work provide clear evidence that not only one, but two, genetically different species *T. longibrachiatum* and *H. orientalis* cause infections, and that clinical and environmental isolates reveal surprisingly consistent PMs (Druzhinina *et al*., 2008).

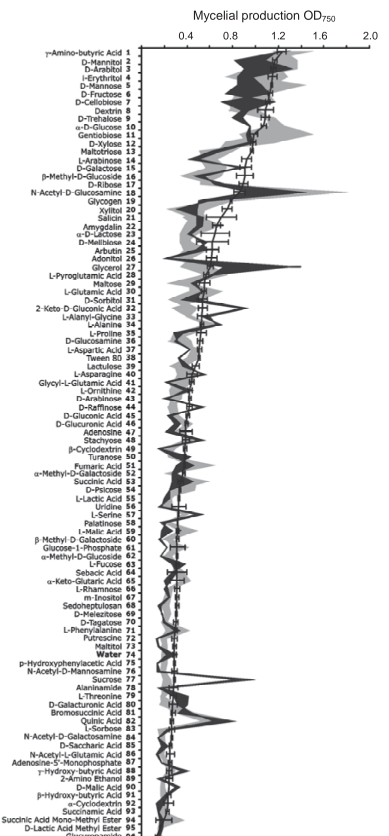

Fig. 3 Metabolic profiles of *H. jecorina* and *H. atroviridis* as inferred by Biolog Phenotype MicroArray analysis after 48 h of incubation
Light grey shadow corresponds to the summed metabolic profile of five wild-type strains of *H. jecorina*. Dark grey shadow indicates the combined profile of three *H. atroviridis* strains. The solid line corresponds to the reference strain *H. jecorina* QM 6a, which has a unique physiological profile. Error bars indicate standard deviation values estimated based on tree independent experiments

In the manner of species identification, Hoyos-Carvajal *et al*. (2009) recently examined the diversity of *Hypocrea/Trichoderma* in neotropic regions using morphological, genetic, as well as metabolic approaches, proving that the latter should not to be omitted in the recognition and characterization of new species or in the analyses of phylogenetic relationships within a fungal genus. Hoyos-Carvajal *et al*. (2009) reported that in *T. asperellum*, one of the most abundant species found in neotropical regions, two distinct genotypes were supported by carbon source utilization profiles: strains of the *T. asperellum* clade A originating from forests grew on very poor substrates, whereas strains from clade B in situ associated with crops preferred rather readily available substrates such as sugars. The predominance of *T. harzianum* can be attributed to a relatively wide variety of utilized carbon substrates, as reported by Hoyos-Carvajal *et al*. (2009), yet three new species morphologically similar to *T. harzianum* showed atypical metabolic profiles in the cluster analysis as well as a separate lineage in the sequence analysis of rRNA markers.

Moreover, a screening test for rapid differentiation of strains of the superior chitinase producing fungi attributed to *T. harzianum* sensu stricto clade with Biolog Phenotype MicroArrays was developed by Nagy *et al*. (2007), revealing that these fungi are unable to grow on *N*-acetyl-D-mannosamine. This fact was used to establish a simple test for directed screening for chitinase-producing strains (Nagy *et al*., 2007).

PM analysis aids, however, in the detection of intra- and inter-specific variability of conidial fungi, thereby offering a means for characterizing species, individual strains, and ecological groups.

2.2 Identification of phenetic differences in non-transformed mutant strains

Many of the classical fungal mutant strains used in biotechnology were selected after chemical or physical mutagenesis solely based on improved product formation, while the biochemical or genetic nature of the mutation has rarely been revealed. An example is *T. reesei* QM 6a (teleomorph *Hypocrea jecorina*), which was subjected to a classical mutagenesis by a series of exposures to radiation with a linear accelerator. The resulting mutants displayed a two- to four-fold increase in cellulolytic activity. We have tested whether PMs would be capable of identifying the possible changes, which had accompanied

this mutagenesis (Druzhinina *et al*., 2006). The data showed that, in general, mutagenesis left the carbon source utilization profile of the respective strains remarkably unchanged, thus indicating that an improvement of cellulase formation was due to mutation of very specific intracellular targets, such as those involved in inducer formation or reception. Some subtle differences, however, could be observed: increased cellulase formation was shown to correlate with a decreased growth on adonitol (=D-ribitol), 2-ketogluconate and γ-aminobutyric acid and increased growth rates on D-sorbitol and saccharic acid. The metabolism of two of these compounds (2-ketogluconate, saccharic acid) in *H. jecorina* is unknown, thereby preventing an interpretation of the observed effect. The two others (γ-aminobutyric acid, D-sorbitol), however, may indeed offer interesting insights: γ-aminobutyric acid formation is up-regulated during the early phase of conidiation in *Trichoderma*, and exogenous addition of γ-aminobutyrate stimulates sporulation in *T. reesei* (unpublished data). Since cellulase formation is triggered by sporulation (Kubicek, 1987), γ-aminobutyrate may constitute a link between cellulase induction and conidiation. On the other hand, the increased utilization of D-sorbitol by the improved producer strain QM 9414 may be linked to enhanced formation of L-sorbose, an inducer of cellulase formation in *H. jecorina* (Nogawa *et al*., 2001), because it is formed from D-sorbitol by the respective nicotinamide adenine dinucleotide phosphate (NADP)-dependent ketose reductase (Seiboth *et al*., 2007). While the importance of these two findings for cellulase production must be verified by reverse genetics, they have nevertheless pointed to two biochemical reactions, which may have a major impact on cellulase formation, and would have remained undetected without PM analysis.

2.3 Biomining for new biocatalysts and improved producer strains

Most of the fungal strains used today in the production of enzymes, secondary metabolites, and other organic chemicals have been screened by testing for either formation of the product itself or for genetic alterations assumed to cause improved production such as carbon catabolite derepression, e.g., by using resistance to 2-desoxiglucose for cellulase overproduction in *H. jecorina* (Montenecourt and Eveleigh, 1979). Improved production, however, may also be due to changes of unknown or unexpected physiological properties, detection of which may therefore offer new methods for the direct screening of improved producer strains. To this end, we have recently used carbon source PMs to correlate the ability to secrete high chitinase activities with the carbon source assimilation profile in *Trichoderma*. In line with the comments made above, we in fact observed a statistically relevant correlation between the assimilation rate of lactic acid methyl ester and chitinase activity (unpublished data). While the physiological meaning of this correlation is not yet understood, lactic acid methyl ester is a valuable indicator of the chitinolytic potential of *Trichoderma* species.

While PM primarily provides information about the growth rate on different nutrients, the integration of these data into a biochemical map could also lead to the detection of pathways or enzymatic steps, which are unique for a particular strain, species or genus. One barrier towards achieving this is that not all of the biochemical pathways occurring in filamentous fungi are as yet understood to the level that the implementation of this type of network would be feasible. Some biotechnologically useful information can, however, be extracted already at this stage. For example, *N*-acetyl-mannosamine is a very poor carbon source for some species and strains of *Hypocrea*/*Trichoderma* such as *H. jecorina*, although they all assimilate *N*-acetyl-glucosamine very well. The majority of strains of *H. lixii*/*T. harzianum* also utilize *N*-acetyl-mannosamine. This is due to the formation of the enzyme *N*-acetylglucosamine epimerase, which converts *N*-acetyl-glucosamine into *N*-acetyl-mannosamine as shown in Fig. 4 (Kowal and Wang, 2002). Since the reaction of this enzyme is essentially in equilibrium, it can, vice versa, be added to the in vitro degradation of chitin by *Trichoderma* chitinase, which would then ultimately lead to the accumulation of *N*-acetyl-mannosamine, a high-value product needed for the biocatalytic synthesis of sialic acid-like compounds used in various therapies (Blayer *et al*., 1999; Schauer, 2000; Keppler *et al*., 2001). Yet, using the PM method for detecting the differentiation between chitinase-overproducing strains among *H. lixii*/*T. harzianum* (Nagy *et al*.,

2007), the lack of *N*-acetyl-β-D-mannosamine utilization was present in the superior chitinase producer strains.

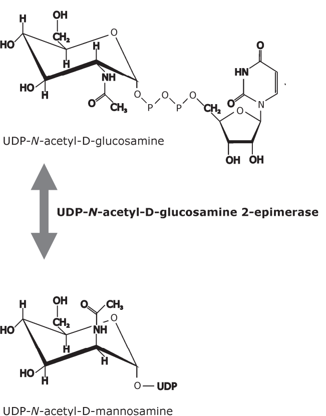

Fig. 4 Conversion of *N*-acetyl-glucosamine into *N*-acetyl-mannosamine by enzyme *N*-acetyl-glucosamine epimerase [modified from Kowal and Wang (2002)]

Furthermore, a screening system consisting of a combination of Biolog PM and specific enzyme activity measurements using a chromogenic substrate (Seidl *et al*., 2006) was developed to identify carbon sources that trigger β-*N*-acetyl-D-glucosaminidase (NAGase) formation in *Hypocrea atroviridis*. Those data were compared with transcript patterns of *nag1* and *nag2*, two genes encoding the NAGase Nag1 and Nag2, in the wild-type and an *H. atroviridis* Δ*nag1* strain (Seidl *et al*., 2006). No differences in the phenotype of these two strains were observed. As the result of the screening Δ*nag1* strain showed a strong reduction of NAGase activity on most carbon sources compared to the wild-type. A number of carbon sources that clearly enhanced NAGase activity were detected, mostly α-glucans, like glycogen, dextrin and maltotriose, and several oligosaccharides. The authors claimed that, since this kind of glycosidic linkage is present in the cell walls of the majority of ascomycetes, these carbon sources can be a part of a mechanism by which *H. atroviridis* senses the presence of a host cell wall containing chitin. Moreover, the comparison of NAGase formation between wild-type and mutant *H. atroviridis* strains showed that the reduction of this enzyme activity varied greatly among different carbon sources, and that the ratio was growth-phase dependant (Seidl *et al*., 2006).

Overall, the screening system of the combination of PMs and an enzymatic assay using a chromogene substrate established by Seidl *et al*. (2006) can be adapted for enzyme activity measurements of a variety of extracellular and cell wall-bound enzymes. Apparently, the differences in regulation between *nag1* and *nag2* would stay undetected without this tool. Seidl *et al*. (2006) propose this method as useful to monitor the expression of specific genes, even for encoding intracellular enzymes, by using appropriate promoter-fusion reporter systems.

2.4 Testing gene knockouts with expected phenotypes

DNA-mediated transformation is now a routine method applied to many fungi, and creation of knockout mutants by homologous integration, therefore, is a standard procedure. In order to obtain reliable knockouts, Southern analyses must be performed to confirm the homologous integration, and a regain of the phenotype of the parent strain by retransformation with the wild-type gene is also essential. Assessment of the wild-type and mutant phenotypes, however, is often laborious. PMs can provide a valuable tool here, as shown with an example of *H. jecorina*: we have previously investigated xylitol dehydrogenase gene (*xdh1*) for its possible involvement in D-xylose catabolism (Seiboth *et al*., 2003). Analysis of an *xdh1*-deletion mutant by PMs (Table 1) reveals confirmations that this mutant is partially defective in growth on L-arabinose and D-xylose, because its activity can be taken over by L-arabinitol dehydrogenase. PMs further show, however, that the mutant has impaired growth on D-sorbitol, whereas it has higher growths on erythritol and glycerol. The latter finding is not understood, but suggests an interplay of xylitol dehydrogenase with catabolism of shorter chain polyols, which needs to be taken into account when the physiology of these mutants is investigated.

Two mutant cellulase-hyperproducing strains NG14 and its direct descendant RUT C30, initially originating from *T. reesei* QM 6a, were newly tested to identify mutations in the genomes that led to cellulase production (le Crom *et al*., 2009). These two strains were identified on the basis of their superior cellulase activity as well as resistance to 2-deoxyglucose in the presence of glycerol (Seidl *et al*., 2008). Biolog PMs were used to compare the mutants with their wild-type strain QM 6a. It was shown that utilization of carbon sources glucose, D-xylose, D-arabinitol, mannitol, and the β-linked disaccharides gentiobiose and cellobiose is correlated with increased cellulose production (le Crom *et al*., 2009). The latter two carbon sources might reflect enhanced β-glucosidase activity, yet the rest of utilized carbon sources are catabolite repressing compounds and at this point, le Crom *et al*. (2009) stressed that it is not clear whether this is a consequence of the *cre1* mutation or it is due to the effect of other affected genes. Previously reported inverse carbon source utilization in correlation with enhanced cellulase production was detected by utilization of α-linked oligosaccharides and glycans (Seidl *et al*., 2008), and was complemented by the correlation between cellulase production and reduced growth on amino acids. le Crom *et al*. (2009) discussed this phenotype to be related to an ability of the superior cellulase production strains to use a higher portion of their amino acid pool for synthesis of secreted proteins versus growth.

Table 1 Utilization of selected carbon sources by *H. jecorina* QM 9414 and Δ*xdh1* transformant strains

Carbon source	48 h			66 h		
	QM 9414	Δ*xdh*1	*P* value	QM 9414	Δ*xdh*1	*P* value
L-arabinose	0.61	0.35	<0.01	1.14	0.46	<0.01
D-cellobiose	0.87	0.91	0.02		Not significant	
i-erythritol	0.86	1.07	<0.01	1.08	1.16	0.04
Glycerol	0.44	0.66	0.03	0.74	1.04	<0.01
D-sorbitol	0.54	0.26	<0.01	0.88	0.31	<0.01
Xylitol	0.49	0.56	<0.01		Not significant	
D-xylose	0.94	0.47	<0.01	0.94	0.75	<0.01

2.5 Analysis of wide-domain regulatory processes

PMs are particularly well suited for the investigation of wide-domain regulatory processes, e.g., carbon catabolite regulation, due to the possibility of measuring growth on 96 different conditions simultaneously (carbon sources, nitrogen sources, stress inducers, pH control, and others). These factors have been intensively studied in *A. nidulans*, *N. crassa*, *H. jecorina*, and *Acremonium chrysogenum* (Bailey and Arst, 1975; Kudla *et al*., 1990; Dowzer and Kelly, 1991; Caddick *et al*., 1994; Strauss *et al*., 1995; Ilmén *et al*., 1996; Platt *et al*., 1996; Ravagnani *et al*., 1997; Marzluf, 1997; Wilson and Arst, 1998; Jekosch and Kück, 2000; Felenbok *et al*., 2001). In this type of experiment, growth of a parent strain and a respective mutant would be compared on every medium composition, which would reveal those particular nutrients whose utilization depends on the mutated wide-domain control gene.

2.5.1 Nitrogen repression

Tanzer *et al*. (2003) used this approach to investigate the effect of the *areA*-dependent nitrogen regulation on the utilization of various nitrogen sources by *A. nidulans*. The *areA* gene encodes a GATA family transcriptional activator mediating nitrogen metabolite repression, thus ensuring the utilization of ammonium and L-glutamine in preference to a variety of alternative nitrogen sources. Mutations in *areA* exhibit an extraordinarily diverse range of phenotypes when monitored for their effects on utilization of nitrogenous compounds. One of these alleles, *areA-102*, displays altered specificity of target promoter activation, which ultimately leads to loss-of-function, gain-of-function, or a wild-type phenotype based on its interaction with the target structural gene promoter (Kudla *et al*., 1990; Ravagnani *et al*., 1997). In comparison to wild-type strains on agar plates, strains carrying *areA*-102 show increased growth when urea, L-histidine, L-citrulline, L-aspartate, or L-glutamate is used as the sole nitrogen source and decreased growth when xanthine or uric acid is used as the sole nitrogen source (Arst and

Cove, 1973; Arst and Scazzocchio, 1975; Hynes, 1975; Kudla *et al*., 1990). Results obtained with PMs were largely in agreement with these observations. In contrast to them, however, growth on L-glutamate or L-aspartate in the *areA*-102 mutant was not higher than that in the wild-type, and growth on xanthine was not detectable. On the other hand, assimilation of quite a number of nitrogen sources was not altered, indicating that they are not a subject to the AreA-mediated nitrogen regulatory system. An interesting finding from this study was that several D-amino acids (D-alanine, D-valine, and D-lysine) were utilized more efficiently by the *areA*-102 mutant strain than by the wild-type strain, suggesting that this *areA* mutation elevates D-amino acid oxidase levels. Consistent data were obtained with another allele (*areA*-30), which is a revertant allele that also shows altered specificity of target promoter activation.

Tanzer *et al*. (2003) further used PMs to perform a large scale analysis of 21 *A. nidulans* strains containing various *areA* mutant alleles. The data from all test wells for each strain were used for cluster analysis based on overall nitrogen source utilization patterns. The data provided excellent insight into the severeness of the individual mutations, e.g., mutant *areA*-1903 or *areA*-2173, which contain the conservative G698A substitution (Wilson and Arst, 1998), clustered with the wild-type strain, because these mutants produce no apparent effect in growth tests on solid medium. Other mutations, particularly those leading to a truncated AreA protein, showed much more serious differences. This work therefore beautifully illustrates the use of PM to screen for essential and dispensable mutations in a regulatory protein.

2.5.2 Carbon catabolite repression

Carbon catabolite repression is another wide-domain regulatory circuit amenable to investigation by PM. Its most observed phenotype is due to the *creA*/*cre1* gene, which encodes a C_2H_2-zinc finger-containing transcriptional repressor (Dowzer and Kelly, 1991; Strauss *et al*., 1995; Ilmén *et al*., 1996). It ensures the utilization of glucose and some other carbon sources in preference to a large variety of other carbon compounds, which either yield less energy during assimilation (e.g., C_2-components), or require a considerable amount of energy for becoming assimilable (e.g., extracellular macromolecules, for which hydrolases must be exported via a secretory pathway).

To determine the utilization of which carbon sources is actually affected by the carbon catabolite repression, Seidl *et al*. (2008) investigated the differences in the growth of a *cre1*-truncated mutant strain of *H. jecorina* (RUT C30) (Ilmén *et al*., 1996) and the respective parent strain NG 14. The results (Fig. 5) show that the carbon catabolite-derepressed mutant strain displays a higher growth rate on D-arabitol, D-mannitol, D-fructose, D-trehalose, D-mannose, D-xylose, D-ribose, *N*-acetyl-D-glucosamine, γ-aminobutyric acid, and glycerol, indicating that all of them are under partial carbon catabolite repression, and that they all repress their own utilization to some extent. As expected, growth on glucose was not different and therefore it can serve as a control for these investigations. Interestingly, the *cre1*

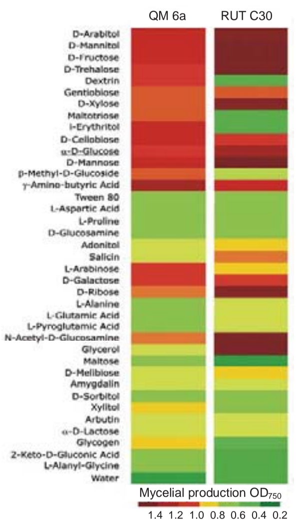

Fig. 5 A phenetic map of carbon utilization patterns of *H. jecorina* QM 6a and the carbon catabolite-derepressed strain RUT C30 after the two-ways joining cluster analysis was applied to (i) carbon sources and (ii) fungal strains as two groups of variables. Only "fast" and "medium" carbon sources are shown. The respective growth (OD_{750} after 48 h) is given by a corresponding color as indicated at the color scale

mutation also results in significant changes in the other direction, e.g., the carbon catabolite-derepressed mutant had decreased the ability to grow on erythritol, dextrin, maltotriose, glycogen, and β-methyl glucoside. The biochemical nature of these observations is unclear, but indicates a dependence of carbon catabolite repression on assimilation of these carbon sources.

Seidl *et al*. (2008) also showed that the *H. jecorina* RUT C30 and its ancestor NG 14 lack an 85-kb genomic fragment, missing additional 29 genes comprising transcription factors, enzymes of the primary metabolism and transport proteins. They reported that these mutations are not linked to the *cre1* locus. Performing PM analysis, it was concluded that a greatly reduced growth of RUT C30 on L-arabinose, L-erythritol and also D-galactose indicates that one of the aldo-/keto-reductases identified as lacking in this strain could be involved in polyol assimilation (Seidl *et al*., 2008).

Tanzer *et al*. (2003) used an alternative, indirect approach to examine the effect of carbon catabolite repression on carbon source utilization in *A. nidulans*, *A. fumigatus*, *M. grisea*, and *M. graminicola*. The approach was based on the CreA-dependent repression of alcohol dehydrogenase, which renders these strains unable to grow on derepressing carbon sources in the presence of allyl alcohol (Bailey and Arst, 1975; Felenbok *et al*., 2001). Obviously, this method can be used only with fungi that are able to use ethanol as a sole carbon source, which is not the case for others, e.g., *H. jecorina* (C.P. Kubicek, personal communication). The validity of the approach for the species investigated, however, arises from the fact that in all four fungal species, the addition of allyl alcohol to the media containing D-glucose had a little effect on the growth levels whereas it was strongly inhibited in media containing the derepressing carbon source L-lactic acid. A closer examination showed that the derepressing class of carbon compounds could be further subdivided by comparing growth in the presence of different concentrations of allyl alcohol. *A. nidulans* and *A. fumigatus* were generally more sensitive to allyl alcohol toxicity than *M. grisea* and *M. graminicola*, the latter two species requiring, respectively, 25- and 2.5-fold higher concentrations of allyl alcohol to reduce growth on derepressing carbon sources (Tanzer *et al*., 2003). These data also revealed contrasting results in different fungi, e.g., while dextrin was derepressing in the *A. nidulans*, it was repressing in *A. fumigatus*, *M. grisea*, and *M. graminicola*. Additionally, several other carbon sources such as maltose, D-sorbitol, and D-trehalose were more strongly repressing in *A. fumigatus* as compared to *A. nidulans*, and a much larger number of carbon sources were strongly repressing in *M. grisea* and *M. graminicola*, e.g., D-trehalose, lactose, and D-raffinose. The fact that *Magnaporthe* and *Mycosphaerella* are less sensitive to allyl alcohol and are derepressed by fewer compounds suggests that the plant pathogens are poised to utilize a number of compounds as carbon sources rapidly whereas the aspergilli are initially more selective and at the same time can use a wider range of carbon sources. Tanzer *et al*. (2003) concluded that the regulation of nutrient utilization may evolve to favour adaptation to the certain environmental niches and thus may provide insights into the nutrients typically encountered by these fungi.

Even though the allyl alcohol resistance system cannot be used for fungi like *Hypocrea*/*Trichoderma*, it is still amenable to this type of global analysis by other means: 2-desoxyglucose (2-DOG) has been used as a nonmetabolizable agent conferring carbon catabolite repression in yeast and filamentous fungi, because it accumulates as 2-desoxyglucose-6-phosphate (Franzusoff and Cirillo, 1982; Randez-Gil *et al*., 1995). Therefore, 2-DOG has been used for the isolation of carbon catabolite derepressed mutants in *H. jecorina* and *Penicillium chrysogenum* (Montenecourt and Eveleigh, 1979; Farkaš *et al*., 1981; Barredo *et al*., 1988). A drawback of the use of 2-DOG, however, is that it also interferes with energy metabolism (e.g., it depletes the cells of adenosine-triphosphate (ATP) because of storage of phosphate in 2-DOG-phosphate and slowing down glycolysis). In order to take that into consideration, the influence of 2-DOG is examined at several different sublethal concentrations.

Brunner *et al*. (2008) analyzed G protein-coupled receptors (GPCRs) in a manner to learn more about the G protein signaling. With the help of in silico exploration of the genome database of the close relative *T. reesei*, four *H. atroviridis* GPCR-encoding genes were isolated and affiliated to the cyclic adenosine monophosphate (cAMP) receptor-like

family. Using Biolog PMs, it was shown that *gpr-1* silenced mutants exhibited reduced growth on several carbon sources, e.g., glucose and glycerol growth of the mutants was reduced by 70%. Furthermore, in comparison to the parental strain, the mutant was not able to grow on lactose and its isomerization product lactulose, and also showed scarce growth on maltose and raffinose. There were also some carbon sources that showed similar growth rate at both strains, indicating that the mutant's reduced growth is carbon source-dependent and generally ineffective. Overall, the characterization of the *H. atroviridis* Gpr-1 revealed that the GPCR influences conidiation, conidial germination and vegetative growth on many carbon sources (Brunner *et al*., 2008). The PM method aids to the exploration of the fundamental role of G protein signaling in *Trichoderma*.

2.6 Application of PMs in fungal photobiology

Contrary to the initial expectation, Friedl *et al*. (2008a) revealed that conidiation in *Hypocrea atroviridis* is primarily carbon source-dependent and that illumination plays a catalytic role in this process. They applied PMs to a wild-type strain of *H. atroviridis* as well as two mutant strains, with each the loss of function of the blue light regulators 1 and 2 (BLR-1 and BLR-2), respectively, responsible for stimulation of transcription activation. In the mutant strains Δ*blr-1* and Δ *blr-2*, conidiation in the darkness was significantly decreased on most carbon sources. Except for strong conidiation of Δ*blr-1* on D-sorbitol and Δ*blr-2* on glucuronic acid, the carbon sources that stimulated low conidiation in the mutant strains were mostly from the pool of those that contribute to strong conidiation in the parent strain (Friedl *et al*., 2008a). While observing this strongly impaired sporulation, it is notable that the mutations do not completely block sporulation but only greatly decrease it, and they still show (light insensitive) sporulation on some carbon sources (notably glucuronic acid and 2-ketogluconic acid). Friedl *et al*. (2008a) therefore hypothesized that the observed differences in levels of conidiation on different carbon sources are due to different redox potentials in the cell during growth. Thus, BLR proteins are not only involved in the photosensing, but also act as redox and oxygen sensors.

Friedl *et al*. (2008a) also concluded that the addition of cAMP induces the sporulation in darkness, as previously reported by Casas-Flores *et al*. (2004), but also showed that effect of cAMP is carbon source-dependent. The addition of cAMP induced conidiation in the parent and both *blr* mutant strains on some, but not all, carbon sources. Thus, the carbon source dependence of mutant strains was different from that of the parent strain. Only a small set of carbohydrates, polyols, and sugar acids allowed conidiation in darkness, whereas for most of them conidiation was strongly expressed in light. Moreover, under every condition tested (darkness, light, addition of cAMP, the loss of function of BLR-1/BLR-2) spore formation occurred only on carbohydrates and their derivates (polyols and sugar acids). Yet, the presence of light always enabled the sporulation only on those carbon sources which also empowered conidiation in darkness by increasing the speed or intensity of sporulation (Friedl *et al*., 2008a). There were a number of carbon sources (e.g., rhamnose, glucosamine and xylitol) detected where light did not further stimulate the spore formation.

Furthermore, Friedl *et al*. (2008b) showed that enhancement of mycelia growth on various carbon sources is highly light-dependent. Carbon source utilization by PMs showed that *Hypocrea atroviridis* growth is light-stimulated on 17 of 95 carbon sources, which are mainly metabolically related to cellulose and hemicelluloses and can be found in the upper soil litter layer (Friedl *et al*., 2008b). The light stimulation depends on the function of two blue light receptor proteins BLR-1 and BLR-2. Utilization of carbon sources in darkness was nearly the same in both knock-out mutants (Δ*blr-1* and Δ*blr-2*), and it was similar to that of the parent strain. But comparing carbon utilization profiles to those exposed to rhythmic and constant illumination, Friedl *et al*. (2008b) concluded that the mutant strains are not photostimulated, but in contrast are inhibited by light, especially in Δ*blr-2*. Evocation of oxidative stress response in darkness imitates the photostimulation on 9 of the mentioned 17 carbon sources, and this effect is fully dependent on the function of BLR-1. The resistance of both *blr* mutants to conditions of oxidative stress is therefore carbon source-dependent and is notably weaker compared to the wild-type strain. Friedl *et al*. (2008b) concluded that light in combination with the availability of litter-specific carbon sources serves as a signal for the fungus to be above

ground, thereby stimulating fast growth in order to produce a maximum of propagules in the shortest time. Furthermore, they deduced that this process involves oxidative stress response and the two blue light receptor proteins BLR-1 and BLR-2, the former playing the major role.

To determine the role of the light regulatory protein ENVOY, Schuster *et al*. (2007) used PMs as an aid to their global screening for genes, which are specifically affected by light in the fungus *H. jecorina*, an important producer of cellulases and hemicellulases. The expression of its cellulase genes is partially susceptible to carbon catabolite repression. They showed that ENVOY acts as a light-independent repressor for several genes and it is crucial for normal growth in light on several carbon sources, but is not able to fully execute its regulatory function when overexpressed in darkness. Higher growth rate of the *H. jecorina* QM 9414 than that of the mutant strain env1^{PAS-} measured in the light indicated that the growth rate is dependent on ENVOY since no such stimulation was observed in the mutant strain (Schuster *et al*., 2007). Moreover, Schuster *et al*. (2007) found that, with the exception of growth on γ-aminobutyric acid, mutant strain growth rate is always lower in light than in the dark. They also reported that the growth rates of two strains compared separately in light and darkness differ significantly more in the light, indicating that light inhibits growth of strain env1^{PAS-}. On the other hand, it was shown that growth in darkness (with the exception of glycerol) was poorly affected. They claimed an *env1*-dependent enhancement of energy metabolism, and thus biomass formation by light as well as a negative effect of light on *H. jecorina* in the absence of functional ENVOY. The inhibitory effect of light in the absence of ENVOY is carbon source-dependent since the inhibition by light in the mutant strain was not observed on all carbon sources (Schuster *et al*., 2007).

2.7 Development and analysis of action of chemical inhibitors

Many fungi are pathogens of plants, animals or human beings. Therefore, screening for novel chemicals capable of inhibiting these species is an important issue (Vitale *et al*., 2005). This is also of particular significance to the increasing number of cases in which non-human-pathogenic fungi such as *Trichoderma* or *Fusarium* have caused fatalities in immunocompromised patients (Yeo and Wong, 2002; Sampathkumar and Paya, 2001). Many of these isolates proved to be resistant to the common clinical fungicides such as fluconazole and 5-fluorocytosine (5FC), amphotericin B, tetraconazole and ketoconazole (Ragnaud *et al*., 1984; Campos-Herrero *et al*., 1996; Tanis *et al*., 1995; Guarro *et al*., 1999; Hennequin *et al*., 2000; Antal *et al*., 2002; Avery, 2004). Thus, the development of new agents is essential. This development also must accompany the identification of action of the compound, for which PM offers an attractive possibility. To show the utility of PM for chemical inhibitor analysis, Tanzer *et al*. (2003) compared the responses of *A. nidulans* and *M. grisea* to the addition of the growth inhibitor glufosinate. Glufosinate (phosphinothricin) is a herbicidal compound that has also been shown to inhibit animal and fungal growth (Avalos *et al*., 1989; Pall, 1993; Kutlesa and Caveney, 2001). It is an analogue of L-glutamate that inhibits glutamine synthetase, thereby preventing the synthesis of glutamine and consequently the synthesis of numerous other nitrogenous compounds required for growth. Based on its mode-of-action, the addition of glutamine should abrogate the inhibitory effects of glufosinate by bypassing the need for glutamine synthetase. Tanzer *et al*. (2003) tested the effect of glufosinate on growth of *A. nidulans* and *M. grisea* in the presence of different nitrogenous compounds. *A. nidulans* showed near wild-type levels of growth on glutamine and glutamate, which successfully competes for the inhibitor, but no growth when ammonium, nitrate or nitrite was used as the nitrogen source. Thus, Tanzer *et al*. (2003) showed that L-aspartate was not an effective competitor and glutamine-containing dipeptides were apparently unable to supply sufficient glutamine to overcome the toxicity. *M. grisea* was able to grow normally in the presence of L-glutamine or L-glutamate, while growth was inhibited when ammonium, nitrate or nitrite was present as the sole nitrogen source. In contrast to *A. nidulans*, *M. grisea* was able to utilize L-aspartate or the glutamine-containing dipeptides as the sole nitrogen source in the presence of glufosinate, thus demonstrating metabolic differences between fungi, differences potentially relevant to the action and screening for inhibitory compounds.

PM also offers an additional advantage for eventual identification of new inhibitory components. Looking at the nutrient source utilization profile, it is evident that for every fungus or strain, there is a cluster of nutrients on which the organism grows even slower than on water and a number of nutrients enabling essentially the same growth rate as water. The observation of growth on water may be mostly due to the utilization of reserve carbohydrates and polyols present in the conidia (d′Enfert *et al*., 1999). Consequently, those nutrients, which result in an even slower growth, are likely inhibitors, at least, of the spore germination process. Examples of this are D-lactic acid methyl ester and glucuronamide for *H. jecorina*, which exhibit a statistically significant inhibitory effect (Druzhinina *et al*., 2006), and hydroxylamine for *A. nidulans* (Tanzer *et al*., 2003). While there may not be too much interest in developing inhibitory agents for *H. jecorina* and *A. nidulans*, similar strategies could be used to screen for biodegradable inhibitors against pathogenic plant and animal fungi.

Gardiner *et al*. (2009) used PMs to assess the role of different carbon and nitrogen sources that could be crucial for regulation of trichothecene synthesis by a plant pathogenic fungus, *Fusarium graminearum*, which causes a head blight disease of wheat. Presence of these toxins has important consequences for human and animal health. Gardiner *et al*. (2009) investigated the application of PMs in combination with a transgenic strain of *F. graminearum* in which the promoter from the TRI5 trichothecene biosynthesis gene was fused to green fluorescent protein (GFP). Using this strain in PM profiling, a variety of amines were identified, which significantly induce TRI5 expression.

2.8 Global inducer screening

The increase in available fungal genome sequences has also led to an increase in the identification of genes for which orthologues in even closely related species are unknown (Dogra and Breuil, 2004; OBrian *et al*., 2003; Schmoll *et al*., 2004). As explained in the introduction, studies of the genome-wide role of these genes are still limited to the use of mutants and their use in DNA-microarray analysis and proteomics (Sims *et al*., 2004). Identifying the physiological conditions under which a newly identified gene is expressed, however, is still restricted to the analysis of its transcript formation in individual experiments. Having an array-type system available for this purpose would greatly facilitate and accelerate the assignment of a function to new genes.

Our interest in this area comes from studies on chitinase formation by *Hypocrea atroviridis* (Mach *et al*., 1999; Brunner *et al*., 2003; Seidl *et al*., 2005). While there are several reports of chitinase gene expression in fungi in response to the availability of chitin or chitin degradation products (Carsolio *et al*., 1999; Mach *et al*., 1999; Donzelli and Harman, 2001; Kim *et al*., 2002), not all chitinases follow this rule. We have recently identified 18 chitinases in *H. jecorina*, and several of them are in fact not induced by chitin (Seidl *et al*., 2005). In order to find conditions leading to their induction, we have constructed recombinant strains containing various promoter-reporter fusions, inoculated them on PM plates, and photometricaly quantified the formation of the reporter enzyme. In our study, we used the *A. niger* glucose oxidase encoding (*goxA*) gene, for which an orthologue is absent in *Hypocrea*, because the enzyme is secreted into the medium and can therefore be assayed without cell breakage (Mach *et al*., 1999). The glucose oxidase assay involves formation of a green color, however, which is difficult to measure in the presence of the violet formazan dye formed by the redox dye implemented in the Biolog FF MicroPlates. Therefore, other non-invasive strategies such as the use of various forms of the green fluorescent protein or the luciferase (March *et al*., 2003; Arnone *et al*., 2004; Piwnica-Worms *et al*., 2004; Gu *et al*., 2004) may be alternatives. Nevertheless, the use of the *goxA* reporter enables us to identify new chitinase inducers in *Hypocrea*/*Trichoderma* (Verena Seidl, personal communication).

3 Concluding remarks and outlook

In this article, we have reviewed recent applications of PM technology as an accessory tool for global and genome-wide investigations on fungi, and reported on early achievements in the area. Although, not all the potential uses of this technology have already been experimentally verified, we anticipate that this broad phenotypic testing will become a simple

standard practice once the method becomes more familiar to scientists working with industrially important or pathogenic fungi. For example, in the past it was impractical to assay more than a few phenotypes and therefore it was impossible to measure phenotypic drift. One still untested use of PM technology is therefore the ability to provide researchers with a practical means to characterize each fungal isolate. To this end, the establishment of a public database of carbon and nitrogen utilization profiles for fungi (wild-type strains and mutants) would be an important instrument in understanding the comparative biochemistry and physiology of these organisms, and aid in the selection of appropriate strains if new products or strain properties are desired.

An advantage of the PM system is that it is much easier to perform than gene expression analysis. It involves only the preparation of a standardized fungal inoculum and its pipetting into the microplates of the standard PM plate. By contrast, to obtain specific and useful data on genes, one needs to perform genetic engineering work to create the strains to be analyzed. At this moment, the commercial availability of a sufficient number of nutrient conditions for fungi is still limited. In theory, one can devise hundreds or thousands of growth conditions in which specific biological pathways or physiological functions are linked to hyphal growth, but only about 2500 different assays are available for bacteria and yeasts, and only half are available for fungi.

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References

Antal, Z., Kredics, L., Dóczi, I., Manczinger, L., Kevei, F., Nagy, E., 2002. The physiological features of opportunistic *Trichoderma* strains. *Acta Microbiol. Immunol. Hung.*, **49**:393.

Arnone, M.I., Dmochowski, I.J., Gache, C., 2004. Using reporter genes to study *cis*-regulatory elements. *Methods Cell Biol.*, **74**:621-652. [doi:10.1016/S0091-679X(04)74025-X]

Arst, H.N.Jr., Cove, D.J., 1973. Nitrogen metabolite repression in *Aspergillus nidulans*. *Mol. Gen. Genet.*, **126**(2):111-141. [doi:10.1007/BF00330988]

Arst, H.N.Jr., Scazzocchio, C., 1975. Initiator constitutive mutation with an up-promoter effect in *Aspergillus nidulans*. *Nature*, **254**(5495):31-34. [doi:10.1038/254031a0]

Avalos, J., Geever, R.F., Case, M.E., 1989. Bialaphos resistance as a dominant selectable marker in *Neurospora crassa*. *Curr. Genet.*, **16**(5-6):369-372. [doi:10.1007/BF00340716]

Avery, R.K., 2004. Prophylactic strategies before solid-organ transplantation. *Curr. Opin. Infect. Dis.*, **17**(4):353-356. [doi:10.1097/01.qco.0000136936.13662.74]

Bailey, C., Arst, H.N.Jr., 1975. Carbon catabolite repression in *Aspergillus nidulans*. *Eur. J. Biochem.*, **51**(2):573-577. [doi:10.1111/j.1432-1033.1975.tb03958.x]

Barredo, J.L., Alvarez, E., Cantoral, J.M., Diez, B., Martin, J.F., 1988. Glucokinase-deficient mutant of *Penicillium chrysogenum* is derepressed in glucose catabolite regulation of both beta-galactosidase and penicillin biosynthesis. *Antimicrob. Agents Chemother.*, **32**(7):1061-1067.

Blayer, S., Woodley, J.M., Dawson, M.J., Lilly, M.D., 1999. Alkaline biocatalysis for the direct synthesis of *N*-acetyl-D-neuraminic acid (Neu5Ac) from *N*-acetyl-D-glucosamine (GlcNAc). *Biotechnol Bioeng.*, **66**(2):131-136. [doi:10.1002/(SICI)1097-0290(1999)66:2<131::AID-BIT6>3.0.CO;2-X]

Bochner, B.R., 1988. New methods aid microbial identification. *Bio/Technology*, **6**(7):756. [doi:10.1038/nbt0788-756]

Bochner, B.R., 1989. Sleuthing out bacterial identities. *Nature*, **339**(6220):157-158. [doi:10.1038/339157a0]

Bochner, B.R., 2003. New technologies to assess genotype-phenotype relationships. *Nature Rev. Genet.*, **4**(4):309-314. [doi:10.1038/nrg1046]

Bochner, B.R., Gadzinski, P., Panomitros, E., 2001. Phenotype MicroArrays for high-throughput phenotypic testing and assay of gene function. *Genome Res.*, **11**(7):1246-1255. [doi:10.1101/gr.186501]

Brunner, K., Peterbauer, C.K., Mach, R.L., Lorito, M., Zeilinger, S., Kubicek, C.P., 2003. The Nag1 *N*-acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin and of major relevance to biocontrol. *Curr. Genet.*, **43**(4):289-295. [doi:10.1007/s00294-003-0399-y]

Brunner, K., Omann, M., Pucher, M.E., Delic, M., Lehner, S., Domnanich, P., Kratochwill, K., Druzhinina, I., Zeilinger, S., 2008. *Trichoderma* G protein-coupled receptors: genome analysis and functional characterization of a cAMP receptor-like protein from *Trichoderma atroviride*. *Curr Genet.*, **54**(6):283-299. [doi:10.1007/s00294-008-0217-7]

Caddick, M.X., Peters, D., Platt, A., 1994. Nitrogen regulation in fungi. *Antonie Van Leeuwenhoek*, **65**(3):169-177. [doi:10.1007/BF00871943]

Campos-Herrero, M.I., Bordes, A., Perera, A., Ruiz, M.C., Fernandez, A., 1996. *Trichoderma koningii* peritonitis in a patient undergoing peritoneal dialysis. *Clin. Microbiol. Newslett.*, **18**(19):150-151. [doi:10.1016/0196-4399(96)83918-3]

Carsolio, C., Benhamou, N., Haran, S., Cortes, C., Gutierrez, A., Chet, I., Herrera-Estrella, A., 1999. Role of the *Trichoderma harzianum* endochitinase gene, *ech42*, in mycoparasitism. *Appl. Environ. Microbiol.*, **65**(3):929-935.

Casas-Flores, S., Rios-Momberg, M., Bibbins, M., Ponce-Noyola, P., Herrera-Estrella, A., 2004. BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*. *Microbiology*, **150**(Pt 11):3561-3569. [doi:10.1099/mic.0.27346-0]

Chaveroche, M.K., Ghigo, J.M., d′Enfert, C., 2000. A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res.*, **28**(22): E97. [doi:10.1093/nar/28.22.e97]

dela Cruz, T.E.E., Schulz, B.E., Kubicek, C.P., Druzhinina, I.S., 2006. Carbon source utilization by themarine *Dendryphiella* species *D. arenaria* and *D. salina*. *FEMS Microbiol. Ecol.*, **58**(3):343-353. [doi:10.1111/j.1574-6941.2006.00184.x]

Dogra, N., Breuil, C., 2004. Suppressive subtractive hybridization and differential screening identified genes differentially expressed in yeast and mycelial forms of *Ophiostoma piceae*. *FEMS Microbiol. Lett.*, **238**(1):175-181. [doi:10.1111/j.1574-6968.2004.tb09753.x]

Donzelli, B.G., Harman, G.E., 2001. Interaction of ammonium, glucose, and chitin regulates the expression of cell wall-degrading enzymes in *Trichoderma atroviride* strain P1. *Appl. Environ. Microbiol.*, **67**(12):5643-5647. [doi:10.1128/AEM.67.12.5643-5647.2001]

Dowzer, C.E.A., Kelly, J.M., 1991. Analysis of the *creA* gene, a regulator of carbon catabolite repression. *Mol. Cell. Biol.*, **11**(11):5701-5709.

Druzhinina, I.S., Schmoll, M., Seiboth, B., Kubicek, C.P., 2006. Global carbon utilization profiles of wild-type strains, mutants and transformants of *Hypocrea jecorina*. *Appl. Environ. Microbiol.*, **72**(3):2126-2133. [doi:10.1128/AEM.72.3.2126-2133.2006]

Druzhinina, I.S., Komon-Zelazowska, M., Kredics, L., Hatvani, L., Antal, Z., Belayneh, T., Kubicek, C.P., 2008. Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. *Microbiology*, **154**(Pt 11):3447-3459. [doi:10.1099/mic.0.2008/021196-0]

d′Enfert, C., Bonini, B.M., Zapella, P.D., Fontaine, T., da Silva, A.M., Terenzi, H.F., 1999. Neutral trehalases catalyse intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. *Mol. Microbiol.*, **32**(3):471-483. [doi:10.1046/j.1365-2958.1999.01327.x]

Farkaš, V., Labudová, I., Bauer, Š., Ferenczy, L., 1981. Preparation of mutants of *Trichoderma viride* with increased production of cellulase. *Folia Microbiol.*, **26**(2):129-132. [doi:10.1007/BF02927368]

Felenbok, B., Flipphi, M., Nikolaev, I., 2001. Ethanol catabolism in *Aspergillus nidulans*: a model system for studying gene regulation. *Prog. Nucleic Acid Res. Mol. Biol.*, **69**:149-204. [doi:10.1016/S0079-6603(01)69047-0]

Fodor, S.A., Rava, R.P., Huang, X.C., Pease, A.C., Holmes, C.P., Adams, C.L., 1993. Multiplexed biochemical assays with biological chips. *Nature*, **364**(6437):555-556. [doi:10.1038/364555a0]

Franzusoff, A., Cirillo, V.P., 1982. Uptake and phosphorylation of 2-deoxy-D-glucose by wild type and single-kinase strains of *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta*, **688**(2):295-304. [doi:10.1016/0005-2736(82)90340-6]

Friedl, M.A., Kubicek, C.P., Druzhinina, I.S., 2008a. Carbon source dependence and photostimulation of conidiation in *Hypocrea atroviridis*. *Appl. Environ. Microbiol.*, **74**(1): 245-250. [doi:10.1128/AEM.02068-07]

Friedl, M.A., Schmoll, M., Kubicek, C.P., Druzhinina, I.S., 2008b. Photostimulation of *Hypocrea atroviridis* growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidativestress. *Microbiology*, **154**(Pt 4):1229-1241.

Gardiner, D.M., Kazan, K., Manners, J.M., 2009. Nutrient profiling reveals potent inducers of trichothecene biosynthesis in *Fusarium graminearum*. *Fungal Genet. Biol.*, **46**(8):604-613. [doi:10.1016/j.fgb.2009.04.004]

Gu, M.B., Mitchell, R.J., Kim, B.C., 2004. Whole-cell-based biosensors for environmental biomonitoring and application. *Adv. Biochem. Eng. Biotechnol.*, **87**:269-305.

Guarro, J., Antolin-Ayala, M.I., Gene, J., Gutierrez-Calzada, J., Nieves-Diez, C., Ortoneda, M., 1999. Fatal case of *Trichoderma harzianum* infection in a renal transplant recipient. *J. Clin. Microbiol.*, **37**(11):3751-3755.

Hamer, L., Adachi, K., Montenegro-Chamorro, M.V., Tanzer, M.M., Mahanty, S.K., Darveaux, B.A., Lampe, D.J., Slater, T.M., Ramamurthy, L., DeZwaan, T.M., *et al.*, 2001. Gene discovery and gene function assignment in filamentous fungi. *Proc. Natl. Acad. Sci. USA*, **98**(9): 5110-5115. [doi:10.1073/pnas.091094198]

Hennequin, C., Chouaki, T., Pichon, J.C., Strunski, V., Raccurt, C., 2000. Otitis externa due to *Trichoderma longibrachiatum*. *Eur. J. Clin. Microbiol. Infect. Dis.*, **19**(8): 641-642. [doi:10.1007/s100960000326]

Hoyos-Carvajal, L., Orduz, S., Bissett, J., 2009. Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions. *Fungal Genet. Biol.*, **46**(9):615-631. [doi:10.1016/j.fgb.2009.04.006]

Hynes, M.J., 1975. Studies on the role of the *areA* gene in the regulation of nitrogen catabolism in *Aspergillus nidulans*. *Aust. J. Biol. Sci.*, **28**(3):301-313.

Ilmén, M., Thrane, C., Penttilä, M., 1996. The glucose repressor gene *cre1* of *Trichoderma*: isolation and expression of a full-length and a truncated mutant form. *Mol. Gen. Genet.*, **251**(4):451-460. [doi:10.1007/s004380050189]

Jekosch, K., Kück, U., 2000. Glucose dependent transcriptional expression of the *cre1* gene in *Acremonium chrysogenum* strains showing different levels of cephalosporin C production. *Curr. Genet.*, **37**(6):388-395.

[doi:10.1007/s002940000121]

Kahmann, R., Basse, C., 2001. Fungal gene expression during pathogenesis-related development and host plant colonization. *Curr. Opin. Microbiol.*, **4**(4):374-380. [doi:10.1016/S1369-5274(00)00220-4]

Keppler, O.T., Horstkorte, R., Pawlita, M., Schmidt, C., Reutter, W., 2001. Biochemical engineering of the *N*-acyl side chain of sialic acid: biological implications. *Glycobiology*, **11**(2):11R-18R. [doi:10.1093/glycob/11.2.11R]

Kim, D.J., Baek, J.M., Uribe, P., Kenerley, C.M., Cook, D.R., 2002. Cloning and characterization of multiple glycosyl hydrolase genes from *Trichoderma virens*. *Curr. Genet.*, **40**(6):374-384. [doi:10.1007/s00294-001-0267-6]

Komon-Zelazowska, M., Bissett, J., Zafari, D., Hatvani, L., Manczinger, L., Woo, S., Lorito, M., Kredics, L., Kubicek, C.P., Druzhinina, I.S., 2007. Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms. *Appl. Environ. Microbiol.*, **73**(22):7415-7426. [doi:10.1128/AEM.01059-07]

Kowal, P., Wang, P.G., 2002. UDP-GlcNAc C4 epimerase involved in the biosynthesis of 2-acetamino-2-deoxy-L-altruronic acid in the O-antigen repeating units of *Plesiomonas shigelloides* O17. *Biochemistry*, **41**(51): 15410-15414. [doi:10.1021/bi026384i]

Kraus, G., Druzhinina, I., Bissett, J., Prillinger, H.J., Szakacs, G., Koptchinski, A., Gams, W., Kubicek, C.P., 2004. *Trichoderma brevicompactum* sp. nov. *Mycologia*, **96**(5): 1059-1073. [doi:10.2307/3762089]

Kubicek, C.P., 1987. Involvement of a conidial endoglucanase and a plasma-membrane bound β-glucosidase in the induction of endoglucanase synthesis by cellulose in *Trichoderma reesei*. *J. Gen. Microbiol.*, **133**(6):1481-1487.

Kubicek, C.P., Bissett, J., Kullnig-Gradinger, C.M., Druzhinina, I.S., Szakacs, G., 2003. Genetic and metabolic diversity of *Trichoderma*: a case study on South-East Asian isolates. *Fungal Genet. Biol.*, **38**(3):310-317. [doi:10.1016/S1087-1845(02)00583-2]

Kudla, B., Caddick, M.X., Langdon, T., Martinez-Rossi, N.M., Bennett, C.F., Sibley, S., Davies, R.W., Arst, H.N.Jr., 1990. The regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. *EMBO J.*, **9**(5):1355-1364.

Kutlesa, N.J., Caveney, S., 2001. Insecticidal activity of glufosinate through glutamine depletion in a caterpillar. *Pest Manage. Sci.*, **57**(1):25-32. [doi:10.1002/1526-4998(200101)57:1<25::AID-PS272>3.0.CO;2-I]

le Crom, S., Schackwitz, W., Pennacchio, L., Magnuson, J.K., Culley, D.E., Collett, J.R., Martin, J.R., Druzhinina, I.S., Mathis, H., Monot, F., *et al.*, 2009. Tracking the roots of cellulase hyperproduction by the fungus *Trichoderma reesei* using massively parallel DNA sequencing. *PNAS*, **106**(38):16151-16156. [doi:10.1073/pnas.0905848106]

Mach, R.L., Peterbauer, C.K., Payer, K., Jaksits, S., Woo, S.L., Zeilinger, S., Kullnig, C.M., Lorito, M., Kubicek, C.P., 1999. Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. *Appl. Environ. Microbiol.*, **65**(5):1858-1863.

March, J.C., Rao, G., Bentley, W.E., 2003. Biotechnological applications of green fluorescent protein. *Appl. Microbiol. Biotechnol.*, **62**(4):303-315. [doi:10.1007/s00253-003-1339-y]

Marzluf, G.A., 1997. Genetic regulation of nitrogen metabolism in the fungi. *Microbiol. Mol. Biol. Rev.*, **61**(1):17-32.

Montenecourt, B.S., Eveleigh, D.E., 1979. Selective Screening Methods for the Isolation of High Yielding Cellulase Mutants of *Trichoderma reesei*. *In*: Brown, R.D.Jr., Jurasek, L. (Eds.), Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis. Advances in Chemistry, Vol. 181. American Chemical Society, p.289-301. [doi:10.1021/ba-1979-0181.ch014]

Nagy, V., Seidl, V., Szakacs, G., Komoń-Zelazowska, M., Kubicek, C.P., Druzhinina, I.S., 2007. Application of DNA bar codes for screening of industrially important fungi: the haplotype of *Trichoderma harzianum* sensu stricto indicates superior chitinase formation. *Appl. Environ. Microbiol.*, **73**(21):7048-7058. [doi:10.1128/AEM.00995-07]

Nogawa, M., Goto, M., Okada, H., Morikawa, Y., 2001. L-sorbose induces cellulase gene transcription in the cellulolytic fungus *Trichoderma reesei*. *Curr. Genet.*, **38**(6): 329-334. [doi:10.1007/s002940000165]

OBrian, G.R., Fakhoury, A.M., Payne, G.A., 2003. Identification of genes differentially expressed during aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genet. Biol.*, **39**(2):118-127. [doi:10.1016/S1087-1845(03)00014-8]

O′Farrell, P.H., 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.*, **250**(10):4007-4021.

Pall, M.L., 1993. The use of ignite (Basta; glufosinate; phosphinothricin) to select transformants of bar-containing plasmids in *Neurospora crassa*. *Fungal Genet. Newslett.*, **40**:58.

Piwnica-Worms, D., Schuster, D.P., Garbow, J.R., 2004. Molecular imaging of host-pathogen interactions in intact small animals. *Cell. Microbiol.*, **6**(4):319-331. [doi:10.1111/j.1462-5822.2004.00379.x]

Platt, A., Langdon, T., Arst, H.N.Jr., Kirk, D., Tollervey, D., Mates Sanchez, J.M., Caddick, M.X., 1996. Nitrogen metabolite signalling involves the C-terminus and the GATA domain of the *Aspergillus* transcription factor AREA and the 3 untranslated region of its mRNA. *EMBO J.*, **15**(11):2791-2801.

Ragnaud, J.M., Marceau, C., Roche-Bezian, M.C., Wone, C., 1984. Infection peritoneale a *Trichoderma koningii* sur dialyse peritoneale continue ambulatoire. *Med. Maladies Infect.*, **14**(7-8):402-405. [doi:10.1016/S0399-077X(84)80067-0]

Randez-Gil, F., Prieto, J.A., Sanz, P., 1995. The expression of

a specific 2-deoxyglucose-6P phosphatase prevents catabolite repression mediated by 2-deoxyglucose in yeast. *Curr. Genet.*, **28**(2):101-107. [doi:10.1007/BF00315774]

Ravagnani, A., Gorfinkiel, L., Langdon, T., Diallinas, G., Adjadj, E., Demais, S., Gorton, D., Arst, H.N.Jr., Scazzocchio, C., 1997. Subtle hydrophobic interactions between the seventh residue of the zinc finger loop and the first base of an HGATAR sequence determine promoter-specific recognition by the *Aspergillus nidulans* GATA factor AreA. *EMBO J.*, **16**(13):3974-3986. [doi:10.1093/emboj/16.13.3974]

Rieger, K.J., Kaniak, A., Coppee, J.Y., Aljinovic, G., Baudin-Baillieu, A., Orlowska, G., Gromadka, R., Groudinsky, O., di Rago, J.P., Slonimski, P.P., 1997. Large-scale phenotypic analysis-the pilot project on yeast chromosome III. *Yeast*, **13**(16):1547-1562. [doi:10.1002/(SICI)1097-0061(199712)13:16<1547::AID-YEA230>3.3.CO;2-P]

Rieger, K.J., El-Alama, M., Stein, G., Bradshaw, C., Slonimski, P.P., Maundrell, K., 1999. Chemotyping of yeast mutants using robotics. *Yeast*, **15**(10B):973-986. [doi:10.1002/(SICI)1097-0061(199907)15:10B<973::AID-YEA402>3.0.CO;2-L]

Ross-Macdonald, P., Coelho, P.S.R., Roemer, T., Agarwal, S., Kumar, A., Jansen, R., Cheung, K.H., Sheehan, A., Symoniatis, D., Umansky, L., *et al.*, 1999. Large-scale analysis of the yeast genome by transposon tagging and gene disruption. *Nature*, **402**(6760):413-418. [doi:10.1038/46558]

Sampathkumar, P., Paya, C.V., 2001. *Fusarium* infection after solid-organ transplantation. *Clin. Infect. Dis.*, **32**(8):1237-1240. [doi:10.1086/319753]

Schauer, R., 2000. Achievements and challenges of sialic acid research. *Glycoconj. J.*, **17**(7-9):485-499. [doi:10.1023/A:1011062223612]

Schmoll, M., Zeilinger, S., Mach, R.L., Kubicek, C.P., 2004. Cloning of genes expressed early during cellulase induction in *Hypocrea jecorina* by a rapid subtraction hybridization approach. *Fungal Genet. Biol.*, **41**(9):877-887. [doi:10.1016/j.fgb.2004.06.002]

Schuster, A., Kubicek, C.P., Friedl, M.A., Druzhinina, I.S., Schmoll, M., 2007. Impact of light on *Hypocrea jecorina* and the multiple cellular roles of ENVOY in this process. *BMC Genomics*, **8**(1):449. [doi:10.1186/1471-2164-8-449]

Seiboth, B., Hartl, L., Pail, M., Kubicek, C.P., 2003. D-xylose metabolism in *Hypocrea jecorina*: loss of the xylitol dehydrogenase step can be partially compensated for by *lad1*-encoded L-arabinitol-4-dehydrogenase. *Eukaryot. Cell*, **2**(5):867-875. [doi:10.1128/EC.2.5.867-875.2003]

Seiboth, B., Gamauf, C., Pail, M., Hartl, L., Kubicek, C.P., 2007. The D-xylose reductase of *Hypocrea jecorina* is the major aldose reductase in pentose and D-galactose catabolism and necessary for β-galactosidase and cellulase induction by lactose. *Mol. Microbiol.*, **66**(4):890-900. [doi:10.1111/j.1365-2958.2007.05953.x]

Seidl, V., Huemer, B., Seiboth, B., Kubicek, C.P., 2005. A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. *FEBS J.*, **272**(22):5923-5939. [doi:10.1111/j.1742-4658.2005.04994.x]

Seidl, V., Druzhinina, I.S., Kubicek, C.P., 2006. A screening system for carbon sources enhancing β-*N*-acetylglucosaminidase formation in *Hypocrea atroviridis* (*Trichoderma atroviride*). *Microbiology*, **152**(Pt 7):2003-2012. [doi:10.1099/mic.0.28897-0]

Seidl, V., Gamauf, C., Druzhinina, I.S., Seiboth, B., Hartl, L., Kubicek, C.P., 2008. The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. *BMC Genomics*, **9**(1):327. [doi:10.1186/1471-2164-9-327]

Sims, A.H., Gent, M.E., Robson, G.D., Dunn-Coleman, N.S., Oliver, S.G., 2004. Combining transcriptome data with genomic and cDNA sequence alignments to make confident functional assignments for *Aspergillus nidulans* genes. *Mycol. Res.*, **108**(Pt 8):853-857. [doi:10.1017/S095375620400067X]

Singh, M.P., 2009. Application of Biolog FF MicroPlate for substrate utilization and metabolite profiling of closely related fungi. *J. Microbiol. Methods*, **77**(1):102-108. [doi:10.1016/j.mimet.2009.01.014]

Strauss, J., Mach, R.L., Zeilinger, S., Hartler, G., Stöffler, G., Wolschek, M., Kubicek, C.P., 1995. Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. *FEBS Lett.*, **376**(1-2):103-107. [doi:10.1016/0014-5793(95)01255-5]

Sweigard, J.A., Carroll, A.M., Valent, B., 1999. Restriction Enzyme-mediated Integration in the Rice Blast Fungus. *In*: *Septoria* on Cereals: A Study in Pathosystems. IACR 15th International Symposium. Long Ashton Research Station, Bristol, p.192-198.

Tanis, B.C., van der Pijl, H., van Ogtrop, M.L., Kibbelaar, R.E., Chang, P.C., 1995. Fatal fungal peritonitis by *Trichoderma longibrachiatum* complicating peritoneal dialysis. *Nephrol. Dial. Transplant.*, **10**(1):114-116.

Tanzer, M.M., Arst, H.N., Skalchunes, A.R., Coffin, M., Darveaux, B.A., Heiniger, R.W., Shuster, J.R., 2003. Global nutritional profiling for mutant and chemical mode-of-action analysis in filamentous fungi. *Funct. Integr. Genomics*, **3**(4):160-170. [doi:10.1007/s10142-003-0089-3]

Vitale, R.G., Afeltra, J., Dannaoui, E., 2005. Antifungal combinations. *Methods Mol. Med.*, **118**:143-152.

Wilson, R.A., Arst, H.N.Jr., 1998. Mutational analysis of AREA, a transcriptional activator mediating nitrogen metabolite repression in *Aspergillus nidulans* and a member of the streetwise GATA family of transcription factors. *Microbiol. Mol. Biol. Rev.*, **62**(3):586-596.

Yeo, S.F., Wong, B., 2002. Current status of nonculture methods for diagnosis of invasive fungal infections. *Clin. Microbiol. Rev.*, **15**(3):465-484. [doi:10.1128/CMR.15.3.465-484.2002]

Appendix

Fig. 1A Carbon sources of Biolog FF MicroPlate

Numbers indicate physiological and biochemical groups: 0: control; 1: monosaccharides: 1.1 heptose, 1.2 hexoses, 1.3 pentoses; 2: monosaccharide-related compounds: 2.1 sugar acids, 2.2 hexosamines, 2.3 polyols; 3: other sugars: 3.1 polysaccharides, 3.2 oligosaccharides, 3.3 glucosides; 4: nitrogen-containing compounds: 4.1 peptides, 4.2 L-amino acids, 4.3 biogene and heterocyclic amines, 4.4 TCA-cycle intermediates, 4.5 aliphatic organic acids; 5: others

Introducing editorial board member: Irina S. Druzhinina, the corresponding author of this invited review, is an editorial board member of *Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)*. Her study is focused on phylogeny and molecular ecology of Hypocreales fungi and on the development of bioinformatic tools for molecular identification of microbes. In the other line of research she is interested in metabolomics of filamentous fungi applying Phenotype MicroArray techniques. In 2001, she gained the doctorate of natural sciences of University of Vienna (Austria). Shortly after, she founded a research group on fungal evolution and functional biodiversity in Vienna University of Technology (Austria) and obtained a position of Assistant Professor in Department of Applied Biochemistry and Gene Technology.

Irina S. Druzhinina

Lea Atanasova, the first author of this invited review, studied biology and ecology at the University of Ljubljana (Slovenia) and has graduated from the University Vienna (Austria). She was awarded for her master thesis by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management as well as by Ecological Society of Germany, Austria and Switzerland. Soon after graduation, she joined the Irina S. Druzhinina's research group of fungal biodiversity and evolution in Vienna University of Technology (Austria) and started to work on her PhD thesis dedicated to the molecular ecology of soil mycobiota.

Lea Atanasova