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1 **Coelomycetous fungi in the clinical setting: Morphological convergence and**
2 **cryptic diversity**

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14 **Running title:** Coelomycetes fungi of clinical origin.
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22 **ABSTRACT**

23 Human infections by coelomycetous fungi are becoming more frequent and range from
24 superficial to systemic dissemination. Traumatic implantation of contaminated plant
25 material is the most common cause. The typical morphological feature of these fungi is
26 the production of asexual spores (conidia) within fruiting bodies called conidiomata.
27 This study aimed to determine the distribution of the coelomycetes in clinical samples
28 by a phenotypic and molecular study of a large set of isolates received from a USA
29 reference mycological institution and by obtaining the *in vitro* antifungal susceptibility
30 pattern of a selected group of strains against nine antifungals. A total of 230 isolates
31 were identified by sequencing the D1 and D2 domains of the LSU nrRNA gene and by
32 morphological characterization. Eleven orders of the phylum *Ascomycota* were
33 identified: *Pleosporales* (the largest group; 66.1%), *Botryosphaeriales* (19.57%),
34 *Glomerellales* (4.35%), *Diaporthales* (3.48%), *Xylariales* (2.17%), *Hysteriales* and
35 *Valsariales* (0.87%), and *Capnodiales*, *Helotiales*, *Hypocreales* and *Magnaporthales*
36 (0.43% each one). The most prevalent species were *Neoscytalidium dimidiatum*,
37 *Paraconiothyrium* spp., *Phoma herbarum*, *Didymella heteroderae* and *Epicoccum*
38 *sorghinum*. The most common anatomical site of isolation was superficial tissue
39 (66.5%), followed by the respiratory tract (17.4%). Most of the isolates tested were
40 susceptible to the majority of antifungals and only flucytosine showed poor antifungal
41 activity.

42

43 **Keywords:** *Colletotrichum*, coelomycetous fungi, coelomycetes, mycosis,
44 *Neoscytalidium*, *Phoma*, *Pyrenochaeta*, antifungal susceptibility.

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46 **INTRODUCTION**

47 The coelomycetous fungi constitute a large number of taxa characterized by the
48 production of conidia (asexual propagules) within a cavity lined by fungal or host tissue
49 called conidiomata (1), and although the majority of the human opportunistic infections
50 are caused by fungi producing conidia on conidiophores (modified hyphae, with one or
51 more conidiogenous cells, which develop free on the substrate) a significant number of
52 mycoses are produced by the former organisms (2–4). Coelomycetous fungi are mostly
53 saprobic and parasites of terrestrial vascular plants, but they can also infect vertebrates
54 and other fungi. They are ubiquitous in soil, salty and freshwater environments, and in
55 sewage (4). Although the term "coelomycetes" is still occasionally used to refer to these
56 fungi, this is an obsolete name and currently is considered an artificial fungal class. The
57 class Coelomycetes, based on the morphological characterization of the asexual
58 reproductive structures, considering the type and the shape of their conidiomata and the
59 ontogeny of their conidia as the most useful characters (5, 6), has been traditionally
60 divided into the orders *Melanconiales* and *Sphaeropsidales*, depending upon the
61 production of either acervular (cup-shaped) or pycnidial (globose to pyriform)
62 conidiomata, respectively; and the *Pycnothyriales*, characterized by the production of
63 pycnothyrial (shield-shape, flattened or hemispherical) conidiomata (5, 6). However,
64 molecular studies have demonstrated that the taxonomy of the Coelomycetes,
65 represented by nearly 1,000 genera and 7,000 species (1), is artificial. Recent studies,
66 have distributed the Coelomycetes into at least three classes of the phylum *Ascomycota*,
67 i.e. *Dothideomycetes*, *Leotiomycetes* and *Sordariomycetes* (7–9).
68 Infections by coelomycetous fungi are mostly acquired by traumatic implantation of
69 plant/woody material or soil particles contaminated by their conidia rather than by
70 inhalation of air-dispersed propagules (2, 4). The coelomycetes are responsible for a

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71 large variety of clinical entities, such as dermatitis, onychomycosis, keratitis,
72 endophthalmitis, subcutaneous phaeohyphomycosis, cysts, mycetoma, sinusitis,
73 osteomyelitis, bursitis, brain abscesses and disseminated infections (4). The appropriate
74 treatment of the infections produced by these fungi is unknown, mainly due to the wide
75 spectrum of taxa involved and by the difficulties in their identification when the typical
76 reproductive structures are not produced. However, the ESCMID and ECMM have
77 provided joint clinical guidelines for the management of phaeohyphomycosis with some
78 recommendations for the treatment of infections due to the most usual genera of
79 Coelomycetes, such as *Neoscytalidium, Phoma* and *Pyrenochaeta*, mainly based in the
80 use of amphotericin B and triazoles (10).
81 For the reasons mentioned above, the spectrum of species of these fungi in the clinical
82 setting is practically unknown (4, 11). Therefore, the objective of this study has been to
83 determine the distribution pattern of the coelomycetous fungi isolated from clinical
84 specimens from the USA using molecular identification of a large set of isolates based
85 on the sequencing of the D1 and D2 (D1-D2) domains of the large subunit (LSU) of the
86 nrRNA gene. In addition, we have characterized those isolates morphologically and
87 determined the antifungal susceptibility of a representative number of them to nine
88 antifungal drugs.
89

90 **MATERIAL AND METHODS**

91 **Fungal isolates and sequences.**

92 Two hundred and thirty isolates of coelomycetous fungi were included in this study,
93 224 from human clinical specimens, 3 from animal sources and 3 from environmental
94 samples. All of the isolates were provided by the Fungus Testing Laboratory of the

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95 University of Texas Health Science Center at San Antonio (UTHSC; San Antonio,
96 Texas, USA). In addition, 92 D1-D2 sequences corresponding to type or reference
97 strains were retrieved from GenBank and CBS databases and included in the
98 phylogenetic analysis.

99 **Morphological and physiological characterization.**

100 For cultural characterization, the isolates were grown on oatmeal agar (OA; 30 g of
101 filtered oat flakes, 15 g of agar-agar, 1 L tap water) and malt extract agar (MEA; 40 g of
102 malt extract, 15 g of agar-agar, 1 L distilled water), at 20 ± 1ºC for 14 days in darkness.
103 The ability of the isolates to grow at 37ºC was determined on potato dextrose agar
104 (PDA; Pronadisa, Madrid, Spain) after seven days of incubation in darkness. The
105 morphological features of the vegetative and reproductive structures were studied using
106 an Olympus CH2 Light Field microscope (Olympus Corporation, Tokyo, Japan) in wet
107 mounts (on water and lactic acid) and slide cultures (by growing the isolates on OA and
108 MEA). The isolates were characterized phenotypically according to traditional criteria
109 (4, 5, 12, 13). Colour standards are from Kornerup and Wanscher (14).
110 Photomicrographs were taken with an Axio-Imager M1 lightfield microscope (Zeiss,
111 Oberkochen, Germany).

112 **DNA extraction, amplification and sequencing.**

113 The total genomic DNA was extracted from colonies grown on PDA after seven days of
114 incubation at 20 ± 1ºC, using FastDNA kit protocol (Bio101, Vista, CA), with a
115 FastPrep FP120 instrument (Thermo Savant, Holbrook, NY) following the
116 manufacturer's protocol. DNA was quantified using the Nanodrop 2000 (Thermo
117 Scientific, Madrid, Spain). The D1-D2 domains were amplified with the primer pair
118 LR0R and LR5 (15). The amplicons were sequenced in both directions with the same

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119 primer pair used for amplification at Macrogen Europe (Macrogen Inc., Amsterdam,
120 The Netherlands). The consensus sequences were obtained using the SeqMan software
121 version 7.0.0 (DNAStar Lasergene, Madison, WI, USA).

122 **Molecular identification and phylogenetic analysis.**

123 Preliminary molecular identification of the isolates was made using the D1-D2
124 nucleotide sequences in $BLAST_N$ searches (http://blast.ncbi.nlm.nih.gov/Blast) and the
125 CBS database (www.cbs.knaw.nl). Only the sequences of type or reference strains
126 deposited in CBS/GenBank databases were considered for identification purposes. A
127 level of identity ≥ 98% was considered for species-level identification.

128 For the phylogenetic study, the sequences were aligned using the ClustalW application
129 (16) of the MEGA 6.06 (17) computer program, refined with MUSCLE (18) and
130 manually adjusted using the same software platform. Phylogenetic reconstructions were
131 made by maximum-likelihood (ML) and Bayesian inference (BI) with MEGA 6.06 and
132 MrBayes 3.2.4 (19), respectively. The best substitution model for the gene matrix
133 (GTR+I+G) was estimated using MrModelTest version 2.3 (20). For ML analyses,
134 nearest-neighbour interchange was used as the heuristic method for tree inference.
135 Support for internal branches was assessed by 1,000 ML bootstrapped pseudoreplicates.
136 Bootstrap support (BS) of ≥70 was considered significant. For BI analyses, Markov
137 chain Monte Carlo (MCMC) sampling was carried out with 23 million generations, with
138 samples taken every 1,000 generations. The 50% majority rule consensus trees and
139 posterior probability values (PP) were calculated after removing the first 25% of the
140 resulting trees for burn-in. A PP value of ≥0.95 was considered significant.
141 *Saccharomyces castellii* (NRRL Y 12630, AY048167) and *Saccharomyces cerevisiae*
142 (NRRL Y 12632, AY048154) were used as outgroup.

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143 **Antifungal susceptibility testing**.

144 Using a broth micro dilution reference method (21), the *in vitro* antifungal susceptibility
145 of eighty-five isolates was determined of selected species of the genera *Colletotrichum*,
146 *Diaporthe*, *Didymella*, *Epicoccum*, *Neoascochyta*, *Neoscytalidium*, *Paraconiothyrium*,
147 *Phoma* sp. and *Pyrenochaetopsis*. The following antifungals were tested: amphotericin
148 B, voriconazole, posaconazole, itraconazole, caspofungin, anidulafungin, micafungin,
149 terbinafine, and flucytosine. The minimal effective concentration (MEC) was
150 determined after 48 h for the echinocandins, and the minimal inhibitory concentration
151 (MIC) was determined after 48 h and 72 h for the other drugs. *Candida parapsilosis*
152 ATCC 22019 and *Paecilomyces variotii* ATCC MYA-3630 were used as controls. The
153 inocula for those coelomycetous fungi that did not sporulate were prepared according to
154 Chowdhary *et al*. (22).

155 **Nucleotide sequence accession numbers.** The DNA sequences determined in this
156 study have been deposited in GenBank (accession LN907285-LN907514).

157

158 **RESULTS**

159 A total of 86 (38%) isolates of the 230 studied were able to produce pycnidial
160 conidiomata, 10 (4%) developed acervuli, and 35 (15%) produced the typical anamorph
161 of *Neoscytalidium*. The other 99 isolates (43%) remained sterile. The most common
162 species was *Neoscytalidium dimidiatum*, representing 15% (35/230) of the isolates,
163 followed by *Paraconiothyrium cyclothyrioides*, with 7% (16/230) of them, both mostly
164 from superficial tissues. The third most common taxon recovered was *Phoma herbarum*
165 (6.5% (15/230)) from superficial and respiratory tract specimens, followed by

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166 *Didymella heteroderae* (5% (12/230)) and *Epicoccum sorghinum* (4% (10/230)), which
167 were isolated from superficial tissues.

168 In the D1-D2 phylogenetic analysis, the isolates were distributed into 11 orders (Fig. 1),
169 most of them belonging to the *Pleosporales* (66.1%) and the *Botryosphaeriales*
170 (19.57%), followed by the *Glomerellales* (4.35%), *Diaporthales* (3.48%), *Xylariales*
171 (2.17%), *Hysteriales* and *Valsariales* (0.87% each one). The orders *Capnodiales*,
172 *Helotiales*, *Hypocreales* and *Magnaporthales* were represented by only one isolate each
173 (0.43%) and the other isolates (0.87%) were *incertae sedis* (of uncertain taxonomic
174 position).

175 Figure 2 shows the phylogenetic tree inferred from the analysis of 322 D1-D2
176 sequences corresponding to our set of isolates and numerous selected type or reference
177 strains phylogenetically related to them. As mentioned above, the *Pleosporales*
178 contained the largest number of isolates (n = 152), which were distributed into 22 clades
179 and belonging, probably, to 61 species of 44 different genera. These clades have been
180 named according to the first taxon historically described.

181 Within the *Pleosporales*, *Phoma* clade I (phylogenetically not supported) included 27
182 isolates, distributed mainly in two genera: *Leptosphaerulina*, with four isolates
183 characterized by a phoma-like asexual morph, which clustered with a reference strain of
184 *L. australis*; and *Phoma*, with 15 isolates placed close to a reference strain of *Phoma*
185 *herbarum* and morphologically characterized by producing pycnidia and hyaline
186 aseptate conidia. The taxonomic position of the other eight isolates of this clade was
187 unresolved; in fact, they formed a separate, unsupported sister clade, and displayed a
188 phoma-like asexual morph. The isolate UTHSC DI16-270 also showed the typical

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189 morphology of *Phoma* (*Phoma* clade II), but has been placed phylogenetically distant
190 from the mentioned genera, and probably belongs to a new genus.

191 The *Didymella* clade included 22 isolates, ten of them clustering with a reference strain
192 of *Epicoccum sorghinum*; unfortunately, the morphological features of these isolates
193 could not be studied because they only produced sterile mycelia in all the culture media
194 tested. Twelve isolates grouped with the type strain of *Didymella heteroderae*,
195 producing a phoma-like asexual morph, but were particularly characterized by the
196 production of chlamydospores in long chains.

197 The *Neoascochyta* clade included seven isolates, six clustering with the type strain of
198 *Neascochyta desmazieri*, and another one placed together with a reference strain of
199 *Ascochyta hordei* var. *hordei*. Morphologically, the species of this clade are mainly
200 characterized by the production of 1-septate conidia that varies in size.

201 The *Paraphoma* clade contained only one isolate, which showed an identical sequence
202 to the type strain of *Paraphoma radicina*, and morphologically was characterized by
203 setose (covered with bristle-like structures) pycnidia and hyaline aseptate conidia.

204 The *Pleospora* clade was made up of five isolates and, with the exception of one of
205 them, was distributed into three well-supported sister clades corresponding to the genera
206 *Edenia*, *Paraphoma* and *Trematophoma*. The isolate that clustered with the type strain
207 of *Paraphoma fimeti* was separate from the type species of *Paraphoma* (*P. radicina*)
208 and showed glabrous pycnidia instead the setose pycnidia produced by the rest of the
209 species. Interestingly, the isolate UTHSC DI16-324 produced fusiform, hyaline, 2-3-
210 septate conidia instead of the ellipsoidal, subhyaline conidia typical of *Edenia* spp. and
211 are probably indicative of a new genus. The other isolates of this clade remained sterile.

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212 The *Coniothyrium* clade included nine isolates, and its topology shows that the genera
213 *Coniothyrium*, *Leptosphaeria* and *Pyrenochaeta* are clearly polyphyletic using this
214 conserved marker. Three of these isolates formed a well-supported sister clade together
215 with a reference strain of *Coniothyrium telephii*, which are characterized by setose
216 pycnidia. The other six isolates were distributed into the genera *Leptosphaeria* and
217 *Pyrenochaeta*. These showed a pyrenochaeta-like anamorph, producing conidiophores
218 within pycnidia and hyaline aseptate conidia.

219 The *Phaeosphaeria* clade grouped nine isolates, four of them clustering with
220 *Neosetophoma* and producing confluent pycnidia and small hyaline conidia. The other
221 five isolates were associated with the genera *Diederichomyces*, *Parastagonospora*,
222 *Phaeosphaeria* and *Phaeosphaeriopsis*. Only one isolate (UTHSC DI16-325),
223 morphologically resembling *Phaeosphaeriopsis* spp., was able to sporulate, displaying
224 small conidiophores within pycnidia and one-septate conidia, variable in shape and
225 pigmented.

226 The *Pyrenochaetopsis* clade included nine isolates, four of them matching the type
227 strain of *Py. leptospora*, another four isolates forming a supported sister clade separate
228 from *Py. leptospora*, and the last one not clustering to any of the type strains included in
229 the analysis. All the isolates displayed the typical phoma-like morphology, i.e. glabrous
230 pycnidia and hyaline aseptate conidia, instead of setose pycnidia of the genus
231 *Pyrenochaetopsis*.

232 The five isolates enclosed in the *Acrocalymma* and *Medicopsis* clades were grouped
233 with the type strains of *A. walkeri* and *M. romeroi*, respectively, but differed in 4.5% of
234 the nucleotide sequences of the respective strains of reference. These isolates remained
235 sterile throughout.

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236 The *Roussoella* clade was made up of eight isolates, two of which were associated with
237 a supposed reference strain of *Arthopyrenia salicis* (CBS 368.94), whose correct
238 identification was questioned by Liu *et al*. (23) and the remaining ones with *Roussoella*
239 spp.; only three isolates were able to sporulate and showed a similar morphology to this
240 genus, i.e., production of glabrous pycnidia and pigmented aseptate conidia.

241 Two isolates nested in the *Biatriospora* clade, but remained sterile. The
242 *Trematosphaeria* clade comprised five sterile isolates, two phylogenetically related with
243 the type strain of *T. pertusa*, and the rest associated with a reference strain of *T. grisea*.

244 The *Keissleriella* clade only had one isolate, which showed a phoma-like morphology
245 and clustered with a reference strain of *K. cladophila*. Another isolate was associated
246 with a reference strain of *Paraconiothyrium flavecens* and displayed a similar
247 morphology to *Paraconiothyrium* (pycnidia, phialidic conidiogenous cells and
248 pigmented aseptate conidia); however, the taxonomic placement of that isolate remains
249 doubtful because it grouped phylogenetically distant from the type species of the genus
250 (*Paraconiothyrium estuarinum*). In the *Camarographium* clade, two sterile isolates
251 were located, related to the genera *Camarographium* and *Pseudochaetosphaeronema*.

252 The *Didymosphaeriaceae* clade comprised 33 isolates, 22 of which were
253 phylogenetically related to *Paraconiothyrium* spp., two to *Montagnula* spp., and two to
254 the type strain of *Paraphaeosphaeria neglecta*. Three isolates were each distributed into
255 the genera *Bimuria*, *Curreya* and *Phaeodothis*, and four isolates formed a well-
256 supported monophyletic sister clade separated from any known taxa of the family. Only
257 three isolates (UTHSC DI16-261, UTHSC DI16-266 and UTHSC DI16-363) were able
258 to sporulate, showing glabrous pycnidia and pale brown conidia, displaying similar
259 morphological features to *Paraconiothyrium* spp. The *Exosporium* clade comprised

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260 only two sterile isolates, one related to the genus *Preussia*, and the other to *Exosporium*.
261 The *Anteaglonium*, *Lophiostoma* and *Phyllosticta* clades comprised only one sterile
262 isolate each one.

263 In the *Valsariales* clade, two isolates matched a reference strain of *Myrmaecium*
264 *rubricosum*. These isolates were characterized by producing free well-differentiated
265 conidiophores instead of simply conidiogenous cells (phialides) inside the pycnidia.
266 The *Hysteriales* clade enclosed two sterile isolates, one related to an unidentified strain
267 of *Chaetophoma*, and the remaining one of uncertain taxonomical placement but
268 phylogenetically related to the former genus and *Gloniopsis* and *Rhytidhysteron*.

269 The second largest clade, corresponding to the order *Botryosphaeriales*, included 45
270 isolates distributed in six clades; with the exception of the *Neoscytalidium* clade. The
271 fungi included in those clades were characterized by the production of stromatic
272 conidiomata (a hard, compact mass of cells or of vegetative hyphae), holoblastic instead
273 of phialidic conidiogenous cells, and aseptate, hyaline to brown, thick-walled conidia.
274 This clade included the genera *Botryosphaeria* (three isolates), *Lasiodiplodia* (two
275 isolates), *Neofusicoccum* (one isolate), *Aplosporella* (two isolates) and
276 *Phaeobotryosphaeria* (two isolates). Additionally, 35 isolates of *Neoscytalidium*
277 *dimidiatum* were also placed in that clade. This fungus is characterized typically by the
278 production of holoarthric conidia (formed by disarticulation of the pre-existing hyphae)
279 in chains.
280 The *Capnodiales*, *Helotiales* and *Magnaporthales* clades each included only one sterile
281 isolate. Only the isolate of the *Helotiales* was not phylogenetically related to any
282 previously known described species. The isolate of the *Capnodiales* was closely related
283 to a reference strain of *Pseudocercospora oenotherae*. This genus is characterized by
284 producing stromata in the (plant) host, subhyaline to brown conidiophores, and small or

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285 large, subhyaline to brown conidia; unfortunately, our isolate failed to sporulate. In the
286 *Magnaporthales*, the isolate matched a reference strain of *Mycoleptodiscus indicus*.
287 That genus is characterized by producing sporodochia (a cushion-like, densely
288 aggregated group of conidiophores) and curved conidia; in this case, the morphological
289 study was not possible due to the lack of sporulation of the isolate.
290 The *Xylariales* clade included five sterile isolates, three of them related to the genus
291 *Diatrype* but phylogenetically distant from a reference strain of *D. disciformis*. The
292 remaining two isolates were associated with the *Peroneutypa* clade, one of them
293 matching a reference strain of *Pe. scoparia*, and the other uncertainly placed
294 taxonomically.
295 The *Diaporthales* clade grouped eight isolates, six of them belonging to the *Diaporthe*
296 clade and characterized by the production of hyaline conidiophores within pycnidial
297 conidiomata, phialidic conidiogenous cells and small conidia. The other two sterile
298 isolates were located in the *Valsa* clade.
299 The *Hypocreales* clade included only a single sterile isolate that matched a reference
300 strain of *Thyronectria austroamericana*.
301 The *Glomerellales* clade comprised ten isolates, all of them belonging to the genus
302 *Colletotrichum* and characterized by the production of acervular conidiomata, phialidic
303 conidiogenous cells, conidia variable in shape, and presence of appressoria. Six of the
304 isolates were identified as *C. gloeosporioides*, two as *C. truncatum*, and the last one as
305 *C. spaethianum*. One isolate (UTHSC DI14-247) was molecularly closely related to a
306 reference strain of *Colletotrichum torulosum*.
307 Two isolates (UTHSC DI16-350 and UTHSC DI16-223) were not located in any of the
308 previously known orders, and consequently were treated as *incertae sedis*. The first one

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309 was enclosed in the *Phomatospora* clade and the other, characterized by the production
310 of sporodochia and hyaline conidia, was identified as *Phialemoniopsis curvata*.
311 From a total of 224 clinical isolates, 153 were recovered mainly from superficial tissues
312 (epidermis and dermis) (66.5%), followed by 40 from the respiratory tract (17.4), 22
313 from miscellaneous deep tissues or fluids (9.6%), and 9 isolates from subcutaneous
314 tissues (3.9%) (Table 1).
315 Approximately half of all the fungi tested (44%; 101/230) were able to grow at 37ºC
316 (Table 1), and distributed within the orders in the following percentages: 100% (10/10)
317 of the *Glomerellales*, 100% (2/2) of *Hysteriales*, 100% (2/2) of the *Valsariales*, 98%
318 (44/45) of the *Botryosphaeriales*, 50% (1/2) of the isolates *incertae sedis*, and 28%
319 (42/152) of the *Pleosporales*.
320 Table 2 summarizes the results of the antifungal susceptibility testing. In general, all the
321 drugs tested, but especially terbinafine and amphotericin B, showed good activity
322 against the coelomycetous fungi, with terbinafine being the most potent (GM MIC 0.04
323 µg/mL; MIC_{90} 0.03 µg/mL). Among the triazoles, itraconazole was the least potent,
324 with an overall GM MIC of 1 µg/mL and a MIC_{90} of 16 µg/mL. *Colletotrichum*
325 *gloeosporioides*, *Neoscytalidium dimidiatum* and *Didymella heteroderae* showed high
326 MICs to all the antifungals tested. Posaconazole and voriconazole demonstrated similar
327 in vitro potency, with the only exceptions of *Colletotrichum gloeosporioides* and
328 *Neoascochyta desmazieri*, in which case voriconazole displayed a GM MIC of 2.64 and
329 2 µg/mL, and against *Neoscytalidium dimidiatum* the posaconazole GM MIC was 2.26
330 µg/mL. All the echinocandins showed good *in vitro* activity against these fungi, with a
331 GM MEC of 0.06 µg/mL. Flucytosine was the least potent antifungal tested with
332 elevated MICs against all isolates.

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334 **DISCUSSION**

335 This is, to our knowledge, the largest taxonomic study on coelomycetous fungi of
336 clinical origin. It has demonstrated, based on DNA sequencing, a wider diversity of taxa
337 than those previously reported. Although two recent reviews have reported
338 approximately 35 species of coelomycetes involved in human infections (3-4), the
339 present study identifies 88 species; unfortunately, many of them still remain uncertain in
340 their role as pathogens for humans, due to the clinical data of the patients are not
341 allowed to be published. In general, the coelomycetes are involved in many kinds of
342 mycoses, superficial to deep infections, onychomycosis, cutaneous infections, keratitis
343 and endophthalmitis being relatively frequent. In general, the most commonly reported
344 species clinically are *Colletotrichum* spp. (24–31), *Neoscytalidium dimidiatum* (32–36)
345 and *Phoma* spp. (11, 37–46). Our study partly confirms the data from those studies, in
346 which *Neoscytalidium dimidiatum* (approx. 15%), *Paraconiothyrium cyclothyrioides*
347 (approx. 7%) and *Phoma herbarum* (approx. 6.5%) were the most common species,
348 mainly having been recovered from superficial tissue and respiratory tract specimens.
349 However, of those fungi, the only species that is relatively easy to identify by
350 phenotypic criteria is *N. dimidiatum*, which is the best known coelomycete found
351 clinically (33, 34, 47). The identification of the other fungi mentioned above, generally
352 requires the use of molecular tools, due to the difficulty it has to sporulate *in vitro*.
353 Although *Paraconiothyrium cyclothyrioides* was relatively common in our studied
354 samples, there are only two clinical reports that refer to this species. Both cases are from
355 immunocompromised patients, the first causing skin lesions of the lower extremities,
356 and the second being a systemic co-infection together with *Phaeoacremonium*
357 *parasiticum* (48, 49). In spite of *Phoma* sporulating easily, it is commonly misidentified
358 as other related genera, such as *Ascochyta*, because they have a similar morphology,

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359 physiology and nucleotide sequences (50, 51). Boerema *et al*. carried out one of the
360 most comprehensive revisions of the taxonomy of the genus *Phoma.* Using systematic
361 criteria that predominated then, approx. 220 species were accepted, distributed into nine
362 sections (13). In a recent multi-locus study based on the sequence data of the 18S
363 nrRNA (SSU) and LSU genes, other authors demonstrated that such classification was
364 totally artificial (52). Currently, *Phoma sensu stricto* is included in the family
365 *Didymellaceae* and the other phoma-like fungi belong to other phylogenetic families i.e.
366 *Cucurbitariaceae*, *Leptosphaeriaceae*, *Phaeosphaeriaceae*, etc. (50-53).

367 It is of note that one of the frequently isolated species in our study, *Didymella*
368 *heteroderae* (5.2% of isolation frequency) has never been mentioned as an etiologic
369 agent of human infections, even though our results reveal its ability to grow and to
370 sporulate at 37°C, uncommon in that genus, suggesting its potential pathogenicity.

371 An important clinical entity produced by the coelomycetous fungi is the eumycetoma,
372 which is restricted to a specific group of pleosporalean species of fungi, namely
373 *Medicopsis romeroi* (formerly, *Pyrenochaeta romeroi*) (54–56), *Biatriospora*
374 *mackinnonii* (formerly *Pyrenochaeta mackinnonii*) (57), and *Trematosphaeria grisea*
375 (formerly, *Madurella grisea*) (57–59) among others. However, in the present study only
376 nine of the isolates, which were isolated from superficial and less frequently from deep
377 tissues, belong to such genera. This might be explained by the fact that the habitat of
378 those fungi is usually restricted to arid zones of East Africa and India, and occasionally
379 from South America (56, 60, 61).

380 Despite several studies in recent years devoted to infections by coelomycetes, there is
381 little clinical data. The first well-documented review of human infections by these fungi
382 was carried out by Punithalingham (11), who referenced a total of 12 species, mostly

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383 belonging to the genera *Botryodiplodia*, *Dothiorella*, *Hendersonula*, *Phoma*,
384 *Phyllosticta*, *Pseudochaetosphaeronema* and *Pyrenochaeta*. In that work, a
385 morphological description of these taxa and their clinical origin was provided, together
386 with a dichotomous key for their identification. However, in our study, only just under
387 12 % of the total isolates identified belong to such genera. In a recent study, Stchigel
388 and Sutton (4) provided detailed information about the species of coelomycetes isolated
389 from clinical samples, described useful tools for their isolation and identification, and
390 gave general guidelines for infection management and treatment. Those authors
391 concluded that these fungi are easy to isolate but it was difficult to induce *in vitro*
392 fructification and sporulation. Our results agree with those authors because 43% of our
393 isolates failed to sporulate and it was only possible to identify them and to determine
394 their phylogenetic relationships by DNA sequencing.

395 The prevalence of coelomycetous fungi find in these clinical specimens – more than 200
396 isolates being recovered in a nine-year period – goes against the fact that so few studies
397 have described infections by them. This highlights the difficulty in conducting a
398 comprehensive study of those fungi and establishing their real occurrence in clinical
399 settings. The taxonomy of these fungi is very complex because numerous isolates are
400 usually unable to sporulate *in vitro* or to produce different synanamorphs, which
401 sometimes predominate over the traditional coelomycetes structures, making their
402 phenotypic recognition difficult; reliable identification can, therefore, only be done by
403 gene sequencing (9, 56, 62). However, even in this case, there are a very high number of
404 genera and species of coelomycetous fungi and the phylogenetic boundaries of
405 numerous taxa are still unresolved. Therefore, we carried out a phylogenetic analysis of
406 a large set of coelomycetous fungi using LSU sequences. This marker proved useful for
407 solving the phylogeny of most of the isolates included in the study, identifying them, at

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408 least at genus level, and showing, over the ITS, the advantage of an easy alignment of
409 sequences.

410 The increasing use of molecular tools in fungal taxonomy has allowed the recognition
411 of numerous new taxa that are impossible to detect by the traditional methods. Recently,
412 several new species of coelomycetes, namely *Roussoella percutanea*, *Truncatella*
413 *angustata*, *Hongkongmyces pedis*, *Rhytidhysteron* spp., *Pseudochaetosphaeronema*
414 *martinelli* and *Emarellia* spp., have been involved in cases of subcutaneous infections
415 and eumycetoma (63–68) and some of our *Pleosporales* isolates, having failed to
416 sporulate, could represent new taxa.

417 Although CLSI breakpoints for coelomycetous fungi have not been defined and *in vitro*
418 antifungal susceptibility studies on these fungi are rare, most of the species seem to be
419 inhibited by amphotericin B (4). Our results show that posaconazole is the most active
420 of the triazoles tested, and results for amphotericin B are similar *in vitro* to those
421 reported by Chowdhary *et al*. (10). Currently, only disseminated infections due to *N.*
422 *dimidiatum* have been conducted in animal models, with amphotericin B, voriconazole
423 and posaconazole being effective in the treatment against this experimental mycosis
424 (47). Guidelines for the management of infections due to coelomycetous fungi only
425 include a small group of taxa (*Neoscytalidium, Phoma* and *Pyrenochaeta* spp.) (10),
426 although our study supports those protocols. A recent study Guégan *et al*. (69) analyzed
427 several coelomycetous fungi that were implicated in human mycosis and concluded that
428 the surgical resection of infected tissues is advisable for treating well-delimited lesions
429 and together with new triazoles could be used if lesions are extensive.

430 In conclusion, this study demonstrates that a wide variety of fungal taxa, identified
431 through their morphology as coelomycetes, are involved in human infections in the
432 USA. However, more studies are necessary to understand the real prevalence of the

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433 coelomycete's infections across the world. The most active antifungal drugs to treat
434 them seem to be terbinafine, echinocandins and amphotericin B, while results for the
435 azoles varied. Although the LSU gene sequence is useful for preliminary identification
436 and to establish phylogenetic relationships between the majority of coelomycetous
437 fungi, future molecular studies, testing a higher number genes, are essential to properly
438 identify at species level the doubtful isolates.
439

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659 **LEGEND OF FIGURE**

660 FIG 1 Distribution by orders of coelomycetous fungi isolates from clinical samples.

661

662 FIG 2 Maximum likelihood tree obtained from the D1-D2 of LSU (555 bp) sequences of the 322
663 strains, where 92 belongs to type or reference strain. In the tree, the branch lengths are proportional
664 to phylogenetic distance. Bayesian posterior probability scores of ≥0.95 and Bootstrap support
665 values of ≥70 are indicated on the nodes. The GenBank accession numbers are given after strains.
666 *Saccharomyces castellii* and *S. cerevisiae* were used to root the tree. The type species and reference
667 strains are shown in bold type. Superscript T, type strain.

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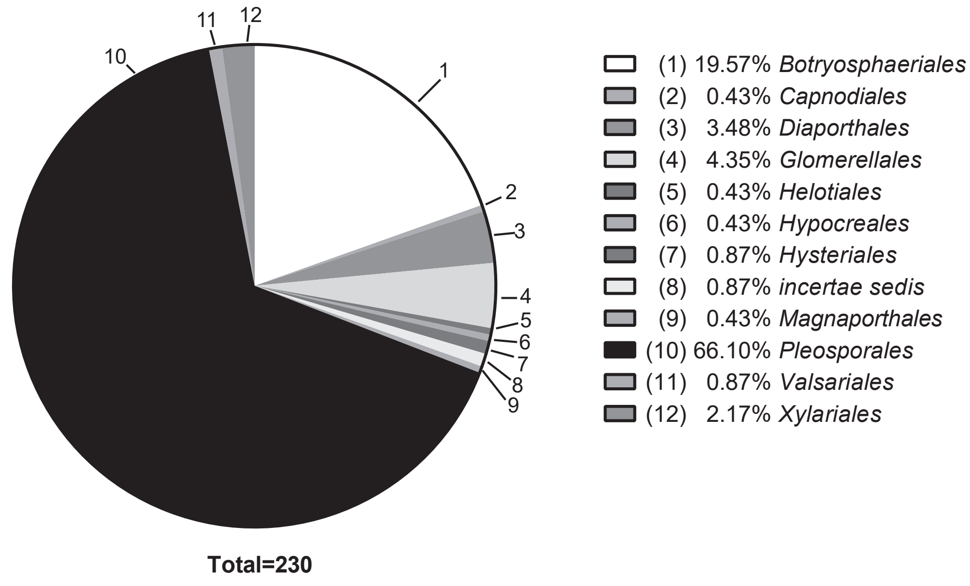

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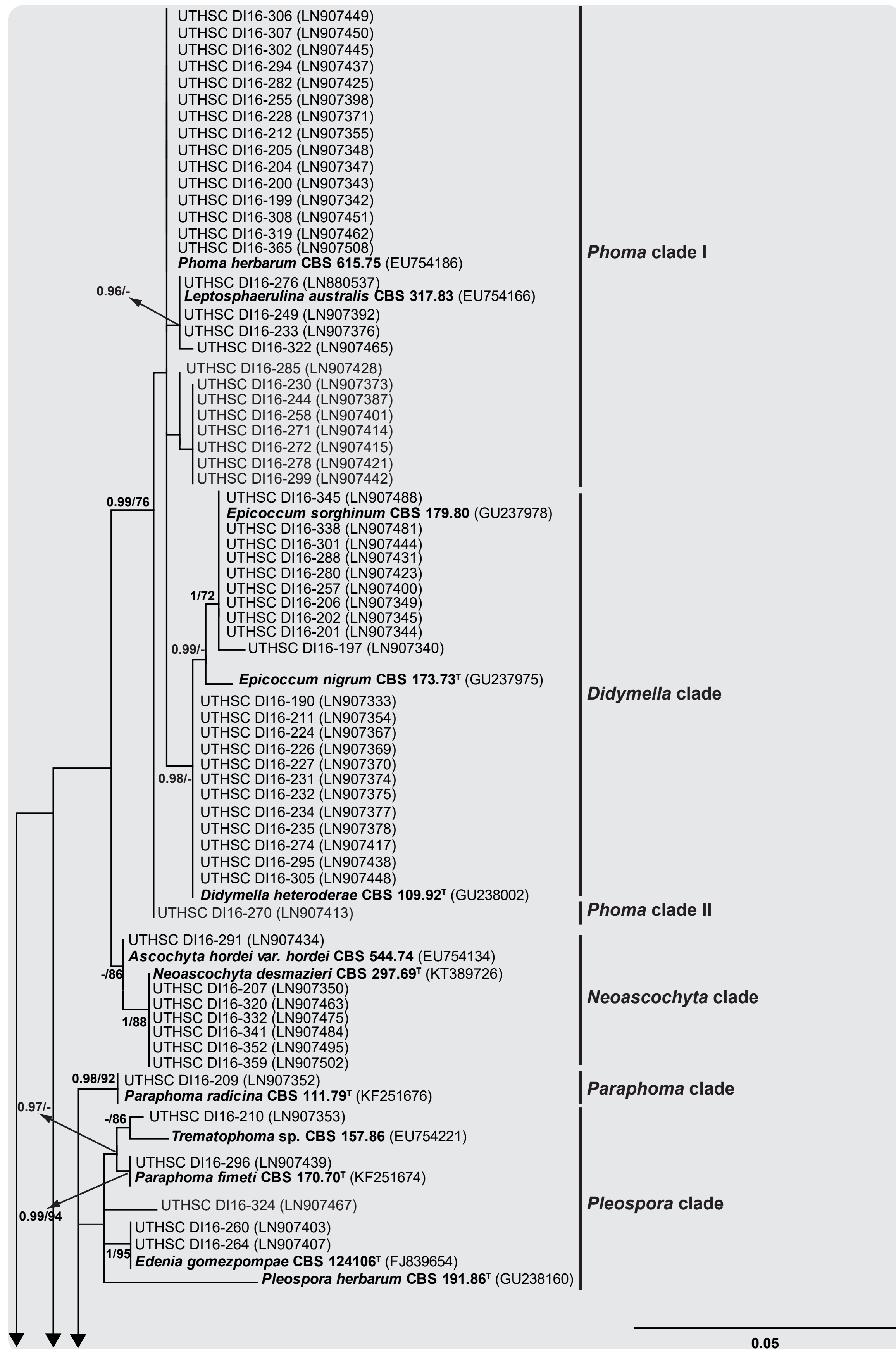

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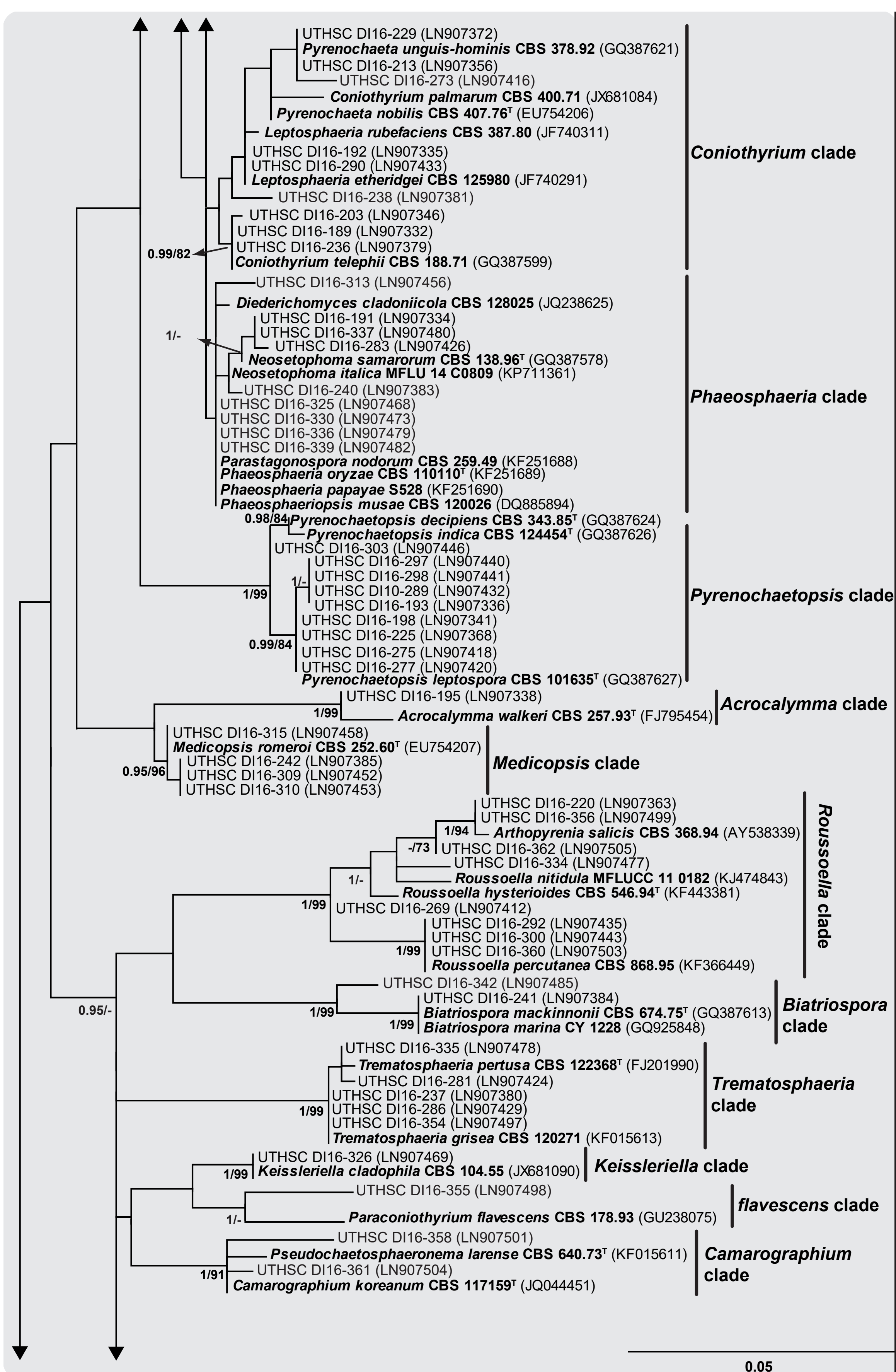

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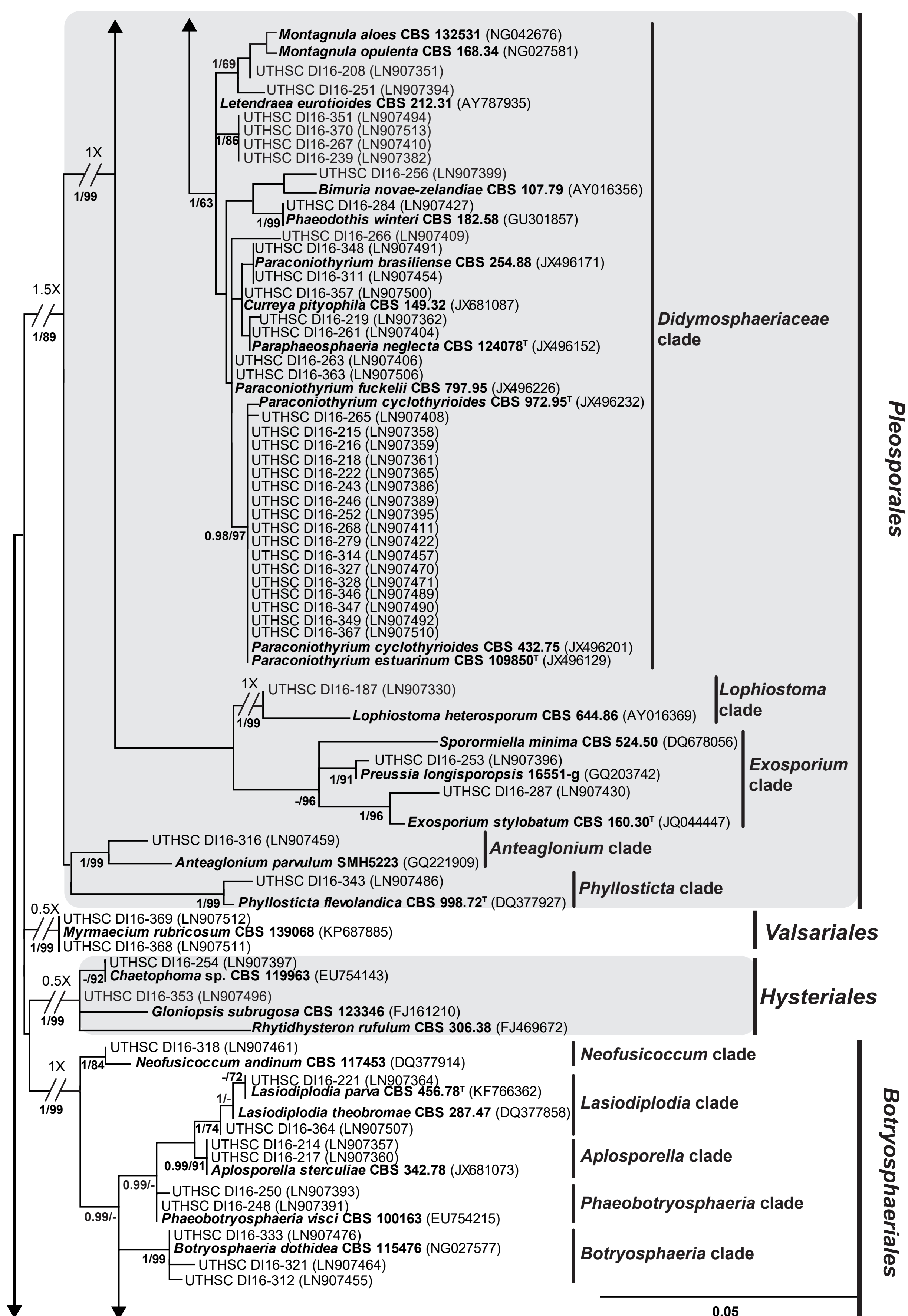

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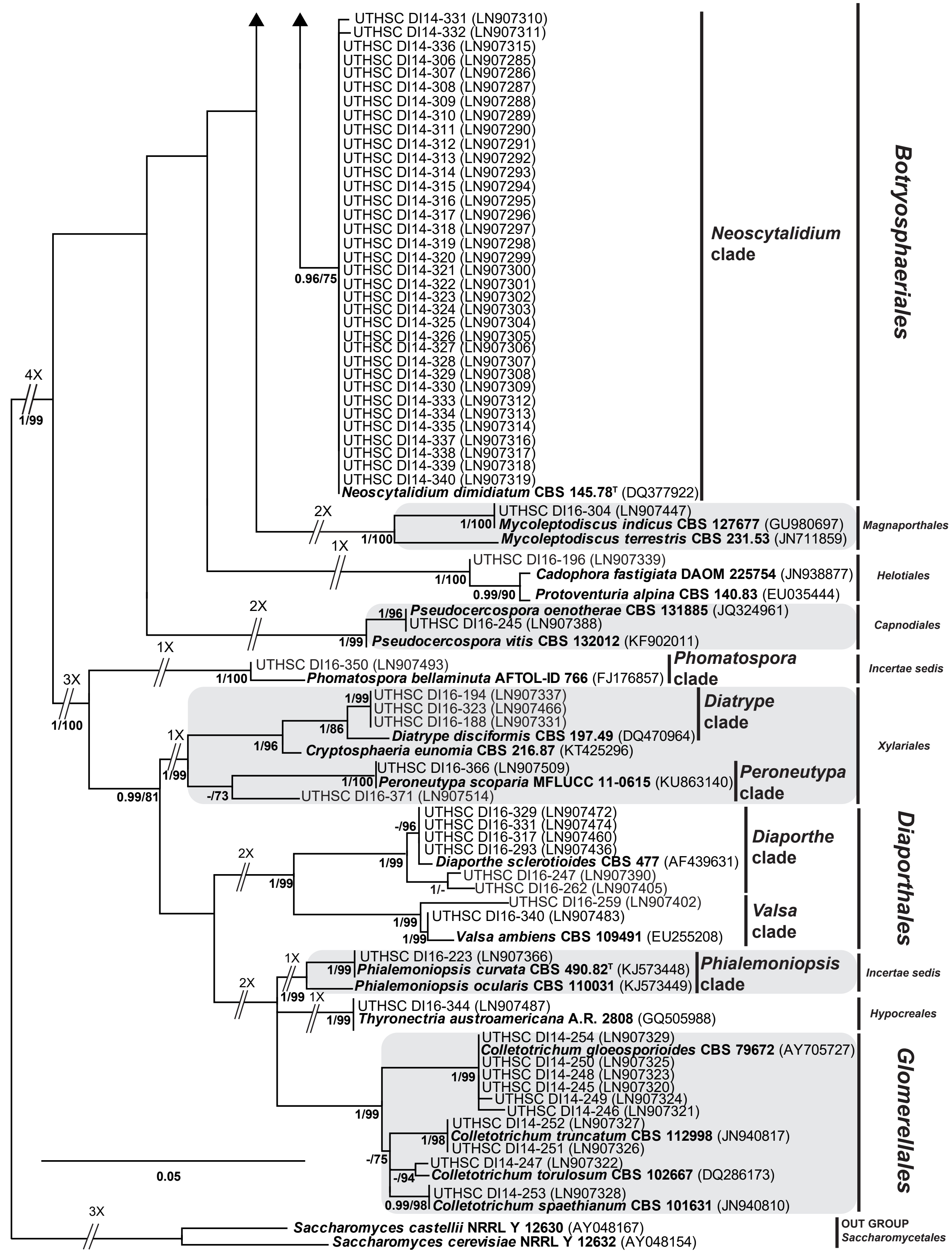

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TABLE 1 Anatomical sites of coelomycetous fungi isolates from clinical specimens.

Orders	Clades	No. of isolates obtained from: Superficial tissue	Subcutaneous tissue	Deep tissue/ fluids	Respiratory tract	Environmental	37ºC Growth	Total no. of isolates
Botryosphaeriales	*Aplosporella* clade	2					+	2
	Botryosphaeria clade	3					+	3
	Lasiodiplodia clade	2					+	2
	Neofusicoccum clade	1					-	1
	Neoscytalidium clade	27		3	5		+	35
	Phaeobotryosphaeria clade	2					+	2
Capnodiales		1					-	1
Diaporthales	*Diaporthe* clade	3		2	1		+	6
	Valsa clade	1			1		+	2
Glomerellales		7	1	1	1		+	10
Helotiales		1					-	1
Hypocreales		1					+	1
Hysteriales			1		1		+	2
incertae sedis	*Phialemoniopsis* clade	1					+	1
	Phomatospora clade			1			-	1
Magnaporthales		1					+	1
Pleosporales	*Acrocalymma* clade	1					-	1
	Anteaglonium clade	1					-	1
	Biatriospora clade	1			1		-	2
	Camarographium clade	2					-	2
	Coniothyrium clade	7	1	1			-	9
	Didymella clade	17		1	4		+	22
	Didymosphaeriaceae clade	26	1	2	3	1	-	33
	Exosporium clade	1				1	-	2

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	flavescens clade	1					-	1
	Keissleriella clade		1				-	1
	Lophiostoma clade		1				-	1
	Medicopsis clade	3		1			+	4
	Neoascochyta clade	6			1		-	7
	Paraphoma clade	1					-	1
	Phaeosphaeria clade	2	1	1	4	1	-	9
	Phoma clade I	13		3	10	1	-	27
	Phoma clade II	1					-	1
	Phyllosticta clade	1					+	1
	Pleospora clade	1		1	3		-	5
	Pyrenochaetopsis clade	6		1	2		-	9
	Roussoella clade	5		2	1		+	8
	Trematosphaeria clade	4		1			+	5
Valsariales						2	+	2
Xylariales	*Diatrype* clade		1	1	1		-	3
	Peroneutypa clade		1		1		-	2
Total no. of isolates (%)		**153 (66.5)**	**9 (3.9)**	**22 (9.6)**	**40 (17.4)**	**6 (2.6)**		**230 (100)**

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TABLE 2 Results of *in vitro* antifungal susceptibility testing of coelomycetous fungi.

Data by taxa (no. of isolates)	Parameter[a]	MIC/MEC (µg/mL):								
		AMB	VRC	ITC	PSC	AFG	CFG	MFG	TRB	5FC
Neoascochyta desmazieri (5)	GM	0.44	2	0.57	0.21	0.03	0.03	0.03	0.03	1.15
	Range	0.25-1	1-4	0.25-1	0.06-0.5	0.03-0.06	≤0.03	≤0.03	≤0.03	0.5-2
	MIC_{90}	0.5	2	1	0.5	0.03	0.03	0.03	0.03	2
Colletotrichum gloeosporioides (5)	GM	0.57	2.64	8	0.87	0.03	0.03	0.03	0.03	16
	Range	0.03-2	0.5-4	1-16	0.5-1	≤0.03	≤0.03	≤0.03	≤0.03	≥16
	MIC_{90}	2	4	16	1	0.03	0.03	0.03	0.03	16
Epicoccum sorghinum (8)	GM	0.25	0.92	0.59	0.30	0.03	0.04	0.03	0.03	2.97
	Range	0.12-1	0.5-2	0.5-1	0.12-0.5	0.03-0.06	0.03-0.5	≤0.03	≤0.03	1-8
	MIC_{90}	0.5	1	1	0.5	0.03	0.03	0.03	0.03	4
Neoscytalidium dimidiatum (16)	GM	0.22	0.59	2.56	2.26	0.13	0.2	0.47	0.08	2.83
	Range	0.06-1	0.03-16	0.06-16	0.03-16	0.03-0.5	0.03-1	0.06-8	0.03-2	0.25-16
	MIC_{90}	0.5	4	16	16	0.25	0.5	4	0.03	8
Paraconiothyrium cyclothyrioides (15)	GM	0.25	0.25	0.3	0.15	0.03	0.03	0.03	0.03	2.61
	Range	0.03-8	0.06-0.5	0.06-0.5	0.03-0.5	≤0.03	≤0.03	≤0.03	≤0.03	1-16
	MIC_{90}	0.5	0.5	0.5	0.25	0.03	0.03	0.03	0.03	4
Didymella heteroderae (11)	GM	1.76	1.87	3.31	1.07	0.34	0.13	0.14	0.03	4
	Range	0.5-8	0.06-16	0.5-16	0.5-2	0.03-8	0.03-4	0.03-2	≤0.03	1-16
	MIC_{90}	4	16	16	2	8	4	2	0.03	16
Phoma herbarum (10)	GM	0.43	0.57	0.81	0.40	0.04	0.04	0.03	0.03	2
	Range	0.12-2	0.06-4	0.25-4	0.12-1	0.03-0.12	0.03-0.12	0.03-0.06	≤0.03	0.5-16
	MIC_{90}	1	1	1	1	0.06	0.12	0.06	0.03	16
Phoma sp. (7)	GM	0.1	0.17	0.17	0.14	0.03	0.03	0.03	0.03	1.78
	Range	0.03-4	0.03-2	0.03-2	0.03-1	≤0.03	≤0.03	≤0.03	≤0.03	0.5-16
	MIC_{90}	0.25	1	0.5	0.5	0.03	0.03	0.03	0.03	4
Diaporthe sclerotioides (4)	GM	0.06	0.21	2	0.5	0.04	0.03	0.03	0.03	4
	Range	0.03-0.12	0.12-0.25	1-4	0.5	0.03-0.06	≤0.03	≤0.03	≤0.03	0.5-16
	MIC_{90}	0.12	0.25	2	0.5	0.03	0.03	0.03	0.03	8
Pyrenochaetopsis leptospora (4)	GM	0.7	0.59	0.7	0.21	0.03	0.03	0.03	0.03	4
	Range	0.03-4	0.25-2	0.06-16	0.03-1	≤0.03	≤0.03	≤0.03	≤0.03	0.5-16
	MIC_{90}	2	1	1	0.5	0.03	0.03	0.03	0.03	16
Overall (85)	GM	0.33	0.61	1	0.46	0.06	0.06	0.06	0.04	2.9
	Range	0.03-8	0.03-16	0.03-16	0.03-16	0.03-8	0.03-4	0.03-8	0.03-2	0.25-32
	MIC_{90}	2	4	16	16	0.25	0.5	2	0.03	16

[a] GM, geometric mean; MIC_{90}, drug concentration that inhibited 90% of isolates.

[b] AMB, amphotericin B;VRC, voriconazole; ITC, itraconazole; PSC, posaconazole; AFG, anidulafungin; CFG, caspofungin; MFG, micafungin; TRB, terbinafine; 5FC, flucytosine.