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

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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.

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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 *Batrachomyces 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'-GAGTCAAGGAGGCC-TTCTCCC-3') and CAL-737R (5'-CATCTTTCTGGCCATCATGG-3') (Chaverri *et al.*, 2003). Purified PCR products for ITS1 and 2, *tef1*, *cal1* and *chi18-5* 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	<i>tef1</i>	<i>chi18-5</i>	<i>cal1</i>
<i>Trichoderma longibrachiatum</i>	C.P.K. 1254	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	<i>Agaricus</i> 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	<i>Pleurotus</i> 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	<i>Agaricus</i> 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	<i>tef1</i>	<i>chi18-5</i>	<i>cal1</i>
<i>Hypocrea orientalis</i>	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
	C.P.K. 166	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
<i>T. sp.</i> PS III	C.P.K. 1817	PPRC J11	<i>Coffea arabica</i> rhizosphere	Jimma, Ethiopia	EU401565	EU401614	EU401533	EU401482
	C.P.K. 1837	PPRC W4	<i>C. arabica</i> rhizosphere	Wellega, Ethiopia	EU401566	EU401615	EU401534	EU401483
	C.P.K. 1841	PPRC H5	<i>C. arabica</i> rhizosphere	Harerga, Ethiopia	NA	EU401616	EU401535	EU401484
<i>H. sp.</i> PS IV	C.P.K. 1261	CBS 243.63	Under <i>Nothofagus</i>	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		
	<i>cal1</i>	<i>chi18-5</i>	<i>tefl</i>
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 $\leftrightarrow$ C	0.06	0.06	0.78
A $\leftrightarrow$ G	0.36	85.84	90.40
A $\leftrightarrow$ T	0.23	0.11	0.30
C $\leftrightarrow$ G	0.08	0.46	0.33
C $\leftrightarrow$ T	80.60	7.11	0.51
G $\leftrightarrow$ 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 *TrichOKey* (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 *tefl*, 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 *tefl* 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 *tefl* 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 *tefl*, *cal1* and *chi18-5* (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 ($\phi=0.32$, $P=0.0005$), whereas the possibility of recombination was rejected for clade PS I (*T. longibrachiatum*) ($\phi=0.14$, $P=0.58$). 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 *tefl*) the

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 shading 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) r^2 (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 (Vizcaino *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 *Batrachomyces 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 it 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 conidiolate *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.

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