ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Molecular Phylogenetics and Evolution 27 (2003) 302–313

MOLECULAR PHYLOGENETICS AND EVOLUTION

www.elsevier.com/locate/ympev

Multilocus phylogenetic structure within the *Trichoderma harzianum/Hypocrea lixii* complex

Priscila Chaverri,[a,b,*] Lisa A. Castlebury,[b] Gary J. Samuels,[b] and David M. Geiser[a]

[a] *Department of Plant Pathology, The Pennsylvania State University, Buckhout Laboratory, University Park, PA 16802, USA*
[b] *United States Department of Agriculture, Agricultural Research Service, Systematic Botany and Mycology Laboratory, Rm. 304, B-011A, BARC-W, Beltsville, MD 20705-2350, USA*

Received 13 March 2002; revised 2 September 2002

Abstract

Trichoderma harzianum is a ubiquitous species in the environment and is effective in the biological control of plant-pathogenic fungi. *T. harzianum* has not been linked unequivocally to its sexual state nor has its phylogeny been studied in detail. It has been suggested that *T. harzianum* is a species complex based on the phenotypic and genotypic variability encountered. On the basis of morphological and cultural characters and DNA sequence data analysis of four genes (ITS rDNA, translation elongation factor 1-α, calmodulin, and α-actin), *Hypocrea lixii* was found to be the sexual state of *T. harzianum*. Both the asexual and sexual states of this species have wide geographic distributions. Phylogenetic analysis of four genes showed that *T. harzianum/H. lixii* is a cohesive group that is supported by bootstrap values higher than 95%. Principles of genealogical concordance indicated that *T. harzianum/H. lixii* is a complex of independent monophyletic lineages, but no diagnostic morphological distinctions were identified that justify formal taxonomic recognition for the different lineages.

1. Introduction

Filamentous ascomycetes (=clade Pezizomycetes (Eriksson, 2000; Eriksson and Winka, 1997)) tend to produce two or more spore types, one of which may be meiotic, sexual spores called ascospores. These sexual stages, referred to as teleomorphs, are recognized taxonomically as members of the phylum Ascomycota. The same fungi may also produce one or more different asexual spore types, usually called conidia or chlamydospores, in stages referred to as anamorphs, which have been classified in a separate phylum, Deuteromycota. As many as 40% of fungi in the clade Pezizomycetes are known only by their anamorphs, and lacking a known sexual stage, are classified separately from their sexual counterparts regardless of phylogenetic relationships. In the interest of developing a natural taxonomic system, and in understanding the full biology of these organisms, connecting anamorphs and teleomorphs is a major goal of fungal systematics (Reynolds and Taylor, 1993). Fungi in the Hypocreaceae (Hypocreales) illustrate this situation particularly well. Many important fungi in this group are known only by their anamorphs and remain unconnected to known teleomorphs. These anamorphs include the cosmopolitan genus *Trichoderma*, which comprises many important biocontrol agents and industrial producers of enzymes. Molecular phylogenetics has proven to be an extremely valuable tool in establishing anamorph–teleomorph connections in this group (e.g., Chaverri et al., 2001; Dodd et al., 2002; Kuhls et al., 1996).

Trichoderma harzianum Rifai (Ascomycota, Hypocreales, Hypocreaceae) is a common soil species and is used in biological control of a variety of plant-pathogenic fungi. *T. harzianum* is effective against pathogenic fungi and diseases such as *Rhizoctonia solani* root rots, *Phytophthora megasperma* f. sp. *glycinea* root rot on soybean, *Gaeumannomyces gramminis* take-all of wheat, *Sclerotinia sclerotiorum* rots, *Sclerotium rolfsii* Southern stem blight of tomato, and *Cylindrocladium scoparium*

* Corresponding author. Fax: 1-301-504-5810.
E-mail address: priscila@nt.ars-grin.gov (P. Chaverri).

1055-7903/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1055-7903(02)00400-1

damping-off of red pines (Al-Heeti and Sinclair, 1988; Bailey and Lumsden, 1998; Mathew and Gupta, 1998; Wells et al., 1972; Yang et al., 1995). In addition to the biocontrol properties of *T. harzianum*, this species has potential for the enhancement of plant growth and resistance to plant pathogens (Bailey and Lumsden, 1998; Gromovich et al., 1998).

In the last decade, *T. harzianum* was reported to cause great losses in commercial mushroom production in Ireland, UK, USA, Canada, and Australia (Fuente et al., 1998; Muthumeenakshi et al., 1994, 1998; Ospina-Giraldo et al., 1998; Seaby, 1996, 1998). Gams and Meyer (1998) suggested that *T. harzianum* sensu stricto was not responsible for losses in mushroom production, based on analyses of morphology, RFLPs and ITS rDNA sequence data. Samuels et al. (2002) determined that the aggressive mushroom parasite previously identified as *T. harzianum* was morphologically and phylogenetically separable from the biocontrol strains of *T. harzianum* and they described the mushroom parasite as *T. aggressivum* Samuels and Gams. Grondona et al. (1997) found a great deal of infraspecific variation in *T. harzianum* in 82 morphological and physiological characters, including 99 isoenzyme bands from seven enzyme systems. Samuels et al. (2002) found three clades within *T. harzianum* s. str. based on translation elongation factor 1-α (EF-1α) sequence data and also suggested that *T. harzianum* was a species complex. They also noted that an unidentified species of *Hypocrea* was the sexual state (teleomorph) of *T. harzianum*.

Trichoderma harzianum was one of nine "aggregate species" described by Rifai (1969) and defined as comprising more than one morphologically cryptic species. *Trichoderma inhamatum* Veerkamp and Gams (1983) was described for a species that was morphologically somewhat different to *T. harzianum*. Bissett (1991b) suggested that *T. inhamatum* was a synonym of *T. harzianum*, based on morphological analyses. Several publications have demonstrated, using molecular tools, the close relationship between *T. harzianum* and *T. inhamatum*, however, they have been maintained as distinct entities (Gams and Meyer, 1998; Hermosa et al., 2000; Samuels et al., 2002). The morphological species concept of *T. harzianum* has been clarified by several authors (Bissett, 1991a, b; Gams and Meyer, 1998; Rifai, 1969; Samuels et al., 2002).

Taylor et al. (2000) described the use of genealogical concordance methods to recognize fungal species (Genealogical Concordance Phylogenetic Species Recognition, GCPSR). A lack of actual reproduction among phylogenetic species will lead to shared phylogenetic partitions in different gene genealogies that would not be observed if recombination occurred between lineages. These methods have been applied to identify cryptic species in a number of fungi, correlated with biogeographic, morphological, biochemical, and ecological traits (Geiser et al., 2000, 2001, 1998; Kasuga et al., 1999; Koufopanou et al., 1997; Kroken and Taylor, 2001; O'Donnell et al., 1998, 2000).

Trichoderma harzianum is an asexually reproducing fungus that has never been unequivocally linked to a teleomorph. However, several cultures derived from ascospores of the sexual fungus *Hypocrea lixii* Pat. produced the morphological species *T. harzianum* in pure culture, suggesting the link between the two (Chaverri and Samuels, 2002).

The research reported in this paper answers two questions: (1) Is *T. harzianum* a complex of morphologically cryptic lineages/species? and (2) Do isolates of *H. lixii* fall within *T. harzianum*? Therefore, is *H. lixii* the teleomorph of *T. harzianum*? In order to answer these questions, analyses of morphological and cultural data, and phylogenies of four genes, internal transcribed spacers ribosomal DNA (ITS rDNA), translation elongation factor-1α (EF-1α), calmodulin (CAL), and actin (ACT), were determined for 33 isolates identified morphologically as *T. harzianum*, including isolates originated from the teleomorph and the anamorph. Common partitions shared among gene genealogies were sought as evidence for reproductive boundaries that could represent useful species concepts (Koufopanou et al., 1997; Taylor et al., 2000).

2. Materials and methods

2.1. Collections and isolates

Thirty-five isolates used for morphological and molecular analysis are listed in Table 1. The majority were maintained by the authors and deposited at the US National Fungus Collection (BPI), but others were obtained from Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS) or from Agriculture and Agri-Food Canada, Eastern Cereals, and Oilseeds Research Centre, Ottawa, Canada (DAOM). Other specimens evaluated only for morphological analyses were: *T. harzianum* DAOM 222121, DAOM 222136, G.J.S. 92-59, G.J.S. 95-20, G.J.S. 95-69, G.J.S. 95-70, G.J.S. 97-261, G.J.S. 97-262, G.J.S. 99-21, G.J.S. 99-22, G.J.S. 99-226, G.J.S. 99-227, G.J.S. 99-23, G.J.S. 00-27, G.J.S. 00-31, G.J.S. 00-32, G.J.S. 00-33, G.J.S. 00-34, G.J.S. 00-36, G.J.S. 00-38, G.J.S. 00-39, G.J.S. 00-40, G.J.S. 00-41, G.J.S. 00-42, G.J.S. 00-43, TR 108, and TR 112; *T. harzianum* originated from *H. lixii* ascospores: G.J.S. 88-47, G.J.S. 90-129, G.J.S. 90-133, G.J.S. 91-137, G.J.S. 92-108, G.J.S. 95-21, and G.J.S. 96-181; and *T. aggressivum* G.J.S. 99-29, G.J.S. 99-30, G.J.S. 99-31, G.J.S. 99-32, and G.J.S. 99-33. Cultures were obtained from fresh collections of *Hypocrea* specimens,

Table 1
Isolates used in this study, including GenBank accession numbers

Species	Isolate No.	Origin	EF-1α	ITS rDNA	CAL	ACT
T. harzianum[a]	G.J.S. 98-183	Austria	AF328560	AF275330	AF442884	AF442851
T. harzianum[a]	G.J.S. 92-110	France	AF443942	AF443924	AF442883	AF442850
T. harzianum[a]	G.J.S. 90-254	Germany	AF443943	AF443926	AF442886	AF442853
T. harzianum[a]	G.J.S. 85-119	Indonesia	AF443941	AF443923	AF442881	AF442848
T. harzianum[a]	G.J.S. 92-135	Switzerland	AF443944	AF443927	AF442887	AF442854
T. harzianum[a]	G.J.S. 97-106	Thailand	AF443939	AF443921	AF442873	AF442840
T. harzianum[a]	G.J.S. 97-96	Thailand	AF443938	AF443920	AF442872	AF442839
T. harzianum[a]	G.J.S. 90-127	USA	AF443936	AF443918	AF442870	AF442837
T. harzianum[a]	G.J.S. 90-22	USA	AF443933	AF443915	AF442867	AF442834
T. harzianum[a]	G.J.S. 91-138	USA	AF443935	AF443917	AF442869	AF442836
T. harzianum[a]	G.J.S. 92-100	USA	AF443937	AF443919	AF442871	AF442838
T. harzianum[a]	G.J.S. 94-53	USA	AF443934	AF443916	AF442868	AF442835
T. harzianum[a]	G.J.S. 98-6	French Guyana	AF469195	AF469189	AF469191	AF469193
T. harzianum	G.J.S. 92-61	Australia	AF443947	AF443925	AF442885	AF442852
T. harzianum	G.J.S. 99-225	Cameroon	AF348106	AY027781	AF442882	AF442849
T. harzianum	G.J.S. 99-230	Cameroon	AF348107	AY027780	AF442875	AF442842
T. harzianum	G.J.S. 99-231	Cameroon	AF348108	AY027783	AF442874	AF442841
T. harzianum	CBS 273.78	Colombia	AF348099	Z68187	AF442891	AF442858
(= *T. inhamatum*)	G.J.S. 95-39					
T. harzianum	CBS 227.95	England	AF348100	AJ222721[b]	AF442866	AF442833
T. harzianum	CBS 226.95 (ex-neotype)	England	AF348101	AJ222720[b]	AF442864	AF442831
T. harzianum	I.M.I. 359823	Northern Ireland	AF348092	X73690[b]	AF442865	AF442832
T. harzianum	G.J.S. 97-263	Japan	AF348091	AF194010	AF442877	AF442844
T. harzianum	G.J.S. 97-264	Japan	AF348103	AF194011	AF442876	AF442843
T. harzianum	G.J.S. 97-265	Japan	AF348104	AF194012	AF442890	AF442855
T. harzianum	G.J.S. 97-266	Japan	AF348090	AF194013	AF442879	AF442846
T. harzianum	G.J.S. 97-268	Japan	AF348105	AF194015	AF442878	AF442845
T. harzianum	G.J.S. 00-06	Mexico	AF443932	AF443914	AF442863	AF442830
T. harzianum	G.J.S. 00-08	Mexico	AF443931	AF443913	AF442862	AF442829
T. harzianum	G.J.S. 00-18	Mexico	AF443946	AF443929	AF442889	AF442857
T. harzianum	G.J.S. 00-21	Mexico	AF443945	AF443928	AF442888	AF442856
T. harzianum	G.J.S. 00-22	Mexico	AF443930	AF443912	AF442861	AF442828
T. harzianum	G.J.S. 00-24	Mexico	AF443940	AF443922	AF442880	AF442847
T. harzianum	G.H. 129522	Biocontrol isolate of unknown origin	AF469194	AF469188	AF469190	AF469192
T. aggressivum	DAOM 222156	Canada	AF348098	AF443911	AF442860	AF442827
T. aggressivum	CBS 100525	England	AF348095	AF057600	AF442859	AF442826

[a] Isolates originated from the teleomorph, *H. lixii*.
[b] Only ITS 1.

which are deposited in BPI. Single ascospore isolations were made on CMD (Difco cornmeal agar + 2% dextrose + antibiotic) with the aid of a micromanipulator.

2.2. Phenotype analyses

Phenotypic characterization of all the isolates was performed in order to unequivocally verify that the isolates studied were *T. harzianum*. The phenotypic evaluation consisted of growth trials, colony appearance, and measurements of the anamorphic parts (as conducted in Samuels et al., 2002).

Crosses were attempted once between all isolates of *T. harzianum*/*H. lixii* to determine compatibility and observe sexual structures if present. One-cm plugs from actively growing colonies of pairs of isolates were placed approx. 3 cm away from each other on a plate of PDA (Difco potato–dextrose–agar), with two plugs from the same isolate used as a control. The crosses were placed in an incubator at 25 °C with alternating darkness and cool white fluorescent light. Observations were made at weekly intervals for four months.

2.3. DNA extraction and sequencing

Extraction of genomic DNA and subsequent PCR followed the protocols described in Samuels et al. (2002). Four gene regions were sequenced: internal transcribed spacers (ITS 1, 5.8S, and ITS 2) of the nuclear ribosomal RNA gene repeat, a portion of the translation elongation

factor (EF-1α) gene including one intron, a portion of the actin (ACT) gene including one intron, and a portion of the calmodulin (CAL) gene including two introns. The primers used for amplification and sequencing ITS were ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). For EF-1α the primers were EF1-728F (5′-CATC GAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TA CTTGAAGGAACCCTTACC-3′), for CAL the primers were CAL-228F (5′-GAGTTCAAGGAGGCCTTCTC CC-3′) and CAL-737R (5′-CATCTTTCTGGCCATCA TGG-3′) and for ACT the primers were ACT-512F (5′-ATGTGCAAGGCCGGTTTCGC-3′) and ACT-783R (5′-TACGAGTCCTTCTGGCCCAT-3′) (Carbone and Kohn, 1999). Sequences were edited and assembled using Sequencher 4.1 (Gene Codes, Wisconsin). Clustal X 1.81 (Thompson et al., 1997) was used to align the sequences, then the alignment was refined by hand. The sequences and alignment were deposited in GenBank (Table 1) and TreeBase (submission number SN 1008, http://herbaria.harvard.edu/treebase/), respectively.

2.4. Analysis of sequence data

Phylogenetic analyses were performed using PAUP* 4.0 b8 using *T. aggressivum* sequences as the outgroup. *Trichoderma aggressivum* is the closest known relative of *T. harzianum* (Samuels et al., 2002). A parsimony analysis was performed using a heuristic search, with a starting tree obtained via stepwise addition, with random addition of sequences with 1000 replicates, tree-bisection-reconnection as the branch-swapping algorithm, and MULTREES off. Analyses treated gaps either as missing or as a fifth character. Bootstrap values (BS) were calculated from 500 replicates. The Incongruence Length Difference Test or Partition Homogeneity Test (PHT) in PAUP* was used to test the congruence among data sets (Cunningham, 1997). For this test, parsimony-uninformative characters were excluded, gaps were treated as missing, and 500 repetitions were performed. A maximum of 100 trees were saved to conserve memory. A Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999) was used to test

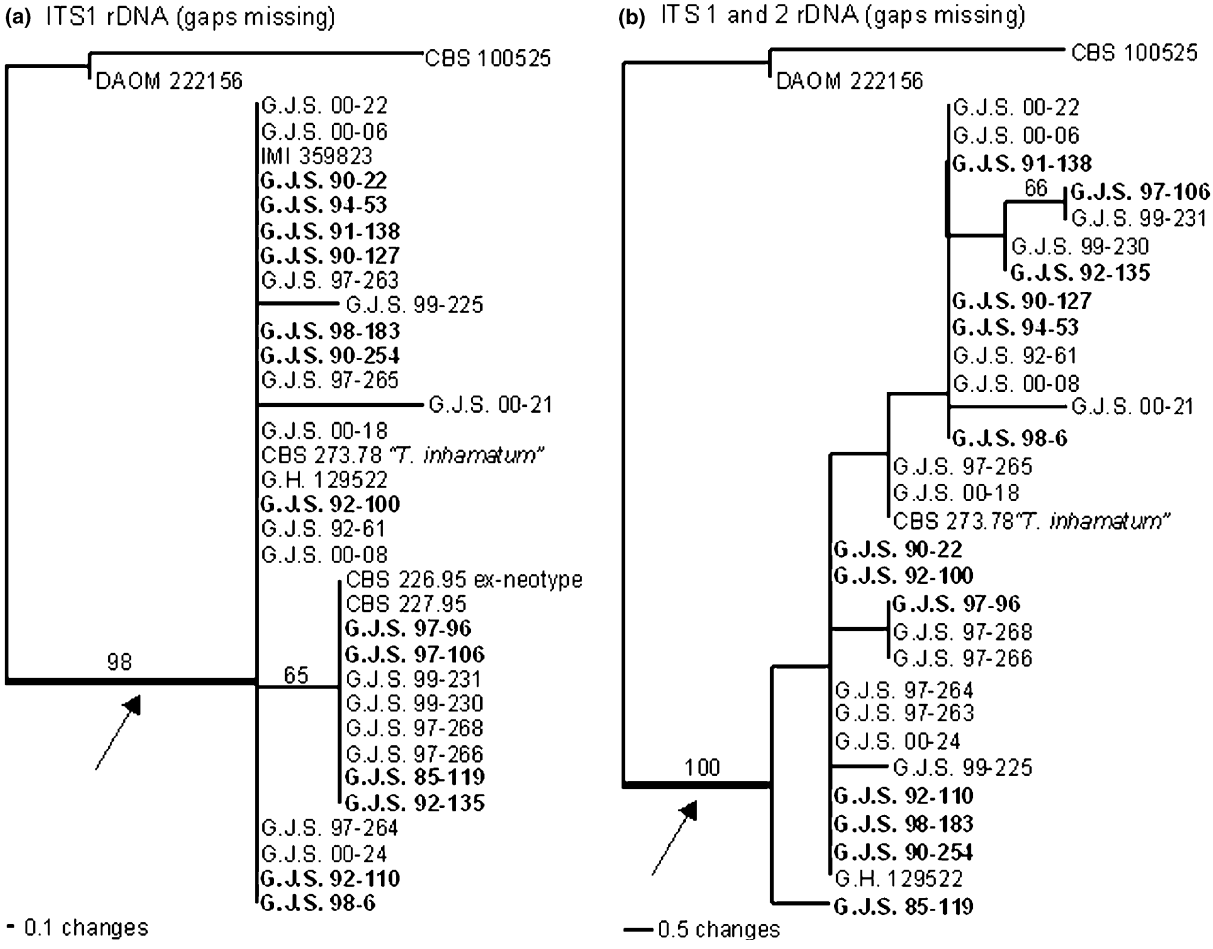

Fig. 1. One equally most parsimonious tree of (a) ITS 1, and (b) combined ITS 1 and 2 sequence data. Isolates that originated from *H. lixii* are in bold. Bootstrap values are shown. Arrow indicates the *T. harzianum*/*H. lixii* complex. *T. aggressivum* CBS 100525 and DAOM 222156 are outgroup isolates.

the significance of alternative phylogenetic hypotheses. The alternative hypothesis was a tree topology constructed by constraining isolates from the same geographic origin to be monophyletic. The likelihood of the resulting constrained tree was then compared to the unconstrained tree. A Kishino–Hasegawa test (Kishino and Hasegawa, 1989) was also performed to compare the results from the two methods. The likelihood settings for each partition were calculated using Modeltest 3.0 (Posada and Crandall, 1998). For ACT, the Kimura-2-parameter model was used, with equal base frequencies, transition/transversion ratio = 2.6115 and a gamma distribution shape parameter = 0.0821. For CAL, the Kimura-2-parameter model was used, with equal frequencies, transition/transversion ratio at 2.6607 and gamma distribution shape parameter at 0.2759. The model used for EF-1α likelihood settings was the Hasegawa–Kishino–Yano, with variable, empirically determined base frequencies ($A = 0.1969$, $C = 0.2847$, $G = 0.1750$, $T = 0.3434$), transition/transversion ratio = 2.002, and gamma distribution shape parameter = 0.6974. RASA (Lyons-Weiler et al., 1996) Web Tool (http://bioinformatics.uml.edu/RASA.shtml) was used to detect potential long-branch attraction (LBA) problems.

3. Results

3.1. Molecular phylogenetic analysis

Molecular sequence data demonstrated that *T. harzianum* is a genetically variable complex, comprised by one morphological species and several phylogenetic species. The ITS rDNA region produced only 18 (2.9%) polymorphic sites and nine parsimony informative characters (1.5%). Parsimony analysis of ITS 1, 5.8S, and ITS 2 rDNA showed *T. harzianum*/*H. lixii* to be monophyletic, supported by a bootstrap value (BS) of 100%. Fig. 1 shows one arbitrarily chosen most parsimonious tree for both ITS 1 alone and combined ITS 1 and 2. The ex-neotype culture of *T. harzianum*, CBS 226.95, has identical ITS 1 sequence to other isolates of *T. harzianum*, several conidial and four ascospore isolates from *H. lixii*. No distinct lineages were supported by high bootstrap values.

The ACT region had more parsimony-informative characters than ITS rDNA, but this number was still low (5.8 vs. 1.5%) (Table 2). The ACT gene tree supported the connection between *T. harzianum* and *H. lixii* with 98% BS, with several monophyletic groups nested within it (Fig. 2). The ex-neotype of *T. harzianum* (CBS 226.95) formed a clade with other isolates from Britain, Ireland, and the USA, supported by 57% BS. The USA isolates originated from ascospores from field-collected specimens of *H. lixii*. Other monophyletic groups observed included a clade with three Mexican isolates, which was highly supported by bootstrapping (98%). This clade was sister to two other clades: one containing mostly Japanese isolates in addition to one from Mexico and one from Cameroon (83% BS), and a second clade containing European isolates (51% BS). Monophyly of isolates from USA, UK, and Western Europe was also observed. The results of the Shimodaira–Hasegawa test (Table 3) show that constrained trees with isolates from Mexico, North America, Europe, Japan, Asia, and Cameroon are significantly less likely. However, the test accepted the hypotheses that constrained trees with isolates from South America and continental Asia are, respectively, not significantly worse explanations of the data than the unconstrained tree. The Kishino–Hasegawa test was consistent with the Shimodaira–Hasegawa test results. Phylogenetic analyses using gaps as fifth characters did not give different results. The only difference was an increase in the number of parsimony informative characters and slightly different bootstrap values (Fig. 2). RASA identified isolate G.J.S. 85-119 as a long branch; however, when it was removed from the analysis, the topology of the tree did not change.

CAL sequence data produced strong bootstrap support (100%) for the *T. harzianum*/*H. lixii* clade (Fig. 3). CAL data produced more parsimony-informative char-

Table 2
Results of the molecular phylogenetic analyses of ITS, ACT, CAL, and EF-1α

Gaps	EF-1α		ACT		CAL		ITS 1	ITS 1 + 2	Combined	
	Missing	Included	Missing	Included	Missing	Included	Missing	Missing	Missing	Included
Number of trees	611	39	26	9	425	266	996	305	197	332
Length	147	281	41	59	123	178	12	20	362	606
Un-informative polymorphic sites	30	63	12	15	20	39	8	9	70	127
Informative polymorphic sites (%)	77 (20.9)	107 (29.0)	20 (5.8)	27 (7.8)	67 (14.8)	78 (17.2)	4 (1.5)	9 (1.5)	168 (11.8)	227 (15.9)
Total included characters	369	369	345	345	452	452	263	612	1429	1429
Consistency index (ingroup)	0.79	0.77	0.85	0.78	0.81	0.80	1.0	0.90	0.73	0.70
Retention index (ingroup)	0.91	0.90	0.95	0.91	0.91	0.88	1.0	0.93	0.87	0.83
Homoplasy index (ingroup)	0.21	0.23	0.15	0.22	0.19	0.21	0.0	0.10	0.27	0.30

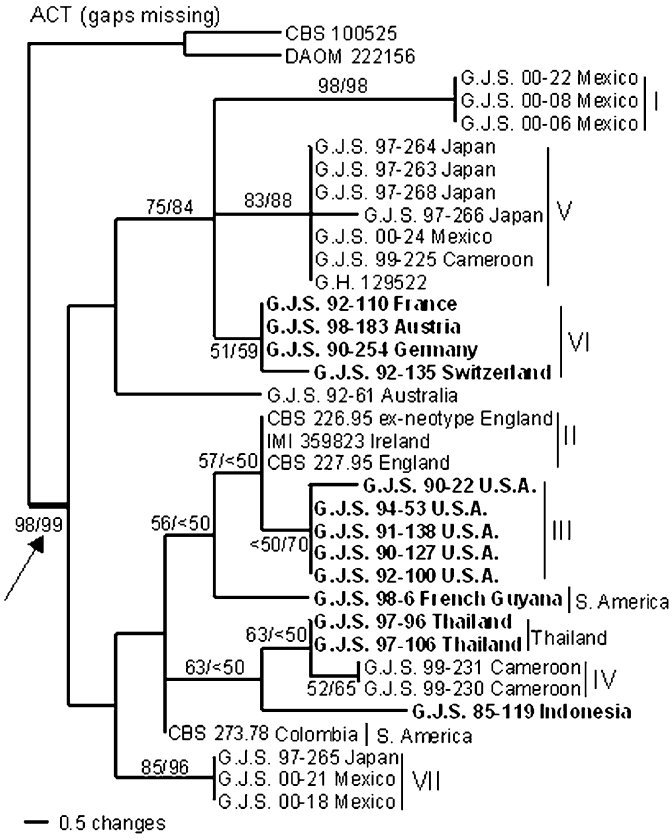

Fig. 2. One equally most parsimonious tree of ACT gene sequence data. Isolates that originated from *H. lixii* are in bold. Bootstrap values are shown (gaps missing/included). Arrow indicates the *T. harzianum*/*H. lixii* complex. *T. aggressivum* CBS 100525 and DAOM 222156 are outgroup isolates.

acters (14.8%) than did ITS rDNA or ACT (Table 2), and showed a high degree of resolution at terminal nodes, with less support for the backbone of the ingroup. Trees resulting from analyses constraining the monophyly of isolates from Japan, Cameroon, and Mexico gave significantly worse explanations than the unconstrained trees (Table 3). The Shimodaira–Hasegawa test accepted the hypothesis that trees constraining isolates from South America and Europe to be monophyletic were equally good explanations of the data. The Kishino–Hasegawa test yielded the same results as Shimodaira–Hasegawa. The CAL phylogenetic tree also showed that ascospore isolates from *H. lixii* are nested within the *T. harzianum*/*H. lixii* clade, strongly confirming the connection between the two. Bootstrap values increased when gaps were included in the CAL analyses, but did not change the topology. RASA detected two taxa as long branches. When gaps are treated as missing, isolates G.J.S. 00-06 and G.J.S. 85-119 showed significant taxon variance ratios; and when gaps are included, only G.J.S. 85-119 appeared to be a long branch. The inferred relationships of other isolates did not change when these isolates were removed from the analyses.

The EF-1α sequence data produced the most parsimony-informative characters (20.9%), producing strongly supported nodes in the termini and slightly higher support in the ingroup backbone (Fig. 4). In this case the ex-neotype isolate of *T. harzianum* is included in a clade with other *T. harzianum* isolates from different geographic origins and with isolates from *H. lixii*. This clade is supported by a bootstrap value of 75%. This clade comprises four sub-clades, three of which are highly supported. The first sub-clade contains three isolates from England and Ireland (100% BS), including the ex-neotype of *T. harzianum*. The second sub-clade contains two isolates from Cameroon (96% BS), the third sub-clade contains all the USA isolates (99% BS), and the fourth sub-clade includes isolates from Thailand (63% BS). Trees resulting from constrained analyses for the isolates from Japan, Cameroon and Mexico were significantly worse than the unconstrained trees (Table 3). The Kishino–Hasegawa test gave the same results as the Shimodaira–Hasegawa except in the test for the tree constraining the continental Asia isolates to be monophyletic. The Shimodaira–Hasegawa test resulted in the rejection of the null hypothesis and the Kishino–Hasegawa test accepted the hypothesis. In some cases, as discussed by Shimodaira and Hasegawa (1999), the use of the Kishino–Hasegawa test can lead to overconfidence in the wrong tree when more than one tree is compared in the analysis. We confirm the importance of being

Table 3
Shimoidara–Hasegawa test results

Hypothesis	Gene	#Trees	Length	−ln *L* best	−ln *L* worst	*p*[a]
Unconstrained	EF	40	281	1359.4902	1359.4902	
	ACT	9	59	753.8343	766.2021	
	CAL	247	176	1359.9340	1370.0057	
Mexico constrained	EF	140	331	1472.8680	1476.6217	0.000*
	ACT	74	69	790.0290	803.0661	0.005–0.001*
	CAL	384	202	1464.7063	1475.4871	0.000*
North America constrained	EF	144	335	1489.7566	1490.6456	0.000*
	ACT	30	70	801.1404	808.4744	0.002*
	CAL	320	203	1467.2004	1478.1380	0.000*
South America constrained	EF	58	295	1377.9550	1385.8343	0.019–0.033*
	ACT	60	61	750.9849	759.2940	**0.331–0.954**
	CAL	246	176	1359.9338	1370.0059	**0.28–0.982**
Europe constrained	EF	174	305	1404.6049	1419.2771	0.000*
	ACT	31	66	780.1602	784.7214	0.004–0.007*
	CAL	479	180	1365.7070	1376.7790	**0.197–0.671**
Japan constrained	EF	172	321	1454.6110	1457.0445	0.000*
	ACT	62	69	785.6603	802.6039	0.002–0.004*
	CAL	191	197	1441.2516	1452.4531	0.000*
Asia constrained	EF	690	179	1482.9729	1496.7949	0.000*
	ACT	18	73	799.8014	813.4337	0.000*
	CAL	698	209	1475.3031	1490.5903	0.000*
Continental Asia constrained	EF	32	303	1386.2628	1386.2628	0.030*
	ACT	4	61	752.2530	762.5441	**0.225–0.803**
	CAL	642	188	1385.1629	1396.5729	0.018–0.039*
Africa constrained	EF	20	309	1430.5877	1430.7060	0.000*
	ACT	18	67	781.7584	793.9723	0.000–0.005*
	CAL	104	188	1400.7351	1413.4852	0.008*
Hypocrea isolates constrained	EF	473	170	1439.9766	1444.8312	0.000*
	ACT	241	50	787.4199	793.0830	0.003*
	CAL	440	132	1394.1720	1401.1011	0.022–0.047*

[a] Significantly less likely at $p = 0.05$, thus, null hypothesis that monophyly of the particular group is not an equally likely explanation of the data.
* The * indicates significantly worst explanations of the data.

cautious in the use of the Kishino–Hasegawa test when comparing many topologies. The inclusion of gaps as a fifth character in the analyses gave higher bootstrap values. Potential long-branch attraction was detected in isolate G.J.S. 97-96 when gaps were included and in CBS 273.78 when gaps were excluded. The topology of the trees did not change when these isolates were deleted.

The Partition Homogeneity Test (PHT) of the combined data set (ITS rDNA, ACT, CAL, and EF-1α) resulted in significant incongruence among data sets (p value = 0.002). Pairwise PHT comparisons also resulted in incongruence (p value = 0.002). Most of the incongruences, however, were encountered in the internal nodes, and the combined data set generally showed much higher bootstrap support for terminal nodes than did the individual data sets alone. Fig. 5 presents one equally parsimonious tree of the combined data set, with a 100% bootstrap value for the *T. harzianum*/*H. lixii* clade. In this tree there are seven lineages supported by bootstrap values of 100%. However the incongruences among the different data sets were evident in the low bootstrap values for the internal nodes. The same seven lineages were present in the individual gene trees but the phylogenetic relationship among lineages was different for each gene tree. The topology of the combined tree is almost identical to the EF-1α gene tree, perhaps partly because of the high proportion of the total ingroup phylogenetic signal (66/143 = 46.1%) coming from this gene. In the ITS rDNA phylogenetic tree, none of the seven lineages are resolved. The CAL gene tree infers CBS 273.78 and G.J.S. 97-96, to be monophyletic (97/99% BS) but this is not indicated in the other gene genealogies. Isolates G.J.S. 85-119 (Indonesia), G.J.S. 92-61 (Australia) and CBS 273.78 (Colombia) did not group with other isolates. Isolates G.J.S. 97-96 and G.J.S. 97-106, both from Thailand, formed a weakly supported clade in the EF-1α and ACT gene phylogenies, but showed a fairly high degree of divergence and

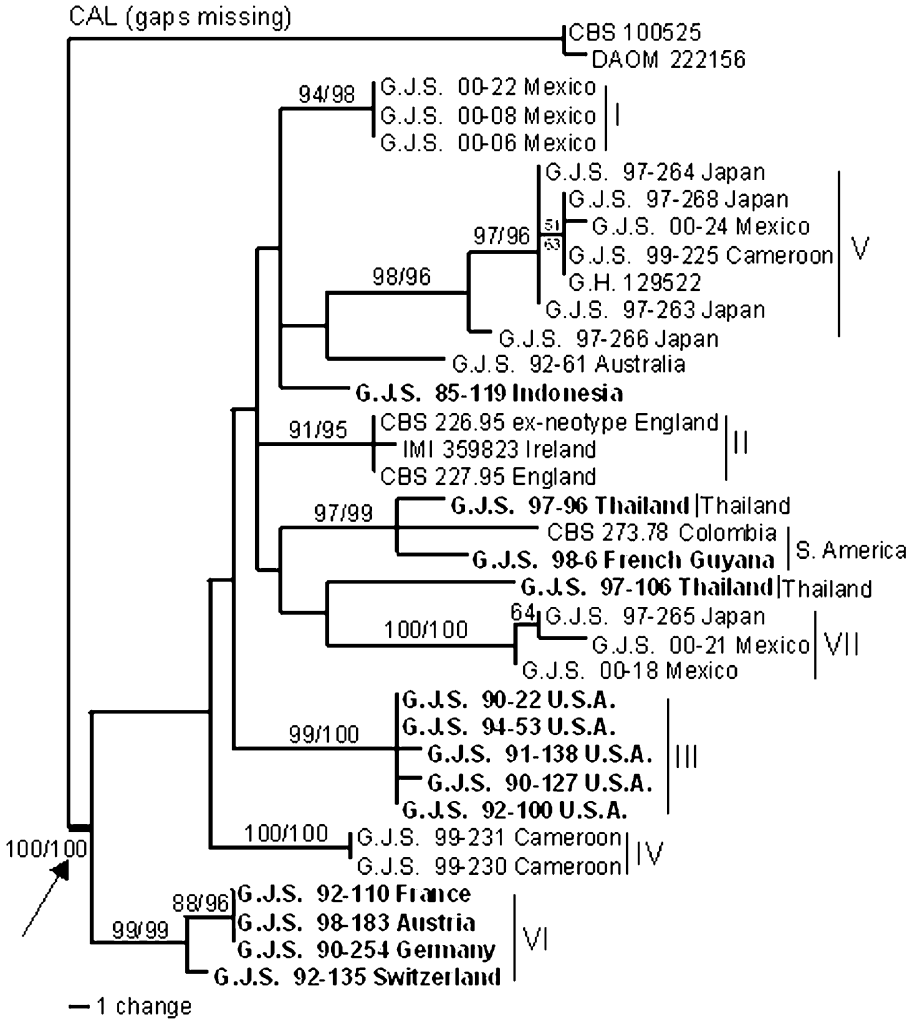

Fig. 3. One equally most parsimonious tree of CAL gene sequence data. Isolates that originated from *H. lixii* are in bold. Bootstrap values are shown (gaps missing/included). Arrow indicates the *T. harzianum*/*H. lixii* complex. *T. aggressivum* CBS 100525 and DAOM 222156 are outgroup isolates.

G.J.S. 97-96 is a long-branch taxon. Another aspect suggested by the combined phylogenetic tree is that isolates originating from ascospores (*H. lixii*) form separate monophyletic groups from conidial isolates (*T. harzianum*). However, in this study there are no conidial isolates from the same geographic origin as the ascospore isolates, so it is not clear if this is real or an artifact of sampling.

The average base pairwise divergence across all four genes, among isolates of *T. harzianum*/*H. lixii* is 3.0%, and between the two isolates of *T. aggressivum* is 0.88%. The average pairwise divergence between *T. aggressivum* and *T. harzianum*/*H. lixii* is 5.6%. The average divergence within each *T. harzianum*/*H. lixii* lineage is 0.26%. Most of the divergence was encountered in the introns. A combined parsimony analysis of ACT, EF-1α and CAL exons produced 15 polymorphic sites, nine of which are parsimony informative. All of the substitutions were synonymous at the third codon position.

3.2. Phenotype analyses

Some heterogeneity in growth rate and colony morphology was observed among isolates of *T. harzianum*. However, these phenotypic differences did not correlate with any of the molecular phylogenetic lineages observed. This study determined that the growth rate and morphology of the ex-type culture of *T. inhamatum* falls within the range for *T. harzianum* (see Bissett, 1991b; Samuels et al., 2002; Veerkamp and Gams, 1983).

After four months in the incubator, three crosses between isolates of *T. harzianum*/*H. lixii* produced deformed sexual fruiting bodies; only one of them produced ascospores that were viable. The cross between conidial isolates G.J.S. 00-18 (Mexico) and G.J.S. 97-265 (Japan), both members of lineage VII, produced fertile sexual fruiting bodies containing green, abnormally shaped ascospores. Single ascospore isolations were made and germination occurred within one day,

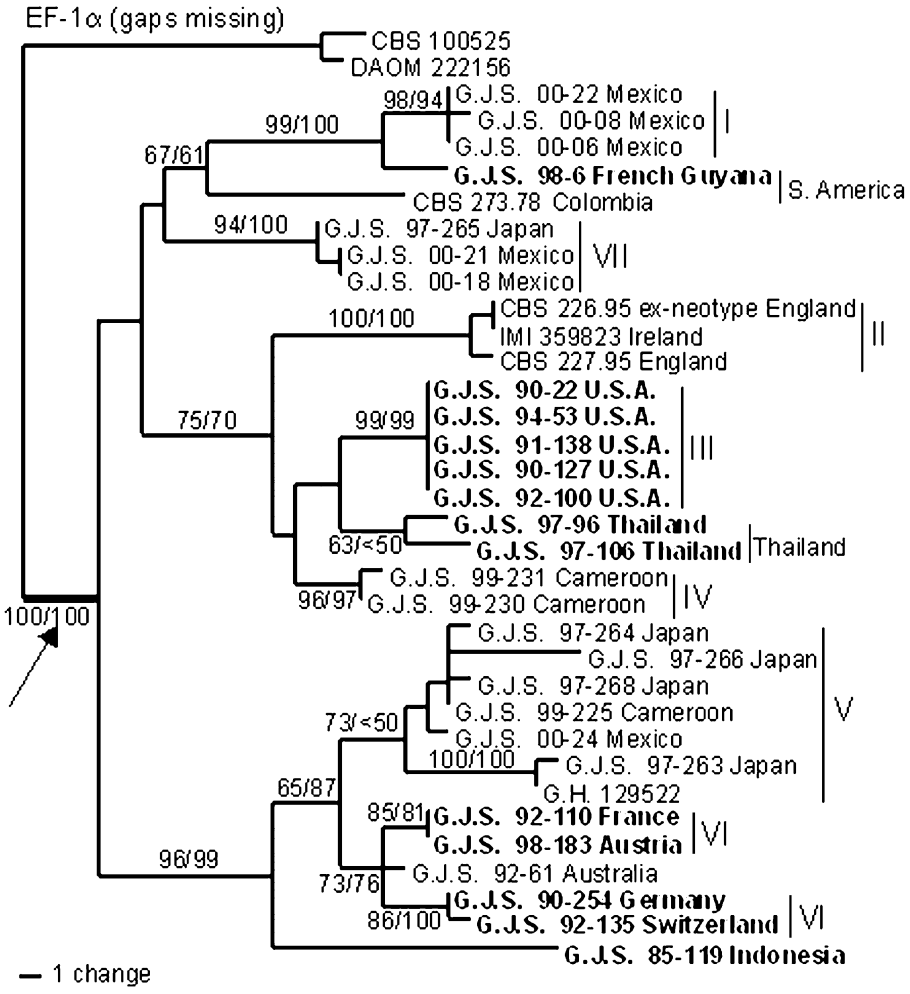

Fig. 4. One most parsimonious tree of EF-1α gene sequence data. Isolates that originated from *H. lixii* are in bold. Bootstrap values are shown (gaps missing/included). Arrow indicates the *T. harzianum*/*H. lixii* complex. *T. aggressivum* CBS 100525 and DAOM 222156 are outgroup isolates.

producing cultures with *T. harzianum* morphology. The crosses between conidial isolate G.J.S. 97-264 (Japan, lineage V) vs. G.J.S. 98-183 (ascospore, Austria, lineage VI) and G.J.S. 97-264 and G.J.S. 92-135 (ascospore, Switzerland, lineage VI) produced deformed fruiting bodies but no ascospores. After four months, sexual fruiting bodies had not formed in cultures that were either 'selfed' or paired with other cultures.

4. Discussion

Trichoderma harzianum/*Hypocrea lixii* is a cosmopolitan species known from all continents except Antarctica. It is found in temperate regions such as USA, Western Europe, UK, Japan, and in tropical and subtropical areas such as Mexico, Thailand, Indonesia, Cameroon, French Guyana, and Colombia.

Isolates that originated from ascospores of *H. lixii* specimens are morphologically and molecularly indistinguishable from *T. harzianum* isolates, including the type, so we unequivocally conclude that *H. lixii* is the teleomorph of *T. harzianum*, and thus are the same species. Molecular phylogenetic analyses of four genes placed teleomorph-derived (*H. lixii*) isolates in numerous places throughout the *T. harzianum* clade. *Hypocrea lixii* isolates from USA appear to be closely related to the ex-neotype of *T. harzianum*, which was isolated in Britain.

Trichoderma inhamatum, consistently grouped within *T. harzianum*. Because *T. inhamatum* clustered in a poorly supported ITS group separate from the type culture of *T. harzianum*, Hermosa et al. (2000) proposed that a name of one segregate from *T. harzianum* could be *T. inhamatum*. Using phenotype and DNA sequence analysis (ITS 1, EF-1α), Samuels et al. (2002) were not able to distinguish *T. inhamatum* from *T. harzianum*. In this study, we found that *T. inhamatum* grouped within the *T. harzianum*/*H. lixii* clade but it did not group with any of the seven lineages. In addition, morphological and cultural data did not distinguish this species from *T. harzianum*. In light of this and the other clear results

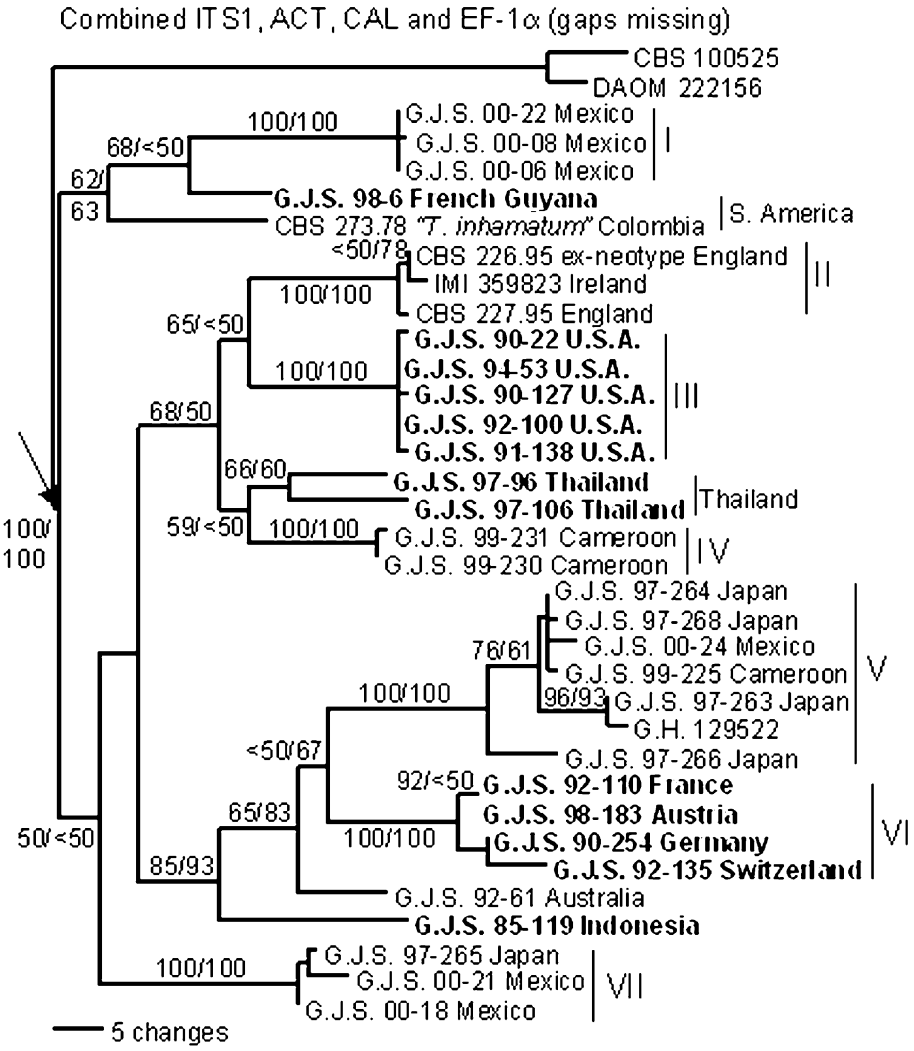

Fig. 5. One most parsimonious tree of combined sequence data. Isolates that originated from *H. lixii* are in bold. Bootstrap values are shown (gaps missing/included). Arrow indicates the *T. harzianum*/*H. lixii* complex. Lineages: I, Mexico; II, UK; III, USA; IV, Cameroon; V, Japanese; VI, Western continental Europe; VII, Japan/Mexico. *T. aggressivum* CBS 100525 and DAOM 222156 are outgroup isolates.

from this study, we conclude that *T. inhamatum* deserves no special recognition within the *T. harzianum*/*H. lixii* complex.

It is important to note that the genes used in this study included mainly non-coding regions, which are probably evolving at high rates, and account for most of the genetic variation encountered. The lack of resolution of internal nodes within topologies suggests a history of rapid diversification, with some possible combination of gene flow or/and lineage sorting within *T. harzianum*/*H. lixii*.

Some case studies have proposed a taxonomy based primarily on molecular data; and in most of these cases phenotypic differences have been found to support these taxa. For example, Samuels et al. (2002) segregated *T. aggressivum* into two *formae*, *T. a.* f. *aggressivum* and *T. a.* f. *europaeum* based on geographic origin, molecular sequences, and cultural data. *Cryptococcus neoformans* var. *grubii* was segregated from *C. n.* var. *neoformans* based on genotypic differences of *URA5* sequences, DNA fingerprinting and serotypes (Franzot et al., 1999). In another example, phylogenetic species were found in a molecular phylogenetic analysis of multiple genes of the *Gibberella fujikoroi* (*Fusarium* spp. anamorph) species complex (O'Donnell et al., 1998). Based on these results, 10 new species of *Fusarium* were described after a meticulous evaluation of morphology (Nirenberg and O'Donnell, 1998). Groups I and II of *Aspergillus flavus* correlate somewhat with aflatoxin production, sclerotium production, and geography (Geiser et al., 2000). Geiser et al. (2001) successfully segregated *Fusarium hostae* from its sister taxon *F. redolens*, by first identifying molecular phylogenetic partitions and then finding distinctive phenotypic characters. Another case is the separation of *Coccidioides posadasii* from *C. immitis* (Fisher et al., 2002). While fairly deep

phylogenetic divergence was observed between these two taxa, slower growth rate in the presence of high salt concentration was the only phenotype found to correlate partially with the phylogenetic distinction. Overlapping of morphological characters ranges indicates a continuous variation of character traits within the *T. harzianum*/*H. lixii* group, suggesting the existence of a "morphological continuum." Despite great phylogenetic, morphological and cultural variability, we did not find any phenotypic diagnostic characters that correlate with the molecular phylogenetic lineages found.

As a result of the Shimodaira–Hasegawa tests, some weak, but not significant, correlations between the seven lineages of *T. harzianum*/*H. lixii* and geography were observed. Lineage I comprises only Mexican isolates, and isolates from Colombia and French Guyana are basal to lineage I. Lineage II comprises isolates from Britain and Ireland, lineage III comprises isolates from USA, lineage IV comprises isolates from Cameroon, and lineage VI comprises isolates from continental Europe. Lineage V includes mostly Japanese isolates, but also isolates from Mexico and Cameroon. The biocontrol isolate G.H. 129522, of unknown geographic origin, is nested within lineage V and closely related to a Japanese isolate. Lineage VII comprises isolates from Japan and Mexico. The two isolates sampled from Thailand show a weakly supported connection in the combined analysis. Isolates from similar geographic sources sometimes showed very different phylogenetic positions. For example, two of the Mexican isolates originated from within a clade that contains a Japanese isolate (lineage VII), indicating the possible movement of isolates from Japan to Mexico or vice-versa. This hypothesis is supported by the fact that a Mexican isolate derived from within lineage V that includes mostly Japanese isolates. The isolates from Cameroon also have at least two evolutionary origins, one of them within the Japanese clade. The European isolates are in two separate lineages, II and VI corresponding to Britain/Ireland and Western Europe, respectively.

The majority of the crosses made between isolates did not produce fertile fruiting bodies; however this is not indicative of homo- or heterothallism, or that it does not occur in nature. Inducing fertile fruiting bodies might require some natural conditions, such as fluctuating temperature and light, presence of various other microorganisms, and recalcitrant nutrients, which are difficult to simulate in artificial pure-culture conditions. Only a single mating test between two isolates from the same lineage produced viable ascospores, while crosses of isolates from different lineages were unsuccessful. Therefore, no conclusions can be drawn with respect to the correlation of biological species to molecular phylogenetic species.

Other than weak correlations with geography and mating, no morphological or other phenotypic characteristics gathered in this study were identified that correlated with the seven phylogenetic lineages/species. While our results indicate strong phylogenetic partitions that satisfy the standards of GCPSR, we suggest that all seven lineages continue to be recognized collectively as *T. harzianum*/*H. lixii* unless diagnostic phenotypic differences are identified. In the meanwhile, we suggest that the lineages be recognized as *T. harzianum*/*H. lixii* lineages I-VII. This usage follows the precedent set in the fungus causing head scab of wheat and barley, *Fusarium graminearum*, which comprises eight species lineages (O'Donnell et al., 2000). Mating tests were inconclusive, yielding no successful crosses within or between lineages, with one exception. While it is possible that additional tests of mating compatibility and other phenotypes may uncover fixed differences among lineages that justify formal taxon recognition, the extensive analyses of morphology performed in this study suggest this is unlikely. Nevertheless, researchers studying these fungi for uses in biological control and other work would be wise to take into account phylogenetic structure within *T. harzianum*/*H. lixii* in choosing potential isolates, as differences in effectiveness may track with phylogeny.

Acknowledgments

We thank Drs. Amy Y. Rossman, Elwin L. Stewart, and Sonja J. Scheffer for the comments given on this paper. The research was supported in part by the U.S. National Science Foundation 'PEET' grant to the Dept. of Plant Pathology, The Pennsylvania State University (Monographic Studies of Hypocrealean Fungi: *Hypocrea* and *Hypomyces* DEB-9712308).

References

Al-Heeti, M.B., Sinclair, J.B., 1988. Antagonism between *Gliocladium roseum*, *Trichoderma harzianum*, or *Trichothecium roseum* and *Phytophthora megasperma* f. sp. *glycinea*. Mycopathologia 103, 135–140.

Bailey, B.A., Lumsden, R.D., 1998. Direct effects of *Trichoderma* and *Gliocladium* on plant growth and resistance to pathogens. In: Harman, G., Kubicek, C. (Eds.), *Trichoderma* and *Gliocladium*. Taylor & Francis Inc., London, UK, pp. 185–204.

Bissett, J., 1991a. A revision of the genus *Trichoderma*. II. Infrageneric classification. Can. J. Bot. 69, 2357–2372.

Bissett, J., 1991b. A revision of the genus *Trichoderma*. III. Section *Pachybasium*. Can. J. Bot. 69, 2373–2417.

Carbone, I., Kohn, L.M., 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91, 553–556.

Chaverri, P., Samuels, G.J., 2002. *Hypocrea lixii* Pat., the teleomorph of *Trichoderma harzianum* Rifai. Mycol. Prog. 1, 283–286.

Chaverri, P., Samuels, G.J., Stewart, E.L., 2001. *Hypocrea virens* sp. nov., the teleomorph of *Trichoderma virens*. Mycologia 93, 1113–1124.

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

Dodd, S.L., Lieckfeldt, E., Samuels, G.J., 2002. *Hypocrea atroviridis* sp nov.: the teleomorph of *Trichoderma atroviride*. Mycologia 94, in press.

Eriksson, O.E., 2000. Outline of Ascomycota—2000. Myconet Available from <http://www.umu.se/myconet/curr/outline.html>.

Eriksson, O.E., Winka, K., 1997. Supraordinal taxa of Ascomycota. Myconet 1, 1–16.

Fisher, M.C., Koenig, G.L., White, T.J., Taylor, J.W., 2002. Molecular and phenotypic description of *Coccidioides posadasii* sp. nov., previously recognized as the non-California population of *Coccidioides immitis*. Mycologia 94, 73–84.

Franzot, S.P., Salkin, I.F., Casadevall, A., 1999. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. J. Clin. Microbiol. 37, 838–840.

Fuente, M.E.d.l., Beyer, D.M., Rinker, D.L., 1998. First report of *Trichoderma harzianum* biotype Th4, on commercial button mushrooms in California. Plant Dis. 82, 1404.

Gams, W., Meyer, W., 1998. What exactly is *Trichoderma harzianum*? Mycologia 90, 904–915.

Geiser, D.M., Dorner, J.W., Horn, B.W., Taylor, J.W., 2000. The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. Fungal Genet. Biol. 31, 169–179.

Geiser, D.M., Juba, J.H., Wang, B., Jeffers, S.N., 2001. *Fusarium hostae* sp. nov., a relative of *F. redolens* with *Gibberella* teleomorph. Mycologia 93, 670–678.

Gromovich, T.I., Gukasian, V.M., Golovanova, T.I., Shmarlovskaya, S.V., 1998. *Trichoderma harzianum* Rifai aggr. as a factor enhancing tomato plants' resistance to the root rotting pathogens. Mikol. Fitopatol. 32, 73–78.

Grondona, I., Hermosa, M.R., Tejada, M., Gomis, M.D., Mateos, P.F., Bridge, P.D., Monte, E., Garcia-Acha, I., 1997. Physiological and biochemical characterization of *Trichoderma harzianum*, a biological control agent against soilborne fungal plant pathogens. Appl. Environ. Microbiol. 63, 3189–3198.

Hermosa, M.R., Grondona, I., Iturriaga, E.A., Diaz-Minguez, J.M., Castro, C., Monte, E., Garcia-Acha, I., 2000. Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. Appl. Environ. Microbiol. 66, 1890–1898.

Kasuga, T., Taylor, J.W., White, T.J., 1999. Phylogenetic relationships of varieties and geographical groups of the human pathogenic fungus *Histoplasma capsulatum* Darling. J. Clin. Microbiol. 37, 653–663.

Kishino, H., Hasegawa, H., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data and the branching order in Hominoidea. J. Mol. Evol. 29, 170–179.

Koufopanou, V., Burt, A., Taylor, J.W., 1997. Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitis*. Proc. Natl. Acad. Sci. USA 94, 5478–5482.

Kroken, S., Taylor, J.W., 2001. A gene genealogical approach to recognize phylogenetic species boundaries in the lichenized fungus *Letharia*. Mycologia 93, 38–53.

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

Lyons-Weiler, J., Hoelzer, G.A., Tausch, R.J., 1996. Relative apparent synapomorphy analysis RASA I: the statistical measurement of phylogenetic signal. Mol. Biol. Evol. 13, 749–757.

Mathew, M.K.A., Gupta, S.K., 1998. Biological control of root rot of French bean caused by *Rhizoctonia solani*. J. Mycol. Plant Pathol. 28, 202–205.

Muthumeenakshi, S., Brown, A.E., Mills, P.R., 1998. Genetic comparison of the aggressive weed mould strains of *Trichoderma harzianum* from mushroom compost in North America and the British Isles. Mycol. Res. 102, 385–390.

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

Nirenberg, H.I., O'Donnell, K., 1998. New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. Mycologia 90, 434–458.

O'Donnell, K., Cigelnik, E., Nirenberg, H.I., 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90, 465–493.

O'Donnell, K., Kistler, H.C., Tacke, B.K., Casper, H.H., 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proc. Natl. Acad. Sci. USA 97, 7905–7910.

Ospina-Giraldo, M.D., Royse, D.J., Thon, M.R., Chen, X., Romaine, C.P., 1998. Phylogenetic relationships of *Trichoderma harzianum* causing green mold in Europe and North America to other species of *Trichoderma* from world-wide sources. Mycologia 90, 76–81.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 917–918.

Reynolds, D.R., Taylor, J.W. (Eds.), 1993. The Fungal Holomorph: Mitotic and Pleiosporic Speciation in Fungal Systematics. CAB International, Wallingford.

Rifai, M.A., 1969. A revision of the genus *Trichoderma*. Mycol. Pap. 116, 1–56.

Samuels, G.J., Dodd, S.L., Gams, W., Castlebury, L.A., Petrini, O., 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia 94, 146–168.

Seaby, D., 1996. Differentiation of *Trichoderma* taxa associated with mushroom production. Plant Pathol. 45, 905–912.

Seaby, D., 1998. *Trichoderma* as a weed mould or pathogen in mushroom cultivation. In: Kubicek, C., Harman, G. (Eds.), *Trichoderma* and *Gliocladium*. Taylor & Francis Inc., London, pp. 267–287.

Shimodaira, H., Hasegawa, H., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.

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 Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Veerkamp, J., Gams, W., 1983. Los hongos de Colombia. VIII. Some new species of soil fungi from Colombia. Caldasia 13, 709–717.

Wells, H.D., Bell, D.K., Jaworski, C.A., 1972. Efficacy of *Trichoderma harzianum* as a biocontrol for *Sclerotium rolfsii*. Phytopathology 62, 442–447.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A. et al. (Eds.), PCR Protocols: A guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.

Yang, D., Bernier, L., Dessureault, M., 1995. *Phaeotheca dimorphospora* increases *Trichoderma harzianum* density in soil and suppresses red pine damping-off caused by *Cylindrocladium scoparium*. Can. J. Bot. 73, 693–700.