DISSERTATION

MOLECULAR ECOLOGICAL ASPECTS OF *TRICHODERMA* THAT SHOULD BE CONSIDERED PRIOR ITS APPLICATION IN AGRICULTURE AND INDUSTRY

ausgeführt zum Zwecke der Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

unter der Leitung von

Priv. 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. Monika Komon-Zelazowska

Matrikelnummer 0627375

Rechte Nordbahngasse 6/ 4/ 31, 1210 Wien

Wien, am 25.03.2014

Mojej Mamie i Tacie za to, ze wierzyli we mnie...

„Look deep into nature,
and then you will understand everything better.”
Albert Einstein

CONTENTS

Kurzfassung 4

Summary 5

Nomenclature and current taxonomic assumptions 6

Abbreviations and web resources 8

Thesis overview 9

1. *Trichoderma* and diversity of its applications 10

2. Aims of the thesis 31

3. *Trichoderma* and *Pleurotus* farming 32

3.1. Biofungicide versus causative agents of the green mold disease of mushrooms 33

3.1.1. Green mould on *Agaricus* 33

3.1.2. Trichoderma on *Plurotus* 35

3.2. Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms 41

3.3. Molecular identification of *Trichoderma* species associated with *Pleurotus ostreatus* and natural substrates of the oyster mushroom. 54

3.4. Further investigations on *T. pleurotum* and *T. pleuroticola* 65

4. Peptaibols of *Trichoderma* – a unique group of Hypocreaceae secondary metabolites 66

4.1. Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma* 67

4.2. The formation of atroviridin by *Hypocrea atroviridis* is conidiation-associated, and positively regulated by blue light and the G-protein GNA3 83

5. Which *Trichoderma* species may cause invasive mycoses in immunocompromised humans? 95

5.1. Inclusion of *Trichoderma longibrachiatum* in the list of opportunistic pathogens of immunocompromised humans 96

5.2. Alternative reproductive strategies of *Hypocrea oreintalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. 100

5.3. Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma* 114

5.4. An unknown species from Hypocreaceae isolated from human lung tissue of a patient with non-fatal pulmonary fibrosis 126

5.5. Taxonomic updates on *Trichoderma* species of putative clinical importance 132

6. Conclusive remarks: *Trichoderma* is a beneficial microorganism which is safe to apply. Almost safe! 134

7. Acknowledgements 139

8. Curriculum Vitae with complete list of publications 140

9. Appendix: Co-authored manuscript that include materials from the thesis 147

Kurzfassung

Dem filamentösen und mykoparasitären Pilz *Trichoderma* kommt eine Schlüsselrolle in der industriellen Produktion von Enzymen zu und wird unter Anderem verwendet, um Cellulasen und Hemicellulasen zu produzieren. *Trichoderma* wird dabei häufig im gleichen Atemzug mit anderen wirtschaftlich relevanten Mikroorganismen erwähnt. Durch seine Rolle als Enzymproduzent wird *Trichoderma* bei der zur Produktion von Biotreibstoffen der zweiten Generation eine bedeutende Rolle zugeschrieben. Darüber hinaus findet der Pilz Anwendung in der Landwirtschaft, wo er als Mittel zur biologischen Kontrolle von pflanzenpathogenen Pilzen (Biocontrol) eingesetzt wird als auch als Biodünger Verwendung findet. In der Gattung *Trichoderma* finden sich aber auch Vertreter, die als mykoparasitäre Pathogene von kommerziell genutzter Pilze bekannt sind. Auch im Humanbereich spielen zwei Arten dieses Genus als opportunistische Krankheitserreger, vor allem bei immungeschwächten Menschen, eine Rolle.

Der Fokus dieser Arbeit ist auf mögliche negative Auswirkungen durch Vertreter der Gattung *Trichoderma* gerichtet. Die Arbeit untersucht (i) den Zusammenhang zwischen den ursächlichen Substanzen die die grüne Schimmel-Krankheit bei Pilzen verusachen, (ii) ob *Trichoderma* potentiell toxische Sekundärmetaboliten bildet und (iii) wie *Trichoderma* invasive Mykosen bei Menschen hervorruft.

Manche negativen Eigenschaften, wie Mykoparasitismus, sind charakteristisch für eine Vielzahl von *Trichoderma* Specien. Andere, wie die opportunistische Pathogenität für immungeschwächte Säugetiere oder die grüne Schimmelpilz-Krankheit, sind widerum auf wenige Taxa beschränkt. Die Vielzahl an Einsatzmöglichkeiten sowie die ökologische Anpassungsfähigkeit von *Trichoderma* machen diesen Pilz ein lohnenswertes Forschungsobjekt, um tieferes Verständnis für seine Ökologie und Vielfältigkeit zu entwickeln.

Summary

The mycoparasitic filamentous fungus *Trichoderma* is frequently listed among the most economically significant microorganisms because of its key role in the production of industrial enzymes such as cellulases and hemicellulases that besides other roles are proposed for the second generation biofuel production. Even more importantly *Trichoderma* is intensively used in agriculture as an agent of biological control of plant pathogenic fungi (biocontrol) and as biofertilizer. On the contrary there are also known adverse effects of the mycoparasitic activity of *Trichoderma* as some species of the genus are pathogenic to the commercial mushrooms. Furthermore, at least two species of the genus are important to mankind as opportunistic pathogens of immunocompromised humans.

The focus of this thesis is put on the possible negative effects of the genus *Trichoderma* such as (i) the investigation of causative agent(s) of the mushroom green mold disease; (ii) production of putatively toxic secondary metabolites and (iii) ability to cause invasive mycoses in humans.

Some properties, like mycoparasitism, are characteristic of a wide variety of species in the genus. Other properties, like opportunistic attacks on mammals with immunodeficiency or green mold disease, seem to be restricted to certain taxa. Thus, the great diversity of applications combined with ecological adaptability of the genus *Trichoderma* makes for a deeper understanding of its ecology and diversity the worthwhile.

Nomenclature and current taxonomic assumptions

The genus *Hypocrea/Trichoderma* is pleomorphic. Taxonomic names *Hypocrea* and *Trichoderma* are manifestations of the same organisms. Recent studies have demonstrated that the majority of the genetic diversity of the genus is represented by its sexual forms (Jaklitsch, 2009, 2011; Druzhinina *et al*., 2011) while 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) (Druzhinina *et al*., 2008, 2010a, 2010b; Samuels *et al.*, 2012). Several changes in the International Code of Botanical Nomenclature which was adopted at the IBC (International Botanical Congress) in Melbourne in July 2011 have important consequences for the way names are applied for fungi. Historically, different names were applied to the sexual and vegetative forms of some fungi, but from now on, only a single older taxonomic name applies to each fungal species: a principle that has been articulated as "one fungus, one name." In addition, starting 1 January 2013, names of new fungi will require the citation of a unique identifier issued by a recognized repository that will register the name (Outcomes of the 2011 Botanical Nomenclature Section at the XVIII International Botanical Congress, James S. Miller).

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. The generic group is termed *Hypocrea/Trichoderma, Hypocrea* or *Trichoderma*.

References:

Jaklitsch WM (2009) European species of *Hypocrea* Part I. The green-spored species. *Stud Mycol* **63**, 1-91.

Jaklitsch WM (2011) European species of *Hypocrea* part II: species with hyaline ascospores. *Fungal Divers* **48**(1), 1-250.

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**, 3447-59.

Druzhinina IS, Kubicek CP, Komoń-Zelazowska M, Mulaw TB & Bissett J (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 IS, Komoń-Zelazowska M, Atanasova L, Seidl V & Kubicek CP (2010b) 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, 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.

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

BCAs	Biological Control Agents
cal1	calmodulin
chi18-5	Endochitinase (former *ech42*)
DNA	deoxyribonucleic acid
GenBank	public DNA/protein sequence database maintained by the National Center for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration (INSDC).
GeneDoc:	sequence editing software http://www.nrbsc.org/gfx/genedoc
IBC	International Botanical Congress
ICBN	International Code of Botanical Nomenclature: http://www.iapttaxon.org/nomen/main.php
INSDC	International Nucleotide Sequence Database Collaboration
ISTH	International Subcommission on taxonomy of *Hypocrea:* http://www.isth.info
JGI	DOE Joint Genome Institute: http://www.jgi.doe.gov
MKZ	Monika Komon-Zelazowska, the author of the thesis
NCBI	National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov
NRPS	Non-Ribosomal Peptide Synthetase
rpb2	RNA polymerase subunit II gene
s.l.	sensu lato
s.s.	sensu stricto
tef1	translation elongation factor 1
USDA	United States Department of Agriculture, also known as the Agriculture Department

Thesis overview

This thesis consists of an introduction that describes the diversity of *Trichoderma* applications followed by the three chapters comprising results of the investigations on (i) the green mould disease of mushrooms caused by *Trichoderma* spp., (ii) diversity of peptaibols secreted by *Trichoderma* and (iii) *Trichoderma* as an opportunistic pathogen of immunocompromised humans; conclusive remarks and an appendix with the manuscript that include materials from the thesis.

The first authorship publications presented in the chapters 3 and 4 have been published in peer-reviewed scientific juornals, *Applied and Environmental Microbiology* and *Eukaryotic Cell* respectively.

The thesis also includes five other research papers and one review co-authored by the applicant; all published in peer-reviewed journals.

In total, the list of applicant's publications consists of 23 peer-reviewed publications (see complete list of publications).

1. *Trichoderma* and diversity of its applications

True fungi or Eumycetes have been traditionally recognised as the kingdom, marked as eukaryotic heterotropic organisms with a low level of thallus diversification and chitin-containing cell walls. They reproduce by means of spores that also function as stress resistant structures. In the modern system Eumycetes is placed on the Eukaryote domain of life where they share the common ancestor with Metazoa (animals).

Species of the mycotrophic filamentous genus *Trichoderma* (teleomorph *Hypocrea*, Hypocreales, Ascomycota, Dykaria) are among the most widely used and widely isolated fungi. They could be found growing on bark, dead wood, other fungi, building materials and animals (Klein and Eveleigh, 1998), in soil and in the rhizosphere of plants. *Trichoderma* strains have been also detected on marine sponges (Paz *et al*., 2010; Gal Hemed *et al*., 2011), as endophytes (Samuels *et al*., 2006b; Zhang *et al*., 2007; Mulaw *et al*., 2010; Chaverri *et al*., 2011; Chaverri and Samuels, 2013) as well as (although rarely) on livivg herbaceous plants (Jaklitsch, 2009). It clearly demonstrates their high opportunistic potential and that they are able very flexibly adapt to various ecological niches (Druzhinina *et al*., 2011).

Among hundreds of fungal genera, *Trichoderma* contains several species which play prodigious role to humankind due to their ability to produce enzymes they can be applied in industry and especially in biotechnology (Figure 1.1). *Trichoderma* enzymes are involved in alteration of plant biomass into soluble sugars and can be used for fossil-fuel production and other coproduction systems (Kubicek and Penttilä, 1998).

In addition, *Trichoderma* spp. are also known for their remarkable potential to antagonize other fungi. This property has been exploited in domestication of *Trichoderma* as biological control agents (BCA) against numerous plant pathogenic fungi. In particular strains of *T*. cf. *harzianum*, *T. atroviride, T. virens* and *T. asperellum* are universally active (Chet, 1987; Kubicek and Penttilä, 1998; Sivasithamparam and Ghisalberti, 1998; Hjeljord and Tronsmo, 1998; Harman *et al*., 2004; Druzhinina *et al*., 2011).

Plant diseases caused by fungal and bacterial pathogens are a major cause of decrease in amount of crops obtained by agriculture every year (Chet *et al.,* 1997). Use of chemical compounds for control of

plant diseases is limited due to the development of resistance to these compounds by pathogens. On the contrary usage of *bio* control agents offers environmentally risk-free approach.

Monte (2001) proposed that a combination of biological control agents applied with addition of lower levels of chemical compounds would result in the same level of crop protection as by using chemicals alone.

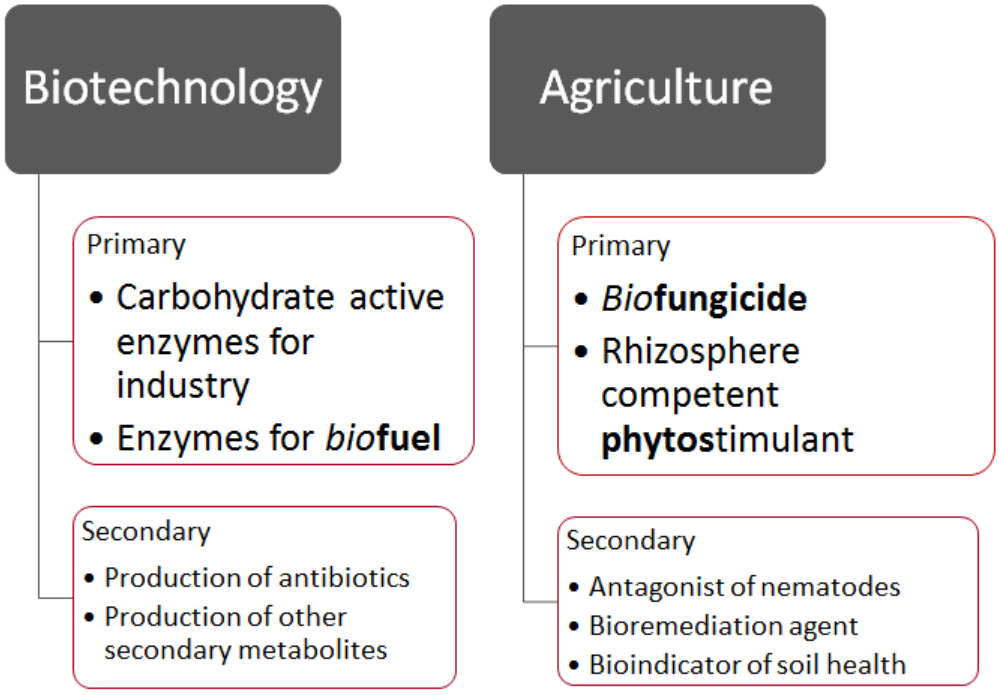


Figure 1.1. Major applications of *Trichoderma* in biotechnology and agriculture

Approach to employ beneficial microorganisms is based on several assumptions. Firstly, growing faster than the plant pathogens, microorganisms can over compete the unwanted fungi or bacteria. Secondly, BCAs can use a plant pathogenic microbe as a nutrient source or can release compounds that inhibit growth and development of such pathogens.

The genus *Trichoderma* spans a big number of species and strains that have properties appropriate for BCAs. Their acting involves the activation of multiple mechanisms (Druzhinina *et al*., 2011; Atanasova *et al*., 2013; Druzhinina and Kubicek, 2014).

The ability to control plant pathogenic fungi – the principal action of BCAs is based on mycoparasitism that is a directed process, which can be divided into several stages: recognition of the presence of a potential prey ("sensing"); induction of the biochemical tools to besiege the prey ("preparing for the prey"); actual attack; and eventual killing and feeding on the prey.

The transition from the commensalism to parasitic state necessitates the molecular dissection of traits responsible for both interactions. The availability of the genome sequence of *T. atroviride* and *T. virens* (Kubicek *et al.,* 2011) has enabled to use full genome arrays to study the sequential events occurring during confrontation of these *Trichoderma* spp. with *Rhizoctonia solani* at a genome-wide transcriptomic level (Atanasova *et al*., 2013). This study revealed that both *T. virens* and *T. atroviride* reacted to the presence of *R. solani* already before physical contact. Yet they showed an essentially different behavior: *T. virens* only overexpressed 78 genes (1 % of all expressed genes), of which those involved in gliotoxin biosynthesis and its precursor metabolites accounted for the largest group. Genes for other secondary metabolites and extracellular enzymes were mostly constitutively expressed. *T. atroviride*, in contrast, overexpresses 400 genes (about 5 % of all expressed genes) which were enriched PTH11-G-protein coupled receptors, lectins and ß-glucanases, small secreted cysteine rich proteins and secondary metabolite synthases. Some of these compounds have been described above, and the potential role of the other gene families is detailed below (given after Druzhinina and Kubicek, 2014).

However, there were also common responses shared by *T. virens* and *T. atroviride* and thus expected to be universal for the genus *Trichoderma* because these species represent the two phylogenetcally distinct clades. One was the overexpression of a high number of genes for proteolytic enzymes and oligopeptide transporters, which is consistent with the findings that the overexpression of the alkaline protease gene *prb1* enhances the mycoparasitic ability (Flores *et al*., 1997). Another event, common to both *T. virens* and *T. atroviride*, is the induction of genes of the heat shock response such as HSP23, HSP70, HSP90 and HSP104, genes of oxidative stress response (cytochrome C peroxidase, proline

oxidase, and ER-bound glutathione-S-transferases), and genes for detoxification processes (ABC efflux transporters, the pleiotropic drug resistance (PDR) transporters and the multidrug resistance MDR-type transporters). *R. solani* has been shown to use radical oxygen species as signaling molecules during sclerotia formation (Papapostolou and Georgiou, 2010), and excrete antifungal components (Aliferis and Jabaji, 2010), both of which may have elicited this response. An ABC-transporter from Ta (TAABC2) has already been shown to be involved in biocontrol of *R. solani* (Ruocco *et al*., 2009).

In this regards it is noteworthy to mention that *Trichoderma* spp. also contain a considerable number of cytochromes P450 proteins (Druzhinina *et al*., 2012). They represent a superfamily of sequence-related heme oxygenases, which are found in most organisms, and whose roles range from carbon-source degradation and the elaboration of metabolites in prokaryotes, lower eukaryotes and plants, to detoxification of xenobiotic compounds in insects and mammals including humans (Kelly *et al*., 2006). Their role in the intracellular detoxification of potentially toxic metabolites is well known in higher organisms (Singh *et al*., 2011) and may also here serve to defend *Trichoderma* against its prey. However, evidence has recently emerged that they are also playing a role in the extracellular inactivation of hazardous materials (Roelofs *et al*., 2012), which may explain the profound ability to grow and develop even in highly contaminated habitats or in kerosene tanks (Klein and Eveleigh, 1998). They may therefore be important components for *Trichoderma's* opportunistic abilities.

Recognition and attachment to the host hyphae is the first essential step in the contact with the prey, although the observed morphological changes depended strongly on the host fungus tested. Lu *et al*. (2004) used a *T. atroviride* strain carrying a green fluorescent protein under a constitutive promoter to study the necrotrophic parasitic interaction between with the oomycete *P. ultimum* and the basidiomycete *R. solani*. Growing alongside the host hyphae and formation of papillae-like structures were observed as the most common events. These authors further showed that the hyphae of *T. atroviride* also frequently branched towards the host suggesting an active, probably chemotactic response to its presence. The formation of helix-shaped hyphae ("coiling") – a morphological response that has most frequently been associated with mycoparasitic attack (Figure 1.2).

Despite the wealth of information on genes that contribute to the mycoparasitic activity of *Trichoderma*, we still know only little about the molecules that are actually used to kill the prey

(Druzhinina and Kubicek, 2014). This is likely also the result of the fact that different strains and species use different strategies for this purpose (Atanasova *et al*., 2013). Secondary metabolites of *Trichoderma* are generally believed to play a role in this process. However, functional genetic evidence is still lacking, and *in vitro* data may be misleading: as an example, the peptaibols can act synergistically with secreted hydrolytic enzymes to promote ingress into pathogen structures suggesting a role in antagonistic actions against plant pathogens (Schirmböck *et al*., 1994). Nonetheless, both NRPS-encoding genes are downregulated during confrontation of *T. atroviride* and *R. solani* (Atanasova *et al*., 2013). Reverse genetic data for an involvement in antagonism have so far only been obtained for trichodermin (in *T. brevicompactum*; Tijerino *et al*., 2011), and gliotoxin (Atanasova *et al*., 2013; Mukherjee *et al*., 2012).

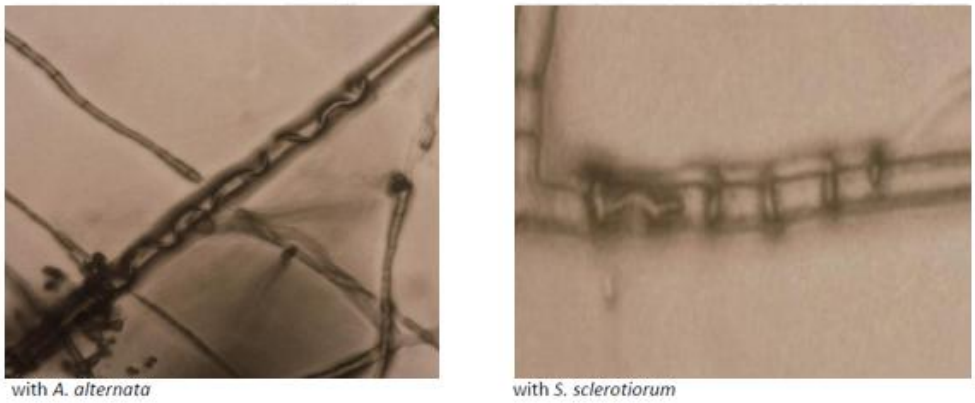


Figure 1.1. Coiling of *Trichoderma koningiopsis* TUCIM D28 in dual confrontations with plant pathogenic fungi *Alternaria alternata* and *Sclerotinia sclerotiorum*, synthetic nutritional agar, 21 days of incubation in darkness, 25^{o}C. Photo: MKZ

It appears appropriate here 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).

Trichoderma strains are applied for biocontrol against fungal phytopathogens on two ways: indirectly by (i) competing for nutrients and space, thereby crowding out the pathogen and taking over, (ii) producing metabolites that may kill the cells (antibiosis), block spore germination (fungistasis) or modifying the rhizosphere preventing the pathogens from growth (acidifying the soil). The direct way occurs when the pathogen itself and BCA interact together in the process which is called parasitism. In this case the physical contact and production of hydrolytic enzymes, toxic compounds and/or antibiotics has to be established in order to work coefficiently with the enzymes (Gajera *et al.*, 2013). It is also known that *Trichoderma* strains can positively influence plant growth by biofertilization and stimulate the plant-defense mechanisms (Haque *et al.*, 2010).

For over 80 years *Trichoderma* species have been investigated as biological control agents but only recently some strains are commercially available. One of the possible reasons could be the impact of harmful side-effect when applying chemical fungicides. BCAs are concerned as “natural” thus not dangerous to the products (Monte, 2001).

For a wide variety of diseases and crops in all climate zones commonly used as biocontrol agents against plant pathogenic fungi are the four *Trichoderma* species. These are strains of *T*. cf. *harzianum*, *T. atroviride* (teleomorph *H. atroviridis), T. virens* (teleomorph *H. virens*) and *T. asperellum* which are applied against plant pathogenic fungi such as *Rhizoctonia (Thanatephorus)*, *Botrytis* (*Botryotinia)*, *Sclerotinia* and *Fusarium (Gibberella)* or fungi-like organisms *Phytophthora*, *Pythium* (Hjeljord and Tronsmo, 1998). Lately, there is growing number of studies facing the biocontrol of nematodes (Dababat *et al*., 2006; Kyalo *et al*., 2007; Goswami *et al*., 2008).

The well known fungal bioagent *Trichoderma* cf. *harzianum* has been investigated for a long time. The biocontrol mechanism of *T.* cf. *harzianum* is a complex process mediated by the secretion of extracellular enzymes, such as chitinases (de la Cruz *et al*., 1992), β-glucanases (Lorito *et al*., 1994) and proteinases (Geremia *et al*., 1993), as well as secondary metabolites (Sivasithamparam and Ghisalberti, 1998).

The role of *Trichoderma* antibiotics in biocontrol is still a matter of discussion. Although some antibiotics may be the major factor for the biocontrol activity of a certain strain, this may not be the case for others (Harman, 2000).

Filamentous fungi are applied in the industry as a valuable source of enzymes due to their extracellular protein production (Kubicek *et al.*, 2009). Among others species of the genus *Trichoderma,* specifically *T. reesei* is considered as a robust enzyme factory due to the fact that it is capable to abundantly secrete enzymes with lytic ability, namely: pectinases (pectin methylesterase, endo and exo-polygalacturonases), cellulases (endo and exoglucan beta-1,3-glucosidases, endoglucan beta-1,6-glucosidases), hemicellulases (xylanases, mannanases), endochitinases, chitin 1,4-beta-chitobiosidases, proteases lipases, amylases, RNases, DNases and phosholipases (Lorito, 1998).
Contrary to other industrially applied hosts for proteins expression, *Trichoderma* can be cultivated on relatively cheap and uncomplicated media thus it is not necessary to use any expensive supplements in the growth medium like: vitamins, amino acids or other additives. Because of the unique properties *Trichoderma reesei* becomes an attractive host organism used for production of its native cellulolytic and hemicellulolytic enzymes and also for the production of heterologous proteins (Saloheimo and Pakula, 2012; Gorsche *et al*., 2013). Another benefit to use *T. reesei* is the evidence of the expansion of all crucial tools required for the expression of heterologous proteins in this organism (Gorsche *et al*., 2013). The fact that *Trichoderma reesei* is entirely known from the single wild type culture QM6a makes it uncommon between fungi which are applied in the industry. The wild type strain QM6a was isolated from the cotton garments and canvas of the US army from Solomon Islands during the World War II (Reese, 1976). It is also worth to mention that nowadays all isolates used in industry and science come from the ancestor strain *Trichoderma reesei* QM6a. Large–scale mutagenesis and screening programs have released entire purebred of isolates with upgraded enzyme production properties (Saloheimo *et al*., 2012). The amount of valuable mutant strains is considerable, among them: hypercellulolytic, cellulose-negative strains or moreover, some partially protease-deficient strains (Mantyla, 1998). Additionally, *T. reesei* can be transformed with a number of diverse selection markers, from auxotrophic markers such as *pyr4* (Gruber *et al*., 1990), to resistance to benomyl (Peterbauer *et al*., 1992; Schuster *et al.,* 2007) or hygromycin (Mach *et al*., 1994) or even to allow

growth on acetamide as sole nitrogen source mediated through the *Aspergillus nidulans amdS* gene (Penttilä *et al*., 1987).

Supplementary to heterologous enzymes already produced on an industrial scale using *Trichoderma* as host organism, great achievements have been initiated to express an amount of other enzymes partly from more distantly related donor organisms (Table 1.1).

The first heterologous protein expressed in *T. ressei* was calf chymosin (Penttilä, 1998), which was expressed in *T. reesei* Rut-C30 under the *cbh1* promoter (Harkki *et al*., 1989; Uusitalo *et al*., 1991). Another example of the first efforts to express heterologously an antibody in *Trichoderma* has been reported by Nyyssönen and co-workers. They successfuly expressed murine Fab fragments in *T. reesei* (Nyyssönen *et al*., 1993; Nyyssönen and Keränen, 1995).

Other impressive examples of the introduction of heterologous proteins are the expression of endopeptidase B from barley under the control of the *cbh1* promoter (Saarelainen *et al*., 1997) and cinnamoyl esterase from *Piromyces equi* (Poidevin *et al*., 2009). From one point of view, the expression of endopeptidase B from barley confirmed that it is feasible to express fully functional proteins from higher eukaryotes in *Trichoderma*. From the another point of view, the expression of cinnamoyl esterase from *Piromyces equi* ensured the production of EstA, a fully functional catalytic domain, capable of releasing ferulic acid, a valuable aromatic compound, from a variety of natural substrates such as maize bran and wheat (Gorsche *et al*., 2013).

As already mentioned, *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is the main industrial producer of lignocellulolytic enzymes, and the secretory behavior of this fungus strongly depends on the carbon sources (Kubicek *et al*., 1996; Jun *et al*., 2013). From the biotechnological point of view some cellulases applications could be found in paper, pulp and textile processing industries (Belghith *et al*., 2001) or in the improvement of animal feed (Bhat, 2000). Since recently, these enzymes are also hired in the degradation of biomass into simple sugars which are in turn useful for biofuel production (Kumar *et al*., 2008).

The wide industrial application of *T. reesei* in industry for enzyme production raises a question whethe this fungus also secreet secondary metabolites that may be potentially dangerous ‘contaminants’ for biotechnological products.

TABLE 1.1. HETEROLOGOUS PROTEINS EXPRESSED IN *TRICHODERMA* (MODIFIED FROM GORSCHE *ET AL*., 2013).

Protein	Donor organism	Host strain	Reference
Acid phosphatase	*Aspergillus niger*	ALKO2221	Miettinen-Oinonen *et al.*, 1997
Aminopeptidase	*Aspergillus* sp.	*Trichoderma reesei* or *longibrachiatum*	Amfep, 2009
Amylase (alpha)	*Aspergillus* sp.	*Trichoderma reesei* or *longibrachiatum*	Amfep, 2009
Antibody Fab fragments	Murine	Rut-C30	Nyyssönen *et al.*, 1993
Beta-Glucosidase	*Talaromyces emersonii*	Rut-C30	Murray *et al.*, 2004
Cellulase	*Trichoderma* sp.	*Trichoderma reesei* or *longibrachiatum*	Amfep, 2009
Chymosin	Calf	Rut-C30	Harkki *et al.*, 1989; Uusitalo *et al.*, 1991
Cinnamoyl esterase EstA	*Piromyces equi*	Rut-C30	Poidevin *et al.*, 2009
Cutinase	*Coprinopsis cinerea*	D-00775 *cbh1*-neg	Kontkanen *et al.*, 2009
DewA	*Aspergillus nidulans*	QM9414	Schmoll *et al.*, 2010
Endochitinase	*Trichoderma harzianum*	Rut-C30	Margolles-Clark *et al.*, 1996
Endopeptidase B	Barley	Rut-C30	
		ALKO2221	Saarelainen *et al.*, 1997
Glucanase (beta)	*Trichoderma* sp.	*Trichoderma reesei* or *longibrachiatum*	Amfep, 2009
Glucoamylase P	*Hormoconis resinae*	Rut-C30	Joutsjoki *et al.*, 1993
		ALKO2221	
Laccase	*Phlebia radiate*	Rut-C30	Saloheimo and Niku-Paavola, 1991
Laccase	*Melanocarpus albomyces*	Rut-C30	Kiiskinen *et al.*, 2004
Pectin lyase	*Aspergillus* sp.	*Trichoderma reesei* or *longibrachiatum*	Amfep, 2009
Phospholipase A	*Thermomyces* sp.	*Trichoderma reesei* or *longibrachiatum*	Amfep, 2009
Steryl esterase	*Melanocarpus albomyces*	Rut-C30	Kontkanen *et al.*, 2006
Xylanase II	*Humicola grisea*	HEP1	De Faria *et al.*, 2002
Xyn VI	*Acrophialophora nainiana*	Rut-C30	Salles *et al.*, 2007
Xyn11A	*Nonomuraea flexuosa*	ALKO3620	Paloheimo *et al.*, 2003

Thus, the genus *Trichoderma* is producing a numerous secondary metabolites such as toxins, potential anti-cancer compounds and antibiotics (Reino *et al*., 2008) that are important in signaling, development and interaction with other living organisms (Hoffmeister and Keller, 2007; Osbourn, 2010; Kubicek *et al*., 2011).

The genes encoding enzymes that synthesize secondary metabolites have recently been reviewed (Mukherjee *et al*., 2012).

The peptaibols are a group of small, linear 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 (Komon-Zelazowska *et al*., 2007). These properties have given rise to the name peptaibol (**pept**ide, **Aib**, and amino alcoh**ol**) (Benedetti,ProcNatAcadSci, 1982). The genomes of all three *Trichoderma* spp. (Martinez *et al*., 2008; Kubicek *et al*., 2011) 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). Mukherjee *et al*. (2011) and Degenkolb *et al*. (2012) showed by gene disruption 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.

Some peptaibol structures are given below (Figure 1.3). Peptaibols form a helical structure with the hydrophobic side chains exposed to the surface which allows them to interact with natural and artificial bilayers and form pores or voltage-dependent ion channels increasing membrane permeability (Rebuffat *et al*., 1999).

The need to sequence more and more genomes of *Trichoderma* species gives us an opportunity to provide better library of enzymes used in biofuel production (Baker *et al*., 2008; Grigoriev *et al*., 2011). On the other hand there are still important unanswered questions in awareness of biocontrol,

identifying new enzymes and exploring the structures of secondary metabolites (Karagiosis and Baker, 2013).

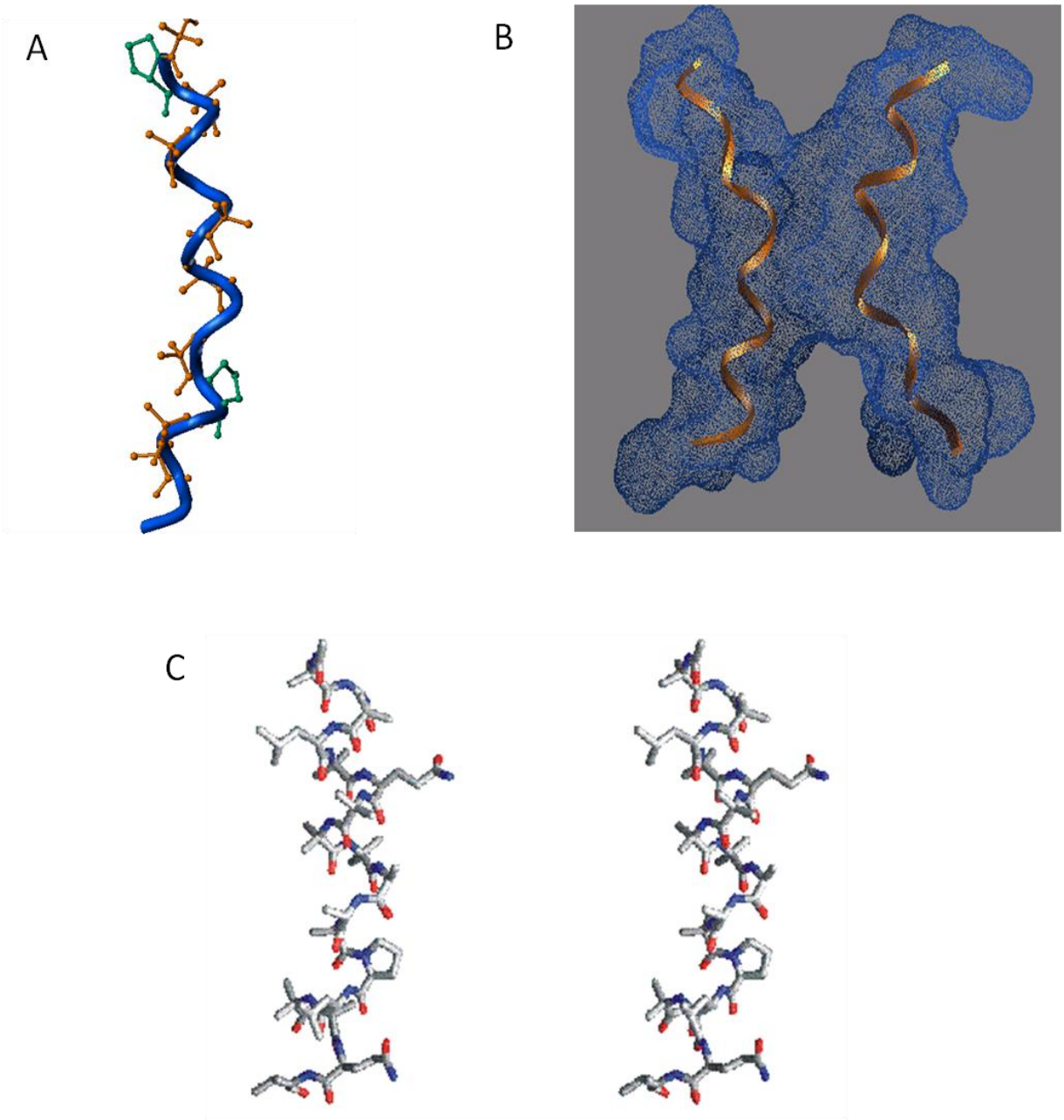


Figure 1.3. Peptaibol structures. (A) An alamethicin (1amt) helix with the eight Aib residues highlighted in orange, thought to promote an alpha-helical character (shown in blue) which is observed in many peptaibols. Two proline residues of alamethicin are highlighted in green. (B) Antiparallel antiamoebin (1joh) dimer (helices highlighted in orange), as found in the asymetric unit. (C) Crystal structure of trichotoxin_a50 (Chugh, Bruckner & Wallace (2002) biochemistry 41: 12934-12941). Pictures taken from http://peptaibol.cryst.bbk.ac.uk/structure.htm

References:

Amfep (2009) *List of commercial enzymes*. Available at http://www.amfep.org/content/list-enzymes (Accessed 10 March 2014).

Aliferis KA & Jabaji S (2010) Metabolite composition and bioactivity of *Rhizoctonia solani* sclerotial exudates. *Journal of Agricultural and Food Chemistry* **58**, 7604-7615.

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.

Baker SE, Thykaer J, Adney WE, Brettin TS, Brockman FJ, DHaeseleer P, Martinez AD, Miller RM, Rokhsar DS, Schadt CW, Torok T, TG, Bennett J, Berka RM, Briggs SP, Heitman J, Taylor J, Turgeon BG, Werner-Washburne M & Himmel ME (2008) Fungal genome sequencing and bioenergy. *Fungal Biology Reviews* **22**, 1-5.

Belghith H, Ellouz-Chaabouni S & Gargouri A (2001) Biostoning of denims by *Penicillium occitanis* (Pol6) cellulases. *Journal of Biotechnology* **89**, 257-262.

Beneditti E, Bavoso A, Di Blasio B, Pavone V, Pedone C, Toniolo C & Bonora GM (1982) Peptaibol antibiotics: a study on the helical structure of the two sequences of emerimicins III and IV. *Proc Natl Acad Sci* USA **79**, 7951–7954.

Bhat MK (2000) Cellulases and related enzymes in biotechnology. *Biotechnology Advances* **18**, 355-383.

Chaverri P, Gazis R & Samuels GJ (2011) *Trichoderma amazonicum*, a new endophytic species on *Hevea brasiliensis* and *H. guianensis* from the Amazon basin. Mycologia **103**, 139-151.

Chaverri P & Samuels GJ (2013) Evolution of hbitat preference and nutrition mode in a cosmopolitan fungal genus with evidence of interkingdom host jumps and major shifts in ecology. Evolution **7**, 2823-2837.

Chet I (1987) *Trichoderma* – application, mode of action and potential as biocontrol agent of soil borne plant pathogenic fungi. In: Chet I (ed.) Innovative Approaches to Plant Disease Control. John Wiley and Sons, New York, 137-160.

Chet I, Inbar J & Hadar I (1997) Fungal antagonists and mycoparasite. In Wicklow DT, Soederstroem B (EDS) *The Mycota* IV: Environmental and microbial relationships, Springer-Verlag, Berlin, 165-184.

Chugh JK, Brückner H & Wallace BA (2002) Model for a helical bundle channel based on the high-resolution crystal structure of trichotoxin_A50E. *Biochemistry* **41**(43), 12934-41.

Dababat AA, Sikora RA & Hauschild R (2006) Use of *Trichoderma harzianum* and *Trichoderma viride* for the biological control of *Meloidogyne incognita* on tomato. *Commun Agric Appl Biol Sci* **71**, 956-961.

De Faria FP, Te'o VSJ, Bergquist PL, Azevedo MO & Nevalainen KMH (2002) Expression and processing of a major xylanase (XYN2) from the thermophilic fungus *Humicola grisea* var. *thermoidea* in *Trichoderma reesei*. *Letters in Applied Microbiology* **34**, 119-123.

Degenkolb T, Karimi Aghcheh R, Dieckmann R, Neuhof T, Baker SE, Druzhinina IS, Kubicek CP, Brückner H & von Döhren H (2012) The production of multiple small peptaibols families by single 14-module peptide synthetases in *Trichoderma/Hypocrea*. *Chem Biodivers* **9**, 499–535.

De la Cruz J, Hidalgo-Gallego A, Lora JM, Benitez T, Pintor-Toro JA & Llobell A (1992) Isolation and characterization of three chitinases from *Trichoderma harzianum*. *European Journal of Biochemistry* **206**, 859-867.

Druzhinina IS, Seidl-Seiboth V, Herrera-Estrella A, Horwitz BA, Kenerley CM, Monte E, Mukherjee PK, Zeilinger S, Grigoriev IV & Kubicek CP (2011) *Trichoderma*: the genomics of opportunistic success. *Nature Reviews Microbiology* **16**, 749-759.

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: The Ecological Genomics of Fungi (ed F Martin), John Wiley & Sons, Inc, Hoboken, NJ. doi: 10.1002/9781118735893.ch5

Flores A, Chet I & Herrera-Estrella A (1997) Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene prb1. *Current Genetics* **31**, 30-37.

Gajera H, Domadiya R, Patel S, Kapopara M & Golakiya B (2013) Molecular mechanism of *Trichoderma* as bio-control agents against phytopathogen system – a review. *Curr Res Microbiol Biotechnol* **1**(4), 133-142.

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

Geremia RA, Goldman GH, Jacobs D, Ardiles W, Vila SB, Van Montagu M & Herrera-Estrella A (1993) prb1, a gene specifically expressed during simulated mycoparasitism by *Trichoderma harzianum*. *Mol Microbiol* **8**, 603-61.

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

Gorsche R, Mach-Aigner AR & Mach RL (2013) *Trichoderma* as cell factories. In: *Trichoderma*: Biology and Applications (eds PK Mukherjee *et al*.), 280-291.

Goswami J, Pandey RK, Tewari JP & Goswammi BK (2008) Management of root knot nematode on tomato through application of fungal antagonists, *Acremonium strictum* and *Trichoderma harzianum*. *J Environ Sci Health* B **43**, 237-240.

Grigoriev IV, Cullen D, Goodwin SB, Hibbett D, Jeffries TW, Kubicek CP, Kuske C, Magnuson JK, Martin F, Spatafora JW, Tsang A & Baker (2011) Fueling the future with fungal genomics. *Mycology* **2**(3), 192-209.

Gruber F, Visser J, Kubicek CP & de Graaff LH (1990) The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a pyrG-negative mutant strain. *Curr Genetics* **18**(1), 71–76.

Hares MC, Hinchliffe SJ, Strong PC, Eleftherianos I, Dowling AJ, ffrench-Constant RH & Waterfield N (2008) The *Yersinia pseudotuberculosis* and *Yersinia pestis* toxin complex is active against cultured mammalian cells. *Microbiology* **154**, 3503–3517.

Harkki A, Uusitalo J, Bailey M, Penttila M & Knowles JKC (1989) A novel fungal expression system: secretion of active calf chymosin from the filamentous fungus *Trichoderma reesei*. *Bio/Technology* **7**, 596–603.

Harman GE (2000) Myths and dogmas of biocontrol. Changes in perceptions derived from research on *Trichoderma harzianum* T22. *Plant Dis* **84**, 377-393.

Harman GE, Lorito M & Lynch JM (2004) Uses of *Trichoderma* spp. To alleviate or remediate soil and water pollution. *Adv Appl Microbiol* **56**, 313-330.

Haque MM, Haque MA, Ilias GNM & Molla AH (2010) *Trichoderma* – enriched biofertilizer: a prospective substitute of inorganic fertilizer for mustard (*Brassica campestris*) production. *The Agriculturists* **8**(2), 66-73.

Hjeljord L & Tronsmo A (1998) *Trichoderma* and *Gliocladium* in biological control: an overview. In: Harman GE and Kubicek CP (eds) *Trichoderma* and *Gliocladium*, Vol. **2**. Enzymes, biological control and commercial applications. Taylor and Francis, London, 131–151.

Hoffmeister D & Keller NP (2007) Natural products of filamentous fungi: enzymes, genes and their regulation. *Nat Prod Rep* **24**, 393-416.

Jaklitsch WM (2009) European species of *Hypocrea* Part I. The green-spored species. *Studies in Mycology* **63**, 1-91.

Joutsjoki VV, Torkkeli TK & Nevalainen KM (1993) Transformation of *Trichoderma reesei* with the *Hormoconis resinae* glucoamylase P (gamP) gene: production of heterologous glucoamylase by *Trichoderma reesei*. *Current Genetics* **24**, 223-228.

Jun H, Guangye H & Daiwen C (2013) Insights into enzyme secretion by filamentous fungi: Comparative proteome analysis of *Trichoderma reesei* grown on different carbon sources. *Journal of Proteomics* **89**, 191-201.

Karagiosis SA & Baker SE (2013) *Trichoderma* and the biorefinery: from plant health to enzymes to biofuel production. In *Trichoderma*: Biology and Applications (eds PK Mukherjee et al.), 222-229.

Kelly SL, Lamb DC & Kelly DE (2006) Cytochrome P450 biodiversity and biotechnology. *Biochemical Society Transactions* **34**, 1159-1160.

Kiiskinen LL, Kruus K, Bailey M, Ylo¨sma¨ki E,Matti Siika-Aho M & Saloheimo M (2004) Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. *Microbiology* **150**, 3065-3074.

Klein D & Eveleigh DE (1998) Ecology of *Trichoderma*. In: *Trichoderma* and *Gliocladium* (Kubicek CP and Harman GE, eds) Vol. **1**. Taylor and Francis, London, Bristol and Philadelphia, 57-69.

Komoń-Zelazowska M, Bissett J, Zafari D, Hatvani L, Manczinger L, Woo S, Lorito M, Kredics L, Kubicek CP & Druzhinina IS (2007a) Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms. *Applied and Environmental Microbiology* **73**, 7415-7426.

Kontkanen H, Westerholm-Parvinen, Saloheimo M, Bailey M, Rättö M, Mattila I, Mohsina M, Kalkkinen N, Nakari-Setälä T & Buchert J (2009) Novel *Coprinopsis cinerea* polyesterase that hydrolyzes cutin and suberin. *Applied and Environmental Microbiology* **75**, 2148-2157.

Kubicek CP, Bölzlbauer UM, Kovacs W, Mach RL, Kuhls K, Lieckfeldt U, Börner T & Samuels GJ (1996) Cellulase formation by species of *Trichoderma* sect. Longibrachiatum and of *Hypocrea* spp. with anamorphs referable to *Trichoderma* sect.Longibrachiatum. *Fung Biol Genet* **20**, 105 – 114.

Kubicek CP & Penttilä ME (1998) Regulation of production of plant polysaccharide degrading enzymes by *Trichoderma*. In: Harman GE, Kubicek CP (Eds.), *Trichoderma* and *Gliocladium*. Vol. **2**. Enzymes, Biological Control and Commercial Applications. Taylor and Francis Ltd., London, 49-71.

Kubicek CP, Zelazowska-Komon M, Sandor E & Druzhinina IS (2007) Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma*. *Chemistry and Biodiversity* **4**, 1068-1082.

Kubicek CP, Mikus M, Schuster A, Schmoll M & Seiboth B (2009) Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. *Biotechnology and Biofuels* **2**, 19.

Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Le Crom S, Martinez DA, Druzhinina IS, Thon M, Zeilinger S, Casas-Flores S, Horwitz BA, Mukherjee P, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, Atanasova L, Cervantes-Badillo MG, Challacombe J, Chertkov O, McCluskey K, Culpier F, Deshpande N, von Döhren H, Ebbole DJ, Esquivel-Naranjo EU, Fekete E, Flipphi M, Glaser F, Gomez-Rodriguez EY, Gruber S, Han C, Henrissat B, Nernandez-Onate M, Karaffa L, Kosti I, Lucas S, Hermosa R, Lübeck M, Lübeck PS, Margeot A, Metz B, Mishra 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 P, 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.

Kumar R, Singh S & Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *Journal of Industrial Microbiology and Biotechnology* **35**, 377-391.

Kyalo G, Affokpon A, Coosemans J & Coynes DL (2007) Biological control effects of *Pochonia chlamydosporia* and *Trichoderma* isolates from Benin (West-Africa) on root-knot nematodes. *Communications in Agricultural and Applied Biological Sciences* **72**, 219-223.

Lorito M, Hayes CK, Di Pietro A, Woo SL & Harman GE (1994) Purification, characterization, and synergistic activity of a glucan 1, 3-β-glucosidase and an N-acetyl-β-glucosaminidase from *Trichoderma harzianum*. *Phytopathology* **84**, 398-405.

Lorito M (1998) Chitinolytic enzymes and their genes. In Harman GE & Kubicek CP (eds) *Trichoderma* and *Gliocladium*, Vol. **2**. Enzymes, biological control and commercial applications. Taylor and Francis, London, 73-99.

Lu Z, Tombolini R, Woo S, Zeilinger S, Lorito M & Jansson JK (2004) In vivo study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Applied and Environmental Microbiology* **70**, 3073-3081.

Mach RL, Schindler M & Kubicek CP (1994) Transformation of *Trichoderma reesei* based on hygromycin B resistance using homologous expression signals. *Current Genetics* **25**, 567-570.

Margolles-Clark E, Hayes CK, Harman GE & Penttilä M (1996) Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei*. *Applied and Environmental Microbiology* **62**, 2145-2151.

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, Schoch CL, Yao J, Barabote R, Nelson MA, Detter C, Bruce D, Kuske CR, Xie G, Richardson P, Rokhsar DS, Lucas SM, Rubin EM, Dunn-Coleman N, Ward M & Brettin TS (2008) Genome sequence analysis of the cellulolytic fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*) reveals a surprisingly limited inventory of carbohydrate active enzymes. *Nature Biotechnology* **26**, 553 – 560.

Mäntylä A, Paloheimo M & Suominen P (1998) Industrial mutants and recombinant strains of *Trichoderma reesei*. In Harman GE & Kubicek CP (eds) *Trichoderma* and *Gliocladium*, Vol. **2**. Enzymes, biological control and commercial applications. Taylor and Francis, London, 291-310.

Miettinen-Oinonen A, Torkkeli T, Paloheimo M & Nevalainen H (1997) Overexpression of the *Aspergillus niger* pH 2.5 acid phosphatase gene in a heterologous host *Trichoderma reesei*. *Journal of Biotechnology* **58**(1), 13-20.

Monte E (2001) Understanding *Trichoderma*: between biotechnology and microbial ecology. *Int Microbiol* **4**, 1-4.

Mukherjee PK, Wiest A, Ruiz N, Keightley A, Moran-Diez ME, McCluskey K, Pouchus YF & Kenerley CM (2011) Two classes of new peptaibols are synthesized by a single non-ribosomal peptide synthetase of *Trichoderma virens*. *J Biol Chem* **286**, 4544–4554.

Mukherjee PK, Buensanteai N, Moran-Diez ME, Druzhinina IS & Kenerley CM (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**, 155-165.

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

Mullaw TB, Kubicek CP & Druzhinina IS (2010) The rhizosphere of *Coffea arabica* in its native highland forests of Ethiopia is associated with a distinguished diversity of *Trichoderma*. *Diversity* **2**, 527-549.

Murray P, Aro N, Collins C, Grassick A, Penttilä M, Saloheimo M & Tuohy M (2004) Expression in *Trichoderma reesei* and characterisation of a thermostable family 3 beta-glucosidase from the moderately thermophilic fungus *Talaromyces emersonii*. *Protein Expression and Purification* **38**, 248-257.

Neuhof T, Dieckmann R, Druzhinina IS, Kubicek CP & von Döhren H (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.

Nyyssönen E, Penttilä M, Harkki A, Saloheimo A, Knowles JKC & Keränen S (1993) Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. *Biotechnology* (NY) **11**, 591–595.

Nyyssönen E & Keränen S (1995) Multiple roles of the cellulase CBHI in enhancing production of fusion antibodies by the filamentous fungus *Trichoderma reesei*. *Curr Genet* **28**, 71-79.

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

Paloheimo M, Mäntylä A, Kallio J & Suominen P (2003) High-yield production of a bacterial xylanase in the filamentous fungus *Trichoderma reesei* requires a carrier polypeptide with an intact domain structure. *Appl Environ Microbiol* **69**, 7073–7082.

Papapostolou I & Georgiou CD (2010) Superoxide radical induces sclerotial differentiation in filamentous phytopathogenic fungi: a superoxide dismutase mimetics study. *Microbiology* **156**, 960-966.

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

Penttilä M, Nevalainen H, Rättö M, Salminen E & Knowles J (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* **61**(2), 155-164.

Penttilä M (1998) Heterologous protein production in *Trichoderma*. In Harman GE & Kubicek CP (eds) *Trichoderma* and *Gliocladium*, Vol. **2**. Enzymes, biological control and commercial applications. Taylor and Francis, London, 365-382.

Peterbauer CK, Heidenreich E, Baker RT & Kubicek CP (1992) Effect of benomyl and benomyl resistance on cellulase formation by *Trichoderma reesei* and *Trichoderma harzianum*. *Canadian Journal of Microbiology* **38**, 1292-1297.

Poidevin L, Levasseur A, Paes G, Navarro D, Heiss-Blanquet S, Asther M & Record E (2009) Heterologous production of the *Piromyces equi* cinnamoyl esterase in *Trichoderma reesei* for biotechnological applications. *Lett Appl Microbiol* **49**, 673–678.

Rebuffat S, Goulard C, Bodo B & Roquebert MF (1999) The peptaibol antibiotics from *Trichoderma* soil fungi; structural diversity and membrane properties. *Recent Res Dev Org Bioorg Chem* **3**, 65–91.

Reese ET (1976) History of the cellulase program at the U.S. Army Natick Development Center. *Biotechnol Bioeng Symp* **6**, 9–20.

Reino JL, Guerrero RF, Hernández-Galan R & Collado IG (2008) Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochemical Reviews* **7**, 89–123.

Roelofs D, de Boer M, Agamennone V, Bouchier P, Legler J & van Straalen N (2012) Functional environmental genomics of a municipal landfill soil. *Frontiers in Genetics* **3**, 85.

Ruocco M, Lanzuise S, Vinale F, Marra R, Turrà D, Woo SL & Lorito M (2009) 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. *Molecular Plant Microbe Interactions* **22**, 291-301.

Saarelainen R, Mantyla A, Nevalainen H & Suominen P (1997) Expression of barley endopeptidase B in *Trichoderma reesei*. *Appl Environ Microbiol* **63**, 4938–4940.

Salles BC, Te'o VS, Gibbs MD, Bergquist PL, Filho EX, Ximenes EA & Nevalainen KMH (2007) Identification of two novel xylanase-encoding genes (xyn5 and xyn6) from *Acrophialophora nainiana* and heterologous expression of xyn6 in *Trichoderma reesei*. *Biotechnol Lett* **29**, 1195–1201.

Saloheimo M & Niku-Paavola ML (1991) Heterologous production of a ligninolytic enzyme: expression of the *Phlebia radiata* laccase gene in*Trichoderma reesei*. *Bio/technology* **9**, 987-990.

Saloheimo M & Pakula TM (2012) The cargo and the transport system: secreted proteins and protein secretion in *Trichoderma reesei* (*Hypocrea jecorina*). *Microbiology* **158**, 46-57.

Samuels GJ, Suarez C, Solis K, Holmes KA, Thomas SE, Ismaiel A & Evans HC (2006b) *Trichoderma theobromicola* and *T. paucisporum*: two new species isolated from cacao in South America. *Mycological Research* **110**, 381-392.

Schirmböck M, Lorito M, Wang YL, Hayes CK, Arisan-Atac I, Scala F, Harman GE & Kubicek CP (1994) Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Applied and Environmental Microbiology* **60**, 4364-4370.

Schmoll M, Seibel C, Kotlowski C, Wollert Genannt Vendt F, Liebmann B & Kubicek CP (2010) Recombinant production of an *Aspergillus nidulans* class I hydrophobin (DewA) in *Hypocrea jecorina* (*Trichoderma reesei*) is promoter-dependent. *Applied Microbiology and Biotechnology* **88**, 95-103.

Schuster A, Kubicek CP, Friedl MA, Druzhinina IS & Schmoll M (2007) Impact of light on *Hypocrea jecorina* and the multiple cellular roles of ENVOY in this process. *BMC Genomics* **8**, 449.

Singh D, Kashyap A, Pandey RV & Saini KS (2011) Novel advances in cytochrome P450 research. *Drug Discovery Today* **16**, 793-799.

Sivasithamparam K & Ghisalberti EL (1998) Secondary metabolism in *Trichoderma* and *Gliocladium*. In: Kubicek CP & Harman GE (eds) *Trichoderma* and *Gliocladium*, Vol. **1**. Basic biology, taxonomy and genetics. Taylor and Francis, London, 139–191.

Strieker M, Tanović A & Marahiel MA (2010) Nonribosomal peptide synthetases: structures and dynamics. *Current Opinions in Structural Biology* **20**, 234-240.

Szekeres A, Leitgeb B, Kredics L, Antal Z, Hatvani L, Manczinger L & Vágvölgyi C (2005) Peptaibols and related peptaibiotics of *Trichoderma*. A review. *Acta Microbiol Immunol Hung* **52**(2), 137-168.

Tijerino A, Cardoza RE, Moraga J, Malmierca MG, Vicente F, Aleu J, Collado IG, Gutiérrez S, Monte E & Hermosa R (2011) Overexpression of the trichodiene synthase gene tri5 increases trichodermin production and antimicrobial activity in *Trichoderma brevicompactum*. *Fungal Genetics and Biology* **48**, 285-296.

Uusitalo JM, Nevalainen KM, Harkki AM, Knowles JK, Penttila ME (1991) Enzyme production by recombinant *Trichoderma reesei* strains. *J Biotechnol* **17**, 35–49.

Wiest A, Grzegorski D, Xu BW, Goulard C, Rebuffat S, Ebbole DJ, Bodo B & Kenerley C (2002) Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. *Journal of Biological Chemistry* **277**, 20862-20868.

Zhang CL, Liu SP, Lin FC, Kubicek CP & Druzhinina IS (2007) *Trichoderma taxi* sp. nov., an endophytic fungus from Chinese yew *Taxus mairei*. *FEMS Microbiology Letters* **270**, 90-96.

2. Aims of the thesis

Trichoderma is a genus of filamentous fungi that contains species with profound potential of environmental opportunism. This property is exploited in a wide range of industrial applications. However, besides the positive role, these fungi also demonstrate some potentially negative impact on mankind.

This study aims to investigate the molecular ecological aspects of *Trichoderma* which should be considered prior its application in agriculture and industry.

To accomplish the goal of the thesis the following questions have been to be answered:

1. Whether mycoparasitic nature of *Trichoderma* may possess a certain danger for mushroom farming?

2. Whether among secondary metabolites secreted by *Trichoderma* there are certain potentially toxic copounds?

3. How diverse are the *Trichoderma* species that may cause mycoses in animals including humans?

3. *Trichoderma* and *Pleurotus* farming

3.1. Biofungicide versus causative agents of the green mold disease of mushrooms

What is a biofungicide? A definition of biofungicides was formulated by Francis and Keinath (2010) that "biofungicides are microorganisms and naturally occurring substances that control diseases (biochemical pesticides) that are approved for organic production".

The number of studies about the biocontrol of plant pathogens is growing rapidly and became more interesting thus more feasible. The exploration on fungal species which are efficient to control plant pathogens revealed *Trichoderma* spp., because their antifungal abilities are known since early 1930's (Weindling, 1934). Most of the opportunistic species are powerful mycoparasites thus are used in agriculture for purpose of plant protection.

On the other hand, the mycoparasitic activity of *Trichoderma* may have also negative effect on humankind. Worldwide there are mushroom farms with the dominant production of *Agaricus bisporus* (champinion), *Pleurotus ostreatus* (oyster mushroom), and *Lentinula edodes* (shiitake) (Chang, 1999). Since over sixty years some *Trichoderma* strains have been notified as causal agent of green mould infection in edible mushroom industry (Sinden and Hauser, 1953).

There are several factors which should be considered by mushroom farmers for improved production. Based on Chen and Moy (2004) these are: carbon and nitrogen source, warm temperatures, high humidity (plus variation of these elements), and darkness. Paradoxically, the same conditions are suitable for mould thus having direct influence towards crop contamination. The favourable conditions, give rise for a faster growth of moulds what in the end effect may cause the competition for nutrients and the space (Williams *et al*., 2003). *Trichoderma* strians could also produce extracellular enzymes, toxic secondary metabolites and volatile organic compounds, which results in drastical crop losses (Kredics *et al*., 2010). The disease appears in the mushroom cultivation substrate or in the casing layer as surfaces covered with *Trichoderma* spores. Emerging mushrooms are severely spotted, often distorted (Seaby, 1989), making them unmarketable.

3.1.1. Green mould on *Agaricus*

In 1988 Geels reported that the mushroom disease has been correlated mainly with either poor hygiene or insufficient quality of the compost. Therefore, until 80's green mould was treated as trivial issue hence easy handled by changing the composting system, reconstruction sanitation or by

applying chemicals (Geels *et al*., 1988). This conception changed significantly after the first reports about green mould infections on *Agaricus* from Northern Ireland in 1985.

Identical infections have been observed in mushroom farms across Europe namely in Spain, Germany, in the United Kingdom, France and the Netherlands (Hermosa *et al*., 1999; Seaby, 1998; Mamoun *et al*., 2000b; Geels, 1997), Poland (Sobieralski *et al.*, 2012), Croatia (Lorant Hatvani, Lea Atanasova, personal communications). In 1998 Castle wrote about similar disease emerged in mushroom crops in Canada and in the United States (Castle *et al*., 1998).

The increased number of green mould cases stimulated researcher to intensify the work towards identification the causative agent.

Despite the fact that several *Trichoderma* spp. (e.g. *T. koningii, T. hamatum, T*. *longibrachiatum*, *T. citrinoviride, T. crassum, T. spirale*)(Castle *et al*., 1998) were found on mushroom compost, only *T. harzianum* has been assigned as a species colonizing cultivation area (Seaby, 1987).

As it has been identified in that time, isolates of *T. harzianum* obtained from British Isles compost belong to three different biological pattern namely Th1, Th2 and Th3 (Seaby, 1987). They behave different in respect to the growth, sporulation and the way of aggressiveness in compost expansion where Th2 has been classified as the most aggressive form responsible for the green mould infection (Seaby, 1987).

In order to assess the intraspecific diversity among *T. harzianum* strains, Muthumeenakshi and colleagues (1994) applied several molecural markers specifically restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analyses and sequencing of the internal transcribed spacer (ITS) 1 of the rRNA gene cluster.

Later on, techniques mentioned above have been enforced for molecular labeling of *Trichoderma* cultures isolated from mushroom farms in North America. They revealed another group of *T. harzianum* called Th4 which was comparable to, but on the other hand noticeable divergent than the Th2 (Castle *et al*., 1998; Muthumeenakshi *et al.,* 1998).

As *T. harzianum* was (and still is) a species often used for biological control of fungal plant pathogens, concerns have emerged regarding the possible involvement of these strains in the development of mushroom green mold. Molecular phylogenetic studies based on RAPD analysis (Ospina-Giraldo *et*

al., 1999) as well as sequence analysis of the ITS 1 and 2 rRNA region disclosed that biocontrol and green mould strains can be clearly differentiated. Supporting these molecular data, Romaine *et al.* (2001) showed in pathogenicity trials that commercial biocontrol *T. harzianum* strains and related ones from the Th1 biotype were not pathogenic on *A. bisporus*, as opposed to Th4 isolates. Specific primer pair has been developed to identify the aggressive biotypes Th2 and Th4 in order to select biocontrol candidates for potential pathogenicity (Chen *et al.,* 1999a). In addition, this PCR-based test has been used for the identification of *Trichoderma* isolates collected in the United States during and prior to the explosion of the green mould plague (Chen *et al*., 1999b). The results showed no evidence for the preepidemic presence of Th4, implying the recent development of highly virulent genotype. Muthumeenakshi *et al*. (1994) suggested, using the molecular differences between the biotypes Th1 – Th3, that they could exhibit three distinct species. Molecular data pointed out afterwords, that the Th3 biotype belongs to *T. atroviride* (Castle *et al.*, 1998; Ospina-Giraldo *et al.,* 1998), whereas Th1 was identified as *T. harzianum* sensu stricto (Gams and Meyer, 1998). In 2002 Samuels and co-workers redescribed two aggressive biotypes, namely Th2 and Th4. Based on the morphological characteristics, and phylogenetic analyses of ITS 1 and the translation elongation factor 1-alpha (*tef1*) gene they were recognized as *T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum*, respectively.

Figure 3.1 shows the separated taxonomic state of *T. aggressivum* in the Harzianum clade (Atanasova *et al*., 2013) as revealed based on the multiloci phylogeny of Druzhinina *et al.* (2010).

3.1.2. Trichoderma on *Plurotus*

A similar infestation occurred almost twenty years ago on the oyster mushroom *Pleurotus ostreatus* caused by *T. viride* (as identified at that time) in North America (Sharma and Vijay, 1996), but the first significant crop losses of cultivated *P. ostreatus* caused by green mould disease were reported in South Korea by Yu (2002). Woo *et al.* (2004, 2006) reported green mould on the oyster mushroom *Pleurotus ostreatus* in Italy followed by Hungary, Romania (Hatvani *et al.,* 2006; Kredics *et al.,* 2006) and South Korea (Park *et al*., 2006).

Based on the observation by Yu (2002) and Woo *et al.* (2004) the presence of *Trichoderma* spp. appears in the initial stage of substrate preparation, with the pasteurization they seem do not exist,

but they could be recognized again in the base after inoculation with *Pleurotus* (spawing), during incubation stage and the fruiting periods.

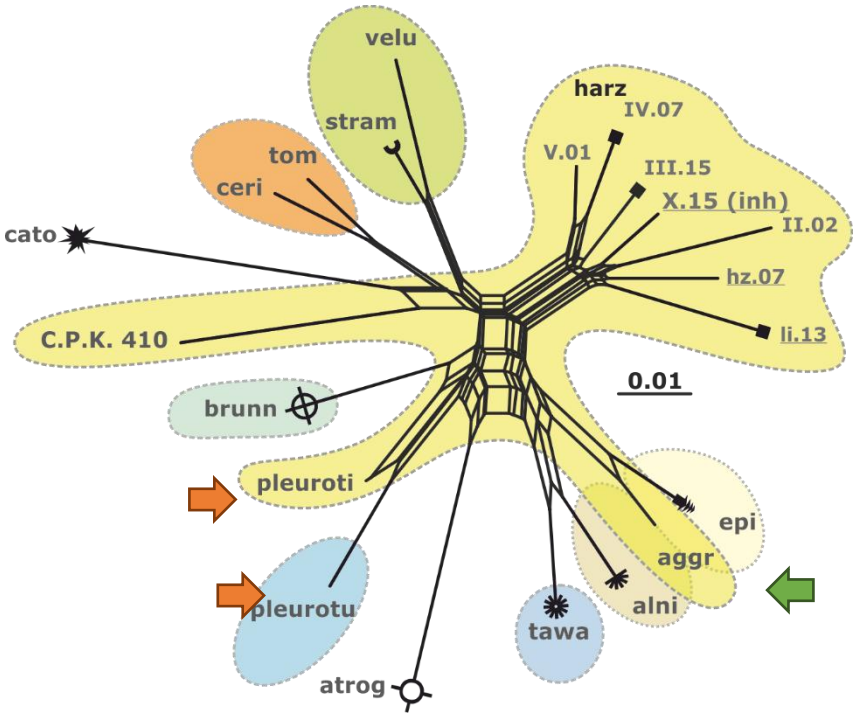


Figure 3.1. The recombination network of *T. harzianum* species complex determined by SplitsTree (NJ mode) from the multilocus phylogenetic dataset of druzhinina *et al.* (2010). taxa with "harzianum"-like morphology are shown by a light yellow background and dotted lines; other anamorph morphologies are shown by different colours and dotted lines; individual teleomorph morphologies are indicated by different end symbols. orange and green arrows show *trichoderma* spp. that are causative agents of *pleurotus* and *agaricus* green mold diseases respectively. Representative sequences for species from Harzianum-Catoptron Clade may be retrieved from NCBI Entrez search engine using [species strain locus] keywords. ceri – *T. cerinum*, velu – *T. velutinum*, harz – *T. harzianum s.l.*, inh – *T. inhamatum*, epi – *H. epimyces*, tom – *T. tomentosum*, stram – *H. straminea*, aggr – *T. aggressivum*, alni – *H. alni*, tawa – *H. tawa*, atro – *H. atrogelatinosa*, pleurotu – *T. pleurotum*, pleuroti – *T. pleuroticola*, brunn – *H. brunneoviridis*, cato – *T. catoptron*. The image is modified from Druzhinina *et al*., 2010.

The causative agent of the oyster mushroom green mold has been reported to be morphologically and culturally distinct from *T. aggressivum* (Park *et al*., 2004; Woo *et al*., 2004). Park *et al.* (2004, 2005a) collected green mould samples originated from South Korea between the years 1997 and 2002. They have examined more than 100 isolates of *Trichoderma* from oyster mushroom substrates. Using the morphology and culture features they were able to classify the isolates into 7 different species, among them two belonging to new taxa (Park *et al.*, 2004, 2005a). The researches claimed

that two new species (*Trichoderma koreana* and *Trichoderma pleuroti*) were responsible for the disease on *Pleurotus* in South Korea, but they did not provide nomenclaturally valid species descriptions. Hatvani *et al*. (2006) reported that the Hungarian oyster mushroom green mold species has the same internal transcribed spacer 1 (ITS1) and ITS2 sequences as an undescribed species of *Trichoderma* (*Trichoderma* sp. strain DAOM 175924) (Kullnig-Gradinger *et al*., 2002) and that its ITS1 and ITS2 sequences were also identical with those deposited for four *Trichoderma* pathogens of *P. ostreatus* from South Korea.

Phylogenetic analysis allowed Park *et al*. (2006) to formally confirm the existence of two new species causing green mould disease in South Korea and described them as *T. pleurotum* and *T. pleuroticola*.

It should be also mentioned that similarly to *T. aggressivum* – the causal agent of *Agaricus* green mould disease - both species were shown to belong to the Harzianum Clade of *Hypocrea/Trichoderma*.

References:

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.

Castle A, Speranzini D, Rghei N, Alm G, Rinker D & Bissett J (1998) Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. *Applied and Environmental Microbiology* **64**, 133-137.

Chang ST (1999) World production of cultivated and medicinal mushrooms in 1997 with emphasis on *Lentinus edodes* (Berk.) Sing. in China. *Int J Med Mushrooms* **1**, 291-300.

Chen X, Romaine CP, Ospina-Giraldo MD & Royse DJ (1999a) A polymerase chain reaction-based test for the identification of *Trichoderma harzianum* biotypes 2 and 4, responsible for the worldwide green mold epidemic in cultivated *Agaricus bisporus*. *Appl Microbiol Biotechnol.* **52**, 246-250.

Chen X, Romaine CP, Tan Q, Schlagnhaufer B, Ospina-Giraldo MD, Royse DJ & Huff DR (1999b) PCR-based genotyping of epidemic and preepidemic *Trichoderma* isolates associated with green mold of *Agaricus bisporus*. *Appl Environ Microbiol* **65**, 2674-2678.

Chen AW & Moy M (2004) Mushroom cultivation: building mold contamination. At: <http://www.mushworld.com> Accessed on 28. October 2008.

Druzhinina IS, Kubicek CP, Komoń-Zelazowska M, Belayneh Mulaw T & Bissett J (2010) 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.

Francis R & Keinath A (2010) Biofungicides and chemicals for managing diseases in organic vegetable production. *Information Leaflet* **88**.

Gams W & Meyer W (1998) What exactly is *Trichoderma harzianum*? *Mycologia* **90**, 904-915.

Geels FJ, van de Geijin J & Rutjens A (1988) Pests and diseases. In: The cultivation of mushrooms. ed van Griensven LJLD (Interlingua, East Grinstead, Sussex, England), 361–422.

Geels FP (1997) Rondetafel- bijeenkomst over *Trichoderma*. *Champignoncultuur* **41**, 13.

Hatvani L, Antal Z, Manczinger L, Szekeres A, Druzhinina IS, Kubicek CP, Nagy A, Nagy E, Vágvölgyi C, Kredics L (2006) Green mold diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderma* species. *Phytopathology* **97**, 532-537.

Hermosa MR, Grondona I & Monte E (1999) Isolation of *Trichoderma harzianum* Th2 from commercial mushroom compost in Spain. *Plant Disease* **83**, 591.

Kredics L, Hatvani L, Antal Z, Manczinger L, Druzhinina IS, Kubicek CP, Szekeres A, Nagy A, Vágvölgyi C & Nagy E (2006) Green mould disease of oyster mushroom in Hungary and Transylvania. *Acta Microbiol Immunol Hung* **53**, 306–307.

Kredics L, García Jimenez L, Naeimi S, Czifra D, Urbán P, Manczinger L, Vágvölgyi C & Hatvani L (2010) A challenge to mushroom growers: the green mould disease of cultivated champignons. In: Méndez-Vilas A (ed.) *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. Badajoz: FORMATEX, 295-305.

Kullnig-Gradinger CM, Szakács G & Kubicek CP (2002) Phylogeny and evolution of the fungal genus *Trichoderma* - A multigene approach. *Mycol Res* **106**, 757-767.

Mamoun ML, Savoie JM & Olivier JM (2000) Interactions between the pathogen *Trichoderma harzianum* Th2 and *Agaricus bisporus* in mushroom compost. *Mycologia* **92**, 233-240.

Muthumeenakshi S, Mills PR, Brown-Averil E & Seaby DA (1994) Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. *Microbiology* (UK) **140**, 769-777.

Muthumeenakshi S, Brown AE & Mills PR (1998) Genetic comparison of the aggressive weed mould strains of *Trichoderma harzianum* from mushroom compost in North America and the British Isles. *Mycological Research* **102**, 385-390.

Ospina-Giraldo MD, Royse DJ, Chen X & Romaine CP (1999) Molecular phylogenetic analyses of biological control strains of *Trichoderma harzianum* and other biotypes of *Trichoderma* spp. associated with mushroom green mold. *Phytopathology* **89**, 308-313.

Park MS, Bae KS & Yu SH (2004) Molecular and morphological analysis of *Trichoderma* isolates associated with green mold epidemic of oyster mushroom in Korea. *J Huazhong Agric Univ* **23**, 157-164.

Park MS, Seo GS, Lee KH, Bae KS & Yu SH (2005a) Morphological and cultural characterization of *Trichoderma* spp. associated with green mold of oyster mushroom in Korea. *Plant Pathol J* **21**, 221–228.

Park MS, Bae KS & Yu SH (2006) Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. *Mycobiology* **34**, 111-113.

Romaine CP, Chen X, Ospina-Giraldo MD & Royse DJ (2001) Molecular genetics and pathogenicity of biocontrol and mushroom *Trichoderma*. *IOBC wprs Bull*. **24**(3), 333-336.

Samuels GJ, Dodd SL, Gams W, Castlebury LA & Petrini O (2002) *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. *Mycologia* **94**, 146-170.

Seaby DA (1987) Infection of mushroom compost by *Trichoderma* species. *Mushroom Journal* **179**, 355-361.

Seaby DA (1989) Further observation on *Trichoderma*. *Mushroom Journal* **197**, 147-151.

Seaby DA (1998) *Trichoderma* as a weed mould or pathogen in mushroom cultivation. In: *Trichoderma* and *Gliocladium*. Vol. **2.** Enzymes, Biological Control and Commercial Applications. Kubicek CP & Harman GE eds. Taylor and Francis, London, 267-287.

Sharma SR & Vijay B (1996) Yield loss in *Pleurotus ostreatus* spp. caused by *Trichoderma viride*. *Mushroom Research* **5**, 19–22.

Sinden J & Hauser E (1953) Nature and control of three mildew diseases of mushrooms in America. *Mushroom Science* **2**, 177–180.Sobieralski K, Siwulski M, Błaszczyk L, Frużyńska-Jóźwiak D & Lisiecka J (2012) The effect of infestation with isolates of *Trichoderma* sp. on mycelium growth and yielding in single spore heterocaryotic cultures of *Agaricus bisporus* (Lange) Imbach. *Acta Sci. Pol., Hortorum Cultus* **11**(6), 47-57.

Weindling R (1934) Studies on lethal principle effective in the parasitic action of *Trichoderma lignorum* on *Rhizoctonia solani* and other fungi. *Phytopathology* **24**, 1153-1179.

Williams J, Clarkson JM, Mills PR & Cooper RM (2003) Saprotrophic and mycoparasitic components of aggressiveness of *Trichoderma harzianum* groups toward the commercial mushroom *Agaricus bisporus. Appl. Environ. Microbiol.* **69**(7), 4192-4199.

Woo SL, Di Benedetto P, Senatore M, Abadi K, Gigante S, Soriente I, Ferraioli S, Scala F & Lorito M (2004) Identification and characterization of *Trichoderma* species aggressive to *Pleurotus* in Italy. *Journal of Zhejiang University*, *Agriculture and Life Sciences* **30**, 469-470.

Woo SL, Scala F, Ruocco M & Lorito M (2006) The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. *Phytopathology* **96**, 181–185.

Yu SH (2002) Integrated Control of Oyster Mushroom Green Mould. At: <http://www.mushworld.com> Accessed on 5. May 2008.

3.2. Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms

Komon-Zelazowska M, Bissett J, Zafari D, Hatvani L, Manczinger L, Woo S, Lorito M, Kredics L, Kubicek CP & Druzhinina IS (2007) Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms. ***AEM*** **73(22), 7415-7426**

OWN CONTRIBUTION:

MKZ coordinated the local research group and the work of international partners, cultivated fungi, extracted genomic DNA, performed PCR and contributed to the analysis of sequences. She also essentially worked out the analysis of the materials and contributed to ms writing.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2007, p. 7415–7426 Vol. 73, No. 22
0099-2240/07/$08.00+0 doi:10.1128/AEM.01059-07

Genetically Closely Related but Phenotypically Divergent *Trichoderma* Species Cause Green Mold Disease in Oyster Mushroom Farms Worldwide▽†

Monika Komoń-Zelazowska,[1] John Bissett,[2] Doustmorad Zafari,[3] Lóránt Hatvani,[4] László Manczinger,[4] Sheri Woo,[5] Matteo Lorito,[5] László Kredics,[4] Christian P. Kubicek,[1] and Irina S. Druzhinina[1]*

Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, A-1060, Vienna, Austria[1]; Agriculture and Agri-Food Canada, Eastern Cereal and Oilseed Research Center, Central Experimental Farm, Ottawa, Ontario, Canada K1A 0C6[2]; Department of Plant Protection, Bu Ali Sina University, Hamadan, Iran[3]; Department of Microbiology, Faculty of Sciences, University of Szeged, Közép fasor 52, H-6726 Szeged, Hungary[4]; and Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Universita degli Studi di Napoli Federico II, Portici, Naples, Italy[5]

Received 11 May 2007/Accepted 27 August 2007

The worldwide commercial production of the oyster mushroom *Pleurotus ostreatus* is currently threatened by massive attacks of green mold disease. Using an integrated approach to species recognition comprising analyses of morphological and physiological characters and application of the genealogical concordance of multiple phylogenetic markers (internal transcribed spacer 1 [ITS1] and ITS2 sequences; partial sequences of *tef1* and *chi18-5*), we determined that the causal agents of this disease were two genetically closely related, but phenotypically strongly different, species of *Trichoderma*, which have been recently described as *Trichoderma pleurotum* and *Trichoderma pleuroticola*. They belong to the Harzianum clade of *Hypocrea*/*Trichoderma* which also includes *Trichoderma aggressivum*, the causative agent of green mold disease of *Agaricus*. Both species have been found on cultivated *Pleurotus* and its substratum in Europe, Iran, and South Korea, but *T. pleuroticola* has also been isolated from soil and wood in Canada, the United States, Europe, Iran, and New Zealand. *T. pleuroticola* displays pachybasium-like morphological characteristics typical of its neighbors in the Harzianum clade, whereas *T. pleurotum* is characterized by a gliocladium-like conidiophore morphology which is uncharacteristic of the Harzianum clade. Phenotype MicroArrays revealed the generally impaired growth of *T. pleurotum* on numerous carbon sources readily assimilated by *T. pleuroticola* and *T. aggressivum*. In contrast, the Phenotype MicroArray profile of *T. pleuroticola* is very similar to that of *T. aggressivum*, which is suggestive of a close genetic relationship. In vitro confrontation reactions with *Agaricus bisporus* revealed that the antagonistic potential of the two new species against this mushroom is perhaps equal to *T. aggressivum*. The *P. ostreatus* confrontation assays showed that *T. pleuroticola* has the highest affinity to overgrow mushroom mycelium among the green mold species. We conclude that the evolutionary pathway of *T. pleuroticola* could be in parallel to other saprotrophic and mycoparasitic species from the Harzianum clade and that this species poses the highest infection risk for mushroom farms, whereas *T. pleurotum* could be specialized for an ecological niche connected to components of *Pleurotus* substrata in cultivation. A DNA BarCode for identification of these species based on ITS1 and ITS2 sequences has been provided and integrated in the main database for *Hypocrea*/*Trichoderma* (www.ISTH.info).

Pleurotus ostreatus is an important edible basidiomycete commonly known as oyster mushroom. This fungus is the third most commercially important edible mushroom worldwide (4). In addition, it is used for the bioconversion of agricultural and industrial lignocellulose debris (2, 32) and as a source of enzymes and other metabolites for industrial and medical applications (13, 26). *P. ostreatus* can be grown on a wide range of agricultural by-products and industrial wastes (29), although pasteurized straw is most commonly used. Many pests and diseases can cause yield losses in *P. ostreatus*, and the association of *Trichoderma* species with the cultivation substratum has long been known to limit production (1). Sharma and Vijay (38) reported green mold of oyster mushroom in North America 10 years ago, and serious cases of green mold diseases of *P. ostreatus* in commercial operations were detected recently in South Korea (30), Italy (41), Hungary (14), and Romania (20).

Trichoderma green mold infection in edible basidiomycetes has been known for a long time (39). An *Agaricus* green mold disease started in Northern Ireland in 1985 and rapidly spread over farms across Europe (15, 25). A similar disease appeared in mushroom crops in the United States and Canada (3). The causative agent was originally believed to be *Trichoderma harzianum* (teleomorph *Hypocrea lixii*) but was later on clarified to be a new species of *Trichoderma*, viz., *Trichoderma aggressivum*, of which two varieties (*T. aggressivum* var. *europaeum* and *T. aggressivum* var. *aggressivum*) were distinguished from Europe and North America, respectively (35).

The causative agent of the oyster mushroom green mold has

* Corresponding author. Mailing address: Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, A-1060 Vienna, Austria. Phone: 43 1 58801 17202. Fax: 43 1 58801 17299. E-mail: druzhini@mail.zserv.tuwien.ac.at.

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

▽ Published ahead of print on 7 September 2007.

been reported to be morphologically and culturally distinct from *T. aggressivum* (30, 41). Park et al. (30) claimed that two new species (*Trichoderma koreana* and *Trichoderma pleuroti*) were responsible for the disease on *Pleurotus* in South Korea, but they did not provide nomenclaturally valid species descriptions. Hatvani et al. (14) reported that the Hungarian oyster mushroom green mold species has the same internal transcribed spacer 1 (ITS1) and ITS2 sequences as an undescribed species of *Trichoderma* (*Trichoderma* sp. strain DAOM 175924) (22) and that its ITS1 and ITS2 sequences were also identical with those deposited for four *Trichoderma* pathogens of *P. ostreatus* from South Korea. Recently, Park et al. (31) have formally described two new species causing *Pleurotus* green mold disease in South Korea, *Trichoderma pleurotum* and *Trichoderma pleuroticola*.

The objective of the present study was to use an integrated approach comprising morphological, physiological, and molecular analyses to investigate the evolution of the *Trichoderma* strains causing *Pleurotus* green mold disease and to examine reasons for the recent disease outbreaks.

MATERIALS AND METHODS

Fungal strains. Strains investigated in this study are given in Table 1. They are maintained in the culture collections of the Division of Gene Technology and Applied Biochemistry, Vienna University of Technology, Vienna, Austria, under assigned CPK numbers; at DAOM (Eastern Cereal and Oilseed Research Centre, Ottawa, Canada); and at the Section of Plant Pathology, University of Naples, Portici (NA), Italy. Representative cultures have also been deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Morphological analysis. Cultures were grown on 2% Oxoid malt extract agar (MA) and Oxoid potato dextrose agar (PDA) at 20°C under ambient daylight conditions or in a 12 h:12 h light:dark cycle under fluorescent and near-UV lamps. Colony descriptions are based on observations on MA under ambient daylight conditions, unless otherwise specified. Color codes and terminology are from the Methuen Handbook of Colours (18). Growth rates from 5°C to 40°C at increments of 5° were determined on PDA using the protocol of Samuels et al. (34). Microscopic observations and measurements were made from preparations mounted in lactic acid. Conidiophore structure and morphology were described from macronematous conidiophores taken from the edge of conidiogenous pustules or fascicles when conidia were maturing, usually after 4 to 7 days of incubation. Conidial morphology and measurements were recorded after 14 days.

Metabolic profiles. Metabolic profiles based on assimilation of carbon sources were performed using Biolog FF MicroPlates (6, 9, 19, 21). Microplates were incubated at 26°C in the dark, and absorbance readings at 490 nm and at 750 nm were analyzed separately. Absorbance data were not corrected for growth in the control well, which was treated as an independent variable in the analyses. Cluster analyses were performed using NTSYS software (33) with a similarity matrix using the product-moment correlation coefficient and employing the unweighted-pair group method using average linkages. SAS was used for analyses of variance (ANOVAs) and canonical variate analyses (36). Univariate ANOVAs were performed on data for each of the 95 different carbon substrata and the control. The substrata were ranked on the ANOVA F values and the degree of significance of the among-species variation in the ANOVAs. The highest ranked variables (probability $> F < 0.0001$) were selected to perform canonical variate analysis. Wilk's Lambda and Pillai's trace were employed to test the significance of the canonical variate analysis. The total standardized canonical coefficients were used to interpret the three significant eigenvectors obtained from the analysis.

Dual confrontation assays. To assess the antagonistic potential of *T. pleuroticola* and *T. pleurotum* against *P. ostreatus* and *A. bisporus*, we isolated pure cultures of the respective mushrooms from the products available on the Austrian market. Three strains of each species were used in dual confrontation tests at 26°C, with *T. aggressivum* CBS 433.95 and *Hypocrea jecorina*/*Trichoderma reesei* QM 6a as positive and negative controls, respectively.

DNA extraction, PCR, and sequencing. After 5 days of growth on MA at 25 ± 1°C, mycelia were harvested, and genomic DNA was isolated using a QIAGEN DNeasy Plant mini kit by following the manufacturer's protocol.

Amplification of the nuclear rRNA gene cluster, containing the ITS1 and ITS2 and the 5.8S rRNA gene, and of a 0.4-kb fragment of endochitinase *chi18-5* (formerly named *ech42*) was done as described previously (16). An approximately 1-kb portion of the *tef1* was amplified and sequenced using primers EF1 [5′-ATGGGTAAGGA(A/G)GACAAGAC-3′] and EF2 [GGA(G/A)GTACCA GT(G/C)ATCATGTT-3′] (28) or as described in Jaklitsch et al. (16).

Purified PCR products for ITS1 and ITS2, *tef1*, and *chi18-5* were subjected to automatic sequencing at MWG (Martinsried, Germany). Sequences were edited manually and deposited in NCBI GenBank and www.ISTH.info.

Phylogenetic analysis. For the phylogenetic analysis, DNA sequences were aligned using ClustalX and visually edited in Genedoc, version 2.6 (27). The interleaved NEXUS file was formatted using PAUP*, version 4.0b10 (40), and manually formatted for the MrBayes program, version 3.0B4. The Bayesian phylogenetic reconstructions have been performed as described in Jaklitsch et al. (16). According to the protocol of Leache and Reeder (23), posterior probability values lower than 0.95 were not considered significant while values below 0.9 were not shown on the consensus phylogram.

Haplotype networks were constructed manually based on detected shared polymorphic sites and confirmed using statistical parsimony analysis as implemented in TCS, version 2.11 (5), and maximum parsimony analysis using PAUP*, version 4.0b10 (40).

RESULTS

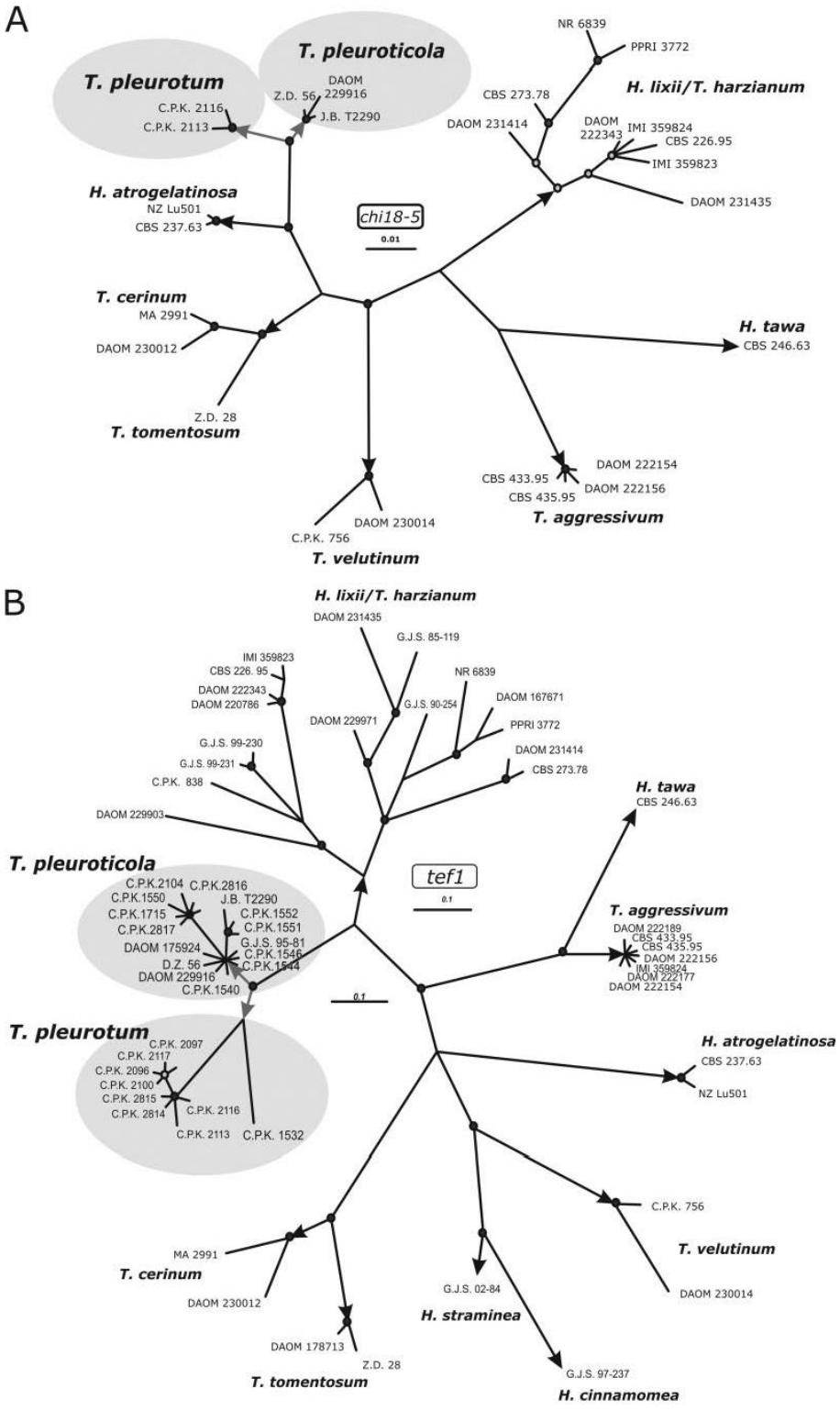
The *P. ostreatus*-associated strains comprise two phylogenetic *Trichoderma* species. Strains from oyster mushroom-producing farms having severe green mold infections from Hungary, Romania, Italy, and South Korea exhibited the same or highly similar ITS1 and ITS2 sequences as the previously recognized putative new species "*Trichoderma* cf. *aureoviride* DAOM 175924" (NCBI GenBank accession no. AY605726) (22) and the recently described *Pleurotus* green mold agent from Korea *T. pleuroticola* CNUMH 601 (NCBI GenBank DQ164409) (31). We have previously documented that strain DAOM 175924 forms a separate phylogenetic branch in the vicinity of *T. harzianum* and *T. aggressivum* in the Harzianum clade (22, 24). We applied two criteria of Dettman et al. (7) employing multilocus genealogies to determine if the various isolates associated with green mold on *Pleurotus* represented a single species or more than one distinct species. The criterion of genealogical concordance requires that the clade must be present in the majority of single-locus trees. The genealogical nondiscordance criterion recognizes a clade as an independent evolutionary lineage when it is reliably supported by at least one single-locus genealogy and if it is not contradicted by any other single gene tree determined by the same methods. To do this, we amplified and sequenced fragments from three different phylogenetic markers, i.e., a fragment spanning the ITS1-5.8S rRNA-ITS2 region of the rRNA gene cluster, a fragment covering the fourth and fifth introns and the last long exon of the translation elongation factor 1-alpha (*tef1*) gene, and a fragment including a portion of the fifth exon of the *chi18-5* gene encoding a family 18 chitinase. In order to compose the sample set for phylogenetic analysis, the resulting sequences were subjected to the sequence similarity search tool implemented in *Tricho*BLAST (17; also www.ISTH.info). No identical hits except "*Trichoderma* sp. strain DAOM 175924" were detected, but the highest sequence homology was shown for species from the Harzianum clade (10). As shown in Fig. 1, the phylogenies obtained from independent analyses of *tef1* and *chi18-5* markers placed the *Pleurotus* green mold isolates and conspecific strains in one supported hypothetical taxonomic unit close to *H. lixii*/*T. harzianum*. On the *chi18-5* tree the hypothetical taxonomic unit node of *Pleurotus*-associated isolates and allied

TABLE 1. *Pleurotus* green mold strains used in this study

Species and strain	Alternative strain no.	Origin	Habitat	NCBI GenBank accession no.[d]		
				ITS1 and ITS2	*tef1*	*chi18-5*
T. pleuroticola strains						
DAOM 175924[a]	CBS 121144	Canada (Ontario)	On *Acer* sp.	*AY605726*	*AY605769*	
DAOM 229916		United States (Wisconsin)	Forest soil, A1 horizon	*AY605738*	*AY605781*	
CPK 1540	CBS 121217	Italy	*P. ostreatus* substratum	EF392782	EF392762	
CPK 1541		Italy	*P. ostreatus* substratum	EF392783		
CPK 1542		Italy	*P. ostreatus* incubating bales	EF392784		
CPK 1543		Italy	*P. ostreatus* incubating bales	EF392785		
CPK 1544		Italy	*P. ostreatus* incubating bales	EF392786	EF392763	
CPK 1545		Italy	*P. ostreatus* incubating bales	EF392787		
CPK 1546		Italy	*P. ostreatus* incubating bales	EF392788	EF392764	
CPK 1547		Italy	*P. ostreatus* incubating bales	EF392789		
CPK 1548		Italy	*P. ostreatus* incubating bales	EF392790		
CPK 1550		Italy	Mushroom farm	EF392791	EF392765	
CPK 1551		Italy	Mushroom farm	EF392792	EF392766	
CPK 1552		Italy	Mushroom farm	EF392793	EF392767	
CPK 2104	CBS 121145	Hungary	*P. ostreatus* substratum	EF392794	EF392769	
CPK 2816		Romania	*P. ostreatus* substratum	EF601676	EF601681	
CPK 2817		Romania	*P. ostreatus* substratum	EF601677	EF601682	
DZ 56	CPK 882, CBS 121146	Iran	Compost for *Agaricus bisporus*	EF392781	EF392761	EF392776
GJS 04-01		United States (Montana)	Biocontrol of *Cercospora*[b]		EF392768	
GJS 95-81		The Netherlands	*Pleurotus* spawn	*AF345948*	*AF348102*	
CBS 628.77		The Netherlands	Foodstuff	*AF194006*		
JB T2290		Canada (Quebec)	Elm log	*AY605746*	*AY605789*	EF392777
BBA 65638		Germany		*AF194007*		
GJS 95-14		New Zealand	Biocontrol of *Armillaria*[c]	*AF055216*		
T. pleurotum strains						
CPK 2113[a]	CBS 121147, DAOM 236051	Hungary	*P. ostreatus* substratum	EF392808	EF392773	EF392779
CPK 2095		Hungary	*P. ostreatus* substratum	EF392796		
CPK 2096		Hungary	*P. ostreatus* substratum	EF392797	EF392770	
CPK 2097		Hungary	*P. ostreatus* substratum	EF392798	EF392771	
CPK 2098		Hungary	*P. ostreatus* substratum	EF392799		EF392778
CPK 2099		Hungary	*P. ostreatus* substratum	EF392800		
CPK 2100		Hungary	*P. ostreatus* substratum	EF392801	EF392772	
CPK 2101		Hungary	*P. ostreatus* substratum	EF392802		
CPK 2102		Hungary	*P. ostreatus* substratum	EF392803		
CPK 2103		Hungary	*P. ostreatus* substratum	EF392804		
CPK 2109		Hungary	*P. ostreatus* substratum	EF392805		
CPK 2110		Hungary	*P. ostreatus* substratum	EF392806		
CPK 2112		Hungary	*P. ostreatus* substratum	EF392807		
CPK 2114		Hungary	*P. ostreatus* substratum	EF392809		
CPK 2116	CBS 121148	Hungary	*P. ostreatus* substratum	EF392810	EF392774	EF392780
CPK 2117		Hungary	*P. ostreatus* substratum	EF392811	EF392775	
CPK 1532	CBS 121216	Italy	*P. ostreatus* substratum	EF392795	EF601678	
CPK 2814		Romania	*P. ostreatus* substratum	EF601674	EF601679	
CPK 2815		Romania	*P. ostreatus* substratum	EF601675	EF601680	

[a] Reference strain.
[b] Obtained from G. J. Samuels as *T. koningii*; used for biocontrol of *Cercospora*.
[c] Indicated as a biocontrol agent by Dodd et al. (8).
[d] Accession numbers in roman type are newly submitted; those in italics were submitted previously and retrieved for this work.

strains further bifurcated into two significantly supported clades. A similar divergence also takes place on the *tef1* tree: one clade comprises strain DAOM 175924, most of the Italian, one of the Hungarian, and two Romanian *Pleurotus* green mold strains, as well as environmental isolates from Iran, North America, and New Zealand; the second clade includes most of the Hungarian and two Romanian green mold strains, together with one strain isolated from soft rot of wood in


A
T. pleuroticola
T. pleurotum
DAOM Z.D. 56 229916
J.B. T2290
C.P.K. 2116
C.P.K. 2113
NR 6839
PPRI 3772
H. lixii/T. harzianum
CBS 273.78
DAOM 231414
DAOM 222343
IMI 359824
CBS 226.95
IMI 359823
DAOM 231435
H. atrogelatinosa
NZ Lu501
CBS 237.63
chi18-5
0.01
T. cerinum
MA 2991
DAOM 230012
H. tawa
CBS 246.63
Z.D. 28
T. tomentosum
DAOM 222154
CBS 433.95
DAOM 222156
CBS 435.95
T. aggressivum
DAOM 230014
C.P.K. 756
T. velutinum
B
H. lixii/T. harzianum
DAOM 231435
G.J.S. 85-119
IMI 359823
CBS 226. 95
NR 6839
DAOM 222343
DAOM 220786
DAOM 167671
G.J.S. 90-254
DAOM 229971
PPRI 3772
G.J.S. 99-230
DAOM 231414
G.J.S. 99-231
CBS 273.78
C.P.K. 838
H. tawa
CBS 246.63
DAOM 229903
T. pleuroticola
C.P.K.2104
C.P.K.2816
C.P.K.1550
J.B. T2290
C.P.K.1715
C.P.K.1552
C.P.K.2817
C.P.K.1551
DAOM 175924
G.J.S. 95-81
C.P.K.1548
D.Z. 56
C.P.K.1544
DAOM 229916
C.P.K.1540
tef1
0.1
T. aggressivum
DAOM 222189
CBS 433.95
CBS 435.95
DAOM 222156
IMI 359824
DAOM 222177
DAOM 222154
0.1
T. pleurotum
C.P.K. 2097
C.P.K. 2117
C.P.K. 2096
C.P.K. 2100
C.P.K. 2815
C.P.K. 2814
C.P.K. 2116
C.P.K. 2113
C.P.K. 1532
H. atrogelatinosa
CBS 237.63
NZ Lu501
C.P.K. 756
T. velutinum
DAOM 230014
MA 2991
T. cerinum
DAOM 230012
G.J.S. 02-84
H. straminea
DAOM 178713
Z.D. 28
T. tomentosum
G.J.S. 97-237
H. cinnamomea

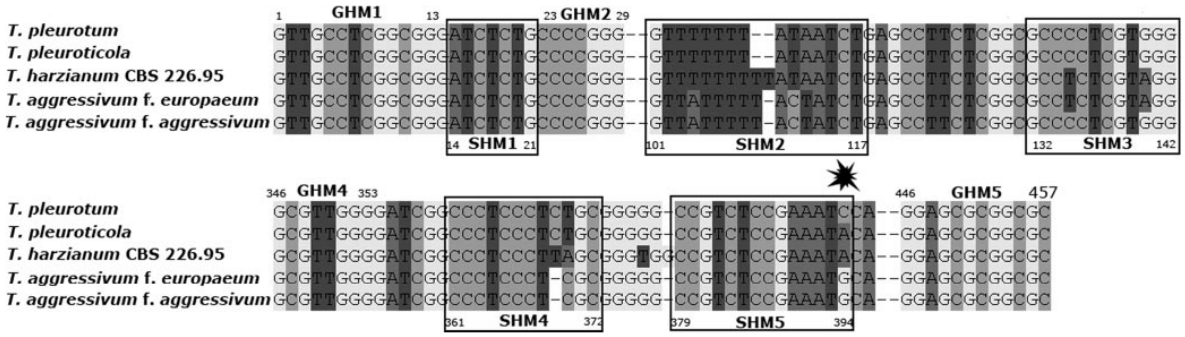


FIG. 2. ITS-based oligonucleotide BarCode for identification of mushroom green mold species. GHM1 to GMH5 and SHM1 to SHM5 indicate positions of genus- and species-specific hallmarks as indicated in Druzhinina et al. (11). The star shows the position of the diagnostic substitution inside SHM5 for *T. pleurotum*, *T. pleuroticola*, *T. aggressivum*, and *H. lixii*/*T. harzianum*. Type sequences were retrieved using accession numbers given in the study of Druzhinina et al. (11).

Germany. A single Italian isolate, CPK 1532, from a *Pleurotus* farm occupies a basal position to this clade.

Visual analysis of ITS1 and ITS2 sequences (Fig. 2) support the relationship of the studied strains to the Harzianum clade. All strains from *Pleurotus* farms were segregated into two ITS2 alleles which differed from each other by one single nucleotide polymorphism (position 394 from the first nucleotide of the first genus-specific hallmark) (11). This divergence strictly corresponds to the two significant clades in the analyses of *tef1* and *chi18-5*. Both of these alleles were 5 to 6 nucleotides different from the type allele of *T. harzianum* (CBS 226.95) and from the two known alleles of *T. aggressivum*.

The concordant divergence of three loci showing two clades of *Pleurotus* green mold strains indicates the presence of two phylogenetic species. Because of the identity of the ITS1 and ITS2 sequences of strains DAOM 175924 and CNUMH 601 and of strains CPK 1532 and CNUMH 501 (NCBI GenBank DQ164405) and based on the similar ecological characterizations, we assume that the detected new species correspond to *T. pleuroticola* type culture CNUMH 601 and *T. pleurotum* type culture CNUMH 501, which have been formally described by Park et al. (31). Since ex-type cultures of these species were not deposited in publicly accessible culture collections and therefore were not available for this study, we refer to strains DAOM 175924 and CPK 1532 as reference strains for *T. pleuroticola* and *T. pleurotum*, respectively. For rapid molecular identification, oligonucleotide BarCodes based on differences in ITS1 and ITS2 sequences among *T. pleurotum*, *T. pleuroticola*, and related species have been implemented in the database for the *Hypocrea*/*Trichoderma* DNA oligonucleotide BarCode program *TrichO*KEY (11, 12; also www.ISTH.info).

Biogeography of *T. pleuroticola* and *T. pleurotum*. We performed a detailed analysis of the *tef1* alleles of *T. pleuroticola* and *T. pleurotum* to investigate possible biogeographic traits in the distribution of the isolates associated with *Pleurotus*. Figure 3 shows the distribution of individual *tef1* alleles among isolates from different locations. The scheme was constructed from one of 100 saved most parsimonious trees obtained using a heuristic search implemented in PAUP*, version 4b10. Six Hungarian and two Romanian strains of *T. pleurotum* showed almost no intraspecific variability since two groups of *tef1* sequences (four isolates each) were separated by only one A↔G transition and one indel in one of several 5′-A_nT_n-3′ spans of the intron. In contrast, two major alleles of *T. pleuroticola* (Fig. 3, I and II) were distinguished based on five diagnostic transitions. Six tested Italian strains of *T. pleuroticola* isolated from cultivated *Pleurotus* substratum were found to be polymorphic; one strain has the *tef1* allele Ia identical to strain GJS 04-01 known to be a biocontrol agent from Montana used against *Cercospora* in sugar beet; three strains share the same allele (IIa) with strain DZ56 isolated from *Agaricus* compost in Iran, and the two remaining strains have the *tef1* allele (IIb) identical to that of reference strain DAOM 175924 isolated from *Acer* sp. in Canada. GJS 95-81 isolated from *Pleurotus* spawn in The Netherlands has one position that differs from the type allele. The only Hungarian isolate of *T. pleuroticola* (CPK 2104) belongs to the first major allele. Thus, the absence of any biogeographical pattern for the distribution of *T. pleuroticola* and, moreover, the mixed composition of the Italian sample suggest the presence of a distribution vector for the species.

Evolution of *T. pleurotum* was accompanied by a loss of certain carbon utilization traits. The results of a cluster analysis based on optical density at 750 nm (assimilation and growth) for 95 carbon sources after 96 h of incubation are presented in Fig. 4A. The strains of *T. pleurotum* formed a "monophenetic" grouping in the cluster analysis, whereas *T. pleuroticola* and *T. aggressivum* were not clearly separated. In univariate ANOVAs, however, highly significant differences

FIG. 1. Bayesian analyses of the phylogenetic position of *Pleurotus* green mold species based on their *chi18-5* and *tef1* sequences. Posterior probability coefficients are given at respective nodes and shown only if the branch was highly supported (>0.95). Arrows indicate branches leading to currently recognized species. GenBank accession numbers for *T. pleurotum* and *T. pleuroticola* are given in Table 1. Accession numbers for other sequences may be retrieved from GenBank using the searches "species+strain+endochitinases 42" and "species+strain+translation elongation" for *chi18-5* and *tef1*, respectively.

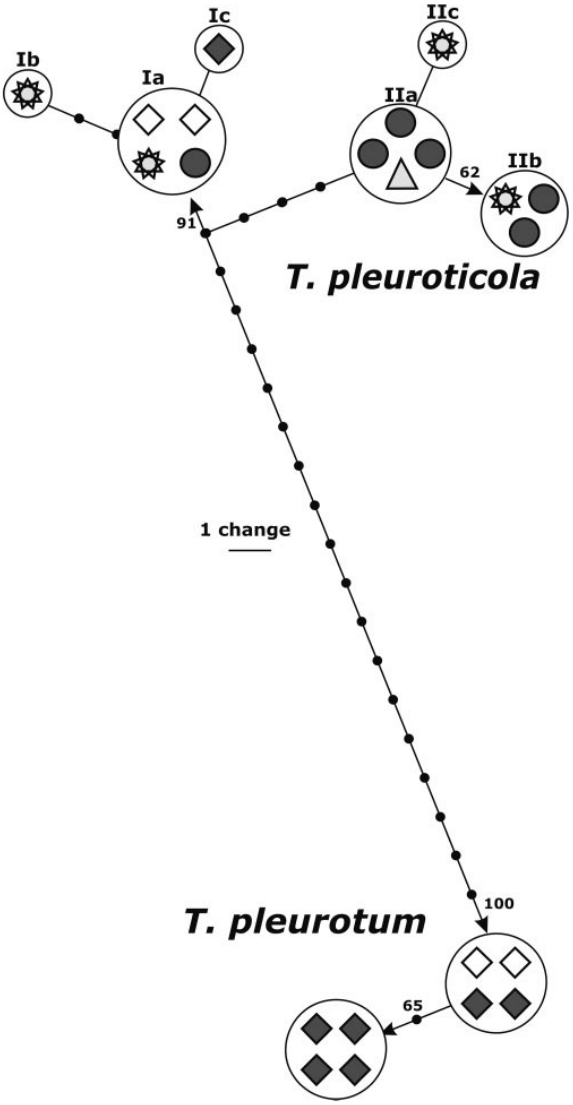


FIG. 3. Distribution of individual *tef1* alleles among isolates from different locations. The scheme was manually constructed based on one of the 100 saved most parsimonious trees obtained using a heuristic search implemented in PAUP*, version 4b10 (39). ◆, isolates from Hungary; ◊, isolates from Romania; ●, isolates from Italy; star, isolates from North America; ▲, single isolate from Iran. Arabic numbers correspond to bootstrap coefficients; roman numbers show main *tef1* alleles.

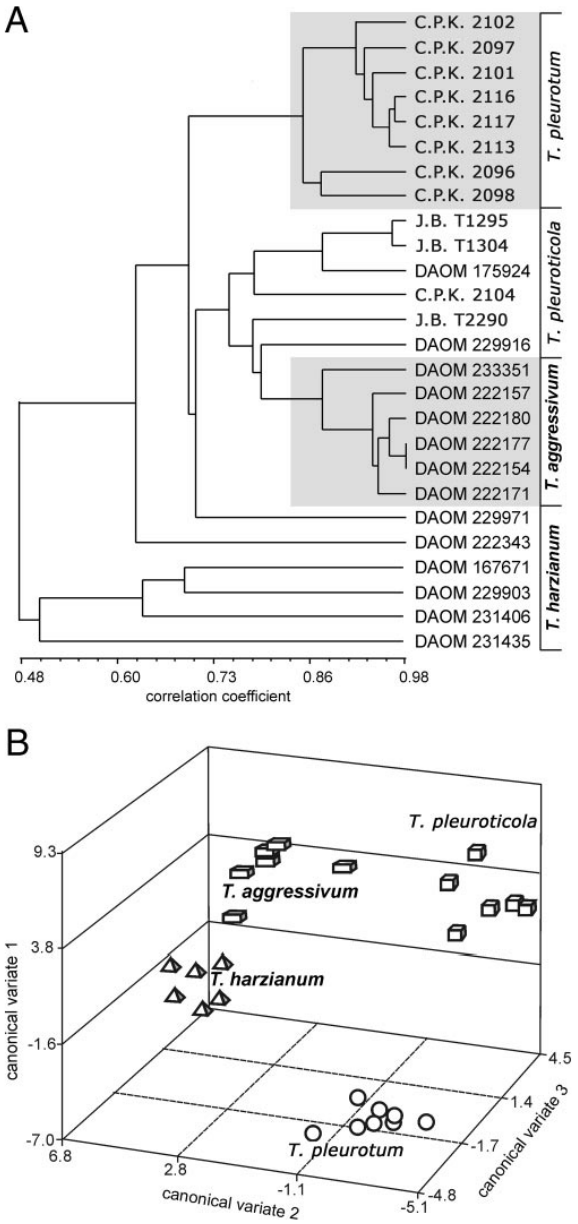


FIG. 4. (A) Cluster analysis of *Trichoderma* strains based on 750-nm optical density readings (mycelial growth) after a 96-h incubation; "monophenetic" taxa are shaded. (B) Canonical variate analysis of 750-nm optical density readings after a 96-h incubation.

among all the species (P of <0.001) were seen for 34 substrata. A canonical variate analysis was performed for the 12 most significant substrata (P of <0.0001). All three canonical variates were highly significant (P of <0.001) (Table 2), and a plot of *T. pleuroticola*, *T. pleurotum*, *T. aggressivum*, and *T. harzianum* on the three canonical variates (Fig. 4B) clearly distinguishes *T. pleuroticola* and *T. pleurotum* from each other, as well as from the closely related species in the Harzianum clade.

Metabolic profiles of *T. pleuroticola*, *T. pleurotum*, and related species in the Harzianum clade on the 34 highly significant substrata (P of <0.001) are compared in Fig. 5 and in the table in the supplemental material. In general, *T. harzianum* had the highest growth rate on a majority of the carbon sources (18/34). However, this did not reflect an overall higher growth rate or preferred temperature optima, since *T. pleuroticola* had

TABLE 2. Total standardized canonical coefficients

Rank	Substrate	Canonical variate 1	Canonical variate 2	Canonical variate 3
1	L-Sorbose	1.11	0.19	1.00
2	*N*-Acetyl-D-glucosamine	0.05	−2.26	1.52
3	L-Threonine	2.99	1.89	−4.36
4	*N*-Acetyl-L-glutamic acid	−2.87	−1.53	−0.28
5	Sebacic acid	−1.85	2.36	0.73
6	Quinic acid	0.94	0.74	0.75
7	D-Tagatose	2.38	0.83	1.02
8	L-Fucose	1.39	1.40	−1.31
9	Succinic acid mono-methyl ester	0.40	−0.50	−2.11
10	Glycyl-L-glutamic acid	1.39	−1.93	−0.22
11	β-Hydroxybutyric acid	1.84	1.14	−0.11
12	D-Glucuronic acid	−4.43	0.21	5.25

the highest growth rates on the most readily assimilated compounds such as *N*-acetyl-D-glucosamine and α-D-glucose. Notably, all three species causing mushroom green mold diseases did not grow on *N*-acetyl-D-mannosamine or on *N*-acetyl-L-glutamic acid (Fig. 5) whereas *T. harzianum* readily assimilated these carbon sources. *T. pleurotum* exhibited generally impaired or slow growth on the majority of the carbon sources, and the species was unable to grow on D-tagatose, succinic acid mono-methyl ester, D-glucuronic acid, α-D-glucose-1-phosphate, and β-methyl-D-galactoside, which were all assimilated by the other species. In addition, growth of *T. pleurotum* on *N*-acetyl-D-glucosamine, sebacic acid, quinic acid, L-phenylalanine, and arbutin was significantly slower than growth of the other three species. The highest assimilation rates for *T. pleuroticola* occurred on *N*-acetyl-D-glucosamine and quinic acid, which could be useful to differentiate between the two causative agents of *Pleurotus* green mold disease. The carbon assimilation profile for *T. pleuroticola* was very similar to that of *T. aggressivum*. The two species are distinguished by the inability of *T. aggressivum* to assimilate α-ketoglutaric acid, L-malic acid, and succinamic acid.

Antagonism of *T. pleurotum* and *T. pleuroticola* against *P. ostreatus* in vitro. Results of dual confrontation tests with *P. ostreatus* and *A. bisporus* are shown in Fig. 6. *A. bisporus* was vulnerable to all *Trichoderma* strains tested. The culture of this mushroom developed slowly and after 10 days was partly overgrown by *Trichoderma reesei* QM 6a (teleomorph *H. jecorina*) used as a negative control since it is a tropical species without notable mycoparasitic ability. All strains of the three green mold species covered the whole plate including the colony of *A. bisporus* after 5 to 7 days. The isolates of *T. pleuroticola* and *T. pleurotum* produced intense conidiation locally over the colony of *A. bisporus*. The *P. ostreatus* culture was fully resistant against *T. reesei* QM 6a, with the contact area between the two opposing colonies characterized by the well-expressed inhibition area. *T. aggressivum* was able to overgrow about one-third of the radius of the *P. ostreatus* colony, but the overgrowth stopped after 12 days of incubation. A clearly melanized barrage reaction in the confrontation zone was seen on the reverse of the plate. *T. pleurotum*

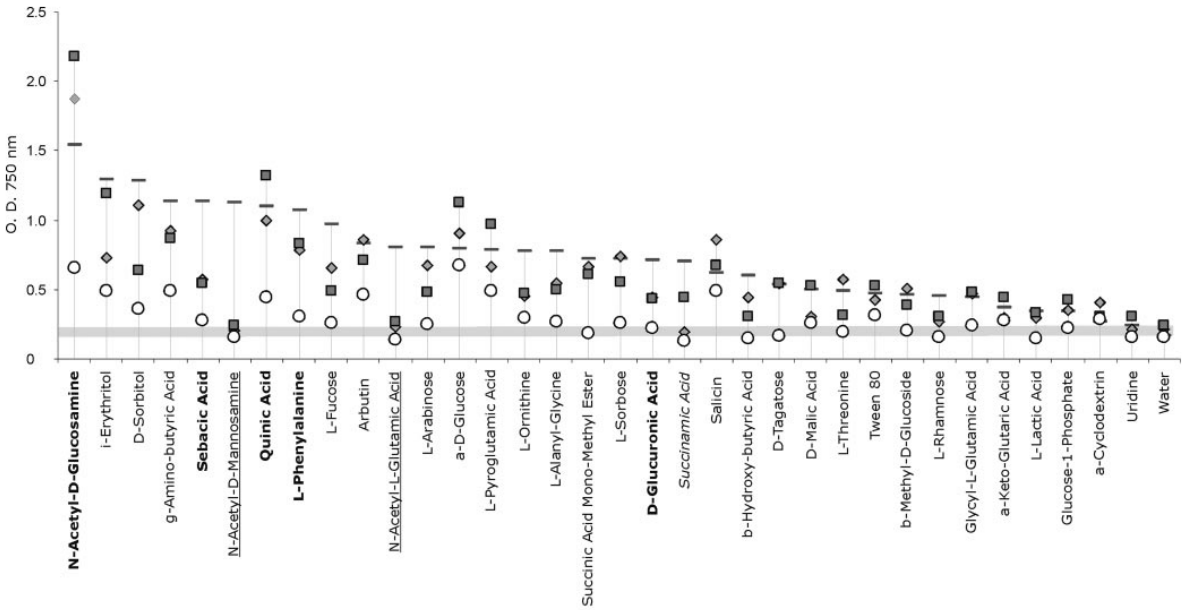


FIG. 5. Mean growth of mushroom green mold species on carbon sources for which statistically significant differences among the species were detected. ◆, *T. aggressivum*; ■, *T. pleuroticola*; ○, *T. pleurotum*; —, *H. lixii/T. harzianum*. The order of the carbon sources is the rank of the growth on 95 carbon sources and water, based on optical density at 750 nm at 96 h for the mean of six strains of *H. lixii/T. harzianum*. Carbon sources utilized differently by *T. pleurotum* and other species are in shown in boldface, underlining indicates cases in which all green mold species were different from *H. lixii/T. harzianum*, and use of italics indicates the case in which *T. aggressivum* and *T. pleurotum* were different from two other species.

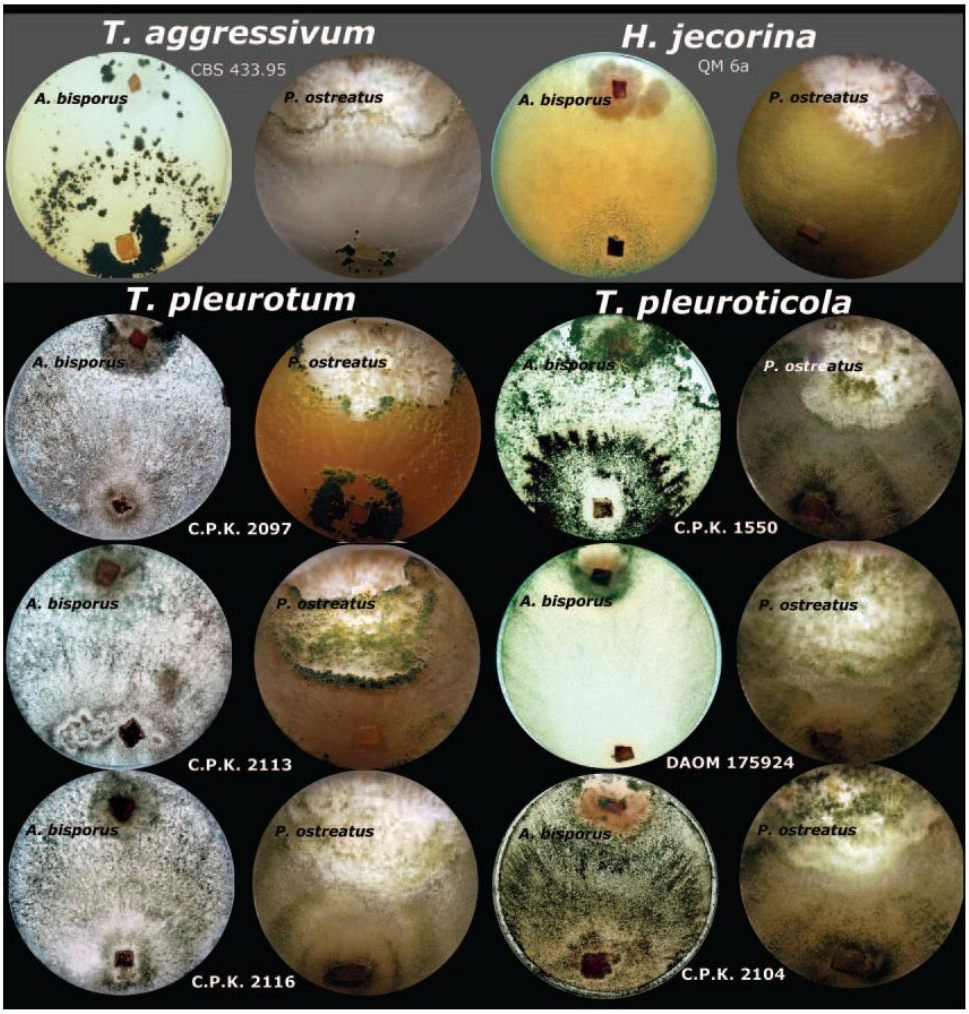


FIG. 6. Dual confrontation assays between cultures of *A. bisporus* and *P. ostreatus* and mushroom green mold species observed after 10 days of incubation on PDA. *H. jecorina*/*T. reesei* QM 6a was used as a negative control.

also caused a strong antagonistic response from *P. ostreatus* although it was able to overgrow the majority of the *Pleurotus* colony by the end of the experiment. The confrontation zone between two opposing colonies was marked by intensive melanization of *P. ostreatus* hyphae and abundant conidiation of *T. pleurotum* (Fig. 6). In contrast to the *T. pleurotum*, *T. pleuroticola* did not cause any visible antagonistic reaction from the *P. ostreatus* culture and was able to overgrow the *Pleurotus* colony within 4 to 6 days.

Morphology of *T. pleuroticola* and *T. pleurotum*. The close genetic relationship of *T. pleuroticola* and *T. pleurotum*, as revealed by gene sequence analysis, led us to expect that these two species would have very similar morphologies. However, and also in accordance with Park et al. (31), this was found not to be the case. The most striking difference was that *T. pleurotum* isolates exhibited a gliocladium-like conidiophore branching (Fig. 7), whereas *T. pleuroticola* showed a typical pachybasium-like conidiophore (Fig. 8) developing in fascicles or pustules more characteristic of species in the Harzianum clade. Subtle differences were also apparent in conidial size and growth rates.

Detailed notes on diagnostic morphology of the two species follow.

***Trichoderma pleurotum* (Fig. 7a to f).** Colonies producing limited aerial mycelium and sparse conidiation from effuse conidiophores on MA, initially greenish white (27:A:2), becoming grayish green (26–27:B-C:3–4), later dull green (26:E-F:4–5), and finally in age on MA dull gray green (25–26:E-F:3) with conidiation evenly distributed in small irregular fascicles; on PDA with more abundant effuse conidiation becoming dark green in age (25:F:3–4). Reverse on MA more or less colorless; on PDA often developing light yellow sectors (4:A:3–5), and in age more or less conspicuously colored dull yellowish brown to reddish brown (5:D:4 to 8:D:5). Conidiophores arising from the substratum, usually

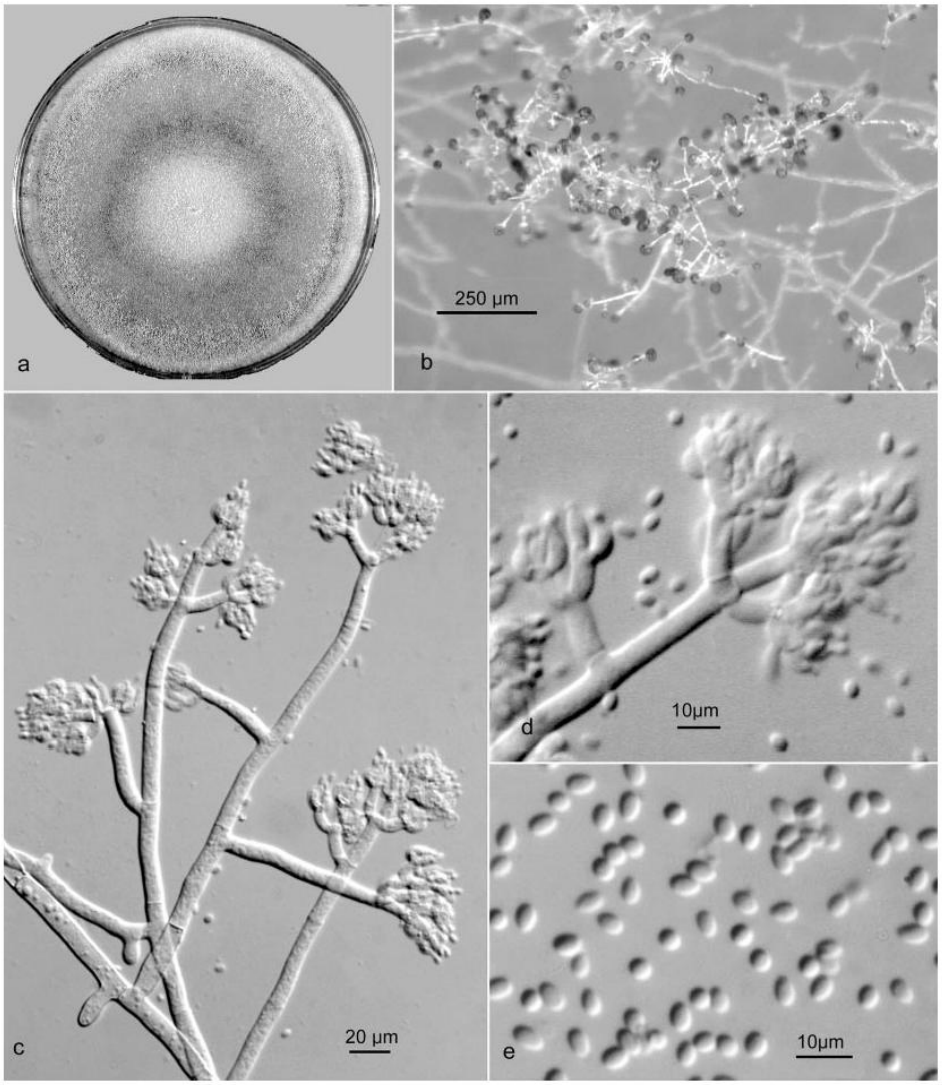


FIG. 7. *T. pleurotum* CPK 2113 (reference culture). (a) Colony on PDA after 10 days at 25°C. (b) Prostrate conidiophore with gliocladium-like branches. (c) Conidiophore with crowded apical branches and phialides. (d) Terminal branches with appressed phialides. (e) Ellipsoid conidia.

unbranched near the base, over most of the length bearing gliocladium-like primary branches at nearly right angles, primary branches approximately equal in length, usually unbranched near the base, with branches arising singly and irregularly or in whorls of up to four near the apex, the apex of the conidiophore and primary branches usually terminated by a whorl of three or four branches rebranching once or twice and bearing crowded whorls of phialides; conidiophore and branches relatively broad, up to 8 μm diameter toward the base, terminal branches mostly 4 to 13 μm long and 3 to 4 μm wide. Phialides arising almost exclusively in verticils of four to seven on terminal branches, nearly ampulliform, often curved, sharply constricted at the conidium-bearing apex, mostly 4.5 to 7.5 by 2.5 to 4.0 μm, solitary phialides rare, intercalary phialides not observed, phialides persistent in age. Conidia predominately ellipsoid, occasionally obovoid with one end pointed, less often subglobose, 2.5 to 4.8 by 1.7 to 2.6 μm (average, 3.6 by 2.1 μm), pale green viewed microscopically, dark green in mass, smooth walled. Chlamydospores few, subglobose.

Morphologically, *T. pleurotum* has a conidiophore branching pattern that is unique within the Harzianum clade, in that conidiophores are mostly solitary and more or less prostrate, branching in an irregular fashion, with branches scattered, arising separately and bearing crowded whorls of appressed phialides at the apex resembling the conidiophore in *Gliocladium*. Conidia are mostly ellipsoid and longer than in most other species in the clade.

Trichoderma pleuroticola **(Fig. 8a to f)**. Colonies producing limited aerial mycelium on MA, on PDA producing fascicu-

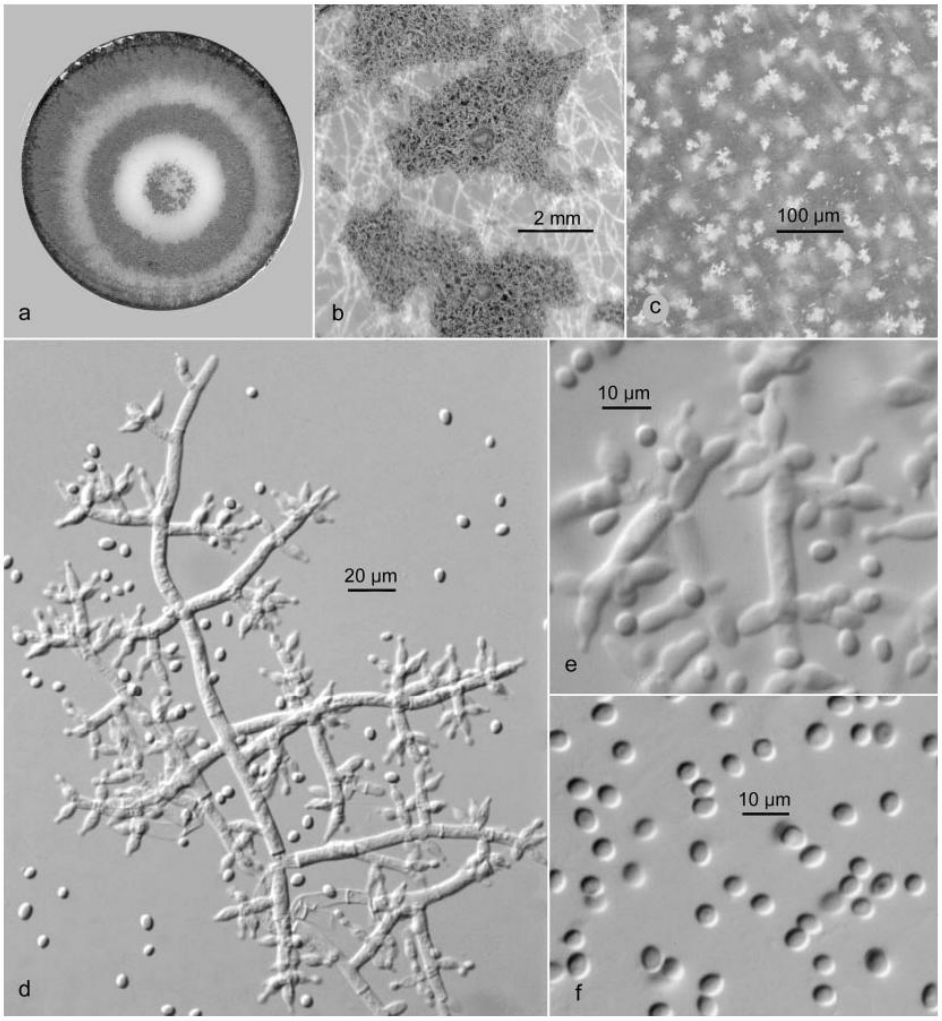


FIG. 8. *T. pleuroticola* strain DAOM 175924 (reference culture). (a) Colony on PDA after 10 days at 25°C. (b) Flat conidiogenous pustules. (c) Crystals in reverse on PDA after 14 days. (d) Conidiophore with paired or verticillate branches and phialides. (e) Phialides. (f) Mostly subglobose conidia.

late, white aerial mycelium, conidiophores forming small pustules that coalesce in broad concentric zones, initially greenish gray (25:B:2–3), soon grayish green (25:C:3–4 to 25:E:4–5), in age on MA dull to dark green (26:E-F:4–5), in age on PDA darker green, or with brighter green, flat pustules fringed by white mycelium and renewed conidiation. Reverse more or less uncolored at first, in age dull yellowish on MA, on PDA typically turning dark brown (8:F:5), abundant, small, yellow crystals often developing in PDA after 7 days of incubation. Conidiophores branching in a more or less pyramidal fashion with branches increasing in length toward the base, branches arising singly or paired toward the base of the conidiophore main axis, near the apex often three to four verticillate, primary branches branching in a pattern similar to the main axis, conidiophore and branches comparatively narrow and flexuous, main axis up to 5.5 µm wide at the base, gradually narrowing to 2.5 to 3.0 µm at the apex, terminal branches cylindrical, 6 to 14 by 2.5 to 3.3 µm. Phialides paired or three to four verticillate at the apex of the terminal branches, or arising separately and scattered along the sides of the conidiophore and branches, ampulliform to lageniform, abruptly narrowing to a conidium bearing collulum less than 1 µm wide, mostly 4.2 to 9.5 by 3.0 to 4.2 µm, or terminal phialides acerose and up to 20 µm long. Short, cylindric intercalary phialides occasionally produced beneath septa on terminal branches or from sides of phialides. Phialides seceding in older cultures. Conidia subglobose to broadly ellipsoid, less often obovoid and pointed at the base, 2.6 to 5.0 by 2.4 to 3.7 µm (average, 3.7 by 2.8 µm), bright green viewed microscopically, dark gray green in mass, smooth walled. Chlamydospores usually in chains or clusters, subglobose, 4 to 10 µm diameter, pale greenish.

Morphologically, the conidiophore in *T. pleuroticola* is orga-

nized in essentially the same fashion as in *T. harzianum* and related species in the Harzianum clade. The conidiophore is branched at regular intervals with branches increasing in length to the base, and branches and phialides arise mostly in uncrowded verticils. Conidia are significantly larger in *T. pleuroticola* than in *T. harzianum*, but the most distinctive morphological feature of *T. pleuroticola* is the production by most strains of a dark brown pigment and yellow crystals in the agar on PDA.

DISCUSSION

In this paper, we provide evidence that the causal agent for the oyster mushroom green mold disease, which recently started to spread in Europe and Asia, is actually two different although genetically closely related species of *Trichoderma* which correspond to recently described taxa *T. pleurotum* and *T. pleuroticola* (31). Both species are also closely related to the *H. lixii*/*T. harzianum* species aggregate and to *T. aggressivum*, which is the causal agent of green mold disease of *Agaricus*. These findings are in accordance with those reported by Park et al. (30), who used parsimony analysis of ITS1 and ITS2, *rpb2*, and *tef1* sequences to separate the *Pleurotus* pathogenic strains into two clusters which they called "*Trichoderma* sp. strain K1" and "strain K2." Unfortunately, sequences from their study, except for ITS1 and ITS2 from six isolates, have not been deposited in public databases and thus could not be included in our analysis. The available ITS1 and ITS2 sequences indicate that their six strains of *Trichoderma* sp. strain K1 isolated from *Pleurotus* substrata (rice straw, cotton waste, and sawdust) from four locations in South Korea correspond to *T. pleuroticola* (reference GenBank accession numbers DQ164409 and DQ164410 for strains CNU601 and CNU646, respectively) subsequently described by the same authors (31). Similarly, strain K2 represents *T. pleurotum*, 14 strains of which were isolated from the same substrata in six locations (reference GenBank accession numbers DQ164405, DQ164406, DQ164407, and DQ164408 for strains CNU501, CNU523, CNU538 and CNU571, respectively). According to Park et al. (30, 31) both species coexist in South Korean *Pleurotus* farms with no clear dominance of one or the other species.

There are several indications that the infection is introduced to farms via the substratum for mushroom cultivation, and differences in species distribution may be due to the use of certain substrata which, depending on the manufacturer, may consist of cereal straw, sawdust, bagasse, or waste cotton. In our sample *T. pleuroticola* dominates in samples from Italian *Pleurotus* farms while *T. pleurotum* is abundant among Hungarian isolates. Although wheat straw is used as a major component for the *Pleurotus* substratum in both countries, the difference in species composition may be due to the addition of pulverized "tufo" in Italian farms, which is a natural calcareous rock of volcanic origin that raises the substratum pH to around 8. To the best of our knowledge, there is no such technological stage in Hungarian farms, where wheat straw is moisturized in the open air before use as *Pleurotus* substratum. The hypothesis of a possible reduction of *T. pleurotum* infection by the alkalization of the substratum may be further supported by the fact that *Pleurotus* green mold is not reported to be a severe problem in the United States, where the addition of lime to increase pH to 7.5 is widely practiced (http://mushroomspawn.cas.psu.edu/). However, this treatment seems to be ineffective against *T. pleuroticola*. There may be another explanation for the occurrence of the two *Pleurotus*-associated green mold species in mushroom farms. *T. pleuroticola* is also frequently isolated from soil and plant debris, and we report environmental strains from Canada, the United States, Europe, and New Zealand. It seems to have a global occurrence, although possibly favoring a temperate climate. *T. pleuroticola* infections may therefore have multiple origins and even be due to introductions from the surrounding environment. In contrast, *T. pleurotum*, just like *T. aggressivum*, has so far never been isolated from areas outside of mushroom farms. Seaby (37) reported evidence that *T. aggressivum* could be carried by red pepper mites into *Agaricus* mushroom farms. Another possibility would be that *T. pleurotum* could be an endophyte of plants used for preparation of the mushroom substratum (possibly wheat, rice, and cotton). The vectors for *T. pleurotum* and *T. pleuroticola* into mushroom farms are currently under investigation in our laboratories. The consistent cooccurrence of these two species in mushroom farms in Romania, Italy, Hungary, and South Korea is interesting. Yet recent metagenomic studies on the occurrence of *Trichoderma* in Austrian soils frequently reveals the presence of *T. pleuroticola* but never of *T. pleurotum* (M. A. Friedl and I. S. Druzhinina, unpublished data), thus suggesting that these two species occupy different ecological and trophic niches in nature.

The large phenetic divergence of *T. pleuroticola* and *T. pleurotum*, morphologically and metabolically, in spite of the very close phylogenetic relationship, is a unique finding, as fungi are believed to develop phenotypic differences only after accumulation of some genetic distance, which gives rise to "cryptic" species which can hardly be phenotypically distinguished. The fact that *T. pleurotum* occupies a more terminal position than *T. pleuroticola* in all gene trees and that the latter exhibits similar morphological and metabolic characteristics as its phylogenetically close members in the Harzianum clade of *Hypocrea*/*Trichoderma* (*T. harzianum* and *T. aggressivum*) suggest that this change in morphology is due to a loss rather than a gain of gene function. Kullnig-Gradinger et al. (22), comparing the morphotypes and phylogeny of *Trichoderma* species have speculated that the switch from fungicolous to saprophytic habitats was accompanied by the expression of the pachybasium-like conidiophore morphology. In this sense, the return to gliocladium-like morphology may be advantageous under the conditions of the natural niche of *T. pleurotum*. The gliocladium-like morphology is rare in the Harzianum clade although it is known for the anamorph of *Hypocrea tawa*.

Even though there is a consistent association between *T. aggressivum* and *Agaricus*, on one hand, and *T. pleuroticola*/*T. pleurotum* and *Pleurotus*, on the other hand, our confrontation assays show that the two newly described species pose a potential threat to mushroom-producing farms: both *T. pleuroticola* and *T. pleurotum* were able to inhibit and then overgrow *Agaricus* culture, while *Pleurotus* showed some resistance toward *T. aggressivum*. Since *T. pleuroticola* is frequently isolated from various soils and plant debris, we consider this species as the most dangerous agent of mushroom green mold disease in general.

Finally, this study also places some caveats on the use of some *Trichoderma* isolates as biocontrol agents. Two of the *T. pleuroticola* isolates of this study were obtained from other researchers as biofungicides against soil-borne diseases (Table 1). In view of the present identification of *T. pleuroticola* as a causative agent of oyster mushroom green mold, this applica-

tion could be problematic. However, the fact that infections by *T. pleuroticola* and *T. pleurotum*—although probably common for decades (see reference 39)—only recently increased dramatically suggests there is a special trigger for the infections, which may involve the source of the substrate used for cultivation, its preparation, or other conditions of mushroom cultivation. This, in turn, implies that if this trigger can be determined, the risk of infection can be managed. With the molecular tools presented in this paper, the processes involved in the preparation of substrata for the cultivation of *P. ostreatus* can be investigated, and infections can be traced back to their sources. Oligonucleotide probes based on diagnostic polymorphisms in *tef1* sequences offer the development of real-time PCR techniques for quantitative detection as well and are in preparation in our laboratories.

ACKNOWLEDGMENTS

This work was supported partly by Austrian Science Fund grants FWF P-12748-MOB and FWF P-16601 to C.P.K. and by grant OTKA F68381 from the Hungarian Scientific Research Fund to L.K., as well as by the Austrian Exchange Service and the Hungarian National Office for Research and Technology under the bilateral project WTZ HU03/2007-TéT A02/2006. L.K. is a grantee of the János Bolyai Research Scholarship (Hungarian Academy of Sciences).

We also thank Parivash Shoukouhi for contributions to the physiological and molecular studies undertaken at the Eastern Cereal and Oilseed Research Center.

REFERENCES

1. **Anonymous.** 2005. Consensus document on the biology of *Pleurotus* spp. (oyster mushroom). Series on harmonisation of regulatory oversight in biotechnology, no. 34 Organisation for Economic Co-operation and Development, Environment Directorate, Paris, France.
2. **Ballero, M., E. Mascia, A. Rescigno, and E. S. D. Tenlada.** 1990. Use of *Pleurotus* for transformation of polyphenols in waste waters from olive presses into proteins. Micol. Ital. **19:**39–41.
3. **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:**133–137.
4. **Chang, R.** 1996. Functional properties of edible mushrooms. Nutr. Rev. **54:**91–93.
5. **Clement, M., D. Posada, and K. A. Crandall.** 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. **9:**1657–1659.
6. **dela Cruz, T. E. E., B. E. Schulz, C. P. Kubicek, and I. S. Druzhinina.** 2006. Carbon source utilization by the marine *Dendryphiella* species *D. arenaria* and *D. salina*. FEMS Microbiol. Ecol. **58:**343–353.
7. **Dettman, J. R., D. J. Jacobson, and J. W. Taylor.** 2003. A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. Evolution **57:**2703–2720.
8. **Dodd, S., R. N. Crowhurst, A. G. Rodrigo, G. J. Samuels, R. A. Hill, and A. Stewart.** 2000. Examination of *Trichoderma* phylogenies derived from ribosomal DNA sequence data. Mycol. Res. **104:**23–34.
9. **Druzhinina, I. S., M. Schmoll, B. Seiboth, and C. P. Kubicek.** 2006. Global carbon utilization profiles of wild-type strains, mutants and transformants of *Hypocrea jecorina*. Appl. Environ. Microbiol. **72:**2126–2133.
10. **Druzhinina, I. S., A. G. Kopchinskiy, and C. P. Kubicek.** 2006. The first one hundred of *Trichoderma* species is characterized by molecular data. Mycoscience **47:**55–64.
11. **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.
12. **Druzhinina, I. S., and A. G. Kopchinskiy.** 2006. *TrichO*KEY v. 2—a DNA oligonucleotide BarCode program for the identification of multiple sequences of *Hypocrea* and *Trichoderma*, p. 53–59. *In* W. Meyer and C. Pearce (ed.), Proceedings of the 8th International Mycological Congress, Cairns, Australia. Medimond-Monduzzi, Bologna, Italy.
13. **Gunde-Cimerman, N.** 1999. Medicinal value of the genus *Pleurotus* (Fr.) P. Karst. (*Agaricales* s.l. Basidiomycetes). Int. J. Med. Mushrooms **1:**69–80.
14. **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 mould diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderma* species. Phytopathology **97:**532–537.
15. **Hermosa, M. R., I. Grondona, and E. Monte.** 1999. Isolation of *Trichoderma harzianum* Th2 from commercial mushroom compost in Spain. Plant Dis. **83:**591.
16. **Jaklitsch, W. M., M. Komon, C. P. Kubicek, and I. S. Druzhinina.** 2006. *Hypocrea crystalligena* sp. nov., a common European species with a white-spored *Trichoderma* anamorph. Mycologia **98:**500–514.
17. **Kopchinskiy, A., M. Komon, C. P. Kubicek, and I. S. Druzhinina.** 2005. *Tricho*BLAST: a multilocus database for *Trichoderma* and *Hypocrea* identifications. Mycol. Res. **109:**657–660.
18. **Kornerup, A., and J. H. Wanscher.** 1978. Methuen handbook of colour, 3rd ed. Eyre Methuen, London, United Kingdom.
19. **Kraus, G., I. Druzhinina, J. Bissett, H. J. Prillinger, G. Szakacs, W. Gams, and C. P. Kubicek.** 2004. *Trichoderma brevicompactum* sp. nov. Mycologia **96:**1057–1071.
20. **Kredics, L., L. Hatvani, Z. Antal, L. Manczinger, I. S. Druzhinina, C. P. Kubicek, A. Szekeres, A. Nagy, C. Vágvölgyi, and E. Nagy.** 2006. Green mould disease of oyster mushroom in Hungary and Transylvania. Acta Microbiol. Immunol. Hung. **53:**306–307.
21. **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.
22. **Kullnig-Gradinger, C. M., G. Szakacs, and C. P. Kubicek.** 2002. Phylogeny and evolution of the fungal genus *Trichoderma*—a multigene approach. Mycol. Res. **106:**757–767.
23. **Leache, A. D., and T. W. Reeder.** 2002. Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood and Bayesian approaches. Syst. Biol. **51:**44–68.
24. **Lieckfeldt, E., C. M. Kullnig, C. P. Kubicek, G. J. Samuels, and T. Börner.** 2001. *Trichoderma aureoviride*: phylogenetic position and characterization. Mycol. Res. **105:**313–322.
25. **Mamoun, M. L., J.-M. Savoie, and J.-M. Olivier.** 2000. Interactions between the pathogen *Trichoderma harzianum* Th2 and *Agaricus bisporus* in mushroom compost. Mycologia **92:**233–240.
26. **Marzullo, L., R. Cannio, P. Giardina, M. T. Santini, and G. Sannia.** 1995. Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and prevents polymerization of laccase-oxidized substrates. J. Biol. Chem. **270:**3823–3827.
27. **Nicholas, K. B., and H. B. Nicholas, Jr.** 1997 Genedoc: a tool for editing and annotating multiple sequence alignments. http://www.psc.edu/biomed/genedoc.
28. **O'Donnell, K., E. Cigelnik, and H. L. Nirenberg.** 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia **90:**465–493.
29. **Pani, B. K., S. N. Panda, and S. R. Das.** 1997. Utilization of some agricultural bioproducts and other wastes for sporophore production of oyster mushroom. Orissa J. Hortic. **25:**36–39.
30. **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. J. Huazhong Agric. Univ. **23:**157–164.
31. **Park, M. S., K. S. Bae, and S. H. Yu.** 2006. Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. Mycobiology **34:**11–113.
32. **Puniya, A. K., K. G. Shah, S. A. Hire, R. N. Ahire, M. P. Rathod, and R. S. Mali.** 1996. Bioreactor for solid-state fermentation of agro-industrial wastes. Indian J. Microbiol. **36:**177–178.
33. **Rolf, F. J.** 1997. NTSYSpc numerical taxonomy and multivariate analysis system, version 2.00. Exeter Software, Setauket, New York.
34. **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.
35. **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.
36. **SAS Institute, Inc.** 1989. SAS/STAT user's guide, version 6, 4th ed., vol. 1. SAS Institute, Inc., Cary, NC.
37. **Seaby, D. A.** 1989. Further observations on *Trichoderma*. Mushroom J. **197:** 147–151.
38. **Sharma, S. R., and B. Vijay.** 1996. Yield loss in *Pleurotus ostreatus* spp. caused by *Trichoderma viride*. Mushroom Res. **5:**19–22.
39. **Sinden, J., and E. Hauser.** 1953. Nature and control of three mildew diseases of mushrooms in America. Mushroom Sci. **2:**177–180.
40. **Swofford, D. L.** 1998. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4. Sinauer, Sunderland, MA.
41. **Woo, S. L., P. Di Benedetto, M. Senatore, K. Abadi, S. Gigante, I. Soriente, S. Ferraioli, F. Scala, and M. Lorito.** 2004. Identification and characterization of *Trichoderma* species aggressive to *Pleurotus* in Italy. J. Zhejiang Univ. Agric. Life Sci. **30:**469–470.

3.3. Molecular identification of *Trichoderma* species associated with *Pleurotus ostreatus* and natural substrates of the oyster mushroom.

Kredics L, Kocsubé S, Nagy L, **Komon-Zelazowska M**, Manczinger L, Sajben E, Nagy A, Vágvölgyi C, Kubicek CP, Druzhinina IS & Hatvani L (2009) Molecular identification of *Trichoderma* species associated with *Pleurotus ostreatus* and natural substrates of the oyster mushroom. ***FEMS Microbiol Lett*** **300, 58-67**

OWN CONTRIBUTION:

MKZ contributed to the molecular and microbiological work at Vienna University of Technology (cultivated fungi, extracted genomic DNA, performed PCR) and participated in the analysis of sequences and ms writing.

FEMS MICROBIOLOGY LETTERS

RESEARCH LETTER

Molecular identification of *Trichoderma* species associated with *Pleurotus ostreatus* and natural substrates of the oyster mushroom

László Kredics[1], Sándor Kocsubé[1], László Nagy[1], Monika Komoń-Zelazowska[2], László Manczinger[1], Enikő Sajben[1], Adrienn Nagy[3], Csaba Vágvölgyi[1], Christian P. Kubicek[2], Irina S. Druzhinina[2] & Lóránt Hatvani[1]

[1]Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary; [2]Research Area Gene Technology and Applied Biochemistry (DGTAB), Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria; and [3]Pilze-Nagy Ltd, Kecskemét, Hungary

Correspondence: Lóránt Hatvani, Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, H-6726 Szeged, Hungary. Tel.: +36 62 544 005; fax: +36 62 544 823; e-mail: lori@szegedkendo.hu

Received 19 May 2009; accepted 12 August 2009.
Final version published online 7 September 2009.

DOI:10.1111/j.1574-6968.2009.01765.x

Editor: Bernard Paul

Keywords
green mold; multiplex PCR; oyster mushroom; *Pleurotus ostreatus*; *Trichoderma*.

Abstract

Green mold of *Pleurotus ostreatus*, caused by *Trichoderma* species, has recently resulted in crop losses worldwide. Therefore, there is an emerging need for rapid means of diagnosing the causal agents. A PCR assay was developed for rapid detection of *Trichoderma pleurotum* and *Trichoderma pleuroticola*, the two pathogens causing green mold of *P. ostreatus*. Three oligonucleotide primers were designed for identifying these species in a multiplex PCR assay based on DNA sequences within the fourth and fifth introns in the translation elongation factor 1α gene. The primers detected the presence of *T. pleurotum* and/or *T. pleuroticola* directly in the growing substrates of oyster mushrooms, without the need for isolating the pathogens. The assay was used to assess the presence of the two species in natural environments in which *P. ostreatus* can be found in Hungary, and demonstrated that *T. pleuroticola* was present in the growing substrates and on the surface of the basidiomes of wild oyster mushrooms. Other *Trichoderma* species detected in these substrates and habitats were *Trichoderma harzianum*, *Trichoderma longibrachiatum* and *Trichoderma atroviride*. *Trichoderma pleurotum* was not found in any of the samples from the forested areas tested in this study.

Introduction

Pleurotus ostreatus (Jacq.) P. Kumm., commonly known as the oyster mushroom, is the third most important commercially grown edible mushroom worldwide (Chang, 1996). In addition, it is used for the bioconversion of agricultural and industrial lignocellulose debris (Ballero *et al.*, 1990; Puniya *et al.*, 1996), and as a source of enzymes and other metabolites for industrial and medical applications (Marzullo *et al.*, 1995; Gunde-Cimerman, 1999). *Pleurotus ostreatus* can be grown on a wide range of agricultural byproducts and industrial wastes (Pani *et al.*, 1997). Many pests and diseases can cause yield losses in *P. ostreatus*. The association of *Trichoderma* species with the cultivation substrate has long been known to limit production (Anonymous, 2005). Sharma & Vijay (1996) reported green mold of oyster mushroom caused by *Trichoderma viride* Pers. in North America, while severe cases of green mold of *P. ostreatus* were detected recently in South Korea (Park *et al.*, 2004a–c), Italy (Woo *et al.*, 2004), Hungary (Hatvani *et al.*, 2007) and Romania (Kredics *et al.*, 2006).

Green mold of oyster mushroom has recently been reported to be caused by two genetically closely related, but phenotypically distinct, new species of *Trichoderma*: *Trichoderma pleuroticola* S.H. Yu & M.S. Park and *Trichoderma pleurotum* S.H. Yu & M.S. Park (Park *et al.*, 2004a–c, 2006; Komoń-Zelazowska *et al.*, 2007). Both species have been found on cultivated *P. ostreatus* and substrates on which the mushroom is grown in Europe, Iran and South Korea, but *T. pleuroticola* has also been isolated from soil and wood in Canada, the United States, Europe, Iran, New Zealand (Park *et al.*, 2004a–c, 2006; Komoń-Zelazowska *et al.*, 2007) and Hungary (Szekeres *et al.*, 2005). It is not yet known whether these species also occur in association with *P. ostreatus* in natural environments.

The objectives of this study were to develop a multiplex PCR assay for the rapid and specific detection of *T. pleuroticola* and *T. pleurotum* and to test for the

occurrence of these two species in substrates used for the cultivation of oyster mushroom, as well as on wood colonized by *P. ostreatus* and on the surface of basidiomes of the mushroom in forested areas in Hungary.

Materials and methods

Fungal strains

All fungal strains used in this study are deposited in the culture collections of the Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary, and the Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria. The strains of *Trichoderma* and other ascomycetes tested with the PCR assay are listed in Table 1. All *T. pleuroticola* and *T. pleurotum* strains in Table 1 were identified previously based on sequence analysis of internal transcribed spacer (ITS)1, ITS2 and translation elongation factor 1α (*tef1*) (Komoń-Zelazowska *et al.*, 2007).

DNA extraction from fungal cultures and cultivation substrates of *P. ostreatus*

Fungal cultures were all grown on a solid MEX medium (30 g L^{-1} malt extract and 20 g L^{-1} agar in distilled water) covered with a cellophane membrane for 1–2 days at 25 °C, from which 70–80 mg mycelium was harvested. After freezing in liquid nitrogen (30 s), mycelia were disrupted in Eppendorf tubes containing glass beads using a TissueLyser RETSCH MM 301 (Retsch GmbH, Haan, Gemany) for 1 min. The freezing and disruption procedure was performed twice. DNA extraction was carried out using the Qiagen DNeasy Plant Mini Kit or the Sigma GenElute™ Plant Genomic DNA Miniprep Kit according to the protocols provided by the manufacturers (Qiagen Vertriebs GmbH, Vienna, Austria; Sigma-Aldrich, Budapest, Hungary). Noninfested and *Trichoderma*-infested substrate samples for cultivation of *P. ostreatus* were each disrupted with a pestle in a mortar filled with liquid nitrogen, which was followed by DNA extraction as described above. DNA extracts from fungal cultures were diluted 1 : 100, while those from oyster mushroom substrates were diluted 1 : 10 with double-distilled water for PCR amplification.

Primer design and validation

DNA sequence alignments of the ITS region (ITS1–5.8S rRNA gene–ITS2) and parts of the *tef1* and endochitinase (*chi18-5*) genes for *T. pleurotum*, *T. pleuroticola* and various isolates of *Trichoderma harzianum, Trichoderma aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum* were screened to identify hallmark sequences appropriate for the development of primers specific for *T. pleurotum* and *T. pleuroticola*.

PCR assays specific for the two target fungi were performed in a final volume of 21 μL, containing 95 mM 5 × Green GoTaq™ reaction buffer, 0.38 mM dNTP mix, 3.57 mM $MgCl_2$, 0.8 U GoTaq™ DNA polymerase (all from Promega GmbH, Mannheim, Germany), 190, 71 and 190 nM of primers FPforw1, FPrev1 and PSrev1, respectively (see Table 2), 0.5 μL double-distilled water and 2 μL template DNA. For each set of samples assayed, a negative control sample with 2 μL double-distilled water substituting for the template DNA was included. *Trichoderma pleurotum* strain A8 and *T. pleuroticola* strain A37 were used as positive control strains; they were identified previously based on ITS and *tef1* sequences that proved to be identical to those of the type strains *T. pleurotum* CBS 121147 and *T. pleuroticola* CBS 121144, respectively (Komoń-Zelazowska *et al.*, 2007; Table 1). Amplification was performed in a Bio-Rad iCycler (Bio-Rad Laboratories, Vienna, Austria) as follows: one cycle at 94 °C for 2 min, 35 cycles at 94 °C for 10 s and 68 °C for 20 s, and one cycle at 72 °C for 30 s. PCR products were subjected to electrophoresis at 80 V for 30 min in a 1.5% agarose gel prepared in TAE buffer (4.84 g L^{-1} Tris base, 1.142 mL L^{-1} glacial acetic acid, 2 mL L^{-1} 0.5 M EDTA, pH 8.0, in distilled water, with the pH then adjusted to 8.5) containing 200 ng mL^{-1} ethidium bromide. The electrophoresis buffer was the same TAE buffer. A GeneRuler™ 1-kb DNA Ladder (Fermentas GmbH, St. Leon-Rot, Germany) was used as a standard with each gel to assess the sizes of the amplicons. DNA was visualized by UV illumination and photographed using a Bio-Rad Gel Doc 2000 device (Bio-Rad Laboratories).

The specificity of the primers was tested with 13 strains of *T. pleurotum* and 17 strains of *T. pleuroticola*, including the type strains of each species (Table 1). To ensure that the primers do not cross-react with other *Trichoderma* spp. or other fungi, DNA extracts of 28 other *Trichoderma* species as well as 12 other fungal species were also tested (Table 1). The strains of *P. ostreatus*, *Penicillium expansum* Link, *Aspergillus* sp., *Mortierella* sp. and *Thermomyces* sp. were obtained from the mushroom farm in Hungary where *T. pleuroticola* and *T. pleurotum* strains had previously been isolated from infested substrate samples (Hatvani *et al.*, 2007).

Isolation of *Trichoderma* strains from the substrates and the surface of wild *P. ostreatus* mushrooms

Basidiomes as well as the natural substrate of *P. ostreatus* were collected from five Hungarian forests (Table 3). Natural substrate samples were taken from wood beside the basidiomes and dispersed directly or inoculated from

Table 1. List of fungal isolates involved in testing of primers specific for *Trichoderma pleuroticola* and *Trichoderma pleurotum*, causal agents of green mold of the oyster mushroom (*Pleurotus ostreatus*)

Species	Strain number*	Origin
T. pleurotum S.H. Yu & M.S. Park	*A1* (C.P.K. 2095), *A8* (C.P.K. 2096), *A11* (C.P.K. 2097), *A16* (C.P.K. 2098), *A25* (C.P.K. 2100), *A28* (C.P.K. 2103), *C4* (C.P.K. 2109), *C5* (C.P.K. 2110), *C14* (C.P.K. 2112), ***C15*** **(C.P.K. 2113, CBS 121147, DAOM 236051)**, *C21* (C.P.K. 2114), *C25* (C.P.K. 2116, CBS 121148), *C27* (C.P.K. 2117)	Substrate of cultivated *P. ostreatus*, Hungary
T. pleuroticola S.H. Yu & M.S. Park	*A37* (C.P.K. 2104, CBS 121145)	Substrate of cultivated *P. ostreatus*, Hungary
	C.P.K. 230 (DAOM 175924, CBS 121144)	Canada
	C.P.K. 882 (CBS 121146)	Iran
	C.P.K. 1401 (DAOM 175924, CBS 628.77)	The Netherlands
	C.P.K. 1540 (CBS 121217) to C.P.K. 1551	From *Pleurotus*, Italy
	C.P.K. 1715 (G.J.S. 04-01)	Biocontrol agent of *Cercospora* in sugar beet, Montana
T. harzianum Rifai	C.P.K. 7 (CBS 960.68)	Unknown
T. aggressivum f. *europaeum* Samuels & W. Gams	C.P.K. 361 (IMI 359824)	United Kingdom
T. aggressivum f. *aggressivum* Samuels & W. Gams	C.P.K. 366 (CBS 435.95)	Mushroom compost, BC, Canada
T. minutisporum Bissett	C.P.K. 22 (G.J.S. 95-216)	Unknown
T. crassum Bissett	C.P.K. 63 (CBS 336.93, DAOM 164916)	Soil under *Picea excelsa*, QC, Canada
T. oblongisporum Bissett	C.P.K. 93 (CBS 343.93)	Wood of *Thuja plicata*, BC, Canada
T. tomentosum Bissett	C.P.K. 97 (CBS 349.93)	Material under bark of *Ulmus* sp., ON, Canada
T. rossicum Bissett, C.P. Kubicek & Szakacs	C.P.K. 223 (DAOM 230008)	Cultivated soil, Krasnoyarsk region, Siberia, Russia
T. fertile Bissett	C.P.K. 232 (DAOM 167161)	Unknown
T. cerinum Bissett, C.P. Kubicek & Szakacs	C.P.K. 293 (DAOM 230012, T.U.B. F-778)	Soil, Annapurna Himal, Nepal
T. velutinum Bissett, C.P. Kubicek & Szakacs	C.P.K. 298 (DAOM 230013, T.U.B. F-784)	Forest soil, Annapurna Himal, Nepal
T. polysporum (Link) Rifai	C.P.K. 462 (G.J.S. 99-159)	Bark, NSW, Australia
T. helicum Bissett, C.P. Kubicek & Szakacs	C.P.K. 414 (DAOM 230016)	Soil near seashore, Malaysia
T. spirale Bissett	C.P.K. 679 (T.U.B. F-825)	Rio de Janeiro, Brazil
T. virens (J.H. Mill., Giddens & A.A. Foster) Arx	C.P.K. 2141 (C.N.R.A. 146)	Rhizosphere of *Theobroma cacao* plantation, Ivory Coast
T. brevicompactum G.F. Kraus, C.P. Kubicek & W. Gams	C.P.K. 1580	Iran
T. hamatum (Bonord.) Bainier	C.P.K. 16	Soil, Florida
T. atroviride Bissett	C.P.K. 626 (T.U.B. F-337)	Soil near seashore, Jamaica
T. asperellum Samuels, Lieckf. & Nirenberg	C.P.K. 674 (T.U.B. F-756)	Brazil
T. viride Pers.	C.P.K. 625 (T.U.B. F-371)	Soil, castle park, Osaka, Japan
T. koningii Oudem.	C.P.K. 1370 (G.J.S. 90-18)	Wisconsin
T. viridescens (A.S. Horne & H.S. Will.) Jaklitsch & Samuels	C.P.K. 2069 (U.N.I.S.S. 3-76 STS)	Soil, Sardinia, Italy
T. gamsii Samuels & Druzhin.	C.P.K. 2070 (U.N.I.S.S., 4-102)	Soil, Sardinia, Italy
T. koningiopsis Samuels, C. Suárez & H.C. Evans	C.P.K. 1813 (PPRC J7)	Soil, Jimma, Ethiopia
T. aureoviride Rifai	C.P.K. 10 (ICMP 3090)	Unknown
T. longibrachiatum Rifai	C.P.K. 45 (IMI 297702)	Unknown
T. citrinoviride Bissett	C.P.K. 343 (T.U.B. F-706)	Soil, Boston
T. ghanense Yoshim. Doi, Y. Abe & Sugiy.	C.P.K. 1255 (NRRL 3091)	Unknown
Fusarium poae (Peck) Wollenw.	C.P.K. 2786 (CBS 115696)	*Triticum aestivum*, Zulawy region, Poland
F. graminearum Schwabe	C.P.K. 1117	Unknown
F. oxysporum E.F. Sm. & Swingle	C.P.K. 1842 (PPRC H6)	Soil, Harerga, Ethiopia
F. culmorum (W.G. Sm.) Sacc.	C.P.K. 2747	Wheat, Austria
F. sporotrichioides Sherb.	C.P.K. 2787 (CBS 115700)	*Fagopyrum esculentum*, Warmia region, Poland

Table 1. Continued.

Species	Strain number*	Origin
Penicillium expansum Link	Sz.M.C. FAM-1, Sz.M.C. FAM-3	Straw used for preparing *Pleurotus* substrate, Hungary
Aspergillus niger Tiegh.	Sz.M.C. 608	Brazil
Aspergillus sp.	Sz.M.C. FDM-5	Straw used for preparing *Pleurotus* substrate, Hungary
Mucor circinelloides Tiegh.	Sz.M.C. 12028	Unknown
Mortierella sp.	Sz.M.C. FDM-7	Straw used for preparing *Pleurotus* substrate, Hungary
Thermomyces sp.	Sz.M.C. FAT-1	Straw used for preparing *Pleurotus* substrate, Hungary
Pleurotus ostreatus (Jacq.) P. Kumm.	Sz.M.C. B7	Mushroom farm, Hungary

*Strain numbers in parentheses are cross-reference numbers of the respective isolates. Type strains of *Trichoderma pleurotum* and *Trichoderma pleuroticola* are indicated in bold font. Strain numbers in italic font refer to isolates from the study of Hatvani *et al.* (2007).
CBS, Centraalbureau vor Schimmelcultures, Utrecht, the Netherlands; C.N.R.A., culture collection of Centre National de Recherche Agronomique, Abidjan, Ivory Coast; C.P.K., culture collection of Christian P. Kubicek, Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria; DAOM, Canadian Collection of Fungal Cultures, Ottawa, Canada; G.J.S., culture collection of Gary J. Samuels, Systematic Mycology and Microbiology, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Beltsville, MD; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; IMI, culture collection of CABI Bioscience UK Centre, Egham, Surrey, UK; NRRL, USDA ARS Culture Collection, Peoria, IL; PPRC, culture collection of the Plant Protection Research Centre, Ambo, Ethiopia; Sz.M.C., culture collection of the University of Szeged, Szeged, Hungary; T.U.B., culture collection of the Technical University of Budapest, Budapest, Hungary; U.N.I.S.S., culture collection of the Università degli Studi di Sassari, Sassari, Italy.

Table 2. Data on *tef1* sequence-based PCR primers designed in this study for the specific detection of *Trichoderma pleurotum* and *Trichoderma pleuroticola*, causal agents of green mold of the oyster mushroom (*Pleurotus ostreatus*)

Primer	Specificity	Sequence	Length in nucleotides	Melting point (T_m) in °C	Amplicon size with FPforw1 in base pairs (bp)
FPforw1	*T. pleurotum* and *T. pleuroticola*	5′-CACATTCAATTGTGCCCGACGA-3′	22	58.22	–
FPrev1	*T. pleurotum* and *T. pleuroticola*	5′-ACCTGTTAGCACCAGCTCGC-3′	20	59.21	447
PSrev1	*T. pleurotum*	5′-GCGACACAGAGCACGTTGAATC-3′	22	58.89	218

suspensions (1 g in 100 mL sterile distilled water) onto a solid yeast extract–glucose (YEG) medium (5 g L^{-1} glucose, 1 g L^{-1} yeast extract, 5 g L^{-1} KH_2PO_4 and 20 g L^{-1} agar in distilled water supplemented with 0.1 g L^{-1} streptomycin and 0.1 g L^{-1} chloramphenicol) for strain isolation. Substrate samples were taken only if the corresponding basidiomes were also observed. Basidiomes were simply picked from the growing substrate. Pieces of hats weighting 1 g were washed in 100 mL sterile distilled water in order to obtain suspensions, and inoculations were performed from the suspensions and by placing the pieces directly onto the medium described above and incubated at 25 °C. After the appearance of conidiating fungal colonies, a conidial suspension was prepared in distilled water for each sample, diluted and plated on YEG agar medium. Agar plugs were then cut from colonies of *Trichoderma* and transferred to new plates of YEG agar. *Trichoderma* isolates derived from the natural *P. ostreatus* substrates as well as the surface of wild oyster mushrooms are listed in Table 3.

Molecular identification of *Trichoderma* species

PCR amplification of the ITS1 region and a 0.7-kb fragment of *tef1* containing the fourth and fifth introns of the original *Trichoderma* isolates and of strains isolated from infested straw samples, and the amplicon purification steps were carried out as described previously (Jaklitsch *et al.*, 2006; Hatvani *et al.*, 2007). Sequencing of the PCR products was performed at Eurofins MWG Operon (Ebersberg, Germany). Sequence analysis of the ITS and *tef1* amplicons was performed with the aid of the TRICHOKEY 2.0 (Druzhinina *et al.*, 2005; Druzhinina & Kopchinskiy, 2006) and TRICHOBLAST (Kopchinskiy *et al.*, 2005) tools available online at http://www.isth.info/.

Table 3. Origin of samples and identification of *Trichoderma* strains isolated from the substrate and the basidiomes of wild-grown *Pleurotus ostreatus* in Hungary

Fungal strain numbers	Specific PCR*	ITS sequence-based identity (GenBank accession number)	*tef1* sequence-based identity (GenBank accession number)
Site 1: *Populus alba* stump, oak-silver lime forest (*Convallario–Quercetum roboris*), Kisújszállás, Hungary			
C.P.K. 3193 to C.P.K. 3197	*T. pleuroticola*	*T. pleuroticola* (EU918140†)	*T. pleuroticola* (EU918160)
C.P.K. 3198 to C.P.K. 3220	*T. pleuroticola*	ND	ND
Site 2: *Populus alba* stump, oak–ash–elm open woodland (*Fraxino pannonicae–Ulmetum*), Tőserdő, Hungary			
C.P.K. 3247 to C.P.K. 3249, C.P.K.3251 to C.P.K. 3253, C.P.K.3258	Negative	*T. harzianum/Hypocrea lixii* (EU918151)	ND
C.P.K. 3250, C.P.K. 3254 to C.P.K. 3257	Negative	*T. harzianum/H. lixii* (EU918149, EU918151)	*T. harzianum* (EU918162–EU918164)
Site 3: *Populus alba* stump, planted poplar forest (*Populetum cult.*), Kecskemét, Nyomási forest, Hungary			
3/A: Surface of basidiome			
C.P.K. 2884, C.P.K. 2888 to C.P.K. 2890, C.P.K. 2898	Negative	*T. longibrachiatum/Hypocrea orientalis* (EU918139)	*T. longibrachiatum* (EU918159)
C.P.K. 2903	Negative	ND	*T. longibrachiatum* (EU918159)
C.P.K. 2885, C.P.K. 2891, C.P.K. 2897, C.P.K. 2899, C.P.K. 2901	*T. pleuroticola*	*T. pleuroticola* (EU918141, EU918143, EU918147, EU918148)	*T. pleuroticola* (EU918160, EU918161)
C.P.K. 2886, C.P.K. 2887, C.P.K. 2894 to C.P.K. 2896, C.P.K. 2900, C.P.K. 2902	*T. pleuroticola*	*T. pleuroticola* (EU918142, EU918144 to EU918146, EU918148)	ND
C.P.K. 2892, C.P.K. 2893	*T. pleuroticola*	ND	ND
3/B: Substrate			
C.P.K. 3271, C.P.K. 3273, C.P.K. 3274, C.P.K. 3276, C.P.K. 3278 to C.P.K. 3281	Negative	*T. longibrachiatum/H. orientalis* (EU918139)	*T. longibrachiatum* (EU918159)
C.P.K. 3275	Negative	*T. longibrachiatum/H. orientalis* (EU918138)	ND
C.P.K. 3272	*T. pleuroticola*	*T. pleuroticola* (EU918148)	*T. pleuroticola* (EU918160)
C.P.K. 3277	Negative	*T. atroviride/H. atroviridis* (EU918133)	*T. atroviride* (EU918154)
Site 4: *Populus canadensis* stump, along the road, Kecskemét, Hunyadiváros, Hungary			
C.P.K. 3259, C.P.K. 3263, C.P.K. 3264, C.P.K. 3269, C.P.K. 3288	Negative	*T. harzianum/H. lixii* (EU918150, EU918152)	*T. harzianum* (EU918165, EU918166)
C.P.K. 3260, C.P.K. 3262, C.P.K. 3265, C.P.K. 3267, C.P.K. 3268, C.P.K. 3282, C.P.K. 3285 to C.P.K. 3287	Negative	*T. harzianum/H. lixii* (EU918150, EU918152, EU918153)	ND
C.P.K. 3261, C.P.K. 3266, C.P.K. 3270, C.P.K. 3283, C.P.K. 3284	*T. pleuroticola*	*T. pleuroticola* (EU918148)	*T. pleuroticola* (EU918160)
Sample 5: *Tilia* sp. stump, inside the city of Szeged, Hungary			
5/A: Surface of basidiome			
No *Trichoderma* found.			
5/B: Substrate			
C.P.K. 3375, C.P.K. 3376, C.P.K. 3378, C.P.K. 3379, C.P.K. 3381, C.P.K. 3387, C.P.K. 3391 to C.P.K. 3394	Negative	ND	*T. harzianum* (EU918165–EU918169)
C.P.K. 3377, C.P.K. 3380, C.P.K. 3382 to C.P.K. 3386, C.P.K. 3388 to C.P.K. 3390	Negative	*T. atroviride/H. atroviridis* (EU918134–EU918137)	*T. atroviride* (EU918155–EU918158)

*Result of the specific multiplex PCR assay developed in this study.
†Identical accession numbers refer to identical sequences.
ND, not determined.

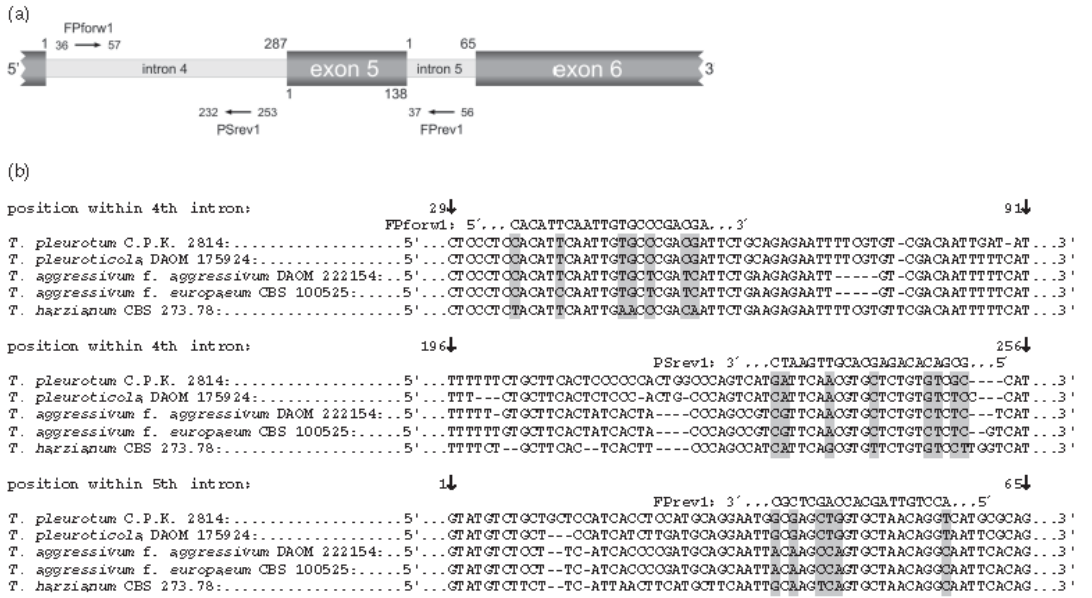


Fig. 1. Binding sites of primers FPforw1, FPrev1 and PSrev1, used in a multiplex PCR assay for detection of two *Trichoderma* spp. pathogenic to *Pleurotus ostreatus*. (a) Schematic presentation of the *tef1* fragment of the target fungi with the primer-binding sites. Numbers indicate nucleotide positions within the introns. (b) Alignment of sequences within the fourth and the fifth introns of the *tef1* gene that were used for primer design. Positions variable for *Trichoderma pleurotum*, *Trichoderma pleuroticola* and related *Trichoderma* species are shaded in gray within the sequences corresponding to the specific primers. The aligned sequences were derived from GenBank (accession numbers: EF601679, AY605769, AY605798, AF348095 and AF348099 for strains C.P.K. 2814, DAOM 175924, DAOM 222154, CBS 100525 and CBS 273.78, respectively).

Results

Design of PCR primers selectively identifying *T. pleurotum* and *T. pleuroticola*

Three areas of *tef1* (Fig. 1a) were identified for development of specific primers FPforw1, FPrew1 and Psrev1 (Table 2), for amplification of a 447-bp fragment from both *T. pleurotum* and *T. pleuroticola* and a 218-bp product specific for *T. pleurotum* (Fig. 1b). PCR assays with these primers produced two major bands for all isolates of *T. pleurotum* evaluated, while only the larger fragment was formed from the DNA extracts of the *T. pleuroticola* strains (Fig. 2a and b). No cross-reaction was observed with the DNA extracts of 28 other *Trichoderma* species and 12 other fungal species (Table 1) (data not shown). Based on these results, the multiplex PCR assay is specific for the detection and identification of the two *Trichoderma* spp. pathogenic to *P. ostreatus*.

Identification of *T. pleuroticola* and *T. pleurotum* directly from substrates used for cultivation of *P. ostreatus*

The primers developed in this study were also tested for the ability to detect the mushroom pathogens directly in the substrates on which oyster mushrooms are cultivated. Samples of straw colonized by *Pleurotus* that was noninfested or infested with green mold were tested. DNA was isolated from the samples as described in Materials and methods and the primers FPforw1, FPrev1 and PSrev1 were used to amplify *tef1* fragment(s) by PCR assay for any *T. pleuroticola* and *T. pleurotum* strains present in the samples. No amplicons of *T. pleurotum* or *T. pleuroticola* were obtained from noninfested straw, but the PCR assay yielded the two bands characteristic of *T. pleurotum* for all samples of infested straw assayed (PSAII/3 and PSAII/4; Fig. 3). This indicated the presence of *T. pleurotum* in the infested substrates, which was confirmed by subsequent ITS sequence analysis of the *Trichoderma* strains isolated from the samples (data not shown).

Identification of *Trichoderma* spp. isolated from natural substrates and from basidiocarps of *P. ostreatus*

A total of 110 *Trichoderma* strains were isolated from the substrates (90 isolates) and basidiomes (20 isolates) of wild *P. ostreatus* growing in five sites in Hungary (Table 3). For Site 1, an oak-silver lime forest, all 28 isolates were identified as *T. pleuroticola*. However, it was not detected in Site 2 (15

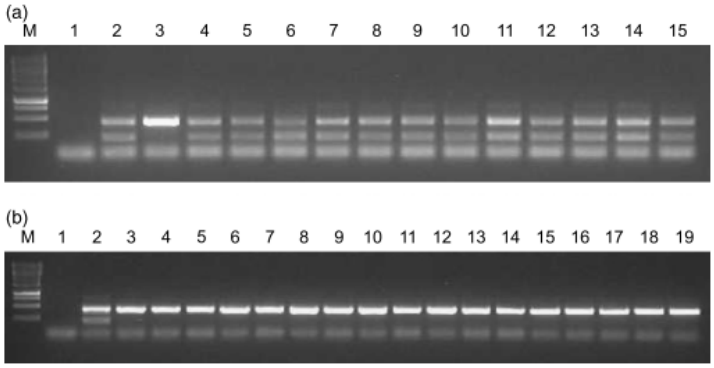


Fig. 2. DNA fragments amplified in a multiplex PCR using primers specific for *Trichoderma pleurotum* and *Trichoderma pleuroticola*. (a) DNA fragments amplified from 13 *T. pleurotum* isolates. Lane M, GeneRuler™ 1-kb DNA Ladder (Fermentas GmbH); lane 1, negative control (no template DNA); lanes 2 and 3, *T. pleurotum* strain A8 and *T. pleuroticola* strain A37, respectively (positive controls); and lanes 4–15, *T. pleurotum* strains A1, A11, A16, A25, A28, C4, C5, C14, **CBS 121147**, C21, C25 and C27, respectively. (b) DNA fragments amplified from 17 *T. pleuroticola* isolates. Lane M, GeneRuler™ 1-kb DNA Ladder; lane 1, negative control (no template DNA); lanes 2 and 3, *T. pleurotum* strain A8 and *T. pleuroticola* strain A37, respectively (positive controls); and lanes 4–19, *T. pleuroticola* strains **CBS 121144**, C.P.K. 882, C.P.K. 1401, C.P.K. 1540, C.P.K. 1541, C.P.K. 1542, C.P.K. 1543, C.P.K. 1544, C.P.K. 1545, C.P.K. 1546, C.P.K. 1547, C.P.K. 1548, C.P.K. 1549, C.P.K. 1550, C.P.K. 1551 and C.P.K. 1715, respectively. Type strains are indicated in bold font.

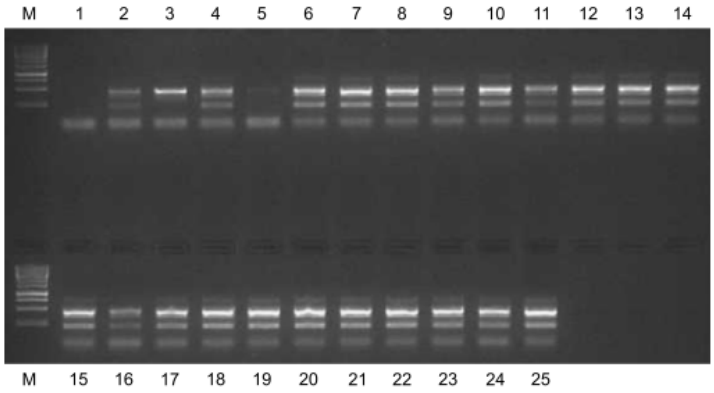


Fig. 3. DNA amplicons detected using primers specific for *Trichoderma pleurotum* and *Trichoderma pleuroticola* in the multiplex PCR assay with DNA extracted directly from green mold-infested wheat straw substrates and from *Trichoderma* strains isolated from these substrates. Lane M, GeneRuler™ 1-kb DNA Ladder (Fermentas GmbH); lane 1, negative control (no template DNA); lanes 2 and 3, *T. pleurotum* strain A8 and *T. pleuroticola* strain A37, respectively (positive controls); lane 4, DNA from green mold-infested substrate PSAII/3; lanes 5–15, DNA from *Trichoderma* strains isolated from substrate PSAII/3; lane 16, DNA from green mold infested substrate PSAII/4; and lanes 17–25, DNA from *Trichoderma* strains isolated from substrate PSAII/4.

isolates), an oak–ash–elm open woodland (*Fraxino pannonicae–Ulmetum*), or in Site 5 from the city of Szeged (20 isolates), whereas it was identified for five of the 19 isolates obtained from Site 4, and a single isolate of 11 found at Site 3B. *Trichoderma pleurotum* was not detected from any of the five sites.

Sequence analyses of the ITS region and/or *tef1* were used to identify the other *Trichoderma* spp. detected from Sites 1–5. The isolates included *T. harzianum* Rifai, *Trichoderma longibrachiatum* Rifai and *Trichoderma atroviride* Bissett exclusively. *Trichoderma harzianum* was the exclusive species found in Site 2 samples, dominated Site 4 (14 of 19 isolates) and made up 50% of the isolates (10 of 20) in Site 5B. *Trichoderma longibrachiatum* was present only in Site 3B, where it appeared to be the dominant species (nine of 11 isolates). *Trichoderma atroviride* was found only in Site 3B (one of 11 isolates) and Site 5, where this species accounted for 50% of the isolates (10 of 20). *Trichoderma* spp. were not

found on *P. ostreatus* basidiomes from Site 5. However, *T. pleuroticola* was the dominant species in Site 3A samples (14 of 20 isolates), while the remaining six isolates from that site were all identified as *T. longibrachiatum*.

Discussion

Fungal pathogens are an emerging problem in mushroom farms, where they can limit mushroom quality and yield. Therefore, there is an increasing need for rapid methods of detecting the pathogens in order to efficiently control the diseases caused by them. Zijlstra *et al.* (2008) used a TaqMan PCR assay for the timely detection of *Verticillium fungicola* var. *aleophilum* W. Gams & Zaayen and *V. fungicola* var. *fungicola* (Preuss) Hassebr., the causal agents of dry bubble disease of *Agaricus bisporus* (J.E. Lange) Imbach in North America and Europe, respectively. Chen *et al.* (1999a, b) described a PCR assay for the identification of *T. harzianum* biotypes 2 and 4, which were later described as *T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum* Samuels & W. Gams, respectively (Samuels *et al.*, 2002), responsible for green mold epidemics in cultivated *A. bisporus* worldwide. This PCR assay has been applied for the comparison of *Trichoderma* strains sampled in the United States during and before the outbreak of the green mold epidemic (Chen *et al.*, 1999a, b), which revealed no evidence of the pre-epidemic existence of *T. aggressivum* f. *aggressivum*, suggesting the recent emergence of a highly virulent genotype. The assay was successfully adopted by Hatvani *et al.* (2007), who identified the causal agent of green mold of cultivated *A. bisporus* in Hungary as *T. aggressivum* f. *europaeum*.

In order to facilitate identification of the two *Trichoderma* spp. pathogenic to *P. ostreatus*, three oligonucleotide primers were developed in this study to identify *T. pleurotum* and *T. pleuroticola* using a multiplex PCR assay. The results demonstrated that these two species can be distinguished from each other, as well as from other fungal species using this assay. The assay was tested with DNA extracted directly from noninfested and *Trichoderma*-infested substrates of cultivated oyster mushroom. PCR amplicons were not detected from noninfested substrates, but DNA extracts from the infested substrates produced the two DNA bands characteristic of *T. pleurotum*. However, the design of the assay was such that the two DNA bands could reflect the presence of either *T. pleurotum* alone or the presence of both *T. pleuroticola* and *T. pleurotum*, i.e. the assay does not differentiate whether the substrate contains only *T. pleurotum* or both species. Therefore, the identity as *T. pleurotum* was confirmed by subsequent ITS sequence analysis. The data show that the primers designed in this study are able to detect the two *Trichoderma* spp. pathogenic to *P. ostreatus* without the need to isolate and culture the pathogens. This assay may help one to identify the presence of green mold disease of *P. ostreatus* caused by *T. pleurotum* and *T. pleuroticola* in the early phases of infection, facilitating the early application of appropriate disease control strategies. In order to prevent contamination from spreading, the application of calcium hydroxide onto the affected area, and the use of fungicides benomyl, thiabendazole and prochloraz are suggested (Won, 2000; Yu, 2002).

The presence and potential damage from *T. pleurotum* and *T. pleuroticola* (Park *et al.*, 2006) in the cultivation of *P. ostreatus* is well known (Yu, 2002; Park *et al.*, 2004a–c, 2005; Woo *et al.*, 2004; Kredics *et al.*, 2006; Hatvani *et al.*, 2007, 2008; Komoń-Zelazowska *et al.*, 2007). However, to the best of our knowledge, the association of these pathogens with oyster mushroom growing in the wild has not been reported previously. Therefore, the presence of these species was tested on wood substrates colonized by wild *P. ostreatus*, as well as on the surface of the mushrooms growing in forested areas in Hungary. The PCR assay developed in this study has revealed that *T. pleuroticola* can be present in both the natural substrates of *P. ostreatus* and on the basidiomes of the mushrooms in the wild.

Szekeres *et al.* (2005) examined the genetic diversity of *Trichoderma* species in the rhizosphere of winter wheat in Hungary and found that approximately 5% of the *Trichoderma* isolates belonged to the species *T. pleuroticola*. In contrast, this study has shown that *T. pleuroticola* may be present at a higher proportion of the *Trichoderma* isolates found in the substrate and on the basidiomes of wild oyster mushrooms. The presence of this species in these habitats suggests that these might be potential sources of infections for mushroom farms. In contrast, *T. pleurotum* was not detected in any of the samples from Hungary examined in this study. Similar to *T. aggressivum*, *T. pleurotum* has never been found in naturally occurring substrates yet. Recent metagenomic studies on the occurrence of *Trichoderma* species in Austrian soils revealed the common presence of *T. pleuroticola*, but never that of *T. pleurotum* (M.A. Friedl & I.S. Druzhinina, unpublished data), suggesting that these two species may occupy different ecological and trophic niches in nature (Komoń-Zelazowska *et al.*, 2007).

The diversity of *Trichoderma* species found in the vicinity of wild *P. ostreatus* was also evaluated in this study. Besides *T. pleuroticola*, other species detected were *T. harzianum*, *T. longibrachiatum* and *T. atroviride*. Hatvani *et al.* (2007) examined *Trichoderma* strains isolated from the substrate used for oyster mushroom cultivation in Hungary. Among 31 strains, *T. pleurotum* was the most prevalent (27 strains). In addition, single isolates of *T. pleuroticola*, *T. atroviride*, *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg and *T. longibrachiatum* were found. However, in contrast to samples of wild *P. ostreatus*, *T. harzianum* was not detected.

The primer set and PCR protocol developed in this study allow for the rapid detection and differentiation of the two

major *Trichoderma* pathogens of oyster mushroom. The ability to detect and track these pathogens could lead to better integrated management tools to reduce losses to green mold in commercial mushroom production.

Acknowledgements

This study was supported by grants OTKA F68381, FWF P-16601, P-17895-B06 and P-19340, and by the János Bolyai Research Scholarship.

References

Anonymous (2005) Consensus document on the biology of *Pleurotus* spp. *Series of Harmonization of Regulatory Oversight in Biotechnology No. 34 OECD Environment, Health and Safety Publications*. Head of Publications Service, OECD, France.

Ballero M, Mascia E, Rescigno A & Teulada ESD (1990) Use of *Pleurotus* for transformation of polyphenols in waste waters from olive presses into proteins. *Micol Ital* **19**: 39–41.

Chang R (1996) Functional properties of edible mushrooms. *Nutr Rev* **54**: 91–93.

Chen X, Romaine CP, Ospina-Giraldo MD & Royse DJ (1999a) A polymerase chain reaction-based test for the identification of *Trichoderma harzianum* biotypes 2 and 4, responsible for the worldwide green mold epidemic in cultivated *Agaricus bisporus*. *Appl Microbiol Biot* **52**: 246–250.

Chen X, Romaine CP, Tan Q, Schlagnhaufer B, Ospina-Giraldo MD, Royse DJ & Huff DR (1999b) PCR-based genotyping of epidemic and preepidemic *Trichoderma* isolates associated with green mold of *Agaricus bisporus*. *Appl Environ Microb* **65**: 2674–2678.

Druzhinina IS & Kopchinskiy AG (2006) *Trich*OKEY v. 2 – a DNA oligonucliotide BarCode program for the identification of multiple sequences of *Hypocrea* and *Trichoderma*. *Proceedings of the 8th International Mycological Congress, Cairns, Australia*, p. 37.

Druzhinina IS, Kopchinskiy AG, Komoń M, Bissett J, Szakács G & Kubicek CP (2005) An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet Biol* **42**: 813–828.

Gunde-Cimerman N (1999) Medicinal value of the genus *Pleurotus* (Fr.) P. Karst. (*Agaricales* s.l. Basidiomycetes). *Int J Med Mushrooms* **1**: 69–80.

Hatvani L, Antal Z, Manczinger L, Szekeres A, Druzhinina IS, Kubicek CP, Nagy A, Nagy E, Vágvölgyi C & Kredics L (2007) Green mold diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderma* species. *Phytopathology* **97**: 532–537.

Hatvani L, Manczinger L, Kocsubé S, Antal Z, Szekeres A, Druzhinina IS, Kubicek CP, Nagy A, Vágvölgyi C & Kredics L (2008) The green mould disease global threat towards the cultivation of oyster mushroom (*Pleurotus ostreatus*): a review. *Science and Cultivation of Edible and Medicinal Fungi: Mushroom Science XVII. Proceedings of the 17th Congress of the International Society for Mushroom Science, Cape Town, South Africa, May 20–24, 2008. CD-ROM* (van Gruening M, ed), pp. 485–495. South African Mushroom Farmers Association, Pretoria, South Africa.

Jaklitsch WM, Komoń M, Kubicek CP & Druzhinina IS (2006) *Hypocrea crystalligena* sp. nov., a common European species with a white-spored *Trichoderma* anamorph. *Mycologia* **98**: 500–514.

Komoń-Zelazowska M, Bissett J, Zafari D, Hatvani L, Manczinger L, Woo S, Lorito M, Kredics L, Kubicek CP & Druzhinina IS (2007) Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms. *Appl Environ Microb* **73**: 7416–7426.

Kopchinskiy A, Komoń M, Kubicek CP & Druzhinina IS (2005) *Trich*oBLAST: a multilocus database for *Trichoderma* and *Hypocrea* identifications. *Mycol Res* **109**: 657–660.

Kredics L, Hatvani L, Antal Z, Manczinger L, Druzhinina IS, Kubicek CP, Szekeres A, Nagy A, Vágvölgyi C & Nagy E (2006) Green mould disease of oyster mushroom in Hungary and Transylvania. *Acta Microbiol Immunol Hung* **53**: 306–307.

Marzullo L, Cannio R, Giardina P, Santini MT & Sannia G (1995) Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and prevents polymerization of laccase-oxidized substrates. *J Biol Chem* **270**: 3823–3827.

Pani BK, Panda SN & Das SR (1997) Utilization of some agricultural by-products and other wastes for sporophore production of oyster mushroom. *Orissa J Hortic* **25**: 36–39.

Park MS, Bae KS & Yu SH (2004a) Molecular and morphological analysis of *Trichoderma* isolates associated with green mold epidemic of oyster mushroom in Korea. *J Huazhong Agric Univ* **23**: 157–164.

Park MS, Bae KS & Yu SH (2004b) Morphological and molecular analysis of *Trichoderma* species associated with green mold epidemic of oyster mushroom in Korea. Available at http://www.mushworld.com (accessed October 11, 2004).

Park MS, Bae KS & Yu SH (2004c) Morphological and molecular analysis of *Trichoderma* species associated with green mold epidemic of oyster mushroom in Korea. *New Challenges in Mushroom Science. Proceedings of the 3rd Meeting of Far East Asia for Collaboration on Edible Fungi Research*, pp. 143–158, Suwon, Korea.

Park MS, Seo GS, Lee KH, Bae KS & Yu SH (2005) Characterization of *Trichoderma* spp. associated with green mold of oyster mushroom by PCR-RFLP and sequence analysis of ITS regions of rDNA. *Plant Pathol J* **21**: 229–236.

Park MS, Bae KS & Yu SH (2006) Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. *Mycobiology* **34**: 111–113.

Puniya AK, Shah KG, Hire SA, Ahire RN, Rathod MP & Mali RS (1996) Bioreactor for solid-state fermentation of agro-industrial wastes. *Indian J Microbiol* **36**: 177–178.

Samuels GJ, Dodd SL, Gams W, Castlebury LA & Petrini O (2002) *Trichoderma* species associated with the green mold epidemic

of commercially grown *Agaricus bisporus*. *Mycologia* **94**: 146–170.

Sharma SR & Vijay B (1996) Yield loss in *Pleurotus ostreatus* caused by *Trichoderma viride*. *Mushroom Res* **5**: 19–22.

Szekeres A, Kredics L, Antal Z, Hatvani L, Manczinger L & Vágvölgyi C (2005) Genetic diversity of *Trichoderma* strains isolated from winter wheat rhizosphere in Hungary. *Acta Microbiol Immunol Hung* **52**: S156.

Won Y (2000) Oyster mushroom disease: green mould. Available at http://www.mushworld.com (accessed November 1, 2000).

Woo SL, Di Benedetto P, Senatore M, Abadi K, Gigante S, Soriente I, Ferraioli S, Scala F & Lorito M (2004) Identification and characterization of *Trichoderma* species aggressive to *Pleurotus* in Italy. *J Zhejiang Univ Agric Life Sci* **30**: 469–470.

Yu SH (2002) Integrated control of oyster mushroom green mould (1–4). Available at http://www.mushworld.com (accessed May 1, 2002).

Zijlstra C, de Weerdt M, Baar J & Baars J (2008) A TaqMan PCR test for timely detection of the causal agents of dry bubble disease. *Abstracts of the 17th Congress of the International Society for Mushroom Science, Cape Town, South Africa, May 20–24, 2008*, p. 56.

3.4. Further investigations on *T. pleurotum* and *T. pleuroticola*

The problematic of green mould infections is also known from Polish mushroom farms. In the work of Sobieralski *et al.* (2012)* we performed experiments where we determined the growth of *T. pleuroticola* and *T. pleurotum* isolates. Additiolnally, we determined the interactions between these strains and several species of *Pleurotus* sp. in *in vitro* conditions. The results showed that all studied *T. pleuroticola* strains exhibited significantly greater growth than *T. pleurotum* isolates after the incubation time. These findings coresponded to those noted by Siwulski *et al*. (2011). In respect to our previous study presented in this thesis (Komon-Zelazowska *et al*., 2007) these results are particularly interesting as up to now there are no wild isolates of *T. pleurotum* known. It may be explained by the association of these two species and fater growth of *T. pleuroticola that* thus hides *T. pleurotum* in isolation plates.

In the work of Sobieralski *et al.* (2012) we analysed also the impact of *T. pleuroticola* and *T. pleurotum* on the mycelium development of different species of *Pleurotus*. The outcome was that the isolates of *T. pleuroticola* demonstrated reduced growth of *P. florida, P. cornucopiae, P. pulmonarius, P. columbinus, P. eryngii* and *P. ostreatus* more than isolates of *T. pleurotum*. These findings correspond to our results obtained by Komon-Zelazowska *et al.* (2007) concerning faster mycelium growth and the overgrowing of P. *ostreatus* mycelium by *T. pleuroticola* isolates.

*Sobieralski K, Siwulski M., **Komon-Żelazowska M**., Błaszczyk L. Górski, R. Spiżewski, T., and I. Sas-Golak1 (2012) Evaluation of the growth of *Trichoderma pleurotum* and *Trichoderma pleuroticola* isolates and their biotic interaction with *Pleurotus* sp. JOURNAL OF PLANT PROTECTION RESEARCH Vol. 52, No. 2 (2012)

Ms presented in the APPENDIX.

4. Peptaibols of *Trichoderma* – a unique group of Hypocreaceae secondary metabolites

4.1. Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma*

Kubicek CP, **Komon-Zelazowska M**, Karaffa-Sandor E & Druzhinina IS (2007) Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma*, a review. ***Chem Biodivers*** **4(6), 1068-1082**

OWN CONTRIBUTION:

MKZ contributed to the writing of this review.

REVIEW

Facts and Challenges in the Understanding of the Biosynthesis of Peptaibols by *Trichoderma*

by **Christian P. Kubicek***[a]), **Monika Komoń-Zelazowska**[a]), **Erzsébet Sándor**[b]), and **Irina S. Druzhinina**[a])

[a]) Division of Gene Technology and Applied Biochemistry (DGTAB), Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, A-1060 Vienna (phone: +43-1-58801-17250; fax: +43-1-58801-17299)

[b]) University of Debrecen, Centre for Agricultural Sciences, Faculty of Agriculture, Department of Plant Pathology, P. O. Box 36, H-4015 Debrecen

Species of the mitosporic filamentous fungal genus *Trichoderma* are prominent producers of both short (7–11 residues) and long (18–20 residues) peptaibols and peptaibiotics, which are thought to be involved in their interaction with other living systems. Numerous reviews are available regarding biodiversity, structure, and mode of action of these peptide derivatives, but little emphasis has been paid to the physiology and genetics of their formation. In this review article, we used the recent knowledge on biosynthesis and production of these components to speculate on some of the unknown points. We also highlight areas where further research is most urgently needed.

Contents

1. Introduction
2. Biosynthesis of Peptaibols
3. Biodiversity of Peptaibols
4. Evolution of Peptaibols
5. Biosynthesis of Modified Peptaibol Residues
6. Biosynthesis of Non-Proteinogenic α,α-Dialkyl Amino Acids
7. Regulation of Peptaibol Biosynthesis
8. Function of *Trichoderma* Peptaibols
9. Conclusions

1. Introduction. – The mitosporic filamentous fungal genus *Trichoderma* (*Hypocrea*, Hypocreales, Ascomycota) contains several species with strains prominent for their economic importance as sources of enzymes and antibiotics, as plant-growth promoters, metabolizers of xenobiotics, and commercial biofungicides [1–3]. One species, *Trichoderma longibrachiatum*, has been shown to infect immunocompromized humans, and is now on the list of emerging fungal pathogens [4]. At least three other species, *T. aggressivum*, *T. pleurotophilum*, and *T. fulvidum*, have been observed to cause significant crop loss in the mushroom industry, producing *Agaricus bisporus* and *Pleurotus ostreatus*, respectively [5]. While the mechanisms involved in all these

processes likely involve a multitude of factors, the formation of secondary metabolites that are antagonistic or signaling molecules is likely an important factor in all processes where *Trichoderma* interacts with other living organisms.

In fact, a plethora of secondary metabolites has been isolated and characterized from various *Trichoderma* species (for a review, see [6]). Among them, peptaibols and related peptaibiotics are a unique class of peptides. They are characterized by molecular weights of 500–2200 Da, a high number of non-proteinogenic, α,α-dialkylated α-amino acids like isovaline (Iva) and α-aminoisobutyric acid (Aib), an acetylated N-terminus, and an amino alcohol, mostly phenylalaninol, at the *C*-terminal end, which is lacking in the peptaibiotics [7]. The name 'peptaibol' originates from parts of the words **pept**ide, **Aib**, and amino alcoh**ol**. Although peptaibols are not only produced by *Trichoderma* [8], most of the peptaibols known today have been found in this genus and in species of closely related genera. Reports on their isolation from evolutionary distant fungi are probably due to contamination (see, *e.g.*, *Boletus edulis* [9]; see also [8]). The structure and properties of more than 300 peptaibols are collected in the Peptaibol Database on the Word Wide Web [10]. The peptaibols formed by *Trichoderma* usually comprise 18–20 amino acid residues, with a single central Pro moiety. The mechanism of action of peptaibols is mostly related to channel formation in lipid membranes, which implies a bundle of hydrophobic transmembrane helices surrounding a central pore [11]. To learn more about their biochemistry, the review of *Duclohier* is recommended [12].

2. Biosynthesis of Peptaibols. – Except for their N- and C-terminal modifications, the peptaibols are peptides, and, consequently, their biosynthesis occurs in the same way as in other microbial peptides, *i.e.*, by non-ribosomal peptide synthases (NRPS). This is a major difference to higher eukaryotes, which synthesize peptides as larger propeptides that are subsequently processed by endoproteases. NRPS are large, multifunctional enzymes whose domain organization resembles fatty acid synthases [13–15]. Genes encoding peptaibol synthases have been cloned from *Trichoderma virens* (*Hypocrea virens*) [16][17], *T. asperellum* [18], and *T. harzianum* [19], and the corresponding genes could be identified in the genome of *T. reesei* (*H. jecorina*) (http://genome.jgi-psf.org/Trire2/Trire2.home.html) and *T. atroviride* (*H. atroviridis*) [20]. The best-characterized gene, *tex1*, does not contain any intron, encodes a mature protein of 20,925 residues (about 2.3 MDa), and is transcribed into an mRNA roughly 63-kb long. *Wiest et al.* [16] stressed that such a large mRNA would span about 10 μm, thus exceeding the diameter of a cell, implying that there must be a special mechanism how the cell handles the processing of such a large molecule, which, in turn, could be of relevance for peptaibol biosynthesis.

Interestingly, *Whilhite et al.* [21] reported the partial cloning and characterization of a peptide synthase from *T. virens*, with a sequence identical to the 3′-terminal 5-kb of *tex1*. The authors reported that a knock-out mutant in this gene (*psy1*) causes partial or complete loss of hydroxymate siderophore production, and, thus, concluded that *psy1* encodes an NRPS responsible for hydroxymate siderophore production. However, the identity of the C-terminus of *psy1* and *tex1* suggests that this is unlikely, because the structure of *tex1* clearly indicates that it synthesizes an 18-residue peptide, which is much larger than expected for an enzyme producing hydroxymate-containing side-

rophores. Also, the genome sequences of *Neurospora crassa* and *Aspergillus fumigatus* (which are known to produce siderophores) do not contain genes encoding NRPS with 18 modules or strong sequence similarity to *tex1* (*psy1*). In fact, *Wiest et al.* [16] reported that, according to unpublished data, the *tex1* delta mutant is not affected in the production of siderophores.

Based on a comparison of the amino acid sequence of the proteins encoded by the peptaibol synthase genes cloned for *Trichoderma* species, and also those of other peptide sythases, some insights into their structures have been obtained: like in other peptide synthases, each amino acid is introduced by an adenylation domain, which is covalently linked to the adjacent carrier domain. Peptide-bond formation takes place at the condensation domains, where adjacent and non-adjacent carrier domains deliver aminoacyl and peptidyl intermediates [22][23].

Nine residues in the active site of peptide synthases have been proposed to play a major role in defining substrate specificity for incorporation of amino acids based on structural data (*Table 1*). These residues are thought to define the signature sequences specifying amino acid incorporation. Interestingly, the signature sequences from the

Table 1. *Amino Acid Signatures of the AMP-Activating Domain of TEX1 from* T. virens *and* T. harzianum, *and Amino Acids Transferred by These.* Abbreviations: U, α-aminoisobutyric acid; J, isovaline; Voh, valinol. Data taken from [16][19], and unpublished data.

Source	Domain	Amino acid	Signature
T. virens	6	Q	DGGMVGGN
	17	Q	DGGMVGGN
	7	J, U	DCGWVVGV
	1	U	DGGYLAGV
	9	U	DLGYLAGC
	12	U	DLGYLAGV
	15	U	DLGFLAGV
	16	U	DLGFLAGL
	5	U, A	DLGWLCGV
	3	A	DVGFVAGV
	8	A	DIFVVAGV
T. harzianum	16	A	DLGLLAGL
	17	A	DGGAVGGN
	18	A	DAIFLGVV
T. virens	16	A	DLGFLAGL
	17	A	DGGMVGVX
	18	A	DAIIIVGV
T. reesei	16	A	DMGWFAGV
	17	A	DGGMVGGN
	18	A	DAAFIMGV
T. virens	11	L	DFLYFGGV
	14	L, V	DAALIGAV
	4	V	DMGFLGGV
	18	Voh	DAIIIVGV
	2	G	DIGMVVGV
	10	S	DVGYLAAV
	13	P	DVLFCGLI

modules of *tex1* are unique and do not exactly match those found in other characterized NRPSs, which is seen best when the phylogenetic relationship of the signature sequences is analyzed (*Fig. 1*). Therefore, the signature sequences alone do not allow the identification for which amino acid substrate they are specific. However, *Wiest et al.* [16] noted a high similarity in the signature sequences of the 1st, 9th, 12th, 15th, and

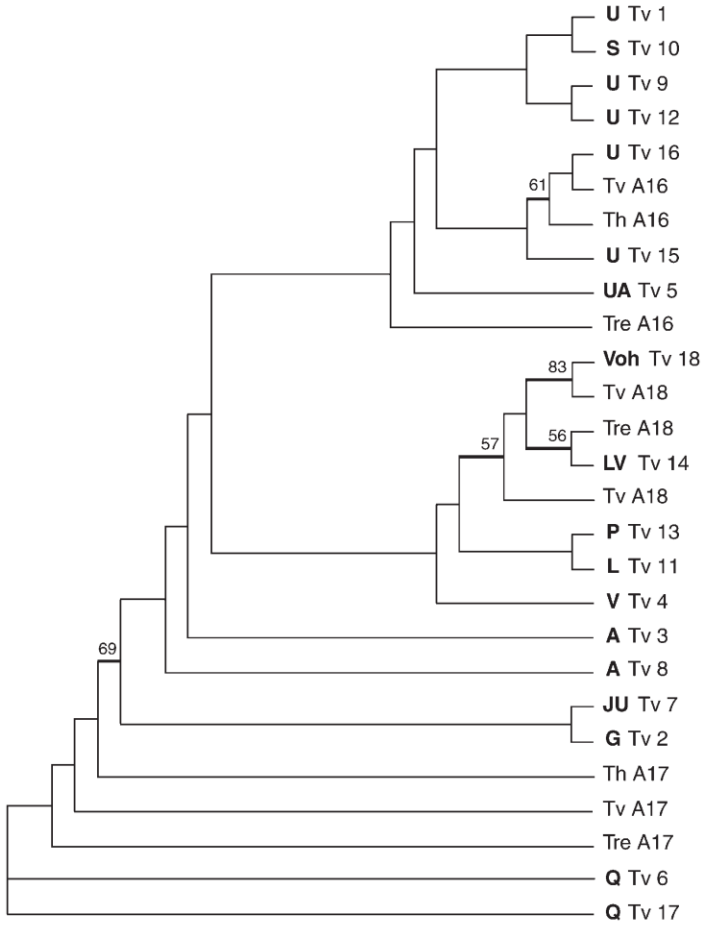


Fig. 1. *Neighbor-joining cladogram of an alignment of the sequence of eight signature amino acids from the AMP-activating domain of three TEX1 proteins* (see *Table 1*). Abbreviations: Tv, *T. virens*; Th, *T. harzianum*; Tre, *T. reesei*. Amino acids are given in the one-letter code, and non-proteinogenic amino acids are abbreviated as explained in *Table 1*. The numbers specify the number of the domain, starting with $n = 1$ at the N-terminus. The analysis was done in PAUP* 4.0b10, using 500 bootstrap replications. The corresponding bootstrap coefficients are given at the respective nodes.

16th modules of TEX1, all of which are – in view of the structure of the correspondingly produced peptaibol – likely responsible for Aib incorporation, and for which also some clustering is observed in the tree (*Fig. 1*).

A characteristic feature of peptaibol synthases is that, unlike most peptide synthases with a thioesterase domain at the C-terminus, they possess a dehydrogenase domain instead. This dehydrogenase leads to the formation of the amino acid alcohol whose presence at the C-terminus is characteristic for the peptaibols (see below). It is suggested that this module effects the release of the product by reductive cleavage.

Because fungal genes for secondary-metabolite biosynthesis, including NRPS, frequently occur in gene clusters [24], it would not be surprising to find such a genomic organization also for the peptaibol synthases. However, comparison of the *tex1* locus in *T. virens*, *T. reesei*, and *T. harzianum* rejects this assumption: in all three species, the locus is synthetic as it contains a gene encoding a retrograde-regulation-protein (RTG2) orthologue, and a gene encoding a protein with high similarity to calcium/proton exchangers 3′-downstream of *tex1*. The preliminary sequence in a 2-kb region upstream of *tex1* has not revealed homology to known proteins in any of the three *Trichoderma* species. This suggests that a gene cluster for peptaibol production does not occur.

3. Biodiversity of Peptaibols. – The peptaibols produced by *Trichoderma* species are considerably heterogeneous, both in size and composition of amino acids at some places in the sequence, and the cultures from a single strain usually comprise several components. In *T. virens* TV29-8, disruption of *tex1* eliminated the production of all of these compounds (*i.e.*, the 18-, 14-, and 11-residue peptaibols) [16]. This would imply that TEX1 has not only a very broad substrate specificity, but also can bypass certain domains, thus producing the shorter peptaibols. However, in the commercially relevant *T. virens* strain G20, disruption of the *tex1* gene (which is 99% similar in its DNA sequence to *tex1* studied in [16]), resulted only in the elimination of production of the 18-residue peptaibol, but not of the 11- and 14-residue congeners [17]. These authors identified AMP-activating domains from additional NRPS of this strain, one of which was likely a second peptaibol synthase. Similar results have been obtained with a *tex1* orthologue from *T. harzianum*, whose disruption also impaired the formation of the 18-residue, but not of and the 11-residue peptaibols [25]. Unfortunately, the sequence of the 11- and 14-residue peptaibols formed by *T. virens* TV 29-8 was not given, and it can, therefore, not be assessed whether they could, indeed, be formed by a 'domain bypassing'; however, the evidence from the other two papers suggests that the shorter peptaibols are formed by a separate enzyme.

There is concordant evidence for a broad substrate specificity of the peptaibol synthases, *i.e.*, some modules are able to bind multiple substrates, albeit with different affinities [26][27], and eventually also with different K_{cat} values. In agreement with such a model, the intracellular abundance of individual amino acids appears to be a major determinant for substrate specificity, because the complexity can be manipulated by supplementing cultures with a specific amino acid [28][29]. Contrary to that, supplement of exogenous Aib, or addition of Glu as a charged amino acid to the medium, resulted in modifications of peptaibol biosynthesis, leading to the simplification of the peptide mixture. The addition of Aib to the cultures of a *T. harzianum*

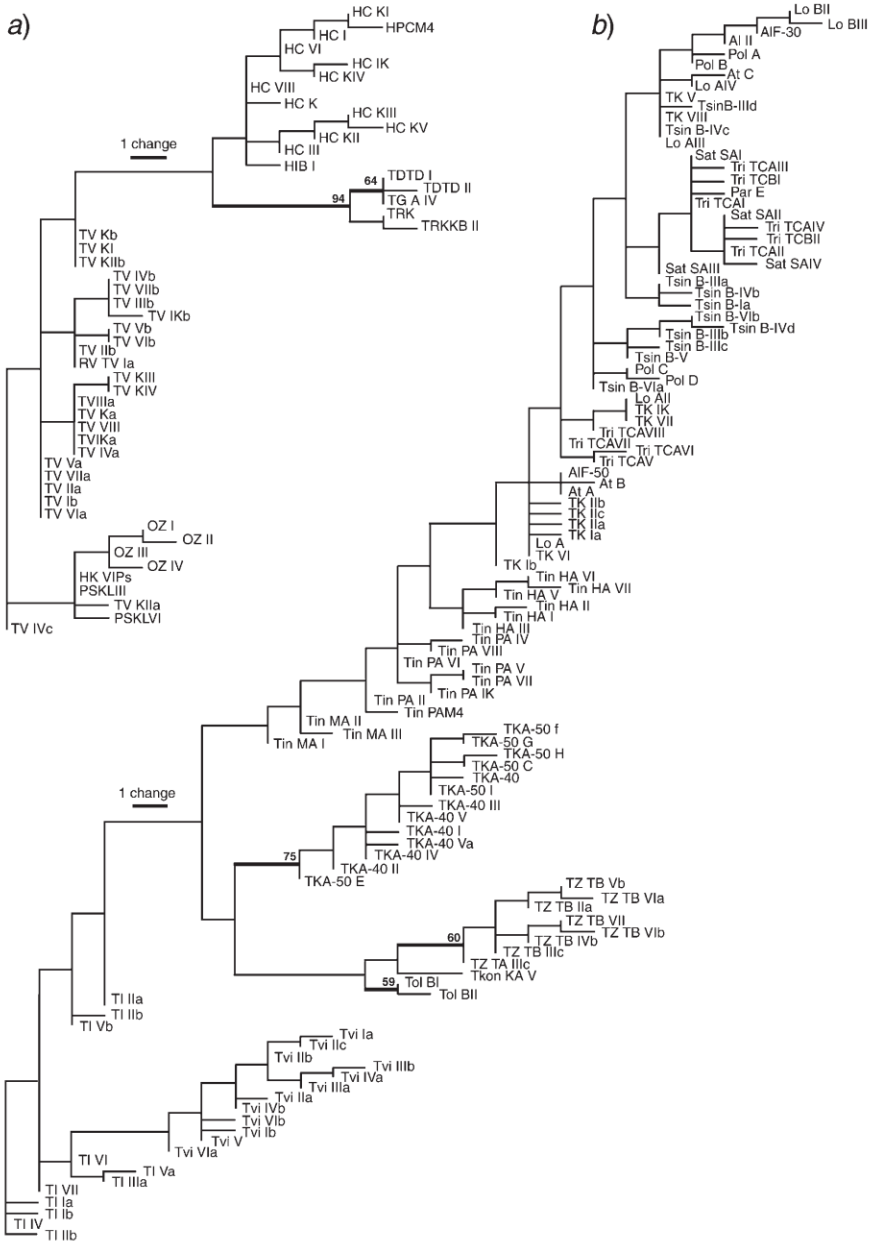
strain generated new peptaibol molecules and led to the production of almost exclusively a single peptide, in which all Iva residues were replaced by Aib, while Glu supply favored the synthesis of peptaibols with increased Glu content in *T. longibrachiatum*. Certain lack of specificity of the amino acid substrate binding to the NRPS was also observed in the case of alamethicin biosynthesis by '*T. viride*' [28]. The observation that extracellular addition of Aib, but not of other amino acids, also increases the rate of peptaibol formation suggests that the substrate specificity of the modules incorporating this amino acid is rather strict though, and that this amino acid is limiting in the intracellular pool (see discussion below).

4. Evolution of Peptaibols. – As described above, peptaibol synthases are members of the NRPS family, which are characterized by a multimodular domain organization. Such modular rearrangements play an important role in protein evolution [30]. Functional modules, often representing structural domains or smaller fragments, are in many cases well-conserved, but re-occur in a different order and across many protein families. The genetic mechanisms responsible for this process are gene duplication, gene fusion, and loss of sequence fragments. This can, therefore, lead to a rearrangement in the sequential order of the domains. *Weiner* and *Bornberg-Bauer* [31] have identified a large number of such rearrangements in multidomain proteins, and analyzed their evolutionary history. For NRPS, they found that 'duplication/deletion' is the most frequent mechanism occurring.

No such studies have yet been performed with peptaibols synthases. We have been addressing the evolution of peptaibols as follows: we assumed that the amino acid sequence of the produced peptaibols (taking the broad substrate specificity into account) would be indicative of the domain order in the respective peptaibol synthases. To this end, we separately aligned all 18-to-20- and 10-to-14-residue peptaibols from the peptaibol database [10][11], and subjected them to phylogenetic analysis. The resulting trees are shown in *Fig. 2*.

As can be seen, on the tree formed by the large peptaibols, some compounds such as trichovirin, trichokonin, trichokindin, or trichorzianine cluster in one, albeit poorly supported, branch, indicating that these peptaibols have already developed a specific sequence that is not corrupted by the relaxed substrate specificity. In contrast, the position of other peptaibols were unresolved, with mixed multiple compounds, indicating that substrate specificity of individual modules and module organization overlap. An even more unresolved picture was obtained for the short peptaibols. Taken together, these data would suggest that the structures of the peptaibols contain no phylogenetic information due to broad substrate specificity of the peptaibol synthases and the involvement of genomic rearrangements in their evolution. In fact, by analyzing a (small) number of *Trichoderma* species with established species identity, *Degenkolb et al.* [32] found that the types of peptaibols produced do not correlate with the taxonomy of these species.

In order to test this hypothesis by a different method, we took the 1st AMP-activating domains of TEX1 [16] and performed a similarity search using the blastn algorithm (www.ncbi.nlm.nih.gov) of the *National Center for Biotechnology Information* (*NCBI*). Thereby, it became evident that the next neighbors of individual domains from one *Trichoderma* species were mostly the corresponding other AMP-activating


a)
b)
1 change
1 change
HC KI
HPCM4
HC I
HC VI
HC IK
HC KIV
HC VIII
HC K
HC KIII
HC KV
HC KII
HC III
HIB I
64
TDTD I
TDTD II
94
TG A IV
TRK
TRKKB II
TV Kb
TV KI
TV KIIb
TV IVb
TV VIIb
TV IIIb
TV IKb
TV Vb
TV VIb
TV IIb
RV TV Ia
TV KIII
TV KIV
TVIIIa
TV Ka
TV VIII
TVIKa
TV IVa
TV Va
TV VIIa
TV IIa
TV Ib
TV VIa
TV IVc
OZ I
OZ II
OZ III
OZ IV
HK VIPs
PSKLIII
TV KIIa
PSKLVI
Lo BII
Lo BIII
AIF-30
Al II
Pol A
Pol B
At C
Lo AIV
TK V
TsinB-IIId
TK VIII
Tsin B-IVc
Lo AIII
Sat SAI
Tri TCAIII
Tri TCBI
Par E
Tri TCAI
Sat SAII
Tri TCAIV
Tri TCBII
Tri TCAII
Sat SAIV
Sat SAIII
Tsin B-IIIa
Tsin B-IVb
Tsin B-Ia
Tsin B-VIb
Tsin B-IVd
Tsin B-IIIb
Tsin B-IIIc
Tsin B-V
Pol C
Pol D
Tsin B-VIa
Lo AII
TK IK
TK VII
Tri TCAVIII
Tri TCAVII
Tri TCAVI
Tri TCAV
AIF-50
At B
At A
TK IIb
TK IIc
TK IIa
TK Ia
Lo A
TK VI
TK Ib
Tin HA VI
Tin HA VII
Tin HA V
Tin HA II
Tin HA I
Tin HA III
Tin PA IV
Tin PA VIII
Tin PA VI
Tin PA V
Tin PA VII
Tin PA IK
Tin PA II
Tin PAM4
Tin MA II
Tin MA III
Tin MA I
TKA-50 f
TKA-50 G
TKA-50 H
TKA-50 C
TKA-40
TKA-50 I
TKA-40 III
TKA-40 V
TKA-40 I
TKA-40 Va
75
TKA-40 IV
TKA-40 II
TKA-50 E
TZ TB Vb
TZ TB VIa
TZ TB IIa
TZ TB VII
TZ TB VIb
60
TZ TB IVb
TZ TB IIIc
TZ TA IIIc
Tkon KA V
59
Tol BI
Tol BII
Tl IIa
Tl IIb
Tl Vb
Tvi Ia
Tvi IIc
Tvi IIb
Tvi IIIb
Tvi IVa
Tvi IIIa
Tvi IIa
Tvi IVb
Tvi VIb
Tvi Ib
Tvi V
Tvi VIa
Tl VI
Tl Va
Tl IIIa
Tl VII
Tl Ia
Tl Ib
Tl IV
Tl IIb

domains of the same peptaibol synthase. The next neighbors were AMP-activating domains from other *Trichoderma* species such as *T. asperellum* or *T. harzianum* (which are present in the database), but, interestingly, also from other fungi such as *Metarhizium*. These findings were substantiated by a phylogenetic analysis, which clearly shows that the AMP-activating domains of different *Trichoderma* species form terminal clusters, indicating that they are orthologues (*Fig. 3*). This is interesting, because these domains do not occur in the same order in different *Trichoderma* species. Currently, a more detailed phylogenetic analysis of peptaibol synthases is performed by *von Döhren* and *Kubicek* [33], but we can already conclude that domain transfer due to recombination and, in part, even by horizontal gene transfer have contributed to the evolution of peptaibol synthases. The clustering of the peptide synthase domain from *Metarhizium anisopliae* and domain 14460 is an example pointing in this direction.

5. Biosynthesis of Modified Peptaibol Residues. – The origin of the acetylated N-terminus and the C-terminal amino alcohol of peptaibols has been studied in detail by *Mohr* and *Kleinkauf* in the case of alamethicine synthase [34], which led to the conclusion that neither of these residues are synthesized by other metabolic pathways, nor are they direct substrates for the synthase. This finding is also substantiated by the domain structure of the peptaibol synthases, which clearly indicates that these modifications are due to posttranslational processing. Also, the peptaibiotics pseudokonin KL III and VI from *T. koningii* (*H. koningii*) have an unusual C-terminal end, with an amide and a hydroxyketopiperazine group, respectively. This might be the result of an alternative processing/chain termination of the Aib-Pro-OH terminus, either by amidation of Pro, or by cyclization of Aib and Pro into the hydroxyketopiperazine ring (probably in two steps: first by reduction of the carboxylic acid residue to an aldehyde, followed by intramolecular cyclization [35]).

6. Biosynthesis of Non-Proteinogenic α,α-Dialkyl Amino Acids. – As mentioned above, peptaibols are rich in Aib and Iva residues. It is intriguing that, despite of this

◀ Fig. 2. *Parsimony phylogenetic analysis of short-chain* (*a*) *and long-chain* (*b*) *peptaibols produced by* Trichoderma. Phylograms show one out of 300 maximum parsimony phylogenetic trees obtained using the heuristic tree-searching algorithm with Maxtree option in effect. Stability of clades was tested with 500 of bootstrap replicates. Note that strain identifications may be incorrect, because the respective isolates have not been identified by DNA-sequence analysis. Trichopolyns I–V were not included in the analysis, because their sequences deviate significantly from that of all other *Trichoderma* peptaibols. It is suggested that this is a product of a *Tolypocladium* sp., which have in earlier times frequently been misidentified as '*T. polysporum*'. **Abbreviations** of peptaibols and producer organisms for tree *a:* TRK, trikoningin (*T. koningii*); HC, harzianin (*T. harzianum*); PS, pseudokinin (*T. pseudokoningii*); TD, trichodecenin (*T. viride*); TG, trichogin (*T. longibrachiatum*); TV, trichorovin (*T. viride*); OZ, trichorozin (*T. harzianum*). **Abbreviations** for tree *b:* TI, trichokindin (*T. harzianum*); Al, alamethicin (*T. viride* NRRL 3199); At, atroviridin (*T. atroviride*); Lo, longibrachin (*T. longibrachiatum*); Par, paracelsin (*T. saturnisporum, H. jecorina*); Pol, polysporin (*T. polysporum*); Sat, saturnisporin (*T. saturnisporum*); Tri, trichocellin (*T. viride*); TKA, trichokonin (*T. koningii*); Tol, tricholongin (*T. longibrachiatum*); TZ, trichorzianine (*T. harzianum*); Tin, trichorzin (*T. harzianum*); tsin, trichosporin (*T. polysporum*); TK, trichotoxin (*T. viride* 5242 = *T. asperellum*); Tvi, trichovirin (*T. viride*); Tkon, trikoningin (*T. koningii*).

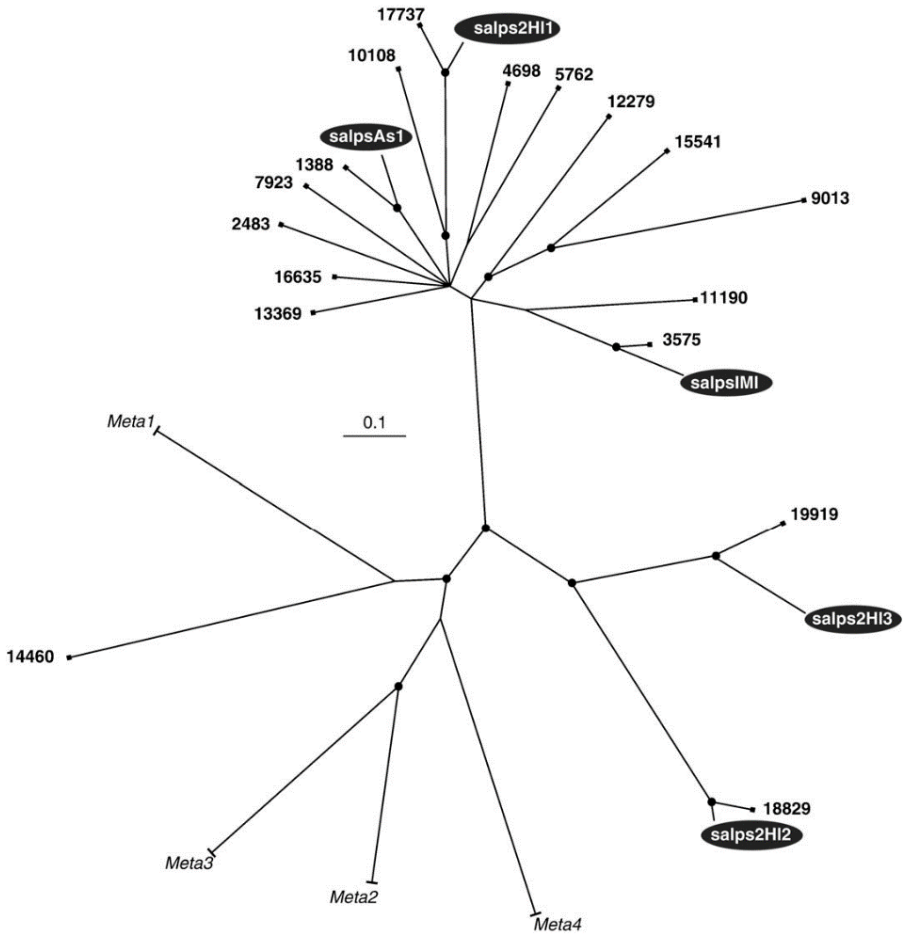


Fig. 3. *Phylogenetic analysis of the AMP-activating domain of TEX1 of* T. virens, and its most similar protein sequences (according to blastn). Domains in TEX1 are indicated by the number of the first amino acid; BLAST-derived domains were: Salps AS1:AY513580, *T. asperellum* (bit score 676, *E* value: 0.0); Salps2 HI1-3: AJ871177, *T. harzianum* (498, 1e-138); Salps IMI: AJ784987, *T. harzianum* (395, 1e-107); AF304355, *T. asperellum* (321, 3e-85); Meta 1–4, 4 matching domains X89442, *M. anisopliae pesA* (319, 9e-85). Phylogenetic analysis was done by bayesian analysis with 10^6 generations and 300 burned trees, and sampling was done after every 100 trees. Black cycles indicate statistically supported nodes ($PP > 0.94$).

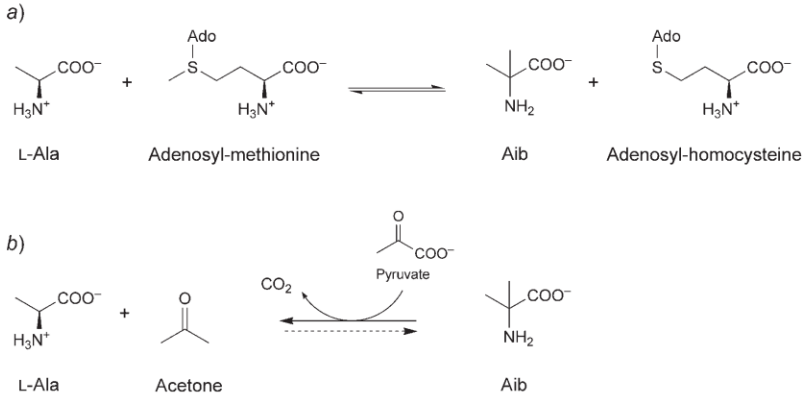
fact, the biosynthesis of these two amino acids has, so far, not been studied in *Trichoderma* or any other organism that produces peptaibols. The only information available is that extracellularly added Aib is *a*) incorporated in the peptaibols and *b*)

stimulates peptaibol formation, which indicates that the naturally occurring intracellular concentration of these two amino acids must be limiting [28][29]. Aib does not occur in mammals, and also seems to be absent from most yeast or filamentous fungi like *Neurospora*, which is evidenced by the fact that it can be used in these species as a non-metabolizable analogue for amino acid transport [36]. No specific investigations towards elucidating the biosynthesis of Aib have, so far, been performed, but we would like to offer some speculations in this respect.

For Aib, the most straightforward possibility would be formation from its stereochemical 'neighbor', *e.g.*, L-alanine, which would imply one or (in the case of L-isovaline) two methyltransferase reactions, using adenosyl-methionine as a Me donor (*Scheme*; part *a*). An alternative way has recently been proposed by *Raap et al.* (*Scheme*, part *b*) [37], although not for *Trichoderma*, but for the fungus *Emericellopsis salmosynnemata*, which produces the 15-residue long peptaibols zervamicins IIA and IIB. When [^{15}N]DL-Iva was added to the culture medium, the ^{15}N-isotope was not only found in the D-Iva residue, but, surprisingly, also in the Aib residues, as well as at the proteinogenic amino acids present in zervamycin. This indicates that Iva can be metabolized and used for the biosynthesis of other amino acids. This is not so unexpected, because bacteria and fungi are known to possess a dialkylglycine decarboxylase (EC 4.1.1.64), a pyridoxal phosphate dependent enzyme of the aminotransferases class-III group. The enzyme is unique in terms of catalyzing both decarboxylation and transamination.

Esaki et al. [38] characterized dialkylglycine decarboxylase from several *Aspergillus* and *Fusarium* species, and confirmed that it performs the reaction shown in part *b* of the *Scheme*. *Adachi et al.* [39] functionally characterized the gene *dgd1* encoding the dialkylglycine decarboxylase in the wheat blotch fungus *Mycosphaerella graminicola*. They showed that *dgd1* mutants cannot utilize Aib as a single nitrogen source. A gene with high similarity was also found in the database for the *H. jecorina* genome (64.2% amino acid similarity; scaffold 8: 1336708–1338033). *Raap et al.* [37] suggested that

Scheme. *Two Hypotheses Regarding the Biosynthetic Reactions Leading to Aib*. For details, see text.



this gene might also be involved in the biosynthesis of Iva and Aib by reversed carboxylation/transamination of acetone and butan-2-one, using peptaibol synthesis as a driving force for reversing the equilibrium of this reaction. A drawback of this hypothesis is that the reverse reaction has, so far, not been demonstrated [40]. Clearly, this is an intriguing hypothesis, which needs to be approved by reversed genetics in *Trichoderma*.

7. Regulation of Peptaibol Biosynthesis. – Regulation of peptaibol biosynthesis is still only poorly understood. Culture conditions used to obtain peptaibols vary widely, using diverse carbon and nitrogen sources, as well as pH values (*Table 2*). However, two findings are noteworthy: the first is that in many studies [16][41–45] surface cultures had to be used to produce peptaibols, and, when grown on the same medium, no peptaibols were produced in submerged cultivation. In addition, in the few cases where submerged cultivations were used with success, it turned out that the addition of an insoluble component (usually a carbon source such as cellulose; see [28]) was in most cases a necessary prerequisite for production. The latter finding was nicely supported by the observation that peptaibol production by *T. atroviride* (named *T. harzianum* in this study) occurred in liquid minimal medium only when *Botrytis cinerea* cell walls were present [46], which suggests a co-regulation of peptaibol biosynthesis and formation of cell-wall-degrading enzymes. Both observations would indicate a developmental regulation of peptaibol biosynthesis. In fact, several authors reported that they have isolated the peptaibols from very old surface cultures of *Trichoderma* (at least 15 d of cultivation; see *Table 2*), which, in our experience, are inevitable strongly sporulating, suggesting the possibility of a correlation between conidiation and peptaibol biosynthesis.

If such a link would actually exist, the promoter of the peptaibol synthase should contain consensus sites for binding of regulatory proteins known to be required for regulation of conidiogenesis [47][48]. We investigated this possibility by two different means: first, we aligned the *tex1* promoter of *T. reesei* with the 5-upstream regions of two hydrophobin genes (*hfb1* and *hfb2*) of *T. reesei*. Hydrophobins of *Trichoderma* as well as of other fungi have been shown to be co-regulated with sporulation [49–51]. Some 48.3% of *hfb1* and 39.6% of *hfb2* were identical with *tex1*. This value is significantly higher than that produced by an alignment of *tex1* with the promoter of the *gpd1* (glyceraldehyde-3-phosphate dehydrogenase) gene (27.4%) or the translation elongation factor 1-alpha (*tef1*; 30.2%), indicating apparent conservation of promoter sequences between *tex1* and *hfb2*.

Second, we manually tested the *T. reesei tex1* promoter for the presence of binding sites of the fluffy regulator (encoded by the *flu* gene) of *Neurospora crassa*, a Zn(2)Cys(6) DNA-binding protein, which is required for asexual sporulation [52]. Fluffy binds to the motif 5′-CGG(N)9CCG-3′, which has also been found in the hydrophobin genes of *N. crassa*. However, despite of the presence of a potential *flu* orthologue in *T. reesei*, we detected such binding sites neither in the *tex1* nor in the promoters of *hfb1* and *hfb2*, eventually suggesting either a different binding specificity of *T. reesei* fluffy or a different regulation of hydrophobin and peptaibol synthase in *Trichoderma*.

Table 2. *Fermentation Conditions for Peptaibol Production in* Trichoderma *Species*

Species	Type of fermentation	Medium			Duration of fermentation	Peptaibols produced	Comments and Refs.
		C source	N source	pH			
T. harzianum	shaken	–	nitrate		20 h	tricharzins	Replacement culture was used with minimal amount of *B. cinerea* cell wall and glutamine [46]
	solid phase	dual culture with *Catharanthus roseus* callus				trichosetin	[41]
		synthetic				tricharzins	[53]
T. koningii	solid phase	complex (wheat bran)			4 d	trichokonins	[45]
	stationary	glucose	nitrate	6.3	21 d	trichosporin	[42]
T. longibrachiatum	stationary	glucose	nitrate		15 d	tricholongins, trichogins	[44]
		glucose	nitrate		15 d	trichogins	[54]
T. pseudokoningii		synthetic			11 d	pseudokonin	[34]
T. virens	stationary	glucose	nitrate		18 d	trichoharzins	[14]
T. viride	shaken	complex (malt extract, soy peptone)		6–6.5	8 d	alamethicins	First neutral, and after *ca.* 5 d acidic alamethicins were produced [55]
	shaken	complex (malt medium)			5 d	trichovirin	[56]
Trichoderma spp.	stirred tank	synthetic, with mixture of nitrate, glutamine, and 2-methylalanine			6 d [a])	alamethicins	Good yield only in the presence of an insoluble carbohydrate [28]
Trichoderma sp.	stationary	complex (malt extract, glucose, yeast extract)			20 d	trichofumins	[38]

[a]) Production from 3rd day.

We have, therefore, recently tested this experimentally, by studying the formation of the long-chain peptaibols atrovirins A–C by *T. atroviride*, using a peptaibiomics approach [57]. We, indeed, found that atroviridins are not formed by vegetative hyphae of the fungus, but are accumulated only when sporulation is induced by one of the triggers (light, starvation, and mechanical injury), thus confirming our conclusions from published data. Interestingly, however, we also found that a mutant in the G-α protein GNA3 (= Tga3) [58], despite of being hypersporulating, is almost completely devoid of peptaibol formation. This result adds the peptaibols to the list of other fungal secondary metabolites that have been shown to be dependent on the positive function of a G-α protein such as penicillin formation by *Aspergillus nidulans* [59], and trichothecene production by *Fusarium sporotrichioides* [60]. The role for this association is not completely understood at this moment: secondary metabolites associated with sporulation are usually divided into three categories [61]: *i*) metabolites with a positive role in sporulation, *ii*) components required for the process of sporulation (melanins being prominent examples), and *iii*) toxic metabolites secreted by the fungus concomitant with sporulation. Because of the membrane-affecting properties of the peptaibols, one would favor the latter explanation. However, a role of the peptaibols in categories *i* or *ii* cannot be ruled out at the moment.

8. Function of *Trichoderma* Peptaibols. – While the above findings are in accordance with a role of the peptaibols as major defensive 'weapons' of *Trichoderma*, there are some points raising doubt that this is the only explanation for their existence. The major one is that spores are, as a whole, already better protected than hyphae; yet the latter do form any peptaibols. We, therefore, wonder whether peptaibols may also serve different functions in *Trichoderma*, and eventually also in the other fungi producing them. In this regard, it is interesting that another ion-channel-forming peptide antibiotic, gramicidin, induces sporulation in its producer bacterium *Bacillus subtilis* [62][63]. Also, in fungi, peptides (whose structures have not been well-characterized yet), are known to induce sporulation [64]. Sporulation of *T. harzianum*, in which the *tex1* orthologue has been knocked-out, appeared to be normal, yet this strain still produced the short-chain peptaibols and, thus, was not completely devoid of their formation. In our opinion, the physiological response of *Trichoderma* to the presence of peptaibols would be a challenging subject worth further studies.

9. Conclusions. – It is intriguing that, despite of the strong interest in application of peptaibols and screening for new components, and the increment in understanding how the respectively involved NRPS work, this review reveals that there exist numerous gaps in our basic understanding of how they are produced. We think that closing this gap for some of these points such as the biosynthesis of the non-proteinogenic amino acids, which may enable to remove the precursor limitation, or understanding the genetic basis for the sporulation-associated nature of their formation, which could offer strategies to produce peptaibols by submerged fermentation, will be important steps in further developments.

The authors own work cited in this review was supported, in part, by grants from the *Austrian Research Foundation* to *C. P. K.* (P 17325) and to *I. S. D.* (P 17895), as well as by grants from the *Hungarian–Austrian Intergovernmental Cooperation Program* (25/2005) to all authors. *E. S.* was awarded by the *János Bólyai* fellowship of the Hungarian Academy of Sciences.

REFERENCES

[1] T. Benitez, A. M. Rincon, M. C. Limon, A. C. Codon, *Int. Microbiol.* **2004**, *7*, 249.
[2] G. E. Harman, L. Lorito, J. M. Lynch, *Adv. Appl. Microbiol.* **2004**, *56*, 313.
[3] G. E. Harman, C. R. Howell, A. Viterbo, I. Chet, M. Lorito, *Nat. Rev. Microbiol.* **2003**, *2*, 43.
[4] L. Kredics, Z. Antal, I. Dóczi, L. Manczinger, F. Kevei, E. Nagy, *Acta Microbiol. Immunol. Hung.* **2003**, *50*, 105.
[5] L. Hatvani, Z. Antal, L. Manczinger, A. Szekeres, I. S. Druzhinina, C. P. Kubicek, A. Nagy, E. Nagy, C. Vágvölgyi, L. Kredics, *Phytopathology* **2007**, *97*, 532.
[6] E. L. Ghisalberti, K. Sivasithamparam, in 'Trichoderma and Gliocladium: Basic Biology, Taxonomy and Genetics', Eds. C. P. Kubicek, G. E. Harman, Taylor & Francis, London, 1998, Vol. 1, p. 191.
[7] A. Szekeres, B. Leitgeb, L. Kredics, Z. Antal, L. Hatvani, L. Manczinger, C. Vagvolgyi, *Acta Microbiol. Immunol. Hung.* **2005**, *52*, 137.
[8] T. Degenkolb, A. Berg, W. Gams, B. Schlegel, U. Gräfe, *J. Pept. Sci.* **2003**, *9*, 666.
[9] S. J. Lee, W. H. Yeo, B. S. Yun, I. D. Yoo, *J. Pept. Sci.* **1999**, *5*, 374.
[10] L. Whitmore, B. A. Wallace, *Nucleic Acids Res.* **2004**, *32*, D593 (see also http://www.cryst.bbk.ac.uk/peptaibol).
[11] L. Whitmore, B. A. Wallace, *Eur. Biophys. J.* **2004**, *33*, 233.
[12] H. Duclohier, *Eur. Biophys. J.* **2004**, *33*, 169.
[13] D. R. Schwarzer, S. Finking, M. A. Marahiel. *Nat. Prod. Rep.* **2003**, *20*, 275.
[14] M. A. Marahiel, T. Stachelhaus, H. D. Mootz, *Chem. Rev.* **1997**, *97*, 2651.
[15] T. Stein, J. Vater, V. Kruft, A. Otto, B. Wittmann-Liebold, P. Franke, M. Panico, R. McDowell, H. R. Morris, *J. Biol. Chem.* **1996**, *271*, 15428.
[16] A. Wiest, D. Grzegorski, B. W. Xu, C. Goulard, S. Rebuffat, D. J. Ebbole, B. Bodo, C. Kenerley, *J. Biol. Chem.* **2002**, *277*, 20862.
[17] X. Wei, F. Yang, D. C. Straney, *Can. J. Microbiol.* **2005**, *51*, 423.
[18] C. Chutrakul, J. F. Peberdy, *FEMS Microbiol. Lett.* **2005**, *252*, 257.
[19] J. A. Vizcaino, R. E. Cardoza, L. Dubost, B. Bodo, S. Gutierrez, E. Monte, *Folia Microbiol.* **2006**, *51*, 114.
[20] S. Baker, C. P. Kubicek, unpublished data.
[21] S. E. Wilhite, R. D. Lumsden, D. C. Straney, *Appl. Environ. Microbiol.* **2001**, *67*, 5055.
[22] G. L. Challis, J. H. Naismith, *Curr. Opin. Struct. Biol.* **2004**, *14*, 748.
[23] R. Finking, M. A. Marahiel, *Annu. Rev. Microbiol.* **2004**, *58*, 453.
[24] J. F. Martin, *J. Ind. Microbiol.* **1993**, *9*, 73.
[25] D. Keszenman-Pereyra (Department of Genetics, University of Sheffield, UK), personal communication.
[26] H. von Döhren, *Adv. Biochem. Eng. Biotechnol.* **2004**, *88*, 217.
[27] W. Kallow, M. Pavela-Vrancic, R. Dieckmann, H. von Döhren, *Biochim. Biophys. Acta* **2002**, *1601*, 93.
[28] D. Brewer, F. G. Mason, A. Taylor, *Can. J. Microbiol.* **1987**, *33*, 619.
[29] G. Leclerc, S. Rebuffat, C. Goulard, B. Bodo, *J. Antibiot.* **1998**, *51*, 170.
[30] J. Weiner 3rd, F. Beaussart, E. Bornberg-Bauer, *FEBS J.* **2006**, *273*, 2037.
[31] J. Weiner 3rd, E. Bornberg-Bauer, *Mol. Biol. Evol.* **2006**, *23*, 734.
[32] T. Degenkolb, T. Gräfenhan, A. Berg, H. I. Nirenberg, W. Gams, H. Brückner, *Chem. Biodiv.* **2006**, *3*, 593; T. Degenkolb, T. Gräfenhan, H. I. Nirenberg, W. Gams, H. Brückner, *J. Agric. Food Chem.* **2006**, *54*, 7047.
[33] H. von Döhren, T. Degenkolb, R. Dieckmann, T. Neuhof, T. Schwecke, I. S. Druzhinina, C. P. Kubicek, H. Brückner, in preparation.

[34] H. Mohr, H. Kleinkauf, *Biochim. Biophys. Acta* **1978**, *526*, 375.
[35] S. Rebuffat, C. Goulard, S. Hlimi, B. Bodo, *J. Pept. Sci.* **2000**, *6*, 519.
[36] S. Ogilvie-Villa, R. M. DeBusk, A. G. DeBusk, *J. Bacteriol.* **1981**, *147*, 944.
[37] J. Raap, K. Erkelens, A. Ogrel, D. A. Skladnev, H. Brückner, *J. Pept. Sci.* **2005**, *11*, 331.
[38] N. Esaki, M. Watanabe, T. Kurihara, K. Soda, *Arch. Microbiol.* **2004**, *161*, 110.
[39] K. Adachi, G. H. Nelson, K. A. Peoples, T. M. DeZwaan, A. R. Skalchunes, R. W. Heiniger, J. R. Shuster, L. Hamer, M. M. Tanzer, *Curr. Genet.* **2003**, *43*, 358.
[40] http://www.brenda.uni-koeln.de/php/result_flat.php4?ecno=4.1.1.64.
[41] A. Berg, P. A. Grigoriev, T. Degenkolb, T. Neuhof, A. Härtl, B. Schlegel, U. Gräfe, *J. Pept. Sci.* **2003**, *9*, 810.
[42] A. Landreau, Y. F. Pouchus, C. Sallenave-Namont, J.-F. Biard, M.-C. Boumard, T. R. du Pont, F. Mondeguer, C. Goulard, J.-F. Verbist, *J. Microbiol. Methods* **2002**, *48*, 181.
[43] E. C. Marfori, S. Kajiyama, E. Fukusaki, A. Kobayashi, *Z. Naturforsch. C* **2002**, *57*, 465.
[44] S. Rebuffat, Y. Prigent, C. Auvin-Guette, B. Bodo, *Eur. J. Biochem.* **1991**, *207*, 661.
[45] X.-Y. Song, Q.-T. Shen, S.-T. Xie, X.-L. Chen, C.-Y. Sun, Y.-Z. Zhang, *FEMS Microbiol. Lett.* **2006**, *260*, 119.
[46] M. Schirmböck, M. Lorito, Y. L. Wang, C. K. Hayes, I. Arisan-Atac, F. Scala, G. E. Harman, C. P. Kubicek, *Appl. Environ. Microbiol.* **1994**, *60*, 4364.
[47] J. H. Yu, N. Keller, *Annu. Rev. Phytopathol.* **2005**, *43*, 437.
[48] D. J. Ebbole, *Fungal Genet. Biol.* **1998**, *25*, 15.
[49] S. Askolin, M. E. Penttila, H. A. Wosten, T. Nakari-Setala, *FEMS Microbiol. Lett.* **2005**, *253*, 281.
[50] P. Rerngsamran, M. B. Murphy, S. A. Doyle, D. J. Ebbole, *Mol. Microbiol.* **2005**, *56*, 282.
[51] G. C. Segers, J. C. Regier, D. L. Nuss, *Eukaryot. Cell* **2004**, *3*, 1454.
[52] L. A. Bailey, D. J. Ebbole, *Genetics* **1998**, *148*, 1813.
[53] D. Duval, S. Rebuffat, C. Goulard, Y. Prigent, M. Becchi, B. Bodo, *J. Chem. Soc., Perkin Trans.* **1997**, 2147.
[54] C. Auvin-Guette, S. Rebuffat, Y. Prigent, B. Bodo, *J. Am. Chem. Soc.* **1992**, *114*, 2170.
[55] J. Kirschbaum, C. Krause, R. K. Winzheimer, H. Brückner, *J. Pept. Sci.* **2003**, *9*, 799.
[56] A. Jaworski, J. Kirschbaum, H. Brückner, *J. Pept. Sci.* **1999**, *5*, 341.
[57] M. Komon-Zelazowska, T. Neuhof, H. von Döhren, C. P. Kubicek, I. S. Druzhinina, manuscript in preparation.
[58] S. Zeilinger, B. Reithner, V. Scala, I. Peissl, M. Lorito, R. L. Mach, *Appl. Environ. Microbiol.* **2005**, *71*, 1591.
[59] A. A. Brakhage, P. Sprote, Q. Al-Abdallah, A. Gehrke, H. Plattner, A. Tuncher, *Adv. Biochem. Eng. Biotechnol.* **2004**, *88*, 45.
[60] A. Tag, J. Hicks, G. Garifullina, C. Ake Jr., T. D. Phillips, M. Beremand, N. Keller, *Mol. Microbiol.* **2000**, *38*, 658.
[61] A. M. Calvo, R. A. Wilson, J. W. Bok, N. P. Keller, *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 447.
[62] W. Pschorn, H. Paulus, J. Hansen, H. Ristow, *Eur. J. Biochem.* **1982**, *129*, 403.
[63] H. Ristow, W. Pschorn, J. Hansen, U. Winkel, *Nature* **1979**, *280*, 165.
[64] G. Soid-Raggi, O. Sanchez, J. Aguirre, *Mol. Microbiol.* **2006**, *59*, 854.

Received November 27, 2006

4.2. The formation of atroviridin by *Hypocrea atroviridis* is conidiation-associated, and positively regulated by blue light and the G-protein GNA3

Komon-Zelazowska M, Neuhof T, Dieckmann R, Döhren H, Herrera-Estrella A, Kubicek CP and Druzhinina IS (2007) The formation of atroviridin by *Hypocrea atroviridis* is conidiation-associated, and positively regulated by blue light and the G-protein GNA3. ***Eukaryotic Cell*** **6(12), 2332-42**

OWN CONTRIBUTION:

MKZ cultivated fungi and contributed to the experimental work and to the bioinformatics analysis of the data. She participated in the interpretation of the results and contributed to ms writing.

EUKARYOTIC CELL, Dec. 2007, p. 2332–2342 Vol. 6, No. 12
1535-9778/07/$08.00+0 doi:10.1128/EC.00143-07

Formation of Atroviridin by *Hypocrea atroviridis* Is Conidiation Associated and Positively Regulated by Blue Light and the G Protein GNA3[▽]

Monika Komon-Zelazowska,[1] Torsten Neuhof,[1,3] Ralf Dieckmann,[4] Hans von Döhren,[2] Alfredo Herrera-Estrella,[3] Christian P. Kubicek,[1]* and Irina S. Druzhinina[1]

Technische Universitat Wien, Institut für Verfahrenstechnik, Umwelttechnik, und Technische Biowissenschaften, FB Gentechnik und Angewandte Biochemie, Getreidemarkt 9-166, 1060 Wien, Austria[1]; Technische Universitat Berlin, Institut für Chemie, FG Biochemie, und Molekulare Biologie, Franklinstr. 29, 10587 Berlin, Germany[2]; National Laboratory of Genomics for Biodiversity, Cinvestav Campus Guanajuato, Apartado Postal 629, Irapuato 36500, Mexico[3]; and Anagnostec GmbH, Am Mühlenberg 11, 14476, Potsdam, OT Golm, Germany[4]

Received 25 April 2007/Accepted 24 September 2007

Species of the mycoparasitic fungal genus *Hypocrea/Trichoderma* are prominent producers of peptaibols, a class of small linear peptides of fungal origin. Some of these peptaibols have been shown to act synergistically with cell-wall-degrading enzymes in the inhibition of the growth of other fungi in vitro and in vivo. Here we present the structure of the *Hypocrea atroviridis* peptaibol synthetase gene (*pbs1*), deduced from the genome sequence of *H. atroviridis*. It consists of 19 typical peptide synthetase modules with the required additional modifying domains at the N and C termini. Phylogenetic and similarity analyses of the individual amino acid-activating modules is consistent with its ability to synthesize atroviridins. Matrix-assisted laser desorption ionization–time of flight mass spectrometry of surface-grown cultures of *H. atroviridis* showed that no peptaibols were formed during vegetative growth, but a microheterogenous mixture of atroviridins accumulated when the colonies started to sporulate. This correlation between sporulation and atroviridin formation was shown to be independent of the pathway inducing sporulation (i.e., light, mechanical injury and carbon starvation, respectively). Atroviridin formation was dependent on the function of the two blue light regulators, BLR1 and BLR2, under some but not all conditions of sporulation and was repressed in a *pkr1* (regulatory subunit of protein kinase A) antisense strain with constitutively active protein kinase A. Conversely, however, loss of function of the Gα-protein GNA3, which is a negative regulator of sporulation and leads to a hypersporulating phenotype, fully impairs atroviridin formation. Our data show that formation of atroviridin by *H. atroviridis* occurs in a sporulation-associated manner but is uncoupled from it at the stage of GNA3.

Peptaibols, a class of linear peptides of fungal origin with 7 to 20 residues, have three structural characteristics: (i) a high proportion of dialkylated amino acids with an abundance of α-aminoisobutyric acid (Aib); (ii) an N-acyl (usually acetyl) terminus; and (iii) a C-terminal amino alcohol, such as phenylalaninol or leucinol. Peptaibols naturally occur as mixtures of isoforms, and more than 300 sequences are now known (http://www.crvstbbk.ac.uk/peptaibol/home.shtml). They are divided into three subclasses, which are (i) the long-sequence peptaibols such as alamethicins or trichorzianins, which contain 18 to 20 amino acid residues; (ii) the short-sequence peptaibols such as harzianins or zervamicins containing 11 to 16 residues; and (iii) the lipopeptaibols with 7 or 11 residues and whose N terminus is acylated by a short fatty acid chain (e.g., trichogin A [11, 43]. Peptaibols generally exhibit antimicrobial activity against gram-positive bacteria and fungi but have also been implicated in the interaction with mammalian cells (39). Their biological activities are believed to be due to their membrane-modifying properties and ability to form transmembrane voltage-dependent channels (23).

Fungi of the anamorphic fungal genus *Trichoderma* (*Hypocreale*, *Ascomycota*), which contains cosmopolitan soilborne fungi with economic importance as biocontrol agents (13, 27), are well known as producers of peptaibols. It has been shown in vitro that peptaibols act synergistically with the cell-wall-degrading enzymes secreted by *Trichoderma* to inhibit the growth of fungal pathogens (22, 32). This antagonism is thought to be due to the action of the peptaibols on the membrane of the target fungus, thereby inhibiting membrane-associated enzymes involved in cell wall synthesis.

Despite their attractive potential as antimycotica and their potential importance in the physiology of the fungus, little information is available on the physiological and molecular regulation of peptaibol formation. Such a knowledge would in turn be a prerequisite to understanding the role of these compounds for the fungus. Like other fungal peptides that are products of secondary metabolism, peptaibols are synthesized by nonribosomal peptide synthetases, and genes encoding the respective peptaibol synthetases are known from *Trichoderma virens* (44, 45) and *T. harzianum* (42). Due to the large molecular mass of the transcript, which makes its intact isolation difficult, almost no studies have been carried out on the regulation of transcript formation. Recently, Vizcaino et al. (42)

* Corresponding author. Mailing address: Technische Universitat Wien, FB Gentechnik und Angewandte Biochemie, Institute of Chemical Engineering, Getreidemarkt 9-166, A-1060 Wien, Austria. Phone: (11) 43-1-58801-17250. Fax: (11) 43-1-581-6226. E-mail: ckubicek@mail.zserv.tuwien.ac.at.

[▽] Published ahead of print on 12 October 2007.

TABLE 1. Comparison of *H. atroviridis* PBS1 to other peptaibol synthases from *Trichoderma/Hypocrea*

Organism	Gene/protein	Product name	Product length	Source or reference
H. atroviridis	*pbs1*/PBS1	Atroviridin	19-mer, 20-mer	This study
H. virens	*tex1*/TEX1	Trichorzin	18-mer	46
H. jecorina	*par1*/PAR1	Paracelsin	20-mer	Unpublished
	har1/HAR1	Harzianin	11-14-mer	Unpublished
H. lixii	*salps2*/SALPS2	Trichorzin (?)	18-mer (?)	42[a]

[a] Only three terminal modules were sequenced; however, because of the presence of a C-terminal alcohol dehydrogenase domain and the amino acid activated by the last three A domains, its identity as a large peptaibol synthase was concluded (42).

reported that the peptaibol synthetase transcript of *T. harzianum* can only be detected under nitrogen starvation.

In the present study, we used a recently developed method for studying peptaibol formation from low amounts of fungal biomass using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) (25) to investigate the formation of *H. atroviridis* peptaibols. This fungus has been chosen because it is a strong biocontrol agent, and its peptaibols may play a role in its antagonistic and mycoparasitic abilities. In order to correctly align the observed masses with the peptaibols, we have cloned in silico the *H. atroviridis pbs1* gene and deduced its protein structure predicting that it produces atroviridin. Here we show that the formation of atroviridin by *H. atroviridis* occurs by a sporulation-coupled pathway, whose physiological implications are discussed. In addition, we show that atroviridins belong to the group of secondary metabolites which require positive regulation by G proteins.

MATERIALS AND METHODS

Nomenclature. The nomenclature for the genes and proteins described here has been partially revised in order to follow the recommendations of the consortium annotating the *Trichoderma* genomes to follow the *Neurospora*/pyrenomycete nomenclature, and—if already known—to use the name of the respective *Neurospora* orthologue. Where this led to a change in the name of an already-cited gene or protein is indicated at the first use.

Reagents. 2,5-Dihydroxibenzoic acid from Sigma Chemicals (St. Louis, MO) was used as matrix for MALDI-TOF experiments. Trifluoroacetic acid, ethanol, acetonitrile, and methanol from Merck were used as solvents.

Strains and culture conditions. The wild-type strains of *H. atroviridis* used throughout the present study were IMI 206040 and ATCC 74058 (= P1). The following mutants, prepared from these strains, were used: the blue-light regulator (BLR1 and -2) Δ*blr-1*, and Δ*blr-2* delta strains (5), the G-α protein GNA-delta Δ*gna3* strain (= *tga3*) (48) and strain AS-*pkr1*, in which the protein kinase A (PKA) regulatory subunit (PKR1) gene is inactivated by expression of an antisense copy (6). With the exception of the *gna3* strain, which was derived from *H. atroviridis* P1, all other mutants were from strain IMI 206040. Cultures were routinely grown at 25°C on malt agar plates. To test the influence of exogenous cyclic AMP (cAMP; Sigma), cAMP was added to potato dextrose agar cooled to 48°C after autoclaving.

Colonies were induced to conidiate as previously described (5) by exposure to white light (25 μmol photons m^{-2} s^{-1}; 1,800 lx). In case of dark-grown cultures, harvesting was done under red safety light, which has been shown to have no effect on the fungus for the short period applied (1 to 2 min).

To induce conidiation by carbon starvation, fungal colonies were grown in darkness for 48 h in MM (1.66 mM $MgSO_4$, 5.16 mM K_2HPO_4, 2.68 mM KCl, 12.5 mM NH_4NO_3, 7.19 μM $FeSO_4$, 6.95 μM $ZnSO_4$, 10.1 μM $MnCl_2$), 111 mM glucose (6), and a piece of agar with mycelium and then transferred to glucose-free MM medium and allowed to grow for a further 24 h in either complete darkness or under illumination. For injury-induced conidiation (6), fungal colonies were grown in total darkness at 25°C for 72 h, cut in strips with a scalpel, and incubated for an additional 24 h in the dark at 25°C.

Extraction and preparation of mycelia for MALDI-TOF analysis. A few micrograms of fungal mycelia, corresponding to 5 mm^2 of the edge of the colonies, were suspended in acetonitrile-methanol-water (1:1:1), and 1 μl of the suspension was directly spotted onto target wells of a 100-position sample plate and immediately mixed with 1 μl of matrix solution (10 mg of 2,5-dihydroxybenzoic acid/ml in acetonitrile-methanol-water [1:1:1] and 0.3% trifluoroacetic acid). The sample matrix mixture was allowed to air dry prior to analysis. Alternatively, freeze-dried mycelium obtained from shaken cultures or fungi grown on plates was homogenized in 60% ethanol and centrifuged. Then, 1 μl of the protein solution was spotted onto a MALDI target plate and mixed with matrix.

MS analysis of low-molecular-mass peptides. Measurements were performed in the delayed extraction mode, allowing the determination of monoisotopic mass values. A low mass gate of 800 Da improved the measurement by filtering out the most intensive matrix ions. The mass spectrometer was used in the positive ion detection and reflector mode.

Semiquantitative analysis of peptaibol formation. Because the biomass weight of the part of the fungal colony that was prepared for analysis could not be measured, the values obtained cannot be related to the biomass amount, and the method therefore does not allow a quantitative analysis. Consequently, only very strong differences (present versus not present or present versus almost not present) were considered relevant and used for the interpretation of results.

Bioinformatic methods. A full-length copy of *H. atroviridis pbs1* was identified in its genome sequence by TBLASTN search with the *T. virens pbs1* gene as a probe. The amino acids activated by individual ATP-binding (amino acid activating)-domains were identified by neighbor joining (NJ) analysis (MEGA 3.1) (20) of individual A domains of *H. atroviridis pbs1* and those from *T. virens* (44), *T. harzianum* (42), and *H. jecorina* (*T. reesei* [unpublished data]) (Table 1). For this purpose, the amino acid sequences were aligned with CLUSTAL X; the alignment was manually improved in GENEDOC and then exported to MEGA 3.1. The NJ tree was constructed with 1,000 bootstrap replicas.

RESULTS

In silico characterization of the *H. atroviridis* 19-residue peptaibol synthetase and its putative product. Using the *T. virens tex1* sequence as a query in TBLASTN, we identified a continuous open reading frame in the genome sequence of *H. atroviridis* IMI 206040, which, when translated, would give rise to a 21,879-amino-acid (aa) protein. This deduced polypeptide consists of 19 complete peptide synthetase modules and the respective additional acetyltransferase and alcohol dehydrogenase domains at the N and C termini of the predicted protein (Fig. 1a), thus clearly identifying it as a peptaibol synthetase. It is the only large peptaibol synthase gene in the genome of *H. atroviridis*. The genes flanking *pbs1* in *H. atroviridis* are shown in Fig. 1b: the immediate downstream region contains sequences putatively encoding proteins similar to *S. cerevisiae* Rtg2p and an *N. crassa* calcium/proton exchanger in the same order and orientation as immediately downstream of *T. virens tex1* and *T. harzianum salps2* (42, 46) (Fig. 1a). The upstream region contains genes encoding proteins of the cytochrome P450 subfamilies and a prenyltransferase (Fig. 1). Both genes are also found in the same orientation upstream of the harzianin synthetase gene of *H. jecorina* (unpublished data) but are absent from the 5′ flanking area of *T. virens tex1*. In fact, these two genes occur at two different scaffolds of the *T. virens* genome database (scaffolds 6 and 17), which are both different

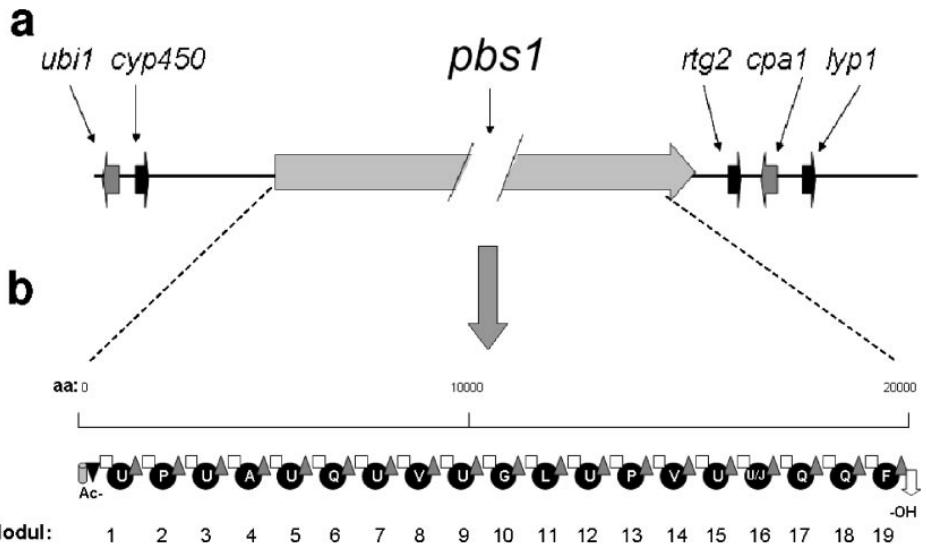


FIG. 1. *pbs1* locus of *H. atroviridis* and modular organization of the predicted PBS1 protein. (a) Chromosomal arrangement of the *pbs1* locus: *ubi1*, UbiA-like prenyltransferase; *cyp450*, cytochrome P450 subfamily protein; *pbs11*, 19-mer peptaibol synthetase; *rgt2*, Rtg2-like protein; cpa1, Ca^{2+}/H^{+} antiporter; *lyp1*, lysine permease. With the exception of *pbs1*, gene lengths are drawn to scale. (b) Modules within the PBS1 protein, numbered consecutively from 1 to 19. The domains within the modules are indicated as follows: gray cylinder, ketoacyl synthetase; black triangle, acyl transferase; white square, condensation domain; black circles: adenylation domains; gray triangles, thiolation domain; white arrow, alcohol dehydrogenase. The amino acids activated by the individual adenylation domains are indicated in the one-letter code (U, α-aminoisobutyric acid; J, isovaline).

from that containing *tex1* (scaffold 12 [unpublished data]). These comparisons show that the locus encoding the large peptaibol synthetases in *Trichoderma* apparently underwent major reorganization during evolution.

Peptide synthetases consist of a conserved iteration of modules, each consisting in 5→3 order of an ATP-binding domain, an amino acid thioesterification domain, and a condensation domain (37). Therein, the ATP-binding domains specify the substrate specificity and thus the sequence of the formed peptide. In order to predict the latter, we first performed a phylogenetic analysis of all 19-aa activating domains and corresponding domains identified in *T. virens* TEX1 and *H. jecorina* paracelsin and harzianin synthetases (PAR1 and HAR1, respectively; H. von Döhren et al., unpublished data) for which the amino acid sequences of the respective peptaibol products are known (Table 1). The rationale for this was the hypothesis that domains activating the same amino acid may be more similar to each other within different *Trichoderma* spp. than to other domains of the same protein. The resulting tree (Fig. 2) verifies this hypothesis in part: there are consistent and well-supported clades for the P-and Q-activating domains, and—albeit poorly resolved at the central nodes—several terminal clades for Aib and Ala. However, a number of clades were mixed. We also noted that domains which activate the same amino acids but are close to either the N or the C terminus of the resulting peptaibol consistently formed different clusters. This analysis allowed some identification but left several domains unidentified.

Therefore, the domains were further analyzed with respect to the presence of the signature sequences proposed by Stachelhaus et al. (38) and Challis et al. (7). For this purpose, we compared them to those present in *H. jecorina* PAR1 because the respective paracelsin is structurally most similar to atroviridin (Table 2) (25). Modules putatively acting on the same aa indeed showed conserved signature sequences, differences often being only conserved changes (e.g., L→V). Based on these combined analyses in Fig. 2 and Table 2, the peptaibols "encoded" by *H. atroviridis* would be compatible with that of atroviridins from a bona fide strain of *H. atroviridis* (26), Ac-UPUAQUVUGLUPVU(U/J)QQF-OH, with the exception that the U between A4 and Q5 is missing.

Identification of atroviridins formed by *H. atroviridis*. In order to compare the structure of the peptaibols putatively formed by PBS1 to those actually formed by *H. atroviridis* IMI 206040 and P1, we used MALDI-TOF analysis of surface-grown cultures of *H. atroviridis*. The rationale for this was that a review of the literature about peptaibol formation by *Trichoderma* revealed that most researchers have used either surface cultures or submerged cultures, to which solid components had been added (19). The results from such an experiment, which yielded consistent results for both strains, are shown in Fig. 3: as long as the culture of *T. atroviride* was in the state of vegetative hyphal growth, no peptaibol formation was detected (data not shown). However, as soon as the fungus started to sporulate, peptaibol formation was evident by the observation of a characteristic island of several mass peaks in the range of 1,935 to 2,010 Da. Table 3 compares the masses of these peaks with those of atroviridins and neoatroviridins of *H. atroviridis*: the main peak at *m/z* 1,963 corresponds to protonated atroviridin A. Masses at 1,920 and 1,934 correspond to atroviridins A and B without the Aib in position 6 and for which the respective module is missing in PBS1. Masses at

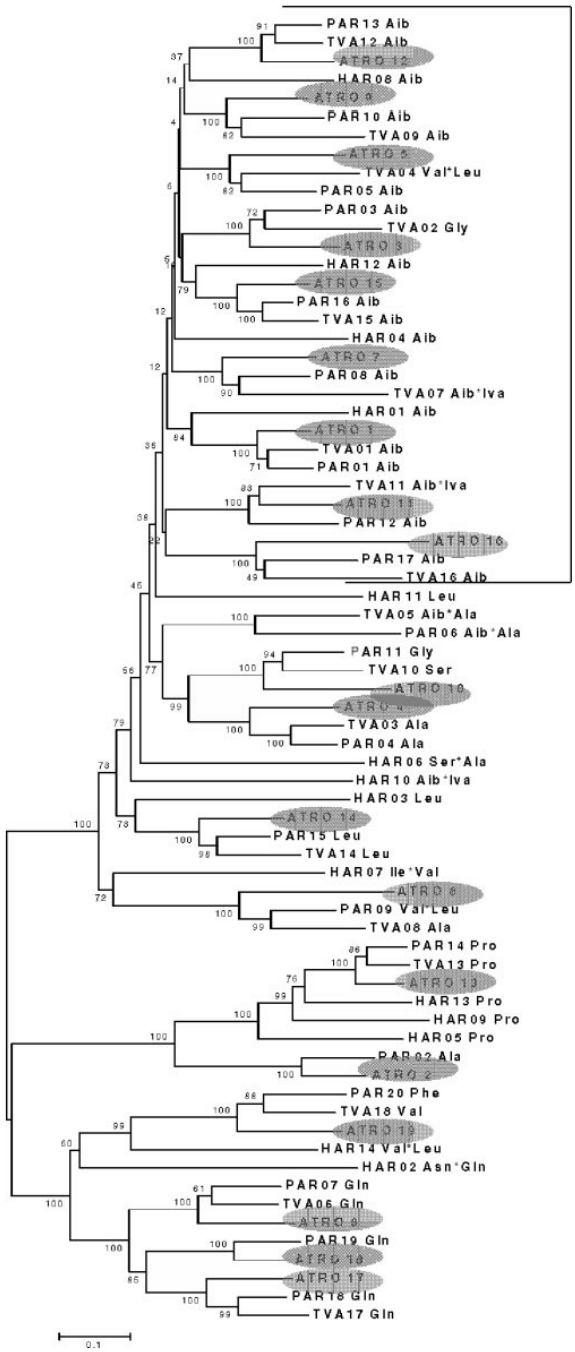


FIG. 2. Phylogenetic relationships of the different adenylation domains of PBS1 to the adenylation domains found in the *H. jecorina* paracelsin synthetase (PAR [unpublished data]), the *H. jecorina* harzianin synthetase (HAR [unpublished data]), and *T. virens* TEX1 (45). Modules in PBS1 (named "ATRO" in this figure) are followed by the respective module number and are shaded in gray. TEX1 is named "TVA" in this figure. In PAR, HAR, and TVA the numbers indicate the positions of the respective modules in the protein, but in addition the amino acid (indicated in the three-letter code) specifies the activated amino acid. When more than a single amino acid is given (linked by an asterisk [*]), this indicates that the module accepts more than a single amino acid. The tree was constructed by NJ analysis, using 1,000 bootstrap replications, whose statistics (percentage of occurrence of the branch in 1,000 trees) are given at the respective nodes. The bracket summarizes all branches leading to modules involved in adenylation of α-aminoisobutyric acid.

1,949, 2,003, and 2,017 may represent new atroviridin variants because they can be explained by the loss or gain of one or two methylene groups from atroviridin A, respectively. While the identification of all of the components of the peptaibol mixture was beyond the scope of the present study, these findings confirm that this "mass island" corresponds to the atroviridins and that both the 20-residue and the 19-residue peptaibols (which would correlate with the module structure) are detectable. We have thus taken the abundance of this peak island, which did not change in its shape (i.e., ratio of individual peaks to each other) throughout this investigation as a semiqualitative measure (see above) for the formation of peptaibols.

Peptaibol formation depends on the BLRl and BLR2 proteins and is stimulated by light. Since atroviridin formation was only detected under conditions of conidiation, we investigated this in more detail. The process of conidiation by fungi is subject to several regulatory influences (47), and the correlation observed above may therefore be simply coincidence, i.e., independent regulation by the same physiological event, or due to a common signal upstream of the conidiation event. We therefore investigated whether different methods for promoting conidiation would always correlate with peptaibol formation or whether the latter specifically occurs only under one of these conditions. As a first condition, we chose white light, which is known to trigger photoconidiation in *H. atroviridis* via the function of the blue light regulator proteins BLR1 and BLR2 (5). Figure 4a shows that the triggering of conidiation by light was indeed paralleled by a strong accumulation of peptaibols, and only traces were seen in dark cultivated cultures. The latter may have been due to some sporulation, since they were completely absent in a parallel set of experiments (data not shown). This accumulation was completely blocked in the *blr1* and *blr2* delta mutants. These data indicate that peptaibol formation under conditions of light-induced sporulation depends on the BLR1/2 proteins.

Starvation-induced sporulation triggers peptaibol formation independently of light and the BLR proteins. Another universal inducer of conidiation in fungi is nutrient starvation, and we therefore tested whether the accumulation of peptaibols would also be stimulated under these conditions. Figure 4b shows that carbon starvation indeed led to the accumulation of peptaibols and that under these conditions the accumulation was independent of the *blr1* and *blr2* genes and, consistent with this, was also independent of light. This would in theory be in contrast to previous reports that carbon deprivation-induced sporulation depends on the BLR1/BLR2 proteins (6). However, more recent data have shown that the BLR1/2-dependent response is only observed when cultures are subjected to a starvation for not more than 12 h and absent during incubation under starvation conditions for a longer time (A. Herrera-

TABLE 2. Signature sequences of the A domains of *H. atroviridis* PBS1 compared to *H. jecorina* PAR1[a]

Domain	Activated aa	Signature aa at position:								
		235	236	239	278	299	301	322	330	331
A1	Aib	D	L	G	Y	L	A	G	V	F
A2	Pro	D	I v	L	I f	C	A g	L	I	C
A3	Aib	D	V l	G	F	L	A	G	V	F
A4	Ala	D	L v	G	F	L v	A	G	V	F l
A4	Aib	D	M l	G	F	I l	A	G	V	V f
A5	Gln	D	G	G	M	V	G	G	N	Y
A6	Aib	D	L	G	Y	V	A	G	V	F
A7	Val	D	A	F	L	L i	G	I g	V	A l
A8	Aib	D	L	G	Y	L	A	G	C v	F
A9	Gly	D	V	G	Y	L	M i	A	V	L f
A10	Leu	**D**	**F**	S l	**Y**	L f	**G**	A g	**V**	M v
A11	Aib	D	L	G	F	L	A	G	V	F
A12	Pro	D	V	L	F	C	G	L	I	C
A13	Val	D	A	A g	L m	V i	V i	G	V	F
A14	Aib	D	L	G	F	L	A	G	V	F
A15	Aib/Iva	D	M	G	W	M f	G a	G	V	I v
A16	Gln	D	G	G	M	V	G	G	N	Y
A17	Gln	D	G	G	M	I v	G	G	N	Y
A18	Phe	D	A	A	I f	L l	V m	G	V	G

[a] Challis et al. (7). Identical signature amino acids are underlined. If the corresponding signature amino acid is different in PAR1, the respective amino acid is indicated by a lowercase letter below that of PBS1. Since no activation domain for leucine (A10) is present in PAR1, its signature was taken from TEX1, and identical amino acids are indicated in boldface to illustrate this difference. aa, amino acids.

Estrella, unpublished data). The present data therefore show that the response to light observed above (i.e., peptaibol accumulation and dependence on the BLR proteins) is due to its sporulation-associated nature and not because of a direct regulatory role of the light triggering signal cascade on peptaibol synthetase.

Induction of conidiation-associated peptaibol formation by mycelial injury is light dependent but BLR independent. As a third, independent approach to study the correlation of peptaibol formation with conidiation, we used mechanical stress (injury with a scalpel (see reference 5) of the mycelia. This conidiation pathway has also been shown to be independent of the BLR proteins (5), and consequently there is also sporulation in the darkness. Interestingly, however, peptaibol formation under these conditions in *H. atroviridis* was completely dependent on the presence of light, and virtually no peptaibols were detectable in its absence in spite of sporulation; essentially the same findings were also observed in the *blr1* and *blr2* mutants (data not shown). While this is consistent with the above findings that injury-promoted conidiation is BLR independent, it documents that there is also a BLR-independent pathway of stimulation of peptaibol formation by light under stress.

The hypersporulating *H. atroviridis* delta-*gna3* mutant is defective in peptaibol formation. Conidiation in *Neurospora crassa* is negatively regulated by the Gα-protein Gna3 (17), and consistent data have also been reported also for *H. atroviridis* (48). Consequently, a *gna3*-delta mutant leads to hypersporulation. We wondered whether this mutant would consequently overproduce peptaibols. However, in contrast to these expectations, the results shown in Fig. 5 document that this mutant is almost completely impaired in peptaibol formation and that this impairment is apparent under all conditions tested above, i.e., light, carbon starvation, and mechanical injury, while the hypersporulating phenotype indeed formed under all of these (with the exception of starvation) conditions. As has been reported previously (48), the addition of exogenous cAMP (1 and 5 mM) did not alter the slowly growing, hypersporulating phenotype of the mutants. We therefore tested whether the addition of cAMP would rescue peptaibol formation in these mutants, but we found that this also was not the case (data not shown). Consequently, these data imply that peptaibol formation is positively controlled by GNA3 via a pathway that must be different from that repressing conidiation and that this control overrides the correlation with sporulation.

Constitutive activation of PKA leads to impairment of peptaibol formation. Despite of the lack of influence of addition of exogenous cAMP, the disparity of the effect of loss of function of GNA3 on conidiation and peptaibol formation prompted us to investigate whether the effect on peptaibol formation would

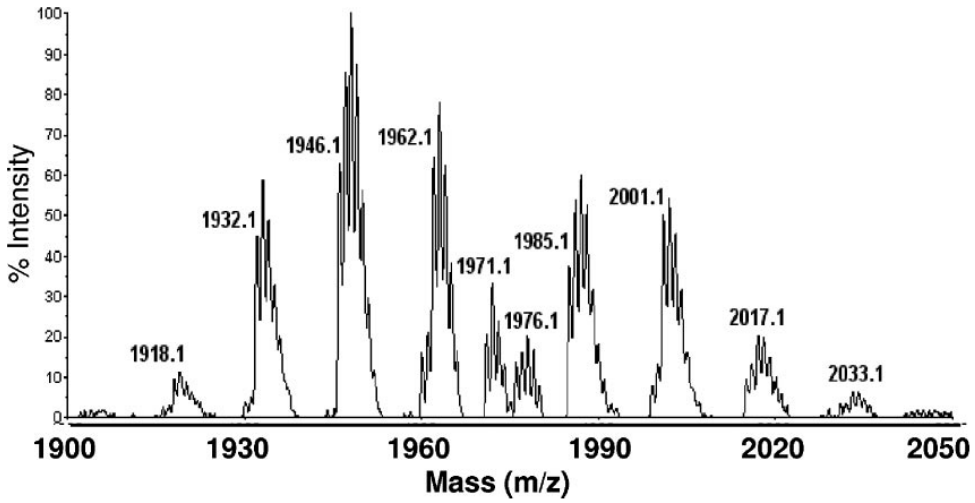


FIG. 3. Detailed view of a MALDI-TOF mass spectrum from a peptaibol producing sporulating culture of the wild-type strain *H. atroviridis* IMI 206040, grown in the presence of light. The *m/z* values of the individual peaks are given.

involve PKA (PKA1). G-protein-mediated inhibition of sporulation is known to involve the downstream action of PKA (35). To this end, we used a strain overexpressing the antisense version of the regulatory subunit of PKA1 (6) and whose PKA1 activity is thus constitutively activated. Consistent with what is known in other fungi, sporulation in this strain was essentially absent (see also reference 6). Also consistent with the findings that the addition of cAMP had no effect, peptaibol formation was not observed in this strain, irrespective of the conditions used (light/darkness, mechanical injury, starvation, etc. [data not shown]). Therefore, the positive action of GNA3 on peptaibol formation does not involve PKA1.

DISCUSSION

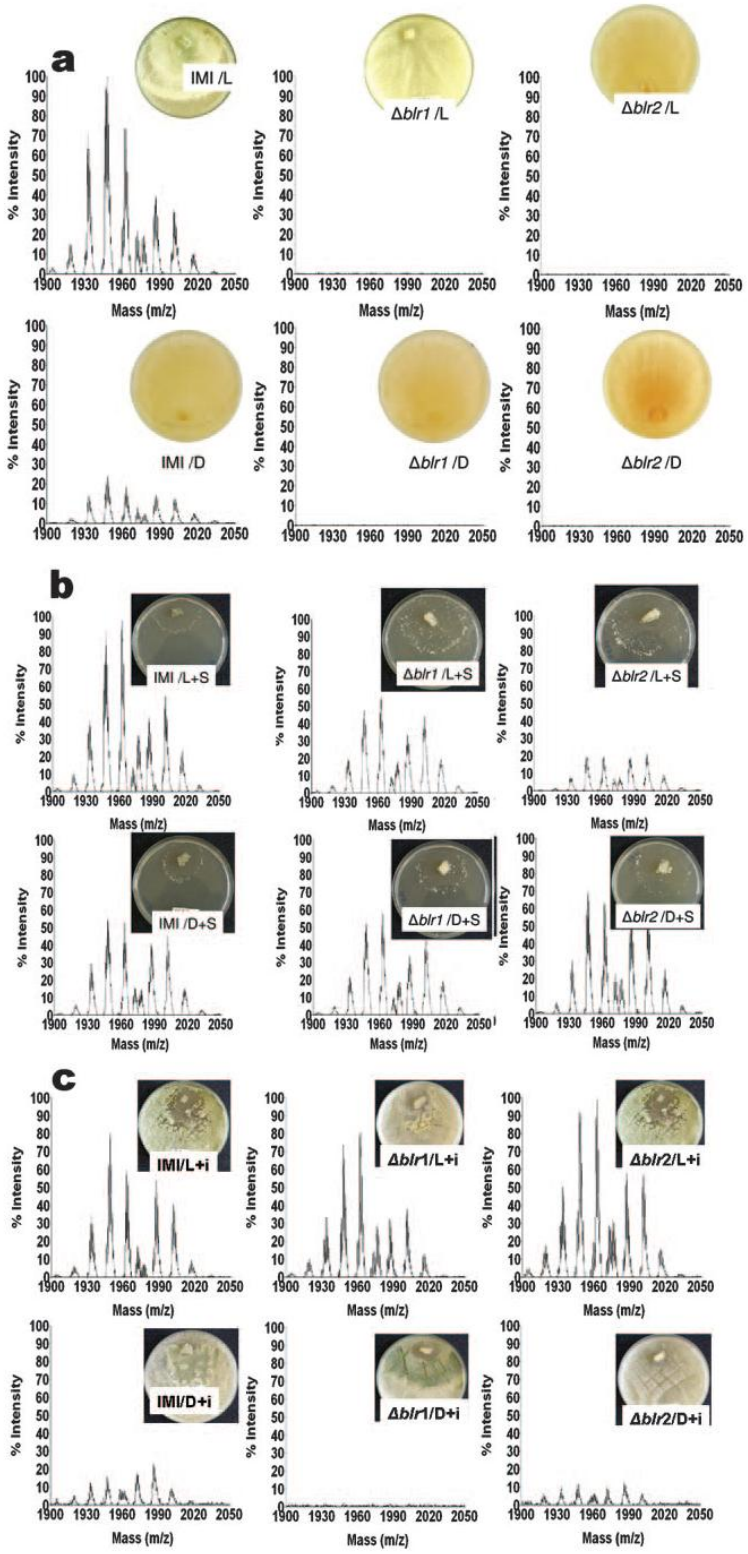
Peptaibols are among the largest peptide-like components formed by fungi and also have a number of interesting applications. However, apart of their isolation and chemical characterization, little information has thus far become available about the reasons for their biodiversity and regulation of formation. Here we used in silico analysis to predict the structure of the peptaibol synthetase of *H. atroviridis*, and we report that the formation of the respective peptaibols—atroviridins—partially correlates with sporulation and is GNA3 dependent. To facilitate the subsequent discussion, these findings and the interaction of components are summarized in Fig. 6.

TABLE 3. Interpretation of the *H. atroviridis* atroviridin by MALDI-TOF analysis

m/z value(s)	Compound identity	Explanation
1,920	Atroviridin A	—Ala + H = 1919; position 6 deletion
1,934	Atroviridin B	–Ala + H = 1933, position 6 deletion
1,948, 1,949	New atroviridin	$-CH_2$ (1961 minus 14)
1,963	Atroviridin A	(1961) + H = 1962
1,987, 1,988	?	
2,001	?	
2,003, 2,004	New atroviridin	$+CH_2$, 1989 + 14
2,017, 2,018	New atroviridin	$+2CH_2$, 1989 + 28

Although we have not produced a delta mutant of *H. atroviridis pbs1*, in silico analysis predicts that the respective gene encodes all of the domains necessary for synthesizing at least 19-residue peptaibols. Amino acid residues 232 to 430 of *H. atroviridis* PBS1 showed 52% identity over 80% of the length of the ketoacyl synthetase domain of *H. jecorina* PAR1, and residues 431 to 815 were 74% identical over 100% of the length of the PAR1 acyltransferase domains and were also highly similar to *T. virens* TEX1. These domains therefore encode the proteins acetylating of the peptaibol N terminus. Amino acid residues 22694 to 23110, on the other hand, were 73% identical over 100% of the length of the alcohol dehydrogenase domain (pfam00106) of PAR1, which is necessary for the reductive cleavage of the final amino acid to generate the C-terminal alcohol. The same domain has been found at the same place in *T. virens* TEX1 and *T. harzianum* SALPS2 (42, 46). Therefore, together with the 19-aa activating, transferring, and condensation domains, the deduced PBS1 protein theoretically contains all of the enzymatic activities necessary and typical to produce peptaibols.

Peptaibols are notoriously microheterogeneous. Wiest et al. (46) emphasized that this multiplicity of products is likely a result of the ability of the activating modules to bind multiple substrates. In addition, this ability may be reflected in different K_m values for different amino acids, because it is known that the microheterogeneity can be influenced by supplementing cultures with a specific amino acid, thereby likely increasing its intracellular concentration (see reference 19). The possibility that multiple peptaibol synthetases would be responsible for generation of this microheterogeneous mixture has been ruled out by Wiest et al. (46), who showed that a *tex1* knockout in *T. virens* eliminated the formation of all 18-residue peptaibols. In


a
IMI /L
Δblr1 /L
Δblr2 /L
IMI /D
Δblr1 /D
Δblr2 /D
b
IMI /L+S
Δblr1 /L+S
Δblr2 /L+S
IMI /D+S
Δblr1 /D+S
Δblr2 /D+S
c
IMI/L+i
Δblr1/L+i
Δblr2/L+i
IMI/D+i
Δblr1/D+i
Δblr2/D+i
% Intensity
Mass (m/z)

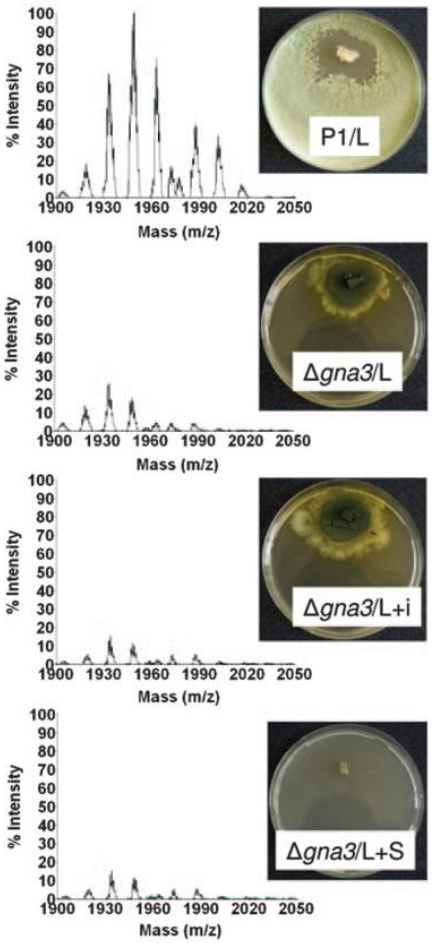


FIG. 5. GNA3 is required for peptaibol production by *H. atroviridis*. *H. atroviridis* P1, and its Δ*gna3* mutant were used. Other symbols are used as explained in Fig. 4. Only experiments performed with illuminated cultures are shown since none of the nonilluminated cultures of the delta-*gna3* mutant showed any peptaibol production. The range of intensity is 0 to 18,000 to make the traces of peptaibols which are still formed more visible. The experiment shown is typical for three independent experiments, whose results were consistent with the data shown here.

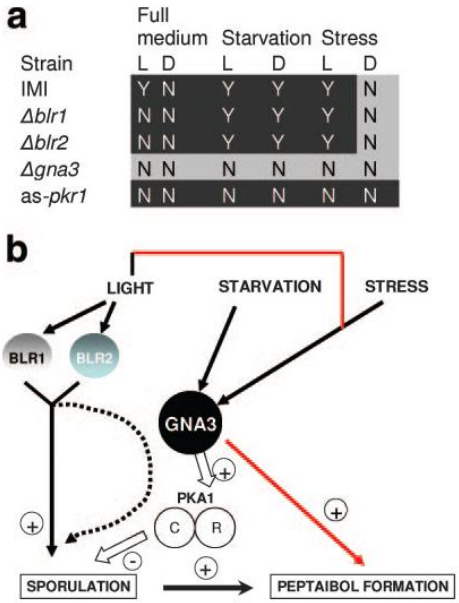


FIG. 6. Schematic summary of the regulation of peptaibol biosynthesis, based on results from the present study. (a) Summary of peptaibol formation under the various conditions in the mutants tested. L, light; D, dark. Strains are abbreviated as indicated in Materials and Methods. "Y" (yes) indicates peptaibol formation; "N" (no) indicates no peptaibol formation. Conditions in which peptaibol formation correlates with sporulation are in white on a black background. (b) Model for the putative interaction of the factors, as studied here, on sporulation and peptaibol formation. Red arrows indicate signaling mechanisms in which peptaibol formation is uncoupled from sporulation. +, activation; −, inhibition. The dotted arrow indicates the possibility of BLR1 and BLR2 action via GNA3 (unpublished data), for which no evidence is presented here.

addition, Wei et al. (45) proved that the small 11-14-mer peptaibols are products of a second, smaller peptaibol synthase. Our genome sequence data support such a claim: in fact, *pbs1* is the only gene in the *H. atroviridis* genome that encodes a peptaibol synthetase consisting of the required number of modules for synthesis of peptaibols with more than 16 residues (C. P. Kubicek, unpublished data). Similar findings have also been made for *H. jecorina* (H. von Doehren, unpublished data). However, our data contribute a new aspect on the multiplicity of peptaibol production: the atroviridins formed contain the sequence Aib5-Aib6-Gln7. while the structure of PBS1 does not contain a second Aib domain preceding the Gln domain. The only explanation that can be offered for this finding is that the U5 module acts twice, forming a hexapeptide intermediate. Such cases of the iterative use of peptide synthetase modules are known in fungal nonribosomal peptide synthetases (43) but have thus far not been reported for peptaibol synthetases. Our data suggest that this could be a further mechanism contributing to peptaibol heterogeneity.

The phylogenetic analysis, which was performed to identify

FIG. 4. BLR1- and BLR2-dependent formation of atroviridin in *H. atroviridis* by different sporulation-inducing pathways. Graphs show the MALDI-TOF spectra of cultures of the wild-type strain (IMI), the delta-*blr1* mutant strain (*blr1*), and the delta *blr2* mutant strain (*blr2*) under illumination (L) and in darkness (D) on ME agar (a), under conditions of starvation (S) (b), and under conditions of mechanical injury (i) (c). See Materials and Methods for details. The ranges of the intensity of the MALDI-TOF spectra were 0 to 28,000 (a), 0 to 23,000 (b), and 0 to 25,000 (c). The insets show photographs of the respective cultures immediately before peptaibol extraction. Data consistent with the claims drawn were obtained in at least one additional, separate experiment.

the individual activating domains, revealed a very high diversity and, with the exception of the domains specific for Pro, Gln, Phe, and in some cases Aib, phylogeny was unable to predict the amino acids bound by these modules. Similar difficulties were observed with the signature sequences proposed by Challis et al. (7) based on a limited set of domains. This high amino acid diversity contrasts with that of the otherwise rather highly conserved transfer and condensation domains (>80% identity). This and the fact that some of the modules activating the same amino acid (e.g., Aib or Ala) occur in different clades of the NJ tree suggests a history of gene duplication and a high mutation rate that ultimately leads to an alteration of the substrate specificity. In addition, the fact that the *pbs1* locus has only partially maintained synteny in *Trichoderma* suggests the possibility of recombination as an additional origin of the diversity of peptaibols in this fungal genus.

The *pbs1* mRNA, in a stretched form, must be about 10 μm long, and it was already emphasized (46) that the mechanism for the transcription of such large mRNAs in eukaryotes is unknown, and the correlation between transcript abundance and expression level is questionable. Therefore, rather than quantifying the *pbs1* transcript, we used MALDI-TOF MS identification of the peptaibols as a means to learn about their regulation of formation. Also, the MALDI-TOF procedure has the advantage that it allows the analysis of a very small part of the growing colony, thereby ensuring the analysis of a homogenous fungal tissue. Unfortunately, it also has the drawback that absolute quantification is impossible since the weight of the fungal material that was extracted could not be determined. Consequently, only extreme changes (present or not present) are used for interpretation. However, using this semiquantitative approach, we could show that the formation of "long" peptaibols by the wild-type of *H. atroviridis* is associated with conidiation. Such an association is not without precedent: as an example, sporulation-deficient mutants of *Aspergillus* spp. (2), *Claviceps purpurea* (28), or *Fusarium verticillioides* (34) have also been shown to be defective in the production of their respective secondary metabolites. Calvo et al. (4) grouped secondary metabolites associated with sporulation into three broad categories: (i) metabolites that are required for the sporulation process (for example, the sporulation-activating linoleic acid-derived compounds produced by *A. nidulans*) (8–10), (ii) metabolites that are required for sporulation structures (for example, pigments such as melanins, which are required for the integrity of sexual and asexual spores) (1, 15), and (iii) metabolites that are toxic and whose formation coincides with the approximate time of sporulation (for example, the biosynthesis of mycotoxins) (14, 41). Based on their fungicidal action, peptaibols would fit into the last group. However, an important difference is that they are not secreted but remain bound to the spores of *Trichoderma*. So could they be important for sporulation in the sense of groups i and ii listed above? Peptaibol synthetase-delta mutants of *T. virens* (46) and of *T. harzianum* (D. Keszenman-Pereyra et al., unpublished data) still sporulate, so the answer would be no. However, both the *T. harzianum* and the *T. virens* delta mutants still form the 11-14 residue peptaibols (46; Keszenman-Pereyra et al., unpublished), and they could compensate for the loss of formation of the 18- to 20-residue peptaibols. In addition, the peptaibols might have subtle effects on sporulation that escape inspection of the morphology only. For example, they are known to function as voltage-gated membrane channels, primarily transporting K^+ (11, 12, 18, 33), which could be important for ion transport and/or homeostasis during sporulation. In fact, gramicidin, another ion channel-forming peptide antibiotic, induces sporulation in its producer *Bacillus subtilis* (24, 29). The necessity for the presence of specific membrane channels in fungal spores has recently been stressed by Sidoux-Walter et al. (36), who showed that sporulation in *S. cerevisiae* is accompanied by the expression of a specific aquaporin, which is responsible for water outflow from spores. Thus, in analogy, one could speculate that the peptaibols are important for K^+ and other monovalent cation homeostasis during the conidiation of *Trichoderma*. We have been unable to find a report describing the effect of peptaibols on the producer organisms, which in the light of the present findings clearly warrants investigation.

Sporulation of *H. atroviridis* is dependent on light (4, 6), and it was therefore not unexpected that the formation of a conidiation-associated secondary metabolite would consequently respond to light as well. However, the present data show that the relationship between light and peptaibol formation is more complex: the fact that mechanical injury can override control by BLR1 and -2 but is still affected by light (i.e., no peptaibol formation was observed in the dark, despite sporulation) suggests that there is a second, light-dependent mechanism that is essential for peptaibol formation. The occurrence of light stimulation of gene expression by an BLR1 and two independent pathways in *H. atroviridis* has been recently shown (31), and it is possible that this pathway may contribute to peptaibol formation under conditions of mechanical injury-induced sporulation.

The G-protein signaling pathway has been shown to be involved in the control of secondary metabolism and conidiation in *Aspergillus* species, particularly in *A. nidulans* (4), but there is also evidence indicating the regulation of trichothecene production in *Fusarium* (40) and γ-pentylpyrone formation in *H. atroviridis* (30) by a similar pathway. Interestingly, not all effects of G proteins (as would be expected from a sporulation-associated process) are negative: while in *A. nidulans* the dominant-activating $fadA^{G42R}$ allele represses conidiation and sterigmatocystin biosynthesis, it stimulates penicillin biosynthesis at the same time (4). Introduction of the $fadA^{G42R}$ allele into *F. sporotrichioides* results in reduced conidiation but increased trichothecene production (40). It is also worth noting that all of the reports on a role of G proteins in fungal secondary metabolism were done with the *Aspergillus* G-protein FadA or its *Trichoderma* orthologue TGA1 (GNA1). In contrast, the present study was undertaken with another G protein, GNA3. Although both GNA1 and GNA3 (previously termed TGA3 [48]) are negative regulators of conidiation in *H. atroviridis* (30, 48), *N. crassa* GNA1 is regulated by the transmembrane receptor protein GPR-4, which is responsible for carbon source signaling (21). The receptor to which GNA3 binds is not known yet. Thus, our data show that not only FadA/GNA1 mediate secondary metabolism, but at least GNA3 also does so. It is tempting to speculate that all G proteins might be able to regulate secondary metabolism, because null mutants in all of them affect sporulation (16). This is understandable in view of the multiple signals (pH, sugar,

nitrogen content, light, and many others) that determine whether a fungal cell can maintain a vegetative mode of growth or whether it is advisable to conidiate.

The subsequent steps required for triggering of peptaibol formation by GNA3 are unclear. If this occurred via activation of adenylate cyclase and thus the formation of cAMP, one would assume that the *H. atroviridis* strain harboring the antisense gene for the regulatory subunit of PKA and thus bearing a constitutively active PKA would overproduce peptaibols. This was shown not to be the case, and indeed, at the level of PKA, peptaibol formation again strictly correlated with sporulation and was therefore absent in the PKA-overproducing mutant. The finding of loss of sporulation is consistent with the hypersporulating phenotype of the delta-*gna3* strain and suggests that the pathway triggering sporulation in *H. atroviridis* is subject to a negative control by a G-protein/PKA pathway similar to that in *A. nidulans* (35). Hence, the positive effect of GNA3 on peptaibol biosynthesis must involve another signaling pathway for which, thus far, only the requirement for GNA3 is known.

ACKNOWLEDGMENTS

This study was supported by the Fifth (EC) Framework program (Quality of Life and Management of Living Resources; project EUROFUNG 2 [QLK3-1999-00729]) and by a grant from the Austrian Science Foundation (P 17325-B17) to C.P.K. The genomic sequence for *H. atroviridis* was provided by the DOE Joint Genome Institute, Walnut Creek, CA. This study was performed under the auspices of the U.S. Department of Energy's Office of Science, Biological, and Environmental Research Program and the by the University of California, Lawrence Livermore National Laboratory, under contract W-7405-Eng-48; the Lawrence Berkeley National Laboratory under contract DE-AC03-76SF00098; and the Los Alamos National Laboratory under contract W-7405-ENG-36.

We thank S. Zeilinger for the *tga3*-delta strain of *H. atroviridis* and S. Baker for help with the *H. atroviridis* genome sequence.

REFERENCES

1. **Alspaugh, J. A., J. R. Perfect, and J. Heitman.** 1997. *Cryptococcus neoformans* mating and virulence are regulated by the G-protein alpha subunit GPA1 and cAMP. Genes Dev. **11:**3206–3217.
2. **Bennett, J. W., and K. E. Papa.** 1988. The aflatoxigenic *Aspergillus*, p. 264–280. *In* D. S. Ingram and P. A. Williams (ed.), Genetics of plant pathology, vol. 6. Academic Press, London, United Kingdom.
3. Reference deleted.
4. **Calvo, A. M., R. A. Wilson, J. W. Bok, and N. P. Keller.** 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. **66:**447–459.
5. **Casas-Flores, S., M. Rios-Momberg, M. Bibbins, P. Ponce-Noyola, and A. Herrera-Estrella.** 2004. BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*. Microbiology **150:**3561–3569.
6. **Casas-Flores, S., M. Rios-Momberg, T. Rosales-Saavedra, P. Martinez-Hernandez, V. Olmedo-Monfil, and A. Herrera-Estrella.** 2006. Cross talk between a fungal blue-light perception system and the cyclic AMP signaling pathway. Eukaryot. Cell **5:**499–506.
7. **Challis, G. L., J. Ravel, and C. A. Townsend.** 2000. Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. Chem. Biol. **7:**211–224.
8. **Champe, S. P., and A. A. E. El-Zayat.** 1989. Isolation of a sexual sporulation hormone from *Aspergillus nidulans*. J. Bacteriol. **171:**3982–3988.
9. **Champe, S. P., P. Rao, and A. Chang.** 1987. An endogenous inducer of sexual development in *Aspergillus nidulans*. J. Gen. Microbiol. **133:**1383–1388.
10. **Chang, P.-K., J. W. Cary, D. Bhatnagar, T. E. Cleveland, J. W. Bennett, J. E. Linz, C. P. Woloshuk, and G. A. Payne.** 1993. Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. Appl. Environ. Microbiol. **59:**3273–3279.
11. **Chugh, J. K., and B. A. Wallace.** 2001. Peptaibols: models for ion channels. Biochem. Soc. Trans. **29:**565–570.
12. **Duclohier, H., G. M. Alder, C. L. Bashford, H. Bruckner, J. K. Chugh, and B. A. Wallace.** 2004. Conductance studies on trichotoxin_A50E and implications for channel structure. Biophys. J. **87:**1705–1710.
13. **Harman, G. E., and C. P. Kubicek.** 1998. *Trichoderma* and *Gliocladium*, vol. 2. Enzymes, biocontrol, and commercial applications. Taylor & Francis, London, United Kingdom.
14. **Hicks, J., J.-H. Yu, N. Keller, and T. H. Adams.** 1997. *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA G-alpha protein-dependent signaling pathway. EMBO J. **16:**4916–4923.
15. **Kawamura, C., T. Tsujimoto, and T. Tsuge.** 1999. Targeted disruption of a melanin biosynthesis gene affects conidial development and UV tolerance in the Japanese pear pathotype of *Alternaria alternata*. Mol. Plant-Microbe Interact. **12:**59–63.
16. **Kays, A. M., and K. A. Borkovich.** 2004. Severe impairment of growth and differentiation in a *Neurospora crassa* mutant lacking all heterotrimeric G alpha proteins. Genetics **166:**1229–1240.
17. **Kays, A. M., P. S. Rowley, R. A. Baasiri, and K. A. Borkovich.** 2000. Regulation of conidiation and adenylyl cyclase levels by the Gα protein GNA-3 in *Neurospora crassa*. Mol. Cell. Biol. **20:**7693–7705.
18. **Kropacheva, T. N., and J. Raap.** 2002. Ion transport across a phospholipid membrane mediated by the peptide trichogin GA IV. Biochim. Biophys. Acta **1567:**193–203.
19. **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. Biodiv. **4:**1068–1082.
20. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5:**150–163.
21. **Li, L., and K. A. Borkovich.** 2006. GPR-4 is a predicted G-protein-coupled receptor required for carbon source-dependent asexual growth and development in *Neurospora crassa*. Eukaryot. Cell **5:**1287–1300.
22. **Lorito, M., V. Farkas, S. Rebuffat, B. Bodo, and C. P. Kubicek.** 1996. Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum*. J. Bacteriol. **178:**6382–6385.
23. **Lucaciu, M., S. Rebuffat, C. Goulard, H. Duclohier, G. Molle, and B. Bodo.** 1997. Interaction of the 14-residue peptaibols, harzianins HC, with lipid bilayers: permeability modifications and conductance properties. Biochim. Biophys. Acta **1323:**85–96.
24. **Marahiel, M. A., M. M. Nakano, and P. Zuber.** 1993. Regulation of peptide antibiotic production in *Bacillus*. Mol. Microbiol. **7:**631–636.
25. **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.
26. **Oh, S. U., B. S. Yun, S. J. Lee, J. H. Kim, and I. D. Yoo.** 2002. Atroviridins A-C and neoatroviridins A-D, novel peptaibol antibiotics produced by *Trichoderma atroviride* F80317. I. Taxonomy, fermentation, isolation, and biological activities. J. Antibiot. **55:**557–564.
27. **Papavizas, G. C.** 1985. *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. Annu. Rev. Phytopathol. **23:**23–54.
28. **Pazoutova, S., V. Pokorny, and Z. Rehacek.** 1977. The relationship between conidiation and alkaloid production in saprophytic strains of *Claviceps purpurea*. Can. J. Microbiol. **23:**1182–1187.
29. **Pschorn, W., H. Paulus, J. Hansen, and H. Ristow.** 1982. Induction of sporulation in *Bacillus brevis*. 2. Dependence on the presence of the peptide antibiotics tyrocidine and linear gramicidin. Eur. J. Biochem. **129:**403–407.
30. **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.
31. **Rosales-Saavedra, T., E. U. Esquivel-Naranjo, S. Casas-Flores, P. Martinez-Hernandez, E. Ibarra-Laclette, C. Cortes-Penagos, and A. Herrera-Estrella.** 2006. Novel light-regulated genes in *Trichoderma atroviride*: a dissection by cDNA microarrays. Microbiology **152:**3305–3317.
32. **Schirmböck, M., M. Lorito, Y.-L. Wang, C. K. Hayes, I. Arisan-Atac, F. Scala, G. E. Harman, and C. P. Kubicek.** 1994. Molecular mechanisms involved in biocontrol by *Trichoderma harzianum:* co-induction and synergism of hydrolytic enzymes and pentaibol antibiotics. Appl. Environ. Microbiol. **60:**4364–4370.
33. **Shenkarev, Z. O., T. A. Balashova, Z. A. Yakimenko, T. V. Ovchinnikova, and A. S. Arseniev.** 2004. Peptaibol zervamicin IIb structure and dynamics refinement from transhydrogen bond J couplings. Biophys. J. **86:**3687–3699.
34. **Shim, W.-B., and C. P. Woloshuk.** 2001. Regulation of fumonisin B1 biosynthesis and conidiation in *Fusarium verticillioides* by a cyclin-like (C-type) gene, *FCC1*. Appl. Environ. Microbiol. **67:**1607–1612.
35. **Shimizu, K., and N. P. Keller.** 2001. Genetic involvement of a cAMP-dependent protein kinase in a G-protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. Genetics **157:**591–600.
36. **Sidoux-Walter, F., N. Pettersson, and S. Hohmann.** 2004. The *Saccharomyces cerevisiae* aquaporin Aqy1 is involved in sporulation. Proc. Natl. Acad. Sci. USA **101:**17422–17427.
37. **Stachelhaus, T., and M. A. Marahiel.** 1995. Modular structure of genes

encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. FEMS Microbiol. Lett. **125:**3–14.
38. **Stachelhaus, T., H. D. Mootz, and M. A. Marahiel.** 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem. Biol. **6:**493–505.
39. **Szekeres, A., B. Leitgeb, L. Kredics, Z. Antal, L. Hatvani, L. Manczinger, and C. Vagvolgyi.** 2005. Peptaibols and related peptaibiotics of *Trichoderma*: a review. Acta Microbiol. Immunol. Hung. **52:**137–168.
40. **Tag, A., J. Hicks, G. Garifullina, C. Ake, T. D. Phillips, M. Beremand, and N. P. Keller.** 2000. G-protein signaling mediates differential production of toxic secondary metabolites. Mol. Microbiol. **38:**658–665.
41. **Trail, F., N. Mahanti, and J. Linz.** 1995. Molecular biology of aflatoxin biosynthesis. Microbiology **141:**755–765.
42. **Vizcaino, J. A., R. E. Cardoza, L. Dubost, B. Bodo, S. Gutierrez, and E. Monte.** 2006. Detection of peptaibols and partial cloning of a putative peptaibol synthetase gene from *Trichoderma harzianum* CECT 2413. Folia Microbiol. **51:**114–120.
43. **von Döhren, H.** 2004. Biochemistry and general genetics of non-ribosomal peptide synthetases in fungi. Adv. Biochem. Eng. Biotechnol. **88:**217–264.
44. **Wallace, B. A.** 2000. Common structural features in gramicidin and other ion channels. Bioessays **22:**227–234.
45. **Wei, X., F. Yang, and D. C. Straney.** 2005. Multiple non-ribosomal peptide synthetase genes determine peptaibol synthesis in *Trichoderma virens*. Can. J. Microbiol. **51:**423–429.
46. **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.
47. **Yoder, O. C., and B. G. Turgeon.** 2001. Fungal genomics and pathogenicity. Curr. Opin. Plant Biol. **4:**315–321.
48. **Zeilinger, S., B. Reithner, V. Scala, I. Peissl, M. Lorito, and R. L. Mach.** 2005. Signal transduction by Tga3, a novel G protein alpha subunit of *Trichoderma atroviride*. Appl. Environ. Microbiol. **71:**1591–1597.

5. Which *Trichoderma* species may cause invasive mycoses in immunocompromised humans?

5.1. Inclusion of *Trichoderma longibrachiatum* in the list of opportunistic pathogens of immunocompromised humans

The number of reports about fungal infections of humans expanding enormously in recent years (Santillan Salas *et al.*, 2011). Infections can appear as a result of a broken normal immune barrier (surgery, traumatic inoculation). Host protection system can be also depleted by medical treatments or when the patients suffer from chronic sinusitis (Antal *et al.*, 2005).

As it has been manifested until now, the genus *Trichoderma* is of widespread relevance (Hatvani *et al.*, 2013). The valuable effects of *Trichoderma* isolates are broadly known but more and more data is being published about the clinical importance of the filamentous fungal genus (Kredics *et al.*, 2003).

These infections are characterized by the presence of fine septate hyphae in tissue sections, the so-called "hyalohyphomycosis pathological entity," for which differential diagnosis with invasive aspergillosis is critical (Chouaki *et al.*, 2002).

Trichoderma shows increasing medical importance as an opportunistic human pathogen particularly in immunocompromised patients. *Trichoderma* species can cause localized infections and fatal disseminated disease. There are several reported cases where *Trichoderma longibrachiatum* was isolated: (i) from an infected skin and subcutaneous tissue of a child with acute aplastic anemia and neutropenia (Munoz *et al.*, 1997), (ii) from a brain abscess in a leukemic patient with prolonged neutropenia (Seguin *et al.*, 1995), (iii) from otitis externa in a pediatric patient (Hennequin *et al.*, 2000) and from a HIV-positive patient (American Type Culture Collection, ATCC 208859; Samuels *et al.*, 1998).

Furukawa (1998) described another case namely the severe invasive sinusitis, as an effect of *T. longibrachiatum* infection, in a liver and small bowel transplant recipient. This one was successfully cured.

T. longibrachiatum had been identified as the causal agent in the majority of reported *Trichoderma* mycoses, therefore the genus is now on the growing list of potential fungal pathogens in immunocompromised humans (Trabelsi *et al.*, 2010).

Antal *et al.* (2005) investigated the potential virulence factors of 12 clinical and 9 environmental *T. longibrachiatum* strains in spite of comparison their ability to cause infections in humans. Based on the results all clinical isolates could grow at physiological pH, what is obligatory to colonize human tissues. All questioned *Trichoderma* isolates were able to grow at inflated temperatures (up to 40ºC). Beyond T. longibrachiatum, other strains (like *H. orientalis*) while being the members of *Longibrachiatum* section of the genus *Trichoderma,* exposed the capability to grow at 37ºC. This finding is a critical issue due to the colonization of human hosts. Experiments of the carbon and nitrogen utilization showed that all of the isolates were able to utilize various basic amino acids both as sole carbon and nitrogen sources. Furthermore, compounds produced by three clinical samples reduced the motility of boar spermatozoa suggesting that they possess toxic effect to mammalian cells as well.

Kredics *et al.* (2004) checked the possible involvement of proteases in the pathogenesis of *Trichoderma* isolates. At the same time, Dóczi *et al.* (2004) reported that *T. longibrachiatum* exhibits overall high levels of resistance to antifungal compounds along with itraconazole, fluconazole and in some instances also amphotericin B.

Remarkable strength of some clinical samples to antifungal medicaments reveals dilemma towards the healing of *Trichoderma* infected patients (Hatvani *et al.*, 2013). In order to find appropriate methods of therapy in some cases of *Trichoderma* infections, Munoz *et al.* (1997) suggested to use combination of amphotericin B and itraconazol or ketoconazole. Another treatment proposed De Miguel *et al.* (2005) as well as Alanio *et al.* (2008) by using caspofungin combined with voriconazole. Nevertheless, before starting with the individual medical treatment, should be clarified the type and the expansion of fungal infection, the health status of patient and the primary predisposing conditions (Hatvani *et al.*, 2013).

References

Alanio A, Brethon B, Feuilhade de Chauvin M, De Kerviler E, Leblanc T,Lacroix C, Baruchel A, Menotti J (2008) Invasive pulmonary infection due to *Trichoderma longibrachiatum* mimicking invasive aspergillosis in a neutropenic patient successfully treated with voriconazole combined with caspofungin. *Clin Infect Dis* **46**, e116–e118.

Antal Z, Szekeres A, Pakarinen J, Dóczi I, Andersson M, Salkinoja-Salonen M, Manczinger L, Szekeres A, Hatvani L, Vágvölgyi C & Nagy E. (2005) Comparative study of potential virulence factors in human pathogenic and saprophytic *Trichoderma longibrachiatum* strains. *Acta Microbiol Immunolog Hung* **52**, 341-350.

Chouaki T, Lavarde V, Lachaud L, Raccurt CP & Hennequin C (2002) Invasive infections due to *Trichoderma* species: report of 2 cases, findings of in vitro susceptibility testing, and review of the literature. *Clin Infect Dis* **35**(11), 1360-7.

De Miguel D, Gómez P, González R, García-Suárez J, Cuadros J A, Bañas MH, Romanyk J, Burgaleta C **(2005)** Nonfatal pulmonary*Trichoderma viride* infection in an adult patient with acute myeloid leukemia: report of one case and review of the literature. *Diagn Microbiol Infect Dis* **53**,33–37.

Dóczi I, Dosa E, Varga J, Antal Z, Kredics L & Nagy E (2004) Etest for assessing the susceptibility of filamentous fungi. *Acta Microbiol Immunolog Hung* **5**, 271-281.

Furukawa H, Kusne S, Sutton DA, Manez R, Carrau R, Nichols L, Abu-Elmagd K, Skedros D, Todo S, Rinaldi MG (1998) Acute invasive sinusitis due to *Trichoderma longibrachiatum* in a liver and small bowel transplant recipient. *Clin Infect Dis* **26,** 487-489.

Hatvani L, Manczinger L, Vágvölgyi C & Kredics L (2013) *Trichoderma* as human pathogen. In book: *Trichoderma* - Biology and Applications, Edition: first, Chapter 17: *Trichoderma* as a human pathogen, Publisher: CABI, Editors: Mukherjee PK, Horwitz BA, Singh US, Mukherjee M, Schmoll M, 292-313.

Hennequin C, Chouaki T, Pichon JC, Strunski V & Raccurt C (2000) Otitis externa due to *Trichoderma longibrachiatum*. *Eur J Clin Microbiol Infect Dis* **19,** 641-642.

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

Kredics L, Antal Z, Szekeres A, Manczinger L, Dóczi I, Kevei F & Nagy E (2004) Production of extracellular proteases by human pathogenic *Trichoderma longibrachiatum* strains. *Acta Microbiol Immunolog Hung* **51**, 283-295.

Munoz FM, Demmler GJ, Travis WR, Ogden AK, Rossmann SN & Rinaldi MG (1997) *Trichoderma longibrachiatum* infection in a pediatric patient with aplastic anemia. *J Clin Microbiol* **35,** 499-503.

Samuels GJ, Petrini O, Kuhls K, Lieckfeldt E & Kubicek CP (1998) The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *Longibrachiatum*. *Stud Mycol* **41,** 1-54.

Santillan Salas CF, Joshi AY, Dhiman N, Banerjee R, Huskins WC, Wengenack NL & Henry NK (2011) Fatal post-operative *Trichoderma longibrachiatum* mediastinitis and peritonitis in a paediatric patient with complex congenital cardiac disease on peritoneal dialysis. *Journal of Medical Microbiology* **60**(12), 1869-1871.

Seguin P, Degeilh B, Grulois I, Gacouin A, Maugendre S, Dufour T, Dupont B & Camus C (1995) Successful treatment of a brain abscess due to *Trichoderma longibrachiatum* after surgical resection. *Eur J Clin Microbiol Infect Dis* **14,** 445-448.

Trabelsi S, Hariga D & Khaled S (2010) First case of *Trichoderma longibrachiatum* infection in a renal transplant recipient in Tunisia and review of the literature. *Tunis Med* **88**(1), 52-7.

5.2. Alternative reproductive strategies of *Hypocrea oreintalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans.

Druzhinina IS, **Komon-Zelazowska M**, Kredics L, Hatvani L, Antal Z, Belayneh T & Kubicek CP (2008) Alternative reproductive strategies of *Hypocrea oreintalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. ***Microbiology*** **154, 3447-3459**

OWN CONTRIBUTION:

MKZ performed strains cultivation, DNA extraction, amplified ITS1 and 2, *tef1* and *chi18-5* loci, participated in sequence analysis and submitted sequences to NCBI GenBank. She also contributed to the writing of the ms.

Microbiology (2008), **154**, 3447–3459 DOI 10.1099/mic.0.2008/021196-0

Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans

Irina S. Druzhinina,[1] Monika Komoń-Zelazowska,[1] László Kredics,[2] Lóránt Hatvani,[2] Zsuzsanna Antal,[2] Temesgen Belayneh[1] and Christian P. Kubicek[1]

Correspondence
Irina S. Druzhinina
druzhini@mail.zserv.tuwien.ac.at

[1]Research Area of Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, A-1060 Vienna, Austria

[2]Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, H-6726 Szeged, Hungary

The common soil fungus *Trichoderma* (teleomorph *Hypocrea*, Ascomycota) shows increasing medical importance as an opportunistic human pathogen, particularly in immunocompromised and immunosuppressed patients. Regardless of the disease type and the therapy used, the prognosis for *Trichoderma* infection is usually poor. *Trichoderma longibrachiatum* has been identified as the causal agent in the majority of reported *Trichoderma* mycoses. As *T. longibrachiatum* is very common in environmental samples from all over the world, the relationship between its clinical and wild strains remains unclear. Here we performed a multilocus (ITS1 and 2, *tef1*, *cal1* and *chit18-5*) phylogenetic analysis of all available clinical isolates (15) and 36 wild-type strains of the fungus including several cultures of its putative teleomorph *Hypocrea orientalis*. The concordance of gene genealogies recognized *T. longibrachiatum* and *H. orientalis* to be different phylogenetic species, which are reproductively isolated from each other. The majority of clinical strains (12) were attributed to *T. longibrachiatum* but three isolates belonged to *H. orientalis*, which broadens the phylogenetic span of human opportunists in the genus. Despite their genetic isolation, *T. longibrachiatum* and *H. orientalis* were shown to be cosmopolitan sympatric species with no bias towards certain geographical locations. The analysis of haplotype association, incongruence of tree topologies and the split decomposition method supported the conclusion that *H. orientalis* is sexually recombining whereas strict clonality prevails in *T. longibrachiatum*. This is a rare case of occurrence of sexual reproduction in opportunistic pathogenic fungi. The discovery of the different reproduction strategies in these two closely related species is medically relevant because it is likely that they would also differ in virulence and/or drug resistance. Genetic identity of environmental and clinical isolates of *T. longibrachiatum* and *H. orientalis* suggests the danger of nosocomial infections by *Hypocrea/Trichoderma* and highlights the need for ecological studies of spore dispersal as source of invasive human mycoses.

Received 7 June 2008
Revised 14 July 2008
Accepted 21 July 2008

INTRODUCTION

Many mould species are capable of causing invasive mycoses of mammals, including humans, under appropriate conditions, but of the more than one million fungal species which are estimated to exist in nature, only a few hundred have been associated with human and animal diseases (Walsh & Groll, 1999). In the last decade, however, case reports on infections by common mould fungi have increased, due to HIV/AIDS and the use of immunosuppressants for organ transplantation and cancer therapies. Species from the fungal genus *Hypocrea/Trichoderma* (Hypocreales, Ascomycota) have recently also joined this emerging list of such opportunistic pathogens. Detailed case reports of *Trichoderma* infections have been summarized by Kredics *et al.* (2003). Typically, these include several isolations from the peritoneal effluent of dialysis patients, infections of immunocompromised transplant recipients, and patients suffering from leukaemia, brain abscesses and HIV (Furukawa *et al.*, 1998; Hennequin *et al.* 2000; Munoz

Abbreviations: ITS, internal transcribed spacer; MCMC, metropolis-coupled Markov chain Monte Carlo; PHT, partition homogeneity test.

et al. 1997; Myoken *et al.* 2002). While *Trichoderma* isolates are still not a major threat, they nevertheless pose difficult diagnostic and therapeutic challenges because (i) without rapid diagnosis and treatment their clinical manifestations can be fatal (Seguin *et al.*, 1995; Tanis *et al.*, 1995; Richter *et al.*, 1999; Chouaki *et al.*, 2002; Myoken *et al.*, 2002; Tang *et al.*, 2003), (ii) they are difficult to identify by morphological analysis (Druzhinina *et al.*, 2005), and (iii) they are resistant to most antifungal agents (Kratzer *et al.*, 2006).

The sources of human and animal infections by *Trichoderma* species, which typically are cosmopolitan soil-borne fungi frequently found in the rhizosphere or as endophytes (Klein & Eveleigh 1998; Harman *et al.*, 2004), are not known at present. Sequence analysis of the internal transcribed spacers 1 and 2 (ITS1 and 2) of the rRNA gene cluster identified all strains isolated from clinical patients – with a few exceptions – as *Trichoderma longibrachiatum* (Kuhls *et al.*, 1999; Kredics *et al.*, 2003). This taxon usually represents a common, albeit minor, 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 appears to be more abundant in indoor environments such as water-damaged buildings or mushroom farms infected by green mould disease (Thrane *et al.*, 2001; Hatvani *et al.*, 2007, respectively). Consequently, *T. longibrachiatum* has also been detected in sputum and sinus ethmoidalis of healthy humans (Kredics *et al.*, 2003).

Many facultative pathogenic fungi such as *Trichophyton rubrum*, *Cryptococcus neoformans* and the pathogenic chytrid *Batrachochytrium dendrobatidis* have been shown to be single worldwide distributed clonal lineages (Gräser *et al.*, 1999; Halliday & Carter, 2003; Morehouse *et al.*, 2003, Zhang *et al.*, 2006). However, some other opportunistic human pathogenic fungi such as *Aspergillus fumigatus* exhibit both clonal and recombining history (Nielsen & Heitman, 2007; Pringle *et al.*, 2005). The reproduction strategy and population structure of *T. longibrachiatum* has not been investigated yet. Phylogenetic analyses of *Trichoderma* section *Longibrachiatum* has been limited to the sequence of the ITS regions of the rRNA genes (Kuhls *et al.*, 1997) and RAPD (random amplified polymorphic DNA) fingerprinting (Turner *et al.*, 1997), which both yielded results suggesting clonality for *T. longibrachiatum*. However, as the ascomycete *Hypocrea orientalis* has been proposed as a teleomorph of *T. longibrachiatum* (Samuels *et al.*, 1998), at least some generations of sexual reproduction should thus be detectable in its population history.

Knowledge of the population structures of *T. longibrachiatum* and *H. orientalis*, their relationship and mode(s) of reproduction would therefore aid our understanding of whether clinical infections are caused by certain lineages only (i.e. whether they share the same recent ancestry), and whether the causative agents are present in all or only some geographical areas. The objectives of this study were therefore to (i) investigate the phylogenetic relatedness of a geographically broad sample of *T. longibrachiatum* and *H. orientalis* including clinical and environmental strains; (ii) to detect the genetic origins of clinical isolates; and (iii) to identify the mode of reproduction of these fungi with special emphasis on that of the clinical isolates.

METHODS

Material studied. The strains, their origin and the sequences 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 (TU Wien). Strains are grouped according to their identification in the present work. For convenience, TU Wien collection codes (C.P.K.) are used for the strains throughout the work, but other collection numbers are also listed in Table 1.

DNA extraction, PCR amplification and sequencing. Mycelia were harvested after 2–4 days growth on 3 % malt extract agar (MEA) at 25 °C and genomic DNA was extracted using the Qiagen DNeasy Plant Maxi kit following the manufacturer's protocol. Amplification of the nuclear rRNA gene cluster, containing ITS1 and 2 and the 5.8S rRNA gene, and of a fragment of the endochitinase gene *chi18-5* (formerly named *ech42*) was done as described previously (Komoń-Zelazowska *et al.*, 2007). The fourth large intron of *tef1* (translation elongation factor 1-α) was amplified using primers EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and TEF1-LLErev (5′-AACTTGCAGGCAATGTGG-3′) (Jaklitsch *et al.*, 2006), and a fragment of *cal1* (calmodulin) using primers CAL-228F (5′-GAGTTCAAGGAGGCCTTCTCCC-3′) and CAL-737R (5′-CATCTTTCTGGCCATCATGG-3′) (Chaverri *et al.*, 2003). Purified PCR products for ITS1 and 2, *tef1*, *cal1* and *chit18-5* were subjected to automatic sequencing at MWG (Martinsried, Germany). NCBI GenBank accession numbers of the corresponding sequences are given in Table 1.

Sequence analysis. DNA sequences were aligned with CLUSTAL X 1.81 (Thompson *et al.*, 1997) and then visually edited using GeneDoc 2.6 (Nicholas & Nicholas, 1997). 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 (Rozas *et al.*, 2003). The neutral evolution of coding sequences (*cal1* and *chi18-5*) was tested by Tajima test implemented in the same software. The interleaved NEXUS file was formatted using PAUP*4.0b10 (Swofford, 2002) and manually formatted for the MrBayes v3.2 program (Ronquist & Huelsenbeck, 2003). The best nucleotide substitution model for each locus was determined using jMODELTEST (Posada, 2008). As Akaike and Bayesian information criteria [AIC (Akaike, 1974) and BIC (Schwarz, 1978), respectively] selected different nucleotide substitution models for every locus and due to the relatively small size of individual datasets (731 characters per 51 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 with two simultaneous runs of four incrementally heated chains that performed either 1 or 3 million generations. The length of run (number of generations) for each dataset was determined using the AWTY graphical system (Wilgenbusch *et al.*, 2004, online at http://ceb.csit.fsu.edu/awty) to check the convergence of MCMC; all analyses were repeated at least twice. 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 'burn' command. The number of 'burned' generations was determined for every run based on visual

Table 1. Clinical and environmental strains of *Trichoderma*, their origin, and NCBI accession numbers of corresponding sequences

Species	TU Wien identifier*	Alternative strain nos	Substratum	Country	ITS1 and 2	*tef1*	*chi18-5*	*cal1*
Trichoderma longibrachiatum	C.P.K. 1254 *ex*-type	CBS 818.68, DAOM 167674	Mud in creek	Ohio, USA	EU401556	EU401591	EU401511	EU401459
	C.P.K. 1685	UAMH7955	Human sinus	NA	AY328040	EU401596	EU401516	EU401464
	C.P.K. 1686	UAMH7956	Lung tissue of a bone marrow transplantation patient	NA	AY328041	EU401597	EU401517	EU401465
	C.P.K. 1687	UAMH 9515	Peritoneal effluent	NA	AY328035	EU401598	EU401518	EU401466
	C.P.K. 1689	ATCC 201044	Neutropenic child	Texas, USA	AY585879	EU401600	EU401520	EU401468
	C.P.K. 1690	ATCC 208859	HIV-positive patient	Texas, USA	AY328042	EU401601	EU401521	EU401469
	C.P.K. 1691	CBS 446.95	Lung tissue	Vienna, Austria	AY328039	EU401602	EU401522	EU401470
	C.P.K. 1692	IP 2110.92	Lung and heart of bone marrow transplantation patient	Villejuif, France	Z82902	EU401603	EU401523	EU401471
	C.P.K. 1693	CNM-CM 382	Peritoneal fluid of CAPD† patient	Las Palmas, Spain	AY328034	EU401604	EU401524	EU401472
	C.P.K. 1695	CNM-CM 1798	Blood culture of patient with liver transplant	Spain	AY920396	EU401605	EU401525	EU401473
	C.P.K. 1696	CNM-CM 2171	Foot skin of premature infant with subcutaneous lesions	Spain	AY920397	EU401606	NA	EU401474
	C.P.K. 1697	CNM-CM 2277	Sputum of tuberculosis patient	Spain	AY920398	EU401607	EU401526	EU401475
	C.P.K. 2882	SzMC IM3	Sinus lavage sample of a rhinosinusitis patient	NA	EU401576	EU401627	EU401546	EU401495
	C.P.K. 2879	SzMC Thg	Water-damaged building	Finland	EU401573	EU401624	EU401543	EU401492
	C.P.K. 1698	CNM-CM 1698, IMI 297702	Biocontrol strain	Spain	AY585880	EU401608	EU401527	EU401476
	C.P.K. 2058	SzMC 3102, B17	*Agaricus* compost	Hungary	NA	EU401620	EU401539	EU401488
	C.P.K. 850	Y 20, CBS 115341	Loamy sand, guava plantation	Alexandria, Egypt	EU401555	EU401590	EU401510	EU401458
	C.P.K. 1749	DAOM 231259	Sandy soil, parkland	Kigali, Rwanda	EU401562	EU401611	EU401530	EU401479
	C.P.K. 1750	DAOM 231258	Sandy soil, parkland	Kigali, Rwanda	EU401563	EU401612	EU401531	EU401480
	C.P.K. 2056	SzMC 3001, A35	*Pleurotus* cultivation substratum	Hungary	EU401569	EU401619	EU401538	EU401487
	C.P.K. 624	TUB F-363	Soil	Lisbon, Portugal	EU401551	EU401582	EU401501	EU401449
	C.P.K. 45	IMI 297702	Soil	Egypt	NA	EU401578	EU401497	EU401445
	C.P.K. 848	Y 17, IMI 155340	Clay, under orange tree	El-Mansura, Egypt	EU401553	EU401588	EU401508	EU401456
	C.P.K. 1889	PPRC S3	Red soil, coffee-growing region	Ethiopia	EU401567	EU401617	EU401536	EU401485
	C.P.K. 1301	IMI 287096	Unknown	India	EU401559	EU401594	EU401514	EU401462
	C.P.K. 1303	IMI 291914	Unknown	Antarctica	EU401560	EU401595	EU401515	EU401463
	C.P.K. 2062	CECT 2412	Mushroom compost	Wales, UK	EU401572	EU401623	EU401542	EU401491
	C.P.K. 2059	SzMC 3103, B19	*Agaricus* compost	Hungary	EU401570	EU401621	EU401540	EU401489
	C.P.K. 1707		Soil	Kazan, Russia	EU401561	EU401610	EU401529	EU401478
	C.P.K. 680	TUB F-828	Flower soil	Mexico City, Mexico	AY857222	EU401583	EU401502	EU401450
	C.P.K. 1890	PPRC S8	Red soil, coffee-growing region	Ethiopia	EU401568	EU401618	EU401537	EU401486
	C.P.K. 710	TUB F-1036	Soil in artificial rain forest	Mexico City, Mexico	AY857241	EU401586	EU401506	EU401454
	C.P.K. 1815	PPRC J9	Red soil, coffee-growing region	Jimma, Ethiopia	EU401564	EU401613	EU401532	EU401481

Table 1. cont.

Species	TU Wien identifier*	Alternative strain nos	Substratum	Country	ITS1 and 2	*tef1*	*chi18-5*	*cal1*
	C.P.K. 842	Y 11, CBS 115338	Sandy loam, clover field	El-Minia, Egypt	EU401552	EU401587	EU401507	EU401455
	C.P.K. 849	Y 19	Loamy soil, wheat field	El-Kharga, Egypt	EU401554	EU401589	EU401509	EU401457
	C.P.K. 42	IAA1	Tea plantation	Kenya	EU401547	EU401577	EU401496	EU401444
Hypocrea orientalis	C.P.K. 166 *ex*-type	G.J.S. 88-81, ATCC 90550	Stump at burn site	Yunnan, China	EU401550	EU401581	EU401500	EU401448
	C.P.K. 704	TUB F-1023	Soil	Iguazo Falls, Argentina	AY857238	EU401585	EU401505	EU401453
	C.P.K. 1294	G.J.S. 91-157	Mud, Hoeloch caves	Switzerland	EU401558	EU401593	EU401513	EU401461
	C.P.K. 109	PPRI 3894	From moss in the Black Forest	South Africa	EU401548	EU401579	EU401498	EU401446
	C.P.K. 1699	CECT 2606, IMI 061758	Soil	Kenema, Sierra Leone	X93929	EU401609	EU401528	EU401477
	C.P.K. 683	TUB F-831	Soil	Costa Rica	AY857225	EU401584	EU401503	EU401451
	C.P.K. 688	TUB F-837	Soil 2500 m elevation	Costa Rica	AY857230	AY857282	EU401504	EU401452
	C.P.K. 112	ICMP 1694	Unknown	New Zealand	EU401549	EU401580	EU401499	EU401447
	C.P.K. 2880	SzMC IM1	Child with acute lymphoblastic leukaemia	NA	EU401574	EU401625	EU401544	EU401493
	C.P.K. 2881	SzMC IM2	Stool of a paediatric patient	NA	EU401575	EU401626	EU401545	EU401494
	C.P.K. 1688	UAMH 9573	Peritoneal catheter tip	Newfoundland, Canada	AY328038	EU401599	EU401519	EU401467
T. sp. PS III	C.P.K. 1817	PPRC J11	*Coffea arabica* rhizosphere	Jimma, Ethiopia	EU401565	EU401614	EU401533	EU401482
	C.P.K. 1837	PPRC W4	*C. arabica* rhizosphere	Wellega, Ethiopia	EU401566	EU401615	EU401534	EU401483
	C.P.K. 1841	PPRC H5	*C. arabica* rhizosphere	Harerga, Ethiopia	NA	EU401616	EU401535	EU401484
H. sp. PS IV	C.P.K. 1261	CBS 243.63	Under *Nothofagus*	New Zealand	EU401557	EU401592	EU401512	EU401460

NA, Not available.
*Bold type highlights clinical strains.
†CAPD, continuous ambulatory peritoneal dialysis.

Table 2. Nucleotide characteristics of loci and parameters of phylogenetic analyses

AIC and BIC, Akaike information criterion (Akaike, 1974) and Bayesian information criterion (Schwarz, 1978) as implemented in jMODELTEST (Posada, 2008); TIM1 (Posada, 2003) and TIM2 (Posada, 2008), nucleotide substitution models with six free parameters and unequal base frequencies; TN (Tamura & Nei, 1993), nucleotide substitution model with five free parameters and unequal base frequencies; K80 (Kimura, 1980), nucleotide substitution model with one free parameter and equal base frequencies; HKY (Hasegawa *et al.*, 1985), nucleotide substitution model with four free parameters and unequal base frequencies; GTR (Tavaré, 1986), general time reversible model with eight free parameters; *I*, proportion of invariable sites; *G*, gamma rates; NS, not significant; NA, not applicable.

Parameters	Phylogenetic marker		
	cal1	***chi18-5***	***tef1***
Fragment features	Exon/intron	Exon	Intron
No. of sequences	51	50	51
No. of characters	429	731	515
Parsimony informative	31	32	49
Constant	390	663	440
	Parameters of MCMC analysis		
Substitution models selected by AIC and BIC	TIM2 + G and K80 + G	TIM1 + G and HKY + G	TrN + G and K80
MCMC generations, millions	1	1	3
No. of chains/Temp. (λ)	4/0.2	4/0.2	4/0.2
Sampling frequency	100	100	100
'Burned'	300	300	300
Mean nt frequencies* A/C/G/T	0.24/0.29/0.27/0.20	0.22/0.33/0.25/0.20	0.21/0.32/0.21/0.26
Substitution rates*			
A↔C	0.06	0.06	0.78
A↔G	0.36	85.84	90.40
A↔T	0.23	0.11	0.30
C↔G	0.08	0.46	0.33
C↔T	80.60	7.11	0.51
G↔T	1	1	1
α*	0.06	0.06	0.08
Total tree length*	7.05	5.09	6.73
	DNA polymorphism analysis		
No. of sites excluding gaps and missing data	377	395	445
Segregation sites	32	34	57
No. of mutations, η	37	35	58
No. of alleles (haplotypes)	11	9	15
Haplotype diversity	0.83	0.84	0.68
Nucleotide diversity, π	0.01	0.03	0.02
θ per site from η	0.02	0.03	0.03
		Neutrality analysis	
Tajima test	NS (-1.18, $P>0.1$)	NS (-1.26, $P>0.1$)	NA

*As estimated after GTR MCMC sampling and burning.

analysis of the plot showing generation versus the log probability of observing the data. According to the protocol of Leache & Reeder (2002) PP values lower than 0.95 were not considered significant while values below 0.9 are not shown on the resulting phylograms. Model parameter summaries after MCMC runs and burning first samplings as well as nucleotide characteristics of the loci used are collected in Table 2.

Detection of recombination. Recombination within individual phylogenetic clades was tested by multiple tools. (a) The index of association (I_A), which measures whether the alleles from the different loci in a population are randomly or nonrandomly associated in the analysed genomes (Maynard Smith, 1992), and which was computed by using the linkage disequilibrium (LD) analysis available on the MLST website (http://linux.mlst.net/link_dis/index.htm); significance was gauged from 1000 random permutations of the data. (b) The partition homogeneity test (PHT) integrated in PAUP*4.0b10 (Swofford, 2002), which estimates the congruence among different loci datasets (Cunningham, 1997). For this test heuristic search under the parsimony optimality criterion was used, parsimony-uninformative characters were excluded, gaps were treated as missing, and 10 000 repetitions were performed. A maximum of 100 trees were saved to conserve memory. (c) Recombination tests implemented in the RecombiTEST package available at http://www.lifesci.sussex.ac.

uk/CSE/test/. (d) The ϕ_w test (pairwise homoplasy index, Phi) as implemented in the SplitsTree software (Huson, 1998). (e) Visual analysis of topologies of phylogenetic trees.

RESULTS

Phylogenetic species in the sample of clinical *Trichoderma* strains

Our sample consisted of 51 strains, all sharing the same sequence of ITS1 and 2 of the rRNA gene cluster. The sequence was identified as the '*H. orientalis–T. longibrachiatum*' species doublet by the oligonucleotide barcode program *TrichO*Key (Druzhinina *et al.*, 2005; online at http://www.ISTH.info). Among these strains, 15 were obtained as clinical isolates. The other 36 were chosen to cover the broadest possible geographical variability of *T. longibrachiatum* including all available strains (7) of its putative teleomorph *H. orientalis*. In order to investigate the evolutionary relations within this sample, we sequenced three phylogenetic markers used in *Hypocrea/Trichoderma*: the long intron of *tef1*, an intron-containing fragment of *cal1* and a partial exon sequence of *chi18-5*. Nucleotide characteristics of these sequences are given in Table 2.

We used Bayesian analysis of the individual gene datasets to infer a phylogenetic structure (Fig. 1a–c). Three statistically supported clades and a single lone branch were present in the *tef1* and *chi18-5* gene trees (Fig. 1a, b): the PS (phylogenetic species) I clade contained more than half the isolates, including the *ex*-type strain of *T. longibrachiatum*; clade PS II included the *ex*-type strain of *H. orientalis*, all but one (C.P.K. 1261) of the other strains of *H. orientalis*, and three strains originally identified as *T. longibrachiatum*; clade PS III contained three strains isolated from coffee rhizosphere in Ethiopia. The single strain isolated from the teleomorph from New Zealand (C.P.K. 1261) formed a branch with unresolved phylogenetic position within the dataset studied. Resolution of the *cal1* tree (Fig. 1c) was less clear as no statistical support was detected for clade PS I, but its topology was in agreement with the other two loci. The phylogenetic position of strains forming clade PS II in *tef1* and *chi18-5* trees was not well resolved by *cal1* analysis as C.P.K. 2880 and C.P.K. 2881 formed a topologically separated subclade (but without statistical support) from other strains of PS II. Positions of C.P.K. 1261 and PS III on the *cal1* tree were concordant with the other two loci.

This analysis shows that the three clades and the single branch are fully supported in two gene trees, and not rejected in the third. Thus, at least three clades (PS I, II and III) fulfil the two criteria of Dettman *et al.* (2003) for multilocus genealogies – i.e. that a clade must be present in the majority of single-locus trees; and that a clade is reliably supported by at least one single-locus genealogy and is not contradicted by any other single-gene tree determined by the same methods (the genealogical nondiscordance criterion). Based on the position of the *ex*-type strains for *T. longibrachiatum* and *H. orientalis* in different clades, we conclude that clade PS I represents *T. longibrachiatum sensu stricto*, and clade PS II consequently represents *H. orientalis*. On the other hand, clade PS III and C.P.K. 1261 are two as yet undescribed species of section *Longibrachiatum* which we will further refer to as *Trichoderma* sp. PS (phylogenetic species) III and *Hypocrea* sp. PS IV, respectively.

The clinical strains of '*T. longibrachiatum*' were only found in clades PS I (12 strains) and II (3 strains) and thus – in contrast to what was believed before – belong to two phylogenetic species (Fig. 1).

Reproductive strategies of *T. longibrachiatum* and *H. orientalis*

In order to test whether the phylogenetically distinct species *T. longibrachiatum* and *H. orientalis* are also separated by a reproductive barrier, we used the split decomposition method provided by the SplitsTree (Huson & Bryant, 2006) package. This analysis enabled us to test for the presence of network relationships within clades PS I, II and III, and various dual and threefold combinations of them, using a concatenated dataset of *tef1*, *cal1* and *chit18-5* (Fig. 2a–c). This method presents conflicting phylogenetic data, presumably arising from recombination, as an interconnected network of lineages. As shown in Fig. 2(a), such a network was evident between the total dataset (four phylogenetic species). However, analyses of individual clades PS I and PS II documented an almost complete lack of a network for *T. longibrachiatum* compared to *H. orientalis*, which is expected to have sexual reproduction in its life cycle as several teleomorphs were collected. When the Phi test (Huson & Bryant, 2006) implemented in the same software package was applied to various concatenated gene combinations of clades PS I, PS II and PS III, evidence for recombination was obtained for every combination (data not shown) and also for clade PS II (*H. orientalis*) alone (ϕ=0.32, P=0.0005), whereas the possibility of recombination was rejected for clade PS I (*T. longibrachiatum*) (ϕ=0.14, P=0.58). These data indicate that in contrast to *H. orientalis*, which represents a sexual population, *T. longibrachiatum* is largely clonal. Strains of PS III could not be analysed individually because they were too low in number.

The partition homogeneity test (PHT; Huelsenbeck *et al.*, 1996) was used to examine the congruence between gene trees. In this test artificial datasets are produced by multiple (10 000) resampling and random swapping of observed datasets and subsequent construction of maximum-parsimony trees for every newly sampled 'gene' sequence. For clonally reproducing populations, the sums of the lengths of the gene trees for the observed and resampled data should be similar, but under recombination the sums of the tree lengths should be longer than that for the actual data because recombination among unlinked sites should introduce homoplasy into the data. When the whole dataset was analysed (PS I–IV; *cal1*, *chi18-5* and *tef1*) the

http://mic.sgmjournals.org

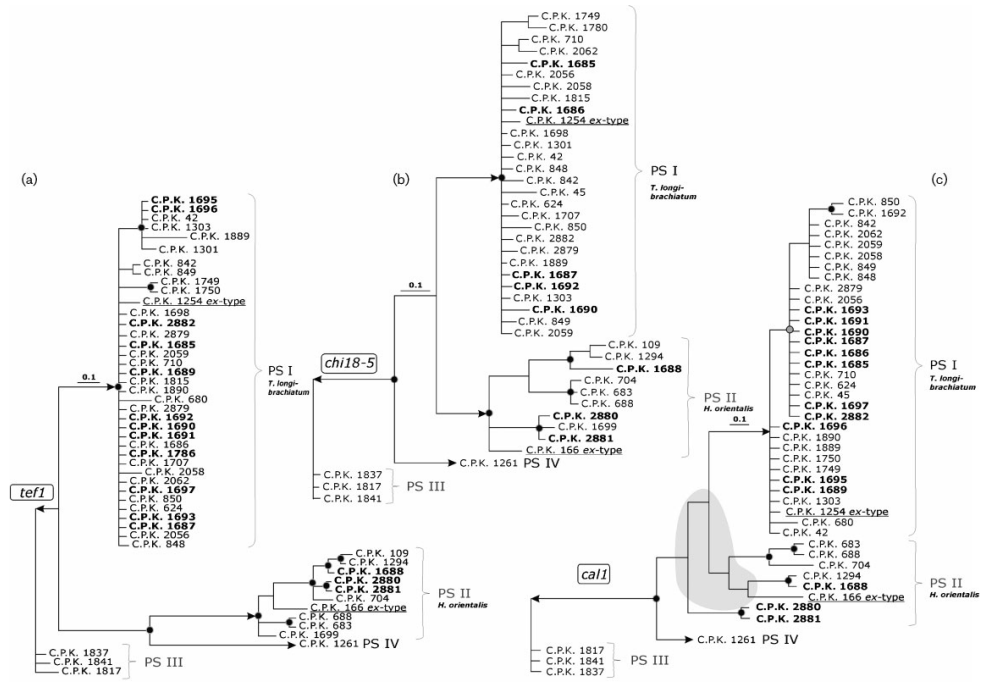


Fig. 1. Bayesian phylogenetic analysis of clinical *Trichoderma* strains (in bold) and environmental isolates of *T. longibrachiatum* and *H. orientalis* based on partial sequences of *tef1* (a), *chi18-5* (b) and *cal1* (c). Nodes marked by black circles indicate posterior probabilities (PP) >0.94; nodes marked by grey circles indicate 0.95>PP>0.90. Arrows mark branches leading to recognized phylogenetic species. *Ex*-type strains of *T. longibrachiatum* and *H. orientalis* are underlined. The shadowing indicates an unresolved area on the *cal1* phylogram.

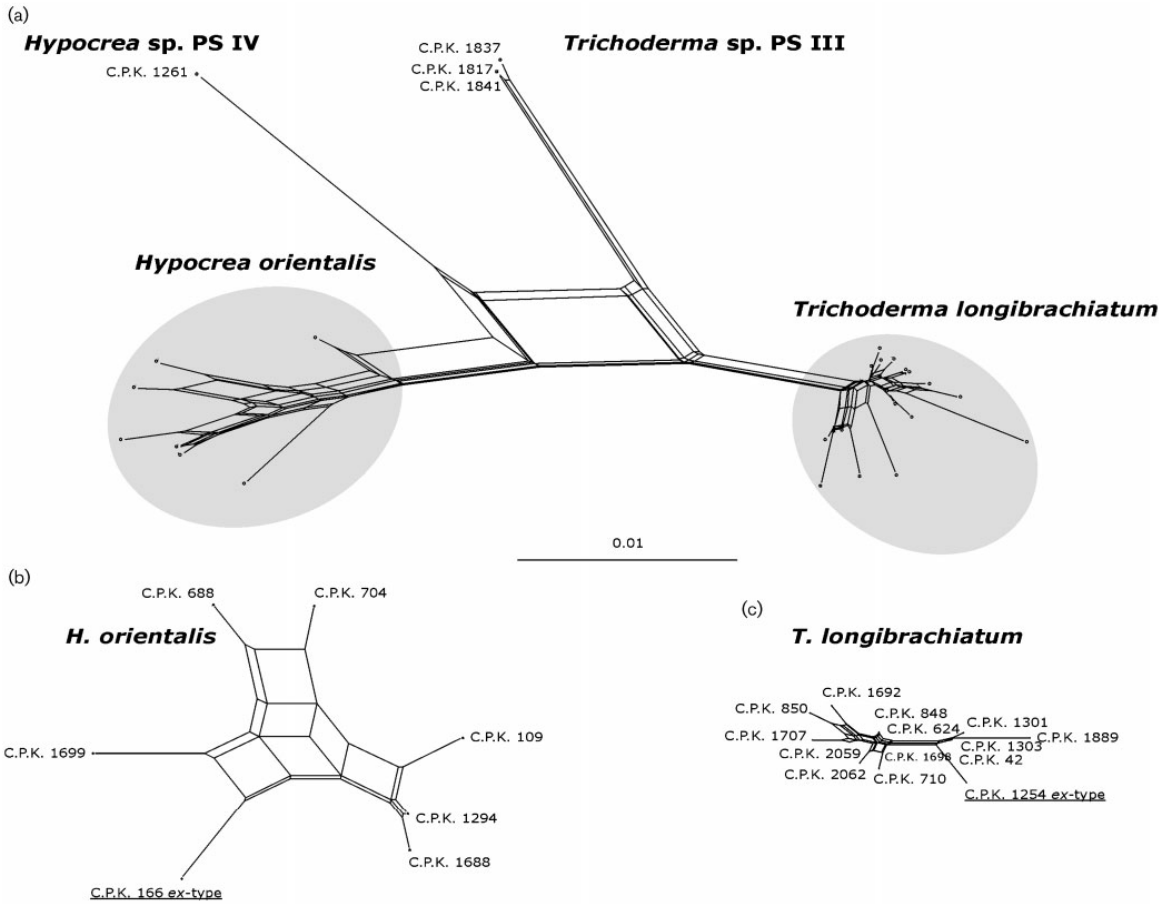


Fig. 2. Reconstruction of possible recombination networks between and within *T. longibrachiatum* and *H. orientalis* by the split decomposition method applied to the concatenated dataset (*tef1*+*cal1*+*chi18-5*). (a) Total sample with four phylogenetic species; (b) strains of *H. orientalis* clade PS II; (c) strains of *T. longibrachiatum* clade PS I. Gaps were treated as missing characters throughout. All networks have been calibrated to fit one scale.

actual summed tree length of 189 steps was exactly at the lowest limit of that produced by any of the 10 000 artificial datasets (P=0.0003), and eight steps shorter than 0.95 % of them, thus indicating incongruence among the different gene trees. In order to test whether this topological conflict appears to be due to incongruence in one particular clade the PHT was applied to the PS I and PS II clades separately. In addition, we ran the analysis with and without partition of the *tef1* gene, which covers the intron sequence and therefore may contain homoplasious characters even in clonal populations, due to high mutation rates. The corresponding data show (Fig. 3) that there is a recombination within the PS II *H. orientalis* clade, the result being independent of *tef1* sequences. At the same time, topologies of *cal1* and *chi18-5* trees for PS I *T. longibrachiatum* are congruent, suggesting the absence of sexual recombination in this clade (Fig. 3). When *tef1* was included in the PHT of PS I the null hypothesis of recombination was not rejected (P=0.046).

In order to verify this result by yet further means, we also used the index of association (IA) test on a subset of 'clone corrected' data (i.e. individuals with identical genotypes at the three loci were excluded so that each multilocus genotype was represented only once; cf. Pringle *et al.*, 2005). The data obtained were in accordance with occurrence of recombination within *H. orientalis* but not in *T. longibrachiatum* and *Trichoderma* sp. PS III, as the IA test did not reject the null hypothesis of recombination in the former (P=0.462) but did so in the latter two datasets.

Finally, the maximum chi-squared test of Maynard Smith (1992), linkage disequilibrium (LD) r2 (Hill & Robertson,

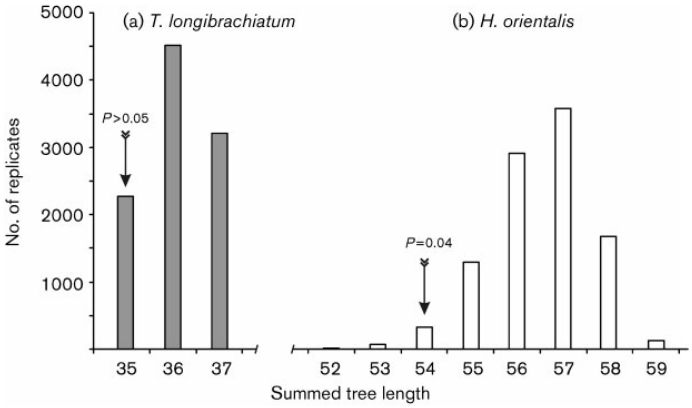


Fig. 3. PHT results for *T. longibrachiatum* (a) and *H. orientalis* (b) applied to *cal1* and *chi18-5* sequences. Arrows indicate the actual summed tree length and corresponding *P* value. For the test parsimony-uninformative characters were excluded, gaps were treated as missing characters, and 10 000 repetitions of heuristic search under parsimony criterion were performed.

1968), and LDD′ (LD versus distance |D|; Lewontin, 1964) estimates available on the RecombiTEST webpage (see Methods) also detected recombination between strains of *H. orientalis,* but not in *T. longibrachiatum* (data not shown).

Inspection of intracladal structures of *H. orientalis* on all trees revealed several conflicts of topologies. Besides the abovementioned unresolved position of C.P.K. 2880 and C.P.K. 2881 on the *cal1* tree, other strains of PS II aggregated in different statistically supported subclades which were unique for every locus. Such 'jumping' behaviour of individual sequences also suggests the presence of sexual recombination. No intracladal patterns were detected for PS I and III.

Haplotype structure and distribution of clinical strains within the *T. longibrachiatum* clade

Since the above data showed that *T. longibrachiatum* behaves essentially clonally, we now tested whether the clinical isolates would occupy specific positions in the structure of clade PS I, i.e. whether clinical occurrence would correlate with specific haplotypes. To this end, we used DnaSP 4.50.3 to collapse individual sequence alignments to haplotypes, and then subjected them to statistical parsimony implemented in TCS (Clement *et al.*, 2000). The results showed that the haplotype with the highest total numbers of environmental isolates also contained the majority of clinical isolates. Haplotypes represented by only a single strain contained only a few individual clinical isolates, but most of them were represented by wild-type strains (data not shown).

Biogeography of *T. longibrachiatum* and *H. orientalis*

The distribution analysis of strains from PS I and II shows that *T. longibrachiatum* and *H. orientalis* are a closely related pair of cosmopolitan and sympatric species, as both of them were detected on almost all continents. *H. orientalis* was frequently isolated from soil or mud samples as the anamorph, so it is clearly a holomorphic species which may coexist with *T. longibrachiatum* in the same ecosystem. The only known teleomorph samples of *H. orientalis* have so far been collected in China, thus making it difficult to speculate on climatic preferences for fruit body formation. Unfortunately the origin of several clinical isolates (6; Table 1) was not available for this study. The other strains were isolated from hospitals in North America (south United States and north-east Canada), temperate Europe (central France and Austria), subtropical Gran Canaria and continental Spain. This dataset shows that there is no bias of clinical strains towards specific geographical location.

DISCUSSION

This study set out to test the hypothesis that opportunistic strains of *T. longibrachiatum* may represent specialized potentially clonal subpopulations within this species. In that case it would be possible to identify specific genetic markers for their diagnosis. Availability of such sequences would enable screening for the source of these infections and so eventually reduce their clinical appearance. In contrast, the results from this work provide clear evidence 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 gene haplotypes or are predominantly associated with the major haplotypes of both species. This leads us to conclude that not a single population of *T. longibrachiatum* is responsible for opportunistic attack on humans but presumably every isolate of *T. longibrachiatum* or *H. orientalis* is potentially able to do so. This conclusion is also supported by phenotype microarray data testing carbon source utilization profiles of these two species, which failed to show any statistically supported metabolic difference (L. Hatvani, L. Kredics & I. S. Druzhinina, unpublished). It is further consistent with data obtained from isoenzyme analysis (Szekeres *et al.*, 2006) and study of mitochondrial

DNA polymorphism (Antal *et al.*, 2006). We have thus demonstrated that both species are a potential threat. It is important to mention that human clinical isolates shared identical multilocus haplotypes with isolates from soil and plant materials, and *Trichoderma* invasive mycoses may therefore be potentially nosocomial.

The finding of this work has important implications also for the biotechnological use of *T. longibrachiatum*, because strains of this fungus have been used as agents of biological control against phytopathogenic fungi (Vizcaíno *et al.*, 2005; Sánchez et al., 2007). In the light of the present results, this application should be abandoned or at least carefully monitored. Also, *T. longibrachiatum* has been reported to be a component 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). We recommend that *T. longibrachiatum* and *H. orientalis* are included on the list of those indoor fungi whose presence is specifically monitored. Primers to be designed for such tests therefore have to take into account the whole genetic variation within *T. longibrachiatum* and *H. orientalis*, as demonstrated in this paper. The lower percentage of *H. orientalis* clinical strains may be a result of the lower sample size. Interestingly, clinical isolates in the *H. orientalis* clade were recovered during the last 5 years, whereas isolates recovered earlier were only *T. longibrachiatum*.

There have so far been no studies dedicated to the ecology of *T. longibrachiatum* and *H. orientalis*. Therefore it is difficult to trace their preferred ecological niches, which would aid prediction of the source of *Trichoderma* infections. Nevertheless, besides being isolated from numerous soil samples worldwide, *T. longibrachiatum* has been consistently detected in association either with wild fruiting bodies of the wood-decaying fungus *Pleurotus ostreatus* (L. Hatvani, L. Kredics, I. S. Druzhinina & C. P. Kubicek, unpublished) or in mushroom farms cultivating *Pleurotus* and *Agaricus* (Hatvani *et al.*, 2007). Another also interesting case of abundant detection of *T. longibrachiatum* was its isolation from the archaeological excavation sites at an Iron Age tomb in the Republic of Tatarstan, Russia (F. Alimova & I. S. Druzhinina, unpublished). An explanation for these findings may be derived from observations that *T. longibrachiatum* occupies the lowest soil horizons but not the upper organic soil layers. Therefore, 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.

The present findings distinguish infections caused by *T. longibrachiatum* and *H. orientalis* from those caused by human-pathogenic fungi from the *Fusarium solani* complex, where the majority of clinical isolates are derived from a single worldwide distributed clonal lineage (Zhang *et al.*, 2006). A clonal structure has also been demonstrated for other pathogenic fungi such as *Trichophyton rubrum*, *Cryptococcus neoformans* or the chytrid pathogen *Batrachochytrium dendrobatidis* (Gräser *et al.*, 1999; Halliday & Carter, 2003; Morehouse *et al.*, 2003), whereas many other human-pathogenic fungi, including *Aspergillus fumigatus* (Pringle *et al.*, 2005), exhibit both clonal and recombining strategies (for reviews see Taylor *et al.*, 1999; Nielsen & Heitman, 2007). The results from this study present evidence that sexual reproduction, indicated by recombination, is an important strategy in one of the two opportunistic pathogenic species of *Hypocrea*/*Trichoderma*, *H. orientalis*. The potential presence of a sexual life cycle in an opportunistic pathogen is a significant finding, because its allows the fungus to respond faster to environmental challenges, thereby combating disease treatment by exchange of antibiotic-resistance genes and virulence factors (Milgroom, 1996; Nielsen & Heitman, 2007; Normak *et al.*, 2003; Paoletti *et al.*, 2005). It is not known whether these two species exploit their animal-pathogenic ability in nature; therefore it is too early to speculate that *H. orientalis* has developed different strategies than *T. longibrachiatum* for its opportunistic attack and subsequent survival. The phylogenetic analysis presented in this paper suggests that *T. longibrachiatum* and *H. orientalis* evolved in parallel from a common ancestor, forming two sympatric species. Thus, their pathogenic ability would be the result of a heritage from a recent ancestor rather than a convergent evolution. Further studies are needed to understand which species exploits more of its pathogenic abilities in nature.

H. orientalis is not the only *Hypocrea* species with potential health risk to humans. Druzhinina *et al.* (2008) reported a case study in which *Hypocrea* sp. CBS 120951 was isolated from the lung tissue of a patient with non-fatal pulmonary fibrosis. This isolate exhibits an uncertain phylogenetic position in the genus *Hypocrea*/*Trichoderma* and is also phenotypically very distinct as it does not conidiate *in vitro* but produces fertile stromata. Nevertheless, cases of sexual reproduction among clinically relevant strains of *Hypocrea* may be more frequent than was previously recognized.

Apart from these clinical implications, the results presented here also provide some new insights into the taxonomy of *T. longibrachiatum* and *H. orientalis*: based on ITS1 sequence analysis and isoenzyme data, Samuels *et al.* (1998) suggested that *T. longibrachiatum* may be the anamorph of *H. orientalis*. The present data reject this hypothesis by clearly showing that these two taxa represent individual phylogenetic species, which have already undergone reproductive isolation. In addition, we provide evidence for a third phylogenetic species, *Trichoderma* sp. PS III, which should be formally described elsewhere. In addition, the branches leading to this species did not form a network in the split decomposition analysis although the three available strains were isolated from three different coffee-growing areas in Ethiopia. These data are supportive of a reproductive barrier between *Trichoderma* sp. PS III, *T. longibrachiatum* and *H. orientalis*.

Hypocrea sp. strain C.P.K. 1261 (=CBS 243.63) formed a separate branch in all gene trees and in the SplitsTree analysis. Samuels *et al.* (1998) had previously noted that this strain differed from the other isolates in their sample both morphologically and in isoenzyme analysis profiles but nevertheless maintained it within their concept of *H. orientalis*. Based on our data, this strain represents a fourth species in our dataset. Since the origin of this strain was from a fruiting body, it is a member of another sexually propagating population which could occupy an as yet unknown ecological niche.

ACKNOWLEDGEMENTS

This work was supported partly by Austrian Science Fund grant FWF P-19340-MOB to C. P. K., partly by Hungarian Scientific Research Fund grant F68381 to L. K.; L. K. is grantee of the János Bolyai Research Scholarship (Hungarian Academy of Sciences). The authors thank John Bissett, Ilona Dóczi, Mirja Salkinoja-Salonen and George Szakacs for the gift of some of the strains. We also acknowledge Katarzyna Szymanska for her help in laboratory work.

REFERENCES

Akaike, H. (1974). A new look at the statistical model identification. *IEEE Trans Automat Contr* **19**, 716–723.

Antal, Z., Varga, J., Kredics, L., Szekeres, A., Hatvani, L., Manczinger, L., Vágvölgyi, C. & Nagy, E. (2006). Intraspecific mitochondrial DNA polymorphism within the emerging filamentous fungal pathogen *Trichoderma longibrachiatum*. *J Med Microbiol* **55**, 31–35.

Chaverri, P., Castlebury, L. A., Samuels, G. J. & Geiser, D. M. (2003). Multilocus phylogenetic structure within the *Trichoderma harzianum/Hypocrea lixii* complex. *Mol Phylogenet Evol* **27**, 302–313.

Chouaki, T., Lavarde, V., Lachaud, L., Raccurt, C. P. & Hennequin, C. (2002). Invasive infections due to *Trichoderma* species: report of 2 cases, findings of in vitro susceptibility testing, and review of the literature. *Clin Infect Dis* **35**, 1360–1367.

Clement, M., Posada, D. & Crandall, K. (2000). TCS: a computer program to estimate gene genealogies. *Mol Ecol* **9**, 1657–1660.

Cunningham, C. W. (1997). Is incongruence between data partitions a reliable predictor of phylogenetic accuracy? Empirical testing an iterative procedure for choosing among phylogenetic methods. *Syst Biol* **46**, 464–478.

Dettman, J. R., Jacobson, D. J. & Taylor, J. W. (2003). A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. *Evolution* **57**, 2703–2720.

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

Druzhinina, I. S., LaFe, K., Willinger, B., Komoń-Zelazowska, M., Ammirati, J., Kubicek, C. P. & Rogers, J. D. (2008). An unknown species from Hypocreaceae isolated from human lung tissue of a patient with non-fatal pulmonary fibrosis. *Clin Microbiol Newsl* **29**, 180–184.

Furukawa, H., Kusne, S., Sutton, D. A., Manez, R., Carrau, R., Nichols, L., Abu-Elmagd, K., Skedros, D., Todo, D. & Rinaldi, M. G. (1998). Acute invasive sinusitis due to *Trichoderma longibrachiatum* in a liver and small bowel transplant recipient. *Clin Infect Dis* **26**, 487–489.

Gräser, Y., Kühnisch, J. & Presber, W. (1999). Molecular markers reveal exclusively clonal reproduction in *Trichophyton rubrum*. *J Clin Microbiol* **37**, 3713–3717.

Halliday, C. L. & Carter, D. A. (2003). Clonal reproduction and limited dispersal in an environmental population of *Cryptococcus neoformans* var. *gattii* isolates from Australia. *J Clin Microbiol* **41**, 703–711.

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

Hasegawa, M., Kishino, K. & Yano, T. (1985). Dating the human–ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**, 160–174.

Hatvani, L., Antal, Z., Manczinger, L., Szekeres, A., Druzhinina, I. S., Kubicek, C. P., Nagy, A., Nagy, E., Vágvölgyi, C. & Kredics, L. (2007). Green mold diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderma* species. *Phytopathology* **97**, 532–537.

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**, 641–642.

Hill, W. G. & Robertson, A. (1968). Linkage disequilibrium in finite populations. *Theor Appl Genet* **38**, 226–231.

Huelsenbeck, J. P., Bull, J. J. & Cunningham, C. W. (1996). Combining data in phylogenetic analysis. *Trends Ecol Evol* **11**, 152–158.

Huson, D. H. (1998). SplitsTree: a program for analyzing and visualizing evolutionary data. *Bioinformatics* **14**, 68–73.

Huson, D. H. & Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* **23**, 254–267.

Jaklitsch, W. M., Komon, M., Kubicek, C. P. & Druzhinina, I. S. (2006). *Hypocrea crystalligena* sp. nov., a common European species with a white-spored *Trichoderma* anamorph. *Mycologia* **98**, 499–513.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Klein, D. & Eveleigh, D. E. (1998). Ecology of *Trichoderma*. *Trichoderma* and *Gliocladium*, vol. 1, *Basic Biology, Taxonomy and Genetics*, pp. 57–74. Edited by C. P. Kubicek & G. E. Harman. London, UK: Taylor & Francis.

Komoń-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**, 7415–7426.

Kratzer, C., Tobudic, S., Schmoll, M., Graninger, W. & Georgopoulos, A. (2006). *In vitro* activity and synergism of amphotericin B, azoles and cationic antimicrobials against the emerging pathogen *Trichoderma* spp. *J Antimicrob Chemother* **58**, 1058–1061.

Kredics, L., Antal, Z., Dóczi, I., Manczinger, L., Kevei, F. & Nagy, E. (2003). Clinical importance of the genus *Trichoderma*. A review. *Acta Microbiol Immunol Hung* **50**, 105–117.

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**, 310–317.

Kuhls, K., Lieckfeldt, E., Samuels, G. J., Meyer, W., Kubicek, C. P. & Börner, T. (1997). Revision of *Trichoderma* sect. *Longibrachiatum* including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. *Mycologia* **89**, 442–460.

Kuhls, K., Lieckfeldt, E., Börner, T. & Guého, E. (1999). Molecular re-identification of human pathogenic *Trichoderma* isolates as

Trichoderma longibrachiatum and *Trichoderma citrinoviride*. *Med Mycol* **37**, 25–33.

Kullnig, C. M., Szakacs, G. & Kubicek, C. P. (2000). Molecular identification of *Trichoderma* species from Russia, Siberia and the Himalaya. *Mycol Res* **104**, 1117–1125.

Leache, A. D. & Reeder, T. W. (2002). Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood and Bayesian approaches. *Syst Biol* **51**, 44–68.

Lewontin, R. C. (1964). The interaction of selection and linkage. I. General considerations: heterozygotic models. *Genetics* **49**, 49–67.

Maynard Smith, J. (1992). Analyzing the mosaic structure of genes. *J Mol Evol* **34**, 126–129.

Milgroom, M. G. (1996). Recombination and the multilocus structure of fungal populations. *Annu Rev Phytopathol* **34**, 457–477.

Morehouse, E. A., James, T. Y., Ganley, A. R. D., Vilgalys, R., Berger, D., Murphy, P. J. & Longcore, J. E. (2003). Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Mol Ecol* **12**, 395–403.

Munoz, F. M., Demmler, G. J., Travis, W. R., Ogden, A. K., Rossmann, S. N. & Rinaldi, M. G. (1997). *Trichoderma longibrachiatum* infection in a pediatric patient with aplastic anemia. *J Clin Microbiol* **35**, 499–503.

Myoken, Y., Sugata, T., Fujita, Y., Asaoku, H., Fujihara, M. & Mikami, Y. (2002). Fatal necrotizing stomatitis due to *Trichoderma longibrachiatum* in a neutropenic patient with malignant lymphoma: a case report. *Int J Oral Maxillofac Surg* **31**, 688–691.

Nicholas, K. B. & Nicholas, H. B., Jr (1997). Genedoc: a tool for editing and annotating multiple sequence alignments. http://www.psc.edu/biomed/genedoc.

Nielsen, K. & Heitman, J. (2007). Sex and virulence of human pathogenic fungi. *Adv Genet* **57**, 143–173.

Normak, B. B., Judson, O. P. & Morgan, N. A. (2003). Genomic signatures of ancient asexual lineages. *Biol J Linn Soc Lond* **79**, 69–84.

Paoletti, M., Rydholm, C., Schwier, E. U., Anderson, M. J., Szakacs, G., Lutzoni, F., Debeaupuis, J. P., Latgé, J. P., Denning, D. W. & other authors (2005). Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Curr Biol* **15**, 1242–1248.

Posada, D. (2003). Using Modeltest and PAUP* to select a model of nucleotide substitution. In *Current Protocols in Bioinformatics*, pp. 6.5.1–6.5.14. Edited by A. D. Baxevanis, D. B. Davison, R. D. M. Page, G. A. Petsko, L. D. Stein & G. D. Stormo. New York: Wiley.

Posada, D. (2008). jModelTest: Phylogenetic Model Averaging. *Mol Biol Evol* (in press).doi:10.1093/molbev/msn083.

Pringle, A., Baker, D. M., Platt, J. L., Wares, J. P., Latge, J. P. & Taylor, J. W. (2005). Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigatus*. *Evolution* **59**, 1886–1899.

Richter, S., Cormican, M. G., Pfaller, M. A., Lee, C. K., Gingrich, R., Rinaldi, M. G. & Sutton, D. A. (1999). Fatal disseminated *Trichoderma longibrachiatum* infection in an adult bone marrow transplant patient: species identification and review of the literature. *J Clin Microbiol* **37**, 1154–1160.

Ronquist, F. & Huelsenbeck, J. P. (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Rozas, J., Sanchez-DelBarrio, J. C., Messeguer, X. & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**, 2496–2497.

Samuels, G. J., Petrini, O., Kuhls, K., Lieckfeldt, E. & Kubicek, C. P. (1998). The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *Longibrachiatum*. *Stud Mycol* **41**, 1–54.

Sánchez, V., Rebolledo, O., Picaso, R. M., Cárdenas, E., Córdova, C., González, O. & Samuels, G. J. (2007). *In vitro* antagonism of *Thielaviopsis paradoxa* by *Trichoderma longibrachiatum*. *Mycopathologia* **163**, 49–58.

Schwarz, G. (1978). Estimating the dimension of a model. *Ann Stat* **6**, 461–464.

Seguin, P., Degeilh, B., Grulois, G., Gacouin, A., Maugendre, S., Dufour, T., Dupont, B. & Camus, C. (1995). Successful treatment of a brain abscess due to *Trichoderma longibrachiatum* after surgical resection. *Eur J Clin Microbiol Infect Dis* **14**, 445–448.

Swofford, D. L. (2002). PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10. Sinauer Associates, Sunderland, MA.

Szekeres, A., Láday, M., Kredics, L., Varga, J., Antal, Z., Hatvani, L., Manczinger, L., Vágvölgyi, C. & Nagy, E. (2006). Rapid identification of clinical *Trichoderma longibrachiatum* isolates by cellulose-acetate electrophoresis-mediated isoenzyme analysis. *Clin Microbiol Infect* **12**, 369–375.

Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* **10**, 512–526.

Tang, P., Mohan, S., Sigler, L., Witterick, I., Summerbell, R., Campbell, I. & Mazzulli, T. (2003). Allergic fungal sinusitis associated with *Trichoderma longibrachiatum*. *J Clin Microbiol* **41**, 5333–5336.

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**, 114–116.

Tavaré, S. (1986). Some probabilistic and statistical problems in the analysis of DNA sequences. In *Some Mathematical Questions in Biology – DNA Sequence Analysis*, pp. 57–86. Edited by R. M. Miura. Providence, RI: American Mathematical Society.

Taylor, J. W., Geiser, D. M., Burt, A. & Koufopanou, V. (1999). The evolutionary biology and population genetics underlying fungal strain typing. *Clin Microbiol Rev* **12**, 126–146.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Thrane, U., Poulsen, S. B., Nirenberg, H. I. & Lieckfeldt, E. (2001). Identification of *Trichoderma* strains by image analysis of HPLC chromatograms. *FEMS Microbiol Lett* **203**, 249–255.

Turner, D., Kovacs, W., Kuhls, K., Lieckfeldt, E., Peter, B., Arisan-Atac, I., Strauss, J., Samuels, G. J., Börner, T. & Kubicek, C. P. (1997). Biogeography and phenotypic variation in *Trichoderma* sect. *Longibrachiatum* and associated *Hypocrea* species. *Mycol Res* **101**, 449–459.

Vizcaíno, J. A., Sanz, L., Basilio, A., Vicente, F., Gutiérrez, F., Hermosa, M. R. & Monte, E. (2005). Screening of antimicrobial activities in *Trichoderma* isolates representing three *Trichoderma* sections. *Mycol Res* **109**, 1397–1406.

Walsh, T. J. & Groll, A. H. (1999). Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. *Transpl Infect Dis* **1**, 247–261.

Wilgenbusch, J. C., Warren, D. L. & Swofford, D. L. (2004). AWTY: a system for graphical exploration of MCMC convergence in Bayesian phylogenetic inference. http://ceb.csit.fsu.edu/awty.

Wuczkowski, M., Druzhinina, I. S., Gherbawy, Y., Klug, B., Prillinger, H. J. & Kubicek, C. P. (2003). Species pattern and genetic diversity of *Trichoderma* in a mid-European, primeval floodplain-forest. *Microbiol Res* **158**, 125–133.

Zhang, C. L., Druzhinina, I. S., Kubicek, C. P. & Xu, T. (2005). Biodiversity of *Trichoderma* in China: evidence for a North to South

difference of species distribution in East Asia. *FEMS Microbiol Lett* **251**, 251–257.

Zhang, N., O'Donnell, K., Sutton, D. A., Nalim, F. A., Summerbell, R. C., Padhye, A. A. & Geiser, D. M. (2006). Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. *J Clin Microbiol* **44**, 2186–2190.

Edited by: H. A. B. Wösten

5.3. Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma*

Druzhinina IS, **Komon-Zelazowska M**, Ismaiel A, Jaklitsch WM, Mulaw T, Samuels GJ & Kubicek CP (2012) Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma*. ***Fungal Genetics and Biology*** **49, 358-368**

OWN CONTRIBUTION:

MKZ performed strains cultivation, prepared selected strains for extracted genomic DNA, performed PCR amplification, analysed sequence data, participated in results discussion and contributed to ms writing.

Fungal Genetics and Biology 49 (2012) 358–368

Contents lists available at SciVerse ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma*

Irina S. Druzhinina [a,*], Monika Komoń-Zelazowska [b,1], Adnan Ismaiel [c,1], Walter Jaklitsch [d], Temesgen Mullaw [a,2], Gary J. Samuels [c], Christian P. Kubicek [a]

[a] Research Area Biotechnology and Microbiology, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/166-5, A-1060 Vienna, Austria
[b] Austrian Institute of Industrial Biotechnology (ACIB), GmBH c/o Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstraße 1a, A-1060 Vienna, Austria
[c] United States Department of Agriculture, ARS, Systematic Mycology & Microbiology Laboratory, B-010a, Rm 213, 10300 Baltimore Ave., Beltsville, MD 20705, USA
[d] Faculty Centre of Biodiversity, University of Vienna, Rennweg 14, A-1030 Vienna, Austria

ARTICLE INFO

Article history:
Received 12 August 2011
Accepted 13 February 2012
Available online 1 March 2012

Keywords:
Hypocrea
Speciation
Genealogical concordance
Phylogeny
4× Rule
Biogeography

ABSTRACT

The phylogenetically most derived group of the genus *Trichoderma* – section *Longibrachiatum*, includes some of the most intensively studied species, such as the industrial cellulase producer *T. reesei* (teleomorph *Hypocrea jecorina*), or the facultative opportunistic human pathogens *T. longibrachiatum* and *H. orientalis*. At the same time, the phylogeny of this clade is only poorly understood. Here we used a collection of 112 strains representing all currently recognized species and isolates that were tentatively identified as members of the group, to analyze species diversity and molecular evolution. Bayesian phylogenetic analyses based on several unlinked loci in individual and concatenated datasets confirmed 13 previously described species and 3 previously recognized phylogenetic species all of which were not yet described formally. When the genealogical concordance criterion, the K/θ method and comparison of frequencies of pairwise nucleotide differences were applied to the data sample, 10 additional new phylogenetic species were recognized, seven of which consisted only of a single lineage. Our analysis thus identifies 26 putative species in section *Longibrachiatum*, what doubles the currently estimated taxonomic diversity of the group, and illustrates the power of combining genealogical concordance and population genetic analysis for dissecting species in a recently diverged group of fungal species.

1. Introduction

Species of the mycotrophic filamentous ascomyceteous genus *Trichoderma* (Hypocreales, Hypocreaceae; teleomorph *Hypocrea*) are among the most commonly encountered fungi (Druzhinina et al., 2011). They are frequently isolated from soil and are found growing on dead wood, bark, other fungi, building materials and animals, including humans, demonstrating a high opportunistic potential and adaptability to ecological conditions (Klein and Eveleigh, 1998; Druzhinina et al., 2011). Taxonomically, *Trichoderma* had been divided into five sections, including section *Longibrachiatum* (for review see Gams and Bissett, 1998), but with increasing molecular phylogenetic analyses the sectional nomenclature of *Trichoderma* was abandoned in favor of naming phylogenetic clades (Samuels, 2006; Kubicek et al., 2008). Interestingly, though, the morphologically and metabolically distinctive section *Longibrachiatum* is one of only two sections that has remained intact following phylogenetic analysis. The comparative analysis of three genomes of diverse *Trichoderma* species has revealed that the *Longibrachiatum* clade is evolutionarily one of the youngest clades (Kubicek et al., 2011) of the genus. Sexual reproduction is common in the *Longibrachiatum* clade: Samuels et al. (1998) defined 10 species within what they called the *'Hypocrea schweinitzii* complex'.

The *Longibrachiatum* clade comprises the most intensively studied *Trichoderma* species, *T. reesei* (teleomorph *Hypocrea jecorina*), which is industrially used for the production of cellulolytic and hemicellulolytic enzymes involved in food and feed industry, textile manufacture and biofuel technology (Harman and Kubicek, 1998; Kubicek et al., 2009). In addition, several members of the clade are used for production of secondary metabolites, particularly strains that were isolated from marine habitats (Sperry et al., 1998; Ruiz et al., 2007; Paz et al., 2009; Gal-Hamed et al., 2011). However, certain strains of three of its species, *T. citrinoviride* (teleomorph *H. schweinitzii*), *T. longibrachiatum* and *H. orientalis*, have caused opportunistic infections of immunocompromized humans (Kuhls et al., 1999; Kredics et al., 2003), and *T. longibrachiatum* and *T. citrinoviride*

* Corresponding author. Address: Microbiology Group, Research Area Biotechnology and Microbiology, Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstrasse 1a, A-1060 Vienna, Austria. Fax: +43 1 58801 17299.
E-mail address: druzhini@mail.zserv.tuwien.ac.at (I.S. Druzhinina).
[1] These authors contributed equally to the research.
[2] Present address: Center for Environmental Science, Institute of Marine and Environmental Technology, University of Maryland, Baltimore, MD, USA.

1087-1845/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2012.02.004

are frequently isolated as indoor contaminants with high allergenic potential for humans (Thrane et al., 2001).

Species delimitation in fungi is still a matter of intensive debate, and several species concepts have been discussed (for review see Giraud et al., 2008). The first molecular phylogenetic analysis of the *Longibrachiatum* clade (Kuhls et al., 1997) was based on the internal transcribed spacer region of the rRNA gene cluster (ITS). Although this region is currently considered to be a universal barcode locus for fungi (Bellemain et al., 2010), it is unable to distinguish all closely related species in many genera of hyphomycetes including *Trichoderma* (Gazis et al., in press). Today, phylogenetic species concept has become most popular, because it bypasses the limitations imposed by the morphological or biological species concepts (such as the requirement for clear phenotypic differences, or the ability to mate the fungus *in vitro*), and because of the simplicity with which gene sequences can be obtained from practically all organisms. Thereby, the GCPSR (Genealogical Concordance Phylogenetic Species Recognition, Taylor et al., 2000) concept, which uses the phylogenetic concordance of multiple unlinked genes to identify the absence of genetic exchange and thus evolutionary independence of lineages, is currently most widely used within the fungal kingdom (e.g. Dettman et al., 2003; Fournier et al., 2005; Johnson et al., 2005; Koufopanou et al., 2001; Le Gac et al., 2007; Pringle et al., 2005). The molecular phylogeny of some species of the *Longibrachiatum* clade was investigated recently using GCPSR (Druzhinina et al., 2008, 2010; Atanasova et al., 2010) with the result that some of the taxa in fact comprised clonal species (or agamospecies) that reproduce exclusively asexually. Druzhinina et al. (2008, 2010) therefore hypothesized that the loss of sexual reproduction may constitute an important mechanism for speciation in the *Longibrachiatum* clade. Yet, whether or not a lineage is indeed a phylogenetic species or e.g. represents demes from a metapopulation that is connected by infrequent migration, can be obscured. In addition, GCPSR can be difficult to apply to truly clonal fungi where no incongruities in multi-locus data are found.

Birky et al. (2010) recently developed a population genetics approach, which can be used to complement species recognition by GCPSR. Their method is based on the theory that in a single species random genetic drift will produce clades and singlets that have all descended from a common ancestor on an average $2N_e$ generations ago (N_e is the effective population size), and their distance from each other will be less than $2N_e$ generations. After the onset of speciation, however, a species will be split into two populations that are completely separated and will thus form clusters separated by a gap exceeding $2N_e$. Thus clusters that are separated by $t \geqslant 4N_e$ generations (the "4× rule" or "K/θ method") 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 K/θ method therefore supports the cluster as an evolutionary species (Birky et al., 2010).

Since the earlier systematic work on the *Longibrachiatum* clade (Bissett, 1984; Kuhls et al., 1997; Samuels et al., 1998) we have received numerous cultures that are members of the clade that cannot be molecularly identified with certainty as any of the recognized species. This uncertainty, combined with the discovery of cryptic species in the clade through the use of GCPSR has leaded us to apply the GCPSR concept and the K/θ method to the enlarged collection of isolates of the *Longibrachiatum* clade.

2. Materials and methods

2.1. Material studied

Fungal strains were independently received by the Vienna University of Technology and USDA labs from colleagues in several research institutions or from personal collections. Most *Trichoderma* cultures were obtained by direct isolation from the substratum. Several collections were derived from stromata of *Hypocrea* teleomorphs. Pure cultures were made by isolating single ascospores or conidia using a micromanipulator or a platinum needle on cornmeal agar (Difco) + 2% (w/v) dextrose (CMD). The strains, their origins and the NCBI GenBank accession numbers of DNA sequences used in this work are listed in Table 1. The isolates are stored at −80 °C in 20–50% glycerol in the laboratory of Vienna University of Technology (Austria) or at the USDA (Beltsville, MD, USA) or the University of Vienna (Austria). Representative strains are deposited in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS).

2.2. DNA extraction, PCR amplification and sequencing

Mycelia were harvested after 2–4 days of growth on 3% malt extract agar (MEA) or up to 7 d in liquid 2% malt extract medium at 25 °C and genomic DNA was isolated using QIAGEN DNeasy® Plant Mini Kit following the manufacturer's protocol. Amplification of fragments of *tef1* (translation elongation factor 1-α), *cal1* (calmodulin), *chi18-5* (endochitinase CHI18-5, former known as *ech42*) and of *rpb2* (RNA polymerase subunit B II) was performed as described previously (Druzhinina et al., 2008, 2010; Atanasova et al., 2010). PCR fragments were purified (PCR purification kit, Qiagen, Hilden, Germany), and sequenced at MWG (Ebersberg, Germany) or cycle-sequenced the University of Vienna after an *in vitro* enzymatic cleanup (Werle et al., 1994). In Beltsville, sequences were obtained using BigDye Terminator cycle sequencing kit v. 3.1 (Applied Biosystems, Foster City, CA, USA), and products were analyzed directly on a 3130 Genetic Analyzer (Applied Biosystems). For each locus both strands were sequenced with the primers used in PCR amplifications.

2.3. Phylogenetic analysis

For the phylogenetic analysis DNA sequences were aligned with Clustal X 1.81 (Thompson et al., 1997) and then visually checked in GeneDoc 2.6 (Nicholas and Nicholas, 1997). Optionally ambiguous areas of the alignment were removed using the gblocks server http://molevol.cmima.csic.es/castresana/Gblocks_server.html (Castresana, 2000). The loci used in this study were previously checked for absence of intragenic recombination (Druzhinina et al., 2008). Neutral evolution was tested by linkage disequilibrium based statistics and Tajima's test as implemented in DnaSP 4.50.3 (Rozas et al., 2003). The interleaved NEXUS file was formatted using PAUP* 4.0b10 (Swofford, 2002). The best nucleotide substitution model for each locus was determined using jMODELTEST (Posada, 2003) and the unconstrained GTR + I + G nucleotide substitution model was applied to all loci. Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling was performed using MrBayes v. 3.0B4 with two simultaneous runs of four incrementally heated chains that performed for 5 millions of generations. The sufficient number of generations for each dataset was determined using the AWTY graphical system (Nylander et al., 2008) to check for convergence of MCMCMC. Bayesian posterior probabilities (PP) were obtained from the 50% majority-rule consensus of trees sampled every 100 generations after removing the first trees. 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 MCMCMC run and burning first samplings as well as nucleotide characteristics of used loci are given in Table 2.

Table 1
Strains used and NCBI GenBank accession numbers.

Taxon	Isolate number	Other numbers	Origin	Recognized as a species in	Published in	NCBI GeneBank accession numbers			
						tef1	*cal1*	*chi18–5*	*rpb2*
Formally described species									
H. andinensis	**G.J.S. 90-140**	CBS 354.97, ATCC 208857	Venezuela	Samuels et al. (1998)	Samuels et al. (1998)	AY956321	JN175412	JN175472	JN175531
H. novae-zelandiae	**G.J.S. 81-265**	CBS 639.92, CBS 496.97, ATCC 28856	New Zealand	Samuels et al. (1998)	Samuels et al. (1998)	AY937448	JN175406	JN175465	DQ641672
	G.J.S. 81-264	CBS 472.97	New Zealand		Samuels et al. (1998)	JQ513358	JN175407	JN175466	DQ641672
	G.J.S. 99-113		New Zealand			JN175582	JN175408	JN175467	JN175526
H. orientalis	G.J.S. 04-321		Peru			JN175573	JN175397	JN175455	JN175517
	G.J.S. 04-332		Peru			JN175574	JN175398	JN175456	JN175518
	G.J.S. 04-333		Peru			JN175575	JN175399	JN175457	JN175519
	G.J.S. 04-316		Peru			JN175576	JN175400	JN175458	JN175520
	DIS 270f		Ecuador			JN175577	JN175401	JN175459	JN175521
	G.J.S. 09-784		Peru			JN175578	JN175402	JN175460	JN175522
	G.J.S. 10-230		Brazil			JN175579	JN175403	JN175461	JN175523
	G.J.S. 88-81		China	Samuels et al. (1998) Druzhinina et al. (2008)	Druzhinina et al. (2008)	EU401581	EU401448	EU401500	n/a
	G.J.S. 91-157		Germany		Druzhinina et al. (2008)	EU401609	EU401693	EU401461	EU401513
	CECT 2606		Sierra Leone		Samuels et al. (1998)	EU401609	EU401477	EU401528	n/a
	C.P.K. 688	TUB F-837	Costa Rica		Druzhinina et al. (2005)	AY857282	EU401452	EU401504	n/a
	C.P.K. 683	TUB-F 831	Costa Rica		Druzhinina et al. (2005)	EU401584	EU401451	EU401503	n/a
	C.P.K. 704	TUB F-1023	Argentina		Druzhinina et al. (2005)	EU401585	EU401453	EU401505	n/a
	G.J.S. 10-253		Tanzania			JN544898	JN388899	JN544899	n/a
	PPRI 3894		South Africa		Druzhinina et al. (2008)	EU401579	EU401446	EU401498	n/a
	UAMH 9573		Canada		Druzhinina et al. (2008)	EU401599	EU401467	EU401519	n/a
H. schweinitzii/T. citrinoviride	**CTR 79-225**		USA		Samuels et al. (1998)	JN175590	JN175418	JN175478	JN175537
	G.J.S. 90-111		USA		Samuels et al. (1998)	JN175591	JN175419	JN175479	JN175538
	CTR 79-290		USA		Samuels et al. (1998)	JN175592	JN175420	JN175480	JN175539
	DAOM 145647		USA		Samuels et al. (1998)	AY937422	JN175421	JN175481	JN175540
	TR 106		USA		Samuels et al. (1998)	JN175593	JN175422	JN175482	JN175541
	G.J.S. 01-18		Russia			JN175594	JN175423	JN175483	JN175542
	DAOM 139758	DAOM 139758	Canada	Samuels et al. (1998)	Samuels et al. (1998)	EU338334	JQ389878	JN175484	JN175543
	G.J.S. 92-8	CBS 636.92, IMI 352472	France		Samuels et al. (1998)	JN175595	JN175424	JN175485	JN175544
	TR 102		USA		Samuels et al. (1998)	JN175596	JN175425	JN175486	JN175545
T. effusum	C.P.K. 254	DAOM230007	India	Bissett et al. (2003)	Bissett et al. (2003)	JN182272	JN182286	JN182295	JQ513368
T. ghanense	ATCC 28019		USA		Samuels et al. (1998)	JN175606	JN175435	JN175496	JN175555
	G.J.S. 07-29		Ghana			JN175607	JN175436	JN175497	JN175556
	G.J.S. 07-28		Ghana			JN175608	JN175437	JN175498	JN175557
	G.J.S. 06-157		Nigeria			JN175609	JN175438	JN175499	JN175558
	G.J.S. 08-208		USA			JN133556	JN133530	JN175500	JN133562
	G.J.S. 95-137	IAM 13109	Ghana	Samuels et al. (1998)	Samuels et al. (1998)	AY937423	JN175439	JN175501	JN175559
	DAOM 165776				Samuels et al. (1998)	JN175610	JN175440	JN175502	JN175560
	G.J.S. 08-114		Argentina			JN175611	JN175441	JN175503	JN175561
	G.J.S. 04-313		Peru			JN175612	JN175442	JN175504	JN175562
	G.J.S. 04-323		Peru			JN175613	JN175443	JN175505	JN175563
	C.P.K. 2057		Hungary		Hatvani et al. (2007)	JN182282	JN182292	JN182307	JN182314
	G.J.S. 05-96		Italy			JN175614	JN175444	JN175506	HQ260617
T. konilangbra	C.P.K. 132		Uganda	Samuels et al. (1998)	Samuels et al. (1998)	JN258681	JN182285	JN182300	JQ513367
	C.P.K. 133		Uganda		Samuels et al. (1998)	JQ513357	JQ513346	JQ513361	n/a
T. longibrachiatum	ATCC 18648		USA	Samuels et al. (1998) Druzhinina et al. (2008)	Samuels et al. (1998)	EU401591	EU401459	EU401511	DQ087242

	G.J.S. 01-121		Netherlands			JN175564	JN175387	JN175445	JN175507
	G.J.S. 08-198		Brazil			JN175565	JN175388	JN175446	JN175508
	G.J.S. 04-31	CGS 118640 , ATCC MYA-3642	Mexico			DQ297069	JN175389	JN175447	JN175509
	G.J.S. 08-104		Argentina			JN175566	JN175390	JN175448	JN175510
	G.J.S. 04-101		Vietnam			JN175567	JN175391	JN175449	JN175511
	G.J.S. 04-53		Vietnam			JN175568	JN175392	JN175450	JN175512
	G.J.S. 07-21		Ghana			JN175569	JN175393	JN175451	JN175513
	G.J.S. 08-119		Argentina			JN175570	JN175394	JN175452	JN175514
	C.P.K. 1707		Russia			EU401610	EU401478	EU401529	JN182315
	C.P.K. 842	CBS 115338	Egypt		Wuczkowski et al. (2003)	EU401587	EU401455	EU401507	JN182316
	C.P.K. 744	TUB F-1237[a]	Fiji			JN182276	JN182288	n/a	JN182308
T. parareesei	G.J.S. 04-41		Brazil		Druzhinina et al. (2010)	GQ354372	GQ354306	HM182989	HM182964
	G.J.S. 07-26		Ghana		Druzhinina et al. (2010)	GQ354373	GQ354307	HM182991	HM182966
	C.P.K. 634	TUB F-430	Sri Lanka		Druzhinina et al. (2010)	GQ354351	GQ354285	HM182993	HM182968
	C.P.K. 717	TUB F-1066	Argentina	Atanasova et al. (2010), Druzhinina et al. (2010)	Druzhinina et al. (2010)	GQ354353	GQ354288	HM182987	HM182963
	C.P.K. 523	TUB F-1034	Taiwan	Druzhinina et al. (2010)	Kubicek et al. (2003)	GQ354349	GQ354283	HM183006	HM182981
	C.P.K. 524	TUB F-1038	Taiwan	Druzhinina et al. (2010)	Kubicek et al. (2003)	GQ354350	GQ354284	HM183007	HM182982
	G.J.S. 04-93		Vietnam			JN175605	JN175434	JN175495	JN175554
T. pseudokoningii	**G.J.S. 81-300**	CBS 254.97, CBS 432.97	New Zealand		Samuels et al. (1998)	AY937429	JN175415	HM183010	HM182985
	NS 19	DAOM 167678, CBS 480.91, ATCC 298861	Australia		Samuels et al. (1998)	JN175588	JN175416	JN175476	JN175535
	G.J.S. 99-149		Australia			JN175589	JN175417	JN175477	JN175536
H. jecorina/T. reesei	**G.J.S. 00-89**		Brazil			JN175599	JN175428	JN175489	JN175548
	G.J.S. 00-09		Mexico			JN175600	JN175429	JN175490	JN175549
	G.J.S. 93-22	ATCC 208850	New Caledonia		Samuels et al. (1998)	GQ354363	GQ354297	HM183001	HM182276
	G.J.S. 09-74		Peru			JN175601	JN175430	JN175491	JN175550
	G.J.S. 06-138		Cameroun		Druzhinina et al. (2010)	GQ354370	GQ354304	HM182997	HM182972
	QM 6a	NS 20	Solomon Islands	Samuels et al. (1998) Druzhinina et al. (2010)	Samuels et al. (1998)	Z23012	JN180917	HM182994	HM182969
	G.J.S. 93-23		New Caledonia		Samuels et al. (1998)	GQ354363	GQ354290	HM183000	HM182975
	G.J.S. 10-189		India			JN175602	JN175431	JN175492	JN175551
	G.J.S. 97-38	CBS 999.97 , ATCC 204423	French Guiana		Lieckfeldt et al. (2000)	JN175603	JN175432	JN175493	JN175552
	G.J.S. 04-115		Vietnam			JN175604	JN175433	JN175494	JN175553
	G.J.S. 06-140		Cameroun		Druzhinina et al. (2010)	GQ354371	GQ354305	HM189226	HM189271
T. saturnisporum	CBS 335.92		Italy		Samuels et al. (1998)	JN182279	JN182291	JN182296	n/a
	CBS 886.72		South Africa		Samuels et al. (1998)	JN182280	JN388898	JN182297	n/a
	ATCC 28023		USA		Samuels et al. (1998)	JN388897	JN180915	JN175462	JN175524
	ATCC 18903		USA		Samuels et al. (1998)	JN182278	JN182290	JN182298	JN182309
	C.P.K. 3406		Dominican Republic			JN258682	JN258683	JN258687	JN258690
T. sinense	DAOM 230004		Taiwan		Bissett et al. (2003)	AY750889	JN175410	JN175469	JN175528
	C.P.K. 530		Taiwan		Bissett et al. (2003)	JN182273	JQ513347	JN182301	JN182310
	C.P.K. 531		Taiwan		Bissett et al. (2003)	JN182274	JQ513348	JN182302	JN182311
Previously recognized phylogenetic species and lone lineages									
H. sp. CBS 243.63	**CBS 243.63**		New Zealand	Druzhinina et al. (2008)	Samuels et al. (1998)	EU401592	EU401460	EU401512	JQ513369
T. sp. PS III	C.P.K. 1817		Ethiopia	Druzhinina et al. (2008)	Mullaw et al. (2010)	EU401614	EU401482	EU401533	n/a
	C.P.K. 1837		Ethiopia	Druzhinina et al. (2008)	Mullaw et al. (2010)	EU401615	EU401483	EU401534	HM182986
	C.P.K. 1841		Ethiopia	Druzhinina et al. (2008)	Mullaw et al. (2010)	EU401616	EU401484	EU401535	n/a
T. sp. C.P.K. 3334	C.P.K. 3503		Ethiopia	Mullaw et al. (2010)		FJ763179	JQ513349	JQ513362	n/a
	C.P.K. 3524		Ethiopia	Mullaw et al. (2010)		FJ763183	JQ513352	JQ513365	n/a

(*continued on next page*)

Table 1 (*continued*)

Taxon	Isolate number	Other numbers	Origin	Recognized as a species in	Published in	NCBI GeneBank accession numbers			
						tef1	*cal1*	*chi18–5*	*rpb2*
	C.P.K. 3522		Ethiopia	Mullaw et al. (2010)		JQ513359	JQ513350	JQ513363	n/a
	C.P.K. 3523		Ethiopia	Mullaw et al. (2010)		JQ513360	JQ513351	JQ513364	n/a
	C.P.K. 3525		Ethiopia	Mullaw et al. (2010)		FJ763184	JQ513353	JQ513366	n/a
	C.P.K. 3334		Ethiopia	Mullaw et al. (2010)		FJ763149	JQ513354	JN258684	JN258688
	C.P.K. 3350		Ethiopia	Mullaw et al. (2010)		FJ763163	JQ513356	JN258686	n/a
	C.P.K. 3345		Ethiopia	Mullaw et al. (2010)		FJ763158	JQ513355	JN258685	JN258689
Phylogenetic species and lone lineages discovered in this study									
T. sp. MA 3642	G.J.S. 99-3	ATCC 20898	Japan			JN175584	JN175411	JN175470	JN175529
	C.P.K. 885	MA 3642	Austria		Wuczkovsky et al. (2003)	JN182277	JN182289	JN182303	n/a
	G.J.S. 06-66		Vietnam			JN175585	n/a	JN175471	JN175530
	C.P.K. 2883		Hungary		Hatvani et al. (2007)	JN182283	JN182293	JN182304	JN182312
	C.P.K. 3412		Taiwan			JN182284	JN182294	JN182305	n/a
H. sp. nov. G.J.S. 02-120	**G.J.S. 04-100**		Vietnam			JN175571	JN175395	JN175453	JN175515
	G.J.S. 02-120		Sri Lanka			JN175572	JN175396	JN175454	JN175516
T. sp. nov. TR175	S19		Italy			JN175580	JN175404	JN175463	
	TR 175		USA			JN182281	JQ349444	JN182299	DQ857348
T. sp. nov. G.J.S. 99-17	G.J.S. 99-17		Japan			JN175581	JN175405	JN175464	JN175525
T. sp. nov. G.J.S. 00-72	G.J.S. 00-72		Reunion			JN175583	JN175409	JN175468	JN175527
T. sp. nov. G.J.S. 10-263	G.J.S. 10-263	TUB 2543	Malaysia			JN175598	JN175427	JN175488	JN175547
T. sp. nov. G.J.S. 08-81	G.J.S. 08-81		Mexico			JN175597	JN175426	JN175487	JN175546
T. sp. nov. G.J.S. 01-355	G.J.S. 01-355		Saudi Arabia			JN175586	JN175413	JN175473	JN175532
T. sp. nov. G.J.S. 09-62	G.J.S. 09-62		Peru			JN175587	JN175414	JN175474	JN175533
T. sp. nov. C.P.K. 667	C.P.K. 667	TUB F-739[a]	USA			JN182275	JN182287	JN182306	JN182313

[a] These strains were obtained from G. Szakacs in the course of a bilateral project between Vienna University of Technology and Budapest University of Technology and Economics; type strains for formally described species are underlined; strains isolated from teleomorphs are given in bold.

2.4. Detection of phylogenetic species

We used three approaches to identify phylogenetic species within our sample. The first was the Genealogical Concordance Phylogenetic Species Recognition concept (GCPSR, Taylor et al., 2000), which identifies a phylogenetic species from the existence of statistically supported phylogenetic clades that are present in the majority (at least two of three) of single-locus trees and that are not contradicted by any other single-gene tree(s) determined by the same method. To identify such clades, we used the approach of Dettman et al. (2003), i.e. production and analysis of a majority-rule consensus tree from the three single-locus trees, which reveals the genealogical patterns shared among loci, regardless of levels of support.

The second criterion was the K/θ method (Birky et al., 2010). Briefly, this involves: (i) estimation of the nucleotide diversity π (using DnaSp v5.0; Rozas et al., 2003) by the mean pairwise difference between sequences multiplied by the sample size correction $n/(n-1)$ where n is the number of sequences in the clade; (ii) calculating θ ($\approx 2N_e\mu$) from $\pi/(1-4\pi/3)$; (iii) testing the nucleotide diversity K between each pair of sister clades; and (iv) calculation of K/θ, which consequently should be >4 in the case of a true evolutionary species.

Third, we compared the frequency of pairwise nucleotide sequence differences within our sample. As shown by Highton (2000), this procedure will result in a bimodal frequency distribution of pairwise sequence differences, among which the lower values represent sequence differences between individuals within species, with an expected mean of $N_e\mu 2N_e\mu$ differences per site, whereas the second mode represents differences between species with an expected mean $>>2N_e\mu$ differences per site (Birky et al., 2010). The pairwise sequence differences were calculated in MEGA 5.0 (Tamura et al., 2011), using the concatenated dataset.

2.5. Detection of recombination

The criterion of incongruence among the four gene genealogies was used to infer the occurrence of sexual recombination among isolates, using the Phi-test implemented in SplitsTree (Huson, 1998), which uses the pairwise homoplasy index, PHI ($=\Phi$) statistic, to detect refined incompatibility indicating recombination (Bruen et al., 2006). In selected cases, also the IA (Index of Association) test, which measures whether the alleles from different loci in a population are randomly or non-randomly associated in the analyzed genomes (Maynard Smith, 1992) was used. The latter method was computed by Multilocus 1.3.b (Agapow and Burt, 2001).

3. Results

3.1. Sample design and phylogenetic markers

The sample (Table 1) consisted of 112 strains, and included strains of putatively new and previously recognized species of the *Longibrachiatum* clade (Samuels et al., 1998, Mullaw et al., 2010; Druzhinina et al., 2008, 2010) and strains that were attributed to this group based on their morphology and/or DNA sequences using *Tricho*BLAST (for *tef1* and *rpb2)* and ITS1 and 2 in *TrichO*Key (Druzhinina et al., 2005; Kopchinskiy et al., 2005) as implemented on www.isth.info or BLAST on the NCBI portal http://blast.ncbi.nlm.nih.gov/. Many of these strains have previously been reported in the literature (Wuczkowski et al., 2003; Bissett et al., 2003; Kubicek et al., 2003; Druzhinina et al., 2005, Druzhinina et al., 2008, 2010; Atanasova et al., 2010; Mullaw et al., 2010). Where possible, strains with the same ITS1 and 2 allele were selected from diverse regions to cover the maximum of geographic distribution.

Individual nucleotide characteristics of the loci are shown in Table 2. Tajima's D test confirmed neutral evolution for all four gene fragments. No conflict was detected between loci and the bivariate plot of bipartitions for the Bayesian analyses suggested convergence between parallel runs.

Table 2
Nucleotide parameters of loci used for phylogenetic analysis.

	tef1	*cal1*	*chi18-5*	Total
Total sites	522	429	704	1655
Sites without gaps	229	179	526	934
Parsimony informative sites	45	53	145	243
nt diversity π	0.0562	0.0261	0.0711	
Tajima's D	NS[a]	NS	NS	

[a] NS, not significant, $P < 0.01$.

3.2. Molecular phylogeny

We first used Bayesian methods to infer genealogies from three single locus alignments. The trees were rooted against *T. virens* (teleomorph *H. virens*), which formed a basal branch to *T. reesei*, a member of the *Longibrachiatum* clade, in a genome-wide phylogeny (Kubicek et al., 2011). Phylograms obtained from *tef1*, *chi18-5* and *cal1* had a well-resolved internal structure with supported internal nodes (Supplementary data 1), while *rpb2* resulted in a poor phylogenetic resolution and therefore was excluded from the subsequent analysis (data not shown). Fifteen terminal phylogenetic clades with posterior probabilities >0.94 and eleven lone lineages were consistently observed in all three trees. Therefore, based on the strict criteria of GCPSR, they can be considered as phylogenetic species. To prove the genealogical concordance of these clades, we used the approach of Dettman et al. (2003) and analyzed a majority-rule consensus tree from the three single-locus trees (Supplementary data 2), which approved these clades. Because of the congruence of the gene trees, we ran a Bayesian analysis with a concatenated dataset of the three genes (Fig. 1). Eleven clades and the lone lineages of *T. effusum* C.P.K. 254 (Bissett et al., 2003) and *H. andinensis* G.J.S. 90-140 contained type strains of formally established taxa (Table 1, Fig. 1). They are indicated by an arrow on the branch leading to the respective node on the concatenated phylograms (Fig. 1). Two clades (*T.* sp. PS III and *T.* sp. C.P.K. 3334) and the lone lineages *H.* sp. CBS 243.63 have been previously considered as putative new species awaiting formal taxonomic description (Druzhinina et al., 2008; Atanasova et al., 2010; Mullaw et al., 2010). They are indicated by a double arrow respectively. The three other clades (*H.* sp. nov. G.J.S. 02-120, *T.* sp. nov. TR175 and *T.* sp. nov. MA 3642), four lone lineages (isolates G.J.S. 08-81, G.J.S. 00-72, G.J.S. 99-17 and G.J.S. 10-263), and a group of strains (C.P.K. 667, G.J.S. 09-62, G.J.S. 01-355) affiliated with the type strain of *H. andinensis* (G.J.S. 90-140) could not be attributed to any known species by GCPSR concept.

3.3. Species recognition

Phylogenetic analysis using GCPSR supported a monophyletic origin of the *Longibrachiatum* clade and all of the species that were recognized by Bissett in 1984 and later authors (Doi et al., 1987; Samuels et al., 1998; Bissett, 1991; Bissett et al., 2003; Atanasova et al., 2010). In order to test whether the additional clades or lone lineages that have not been formally described may represent putatively species, we measured their phylogenetic distance from the neighboring clades by the K/θ method (Birky et al., 2010). As can be seen in Table 3, all of the previously

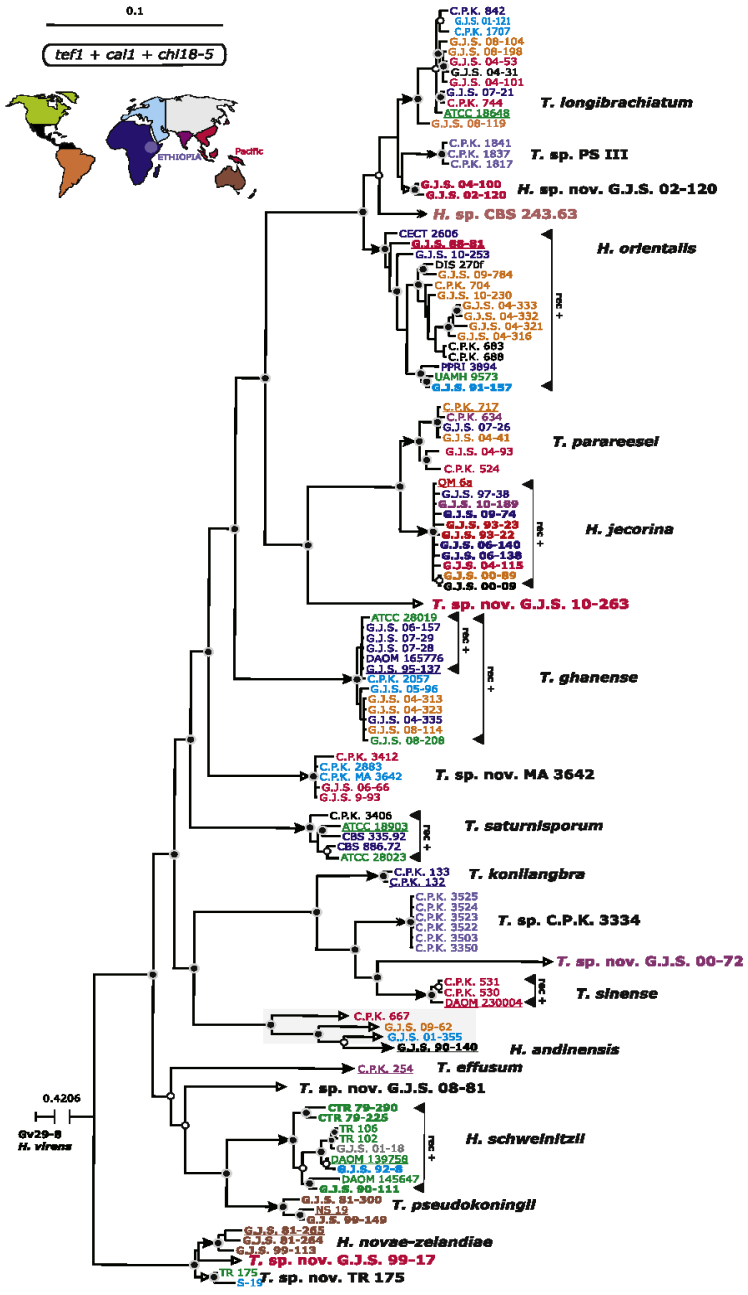


Fig. 1. Bayesian phylograms obtained from the concatenated alignment of *tef1*, *cal1* and *chi18-5* loci. Branches leading to formally described or previously recognized phylogenetic species are marked by filled single and double arrows respectively; phylogenetic species recognized in this study are shown by open arrows. The color code corresponds to the map insert and indicates geographic origin of isolates. Nodes supported by posterior probability >0.94 are shown in circles: black circles indicate supports obtained in both analyses after removal of ambiguous areas of the alignment using unconstrained gblocks (Castresana, 2000) and without such treatment, while white circles indicate supports obtained based on the complete concatenated alignment only. Sexual recombination is shown by vertical bars with a 'rec+' sign. Type strains of formally described species are underlined. Strains isolated from teleomorphs are given in bold.

known species were supported by values of >4, and this also turned out to be true for all but one of the new clades of unknown species identity. The only exception was the clade containing *H. novae-zelandiae*, where the branch to *T.* sp. TR175 was not supported. Also, the hypothesis that *T. ghanense* would consist of two cryptic species received no support.

As inferred from Birky et al. (2010), the species identified by the 4× rule could theoretically also be metapopulations that consist of two or more local populations connected by migration or by periodic extinction and re-colonization. An additional problem, particularly relevant in this case, are the lone lineages for which no π or θ could be determined and therefore their species status could not be clarified. To solve such cases, Birky et al. (2010) introduced a further criterion to distinguish between species and populations of species by plotting the sequence differences versus the respective number of pairs of strains. As already shown by Highton (2000), this will result in a bimodal frequency distribution of pairwise sequence differences, among which the lower values represent sequence differences between individuals within species, with an expected mean of $2N_e\mu$ differences per site, whereas the second mode represents differences between species with an expected mean $\gg 2N_e\mu$ differences per site (Birky et al., 2010). We therefore plotted the sequence differences in *tef1*, *cal1* and *chi18-5* of the investigated 104 isolates versus the nucleotide differences of all pairs (Fig. 2). As can be seen, this resulted in a bimodal distribution, although the distribution in the second mode (which represents higher diversities) was not perfectly bell shaped. Nevertheless, the first mode (supposed to represent the sequence diversity between individuals within species occurred at diversities of 0–0.01. This fits nicely to the mean $2N_e\mu = \theta$ value of 0.00754 calculated for our sample. Hence we consider values >0.02 to be indicative of differences between species, which is supported by the fact that 0.02 already lies within the onset of the second mode.

All of the species that were detected by the 4× rule showed values >0.02 when compared with other species. All clades identified above were approved by pairwise sequence differences of their isolates with those from other clades of >0.02, whereas intraspecific pairwise differences were always <0.02. This method also showed that the sister species to *H. novae-zelandiae*, *T.* sp. nov. are separate species (pairwise sequence difference 0.028).

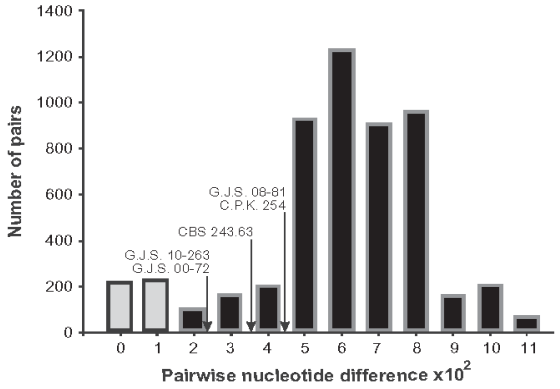


Fig. 2. Frequency distribution of uncorrected pairwise sequence differences, in bins of 1%. The gray bars show values detected between isolates of the same species. Arrows with strain numbers identify the lowest difference value detected for this isolate against isolates from any other taxon.

Consequently, we also tested the nucleotide differences between the lone lineages and other isolates (Fig. 2). This analysis confirmed G.J.S. 10-263, G.J.S. 08-81, G.J.S. 00-72, G.J.S. 99-17, CBS 243.63 and C.P.K. 254 (*T. effusum*) to be individual species, but did not support it for C.P.K. 524 and G.J.S. 04-93. These therefore should be attributed to *T. parareesei*.

3.4. Evolution of phenotypical traits

There is a high degree of phenotypic consistency among the members of the *Longibrachiatum* clade, which is reflected e.g. in a strong tendency for species to be thermotolerant (i.e. they still grow at 37 or 40 °C), and that the morphology of the conidiophores and conidia is largely homogeneous and in agreement with earlier descriptions by Bissett (1984). Thus it is not surprising that the main clades that are supported by GCPSR and the K/θ method, show only subtle internal phenotypic variation, and differences

Table 3
Pairwise calculations of 4× rule for clades recognized based on genealogical concordance.

Species	Next neighbor	θ	K	K/θ
T. sp. G.J.S. 10-263	*T. reesei*	0.00127[a]	0.045	**35.7**[b]
T. parareesei	*T. reesei*	0.00199	0.176	**88.4**
H. sp. G.J.S. 02-120	*T.* sp. PS III	0.00234	0.177	**75.7**
H. sp. CBS 243.63	*H. orientalis*	0.00127[a]	0.180	**141.7**
H. sp. G.J.S. 02-120	*T. longibrachiatum*	0.00234	0.124	**53.0**
H. sp. CBS 243.63	*H.* sp. G.J.S. 02-120	0.00127[a]	0.078	**61.41**
T. ghanense type subclade	*T. ghanense* none type subclade	0.00485	0.004	0.8
T. ghanense none type subclade	*T. ghanense* type subclade	0.00056	0.004	**7.3**
T. sp. MA 3642	*T. ghanense*	0.00238	0.010	**43.7**
T. saturnisporum	*T.* sp. MA 3642	0.01375	0.089	**70.1**
T. sinense	*T.* sp. G.J.S. 00-72	0.00403	0.035	**8.6**
T. konilangbra	*T. sinense*	0.00154	0.021	**13.8**
T. sp. C.P.K. 3334	*T. konilangbra*	0.00127[a]	0.195	**153.2**
T. sp. C.P.K. 3334	*T. sinense*	0.00127[a]	0.042	**33.1**
T. effusum	*H. schweinitzii*	0.00127[a]	0.230	**181.1**
T. effusum	*T.* sp. G.J.S. 08-81	0.00127[a]	0.087	**68.5**
T. sp. G.J.S. 08-81	*H. schweinitzii*	0.00127[a]	0.148	**116.5**
H. schweinitzii	*T. pseudokoningii*	0.01389	0.062	**4.5**
H. novae-zelandiae	*T. saturniopsis*	0.01006	0.01736	1.71
H. novae-zelandiae	*T.* sp. G.J.S. 99-17	0.01006	0.01616	1.58
T. sp. S 19	*T.* sp. G.J.S. 99-17	0.00965	0.02234	2.31

[a] θ was calculated based on a single strain, see Section 2.4 for details.
[b] Bold font highlights values >4.

Table 4
Recombination and evolution of species from the *Longibrachiatum* clade.

	n	Tajima's *D*	Fu and Li's *D*	*Φ*-test	IA test[c]
H. andinensis	4	−0.494	−0.436	0.3029	0.86[c]
T. sp. MA 3642	5	−1.161	−1.167	NA[a]	NA
T. sp. PS III	3	NP[b]	NP	0.223	0.96
T. sp. C.P.K. 3334	6	NP	NP	NA	NA
T. ghanense					
Subclade with the type strains	8	−0.509	−1.168	0.0361	0.37
All strains		−0.722	−0.933		0.41
Subclade without the type strain	5	−0.972	−0.972	0.233	1.335[c]
H. jecorina	11	−1.256	−1.256	0.006	0.175
T. konilangbra	2	NP	NP	NA	NA
T. longibrachiatum	12	−1.7	−2.025	0.58	0.88
H. novae-zelandiae	3	NP	NP	0.36	0.79[c]
H. orientalis	17	−0.164	0.087	0.0005	0.12
T. parareesei	4	−0.212	−0.212	0.126	0.93
H. sp. G.J.S. 02-120	2	NP	NP	NA	NA
H. pseudokoningii	3	NP	NP	NA	NA
T. saturnisporum	5	−0.641	−0.573	0.0313	0.42
T. sinense	3	NP	NP	0.02	NA
H. schweinitzii	9	−0.537	−0.561	0.00058	0.22

[a] NA, not analysed; species or putative species known only from a single isolate were not included.
[b] NP, not possible: calculation not done because of insufficiently large sample.
[c] In these cases p was > 0.05, and the data are thus questionable.

are mainly reflected in dimensions of conidia or rates of growth. There are, however, notable exceptions: *Hypocrea novae-zelandiae* is apparently endemic to New Zealand, where it has been collected as teleomorph. Its *Trichoderma* anamorph is unremarkable in the *Longibrachiatum* clade. Most species in the *Longibrachiatum* clade have smooth, ellipsoidal to oblong conidia, but conidia of *T. ghanense*, *T. saturnisporum* and *T.* sp. TR 175 are typically tuberculate to a greater or lesser degree. The basal position of the *H. novae-zelandiae* clade in Fig. 1 may indicate that tuberculate conidia, which are also found in the Viride clade (Jaklitsch et al., 2006) may be an ancestral trait of the *Longibrachiatum* clade. *Trichoderma effusum* and the phylogenetic species *T.* sp. G.J.S. 08-81 respectively, are phenotypically divergent (Samuels et al., in press) to such an extent that, based on their morphology alone, they would not have been considered as members of the clade.

Stromata of most members of the *Longibrachiatum* clade are brown but in one single subclade, which includes the sexually reproducing species *H. schweinitzii* (anamorph *T. citrinoviride*) and *H. pseudokoningii*, stromata are black or nearly so.

3.5. Sexual recombination

We have previously reported that closely related species of the *Longibrachiatum* clade can survive based on alternative (combined sexual and asexual or exclusively asexual) reproduction strategies (Druzhinina et al., 2008, 2010). In order to identify clonal and mainly sexually recombining species in the whole clade, we used the Phi-test built on the pairwise homoplasy index (PHI, *Φ*) to detect refined incompatibility even in the presence of recurrent mutation (Bruen et al., 2006). This method assumes the infinite sites model of evolution, in which the detection of incompatibility for a pair of sites indicates recombination. It detected recombination within *H. schweinitzii/T. citrinoviride*, *T. sinense, T. saturnisporum, H. jecorina/T. reesei, H. orientalis* and the subclade within *T. ghanense* that contains the type strain G.J.S. 95-137 (Table 4), but not in any of the other clades shown in Fig. 1. Recombination was also evident from the topology of single locus trees, which showed incongruent positions of individual isolates within these species (cf. Supplementary data 1). Interestingly, no recombination was detected between strains basal to the type strains of either *H. andinensis* or *H. novae-zelandiae*, which both were isolated from their teleomorphs.

In addition, we tested these strains by the index of association test, which confirmed all the recombining taxa that were identified by the PHI-test (Table 4).

4. Discussion

In the present paper, we extended and complemented the well-known GCPSR concept for species delimitation by the addition of the population genetics-based K/θ method to identify species within the *Longibrachiatum* clade of *Hypocrea/Trichoderma*. We show that the results obtained by the two methods agree with each other well, and detected 26 phylogenetic species which is more than a doubling of the species inventory already known for this group (Samuels et al., 1998). All of the previously described taxa and all but one of the proposed phylogenetic species were confirmed. In addition, two new phylogenetic species were identified.

However, we also noted some problematic cases: the four isolates that according to GCPSR represent *H. novae-zelandiae* and the two isolates that represent *T.* sp. nov. TR 175, respectively, were not supported by the K/θ method. This finding was particularly puzzling in view of the fact that they have already acquired a number of distinct phenotypical characters that would be consistent with their nature as a separate species. Also, their pairwise nucleotide differences (0.026–0.032) placed them into the mode typical for separate species (cf. Fig. 2). Birky et al. (2010) observed a similar case in some *Penicillium* clades. It is possible that in the present case, the failure to pass the K/θ method is either due to a still incomplete sampling of the genetic diversity of *H. novae-zelandiae* and phylogenetic species *T.* sp. nov. TR 175, or, less likely, to an unusually high plasticity of phenotypic characters. If two, recently diverged clades are now genetically isolated but share retained ancestral variation, their divergence and genetic isolation follow a continuum and no single percentage is going to work in all cases. Thus the K/θ method will not work in such cases.

Another interesting case was the branch containing the type strain of *H. andinesis*. Based on the principles of GCPSR and the strong statistic support for this branch, all isolates in this clade would be identified as the same species. Yet, it was suspicious that the genetic distances between these isolates were much greater than those observed among isolates of other species within the *Longibrachiatum* clade. A calculation of the pairwise sequence differences between the four isolates of the *H. andinensis* clade revealed

values between 0.028 and 0.062 what corresponds to different species in the sense of our sample. In addition, tests for recombination within this clade frequently gave negative result what contrasts with the findings that the type strain of *H. andinensis* (G.J.S. 90-140) was sampled as a teleomorph. We therefore consider these strains to represent closely related but rare species, for which we find it practicable to continue calling these strains "*H. andinensis* complex" until more isolates of them have been found.

It was also conspicuous that 25% of the identified phylogenetic species were represented only by a single isolate, i.e. they formed lone lineages. All of them exhibited a basal position to the species clusters they were associated with, and were characterized by long genetic distances and nucleotide sequence diversities >0.025, thus implying an already long history of existence as a separate species. Theoretically, they could be species with growth requirements that exceed those fulfilled by the media used for isolation of *Trichoderma* from the environment; yet none of these has so far yet been detected in metagenomic studies on *Trichoderma* (Hagn et al., 2007; Friedl and Druzhinina, 2012). Alternatively it is possible that they represent relict species that are in progress of extinction. However, the most likely interpretation is that these species are strongly biased in their habitat and geographic distribution and have therefore not been found so far. Similar cases were observed for the genetically diverse *Harzianum* clade of *Trichoderma* by Druzhinina et al. (2010).

Six of ten species of the *Longibrachiatum* clade, for which enough isolates were available to test for a history of recombination, were shown to exhibit evidence for sexual recombination. With the exception of *H. andinensis* and *H. novae-zelandiae* (which was above explained as a sampling problem), all of the species for which also sexual stages were sampled, in fact confirmed recombination, thereby also verifying the validity of our approach. In addition, two species for which so far no teleomorph has been found (*T. ghanense, T. saturnisporum*) were also positive in this test. An interesting finding from the recombination tests was that there are some phylogenetic clades in *Longibrachiatum*, which contain a sexual and an apparently asexual species (e.g. *T. longibrachiatum* versus *H. orientalis*; *T. reesei* versus *T. parareesei*; *T. sinense* versus *T.* sp. nov. C.P.K. 3334), suggesting that speciation in these cases involved loss or gain of sexual reproduction. This phenomenon is also seen in *T. ghanense*, which was shown to split into two phylogenetic groups: the clade containing the type strain showed a history of recombination, the other clade did not. It is possible that the latter clade represents a species in progress. One must apply caution to these analyses, however, because undetected population structure, or lack of sufficient variation among the individuals may obscure the detection of recombination. So the inability to detect recombination does not necessarily equate with asexuality.

Summarizing, our data show that the combination of GCPSR with the K/θ method represents a robust test to identify phylogenetic species in fungi. Although Birky et al. (2010) propose the K/θ method only for asexual fungi, our current results demonstrate that this approach is also applicable to a phylogenetic analysis of fungi which consist of a mixed batch of sexual and asexual taxa. In addition, combining the K/θ method with GCPSR helped to deepen the analysis and to exclude false positives. We therefore recommend combining these two methods also in future studies with other fungi.

Acknowledgments

This work was supported partly by Austrian Science Fund (FWF): P-19340-MOB to C.P.K., P-17859 to I.S.D. and P22081-B17 to W.M.J. We express our thanks to Farida Alimova and Rezeda Tukhbatova (Kazan State University, Kazan, Russia) for the gift of isolate C.P.K. 1707, to Katja Fisch (University of Bonn, Bonn, FRG) for the gift of isolates C.P.K. 3406 and C.P.K. 3412, and to George Szakacs (Budapest University of Technology and Economics, Budapest, Hungary) for the gift of isolate TUB 2543. The help of Benigno Aquino with PCR amplification is warmly appreciated.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2012.02.004.

References

Agapow, P.M., Burt, A., 2001. Indices of multiloci linkage disequilibrium. Mol. Ecol. Notes 1, 101–102.
Atanasova, L., Jaklitsch, W.M., Komoń-Zelazowska, M., Kubicek, C.P., Druzhinina, I.S., 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.
Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., Kauserud, H., 2010. ITS as an environmental DNA barcode for fungi: an *in silico* approach reveals potential PCR biases. BMC Microbiol. 10, 189.
Birky Jr., C.W., Adams, J., Gemmel, M., Perry, J., 2010. Using population genetic theory and DNA sequences for species detection and identification in asexual organisms. PLoS One 5, e10609.
Bissett, J., 1984. Revision of the genus *Trichoderma*: I. Section *Longibrachiatum*, new section. Can. J. Bot. 62, 924–931.
Bissett, J., 1991. Revision of the genus *Trichoderma:* II. Infrageneric classification. Can. J. Bot. 69, 2357–2372.
Bissett, J., Szakacs, G., Nolan, C.A., Druzhinina, I., Gradinger, C.M., Kubicek, C.P., 2003. New species of *Trichoderma* from Asia. Can. J. Bot. 81, 570–586.
Bruen, T.C., Philippe, H., Bryant, D., 2006. A simple and robust statistical test for detecting the presence of recombination. Genetics 172, 2665–2681.
Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.
Dettman, J.R., Jacobson, D.J., Turner, E., Pringle, A., Taylor, J.W., 2003. Reproductive isolation and phylogenetic divergence in *Neurospora*: comparing methods of species recognition in a model eukaryote. Evolution 57, 2721–2741.
Doi, Y., Abe, I., Sugiyama, J., 1987. *Trichoderma* sect. *Saturnisporum*, sect. nov. and *Trichoderma ghanense*, sp. nov. Bull. Nat. Sci. Museum, Ser. B (Botany) 13, 1–9.
Druzhinina, I.S., Kopchinskiy, A.G., Komoń, M., Bissett, J., Szakacs, G., Kubicek, C.P., 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet. Biol. 42, 813–928.
Druzhinina, I.S., Komoń-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. Microbiol. UK 154, 3447–3459.
Druzhinina, I.S., Komoń-Zelazowska, M., Atanasova, L., Seidl, V., Kubicek, C.P., 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.
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., Kubicek, C.P., 2011. *Trichoderma*: the genomics of opportunistic success. Nat. Microbiol. Rev. 16, 749–759.
Fournier, E., Giraud, T., Albertini, C., Brygoo, Y., 2005. Partition of the *Botrytis cinerea* complex in France using multiple gene genealogies. Mycologia 97, 1251–1267.
Friedl, M.A., Druzhinina, I.S., 2012. The tribal dwelling in soil: taxon-specific metagenomics of *Trichoderma* reveals a narrow community of opportunistic species regulating the development of each other. Microbiol. UK November 10 (Epub ahead of print).
Gal-Hemed, I., Atanasova, L., Komon-Zelazowska, M., Druzhinina, I.S., Viterbo, A., Yarden, O., 2011. Marine isolates of *Trichoderma* as potential halotolerant agents of biological control for arid-zone agriculture. Appl. Environ. Microbiol. June 10 (Epub ahead of print).
Gams, W., Bissett, J., 1998. Morphology and identification of *Trichoderma*. In Kubicek, C.P., Harman, G.E. (Eds.), *Trichoderma* and *Gliocladium*. Basic Biology, Taxonomy and Genetics, vol. 1. Taylor & Francis, London, UK, pp. 3–34.
Gazis, R., Rehner, S.R., Chaverri, P., in press. Species delimitation in fungal endophyte diversity studies and its implications in ecological and biogeographic inferences. Mol. Ecol. doi:10.1111/j.1365-294X.2011.05110.x.
Giraud, T., Refrégier, G., Le Gac, M., de Vienne, D.M., Hood, M.E., 2008. Speciation in fungi. Fungal Genet. Biol. 45, 791–802.
Hagn, A., Wallisch, S., Radl, V., Charles Munch, J., Schloter, M., 2007. A new cultivation independent approach to detect and monitor common *Trichoderma* species in soils. J. Microbiol. Methods 69, 86–92.
Harman, G.E., Kubicek, C.P., 1998. *Trichoderma* and *Gliocladium*, vol. 2. Taylor & Francis, London.
Hatvani, L., Antal, Z., Manczinger, L., Szekeres, A., Druzhinina, I.S., Kubicek, C.P., Nagy, A., Nagy, E., Vágvölgy, C., Kredics, L., 2007. Green mold diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderma* species. Phytopathology 97, 532–537.

Highton, R., 2000. Detecting cryptic species using allozyme data. In: Bruce, R.C., Jaeger, R.G., Houck, L.D. (Eds.), The Biology of Plethodontid Salamanders. Kluwer Academic/Plenum Publishers, New York, pp. 215–241.

Huson, D.H., 1998. SplitsTree: a program for analyzing and visualizing evolutionary data. Bioinformatics 14, 68–73.

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

Johnson, J.A., Harrington, T.C., Engelbrecht, C.J.B., 2005. Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. Mycologia 97, 1067–1092.

Klein, E., Eveleigh, D.E., 1998. Ecology of *Trichoderma*. In: Kubicek, C.P., Harman, G.E. (Eds.), *Trichoderma* and *Gliocladium*, Basic Biology, Taxonomy and Genetics, vol. 1. Taylor & Francis, London, UK, pp. 57–74.

Kopchinskiy, A., Komoń, M., Kubicek, C.P., Druzhinina, I.S., 2005. *Tricho*BLAST: a multiloci database of phylogenetic markers for *Trichoderma* and *Hypocrea* powered by sequence diagnosis and similarity search tools. Mycol. Res. 109, 658–660.

Koufopanou, V., Burt, A., Szaro, T., Taylor, J.W., 2001. Gene genealogies, cryptic species, and molecular evolution in the human pathogen *Coccidioides immitis* and relatives (Ascomycota, Onygenales). Mol. Biol. Evol. 18, 1246–1258.

Kredics, L., Antal, Z., Dóczi, I., Manczinger, L., Kevei, F., Nagy, E., 2003. Clinical importance of the genus *Trichoderma*. A review. Acta Microbiol. Immunol. Hung. 50, 105–117.

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, 310–317.

Kubicek, C.P., Komon-Zelazowska, M., Druzhinina, I.S., 2008. Fungal genus *Hypocrea/Trichoderma*: from barcodes to biodiversity. J. Zhejiang Univ. Sci. B 9, 753–763.

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

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., Grigoriev, I.V., 2011. Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol. 12, R40.

Kuhls, K., Lieckfeldt, E., Samuels, G.J., Börner, T., Meyer, W., 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.

Kuhls, K., Lieckfeldt, E., Börner, T., Guého, E., 1999. Molecular re-identification of human pathogenic *Trichoderma* isolates as *Trichoderma longibrachiatum* and *Trichoderma citrinoviride*. Med. Mycol. 37, 25–33.

Le Gac, M., Hood, M.E., Fournier, E., Giraud, T., 2007. Phylogenetic evidence of host-specific cryptic species in the anther smut fungus. Evolution 61, 15–26.

Lieckfeldt, E., Kullnig, C.M., Samuels, G.J., Kubicek, C.P., 2000. Sexually competent, sucrose- and nitrate-assimilating strains of *Hypocrea jecorina* (*Trichoderma reesei*) from South American soils. Mycologia 92, 374–380.

Maynard Smith, J., 1992. Analyzing the mosaic structure of genes. J. Mol. Evol. 35, 126–129.

Mullaw, T.B., Kubicek, C.P., Druzhinina, I.S., 2010. The rhizosphere of *Coffea arabica* in its native highland forests of Ethiopia is associated with a distinguished diversity of *Trichoderma*. Diversity 2, 527–549.

Nicholas, K.B., Nicholas Jr., H.B., 1997. Genedoc: a tool for editing and annotating multiple sequence alignments. <http://www.psc.edu/biomed/genedoc> (accessed 21.08.09).

Nylander, J.A., Wilgenbusch, J.C., Warren, D.L., Swofford, D.L., 2008. AWTY: a system for graphical exploration of MCMC convergence in Bayesian phylogenetics. Bioinformatics 25, 581–583.

Paz, Z., Komon-Zelazowska, M., Druzhinina, I.S., Aveskamp, M.M., Schnaiderman, A., Aluma, Y., Carmeli, S., Ilan, M., Yarden, O., 2009. Diversity and potential antifungal properties of fungi associated with a Mediterranean sponge. Fungal Diversity 1, 17–26.

Posada, D., 2003. Using MODELTEST and PAUP* to select a model of nucleotide substitution. In: Baxevanis, A.D., Davison, D.B., Page, R.D.M., Petsko, G.A., Stein, L.D., (Eds.), Current Protocols in Bioinformatics. John Wiley & Sons, Hoboken, New Jersey, pp. 6.5.1–6.5.14.

Pringle, A., Baker, T., Platt, J., Wares, J., Latgé, J.P., Taylor, J.W., 2005. Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigates*. Evolution 59, 1886–1899.

Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.

Ruiz, N., Wielgosz-Collin, G., Poirier, L., Grovel, O., Petit, K.E., Mohamed-Benkada, M., du Pont, T.R., Bissett, J., Vérité, P., Barnathan, G., Pouchus, Y.F., 2007. New Trichobrachins, 11-residue peptaibols from a marine strain of *Trichoderma longibrachiatum*. Peptides 28, 1351–1358.

Samuels, G.J., 2006. *Trichoderma*: systematics, the sexual state, and ecology. Phytopathology 96, 195–206.

Samuels, G.J., Petrini, O., Kuhls, K., Lieckfeldt, E., Kubicek, C.P., 1998. The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *Longibrachiatum*. Stud. Mycol. 41, 1–54.

Samuels, G.J., Ismaiel, A., Mullaw, T.B., Szakacs, G., Druzhinina, I.S., Kubicek, C.P., Jaklitsch, W.M., in press. The *Longibrachiatum* clade of *Trichoderma*: a revision with new species. Fungal Div. doi:10.1007/s13225-012-0152-2.

Sperry, S., Samuels, G.J., Crews, P., 1998. Vertinoid polyketides from the saltwater culture of the fungus *Trichoderma longibrachiatum* separated from a *Haliclona* marine sponge. J. Org. Chem. 63, 10011–10014.

Swofford, D.L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10. Sinauer Associates, Sunderland, MA.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28, 2731–2739.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31, 21–32.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25, 4876–4882.

Thrane, U., Poulsen, S.B., Nirenberg, H.I., Lieckfeldt, E., 2001. Identification of *Trichoderma* strains by image analysis of HPLC chromatograms. FEMS Microbiol. Lett. 203, 249–255.

Werle, E., Schneider, C., Renner, M., Völker, M., Fiehn, W., 1994. Convenient single-step, one tube purification of PCR products for direct sequencing. Nucl. Acids Res. 22, 4354–4355.

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

5.4. An unknown species from Hypocreaceae isolated from human lung tissue of a patient with non-fatal pulmonary fibrosis

Druzhinina IS, LaFe K, Willinger B, **Komon-Zelazowska M**, Ammirati J, Kubicek CP & Rogers JD (2007) An unknown species from *Hypocreaceae* isolated from human lung tissue of a patient with non-fatal pulmonary fibrosis. ***Clinical Microbiology Newsletters*** **29(23), 180-184**

OWN CONTRIBUTION:

MKZ performed strains cultivation, prepared selected strains for susceptibility tests, amplified ITS1 and 2, *tef1* and *rpb2* loci, participated in sequence analysis and submitted sequences to NCBI GenBank.

Case Report

An Unknown *Hypocreaceae* Species Isolated from Human Lung Tissue of a Patient with Non-Fatal Pulmonary Fibrosis

Irina S. Druzhinina, Dr. Sc.,[1] Karen LaFe, B. Sc.,[2] Birgit Willinger, Dr. Med. Sc.,[3] Monika Komoń-Zelazowska, M. Sc.,[1] Joseph Ammirati, Dr. Sc.,[4] Christian P. Kubicek, Dr. Sci.,[1] and Jack D. Rogers, Dr. Sci.,[5] [1]Institute of Chemical Engineering, Research Area of Gene Technology and Applied Biochemistry, Vienna University of Technology, Vienna, Austria, [2]Mycology Section, Clinical Microbiology Laboratory, University of Washington Medical Center, Seattle, Washington, [3]Department of Clinical Microbiology, Medical University of Vienna, Vienna, Austria, [4]Department of Biology, University of Washington, Seattle, Washington, and [5]Department of Plant Pathology, Washington State University, Pullman, Washington

Introduction

We describe the first human case in which a fungus in the family *Hypocreaceae* (*Hypocreales*, *Ascomycota*) was isolated in culture. The organism was isolated from lung tissue of a 19-year-old patient diagnosed with non-fatal pulmonary fibrosis. The fungus occasionally produced the hypocrea-like sexual state in culture and lacked conidial sporulation. The perithecia that formed from the primary isolate contained asci with eight bicellular ascospores, which soon disarticulated. Thus, mature asci contained 16 part spores. Examination of the culture showed chlamydospores and arthrospores resulting from disarticulated hyphae, but no aerial condiophores. Phylogenetic analysis of nucleotide sequences from the internal transcribed spacers ITS1 and ITS2 of the rRNA gene cluster (a large exon and a short intron of the translation elongation factor 1-alpha-encoding gene *tef1* and of a fragment of the RNA polymerase subunit B-encoding gene *rpb2*) revealed that this fungus occupies a unique phylogenetic position close to the genus *Hypocrea* and its anamorph, *Trichoderma*. The absence of anamorphic characteristics and the conflicting phylogeny of *tef1* and *rpb2* do not allow its reliable classification to the genus level. The MICs of most antifungal agents were relatively high for this organism, except for amphotericin B (MIC, 0.125 µg/ml) and voriconazole (MIC, 1.0 µg/ml).

Mailing Address: Irina S. Druzhinina, Dr. Sc., Institute of Chemical Engineering, Research Area of Gene Technology and Applied Biochemistry, Vienna University of Technology, Getreidemarkt 9-166.5, A-1060 Vienna, Austria. E-mail: druzhini@mail.zserv.tuwien.ac.at

This case illustrates the widening spectrum of potential opportunistic pathogenic fungi and underscores problems encountered in diagnosing invasive fungal diseases.

Background

For many years, filamentous fungi have been recognized as opportunistic human pathogens. They are important causes of morbidity and mortality in immunocompromised patients, including those who are HIV infected (1). Hyaline septate mycelia of fungi, such as *Fusarium* spp., *Acremonium* spp., and *Paecilomyces* spp., are increasingly reported as causing invasive mycoses that do not respond to conventional therapy. One newly recognized but already prominent agent causing hyalohyphomycosis is *Trichoderma longibrachiatum*, a member of the mitosporic fungal genus *Trichoderma* (anamorph of *Hypocrea*, phylum *Ascomycota*). Although other species were named, later molecular diagnosis identified them all as *T. longibrachiatum*. This fungus causes a range of symptoms from localized infections to fatal disseminated diseases. Most cases have been reported in immunocompromised patients, particularly those undergoing peritoneal dialysis, and in transplant recipients (2).

Many species of *Trichoderma* have applications in biotechnology, as producers of enzymes, and in agriculture, as agents for biological pest control (3). To date, only *Trichoderma* (i.e., the anamorph stage) has been cultured from clinical samples, whereas *Hypocrea*, its sexual form, has never been observed in culture. In our case, an unknown fungal species with the hypocrea-like teleomorph morphology was isolated from human lung tissue. Phylogenetic analysis of molecular markers showed that this species occupies a unique taxonomic position close to the genus *Hypocrea* and its anamorph, *Trichoderma*, emphasizing the widening spectrum of opportunistic human-pathogenic fungi.

Clinical History

In January 1999, a 19-year-old female was diagnosed with pulmonary fibrosis at the University of Washington Medical Center. She had a history of asthma and interstitial lung disease with mediastinal lymphadenopathy and bilateral interstitial infiltrates. On pulmonary function testing, a restrictive pattern with a diminished diffusing capacity of the lung for carbon monoxide was also found. Bronchoscopy was performed to check for sarcoidosis, interstitial pulmonary fibrosis, and hypersensitivity pneumonitis. The upper airways, both vocal cords, and the trachea appeared normal. The right and left airways were without endobronchial lesions or abnormal secretions. Bronchoalveolar lavage (BAL) of the right middle lobe was performed, and transbronchial biopsy specimens from the right lower lobe basilar segments and an endobronchial biopsy specimen from the right lower lobe carina were obtained. Cellular analysis of the BAL showed alveolitis with a preponderance of macrophages and 15% eosinophils. T lymphocytes comprised 11% of the cells, and the CD4/CD8 ratio was 1:1, indicating a nonspecific alveolitis with features atypical for sarcoidosis. The transbronchial lung biopsy specimen revealed no diagnostic features specific for granuloma or vasculitis but reflected nonspecific fibrosis and inflammation. All laboratory values were in the normal range, including liver function tests. Antinuclear antibody serology showed antibodies reacting positively to nuclear antigen, with a

 0196-4399/00 (see frontmatter)

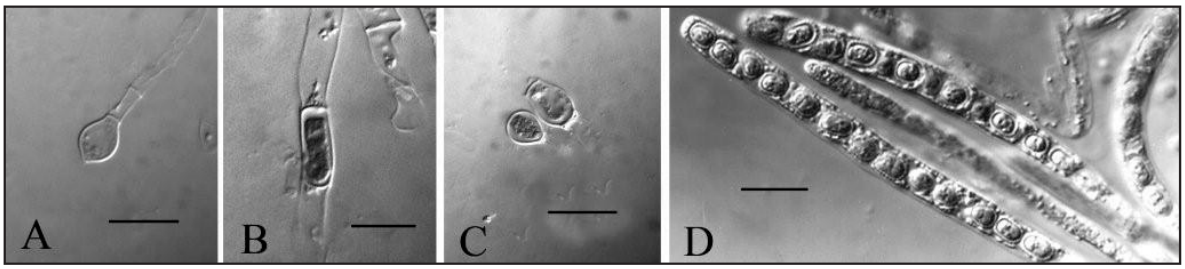


Figure 1. Morphology of CBS 120951. Terminal chlamydospore (A); endoarthric arthrospore (B); chlamydospores, the lower one terminal, the upper one intercalary (C); and two mature asci (stipes not shown), where the ascospores are mostly disarticulated into part spores (D). Panels B and C are from material stained with aniline blue in lactic acid; panels A and D are from unstained material. All photos were taken by differential interference microscopy. Bars, 10 µm.

speckled pattern at a titer of 160. The test for anti-neutrophil cytoplasmic antibodies was negative, indicating no sign of vasculitis. Serology for HIV was negative. Pulmonary function studies performed at the Womens Health Care Center showed forced experatory volume in the first second (FEV1) of 1.58 (51% of that predicted) and an FEV1/forced vital expiratory capacity ratio of 70%. After receiving bronchodilators, she improved dramatically. Chest X rays from January 1999 and 6 months later showed bilateral hilar lymphadenopathy and diffuse interstitial markings. A chest CT showed the same findings. Ground-glass opacities, especially in the lower posterior lung areas, were described.

Because of suspected sarcoidosis, a biopsy of the mediastinal lymph nodes and a thoracoscopic lung biopsy were performed. Transbronchial biopsies revealed alveolar lung tissue with interstitial fibrosis and mild chronic inflammation. Neither the lymph nodes nor the bronchial mucosa showed any diagnostic alterations. The right posterior base of the lung revealed interstitial pulmonary fibrosis, with a pattern consistent with moderate to severe (grade II to III of IV) interstitial pneumonia and moderate lymphoplasmacytic and eosinophilic inflammatory infiltrates. Movat stains were performed to help differentiate fibrous matter in histologic preparations.

Mycological Investigation

Tissue from the right upper pulmonary lobe was cultured, and one colony of fungal growth was observed during incubation on Sabouraud chloramphenicol agar (Emmons' modification). The organism was deposited in the Centraalbureau voor Schimmelcultures strain collection (Utrecht, The Netherlands) as CBS 120951. The colony was white and lanose, with no noticeable color on the reverse. Only septate mycelia were noted on microscopic examination. The fungus was subsequently subcultured onto 2% potato dextrose agar (PDA) and mycobiotic agar (containing 0.04% cycloheximide) incubated at 30°C and onto Sabouraud agar incubated at 35°C and 45°C. No growth occurred on mycobiotic agar or on Sabouraud agar at 45°C. Cultures on PDA at 30°C and Sabouraud agar at 35°C were similar to the original isolate in macroscopic and microscopic appearance. A sandwich slide culture was initiated, using potato flake agar. Coverslips were removed and examined at 7, 14, and 21 days for the presence of chlamydospores.

Plates of 2% PDA with 5 g/L of yeast extract (PDYA) were inoculated. Colony growth on PDYA, incubated in a natural cycle of sunlight-darkness at 21°C, covered a 9-cm-diameter petri plate within 7 days and was white and lanose with no color on the reverse. After 14 days of incubation, tan-to reddish-tan pulvinate stromata (up to 1 cm in diameter) were produced at the periphery of the plate. After 28 days, sections of stromata showed abundant perithecia that were 0.1 to 0.2 mm in diameter and contained short-stipitate asci, 85 to 95 by 4.5 µm, with iodine-negative apical apices. The perithecia contained eight bicellular ascospores, each of which soon disarticulated, giving rise to 16 subglobose to rectangular part spores, 4.5 to 6 by 3 to 3.5 µm in size (Fig. 1). Part spores were discharged in great numbers on overlying petri plate lids.

Asexual structures had terminal and intercalary chlamydospores that were globose, pyriform, or irregular, 7 to 15 by 4.5 to 8 µm, and arthrospores, 9 to 14 by 5 to 6 µm. The arthrospores were holoarthric (hypha disarticulates at the septum) and enteroarthric (propagule remains in the hypha devoid of contents). No aerial conidiophores or conidia were observed.

Thirty-two single ascospores (half ascospores) discharged from the perithecia onto petri plate lids were isolated by a micromanipulator and placed on PDYA. Of these 32 cultures, 11 produced perithecial stromata, and of these, 3 discharged half ascospores. Asci produced from single-spore cultures produced eight typical bicellular ascospores. Thus, this fungus appears to have four fertile ascospores (eight half ascospores) and four sterile ascospores (eight half ascospores) per generation. This production is similar to the mating-type gene switching first described in *Hypocrea (Chromocrea) spinulosa* (4) and *Hypocrea citrina* (5). After a few weeks, production of perithecial stromata ceased altogether, and all attempts to restore it failed.

Molecular Identification of the Fungus

To identify CBS 120951 by molecular means, we first used PCR to amplify and sequence the internal transcribed spacers of the rRNA gene cluster (ITS1 and ITS2). Since its morphology was consistent with *Hypocrea*, we submitted the sequence (GenBank accession number EF392732) to *TrichO*KEY, the oligonucleotide BarCode program for identification of *Hypocrea/Trichoderma* species (6, 7) (http://www.isth.info/molkey). Although the program detected all five genus-specific BarCodes for

  0196-4399/00 (see frontmatter)

Hypocrea/ Trichoderma, no oligonucleotide hallmark(s) specific for any known species, section, or clade within the genus was found. On the basis of *TrichO*KEY, it is likely that CBS 120951 represents a new species or possesses an as-yet-unknown ITS1 and ITS2 allele of a described species. The sequence similarity search performed for ITS1 and ITS2 against the National Center for Biotechnology Information GenBank, using the BLASTN program, resulted in three moderately related species of *Hypocrea* as the three best hits (88 to 98% similarity): *H. flaviconidia* and *H. pezizoides* from *Trichoderma* section *Trichoderma* (7) and *H. megalocitrina* from the *Megalocitrina* clade (8) (Table 1). This similarity occurred primarily in the 5.8S rRNA gene region, which is highly conserved in the genus, whereas the main diagnostic areas of ITS1 and ITS2 did not show significant alignment (expected E value, 10^{-4}).

The phylogenetic position of CBS 120951 was further examined by amplifying three other phylogenetic markers used in the molecular taxonomy of *Hypocrea/Trichoderma*: the highly variable fifth (short) intron of the translation elongation factor 1-alpha-encoding gene *tef1*, the sixth exon of *tef,* and a fragment of the RNA polymerase subunit B-encoding gene *rpb2*. Short sequence stretches (~30 and ~40 nucleotides [nt]) of the 126 nt-long *tef1* intron were identical to that of *H. crystalligena* from the *Megalocitrina* clade (8) and *H. sulawesensis* and *H. chromosperma* (Table 1), both of which represent lone lineages of *Hypocrea/ Trichoderma* phylogeny (7). The BLASTN analysis of the *tef1* exon produced a heterogeneous mix of mostly similar sequences (Table 1): *H. farinosa* (8) (94% similarity), *H. microcitrina* from *Hypocrea* section *Hypocreanum* (9), (93%), and *Hypomyces stephanomatis*, which represents a neighboring genus (93%). The similarity search applied to the partial coding sequence of the *rpb2* gene showed 84 to 85% similarity to several species of *Hypocrea/Trichoderma* sensu stricto (*H. schweinitzii* and *T. rossicum*) as well as to *H. cinereoflava*, which has an unclear phylogenetic position on the genetic edge of the genus (9).

These results were used to compose a data set for a phylogenetic analysis of the *tef1* exon and *rpb2*, which contain the main diversity within the genera *Hypocrea/Trichoderma*, and the available sequences of the most closely related genera. Neither gene provided a phylogram with high resolution of internal nodes (Fig. 2). In the *rpb2* tree, CBS 120951 occupies a statistically supported position (posterior probability, 0.99) outside of a large clade that combines all included *Hypocrea/ Trichoderma* species, as well as sequences of fungi that are believed to be outside of the genera (e.g., *H. pallida*, *Hypomyces stephanomatis*, *Sphaerostilbella* sp., and two *Arachnocrea* spp. (Fig. 2A). In contrast, analysis of the *tef1* exon places CBS 120951 into a statistically supported multifurcating clade (posterior probability, 0.95), which combines *Trichoderma* section *Longibrachiatum*, the *Pachybasioides*, the *Citrina* and *Lutea* clades; and lone lineages, such

Table 1. NCBI GenBank sequences[a]

Sequence	Length of fragment (nt)	Aligned fragments (5'-3')	Similarity (%)	3 best hits[b]	Accession no.
ITS1 - 5.8S - ITS2	558	161-547 40-83	94 90	*Hypocrea flaviconidia* G.J.S. 99-49	DQ023301
		161-542 40-78	94 88	*H. pezizoides* G.J.S. 01-257	DQ000632
		139-371 385-489 7-86	98 92 93	*H. megalocitrina* B.E.O. 00-09	DQ835511
tef1 intron (5th short)	126	86-126 1-28	100 100	*H. crystalligena* C.P.K. 2134	DQ345346
		86-126 1-29	100 100	*H. sulawesensis* G.J.S. 85-228	AY737730
		86-126 1-27	100 100	*H. chromosperma* G.J.S. 94-67	AY737728
tef1 exon	592	1-591 1-588 3-592	94 93 93	*H. farinosa* G.J.S. 89-139 *H. microcitrina* G.J.S. 97-248 ***Hypomyces stephanomatis* G.J.S. 88-50**	DQ835477 DQ835479 AF534632
rpb2	676	1-676 29-666 16-675	85 84 84	*H. schweinitzii* CTR 79-225 ***H. cinereoflava* G.J.S. 92-102** *Trichoderma rossicum* DAOM 230009	AY015639 DQ834461 DQ087240

[a]Total core nucleotide sequences 4,658,713; 12.12.2006.
[b]Non-*Hypocrea/Trichoderma* BLAST hits shown in boldface.

as *H. voglmayrii*, *H. farinosa* (9), and *Aphysiostroma stercorarium*, all of which are considered to belong to *Hypocrea* sensu stricto. In this tree, the last two species are the closest neighbors to CBS 120951 (Fig 2B). Thus, the phylogenetic analysis of two unlinked loci confirms that CBS 120951 is close to the genus *Hypocrea/Trichoderma*, although the exact taxonomic position of the isolate remains uncertain.

Susceptibility Tests

Susceptibility tests were performed by means of the Etest, as described by the manufacturer (AB Biodisk, Solna, Sweden). CBS 120951 did not grow on the recommended RPMI 1640 medium (Sigma-Aldrich, Vienna, Austria), and therefore, yeast nitrogen base (Sigma-Aldrich, Vienna, Austria) was used instead. This medium was the only one that provided sufficient growth to provide reliable results in susceptibility testing. As controls, reference strains of *Candida albicans* ATCC 90028 and *Candida krusei* ATCC 6258 with known MIC ranges were also tested. All plates were incubated at 30 and 37°C, and the results were read after 24, 48, 72, and 96 h of incubation.

The reference strains showed good growth at both incubation temperatures at all time points, and the MICs of all antifungal agents were within the ranges indicated by CLSI. For CBS 120951, results could be read only after 48, 72, and 96 h at an incubation temperature of 30°C. At 37°C, no growth was observed. The MICs of most antifungal agents were relatively high, with the exception of amphotericin B (MIC, 0.125 µg/ml) and voriconazole (MIC, 1.0 µg/ml). MICs for capsofungin, flucytosine, ketoconazole, and posaconazole were greater than 32 µg/ml. The MICs of fluconazole and itraconazole were >256 µg/ml and 16 µg/ml, respectively. As judged by the MICs, the isolate appears to be susceptible only to amphotericin B. As no interpretive breakpoints exist for this strain, it is uncertain whether CBS 120951 is susceptible to voriconazole. However, it is clearly resistant to the other azoles and flucytosine and caspofungin.

Conclusions

In this report, we describe an attempt to identify an unknown *Hypocreaceae* species from the lung tissue of a patient with pulmonary fibrosis. Although the clinical and mycological investigations did not determine whether this fungus did indeed cause the infection or contribute to pulmonary fibrosis, and the outcome was not fatal, we wish to alert physicians and clinical microbiologists to the possible further emergence of such fungal opportunists. The high resistance to most antifungal agents in use is comparable to that of other opportunistic pathogens, including *Trichoderma* (2). CBS 120951 is a newly described fungus whose habitat is unknown. Although the fungus was isolated originally in its sexual form, it has now ceased to grow in this form and can be identified only by cultural features and gene sequence analysis. From a mycological perspective, it is notable that CBS 120951 produces only chlamydospores and arthrospores as asexual propagules in vitro. If the organism becomes more widely recognized as an animal or human pathogen, it might be disseminated by either ascospores that come from some as-yet-unknown source where sexual fruiting

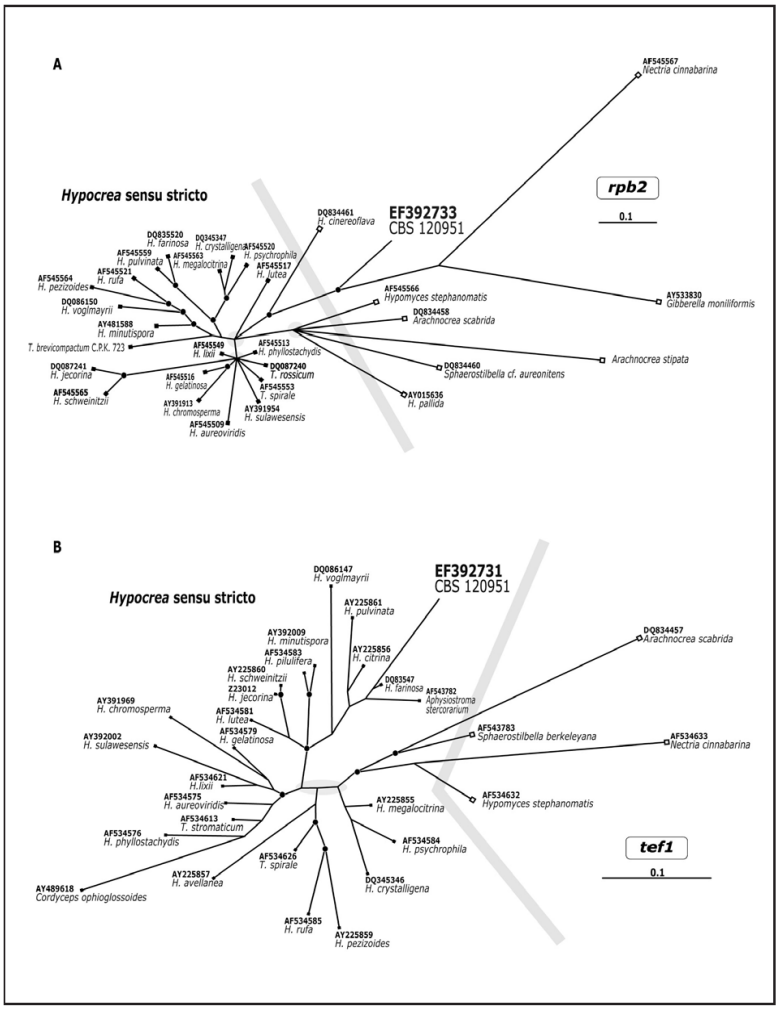


Figure 2. Results of Bayesian phylogenetic analyses of *rpb2* (A) and *tef1* (B) gene sequences (GTR model, 1,000,000 generations, repeated in triplicate). Black circles above nodes indicate posterior probability above 0.94; grey shadows show unresolved areas on both trees; filled and open terminal squares indicate species from *Hypocrea* sensu stricto and related genera, respectively. The numbers correspond to the NCBI GenBank accession numbers of the sequences used in the analysis.

  0196-4399/00 (see frontmatter)

is common or inhalation of asexual propagules by the host.

Apart from its isolation from human tissue, the evolutionary pathway of CBS 120951 remains unknown. The only point that can be claimed with certainty is that this fungus is unrelated to all known agents of hyalohyphomycoses. On the basis of sequencing, this fungus is not a member of *Trichoderma* section *Longibrachiatum,* a section from which all clinical strains of *Trichoderma* have originated. Moreover, our analysis even casts doubt on whether CBS 120852 is a member of the genera *Hypocrea/ Trichoderma* despite its morphology. In part, this is due to the fact that the genetic borders of the genus are not known at present, although it appears that more arguments can be made for its assignment to *Hypocrea/Trichoderma* than against it. The presence of five genus-specific BarCodes in the ITS1 and ITS2 sequences, *tef1* phylogeny, mating-type switching, and hypocrea-like morphology of the teleomorph form all support this idea. Moreover, the isolation from such an unusual habitat for a species of *Hypocrea* may indicate that as-yet-undiscovered ecological niches are inhabited by the genus. The main argument against attributing CBS 120951 to *Hypocrea/Trichoderma* is its extrageneric topology on the *rpb2* phylogenetic tree and the relatively long genetic distance from *Hypocrea* sensu stricto. These inconsistencies may be explained by the lack of sequence information from *Hypocrea/Trichoderma*-related genera.

Acknowledgements

I.S.D., M.K-Z., and C.P.K. were supported by the Austrian Science Foundation grant FWF P-16601 to C.P.K. and FWF P-17895 to I.S.D. We thank Lorant Hatvani for his help with laboratory work. K.L. was supported by the University of Washington Medical Center. J.A. was supported by the Department of Biology, University of Washington. J.D.R. was supported by the College of Agricultural, Human, and Natural Resources Sciences Research Center, Project 1767, Washington State University.

References

1. Walsh, T.J. and A. H. Groll. 1999. Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. Transplant. Infect. Dis. 1:247-261.
2. Kredics, L. et al. 2003. Clinical importance of the genus *Trichoderma*. A review. Acta. Microbiol. Immunol. Hung. 50:105-117.
3. Harman, G.E. and C.P. Kubicek. 1998. *Trichoderma* and *Gliocladium*, vol. 2. Enzymes, biological control and commercial applications. Taylor and Francis, London, UK.
4. Fincham, J.R.S., P.R. Day, and A. Radford. 1979. Fungal genetics, 4th ed. University of California Press, Berkeley, CA.
5. Canham, S.C. 1969. Taxonomy and morphology of *Hypocrea citrina*. Mycologia 61:315-331.
6. Druzhinina, I.S. and A.G. Kopchinskiy. 2006. *TrichO*KEY v. 2 — a DNA oligonucleotide BarCode program for the identification of multiple sequences of *Hypocrea* and *Trichoderma*. Proc. 8th Int. Mycolog. Cong., Cairns, Australia.
7. Druzhinina, I.S. et al. 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet. Biol. 42:813-828.
8. Jaklitsch, W.M. et al. 2006. *Hypocrea crystalligena* sp. nov., a common European species with a white-spored *Trichoderma* anamorph. Mycologia 98:500-514.
9. Overton, B.E., E.L. Stewart, and D.M. Geiser. 2006. Taxonomy and phylogenetic relationships of nine species of *Hypocrea* with anamorphs assignable to *Trichoderma* section *Hypocreanum*. Stud. Mycol. 56:39-65.

5.5. Taxonomic updates on *Trichoderma* species of putative clinical importance

In the work presented in the thesis (Druzhinina *et al.*, 2012), we recognized several putative new species with direct genetic link to the clinically important *T. longibrachiatum* and *H. orientalis* (Druzhinina *et al.*, 2008). These taxa have been later on named in the subsequent ms of Smauels *et al.* (2012: as *Trichoderma aethiopicum* (former *T.* sp. PS III) and *Trichoderma pinnatum* (former *H*. sp. nov. G.J.S. 02-120). Fungal strains were independently received by the Vienna University of Technology and USDA labs from colleagues in several research institutions or from personal collections. Most *Trichoderma* cultures were obtained by direct isolation from the substratum. Several collections were derived from stromata of Hypocrea teleomorphs.

It is also worth to mention that both species are plants-associated (isolated from soil) but their closest relationship is with *T. longibrachiatum* which is also known as opportunistic species. That is why more studies are required in order to find out whether these species also have a potential to cause diseases in immonocompromised humans.

We also reported on another *Trichoderma*-related isolate CBS 120951 that was associated with a cystic fibrosis (Druzhinina *et al.*, 2007). This isolate has been later included in the taxonomic study of Samuels and Ismaiel (2011). They have indicated that the so far "unidentified Hypocreaceae" noted in the literature as being isolated from lung of a patient with non-fatal pulmonary fibrosis belongs to the novel taxon *Hypocrea peltata*. This statement has been proved by the phylogenetic analysis based on of nucleotide sequences from the internal transcribed spacer region (ITS), 28S nuclear large subunit (LSU) of rDNA and RNA polymerase subunit B-encoding gene (rpb2). In all cases, fungal isolates of *H. peltata* (assembled by Samuels) and the strain CBS 120951 formed a highly supported clade (Samuels and Ismaiel, 2011).

References:

Druzhinina IS, LaFe K, Willinger B, Komon-Zelazowska M, Ammirati J, Kubicek CP & Rogers JD (2007) An unknown species from *Hypocreaceae* isolated from human lung tissue of a patient with non-fatal pulmonary fibrosis. *Clinical Microbiology Newsletters* **29**(23), 180-184.

Druzhinina IS, Komon-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 humas. *Mycrobiology* **154**, 3447-3459.

Druzhinina IS, Komon-Zelazowska M, Ismaiel A, Jaklitshc WM, Mulaw T, Samuels GJ & Kubicek CP (2012) Molecular phylogeny and species delimination in the *Longibrachiatum* Clade of *Trichoderma*. *Fung Genet Biol* **49**(5), 358-368.

Samuels GJ & Ismaiel A (2011) *Hypocrea peltata*: a mycological Dr Jekyll and Mr Hyde?. *Mycologia* **103** (3), 616-630.

6. Conclusive remarks: *Trichoderma* is a beneficial microorganism which is safe to apply. Almost safe!

Species of the genus *Trichoderma* are part of the most valuable groups of microorganisms which have had an impact on human prosperity in recent times. The number of literature (Google Scholar database retrieved at http://www.scholar.google.com ~ 182,000 hits, March 2014) and the amount of patents (Google patent database retrieved at http://www.google.com/patents ~ 293,000 hits, March 2014) being filed/granted on *Trichoderma* is still growing (Mukherjee *et al.,* 2013).

Aeons ago, the genus was raised by Persoon (1794) nevertheless the adventages of application of *Trichoderma* as plant disease biocontrol agents, mycoparasites and producers of antibiotic have been presented in the 1930s by Weindling (1932, 1934). Today, *Trichoderma* strains are the most widely used microbs all over the world.

In this thesis we have investigated potentially negative molecular ecological aspects of the genus *Trichoderma*. We have demonstrated that the fungus can cause a devastationg green mold disease on musroom farms world-wide. Althought many different *Trichoderma* species are usually present in mushroom substrates, only several species seem to be really specific causative agents of the green mold disease. The initial recognition of *T. pleuroticola* was made in the pioneering phylogenetic work of *Hypocrea/Trichoderma* (Kullnig-Gradinger *et al.*, 2002) where it was detected as *T.* cf. *aureoviride* DAOM 175924, a sister taxon to *T. harzianum* sensu lato. The first indications of the ability of this species to cause a *Pleurotus* green mold disease were obtained from Hungary and later on from Italy Poland and Romania. We recognized that although there are other *Hypocrea/Trichoderma* species detected in *Pleurotus* farms, *T.* cf. *aureoviride* DAOM 175924 is likely specific for this environment. The detailed phenetic and micromorphological analysis revealed the existence of the two distinct morphotypes, which were subsequently recognized on the basis of multiloci phylogeny. The first version of the manuscript presented in the thesis (Part 3.2. Komon-Zelazowska *et al.,* 2007a) contained the formal taxonomic descriptions of the two new species with the new names proposed. However in a course of the revision we learned that the South Korean scientists (Park *et al.,* 2006) have already published formal descriptions of these two species although presenting limited molecular data. As our study was based on a large sample of worldwide isolates and also contained

the detailed phylogenetic and phenetic characterization of the taxa, the editor found the publication worthwhile but suggested us to adapt the taxonomy of Park *et al.* (2006).

Although this study has certain importance for mushroom industry, from the perspective of fungal biology the following outcomes seem to be the most interesting: **(i)** despite that the variability of morphological characters is known to be very low in *Trichoderma* (the number of morphological species is several folds lower than the number of currently recognized phylogenetic species) the two closely related and strictly sympatric species *T. pleuroticola* and *T. pleurotum* have distinctively different phenotypes. Moreover, the morphology of *T. pleurotum* is not known to be present in the Harzianum Clade. Therefore the next challenge will be the search for the selective pressure, which could drive the evolution of *T. pleurotum* towards such differentiation. **(ii)** The source of *T. pleurotum* infection remains mysterious as this taxon has not been reported from any other environment so far. In this respect *T. pleurotum* resembles *T. aggressivum*, the causative agent of *Agaricus* green mold disease, which is also not known from nature. The application of *tef1*-based specific PCR primers developed for all three green mold species (Part 3.3. Kredics *et al.,* 2009) to total soil DNA extracted from different temperate samples did not reveal presence of *T. aggressivum* and *T. pleurotum*. In contrast to that, *T. pleuroticola* is frequent in environmental samples from North America and Europe. The search for the source of green mold infection constitutes another challenge for our colleagues and us. **(iii)** The dual confrontations of the *Trichoderma* species causing green mold diseases of mushrooms showed that *Agaricus bisporus* (at least the strain studied) is even more vulnerable to *T. pleurotum* and *T. pleuroticola*, however the distribution of these species is biased towards *Pleurotus* farms. The nature of such specialization may be explained by different substrata used for the two mushrooms but its biology remains unclear.

The ability of *Trichoderma* to cause invasive mycoses of immunocompromised humans should be considered as another potential risk factor associated with *Trichoderma*-based products for agriculture (*bio*fertilizers and *bio*fungicides). In the research presented in the thesis (Part 5.2 Druzhinina *et al*., 2008) we have got a possibility to analyze a nearly complete collection of clinical isolates of *Trichoderma*. Despite that their attribution to *T. longibrachiatum* was already known we assumed that there might be a certain specialization of these strains, which put them in a winning position in clinical environment. Moreover, the relatedness of *T. longibrachiatum* to *H. orientalis* was

not resolved at that time. As *T. longibrachiatum* is one of the most frequently isolated species the purpose of this study was obvious: we wanted to investigate the relation between the geographically broad sample of *T. longibrachiatum* and *H. orientalis* in order to trace the origin of clinical isolates. The first results showed that *T. longibrachiatum* and *H. orientalis* are genetically isolated taxa as they fulfill the criteria of the genealogical concordance phylogenetic species concept, and, more importantly, that the ability to attack immunocompromised humans is an unspecific property of both species. We also revealed that the isolates from rhizosphere of *Coffea arabica* from Ethiopia represent a distinct taxon that has been recently named as *Trichoderma aethiopicum* (Samuels *et al.,* 2012). Druzhinina et al. (2012) and the later group (Samuels *et al.,* 2012) also recognized *Trichoderma pinnatum* and a yet undescribed species *Trichoderma* sp. CBS 243.63, all three located in *T. longibrachiatum – H. orientalis* phylogenetic clade. From the phylogenetic perspective we may assume that all these three novel species may be also opportunistic causative agents of human mycoses. However there are no cases repoted so far.

Interestingly a considerable experimental effort was dedicated to the phenotypic profiling of clinical and none-clinical strains in respect to their ability to utilize different carbon sources at low, optimal and human body temperatures. As these data revealed no difference between clinical and environmental isolates they were not included in the manuscript but only mentioned in the discussion part.

Thus, we suggest to urge, that all strains planned for agricultural applications should be tested for growth at 37°C as a safety precaution, in order to minimize potential health risks. Additionally, the application of all five known species from *T. longibrachiatum – H. orientalis* phylogenetic clade and actually from the entire Section *Longibrachiatum* in biotechnology and agriculture should be controlled with special care or rather neglected, as the most common human pathogen within the genus *Trichoderma* is *T. longibrachiatum* (Druzhinina *et al.,* 2008).

The use of some *Trichoderma* species in contemporary biotechnologies is because of their ability to hydrolyze cellulose. The production of hydrolytic enzymes (cellulases and hemicellulases) has been mostly examined and commercially exploited in *T. reesei* (teleomorph *H. jecorina*). Mutants of the isolate QM 6a have been used for years for cellulase and heterologous protein production and, more recently, for the production of biofuels (Kubicek and Penttilä, 1998; Kumar *et al.,* 2008; Kubicek *et al.,*

2009). Although our study has demonstrated that *Trichoderma* contains some potentially toxic metabolites such as peptaibols (Part 4, Komon-Zelazowska *et al.,* 2007b), their toxicity to humans is quite low.

Thus, our study shows that the fungal genus *Trichoderma* is certainly a highly beneficial microorganism that is safe to apply in industry in agriculture if the applied strains are screened against their ability to cause green mold disease and grow at the temperature of human body. We would like to specially note that the correct molecular identification of every *Trichoderma* strain is required prior its application is proposed.

References:

Druzhinina IS, Komon-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**, 3447–3459.

Druzhinina IS, Komon-Zelazowska M, Ismaiel A, Jaklitsch WM, Mulaw T, Samuels GJ & Kubicek CP (2012) Molecular phylogeny and species delimination in the *Longibrachiatum* Clade of *Trichoderma*. *Fung Genet Biol* **49**(5), 358-368.

Komoń-Zelazowska M, Bissett J, Zafari D, Hatvani L, Manczinger L, Woo S, Lorito M, Kredics L, Kubicek CP & Druzhinina IS (2007a) Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms. *Applied and Environmental Microbiology* **73**, 7415-7426.

Komon-Zelazowska M, Neuhof T, Dieckmann R, Döhren H, Herrera-Estrella A, Kubicek CP & Druzhinina IS (2007b) The formation of atroviridin by *Hypocrea atroviridis* is conidiation-associated, and positively regulated by blue light and the G-protein GNA3. *Eukaryotic Cell* **6**(12), 2332-42.

Kredics L, Kocsubé S, Nagy L, Komon-Zelazowska M, Manczinger L, Sajben E, Nagy A, Vágvölgyi C, Kubicek CP, Druzhinina IS & Hatvani L (2009) Molecular identification of *Trichoderma* species associated with *Pleurotus ostreatus* and natural substrates of the oyster mushroom. *FEMS Microbiol Lett* **300**, 58-67.

Kubicek CP& Penttilä ME (1998) In Harman GE & Kubicek CP (eds) *Trichoderma and Gliocladium*, Vol. **2.** Enzymes, biological control and commercial applications. Taylor and Francis, London, 49-71.

Kubicek CP, Mikus M, Schuster A, Schmoll M & Seiboth B (2009) Metabolic engineering strategies for improvement of cellulase production by *Hypocrea jecorina. Biotechnol Biofuels* **2**, 19.

Kullnig-Gradinger CM, Szakács G & Kubicek CP (2002) Phylogeny and evolution of the fungal genus *Trichoderma* - A multigene approach. *Mycol Res* **106**, 757-767.

Kumar R, Singh S & Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *J Ind Microbiol Biotechnol* **35**, 377-391.

Mukherjee PK, Horwitz BA, Singh US, Mukherjee M & Schmoll M (2013) *Trichoderma* in agriculture, industry and medicine: an overview. In *Trichoderma*: Biology and Applications (eds PK Mukherjee *et al*.), 1-9.

Park MS, Bae KS & Yu SH (2006) Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. *Mycobiology* **34**, 111-113.

Persoon CH (1794) Disposita methodica fungorum. *Römer's Neues Mag Bot* **1**, 81–128.

Samuels GJ, Ismaiel A, Mulaw TB, Szakacs G, Druzhinina IS, Kubicek CP & Jaklitsch WM (2012) The Longibrachiatum Clade of *Trichoderma*: a revision with new species. *Fungal Diversity* **55**, 77-108.

Weindling R (1932) *Trichoderma lignorum* as a parasite of other soil fungi. *Phytopathology* **22**, 837-845.

Weindling R (1934) Studies on lethal principle effective in the parasitic action of *Trichoderma lignorum* on *Rhizoctonia solani* and other fungi. *Phytopathology* **24**, 1153-1179.

7. Acknowledgements

First and foremost, I owe my deapest gratitude to my supervisor and an enthusiastic scientist Irina Druzhinina for her invaluable guidance, trust, constant support, encouragement and for her friendship. Ira, thank you for protecting me from all Evil of the world. We have had many fruitful discussions and your feedback has always been helpful, insightful and right to the point.

Besides my advisor, I would like to express my special thanks to Christian Kubicek for his patience, motivation and immense knowledge.

My sincere thanks also goes to my beloved husband Radek Zelazowski for his support, understanding, care and standing behind me.

I greatly appreciate the collaboration with Walter Jaklitsch (University of Vienna), László Kredics (University of Szeged) and Quirico Migheli (University of Sassari).

I would also thank to my colleagues Alexey Kopchinskiy, Liliana Espino Tenorio de Rammer, Lea Atanasova, Temesgen Mulaw Belayneh, Marym Marzouk for their support, help and friendship. They provided a friendly and cooperative atmosphere at work. I would be remiss if I did not thank Rita Linke, Lorant Hatvani, Christian Seibel, Sabine Gruber, Marinna Mikus, Eda Akel, Małgorzata Boraca and Gabriele Maier-Grüner who deserves credit for general support, useful comments and beneficial discussions.

Last but not the least; I would like to thank my parents for supporting me throughout all my studies. Words cannot express how grateful I am to my mother, who looked after my daugther, Maja, while I was writing the thesis.

8. Curriculum Vitae with complete list of publications

Personal information

Name:	Mag. Monika Komon-Zelazowska
Date and place of birth:	25.08.1977, Radzyn Podlaski, Poland
Address:	Rechte Nordbahngasse 6/ 4/ 31, 1210 Vienna, Austria
E-mail:	monika.komon@gmx.at

Education

10/2006 – 04/2014	PhD thesis (Doz. Dr. Irina Druzinina). Specialization in Molecular Biology, Vienna University of Technology, Institute of Chemical Engineering, Austria
10/2004	Training at the University of Sassari, Institute of Plant Protection, Sardinia
06/2002	Diploma thesis "The determination of optimal fermentation conditions for superior lipase production by *Aspergillus candidus*" (Prof. Jerzy Lobarzewski)
08/2000	Personal development at the Polish Academy of Science, Institute of Biochemistry and Biophysics, Warsaw, Poland
1997 – 2002	Study at the Maria Curie-Sklodowska University, Institute of Biology and Biotechnology, Lublin, Poland
1992 – 1996	High School, Matura, Komarowka Podlaska, Poland

Research experience

Since 10/2013	Job hunter
01/2013 – 09/2013	Scientific Assistant at ACIB GmbH, Austria
09/2011 – 12/2012	Maternity leave
03/2010 – 12/2012	PhD Student at ACIB GmbH, Austria

11/2003 – 02/2010	Project Assistant at Vienna University of Technology, Institute of Chemical Engineering, Austria
11/2007 – 04/2009	University Assistant and additionally scientific secretary of the "IUMS Subcommision on Taxonomy of Trichoderma and Hypocrea" (www.ISTH.info)
01/2004 – 09/2013	Curator of TU Collection of Industrially Important Microorganisms (TUCIM) at Vienna University of Technology, Austria
09/2000 – 08/2001	Work experience as biology teacher at Compulsory School, Poland

Teaching experience

Institute of Chemical Ingineering, Vienna University of Techology, Vienna, Austria:

Teaching Assistant in the lab course of Microbiology (2008-2010)

Teaching Assistant in the lab course of Advanced Biotechnology (2008-2009)

Additional skills

Methods

Techniques of molecular biology:	Isolation of DNA and RNA
	Synthesis of cDNA
	PCR, primer construction and cloning
	Sequence analysis
Techniques of protein biochemistry:	Protein overexpression, protein purification
	Gel-electrophoresis of proteins
	Western-Blot
Bioinformatics:	GenBank search: BLAST
	Multiple sequences alignment

Language skills

Polish	Mother language
English	Fluent in writing and speaking
German	Fluent in writing and speaking
Russian	Basic level

Publications

Molecular phylogeny and species concept:

1. Druzhinina IS, **Komon-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* **10**, 1016.
2. Atanasova L, Jaklitsch WM, **Komon-Zelazowska M**, Kubicek CP & 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 – 7267.
3. Druzhinina IS, Kubicek CP, **Komon-Zelazowska M**, Belayneh Mulaw T & Bissett J (2010) The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. *BMC Evolutional Biology* **10**, 94.
4. Druzhinina IS, **Komon-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. doi:10.1371/journal.pone.0009191
5. Zhang CL, Wang GP, Mao LJ, **Komon-Zelazowska M**, Yuan ZL, Lin FC, Druzhinina IS & Kubicek CP (2010) *Muscodor fengyangensis* sp. nov. from southeast China: morphology, physiology and production of volatile compounds. *Fungal Biol* **114**(10), 797-808. doi: 10.1016/j.funbio.2010.07.006
6. Druzhinina IS, **Komon-Zelazowska M,** Kredics L, Hatvani L, Antal Z, Mulaw Belayneh T & Kubicek CP (2008) Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable to cause invasive mycoses of humans. *Microbiology* UK, **154**, 3447-3459.
7. Kubicek CP, **Komon-Zelazowska M** & Druzhinina IS (2008) Fungal genus *Hypocrea/Trichoderma*: from barcodes to biodiversity. *J Zhejiang Univ Sci B* **9**, 753-763.

8. Druzhinina IS, LaFe K, Willinger B, **Komon-Zelazowska M**, Ammirati J, Kubicek CP & Rogers JD (2007) An unknown species from *Hypocreaceae* isolated from human lung tissue of a patient with non-fatal pulmonary fibrosis. *Clinical Microbiology Newsletters* **29**(23), 180-184.
9. Jaklitsch WM, **Komon M**, Kubicek CP & Druzhinina IS (2006) *Hypocrea crystalligena* sp. nov., a common European species with a white-spored *Trichoderma* anamorph. *Mycologia* **98**(3), 499-513.
10. Druzhinina IS, Kopchinskiy AG, **Komon M**, Bissett J, Szakacs G & Kubicek CP (2005) An oligonucleotide barcode for species identification on *Trichoderma* and *Hypocrea*. *Fungal Genetics and Biology* **42**, 813-828.
11. Jaklitsch WM, **Komon M**, Kubicek CP & Druzhinina IS (2005) *Hypocrea voglmayrii* sp. nov. from the Austrian Alps represents a new phylogenetic clade in *Hypocrea/Trichoderma*. *Mycologia* **97**, 1365-1378.

Molecular diversity of *Trichoderma* and other fungi

12. López-Quintero CA, Atanasova L, Franco-Molano AE, Gams W, **Komon-Zelazowska M**, Theelen B, Müller WH, Boekhout T & Druzhinina IS (2013) DNA barcoding survey of *Trichoderma* diversity in soil and litter of the Colombian lowland Amazonian rainforest reveals *Trichoderma strigosellum* sp. nov. and other species. *Antonie Van Leeuwenhoek* **104**(5), 657-74.
13. Gal-Hemed I, Atanasova L, **Komon-Zelazowska M**, Druzhinina IS, Viterbo A & Yarden O (2011) Marine isolates of *Trichoderma* as potential halotolerant agents of biological control for arid-zone agriculture. *Appl Environ Microbiol* **77**, 5100-5109.
14. Migheli Q, Balmas V, **Komon-Zelazowska M**, Scherm B, Caria R, Kopchinskiy AG, Kubicek CP & Druzhinina IS (2009) Soils of a Mediterranean hotspot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European and likely invasive species of *Hypocrea/Trichoderma*. *Environmental Microbiology* **1**(1), 35-46.
15. Paz Z, **Komon-Zelazowska M**, Druzhinina IS, Aveskamp MM, Schnaiderman A, Aluma Y, Carmeli S, Ilan M & Yarden O (2009) Diversity and potential antifungal properties of fungi associated with a Mediterranean sponge. *Fungal Diversity*, DOI 10.1007/s13225-010-0020-x
16. Zachow C, Berg C, Müller H, Meincke R, **Komon-Zelazowska M**, Druzhinina IS, Kubicek CP & 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.

Applied physiology of *Trichoderma*

17. Mikus M, Hatvani L, **Komon-Zelazowska M**, Neuhof T, Dieckmann R, Druzhinina IS, von Döhren & Kubicek CP (2009) Differential regulation and posttranslational processing of the class II hydrophobin genes from the biocontrol fungus *Hypocrea atroviridis*. *Appl Environ Microbiol* **75**(10), 3222 – 3229.
18. **Komon-Zelzowska M**, Neuhof T, Dieckmann R, Döhren H, Herrera-Estrella A, Kubicek CP & Druzhinina IS (2007) The formation of atroviridin by *Hypocrea atroviridis* is conidiation-associated, and positively regulated by blue light and the G-protein GNA3. *Eukaryotic Cell* **6**(12), 2332-42.
19. Kubicek CP, **Komon-Zelzowska M**, Karaffa-Sandor E & Druzhinina IS (2007) Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma*, a review. *Chem Biodivers* 4(6), 1068-1082.
20. Nagy V, Seidl V, Szakacs G, **Komon-Zelazowska M**, Kubicek CP & Druzhinina IS (2007) Application of DNA BarCodes for screening of industrially important fungi: the haplotype of *Trichoderma harzianum* sensu stricto indicates superior chitinase formation. *Appl Environ Microbiol* **73**(21), 7048-7058.

Trichoderma green mold disease of mushrooms

21. Sobieralski K, Siwulski M, **Komon-Żelazowska M**, Błaszczyk L, Górski R, Spiżewski T & Sas-Golak I (2012) Evaluation of the growth of *Trichoderma pleurotum* and *Trichoderma pleuroticola* isolates and their biotic interaction with *Pleurotus* sp. *Journal of Plant Protection* **52(2)**, 235-239.
22. Kredics L, Kocsubé S, Nagy L, **Komon-Zelazowska M**, Manczinger L, Sajben E, Nagy A, Vágvölgyi C, Kubicek CP, Druzhinina IS & Hatvani L (2009) Molecular identification of *Trichoderma* species associated with *Pleurotus ostreatus* and natural substrates of the oyster mushroom. *FEMS Microbiol Lett* **300**, 58-67.
23. **Komon-Zelazowska M**, Bissett J, Zafari D, Hatvani L, Manczinger L, Woo S, Lorito M, Kredics L, Kubicek CP & Druzhinina IS (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.

Conference participations – lectures

1. Komon-Zelazowska M, Mpika J, Kubicek CP & Druzhinina IS. **Icommensurably low diversity of *Hypocrea/Trichoderma* in the rhizosphere of *Theobroma cacao L*. infected by black pod in Ivory Coast cacao plantations.** Biological Control Symposium, Graz, Austria, March 2007

2. Komon-Zelazowska M, Mulaw Belayneh T, Kubicek CP & Druzhinina IS. **Indigenous *Trichoderma/Hypocrea* species as efficient agents for control of coffee wilt disease in Ethiopia.** 9th International Workshop on on *Trichoderma* and *Gliocladium*, Vienna, Austria, April 2006

Conference participations – posters

1. Espino Tenorio de Rammer L, Komon-Zelazowska M, Ribitsch D, Greimel K, Herrero Acero E, Guebitz G, Kubicek CP & Druzhinina IS. **The HFB4 family : novel class II hydrophobins of *Trichoderma* with universal infrageneric distribution and potential for industrial applications.** ECFG 11 - 11th European Conference on Fungal Genetics, Marburg, Deutschland, March 2012
2. Komon-Zelazowska M, Kopchinskiy A, Kubicek CP & Druzhinina IS. **Genus-wide screening for class II hydrophobins in *Hypocrea/Trichoderma* using phylogenetic and metagenomic approaches.** XVI Congress of European Mycologists, Halkidiki, Griechenland, September 2011
3. Atanasova L, Jaklitsch WM, Komon-Zelazowska M, Kubicek CP & Druzhinina IS. **A new cellulose producing species *Trichoderma parareesei* nom. prov. reveals high antagonistic potential against soil and plant pathogenic fungi.** IOBC/WPRS Working Group "Biological control of fungal and bacterial plant pathogens", Graz, Austria, June 2010
4. Komon-Zelazowska M, Kredics L, Hatvani L, Antal Z, Belayneh T, Kubicek CP & Druzhinina IS. **Human opportunistic *Trichoderma* spp. comprise phylogenetically related holomorph *Hypocrea orientalis* and the stricly clonal species *Trichoderma longibrachiatum*.** 11th International Congress of Mycology, Istanbul, Turkey, August 2008
5. Druzhinina IS, Komon-Zelazowska M, Neuhoff T, von Döhren & Kubicek CP. **Peptaibols, *Trichoderma* secondary metabolites with versatile application: identification of biochemical traits for submerged production.** LISA, Vienna, Austria, October 2006
6. Dela Cruz TE, Schulz B, Komon-Zelazowska M & Druzhinina IS. **Conidial morphology: homology or homoplasy? The analysis of molecular data shows that marine *Dendryphiella* species do not belong to the genus *Scolecobasidium.*** 8th International Mycological Congress, Crains, Australia, August 2006
7. Komon-Zelazowska M, Bisset J, Zafari J, Hatvani L, Woo SL, Manczinger L, Lorito M, Kredics L, Kubicek CP & Druzhinina IS. **A recently emerging green mold disease in Eurasian oyster mushroom farms is caused by new species of *Trichoderma*.** 8th International Mycological Congress, Cairns, Australia, August 2006
8. Dela Cruz TE, Schulz B, Komon-Zelazowska M, Kubicek CP & Druzhinina IS. **DNA sequence analysis and metabolic profiles demonstrate existence of two marine *Dendryphiella* species that do not belong to**

the genus *Scolecobasidium.* 9th European Conference of Fungal Genetics (ECFG8), Vienna, Austria, April 2006

9. Karimova LY, Komon-Zelazowska M, Druzhinina IS, Tukhbatova R, Tazetdinova D, Kubicek CP & Alimova FK. **Biodiversity and ecophysiology of *Hypocrea/Trichoderma* strains isolated from the human skull dated as VIII-VI B.C. discovered in the Murzichinsk II Tomb (Republic of Tatarstan, Russia).** 9th International Workshop on *Trichoderma* and *Gliocladium*, Vienna, Austria, April 2006
10. Komon-Zelazowska M, Mpika J, Kebe Boubacar I, Kubicek CP & Druzhinina. **Diversity of *Hypocrea/Trichoderma* in the rhizosphere *of Theobroma cacao L*. in Ivory Coast.** 9th International Workshop on *Trichoderma* and *Gliocladium*, Vienna, Austria, April 2006

9. Appendix: Co-authored manuscript that includes materials from the thesis

Sobieralski K, Siwulski M, **Komon-Żelazowska M**, Błaszczyk L, Górski R, Spiżewski T & Sas-Golak I (2012) Evaluation of the growth of *Trichoderma pleurotum* and *Trichoderma pleuroticola* isolates and their biotic interaction with *Pleurotus* sp. *Journal of Plant Protection Research* **52**(2), 235-239.

JOURNAL OF PLANT PROTECTION RESEARCH Vol. 52, No. 2 (2012)

EVALUATION OF THE GROWTH OF *TRICHODERMA PLEUROTUM* AND *TRICHODERMA PLEUROTICOLA* ISOLATES AND THEIR BIOTIC INTERACTION WITH *PLEUROTUS* SP.

Krzysztof Sobieralski[1], Marek Siwulski[1], Monika Komon-Żelazowska[2], Lidia Błaszczyk[3] Romuald Górski[4], Tomasz Spiżewski[1], Iwona Sas-Golak[1]

[1] Department of Vegetable Crops, Poznań University of Life Sciences
Dąbrowskiego 159, 60-594 Poznań, Poland
[2] Division Applied Biochemistry and Gene Technology, Institute of Chemical Engineering Vienna University of Technology
Getreidemarkt 9/1665, A-1060, Vienna, Austria
[3] Institute of Plant Genetics, Polish Academy of Science
Strzeszyńska 34, 60-479 Poznań, Poland
[4] Department of Plant Protection Methods, Poznań University of Life Sciences
Zgorzelecka 4, 60-198 Poznań, Poland

Received: April 26, 2011
Accepted: January 27, 2012

Abstract: Growth of *Trichoderma pleurotum* and *T. pleuroticola* isolates on the Potato Dextrose Agar (PDA) medium was investigated. *T. pleuroticola* isolates showed a significantly greater diameter of their mycelium colonies after 5 days of incubation than the *T. pleurotum* isolates. In addition, biotic interactions between *T. pleurotum* and *T. pleuroticola* isolates and species of *Pleurotus* sp. were determined. The following six species of oyster mushroom were used: *P. florida, P. cornucopiae, P. pulmonarius, P. columbinus, P. ostreatus* and *P. eryngii*. It was demonstrated that isolates of the *T. pleuroticola* species limited the growth of the examined species of oyster mushroom to a much greater extent than the isolates of the *T. pleurotum* species.

Key words: *Trichoderma*, oyster mushroom, mycelium growth, biotic interaction

INTRODUCTION

Aggressive species of *Trichoderma* fungi may cause green mould diseases – a serious problem in mushroom production. In *Agaricus bisporus* cultivation the pathogenic *Trichoderma* were designated as *T. aggressivum* f. *europaeum* (Th2) and *T. aggressivum* f. *aggressivum* (Th4) (Williams *et al.* 2003). Green moulds occur on a massive scale in oyster mushroom plantations in North America (Sharma and Vijay 1996), South Korea (Park *et al.* 2004a, b, c, 2006), Italy (Woo *et al.* 2004), Hungary (Hatvani *et al.* 2007) as well as in Romania (Kredics *et al.* 2006). Bałaszczyk *et al.* (2011) investigated molecular variability of *Trichoderma* strains occurring in Poland, including those isolated from the substrate used in mushroom cultivations. In the case of oyster mushroom cultivations, the following two closely related genetically *Trichoderma* species were identified as pathogens: *T. pleuroticola* and *T. pleurotum* (Kommon-Żelazowska *et al.* 2007). However, phenotypically, the above species exhibit considerable differences. They were identified in cultivation media in Europe, Iran, and South Korea. Recently, *T. pleuroticola* and *T. pleurotum* species were identified in Spain as well (Gea 2009). In 2010, *T. pleuroticola* and *T. pleurotum* strains were also identified in oyster mushroom cultivations in Poland (Siwulski *et al.* 2011). The wild growing *P. ostreatus* species was accompanied by a number of *Trichoderma* species, most commonly by *T. pleuroticola* but also by *T. harzianum*, *T. longibrachiatum* and *T. atroviride* (Kredics *et al.* 2009).

The first aim of the performed investigations was to determine growth of *T. pleuroticola* and *T. pleurotum* isolates. The second aim was to determine the interactions between these isolates and several species of *Pleurotus* sp. in *in vitro* conditions.

MATERIALS AND METHODS

The oyster mushroom species used in the experiments were derived from the collection of cultivated and medicinal mushrooms of the Department of Vegetable Crops at Poznań University of Life Sciences, Poland (Table 1). Isolates of the *Trichoderma* genus: four *T. pleurotum* isolates and four *T. pleuroticola* isolates are described in table 2.

*Corresponding address:
sobieralski@up.poznan.pl

Table 1. List of *Pleurotus* sp. strains used in the experiment

Species	Strain number	Origin
P. florida	Pf149/B	Collection of cultivated and medicinal mushrooms from the Department of Vegetable Crops, Poznań University of Life Sciences, Poland
P. cornucopiae	Pc74/C	
P. pulmonarius	Pp47/A	
P. columbinus	Pc88/F	
P. ostreatus	Po44/S	
P. eryngii	Pe132/P	

Table 2. List of *Trichoderma* isolates used in the experiment

Isolate	Strain number	Origin
T. pleurotum	E136	Vienna University of Technology, Institute of Chemical Engineering, Division Applied Biochemistry and Gene Technology, Austria
T. pleurotum	E139	
T. pleurotum	T53/B	Institute of Genetics Polish Academy of Science, Poznań, Poland
T. pleurotum	T270/C	
T. pleuroticola	M141	Vienna University of Technology, Institute of Chemical Engineering, Division Applied Biochemistry and Gene Technology, Austria
T.pleuroticola	M143	
T. pleuroticola	T6/PR	Institute of Genetics Polish Academy of Science, Poznań, Poland
T. pleuroticola	T52/2D	

The trial was conducted on Potato Dextrose Agar (PDA) medium (Oxoid Ltd., England) in the biological laboratory of the Department of Vegetable Crops at Poznań University of Life Sciences. During the first stage of the investigations, growth of the examined *T. pleurotum* and *T. pleuroticola* isolates on the above-mentioned PDA medium was determined. Inoculations were performed in a laminar-airflow cabinet by putting mycelia discs (5 mm diameter) of the examined *Trichoderma* isolate in the centre of the medium in a Petri dish. The dish was 9 cm in diameter. Discs were cut out from the PDA medium overgrown with the mycelium of the examined strains. Incubation was carried out in an incubator with no light access, at a temperature of 24–25C and relative air humidity ranging from 80 to 85%. The diameter of the fungus colony was measured after 5 days of incubation.

In the course of the second stage of the experiment, the individual biotic effect (IBE) was estimated with the index of biotic relations developed by Mańka (1974) (Table 3). For this purpose, mycelia discs of *Pleurotus* strains and competitive *Trichoderma* were placed on a Petri dish at a distance of 4 cm from each other. Using a plastic pipe of 5 mm diameter, the discs were cut out from PDA media overgrown with the mycelium of the examined mushroom. The mycelia discs of examined *T. pleurotum* and *T. pleuroticola* isolates, were inoculated 7 days after the inoculation of the tested *Pleurotus* strain. Incubation was carried out in the conditions described above. Mycelium growth measurements of the investigated fungus were taken every 24 hours. Assessments of interactions between the developing mycelia were determined. The observations noted: the degree of one colony surrounding the other, width of the inhibition zone, and growth limitation or infestation of one colony by the other. A precise description of the method of conducting the experiment was given by Frużyńska-Jóźwiak *et al.* (2010).

The experiment was established in six replications in a random design. Two cycles of the experiments were conducted. No significant differences between cycles were found.

Table 3. Score scale for the determination of individual biotic effect (IBE) (acc. to Mańka 1974)

Type of interaction between colonies	Points
Both colonies are in contact along a straight line	0
Colony A remains in contact with colony B along a slightly curved line so that it surrounds less than 1/3 of colony A	+1
Colony A remains in contact with colony B along a curved line so that it surrounds at least 1/3 but less than 1/2 of colony A	+2
Colony A remains in contact with colony B along a curved line so that it surrounds at least 1/2 but less than 2/3 of colony A	+3
Colony A remains in contact with colony B along a curved line so that it surrounds at least 2/3 or more of colony A	+4
Each millimetre of the inhibition zone is occupied by colony A	+1
Colony B at least by 1/3 but less that ½ smaller than its control colony developed individually on a separate plate	+2
Colony B at least by 2/3 smaller than its control colony developed individually on a separate plate	+3
Colony B completely undeveloped	+4

RESULTS

Figure 1 presents growth of *T. pleurotum* and *T. pleuroticola* isolates on the PDA medium. The diameters of colonies of different strains of *T. pleurotum* after 5 days of incubation were similar and ranged from 72 to 75 mm. *T. pleuroticola* isolates exhibited significantly greater diameters of colonies after the above-mentioned period of incubation. The colony diameter of these isolates ranged from 83 to 88 mm. There were no significant differences between isolates.

When analysing IBE indices for *T. pleurotum* and *T. pleuroticola* isolates, this index assumed a different value depending on the tested *Pleurotus* species. The examined *T. pleurotum* isolates exhibited a very similar IBE index in their interaction with all the species of *Pleurotus*. This index, in the case of all the examined isolates, varied from +3 to +5. No difference was found in the IBE index value for *T. pleurotum* isolates in their interaction with *P. cornucopiae*. In all the cases, the IBE index reached +5. The greatest sensitivity to the effect of *T. pleurotum* was shown by *P. cornucopiae*. A very similar value was also exhibited by the index in the case of the *T. pleurotum* isolate in its interaction with *P. columbinus*. The IBE index amounted to +4 with the exception of the *T. pleurotum* E136 isolate where the value was higher and reached +5. Considerable variation in the IBE index was determined in the case of *T. pleurotum* isolates in their interactions with *P. ostreatus* and *P. eryngii* (Table 4).

Table 4. Individual biotic effect index for *T. pleurotum* and *T. pleuroticola* isolates

Trichoderma species	Isolate designation	*Pleurotus*											
		florida		*cornucopiae*		*pulmonarius*		*columbinus*		*ostreatus*		*eryngii*	
T. pleurotum													
T. pleurotum	E136 E139	+4	+4	+5	+5	+4	+4	+5	+4	+4	+5	+5	+4
T. pleurotum	T53/B T270/C	+5	+4	+5	+5	+4	+5	+4	+4	+4	+3	+3	+4
T. pleurotum													
T. pleuroticola													
T. pleuroticola	M141 M143 T6/	+6	+6	+6	+7	+6	+6	+6	+6	+5	+6	+6	+6
T. pleuroticola	PR T52/2D	+5	+6	+7	+7	+7	+6	+5	+7	+5	+7	+6	+6
T. pleuroticola													

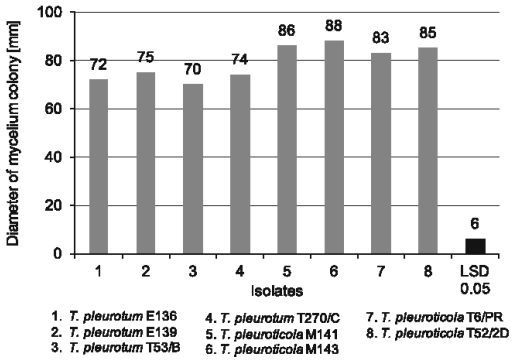


Fig. 1. Diameter of the colonies of *T. pleurotum* and *T. pleuroticola* isolates after 5 days of incubation on PDA medium

The IBE index for the examined *T. pleuroticola* isolates and *Pleurotus* sp. strains was significantly higher and ranged from +5 to +7. For the interaction between the *T. pleuroticola* isolate and *P. florida*, the IBE index was similar and amounted to +6, with the exception of the T6/PR isolate where the IBE index was found to be lower (+5). *T. pleuroticola* isolates in their interaction with *P. cornucopiae* showed a similar IBE index amounting to +7, with the exception of the M141 strain for which the IBE index reached the value of +6. This species of *Pleurotus* showed the greatest sensitivity to *T. pleuroticola* isolates. The IBE index for the examined *T. pleuroticola* isolates and *P. pulmonarius* as well as *P. eryngii* amounted to +6, with the exception of the T6/PR isolate for which the IBE value was higher - reaching a value of +7. The IBE index for interactions between the *T. pleuroticola* isolates and *P. columbinus* as well as *P. ostreatus* fluctuated within wider limits from +5 to +7.

DISCUSSION

Experiments involving the growth of *T. pleurotum* and *T. pleuroticola* isolates on PDA medium did not show any significant differences in colony diameters of the isolates after 5 days of incubation. All the examined *T. pleuroticola* isolates exhibited a significantly greater colony diameter after the incubation period than *T. pleurotum* isolates. The above results correspond with those reported by Siwulski *et al.* (2011).

In the available literature on the subject, there is no precise information regarding the impact of *T. pleurotum* and *T. pleuroticola* species on the mycelium development of different species of *Pleurotus* sp. In the described investigations, the authors used six different *Pleurotus* species as well as eight isolates of *T. pleurotum* and *T. pleuroticola*. The analysis of IBE indices for individual *T. pleurotum* and *T. pleuroticola* isolates demonstrates that isolates of the *T. pleuroticola* species reduced growth of the examined *Pleurotus* sp. species considerably more than isolates of the *T. pleurotum*. In the case of all examined *T. pleurotum* isolates, the value of the IBE index in their interactions with *Pleurotus* species ranged from +3 to +5. The IBE index for *T. pleuroticola,* on the other hand, reached a value from +5 to +7. When analysing table 3, it can be said that the IBE index of *T. pleurotum* isolates in interaction with individual species of *Pleurotus,* was fairly similar within species and isolates. *T. pleurotum* to the greatest extent inhibited the development of *P. cornucopiae,* which was the most sensitive species to the effect of this fungus. The relatively highest variability of the IBE index for *T. pleurotum* isolates and the *Pleurotus* species occurred in the case of *P. eryngii*. A similar variability in the IBE index was determined for the *T. pleurotum* isolates in interaction with *P. ostreatus*. Greater differences were found in the value of the IBE index for *T. pleuroticola* isolates in interaction with the *Pleurotus* species. The species most sensitive to *T. pleuroticola* was *P. cornucopiae*

The obtained results support the observations of Kommon-Żelazowska *et al*. (2007) regarding faster mycelium growth and the overgrowing of the *P. ostreatus* mycelium by *T. pleuroticola* isolates. The above-mentioned researchers demonstrated that *T. pleuroticola* can overgrow *P. ostreatus* mycelium, whereas the *T. pleurotum* isolates penetrate up to 3 mm into *P. ostreatus* mycelium and then it ceases to overgrow mycelium of the oyster mushroom. The performed investigations indicated that mycelium of the examined oyster mushroom species exhibited a certain antagonistic response in relation to *T. pleurotum* isolates. The above experiments confirmed the fact that none of the experimental mycelia of oyster mushroom exhibited an antagonistic response to *T. pleuroticola* isolates, which were characterized by a very high IBE index in their interactions with nearly all *Pleurotus* species. Only in some cases did the value of the IBE index for the examined *T. pleuroticola* isolates in their interaction with *Pleurotus* sp. species amount to +5. In the majority of the cases, the index reached a value of +6 or +7 indicating the absence of antagonistic responses in relation to *T. pleuroticola* isolates.

Information can be found in the literature about some mushroom species which exhibit a defense response to some aggressive *Trichoderma* isolates. This kind of response was shown by the shiitake (*Lentinula edodes*) as well as oyster mushrooms (*P. ostreatus* and *P. eryngii*) (Savoie *et al*. 2001). *Trichoderma aggressivum* f. *europaeum* is one of the most dangerous pathogens in mushroom production. This species causes large losses in edible and medicinal mushroom cultivation. Earlier investigations by the authors on interaction of edible mushroom species with pathogenic fungi revealed that certain antagonistic responses to *T. aggressivum* f. *europaeum* isolates were shown by some strains of *Coprinus comatus*. (Frużyńska-Jóźwiak *et al*. 2011). Other investigations carried out by the authors (Siwulski *et al*. 2009; Sobieralski *et al*. 2009) correspond with the results obtained in our described experiment. The tested species and strains of *Agaricus* and *Pleurotus* were very sensitive to the effect of *Trichoderma*. They did not exhibit any defensive response in relation to *T. aggressivum* f. *europaeum* isolates. The studies also revealed a significant ability of *Trichoderma* to inhibit mycelium development in the case of some *Pleurotus* species. The most sensitive to *Trichoderma* impact was *P. cornucopiae*. The results clearly indicated greater inhibition of *Pleurotus* growth by *T. pleuroticola* isolates.

REFERENCES

Błaszczyk L., Popiel D., Chełkowski J., Koczyk G., Samuels G. J., Sobieralski K., Siwulski M. 2011. Species diversity of *Trichoderma* in Poland. J. Appl. Genetics – on line (DOI 10.1007/s13353-011-0039-z)

Frużyńska-Jóźwiak D., Siwulski M., Sobieralski K., Sas-Golak I., Błaszczyk L. 2011. Impact of *Trichoderma* isolates on the mycelium development of wild strains of *Coprinus comatus* (Müll.) S.F. Gray. J. Plant Prot. Res. 51 (2): 163–166.

Gea F.J. 2009. First report of *Trichoderma pleurotum* on oyster mushroom crops in Spain. J. Plant Pathol. 91 (2). p. 504.

Hatvani L., Antal L., Manczinger L., Szekeres A., Druzhinina I.S., Kubicek C.P., Nagy E., Vagvolgyi C., Kredics L. 2007. Green mold diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderema* species. Phytopathology 97 (4): 532–537.

Komon-Zelazowska M., Bisset 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 green mold disease in oyster mushroom farms worldwide. Appl. Environ. Microbiol. 73 (22): 7415–7426.

Kredics L., Hatvani L., Antal L., Manczinger L., Druzhinina I.S., Kubicek C.P., Szekeres A., Nagy A., Vagvolgyi C., Nagy E. 2006. Green mold disease of oyster mushroom in Hungary and Transylvania. Acta Microbiol. Immunol. Hung. 53: 306–307.

Kredics L., Kocsube S., Nagy L., Komon-Zelazowska M., Manczinger L., Sajben E., Nagy A., Vagvolgyi C., Kubicek C.P., Druzhinina I.S., Hatvani L. 2009. Molecular identification of *Trichoderma* species associated with *Pleurotus osteratus* and natural substrates of the oyster mushroom. Microbial. Lett. 300: 58–67.

Mańka K. 1974. Zbiorowiska grzybów jako kryterium oceny wpływu środowiska glebowego na choroby roślin. Zesz. Probl. Post. Nauk Rol. 160: 9–23.

Park M.S., Bae K.S., Yu S. H. 2004a. Molecular and morphological analysis of *Trichoderma* isolates associated with green mold epidemic of oyster mushroom in Korea. J. Huazhong Agric. Univ. 23: 157–164.

Park M.S., Bae K.S., Yu S.H. 2004b. Morphological and molecular of *Trichoderma* isolates associated with green mold epidemic of oyster mushroom in Korea. Accessed on: www.mushworld.com

Park M.S., Bae K.S., Yu S.H. 2004c. Morphological and molecular of *Trichoderma* isolates associated with green mold epidemic of oyster mushroom in Korea. New Challenges in Mushroom Science. p. 143–158. In: Proc. 3rd Meeting of Far East Asia for Collaboration of Edible Fungi Research. Suwon, Korea.

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.

Savoie J-M., Iapicco R., Largeteau-Mamoun M. 2001. Factors influencing the competitive saprophytic ability of *Trichoderma harzianum* Th2 in mushroom compost. Mycol. Res. 105: 1348–1356.

Sharma S.R., Vijay B. 1996. Yield loss in *Pleurotus ostreatus* spp. caused by *Trichoderma viride*. Mushroom Res. 5: 19–22.

Siwulski M., Sobieralski K., Buśko S., Górski R. 2009. Wpływ różnych szczepów grzybów z rodzaju *Trichoderma* na rozwój grzybni dwóch odmian boczniaka ostrygowatego –*Pleurotus ostreatus* (Jacq.) Queilet. [Influence of different isolates of Trichoderma genus fungi on mycelium development of two strains of oyster mushroom – Pleurotus ostraentus (J. acq.) Queilet]. Prog. Plant Prot./Post. Ochr. Roślin 49 (2): 714–718.

Siwulski M., Sobieralski K., Błaszczyk L., Frąszczak B., Frużyńska-Jóźwiak D., Sas-Golak I. 2011. Evaluation of mycelium growth of several *T. pleurotum* and *T. pleuroticola* isolates and their biotic interaction with *Pleurotus florida*. Phytopathologia 59: 43–48.

Sobieralski K., Siwulski M., Frużyńska-Jóźwiak D., Górski R. 2009. Impact of *Trichoderma aggressivum* f. *europaeum* Th2 on the yielding of *Agaricus bisporus*. Phytopathol. Pol. 53: 5–10.

Woo S.L., Di Benedetto P., Senator M., Abadi K., Gigante S., Soriente I., Ferraioli S., Scala F., Lorito M. 2004. Identification and characterization of *Trichoderma* species aggressive to *Pleurotus* in Italy. J. Zhejiang Univ. Agric. Life Sci. 30: 469–470.

Williams J., Clarkson J.M., Mils P.R., Cooper R.M. 2003: Saprotrophic and mycoparasitic components of aggressiveness of *Trichoderma harzianum* groups toward the commercial mushroom *Agaricus bisporus*. Appl. Environm. Microbiol. 69 (7): 4192–4199.