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Phylogeny of the Clinically Relevant Species of the Emerging Fungus *Trichoderma* and Their Antifungal Susceptibilities

Marcelo Sandoval-Denis,[a] Deanna A. Sutton,[b] José F. Cano-Lira,[a] Josepa Gené,[a] Annette W. Fothergill,[b] Nathan P. Wiederhold,[b] Josep Guarro[a]

Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain[a]; Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas, USA[b]

A set of 73 isolates of the emerging fungus *Trichoderma* isolated from human and animal clinical specimens were characterized morphologically and molecularly using a multilocus sequence analysis that included the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA and fragments of the translation elongation factor 1 alpha (*Tef1*), endochitinase CHI18-5 (*Chi18-5*), and actin 1 (*Act1*) genes. The most frequent species was *Trichoderma longibrachiatum* (26%), followed by *Trichoderma citrinoviride* (18%), the *Hypocrea lixii/Trichoderma harzianum* species complex (15%), the newly described species *Trichoderma bissettii* (12%), and *Trichoderma orientale* (11%). The most common anatomical sites of isolation in human clinical specimens were the respiratory tract (40%), followed by deep tissue (30%) and superficial tissues (26%), while all the animal-associated isolates were obtained from superficial tissue samples. Susceptibilities of the isolates to eight antifungal drugs *in vitro* showed mostly high MICs, except for voriconazole and the echinocandins.

The genus *Trichoderma* of the order *Hypocreales* (*Ascomycota*) comprises a large number of saprobic species with a worldwide distribution (1). They are commonly found in soil and play an important role as decomposers of decaying plant material and insect pathogens (2). In addition, members of this genus are important biotechnologically due to their ability to produce a wide spectrum of bioactive compounds (3).

Trichoderma species are infrequent but emergent human pathogens. *Trichoderma* infections in humans have been related with several risk factors, being associated mostly with peritoneal dialysis, organ transplantation, and hematologic disorders (4). They cause severe and persistent disseminated infections that usually fail to respond to treatment with amphotericin B (AMB) or voriconazole (VRC) (5–9). Other diseases attributed to members of this genus are allergic and acute invasive sinusitis (10, 11), keratitis (12), otitis externa (13), skin and subcutaneous infections (14), peritonitis (9, 15–20), deep pulmonary infections (21–23), endocarditis (24), and brain abscess (25). Most infections are caused by *Trichoderma longibrachiatum*, which is recognized as the main human pathogen of the genus (4, 11, 26), but eight other species (i.e., *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. koningii*, *T. orientale*, *T. pseudokoningii*, *T. reesei*, and *T. viride*) have also been reported occasionally (4, 26–28). On the other hand, data on animal infections by *Trichoderma* spp. are very limited. The few case reports available mostly involve superficial infections in cold-blooded animals (29, 30), and more recently, there were three cases of pulmonary infection by *T. pseudokoningii* (31).

Trichoderma isolates can be easily recognized by their characteristic green and rapidly growing colonies, as well as the typical branching patterns of the conidiophores. Traditionally, those species that developed the sexual morph were included in the genus *Hypocrea*. Despite the recent change in fungal nomenclature in favor of "one fungus, one name" (32), in this group of fungi both generic names are still used (33). Classically, species in the genus have been organized in "species aggregates" (34) and later in sections and clades (35–38). This latter taxonomical scheme reflects the modern phylogenetic-based classification of the genus (39). Most clinical reports have based identification of the etiological agents on their morphological characteristics (17). However, morphological identification of *Trichoderma* species can be problematic, because some species show great homoplasy in their conidial structures (39, 40). DNA sequence analysis has helped clarify the taxonomy of *Trichoderma*, and several new species have been described based on multigene phylogenies (39, 41, 42). In the clinical setting, species identification has been based mainly on ribosomal DNA (rDNA) sequences (8, 23, 28, 40), although it has been shown that the use of this locus does not provide sufficient resolution to accurately distinguish closely related species (4, 26, 41, 43). This, together with the poor *in vitro* activities of commonly used antifungals against *Trichoderma* isolates, is an impediment for the treatment of *Trichoderma* infections (11, 43) and limits our understanding of the epidemiology of the species.

The objective of this study was to assess the spectrum of *Trichoderma* species in clinical samples. Herein, a large set of clinical isolates was identified by using morphological and molecular data, via comparison of the multilocus sequences with those of reference strains. In addition, the *in vitro* antifungal susceptibilities of these isolates to eight currently used antifungal drugs were determined.

MATERIALS AND METHODS

Fungal isolates and sequences. A total of 73 isolates of *Trichoderma* were included in this study; 63 were obtained from human clinical specimens and 10 from animal clinical sources (Table 1). Most of them were from the

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Editor: D. W. Warnock
Address correspondence to Josepa Gené, josepa.gene@urv.cat.

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TABLE 1 Origin and GenBank accession numbers of the sequences of the *Trichoderma* isolates identified in this study

Species	Isolate no.[a]	Origin[b]	GenBank accession no.			
			ITS	*Tef1*	*Chi18-5*	*Act1*
H. lixii/*T. harzianum* species complex	UTHSC 02-2663	Human maxillar sinus, USA	KJ174168	HG931199	HG931272	
	UTHSC 03-2105	Marine sponge, USA	KJ174171	HG931202	HG931275	
	UTHSC 05-2749	Human sputum, USA	KJ174170	HG931200	HG931274	
	UTHSC 04-134	Manatee, USA	KJ174169	HG931200	HG931273	
	UTHSC 07-2109	Human blood, USA	KJ174172	HG931203	HG931276	
	UTHSC 08-418	Sea turtle, USA	KJ174173	HG931204	HG931277	
	UTHSC 09-3558	Human cornea, USA	KJ174174	HG931205	HG931278	
	UTHSC 10-1527	Human BAL, USA	KJ174175	HG931206	HG931279	
	UTHSC 11-2939	Goat hair, USA	KJ174176	HG931207	HG931280	
	UTHSC 11-3209	Marine sponge, USA	KJ174177	HG931208	HG931281	
	UTHSC 11-3234	Human stool, USA	KJ174178	HG931209	HG931282	
T. asperelloides	UTHSC 07-2264	Human nails, USA	KJ174188	HG931219		HG931187
	UTHSC 09-1326	Marine sponge, USA	KJ174189	HG931220		HG931188
T. asperellum	UTHSC 07-1832	Human sputum, USA	KJ174187	HG931218		HG931186
T. atroviride	UTHSC 03-1690	Manatee skin, USA	KJ174190	HG931221		HG931189
	UTHSC 08-2439	Marine sponge, USA	KJ174191	HG931222		HG931190
	UTHSC 10-2682	Turtle shell, USA	KJ174192	HG931223		HG931191
	UTHSC 11-1239	Human lung mass, USA	KJ174193	HG931224		HG931192
T. bissettii sp. nov.	UTHSC 07-852	Human sinus, USA	KJ174232	HG931263	HG931323	
	UTHSC 07-2998	Human nails, USA	KJ174233	HG931264	HG931324	
	UTHSC 08-615	Human wound, USA	KJ174234	HG931265	HG931325	
	UTHSC 08-2443^T = CBS 137447	Human sinus, USA	KJ174235	HG931266	HG931326	
	UTHSC 09-2160	Human BAL, USA	KJ174236	HG931267	HG931327	
	UTHSC 11-455	Human foot, USA	KJ174237	HG931268	HG931328	
	UTHSC 12-337	Human bone, USA	KJ174238	HG931269	HG931329	
	UTHSC 12-944	Human vertebral body, USA	KJ174239	HG931270	HG931330	
	UTHSC 12-1543	Human nails, USA	KJ174240	HG931271	HG931331	
T. citrinoviride	UTHSC 03-1479	Human BAL, USA	KJ174194	HG931225	HG931291	
	UTHSC 03-3702	Human blood, USA	KJ174196	HG931227	HG931293	
	UTHSC 06-3324	Human toe nail, USA	KJ174195	HG931226	HG931292	
	UTHSC 08-1945	Human ascitic fluid, USA	KJ174197	HG931228	HG931294	
	UTHSC 09-959	Human pleural fluid, USA	KJ174198	HG931229	HG931295	
	UTHSC 09-2229	Human eye, USA	KJ174199	HG931230	HG931296	
	UTHSC 10-434	Human abdominal wound, USA	KJ174200	HG931231	HG931297	
	UTHSC 10-1704	Human BAL, USA	KJ174201	HG931232	HG931298	
	UTHSC 10-2923	Human lung, USA	KJ174202	HG931233	HG931299	
	UTHSC 11-1116	Human sputum, USA	KJ174203	HG931234	HG931300	
	UTHSC 11-1353	Human blood, USA	KJ174204	HG931235	HG931301	
	UTHSC 11-3314	Human BAL, USA	KJ174205	HG931236	HG931302	
	UTHSC 12-536	Human BAL, USA	KJ174206	HG931237	HG931303	
T. erinaceus	UTHSC 07-1088	Human nails, USA	KJ174207	HG931238		HG931193
T. gamsii	UTHSC 09-135	Human sputum, USA	KJ174208	HG931239		HG931194
T. koningiopsis	UTHSC 06-1272	Human nails, USA	KJ174209	HG931240		HG931195
	UTHSC 11-2740	Human BAL, USA	KJ174210	HG931241		HG931196
	UTHSC 11-3372	Canine footpad, USA	KJ174211	HG931242		HG931197
T. longibrachiatum	UTHSC 06-2352	Human ear, USA	KJ174212	HG931243	HG931304	
	UTHSC 06-2504	Human BAL, USA	KJ174213	HG931244	HG931305	
	UTHSC 06-3659	Human CSF, USA	KJ174214	HG931245	HG931306	
	UTHSC 07-2530	Human BAL, USA	KJ174215	HG931246	HG931307	
	UTHSC 07-3636	Human nail, USA	KJ174216	HG931247	HG931308	
	UTHSC 07-3704	Human nail, USA	KJ174217	HG931248	HG931309	
	UTHSC 07-3821	Human BAL, USA	KJ174218	HG931249	HG931310	

(Continued on following page)

TABLE 1 (Continued)

Species	Isolate no.[a]	Origin[b]	GenBank accession no.			
			ITS	*Tef1*	*Chi18-5*	*Act1*
	UTHSC 08-1222	Human lung tissue, USA	KJ174219	HG931250	HG931311	
	UTHSC 09-2900	Human sputum, USA	KJ174220	HG931251	HG931312	
	UTHSC 09-3339	Human BAL, USA	KJ174221	HG931252	HG931313	
	UTHSC 10-244	Human pleural fluid, USA	KJ174222	HG931253	HG931314	
	UTHSC 10-457	Human maxillar sinus, USA	KJ174223	HG931254	HG931315	
	UTHSC 11-589	Human BAL, USA	KJ174224	HG931255	HG931316	
	UTHSC 11-942	Human peritoneal fluid, USA	KJ174225	HG931256	HG931317	
	UTHSC 11-997	Human mediastinal mass, USA	KJ174226	HG931257	HG931318	
	UTHSC 11-3265	Human blood, USA	KJ174227	HG931258	HG931319	
	UTHSC 11-3571	Human sputum, USA	KJ174228	HG931259	HG931320	
	UTHSC 11-3808	Human sputum, USA	KJ174229	HG931260	HG931321	
	UTHSC 12-264	Human BAL, USA	KJ174230	HG931261	HG931322	
T. orientale	UTHSC 03-91	Human sputum, USA	KJ174179	HG931210	HG931283	
	UTHSC 04-373	Human sinus, USA	KJ174181	HG931212	HG931285	
	UTHSC 06-2183	Human blood, USA	KJ174180	HG931211	HG931284	
	UTHSC 07-285	Human BAL, USA	KJ174182	HG931213	HG931286	
	UTHSC 07-1541	Human peritoneal fluid, USA	KJ174183	HG931214	HG931287	
	UTHSC 09-1967	Human arm, USA	KJ174184	HG931215	HG931288	
	UTHSC 09-2386	Human blood, USA	KJ174185	HG931216	HG931289	
	FMR 12739	Human vascular protesis, Spain	KJ174186	HG931217	HG931290	
T. sinuosum	UTHSC 07-3543	Human skin, USA	KJ174231	HG931262		HG931198

[a] FMR, Facultat de Medicina i Ciències de la Salut, Reus, Spain; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center.
[b] CSF, cerebrospinal fluid; BAL, broncheoalveolar lavage fluid.

United States and were received at the Fungus Testing Laboratory of the University of Texas Health Science Center at San Antonio (UTHSC) mainly for identification purposes. In addition, 248 sequences corresponding to type or reference strains of *Trichoderma* species or related genera retrieved from GenBank were also included in the phylogenetic analyses.

Morphological identification. The isolates were subcultured onto potato-dextrose agar (PDA; Pronadisa, Spain) and cornmeal agar (cornmeal, 50 g; agar, 15 g; water, 1 liter) with 2% glucose (CMD) (42), incubated in the dark at different temperatures (15, 25, 30, 35, 37, and 40°C), and measured daily to determine colony growth rates. Microscopic observations were made from plate and slide cultures on PDA or CMD plates incubated for 7 to 14 days at 25°C. Slides were mounted on lactic acid or 3% potassium hydroxide and examined using an Olympus CH2 light microscope (Olympus Corporation, Tokyo, Japan). All isolates were identified morphologically after the methods described by Samuels et al. (39, 42, 44) and Jacklitsch (45) and by using an interactive *Trichoderma* identification key (http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm [46]). Color standards followed those of Kornerup and Wanscher (47). Photomicrographs were made with an Axio-Imager M1 light microscope (Zeiss, Oberkochen, Germany), using Nomarski differential interference.

DNA extraction, amplification, and sequencing. PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA) was used to extract total genomic DNA from mycelia that were scraped from colonies grown on YES agar (yeast extract, 20 g; sucrose, 150 g; agar, 20 g; distilled water, 1 liter) after 3 to 5 days of incubation at 25°C. DNA was quantified using a Nanodrop 3000 apparatus (Thermo Scientific, Madrid, Spain).

Four different nuclear DNA targets were amplified by PCR and sequenced using the following primer pairs: ITS5/ITS4 for the internal transcribed spacer 1 and 2 (ITS1 and ITS2) and the 5.8S gene of the rRNA (48), EF-1H/EF-2T for a fragment of the translation elongation factor 1 alpha gene (*Tef1*) (49), Chit42-1a/Chit42-2a for a fragment of the endochitinase CHI18-5 gene (*Chi18-5*) (50), and Act-1/Act4R for a fragment of the actin 1 gene (*Act1*) (51), according to the protocols described by the respective authors. The amplified products were purified using the Diffinity rapid tip purification system (Sigma-Aldrich, St. Louis, MO) and stored at −20°C until sequencing.

Amplicons were sequenced in both directions, using the PCR primers at Macrogen Europe (Macrogen Inc., Amsterdam, The Netherlands). Consensus sequences were assembled using SeqMan version 7.0.0 (DNASTAR, Madison, WI).

Molecular identification and phylogenetic analysis. An initial identification was made by comparing ITS sequences with those in *Trich*OKEY (http://isth.info/) (52). Multiple sequence alignments were made in MEGA version 5.05 (53) using the ClustalW application (54), refined with MUSCLE (55), and manually adjusted using the same software platform. Phylogenetic reconstructions were made using the individual loci via maximum-likelihood (ML) and Bayesian inference (BI) with MEGA version 5.05 and MrBayes version 3.1.2 (56), respectively. The best substitution model for all gene matrices (GTR+I+G) was estimated using MrModelTest version 2.3 (57). For ML analyses, nearest-neighbor interchange was used as the heuristic method for tree inference. Support for internal branches was assessed by 1,000 ML bootstrapped pseudoreplicates of data. Bootstrap support (bs) of ≥70 was considered significant. For BI analyses, Markov chain Monte Carlo (MCMC) sampling was performed with two simultaneous runs for 3 million generations, with samples taken every 100 generations. The 50% majority rule consensus trees and posterior probability values (pp) were calculated after removing the first 25% of the resulting trees for burn-in. A pp value of ≥0.95 was considered significant.

The phylogenies obtained from each locus were compared in order to assess for incongruent results among the different DNA matrices, by comparing the phylogenetic placement and internal nodes with significant bs/pp support. Given that no incongruence was observed, the different matrices were combined for the final phylogenetic analyses. Given the high genetic variability observed between the different clades and the in-

ability to obtain a reliable alignment that included all of the species, the different *Trichoderma* clades were analyzed individually using different gene combinations according to previous phylogenetic studies (41, 44, 58, 59): ITS, *Tef1*, and *Chi18-5* for the clades *Longibrachiatum* and *Harzianum* and ITS, *Tef1*, and *Act1* for the clades *Viride*, *Hamatum*, and *Chlorospora*. Analyses of the combined data set were performed using the same parameters mentioned above. *Hypocrea pachybasioides* and *Trichoderma minutisporum* were selected as outgroup taxa.

Antifungal susceptibility testing. Antifungal susceptibility testing was performed according to CLSI document M38-A2 (60) with AMB, VRC, posaconazole (PSC), itraconazole (ITC), caspofungin (CFG), anidulafungin (AFG), micafungin (MFG), and terbinafine (TBF). The minimal effective concentration (MEC) was determined at 24 h for the echinocandins, and the MIC was determined at 48 h for the remaining drugs. The MIC was defined as the lowest concentration exhibiting 100% visual inhibition of growth for AMB, VRC, ITC, and PSC and an 80% reduction in growth for TRB. *Paecilomyces variotii* ATCC MYA-3630 and *Aspergillus fumigatus* ATCC MYA-3626 were used as quality control strains. Statistical analyses of the MIC/MEC data were performed using the Kruskal-Wallis test in the Prism program for Windows, version 6.0 (GraphPad Software, San Diego, CA).

Nucleotide sequence accession numbers. DNA sequences determined in this study have been deposited in GenBank; the accession numbers are reported in Table 1.

RESULTS

Analysis of the ITS sequences showed that our isolates were distributed among five clades of four different sections of the genus *Trichoderma* (data not shown). Most of them (67.1%) belonged to section *Longibrachiatum* (35, 36), clade *Longibrachiatum*; 15.1% belonged to section *Pachybasium* (37, 38), clade *Harzianum*; 12.3% and 4.1% belonged to section *Trichoderma* (37), clades *Viride* and *Hamatum*, respectively; 1.4% of the isolates belonged to section *Hypocreanum* (37), clade *Chlorospora*. Distinct morphological features of the isolates nested in clade *Longibrachiatum* included formation of yellow to green colonies on PDA, sometimes with a diffusible yellow pigment, rapid sporulation at 30 to 35°C, and abundant growth at 40°C. The conidiophores that arose mainly from the aerial hyphae were hyaline and composed of a long, thick central axis from which secondary branches emerged. The phialides were lageniform, cylindrical or with a slight swelling in the middle, hyaline, and smooth walled. They arose singly and directly from the main axis or from the branches or formed whorls on a supporting cell. Intercalary phialides were common. Conidia were oblong to ellipsoidal, green, and smooth walled.

The isolates that belonged to the *Harzianum* clade on the same medium formed flat, white, greyish or pale yellowish colonies that grew and sporulated well at 25 to 30°C, growth was restricted at 35°C, and they were unable to grow at 40°C. The conidiophores were irregularly branched and formed on the aerial hyphae. The phialides were short and ampulliform, often arranged in whorls. Intercalary phialides were not present. The conidia were globose to ovoidal, green, and smooth walled.

The isolates included in the *Viride* and *Hamatum* clades produced yellow or green colonies with different growth rates between 25 and 40°C. A strong coconut odor was present in some isolates. The conidiophores exhibited a pyramidal branching pattern or were verticillate and were formed on the aerial hyphae. The phialides were mostly ampulliform and grouped in whorls. Intercalary phialides were observed in isolates of *T. gamsii* and *T. koningiopsis*. The conidia were yellow or green, globose or ellipsoidal, smooth walled or finely roughened.

The only isolate that belonged to the *Chlorospora* clade was unable to sporulate under the culture conditions used in this study and only could be identified by using molecular methods.

The tree topologies obtained with ML and BI analyses were nearly identical, except for minor differences in internal nodes with low or insignificant statistical support. Phylogenetic analysis of the *Longibrachiatum* clade included 1,388 bp (ITS, *Tef1*, and *Chi18-5*), corresponding to 23 species of *Trichoderma* (Fig. 1). The isolates mainly belonged to three species (i.e., *T. longibrachiatum*, *T. citrinoviride*, and *T. orientale*), while 9 isolates formed a well-supported subclade (bs 100/pp 1.00), which corresponded to a new species-level lineage. Since these isolates were morphologically and phylogenetically different from any previously known species of *Trichoderma*, they are described below as a new species named *Trichoderma bissettii*. Phylogenetic analysis of the *Harzianum* clade included 1,055 bp, based on the same three loci and representing 15 *Trichoderma* species, revealed that all of the isolates were nested with low support in the *H. lixii*/*T. harzianum* species complex sensu Druzhinina et al. (58) (Fig. 2). The majority of the human clinical isolates were nested with the type strain of *H. lixii* (CBS 110080), while only two isolates (UTHSC 07-2109 and UTHSC 11-3234) grouped with the type strain of *T. harzianum* (CBS 226.95). A single isolate (UTHSC 11-3209) grouped with the reference strain *T. afroharzianum* DAOM 231421. Two isolates of animal origin (UTHSC 03-2105 and UTHSC 08-418) grouped with the reference strain of *H. lixii* DAOM 231412, while isolate UTHSC 10-1527 grouped in a different clade with the reference strain of *H. lixii* CBS 115343. Phylogenetic analysis of the *Viride* clade included 1,253 bp (ITS, *Tef1*, and *Act1*) from 22 *Trichoderma* species (Fig. 3). Four isolates grouped with a reference strain of *T. atroviride* (CBS 142.95), with only one of them from human origin. One isolate nested with the type strain of *T. gamsii* (CBS 120075) and another isolate grouped with two reference strains of *T. erinaceus* (DIS 7 and CBS 117088), while two isolates of human origin and one animal-associated isolate nested with the type strain of *T. koningiopsis* (CBS 119075). Phylogenetic analysis of the *Hamatum* clade included 1,274 bp (ITS, *Tef1*, and *Act1*) and 10 species (Fig. 4). One isolate nested with the type strain of *T. asperellum* (CBS 433.97), while two isolates (one of human and one of animal origin) grouped with a reference strain of *T. asperelloides* (CBS 125399). Phylogenetic analysis of the *Chlorospora* clade included 1,917 bp (ITS, *Tef1*, and *Act1*) and 8 species (Fig. 5). The single isolate included in this clade was from a human clinical specimen and grouped with a reference strain of *T. sinuosum* (CBS 114247).

In summary, the molecular identification showed that the isolates belonged to 11 different *Trichoderma* species (Table 2), in addition to those belonging to the *H. lixii*/*T. harzianum* complex. The correlation between the molecular and the morphological identification is shown in Table 3. An agreement between both methods was found for 49.3% of the isolates. All of the isolates within the *T. longibrachiatum* and *H. lixii*/*T. harzianum* species complex were correctly identified morphologically. In contrast, most of the isolates of *T. citrinoviride*, *T. bissettii*, and *T. orientale* were identified morphologically as *T. longibrachiatum* or *T. pseudokoningii*. Most of the isolates identified morphologically as *T. atroviride* corresponded phylogenetically to other species of the *Viride* clade; however, two isolates belonged to the *Hamatum* clade (*T. asperelloides* and *T. asperellum*).

All the *Longibrachiatum* clade isolates were recovered from hu-

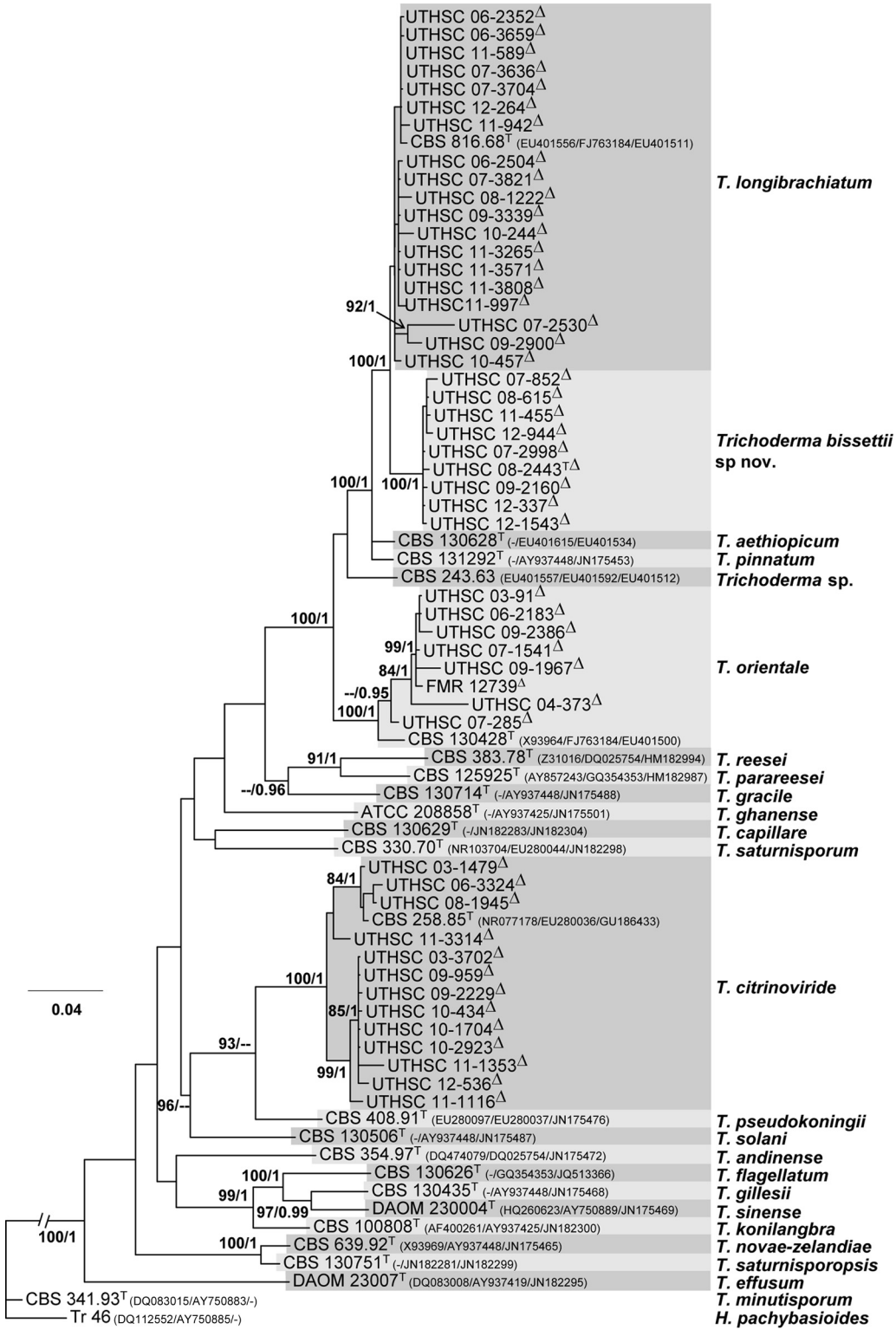

FIG 1 Bayesian tree inferred from combined ITS (456-bp), *Tef1* (466-bp), and *Chi18-5* (466-bp) sequences of *Trichoderma* and *Hypocrea* isolates belonging to the *Longibrachiatum* clade. Branch lengths are proportional to phylogenetic distance. ML bootstrap support values of >70% and posterior probability values of >0.95 are shown above the branches. The GenBank accession numbers given after some strains are those of the ITS/*Tef1*/*Chi18-5* genes. Missing sequences are indicated by a dash. *Hypocrea pachybasioides* and *T. minutisporum* were used to root the tree. Superscript T, type strain; Δ, strain of human origin. ATCC, American Type Culture Collection; CBS-KNAW, Fungal Biodiversity Centre culture collection, The Netherlands; DAOM, Agriculture and Agri-Food Canada National Mycological Culture Collection; FMR, Facultat de Medicina i Ciències de la Salut, Reus, Spain; Tr, collection of Earl Nelson maintained at the USDA-ARS Beltsville collection.

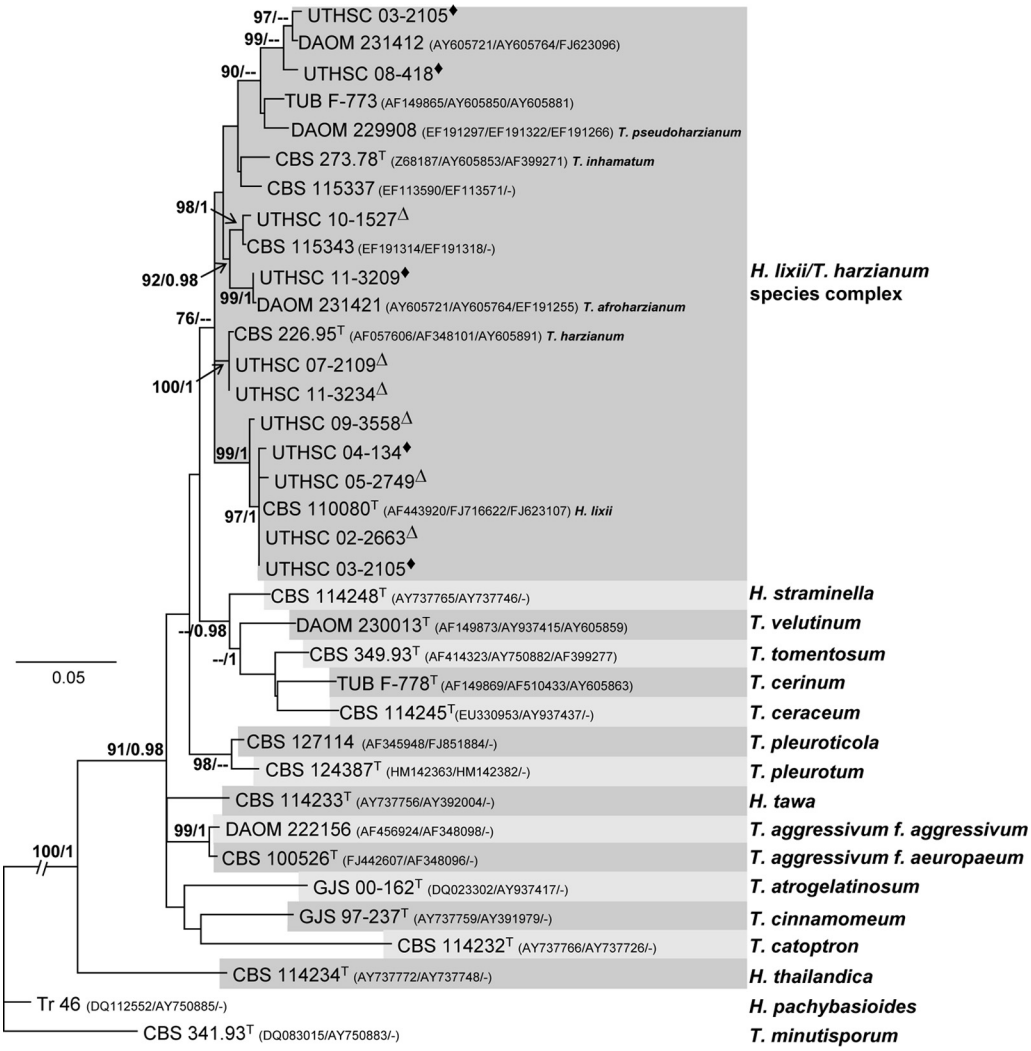

FIG 2 Bayesian tree inferred from combined ITS (407-bp), *Tef1* (238-bp), and *Chi18-5* (410-bp) sequences of *Trichoderma* and *Hypocrea* isolates belonging to the *Harzianum* clade. Branch lengths are proportional to phylogenetic distance. ML bootstrap support values of >70% and posterior probability values of >0.95 are shown above the branches. GenBank accession numbers given after some strains are those of the ITS/*Tef1*/*Chi18-5* genes. Missing sequences are indicated by a dash. *Hypocrea pachybasioides* and *T. minutisporum* were used to root the tree. Superscript T, type strain; Δ, strain of human origin; ◆, strain of animal origin. CBS-KNAW, Fungal Biodiversity Centre culture collection, The Netherlands; DAOM, Agriculture and Agri-Food Canada National Mycological Culture Collection; GJS, collection of Gary J. Samuels maintained at the USDA-ARS Beltsville collection; Tr, collection of Earl Nelson maintained at the USDA-ARS Beltsville collection; TUB, Technical University of Budapest Microbial Culture Collection.

man specimens, mainly from the respiratory tract. Isolates of human origin that belonged to the other clades were mainly from superficial tissues. All the animal-associated isolates were obtained from superficial tissue specimens.

Results of the antifungal susceptibility testing are summarized in Table 4. The geometric mean (GM) MIC and MIC_{90} values for AMB were 1.05 μg/ml and 2 μg/ml, respectively. Among the azoles, VRC was the most active, with an overall GM MIC and MIC_{90} of 1.62 μg/ml and 4 μg/ml, respectively, while PSC and ITC showed GM MIC values of 9.98 μg/ml and 16.15 μg/ml, respectively ($P < 0.0001$). The echinocandins showed the most potent *in vitro* activities, with overall GM MIC values of 0.23, 0.24, and 0.09 μg/ml for CFG, AFG, and MFG, respectively ($P < 0.05$). Terbinafine showed variable activity, with overall GM MIC and MIC_{90} values of 0.75 μg/ml and 4 μg/ml, respectively. All the *Trichoderma* species tested exhibited similar susceptibility patterns without showing statistical significance.

Taxonomy and related information. *Trichoderma bissettii* Sandoval-Denis & Guarro, sp. nov. (Fig. 6). MycoBank accession number MB807940. The etymology is in honor of John Bissett for his extensive work on *Hypocrea* and *Trichoderma*. The new species differs from *T. longibrachiatum* by having slightly longer conidia and narrower phialides borne on longer basal cells.

Colonies on PDA at 30°C to 35°C attaining 84 to 112 mm in diameter in 48 h, covering the plate (9 cm diameter) after 72 h, flat, forming concentric rings of whitish mycelium, fluffy and dense, with velvety to slightly granular zones, light yellow (2-A5) to olive-yellow (3-D7), margin irregular; reverse pale yellow (3-A3) to vivid yellow (3-A6); yellow diffusing pigment formed within 24 h at 15, 25, 30, and 35°C and within 48 h at 40°C. On CMD at 30 to

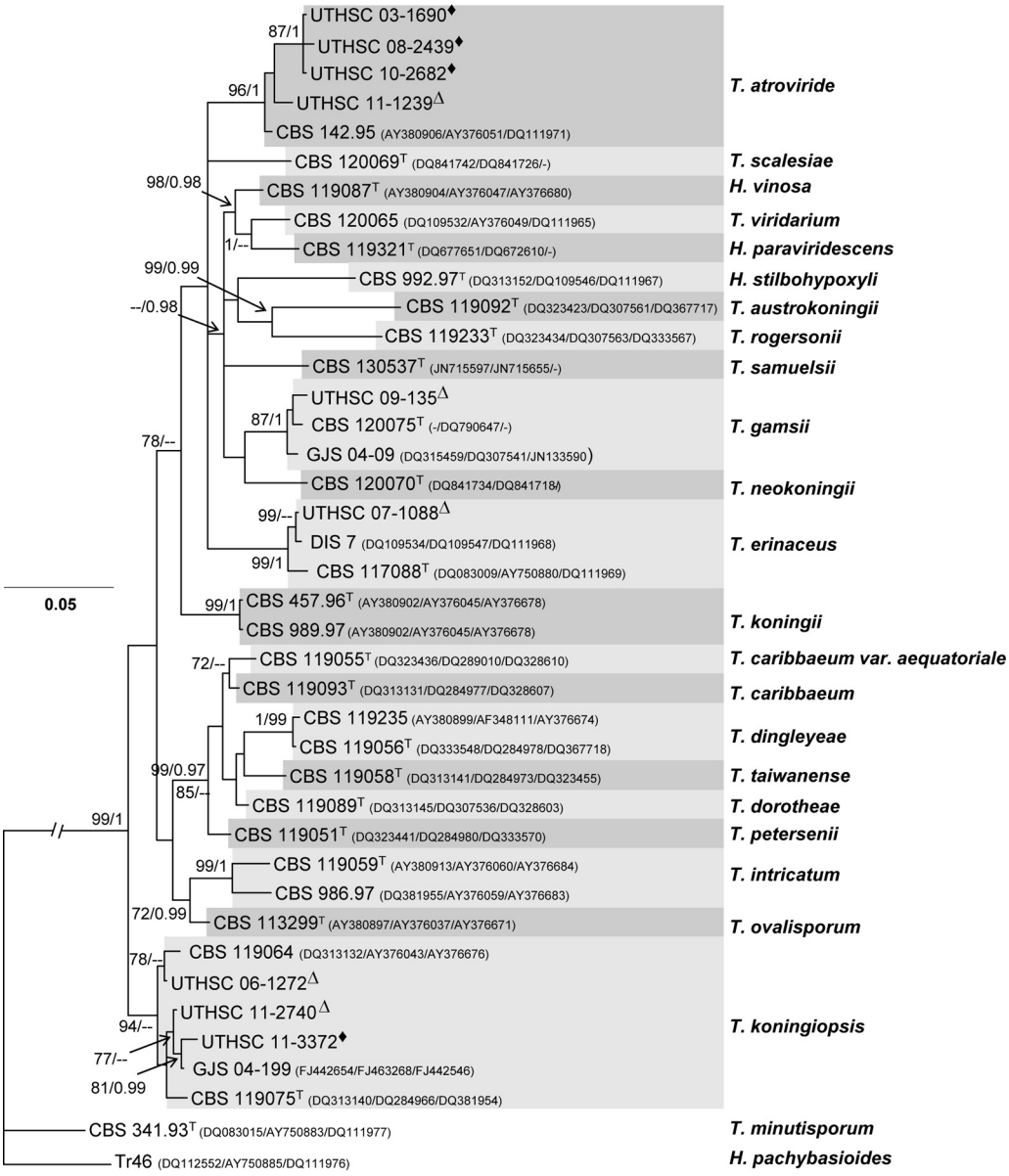

FIG 3 Bayesian tree inferred from combined ITS (389-bp), *Tef1* (304-bp), and *Act1* (560-bp) sequences of *Trichoderma* and *Hypocrea* isolates belonging to the *Viride* clade. Branch lengths are proportional to phylogenetic distance. ML bootstrap support values of >70% and posterior probability values of >0.95 are shown above the branches. GenBank accession numbers given after the some strains are those of the ITS/*Tef1*/*Act1* genes. Missing sequences are indicated by a dash. *Hypocrea pachybasioides* and *T. minutisporum* were used to root the tree. Superscript T, type strain; Δ, strain of human origin; ◆, strain of animal origin. CBS-KNAW, Fungal Biodiversity Centre culture collection, The Netherlands; DIS, CABI-Bioscience cultures held by GJS; GJS, collection of Gary J. Samuels maintained at the USDA-ARS Beltsville collection; Tr, collection of Earl Nelson maintained at the USDA-ARS Beltsville collection.

35°C attaining 70 to 100 mm in diameter in 48 h, completely filling the plate after 72 h, flat, forming concentric rings of whitish, often submerged mycelia, with granular deep green (26-D8) zones, minute deep green (26-D8) pustules (<1 mm) often formed, margin irregular; reverse deep green (26-D8); diffusible pigment not formed. Vegetative hyphae septate, hyaline, smooth and thin walled. Conidiophores usually consisting of a distinctive main axis, 3 to 5 μm wide, 2 or 3 side branches at right angles, straight or slightly bent toward upwards, up to 200 μm long, hyaline, smooth walled. Sterile hairs not formed. Phialides borne singly and laterally on the main axis or from side branches, or divergent in small whorls of 2 to 3 phialides arising from supporting cells (5.0)5.7 to 9.1(10.0) μm. Phialides cylindrical to lageniform, often curved or flexuous, (6.0)7.0 to 10.4(13.0) μm long, (2.0)2.1 to 2.9(3.5) μm at the widest point, length/width ratio (L/W) of (2.0)2.6 to 4.6(6.5), (1.4)1.5 to 1.8(2.0) μm wide at the base, hyaline, smooth walled. Intercalary phialides abundant, with lateral cylindrical conidiogenous opening 1.5 to 3 by 1 to 1.5 μm. Conidia unicellular, broadly ellipsoidal to nearly oblong, (3.5)3.9 to 5.1(6.0) by (2.0)2.3 to 2.9(4.0) μm, L/W (1.3)1.5 to 2.1(3.0), at first white, rapidly becoming yellowish-green to dark green, smooth and thin walled. Chlamydospores abundant, terminal and intercalary, subglobose or ellipsoidal, (6.5)6.8 to 8.4(9.5) by (5.5)6.6 to 8.2(8.5) μm, smooth and thick walled. Sexual morph not observed. Car-

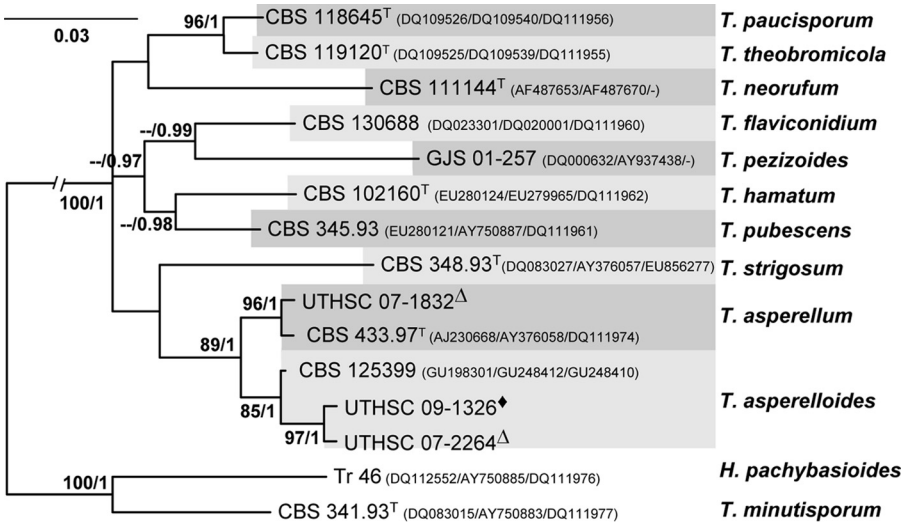

FIG 4 Bayesian tree inferred from combined ITS (405-bp), *Tef1* (310-bp), and *Act1* (559-bp) sequences of *Trichoderma* and *Hypocrea* isolates belonging to the *Hamatum* clade. Branch lengths are proportional to phylogenetic distance. ML bootstrap support values of >70% and posterior probability values of >0.95 are shown above the branches. GenBank accession numbers given after some strains are those of the ITS/*Tef1*/*Act1* genes. Missing sequences are indicated by a dash. *Hypocrea pachybasioides* and *T. minutisporum* were used to root the tree. Superscript T, type strain; Δ, strain of human origin; ◆, strain of animal origin. CBS-KNAW, Fungal Biodiversity Centre culture collection, The Netherlands; GJS, collection of Gary J. Samuels maintained at the USDA-ARS Beltsville collection; Tr, collection of Earl Nelson maintained at the USDA-ARS Beltsville collection.

dinal temperature for growth: optimum, 30 to 35°C; maximum, 40°C; minimum, 15°C.

Holotype. USA, from human sinusal cavity, 2008, D. A. Sutton (CBS H-21626; ex-type cultures CBS 137447 = UTHSC 08-2443 = FMR 12635).

DISCUSSION

In the present study, a large set of human and animal clinical isolates of *Trichoderma* was identified molecularly by using different gene combinations depending on the clade. The most common species were members of the *Longibrachiatum* clade, which is in agreement with findings reported by Kuhls et al. (40) who, by using ITS sequences, concluded that the spectrum of human pathogenic species of *Trichoderma* appeared to be restricted to this clade, particularly to *T. longibrachiatum*. Nevertheless, those authors' conclusions might have been biased, since in that study only six clinical isolates were included. More recently, several studies have emphasized the importance of this species as the causal agent of human infections (4, 26, 27). Three other species within the clade have been associated with human disease, i.e., *T. citrinoviride*, *T. orientale*, and *T. reesei* (21, 27, 40) The latter species has not been identified in this study, but the new species *T. bissettii* might be considered an opportunistic human pathogen of the *Longibrachiatum* clade. This is supported by the fact that *T. bisset-*

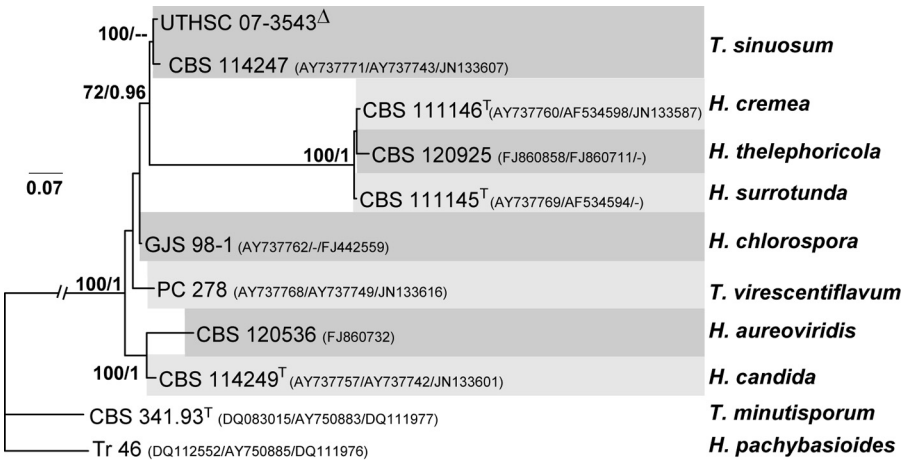

FIG 5 Bayesian tree inferred from the combined ITS (580-bp), *Tef1* (629-bp), and *Act1* (708-bp) sequences of *Trichoderma* and *Hypocrea* isolates belonging to the clade *Chlorospora*. Branch lengths are proportional to phylogenetic distance. ML bootstrap support values of >70% and posterior probability values of >0.95 are shown above the branches. GenBank accession numbers given after some strains are those of the ITS/*Tef1*/*Act1* genes. Missing sequences are indicated by a dash. *Hypocrea pachybasioides* and *T. minutisporum* were used to root the tree. Superscript T, type strain; Δ, strain of human origin. CBS-KNAW, Fungal Biodiversity Centre culture collection, The Netherlands; GJS, collection of Gary J. Samuels; PC, collection of Priscilla Chaverri; Tr, collection of Earl Nelson maintained at the USDA-ARS Beltsville collection.

TABLE 2 Numbers and sites of *Trichoderma* spp. isolated from human and animal sources

Species	No. of isolates from: Human			Animal	Total (%) no. of isolates
	Superficial	Respiratory	Deep tissue	Superficial tissue	
T. longibrachiatum	3	10	6	0	19 (26.0)
T. citrinoviride	3	5	5	0	13 (18.0)
H. lixii/T. harzianum species complex	2	3	1	5	11 (15.0)
T. bissettii	4	3	2	0	9 (12.0)
T. orientale	1	3	4	0	8 (11.0)
T. atroviride	0	0	1	3	4 (5.4)
T. koningiopsis	1	1	0	1	3 (4.0)
T. asperelloides	1	0	0	1	2 (3.0)
T. asperellum	0	1	0	0	1 (1.4)
T. erinaceum	1	0	0	0	1 (1.4)
T. gamsii	0	1	0	0	1 (1.4)
T. sinuosum	1	0	0	0	1 (1.4)
Total	17	27	19	10	73 (100)

tii was recovered from several different parts of the body, including deep tissue samples, and that it could grow at temperatures as high as 40°C, like the other clinically relevant species of the clade. Remarkably, *T. bissettii* was the fourth most common species identified. This species was probably not detected previously in clinical settings because its morphology is very similar to that of *T. longibrachiatum* and *T. orientale*. However, some subtle morphological differences were observed between them. In *T. bissettii*, the length and L/W ratio of the conidia were higher, the width of the basal portion of the phialides was narrower, and the length of the basal cell was longer than in *T. longibrachiatum* and *T. orientale*. It should also be noted that *T. bissetti* showed slightly higher MICs to AMB than *T. longibrachiatum* and *T. orientale*. In addition to these morphological differences, sequencing of the *Tef1* locus was a suitable phylogenetic marker that allowed correct discrimination of *T. bissettii* from its closest relatives.

Apart from species of the *Longibrachiatum* clade, several clinical cases have been attributed to other *Trichoderma* species (4, 8, 26, 61). Three clinical cases have been associated with *T. harzianum*, although the fungus was identified by ITS sequencing in only two of them (5, 8, 62). The *Harzianum* clade was the second most common in our study and, unlike the *Longibrachiatum* clade, it included isolates from humans and animals. Although this clade comprises more than 15 species, our clinical isolates only belonged to the *H. lixii*/*T. harzianum* species complex. This complex is formed by several phylogenetic species whose boundaries have not yet been delimited. Thus, the taxonomy of these species is unsettled (58, 63).

The *Viride* clade is the largest and most diverse group of species in *Trichoderma*, and it is composed of many phylogenetic species isolated from very diverse sources with a wide geographic distribution. Most of these species show high morphological homoplasy, which makes identification difficult (39). Three species of this clade have been linked to human disease. Ranque et al. (28) described an infection by *T. atroviride* in a liver transplant recipient, and morphological identification was later confirmed by molecular techniques (4). On the other hand, isolates recovered from two patients with peritonitis attributed to *T. koningii* (19, 20) were later reidentified as *T. longibrachiatum* (40, 62). Several cases have been attributed to *T. viride*, but the identification of the causal agent has never been confirmed molecularly (4). From the set of isolates studied, only *T. atroviride* was identified from both human and animal origins. *Trichoderma koningii* was identified morphologically on only one occasion, but molecularly that isolate corresponded to the closely related species *T. koningiopsis*. This latter species is commonly isolated from soil and has been described as an endophyte of *Theobroma* sp. and as a mycoparasite (44), but it has never been reported from clinical samples. The three isolates identified here as *T. koningiopsis* were from both human and animal origins. Two isolates identified morphologically as *T. atroviride* corresponded molecularly to *T. gamsii* and *T. erinaceus*. Both species are known to be soil saprophytes and endophytes (44, 45), but this is the first report of their isolation from clinical samples.

Only two species of the *Hamatum* clade were found in this study, i.e., *T. asperellum* recovered from a human sputum sample and *T. asperelloides* obtained from human nail and from a marine sponge. Both species are indistinguishable morphologically and are only known as saprophytes from agricultural soils, particularly from tropical regions (59). None of the isolates could be correctly

TABLE 3 Correlation between the morphological and molecular identification of the *Trichoderma* isolates from human and animal sources

Molecular identification	Morphological identification									% agreement
	T. atroviride	*T. citrinoviride*	*T. harzianum*	*T. koningii*	*T. longibrachiatum*	*T. pseudokoningii*	*T. strigosum*	*Trichoderma* spp.[a]	Total	
T. longibrachiatum					19				19	100
T. citrinoviride		3			7	3			13	23.1
H. lixii/T. harzianum species complex			11						11	100
T. bissettii					7	2			9	0
T. orientale					7	1			8	0
T. atroviride	3							1	4	75
T. koningiopsis	1			1				1	3	0
T. asperelloides	1						1		2	0
T. asperellum	1								1	0
T. erinaceus	1								1	0
T. gamsii	1								1	0
T. sinuosum								1	1	0
Total	8	3	11	1	40	6	1	3	73	49.3

[a] Isolates that failed to sporulate on the culture media.

TABLE 4 Results of *in vitro* antifungal susceptibility testing of the 73 clinical isolates of *Trichoderma* spp. included in the study

Species (no. of isolates tested)		MIC or MEC (μg/ml)							
		AMB	VRC	PSC	ITC	CFG	AFG	MFG	TBF
T. longibrachiatum (19)	GM	1.19	1.67	14.87	22.21	0.27	0.38	0.12	0.75
	MIC range	0.06–2	0.5–8	2–32	1–32	0.06–4	0.06–2	0.03–0.5	0.125–4
	MIC_{90}	2	4	32	32	0.5	1	0.5	2
T. citrinoviride (13)	GM	0.29	1.80	17.80	18.78	0.28	0.30	0.07	0.25
	MIC range	0.03–2	0.5–4	2–32	2–32	0.03–2	0.03–8	0.03–2	0.25–1
	MIC_{90}	1	4	32	32	1	2	0.25	1
H. lixii/*T. harzianum* species complex (11)	GM	1.55	1.77	3.76	9.66	0.16	0.07	0.06	0.78
	MIC range	0.5–4	0.25–16	1–32	1–32	0.06–0.5	0.03–0.25	0.03–0.25	0.25–4
	MIC_{90}	2	8	32	32	0.5	0.25	0.25	1
T. bissettii (9)	GM	2.33	1.17	17.28	14.81	0.21	0.29	0.10	1.17
	MIC range	2–4	0.5–4	4–32	1–32	0.125–0.25	0.125–2	0.06–0.25	0.25–2
	MIC_{90}	4	2	32	32	0.25	1	0.125	1
T. orientale (8)	GM	1.41	1.09	5.19	12.34	0.27	0.27	0.09	0.60
	MIC range	0.5–4	0.5–4	1–32	1–32	0.06–2	0.06–1	0.03–0.5	0.25–1
	MIC_{90}	4	4	32	32	2	1	0.125	1
T. atroviride (4)	GM	2.83	4.76	16.00	22.63	0.25	0.21	0.12	4.00
	MIC range	1–8	2–32	2–32	8–32	0.03–0.5	0.03–1	0.03–0.5	1–16
	MIC_{90}								
T. koningiopsis (3)	GM	0.25	4.00	11.31	32.00	0.16	0.08	0.08	0.79
	MIC range	0.06–2	1–4	4–32	32	0.06–0.5	0.03–0.5	0.06–0.125	0.5–1
	MIC_{90}								
T. asperelloides (2)	GM	1.41	2.00	2.00	11.31	0.06	0.35	0.04	2.00
	MIC range	1–2	2	2	8–16	0.03–0.125	0.125–1	0.03–0.06	2
	MIC_{90}								
T. asperellum (1)	GM	2.00	2.00	4.00	16.00	0.25	2.00	0.06	4.00
	MIC range								
	MIC_{90}								
T. erinaceus (1)	GM	0.50	1.00	4.00	32.00	0.25	0.25	0.06	1.00
	MIC range								
	MIC_{90}								
T. gamsii (1)	GM	1.00	1.00	32.00	32.00	0.25	1.00	0.25	4.00
	MIC range								
	MIC_{90}								
T. sinuosum (1)	GM	0.25	0.13	1.00	1.00	1.00	0.06	0.03	0.25
	MIC range								
	MIC_{90}								
Overall (73)	GM	1.05	1.62	9.98	16.15	0.23	0.24	0.09	0.75
	MIC range	0.03–8	0.125–32	1–32	1–32	0.03–4	0.03–8	0.03–2	0.125–16
	MIC_{90}	2	4	32	32	0.5	1	0.25	4

identified morphologically, because they could not be distinguished from *T. atroviride*, *T. harzianum*, and *T. strigosum*.

The *Chlorospora* clade is composed of a few species that show high morphological similarity to each other, and they are commonly found in soil or as endophytes (63). *Trichoderma sinuosum* was the only isolate from this clade, a species never reported found in a clinical setting.

Trichoderma spp. have been reported from deep tissue infections, mainly affecting the peritoneum and brain, followed by respiratory and other infection sites (4, 6, 8, 17, 61). The species studied here were mainly found in human respiratory specimens, followed by deep tissue and superficial tissue samples in similar amounts. Animal isolates were only from superficial tissue samples. While the isolates of the species of the *Longibrachiatum* clade were exclusively from human clinical specimens, most of the animal-associated isolates were found to belong to the *H. lixii*/*T. harzianum* species complex (Table 2). The isolation of *Trichoderma* spp. from animal sources (i.e., *T. asperel-*

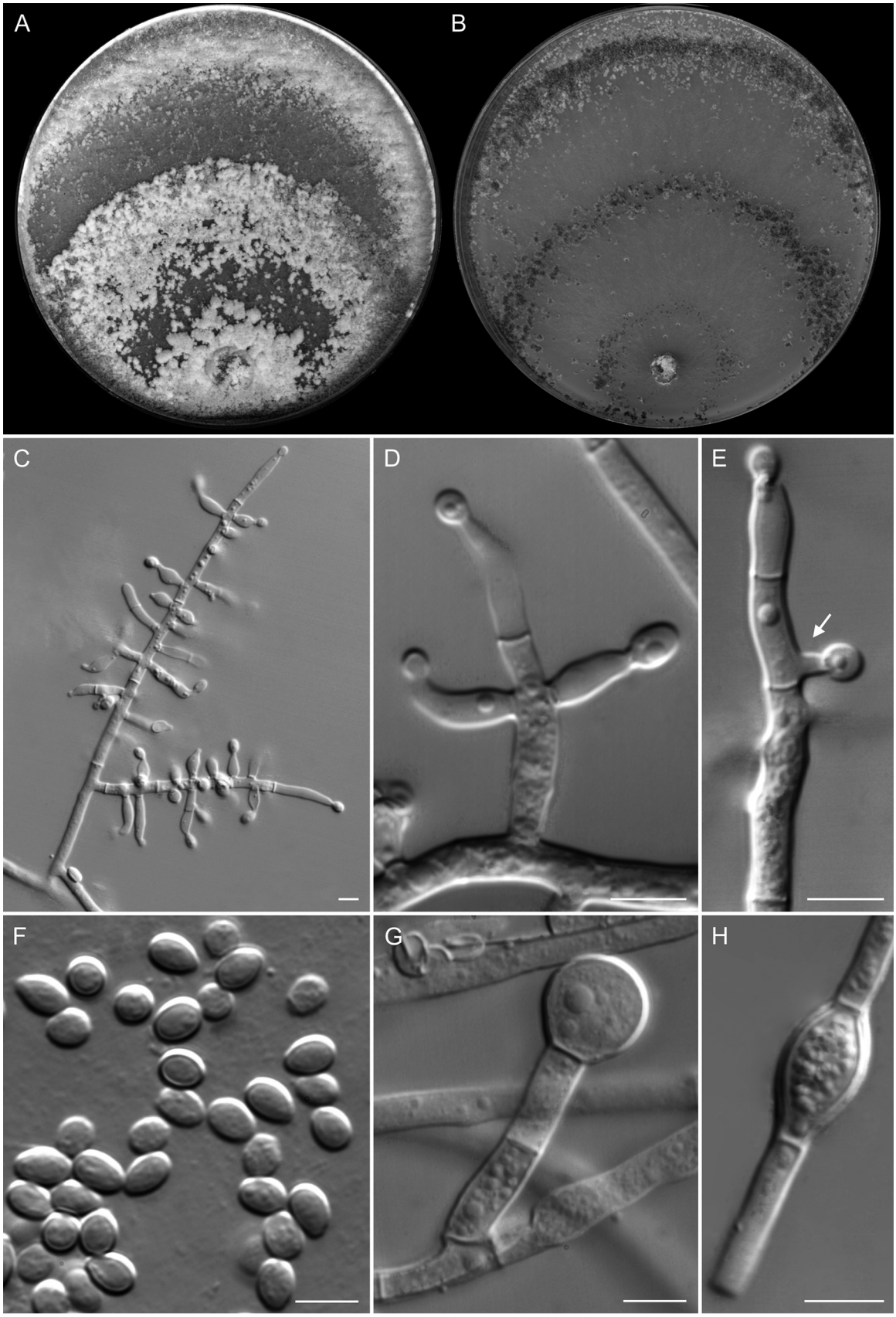

FIG 6 *Trichoderma bissettii* sp. nov. (UTHSC 08-2443). (A and B) Colonies on PDA and CMD, respectively, after 3 days at 25°C. (C) Conidiophore; (D) phialides; (E) intercalary phialide (arrow); (F) conidia; (G and H) chlamydospores. Bars, 5 μm.

loides, *T. atroviride*, *T. harzianum*, *T. longibrachiatum*, and *T. pseudokoningii*) has been previously reported (4, 31, 64), especially from marine sponges, although their role(s) have not been yet elucidated (64).

Among the human isolates, the most prevalent species identified in our study were *T. longibrachiatum* and *T. citrinoviride*, which were mainly isolated from respiratory and deep tissue samples; the other species identified were mainly from superficial tissue samples. Most of the species found here have previously been associated with opportunistic human diseases. However, *T. asperelloides*, *T. asperellum*, *T. erinaceum*, *T. gamsii*, *T. koningiopsis*, and *T. sinuosum* were found in clinical specimens for the first time. Despite the fact that these species have not been proven to be etiological agents of infections, their ability to grow at 40°C (in the first two species) and at 35 to 37°C (in the other four) and their prevalence in clinical specimens could indicate their possible role as human pathogens.

Current knowledge on the *in vitro* antifungal susceptibilities of pathogenic species of *Trichoderma* is mainly inferred from clinical cases that have shown variable results, probably due to the use of different methodologies (8, 26, 61). Our isolates showed similar antifungal patterns to those previously reported (6, 8, 23, 61); most of the antifungal agents tested showed high MICs. Although some isolates showed low MICs for AMB, especially against the less frequent species, generally the AMB MICs were considerably high. Among the azoles, VRC was the most active drug *in vitro* for most species, while PSC and ITC showed practically no clinically relevant activity; this correlates with the previous data of other authors (4). The activities of the echinocandins against *Trichoderma* have been proven before *in vitro*, especially for AFG, which has been shown to be at least four times more active than CFG (4, 65, 66). However, no data are available on the activity of MFG which, according to our study, showed the best *in vitro* activity against all the species tested. Similar to the previously published data, the activity of TBF varied, with relatively low MICs (4, 67). Clinical cases have reported unpredictable results regardless of the antifungal drug used. Most of the successfully resolved clinical cases were treated with AMB or its lipid formulations plus surgical debridement (61). The clinical experience with azole-based therapies also shows variable outcomes; VRC used mostly in combination with CFG has shown some positive results against deep infections by *T. longibrachiatum*, *T. reesei*, and *T. viride* (7, 21–23, 68). ITC has shown success alone and in combination with AMB and surgical resection against *T. longibrachiatum* (10, 11, 25), although according to our susceptibility results the apparent effectiveness of ITC might be only anecdotical. Ketoconazole was successful in a peritonitis case attributed to an unidentified *Trichoderma* species (15), while there are no reports on the use of PSC against *Trichoderma* infections. Our results suggest that this latter drug should be avoided, because most of the pathogenic species showed high MIC values. The use of echinocandins has been reported only for CFG in combination with VRC (21–23).

To date, this is the largest study involving clinical isolates of *Trichoderma*. The spectrum of *Trichoderma* species reported from clinical specimens has been expanded to 12 species, belonging to four different sections of the genus, including the new species *T. bissettii*, which was represented by nine isolates.

ACKNOWLEDGMENT

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