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Cryptic and Rare *Aspergillus* Species in Brazil: Prevalence in Clinical Samples and *In Vitro* Susceptibility to Triazoles

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***Aspergillus* spp. are among the most common causes of opportunistic invasive fungal infections in tertiary care hospitals. Little is known about the prevalence and *in vitro* susceptibility of *Aspergillus* species in Latin America, because there are few medical centers able to perform accurate identification at the species level. The purpose of this study was to analyze the distribution of cryptic and rare *Aspergillus* species among clinical samples from 133 patients with suspected aspergillosis admitted in 12 medical centers in Brazil and to analyze the *in vitro* activity of different antifungal drugs. The identification of *Aspergillus* species was performed based on a polyphasic approach, as well as sequencing analysis of the internal transcribed spacer (ITS) region, calmodulin, and β-tubulin genes and phylogenetic analysis when necessary. The *in vitro* susceptibility tests with voriconazole, posaconazole, and itraconazole were performed according to the CLSI M38-A2 document (2008). We demonstrated a high prevalence of cryptic species causing human infection. Only three isolates, representing the species *Aspergillus thermomutatus*, *A. ochraceus*, and *A. calidoustus*, showed less *in vitro* susceptibility to at least one of the triazoles tested. Accurate identifications of *Aspergillus* at the species level and with *in vitro* susceptibility tests are important because some species may present unique resistance patterns against specific antifungal drugs.**

Aspergillus is a ubiquitous fungus that is responsible for a wide spectrum of infections. One of the most important clinical manifestations of *Aspergillus* is invasive aspergillosis (IA), which is associated with high morbidity and mortality rates (1, 2). The genus *Aspergillus* is divided into eight subgenera that in turn are subdivided into several sections that include a large variety of closely related species (3, 4). The most clinically relevant sections are *Fumigati*, *Flavi*, *Terrei*, *Usti*, *Nigri*, and *Nidulantes* (5). Molecular studies have revealed numerous cryptic species within the different sections of the genus *Aspergillus* (6).

Historically, *Aspergillus* has been identified in the laboratory by conventional methods such as colony morphology and microscopic characteristics. However, there is a consensus that morphological characteristics may not be reliable for distinguishing between *Aspergillus* species (7). Despite its clinical relevance and several comprehensive studies dealing with the taxonomy of *Aspergillus* in the last few years, the taxonomy of *Aspergillus* remains somewhat ill defined. For consistent species identification, analyses of morphological, physiological, and molecular characteristics are required (7, 8). As this process is not suitable for routine testing by clinical microbiological laboratories, identification of *Aspergillus* clinical isolates at the species level has been scarcely reported (9). The accurate identification of species is critical given that different species may present peculiarities in terms of reservoir, virulence factors, natural history of infection, and *in vitro* susceptibility to antifungal drugs (10, 11).

The aim of this study was to analyze the distribution of *Aspergillus* species among clinical samples isolated from 133 patients with suspected aspergillosis admitted to 12 medical centers in Brazil and to analyze the *in vitro* antifungal susceptibility profiles of rare and cryptic species within the genus.

MATERIALS AND METHODS

Fungal isolates. We selected 133 isolates previously identified as *Aspergillus* spp. obtained from 133 different patients admitted to 12 medical centers in Brazil between 2006 and 2013. All isolates were interpreted as pathogens by the clinicians following the criteria suggested by the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) before being sent for further identification in our reference lab. The isolates were grown on slanted potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) for 7 days at 25°C and were covered with mineral oil for long-term room temperature storage until analysis.

Morphological examination and thermotolerance. The isolates were grown on PDA, malt extract agar (MEA) (Difco Laboratories, Detroit, MI, USA), and Czapek agar (CZK) (Difco Laboratories, Detroit, MI, USA). The fungi were inoculated at three points on duplicate plates of each medium and incubated at 15, 25, 37, 42, and 50°C for 14 days in the dark (12). Micromorphology observations were performed on microscopic mounts prepared in lactic acid from MEA colonies. The thermotolerance test involved assessment of the presence or absence of fungal growth at different temperatures (8).

Molecular identification: DNA extraction, amplification, and sequencing of ITS, calmodulin, and β-tubulin genes. The isolates were grown on yeast extract sucrose agar (YES) (10 g yeast extract, 75 g sucrose, 10 g agar, and 500 ml distilled water). Then, DNA was extracted with the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The DNA concentration and purity (relative to proteins and salts) were determined by optical density at 260 nm (OD_{260}) and ratios of $OD_{260/280}$ and $OD_{260/230}$, respectively. Fragments of the calmodulin (CAL) and β-tubu-

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TABLE 1 GenBank accession numbers of the nucleotide sequences representatives of each identified species and reference strains included in this study

Aspergillus species	Strain[a]	GenBank accession no. for: β-Tubulin	Calmodulin
A. flavus	CBS 100927^{NT}	AY819992.1	EF202063.1
A. flavus	LEMI803	KJ767726	KJ766990
A. flavus	LEMI925	KJ767727	KJ766989
A. flavus	LEMI953	KJ767728	KJ766988
A. flavus	LEMI896	KJ767729	KJ766987
A. arachidicola	CBS 117610^{T}	EF203158	EF202049.1
A. arachidicola	LEMI760	KJ767720	KJ767736
A. minisclerotigenes	CBS 117620	EF203150.1	EF202073.1
A. parasiticus	CBS 100926^{NT}	EF203155.1	EF202043.1
A. sergii	MUM 10.219^{T}	HM803082	HM803029
A. transmontanensis	MUM 10.214^{T}	HM803101	HM803020
A. transmontanensis	LEMI800	KJ766997	KJ767732
A. nomius	NRRL 13137^{T}	EF661494.1	EF661531.1
A. nomius	LEMI878	KJ767725	KJ767731
A. oryzae var. *effusus*	NRRL 506^{T}	JN185446.1	JN185447.1
A. oryzae	NRRL 447^{T}	EF661483.1	EF661506.1
A. sojae	CBS 100928^{T}	EF203168.1	EF202041.1
A. tamarii	NRRL 20818^{T}	EF661474.1	EF661526.1
A. tamarii	LEMI436	KJ767721	KJ767722
A. tamarii	LEMI999	KJ767733	KJ767735
A. caelatus	NRRL 25528^{T}	EF661470.1	AF255036.1
A. tennesseensis	NRRL 13150^{T}	JN853976.1	JN854017.1
A. tennesseensis	LEMI875	KJ766999	KJ766995
A. tennesseensis	LEMI917	KJ766998	KJ766994
A. tennesseensis	LEMI870	KJ767000	KJ766996
A. puulaauensis	NRRL 35641^{T}	JN853979.1	JN854034.1
A. puulaauensis	NRRL 58602	JN853999.1	JN854048.1
A. cvjetkovicii	NRRL 227^{T}	EF652264.1	EF652352.1
A. cvjetkovicii	NRRL 58593	JN853998.1	JN854044.1
A. cvjetkovicii	NRRL 4642	EF652291.1	EF652379.1
A. creber	NRRL 58592^{T}	JN853980.1	JN854043.1
A. subversicolor	NRRL 58999^{T}	JN853970.1	JN854010.1
A. asperescens	NRRL 4770^{T}	EF652299.1	EF652387.1
A. multicolor	NRRL 4775^{T}	EF652301.1	EF652389.1
A. awamori	CCF 4068^{T}	HE661602.1	FR751414.1
A. awamori	LEMI1010/LEMI993	KJ777804	KJ777809
A. foetidus	CBS564.65	GU296697.1	FN594547.1
A. foetidus	LEMI891	KJ777808	KJ777811
A. tubingensis	CBS 134.48^{T}	FJ629305.1	FN594558.1
A. creber	NRRL 58673^{T}	JN853993.1	JN854056.1
A. creber	NRRL 58670	JN853991.1	JN854053.1
A. creber	NRRL 58672	JN853992.1	JN854055.1
A. creber	LEMI984	KJ767001	KJ766991
A. jensenii	NRRL 58600^{T}	JN854007.1	JN854046.1
A. jensenii	NRRL 225	JN854000.1	JN854020.1
A. jensenii	NRRL 235	JN854001.1	JN854027.1
A. jensenii	NRRL 240	JN854002.1	JN854030.1
A. venenatus	NRRL 13147^{T}	JN854003.1	JN854014.1
A. venenatus	NRRL 13148	JN854004.1	JN854015.1
A. venenatus	NRRL 13149	JN854005.1	JN854016.1
A. sydowii	NRRL 250^{T}	EF652274.1	EF652362.1
A. sydowii	NRRL 254	EF652275.1	EF652363.1
A. sydowii	NRRL 4768	EF652297.1	EF652385.1
A. sydowii	NRRL 5585	JN853936.1	JN854039.1
A. austroafricanus	NRRL 233^{T}	JN853963.1	JN854025.1
A. protuberus	NRRL 3505^{T}	EF652284.1	EF652372.1
A. protuberus	NRRL 58942	JN853956.1	JN854061.1
A. protuberus	NRRL 58748	JN853967.1	JN854060.1
A. amoenus	NRRL35600	JN853952.1	JN854033.1
A. amoenus	NRRL 4838^{T}	EF652304.1	EF652392.1
A. amoenus	NRRL 226	JN853939.1	JN854021.1
A. amoenus	NRRL 236	JN853940.1	JN854028.1
A. tabacinus	NRRL 4791^{T}	EF652302.1	EF652390.1
A. tabacinus	NRRL A-23173	JN853960.1	JN854065.1
A. tabacinus	NRRL 5031	JN853947.1	JN854036.1
A. tabacinus	LEMI968	KJ767002	KJ766992
A. versicolor	NRRL 13145	JN853950.1	JN854012.1
A. versicolor	NRRL 13144	JN853949.1	JN854011.1
A. versicolor	NRRL 13146	JN853951.1	JN854013.1
A. fructus	NRRL 241	JN853943.1	JN854031.1
A. niger	CBS 554.65^{T}	FJ629288.1	FN594540.1
A. niger	LEMI975	KJ777807	KJ777813
A. thermomutatus	LEMI918	KJ767003	KJ766993
A. ochraceus	LEMI966	KJ767724	KJ767730
A. clavatus	LEMI40	KJ767723	KJ767734
A. terreus	LEMI941	KJ777806	KJ777812
A. calidoustus	LEMI749	KJ777803	KJ790258
A. fumigatus	LEMI864	KJ777805	KJ777810

[a] T, type strain; NT, neotype strain.

lin (BT2) genes and the internal transcribed spacer (ITS) region of rDNA were amplified with the primer pairs cl1/cl2a, bt2a/bt2b (13), and ITS1/ITS4, respectively. The reactions were performed with PCR master mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. After amplification, the fragments were sequenced following the protocol provided with the BigDye reagent kit (Applied Biosystems, Foster City, CA, USA) in an ABI 3130 (Applied Biosystems, Foster City, CA, USA) automatic sequencer. PCR products were sequenced with the same primers used for amplification. Contig assembly and editing were performed with Sequencher DNA sequence assembly software 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Successful assembly of the contigs required a minimum match percentage of ≥85 and a minimum overlap of 20.

BLAST analysis. Complete CAL, BT2, and ITS consensus sequences were used to conduct BLAST search analysis (BLASTn) for species identification from the NCBI genomic database (http://blast.ncbi.nlm.nih.gov/). For all regions analyzed by BLAST search, the sequences that presented with high identity (≥99%), queries and E values of $e10^{-5}$ were considered for the final species identification using the sequencing method.

The identification of *Aspergillus* species was performed based on macromorphology, micromorphology, and thermotolerance of the colonies, as well as on sequence comparisons of the ITS region and the calmodulin and β-tubulin genes with published sequences in genomic databases. Phylogenetic analyses using Bayesian inference and maximum parsimony methodologies were performed to characterize isolates with inconsistent identification by morphological and genotypic analysis.

Phylogenetic analysis. Most-parsimonious analysis was carried out for all data sets using PAUP* version 4.0b10 (14). One hundred heuristic searches were conducted using random sequence addition and tree bisection-reconnection branch-swapping algorithms, collapsing zero-length branches, and saving all minimal-length trees (MulTrees) on different sets of data. Gaps were treated as missing data. Support for internal branches was assessed by a heuristic parsimony search of 1,000 bootstrapped sets of data. Other measures were also taken, including tree length, consistency index (CI), homoplasy index (HI), and retention index (RI). The combined data set was tested for incongruence with the partition homogeneity test as implemented in PAUP*. The alignments used in the phylogenetic analysis were deposited in TreeBASE (www.treebase.org).

Bayesian posterior probabilities were calculated using MrBayes 3.12

(15). A neighbor-joining (NJ) tree was analyzed in the ModelTest program to estimate the best model of nucleotide substitution for application on the phylogeny inference. A general time-reversible (GTR) model was used with a proportion of invariant sites and a gamma-shaped distribution of rates across the sites. Markov chain Monte Carlo (MCMC) analysis was conducted for up to 1×10^6 generations until the chain converged. Concordance analysis was based on the exclusionary principle of Baum and Shaw (16) and the genealogical concordance phylogenetic species recognition concepts of Taylor et al. (17). Clades were recognized as independent evolutionary lineages if a clade was strongly supported by both parsimony and Bayesian analysis in at least one locus and the result was not contradicted by another strongly supported locus. Strong support was assessed at >70% bootstrap and >0.95 Bayesian posterior probability (11, 18, 19).

Nucleotide sequence accession numbers. The nucleotide sequence representatives of each identified species in this study were deposited in the GenBank database under the accession numbers given in Table 1.

Antifungal susceptibility tests. Antifungal susceptibility testing was performed as outlined in the Clinical and Laboratory Standards Institute (CLSI) M38-A2 protocol (20). The isolates were cultured on PDA and incubated at 25°C for 7 days to prepare the fungal inocula. Briefly, 100-μl culture preparations in RPMI 1640 (Vitrocell, Campinas, São Paulo, Brazil) were inoculated into the flat-bottom wells of 96-well microtiter plates containing 100 μl of the drug dilutions. The final inoculum concentration ranged from 0.4×10^4 to 5×10^4 CFU/ml. The drugs tested were provided by the manufacturers as pure powders and included itraconazole (ITC) (Sigma, Janssen Pharmaceutica, Beerse, Antwerp, Belgium), voriconazole (VRC) (Sigma, Pfizer, Inc., New York, NY, USA), and posaconazole (PSC) (Schering-Plough, Inc., Kenilworth, IL, USA). The MIC values were determined visually as the lowest concentrations that resulted in complete growth inhibition. Tests were performed in duplicate, and when the results did not concur, the test was repeated and the mode of the MICs was considered (11, 21).

RESULTS

Screening of *Aspergillus* sections. Based on morphological characterization, 133 isolates were classified into 9 different sections: *Fumigati* ($n = 72$), *Flavi* ($n = 37$), *Nigri* ($n = 13$), *Nidulantes* ($n = 5$), *Terrei* ($n = 2$), *Circumdati* ($n = 1$), *Usti* ($n = 1$), *Flavipedes* ($n = 1$), and *Clavati* ($n = 1$).

BLAST analysis of the ITS region sequences confirmed our morphological findings and the phenotypic identification of those sections.

Identification of species by polyphasic approach. BLAST of calmodulin and β-tubulin gene sequences together with morphological and ITS characterization provided consistent identification at the species level of 82 out of the 133 isolates tested. All of the species from *Fumigati*, *Circumdati*, *Usti*, *Terrei*, *Flavipedes*, and *Clavati* sections were identified using the polyphasic approach (morphology, thermotolerance, and sequencing) without requiring further phylogeny analyses. However, those tools did not identify 35 isolates of the *Flavi* section, 11 isolates from the *Nigri* section, and 5 isolates from the *Nidulantes* section.

Thermotolerance testing was useful in the discrimination of closely related species within the sections *Circumdati* (*A. ochraceus* versus *A. westerdijkiae*), *Usti* (*A. ustus* versus *A. calidoustus*), and *Fumigati* (*A. fumigatus* versus *A. thermomutatus*). Another interesting finding was that only the *A. fumigatus* strains grew at 50°C. The 51 isolates from the *Flavi*, *Nigri*, and *Nidulantes* sections that were not identified at the species level using the strategies outlined above underwent further phylogenetic analysis.

Phylogenetic analysis of the *Flavi*, *Nigri*, and *Nidulantes* sections. The results of the partition homogeneity test on sections *Flavi*, *Nigri*, and *Nidulantes* showed that the sequence data sets for the two selected loci (BT2 and CAL) were congruent and could therefore be combined ($P = 0.1667$, 0.1667, and 0.333, respectively).

The combined gene fragments generated by BT2 and CAL sequencing from *Flavi*, *Nigri*, and *Nidulantes* sections were 990 bp, 1,047 bp, and 1,372 bp, respectively. Unambiguous sequences of type and neotype strains corresponding to potential species to be identified were inserted into the analysis (Table 1). The analysis also included previously identified strains to function as outgroups.

Section *Flavi*. A most-parsimonious tree of 235 steps in length with a CI of 0.8340, HI of 0.1660, and RI of 0.9286 was produced from a heuristic search using the combined data set of 990 characters from two loci (Fig. 1), including 811 constant, 130 variable parsimony-informative, and 49 variable and parsimony-uninformative characters. After the analysis, we noted the presence of 3 main groups with well-supported clades. (i) Group I, denominated *A. parasiticus* complex, with 85% of bootstrap (bs) and Bayesian posterior probability (bpp) of 0.95 included 5 clinical isolates, type and neotype strains of *A. parasiticus*, *A. sojae*, *A. transmontanensis*, and *A. arachidicola*. One isolate (LEMI760) allocated as *A. arachidicola* and another isolate (LEMI800) as *A. transmontanensis*. Three isolates grouped apart from the reference strains and were named *Aspergillus* sp. 1. (ii) Group II, named *A. flavus* complex (100% bs and 1 bpp), contained 29 clinical isolates and type and neotype strains of *A. flavus*, *A. oryzae*, and *A. oryzae* var. *effusus*. The type strain sequences of *A. minisclerotigenes* and *A. sergii* were phylogenetically close to the *A. flavus* complex but were not grouped with any isolate. (iii) Group III (100% bs and 1 bpp) was subdivided into two subgroups. One subgroup included two clinical isolates and a type strain of *A. tamarii*. The other group included one clinical isolate and a type strain of *A. nomius*. Notably, *A. tamarii* and *A. nomius* were previously identified using morphological features and BLAST analysis.

Section *Nigri*. A most-parsimonious tree of 117 steps in length with a CI of 0.9316, HI of 0.0684, and RI of 0.9535 was produced from a heuristic search using the combined data set of 1,047 characters from two loci (Fig. 2), including: 943 constant, 65 variable parsimony informative, and 39 variable and parsimony uninformative. After analysis, we noted the presence of 2 main groups with well-supported clades. Group I (100% bs and 1 bpp) was subdivided into other 2 subgroups, (i) the subgroup *A. niger* (100% bs and 0.96 bpp), which contained 4 clinical isolates and one type strain of *A. niger*, and (ii) the subgroup *A. awamori* (100% bs and 0.95 bpp), which contained 7 clinical isolates and one type strain of *A. awamori*. This subgroup was also subdivided into 2 groups. One of them contained 4 clinical isolates and no reference sequences (*Aspergillus* sp. 2), and the other contained 3 clinical isolates and one type strain of *A. awamori*. The isolate LEMI926 was related to subgroup *A. awamori* but was not identified. We referred to this isolate as *Aspergillus* sp. 3. Group 2, also with 100% bs and 1 bpp, included one clinical isolate, one type strain of *A. foetidus*, and one separate branch with one type strain sequence of *A. tubingensis*.

Section *Nidulantes*. A most-parsimonious tree of 535 steps long with a CI of 0.8224, HI of 0.1776, and RI of 0.9251 was produced from a heuristic search using the combined data set of 1,372 characters from two loci (Fig. 3), including 1,009 constant, 188 variable parsimony informative, and 175 variable and parsimony uninformative. After the analysis, we noted the presence of

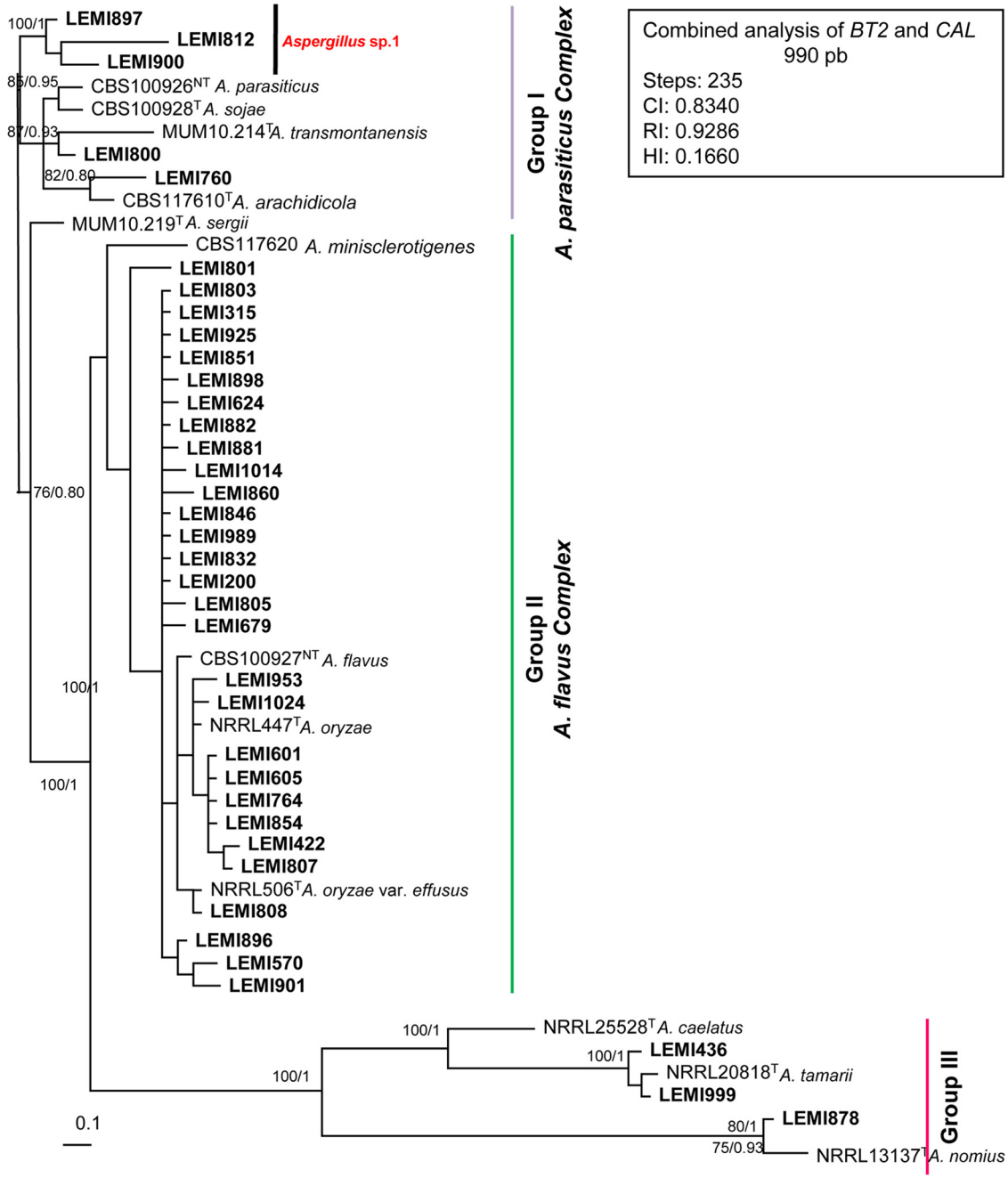

FIG 1 *Aspergillus* section *Flavi*. Most-parsimonious tree obtained from a heuristic search based on parsimony analysis of the data produced from the BT2 and CAL genes combined. The numbers at the nodes of the branches are bootstrap values/Bayesian posterior probability. Clinical isolates are designated in bold. T, type strain; NT, neotype strain. Bar, 0.1 nucleotide changes between taxa.

2 main groups. Group I was a well-supported clade (100% bs and 1 bpp) subdivided into two other subgroups, *versicolor* and *sydowii*. The subgroup *versicolor* (100% bs and 1 bpp) contained one clinical isolate and one type strain from each of the following species: *A. tabacinus*, *A. fructus*, *A. versicolor*, *A. austroafricanus*, and *A. protuberus*. The LEMI968 isolate grouped with the *A. tabacinus* type strain. The subgroup *sydowii* (100% bs and 1 bpp) contained 4 clinical isolates and 1 type strain of *A. sydowii*, *A. venenatus*, *A. cvjetkovicii*, *A. creber*, *A. puulaauensis*, *A. tennesseensis*, and *A. jensenii*. The clinical isolate LEMI984 clustered with the reference sequences of *A. creber* with 100% bs and 1 bpp. The other 3 clinical isolates (LEMI875, LEMI917, and LEMI870) grouped with reference sequences of *A. tennesseensis*. Group II, with 100% bp and 0.97 bpp, consisted of *A. asperescens* and *A. multicolor* sequences. The reference sequences of *A. subversicolor* were used as outgroups.

Final species identification and susceptibility profiles to triazoles. According to data generated by conventional molecular tools and phylogenetic analysis, *A. fumigatus* was found to be the most prevalent species, with 71 (53%) isolates, followed by *A.*

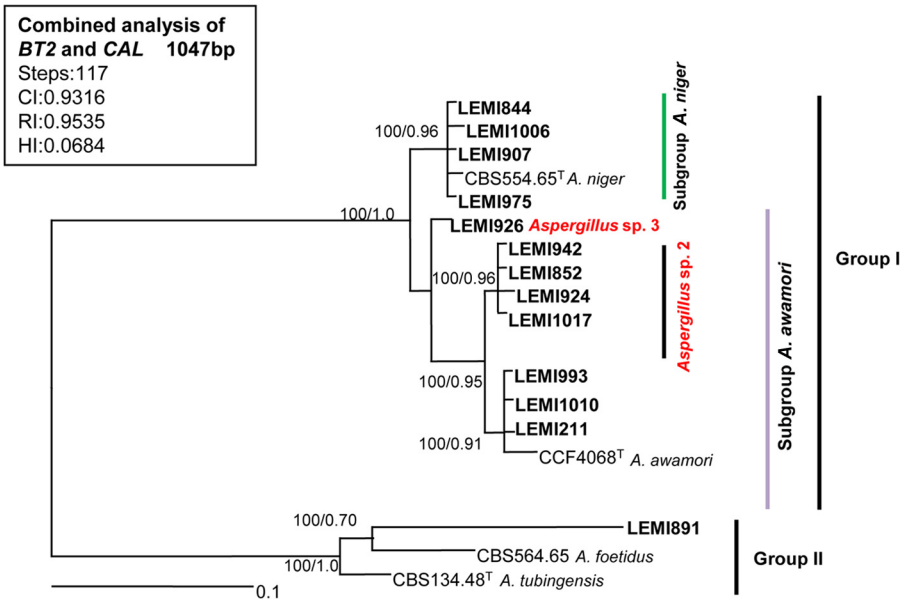

FIG 2 *Aspergillus* section *Nigri.* Most-parsimonious tree obtained from a heuristic search based on parsimony analysis of the data produced from the BT2 and CAL genes combined. The numbers at the nodes of the branches are bootstrap values/Bayesian posterior probability. Clinical isolates are highlighted in bold type. T, type strain; NT, neotype strain. Bar, 0.1 nucleotide changes between taxa.

flavus with 29 (22%) isolates and *A. niger* with only 4 (3%) isolates. The following less frequent or rare species were also found: *A. clavatus* ($n = 1$), *A. flavipes* ($n = 1$), and *A. terreus* ($n = 2$). Among the cryptic species found in Brazilian clinical samples, the most frequent was *Aspergillus* sp. 2 undescribed species (4 isolates, section *Flavi*), followed by *A. awamori* (section *Nigri*), *Aspergillus* sp. 1 undescribed species (section *Flavi*), and *A. tennesseensis* (section *Nidulantes*), with 3 isolates each. Other species found were related to *Flavi* (2 *A. tamarii* strains, 1 *A. arachidicola* strain, 1 *A. transmontanensis* strain, and 1 *A. nomius* strain), *Fumigati* (1 A. *thermomutatus* strain), *Circumdati* (1 *A. ochraceus* strain), *Nidulantes* (1 *A. tabacinus* strain and 1 *A. creber* strain), *Nigri* (1 *A. foetidus* strain and 1 *Aspergillus* sp. 3 strain), and *Usti* (1 *A. calidoustus* strain). We observed that 8 isolates from our study (32% of cryptic species) were not identified by any of our 3 applied methodologies and possibly represent new *Aspergillus* species (Table 2).

Of note, only three cryptic species isolates (*A. calidoustus*, *A. thermomutatus*, and *A. ochraceus*) presented high MIC values against at least one of the triazoles tested (Table 2). The *A. calidoustus* isolate presented high MIC values against all triazoles tested (MICs of 4 μg/ml against voriconazole and posaconazole and >32 μg/ml against itraconazole). The isolate of *A. thermomutatus* showed high MIC values against itraconazole and voriconazole, 2 and 16 μg/ml, respectively. The *A. ochraceus* isolate presented a high MIC value (4 μg/ml) only against itraconazole. The other isolates of *Aspergillus* cryptic species showed MIC values ranging from 0.03 and 2 μg/ml for all triazoles studied.

DISCUSSION

Advances in genomic and molecular tools have provided improved conditions for the classification of all microorganisms, including fungal species. Following the application of new molecular tools in taxonomic studies of the *Aspergillus* genus, a large number of new species have been described in the past few decades (6). This new information has driven the attention of the medical community to an increasing number of invasive fungal infections caused by rare and cryptic species of *Aspergillus* that in the recent past had been misidentified and most likely underestimated (6).

A variety of factors have all contributed to an increase in the number of people at risk of developing fungal infections, including by agents that had never been described as human pathogens. These factors include an aging population, large numbers of patients with degenerative and neoplastic diseases, patients who have had solid organ and hematopoietic stem cell transplantations, and patients under immunosuppressive therapy (22, 23).

The most common species implicated in IA is *Aspergillus fumigatus*. Other *Aspergillus* species, including *A. flavus*, *A. niger*, *A. terreus*, and *A. ustus*, have also been reported as pathogens. *Aspergillus flavus* has been described as the second most common *Aspergillus* species in several medical centers from Europe and the United States, whereas *A. terreus* is particularly frequent in Austria (6th Trends in Medical Mycology Workshop, w 10.1) (24, 25). In countries such as Saudi Arabia, Sudan, and Taiwan, with semi-arid, arid dry, and tropical weather conditions, *A. flavus* appears to be the main etiological agent of invasive aspergillosis (26, 27).

In the study samples described here, *A. flavus* was the second most common species, responsible for 22% of all clinical isolates. The prevalence of *A. flavus* in our collection is substantially higher than the rates demonstrated in recent multicenter studies from the United States and Spain (9 to 10%) (28, 29). Indeed, this observed prevalence is also considerably higher than the 10% rates of *A. flavus* found in a worldwide collection represented by 771 *Aspergillus* clinical samples obtained from 62 medical centers (30).

In addition to the different species we found within the section *Flavi*, we characterized a large spectrum of cryptic species related to sections *Circumdati*, *Fumigati*, *Nigri*, *Usti*, and *Nidulantes*. Overall, cryptic species of *Aspergillus* represented 19% of our collection. This number agrees closely with the rate recently found by

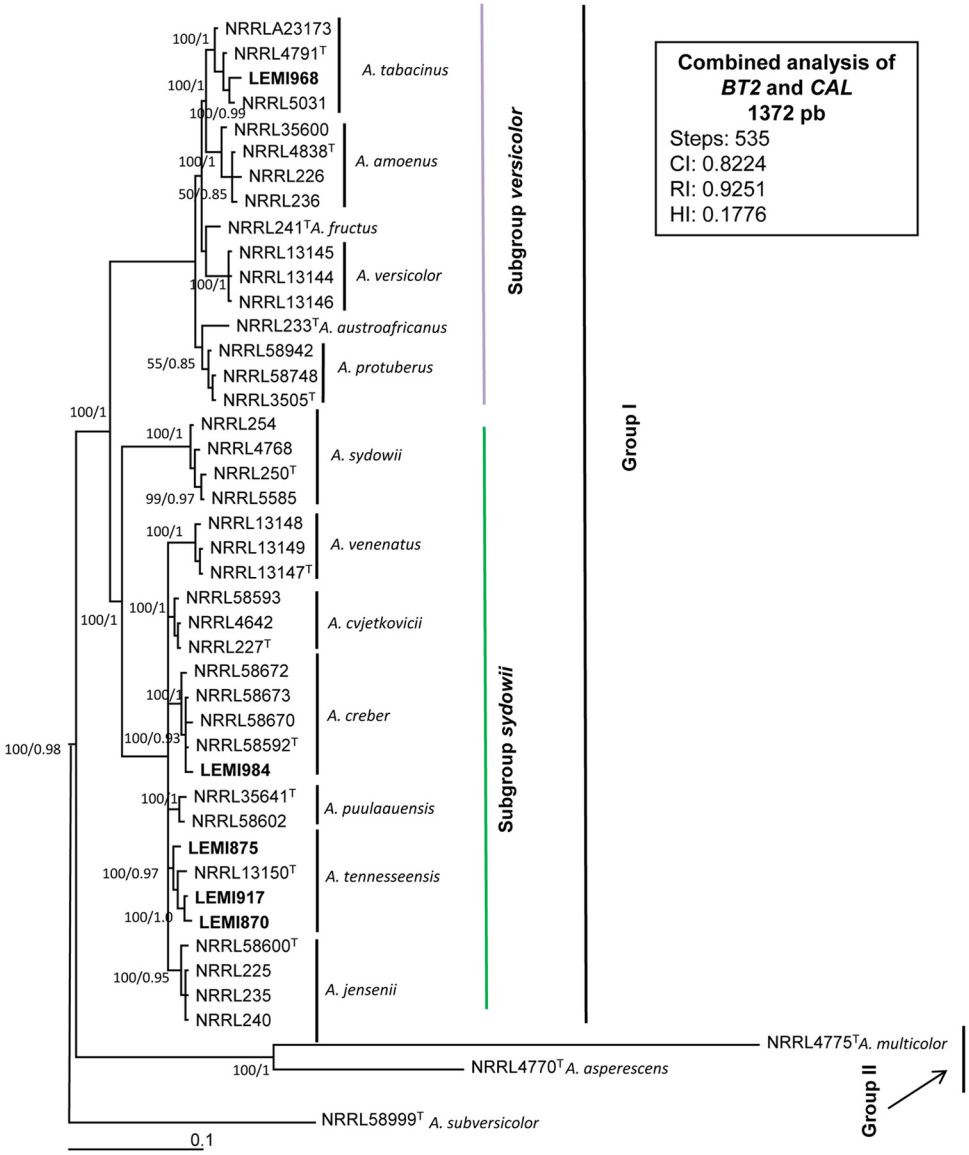

FIG 3 *Aspergillus* section *Nidulantes*. Most-parsimonious tree obtained from a heuristic search based on parsimony analysis of the data produced from the BT2 and CAL genes combined. The numbers at the nodes of the branches are bootstrap values/Bayesian posterior probability. Clinical isolates are highlighted in bold type. T, type strain; NT, neotype strain. Bar, 0.1 nucleotide changes between taxa.

Alastruey-Izquierdo et al. (29) by testing 325 isolates in filamentous fungi. The most frequently identified cryptic species being reported in both Europe and the United States are *Aspergillus alliaceus* (section *Flavi*), *Aspergillus tubingensis* (section *Nigri*), *Aspergillus calidoustus* (section *Usti*), and *Aspergillus lentulus* (section *Fumigati*) (28, 29, 31). In Brazil, unlike in Europe and the United States, *A. tamarii* and *A. awamori* were the two cryptic species most commonly found within the sections *Flavi* and *Nigri*, respectively.

Of note, some cryptic species described in our study had never been reported in human hosts such as *A. arachidicola*, *A. tabacinus*, *A. tennesseensis*, *A. creber*, and *A. transmontanensis*. As illustrated in Table 2, all of these strains were related to episodes of colonization and/or infection of the respiratory tract. In addition, we also found 5 other cryptic species already described in the literature as agents of human infections (*A. ochraceus*, *A. tamarii*, *A. nomius*, *A. thermomutatus*, and *A. foetidus* [3, 11, 32]).

In terms of antifungal susceptibility, it is notable that almost 90% of all cryptic species exhibited susceptibility to the 3 triazoles tested. The exceptions were strains representative of *A. calidoustus*, *A. ochraceus*, and *A. thermomutatus* that were less susceptible *in vitro* against at least one of the three triazoles tested. The reduced *in vitro* susceptibility to triazoles of *A. thermomutatus* and *A. calidoustus* has also been demonstrated in other studies (10, 33).

We conclude that there is a great diversity of species belonging to the *Aspergillus* genus causing human colonization and/or infections in Brazil, with a higher occurrence of *Aspergillus* section *Flavi* compared to U.S. and European medical centers. We emphasized the importance of accurate identification and *in vitro* susceptibil-

TABLE 2 Cryptic and rare *Aspergillus* species distributions, *in vitro* susceptibility profiles, and sources of isolates

Section (no. of isolates)	Species identification (no. of isolates)	Source (no. of isolates)	MIC data (μg/ml) for:					
			Itraconazole		Voriconazole		Posaconazole	
			Range	GM[a]	Range	GM	Range	GM
Clavati (1)	*A. clavatus* (1)	Respiratory tract biopsy specimen (1)	1.0		1.0	1.0	0.5	0.5
Circumdati (1)	*A. ochraceus* (1)	Respiratory secretion (1)	4.0		1.0	1.0	0.5	0.5
Flavi (8)	*A. arachidicola* (1)	Respiratory tract biopsy specimen (1)	0.5		1.0	1.0	0.125	0.125
	Aspergillus sp. 1 (3)	Respiratory secretion (2), respiratory tract biopsy specimen (1)	0.5–1.0	0.63	0.5–1.0	0.63	0.125–0.25	0.16
	A. tamarii (2)	Tissue biopsy specimen (1), respiratory tract biopsy specimen (1)	0.25–0.5	0.35	0.125–0.5	0.25	0.03–0.125	0.06
	A. transmontanensis (1)	Respiratory tract biopsy specimen (1)	0.25		1.0		0.125	
	A. nomius (1)	Respiratory secretion (1)	0.5		1.0		0.25	
Flavipedes (1)	*A. flavipes* (1)	Tissue biopsy specimen (1)	0.125		0.125		0.25	
Fumigati (1)	*A. thermomutatus* (1)	Tissue biopsy specimen (1)	2.0		16.0		0.5	
Nidulantes (5)	*A. tennesseensis* (3)	Respiratory tract secretion (2), skin biopsy specimen (1)	0.5–1.0	0.63	0.5–1.0	0.63	0.25	0.25
	A. tabacinus (1)	Respiratory secretion (1)	1.0		1.0		0.25	
	A. creber (1)	Respiratory secretion (1)	0.5		0.25		0.5	
Nigri (9)	*A. awamori* (3)	Respiratory secretion (2), skin biopsy specimen (1)	0.25–2.0	0.5	0.25–0.5	0.4	0.125–0.25	0.16
	Aspergillus sp. 2 (4)	Respiratory secretion (3), ear secretion (1)	0.5–1.0	0.84	0.25–1.0	0.6	0.25–0.5	0.3
	Aspergillus sp. 3 (1)	Respiratory secretion (1)	1.0		1.0		0.25	
	A. foetidus (1)	Respiratory secretion (1)	2.0		0.25		0.25	
Terrei (2)	*A. terreus* (2)	Tissue biopsy specimen (1), ear secretion (1)	0.25–0.5	0.35	0.25–1.0	0.5	0.25	0.25
Usti (1)	*A. calidoustus* (1)	Tissue biopsy specimen (1)	>32		4.0		4.0	

[a] GM, geometric mean.

ity tests of clinical *Aspergillus* species. This will allow the generation of consistent data about potential peculiarities of infections caused by rare and cryptic species with regard to reservoirs, natural history, and clinical response to antifungal drugs.

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