Whole-genome sequencing of *Candida haemulonii* species complex from Brazil and the United States: Genetic diversity and antifungal susceptibility

Dality Keffelen de Barros Rodrigues[1], **Shawn R. Lockhart**[2], **Elizabeth L Berkow**[2], **Lalitha Gade**[2], **Lucas Xavier Bonfietti**[3], **Viviane Mazo Fávero Gimenes**[4], **Luciana da Silva Ruiz**[5], **Milena Bronze Macioni**[3,6], **Marcia de Souza Carvalho Melhem**[1,3]

[1]Programa de Pós-graduação em Doenças Infecciosas e Parasitárias, Universidade Federal do Mato Grosso do Sul, Mato Grosso do Sul, MS, Brazil

[2]Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

[3]Núcleo de Micologia do Instituto Adolfo Lutz, Secretaria de Saúde, São Paulo, SP, Brazil

[4]Laboratório de Micologia Médica-LIM 53, Instituto de Medicina Tropical, Universidade de São Paulo, São Paulo, SP, Brazil

[5]Núcleo de Ciências Biomédicas, Instituto Adolfo Lutz, Regional Bauru, São Paulo, SP, Brazil

[6]Universidade Paulista, São Paulo, SP, Brazil

Abstract

Candida haemulonii complex species can be multidrug-resistant and cause infections such as candidemia. This study determined the genetic relationship between isolates from Brazil and the United States through whole-genome sequencing and performed antifungal susceptibility testing to investigate drug resistance. Contrary to what is widely described, most isolates were susceptible to azoles. However, an atypical susceptibility profile was found in 50% of *Candida pseudohaemulonii* strains, including resistance to the three echinocandins. Isolates from both countries formed distinct clusters with wide genetic diversity. Isolates from three hospitals in Brazil were clonal and involved in candidemia cases, pointing to the importance of improving hospital infection control measures and molecular identification.

Lay Summary

Candida haemulonii complex species is worldwide distributed, and this study aimed to evaluate the resistance to antifungal drugs in cases from Brazil and the United States, and also compare their genetic relationships. A total of 50 strains were studied; most of them from Brazil were from cases of bloodstream infections, while the strains from the United States came from cases of wounds and may be associated with diabetic patients. The vast majority of strains were resistant to amphotericin B, one of the most effective drugs, and susceptible to fluconazole. In addition, 50% of *C. pseudohaemulonii* strains were resistant to echinocandins. The strains from Brazil and the United States had no genetic relationship and formed two distinct groups. In three Brazilian hospitals, strains were clonal, indicating an intra-hospital transmission. Our findings contribute to guiding therapy in bloodstream fungal infections caused by *C. haemulonii* species and alerting for nosocomial transmission of this yeast complex species.

Introduction

Candida haemulonii was first isolated from Pacific Ocean seawater and fish in 1962.[1] Since 2012, the taxonomic classification of *C. haemulonii* has been changed from a single species to a species complex (SC), which includes the cryptic species *C. haemulonii sensu stricto* (ss) *C. duobushaemulonii, C. vulturna*, and *C. haemulonii* var. *vulnera*.[2,3] *C. pseudohaemulonii* is phylogenetically close but not currently considered part of the complex.[4] All these species belong to the *Metschnikowia* clade known to host multidrug-resistance *C. auris*. Systems that rely on biochemical analysis like VITEK generally cannot distinguish between the species in the complex, so the sequencing of rDNA intragenic spacer region is the gold standard methodology for identification of this SC.[2] Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has also been successfully used, but accurate identification depends on the creation of robust databases, and some studies have reported misidentification or low scores in the identification of *C. haemulonii* var. *vulnera* and *C. vulturna*.[5–7] Furthermore, a multiplex PCR for differentiating *C. auris, C. haemulonii* ss, *C. duobushaemulonii*, and *C. pseudohaemulonii* was developed and might be a cheaper alternative for places that do not have access to other molecular tool.[8,9]

The first clinical case of *C. haemulonii* was described from a blood culture of a patient with renal failure.[10] Since then, other infections by *C. haemulonii* SC like fungemia, cutaneous infections, and especially wounds in diabetic patients have been reported,[8,11] as well as outbreaks such as the one in a neonatal unit in Kuwait,[12] and transmission within a health care facility.[13]

Many of the genes associated with resistance in *C. albicans* are conserved in the *C. haemulonii* SC.[14] In addition, resistance to azoles and amphotericin B has been frequently documented.[9,15–17] Although echinocandins have good in vitro activity against most isolates, there are reports of high minimum inhibitory concentrations (MICs) for echinocandins in *C. haemulonii* SC isolates.[2,11] This indicates that there is some species specificity to susceptibility profiles, and more molecular studies may be necessary to identify the mechanisms of resistance.

The genetic relationship between strains from the American continent has been researched previously, but there are no studies that include Brazilian strains.18 Thus, strains from Brazil and the United States were collected through surveillance laboratories, analyzed phylogenetically, and performed antifungal susceptibility profiles between them to look for clonal strains and better understand resistance in this SC.

Methods

Isolates

Adolfo Lutz Institute in Brazil and the Mycotic Diseases Branch Reference Laboratory at the CDC in the United States received isolates for routine fungal identification. The isolates from Brazil were previously identified using MALDI-TOF MS (Bruker, Bremen, Germany) in the Adolfo Lutz Institute and after confirming that they belonged to the *C. haemulonii* SC, they were included in the study and sent to the Mycotic Diseases Branch at CDC. There, their identification was reconfirmed through Sanger Sequencing. Isolates from Brazil were collected between 2011 and 2019 and those of the United States from 2017 to 2019 and all belonged to different patients. Isolates were identified by Sanger sequencing of the ITS2 region of the rDNA using the primers ITS3/ITS4, D1/D2 domain.19,20 Furthermore, MALDI-TOF MS (Bruker, Bremen, Germany) using a CDC-developed database, MicrobeNet (https://www.cdc.gov/microbenet/index.html) also identified the species and a minimum score of 2.3 to 3.0 was required.

Whole-genome sequencing

The extraction of DNA was performed with the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) was used to construct and barcode genomic libraries following the manufacturer's instructions. Libraries were sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) using the HiSeq Rapid SBS Kit v2 500-cycles. HiSeq 500-cycle kit generated 251 bp paired and reads.18

Single-nucleotide polymorphism analysis

The methodology was carried out according to Gade et al., 2020.18 Paired-end sequences that had at least 50X coverage were used for downstream analyses. For read quality, FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was assessed and for filtering low-quality sequences, PRINSEQ v0.20.3 (http://prinseq.sourceforge.net/manual.html) was performed using the following command: '-trim_left 15 -trim_qual_left 20 -trim_qual_right 20 -min_len 100 -min_qual_mean 25 -derep 14'. For identifying single-nucleotide polymorphisms (SNPs), paired-end reads of each species were aligned using BWA mem v0.7.1221 to their respective previously published assemblies [*C. haemulonii* strain B11899, GenBank accession PKFO0000000022; *C. duobushaemulonii* strain B09383, GenBank accession PKFP0000000023; *C. pseudohaemulonii* strain B12108, GenBank accession PYFQ00000000,14 *C. vulturna* strain CBS14366,7 and *C. auris* reference strain B8441.14 SNPs were identified and filtered using the publicly available pipeline NASP (http://tgennorth.github.io/NASP/) to remove SNPs that had <10× coverage, <90% variant

allele calls, or that were identified by Nucmer[24] as being within duplicated regions in the reference (Supplementary Table 1).

Phylogenetic analyses

Maximum parsimony phylogenies were constructed using the subtree-pruning-regrafting (SPR) algorithm, bootstrapped using 500 reiterations, and pairwise distance was done in MEGA X Software[25] and visualized in Interactive Tree of Life (iTOL) v4.2[6]

Antifungal susceptibility testing

Antifungal susceptibility testing was performed according to Clinical and Laboratory Standards Institute (CLSI) standard M27-A4.[27] Custom-prepared frozen panels (Trek Diagnostics, Thermo Fisher Scientific, Oakwood Village, OH, USA) were used for echinocandins (anidulafungin, caspofungin, and micafungin) and the azoles (fluconazole, voriconazole, itraconazole, posaconazole, and isavuconazole). For amphotericin B, E-test™ strips (BioMérieux, Marcy l'Etoile, France) were used. As suggested for CLSI M27-A4, plates were read at 48 h due to the slow growth of *C. haemulonii*.

There are no breakpoints for *C. haemulonii* complex. In order to interpret the results, we followed the epidemiological cutoff values (ECVs) in the CLSI document M57S.[28] The ECVs for *C. duobushaemulonii* are: anidulafungin (1 μg/ml), caspofungin (0.25 μg/ml), micafungin (0.5 μg/ml), fluconazole (32 μg/ml), voriconazole (0.5 μg/ml), itraconazole (1 μg/ml), posaconazole (1 μg/ml), and isavuconazole (0.25 μg/ml). For *C. haemulonii* ss the ECVs are: anidulafungin (0.5 μg/ml), fluconazole (128 μg/ml), posaconazole (1 μg/ml), and voriconazole (2 μg/ml).

For the other species, there are no proposed EVCs. Since there are also no breakpoints and ECVs for amphotericin B, we considered >1 μg/ml as resistant as it is for most *Candida* species.

Results

Species distribution

Overall, 50 isolates within the *C. haemulonii* SC were identified as *C. haemulonii* ss (64%; $n = 32$), *C. duobushaemulonii* (24%; $n = 12$), *C. pseudohaemulonii* (8%; $n = 4$), and *C. vulturna* (4%; $n = 2$). The 25 isolates from Brazil were identified as *C. haemulonii* (76%; $n = 19$) and *C. duobushaemulonii* (24%; $n = 6$). The 25 US isolates were identified as *C. haemulonii* (52%; $n = 13$), *C. duobushaemulonii* (24%; 6), *C. pseudohaemulonii* (16%; $n = 4$), and *C. vulturna* (8%; 2) (Table 1). Isolates from Brazil came from blood (76%; $n = 19$), urine (8%; $n = 2$), body fluid (8%; $n = 2$), bone marrow (4%; $n = 1$), and skin (4%; $n = 1$). The isolates from the United States came from foot or toes (24%; $n = 6$), tissue or skin (20%; $n = 5$), wound (12%; $n = 3$), blood (12%; $n = 3$), bronchial washing (4%; $n = 1$), or no identified source (28%; $n = 7$).

Phylogenetic relationships

Figure 1 shows the relationships within the SC. *C. vulturna* has a distinct monophyletic branch on the phylogenetic tree, and *C. duobushaemulonii* is closely related to *C. pseudohaemulonii.*

Genetic relationships among *C. haemulonii* isolates in Brazil and the United States are shown in Fig. 2. The average pairwise difference between the isolates was 241 SNPs (range 0–437), and there was no distinct phylogeographic population structure. Most isolates were genetically distinct. However, three pairs of Brazilian *C. haemulonii* ss isolates were closely related with 0–2 SNPs, and formed small, well-supported clusters in the phylogenetic tree based on bootstrap analysis (Fig. 2). Isolates IAL6905/IAL6839 from hospital A were genetically identical with 0 SNP difference, whereas IAL6842/IAL6843 from hospital B and IAL6895/IAL6897 from hospital C were different with 1 and 2 SNPs, respectively. All of the identical pairs were recovered from blood, and each pair was collected during the same period of time.

The average pairwise distance between *C. duobushaemulonii* isolates was 585 SNPs (range 51–1257). The isolates can be separated into three genetically distinct clades separated by more than 201 SNPs. Interestingly, all Brazilian isolates were included in only one of these clades. No *C. duobushaemulonii* isolates showed a high degree of relatedness (Fig. 3).

Antifungal susceptible testing

Table 1 shows the distribution of MIC values for the isolates, the MIC_{50}, and the MIC_{90}. The MICs for *C. haemulonii* complex ranged for fluconazole from 0.5 to 128 μg/ml and most strains were susceptible to all azoles with exception of one non-wild *C. duobushaemulonii* isolate for fluconazole (128 μg/ml), isavuconazole (1 μg/ml), voriconazole (1 μg/ml), and amphotericin B (32 μg/ml).

A large proportion of isolates had elevated MICs to amphotericin B (86%; 43/50) ranged from 0.094 to >32 μg/ml. The MIC_{50} of *C. haemulonii* SC strains in amphotericin B was 3 μg/ml for Brazilian strains versus 32 μg/ml for US strains. All strains of *C. duobushaemulonii* have MICs 32 μg/ml. Both *C. vulturna* isolates (2/50) were non-wild types for amphotericin B (24 to >32 μg/ml) and susceptible to other drugs.

Candida pseudohaemulonii was identified only among US strains and two of the four isolates exhibited high MICs for caspofungin (>16 μg/ml), micafungin (1 μg/ml), and anidulafungin (4 μg/ml), but all four were susceptible to amphotericin B and azoles.

Discussion

The variety of species was greater among US isolates with the inclusion of *C. pseudohaemulonii* and *C. vulturna*, the latter of species that has not yet been reported in Brazil. Unfortunately, the ability to discriminate cryptic species may not be routinely available in Brazil, as it is estimated that only about 20% of health centers in Brazil and South America have access to MALDI-TOF MS or sequencing.[29,30] The prevalence of infections caused by the *C. haemulonii* complex increased to 1.7% in the years 2014–2019

in hospitals from São Paulo State, Brazil.31 Furthermore, a study conducted in Brazil found a high prevalence (>4%) for this SC.32 However, the fact that *C. auris* isolates are often misidentified as species within the *C. haemulonii* complex has caused surveillance measures to monitor *C. auris* to impact the increase in reported *C. haemulonii* infections.33

Two strains identified by Bruker MALDI-TOF MS as *C. haemulonii* var. *vulnera* in Brazil were re-identified as *C. haemuloni* ss by CDC MALDI-TOF and ITS sequencing in the United States reinforcing the need to update the MALDI-TOF MS databases for an accurate identification. Our study used MALDI-TOF, ITS, and the D1/D2 domain to identify the isolates. There was 98% agreement between the ITS and MALDI-TOF and 96% between the ITS and the D1/D2 domain. The results of our sequencing were BLASTed in GenBank, and as it is known that this database is not curated, it is important, especially in cases of low confidence identification to resort to databases with greater reliability, such as Mycobank, and phylogenetic analyzes to determine the true identity of the species.

Brazilian isolates were predominantly from bloodstream infections; however, it is difficult to estimate the prevalence of fungal infections caused by *Candida* spp. in Brazil because cases of non-invasive infection are often underreported and isolates from non-sterile body site infections are rarely referred to reference centers in Brazil. The US strains were from various body sites, especially wounds. The ability of *C. haemulonii* SC to produce hyphae and switch morphology at lower temperatures has already been described, and together with uncontrolled diabetes as a risk factor and other comorbidities may explain skin commensalism and why many cases of infection by this species are described from wounds.11,34 Patients who have suffered extensive burns are also at risk for invasive infections, as their skin and mucosal barriers have been breached, facilitating the invasive infection of *Candida* spp. that were previously commensal in the skin.35 Some authors also believe that the use of topical azole antifungals in the treatment of ulcers may have influenced the selective pressure of some species.13 All US strains of *C. duobushaemulonii* came from non-invasive sites such as the skin. Although our study did not have clinical data and we can know whether the strains isolated from wounds and tissues are infections or colonization, it is *C. duobushaemulonii* that should draw attention due to its potential for multidrug resistance, as observed by other authors.

The majority of the *C. haemulonii* SC isolates from both countries were found to be susceptible to fluconazole (MICs < 32 μg/ml; MIC_{50} 4 μg/ml) in contrast to what has been reported previously.9,12,36–38 The MIC_{50} calculated separately for both Brazil and US strains was also 4 μg/ml. All isolates were susceptible to fluconazole, with the exception of one isolate of *C. duobushaemulonii* (128 μg/ml) from US and isolated from wound. MIC_{50}s of 4 μg/ml and MIC_{90} 16 μg/ml for both *C. haemulonii* ss and *C. duobushaemulonii* were below those described by other authors who found an MIC_{50} of 64 μg/ml for both species13,15,31,33 and a higher susceptibility rate to azoles than previously described.37,39 The fluconazole non-wild type *C. duobushaemulonii* isolate was also non-wild type for isavuconazole (1 μg/ml), voriconazole (1 μg/ml), and amphotericin B (32 μg/ml).

All *C. duobushaemulonii* (MIC_{50} > 32 μg/ml), *C. vulturna*, and the majority of *C. haemulonii* (MIC_{50} 6 μg/ml) isolates had elevated MIC values to amphotericin B (MICs

24 to >32 μg/ml). In contrast to previous reports, we found *C. pseudohaemulonii* isolates were susceptible to amphotericin B (MIC < 1 μg/ml).[11,36]

Interestingly, half of *C. pseudohaemulonii* isolates (2/4) had decreased susceptibility to caspofungin (16 μg/ml), anidulafungin (4 μg/ml), and micafungin (1 μg/ml). This may be the first report of such high MICS for this species to the echinocandins, which is concerning as it was associated with an atypical profile of susceptibility to amphotericin B and fluconazole.[4,18,36] In Brazil, about 50% of hospitals may not have access to echinocandins, which makes the treatment of species resistant to fluconazole or amphotericin B a public health problem.[29,30] One isolate that was non-wild type to echinocandins came from a bloodstream infection, which could pose a treatment problem as echinocandins are the recommended first-line treatment. Unfortunately, three out of four strains of *C. pseudohaemulonii* did not have records from which body site they were isolated.

Whole-genome sequencing (WGS) of the 50 isolates showed that the American and Brazilian isolates of *C. haemulonii* SC were phylogenetically distant. This heterogeneity was also observed in another study that used WGS to determine the genetic relationship between isolates from the United States, Latin America, and Central America.[18] The overall population shows a high degree of diversity, but clonal bloodstream isolate pairs were found in three different hospitals in Brazil, which is an indicator of the transmissibility of this species within healthcare settings.[40] These six isolates were all *C. haemulonii* ss and were mostly susceptible to fluconazole and echinocandins but not amphotericin B, the exceptions being one isolate that was susceptible to amphotericin B and another that was resistant to fluconazole (64 μg/ml).

Studies that define the clonality and formation of clusters of strains of genus *Candida* obtained from hospitalized patients may help us to understand the occurrence of endemic genotypes that are transmitted from patient to patient. Most intra-hospital transmission of yeast infections is related to adult and neonatal intensive care units. Outbreaks are mainly caused by strains that spread within the same ward, while outbreaks containing unrelated clusters usually originate outside the hospital.[41,42] In addition, a study by Guinea et al., designated the term 'generalized clusters' for those that do not have an epidemiological relationship, such as patients hospitalized in different wards and which are difficult to interpret because they may be better-adapted strains that persist in hospitals for a long time, and their spread also depends on adherence to infection controls and other factors.[43,44] Our study did not use epidemiological data that could infer how the strains of *C. haemulonii* were circulated within the three Brazilian hospitals.

We also did not look for the origin of the resistance, considering that there could be several causes, such as genetic mutations, overexpression of efflux pumps, and formation of biofilms, among others.

We conclude that the *C. haemulonii* SC is an important cause of fungemia, and cases of non-invasive candidiasis, such as wound infections, should also be closely monitored and reported to improve the epidemiological data of this SC, especially in Brazil. The strains circulating in Brazil and the United States are diverse and may have different susceptibility

profiles. Due to the diversity of susceptibilities not only among complexes but also among isolates, antifungal susceptibility testing of isolates is recommended.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Data availability

All Illumina sequence data generated by this project are available in the NCBI SRA under BioProject accession numbers are PRJNA938413.
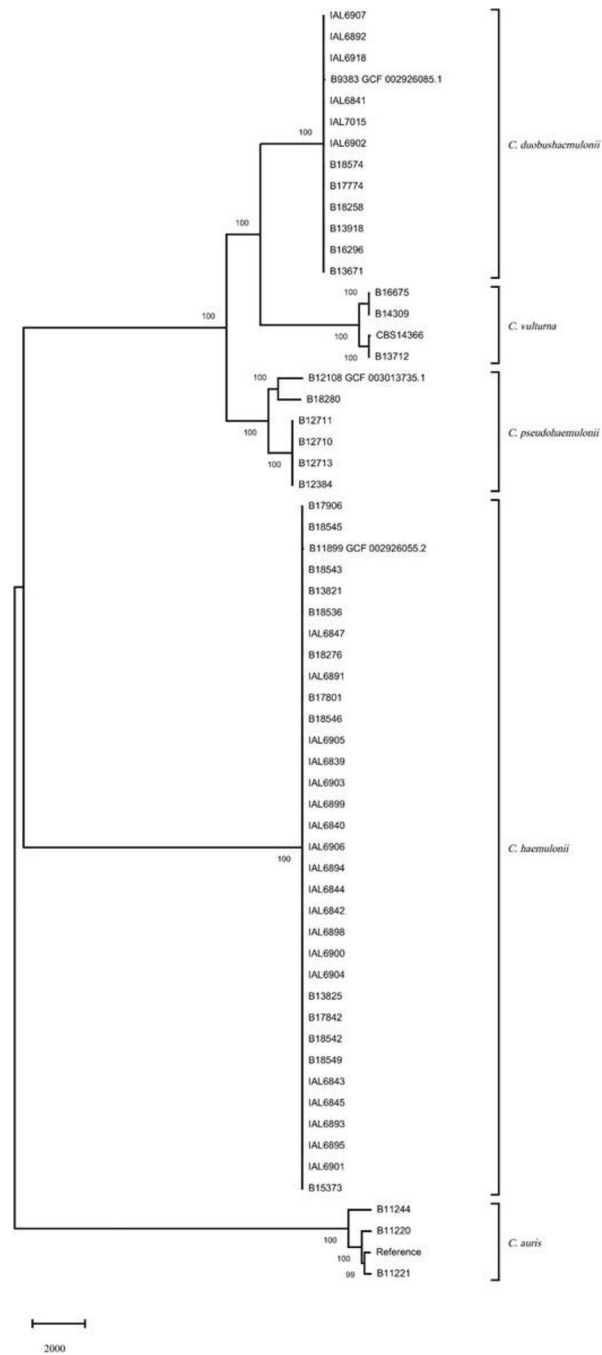
References

1. van Uden N, Kolipinski MC, *Torulopsis haemulonii* nov. Spec. A yeast from the Atlantic Ocean. Antonie Van Leeuwenhoek. 1962; 28: 78–80.

2. Cendejas-Bueno E, Kolecka A, Alastruey-Izquierdo A et al. Reclassification of the Candida haemulonii complex as Candida haemulonii (C. haemulonii group I), C. duobushaemulonii sp. Nov. (C. haemulonii group II), and C. Haemulonii var. vulnera var. Nov.: three multiresistant human pathogenic yeasts. J Clin Microbiol. 2012; 50: 3641–3651.

3. Sipiczki M, Tap RM. *Candida vulturna* pro tempore sp. Nov., A dimorphic yeast species related to the *Candida haemulonis* species complex isolated from flowers and clinical sample. Int J Syst Evol Microbiol. 2016; 66: 4009–4015.

4. Sugita T, Takashima M, Poonwan N, Mekha N. *Candida pseudohaemulonii* sp. Nov., an amphotericin B- and azole-resistant yeast species, isolated from the blood of a patient from Thailand. Microbiol Immunol. 200650: 469–473.

5. Rodrigues LS, Gazara RK, Passarelli-Araujo H et al. First genome sequences of two multidrug-resistant *Candida haemulonii* var. *vulnera* isolates from pediatric patients with candidemia. Front Microbiol. 2020; 11: 1–12.

6. Grenfell RC, Da Silva Junior AR, Del Negro GMB et al. Identification of Candida haemulonii complex species: use of ClinProToolsTM to overcome limitations of the Bruker BiotyperTM, VITEK MSTM IVD, and VITEK MSTM RUO databases. Front Microbiol. 2016; 7: 1–10.

7. Navarro-Muñoz JC, de Jong AW, Gerrits van den Ende B et al. The high-quality complete genome sequence of the opportunistic fungal pathogen Candida vulturna CBS 14366T. Mycopathologia. 2019; 184: 731–734.

8. Arastehfar A, Fang W, Badali H et al. Low-cost tetraplex PCR for the global spreading multi-drug resistant fungus, Candida auris and its phylogenetic relatives. Front Microbiol. 2018; 9: 1–8.

9. Frías-De-León MG, Martínez-Herrera E, Acosta-Altamirano G, Arenas R, Rodríguez-Cerdeira C. Superficial candidosis by *Candida duobushaemulonii*: an emerging microorganism. Infect Genet Evol. 2019; 75: 1–5.

10. Lavarde V, Daniel F, Saez H, Arnold MFB. Peritonite mycosique a Torulopsis haemulonii. Bull Soc Fr Mycol Med. 1984; 13: 173.

11. Kumar A, Prakash A, Singh A et al. *Candida haemulonii* species complex: an emerging species in India and its genetic diversity assessed with multilocus sequence and amplified fragment-length polymorphism analyses. Emerg Microbes Infect. 2016; 5: 49–51.

12. Khan ZU, Al-sweih NA, Ahmad S et al. Outbreak of fungemia among neonates caused by *Candida haemulonii* resistant to amphotericin B, itraconazole, and fluconazole. J Clin Microbiol. 2007; 45: 2025–2027. [PubMed: 17428940]

13. Ben-ami R, Berman J, Novikov A et al. Multidrug-resistant *Candida haemulonii* and *C. auris*, Tel Aviv, Israel. Emerg Infect. Dis. 2018; 23: 195–203.

14. Muñoz JF, Gade L, Chow NA et al. Genomic insights into multidrug-resistance, mating and virulence in *Candida auris* and related emerging species. Nat Commun. 2018; 9: 1–13. [PubMed: 29317637]

15. de Almeida JN, Assy JGPL, Levin AS et al. Candida haemulonii complex species, Brazil, January 2010–March 2015 João. Emerg Infect. Dis. 2016; 22: 561–563. [PubMed: 26891028]

16. Kim M, Shin JH, Sung H et al. *Candida haemulonii* and closely related species at 5 university hospitals in Korea: identification, antifungal susceptibility, and clinical features. Clin Infect Dis. 2009; 48: e57–e61. [PubMed: 19193113]

17. Ramos R, Caceres DH, Perez M et al. Emerging multidrug-resistant *Candida duobushaemulonii* infections in Panama hospitals: importance of laboratory surveillance and accurate identification. J Clin Microbiol. 2018; 56: e00371–18.

18. Gade L, Muñoz JF, Sheth M et al. Understanding the emergence of multidrug-resistant Candida: using whole-genome sequencing to describe the population structure of *Candida haemulonii* species complex. Front Genet. 2020; 11: 1–15. [PubMed: 32117431]

19. Lott TJ, Burns BM, Zancope-Oliveira R, Elie CM, Reiss E. Sequence analysis of the internal transcribed spacer 2 (ITS2) from yeast species within the genus Candida. Curr Microbiol. 1998; 36: 63–69. [PubMed: 9425241]

20. Nagatsuka Y, Kiyuna T, Kigawa R, Sano C, Miura S, Sugiyama J. *Candida tumulicola* sp. Nov. and *Candida takamatsuzukensis* sp. Nov., novel yeast species assignable to the *Candida membranifaciens* clade, isolated from the stone chamber of the Takamatsuzuka tumulus. Int J Syst Evol Microbiol. 2009; 59: 186–194. [PubMed: 19126745]

21. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics. 2009; 25: 1754–1760. [PubMed: 19451168]

22. Chow NA, Gade L, Batra D et al. Genome sequence of a multidrugresistant *Candida haemulonii* isolate from a patient with chronic leg ulcers in Israel. Genome Announc. 2018; 6: 10–11.

23. Strain B, Chow NA, Gade L et al. Genome sequence of the amphotericin B-resistant Candida duobushaemulonii strain B09383. Genome Announc. 2018; 6: 1–2.

24. Kurtz S, Phillippy A, Delcher AL et al. Versatile and open software for comparing large genomes. Genome Biol. 2004; 5: R12. [PubMed: 14759262]

25. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018; 35: 1547–1549. [PubMed: 29722887]

26. Letunic I, Bork P. Interactive Tree of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 2019; 47: 256–259.

27. CLSI. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, volume 4. Wayne, PA: Clinical and Laboratory Standards Institute. 2017: 46. https://clsi.org/media/1461/m27a3_sample.pdf

28. CLSI. Epidemiological Cutoff Values for Antifungal Susceptibility Testing, volume 4. Wayne, PA: Clinical and Laboratory Standards Institute. 2022: 136–137.

29. Falci DR, Pasqualotto AC. Clinical mycology in Latin America and the Caribbean: a snapshot of diagnostic and therapeutic capabilities. Mycoses. 2019; 62: 368–373. [PubMed: 30614600]

30. Chakrabarti A, Meis JF, Cornely O. International society for human and animal mycology (Isham)-new initiatives. J Fungi. 2020; 6: 1–6.

31. Lima SL, Francisco EC, de Almeida Júnior JN et al. Increasing prevalence of multidrug-resistant Candida haemulonii species complex among all yeast cultures collected by a reference laboratory over the past 11 years. J Fungi. 2020; 6: 1–7.

32. Rodrigues DKB, Bonfietti LX, Garcia RA et al. Antifungal susceptibility profile of Candida clinical isolates from 22 hospitals of São Paulo State, Brazil. Brazilian J Med Biol Res. 2021; 54: 1–5.

33. Kathuria S, Singh PK, Sharma C et al. Multidrug-resistant *Candida auris* misidentified as *Candida haemulonii*: characterization by matrix-assisted laser desorption ionization-time of flight mass spectrometry and DNA sequencing and its antifungal susceptibility profile variability by vitek 2, CL. J Clin Microbiol. 2015; 53: 1823–1830.

34. Deng Y, Li S, Bing J, Liao W, Tao L. Phenotypic switching and filamentation in Candida haemulonii, an emerging opportunistic pathogen of humans. Microbiol Spectr. 2021; 9: 1–14.

35. Fan C, Tian Q, Huang G, Zhang L, Wu Q, Zhang K. Candida tropicalis burn wound sepsis: a series of histopathology-confirmed cases. Intensive Crit Care Nurs. 2018; 46: 6–9.

36. Kim M, Shin JH, Sung H et al. *Candida haemulonii* and closely related species at 5 university hospitals in Korea: identification, antifungal susceptibility, and clinical features. Clin Infect Dis. 2009; 48: 57–61.

37. Hou X, Xiao M, Chen SCA et al. Identification and antifungal susceptibility profiles of *Candida haemulonii* species complex clinical isolates from a multicenter study in China. J Clin Microbiol. 2016; 54: 2676–2680.

38. Ramos LS, Figueiredo-Carvalho MHG, Barbedo LS et al. *Candida haemulonii* complex: species identification and antifungal susceptibility profiles of clinical isolates from Brazil. J Antimicrob Chemother. 2015; 70: 111–115.

39. de Souza Ramos L, Barbedo LS, Braga-Silva LA, Souza dos Santos AL, Pinto MR, de Graça Sgarbi DB. Protease and phospholipase activities of Candida spp. isolated from cutaneous candidiasis. Rev Iberoam Micol. 2015; 32: 122–125.

40. Thomaz DY, de Almeida JN, Sejas ONE et al. Environmental clonal spread of azole-resistant *Candida parapsilosis* with Erg11-Y132F mutation causing a large candidemia outbreak in a Brazilian cancer referral center. J Fungi. 2021; 7: 259.

41. Escribano P, Rodríguez-Créixems M, Sánchez-Carrillo C, Muñoz P, Bouza E, Guinea J. Endemic genotypes of *Candida albicans* causing fungemia are frequent in the hospital. J Clin Microbiol. 2013; 51: 2118–2123.

42. Goemaere B, Lagrou K, Spriet I, Hendrickx M, Becker P. Clonal spread of Candida glabrata bloodstream isolates and fluconazole resistance affected by prolonged exposure: a 12-year single-center study in Belgium. Antimicrob Agents Chemother. 2018; 62: 1–11.

43. Guinea J, Arendrup MC, Cantón R et al. Genotyping reveals high clonal diversity and widespread genotypes of *Candida* causing candidemia at distant geographical areas. Front Cell Infect Microbiol. 2020; 10: 1–12.

44. Asadzadeh M, Ahmad S, Al-Sweih N, Khan Z. Molecular fi7ngerprinting studies do not support intrahospital transmission of Candida albicans among candidemia patients in Kuwait. Front Microbiol. 2017; 8: 1–12.

Figure 1.
Genetic relationship of isolates among *C. haemulonii* SC. The maximum parsimony tree
was constructed using 37 391 SNPs called against B8441 *Candida auris* reference genome
(GenBank accession PEKT00000000.2).
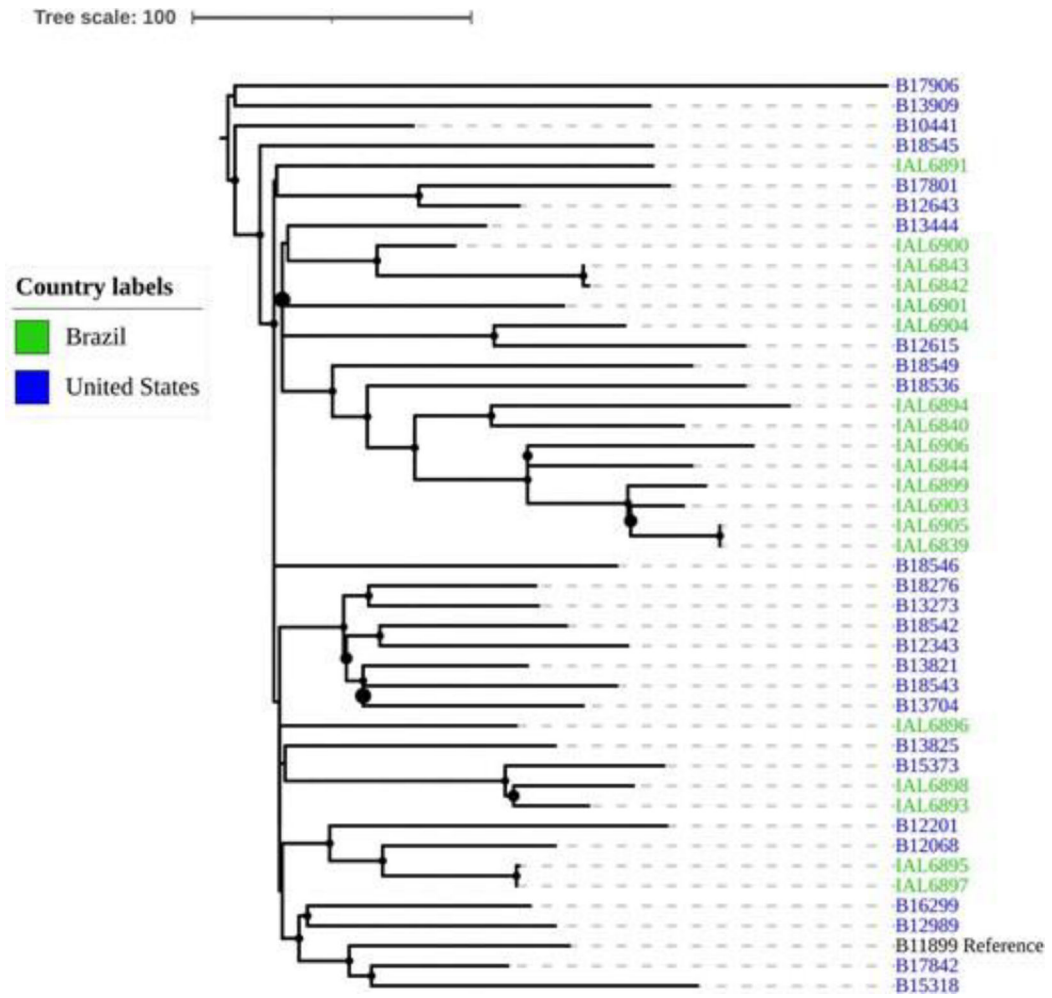
Figure 2.
Genetic relationship among *C. haemulonii sensu stricto isolates*. The maximum parsimony
tree was constructed using 5912 SNPs called against B11899 *C. haemulonii* reference
genome (GenBank accession PKFO00000000). Scale bar shows pairwise SNPs.

Tree scale: 100
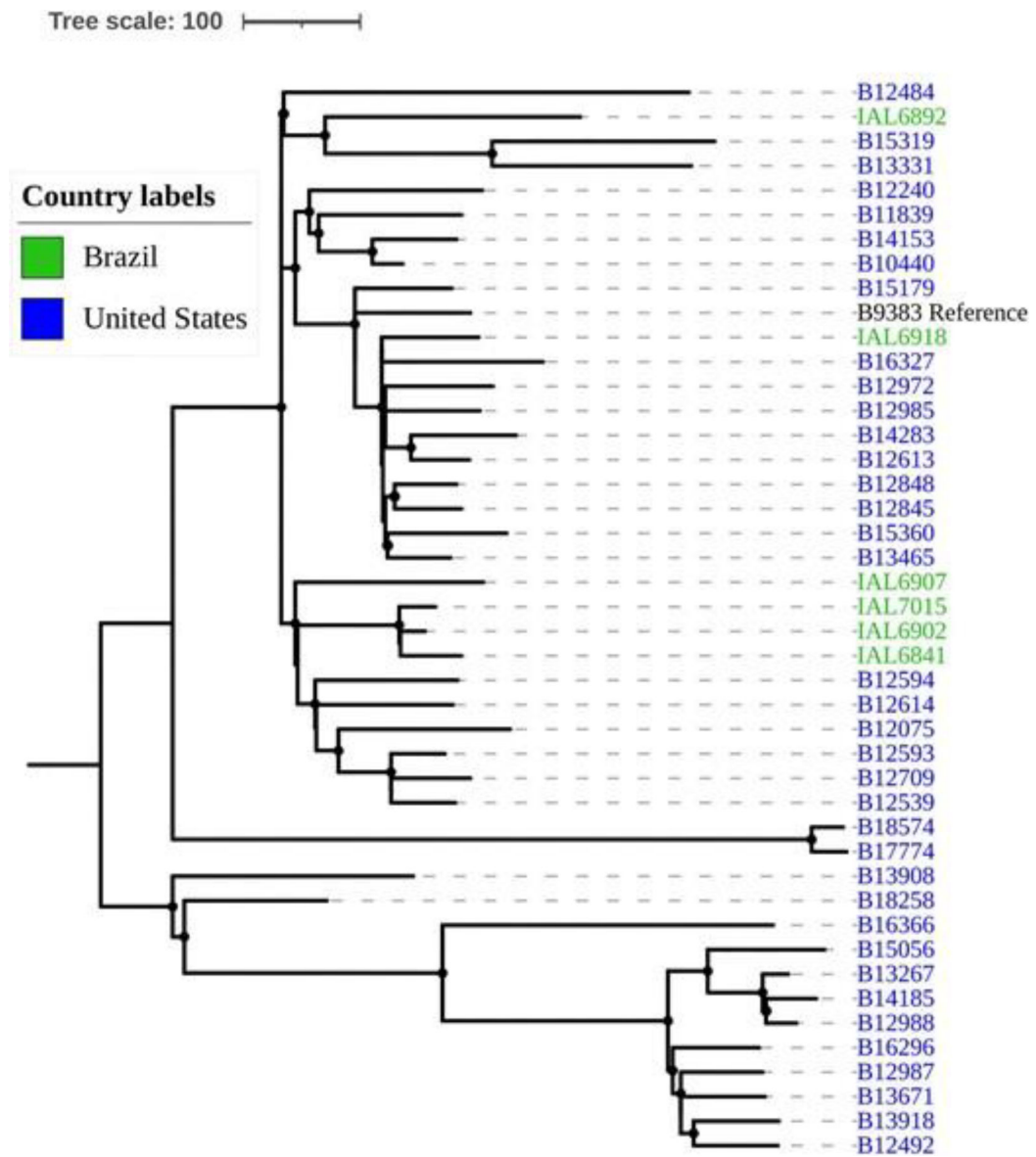
Figure 3.
Genetic relationship among *C. duobushaemulonii* isolates. The maximum parsimony tree was constructed using 8374 SNPs called against B09383 *C. duobushaemulonii* reference genome (GenBank accession PKFP00000000). Scale bar shows pairwise SNPs.

Table 1.

Origin and antifungal susceptibility profile of 50 isolates from Brazil and the United States.

| Isolate | Location | Year | Speciment | MIC (μg/ml) | | | | | | | | Etest |
| | | | | Broth microdilution method | | | | | | | | |
				MCF	AND	CAS	FZ	ITR	ISA	POS	VRC	AMB
Candida haemulonii sensu stricto												
IAL 6906	Brazil	2012	Urine	0.06	0.06	0.03	2	0.25	0.016	0.06	0.06	2
IAL 6895	Brazil	2012	Blood	0.06	0.06	0.03	4	0.12	0.03	0.06	0.03	>32
IAL 6896	Brazil	2012	Blood	0.06	0.12	0.03	4	0.06	0.03	0.016	0.03	>32
IAL 6897	Brazil	2012	Blood	0.06	0.06	0.03	8	0.5	0.12	0.25	0.12	32
IAL 6903	Brazil	2013	Blood	0.06	0.25	0.06	4	0.25	0.06	0.12	0.06	2
IAL 6891	Brazil	2014	Blood	0.06	0.12	0.06	2	0.25	0.016	0.12	<0.008	6
IAL 6900	Brazil	2014	Blood	0.06	0.25	0.06	4	0.5	0.06	0.06	0.25	2
IAL 6901	Brazil	2014	Fluid body	0.12	0.25	0.06	1	0.25	0.016	0.06	0.03	3
IAL 6904	Brazil	2014	Blood	0.25	0.06	0.06	4	1	0.016	0.06	0.25	12
IAL 6893	Brazil	2014	Blood	0.03	0.06	0.06	4	0.25	0.03	0.12	0.06	32
IAL 6898	Brazil	2016	Tissue	0.06	0.03	0.03	4	0.5	0.03	0.25	0.06	32
IAL 6899	Brazil	2016	Urine	0.12	0.06	0.06	16	0.5	0.06	0.25	1	4
IAL 6905	Brazil	2017	Blood	0.06	0.12	0.03	4	0.25	0.016	0.06	0.016	2
IAL 6839	Brazil	2017	Blood	0.12	0.25	0.06	16	0.5	0.06	0.12	0.12	1.5
IAL 6844	Brazil	2017	Blood	0.06	0.06	0.03	2	0.25	0.016	0.12	0.03	6
IAL 6840	Brazil	2017	Blood	0.12	0.25	0.12	8	0.5	0.06	0.12	0.06	4
IAL 6894	Brazil	2017	Fluid body	0.06	0.06	0.03	2	0.25	0.016	0.06	0.03	2
IAL 6842	Brazil	2018	Blood	0.06	0.12	0.06	8	0.25	0.06	0.06	<0.008	1
IAL 6843	Brazil	2018	Blood	0.03	0.12	0.12	64	1	0.5	0.5	0.5	1,5
B13821	Ohio, USA	2017	Foot	0.125	0.06	0.06	2	0.25	0.125	0.125	0.06	32
B13825	Alabama, USA	2017	Arm	0.125	0.25	0.125	16	1	0.5	0.25	0.125	>32
B15373	Texas, USA	2018	Tissue skin (biopsy)	0.03	0.125	0.06	4	0.125	0.06	0.06	0.03	32
B17801	Florida, USA	2019	NA	0.06	0.016	0.03	1	0.06	0.03	0.03	0.03	8
B17842	Washington,USA	2018	NA	0.125	0.06	0.125	4	0.25	0.03	0.125	0.03	32

MIC (μg/ml)

| Isolate | Location | Year | Speciment | Broth microdilution method | | | | | | | | Etest |
				MCF	AND	CAS	FZ	ITR	ISA	POS	VRC	AMB
B17906	Florida, USA	2019	Wound	0.06	0.12	0.03	4	0.25	0.06	0.12	0.03	>32
B18276	Hawaii, USA	2019	NA	0.016	0.06	0.06	2	0.06	0.03	0.06	0.016	>32
B18536	Florida, USA	2019	Foot	0.06	0.25	0.06	0.5	0.06	2	0.03	0.06	3
B18542	Mississipi, USA	2019	Skin swab	0.03	0.06	0.06	16	0.5	0.25	0.25	0.25	16
B18543	Florida, USA	2019	Foot	0.25	0.125	0.125	16	0.5	0.25	0.25	0.06	>32
B18544	Florida, USA	2019	Blood	0.125	0.06	0.06	32	1	0.5	0.5	0.25	0.75
B18545	Georgia, USA	2019	Wound	0.125	0.25	0.125	2	0.5	0.25	0.25	0.25	>32
B18546	Florida, USA	2019	Blood	0.25	0.125	0.25	8	0.03	0.06	0.125	0.008	2
MIC50				0.06	0.125	0.06	4	0.25	0.06	0.12	0.06	6
MIC90				0.125	0.25	0.125	16	1	0.5	0.25	0.25	>32
Candida duobushaemulonii												
IAL 6907	Brazil	2011	Blood	0.06	0.06	0.06	16	0.5	0.06	0.12	0.06	>32
IAL 6892	Brazil	2015	Blood	0.06	0.06	0.03	8	0.25	0.03	0.06	0.12	>32
IAL 6902	Brazil	2016	Bone marrow	0.06	0.06	0.03	8	0.5	0.03	0.12	0.12	>32
IAL 6841	Brazil	2018	Blood	0.03	0.06	0.03	4	0.25	0.03	0.06	0.06	>32
IAL 6918	Brazil	2019	Blood	0.12	0.25	0.12	4	0.5	0.03	0.12	0.06	>32
IAL 7015	Brazil	2019	Blood	0.03	0.016	0.016	4	0.25	0.03	0.06	0.06	>32
B13671	Connecticut, USA	2017	Tissue	0.06	0.125	0.03	4	0.125	0.06	0.125	0.06	32
B13918	Virginia, USA	2017	Wound	0.03	0.25	0.06	128	0.25	1	0.25	1	32
B16296	Ohio, USA	2018	Foot	0.03	0.06	0.03	16	0.25	0.06	0.125	0.5	>32
B17774	Georgia, USA	2019	Foot	0.008	0.008	0.008	0.125	0.25	0.03	0.06	0.06	>32
B18258	Alabama, USA	2019	Tissue	0.016	0.06	0.03	1	0.016	0.008	0.008	0.016	>32
B18574	Georgia, USA	2019	Toe	0.06	0.03	0.06	8	0.25	0.125	0.25	0.25	>32
MIC50				0.03	0.06	0.03	4	0.25	0.03	0.12	0.06	>32
MIC90				0.06	0.25	0.06	16	0.5	0.125	0.25	0.5	>32
Candida pseudohaemulonii												
B12710	Not available	NA	NA	1	4	>16	2	0.125	0.016	0.06	0.125	0.19

MIC (µg/ml)

Isolate	Location	Year	Speciment	Broth microdilution method								Etest
				MCF	AND	CAS	FZ	ITR	ISA	POS	VRC	AMB
B12711	Not available	NA	NA	0.5	0.5	0.125	2	0.25	0.016	0.06	0.06	0.094
B12713	Not available	NA	NA	1	4	>16	4	0.25	0.016	0.06	0.125	0.094
B18280	Michigan, USA	2019	Blood	0.12	0.06	0.12	2	0.12	0.016	0.016	0.03	0.38
Candida vulturna												
B13712	Wisconsin, USA	2017	Bronchial washings	0.03	0.016	0.016	1	0.06	0.008	0.03	0.03	24
B16675	Florida, USA	2018	Tissue	0.06	1	0.125	8	0.25	0.125	0.25	0.06	>32

Table 3.

Antifungal susceptibility profiles

Strain or Isolate	Location	MIC (µg/mL) Broth Microdilution						
		VOR	AND	CAS	FZ	IZ	ISA	PZ
C. haemulonii								
IAL 6906	Brazil	0.06	0.06	0.03	2	0.25	0.016	0.06
IAL 6895	Brazil	0.03	0.06	0.03	4	0.12	0.03	0.06
IAL 6896	Brazil	0.03	0.12	0.03	4	0.06	0.03	0.0016
IAL 6897	Brazil	0.12	0.06	0.03	8	0.5	0.12	0.25
IAL 6903	Brazil	0.06	0.25	0.06	4	0.25	0.06	0.12
IAL 6891	Brazil	<0.008	0.12	0.06	2	0.25	0.016	0.12
IAL 6900	Brazil	0.25	0.25	0.06	4	0.5	0.06	0.06
IAL 6901	Brazil	0.03	0.25	0.06	1	0.25	0.016	0.06
IAL 6904	Brazil	0.25	0.06	0.06	4	1	0.016	0.06
IAL 6893	Brazil	0.06	0.06	0.06	4	0.25	0.03	0.12
IAL 6898	Brazil	0.06	0.03	0.03	4	0.5	0.03	0.25
IAL 6899	Brazil	1	0.06	0.06	16	0.5	0.06	0.25
IAL 6905	Brazil	0.016	0.12	0.03	4	0.25	0.016	0.06
IAL 6839	Brazil	0.12	0.25	0.06	16	0.5	0.06	0.12
IAL 6844	Brazil	0.03	0.06	0.03	2	0.25	0.016	0.12
IAL 6840	Brazil	0.06	0.25	0.12	8	0.5	0.06	0.12
IAL 6894	Brazil	0.03	0.06	0.03	2	0.25	0.016	0.06
IAL 6842	Brazil	<0.008	0.12	0.06	8	0.25	0.06	0.06
IAL 6843	Brazil	0.5	0.12	0.12	64	1	0.5	0.5
B13821	Ohio, USA	0.06	0.06	0.06	2	0.25	0.125	0.125
B13825	Alabama, USA	0.125	0.25	0.125	16	1	0.5	0.25
B15373	Texas, USA	0.03	0.125	0.06	4	0.125	0.06	0.06
B17801	Florida, USA	0.03	0.016	0.03	1	0.06	0.03	0.03
B17842	Washington,USA	0.03	0.05	0.125	4	0.25	0.03	0.125
B17906	Florida, USA	0.03	0.12	0.03	4	0.25	0.06	0.12
B18276	Hawaii, USA	0.016	0.06	0.06	2	0.06	0.03	0.06
B18536	Florida, USA	0.06	0.25	0.06	0.5	0.06	2	0.03
B18542	Mississipi, USA	0.25	0.06	0.06	16	0.5	0.25	0.25
B18543	Florida, USA	0.06	0.125	0.125	16	0.5	0.25	0.25
B18544	Florida, USA	0.25	0.06	0.06	32	1	0.5	0.5
B18545	Georgia, USA	0.25	0.25	0.125	2	0.5	0.25	0.25
B18546	Florida, USA	0.008	0.125	0.25	8	0.03	0.06	0.125
C. duobushaemulonii								
IAL 6907	Brazil	0.06	0.06	0.06	16	0.5	0.06	0.12
IAL 6892	Brazil	0.12	0.06	0.03	8	0.25	0.03	0.06
IAL 6902	Brazil	0.12	0.06	0.03	8	0.5	0.03	0.12

Strain or Isolate	Location	MIC (µg/mL) Broth Microdilution						
		VOR	AND	CAS	FZ	IZ	ISA	PZ
IAL 6841	Brazil	0.06	0.06	0.03	4	0.25	0.03	0.06
IAL 6918	Brazil	0.06	0.25	0.12	4	0.5	0.03	0.12
IAL 7015	Brazil	0.06	0.016	0.016	4	0.25	0.03	0.06
B13671	Connecticut, USA	0.06	0.125	0.03	4	0.125	0.06	0.125
B13918	Virginia, USA	1	0.25	0.06	128	0.25	1	0.25
B16296	Ohio, USA	0.5	0.06	0.03	16	0.25	0.06	0.125
B17774	Georgia, USA	0.06	0.008	0.008	0.125	0.25	0.03	0.06
B18258	Alabama, USA	0.016	0.06	0.03	1	0.016	0.008	0.008
B18574	Georgia, USA	0.25	0.03	0.06	8	0.25	0.125	0.25
C. pseudohaemulonii								
B12710	Not available	0.125	4	>16	2	0.125	0.016	0.06
B12711	Not available	0.06	0.5	0.125	2	0.25	0.016	0.06
B12713	Not available	0.125	4	>16	4	0.25	0.016	0.06
B18280	Michigan, USA	0.03	0.06	0.12	2	0.12	0.016	0.016
C. vulturna								
B13712	Wisconsin, USA	0.03	0.016	0.016	1	0.06	0.008	0.03
B16675	Florida, USA	0.06	1	0.125	8	0.25	0.125	0.25

Abbreviations: MIC, minimum inhibitory concentration; VOR voriconazole; AND anidulafungin; CAS caspofungin; FZ fluconazole; IZ itraconazole; ISA isavuconazole; PZ posaconazole; MF micafungin; AMB amphotericin B;