Title

Multilocus phylogeny and antifungal susceptibility of *Aspergillus* section *Circumdati* from clinical samples and description of *A. pseudosclerotiorum* sp. nov.

Running title

Clinical isolates of *Aspergillus* section *Circumdati*

Authors

J. P. Z. Siqueira[1,2], D.A. Sutton[3], J. Gené[1], D. García[1], N. Wiederhold[3], S. W. Peterson[4], J. Guarro[1]

[1]Unitat de Micologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain. [2]Laboratório de Microbiologia, Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, Brazil. [3]Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX, USA. [4]Mycotoxin Prevention and Applied Microbiology Research Unit, National Center for Agricultural Utilization Research, Peoria, IL, USA.

Abstract

A multilocus phylogenetic study was carried out to assess species identity of a set of 34 clinical isolates from *Aspergillus* section *Circumdati* from the USA, and to determine their *in vitro* antifungal susceptibility against eight antifungal drugs. The genetic markers used were ITS, *BenA*, *CaM* and *RPB2*, and the drugs tested were amphotericin B, itraconazole, posaconazole, voriconazole, anidulafungin, caspofungin, micafungin, and terbinafine. The most common species sampled was *A. westerdijkiae* (29.4%), followed by a novel species, which was described here as *A. pseudosclerotiorum* (23.5%). Other species identified were *A. sclerotiorum* (17.6%), *A. ochraceus* (8.8%), *A. subramanianii* (8.8%), and *A. insulicola* and *A. ochraceopetaliformis* with two

isolates (5.9%) each. The drugs that showed the most potent activity were caspofungin, micafungin, and terbinafine, while amphotericin B showed the least activity.

Introduction

Section *Circumdati* includes aspergilli with biseriate conidial heads in shades of yellow to ochre, with mostly globose vesicles, and sclerotia variable in shape and color (1–3). It contains 26 species (3), with *A. ochraceus* being the best known and described as an important producer of many extrolites, including the mycotoxin ochratoxin A (3–5). This metabolite has nephrotoxic, immunosuppressive, teratogenic, and carcinogenic properties (6, 7), and is commonly found in coffee, rice, beverages and other contaminated foodstuffs (3, 8). Several species in this section have been involved in different types of infections, such as: onychomycosis caused by *A. insulicola*, *A. melleus*, *A. ochraceopetaliformis*, *A. persii*, *A. sclerotiorum*, and *A. westerdijkiae* (9–14); otomycosis by *A. sclerotiorum* (15); skin infection by *A. westerdijkiae* (12); and pulmonary aspergillosis and osteomyelitis by *A. ochraceus* (16, 17). Moreover, *A. ochraceus*, *A. sclerotiorum*, and *A. westerdijkiae* have been repetitively isolated from clinical specimens of immunocompromised patients, although, in such cases, their pathogenic role is uncertain (18–22).

There are few data on the *in vitro* antifungal susceptibility of species within section *Circumdati*. The azoles, especially itraconazole, appear to have good activity against *A. ochraceus* and *A. sclerotiorum* (18, 23). In contrast, amphotericin B shows limited activity against species in this section (18, 23, 24), particularly against *A. westerdijkiae* (25).

Identification of *Aspergillus* species, traditionally based on morphological and physiological aspects (2), has changed recently with the use of DNA sequencing and multilocus analyses (26). Therefore, to assess the diversity of clinically relevant species

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within this section, a set of isolates with features characteristic of *Circumdati* section were identified molecularly. These clinical isolates were recovered between 2003 and 2015 in a USA reference laboratory. Moreover, the antifungal susceptibility of the most frequent species was determined against eight antifungal drugs.

Materials and Methods

Fungal isolates

A total of 34 *Aspergillus* isolates received from the Fungus Testing Laboratory at the University of Texas Health Science Center (San Antonio, USA) were investigated. Based on morphological features the isolates were identified as belonging to section *Circumdati*. Most of isolates studied were from human clinical specimens, mainly from the respiratory tract (n= 22, 64.7%), although other human clinical sources were noted as well (n= 8, 23.5%). In addition, four isolates were from marine animals (Table 1).

Morphological characterization

The isolates were characterized morphologically following the criteria recommended by Samson *et al.* (1). Briefly, colony morphology and growth rates were determined after 7 days of incubation on Czapek Yeast Autolysate agar (CYA; Becton, Dickinson and Company®, Sparks MD, USA) at 25ºC and 37ºC, and on Malt Extract agar (MEA; Pronadisa®, Madrid, Spain) at 25ºC. After 10 to 14 days of incubation, microscopic structures were examined and measured from MEA cultures, in wet mounts with 60% lactic acid and a drop of ethanol 70% to wash out the excess conidia. A minimum of 20 of each structure was measured in order to cover all the size ranges. Photographs were made using a Zeiss Axio Imager M1 light microscope (Zeiss, Oberkochen, Germany) with a mounted DeltaPix Infinity X digital camera, using Nomarski differential interference contrast and phase contrast optics.

DNA extraction, amplification, and sequencing

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Total genomic DNA was extracted from MEA cultures after 7 days of incubation at 25ºC, using the FastDNA® Kit and the FastPrep® Instrument (MP Biomedicals, Irvine CA, USA), according to the manufacturer's specifications. Four genetic markers were amplified, i.e., the internal transcribed spacer (ITS) region of the rRNA, which comprises ITS1, the 5.8S gene, and ITS2 regions, and fragments of the β-tubulin (*BenA*), calmodulin (*CaM*), and RNA polymerase II second largest subunit (*RPB2*) genes (1, 26). The primers used were ITS5 and ITS4 for the ITS region (27), Bt2a and Bt2b for *BenA* (28), Cmd5 and Cmd6 for *CaM* (29), and 5F and 7CR for *RPB2* (30). PCR products were sequenced in both directions, using the same primers, at Macrogen Europe (Macrogen Inc., Amsterdam, Netherlands). Sequences were assembled and edited using SeqMan v.7.0.0 (DNASTAR, Madison, WI, USA).

Molecular identification and phylogenetic analysis

Phylogenetic analyses were first performed individually for each gene. Since the topologies proved to be congruent with the incongruence length difference test (31), a concatenated analysis was performed. Sequences of the ex-type strains of all the species in section *Circumdati* were obtained from GenBank and added to the analyses. *Aspergillus tanneri* (section *Tanneri*) and *A. robustus* (section *Robusti*) were used as outgroups. In addition, GenBank sequences of two strains identified only as *Aspergillus* sp. (NRRL 35028 and NRRL 35026) were also added to the analyses because they formed a distinct lineage in section *Circumdati* (26). For multiple sequence alignment, ClustalW was used together with MUSCLE in MEGA v.6 (32), followed by manual adjustments. The Maximum Likelihood (ML) analysis was conducted with MEGA v.6, as well as to estimate of the best nucleotide substitution model. Support of the internal branches was assessed by the bootstrap method with 1,000 replications, where values ≥70 were considered significant. Bayesian Inference (BI) was performed using

MrBayes v.3.1.2 (33). The evolutionary model that best fit each gene was assessed by MrModelTest (34). Markov chain Monte Carlo (MCMC) sampling was performed with two simultaneous runs for 1 million generations, with samples taken every 100 generations. The 50% majority rule consensus trees and posterior probability values (pp) were calculated after removing the first 25% of the resulting trees for burn-in. A pp value of ≥0.95 was considered significant.

Antifungal susceptibility testing

Isolates of the most frequent *Aspergillus* species identified here were tested against eight antifungal drugs using the methods in the CLSI M38-A2 reference standard (35). The antifungal agents, obtained as pure powders, were amphotericin B (AMB) (Sigma Aldrich Quimica S.A., Madrid, Spain), itraconazole (ITC) (Jansen Pharmaceuticals, Beerse, Belgium), posaconazole (PSC) (Schering-Plough Res., Inst., NJ, USA), voriconazole (VRC) (Pfizer S.A., Madrid, Spain), anidulafungin (AFG) (Pfizer S.A., Madrid, Spain), caspofungin (CFG) (Merk & Co., Inc., Rahway, USA), micafungin (MFG) (Astellas Pharma, Madrid, Spain), and terbinafine (TBF). Minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that produced 100% inhibition of visible fungal growth for AMB and the azoles (ITC, PSC and VRC), and 80% for TBF. The minimum effective concentration (MEC) was determined for the echinocandins (AFG, CFG and MFG) and was defined microscopically as the lowest concentration of drug that permitted growth of small, rounded, compact hyphal forms, opposed to the long, unbranched hyphal clusters that were seen in the growth control. The quality control strain *Candida krusei* ATCC 6258 was used in each test and the MIC values were according to CLSI guidelines range. All tests were carried out in duplicate, on different days, to assess reproducibility. Statistical analyses were

performed using Prism software for Windows v.6.0 (GraphPad Software, San Diego, CA).

Nucleotide sequence accession numbers

Newly-generated sequences from this study were deposited in GenBank/EMBL databases under accession numbers listed on Table 1.

Results

Single gene analyses of sequences revealed similar topologies for all them, especially for the terminal branches. The ITS marker was the least informative, unable to discriminate among closely related species. However, the most basal clades could still be discerned in the analysis of this region, providing useful data in the concatenated tree. A limitation of the concatenated analysis that included all of the species in *Circumdati* section was the lack of *RPB2* sequences for the ex-type strains of *A. affinis*, *A. occultus*, *A. pulvericola*, *A. salwaensis*, *A. sesamicola*, and *A. westlandensis*. However, analyses of the other three markers, i.e. ITS, *BenA* and *CaM,* unequivocally demonstrated that none of the strains studied here corresponded to any of those above-mentioned species.

The final concatenated sequence alignment, with 58 strains and the 4 sequenced markers, consisted of 2451 base pairs (ITS, 482 bp; *BenA*, 470 bp; *CaM*, 481 bp; *RPB2*, 1018 bp), of which 941 sites were variable (ITS, 85; *BenA*, 250; *CaM*, 231; *RPB2*, 375) and 686 parsimony informative (ITS, 57; *BenA*, 182; *CaM*, 159; *RPB2*, 288). Topology trees inferred by the two phylogenetic methods were basically the same, with only minor differences in the support values of the internal nodes. The ML phylogenetic tree and the bootstrap and posterior probabilities values (Figure 1) show that 26 of the strains included in this study clustered with the ex-type strains of six species from section *Circumdati*, i.e. *A. westerdijkiae* (n = 10; 29.4%), *A. sclerotiorum* (n = 6;

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17.6%), *A. ochraceus* (n = 3; 8.8%), *A. subramanianii* (n = 3; 8.6%), *A. insulicola* (n = 2; 5.7%), and *A. ochraceopetaliformis* (n = 2; 5.9%). Interestingly, a group of eight isolates (25.7%) formed a well-supported clade together with sequences of two unidentified *Aspergillus* strains (NRRL 35028 and NRRL 35056). This clade represents an undescribed species, proposed here as *Aspergillus pseudosclerotiorum*.

The isolates examined here showed typical morphology of section *Circumdati* and matched those of the respective species. We found, however, that identification to the species level based only on phenotypic characteristics is difficult, but combining some of the phenotypic characteristics can make this feasible (Table 2). Among the species identified here, *A. westerdijkiae* and *A. ochraceus* were the only ones with finely roughened conidia; these two species could be distinguished from each other by the lack of or only slight growth at 37 ºC (0 to 9 mm) for *A. westerdijkiae*, while *A. ochraceus* reached 23 to 26 mm diam in 7 days at the same temperature. The other species identified here had smooth-walled conidia. In addition, *A. insulicola* was the only species that did not produce sclerotia but did produce a reddish brown soluble pigment on CYA; *A. subramanianii* showed good growth at 37 ºC (39 to 46 mm in 7 days); the colonies of *A. ochraceopetaliformis* had dense white mycelial areas and poor sporulation after 7 days; and *A. sclerotiorum* produced yellow (3A7) to brownish orange (6C3) colonies, which reached 56 to 58 mm diam in 7 days on CYA, with white sclerotia, abundant sporulation, and profuse growth at 37 ºC (32 to 36 mm). *Aspergillus pseudosclerotiorum* shares similar morphological features with *A. sclerotiorum* but with a slightly slower growth rate at 25ºC (45 to 55 diam in 7 days) and at 37ºC (22 to 38 mm), smaller metulae (3 to 9 by 2.5 to 6 µm, compared with 7 to 15 by 4 to 7 µm in *A. sclerotiorum*), and its sclerotia become yellow to orange yellow with age.

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In vitro susceptibility testing showed that the drugs with the most potent activity against all of the isolates tested were CFG, MFG and TBF, while AMB showed the lowest activity. The azoles (ITC, PSC, VRC), in general, showed good activity, with the exception of ITC against *A. sclerotiorum*. Interestingly, according to statistical analyses based on the Mann-Whitney test, the ITC MIC values showed significant differences between *A. sclerotiorum*, *A. ochraceus*, and *A. westerdijkiae* (GM of 11.31 µg/ml, 1.0 µg/ml, and 0.46 µg/ml, respectively; $p < 0.05$); however, differences were not significant between *A. sclerotiorum* and *A. pseudosclerotiorum* (0.89 µg/ml; $p = 0.06$) and *A. subramanianii* (4.0 µg/ml; $p = 0.43$). Regarding the new species, in general the drugs tested showed good activity against *A. pseudosclerotiorum*. Higher MIC values were observed only for AMB and VRC. Results of the *in vitro* susceptibility test are summarized in Table 3.

Taxonomy

Aspergillus pseudosclerotiorum J.P.Z. Siqueira, Deanna A. Sutton & Gené sp. nov. (MycoBank MB818572, Fig. 2). Etymology: the name refers to the morphological similarity with *A. sclerotiorum*. Holotype: USA, Pennsylvania, isolated from lung biopsy (human), D.A. Sutton, 2014 (CBS H-22808; cultures ex-type: UTHSCSA DI15-13, FMR 14449, CBS 141845).

Colonies on CYA at 7 days reached 45 to 55 mm diam at 25ºC; at 30º exhibited optimum growth, reaching 55 to 64 mm diam; at 37ºC reached 22 to 38 mm diam, and at 40ºC showed restricted growth. Colonies on CYA were pale yellow (3A3) to reddish white (7A3) at the center, white towards the periphery, cottony to floccose and usually granulose due to the presence of abundant sclerotia, margin fimbriate; reverse yellow (3A7) to greyish yellow (3B5); colorless exudates present in most isolates; little soluble pigment produced, yellow (3A6), or absent. On MEA colonies similar to CYA but with

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slower growth, reaching 34 to 42 mm at 7 days. On YES colonies showed fastest growth, reaching 56 to 66 mm at 7 days, white, cottony to floccose, with abundant sclerotia; reverse yellow (3A6) to greyish yellow (4B5), sulcate; exudates abundant, colorless to yellowish white (3A2). On DG18 colonies reaching 28 to 34 mm at 7 days, with white to light orange (5A4) compact center, and white fluffy mycelium towards periphery; reverse yellowish white (3A2) to pale yellow (3A3); sporulation sparsely produced only in age; sclerotia absent. On OA colonies reaching 24 to 27 mm at 7 days, yellowish white (3A2) to greyish yellow (4B4), sandy to dusty, with a more compact center, margin regular; reverse yellowish white (4A2) to greyish yellow (4B6). On CREA, colonies reaching 22 to 28 mm at 7 days, white, dense at the center, sparse aerial mycelium towards the periphery; acid production absent. Micromorphology consisting of conidiophores with biseriate and radiating conidial heads; stipes septate with rough walls, sub-hyaline to pale brown, 120 to 980 µm long by 2.5 to 8 µm wide; vesicles mainly globose, occasionally subglobose, 7 to 31 µm diam; metulae cylindrical, 3 to 9 by 2.5 to 6 µm, usually covering 100% of vesicle, with exception of the strain UTHSCSA DI16-383 which covered 75% of vesicle; phialides ampulliform, 4.5 to 8 by 1.25 to 3 µm; conidia globose, smooth-walled, 1.5 to 3 µm diam; sclerotia present (except in UTHSCSA DI16-380), 150 to 507 µm diam, white to light orange (5A4), becoming yellow (3A6) to orange yellow (4A6) in age.

Discussion

In this study we identified a total of six species in the section *Circumdati* from clinical samples, some of which contained a relatively high number of isolates. Although their role as etiologic agents in these cases is unknown, detection of 34 isolates of this section over a period of 12 years in a single reference center, together with some reports on infections produced by members of this section in the same period (15, 17, 18, 22, 36),

highlights the importance of these fungi in the clinical setting. The degree of morphological similarity among the species of *Circumdati* section, as with other groups of *Aspergillus*, requires DNA sequencing analysis for a definitive identification.

As was mentioned, the most common *Aspergillus* in the set of isolates studied here was *A. westerdijkiae*, a species described in 2004 and known to produce ochratoxin (37). It is noteworthy that the *A. ochraceus* strain from which ochratoxin A was discovered was later re-identified as *A. westerdijkiae*. This means that some isolates reported as *A. ochraceus*, especially the ones identified before 2004, may be in fact *A. westerdijkiae* (38). Growth rates at 37 ºC can be a useful feature to differentiate between these species without sequencing (3). *Aspergillus westerdijkiae* is commonly found in environmental samples (39), and as a food (40) and indoor contaminant (40–44). In the clinical setting, *A. westerdijkiae* has been linked to superficial infections (12) and isolated from sputum of immunocompromised patients in Tunisia (19). In our case, this species was mainly identified from respiratory specimens, but also from a nail and in a sample from a marine animal (Table 1).

It is worth noting that the second most frequent species identified in the present study was a novel one, *A. pseudosclerotiorum*. This species is closely related to *A. bridgeri*, *A. persii*, *A. salwaensis*, *A. sclerotiorum* and *A. subramanianii*. While these species could not be discriminated from each other using the ITS-based fungal barcode, *A. pseudosclerotiorum* was noted to have unique sequences for the other three markers (*BenA*, *CaM* and *RPB2*). Phenotypically, *A. pseudosclerotiorum* can generally be distinguished from the above-mentioned aspergilli by its growth rate on different media and temperatures, colony pigmentation and degree of sporulation, as well as sclerotia and conidiophores features. *Aspergillus bridgeri* produces brown colonies (3, 45); *A. persii* grows faster on oat-meal agar (35 to 38 mm diam in 7 days) and DG18 (45 to 50

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mm diam in 7 days) (3); *A. salwaensis* produces a characteristic yellowish orange soluble pigment and usually has conidiophores with vesicles flattened at the apex (3); *A. subramanianii* grows faster on CYA at 37 ºC (39 to 46 mm diam in 7 days); and *A. sclerotiorum* grows faster on CYA at 25ºC (54 to 57 mm diam in 7 days) and at 37 ºC (32 to 36 mm diam at 37 ºC), it shows a higher level of sporulation, and its sclerotia are white to cream colored. However, one of the eight isolates of *A. pseudosclerotiorum* (UTHSCSA DI16-380), which showed 99.6% similarity with the other isolates, produced atypical colonies (i.e. brownish and profusely sporulated). The size of metulae is also a diagnostic feature for *A. pseudosclerotiorum*, because they are smaller (3 to 9 by 2.5 to 6 µm) than those of the related species (6.5 to 10 by 3.5 to 5.5 µm in *A. bridgeri*; 9 to 17.5 by 4 to 7.5 µm in *A. persii*; 8 to 21 by 3.5 to 6 µm in *A. salwaensis*; 8 to 16 by 4.5 to 7 µm in *A. sclerotiorum*, 9 to 14 by 4 to 6.5 µm in *A. subramanianii*) (3). Although all isolates of *A. pseudosclerotiorum* were from the human respiratory tract (i.e., BAL fluid samples, sputum and lung tissue), further studies are needed to determine the pathogenic role of this new fungus.

The third most common species sampled was *A. sclerotiorum*, which has been reported to cause superficial infections, such as onychomycosis and otomycosis (10, 14, 15). Here, most of the isolates were also from the human respiratory tract. *Aspergillus sclerotiorum* is found worldwide, commonly isolated from soil, and reported as a species of biotechnological importance due to its ability to produce a wide range of compounds (46–48).

The best-known species in the section, *A. ochraceus*, was poorly represented in this study (8.8%). By contrast, it is commonly found on coffee, rice, dried fruits and nuts (8, 49, 50) and is capable of producing different metabolites (51–53). Previously, it was reported in pulmonary infections, based on morphological identifications (16, 20).

More recently, it has been identified in a case of osteomyelitis (17), and has also been isolated from immunocompromised patients (18, 19). Carpagnano et al. often found *A. ochraceus* in exhaled breath condensate of lung cancer patients (36). In other mammals, it was associated in a case of otomycosis in a dog (54). Here, the three isolates were from different clinical origins (i.e., BAL fluid, ear, and heart valve).

Of the three other species identified, *A. insulicola* and *A. ochraceopetaliformis* have been reported from cases of onychomycoses (9, 12), while *A. subramanianii* was recovered for the first time from clinical specimens. Concerning the latter species, it is noteworthy that two isolates (UTHSCSA DI16-378 and UTHSCSA DI16-389) formed a clade slightly separate from the other *A. subramanianii* isolates (Figure 1); however, the genetic identity (99.3%) with the ex-type strain and phenotypic similarity confirm their identification as *A. subramanianii*. This species could be considered as a potential agent of human infections because of its ability to grow at 37 ºC, and the deep tissue origin of the isolates (lung tissue and wound).

Data available on the *in vitro* susceptibility of section *Circumdati* aspergilli against antifungal drugs are limited to a few reports with a low number of isolates tested. Here, the three echinocandins and TBF exhibited potent activity against the fungi tested. Similar results were obtained in our previous study on *Aspergillus* section *Versicolores* (55). TBF has been also reported highly *in vitro* effective against clinically relevant *Aspergillus* species such as *A. flavus*, *A. niger*, *A. nidulans* or *A. terreus*, even against numerous isolates of *A. fumigatus sensu stricto* (56–58). To our knowledge, however, there is no previous information available on the activity of TBF against section *Circumdati* species. Results observed for echinocandins, especially MFG and AFG, could be expected since, in general, they have been reported *in vitro* effective on *Aspergillus* species (59, 60). Respect *Circumdati* aspergilli, Arabatzis et al. (18) tested

three echinocandins against two isolates of *A. ochraceus* and one of *A. sclerotiorum,* and reported high MICs only for CFG. By contrast, Gheith et al. (21) tested CFG against one isolate of *A. ochraceus* and one of *A. westerdijkiae* and reported low MICs, which is similar to our findings. AMB showed the least activity against the isolates tested, especially for *A. ochraceus*, *A. subramanianii*, and *A. westerdijkiae*. The high AMB MICs were also observed for species in section *Circumdati* (i.e. *A. melleus*, *A. ochraceous* and *A. pallidofulvus*) recently identified from human clinical specimens in India, in contrast to the results obtained in the same study for most isolates of *A. fumigatus*, *A. flavus* and *A. terreus* which were susceptible to antifungals tested there (60). PSC was the azole with the most potent activity against the strains tested, which agrees with Alastruey-Izquierdo et al. (25), Gheith et al. (21), and Masih et al. (60); however, the study of Arabatzis et al. (18) showed higher MICs for PSC. Recently, Babamahmoodi et al. (17) reported a case of osteomyelitis by *A. ochraceus*, for which the strain showed azole MICs (PSC, 0.032 µg/mL; VRC and ITC, 1.0 µg/ml) similar to ours (Table 3), and the patient improved after 4 months of treatment with VRC.

In conclusion, taxonomic studies are very important to assess the distribution of fungal species and their identity in clinical settings. In our study of clinical isolates within section *Circumdati* from a reference collection in the USA, we not only identified *A. subramanianii* for the first time associated with human specimens, but we also described a new taxon, *Aspergillus pseudosclerotiorum*, as one of the most frequent species of the section in this set of isolates. However, data from more isolates are needed to determine more reliable MICs of the different antifungal drugs against the species of this section, and to determine the pathogenic role of these fungi in human and animal infections.

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Fig 1 − Maximum likelihood tree obtained from anaylis of combined ITS, *BenA*, *CaM* and *RPB2* data set. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Fully supported branches (100/1) and ex-type strains are shown in bold. UTHSCSA, University of Texas Health Science Center (San Antonio, USA).

Fig 2 − Morphological features of *Aspergillus pseudosclerotiorum* sp. nov. (a to n, UTHSCA DI 15-13; o, UTHSCSA DI16-383). Panels: a, b, e, f, Front and reverse of colonies on CYA and MEA, respectively, after 7 days at 25 ºC; c, d, g, h, Front of colonies on DG18, OA, YES and CREA, respectively, after 7 days at 25 ºC; i, enlarged view of conidial heads on CYA after 7 days at 25 ºC; j, sclerotia on CYA after 14 days at 25 ºC; k, conidia; l, conidiophores and a sclerotium; m, detail of conidiophore stipe; n, o, detail of conidial heads. Scale bars: k, m, n, and o = 10 µm; l = 100 µm.

TABLE 1 – List of *Aspergillus* section *Circumdati* species, their isolate information and sequences generated in this study (in **bold**) and those retrieved from GenBank.

Species	Isolate number[a]	Origin[b]	Year	GenBank/EMBL accession number[c]			
				ITS	BenA	CaM	RPB2
A. affinis	ATCC MYA-4773^T			GU721090	GU721092	GU721091	
A. auricomus	NRRL 391^T			EF661411	EF661320	EF661379	EF661301
A. bridgeri	NRRL 13000^T			EF661404	EF661335	EF661358	EF661290
A. cretensis	NRRL 35672^T			FJ491572	AY819977	FJ491534	EF661311
A. elegans	NRRL 4850^T			EF661414	EF661349	EF661390	EF661316
A. fresenii	NRRL 407^T			EF661409	EF661341	EF661382	EF661296
A. insulicola	NRRL 6138^T			EF661430	EF661353	EF661396	EF661286
	UTHSCSA DI16-374	**Marine**	**2003**	**LT574681**	**LT574716**	**LT574751**	**LT574786**
	UTHSCSA DI16-402	**Marine**	**2009**	**LT574682**	**LT574717**	**LT574752**	**LT574787**
A. melleus	NRRL 5103^T			EF661425	EF661326	EF661391	EF661309
A. muricatus	NRRL 35674^T			EF661434	EF661356	EF661377	EF661314
A. neobridgeri	NRRL 13078^T			EF661410	EF661345	EF661359	EF661298
A. occultus	CBS 137330^T			KJ775443	KJ775061	KJ775239	
A. ochraceopetaliformis	NRRL 4752^T			EF661429	EF661350	EF661388	EF661283
	UTHSCSA DI16-387	**BAL**	**2006**	**LT574683**	**LT574718**	**LT574753**	**LT574788**
	UTHSCSA DI16-392	**Marine**	**2007**	**LT574684**	**LT574719**	**LT574754**	**LT574789**
A. ochraceus	NRRL 398^T			EF661419	EF661322	EF661381	EF661302
	UTHSCSA DI15-10	**BAL**	**2012**	**LT574686**	**LT574721**	**LT574756**	**LT574791**
	UTHSCSA DI15-11	**Heart valve**	**2013**	**LT574687**	**LT574722**	**LT574757**	**LT574792**
	UTHSCSA DI16-384	**Ear**	**2006**	**LT574685**	**LT574720**	**LT574755**	**LT574790**
A. ostianus	NRRL 420^T			EF661421	EF661324	EF661385	EF661304
A. pallidofulvus	NRRL 4789^T			EF661423	EF661328	EF661389	EF661306
A. persii	NRRL 35669^T			FJ491580	AY819988	FJ491559	EF661295
A. pseudoelegans	CBS 112796^T			FJ491590	AY819962	FJ491552	EF661282

A. pseudosclerotiorum	NRRL 35028			EF661407	EF661343	EF661362	EF661293
	NRRL 35056			EF661405	EF661344	EF661364	EF661294
	UTHSCSA DI15-13 [T]	**Lung biopsy**	**2014**	**LT574713**	**LT574748**	**LT574783**	**LT574818**
	UTHSCSA DI15-14	**BAL**	**2014**	**LT574714**	**LT574749**	**LT574784**	**LT574819**
	UTHSCSA DI15-15	**Lung tissue**	**2015**	**LT574715**	**LT574750**	**LT574785**	**LT574820**
	UTHSCSA DI16-373	**Sputum**	**2003**	**LT574707**	**LT574742**	**LT574777**	**LT574812**
	UTHSCSA DI16-380	**BAL**	**2006**	**LT574708**	**LT574743**	**LT574778**	**LT574813**
	UTHSCSA DI16-383	**BAL**	**2006**	**LT574709**	**LT574744**	**LT574779**	**LT574814**
	UTHSCSA DI16-385	**Sputum**	**2006**	**LT574710**	**LT574745**	**LT574780**	**LT574815**
	UTHSCSA DI16-386	**Lung mass**	**2006**	**LT574711**	**LT574746**	**LT574781**	**LT574816**
A. pulvericola	CBS 137327 [T]			KJ775440	KJ775055	KJ775236	
A. robustus	NRRL 6362 [T]			EF661176	EU014101	EF661357	EF661033
A. roseoglobulosus	NRRL 4565 [T]			FJ491583	AY819984	FJ491555	EF661299
A. salwaensis	DTO 297B3 [T]			KJ775447	KJ775056	KJ775244	
A. sclerotiorum	NRRL 415 [T]			EF661400	EF661337	EF661384	EF661287
	UTHSCSA DI15-12	**Sputum**	**2014**	**LT574693**	**LT574728**	**LT574763**	**LT574798**
	UTHSCSA DI16-395	**Sputum**	**2007**	**LT574688**	**LT574723**	**LT574758**	**LT574793**
	UTHSCSA DI16-398	**BAL**	**2008**	**LT574689**	**LT574724**	**LT574759**	**LT574794**
	UTHSCSA DI16-404	**Sputum**	**2009**	**LT574690**	**LT574725**	**LT574760**	**LT574795**
	UTHSCSA DI16-399	**BAL**	**2009**	**LT574691**	**LT574726**	**LT574761**	**LT574796**
	UTHSCSA DI16-409	**Eye**	**2014**	**LT574692**	**LT574727**	**LT574762**	**LT574797**
A. sesamicola	CBS 137324 [T]			KJ775437	KJ775063	KJ775233	
A. steynii	NRRL 35675 [T]			EF661416	EF661347	EF661378	JN121428
A. subramanianii	NRRL 6161 [T]			EF661403	EF661339	EF661397	EF661289
	UTHSCSA DI16-378	**Lung tissue**	**2005**	**LT574694**	**LT574729**	**LT574764**	**LT574799**
	UTHSCSA DI16-389	**Wound**	**2006**	**LT574695**	**LT574730**	**LT574765**	**LT574800**
	UTHSCSA DI16-390	**Foot**	**2006**	**LT574696**	**LT574731**	**LT574766**	**LT574801**
A. tanneri	NRRL 62425 [T]			JN853798	JN896582	JN896583	JN896585

A. westerdijkiae	NRRL 3174 [T]			EF661427	EF661329	EF661360	EF661307
	UTHSCSA DI15-5	**BAL**	**2014**	**LT574703**	**LT574738**	**LT574773**	**LT574808**
	UTHSCSA DI15-6	**Sputum**	**2014**	**LT574704**	**LT574739**	**LT574774**	**LT574809**
	UTHSCSA DI15-7	**Nail**	**2015**	**LT574705**	**LT574740**	**LT574775**	**LT574810**
	UTHSCSA DI15-8	**Marine**	**2011**	**LT574706**	**LT574741**	**LT574776**	**LT574811**
	UTHSCSA DI16-376	**Unknown**	**2004**	**LT574697**	**LT574732**	**LT574767**	**LT574802**
	UTHSCSA DI16-377	**Unknown**	**2004**	**LT574698**	**LT574733**	**LT574768**	**LT574803**
	UTHSCSA DI16-379	**BAL**	**2005**	**LT574699**	**LT574734**	**LT574769**	**LT574804**
	UTHSCSA DI16-388	**Lung mass**	**2006**	**LT574700**	**LT574735**	**LT574770**	**LT574805**
	UTHSCSA DI16-391	**Lung nodule**	**2007**	**LT574701**	**LT574736**	**LT574771**	**LT574806**
	UTHSCSA DI16-393	**Sputum**	**2007**	**LT574702**	**LT574737**	**LT574772**	**LT574807**
A. westlandensis	CBS 137321 [T]			KJ775434	KJ775066	KJ775230	

[a]ATCC, American Type Culture Collection; CBS, CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands); DTO, Applied and Industrial Mycology Department Collection (Utrecht, Netherlands); NRRL, Agriculture Research Service Culture Collection (Peoria, USA); UTHSCSA, University of Texas Health Science Center (San Antonio, USA). [T], ex-type strain.
[b]BAL, fluid, bronchoalveolar lavage fluid specimens.
[c]ITS: internal transcribed spacer regions of the rDNA and 5.8S region; *BenA*: β-tubulin; *CaM*: calmodulin; *RPB2*: partial RNA polymerase II second largest subunit.

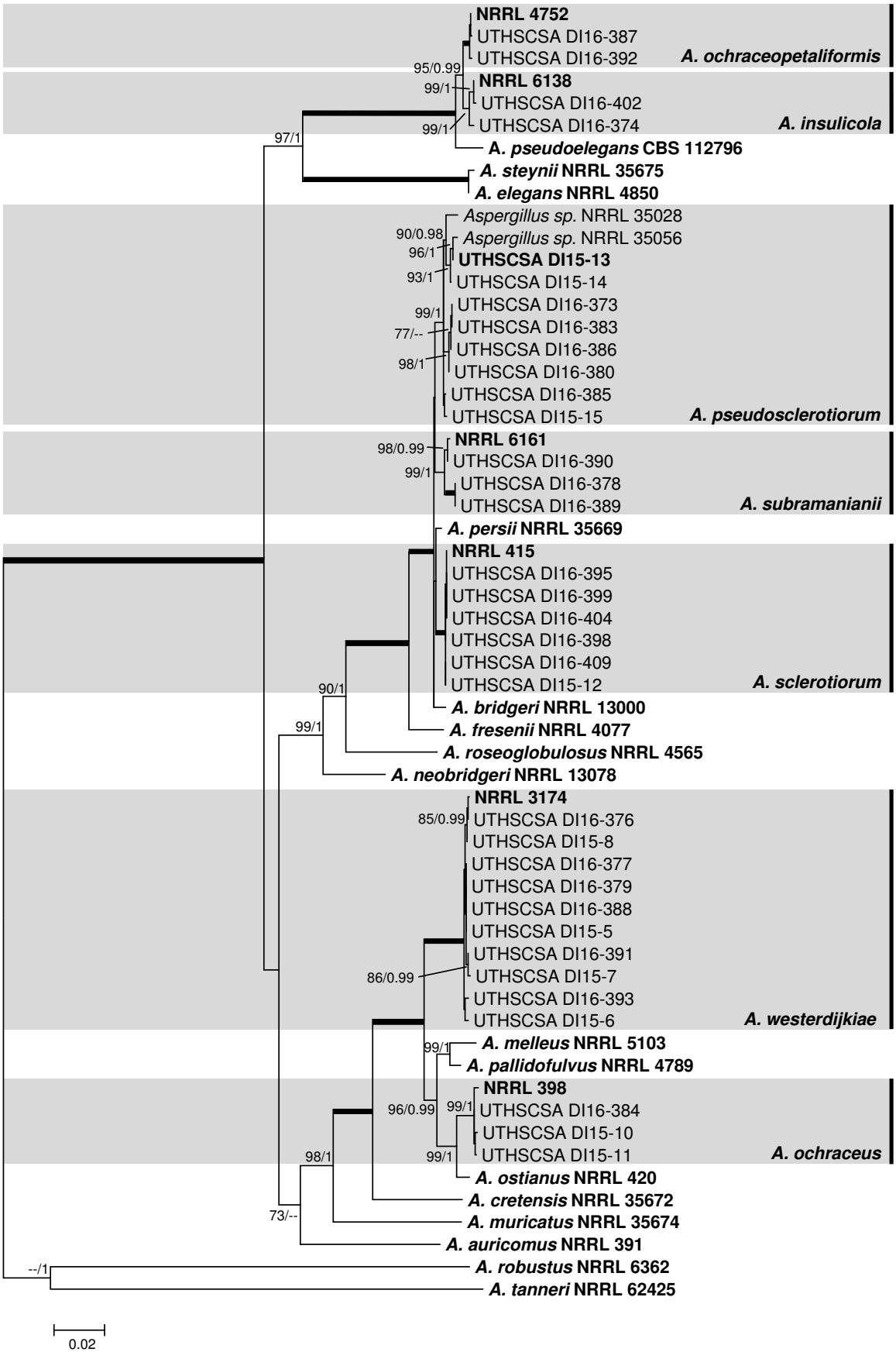
TABLE 2 – Key morphological features of *Aspergillus* section *Circumdati* species identified in this study

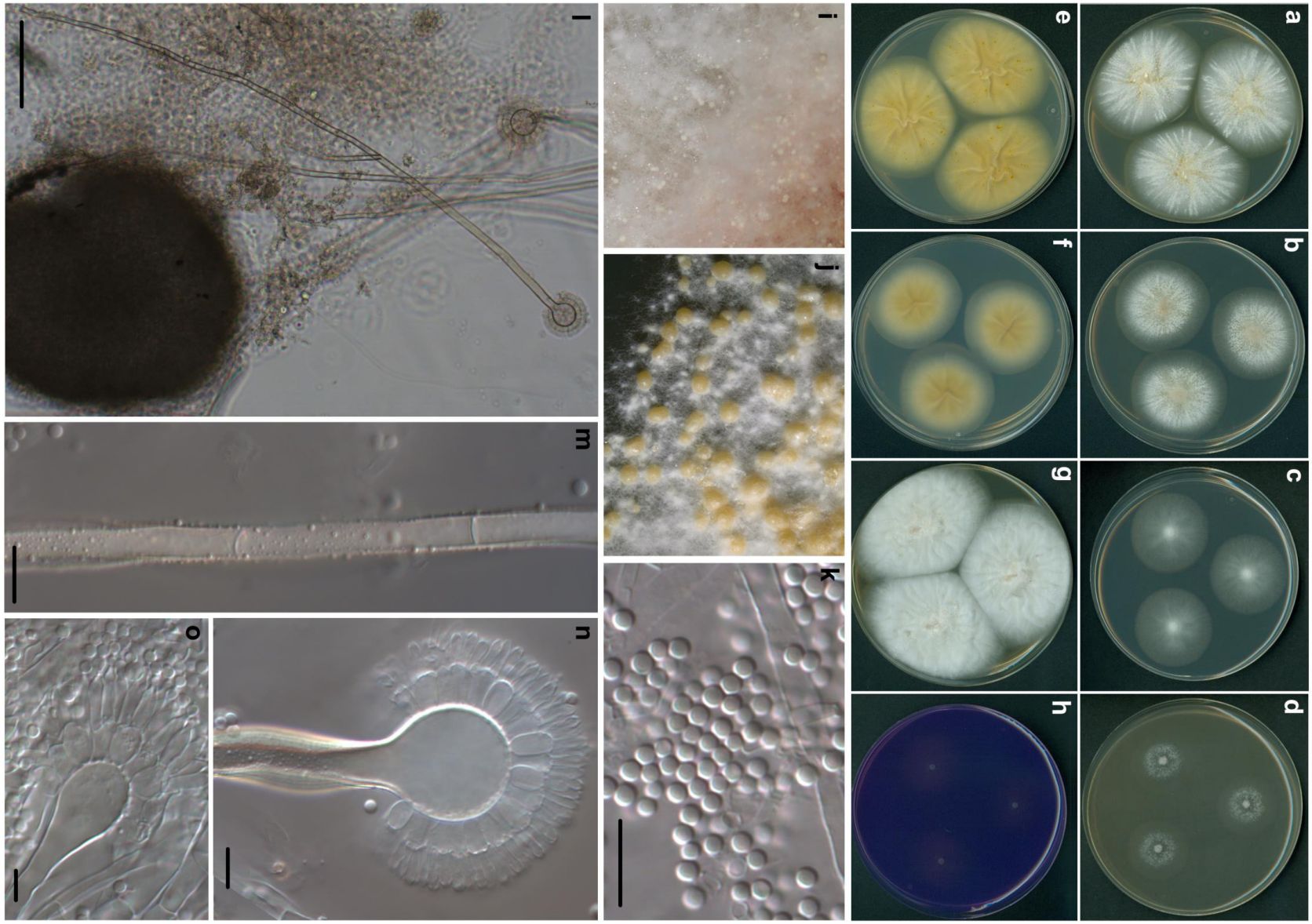
Species	Sclerotia	Metulae (µm)	Conidial ornamentaion	Colony diameter (mm) in 7 days	
				CYA 25 ºC	CYA 37 ºC
A. insulicola	absent	6.5–12 x 3–5	smooth	46–49	14–15
A. ochraceopetaliformis	present	9–18 x 3.5–6	smooth	38–46	27–29
A. ochraceus	present	7–14 x 3–6	finely roughened	44–49	23–26
A. pseudosclerotiorum	present	3–9 x 2.5–6	smooth	45–55	22–38
A. sclerotiorum	present	7–15 x 4–7	smooth	56–58	32–36
A. subramanianii	present	8.5–14 x 3.5–6.5	smooth	52–53	39–46
A. westerdijkiae	present	8–18 x 4–7	finely roughened	41–51	0–9

TABLE 3 − Results of *in vitro* antifungal susceptibility test for 30 isolates of *Aspergillus* section *Circumdati*

Species (no. of isolates)		MIC or MEC (µg/ml) for:							
		AMB	AFG	CFG	MFG	ITC	PSC	VRC	TBF
A. ochraceus	GM	16.0	0.25	0.04	0.03	1.0	0.31	2.0	0.03
(3)	MIC range	16.0	0.12–0.5	0.03–0.06	0.03	1.0	0.25–0.5	2.0	0.03
	Mode	16.0	0.5	0.03	0.03	1.0	0.25	2.0	0.03
A. subramanianii	GM	>16.0	0.10	0.03	0.03	4.0	0.79	4.0	0.03
(3)	MIC range	16.0–>16.0	0.03–0.25	0.03	0.03	4.0	0.5–1.0	4.0	0.03
	Mode	>16.0	0.25	0.03	0.03	4.0	1.0	4.0	0.03
A. sclerotiorum	GM	4.76	0.03	0.04	0.03	11.31	1.0	3.36	0.03
(6)	MIC range	4.0–8.0	0.03	0.03–0.06	0.03	4.0–>16.0	1.0	2.0–4.0	0.03
	Mode	4.0	0.03	0.03	0.03	>16.0	1.0	4.0	0.03
A.pseudosclerotiorum	GM	5.04	0.04	0.03	0.03	0.89	0.25	1.41	0.03
(8)	MIC range	2.0–>16	0.03–0.12	0.03–0.06	0.03	0.25–>16.0	0.12–0.5	1.0–2.0	0.03
	Mode	4.0	0.03	0.03	0.03	0.5	0.25	2.0	0.03
A. westerdijkiae	GM	>16.0	0.14	0.03	0.03	0.46	0.29	1.08	0.03
(10)	MIC range	>16.0	0.03–1.0	0.03–0.06	0.03–0.06	0.12–1.0	0.12–0.5	1.0–2.0	0.03
	Mode	>16.0	0.25	0.03	0.03	0.5	0.25	1.0	0.03
	MIC90	>16.0	0.5	0.06	0.06	0.5	0.5	1.0	0.03
Total	GM	12.82	0.08	0.03	0.03	1.28	0.39	1.74	0.03
(30)	MIC range	2.0–>16.0	0.03–1.0	0.03–0.06	0.03–0.06	0.12–>16.0	0.12–1.0	1.0–4.0	0.03
	Mode	>16.0	0.03	0.03	0.03	0.5	0.25	1.0	0.03
	MIC90	>16.0	0.5	0.06	0.03	4.0	1.0	4.0	0.03

AMB, amphotericin B; AFG, anidulafungin; CFG, caspofungin; MFG, micafungin; ITC, itraconazole; PSC, posaconazole; VRC, voriconazole; TBF, terbinafine; MIC, minimum inhibitory concentration; MEC, minimum effective concentration, for AFG, CFG, and MFG; GM, geometric mean.

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