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Aspergillus Section *Fumigati*: Antifungal Susceptibility Patterns and Sequence-Based Identification▽

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This study analyzed 28 *Aspergillus* strains belonging to the section *Fumigati* that were isolated from clinical samples in Spain. All isolates sporulated slowly and were unable to grow at 48°C. Phylogenetic analysis based on sequencing of partial sequences of the β-tubulin and rodlet A genes was used to classify the 28 strains into six different clades (*Neosartorya hiratsukae*, *Neosartorya pseudofischeri*, *Aspergillus viridinutans*, *Aspergillus lentulus*, *Aspergillus fumigatiaffinis*, and *Aspergillus fumisynnematus*). Antifungal susceptibility testing showed heterogeneous patterns and grouped the strains together by species. Most *A. lentulus* and *A. fumigatiaffinis* isolates showed high MICs of amphotericin B (geometric mean [GM] MICs, ≥4.5 μg/ml), itraconazole (GM MICs, ≥6 μg/ml), voriconazole (GM MICs, ≥3 μg/ml), and ravuconazole (GM MICs, ≥3 μg/ml); *N pseudofischeri* and *A. viridinutans* showed high MICs of itraconazole (GM MICs, ≥8 μg/ml), voriconazole (GM MICs, ≥3.33 μg/ml), and ravuconazole (GM MICs, ≥2 μg/ml); and *N. hiratsukae* and *A. fumisynnematus* were susceptible to all the antifungals tested. In conclusion, a number of different species whose morphological features resemble those of *Aspergillus fumigatus* could succeed in producing invasive infections in the susceptible host. In addition, some of them showed high MICs for most of the antifungals available for the treatment of patients infected with these organisms. The epidemiology and clinical relevance of these species should therefore be addressed.

The incidence of invasive aspergillosis continues to increase, due to the rising number of patients undergoing bone marrow or solid organ transplantation or corticosteroid treatment and those with hematological malignancies or pulmonary disease (18).

Invasive aspergillosis is mainly caused by *Aspergillus fumigatus*, although other species, such as *Aspergillus terreus*, *Aspergillus niger*, and *Aspergillus flavus* can also cause invasive infections (10, 15, 22). Furthermore, recent studies have reported on cases of aspergillosis caused by other *Aspergillus* species that belong to *Aspergillus* section *Fumigati* (4, 12, 14, 17).

Aspergillus section *Fumigati* has recently been reclassified by Samson et al. (36). It currently contains 25 different species, with 8 anamorphs (*Aspergillus brevipes*, *Aspergillus duricaulis*, *A. fumigatus*, *Aspergillus fumigatiaffinis*, *Aspergillus lentulus*, *Aspergillus novofumigatus*, *Aspergillus unilateralis*, *Aspergillus viridinutans*) and 17 telemorphs (*Neorsartorya aurata*, *Neorsartorya aureola*, *Neorsartorya coreana*, *Neorsartorya fennelliae*, *Neorsartorya fischeri*, *Neorsartorya glabra*, *Neorsartorya lacinosa*, *Neorsartorya spinosa*, *Neorsartorya quadricincta*, *Neorsartorya stramenia*, *Neorsartorya spathulata*, *Neorsartorya hiratsukae*, *Neorsartorya pseudofischeri*, *Neorsartorya tetenoi*, *Neorsartorya mulplicata*, *Neorsartorya udagawae*, and *Neorsartorya sublevispora*).

In the section *Fumigati*, besides *A. fumigatus*, other species, such as *Neorsartorya fischeri*, *Neorsartorya pseudofischeri*, *Neorsartorya hiratsukae*, and *A. lentulus*, have been reported to be human pathogens (3, 4, 12, 14, 17). This implies that in the appropriate human host, all of them could cause disease.

The conventional means of identification of *A. fumigatus* is based on its morphological characteristics and microscopic features. Several morphological characteristics for differentiation between species of the *Aspergillus* section *Fumigati* have been described. However, many species sporulate very slowly, and an extremely high level of expertise and long-term observation are also required to identify the species. Other species, such as those of the genus *Neosartorya*, are able to produce ascospores; however, a considerable length of time is usually required for the production of ascospores, and ascospore production is not a practical method of identification for clinical microbiology laboratories (14, 16, 17, 36). Although for the very experienced taxonomist the species included in the section *Fumigati* are not morphologically uniform, morphological observation is not sufficient to distinguish between them. This fact has led to species misidentification and also to the discarding of organisms as contaminants (6, 44).

In order to resolve this issue, a number of different techniques have been developed and used to identify the species belonging to this section. These include analysis of the profiles of secondary metabolites, isozyme electrophoretic pattern analysis (23, 27, 33, 38, 39, 40), and molecular data analysis (41, 44).

Although secondary resistance to azole drugs has been described in *A. fumigatus* strains (7, 8, 11, 25, 26, 28, 42), most *A. fumigatus* strains are susceptible to the antifungals available for the treatment of patients with invasive infections (13). Because few clinical laboratories routinely perform antifungal susceptibility testing of molds and resistance in the section *Fumigati* has already been reported (5, 24), the misidentification of these species is a matter of concern.

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TABLE 1. Origin, molecular identification, MICs, and MECs of different antifungal against clinical isolates of *Aspergillus* section *Fumigati*

Isolate	Molecular identification			MIC (μg/ml)[a]						MEC (μg/ml)[b]	
	Origin[c]	β-Tubulin	Rodlet A	AMB	ITC	VCZ	RVC	POS	TRB	CAS	MICA
ATCC 2004305	Reference strain	*A. fumigatus*	*A. fumigatus*	0.25–1.0	0.125–1.0	0.25–1.0	0.25–1.0	0.03–0.25	2.0–8.0	0.12–0.5	0.03
CNM-CM-1290	Sputum	*A. lentulus*	*A. lentulus*	4.5	9.25	4.5	3.5	0.40	1.33	0.28	0.05
CNM-CM-3134	Sputum	*A. lentulus*	*A. lentulus*	10	10.25	6	6	0.56	1.75	0.38	0.07
CNM-CM-3364	BAL	*A. lentulus*	*A. lentulus*	8.4	8	5.6	5.6	2	0.5	0.18	0.03
CNM-CM-3537	Sputum	*A. lentulus*	*A. lentulus*	7	8.25	3.5	3	0.28	1	0.42	0.04
CNM-CM-3538	Sputum	*A. lentulus*	*A. lentulus*	7.25	8.25	7.5	7	0.75	1	0.11	0.10
CNM-CM-3583	Sputum	*A. lentulus*	*A. lentulus*	8	0.43	3.5	2	0.25	1.25	1	0.03
CNM-CM-3599	Sputum	*A. lentulus*	*A. lentulus*	2.66	0.5	4	2	0.5	1	0.26	0.03
CNM-CM-4330	Sputum	*A. lentulus*	*A. lentulus*	8	6	3	1.5	0.12	0.5	1.2	0.03
CNM-CM-4370	Sputum	*A. lentulus*	*A. lentulus*	7.33	8	5.33	5.33	0.18	0.5	0.91	0.03
CNM-CM-4387	Sputum	*A. lentulus*	*A. lentulus*	6	8	4	3	0.25	0.5	0.25	0.03
CNM-CM-4415	Sputum	*A. lentulus*	*A. lentulus*	6	16	5.33	4.66	0.33	0.5	0.25	0.03
CNM-CM-4420	Nail	*A. lentulus*	*A. lentulus*	10.66	8	5.33	4	0.33	1	0.33	0.03
CNM-CM-4426	Sputum	*A. lentulus*	*A. lentulus*	12	6.66	5.33	5.33	0.20	0.83	0.22	0.03
CNM-CM-4428	Skin	*A. lentulus*	*A. lentulus*	12	7	5	5	0.62	0.66	0.29	0.03
CNM-CM-4063	BAS	*A. fumisynnematus*	*A. fumisynnematus*	1	0.25	0.83	0.83	0.12	1.66	0.5	0.03
CNM-CM-2280	Sputum	*A. fumigatiaffinis*	*A. fumigatiaffinis*	16	6.66	6.66	6	1.16	1.66	1.3	0.04
CNM-CM-3227	BAL	*A. fumigatiaffinis*	*A. fumigatiaffinis*	8	8	5.6	5	0.625	1.4	0.16	0.08
CNM-CM-3303	Skin	*N. hiratsukae*	*N. hiratsukae*	0.5	0.12	0.5	0.375	0.06	0.09	0.34	0.03
CNM-CM-3305	Skin	*N. hiratsukae*	*N. hiratsukae*	0.31	0.185	0.375	0.25	0.045	0.06	0.03	0.14
CNM-CM-3764	OPE	*N. hiratsukae*	*N. hiratsukae*	1	0.33	1.66	1.33	0.16	0.25	0.26	0.04
CNM-CM-3740	OPE	*N. hiratsukae*	*N. hiratsukae*	0.66	0.16	1	0.66	0.10	0.20	0.66	0.03
CNM-CM-4328	Corneal	*N. hiratsukae*	*N. hiratsukae*	0.75	0.25	1.25	0.5	0.09	0.09	0.03	0.03
CNM-CM-3769	Sputum	*N. pseudofischeri*	*N. pseudofischeri*	1	8	3.33	2	0.25	1.33	0.12	0.06
CNM-CM-2270	Sputum	*N. pseudofischeri*	*N. pseudofischeri*	0.17	8	3	3.5	0.31	1	0.51	0.26
CNM-CM-4060	Sputum	*N. pseudofischeri*	*N. pseudofischeri*	0.25	11	3.33	3.33	0.29	0.5	0.62	0.03
CNM-CM-3914	Nail	*N. pseudofischeri*	*N. pseudofischeri*	0.25	16	6.66	4	0.5	0.5	1	0.03
CNM-CM-3147	OPE	*A. viridinutans*	*A. viridinutans*	0.58	14.4	4	5.66	0.41	1.2	0.67	0.20
CNM-CM-4518	Nail	*A. viridinutans*	*A. viridinutans*	0.37	16	4	4	0.25	0.75	1	0.03

[a] The MICs of amphotericin B (AMB), itraconazole (ITC), voriconazole (VCZ), ravuconazole (RVC), posaconazole (POS), and terbinafine (TRB) are GMs. The MICs for the reference strain are ranges.
[b] The MECs of caspofungin (CAS) and micafungin (MICA) are GMs. The CAS MEC for the reference strain is the range.
[c] BAL, bronchoalveolar lavage fluid; BAS, bronchoalveolar aspirate; OPE, oropharyngeal exudate.

The aim of this study was to analyze clinical strains of *Aspergillus* section *Fumigati.* To date, we have analyzed a collection of 28 *Aspergillus* clinical strains that had previously been identified as atypical *A. fumigatus* isolates. We report on the results of the molecular identification by sequencing of both the β-tubulin and the rodlet A genes and the antifungal susceptibility testing profiles of the strains.

MATERIALS AND METHODS

Fungal strains and media. A total of 37 strains were included in this study; 28 *Aspergillus* section *Fumigati* strains (Table 1) were independent clinical isolates from different patients and belong to the Mold Collection of the Centro Nacional de Microbiologia (CNM). Control strains included a set of five *A. fumigatus* strains belonging to the CNM (strains CNM-CM-3248, CNM-CM-3254, CNM-CM-3258, CNM-CM-3652, and CNM-CM-3722) and four strains obtained from the Centralbureau voor Schimmelcultures (CBS) (*N. hiratsukae* CNM-CM-4551 [CBS 109356] and CNM-CM-4554 [CBS 117067] and *N. pseudofischeri* CNM-CM-4487 [CBS 404.67] and CNM-CM-4488 [CBS 208.92]).

The fungi were grown at 37°C in potato dextrose agar (Oxoid, Madrid, Spain) or malt extract agar. The conidial stocks were preserved in sterile distilled water at 4°C (2).

Fungal morphology and growth conditions. Fungal morphological features were examined by conventional methods (9). The differential temperature growth was determined by the presence or absence of growth at 37°C and 48°C for 3 days (4).

Antifungal susceptibility testing. Broth microdilution susceptibility testing was performed as described in the CLSI (formerly NCCLS) reference method (29), with minor modifications. The modifications included the use of RPMI 1640 with L-glutamine buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid and 10 M NaOH and supplemented with 2% glucose (Oxoid) and the use of an inoculum size of 1×10^5 to 5×10^5 CFU/ml (20, 31, 34).

The antifungal agents used in the study were amphotericin B (concentration range, 16 to 0.03 μg/ml; Sigma Aldrich Química), itraconazole (concentration range, 8 to 0.015 μg/ml; Janssen S.A., Madrid, Spain), voriconazole (concentration range, 8 to 0.015 μg/ml; Pfizer, S.A.), ravuconazole (concentration range, 8 to 0.015 μg/ml; Bristol-Myers, Squibb, Princeton, NJ), posaconazole (concentration range, 8 to 0.015 μg/ml; Schering-Plough Research Institute, Kenilworth, NJ), terbinafine (concentration range, 16 to 0.03 μg/ml; Novartis, Basel, Switzerland), caspofungin (concentration range, 16 to 0.03 μg/ml; Merck & Co, Inc., Rahway, NJ), and micafungin (concentration range, 16 to 0.03 μg/ml; Astellas Pharma Inc, Tokyo, Japan). Inoculum suspensions were prepared from fresh, mature (3- to 5-day-old) cultures by the use of a previously reported methodology (34). The plates were incubated at 35°C for 48 h in a humid atmosphere. Visual readings were performed with the help of a mirror. The endpoint for the MIC determination was the antifungal concentration that produced the complete inhibition of visual growth at 48 h. For the echinocandins, the minimal effective concentration (MEC) was used for endpoint determination. The MEC was defined as the minimal antifungal concentration that produced morphological alterations of hyphal growth at 48 h.

A. fumigatus ATCC 2004305 was used as the quality control strain to validate the MICs and MECs (reference values are shown in Table 1). Antifungal susceptibility testing was repeated at least three times on different days.

PCR amplification and sequencing. Partial sequences of the β-tubulin and the rodlet A genes were amplified with primer set βtub3 (5′-TTCACCTTCAGAC CGGT-3′) and βtub2 (4) and primer set RodA1 and RodA2, respectively (4).

All primers were synthesized by Sigma Genosys (Madrid, Spain). PCRs were carried out with a 50-μl volume containing 1× PCR buffer (Applied Biosystems, Madrid, Spain); 2 mM $MgCl_2$ (Applied Biosystems); 250 μM each of dATP, dGTP, dCTP, and dTTP (Applied Biosystems); 1 μM of each primer; 2.5 U of *Taq* DNA polymerase (Applied Biosystems); and 25 to 50 ng of *A. fumigatus* genomic DNA. Amplification was performed in a thermal cycler (GeneAmp PCR system 9700; Applied Biosystems) for 1 cycle of 5 min at 94°C and then 30 cycles of 30 s at 94°C, 45 s at 55°C, and 1 to 2 min at 72°C, followed by 1 final cycle of 10 min at 72°C. The PCR products were analyzed by electrophoresis on 0.8% agarose gels and were visualized by transillumination (Gel Doc 2000; Bio-Rad Laboratories, Madrid, Spain) after they were stained with ethidium bromide (Sigma, Madrid, Spain).

The sequencing reactions were undertaken as described before (1) with primers βtub1 (4) and βtub4 (5′-AGCGTCCATGGTACCAGG-3′) for the β-tubulin gene and primers RodA3 (5′-AACGTCCGCTTCCCCGTTC-3′), RodA4 (5′-TACGGCATCGGAAGGAGAG-3′), and RodA5 (5′-TACGGCATCGGAGGG AGAG-3′) for the rodlet A gene.

TABLE 2. GenBank sequences and accession numbers of the genes used in this study

GenBank accession no.	Gene	Isolate
DQ094884	β-Tubulin	*A. fumigatiaffinis* IBT 13131
DQ094885	β-Tubulin	*A. fumigatiaffinis* IBT 12703
AB248076	β-Tubulin	*A. fumisynnematus* IFM 42277
AB249897	Rodlet A	*A. fumisynnematus* IFM 42277
AB248077	β-Tubulin	*A. fumisynnematus* 90-BP-70
AB249898	Rodlet A	*A. fumisynnematus* 90-BP-70
AB248078	β-Tubulin	*A. fumisynnematus* 90-BP-177
AB249899	Rodlet A	*A. fumisynnematus* 90-BP-177
AY738513	β-Tubulin	*A. lentulus* FH 5
AY738514	Rodlet A	*A. lentulus* FH 5
AY738517	β-Tubulin	*A. lentulus* FH 4
AY738519	Rodlet A	*A. lentulus* FH 4
AY738520	β-Tubulin	*A. lentulus* FH 7
AY738522	Rodlet A	*A. lentulus* FH 7
AY738523	β-Tubulin	*A. lentulus* FH 220
AY738525	Rodlet A	*A. lentulus* FH 220
DQ094886	β-Tubulin	*A. novofumigatus* IBT 16806
DQ094887	β-Tubulin	*A. novofumigatus* IBT 16755
AB248299	β-Tubulin	*A. viridinutans* IFM 54303
AY590130	β-Tubulin	*A. viridinutans* MK284
AB250103	Rodlet A	*A. viridinutans*
AF057312	β-Tubulin	*A. clavatus* H 522
AF057322	Rodlet A	*A. clavatus* H 522

Sequence analysis. The sequences were assembled and edited with the SeqMan II and EditSeq software packages (Lasergene; DNAStar, Inc., Madison, WI). Sequence analysis was performed by comparing the DNA sequences with those of the control strains included in this study and with the sequences obtained from the GenBank database. Fourteen β-tubulin gene partial sequences and nine rodlet A gene partial sequences were used and are listed in Table 2.

Phylogenetic analysis. All phylogenetic analyses were conducted with InfoQuest FP software (version 4.50; Bio-Rad Laboratories). The methodology used was maximum-parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2,000 simulations. The *Aspergillus clavatus* β-tubulin and rodlet A gene sequences were used as the outgroups (Table 2).

Nucleotide sequence accession numbers. The following are the GenBank accession numbers for the β-tubulin and rodlet A gene fragment sequences from all the strains used in this work: for the β-tubulin gene fragments, CM-1290, EU310839; CM-2270, EU310840; CM-2280, EU310841; CM-3134, EU310842; CM-3147, EU310843; CM-3227, EU310844; CM-3248, EU310845; CM-3254, EU310846; CM-3258, EU310847; CM-3303, EU310848; CM-3305, EU310849; CM-3364, EU310850; CM-3537, EU310851; CM-3538, EU310852; CM-3583, EU310853; CM-3599, EU310854; CM-3652, EU310855; CM-3722, EU310856; CM-3740, EU310857; CM-3764, EU310858; CM-3769, EU310859; CM-3914, EU310860; CM-4060, EU310861; CM-4063, EU310862; CM-4328, EU310863; CM-4330, EU310864; CM-4370, EU310865; CM-4387, EU310866; CM-4415, EU310867; CM-4420, EU310868; CM-4426, EU310869; CM-4428, EU310870; and CM-4518, EU310871; for the rodlet A gene fragments, CM-1290, EU310806; CM-2270, EU310807; CM-2280, EU310808; CM-3134, EU310809; CM-3147, EU310810; CM-3227, EU310811; CM-3248, EU310812; CM-3254, EU310813; CM-3258, EU310814; CM-3303, EU310815; CM-3305, EU310816; CM-3364, EU310817; CM-3537, EU310818; CM-3538, EU310819; CM-3583, EU310820; CM-3599, EU310821; CM-3652, EU310822; CM-3722, EU310823; CM-3740, EU310824; CM-3764, EU310825; CM-3769, EU310826; CM-3914, EU310827; CM-4060, EU310828; CM-4063, EU310829; CM-4328, EU310830; CM-4330, EU310831; CM-4370, EU310832; CM-4387, EU310833; CM-4415, EU310834; CM-4420, EU310835; CM-4426, EU310836; CM-4428, EU310837; and CM-4518, EU310838.

RESULTS

Clinical isolate morphology and growth temperature. The clinical origins of the 28 *Aspergillus* section *Fumigati* strains are shown in Table 1. These were isolated from 21 respiratory specimens, 1 ocular swab specimen, and 6 skin or nail samples.

All strains were first identified as atypical *A. fumigatus* strains by conventional macroscopic and microscopic morphological analysis methods. Identification to the *Aspergillus* genus level was straightforward; however, we were not able to discriminate between the different species of *Aspergillus* section *Fumigati*. While *A. fumigatus* grew at 37°C and 48°C, all the other strains analyzed in this study grew at 37°C but were not able to grow at 48°C.

Molecular identification of *Aspergillus* section *Fumigati*. Partial DNA sequences of the β-tubulin and rodlet A genes were obtained and analyzed. Four CBS strains were amplified with the primers described above in order to compare the 28 atypical *Aspergillus* with members of the section *Fumigati*. We also included a set of β-tubulin and rodlet A sequences from members of *Aspergillus* section *Fumigati* available from GenBank (Table 2).

The phylogenetic tree produced by maximum parsimony of the β-tubulin sequences (Fig. 1) grouped the 28 clinical isolates into six different clades (*N. hiratsukae*, *N. pseudofischeri*, *A. viridinutans*, *A. lentulus*, *A. fumigatiaffinis*, and *A. fumisynnematus*).

On the basis of these results, 14 atypical *A. fumigatus* strains were identified as *A. lentulus*, supported by bootstrap values from 70% to 99%. Five isolates were identified as *N. hiratsukae* with a bootstrap value of 100%, four were identified as *N. pseudofischeri* (bootstrap value, 100%), two were identified as *A. viridinutans* (bootstrap value, 97%), two were identified as *A. fumigatiaffinis* (bootstrap value, 92%), and one was identified as *A. fumisynnematus* (bootstrap value, 98%). None of the *Aspergillus* section *Fumigati* sequences analyzed matched the *A. fumigatus* sequences.

Figure 2 shows the results of a phylogenetic analysis obtained by maximum parsimony of the rodlet A gene sequences. According to those results, the 28 clinical strains fell into the same six clades. Fourteen samples were grouped with *A. lentulus* with bootstrap values of 63% and 81%. Five isolates were identified as *N. hiratsukae* with a bootstrap of 100%. Four isolates were identified as *N. pseudofischeri*, two were identified as *A. viridinutans*, two were identified as *A. fumigatiaffinis*, and one was identified as *A. fumisynnematus*, supported by bootstrap values of 99%, 100%, 46%, and 83%, respectively. All *A. fumigatus* sequences were divided into a single group according to their rodlet A sequences.

The identities and classification results for the *Aspergillus* section *Fumigati* strains on the basis of their β-tubulin and rodlet A sequences are summarized in Table 1.

Antifungal susceptibility testing. The MICs and MECs of the antifungal agents for the collection of clinical isolates are shown in Table 1 and are expressed as geometric means (GMs). The species were identified by β-tubulin and rodlet A gene sequencing. Analyses of the susceptibility phenotypes (Table 1) resulted in clear differences between *Aspergillus* species of the section *Fumigati.* We were able to differentiate three different antifungal phenotypes. Twelve of the 14 *A. lentulus* isolates and the two *A. fumigatiaffinis* isolates showed high MICs of amphotericin B (GM MICs, 4.5 μg/ml to 16 μg/ml), itraconazole (GM MICs, 6 μg/ml to 10.25 μg/ml), voriconazole (GM MICs, 3 μg/ml to 7.5 μg/ml), and ravuconazole (GM

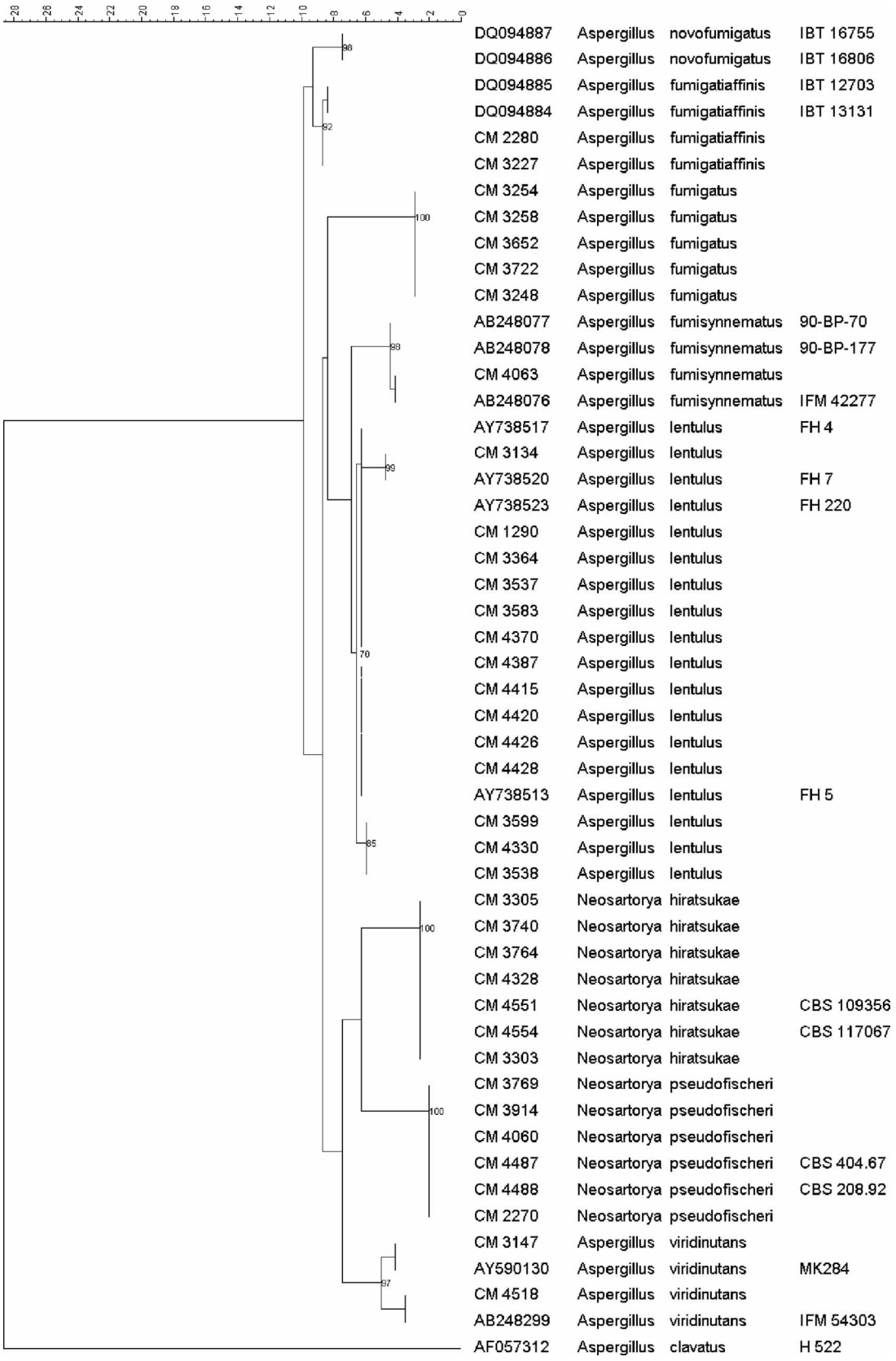

FIG. 1. Phylogenetic tree obtained by maximum-parsimony phylogenetic analysis with 2,000 bootstrap simulations on the basis of the β-tubulin sequences from all the strains included in the study.

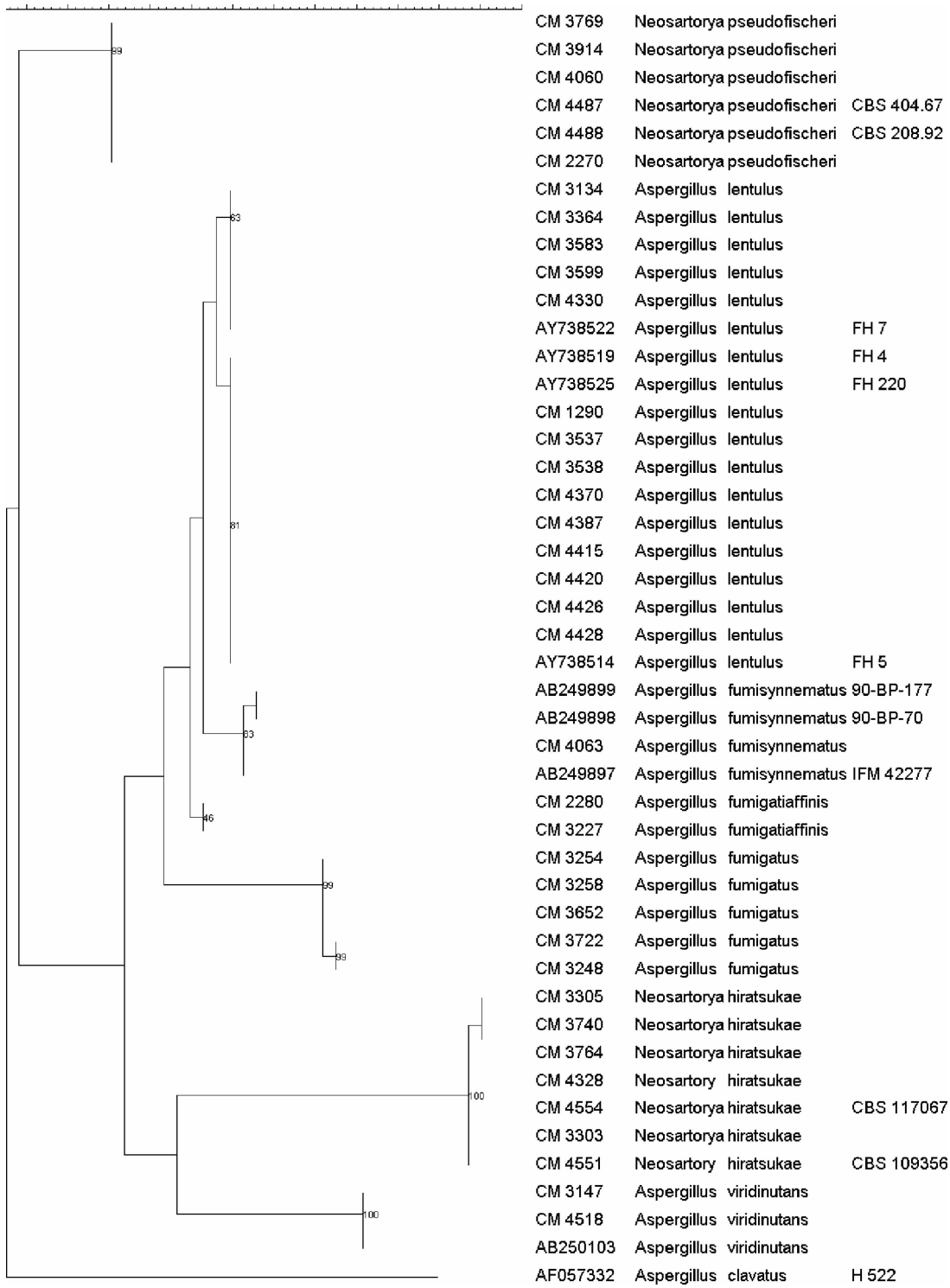

FIG. 2. Phylogenetic tree obtained by maximum-parsimony phylogenetic analysis with 2,000 bootstrap simulations on the basis of the rodlet A sequences from all the strains included in the study.

MICs, 1.5 μg/ml to 7 μg/ml). Although strains CNM-CM-3583 and CNM-CM-3599 did not follow this pattern for itraconazole (GM MICs, 0.43 μg/ml and 0.5 μg/ml, respectively), they retained the high MICs of amphotericin B (GM MICs, ≥2.66 μg/ml), voriconazole (GM MICs, ≥3.5 μg/ml), and ravuconazole (GM MICs, 2 μg/ml). A second profile was related to the species *N. pseudofischeri* and *A. viridinutans*. They showed high MICs of itraconazole (GM MICs, ≥8 μg/ml), voriconazole (GM MICs, ≥3.33 μg/ml), and ravuconazole (GM MICs, ≥2 μg/ml) but were susceptible to amphotericin B (GM MICs, ≤1

μg/ml). In contrast, strains identified as *N. hiratsukae* and *A. fumisynnematus* were more susceptible in vitro to all the antifungal compounds tested.

Among the azoles, posaconazole showed better activity in vitro (GM MICs, ≤0.75 μg/ml) against all clinical isolates analyzed in this study. Moreover, all strains were susceptible to terbinafine (GM MICs, ≤1.75 μg/ml) and the echinocandins, showing GM MECs of caspofungin and micafungin of ≤1.3 μg/ml and ≤0.26 μg/ml, respectively.

DISCUSSION

The present study highlights the limitations of phenotypic methods for the identification of some genera of molds. The use of molecular methods to partially sequence the β-tubulin and rodlet A genes enabled us to identify to the species level all clinical strains included in the study and previously classified as "atypical" *A. fumigatus* isolates. The majority of molecular methods use either specific probes or universal primers that are normally directed to conserved regions of the rRNA gene, particularly to the internal transcribed spacer regions (32, 35). However, internal transcribed spacer regions do not have enough phylogenetic strength to resolve the evolutionary relationship with strong bootstrap support for *Aspergillus* species from the section *Fumigati* (4, 5, 24, 41). Sequence analysis of both the β-tubulin and the rodlet A genes revealed that this method accurately differentiated the non-*A. fumigatus* isolates from the *A. fumigatus* isolates (4, 5). Therefore, all clinical isolates used in this study were identified to the species level by maximum-parsimony analysis of the β-tubulin and the rodlet A gene sequences.

On the basis of the identities of β-tubulin and rodlet A DNA sequences, the 28 strains were divided into six monophyletic clades supported by bootstrap values that differentiated the species (Fig. 1 and 2). The clades identified the isolates as belonging to the following species: 14 strains were *A. lentulus*, 5 strains were *N. hiratsukae*, 4 strains were *N. pseudofischeri*, 2 strains were *A. viridinutans*, 2 strains were *A. fumigatiaffinis*, and 1 strain was *A. fumisynnematus*.

A. lentulus has been isolated from soil and air, and it has also been isolated from patients with invasive infections (4). In this regard, most *A. lentulus* isolates recovered in this study were cultured from respiratory samples. This fact demonstrates the potential invasiveness of this pathogen in a susceptible host because *A. fumigatus* is able to colonize the human respiratory tract. *A. viridinutans*, *N. hiratsukae*, and *N. pseudofischeri* have also been isolated from humans (44), although only *N. hiratsukae* and *N. pseudofischeri* have been associated with human invasive fungal infections (3, 14, 17).

A. fumisynnematus was described as a separate taxon on the basis of the partial cytochrome *b* gene sequences of the species (43). However, this species was not included in the last classification of *Aspergillus* section *Fumigati* because the type strain of the species was not available for phenotypic characterization (36). To our knowledge, *A. fumisynnematus* and *A. fumigatiaffinis* had always been isolated from environmental sources. We describe for the first time the isolation of *A. fumisynnematus* and *A. fumigatiaffinis* strains from human hosts, which raises the intriguing issue of whether these species should be considered pathogenic fungi.

The common antifungal susceptibility phenotype of *A. fumigatus* is characterized by low MICs of the azole drugs, amphotericin B, and echinocandins. *A. fumigatus* strains showing resistance to azole drugs have been reported, although they have always maintained low amphotericin B MICs (11, 25, 26). In contrast, most *A. lentulus* and *A. fumigatiaffinis* isolates analyzed here showed extremely high MICs of amphotericin B, itraconazole, voriconazole, and ravuconazole.

Although 2 of the 14 *A. lentulus* isolates seemed to be susceptible to itraconazole, it is important to emphasize that we have not observed a uniform itraconazole susceptibility pattern, even after repeating the susceptibility testing more than eight times (data not shown). Due to the slow growth of most of these species, endpoint reading was easier at 72 h than at 48 h.

Since it has been demonstrated that elevated MICs of amphotericin B are associated with poor clinical outcomes (19, 30), the high MICs of amphotericin B for *A. lentulus* and *A. fumigatiaffinis* could have a remarkable clinical impact that merits research. In addition, it is well known that *A. terreus* strains have higher amphotericin B MICs than *A. fumigatus* strains, and this fact has been associated with a poorer response to amphotericin B in patients infected with this species (21, 37).

Even though *A. fumisynnematus* seems to be very closely related to *A. lentulus*, it had a different profile of susceptibility to all drugs tested. However, more isolates need to be analyzed in order to establish the antifungal susceptibility profile for this species.

Consistent with previous data (3, 44), the antifungal phenotypes of *N. pseudofischeri* and *A. viridinutans* showed that they had high itraconazole, voriconazole, and ravuconazole MICs.

All *N. hiratsukae* isolates were susceptible to all the antifungal drugs tested. There has been only one report of cerebral aspergillosis caused by *N. hiratsukae* (14), in which the strain cultured from that patient showed a pattern of MICs similar to the patterns described in this study.

Among the azoles, posaconazole showed the highest level of activity against all species analyzed. Terbinafine also had good activity in vitro against all clinical isolates compared with the MICs for *A. fumigatus*.

Finally, our antifungal susceptibility testing results for the echinocandins showed that all strains were susceptible to both caspofungin and micafungin (MECs, ≤1.3 μg/ml). This result contradicts the findings of other authors, but since a reference method for testing the susceptibilities of filamentous fungi to echinocandins has not yet been defined, conclusions about the echinocandin susceptibility or resistance of *Aspergillus* spp. should be made carefully.

This study emphasizes that molecular methods are needed for the correct identification of members of *Aspergillus* section *Fumigati* to the species level. Moreover, the members of this section have different antifungal susceptibility profiles. The identification of species or isolates showing high MICs of the antifungals used clinically is therefore mandatory. It should be noted that high in vitro MICs may not necessarily reflect decreased susceptibility in vivo, especially because these species have growth differences that could affect the results. Therefore, to ascertain the true clinical importance of these species, epidemiological studies must be performed together with in

vitro-in vivo correlation studies. In the meantime, it is difficult to give practical advice for clinical laboratories, but we suggest that those isolates which appear to be *A. fumigatus* under the microscope but which have poor sporulation or slow growth be sent to reference laboratories. An alternative approach could be to send any *A. fumigatus*-like strain which does not grow at 48°C to a reference laboratory. Due to the shortage of data and the unpredictable susceptibility profiles of some isolates, we highly recommend that antifungal susceptibility testing be performed by a standardized methodology for all isolates associated with human infections.

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