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Pithomyces species (Montagnulaceae) from clinical specimens: identification and antifungal susceptibility profiles

Keith Cássia da Cunha[1], Deanna A. Sutton[2], Josepa Gené[1,*], Josep Cano[1], Javier Capilla[1], Hugo Madrid[3], Cony Decock[4], Nathan P. Wiederhold[2] and Josep Guarro[1]

[1]Unitat de Micologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain, [2]Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center, San Antonio, Texas, USA, [3]Centraalbureau voor Schimmelcultures (CBS)-an Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW) Fungal Biodiversity Centre, Utrecht, The Netherlands and [4]Mycothéque de l'Université catholique de Louvain (MUCL), Université Catholique de Louvain, Louvain-la-Neuve, Belgium

*To whom correspondence should be addressed. Josepa Gené, Mycology Unit, Medical School, Universitat Rovira i Virgili, C/ Sant Llorenç 21, 43201-Reus-Tarragona-Spain. Tel: 34 977 759359; Fax: 34 977 759322; E-mail: josepa.gene@urv.cat

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Abstract

The fungal genus *Pithomyces* comprises numerous dematiaceous saprobic species commonly found on dead leaves and stems of a great variety of plants. Occasionally, they have been recovered from clinical specimens. We morphologically and molecularly (rDNA sequences) investigated a set of 42 isolates tentatively identified as *Pithomyces* recovered from clinical specimens in the United States. The predominant species were *P. chartarum* and *P. sacchari* (33.3% each), followed by *Pithomyces* sp. I (28.6%) and *P. maydicus* (4.8%). Most of the isolates were obtained from samples of superficial tissue (50%), the respiratory tract (21.4%), and the nasal region (19%). In general, these fungi were highly susceptible *in vitro* to the eight antifungal agents tested.

Key words: *Pithomyces*, DNA analysis, clinical specimens, morphological identification, antifungal susceptibility.

Introduction

Pithomyces is a large ascomycetous genus of Pleosporales with species commonly colonizing dead leaves and stems of many different plants [1]. Some species have been isolated from mammals with various disease symptoms. *Pithomyces chartarum*, the most widespread species, has been reported to cause facial eczema in some animals (ie, sheep, cattle, goats, and deer) due to liver damage caused by a mycotoxin (sporidesmin) produced by the fungus [2–6]. This species has also been reported as an agent of human onychomycosis [7] (Deplano M, Fadda ME, Pisano MB, et al. Detection and identification of non-dermatophyte filamentous fungi in onycomycosis: conventional and PCR methods. 18th Congress of the International Society for Human and Animal Mycology (ISHAM), Berlin, 10–14 Jun 2012, P563), while an unidentified *Pithomyces*

isolate was involved in a case of peritonitis in a patient with vulvar cancer [8]. Several studies have also described the presence of *Pithomyces* conidia in the indoor air environment where asthma patients reside [9–11]. However, an accurate identification of the *Pithomyces* isolates at the species level has usually not been attempted.

The genus *Pithomyces* is morphologically characterized by subspherical to clavate, brown, commonly verruculose or echinulate, and variably septate (ie, transverse, oblique, or/and longitudinal septa) conidia, borne at the apices of short peg-like conidiophores that arise laterally on the hyphae [1,12]. While the genus comprises close to 50 species, only *P. chartarum* is associated with a sexual stage, *Leptosphaerulina chartarum* [13]. Since *Leptosphaerulina* belongs to the family Didymellaceae (Pleosporales), *Pithomyces* has been taxonomically placed in that family [14].

Considering the few studies of *Pithomyces* isolates from clinical sources and the availability of numerous isolates of the genus obtained from human specimens in a mycology reference laboratory in the United States, our aim was to identify a set of those isolates by both morphological and molecular methods and to determine the *in vitro* susceptibility to different antifungal agents.

Materials and methods

Fungal isolates

A total of 42 isolates obtained from clinical specimens, received at the Fungus Testing Laboratory of the University of Texas Health Science Center, San Antonio, from different regions of the United States were investigated (Table 1). In addition, the ex-type strain of *L. chartarum* and reference

Table 1. Clinical isolates, reference strains, and sequences of *Pithomyces* spp. included in the study.

Species	Strain	Origin	GenBank (or NRBC) accession number	
			ITS	D1/D2
P. chartarum	MUCL 15905	Unknown substrate, Belgium	–	–
	MUCL 9393	Unknown substrate, France	–	–
	UBC F15184	Conidial drop from *Rubus spectabilis*, Canada	DQ384571.1	DQ384571.1
	UTHSC 03-2472	Skin, Minnesota	**HG518059**	**HG518064**
	UTHSC 04-678	Maxillary sinus, Tennessee	**HG518060**	**HG518065**
	UTHSC 04-2495	Foot, Montana	**HG518061**	HG518066
	UTHSC 05-2460	Maxillary sinus, Tennessee	**HG518062**	**HG518067**
	UTHSC 06-214	Toe, Colorado	**HG518063**	**HG518068**
	UTHSC 07-692	Scalp, Texas	–	–
	UTHSC 07-2045	Skin, Utah	–	–
	UTHSC 07-2802	Toenail, Massachusetts	–	–
	UTHSC 07-3664	Unknown, Illinois	–	–
	UTHSC 08-331	Bronchoalveolar lavage, Pennsylvania	–	–
	UTHSC 08-2855	Bronchoalveolar lavage, Pennsylvania	–	–
	UTHSC 10-2361	Sinus, Minnesota	–	–
	UTHSC 10-3088	Bronchoalveolar lavage, Washington	–	–
	UTHSC 11-2179	Pectoralis muscle, Michigan	–	–
P. maydicus	CBS 400.73	Dead leaf in *Cocos nucifera*, Sri Lanka	–	–
	UTHSC 06-1549	Toenail, South Carolina	**HG933801**	**HG933818**
	UTHSC 06-3954	Maxillary sinus, Texas	**HG933802**	**HG933819**
P. sacchari	CBS120504	Millet, Namibia	–	–
	CBS 803.72	Seed, South Africa	–	–
	MUCL 15288	Air, South Africa	–	–
	UTHSC 03-1337	Bronchoalveolar lavage, Florida	**HG933796**	**HG933813**
	UTHSC 03-3221	Nasal passage, Tennessee	**HG933797**	**HG933814**

Table 1. *Continued*

Species	Strain	Origin	GenBank (or NRBC) accession number	
			ITS	D1/D2
P. sacchari	UTHSC 04-2483	Bronchoalveolar lavage, Texas	**HG933798**	**HG933815**
	UTHSC 04-2746	Maxillary sinus, Texas	**HG933799**	**HG933816**
	UTHSC 05-3251	Bronchoalveolar lavage, South Carolina	**HG933800**	**HG933817**
	UTHSC 06-3688	Leg, Arizona	–	–
	UTHSC 06-3844	Scalp, Texas	–	–
	UTHSC 07-1285	Nail, South Carolina	–	–
	UTHSC 08-426	Maxillary sinus, Tennessee	–	–
	UTHSC 09-1473	Leg, Texas	–	–
	UTHSC 10-670	Lung, Florida	–	–
	UTHSC 10-1977	Sputum, North Carolina	–	–
	UTHSC 10-2143	Sinus, Tennessee	–	–
	UTHSC 12-56	Cornea, Louisiana	–	–
Pithomyces sp. I	CBS 244.96 (received as *P. atro-olivaceous*)	Skin scrapings of female diabetic, USA	–	–
	CBS 925.87 (received as *P. cynodontis*)	Stem of *Spartocytisus supranubius*, Spain	–	–
	UTHSC 05-3161	Nail, South Carolina	**HG933806**	**HG933823**
	UTHSC 05-3373	Bronchoalveolar lavage, Florida	**HG933807**	**HG933824**
	UTHSC 06-3492	Unknown, Florida	**HG933808**	**HG933825**
	UTHSC 06-3706	Cornea, Florida	**HG933809**	**HG933826**
	UTHSC 06-4528	Foot, Florida	**HG933810**	**HG933827**
	UTHSC 07-578	Toenail, Florida	–	–
	UTHSC 07-995	Toenail, Florida	–	–
	UTHSC 07-1535	Scalp, Florida	–	–
	UTHSC 08-535	Skin feet, Texas	–	–
	UTHSC 09-3190	Asian elephant eye, Massachusetts	–	–
	UTHSC 10-2776	Bronchoalveolar lavage, South Carolina	–	–
	UTHSC 11-3528	Leg tissue, Maryland	–	–
Leptosphaerulina chartarum	CBS 329.86 [T]	*Galenia procumbens*, South Africa	**HJ796400**	**HJ796401**
Massarina phragmiticola	NBRC105268	Decayed drift wood in the intertidal zone, Japan	(12117501)	(12117502)
Bipolaris maydis	CBS 136.29	Unknown, Japan	HG326306	HG326307

CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; MUCL, Mycothèque de L'Université Catholique de Louvain, Faculté des Sciences Agronomiques, Louvain-la-Neuve, Belgium; NRBC, National Institute of Technology and Evaluation (NITE) Biological Resource Center, Japan; UBC Herbarium—Beaty Biodiversity Museum University of British Columbia, Canada; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, USA.

[T] Type. New generated sequences for this study are in bold.

strains of different *Pithomyces* species, obtained from the Mycothèque de L'Université Catholique de Louvain (Belgium) and from the Centraalbureau voor Schimmelcultures (CBS)-an Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW) Fungal Biodiversity Centre (The Netherlands), were included in the study for morphological and molecular comparison (Table 1).

Morphological study

The isolates were grown on potato carrot agar (20 g of potatoes, 20 g of carrots, 20 g of agar, 1 l of distilled water) and oatmeal agar (30 g of filtered oat flakes, 20 g of agar, 1 l of distilled water) for 7–21 days at 25°C. Microscopic features were examined by making direct wet mounts in 85% lactic acid of portions of fungi growth on the two media. The isolates were morphologically identified following the criteria of Ellis [1,12,15] and Rao and de Hoog [16].

Molecular study

Isolates were grown on yeast extract sucrose (2% yeast extract, 15% sucrose, 2% agar, 1 l of water) for 3 days at 25°C; DNA was extracted and purified directly from fungal colonies following the FastDNA kit protocol (Bio101,Vista, CA, USA), with a minor modification, that is, a homogenization step was repeated three times with a FastPrep FP120 instrument (Thermo Savant, Holbrook, NY, USA). The internal transcribed spacer (ITS) region and D1/D2 domains of the 28S rDNA were amplified and sequenced with the primer pairs ITS5 and NL4b following previously described protocols [17,18]. The ITS polymerase chain reaction products were purified and sequenced at Macrogen Europe (Amsterdam) using a 3739XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). SeqMan (Lasergene, Madison, WI, USA) was used to obtain consensus from the complementary sequences of each isolate. BLAST sequence identity searches with ITS and D1/D2 regions were carried out to compare data from the isolates studied with those deposited in the GenBank database.

Two alignments were performed in the present study using Clustal X, version 1.81 [19]. The first alignment included ITS and D1/D2 sequences of the clinical isolates and a set of seven sequences representing five species of *Pithomyces* (one of these retrieved from GenBank; Table 1). The second alignment included D1/D2 representative sequences of our clinical isolates, the sequence of the ex-type strain of *L. chartarum*, and species representative of different families within the order Pleosporales [14,20]. Phylogenetic analyses were conducted using MEGA, version 5.05, with the maximum likelihood method. A search for the best model of nucleotide substitution was performed for both alignments. In the first analysis, the best model was Kimura 2-parameter; in the second, it was Tamura-Nei. The analyses were performed with pairwise deletion of gaps [21]. The robustness of branches was assessed through the use of bootstrap analyses of 1000 replicates. Sequences of *Massarina phragmiticola* (National Institute of Technology and Evaluation (NITE), Biological Resource Center (NBRC) 105268: 12117501 and 12117502) and *Bipolaris maydis* (CBS 136.29: HG326306 and HG326307 from GenBank) were used as out groups in the combined analysis.

Antifungal susceptibility

The *in vitro* antifungal susceptibility of 31 isolates with sufficient conidial formation to prepare standardized conidial inocula was evaluated according to reference guidelines [22], with a minor modification (ie, incubation at 30°C). The drugs tested were anidulafungin, amphotericin B (AMB), caspofungin (CAS), itraconazole (ITC), voriconazole (VRC), micafungin (MFG), posaconazole (PSC), and terbinafine (TBF). *Paecilomyces variotii* American Type Culture Collection MYA-3630 was used as a quality control strain. The minimal inhibitory concentration (MIC) endpoint for AMB, ITC, VRC, and PSC was 100% inhibition of growth. The minimum effective concentration (MEC) for the echinocandins was the lowest concentration at which a visible change in the growth characteristics compared with the growth control was observed. Endpoint determinations were read at 48 h.

Results

Of the 42 clinical isolates studied, 35 were morphologically identified as members of the genus *Pithomyces*. Although the remaining seven isolates did not form conidia, their ITS and D1/D2 sequences clustered with the rest of the *Pithomyces* clinical isolates investigated.

Figure 1 shows the maximum likelihood phylogenetic tree inferred from the combined analysis of ITS and D1/D2 sequences. The length of the alignment used in the analysis was 976 bp (545 bp corresponding to ITS and 431 bp to D1/D2 regions), which demonstrated that all clinical isolates were distributed into four clades taxa, of which three were found to correspond to the following species: *P. chartarum* (14 isolates, 33%), *P. sacchari* (14 isolates, 33.3%), and *P. maydicus* (2 isolates, 4.8%). The remaining 12 isolates (28.6%) formed an isolated clade that morphologically was not similar to any known species and were referred to as *Pithomyces* sp. I.

The *P. sacchari* clade (96% bootstrap support) included 3 reference strains and 14 clinical isolates. The similarity among these 17 isolates ranged from 99.2% to 100%; they

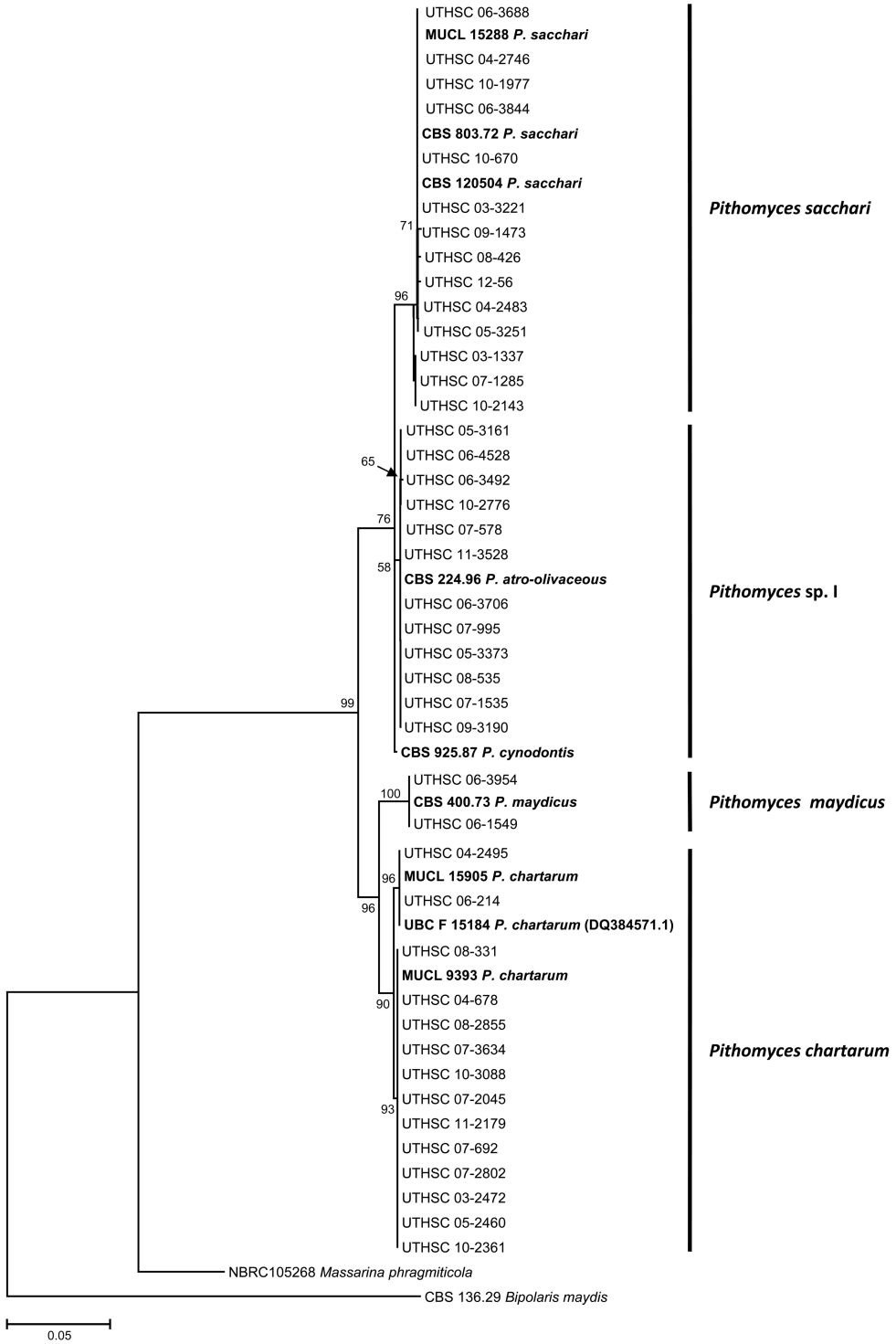

Figure 1. Maximum likelihood tree inferred from combined internal transcribed spacer and D1/D2 region of the 28S gene sequences of the isolates listed in Table 1. Branch lengths are proportional to the distance. Reference strains are in bold.

were characterized by having beige, gray, or olivaceous brown colonies. The majority (four did not form conidia) produced smooth to verruculose, brown, ellipsoidal or slightly clavate conidia, with two to five transverse septa and one oblique or longitudinal septum, and measuring 12–31 × 5–10 μm in the broadest part (Fig. 2A, B).

The *Phytomyces* sp. I clade comprised 12 clinical isolates and 2 reference strains, previously identified as *P. atro-olivaceous* (CBS 244.96) and *P. cynodontis* (CBS 925.87), respectively. The similarity among the isolates of this clade ranged from 99.1% to 100%. They were characterized by yellowish white to brownish gray colonies and

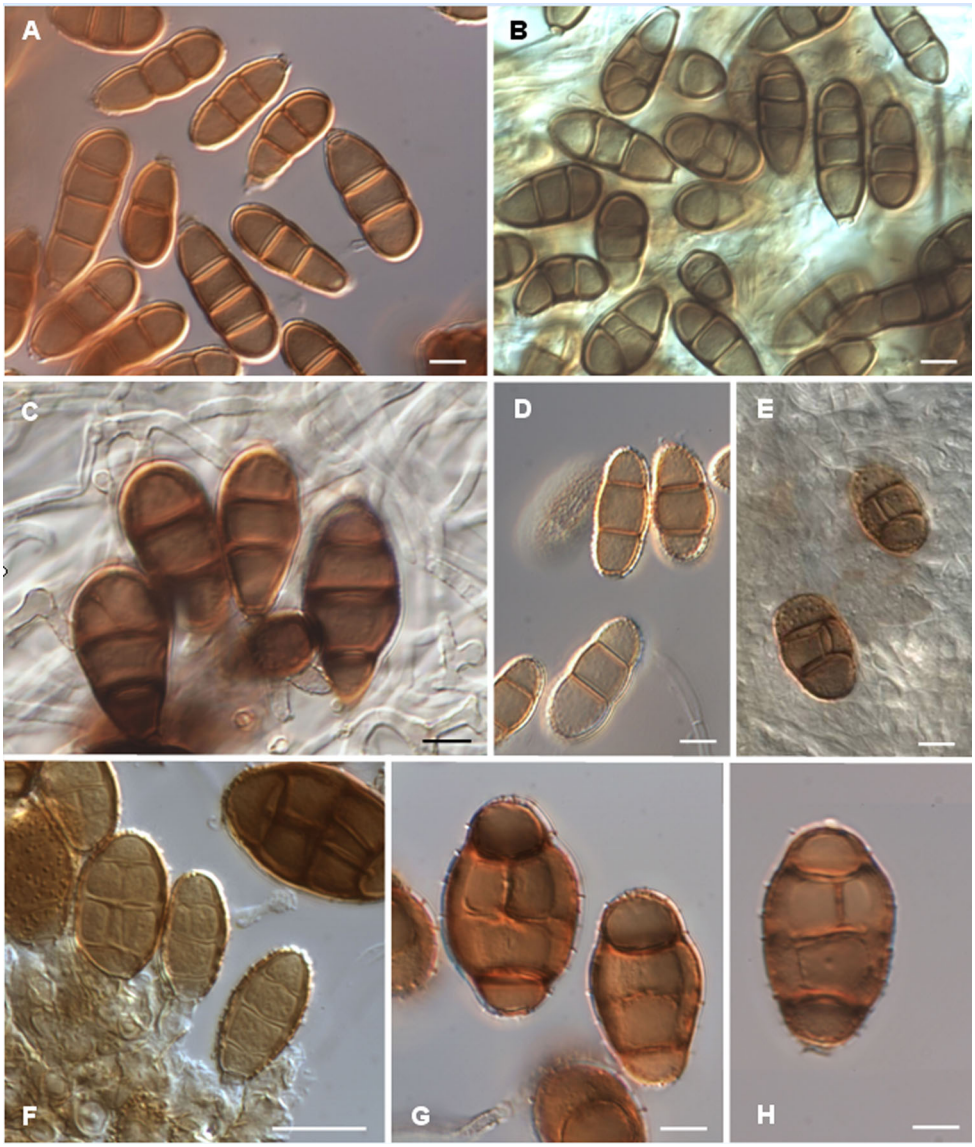

Figure 2. Conidia of *Pithomyces* spp. on oatmeal agar at 25°C after 14 days. (A, B) *Pithomyces sacchari* (A, UTHSC 07-1285; B. UTHSC 10-2143). (C) *Pithomyces* sp. I (UTHSC 06-3492). (D, E) *Pithomyces maydicus* (UTHSC 06-3954). (F–H) *Pithomyces chartarum* (F, UTHSC 05-2460; G, H, UTHSC 03-2472). Scale bar: A–E, G, H = 5 μm; F = 10 μm.

conidia of variable shape—clavate, navicular, pyriform, or turbinate—with one to six transverse septa and, occasionally, one to two oblique or longitudinal septa, verruculose to verrucose, measuring 11–36 × 5–15 μm (Fig. 2C).

The *P. maydicus* clade included two clinical isolates and one reference strain of the species and were 100% similar. The species was characterized by white to grayish yellow colonies and conidia with two to three transverse septa, rarely with one oblique or longitudinal septum, cylindrical or slightly clavate, pale brown, verrucose, and measuring 12–19 × 4–8 μm (Fig. 2D, E).

Finally, the *P. chartarum* clade (90% bootstrap support) included nine clinical isolates and three more sequences of that species, two of which corresponded to reference strains and one sequence retrieved from GenBank (UBC F15184). They showed a similarity of 99.5% to 100% and formed white to dark gray colonies and produced conidia that were usually broadly ellipsoidal, with three transverse septa often constricted, and one to two longitudinal septa at the middle cells, brown to mid brown, verruculose to echinulate, 12–33 × 9–19 μm (Fig. 2F–H).

Leptosphaerulina chartarum was described as the teleomorph of *P. chartarum* [13]. However, our phylogenetic analysis revealed that the ex-type strain of *L. chartarum* did not belong to the *P. chartarum* clade or to be related to

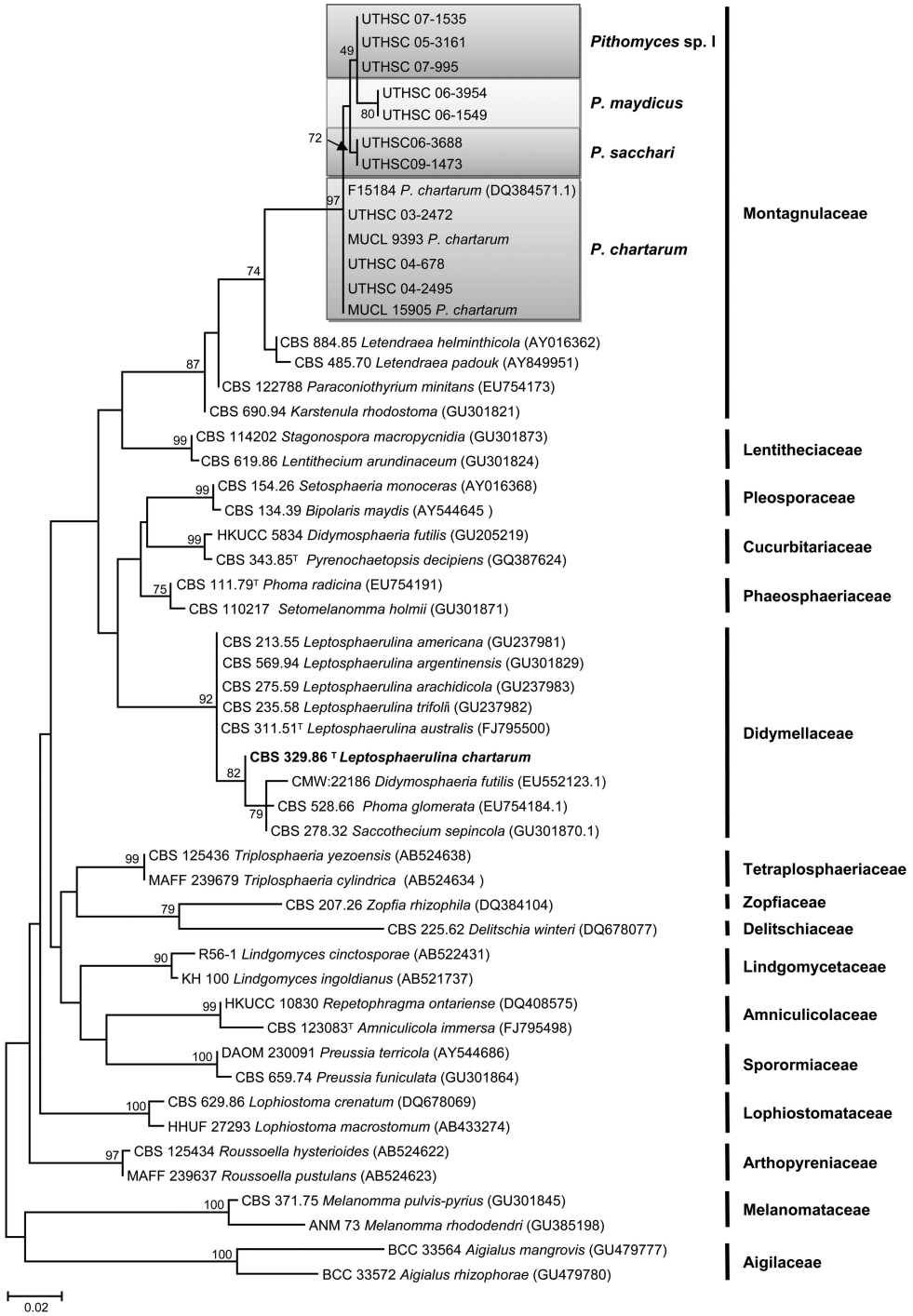

Figure 3. Maximum likelihood tree inferred from D1/D2 region of the 28S gene sequences of species of different Pleosporalean families and representatives of our clinical isolates listed in Table 1. Branch lengths are proportional to the distance.

any of the *Pithomyces* lineages (data not shown). An additional phylogenetic analysis of the D1/D2 domain sequences was carried out to determine affinities of this ex-type strain (Fig. 3). The alignment used in the analysis was 433 bp; analysis indicated that this strain of *L. chartarum* was related to members of the family Didymellaceae, as well as other species of the genus *Leptosphaerulina*. However, the *Pithomyces* species investigated were close to species of the genus *Letendraea*, which belong to the family Montagnulaceae.

The antifungal susceptibility test showed, in general, a high activity for the eight drugs tested against the isolates tested, with PSC, CAS, and TBF being the most potent agents (Table 2).

Table 2. Results of *in vitro* antifungal susceptibility testing for *Pithomyces* spp.

Species (n° of isolates)		MIC/MEC (μg/ml) 48 h							
		AMB	VRC	PSC	ITC	CFG	AFG	MFG	TRB
Pithomyces chartarum (14)	GM	0.06	0.12	0.06	0.06	0.04	0.1	0.1	0.06
	MIC range	<0.03–0.12	0.03–0.5	<0.03–0.12	<0.03–0.25	0.03–0.06	0.03–0.25	0.03–0.25	0.03–0.12
	MIC_{90}	0.05	0.12	0.12	0.12	0.06	0.12	0.12	0.06
Pithomyces sp. I (8)	GM	0.02	0.03	0.01	0.02	0.04	0.04	0.04	0.02
	MIC range	<0.03–0.03	<0.03–0.06	<0.03–0.03	<0.03–0.03	<0.03–0.03	0.03–0.12	0.03–0.06	<0.03–0.06
	MIC_{90}	—	—	—	—	—	—	—	—
Pithomyces sacchari (7)	GM	0.39	0.66	0.04	0.04	0.05	0.03	0.06	0.03
	MIC range	<0.03–2	<0.03–4	<0.03–0.12	<0.03–0.12	<0.03–0.12	0.03	0.03–0.12	0.03–0.06
	MIC_{90}	—	—	—	—	—	—	—	—
Pithomyces maydicus (2)	GM	0.04	0.04	<0.03	<0.03	0.03	0.03	0.03	<0.03
	MIC range	0.03–0.06	0.03–0.06	<0.03	<0.03	<0.03	0.03	0.03	<0.03
	MIC_{90}	—	—	—	—	—	—	—	—
Total (31)	GM	0.12	0.21	0.04	0.06	0.04	0.06	0.07	0.04
	MIC range	<0.03–2	<0.03–4	<0.03–0.12	<0.03–0.25	0.03–0.12	0.03–0.25	0.03–0.025	<0.03–0.12
	MIC_{90}	0.12	0.25	0.06	0.12	0.06	0.12	0.12	0.06

GM, geometric mean; AFG, anidulafungin; AMB, amphotericin B; CFG, caspofungin; ITC, itraconazole; VRC, voriconazole; MFG, micafungin; PSC, posaconazole; TRB, terbinafine.

Discussion

Species of *Pithomyces* have been rarely investigated as animal pathogens. Although some species have been isolated from mammals, including humans, and form lesions on the host, it is not known whether they are real pathogens. Their relevance as possible human pathogens has been rarely raised and never tested. However, since an increasing number of clinical isolates of *Pithomyces* have been received at the Fungus Testing Laboratory in recent years, it seems relevant to explore more closely their possible pathogenic role. This is the largest study of *Pithomyces* isolates from clinical samples.

In the majority of previous studies, *Pithomyces* identifications were based solely on morphological features, and only rarely were they identified to the species level [7,23–25]. A BLAST search based on ITS sequences is currently a limited tool for *Pithomyces* identification because there are only a few sequences deposited in GenBank and most of them belong to *P. chartarum* labeled under the name *Leptosphaerulina chartarum*.

In our study, four species were recognized, the most commonly identified being, in decreasing order, *P. chartarum, P. sacchari*, and *Pithomyces sp.* I, with *P. maydicus* represented by only two clinical isolates. In the literature, *P. chartarum* is the only species related to clinical cases [2,3,6,26]. *Pithomyces maydicus* and *P. sacchari* have been reported only as plant saprobes [1,15]. Nevertheless, 12 clinical isolates, labeled as *Pithomyces* sp. I, could not be identified as any known species within the genus. These isolates were highly variable in terms of the shape and number of septa in their conidia, with no other known *Pithomyces* species showing such morphological variation. Therefore, our results suggest that *Pithomyces* sp. I could represent a new species. To ascertain this, an extensive taxonomical study should be carried out with more reference and ex-type strains of the different species described. However, as mentioned previously, *Pithomyces* comprises close to 50 species and, unfortunately, only a few ex-type or reference strains exist. In addition, the type of *P. flavus*, the type species of the genus, is not available. This is an important handicap to clarifying the taxonomy of this genus.

Pithomyces belongs to the Pleosporales, a large order that includes numerous plant and animal pathogens. Species of Pleosporales are frequently characterized by dark brown, often septate (phragmo- or dictyoseptate) conidia. Well-known genera within the order include *Alternaria, Curvularia, Exserohilum*, and *Stemphylium* [27]; morphologically, they differ from *Pithomyces* in their conidiogenesis method, which is mono- or polytretic, and in the conidial secession, which is schizolytic in the above-mentioned genera. *Pithomyces* has monoblastic conidia with

rhexolytic secession. As mentioned previously, since the teleomorph of *P. chartarum* was identified as a member of *Leptosphaerulina* [13], *Pithomyces* was considered an anamorphic genus of the family Didymellaceae [14,20]. However, in our study, none of the *Pithomyces* isolates, including those received from different culture collections and the sequences of *L. chartarum* retrieved from GenBank, were grouped with *Leptosphaerulina* species or other members of Didymellaceae. According to our D1/D2 analysis, all the *Pithomyces* isolates studied, even those identified as *P. chartarum*, grouped with members of the family Montagnulaceae (Fig. 3). To confirm that *L. chartarum* was related to that family, we sequenced the ex-type of *L. chartarum* (CBS 329.86); surprisingly, its sequence fell with other *Leptosphaerulina* species in the Didymellaceae clade (Fig. 3). We morphologically examined the ex-type strain of *L. chartarum* and found that it developed ascomata and ascospores, which matched with the protologue description [13] but did not produce the anamorph in any of the culture media and incubation temperatures tested. Considering that the morphology of ascospores of *L. chartarum* and conidia of *P. chartarum* are very similar, some confusion in the interpretation of such structures could occur during the study of the former fungus. What is clear is that our data suggest that *L. chartarum* is not the sexual stage of *P. chartarum*; however, a more extensive study with more strains of both taxa should be done to clarify this issue.

In our study, the *Pithomyces* isolates originated mainly from superficial tissue, the respiratory tract and nasal region, and anatomic sites where other melanized anamorphic fungi such as *Curvularia* or *Exserohilum* are also commonly found [28]. The potent *in vitro* activity of most of the antifungal drugs tested against *Pithomyces* species was remarkable, which could offer different therapeutic options for the treatment of infections caused by these fungi.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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