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# Cryptic Species and Azole Resistance in the *Aspergillus niger* Complex▽†

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***Aspergillus niger* is a common clinical isolate. Multiple species comprise the *Aspergillus* section *Nigri* and are separable using sequence data. The antifungal susceptibility of these cryptic species is not known. We determined the azole MICs of 50 black aspergilli, 45 from clinical specimens, using modified EUCAST (mEUCAST) and Etest methods. Phylogenetic trees were prepared using the internal transcribed spacer, beta-tubulin, and calmodulin sequences to identify strains to species level and the results were compared with those obtained with *cyp51A* sequences. We attempted to correlate *cyp51A* mutations with azole resistance. Etest MICs were significantly different from mEUCAST MICs ($P < 0.001$), with geometric means of 0.77 and 2.79 mg/liter, respectively. Twenty-six of 50 (52%) isolates were itraconazole resistant by mEUCAST (MICs > 8 mg/liter), with limited cross-resistance to other azoles. Using combined beta-tubulin/calmodulin sequences, the 45 clinical isolates grouped into 5 clades, *A. awamori* (55.6%), *A. tubingensis* (17.8%), *A. niger* (13.3%), *A. acidus* (6.7%), and an unknown group (6.7%), none of which were morphologically distinguishable. Itraconazole resistance was found in 36% of the isolates in the *A. awamori* group, 90% of the *A. tubingensis* group, 33% of the *A. niger* group, 100% of the *A. acidus* group, and 67% of the unknown group. These data suggest that *cyp51A* mutations in section *Nigri* may not play as important a role in azole resistance as in *A. fumigatus*, although some mutations (G427S, K97T) warrant further study. Numerous cryptic species are found in clinical isolates of the *Aspergillus* section *Nigri* and are best reported as "*A. niger* complex" by clinical laboratories. Itraconazole resistance was common in this data set, but azole cross-resistance was unusual. The mechanism of resistance remains obscure.**

All black-spored aspergilli are grouped into *Aspergillus* section *Nigri* (12). Black aspergilli are often reported to be the third most frequently occurring *Aspergillus* spp. associated with invasive disease and aspergillomas (1, 9, 28, 29). Aspergillomas may subsequently produce oxalic acid *in situ*, which can result in renal complications (43). More commonly, however, the species cause otomycosis. In addition to their clinical significance, they also have agricultural importance as a common food spoilage organism (primarily grapes and coffee) (24, 26). The detection of ochratoxin, a potent nephrotoxin and potential carcinogen produced by some species in the section, has raised concerns about incorporation into the food chain (7, 31, 33). Black aspergilli are also used in biotechnology for the production of enzymes (such as amylases), acids (in particular, citric acid), and pectinases for fermentation (4, 54). Products of *Aspergillus niger* are considered generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) for use in the food industry (42).

Despite their importance, the taxonomy of *Aspergillus* section *Nigri* remains somewhat ill defined. It comprises a closely related group of organisms which are difficult to distinguish morphologically (1). As a result, in the clinical laboratory, reporting of all black aspergilli as *A. niger* on the basis of classical culture techniques (colony morphology, conidia size/ornamentation, etc.) is almost universal, yet isolates may not be *A. niger* but a closely related species. More recently, the results of non-culture-based methods have been utilized to differentiate between these species, including extrolite patterns, amplified fragment length polymorphisms, and restriction fragment length polymorphisms (11, 17, 41). However, the taxonomy of this section has principally been refined by DNA sequencing of the internal transcribed spacer (ITS) region, beta-tubulin, calmodulin, and actin genes, and a polyphasic approach using these targets has been shown to be optimal (11, 26). Other targets have also been investigated, including pyruvate kinase, pectin lyase, intergenic spacer, and partial mitochondrial cytochrome *b* gene, with varying but often limited success (13, 41, 56, 57). Since the 1960s (37) there have been several suggested taxonomic revisions. Currently, there are 19 recognized taxa: *A. aculeatinus*, *A. aculeatus*, *A. brasiliensis*, *A. carbonarius*, *A. costaricaensis*, *A. ellipticus*, *A. acidus* (*A. foetidus* var. *acidus* [19, 23]), *A. heteromorphus*, *A. homomorphus*, *A. ibericus*, *A. japonicus*, *A. lacticoffeatus*, *A. niger*, *A. piperis*, *A. sclerotiicarbonarius*, *A. sclerotioniger*, *A. tubingensis*, *A. uvarum*, and *A. vadensis* (1, 41). Of these, several belong to the *A. niger* "aggregate" and are morphologically indistinguishable, including, e.g., *A. brasiliensis*, *A. acidus*, *A. awamori*, *A. niger*, and *A. tubingensis* (41). Unsurprisingly, there are limited taxonomic data available for clinical strains (3).

Azole resistance has been shown to be increasing and an

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important factor in the outcome of *A. fumigatus* infections (18, 45). The most commonly reported mechanism of azole resistance in *A. fumigatus* is alterations to the azole target protein (Cyp51Ap), as a result of mutations in the gene encoding it (*cyp51A*). Other reported mechanisms are overexpression of *cyp51A* and upregulation of efflux pumps, although the influence of these and other possible mechanisms has yet to be determined (18, 45, 53). Raised itraconazole MICs have also been reported in *Nigri* isolates, although susceptibility data are relatively scarce (10, 15, 20, 35, 44). To our knowledge, no reports describing resistance mechanisms in this complex have been published to date. Triazole breakpoints/epidemiological cutoff values (ECVs) have been proposed for *A. fumigatus* (36, 38, 53) and more recently for *A. niger* (10).

The aims of this study were to identify the species of a clinical collection of black-sporing aspergilli using three molecular targets (the ITS, beta-tubulin, and calmodulin regions), identify any links between susceptibility and species, and investigate potential mechanisms of resistance in azole-resistant isolates by sequencing the *cyp51A* gene.

## MATERIALS AND METHODS

**Isolates.** The itraconazole susceptibility and taxonomy of 50 black aspergilli were investigated: 45 were clinical isolates (all initially identified as *A. niger* using macro- and micromorphological techniques), 3 were from the Northern Regional Research Laboratory (NRRL; Peoria, IL), and 2 were from the American Type Culture Collection (ATCC; Manassas, VA). Clinical strains were isolated between 1992 and 2007 and deposited in the Mycology Reference Centre, Manchester, United Kingdom, culture collection. These isolates were from 43 patients: 20 were isolated from ear swabs, 16 from respiratory samples, 6 from unknown specimens, and 3 from sterile sites (2 blood cultures and 1 mitral valve). Two samples revealed mixed macromorphology and were tested separately (suffixed a and b). A subset of 24 of these isolates was selected for further analysis to include at least 2 from each clade (Fig. 2); susceptibility to additional antifungal agents was tested, and the *cyp51A* gene was sequenced.

**Susceptibility.** MICs were determined for itraconazole (Research Diagnostics Inc., Concord, MA) by a modified EUCAST (mEUCAST) method (the modification being a lower final inoculum concentration of $0.5 \times 10^5$ CFU/ml) (18, 46) and by Etest (bioMérieux, Basingstoke, United Kingdom) performed according to the manufacturer's instructions. The mEUCAST method was used to facilitate comparison with prior *A. fumigatus* data (18). *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains, and all results were within the target range.

For the purposes of analysis, mEUCAST values of >8 mg/liter were classified as 16 mg/liter, and Etest values of >32 mg/liter were classified as 64 mg/liter.

A subset of 24 isolates was also tested against voriconazole (Pfizer Ltd., Sandwich, United Kingdom), posaconazole (Schering-Plough, NJ), econazole (Sigma, Poole, United Kingdom), ravuconazole (Bristol-Myers Squibb, NY), and amphotericin B (Sigma) by both the mEUCAST (18) and EUCAST methods (46) in duplicate.

In the absence of clinical breakpoints for *Aspergillus*, proposed ECVs (10, 53) were applied to this data set for itraconazole (>2 mg/liter), voriconazole (>2 mg/liter), and posaconazole (>0.5 mg/liter).

**Taxonomy.** DNA was extracted using an Ultraclean soil DNA isolation kit (MoBio Laboratories Inc., Cambridge, United Kingdom) following the manufacturer's instructions. The PCR/sequencing primers used to amplify the ITS region, partial calmodulin, and partial beta-tubulin genes were ITS5 and ITS4 (55), CL1 and CL2A (25), and Bt2a and Bt2b (14), respectively. Forward primer ITS5 was chosen over ITS1, as ITS1 contains an additional guanine base (5′-TCCGTAG**G**GTGAACCTGCGG versus TCCGTAGGTGAACCTGCGG-3′) in the primer region, leading to failed sequencing reactions. PCRs were performed in 25 μl, with 2 μM primer (4 μM for calmodulin), approximately 10 ng DNA, and 1× PCR Master Mix (providing final concentrations of 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate, and 0.625U *Taq* DNA polymerase; Promega Southampton, United Kingdom). Thermal cycling profiles for ITS and beta-tubulin amplification were as follows: 2 min at 94°C, followed by 35 cycles of 94°C for 1 min, 54°C for 1 min, and 68°C for 1 min, with a final extension step at 68°C for 10 min. The calmodulin thermal cycling profile was 94°C for 10 min, 35 cycles at 94°C for 50 s, 55°C for 50 s, and 72°C for 1 min, followed by 72°C for 7 min. Approximately 20 to 40 ng of purified PCR product and 4 μM primer were suspended in 10 μl water and sequenced by BigDye Terminator ready reaction mix (version 1.1) on an ABI 3730 genetic analyzer (Applied Biosystems, Warrington, United Kingdom).

TABLE 1. PCR primers used to amplify the *cyp51A* gene in *Aspergillus* section *Nigri*

| Primer[a] | $T_m$[c] (°C) | Sequence (5′–3′) |
|---|---|---|
| An*cyp51A*1F | 60.8 | TKT YCC TGC CTA CRG TCG CTT[b] |
| An*cyp51A*2F | 60.8 | GTC CGA YGT TGT GTA CGA CTG[b] |
| An*cyp51A*3F | 62.2 | GGA CAA AGA GAT TGC YCA CAT GAT G[b] |
| An*cyp51A*4F | 61.4 | GGA GAG ATG GTG GAC TAC GG |
| An*cyp51A*5R | 54.7 | GAT GCT TAT TAC AAG GTA CTA G |
| An*cyp51A*6R | 61.4 | CCT GGT GAG GCG AGT AGA AC |
| An*cyp51A*7R | 61.2 | CTT MTC CTC GTC TGG GTT CTT G[b] |
| An*cyp51A*8R | 61.4 | CTG TAG ACC TCT TCC GCG CT |

[a] PCR and sequencing primers. F, forward strand; R, reverse strand.
[b] Degenerate primer, where K is G or T, Y is C or T, R is A or G, and M is A or C.
[c] $T_m$, melting temperature.

Sequence alignments, including both exons and introns, for each molecular target were conducted using the ClustalW program (50) in the BioEdit package (version 7.0.5.3) (16). Subsequently, calmodulin and beta-tubulin sequences and then *cyp51A* sequences were combined and realigned. Additional GenBank sequences of *Aspergillus* section *Nigri* were incorporated for comparison (prefixed AJ/AY). Phylogenetic trees were prepared from alignments in the MEGA (version 4.1) program (47), using maximum parsimony and neighbor-joining methods. Gaps were treated as relevant for calculation of branch length. The support for each clade was determined by bootstrap analysis with 1,000 replications. Higher-resolution trees were made by realigning the combined sequence data from different genes.

**Sequencing of *cyp51A* gene.** The entire coding region of the *cyp51A* gene was amplified. Initially, primers designed from the *A. niger* ATCC 1015 genome sequence were used; however, these primers produced insufficient or no yield in some isolates, despite PCR optimization. From these data, partial or complete *cyp51A* sequences were obtained for 12 isolates, from which degenerate primers were designed (Table 1). Twenty-five-microliter reaction mixtures were set up for each isolate with 1× PCR Master Mix (Promega), 0.5 μM primer, and approximately 10 ng genomic DNA. In addition, 5% dimethyl sulfoxide was added to some PCR mixtures where the yield was initially poor. The *cyp51A* 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 was conducted as described above in the "Taxonomy" section with the primers listed in Table 1 and 10 to 30 ng of purified PCR product. Mismatches in the *cyp51A* gene were investigated by alignment against sequences in the same clade from this data set, as no *Nigri cyp51A* sequences were available in GenBank.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the ITS, beta-tubulin, and calmodulin sequences are JF450750 to JF450799, JF450850 to JF450899, and JF450800 to JF450849, respectively. The GenBank accession numbers for *cyp51A* sequences determined in this study are JF450900 to JF450925.

## RESULTS

**Susceptibility.** The distribution of itraconazole MICs by the mEUCAST method is shown in Fig. 1. Etest MICs (see the table in the supplemental material) were significantly different (generally lower) from mEUCAST MICs (paired samples *t* test, $P < 0.001$), with geometric means of 0.77 and 2.79 mg/liter, respectively. Isolates with itraconazole MICs of >8 mg/liter (26/50, 52%) by the mEUCAST method also showed raised Etest MICs: the resistant group had a geometric mean Etest MIC of 1.13 mg/liter (range, 0.32 to 64 mg/liter), whereas the geometric mean MIC was 0.5 mg/liter (range, 0.06 to 3 mg/liter) for those with mEUCAST MICs of <8 mg/liter. All 3 NRRL strains (NRRL341, NRRL4770, and NRRL4875) had

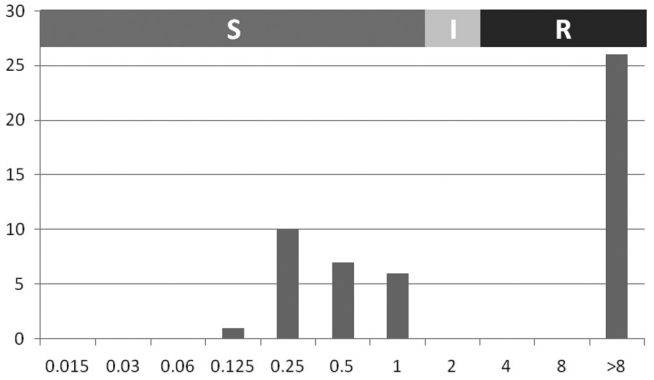


FIG. 1. Itraconazole MICs of *Aspergillus* section *Nigri* from the Mycology Reference Centre Manchester culture collection and proposed ECVs (S, susceptible; I, intermediate; R, resistant). The MIC is shown on the *x* axis, and the number of isolates is shown on the *y* axis.

high itraconazole MICs, whereas the MICs of both ATCC strains (ATCC 1015 and ATCC 9029) were low.

Susceptibilities determined for the subset of 24 isolates studied in more detail are shown in Table 2. As one might expect, MICs by the mEUCAST and EUCAST methods were generally comparable. Posaconazole and amphotericin B were the most active compounds *in vitro*, and itraconazole was the least active in this data set. Isolates with itraconazole MICs of >8 mg/liter by the mEUCAST method arguably showed reduced susceptibility to the other azoles. Comparing the itraconazole-resistant group to the itraconazole-susceptible group, the voriconazole geometric mean MICs were 2.08 and 0.91 mg/liter, respectively, and the posaconazole geometric mean MICs were 0.28 and 0.12 mg/liter, respectively. Of those resistant to itraconazole, 12% and 6% were resistant to voriconazole and posaconazole, respectively, and 59% and 6% fell into the intermediate range, respectively (53). Only one isolate (F12140) was highly cross-resistant to all azole drugs tested.

A significant color change of RPMI medium, indicating acidification, was observed during susceptibility testing of these isolates for all drugs by the mEUCAST, EUCAST (both RPMI broth), and Etest (solid RPMI agar) methods. This occurred to a greater extent in the broth microdilution wells containing lower drug concentrations, where there was more fungal growth. The higher inoculum of the EUCAST method than the mEUCAST method will likely accentuate the pH shift during incubation and, if relevant to the final MIC reading, could account for slightly higher MICs with the EUCAST method than the mEUCAST method.

**Taxonomy.** The ITS region proved too similar to give sufficient resolution between this group of closely related organisms. Bootstrap values were poor (all values were 0), and branch lengths were short (data not shown). However, beta-tubulin and calmodulin were good targets in this setting, and the phylogenetic trees were largely in agreement. The calmodulin tree was more supported than the beta-tubulin tree, with an average bootstrap value of 98, compared to an average bootstrap value of 86 for the main clades.

Beta-tubulin and calmodulin sequences were then combined for each isolate and realigned. The resulting maximum parsimony tree is shown in Fig. 2. Bootstrap values are shown above the branches, and the number of nucleotide changes between taxa is represented by branch length. The topology of the neighbor-joining tree was comparable (data not shown). Some clades were more strongly supported than others, with less resolution on the *A. awamori*/*A. niger* branch.

Using the combined beta-tubulin/calmodulin data, the 45 clinical isolates grouped into 5 clades: a group of 25 *A. awamori* isolates (55.6%), a group of 8 *A. tubingensis* isolates (17.8%), a group of 6 *A. niger* isolates (13.3%), a group of 3 *A. acidus* isolates (6.7%), and a group of 3 unknown species (6.7%). Isolates from patients with repeat specimens (2 patients, 2 isolates each) and those separated due to mixed morphology (F5412a and F5412b, F6705a and F6705b) had identical sequences. Of these 36% *A. awamori*, 90% *A. tubingensis*, 33% *A. niger*, 100% *A. acidus*, and 67% unknown group isolates were resistant to itraconazole (MICs, ≥8 mg/liter by mEUCAST).

TABLE 2. *In vitro* susceptibilities of a subset of 24 *Aspergillus* section *Nigri* isolates to commonly used antifungal agents

| Drug | Method | Cumulative % of isolates with MICs (mg/liter) of: | | | | | | | | | | Geometric mean MIC (mg/liter) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.03 | 0.06 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | >8 | |
| Itraconazole | mEUCAST[a] | | | 4 | 8 | 17 | 29 | 29 | 29 | 29 | 100 | 5.82 |
| | EUCAST | | | 4 | 8 | 13 | 21 | 21 | 21 | 21 | 100 | 7.55 |
| Voriconazole | mEUCAST | | | | | 4 | 58 | 92 | 92 | 92 | 100 | 1.63 |
| | EUCAST | | | | | 4 | 50 | 79 | 79 | 79 | 100 | 1.89 |
| Posaconazole | mEUCAST | 4 | 8 | 33 | 92 | 96 | 96 | 96 | 96 | 96 | 100 | 0.22 |
| | EUCAST | | 8 | 25 | 88 | 96 | 96 | 96 | 96 | 96 | 100 | 0.23 |
| Ravuconazole | mEUCAST | | | | | 8 | 25 | 71 | 92 | 96 | 100 | 2.12 |
| | EUCAST | | | | | 8 | 17 | 71 | 92 | 92 | 100 | 2.31 |
| Econazole | mEUCAST | | | | 4 | 25 | 79 | 83 | 96 | 96 | 100 | 1.11 |
| | EUCAST | | | | 4 | 8 | 67 | 79 | 92 | 92 | 100 | 1.50 |
| Amphotericin B | mEUCAST | | 8 | 29 | 88 | 100 | | | | | | 0.21 |
| | EUCAST | | 8 | 17 | 92 | 100 | | | | | | 0.22 |

[a] mEUCAST, modified EUCAST standard (46) method (with the modification being a lower final inoculum concentration of $0.5 \times 10^5$ CFU/ml) (18).

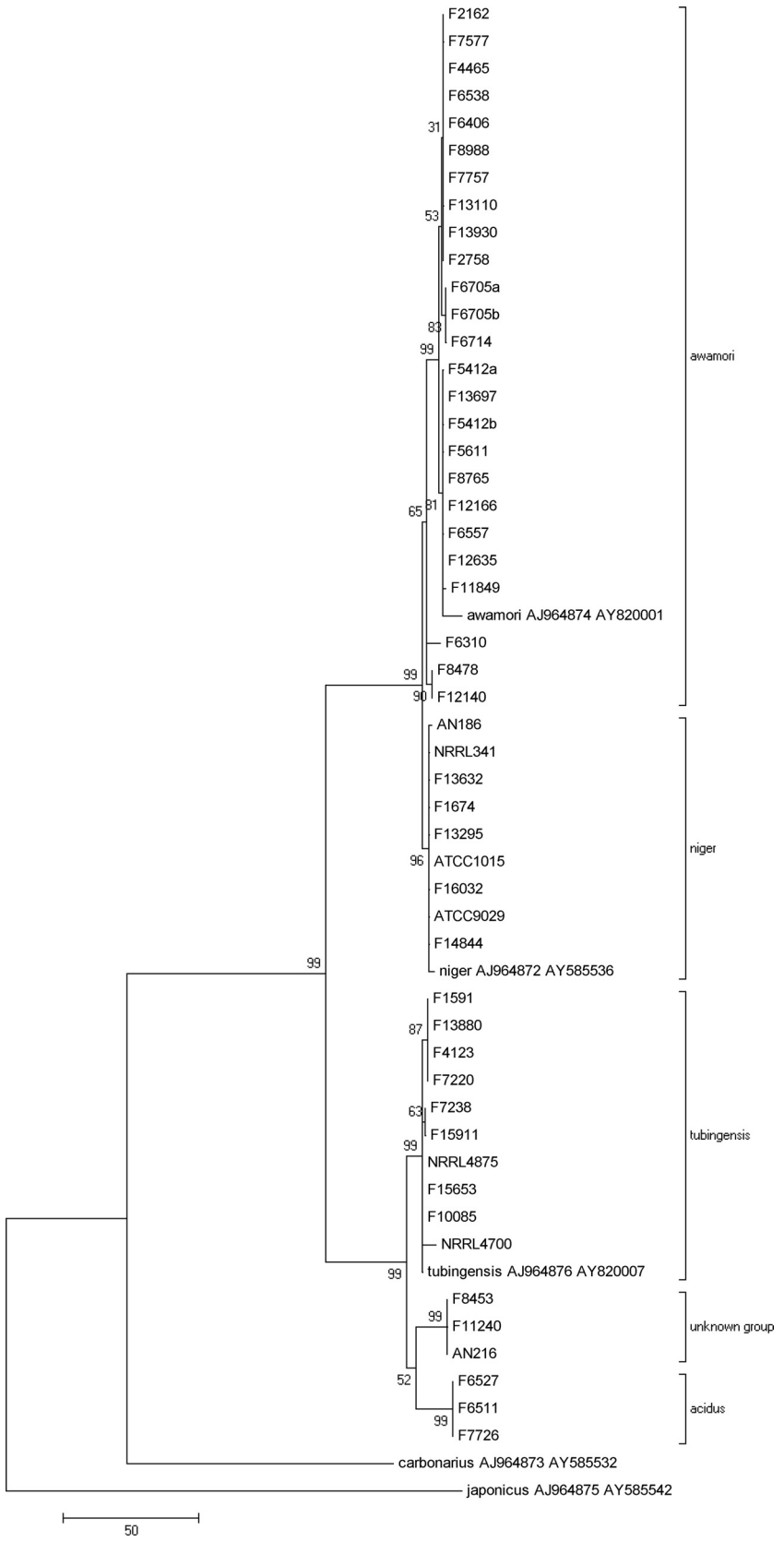


FIG. 2. Maximum parsimony tree based on combined partial beta-tubulin and calmodulin sequences.

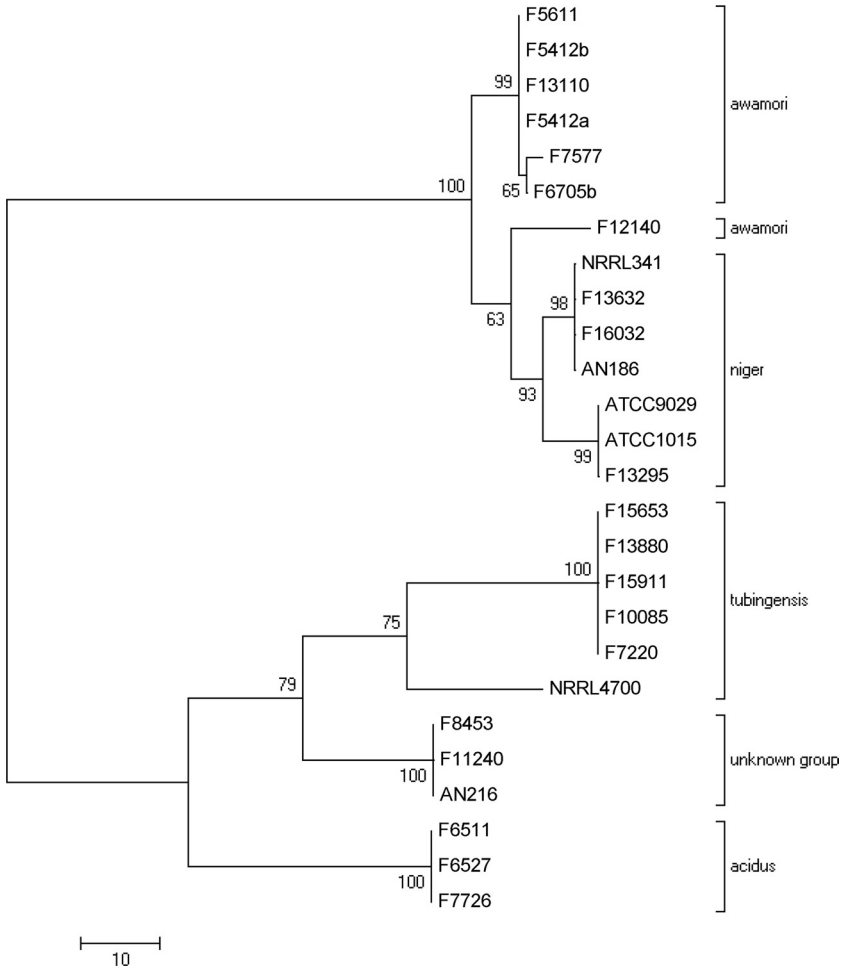


FIG. 3. Maximum parsimony tree based on *cyp51A* sequences.

***cyp51A* sequencing.** The *cyp51A* gene in *Aspergillus* section *Nigri* is 1,539 bp in length, 9 bp shorter than *cyp51A* in *A. fumigatus*. A DNA sequence alignment of *A. niger* (ATCC 1015) and *A. fumigatus* revealed 70% identity (GenBank numbers JF450900 and AF338659, respectively). The intron in *Nigri* is 57 bases in length (*A. tubingensis* has 56 bases), positioned after codon 65.

An alignment of the *cyp51A* gene (data not shown) revealed consistent sequence differences between clades (discussed later), although there were a few isolates with notable nonsynonymous single nucleotide polymorphisms, as follows. Compared to a susceptible strain of the same species, azole-resistant *A. awamori* F7577 revealed alterations at codons 97 (K97T) and 512 (N512K). *A. awamori* F12140, the only strain in this study with high-level azole cross-resistance, had a mutation at codon 427 (G427S). NRRL4700 (also a resistant strain) differed from the other *A. tubingensis* strains tested by five nonsynonymous mutations (A9V, T321A, P413L, I503V, and L511S): at codon 9 it was identical to the *A. niger*/*A. awamori* group, while at codon 321 it was identical to all *Nigri* groups other than *A. tubingensis*. Conversely, no nonsynonymous mutations were found in resistant *A. niger* isolates. The *cyp51A* sequences of the three *A. acidus* isolates were identical to each other, although comparisons were hindered by the lack of a susceptible strain. All three isolates in the unknown species group had identical *cyp51A* sequences, including F11240, which was azole susceptible. This suggests that there were no resistance-linked *cyp51A* mutations in these clades.

The *cyp51A* data revealed species-specific divergence and thus were reassessed as a taxonomic tool (Fig. 3). The *A. niger* clade divided into two groups differing by 11 base changes (6 synonymous), resulting in 4 amino acid alterations. Interestingly, the isolates in the first *A. niger* group (F13295, ATCC 1015, and ATCC 9029) were identical to *A. awamori* at codons 228 and 506, whereas those in the second group (F13632, F16032, NRRL341, and AN186) were identical to *A. awamori* at codons 507 and 511, so the evolutionary path is unclear. All *A. niger* isolates differed from *A. awamori* by a single amino acid at position 57. The *cyp51A* sequence of the unknown group differed from the sequences of the other clades at codons 140 and 413. Taxonomic findings from combined calmodulin and beta-tubulin sequencing (Fig. 2) suggest that F12140 is most closely related to *A. awamori*; however, by *cyp51A* sequencing (Fig. 3), the isolate was most similar to *A. niger*, although bootstrap values were low (<70) for all. This isolate differed from the *A. awamori* and *A. niger* groups by 19

and 12 base changes, respectively. Generally, *A. tubingensis cyp51A* sequences were identical, including the *cyp51A* sequence of F15911 (which was itraconazole susceptible by the mEUCAST method but resistant by the EUCAST method). NRRL4700 differed from the other five *A. tubingensis* sequences by 41 *cyp51A* base changes, which supports the combined calmodulin and beta-tubulin data (Fig. 2), suggesting that this isolate is dissimilar from the others in the clade.

Subsequently, *cyp51A* sequences were combined with partial calmodulin and beta-tubulin sequences and realigned (data not shown). Branch length and bootstrap values largely corroborated the combined calmodulin and beta-tubulin data, although with the addition of *cyp51A* sequences, the *A. niger* isolates split into two distinct groups. Furthermore, strain F12140 appears to be even more divergent from *A. awamori* and *A. niger* than it was previously.

## DISCUSSION

There was a particularly high frequency of itraconazole resistance in this collection of black aspergilli, the reason for which remains unclear. Overall, 51% of clinical isolates ($n$ = 45) but only 5% of *A. fumigatus* isolates from the same collection had itraconazole MICs of ≥8 mg/liter, determined using the same mEUCAST methodology (18). The mEUCAST method was used primarily to enable this comparison.

During this study ECVs proposed for the broth microdilution method were applied to the entire data set (10, 36, 38, 53), although much of that data set is based on *A. fumigatus* data, and they have yet to be clinically validated. MIC data collated during this study suggest that *A. fumigatus* itraconazole ECVs may be applicable to *Aspergillus* section *Nigri*, as the MIC distributions of the two complexes are similar (Fig. 1), although more data for black aspergilli are required to confirm this (36, 38, 53). Etest MICs were significantly lower than mEUCAST MICs. It is possible that the mEUCAST method is overestimating the MIC. However, where reduced susceptibility was observed, this was apparent by both techniques. This suggests not only that the high MICs were reproducible but also that ECVs are method dependent. Only 11% (5/45) of clinical isolates would have been itraconazole resistant by Etest using the same ECVs.

Interestingly, very little azole cross-resistance was observed in isolates with high itraconazole MICs in this study (Table 2), consistent with other some reports (10) but not others (3). This is in contrast to the findings for the comparable *A. fumigatus* data set, however (18). This could be mechanism related, or the ECVs may require further consideration, as other azole MICs were raised in itraconazole-resistant isolates but not necessarily in the resistant range.

Another potentially important observation was the color change of the RPMI medium during susceptibility testing of the *Nigri* group, which was far more than that seen with other *Aspergillus* species. RPMI contains the pH indicator phenol red; the more yellow that the medium is, the more acidic that the solution is. This factor could be critical for susceptibility testing in this setting, as pH has previously been shown to have a profound effect on MICs (21, 48, 49). In industry, *A. niger* is a citric acid producer, so it is not entirely surprising that the organism might produce acid during incubation. This phenomenon requires further study to explore the optimal susceptibility testing format in the *A. niger* complex.

The taxonomic work during this study was conducted to allow analysis of *cyp51A* sequences (discussed later) but revealed noteworthy findings. All isolates in this clinical collection were found to belong to the morphologically indistinguishable *A. niger* aggregate. Of these, only three are currently accepted *Nigri* species: *A. acidus*, *A. niger*, and *A. tubingensis* (40). *A. awamori* has been described to be a variety of *A. niger* (1, 57) and has only recently been suggested to represent a separate species (32). Assuming that *A. awamori* is a subgroup of *A. niger*, then approximately 70% of isolates were found to be *A. niger*. In the literature, *A. niger* is the most commonly reported human pathogen in this complex (39, 52). Reports of other *Nigri* species are scarce in this setting, although it is possible that these closely related organisms have been misidentified as *A. niger* using morphological techniques (1, 39). Similarly, *A. niger* predominated (68%) in a U.S. study ($n$ = 19), with 32% being *A. tubingensis* (6), whereas in a recent Spanish report ($n$ = 34), *A. tubingensis* was the most common (53%), followed by *A. niger* (38%) and *A. acidus* (9%) (3). Much of the molecular taxonomic work in the *Nigri* section has been conducted on plant pathogens due to their agricultural significance, although geographical environmental exposure may be medically relevant. *A. niger*, *A. carbonarius*, and *A. tubingensis* have been shown to be common causes of European grape spoilage (26, 30). The lack of wine production in the United Kingdom means that the frequency and environmental distribution of *Nigri* species are unknown.

This study revealed a discrete clade containing three isolates of unknown species. Calmodulin sequences were aligned against those in the extensive Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands) *Nigri* database and were found to be distinct from those of any currently recognized species. It is possible that these represent a new species, although this requires further investigation. Due to the lack of morphological differences within the *A. niger* aggregate, this is likely to require additional genome sequence data.

High itraconazole susceptibilities were more common in *A. acidus*, *A. tubingensis*, and the unknown group, as has been shown by other centers (3), suggesting that itraconazole resistance may be more common in certain *Nigri* species. There did not appear to be any link with the site of isolation and species. Isolates from all clades were cultured from a mix of specimens from lower respiratory, ear swab, and other sites. Species isolated from sterile sites were *A. niger* and *A. awamori*, both from blood culture, and an unknown group from a mitral valve.

ATCC and NRRL strains grouped as expected according to their species, with the exception of NRRL341. Isolate NRRL341 is held in the NRRL collection as an *A. foetidus* strain, and it is also held in the ATCC collection as the *A. foetidus* type strain (ATCC 16878). Results from this study suggest that NRRL341 is an *A. niger* strain, in accordance with the data of Peterson (34).

ITS sequences provided insufficient resolution (presumably due to the particularly close genetic relatedness of these isolates), as has been shown previously (3), whereas calmodulin and beta-tubulin were found to be good molecular taxonomic targets in this collection. Calmodulin sequencing traces were more problematic to decipher than those of either beta-tubulin

or ITS due to background noise (presence of multiple peaks in the same position) and irregular spacing. One of the limitations of this study was the small numbers in some clades, reducing confidence in the phylogeny. Furthermore, GenBank sequences were added to the data set to help resolve identification, and it is theorized that up to 20% of GenBank submissions may be incorrect (5).

This is the first report, to our knowledge, describing *cyp51A* data in *Aspergillus* section *Nigri*. The alignment of *cyp51A* sequences suggests that the gene may be useful as a taxonomic target to distinguish between the *Nigri* clades. Results largely mirrored those obtained with calmodulin and beta-tubulin sequences. However, *cyp51A* is a large gene and requires four primer pairs to amplify the entire coding region. This provides full sequence data but increases costs and time; potentially specific regions of interest could be targeted for molecular typing or taxonomy studies.

Some isolates also revealed alterations which may be of potential interest in terms of resistance. The *cyp51A* gene of isolate F12140 (*A. awamori*) had an alteration at codon 427, which is positioned at the start of a highly homologous region containing the heme-binding site. Amino acid substitutions have been identified at this position in *A. fumigatus*; however, they have also been found in azole-susceptible isolates and so are unlikely to be associated with resistance in *A. fumigatus* (18). One mutation at codon 97 in *A. awamori* isolate F7577 is in a highly conserved region and all other *Nigri* isolates in this study had identical sequences at this codon, so it may be significant. Codon 98 mutations have been shown to be associated with azole resistance in *A. fumigatus* (22). Codon 512, however, is positioned in a variable region at the end of the gene just prior to the stop codon so is less likely to be significant.

The alterations identified have yet to be proven to be associated with azole resistance, although these preliminary data suggest that *cyp51A* mutations in *Nigri* may not play as important a role in azole resistance as in *A. fumigatus*. Perhaps *cyp51A* overexpression is more important, as demonstrated for some strains of *A. fumigatus* (2, 8, 22, 27), although cross-resistance might be expected more often if this were the primary resistance mechanism. Azole resistance has been engineered by overexpression of a *cyp51A* homologous gene from *Penicillium italicum* (51).

A high frequency of itraconazole resistance was found in our clinical isolates, although the clinical significance of this is unclear. Itraconazole resistance was more common in certain *Nigri* species, signifying that species identification may assist with the selection of antifungal therapy. Few, if any, *cyp51A* single nucleotide polymorphisms were identified to explain elevated MICs. Species identification using ITS sequences was found to be imprecise, but variations in the beta-tubulin, calmodulin, and *cyp51A* genes were all useful for the differentiation of this group of organisms. *A. niger* was the most common (assuming that *A. awamori* is a member of the *A. niger* subgroup). Several cryptic species, including an unknown group which may be a new species, were found, indicating that clinical reporting of *A. niger* isolate without molecular confirmation of identity is potentially misleading. We suggest that clinical laboratories report these isolates as "*A. niger* complex" if molecular identification is not undertaken. Despite their medical, agricultural, and industrial importance, the taxonomy of *Aspergillus* section *Nigri* remains relatively poorly defined. This is no doubt testament to the complexity of differentiation of these closely related species. Further study of resistance mechanisms and for an optimal susceptibility testing methodology is required in this group.

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