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Species Distribution and *In Vitro* Azole Susceptibility of *Aspergillus* Section *Nigri* Isolates from Clinical and Environmental Settings

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***Aspergillus* section *Nigri* includes species of interest for animal and human health, although studies on species distribution are limited to human cases. Data on the antifungal susceptibilities and the molecular mechanism of triazole resistance in strains belonging to this section are scant. Forty-two black *Aspergillus* strains from human patients (16 isolates), animals (14 isolates), and the environment (12 isolates) were molecularly characterized and their *in vitro* triazole susceptibilities investigated. *Aspergillus tubingensis* was isolated from humans, animals, and environmental settings, whereas *Aspergillus awamori* and *Aspergillus niger* were isolated exclusively from humans. Phylogenetic analyses of β-tubulin and calmodulin gene sequences were concordant in differentiating *A. tubingensis* from *A. awamori* and *A. niger*. Voriconazole and posaconazole (PSZ) were the most active triazoles. One *A. tubingensis* strain was resistant to itraconazole and PSZ and one *A. niger* strain to PSZ. Sequence analysis of the *cyp51A* gene revealed different sequence types within a species, and *A. tubingensis* strains were also phylogenetically distinct from *A. awamori*/*A. niger* strains according to the strain origin and susceptibility profile. Genetic analysis of the *cyp51A* sequences suggests that two nonsynonymous mutations resulting in amino acid substitutions in the CYP51A protein (changes of L to R at position 21 [L21R] and of Q to R at position 228 [Q228R]) might be involved in azole resistance. Though azole resistance in black *Aspergillus* isolates from animals and rural environments does not represent a threat to public health in Southern Italy, the use of triazoles in the clinical setting needs to better monitored. The *cyp51A* sequence is useful for the molecular identification of black *Aspergillus*, and point mutations in protein sequences could be responsible for azole resistance phenomena.**

Aspergillus section *Nigri* includes 19 species of black *Aspergillus*, which are widely known for being able to cause food spoilage and to produce enzymes and organic acids used in the fermentation industry, as well as mycotoxins (1). Nevertheless, *Aspergillus* section *Nigri* includes species causing pulmonary aspergillosis and otomycosis in humans, as well as localized and disseminated disease in domestic and wild animals (2, 3). Species belonging to *Aspergillus* section *Nigri* have been difficult to classify due to their phenotypic similarities (4), whereas molecular tools such as the sequencing of calmodulin and β-tubulin genes have been successfully employed for species identification and discrimination within section *Nigri*. Molecular data have shown that *Aspergillus tubingensis* is the species most frequently distributed in various environments (5–7). The management and prophylaxis of aspergillosis is mainly performed using triazole drugs, although long-term therapy and the indiscriminate use of azoles in agriculture have raised concerns about resistance to these compounds (8–10). In spite of the large number of reports of triazole resistance phenomena worldwide, mainly in *Aspergillus fumigatus* isolates from environmental and clinical settings (9, 10), data on the *in vitro* antifungal susceptibilities of members of *Aspergillus* section *Nigri* are scant and those available show that clinical isolates from different geographical regions exhibited remarkably different azole susceptibilities (5, 7, 11, 12). Azole resistance mechanisms have been described mainly in *A. tubingensis* (5), the most common being attributed to point mutations in the *cyp51A* gene encoding the azole target protein (14α-sterol demethylase [CYP51A]) (9, 10). However, the molecular mechanisms of azole resistance in species of *Aspergillus* section *Nigri* are controversial and not well defined (5).

In light of this situation, accurate species identification of black *Aspergillus* isolates and their susceptibility testing are instrumental to assess the occurrence and molecular mechanisms of azole resistance phenomena and fungal species distribution among different clinical settings and environments. Thus, the aims of this study were to (i) identify the species of black *Aspergillus* isolates from different clinical and environmental sources based on sequence analysis of the calmodulin and β-tubulin genes, (ii) evaluate their *in vitro* susceptibilities to triazoles, and (iii) investigate the potential mechanisms of azole resistance.

MATERIALS AND METHODS

Isolates. A total of 42 black *Aspergillus* isolates were tested in the study. The isolates came from 567 human patients, 125 squirrels, and 57 air samples from 19 sheds from laying hen farms, as follows. Sixteen clinical isolates were from human patients with hematological disorders ($n = 5$), cystic fibrosis ($n = 6$), or lung disease ($n = 5$) admitted to different wards of Azienda Ospedaliera-Universitaria, Ospedale Policlinico Consorziale di Bari, between September 2015 and March 2016. Fourteen isolates were from the respiratory tract of squirrels (*Callosciurus finlaysonii*; Basilicata, Southern Italy) trapped during a campaign to control allochthonous

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wildlife populations (13). Twelve isolates were from the environment of laying hen farms (14).

All animal and human cases of probable/possible invasive aspergillosis (IA) were defined according to the criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) (15). All isolates were morphologically identified as belonging to *Aspergillus* section *Nigri* using macro- and microscopic features (16). The strains were stored at −80°C in the fungal collection of the Department of Veterinary Medicine at the University of Bari, Italy. Prior to testing, each isolate was subcultured at least twice onto Sabouraud dextrose agar (SDA) plates to ensure purity and viability.

Molecular identification and phylogenetic analysis. DNA was extracted from isolates grown on SDA for 5 days at 35°C by using the ArchivePure DNA yeast kit (5-Prime, Inc., USA) according to the manufacturer's instructions.

The isolates were molecularly identified by PCR and sequencing of partial calmodulin and β-tubulin genes using the primer pair Cmd5 (5′-CCGAGTACAAGGAGGCCTTC-3′) and Cmd6 (5′-CCGATAGAGGTCATAACGTGG-3′) and the primer pair BT2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and BT2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′), respectively (17, 18). The thermocycling conditions were an initial denaturation at 95°C for 7 min, followed by 35 cycles of 95°C for 30 s, 61°C for 30 s (for the β-tubulin gene) or 57°C for 30 s (for the calmodulin gene), and 72°C for 1 min, followed by a final extension step of 72°C for 10 min.

The resultant PCR amplicons were purified using the ExoI/FAST AP enzyme systems (Thermo Scientific). Purified PCR products were sequenced using the TaqDyeDoxy terminator cycle sequencing kit (version 2; Applied Biosystems) in an automated sequencer (ABI Prism 377). Sequences were aligned using the ClustalX program and compared with those available in the GenBank database by using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis was performed using the calmodulin and β-tubulin gene sequences of all black *Aspergillus* isolates. Phylogenetic trees were produced from ClustalX-aligned sequences by the maximum-likelihood (ML) method (Kimura 2-parameter model) (19) and neighbor-joining (NJ) (20), based on the *p*-distance of each gene (calmodulin and β-tubulin). Evolutionary distances were computed using the software package MEGA 6.0 (Center for Evolutionary Medicine and Informatics, Tempe, AZ). The reliability of internal branches was assessed using the bootstrap method with 1,000 replicates, and bootstrap values of >50 were considered significant in this analysis. *Aspergillus carbonarius* was chosen as the outgroup in both sequence analyses.

***In vitro* antifungal susceptibility.** *In vitro* susceptibility testing of black *Aspergillus* isolates to determine their MICs of itraconazole (ITZ), voriconazole (VRZ), and posaconazole (PSZ) was performed by the broth microdilution (BMD) method as described in CLSI document M38-A2 (21). The triazole MICs were determined as described by the CLSI reference method (21). *In vitro* susceptibility testing was performed in triplicate for each isolate. The proposed epidemiological cutoff values (ECVs) for ITZ (2 mg/liter), VRZ (2 mg/liter), and PSZ (0.5 mg/liter) were used for the interpretation of results (22). Quality control was performed as recommended in the M38-A2 CLSI document using *Candida krusei* (strain ATCC 6258) and *Candida parapsilosis* (strain ATCC 22019) (American Type Culture Collection, Manassas, VA, USA). The MIC data obtained were reported as the ranges, mean values, and MIC_{50} and MIC_{90}.

Sequencing of *cyp51A*. The *cyp51A* sequences were obtained for 24 isolates (16 clinical human isolates, 4 from squirrels, and 4 from laying hen farms).

The partial *cyp51A* gene was amplified by PCR using the primers Ancyp51A-F (5′-TKTYCCTGCCTACRGTCGCTT-3′) and Ancyp51A-R (5′-CCGTAGTCCACCATCTCTCC-3′) (5). The thermal cycling profile for amplification was as follows: 94°C for 5 min, followed by 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min, with a final step of 72°C for 10 min. Sequencing and phylogenetic analysis were conducted as described above.

Phylogenetic analysis was performed using the *cyp51A* sequences of 24 black *Aspergillus* isolates from this study and those available in the GenBank database.

Nucleotide sequence accession numbers. All representative sequence types of *A. tubingensis*, *Aspergillus niger*, and *Aspergillus awamori* have been deposited in the GenBank database (for β-tubulin, accession numbers KX231820 to KX231822, and for calmodulin, accession numbers KX231823 to KX231827). The GenBank accession numbers for representative *cyp51A* sequences are KX245375 to KX245382.

RESULTS

Isolates. The clinical data, diagnoses, antifungal prophylaxis or therapy, and clinical outcomes of the 16 patients that scored positive for *Aspergillus* spp. are summarized in Table 1. In particular, *Aspergillus* species were isolated from patients who suffered from hematological disorders ($n = 5$) and cystic fibrosis ($n = 6$) with high and low respiratory tract fungal colonization, respectively, and from patients with probable IA ($n = 5$) (Table 1). *Aspergillus* spp. were isolated from squirrels with possible IA ($n = 2$) or colonization of the nasal cavity ($n = 12$).

Molecular identification and phylogenetic analysis. The PCR amplification of individual DNA samples resulted in amplicons of the expected size (~550 bp for the β-tubulin gene and ~580 bp for the calmodulin gene). Three distinct species were identified among all 42 samples according to the β-tubulin and calmodulin gene sequence analysis. All β-tubulin and calmodulin gene sequences displayed a 99 to 100% nucleotide identity with sequences available in GenBank (Table 1). Interestingly, *A. tubingensis*, the most common *Aspergillus* species identified (76.2%) (Table 1), was the only species isolated from human patients, animals, and environmental settings, whereas *A. awamori* and *A. niger* were isolated exclusively from human patients.

Pairwise comparisons revealed a single sequence type (ST) for β-tubulin genes within each species, herein designated $At_{\beta T}$ for *A. tubingensis*, $An_{\beta T}$ for *A. niger*, and $Aa_{\beta T}$ for *A. awamori*. Three calmodulin STs were detected among *A. tubingensis* strains (herein designated At_{C1}, At_{C2}, and At_{C3}), with nucleotide variation ranging from 0.2 to 0.3%. A single ST was identified among *A. niger* and *A. awamori* isolates (herein designated An_{C1} and Aa_{C1}, respectively). There was consistency in the topology of the trees inferred by the ML and NJ methods (for each locus; data not shown), and the phylogenetic analyses of β-tubulin and calmodulin sequences were concordant in grouping the 42 isolates into two different clades, dividing *A. tubingensis* isolates from *A. niger*/*A. awamori* (Fig. 1 and 2). In the calmodulin tree, three sister subclades grouped *A. tubingensis* STs (At_{C1}, At_{C2}, and At_{C3}), and within the clade there is support (bootstrap value of >50) for clustering of isolates according to their origin (squirrels, humans, and laying hen farms) (Fig. 2).

***In vitro* antifungal susceptibility.** The antifungal susceptibilities of the 42 isolates in this study are shown in Table 2. VRZ and PSZ were the triazoles most active against the three *Aspergillus* species tested. Higher MICs of ITZ were recorded in *A. tubingensis* than in the other *Aspergillus* species. Only two *Aspergillus* strains revealed azole resistance phenomena, and the corresponding data on the isolates and patients are summarized in Table 1. In particular, one strain (strain CD1199) of *A. tubingensis* was resistant to ITZ (MIC of 64 μg/ml) and PSZ (MIC of 1 μg/ml) and one strain of *A. niger* (strain CD1200) to PSZ (MIC of 1 μg/ml). These two strains were isolated from two patients admitted to the hematology unit with nasal colonization.

TABLE 1 Clinical data, diagnosis, antifungal prophylaxis or therapy, and clinical outcome of the 16 patients that scored positive for *Aspergillus* spp.

Patient code	Gender	Age (yr)	Underlying disease[a]	Diagnosis[b]	Antifungal prophylaxis/therapy[c]	Clinical outcome	Strain code	*Aspergillus* sp.	Sample type[d]	Azole susceptibility[e]			Nonsynonymous mutation in *cyp51A*
										ITZ	PSZ	VRZ	
ET16	F	51	AML	RTC	None	Alive	CD1450	*A. tubingensis*	NS	S	S	S	None
ET21	F	37	ALL	RTC	None	Alive	CD1445	*A. tubingensis*	NS	S	S	S	None
ET19	F	66	MM	RTC	P: FLZ 400 mg/day for 10 days	Alive	CD1443	*A. tubingensis*	PS	S	S	S	None
ET22	M	42	AML	RTC	P: PSZ 600 mg/day for 15 days	Alive	CD1199	*A. tubingensis*	NS	R	R	S	L21F
ET23	F	50	ALL	RTC	P: PSZ 600 mg/day for 18 days	Alive	CD1200	*A. niger*	NS	S	R	S	R228Q
FC5	F	20	CF	RTC	None	Alive	CD1461	*A. awamori*	Sp	S	S	S	None
FC13	F	21	CF	RTC	None	Alive	CD1452	*A. awamori*	Sp	S	S	S	None
FC15	F	38	CF	RTC	None	Alive	CD1455	*A. niger*	Sp	S	S	S	None
FC16	M	11	CF	RTC	None	Alive	CD1456	*A. tubingensis*	Sp	S	S	S	None
FC21	M	5	CF	RTC	None	Alive	CD1498	*A. awamori*	Sp	S	S	S	None
FC	F	33	CF	RTC	None	Alive	CD1201	*A. tubingensis*	Sp	S	S	S	None
R8	F	40	Lung disease	PIA	T: VRZ 200 mg/day	Alive	CD1202	*A. awamori*	Sp	S	S	S	None
R9	M	61	Lung disease	PIA	T: VRZ 200 mg/day	Alive	CD1497	*A. awamori*	Sp	S	S	S	None
R10	M	56	Lung disease	PIA	T: VRZ 200 mg/day	Alive	CD1463	*A. awamori*	Sp	S	S	S	None
A8	M	70	Lung disease	PIA	T: VRZ 200 mg/day	Alive	CD1449	*A. niger*	Sp	S	S	S	None
A9	M	59	Lung disease	PIA	T: PSZ 800 mg/day	Died	CD1462	*A. awamori*	Sp	S	S	S	None

[a] AML, acute myeloid leukemia; ALL, acute lymphatic leukemia; MM, multiple myeloma; CF, cystic fibrosis.
[b] RTC, respiratory tract colonization; PIA, probable invasive aspergillosis.
[c] P, prophylaxis; T, therapy; FLZ, fluconazole; VRZ, voriconazole; PSZ, posaconazole.
[d] NS, nasal swab; PS, pharyngeal swab; Sp, sputum.
[e] ITZ, itraconazole; S, susceptible; R, resistant.

***cyp51A* sequencing and phylogenetic analysis.** The sequence analysis of the partial *cyp51A* gene (~1,230 bp) revealed 3 distinct species with 3 or 2 STs within a single species (Fig. 3). In particular, three *cyp51A* STs were detected among *A. tubingensis* (At_{cy1}, At_{cy2}, and At_{cy3}) and *A. awamori* (Aa_{cy1}, Aa_{cy2}, and Aa_{cy3}), respectively, and two STs were detected for *A. niger* (An_{cy1} and An_{cy2}). Pairwise comparison among the different STs revealed nucleotide variation ranging from 1.3 to 2.8% in *A. tubingensis* isolates and from 0.3 to 0.9% in *A. awamori* isolates and nucleotide variation of 0.3% in *A. niger* isolates (Table 3).

In *A. tubingensis*, three nonsynonymous amino acid substitutions (changes of A to V at position 9 [A9V], T to A at position 321 [T321A], and L to F at position 21 [L21F]) were detected in the At_{cy1} ST and were characteristic of the ITZ- and PSZ-resistant strain (strain CD1199). In particular, the A9V and T321A mutations were detected in strain CD1199 and in the reference resistant strain (strain NRRL4700), as well as in *A. niger*/*A. awamori* susceptible strains (strains CD1449, CD1461, and CD1498), whereas the L21F mutation was present only in strain CD1199. The At_{cy2} ST, having only the T321A mutation, characterized the susceptible strains collected from laying hen farms. The At_{cy3} ST was associated with susceptible strains originating from human patients and animals. In *A. niger*, the An_{cy1} ST, characterizing the PSZ-resistant strain (strain CD1200), differed from the ST in the susceptible strains (ST An_{cy2}) by a nonsynonymous mutation (R228Q). The R228Q mutation has also been detected in reference resistant strains (strains NRRL341 and AN186). In *A. awamori*, all three STs (Aa_{cy1}, Aa_{cy2}, and Aa_{cy3}) characterized the susceptible strains and only the Aa_{cy1} ST showed a nonsynonymous variation (F343L). The F343L mutation has not been detected in the reference resistant strain (strain F7577), which was characterized by the nonsynonymous mutation K97T.

Phylogenetic analysis of the partial *cyp51A* gene sequences revealed concordance with the β-tubulin and calmodulin sequences in grouping *Aspergillus* spp. into two clades, differentiating *A. tubingensis* from *A. awamori*/*A. niger*. The *cyp51A* data separated the strains according to their azole susceptibility profiles within the species. In particular, *A. tubingensis* strain CD1199 (At_{cy1}) clustered separately from the susceptible strains (Fig. 3) and from the resistant strain NRRL4700. Similarly, in the *A. niger* subclade, the azole-resistant (CD1200, NRRL341, and AN186) and azole-susceptible (ATCC 1015, CD1455, and CD1449) strains clustered separately (Fig. 3), and the *A. awamori* susceptible strains clustered separately from the corresponding resistant strain (strain F7577).

DISCUSSION

This study presents, for the first time, an overview of the occurrence of black *Aspergillus* from clinical and environmental sources in Southern Italy and a molecular characterization at the species level by sequencing the β-tubulin and calmodulin genes. In addition, to our knowledge, this is the first study that has characterized black *Aspergillus* from animals (squirrels) and rural environments (laying hen farms). In particular, *A. tubingensis* is one of the most prevalent species isolated from human patients (5, 11, 23, 24), but it was never isolated previously from animals and the environment. Albeit the sampling of squirrels and rural environments performed in this study was limited, the presence of *A. awamori* and *A. niger* was recorded only in human patients (5 with probable IA, 4 who suffered from cystic fibrosis, and 1 with a hematological disorder), thus indicating that these species might circulate in nosocomial environments and be encountered mainly in cystic fibrosis patients, as previously reported (5, 23, 24). Phylogenetic analyses based on β-tubulin and calmodulin gene sequences grouped the *Aspergillus* species consistently, indicating that these genes are good targets for molecular characterization of black *Aspergillus* at

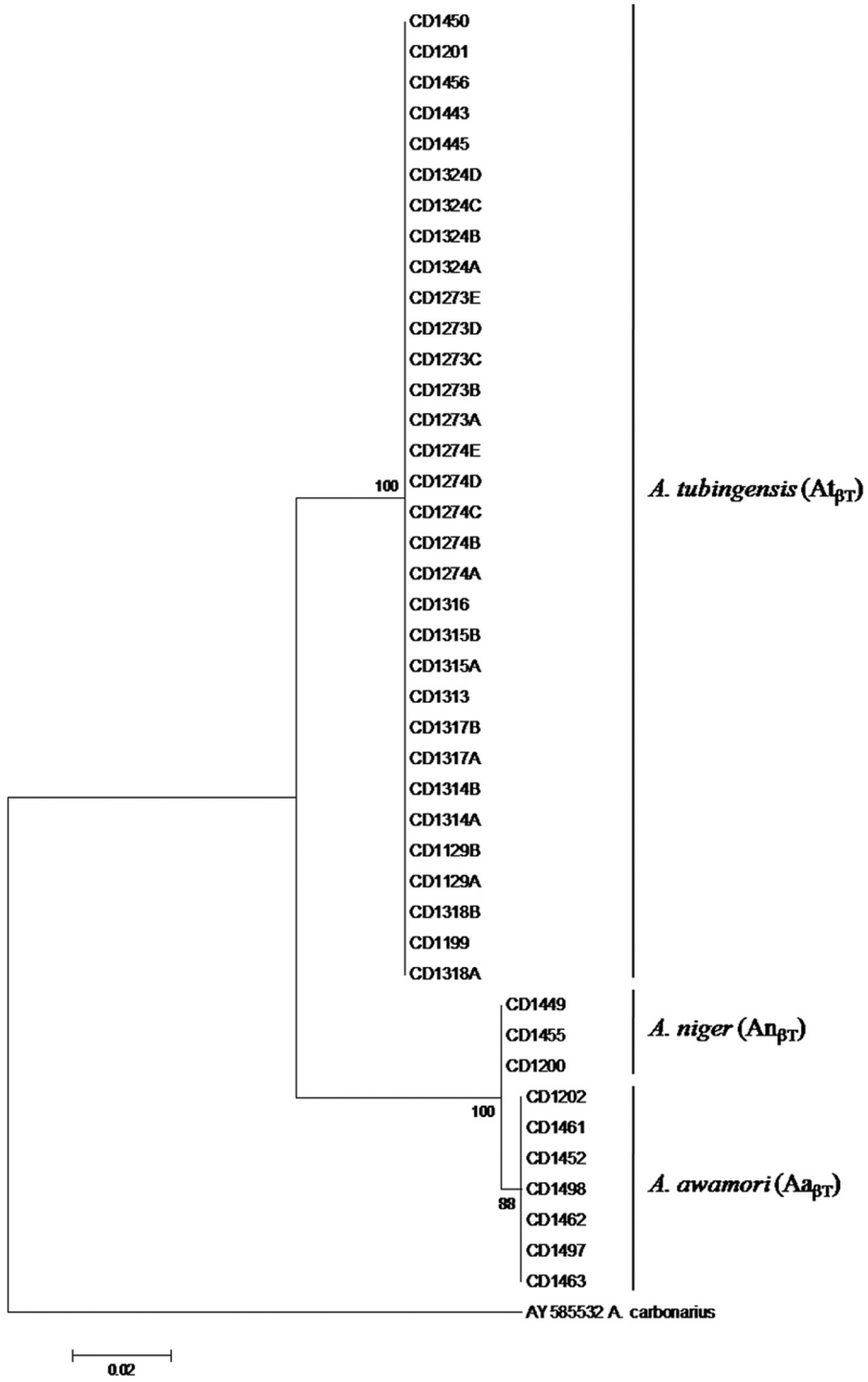

FIG 1 Maximum-parsimony tree based on partial β-tubulin sequences. The laboratory code is reported for each strain, and the sequence type is indicated in parentheses.

the species level (5). However, the calmodulin sequences were more suitable for molecular epidemiologic studies, since they allowed segregation of *A. tubingensis* strains according to their origin.

High VRZ and PSZ activities against *Aspergillus* spp. were confirmed (25–28) and are reported here for the first time in strains from animals and rural environments. *A. tubingensis* revealed lower susceptibility to ITZ than to VRZ and PSZ, as previously suggested (2, 29), and azole-resistant and cross-resistant isolates were detected only in strains from human patients (5). Indeed, in this study, *A. tubingensis* and *A. niger* isolates with low susceptibilities to ITZ and/or PSZ were isolated from hematological patients who received PSZ as prophylaxis in preventing IA. There-

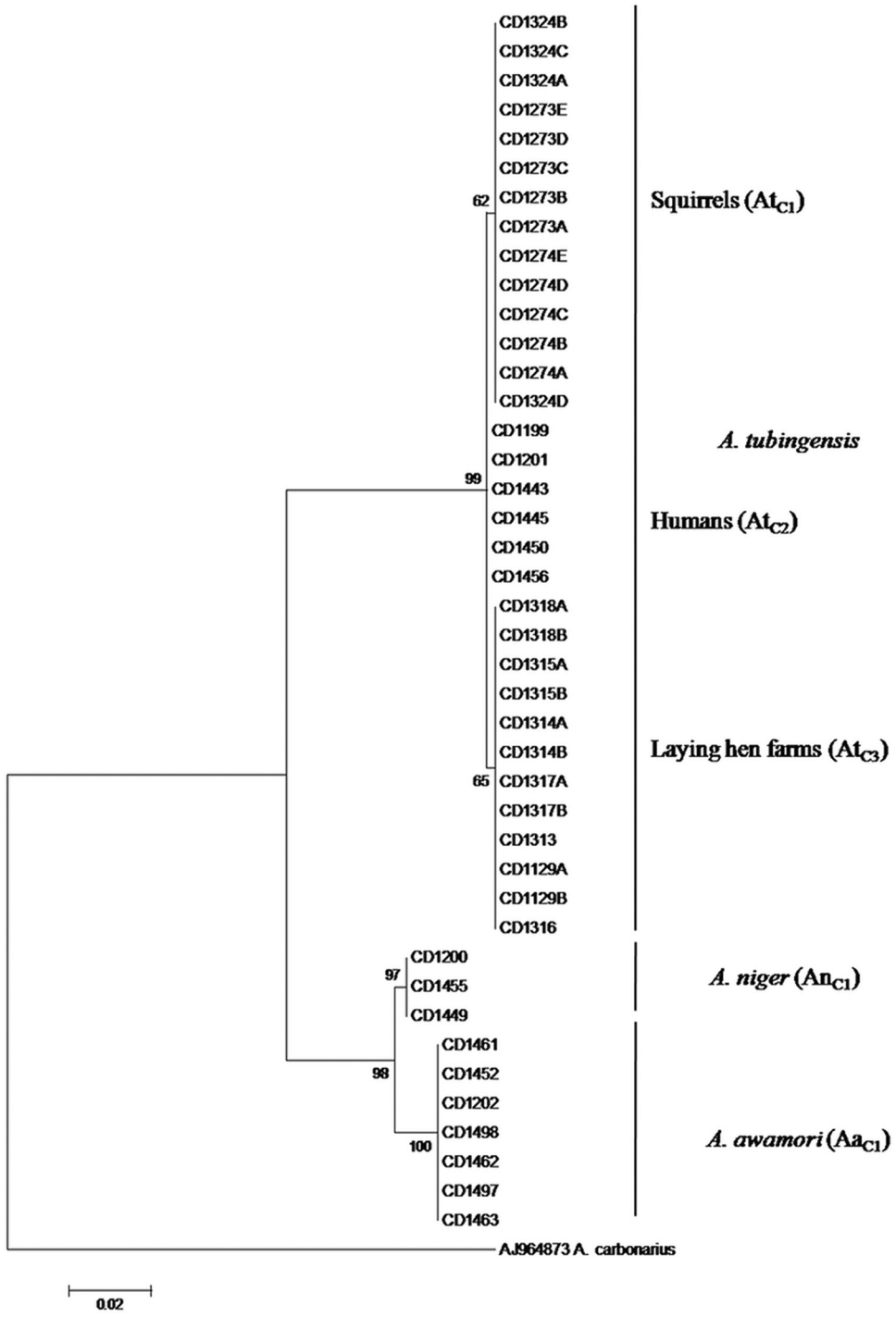

FIG 2 Maximum-parsimony tree based on partial calmodulin sequences. The laboratory code is reported for each strain, and the strain origin and sequence type, in parentheses, are indicated.

fore, the use of PSZ in hematopoietic stem cell transplant recipients might have induced or selected resistant strains in these patients (2, 29). The low prevalence of triazole resistance in black *Aspergillus* isolates (2.4%) detected in this study might be due to the low number of *Aspergillus* strains tested.

The finding of three nonsynonymous mutations (A9V, T321A, and L21F) in the CYP51A sequence of the *A. tubingensis*-resistant strain (CD1199) suggests a role in azole resistance or cross-resistance phenomena. Nonetheless, since the A9V and T321A mutations were previously detected both in the *A. tubingensis* resistant strain (NRRL4700) and in *A. niger*/*A. awamori* susceptible strains (5), further studies are necessary to elucidate their role in azole resistance mechanisms. Contrarily, the L21F mutation, detected only in the ITZ- and PSZ-resistant *A. tubingensis* strain CD1199,

TABLE 2 Numbers and percentages of *Aspergillus* sp. isolates obtained from human patients, squirrels, and laying hen farms

Aspergillus sp.	No. (%) of isolates from:			
	Human patients	Squirrels	Laying hen farms	Total
A. tubingensis	6 (14.3)	14 (33.3)	12 (28.6)	32 (76.2)
A. awamori	7 (16.7)			7 (16.7)
A. niger	3 (7.1)			3 (7.1)
Total	16 (38.1)	14 (33.3)	12 (28.6)	42 (100)

was reported here for the first time, suggesting its role in azole resistance or cross-resistance phenomena in this species. Likewise, PSZ resistance could also be caused by the nonsynonymous mutation Q228R that was detected only in the resistant *A. niger* strain CD1200 (see also strains NRRL341 and AN186 in Howard et al. 5).

Since in *A. awamori*, the nonsynonymous mutation F343L was associated with susceptible strains, the mutation K97T, previously recorded in the reference resistant strain F7577 and lacking in the susceptible strains tested here, might play a role in azole resistance, as suggested by Howard and colleagues (5).

The phylogeny obtained with *cyp51A* largely reflected those obtained with the calmodulin and β-tubulin sequences, indicating that the gene may be useful as a taxonomic target to differentiate the *Nigri* clades, as previously reported (5). In that phylogeny, the two azole-resistant *A. tubingensis* and *A. niger* strains (CD1199 and CD1200) were clearly separated from the susceptible ones, thus indicating the relationship between their phylogeny and azole susceptibility profiles.

This study provides new data on the prevalence of black *Aspergillus* collected from different sources in Southern Italy and shows that the β-tubulin, calmodulin, and *cyp51A* genes were all useful for the molecular identification of this group of microorganisms,

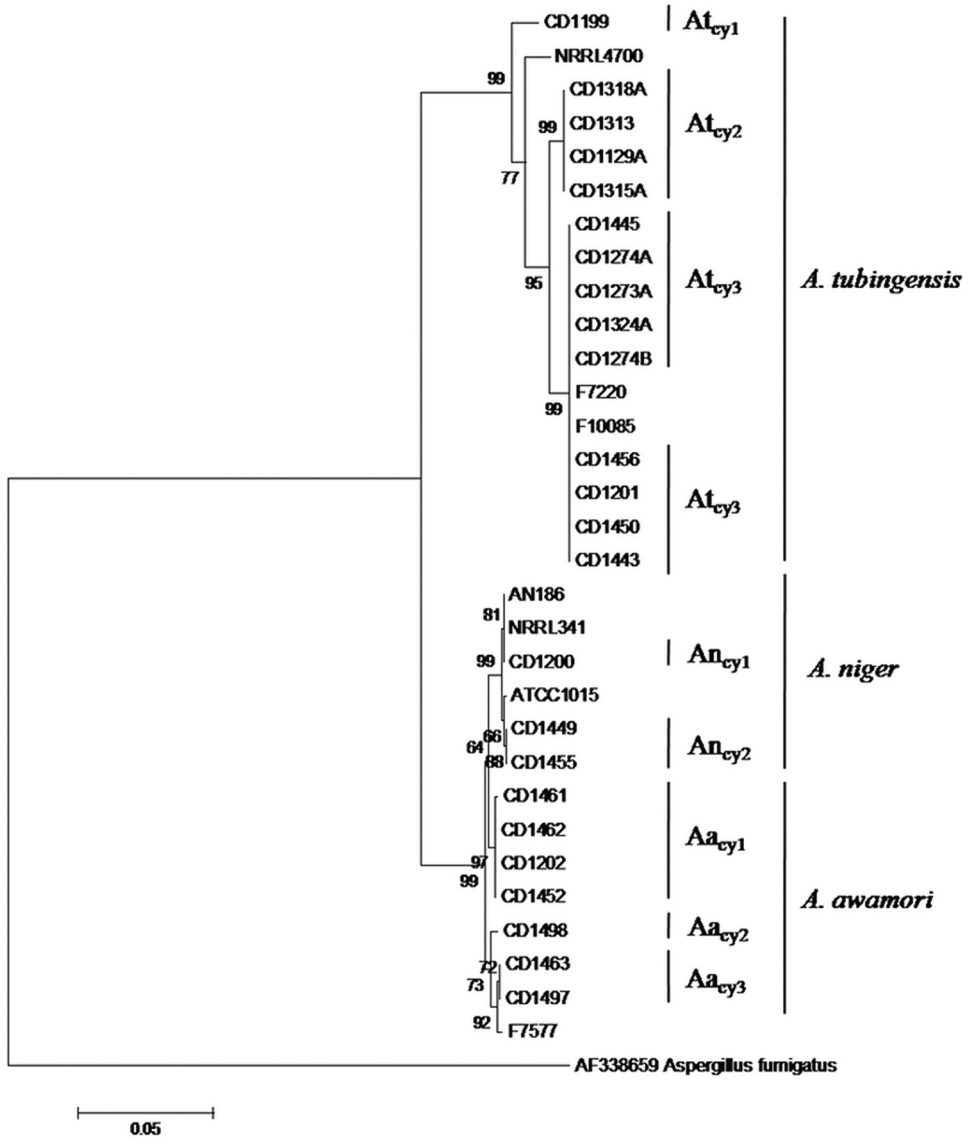

FIG 3 Maximum-parsimony tree based on partial *cyp51A* sequences. The laboratory code is reported for each strain, and sequence types are shown.

TABLE 3 Itraconazole, voriconazole, and posaconazole MIC data for 42 *Aspergillus* species isolates

Aspergillus sp. (no. of isolates)	MIC (μg/ml) value(s) for[a]:											
	ITZ				VRZ				PSZ			
	Range	Mean (SD)	MIC_{50}	MIC_{90}	Range	Mean (SD)	MIC_{50}	MIC_{90}	Range	Mean (SD)	MIC_{50}	MIC_{90}
A. tubingensis (32)	0.25–64	3 (11.14)	1	2	0.5–2	0.69 (0.38)	0.5	1	0.25–1	0.49 (0.12)	0.5	0.5
A. awamori (7)	0.5–2	1 (0.5)	1	1	0.25–1	0.46 (0.27)	0.5	0.5	0.25–0.5	0.36 (0.27)	0.5	0.5
A. niger (3)	0.25–2	1.08 (0.88)			0.25–2	0.91 (0.94)			0.25–1	0.58 (0.38)		

[a] ITZ, itraconazole; VRZ, voriconazole; PSZ, posaconazole.

although the latter gene was more informative on strain origin and antifungal susceptibility.

The lack of recorded azole resistance in black *Aspergillus* isolates from animals and rural environments indicates that these sources do not currently represent a threat to public health in Southern Italy, whereas the use of triazoles in clinical settings might have induced or selected resistant strains; thus, in order to minimize the spread of these strains in nosocomial environments, as well as to ensure good management of *Aspergillus* species infections, the antifungal susceptibility profiles of isolates should be accurately monitored. Finally, not all of the amino acid substitutions identified in CYP51A protein sequences are responsible for azole resistance phenomena in black *Aspergillus*. However, the F21L and Q228R amino acid substitutions might have a role in triazole resistance of black *Aspergillus* and, since the number of isolates tested in this study is limited, confirmatory studies should be performed in order to support our data. Other molecular mechanisms of antifungal resistance, such as the overexpression of the *cyp51A* gene and of the multidrug efflux transporter genes, need to be investigated to clarify the molecular mechanisms involved in azole resistance of this group of microorganisms.

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