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Species Identification and Antifungal Susceptibility Patterns of Species Belonging to *Aspergillus* Section *Nigri*▽

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A phylogenetic analysis was performed for 34 *Aspergillus* strains belonging to section *Nigri*. Molecular methods allowed for the correct classification into three different clades (*A. niger*, *A. tubingensis*, and *A. foetidus*). Correlation with in vitro itraconazole susceptibility distinguished the following three profiles: susceptible, resistant, and showing a paradoxical effect. A number of different species whose morphological features resemble those of *A. niger* showed unusual MICs to itraconazole that have never been described for the *Aspergillus* genus.

Black aspergilli are widely distributed in nature (16); they are common food spoilers but are also well used for industrial purposes (15). Among *Aspergillus* species of the *Nigri* group, *A. niger* constitutes the most frequent etiological agent of otomycosis (13) and is considered the third cause of pulmonary aspergillosis (10). Nevertheless, the clinical implications of other species are rarely reported, and they are generally identified as *A. niger* (14, 22).

Clinically, identification of unknown *Aspergillus* clinical isolates to the species level may be important given that different species have dissimilar susceptibilities to antifungal drugs. Thus, the knowledge of the species identity may influence the choice of appropriate antifungal therapy (2, 4). Furthermore, since the antifungal susceptibility patterns for most of the species within section *Nigri* have been poorly investigated, their identification and antifungal susceptibility profiles appear to be of clinical interest for further research.

Black aspergilli belong to one of the most difficult groups concerning classification and identification (18), and so a number of different techniques have been developed in order to solve this issue. Among them, molecular tools are the gold standard (1, 18), as the sequencing of the β-tubulin or calmodulin gene is suitable, and enough, to discriminate between species within section *Nigri* (3, 18, 21).

Thirty-four *Aspergillus* section *Nigri* strains belonging to the Mold Collection of the Centro Nacional de Microbiologia and collected since 2004 were analyzed. Thirty-three strains were independent clinical isolates, and 1 had an environmental origin. All strains were identified as *A. niger* using conventional methods of morphology at the macroscopic as well as microscopic levels (9). Species identification analysis was addressed using sequences of the β-tubulin gene from all the strains included in this study together with the sequences of different *Aspergillus* section *Nigri* type strains and others that were available at GenBank as follows: *A. tubingensis* AY820007^T, AY820009, and AY585527; *A. foetidus* AY585533^T, AY585534, and DQ768454; and *A. niger* FJ629288^T, EF422213, and AY585537. Partial sequences of the β-tubulin gene were amplified using the primer set βtubAniger1 and βtubANiger2 (11) and were carried out according to standard PCR guidelines (Applied Biosystems). Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene 8.0; DNAStar, Inc., Madison, WI).

All phylogenetic analyses were conducted with InfoQuest FP software, version 4.50 (Bio-Rad). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by using parsimony bootstrapping with 2,000 simulations and by using the *Aspergillus clavatus* AY214441^T sequence as the out-group.

The phylogenetic tree grouped the 34 clinical isolates into three different clades consisting of 13 *A. niger* isolates, 18 *A. tubingensis* isolates, and 3 *A. foetidus* isolates (Fig. 1). Table 1 shows β-tubulin gene identification, as well as the origin and susceptibility profiles of the isolates.

Antifungal susceptibility testing (AST) was performed following the EUCAST Definitive Document E.DEF 9.1 method for the determination of broth dilution MICs of antifungal agents for conidium-forming molds (17). Antifungal ranges used in the microdilution assays have been described previously (2). Endpoints were determined at 48 h. The endpoint for MEC determination was the minimal antifungal concentration that produced morphological alterations of hyphal growth at 48 h. The paradoxical effect to itraconazole was defined as an increase in growth occurring at least 2 drug dilutions above the MIC. AST was repeated at least twice on different days.

Three different antifungal patterns were clearly distinguishable based on the itraconazole MIC values (Table 1): low and high itraconazole MICs and a third group (12 strains) showing an uncommon paradoxical effect of this antifungal (5). Either those strains classified as paradoxical strains or those showing much higher itraconazole MICs also had higher MIC values to voriconazole and ravuconazole.

Posaconazole showed better activity in vitro. Moreover, all

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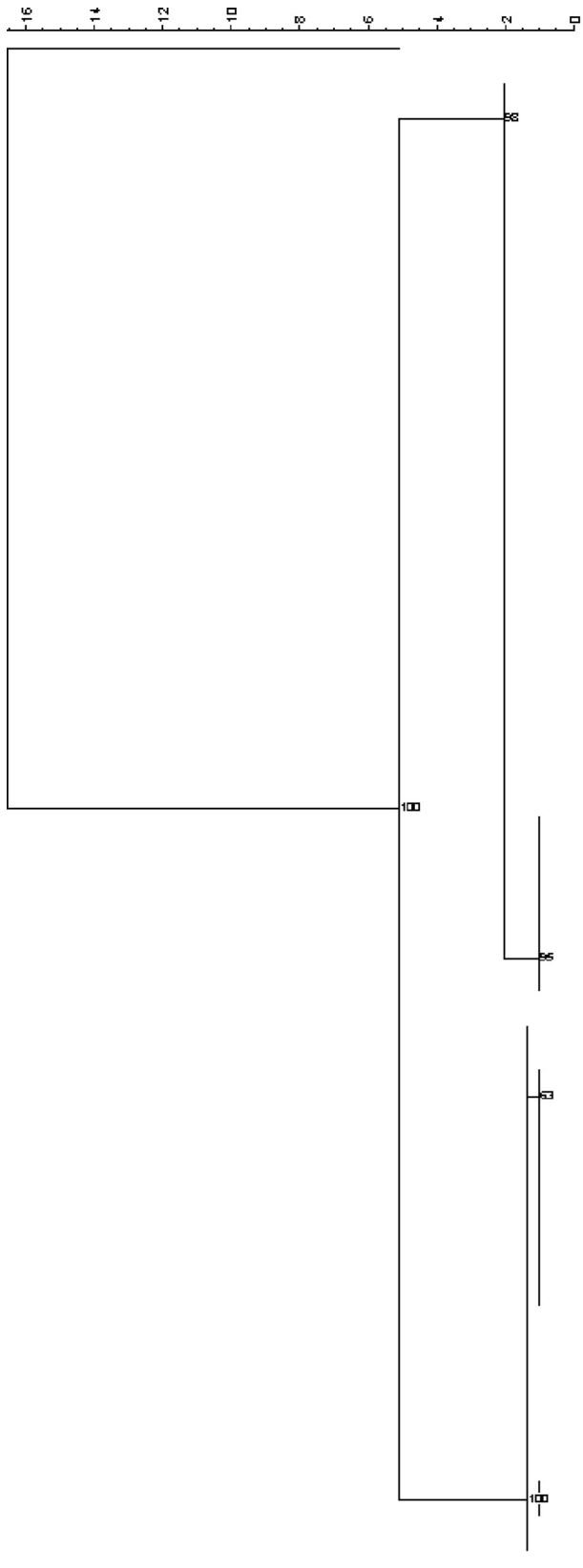

AY214441 (T)	Aspergillus	clavatus	CBS 513.65
CM 3123	Aspergillus	tubingensis	
CM 3125	Aspergillus	tubingensis	
CM 3177	Aspergillus	tubingensis	
CM 3810	Aspergillus	tubingensis	
CM 4352	Aspergillus	tubingensis	
CM 4688	Aspergillus	tubingensis	
CM 4899	Aspergillus	tubingensis	
AY820009	Aspergillus	tubingensis	CBS 425.65
AY585527	Aspergillus	tubingensis	CBS 16179
AY820007 (T)	Aspergillus	tubingensis	CBS 134.48
CM 3507	Aspergillus	tubingensis	
CM 3585	Aspergillus	tubingensis	
CM 3654	Aspergillus	tubingensis	
CM 4000	Aspergillus	tubingensis	
CM 4001	Aspergillus	tubingensis	
CM 4003	Aspergillus	tubingensis	
CM 4005	Aspergillus	tubingensis	
CM 4264	Aspergillus	tubingensis	
CM 4296	Aspergillus	tubingensis	
CM 4897	Aspergillus	tubingensis	
CM 5094	Aspergillus	tubingensis	
AY585533 (T)	Aspergillus	foetidus	CBS 56465
AY585534	Aspergillus	foetidus	CBS 56565
DQ768454	Aspergillus	foetidus	CBS 107.47
CM 4002	Aspergillus	foetidus	
CM 4995	Aspergillus	foetidus	
CM 5264	Aspergillus	foetidus	
CM 4213	Aspergillus	niger	
CM 4262	Aspergillus	niger	
CM 3506	Aspergillus	niger	
EF422213	Aspergillus	niger	CBS 113.33
FJ629288 (T)	Aspergillus	niger	CBS 554.65T
CM 3236	Aspergillus	niger	
CM 3672	Aspergillus	niger	
CM 4316	Aspergillus	niger	
CM 3636	Aspergillus	niger	
AY585537	Aspergillus	niger	CBS 101699
CM 3257	Aspergillus	niger	
CM 3551	Aspergillus	niger	
CM 3586	Aspergillus	niger	
CM 5095	Aspergillus	niger	
CM 3641	Aspergillus	niger	
CM 4004	Aspergillus	niger	

FIG. 1. Phylogenetic tree using maximum parsimony phylogenetic analysis and 2,000 bootstrap simulations based on β-tubulin gene sequences from all the *Aspergillus* section *Nigri* strains included in the study. Percentages indicate the bootstrap support for each group of sequences. (T), type strain.

TABLE 1. Source, molecular identification, MICs, and MECs for species of *Aspergillus* section *Nigri*[a]

Isolate	Source	Molecular identification (β-tubulin gene)	MIC (mg/liter)[b]						MEC (mg/liter)[c]	
			AMB	ITC	VCZ	RVC	POS	TRB	CAS	MICA
Isolates of *Aspergillus* section *Nigri* showing low ITC MICs										
CM-3236	Respiratory	*A. niger*	0.19	0.5	0.5	1.0	0.12	1.0	0.25	0.03
CM-3257	Respiratory	*A. niger*	0.25	1.0	1.0	1.0.	0.25	1.0	0.25	0.03
CM-3506	Respiratory	*A. niger*	0.19	0.5	0.75	1.0	0.12	0.31	0.15	0.03
CM-3507	Respiratory	*A. tubingensis*	0.19	0.5	1.0	1.5	0.15	0.62	0.06	0.03
CM-3585	Environmental	*A. tubingensis*	0.19	0.5	1.0	1.67	0.12	0.42	0.37	0.03
CM-3586	Catheter	*A. niger*	0.25	0.5	1.0	2.0	0.12	0.12	1.0	0.03
CM-3636	Respiratory	*A. niger*	0.25	0.5	0.5	1.0	0.19	1.0	0.25	0.03
CM-3641	Respiratory	*A. niger*	0.25	0.5	1.0	1.0	0.125	0.25	0.5	0.03
CM-3672	Cutaneous	*A. niger*	0.12	0.5	1.0	1.5	0.19	0.07	0.15	0.03
CM-4004	Unknown	*A. niger*	0.25	1.0	1.0	1.67	0.25	0.13	0.10	0.03
CM-4213	Respiratory	*A. niger*	0.33	0.14	0.33	0.58	0.03	0.22	0.39	0.03
CM-4264	Blood culture	*A. tubingensis*	0.12	0.5	1.0	1.5	0.12	0.50	0.03	0.03
CM-4296	Respiratory	*A. tubingensis*	0.12	0.75	1.0	1.5	0.19	0.62	0.25	0.03
CM-4316	Respiratory	*A. niger*	0.19	0.5	0.5	1.0	0.125	0.5	0.25	0.03
CM-5094	Respiratory	*A. tubingensis*	0.12	0.5	0.75	2.0	0.06	0.62	0.19	0.03
CM-5095	Respiratory	*A. niger*	0.19	0.5	0.75	1.5	0.12	0.62	0.25	0.03
GM for group			0.20	0.56	0.82	1.31	0.14	0.50	0.28	0.03
Isolates of *Aspergillus* section *Nigri* showing much higher ITC MICs										
CM-3123	Respiratory	*A. tubingensis*	0.25	11	1.67	2.67	0.25	1.17	0.25	0.03
CM-3810	Respiratory	*A. tubingensis*	0.25	4.0	2.0	2.0	0.12	1.0	0.5	0.03
CM-4003	Unknown	*A. tubingensis*	0.12	16	2.0	4.0	0.25	1.0	0.25	0.03
CM-4005	Unknown	*A. tubingensis*	0.12	16	2.0	4.0	0.5	0.25	0.5	0.03
CM-4688	Respiratory	*A. tubingensis*	0.21	3.67	2.0	3.33	0.25	1.50	0.18	0.03
CM-5264	Respiratory	*A. foetidus*	0.12	16	2.0	8.0	0.5	0.5	0.06	0.03
GM for group			0.18	11.11	1.95	4.0	0.31	0.90	0.29	0.03
Isolates of *Aspergillus* section *Nigri* showing paradoxical effect against ITC										
CM-3125	Respiratory	*A. tubingensis*	0.12	0.5	1	1.67	0.12	0.5	0.05	0.03
CM-3177	Respiratory	*A. tubingensis*	0.16	1	2	3.33	0.25	0.67	0.05	0.03
CM-3551	Respiratory	*A. niger*	0.5	4.75	1	2	0.12	0.25	0.03	0.03
CM-3654	Blood culture	*A. tubingensis*	0.19	1	2	2.50	0.25	0.63	0.14	0.03
CM-4000	Unknown	*A. tubingensis*	0.16	1	2	2	0.25	0.33	0.10	0.03
CM-4001	Unknown	*A. tubingensis*	0.19	1	1.75	2.50	0.25	0.56	0.11	0.03
CM-4002	Unknown	*A. foetidus*	0.25	1	2	2.67	0.12	0.33	0.14	0.03
CM-4262	Ophthalmic	*A. niger*	0.25	1	2	2	0.25	0.29	0.13	0.03
CM-4352	Respiratory	*A. tubingensis*	0.28	1	0.88	2	0.25	0.31	0.15	0.03
CM-4897	Blood culture	*A. tubingensis*	0.16	1	2	2	0.25	0.42	0.10	0.03
CM-4899	Respiratory	*A. tubingensis*	0.16	1	2	2.67	0.25	0.33	0.10	0.05
CM-4995	Prosthesis	*A. foetidus*	0.21	1	2	2	0.16	0.33	0.14	0.03
GM for group			0.2	1.3	1.72	2.28	0.21	0.41	0.10	0.03

[a] GM, geometric means of MICs and MECs for the strains within each group.
[b] MIC geometric mean of amphotericin B (AMB), itraconazole (ITC), voriconazole (VCZ), ravuconazole (RVC), posaconazole (POS), and terbinafine (TRB).
[c] MEC geometric mean of caspofungin (CAS) and micafungin (MICA).

strains were susceptible to the rest of the following antifungals tested: amphotericin B, terbinafine, and echinocandins.

In summary, *A. niger* MICs for itraconazole, voriconazole, and ravuconazole were slightly higher than *A. fumigatus* MICs and even more so for *A. tubingensis* and *A. foetidus* MICs. Identification of clinical isolates belonging to *Aspergillus* section *Nigri* and involved in proven or probable infections should be to the species level because it is the only way to monitor the development of secondary resistances of these molds (7, 8).

The paradoxical effect or "Eagle effect" (12) has been previously described for yeasts or *A. fumigatus* but always in relation to echinocandins (5, 6, 19, 20). This is the first report

showing the paradoxical effect of azole drugs against *Aspergillus* spp. The link between the paradoxical effect against itraconazole and a molecular mechanism responsible for it is yet to be determined, as is the clinical impact of those findings. Therefore, further studies including experimental models of aspergillosis to address any in vitro/in vivo correlations are warranted.

Nucleotide sequence accession numbers. GenBank accession numbers for β-tubulin gene fragment sequences from all the strains used in this work are as follows: CM-3123:FJ828892, CM-3125:FJ828893, CM-3177:FJ828894, CM-3236:FJ828895, CM-3257:FJ828896, CM-3506:FJ828897, CM-3507:FJ828898, CM-3551:FJ828899, CM-3585:FJ828900, CM-3586:FJ828901, CM-3636: FJ828902, CM-3641:FJ828903, CM-3654:FJ828904, CM-3672: FJ828905, CM-3810:FJ828906, CM-4000:FJ828907, CM-4001: FJ828908, CM-4002:FJ828909, CM-4003:FJ828910, CM-4004: FJ828911, CM-4005:FJ828912, CM-4213:FJ828913, CM-4262: FJ828914, CM-4264FJ828915, CM-4296:FJ828916, CM-4316: FJ828917, CM-4352:FJ828918, CM-4688:FJ828919, CM-4897: FJ828920, CM-4899:FJ828921, CM-4995:FJ828922, CM-5094: FJ828923, CM-5095:FJ82892, and CM-5264:FJ828925.

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