Acrophialophora a poorly known fungus with clinical significance

RUNNING TITLE: *Acrophialophora* species in clinical specimens

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ABSTRACT

Acrophialophora fusispora is an opportunistic emerging fungus causing human infections. Because the taxonomy of the genus is not yet resolved, and in order to facilitate identification of clinical specimens, we have studied morphologically and molecularly a set of clinical and environmental *Acrophialophora* isolates comprising the available type strains of *Acrophialophora* species and similar fungi, some considered by various authors synonyms of *A. fusispora*. The analysis of the sequences of the large-subunit (LSU) and the internal transcribed spacer (ITS) regions of the nuclear rDNA, and a fragment of the β-tubulin gene (*Tub*) revealed that *Acrophialophora* belongs to the family *Chaetomiaceae* and comprises three different species, i.e., *A. fusispora*, *A. levis* and *A. seudatica*, the latter previously included in the genus *Ampullifera.* The most prevalent species among clinical isolates was *A. levis* (72.7%), followed by *A. fusispora* (27.3%), both species mostly isolated from respiratory (72.7%), subcutaneous and corneal tissues samples. In general, the eight antifungal drugs tested, with the exception of voriconazole, showed low in vitro activity against these fungi.

INTRODUCTION

Acrophialophora is a thermotolerant soil fungus widely distributed in temperate and tropical regions. Given its capacity to produce high quantities of cellulases and xylanases, it is also commonly isolated as decomposer of compost and other self-heating substrates (1, 2).

The genus *Acrophialophora* was erected by Edward (3) with a single species, *Acrophialophora nainiana.* This fungus forms grayish colonies with a black reverse with age, microscopically it produces darkly pigmented, straight, septate and unbranched setae-like conidiophores with thick and verrucose walls, which are fertile towards the apex and, flask-shaped, hyaline phialides grouped in verticills. Single flask-shaped phialides are also formed directly from the aerial hyphae. However, *Acrophialophora* was not fully accepted as a distinct genus until the work of Samson & Mahmood (4), who, after studying a large set of isolates, demonstrated that the mentioned morphological features were stable, thereby supporting the differentiation of *Acrophialophora* from morphologically similar genera such as *Paecilomyces* or *Masonia*. These authors accepted three species based mainly on the size, pigmentation and ornamentation of their conidia. These were, *A. nainiana* (4–10.5 x 2–5 µm, hyaline, and finely echinulate), *A. fusispora* (5–12 x 3–6 µm, brown, finely echinulate forming spiral bands), which had been described earlier as *Paecilomyces fusisporus* (4), and *A. levis* (4.5–8 x 2–3.5 µm, hyaline, and smooth to slightly roughened). However, while Ellis (5) regarded *A. nainiana* conspecific with *A. fusispora* and the latter as the type species of the genus, Al-Mohsen et al (1) considered the three species synonyms and conserved the single species name *A. fusispora.* In this wide concept of the species, other taxa were considered conspecific with *A. fusispora*, i.e. *Masoniella indica* and *Ampullifera seudatica* (4).

Acrophialophora fusispora is currently reported an emerging human opportunistic pathogen (6, 7), responsible of cases of keratitis (6, 8, 9), pulmonary

colonization and infection (6, 10-12), and devastating cerebral infections requiring intensive antifungal therapy (1, 13-15). Antifungal susceptibility data for *Acrophialophora* is scarce and based mostly on a few clinical reports (1, 15).

The species delimitation on *Acrophialophora,* using a modern phylogenetic approach, has not been properly revised, and the taxonomic position and boundaries of the genus are unknown. Therefore, we have carried out a phenotypic and molecular study of a set of clinical and environmental isolates, including all the available type strains of the species historically included in the genus. In addition, the antifungal susceptibility of these isolates was determined against eight antifungal agents.

MATERIALS AND METHODS

Fungal isolates and sequences. A total of 39 isolates were included in this study; 32 from human clinical samples, 1 from an animal clinical sample, and 6 from environmental sources, including all the available type strains of the genus (Table 1). Most of the clinical isolates were from the United States, received in the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio (UTHSCSA) from different parts of the country. In addition, 39 sequences retrieved from GenBank or NBRC were also included in the phylogenetic analyses.

Phenotypic studies. The isolates were grown on Malt Extract Agar (MEA; 30 g of malt extract, 5 g of peptone, 15 g of agar, 1 l of distilled water) and Oatmeal Agar (OA; 30 g of filtered oat flakes, 20 g of agar, 1 l of distilled water). Colonial

features and growth rates were determined at 7 and 14 d of incubation at different temperatures (5, 15, 25, 35, 37, 40, 45, 50 and 52ºC). The microscopic features were examined on both media after 14 d of incubation at 25ºC in wet mounts with 85% lactic acid using light microscopy. All the isolates were identified based on Edward (3), Samson & Mahmood (4) and Ellis (5). Photomicrographs were obtained with a Zeiss Axio-Imager M1 light microscope, using phase contrast and Nomarski differential interference optics.

DNA extraction, amplification and sequencing. FastPrep (MP Biomedicals, Santa Ana, California, USA) was used to extract total genomic DNA from fungal mycelia harvested from colonies growing on PDA agar for 7d at 25ºC, following the manufacturer's protocol. DNA was quantified using a Nanodrop 3000 apparatus (Thermo Scientific, Madrid, Spain).

Three nuclear DNA targets were amplified by PCR and sequenced using the following primer pairs: ITS4/ITS5 (16) for a region spanning the internal transcribed spacers (ITS) 1 and 2 and the 5.8S gene of the rDNA, LR0R/LR5 (17, 18) for a fraction of the 5' end of the large-subunit (LSU) gene of the rDNA, and BT2a/BT2b (19) for a fragment of the β-tubulin gene (*Tub*). The amplified products were purified with the Diffinity RapidTip purification system (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20°C until sequencing.

Sequencing was made in both directions with the same primer pair used for amplification at Macrogen Europe (Macrogen Inc., Amsterdam, The Netherlands). The consensus sequences were obtained using the SeqMan software version 7.0.0 (DNAStar Lasergene, Madison, WI, USA). Sequences newly generated in

this study were deposited in GenBank under the accession numbers KM995840-KM995877 and LN736031 (LSU), KM995878-KM995915 and LN736030 (ITS) and LN624419-LN624456 and LN736032 (*Tub*) (Table 1).

Molecular identification and phylogenetic analysis. In order to assess the taxonomic position of the genus *Acrophialophora*, a first phylogenetic analysis was carried out using partial LSU sequences of the available type strains of *Acrophialophora* species complemented with 15 sequences retrieved from public databases, selected on the basis of BLAST homology searches and representing 8 different genera from the families *Chaetomiaceae* and *Sordariaceae*, of the subclass *Sordariomycetidae*.

A second phylogenetic analysis directed to assess the species distribution on *Acrophialophora* was conducted using partial LSU, ITS and *Tub* sequences and included all the type strains of *Acrophialophora* species, the type strains of the putative synonyms *Ampullifera seudatica* and *M. indica,* and several clinical and environmental isolates morphologically identified as *Acrophialophora* spp. Multiple sequence alignments were made for each individual locus using MEGA version 6.06 (20), with the ClustalW function and manually refined when necessary. The nucleotide substitution models for each dataset (GTR+G+I for LSU, JC+G for ITS and T92+G for *Tub*) were calculated using the Find best DNA/protein model tool on Mega 6.06. In order to compare the concordance of the different loci, individual phylogenetic analyses were first carried out using the Maximum likelihood algorithm under MEGA and the resulting trees were compared visually using a 70% bootstrap cutoff and complemented with the Partition Homogeneity Test

carried out as implemented in PAUP* (Phylogenetic Analysis Using Parsimony, v. 4.0b10) (21). Since no incongruence was found (p=0.180), the three genes were combined into a single dataset. The combined phylogenetic analyses were made using Maximum-Likelihood (ML) and Bayesian Inference (BI) under MEGA and MrBayes version 3.1.2 (22), respectively. For the ML analysis, nearest-neighbor interchange (NNI) was used as heuristic method for tree inference. Support for the internal branches was assessed by a search of 1,000 bootstrapped sets of data. A bootstrap support (bs) of ≥70 was considered significant. For BI analysis, two simultaneous runs of 3,000,000 generations were performed and samples were stored every 100 generations. The 50% majority-rule consensus tree and posterior probability values (pp) were calculated after discarding the first 25% of the samples. A pp value ≥0.95 was considered significant.

Antifungal susceptibility testing. Antifungal susceptibility testing was performed following the protocols of the CLSI document M38-A2 (23). The antifungal drugs tested were: amphotericin B (AMB), voriconazole (VRC), itraconazole (ITC), posaconazole (PSC), terbinafine (TRB), anidulafungin (AFG), caspofungin (CFG) and micafungin (MFG). 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. Geometric mean (GM) MICs were compared using the Mann-Whitney test under GraphPad Prism version 6 for Windows (GraphPad Software, La Jolla California, USA).

RESULTS

Figure 1 shows the results of the analysis of the LSU sequences (431 base pairs [bp]) of *Acrophialophora* species and related fungi. The type strain of *A. nainiana* (CBS 100.60) clustered with the type strains of *A. levis* (CBS 484.70), and *A. fusispora* (CBS 380.55), being included in a well-supported clade (bs 85%, pp 0.95) containing several members of the family *Chaetomiaceae* of the order *Sordariales*. The closest species to *Acrophialophora* were members of *Achaetomium*, *Botryotrichum*, *Chaetomidium* and *Chaetomium*. The latter genus was also found to be polyphyletic.

Figure 2 shows the result of the phylogenetic analysis of the species of the genus *Acrophialophora,* using concatenated LSU, ITS and *Tub* sequences. The final alignment consisted of 1804 bp (LSU 843 bp, ITS 502 bp, and *Tub* 459 bp) of 41 isolates, i.e, one from an environmental source, 33 from clinical origin and the type strains of *A. fusispora, A. levis, A. nainiana,* the putative synonyms *Ampullifera seudatica* and *M. indica*. *Chaetomium globosum* and *Chaetomium angustispirale* were used to root the tree. The tree showed two fully-supported main clades, one which included the type strain of *A. levis* and the other that of *A. fusispora.* The latter clustered in a fully-supported clade with the type strains of *A. nainiana* and *M. indica*, which demonstrated them to be conspecific since their sequences were practically identical. The type strain of *Ampullifera seudatica* formed a single lineage, basal and distant to the *A. fusispora* clade (98.5%

183 sequence similarity with *A. fusispora* in the combined analysis), and is here
184 considered as a different species of the genus named *Acrophialophora seudatica.*

185 The isolates identified as *A. fusispora* and *A. levis* showed similar
186 macroscopic features on all media tested. The colonies on MEA (Fig. 3: a, h)
187 ranged from 30 to 60 mm in 14 d at 25ºC, and were flat to slightly umbonate, at
188 first white soon becoming pale yellow to brownish grey, velvety to felty, with
189 irregular margins and a yellow, brown or black reverse. *Acrophialophora seudatica*
190 grew rather slower (20 to 25 mm in 14 d) and its colonies were flat, at first white
191 rapidly turning to pale orange, velvety, with pale orange reverse (Fig 3: p). The
192 optimal temperature for growth was between 35 and 40ºC, with a minimum of 15ºC
193 and a maximum of 50ºC for all the species. Microscopically, the isolates of *A.*
194 *fusispora* were characterized by the abundant production of flask-shaped phialides
195 and polyphialides measuring 5–19 x 1.5–5 µm (Fig 3: d, e), swollen at the base
196 and tapering abruptly to a narrow neck, mostly formed directly from the aerial
197 hyphae or in the apex of well differentiated conidiophores, which were erect,
198 unbranched, dark-coloured and with a spiny to warted wall surface (Fig 3: c).
199 Conidia were produced on basipetal chains and were one-celled, subhyaline to
200 brownish, ovoid to fusiform, finely echinulate or forming spiral bands and
201 measuring 5–12 x 2–5 µm, (Fig 3: d-g) The isolates of *A. levis* also produced
202 abundant flask-shaped phialides, frequently polyphialides, measuring 4–13 x 1.5–5
203 µm (Fig 3: i, m), and hyaline to subhyaline ellipsoidal to cylindrical conidia, smooth
204 to finely echinulate and measuring 4–9 x 2–6 µm (Fig 3: m-o). The isolate of *A.*
205 *seudatica* exhibited flask-shaped phialides measuring 8–22 x 2.5–4.5 µm, with long

206 necks (Fig 3: r, s) and ovoid to fusiform conidia measuring 6–8 x 3–4 µm (Fig 3: t,
207 u), with thick and finely verruculose walls, subhyaline or turning pale yellow when
208 mature. This isolate was unable to produce the typical pigmented conidiophores of
209 *Acrophialophora*. Table 2 summarizes the key morphological features that
210 distinguish the three *Acrophialophora* species.

211 The majority of isolates from clinical sources belonged to *A. levis* (72.7%),
212 while *A. fusispora* account for the remaining 34.3% of the isolates. The main
213 source of isolation was the respiratory tract (72.7%), mostly from sputum and
214 bronchoalveolar lavage specimens, followed by subcutaneous tissues (9.1%),
215 brain and cornea (6.1% each), and other localizations such as sphenoid sinus and
216 a chest mass (3% each). No major differences regarding the origin of isolation
217 were observed between *A. levis* and *A. fusispora*.

218 The antifungal susceptibility of the isolates belonging to *A. levis* and *A.*
219 *fusispora* is shown in Table 3. Overall, the highest MIC values were observed for
220 AMB with a geometric mean (GM) MIC and MIC_{90} of 5.66 µg/ml and 16 µg/ml,
221 respectively. The azole drugs exhibited the best *in vitro* activity, VRC being the
222 most active, with an overall GM MIC and MIC_{90} of 0.17 µg/ml and 0.25 µg/ml,
223 respectively; followed by PSC and ITC. The echinocandins exhibited poor *in vitro*
224 activity, with AFG showing the lowest GM MIC and MIC_{90} (1.86 µg/ml and 4 µg/ml,
225 respectively). TRB showed a GM MIC and MIC_{90} of 0.51 µg/ml and 1 µg/ml,
226 respectively. Although the differences were subtle, the MICs for VRC, ITC, CSP,
227 AFG, MFG, and TBF were significantly lower against *A. levis* than *A. fusispora*
228 ($p>0.0001$).

TAXONOMY

According to the results of our phylogenetic and morphological analyses, the following new combination is proposed: *Acrophialophora seudatica* (Subrahm.) Sandoval-Denis, Gené & Guarro *comb. nov*. MB811225. Basionym: *Ampullifera seudatica* Subrahmanyam, Nova Hedwigia 31: 159 (1979).

DISCUSSION

To our knowledge, this is the first study involving the molecular assessment of the fungal genus *Acrophialophora*, a rare opportunistic human and animal pathogen. It also includes the largest set of clinical isolates of the species studied to date. The taxonomy of the genus has been revised and the spectrum of species associated with human disease determined.

According to our results the genus *Acrophialophora,* belonging to the sordariomycetous family *Chaetomiaceae,* comprises three species, *A. fusispora, A. levis* and *A. seudatica*. This family groups mostly soil-borne cellulose decomposers, but also thermotolerant opportunistic pathogens including neurotropic species such as *Achaetomium strumarium* and *Chaetomium atrobrunneum* (24, 25). Although historically, the family *Chaetomiaceae* encompassed mainly essentially ascosporulating fungi, *Acrophialophora* is not the first genus of the family showing a strictly asexual reproduction. Recently, de Hoog et al (25) demonstrated that agents of black-grain mycetoma such as *Madurella* species, which fail to produce fertile sexual morphs, also belong to the

Chaetomiaceae. According to our LSU phylogeny, members of the genera *Acrophialophora, Chaetomidium* and *Thielavia* nested unambiguously in highly supported terminal clades, while the position of the genera *Achaetomium*, *Botryotrichum*, *Chaetomium* and *Madurella* is unclear, the genus *Chaetomium* forming two paraphyletic clades. The classification of latter genus however has been showed to differ significantly when molecular and conventional approaches are compared (25)

As the morphological features used to distinguish the three *Acrophialophora* species recognized by Samson & Mahmood (4) tended to overlap, Al-Mohsen et al (1) considered them morphological variations from a single species*, A. fusispora*. However, our phylogenetic analysis of the different type strains does not support this conclusion. Our results confirm that *A. fusispora*, *A. nainiana* and *M. indica* are conspecific*,* while *A. levis* and *A. seudatica* are two different species. In contrast to the observations of Al-Mohsen et al (1), our phylogenetic results support that subtle morphological evidence in these fungi, such as conidial size, shape, colour and ornamentation are consistent differences for distinguishing *A. fusispora, A. levis* and *A. seudatica* (Table 2). The presence, mainly on OA media, of erect pigmented conidiophores is typical of cultures of the two clinically relevant species of *Acrophialophora*, i.e. *A. fusispora* and *A. levis,* and can be important for the initial generic diagnosis. However, these pigmented conidiophores are absent in *A. seudatica*. This species was originally described with simple, hyaline and straight conidiophores (26), a feature that is also confirmed here. However, *A. seudatica* is only known from its type specimen isolated from a soil sample in India. Therefore,

275 confirmation of presence/absence of the typical conidiophores of *Acrophialophora*
276 will be only possible studying more isolates of this rare species.

277 The three species of *Acrophialophora* shared very similar LSU sequences
278 (99.9%) but the big differences in their ITS and Tub sequences (<96.1% and
279 <96.6% sequence similarity, respectively) show that both loci can discriminate the
280 three species, making them good candidates for barcoding targets in
281 *Acrophialophora*.

282 Some authors stated that *Acrophialophora* infections may have been
283 underdiagnosed due to the rarity of these fungi and the potential confusion with
284 similar opportunistic moulds such as *Lomentospora prolificans* and *Scopulariopsis*
285 *chartarum* (9, 13, 27, 28). Only six well-documented cases of human infection exist
286 in the literature, most of which lacks molecular confirmation of the etiologic agent.
287 Only in one case was the fungus confirmed as *A. fusispora* by sequencing the ITS
288 region (15). This also responds to the scarcity of reference sequences for
289 comparison in fungal databases which do not include any type or correctly
290 identified reference strains.

291 Since the synonymy of the species of *Acrophialophora* was formally
292 proposed by Al-Mohsen et al (1), *A. fusispora* has remained the only accepted
293 species of the genus and, as such, has been cited as the causative agent in the
294 different reported clinical cases. (1, 7, 14, 24). However, according to our results *A.*
295 *levis* seems to be the most common species isolated from human clinical samples.
296 The identification of some of the isolates included in the study of Guarro et al (6)
297 was reassessed here by sequence comparison. One clinical isolate (FMR 8888,

298 from corneal infection) was confirmed as *A. fusispora*, while another one (FMR
299 6662, isolated from sputum) was reidentified here as *A. levis*. The third clinical
300 isolate included in that study (FMR 6404) was not available for analysis and thus
301 its final identification remains unknown.

302 Most published cases refer to pulmonary involvement with or without
303 systemic dissemination (6, 10-12). Similarly, the majority of our clinical isolates
304 were obtained from respiratory specimens, half of them being from BAL samples;
305 however, it was not possible to distinguish between true infectious agents,
306 colonizers or merely environmental contaminants given the nature of the samples
307 and the absence of appropriate clinical or histopathological data. The second most
308 common infection in clinical reports is keratitis (6, 9), while that in our study it was
309 soft tissue, particularly leg tissue, corneal and cerebral samples being the third
310 most common sites of isolation in equal proportion. The lack of isolates from CNS
311 does not allow us to confirm the potential neurotropism attributed to
312 *Acrophialophora* (14).

313 The antifungal treatment of *Acrophialophora* infections has been hampered
314 by the paucity of *in-vitro* susceptibility data and the lack of specific treatment
315 guidelines. The clinical cases have reported variable results. Arthur et al (9)
316 reported a favourable outcome with the use of AMB and surgical debridement in a
317 case of keratouveitis. In a pulmonary infection, a monotherapy with liposomal AMB
318 (LAMB) was not effective, the patient recovering with a combined therapy including
319 LAMB and ITC (1). Guarro et al (6) reported the use of VRC in two clinical cases, a
320 case of keratitis that responded favourably to the drug and another of pulmonary

infection with a fatal outcome. In addition, Li et al (15) described a negative outcome using VRC in a case of cerebral infection. The two latter cases, however, were in highly compromised patients with systemic involvement. Our susceptibility results showed that while AMB and the echinocandins have almost no activity against *Acrophialophora* species, VRC exhibits *in-vitro* activity. This confirms the observations of Guarro et al (6), suggesting VRC as a potential treatment for *Acrophialophora* infections.

In conclusion, *Acrophialophora* includes three closely related species *A. fusispora*, *A. levis* and *A. seudatica*, that can be accurately identified on the basis of ITS or *Tub* sequencing and a detailed morphological study. *Acrophialophora levis* appears to be the most frequent species in clinical samples. VRC shows *in vitro* activity against these fungi.

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425 **Table 1** Origin and GenBank accession numbers of the sequences of the *Acrophialophora* spp. included in this study

Species	Strain number	Origin	Genbank accession number		
			LSU	ITS	*Tub*
A. levis	CBS 484.70 (ex-type)	Composted domestic waste	KM995840	KM995878	LN624419
	FMR 6662 = CBS 120407	Spain, Sputum	KM995841	KM995879	LN624420
	FMR 12780	Spain, Sputum	KM995842	KM995880	LN624421
	UTHSCSA DI-13-134	USA, Sputum	KM995843	KM995881	LN624422
	UTHSCSA DI-13-137	USA, Sputum	KM995844	KM995882	LN624423
	UTHSCSA DI-13-138	USA, Bronchoalveolar wash fluid	KM995845	KM995883	LN624424
	UTHSCSA DI-13-139	USA, Bronchoalveolar wash fluid	KM995846	KM995884	LN624425
	UTHSCSA DI-13-142	USA, Sputum	KM995847	KM995885	LN624426
	UTHSCSA DI-13-144	USA, Sputum	KM995848	KM995886	LN624427
	UTHSCSA DI-13-145	USA, Brain	KM995849	KM995887	LN624428
	UTHSCSA DI-13-146	USA, Bronchoalveolar wash fluid	KM995850	KM995888	LN624429
	UTHSCSA DI-13-147	USA, Bronchoalveolar wash fluid (canine)	KM995851	KM995889	LN624430
	UTHSCSA DI-13-148	USA, Lung, right upper lobe	KM995852	KM995890	LN624431
	UTHSCSA DI-13-150	USA, Sputum	KM995853	KM995891	LN624432
	UTHSCSA DI-13-151	USA, Sputum	KM995854	KM995892	LN624433
	UTHSCSA DI-13-152	USA, Leg tissue	KM995855	KM995893	LN624434
	UTHSCSA DI-13-153	USA, Tissue	KM995856	KM995894	LN624435
	UTHSCSA DI-13-154	USA, Bronchoalveolar wash fluid	KM995857	KM995895	LN624436
	UTHSCSA DI-13-155	USA, Sputum	KM995858	KM995896	LN624437
	UTHSCSA DI-13-156	USA, Knee tissue	KM995859	KM995897	LN624438
	UTHSCSA DI-13-157	USA, Bronchoalveolar wash fluid	KM995860	KM995898	LN624439
	UTHSCSA DI-13-158	USA, Sputum	KM995861	KM995899	LN624440
	UTHSCSA DI-13-159	USA, Bronchoalveolar wash fluid	KM995862	KM995900	LN624441
	UTHSCSA DI-13-162	USA, Bronchoalveolar wash fluid	KM995863	KM995901	LN624442

	UTHSCSA DI-13-163	USA, Bronchoalveolar wash fluid	KM995864	KM995902	LN624443
A. fusispora	CBS 100.60 (*A. nainiana* ex-type)	Farm soil	KM995865	KM995903	LN624444
	CBS 149.64 (*M. indica* ex-type)	Forest soil	KM995866	KM995904	LN624445
	CBS 380.55 (*P. fusisporus* ex-type)	Forest soil	KM995867	KM995905	LN624446
	FMR 6258 = CBS 120406	India, Soil	KM995868	KM995906	LN624447
	FMR 8888 = CBS 120409	India, Corneal	KM995869	KM995907	LN624448
	UTHSCSA DI-13-135	USA, Left sphenoid sinus	KM995870	KM995908	LN624449
	UTHSCSA DI-13-136	USA, Brain abscess	KM995871	KM995909	LN624450
	UTHSCSA DI-13-140	USA, Bronchoalveolar wash fluid	KM995872	KM995910	LN624451
	UTHSCSA DI-13-141	USA, Sputum	KM995873	KM995911	LN624452
	UTHSCSA DI-13-143	USA, Chest mass	KM995874	KM995912	LN624453
	UTHSCSA DI-13-149	USA, Corneal	KM995875	KM995913	LN624454
	UTHSCSA DI-13-160	USA, Bronchoalveolar wash fluid	KM995876	KM995914	LN624455
	UTHSCSA DI-13-161	USA, Sputum	KM995877	KM995915	LN624456
A. seudatica	CBS 916.79 (*Ampullifera seudatica* ex-type)	India, Soil	LN736031	LN736030	LN736032

CBS-KNAW, Fungal Biodiversity Centre culture collection, Utrecht, The Netherlands; FMR, Facultat de Medicina, Universitat Rovira i Virgili, Reus, Spain; UTHSCSA, Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio, San Antonio, USA.

429 **Table 2.** Key differential features of the three species of *Acrophialophora.*

Characters*	**Species**		
	A. fusispora	***A. levis***	***A. seudatica***
Colony diameter (mm)	30–50	35–60	20–25
Colony colour	White, pale yellow or grey	White, pale yellow or grey	White to pale orange
Colony texture	Velvety to felty	Velvety to felty	Velvety
Phialides	5–19 x 1.5–5 µm	4–13 x 1.5–5 µm	8–22 x 2.5–4.5 µm
Conidia	5–12 x 2–5 µm	4–9 x 2–6 µm	6–8 x 3–4 µm
Conidial shape	Ovoid to fusiform	Ellipsoidal to cylindrical	Ovoid to fusiform
Conidial ornamentation	Finely echinulate to spiral sculpted	Smooth to finely echinulate	Finely verruculose
Conidial color	Subhyaline to brown	Hyaline to subhyaline	Subhyaline to pale yellow

430 *Characters obtained on MEA at 25ºC after 14d.

431

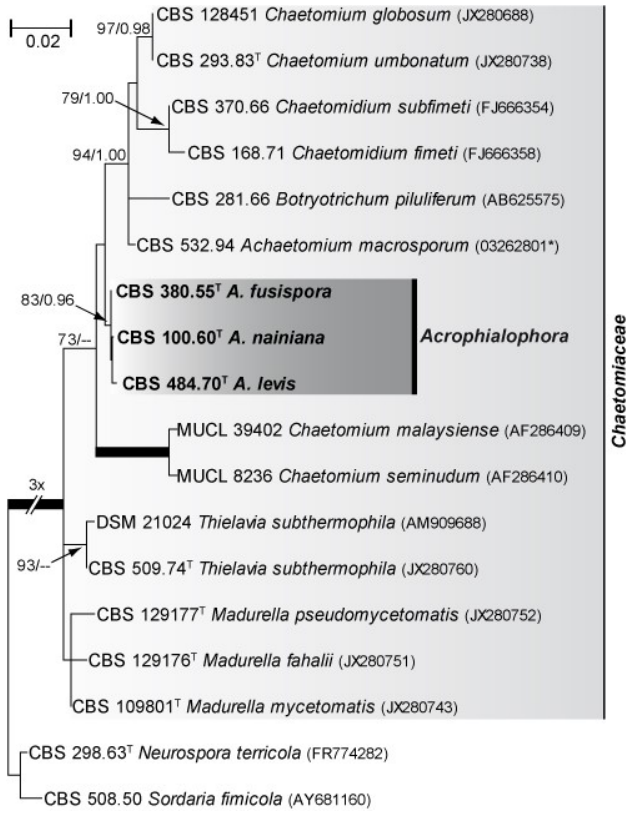
Table 3. Results of *in vitro* antifungal susceptibility testing of the 33 clinical isolates of *Acrophialophora* spp. included in the study.

Species (No. of isolates tested)		MIC or MEC (µg/ml)							
		AMB	**VRC**	**PSC**	**ITC**	**CFG**	**AFG**	**MFG**	**TRB**
A. levis	**GM**	6.77	0.16	0.50	1.15	3.58	1.65	2.64	0.42
(24)	**RANGE**	1-32	0.06-05	0.25-1	0.5-4	0.25-32	0.25-8	0.25-32	0.125-4
	MIC_{90}	32	0.25	1	2	16	4	32	1
A. fusispora	**GM**	4.22	0.18	0.50	0.85	11.02	2.61	6.46	0.75
(9)	**RANGE**	2-32	0.125-0.25	0.25-1	0.125-1	4-32	2-8	0.125-32	0.5-1
	MIC_{90}	16	0.25	1	1	16	4	32	1
Overall	**GM**	5.66	0.17	0.49	1	4.98	1.86	3.21	0.51
(33)	**RANGE**	1-32	0.06-05	0.25-1	0.125-4	0.25-32	0.25-8	0.125-32	0.125-4
	MIC_{90}	16	0.25	1	1	16	4	32	1

AMB, Amphotericin B; VRC, Voriconazole; PSC, Posaconazole; ITC, Itraconazole; CFG, Caspofungin; AFG, Anidulafungin; MCF, Micafungin; TRB, Terbinafine. MIC_{90}, drug concentration that inhibited 90% of the isolates tested.

436 **Figure 1** Maximum-Likelihood (ML) tree constructed with partial LSU sequences
437 (431bp) of *Acrophialophora* spp. and members of the *Chaetomiaceae*. Branch
438 lenghts are proportional to the phylogenetic distance. ML bs/Bayesian pp values
439 over 70% and 0.95, respectively, are shown on the nodes. Thickened branches
440 indicate full statistical support. GenBank accesion numbers are shown between
441 parenthesis; *, sequence retrieved from the NBRC (NITE, Biological Resource
442 Center, Chiba, Japan). The tree is rooted with *Neurospora terricola* and *Sordaria*
443 *fimicola.* ^{T}, type strain; CBS-KNAW, Fungal Biodiversity Centre culture collection,
444 Utrecht, The Netherlands; DSM, Libniz Institute DSMZ-German Collection of
445 Microorganisms and Cell Cultures, Braunschweig, Germany; MUCL, Mycothèque
446 de l´Université Catholique de Louvain, Louvain-la-Neuve, Belgium.
447
448 **Figure 2** Maximum likelihood (ML) tree constructed with combined LSU (843 bp),
449 ITS (502 bp) and *Tub* (459 bp) sequences of *Acrophialophora* clinical and
450 environmental isolates. ML bs/Bayesian pp values, respectively are shown on the
451 nodes. Thickened branches indicate full statistical support. The tree is rooted with
452 *Chaetomium globosum* and *Chaetomium angustispirale.* GenBank accesion
453 numbers for LSU, ITS and *Tub* are shown between parenthesis. ^{T}, type strain;
454 CBS-KNAW, Fungal Biodiversity Centre culture collection, Utrecht, The
455 Netherlands; FMR, Facultat de Medicina i Ciències de la Salut, Reus, Spain;
456 UTHSCSA, Fungus Testing Laboratory of the University of Texas Health Science
457 Center at San Antonio, San Antonio, USA.
458

459 **Figure 3** Key morphological features of *Acrophialophora fusispora* (a-g), *A. levis*
460 (h-o) and *A. seudatica* (p-u). Colonies on MEA after 14d at 25 °C (a, h, p). Colonies
461 on OA after 14d at 25 °C (b, i, q). Conidiophores (c, j). (d-g) Phialides and conidia
462 (f, g, n, o, t, u). – Scale bars: white = 10 µm, black = 5 µm.
463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97/0.98
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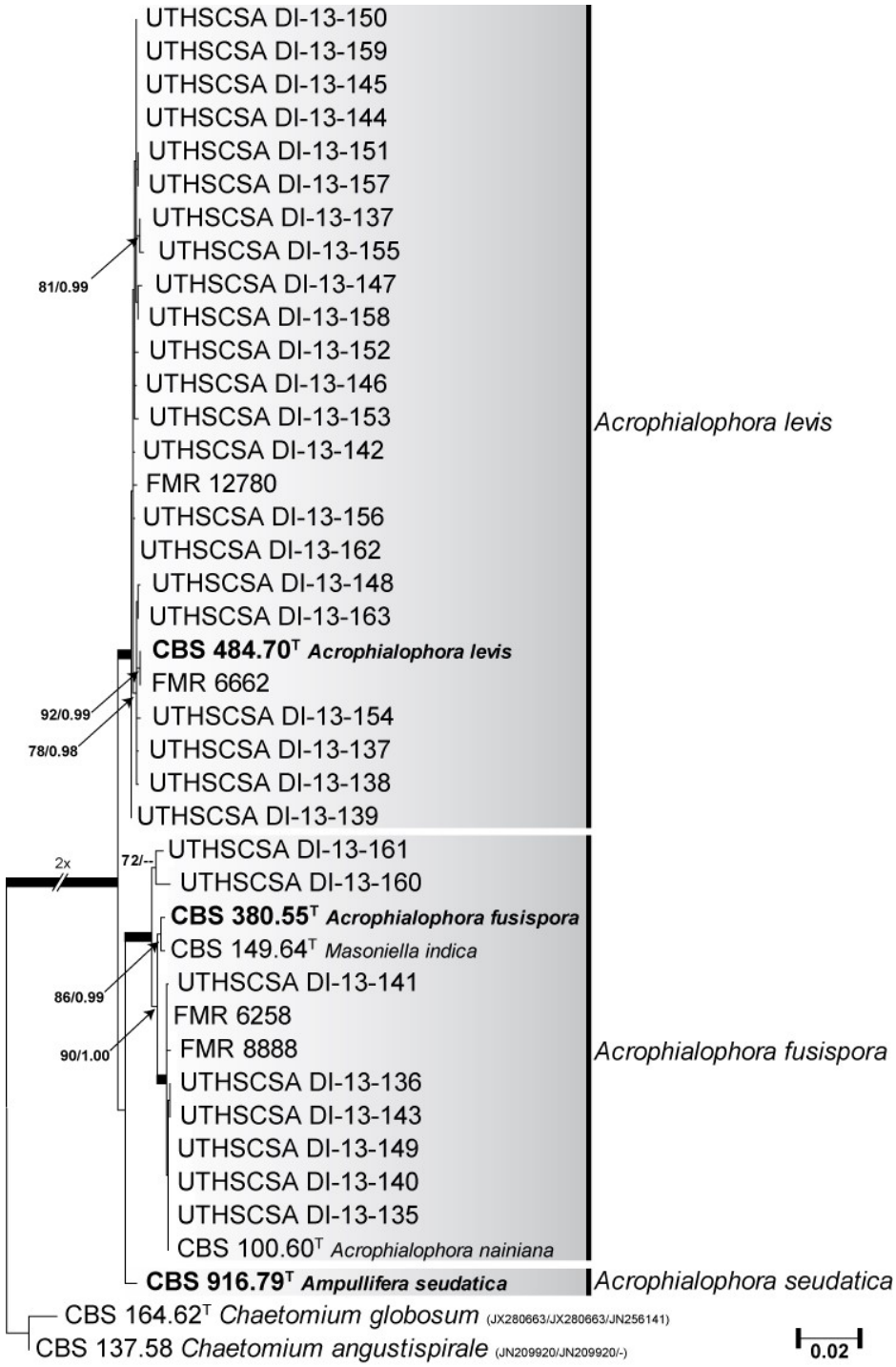
79/1.00
CBS 370.66 Chaetomidium subfimeti (FJ666354)
94/1.00
CBS 168.71 Chaetomidium fimeti (FJ666358)
CBS 281.66 Botryotrichum piluliferum (AB625575)
CBS 532.94 Achaetomium macrosporum (03262801*)
83/0.96
CBS 380.55T A. fusispora
73/--
CBS 100.60T A. nainiana
CBS 484.70T A. levis
Acrophialophora
Chaetomiaceae
MUCL 39402 Chaetomium malaysiense (AF286409)
MUCL 8236 Chaetomium seminudum (AF286410)
3x
DSM 21024 Thielavia subthermophila (AM909688)
93/--
CBS 509.74T Thielavia subthermophila (JX280760)
CBS 129177T Madurella pseudomycetomatis (JX280752)
CBS 129176T Madurella fahalii (JX280751)
CBS 109801T Madurella mycetomatis (JX280743)
CBS 298.63T Neurospora terricola (FR774282)
CBS 508.50 Sordaria fimicola (AY681160)

Figure 1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UTHSCSA DI-13-155
81/0.99
UTHSCSA DI-13-147
UTHSCSA DI-13-158
UTHSCSA DI-13-152
UTHSCSA DI-13-146
UTHSCSA DI-13-153
UTHSCSA DI-13-142
FMR 12780
UTHSCSA DI-13-156
UTHSCSA DI-13-162
UTHSCSA DI-13-148
UTHSCSA DI-13-163
CBS 484.70T Acrophialophora levis
FMR 6662
92/0.99
UTHSCSA DI-13-154
78/0.98
UTHSCSA DI-13-137
UTHSCSA DI-13-138
UTHSCSA DI-13-139
Acrophialophora levis
2x
72/--
UTHSCSA DI-13-161
UTHSCSA DI-13-160
CBS 380.55T Acrophialophora fusispora
CBS 149.64T Masoniella indica
86/0.99
UTHSCSA DI-13-141
FMR 6258
90/1.00
FMR 8888
UTHSCSA DI-13-136
UTHSCSA DI-13-143
UTHSCSA DI-13-149
UTHSCSA DI-13-140
UTHSCSA DI-13-135
CBS 100.60T Acrophialophora nainiana
Acrophialophora fusispora
CBS 916.79T Ampullifera seudatica
Acrophialophora seudatica
CBS 164.62T Chaetomium globosum (JX280663/JX280663/JN256141)
CBS 137.58 Chaetomium angustispirale (JN209920/JN209920/-)
0.02

Figure 2

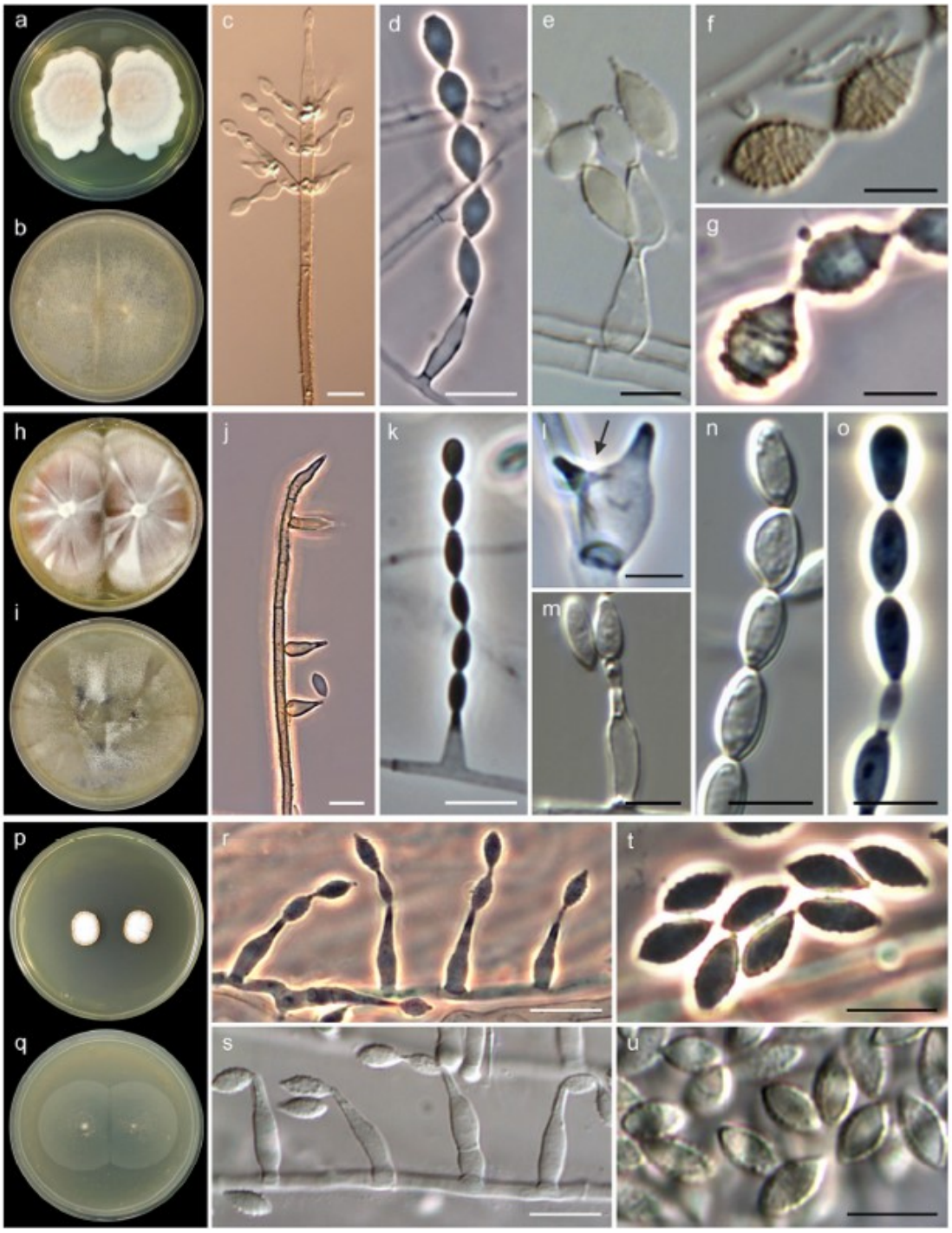

Figure 3