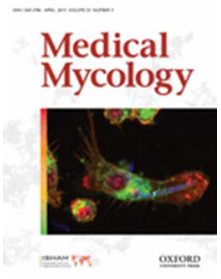

Species of Aspergillus section Aspergillus from clinical samples in USA

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Abstract:	The diversity of Aspergillus species in clinical samples is continuously increasing. Species under the former name Eurotium, currently accommodated in section Aspergillus of the genus, are xerophilic fungi widely found in the human environment and able to grow on substrates with low water activity. However, their prevalence in the clinical setting is poorly known. We have studied the presence of these species in a set of clinical samples from the USA using a multilocus sequence analysis based on the internal transcribed spacer (ITS) region of the rRNA,and fragments of the genes β-tubulin (BenA), calmodulin (CaM), and polymerase II second largest subunit (RPB2). A total of 25 isolates were studied and identified as follows:A. montevidensis (44%), A. chevalieri (36%), A. pseudoglaucus (8%), and A. costiformis (4%).A new species Aspergillus microperforatus is also proposed, which represented 8% of the isolates studiedand is characterized by uniseriate conidial heads, subglobose to pyriform vesicles, rough conidia, globose to subglose cleistothecia, and lenticular and smooth ascospores.The in vitro antifungal activity of 8 clinically available antifungals was also determined against these isolates,

	with the echinocandins and posaconazole having the most potent activity.

Title: Species of *Aspergillus* section *Aspergillus* from clinical samples in USA

Runing title: *Aspergillus* section *Aspergillus* in clinical setting

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Abstract

The diversity of *Aspergillus* species in clinical samples is continuously increasing. Species under the former name *Eurotium*, currently accommodated in section *Aspergillus* of the genus, are xerophilic fungi widely found in the human environment and able to grow on substrates with low water activity. However, their prevalence in the clinical setting is poorly known. We have studied the presence of these species in a set of clinical samples from the USA using a multilocus sequence analysis based on the internal transcribed spacer (ITS) region of the rRNA, and fragments of the genes β-tubulin (*BenA*), calmodulin (*CaM*), and polymerase II second largest subunit (*RPB2*). A total of 25 isolates were studied and identified as follows: *A. montevidensis* (44%), *A. chevalieri* (36%), *A. pseudoglaucus* (8%), and *A. costiformis* (4%). A new species *Aspergillus microperforatus* is also proposed, which represented 8% of the isolates studied and is characterized by uniseriate conidial heads, subglobose to pyriform vesicles, rough conidia, globose to subglose cleistothecia, and lenticular and smooth ascospores. The in vitro antifungal activity of 8 clinically available antifungals was also determined against these isolates, with the echinocandins and posaconazole having the most potent activity.

Introduction

The number of species of *Aspergillus* involved in human infections is continuously increasing, and most of these species have nowadays been identified using modern molecular techniques. Until recently, the dual nomenclature system permitted different names for the sexual and asexual forms of *Aspergillus*. One such example is the genus *Eurotium*, the name for the sexual state for species in the former *Aspergillus glaucus* group[1]. However, following the recent changes in fungal nomenclature[2,3] and based on phylogenetic studies[4,5], all generic names for sexual states of *Aspergillus* are now included under the name *Aspergillus,* and former species of *Eurotium* now comprise the aspergilli in the section *Aspergillus*[4]. A new approach to phylogenetic study supports the current wide range of *Aspergillus*[5], with *A. glaucus* (= *E. herbariorum*) being the type species of the genus.

Species in the section *Aspergillus* are usually osmophilic, with optimum growth on substrates with high sugar or salt concentrations. Commonly the asexual morph has smooth conidiophores, with uniseriate, radiate to somewhat columnar conidial heads, and ellipsoidal to globose echinulate conidia[1,6]. The sexual morph is usually characterized by globose to subglobose, thin-walled cleistothecia, eight-spored asci, and lenticular, smooth to rough-walled ascospores, generally showing an equatorial line or furrow[1,7]. These species are found worldwide, and often on organic materials, dust, and stored cereals and other food products[1,7]. Although these aspergilli are of minimal clinical importance, some, such as *A. glaucus*, has been reported in orofacial[8] and brain infections[9]. In addition, *A. montevidensis* has been involved in cases of otitis, mycetoma, cerebral abscess, keratitis, and pulmonary infections[10] and *A. glaucus* and *A. montevidensis* can also cause hypersensitive pneumonitis[11,12]. *Aspergillus chevalieri* and *A. pseudoglaucus* have been linked to cutaneous aspergillosis[13] and maxillary sinusitis[14], respectively. Hubka et al.[15] recovered five species of section *Aspergillus* among isolates from probable cases of superficial infections (e.g., skin

and nails), including *A. montevidensis*, *A. costiformis*, *A. pseudoglaucus*, *A. proliferans*, and *A. ruber* in Czech Republic. The antifungal susceptibility patterns of members of section *Aspergillus* are largely unknown[16], with little published data. Masih et al.[17] demonstrated potent activity of posaconazole, anidulafungin, and micafungin against 3 strains of *A. montevidensis* (GM MICs of 0.04 µg/mL, 0.015 µg/mL, and 0.015 µg/mL, respectively) and one of *A. chevalieri* (MICs of 0.015 µg/mL, 0.03 µg/mL, and 0.015 µg/mL, respectively). García-Martos et al.[18] also demonstrated low MIC values for amphotericin B, itraconazole, and voriconazole against 3 strains of *A. glaucus* (MIC ranges of 0.125–0.5 µg/mL, 0.25–0.5 µg/mL, and 0.125–0.25 µg/mL, respectively) and 2 strains of *A. chevalieri* (MIC ranges of 0.125–0.5 µg/mL, 0.125–0.25 µg/mL, and 0.125–0.25 µg/mL, respectively). Wildfeuer et al.[19] tested 8 strains of *A. glaucus* against 4 drugs and observed that itraconazole exhibited the most potent activity (GM MIC of 0.39 µg/mL).

In order to assess the diversity of species from *Aspergillus* section *Aspergillus* in the clinical setting and to observe their response to antifungal drugs, the aim of this study was to identify to the species level a set of clinical isolates from the USA using a multilocus phylogenetic study, and to determine the susceptibility pattern of 8 clinically available antifungals against these species.

Materials and Methods

Fungal isolates

A total of 25 isolates of section *Aspergillus* were investigated in this study. Most of them were from human clinical samples, primarily from the respiratory tract (BAL, sputum, and sinus), but also in fewer numbers from corneas, nails, stool, and lymph nodes. One of them was of environmental origin and the origin of four were unknown (Table 1). These isolates were received at the Fungus Testing Laboratory of the University of Texas Health Science

Center (San Antonio, TX, USA) from different institutions across the USA over a period of 11 years (2004-2015), for identification and/or antifungal susceptibility testing.

Morphological characterization

The morphology of the fungi was characterized by the traditional criteria[4,20]. Briefly, this is determined after 7 days of incubation on Czapek Yeast Autolysate agar (CYA, Becton, Dickinson and Company®, Sparks MD, USA), CYA supplemented with 20% sucrose (CY20S), and Malt Extract agar (MEA, Pronadisa®, Madrid, Spain) at 25 ºC; and CY20S and Harrold's Agar containing 60% sucrose[1] (M60Y) at 37 ºC. Colors match Kornerup & Wanscher[21]. Microscopic features were examined and measured on MEA and CY20S cultures, after 10–14 days of incubation, on wet mounts with 60% lactic acid and a drop of ethanol 70% to wash out the excess conidia. Photomicrographs were taken with a DeltaPix Infinity X digital camera mounted on a Zeiss Axio Imager M1 light microscope (Zeiss, Oberkochen, Germany), using a Nomarski differential interference contrast and phase contrast optics. Scanning electron microscope (SEM) photographs were taken with a Jeol JSM- 6400 using techniques described previously[22].

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from MEA cultures after 7 days of incubation at 25 ºC, using the FastDNA® Kit and the FastPrep® Instrument (MP Biomedicals, Irvine CA, USA), according to the manufacturer's specifications. After extraction, four different genetic regions were amplified for each strain[20,23]; i.e., the internal transcribed spacer (ITS) region of the rRNA, comprising ITS1, 5.8S gene, and ITS2 regions, using ITS5 and ITS4 primers[24]; a portion of β-tubulin gene (*BenA*), using Bt2a and Bt2b primers[25]; a portion of calmodulin gene (*CaM*), using Cmd5 and Cmd6 primers[26]; and a portion of RNA polymerase II second largest subunit gene (*RPB2*), using 5F and 7CR primers[27]. PCR products were sequenced in both directions, using the same primers, at Macrogen Europe (Macrogen Inc., Amsterdam,

the Netherlands). Sequences were assembled and edited using SeqMan v.7.0.0 (DNASTAR, Madison, WI, USA).

Molecular identification and phylogenetic analysis

The phylogeny was analyzed first individually for each partition and then in a concatenated study, once the topologies proved to be congruent. To give support to our analyses, sequences of the ex-type strains of all species of the section *Aspergillus* obtained from GenBank were also included, and *A. halophilicus* (section *Restricti*) was used as the outgroup. To increase the robustness of the *A. pseudoglaucus* clade, sequences of 15 other strains of this species were additionally retrieved from GenBank and included in the analyses. A multiple sequence alignment was performed using ClustalW inside MEGA v.6 software[28]. When necessary, the MUSCLE tool and manual adjustments were used to refine the alignment. Maximum Likelihood (ML) was conducted with MEGA v.6 software, as well as the estimation of the best nucleotide substitution method. Support values of the internal branches were assessed using the Bootstrap method with 1,000 replications (values equal or higher than 70% were considered significant). Bayesian Inference (BI) was performed using MrBayes v.3.1.2 software[29]. The evolutionary models that best fit each partition were assessed by MrModel Test software[30]. 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. Values of 0.95 or higher were considered significant.

Antifungal susceptibility testing

The isolates were tested against eight antifungals, following the microdilution broth method[31]. The antifungal agents tested 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) (Sigma Aldrich Química S.A., Madrid, Spain). Readings were taken after 72 h to allow the strains to grow properly. Strains of *A. pseudoglaucus* (UTHSCSA DI15-17 and UTHSCSA DI16-410) and *A. microperforatus* (UTHSCSA DI16-400 and UTHSCSA DI16-407) were incubated at 30 ºC, while the others were incubated at 35 ºC to fit the growth requirements of the isolates under the CLSI protocol. Minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that produced 100% inhibition of visible fungal growth for the AMB and azoles (ITC, PSC and VRC) and 80% for TBF. For echinocandins (AFG, CFG, and MFG), the minimum effective concentrations (MEC) were determined microscopically as the lowest concentration of drug that allowed the growth of small, rounded, compact hyphal forms, as opposed to the long, unbranched hyphal clusters that are seen in the growth control. *Candida krusei* ATCC 6258 was used as the quality control strain in each test and the MIC values were within the acceptable MIC range per the CLSI standard. All tests were carried out in duplicate, on different days, for reproducibility. Statistical analyses of the results were performed using the Prism software for Windows v.6.0 (GraphPad Software, San Diego, CA).

Nucleotide sequence accession numbers and alignments

Newly-generated sequences from this study have been deposited in GenBank/EMBL databases under the accession numbers listed on Table 1. The alignments were deposited in TreeBASE (submission number S20583).

Results

In the present study, as expected, the ITS region was the least informative marker, being unable to discriminate some of the species included in the analysis (Figure S1 in supplemental material). Two main clades were defined using this genetic marker, one grouping the species *A. chevalieri*, *A. intermedius*, *A. montevidensis*, *A. cristatus*, and *A. costiformis,* and the second one grouping the species *A. pseudoglaucus*, *A. glaucus*, *A. neocarnoyi*, *A. niveoglaucus*, *A. brunneus*, *A. proliferans*, *A. ruber*, *A. appendiculatus*, *A. cibarius*, *A. tonophilus*, and *A. sloanii*. The other markers (*BenA*, *CaM*, and *RPB2*) were more informative, with better delineation in well-supported monophyletic groups (Figures S2–S4 in supplemental material). The single phylogenetic analysis corresponding to the different genes showed very similar tree topologies, and a concatenated study was performed. The final concatenated sequence alignment consisted of 2,653 bases (ITS, 641; *BenA*, 433; *CaM*, 596; *RPB2*, 983), of which 794 were variable sites (ITS, 105; *BenA*, 176; *CaM*, 255; *RPB2*, 258) and 461 parsimony informative (ITS, 31; *BenA*, 104; *CaM*, 160; *RPB2*, 166). The ML tree (Figure 1) shows significant support values for both phylogenetic methods (bootstrap/posterior probabilities).

The clinical isolates grouped together with the following species: *A. montevidensis* (11 isolates, 44%), *A. chevalieri* (9 isolates, 36%), and *A. pseudoglaucus* (2 isolates, 8%). The environmental isolate was identified as *A. costiformis* (4%). The isolates UTHSCSA DI16-400 (= CBS 142377) and UTHSCSA DI16-407 (= CBS 142376), from toenail and lymph node samples, respectively, and sequences of two environmental isolates (CCF 5387 and CCF 5388) retrieved from GenBank, formed a full-supported clade close to the *A. pseudoglaucus* clade, which represents an undescribed phylogenetic lineage for the section *Aspergillus*. Therefore, we propose the new species *A. microperforatus*.

The morphology of the isolates shows the expected phenotypic characters that agree with the previous species descriptions[1,4,32–34]. *Aspergillus montevidensis* exhibits rough ascospores,

with irregular crests; *A. chevalieri* shows smooth ascospores, with prominent crests; *A. costiformis* is the only species with smooth conidia; and *A. pseudoglaucus* and *A. microperforatus* demonstrated smooth ascospores, with no crests, and rough conidia. In fact, these last two species have a similar morphology, being differentiated by the slow growth and restricted sporulation of the novel species on CYA at 25 ºC and on M60Y at 37 ºC and the absence of soluble pigment in any of the culture media tested. *Aspergillus pseudoglaucus* isolates identified here grew and sporulated well on both media and temperatures, and produced a brownish soluble pigment on CYA at 25 ºC in 14 days of incubation. Table 2 shows the key phenotypic features of the species of *Aspergillus* section *Aspergillus* already reported in clinic, including those recovered in this study.

In general, all isolates were inhibited by each of the antifungal drugs tested, with overall geometric mean (GM) values lower than 1.0 µg/ml. The most potent activity was observed with the echinocandins (GM of 0.03 µg/ml), while VRC had the highest MIC values (GM of 1.0 µg/ml for *A. pseudoglaucus*, and 0.77 µg/ml for *A. montevidensis*, with individual values up to 2.0 µg/ml). The results of the *in vitro* susceptibility test are summarized in Table 3.

Taxonomy

Aspergillus microperforatus J.P.Z. Siqueira, Deanna A. Sutton & Gené sp. nov. (MycoBank MB 820080, Fig. 2).

Colonies on CYA 8–15 mm diam in 7 days at 25 ºC, floccose, yellowish white (3A2) at the center, white towards the periphery, sporulation scarce, margin entire; reverse pale (2A2) to olive (3A3); exudate and soluble pigment absent. On CY20S, colonies 30–45 mm diam in 7 days at 25 ºC, granulose due to the presence of ascomata, sporulation abundant, conidial masse greyish green (25E5); reverse brownish orange (7C5) to olive (3A6) at the centre, pale yellow (2A3) to yellow (2A6) towards the periphery; exudate and soluble pigment absent. On MEA, colonies 11 mm diam in 7 days at 25 ºC; sporulation absent in UTHSCSA DI16–407,

abundant in UTHSCSA DI16–400, with conidial masse brown (5A5), margin entire; reverse pale (2A2) to brownish orange (5C3); exudate and soluble pigment absent. On YES, colonies 33–40 mm in 7 days at 25 ºC, velutinous to downy, slightly granulose at the centre due to the presence of ascomata, sporulation abundant, with conidial masse dark green (27F5), margin entire; reverse pale yellow (4A3) to light yellow (4A4). On DG18, colonies 30–45 mm in 7 days at 25 ºC, fuzzy, white to light orange (5A5), sporulation abundant, conidial mass honey yellow (4D6); reverse light yellow (1A4) to yellow (3A7). On CREA, colonies up to 5 mm in 7 days at 25 ºC, acid production absent. No growth on OA at 25 ºC, or on CY20S at 37 ºC. Conidiophores up to 550 µm long, with uniseriate and radiating conidial heads; stipes occasionally septate, 260–500 x 6.5–9.5 µm, hyaline to subhyaline, smooth to finely roughened; vesicles subglobose to pyriform, 24–36 µm diam; phialides variable in shape and size, ampulliform to cylindrical, 7–18 (30) x 2–5 µm; conidia globose to elongate, sometimes pyriform, 6–9.5(–11) x 4.5–9 µm, in shades of brown, rough. Cleistothecia globose to subglobose, 90–130 µm diam, light yellow (2A5) to deep yellow (4A8); asci globose, 10–14 µm in diam; ascospores lenticular, 4–5.5 x 2.5–4.5 µm, hyaline, with a slight furrow in the equatorial region, convex surface smooth with very small pits only visible under SEM.

Etymology: Referring to the presence of small pits in the ascospore wall under SEM.

Type: USA, Texas, isolated from human lymph node, D.A. Sutton, 2011 (CBS H-22998 holotype; cultures ex-type: UTHSCSA DI16-407, CBS 142376, FMR 14071).

Discussion

Although the diversity of *Aspergillus* section *Aspergillus* species is well known in osmophilic substrates, house dust, indoor air or stored products, in the clinical setting it is poorly documented. As previously noted, the taxonomy and nomenclature of the species of section *Aspergillus* has recently changed. In addition to that, recent advances in molecular tools have allowed for the description of new cryptic species that are almost impossible to differentiate

using classical morphological tools[35]. Clinically, identification of *Aspergillus* isolates at the species level may be important given that susceptibilities to antifungal drugs vary for different species and that species identity can influence the choice of appropriate antifungal therapy[36]. In the present study, a total of 25 isolates of section *Aspergillus* were used, most of them being recovered from human respiratory specimens, although further studies are needed to elucidate the role of these fungi as pathogens. Five different species of the section have been identified here, including a novel one (i.e., *A. chevalieri*, *A. costiformis, A. microperforatus, A. montevidensis* and *A. pseudoglaucus*). To facilitate comparison, their key morphological features are presented in Table 2.

Aspergillus montevidensis was the most prevalent (44%). Clinically, this species may be the most relevant pathogen of this group because it has been isolated from different bodies sites, from superficial to deep tissue infections[10,15,37]. It was first reported and described from a case of human otomycosis[38]. This species currently includes strains that were formerly accepted as different but now considered conspecific. *Aspergillus hollandicus* (incorrectly associated to the sexual state *Eurotium amstelodami*), *A. heterocaryoticus*, and *A. vitis* are all synonyms of *A. montevidensis* and these names should no longer be used[4]. The nine isolates of *A. montevidensis* in the present study, with the exception of two of unknown origins, were from respiratory specimens (i.e. sputum, sinuses, and lung biopsies).

The second most prevalent species in the present study is *A. chevalieri* (36%), known until recently by its teleomorph name, *Eurotium chevalieri*. This species has been reported from a case of cutaneous aspergillosis[13] and more recently was the cause of fatal cerebral aspergillosis acquired by traumatic inoculation[17]. It is worth noting that *A. chevalieri* and *A. montevidensis* represent 80% of the isolates included in this study, in fact they are also some of the most commonly species found in indoor environments[4]. These two species, and the

others reported here, were able to grow well at 37 ºC in vitro (Table 2), the basic pathogenic feature that enables them to invade deep tissue[39].

The isolate of *A. costiformis* identified in this study is the third known strain of this species since its description. It was originally recovered from a moldy paper-box in China[34]. The second strain was isolated from a human nail in the Czech Republic[15], and the present study has recovered a third strain from hospital environment. The difficulty in the phenotypic characterization of this species might explain why it is rarely reported. The key characteristic that identifies *A. costiformis* is the presence of smooth conidia (Table 2) and the asexual morph is not usually formed in standard culture conditions. To overcome this problem, the production of conidial heads can be induced on M60Y at 37 ºC, as previously reported[4].

The remaining isolates included in this study were genetically and morphologically similar but they could be distinguished as two different species (each representing 8% of the isolates). Firstly, isolates UTHSCSA DI15-17 and UTHSCSA DI16-410 were identified as *A. pseudoglaucus*, a species described by Blochwitz in 1929[33]. This species was more recently delineated phylogenetically by Hubka et al.[4] in whose study other species were shown to be conspecific with *A. pseudoglaucus*, i.e. *A. glaucoaffinis*/*E. pseudoglaucum*[40], *A. glaber*[40], *A. fimicola*[41], and *A. reptans*/*E. repens*[40]. The phylogenetic tree constructed in the present study includes sequences of the ex-type strains of synonymous species and of numerous reference strains, giving more support to the *A. pseudoglaucus* clade. Our analysis shows that the genetic similarity among all those strains is 99.9% or higher in the concatenated alignment, in agreement with the proposal mentioned above. *Aspergillus pseudoglaucus* is commonly found in stored products[7] and produces metabolites that are potentially toxic[42]. There are few clinical reports involving *A. pseudoglacus*; it has been reported from a mixed infection in a case of maxillary sinusitis[14] and occasionally recovered from human skin and nails[15], although its pathogenicity has not been confirmed. The two isolates of *A. pseudoglaucus*

identified in the present study were from nasal and stool samples. Secondly, the isolates UTHSCSA DI16-400 and UTHSCSA DI16-407, recovered from toenail and lymph node, respectively, and sequences of two isolates retrieved from GenBank group in a clade close to *A. pseudoglaucus*. Although these latter isolates were labelled as *A. pseudoglaucus*, both phylogenetic methods (ML and BI) and the three most informative markers (*BenA*, *CaM*, and *RPB2*) all show that they represent together with our isolates investigated a distinct lineage in the section[4]. Thus, they have been proposed as the novel species *A. microperforatus*. The possible role of this species in the clinical disease is yet unknown. Although section *Aspergillus* includes some species that are closely related, some characteristics can be useful for discriminating *A. microperforatus* (Table 2). For example, the novel species shows restricted growth on CYA at 25 ºC (up to 15 mm), in contrast to *A. pseudoglaucus* (up to 24 mm), which also differs by growing better on M60Y at 37 ºC (41 to 46 mm, vs. 28 to 32 mm diam in *A. microperforatus*) and, according to our results, on CYA at 25º C it produces a diffusible brown pigment that is absent in *A. microperforatus*. The novel species can be differentiated from *A. glaucus* and *A. proliferans* by its ability to grow on M60Y at 37 ºC and *A. glaucus* has larger ascospores (6.0 to 7.5 µm, vs. 4.0 to 5.5 µm in *A. microperforatus*). Based on the descriptions and other reports, it might be difficult to differentiate the morphologies of *A. microperforatus* and *A. ruber* although the ascospores of *A. ruber* have an evident furrow and the conidia are usually ellipsoidal[1,4].

Although *A. glaucus* is a known opportunistic pathogen, being reported from many types of infections[8,9,11], it was not recovered in the present study, as was the case in a study of clinical aspergilli in the Czech Republic[15]. Therefore, it is possible that the clinical prevalence of this species has been overestimated, probably due to the limitations of diagnostic tools for *Aspergillus* identification and for filamentous fungi in general.

The CLSI have established epidemiological cut-off values for triazoles (ITC, VRC, and POS) and AMB for only six *Aspergillus* species, i.e., *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. nidulans*, and *A. versicolor*[43,44]. However, the limited number of isolates of other species available in different clinical laboratories precludes the determination of epidemiologic cutoff values, and members of section *Aspergillus* have only been rarely tested for antifungal susceptibility[16]. With few exceptions, the eight antifungals used in this study showed good activity against the aspergilli tested, with MIC values equal to or less than 1.0 µg/ml (Table 3). Recently, Masih et al.[17] provided the in vitro antifungal susceptibility profiles of rare *Aspergillus* species in clinical samples from India. Although they did not test TBF, the MIC values for the other seven antifungals against three isolates of *A. montevidensis* and one strain of *A. chevalieri* were similar to the values observed in the current study. Most available in vitro data is with *A. glaucus*. Wildfeuer et al.[21] and García-Martos et al.[22] include eight and three clinical isolates of *A. glaucus*, respectively, and both reported good activity for ITC (MIC range of 0.25–0.5 µg/ml), VRC (0.125–0.78 µg/ml), and AMB (0.125–1.56 µg/ml). Although we did not study any *A. glaucus* strains, these values are similar to the overall values observed for the strains tested here. However, our MIC values were slightly lower for AMB and higher for VRC. Furthermore, García-Martos et al.[21] also included two isolates of *A. chevalieri* and report the same MIC range (0.125–0.25 µg/ml) for ITC, VRC, and AMB. These results are very similar to those found in our *A. chevalieri* isolates, with the exception of one isolate that had an ITC MIC of 1.0 µg/ml.

In summary, this study has assessed the species diversity of *Aspergillus* section *Aspergillus* from a set of clinical isolates from the USA and demonstrated that *A. montevidensis* and *A. chevalieri* were the most frequently identified species. We also describe *A. microperforatus* as a new species. The antifungals tested showed potent activity against these isolates, especially the echinocandins and PSC.

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Fig 1 − Maximum likelihood tree obtained from the combined ITS, *BenA*, *CaM* and *RPB2* sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. The new species is shown in the colored box.

Fig 2 − Morphological features of *Aspergillus microperforatus* sp. nov. (UTHSCSA DI16-407). Panels: a, b, c, f, g, h, front and reverse of colonies on CY20S, DG18, and YES, respectively, after 7 days at 25 ºC; d, e, front of colonies on CYA, and MEA, respectively, after 7 days at 25 ºC; i, front of colonies on M60Y after 7 days at 37 ºC; j, front of colonies on CYA after 14 days at 25º; k, l, ascoma; m, n, asci; o, p, ascospores; q, r, s, conidial heads; t, u, conidia. Scale bars: k, 100 µm, l–u, 10 µm.

TABLE 1 – Origins, year of isolation, and GenBank/EMBL accession numbers of the *Aspergillus* strains included in this study

Species	Isolate number	Origin	Year	GenBank/EMBL accession number			
				ITS	*BenA*	*CaM*	*RPB2*
A. chevalieri (9)	UTHSCSA DI15-18	BAL	2014	LT627247	LT627272	LT627297	LT627322
	UTHSCSA DI16-375	Sputum	2004	LT627248	LT627273	LT627298	LT627323
	UTHSCSA DI16-381	BAL	2006	LT627249	LT627274	LT627299	LT627324
	UTHSCSA DI16-382	BAL	2008	LT627250	LT627275	LT627300	LT627325
	UTHSCSA DI16-394	BAL	2007	LT627251	LT627276	LT627301	LT627326
	UTHSCSA DI16-396	Corneal	2008	LT627252	LT627277	LT627302	LT627327
	UTHSCSA DI16-397	Sinus	2008	LT627253	LT627278	LT627303	LT627328
	UTHSCSA DI16-413	Unknown	2008	LT627254	LT627279	LT627304	LT627329
	UTHSCSA DI16-414	Unknown	2008	LT627255	LT627280	LT627305	LT627330
A. costiformis (1)	UTHSCSA DI15-16	Environmental	2014	LT627256	LT627281	LT627306	LT627331
A. microperforatus (2)	UTHSCSA DI16-400	Toe nail	2009	LT627270	LT627295	LT627320	LT627345
	UTHSCSA DI16-407	Lymph node	2011	LT627271	LT627296	LT627321	LT627346
A. montevidensis (11)	UTHSCSA DI15-19	Ethmoid sinus	2014	LT627257	LT627282	LT627307	LT627332
	UTHSCSA DI15-20	Sputum	2014	LT627258	LT627283	LT627308	LT627333
	UTHSCSA DI15-21	BAL	2015	LT627259	LT627284	LT627309	LT627334
	UTHSCSA DI15-22	Sputum	2015	LT627260	LT627285	LT627310	LT627335
	UTHSCSA DI16-401	Lung tissue	2009	LT627261	LT627286	LT627311	LT627336
	UTHSCSA DI16-403	Sputum	2009	LT627262	LT627287	LT627312	LT627337
	UTHSCSA DI16-405	Sinus	2010	LT627263	LT627288	LT627313	LT627338
	UTHSCSA DI16-406	Lung tissue	2010	LT627264	LT627289	LT627314	LT627339
	UTHSCSA DI16-408	Paranasal	2013	LT627265	LT627290	LT627315	LT627340
	UTHSCSA DI16-411	Unknown	2008	LT627266	LT627291	LT627316	LT627341
	UTHSCSA DI16-412	Unknown	2008	LT627267	LT627292	LT627317	LT627342
A. pseudoglaucus (2)	UTHSCSA DI15-17	Nasal	2011	LT627268	LT627293	LT627318	LT627343
	UTHSCSA DI16-410	Stool	2014	LT627269	LT627294	LT627319	LT627344

UTHSCSA: University of Texas Health Science Center (San Antonio, USA); 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 – Relevant features of the *Aspergillus* section *Aspergillus* already reported in clinical setting.

	Growth rate (mm) at 7d				Ascospores			Conidia		
	25ºC		37ºC							
Species	CYA	CY20S	CY20S	M60Y	Long axis (µm)	Equatorial region	Ornamentation	Size (µm)	Ornamentation	References
A. chevalieri	16–24	45–65	30–49	65–>70	4.5–6.5	crests prominent	smooth	3.5–5.5	rough	this study
A. costiformis	18–21	33–38	36–39	>70	6–8	crests irregular	rough	4–8 (12)	smooth	this study
A. glaucus	3–20	30–45	0	0	6–7.5	crests absent	smooth	4.5–8.5	rough	[1, 4]
A. microperforatus	8–15	40–46	0	28–32	4–5.5	crests absent	smooth	6–9.5(11)	rough	this study
A. montevidensis	17–21	36–48	39–55	68–>70	3.5–5.5	crests irregular	rough	4.5–5.5	rough	this study
A. proliferans	n.a.	15–22	0	0	4.5–6	crests absent	smooth	5–9	rough	[1, 4]
A. pseudoglaucus	22–24	38–44	0	41–46	3.5–5.5	crests absent	smooth	7–8.5	rough	this study
A. ruber	n.a.	>30	0	n.a.	5–6	crests absent	smooth	5–7.5	rough	[1, 4]

CYA: Czapek yeast autolysate agar; MEA: malt extract agar; CY20S: CYA supplemented with 20% sucrose; M60Y: Harrold's agar; n.a.: not available.

TABLE 3 − Results of in vitro antifungal susceptibility test for 25 isolates of *Aspergillus* section *Aspergillus*

Species (no. of isolates)		MIC or MEC (μg/ml) for: AMB	AFG	CFG	MFG	ITC	PSC	VRC	TBF
A. chevalieri (9)	GM	0.14	0.03	0.03	0.03	0.24	0.03	0.37	0.09
	MIC range	0.06–0,5	0.03	0.03	0.03	0.12–1.0	0.03	0.12–0.5	0.03–0.12
	Mode	0.12	0.03	0.03	0.03	0.5	0.03	0.5	0.12
A. costiformis (1)	Values	0.25	0.03	0.03	0.03	0.25	0.06	0.5	0.12
A. microperforatus (2)	GM	0.03	0.03	0.03	0.03	0.12	0.03	1.0	0.06
	MIC range	0.03	0.03	0.03	0.03	0.12	0.03	1.0	0.06
A. montevidensis (11)	GM	0.25	0.03	0.03	0.03	0.19	0.03	0.77	0.13
	MIC range	0.12–0.5	0.03–0.06	0,03	0.03	0.12–0.5	0.03–0.06	0.5–2.0	0.06–0.25
	Mode	0.25	0.03	0.03	0.03	0.12	0.03	0.5	0.12
	MIC90	0.5	0.03	0.03	0.03	0.25	0.03	1.0	0.25
A. pseudoglaucus (2)	GM	0.03	0.03	0.03	0.03	0.12	0.03	1.0	0.12
	MIC range	0.03	0.03	0.03	0.03	0.12	0.03	1.0	0.12
Total (25)	GM	0.14	0.03	0.03	0.03	0.2	0.03	0.57	0.1
	MIC range	0.03–0.5	0.03–0.06	0.03	0.03	0.12–1.0	0.03–0.06	0.12–2.0	0.03–0.25
	Mode	0.12	0.03	0,03	0.03	0.12	0.03	0.5	0.12
	MIC90	0.5	0.03	0.03	0.03	0,5	0.03	1.0	0.12

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.

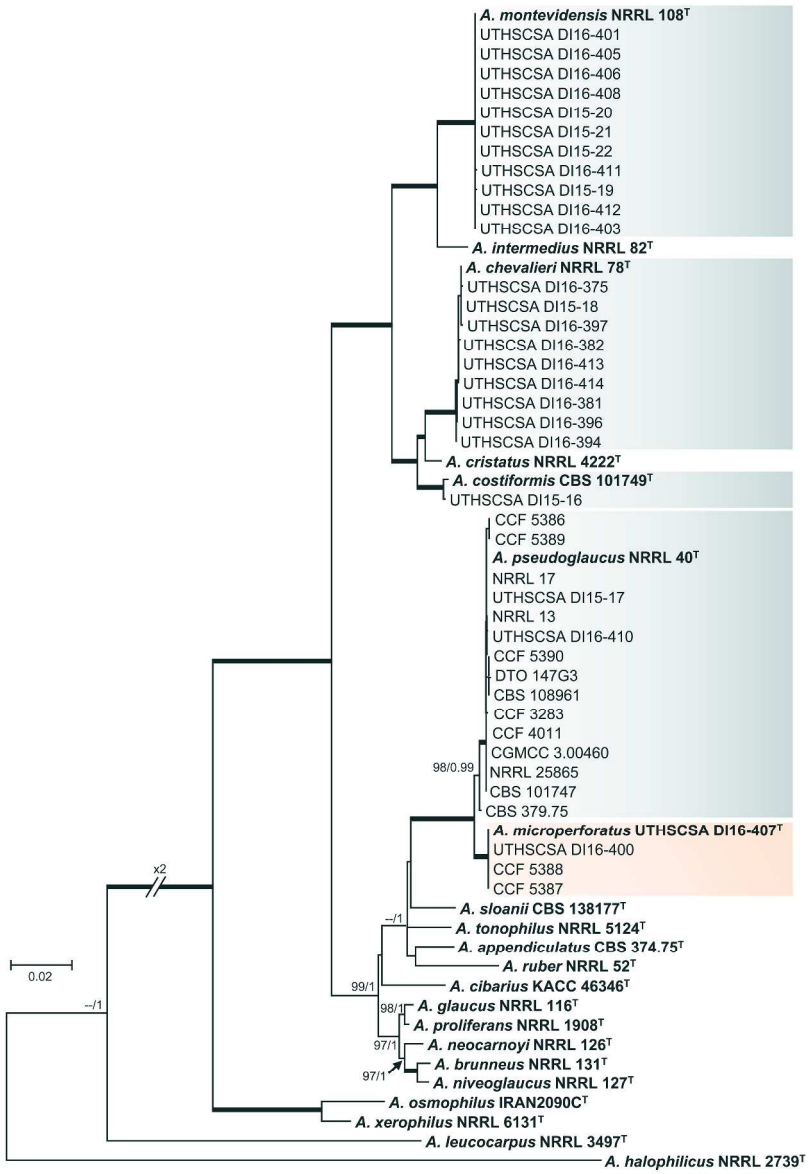
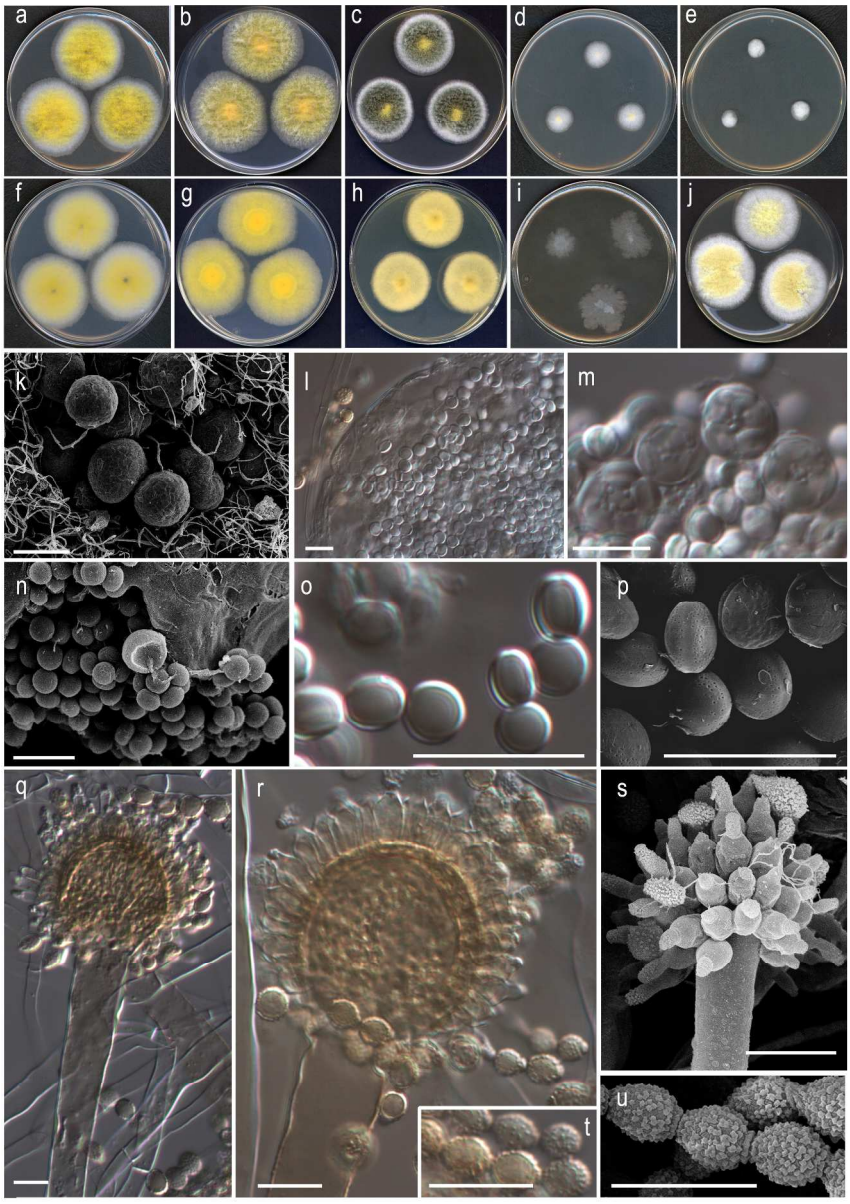


Fig 1 – Maximum likelihood tree obtained from the combined ITS, BenA, CaM and RPB2 sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. The new species is shown in the colored box.



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209x297mm (300 x 300 DPI)

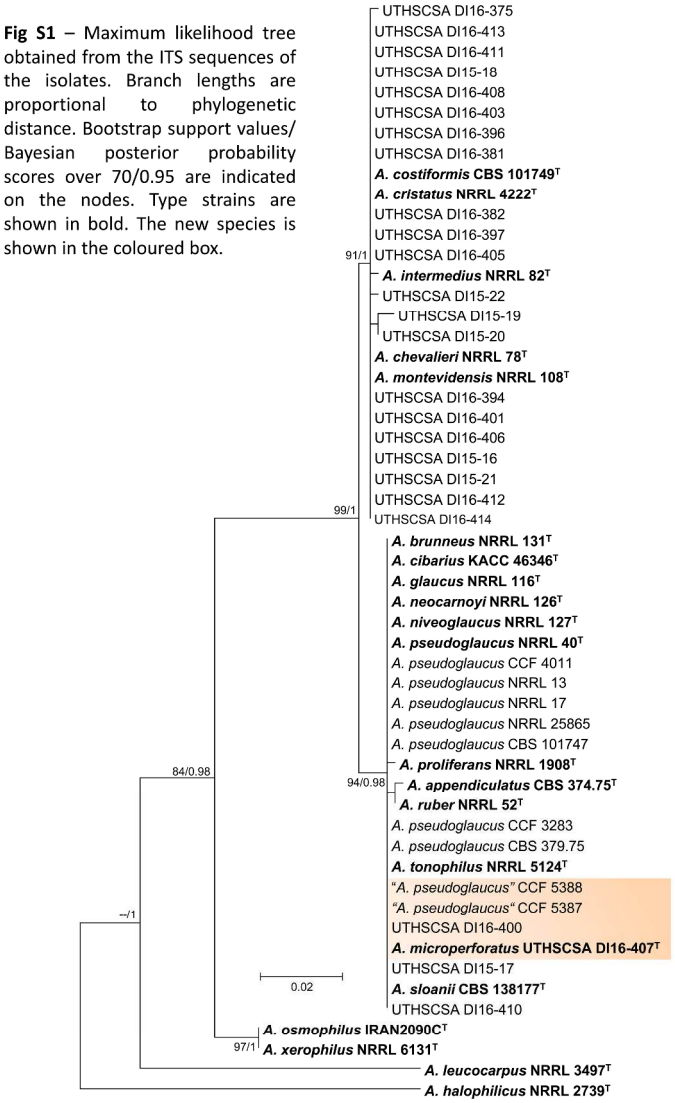

Fig S1 – Maximum likelihood tree obtained from the ITS sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/ Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. Type strains are shown in bold. The new species is shown in the coloured box.

Fig S2 – Maximum likelihood tree obtained from the *BenA* sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/ Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. The new species is shown in the coloured box.

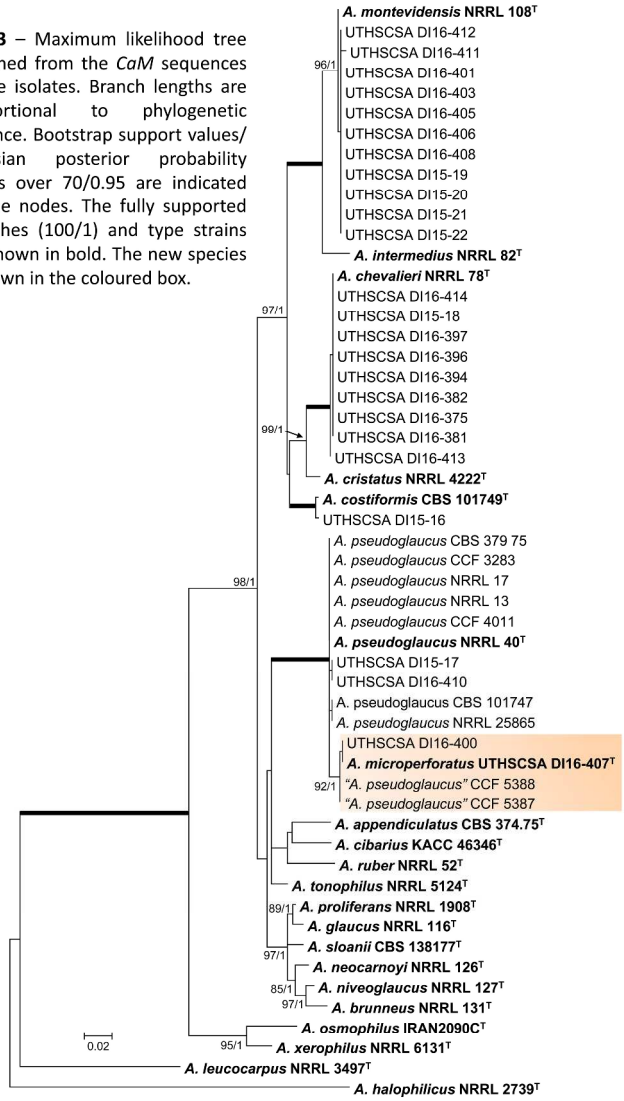

Fig S3 – Maximum likelihood tree obtained from the *CaM* sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/ Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. The new species is shown in the coloured box.

Fig S4 – Maximum likelihood tree obtained from the *RPB2* sequences of the isolates. Branch lengths are proportional to phylogenetic distance. Bootstrap support values/ Bayesian posterior probability scores over 70/0.95 are indicated on the nodes. The fully supported branches (100/1) and type strains are shown in bold. The new species is shown in the coloured box.