Molecular Phylogenetic Diversity, Multilocus Haplotypes, and In Vitro Antifungal Resistance within the *Fusarium solani* Species Complex

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Members of the species-rich *Fusarium solani* species complex (FSSC) are responsible for approximately two-thirds all fusarioses of humans and other animals. In addition, many economically important phytopathogenic species are nested within this complex. Due to their increasing clinical relevance and because most of the human pathogenic and plant pathogenic FSSC lack Latin binomials, we have extended the multilocus haplotype nomenclature system introduced in a previous study (D. C. Chang, G. B. Grant, K. O’Donnell, K. A. Wannemuehler, J. Noble-Wang, C. Y. Rao, L. M. Jacobson, C. S. Crowell, R. S. Sneed, F. M. T. Lewis, J. K. Schaffzin, M. A. Kainer, C. A. Genese, E. C. Alfonso, D. B. Jones, A. Srinivasan, S. K. Fridkin, and B. J. Park, JAMA 296:953–963, 2006) to all 34 species within the medically important FSSC clade 3 to facilitate global epidemiological studies. The typing scheme is based on polymorphisms in portions of the following three genes: the internal transcribed spacer region and domains D1 plus D2 of the nuclear large-subunit rRNA, the translation elongation factor 1 alpha gene (*EF-1a*), and the second largest subunit of RNA polymerase II gene (*RPB2*). Of the 251 isolates subjected to multilocus DNA sequence typing, 191 sequence types were differentiated, and these were distributed among three strongly supported clades designated 1, 2, and 3. All of the mycosis-associated isolates were restricted to FSSC clade 3, as previously reported (N. Zhang, K. O’Donnell, D. A. Sutton, F. A. Nalim, R. C. Summerbell, A. A. Padhye, and D. M. Geiser, J. Clin. Microbiol. 44:2186–2190, 2006), and these represent at least 20 phylogenetically distinct species. Analyses of the combined DNA sequence data by use of two separate phylogenetic methods yielded the most robust hypothesis of evolutionary relationships and genetic diversity within the FSSC to date. The in vitro activities of 10 antifungals tested against 19 isolates representing 18 species that span the breadth of the FSSC phylogeny show that members of this complex are broadly resistant to these drugs.

*Fusarium* species have emerged as one of the more important groups of clinically important filamentous fungi, causing localized and life-threatening invasive infections with high morbidity and mortality. Fusarioses that become disseminated hematogenously in immunologically impaired patients typically result in 100% mortality (12). The high mortality rate within hematogenously in immunologically impaired patients typically is due in part to the fact that fusarioses are typically resistant to virtually all antifungal drugs. Fusarioses in the growing population of immunocompromised and artificial immunosuppressed patients is due in part to the fact that fusarioses are typically resistant to virtually all antifungal drugs currently available (5, 15, 41). Liposomal amphotericin B (AMB) or voriconazole (VRC), however, has proven to be efficacious in some cases (43). Results of antifungal susceptibility testing in vitro indicate broad resistance within the species-rich *Fusarium solani* species complex (FSSC) (6), whose members account for approximately two-thirds of all fusarioses worldwide. Members of the FSSC were also the predominant fusaria in the 2005 and 2006 contact lens-associated keratitis outbreaks in the United States (11, 37), and they comprised all of the keratitis outbreak isolates from Asia (24).

Although the traditional taxonomic practice has been to refer to members of this morphologically cryptic species complex as *Fusarium solani* or under the informal subspecific taxonomic rank forma specialis (f. sp.) for putatively host-specific plant pathogens within this polytypic morphospecies (18, 25, 33), molecular phylogenetic studies have clearly demonstrated that the FSSC comprises at least 45 phylogenetically distinct species distributed among three major clades (35, 63). Zhang et al. (63) first reported that all of the species from clinical or veterinary sources were nested within clade 3 of the FSSC. In contrast to the geographically restricted and exclusively plant-associated species within FSSC clades 1 and 2, which appear to be endemic to New Zealand and South America, respectively (35), members of clade 3 appear to be more common in populous areas and they may have greater fecundity in that members of this clade have been shown to grow significantly faster and produce more conidia than members of clades 1 and 2 (3). In addition, clade 3 fusaria grow well at 37°C and many produce the immunosuppressive drug cyclosporine in vitro (51). However, it has not been determined whether clinically significant amounts of this compound are produced in infected humans.

Due to their increasing clinical relevance and because most human pathogenic fusarioses lack Latin binomials, Chang et al. (11) introduced a multilocus haplotype nomenclature for the phylogenetically diverse fusarioses associated with the 2005 and
2006 U.S. Centers for Disease Control and Prevention (CDC) keratitis outbreak investigation to facilitate accurate communication of the epidemiological data within the public health community. To further this endeavor, the present molecular phylogenetic study of the FSSC was initiated (i) to develop a more robust hypothesis of molecular evolutionary relationships and species limits within the FSSC by collecting and analyzing twice as much multilocus DNA sequence data per isolate as used in previous studies that spanned the breadth of the FSSC (35, 63); (ii) to determine the utility of a three-locus typing scheme to differentiate mycosis-associated isolates within the FSSC; (iii) to expand the haplotype nomenclature to include every genetically unique isolate within the clinically important FSSC clade 3, including 26 isolates/sequence types (STs) from a recently published study on the FSSC (6); and (iv) to determine in the vitro susceptibilities of phylogenetically diverse members of the FSSC to a wide spectrum of antifungal drugs.

MATERIALS AND METHODS

Isolates. Of the 251 isolates selected for study (Table 1), 176 were cultured originally from human clinical sources. The remaining isolates were recovered mostly as pathogens of other animals or plants and were chosen to represent the breadth of the FSSC based on published phylogenetic analyses (6, 11, 35, 37, 63). All isolates were identified as members of the FSSC by conducting BLAST searches of the Fusarium ID database (http://fusarium.cbio.psu.edu/), with partial translation elongation factor sequences as the query (16). To obtain dense taxon sampling of clinically important members of the FSSC, isolates representing all unique multilocus haplotypes associated with mycotic infections (6, 11, 37, 63) were included in this study together with newly recovered clinical isolates. With the exception of Fusarium falciforme CBS 475.67 and CBS 101427, all isolates are available for distribution from the Agricultural Research Service (NRRL) Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, where they are stored cryogenically.

DNA manipulations. Total genomic DNA was isolated from freeze-dried mycelia as described previously (36). Portions of the following three gene fragments were chosen for multilocus sequence typing (MLST) based on published phylogenetic analyses (11, 37, 63): the internal transcribed spacer (ITS) region and domains D1 plus D2 of the nuclear large-subunit (LSU) rRNA, EF-1α, and the second-largest subunit of RNA polymerase (RPB2). All PCR and sequencing primers used in the MLST scheme are listed in Table 2. PCRs employed Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) in an Applied Biosystems 9700 thermocycler (Emeryville, CA) using the following program: 1 cycle of 94°C for 2 min and 40 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min at 68°C, followed by 1 cycle of 5 min at 68°C and a 1°C soak. Amplicons were size fractionated using agarose gel electrophoresis and then visualized over an Applied Biosystems 9700 thermocycler (Emeryville, CA) using the following Platinum primers used in the MLST scheme are listed in Table 2). PCRs employed Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) in an Applied Biosystems 9700 thermocycler (Emeryville, CA) using the following program: 1 cycle of 94°C for 2 min and 40 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min at 68°C, followed by 1 cycle of 5 min at 68°C and a 1°C soak. Amplicons were size fractionated using agarose gel electrophoresis and then visualized over a UV transilluminator following ethidium bromide staining. Prior to sequencing, amplicons were purified using Montage96 filter plates (Millipore Corp. Billerica, MA). Sequencing reactions were conducted in a 10-μl volume with 2 to 4 pmol of each sequencing primer, 2 μl of Applied Biosystems BigDye version 3.1 Terminator reaction mix, and approximately 50 ng of amplicon as described previously (35). Sequencer version 4.1.2 (Gene Codes, Ann Arbor, MI) was used to edit and align the raw sequence data, after which the alignments were improved manually.

Phylogenetic analysis. Of the 225 isolates subjected initially to the MLST scheme, COLLAPSE version 1.1 (http://inbio.byu.edu/Faculty/kac/cranial_lab/Computer.html) identified 180 unique three-locus haplotypes (EF-1α, ITS plus LSU, and RPB2). Subsequently, with the addition of 26 isolates/STs from the recent study by Azor et al. (6), 191 unique haplotypes were identified in the 251-isolate data set. However, the 180-haplotype data set was used for all of the phylogenetic analyses reported herein, except for a maximum parsimony (MP) analysis of the 191-haplotype data set that was used to assign the 26 isolates from the study of Azor et al. (6) to phylogenetic species. Sequences of Fusarium staphyleae were chosen to root the phylogeny based on a prior analysis (35). The Wilcoxon signed-rank Templeton test implemented in PAUP* 4.0b10 (56), using 90% bootstrap consensus trees as constraints, indicated that the individual data partitions could be combined. MP analyses were conducted on the combined partitions for the 180-isolate data set by use of PAUP* (56). Searches for the shortest trees employed five independent parsimony ratchet runs of 200 iterations, using tree bisection and reconnection branch swapping and 1,000 random sequence addition replicates. Nonparametric bootstrap was used to assess clade support with PAUP* and employing 1,000 pseudoreplicates of the data, 10 random addition sequences per replicate, and tree bisection and reconnection branch swapping. The best-fit model of nucleotide substitution for the combined data set selected by the hierarchical likelihood ratio tests in MrModeltest version 2.2 (45, 46), using PAUP*, was the general time-reversible model with a proportion of invariant sites and gamma-distributed rate heterogeneity. The best maximum likelihood (ML) tree received a negative-log likelihood (−lnL) score of −20,619.03094 based on the results of 10 independent ML heuristic phylogenetic analyses, using the general time-reversible model with a proportion of invariant sites and gamma-distributed rate heterogeneity for nucleotide substitution in GARLI (64). Nonparametric bootstrap analysis of the 180-isolate data set was conducted in GARLI (64), with a dual 2-GHz Power Mac G5 system, using 5,000 generations without improving the topology parameter and 250 ML pseudoreplicates of the data (Fig. 1).

Antifungal susceptibility testing in vitro. Antifungal susceptibility testing was conducted to determine the activity of AMB, natamycin (NAT), fluconazole (5FC), itraconazole (ITC), posaconazole (POS), VRC, anidulafungin (ANID), caspofungin (CAS), micafungin (MICA), and terbinafine (TRB) against our panel of FSSC members. Testing was accomplished via the microtiter method outlined in CLSI document M38-A (31). This included preparation of the inoculum spectrophotometrically by adjusting the turbidity to 68 to 72% and the use of either RPMI-1640 (5FC, ITC, POS, VRC, and TRB) or antibiotic medium 3 (M3) (AMB, NAT, ANID, CAS, and MICA) as the test medium. Although M3 is an adaptation from the M9 defined medium, it is used as an alternative medium for certain drugs (31). Test samples were incubated at 35°C for up to 72 h. For a few isolates, extended incubation was required. When isolates did not grow after 48 h, they were allowed to incubate at 25°C until growth was noted. The MICs for AMB, NAT, and the azoles were the lowest concentrations for which complete inhibition of growth was noted. For 5FC, the MIC was determined to be the lowest concentration that gave a 50% reduction in growth compared to what was seen for the drug-free control tube, whereas for other agents, the MIC for TRB was the lowest concentration that resulted in an 80% reduction in growth compared to that for the control. Endpoints for the candins were called at the point where a noticeable change in the growth patterns occurred. This is termed the minimum effective concentration. Since the candins are static agents attack the growing tips of the hyphae, considerable albeit distorted growth may be seen at all test concentrations. This distorted growth is easily distinguished from the healthy growth within the drug-free control tube, and the lowest concentration with such growth is the minimum effective concentration. Minimum lethal concentrations (MLCs) were determined by plating 50 μl of the MIC well and each well above the MIC to Sabouraud dextrose agar plates. The MLC was defined as the lowest concentration that allowed the growth of two or fewer colonies, equating to a 99.5% killing effect of the drug (54).

Nucleotide sequence accession numbers. The DNA sequence data reported in this study have been deposited in GenBank under accession numbers EU329487 to EU329717. Sequences previously published (11, 37, 63) are available from GenBank (http://www.ncbi.nlm.nih.gov/).

RESULTS

Phylogenetic diversity of FSSC clinical isolates. Multilocus DNA sequence data were used to assess the phylogenetic relationships and species limits of a comprehensive collection of isolates of clinical and veterinary importance within the FSSC. The 224 isolates within the in-group also included isolates which are pathogenic to plants and putative saprobes to provide dense taxon sampling within the three major clades of the FSSC (35). The aligned partial nuclear ITS plus LSU 28S rRNA gene, EF-1α gene, and the second-largest subunit of RNA polymerase gene (RPB2) partitions consisted of 986, 716, and 1,738 characters, respectively, totaling 3,440 bp of aligned DNA sequence per isolate. The data set was collapsed from 225 isolates to 180 unique multilocus haplotypes by use of COLLAPSE version 1.1 to facilitate all subsequent phylogenetic analyses. Comparisons of bipartitions with parsimony bootstrap values of ≥90% for trees obtained from the three
TABLE 1. FSSC isolates subjected to DNA MLST

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<td>34123 IMI 313108</td>
<td>Human eye</td>
<td>India</td>
<td></td>
</tr>
<tr>
<td>17-a</td>
<td>22157 ATCC 18689</td>
<td>Morus alba</td>
<td>Japan</td>
<td></td>
</tr>
<tr>
<td>17-b</td>
<td>22230 ATCC 44934</td>
<td>Morus alba</td>
<td>Japan</td>
<td></td>
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<tr>
<td>18-a</td>
<td>31158 MDA 1</td>
<td>Human</td>
<td>Texas</td>
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<tr>
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<td>32172 MDA F1</td>
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<td>Texas</td>
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</table>
partitions did not reveal any incongruence. Therefore, the individual data sets were combined and analyzed phylogenetically by MP in PAUP* version 4.0b10 (56) and ML in GARLI. Individual data sets were combined and analyzed phylogenetically by MP in PAUP* version 4.0b10 (56) and ML in GARLI. The most parsimonious trees were 2,857 trees that were highly concordant topologically (Fig. 1; only the set conducted with PAUP* and GARLI, respectively, yielded version 0.951 (64). MP and ML analyses of the combined data were conducted with PAUP* and GARLI, respectively, yielding trees that were highly concordant topologically (Fig. 1; only the ML tree is shown). The most parsimonious trees were 2,857 steps in length, whereas the ML tree with the best −lnL score was −20,619.03094, based on 10 independent analyses. The major difference between the MP and ML topologies was that five internodes along the backbone of the MP phylogeny received bootstrap values that were 17 to 31% higher than those in the ML analysis. The MP and ML phylogenies, however, received comparable levels of clade support overall, as re-
flected by relatively similar numbers of internodes that received \( \geq 70\% \) (MP = 62; ML = 63) and \( \geq 90\% \) (MP = 44; ML = 38) bootstrap support. Summary sequence and tree statistics for the individual and combined data sets are shown in Table 3. The nuclear ITS plus LSU 28S rRNA gene and EF-1\( \alpha \) gene fragments were the least and most informative, respectively, resolving 70 and 138 unique haplotypes when analyzed individually using COLLABSE version 1.1 (Table 3). Separate analyses of the nuclear ITS and LSU rRNA gene regions, and analyses of the combined data set in which the nuclear LSU rRNA genes were excluded (Table 2), showed that the highly conserved LSU region resolved only one haplotype not resolved by the other partitions.

In all phylogenetic analyses, the root of the out-group joined the tree with the plant-associated clades 1 and 2, with 2 and 11 species, respectively, always forming the two most basal branches within the FSSC phylogeny (Fig. 1). The placement of isolate NRRL 22396, *Fusarium* sp. ex bark, from French Guiana was unresolved in that it oscillated between clade 2 (MP = 50\% and clade 3 (ML = 58\%). The remaining core FSSC, including all of the isolates of clinical and veterinary importance, were strongly supported as belonging to the later-evolving clade 3 (MP = 100\%; ML = 83\%), by far the most evolutionarily diverse and species-rich clade. Consistent with previous phylogenetic analyses of medically important fusaria (37, 63), the 26 isolates that were genotyped in the work of Azor et al. (6) were nested within clade 3 (Table 1). Results of the 191-haplotype multilocus MP phylogenetic analysis (data not shown) indicate that clade 3 comprises at least 34 phylogenetically distinct species, 20 of which were associated with mycotic infections of humans and other animals (Fig. 2; Table 1). However, only 3 of the 20 clinically relevant species have been described formally with Latin binomials, and these include *Fusarium lichenicola* (FSSC 16); *Neocosmospora vasinfecta* (FSSC 8), which produces an undescribed microconidial *Fusarium* anamorph (35); and *F. falciforme* (FSSC 3+4, a single putative phylogenetic species that combines FSSC groups 3 and 4, first identified by Zhang et al. (63) but unresolved as reciprocally monophyletic by the present MP and ML analyses). All 18 species represented by two or more unique haplotypes were strongly supported as monophyletic in the MP and ML analyses (bootstrap values of 83 to 100\%) (Fig. 1). Twelve clade 3 species in the 191-haplotype data set were represented by a single isolate, and these included five human pathogens and five plant pathogens (Fig. 2; Tables 1 and 2). It is worth noting that only two species within the FSSC have been shown to be pathogenic to humans and plants (Fig. 2), and these include FSSC 1 (informally known as *F. solani* f. sp. *cucurbitae* race 2 [29]) and FSSC 8 (*Neocosmospora vasinfecta*).

**Multilocus species and haplotype nomenclature for the FSSC.** We have extended the multilocus species and haplotype nomenclature proposed previously for species within the FSSC (11, 37) to all 34 species within clade 3, including 14 phytopathogenic or putatively saprobic species that are currently not known to be clinically important (Fig. 1 and Table 1). Because FSSC groups 3 and 4, first identified by Zhang et al. (63), were not resolved as reciprocally monophyletic in the present MP and ML analyses, they were combined as a single putative phylogenetic species designated FSSC 3+4. In analyses of the 180-haplotype data set, FSSC 3+4 received strong ML and MP (100\%) bootstrap support. With the addition of the 26 isolates from the work of Azor et al. (6), FSSC 3+4 accounted for approximately one-third \((n = 63)\) of the 191 unique multilocus haplotypes within clade 3 (i.e., FSSC 3+4-a to -kkk) and 46.7\% of the FSSC STs associated with mycotic infections of humans and other vertebrates. Three other species were represented by 10 or more clinically derived STs (Fig. 2; Table 1), and these include FSSC 2 \((n = 24)\), FSSC 5 \((n = 16)\), and FSSC 6 \((n = 10)\).

Based on the frequency with which they were recovered from human infections, FSSC 1-b and FSSC 2-d appear to represent the two most important clinical haplotypes within the FSSC (11, 37), suggesting that they may represent cosmopolitan clones or clonal lineages (38). In addition to these 2 haplotypes, at least 14 other STs associated with mycoses of humans and other vertebrates exhibited transoceanic distribu-
tions (i.e., FSSC 1-a, 2-f, 2-h, 2-i, 3+4-bb, 3+4-hh, 3+4-vv, 3+4-w, 5-c, 5-f, 5-h, 5-n, 6-j, and 9-a; Table 1). Lastly, although 15 of the 26 isolates from the study of Azor et al. (6) that we genotyped were already represented in the 180-haplotype data set, 11 STs and two species (FSSC 34-a ex human cornea in Brazil and FSSC 35-a from a nematode in Spain; Fig. 2 and Table 1) appeared to represent novel phylogenetic species.

**FSSC antifungal susceptibility testing in vitro.** Based on the molecular phylogenetic results, 19 isolates representing 18 species distributed among all three clades of the FSSC were tested for susceptibility to 10 antifungals in vitro (Tables 4 and 5). Of the 19 isolates tested, all were from clinical sources (Table 1) appeared to represent novel phylogenetic species. AMB appeared to be the most active antifungal, in that MICs for nine stains were \( \leq 1 \) \( \mu \)g/ml at the first time point. Also, the MICs for AMB were the most variable, ranging between 0.5 and \( > 16 \) \( \mu \)g/ml at 24 h. Isolates with high MICs for AMB did not form exclusive clusters within the multilocus phylogeny (Fig. 1). Similarly, isolates with MICs of \( \leq 1 \) \( \mu \)g/ml for AMB were widely distributed throughout clade 3, and they included the only isolate within clades 1 and 2 tested for antifungal susceptibility (Fig. 1).

In addition to the MICs, MLSs for AMB and NAT were determined for the same set of 19 FSSC isolates in vitro (Table 5). Except for NRRL 22090 *F. illudens* and NRRL 32309 FSSC 12-d, which had to be read at 48 and 72 h, and NRRL 31096 *F. tucumaniae*, read at 144 and 168 h, all of the MLSs were assayed at 24- and 48-h time points. MLSs were highly variable for both antifungals, ranging from 1 to \( > 16 \) \( \mu \)g/ml for AMB at 24 and 48 h and from 2 to 16 \( \mu \)g/ml at 24 h and from 2 to \( > 32 \) \( \mu \)g/ml at 48 h for NAT. MLSs for NAT were more variable than those for AMB at the two time points in that seven increased by two- to fourfold for the former compared with only three for the latter (Table 5). The 8 isolates with MLSs of \( \geq 8 \) \( \mu \)g/ml for AMB and the 11 isolates with MLSs of \( \geq 8 \) \( \mu \)g/ml for NAT were all nested in and broadly distributed across the medically relevant clade 3 (Fig. 1). Lastly, seven of the eight isolates with MLSs of \( \geq 8 \) \( \mu \)g/ml for AMB also had high MLSs for NAT, ranging from 8 to \( > 32 \) \( \mu \)g/ml.

**DISCUSSION**

**Phylogenetic diversity of FSSC clinical isolates.** One of the primary objectives of the present study was to use MLST data to improve our understanding of the genetic diversity of clin-

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**TABLE 3. Primers used for PCR and DNA sequencing**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene product</th>
<th>Length of sequence obtained (bp)</th>
<th>Primer: Use&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PCR Sequencing</th>
</tr>
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<tbody>
<tr>
<td>EF-1α</td>
<td>EF-1α</td>
<td>716</td>
<td>EF1 ATGGTAAAGGARCAAGAC</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EF2 GGARGTACGTSATCATG</td>
<td>●</td>
</tr>
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<td>EF3 GTAAGGAGGASAAAGACTAC</td>
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<td></td>
<td></td>
<td></td>
<td>EF22T AGGAACCCCTACGGAGCTC</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITS5 GGAAGTAAGACTGTAAC</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AAGG</td>
<td>●</td>
</tr>
<tr>
<td>rRNA genes</td>
<td>Nuclear ribosomal rRNA gene product</td>
<td>986</td>
<td>NL4 GGTCCGTTCTTCAGAGGG</td>
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<td></td>
<td></td>
<td></td>
<td>S2 GGGGGWGAACGAAGAAAGGC</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7cr CCCATGCGTGGTTRCCCAT</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7cf ATGGGAAAACCAAGCUTGGG</td>
<td>●</td>
</tr>
<tr>
<td>RPB2</td>
<td>RNA polymerase (second-largest subunit)</td>
<td>1,738</td>
<td>11ar GCRGATGCTTTRCTCSACC</td>
<td>●</td>
</tr>
</tbody>
</table>

<sup>a</sup> R, A or G; S, C or G; W, A or T; Y, C or T.

<sup>b</sup> ●, primer was used for indicated purpose.

---

**FIG. 1.** Bootstrapped ML out-group-rooted cladogram inferred from the combined DNA sequence data from three loci for 180 unique STs. Arabic numbers and lowercase roman letters identify the species and STs, respectively, within clade 3. Dark shading is used to identify isolates from nonclinical sources; light shading identifies clade 3. Note that only three of the clinically relevant species within clade 3 have Latin binomials. Eight putatively plant host-specific forms speciales of the polytrophic morphospecies *Fusarium solani* are indicated with the f. sp. prefix. Numbers placed above and below the internodes represent the frequency (%) with which they were recovered, respectively, from 1,000 MP and 250 ML bootstrap pseudoreplicates of the data. An asterisk by a bootstrap value indicates that the ML and MP bootstrap support values are identical.
isolate as used in previous studies that encompassed the phylogenetically important members of the FSSC. Toward this end, we each species according to the haplotype nomenclature system of Chang et al. (11). FSSC 3 the number of STs within each species, except for 12 species, for which this number is given above the bars. The number below each bar identifies each species according to the haplotype nomenclature system of Chang et al. (11). FSSC 3+4 (Fusarium falciforme) was formerly known as FSSC groups 3 and 4 (63).

The three-locus phylogeny was used to identify phylogenetically distinct species (Fig. 1). A number followed by a letter to identify each unique multilocus haplotype.

FIG. 2. Histogram showing the sources of the 231 clade 3 FSSC isolates subjected to MLST. Twenty of the 34 phylogenetically distinct species within this clade appear to be clinically relevant, and these accounted for 135 of the 176 unique STs (Table 1). FSSC 3 and 4 (63).

<table>
<thead>
<tr>
<th>Species</th>
<th>Clade</th>
<th>AMB</th>
<th>5FC</th>
<th>ITC</th>
<th>VRC</th>
<th>POS</th>
<th>NAT</th>
<th>MICR</th>
<th>ANID</th>
<th>TRB</th>
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<td>1,1</td>
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<td>&gt;8</td>
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<td>8,8</td>
<td>2,4</td>
<td>16,16</td>
<td>&gt;16,&gt;16</td>
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<tr>
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<td>0,5,0,5</td>
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<td>16,&gt;16</td>
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TABLE 4. In vitro MICs of 10 antifungal agents against members of the FSSC

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<th>Species</th>
<th>AMB</th>
<th>5FC</th>
<th>ITC</th>
<th>VRC</th>
<th>POS</th>
<th>NAT</th>
<th>MICR</th>
<th>ANID</th>
<th>TRB</th>
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<td>&gt;8</td>
<td>&gt;8</td>
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<td>&gt;16,&gt;2,&gt;2</td>
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a MICs were determined using standard methods as described in Materials and Methods. All MICs were read at 24 and 48 h except for the following (indicated with asterisks in the leftmost column): for NRRL 22090, MICs were read at 72 and 96 h; for NRRL 32309, at 48 and 72 h; and for NRRL 31096 and 37625, at 144 and 168 h. Dashes represent the second MIC readings which are not recorded when the initial MIC exceeds the highest concentration tested.

b The three-locus phylogeny was used to identify phylogenetically distinct species (Fig. 1). A number followed by a letter to identify each unique multilocus haplotype.

c The FSSC comprises three clades designated 1, 2, and 3 (35).
TABLE 5. In vitro MLCs of AMB and NAT against members of the FSSC

<table>
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<th>Speciesa</th>
<th>Cladeb</th>
<th>AMB</th>
<th>NAT</th>
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<td>4</td>
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<tr>
<td>F. tucumaniae</td>
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<td>1, 4</td>
<td>4</td>
</tr>
<tr>
<td>Fusarium sambucinum</td>
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<td>4, 4</td>
<td>4</td>
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<td>Fusarium solani</td>
<td>18-b</td>
<td>2, 8</td>
<td>8, 32</td>
</tr>
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<td>F. oxysporum</td>
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<td>8, 16</td>
<td>16, 32</td>
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<td>2-v</td>
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<td>4, 8</td>
</tr>
<tr>
<td>F. lichenicola/16-c</td>
<td>3</td>
<td>16, 16</td>
<td>8, 8</td>
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<tr>
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<td>27-a</td>
<td>3</td>
<td>&gt;16, —</td>
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<td>N. vasinfecta/8-a</td>
<td>3</td>
<td>8, 8</td>
<td>8, &gt;32</td>
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<td>F. falciforme</td>
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<td>3</td>
<td>16, 16</td>
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<tr>
<td>F. falciforme</td>
<td>7-a</td>
<td>3</td>
<td>&gt;16, —</td>
</tr>
<tr>
<td>F. falciforme/3+4-c</td>
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<td>4, 4</td>
</tr>
<tr>
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<tr>
<td>F. falciforme</td>
<td>11-c</td>
<td>3</td>
<td>1, 1</td>
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</table>

a MLCs were determined using standard methods as described in the Materials and Methods. All MLCs were read at 24 and 48 h, except for the following (indicated with asterisks in the leftmost column): for NRRL 22090 Fusarium illudens, MLCs were read at 72 and 96 h; for 32309 FSSC 12-d, at 48 and 72 h; and for NRRL 31096 Fusarium tucumaniae and NRRL 37625, at 144 and 168 h. Dashes represent the second MLC readings which are not recorded when the initial MLC exceeds the highest concentration tested.

b The three-locus phylogeny was used to identify phylogenetically distinct species. A number followed by a letter to identify each unique multilocus haplotype identifies all of the species in the medically relevant clade 3.

c The FSSC comprises three clades designated 1, 2, and 3 (35).

In individuals who are persistently neutropenic. Our results add to a growing number of molecular phylogenetic studies that have identified cryptic species within morphologically defined species of medically important (references 8, 21, 47, and 60 and references therein), agriculturally important (17, 23, 39), and model system (reference 61 and references therein) fungi. The present study extended our knowledge of species boundaries within the FSSC by identifying three additional human pathogenic species within clade 3 not included in the comprehensive study by Zhang et al. (63). Consistent with the original report that FSSC groups 3 and 4 appear to represent unresolved phylogenetic species with short internodes and low bootstrap support (63), results of the ML and MP analyses indicate that these groups are not reciprocally monophyletic. Therefore, we combined these two groups as a single phylogenetic species (FSSC 3+4, i.e., Fusarium falciforme). Results of the present study show that FSSC 1, 2, and 3+4, initially reported as groups 1 to 4 in the work of Zhang et al. (63), along with FSSC 5 and 6, are the most important clinical species within this complex, based on the frequency with which they have been recovered from humans and other animals. Not surprisingly, these five species possess the greatest number of genetically distinct STs, accounting for 77.8% (105 of 135) of all of the unique STs associated with mycotic infections of humans in the present study (Fig. 2). Because FSSC 2 and 3+4 are unusually diverse genetically, collectively comprising 57.8% (78 of 135) of all clinically important STs within the FSSC, we theorize that species-level studies employing additional phylogenetically informative MLST data may lead to the discovery of additional species partitions within these taxa.

It is noteworthy that FSSC 1, known by the informal name F. solani f. sp. cucurbitae race 2 by plant pathologists (35), and FSSC 8 (Neocosmospora vasinfecta) are the only two species within this complex that have been shown to be pathogenic both to plants and to humans (29, 63). As such, they possess the potential to be developed as model system organisms to investigate whether common virulence factors are involved in plant and human pathogenesis (40).

Multilocus species and haplotype nomenclature for the FSSC. Prior to the recent introduction of multilocus-based phylogenetic species recognition, most of the species of clinical, veterinary, and agricultural importance within the FSSC have been reported in the literature under the polytypic morphospecies name Fusarium solani (19, 20, 32). Notable exceptions include F. lichenicola (FSSC 16) and F. falciforme (FSSC 3+4); but these species were only recently transferred to Fusarium from Cylindrocarpon and Acremonium, respectively, based on the results of recently published morphological and molecular phylogenetic analyses (53). Because most clinically and agronomically important fusaria lack Latin binomials, the communication of epidemiologically based MLST data among public health and agricultural scientists has been impeded. To address this problem, Chang et al. (11) first proposed a standardized haplotype nomenclatural system for Fusarium in a report of the 2005 and 2006 contact lens-associated keratitis outbreak investigation that included capitalized roman letters in abbreviated form for the major species complexes, Arabic numerals for the species within each complex, and lowercase roman letters for unique STs within each species. Consistent with the finding of Zhang et al. (63) and others (2, 19, 20, 30,
52) that members of the FSSC comprise the majority of clinically relevant opportunists within *Fusarium*, Chang et al. (11) reported that 6 of the 10 species and two-thirds of all STs from the confirmed keratitis outbreak cases investigated by the CDC were nested within the FSSC. Similarly, all of the keratitis isolates from the outbreaks in Singapore and Hong Kong were members of this complex (11, 24, 37). As an extension of the CDC’s U.S. keratitis outbreak investigation, close to two-thirds of the corneal and environmental isolates genotyped (118 of 191) were members of the FSSC, and these included a total of nine species representing 24 STs (37). The standardized haplotype nomenclatural system proposed by Chang et al. (11) was extended in the aforementioned study to the fusaria within four of the five monophyletic species complexes involved in the keratitis outbreaks.

Although the haplotype system of Chang et al. (11) was not used for members of the *Gibberella fujikuroi* species complex, primarily because all but one of the clinically important species within this complex have Latin binomials, this system should be useful in identifying clinically relevant STs within the *Gibberella fujikuroi* species complex should the necessity arise. In addition, the growing MLST databases for medically and agriculturally important fusaria will provide a wealth of discrete nucleotide polymorphism data needed to expand current allele-specific microsphere-array-based genotyping assays (37, 62).

One of the major objectives of the present study was to lay the foundation for an FSSC MLST database by extending the multilocus species/haplotype nomenclature to all clinically relevant genotypes identified in the comprehensive molecular phylogenetic analysis of the FSSC conducted by Zhang et al. (63). By expanding the system of Chang et al. (11) to all 34 phylogenetically distinct species within FSSC clade 3, including the 20 species and 141 STs associated with opportunistic infections in humans and other vertebrates, we have proposed a standardized haplotype nomenclature that should facilitate sharing of electronically portable genotypic data globally via the Internet, and we have provided a system that can be easily expanded as new clinically relevant STs are discovered (28). To further this objective and to increase the utility of the MLST data, plans are in progress to make this database Web accessible at the CDC. Ideally, this database should include all associated electropherograms so that all new STs can be verified before they are accessioned in the haplotype system (10). Future improvements in this database should benefit from the development of high-resolution MLST and multilocus microsatellite typing schemes (59) for the most important mycosis-associated species, similar to those currently available for *Candida* spp. (http://calbicans.mlst.net/) (9, 13, 22, 34, 57, 58), *Aspergillus fumigatus* (7), and *Cryptococcus neoformans* (http://cneformans.mlst.net/) (27), to facilitate their global molecular surveillance and to increase our understanding of their population biology and reproductive modes. As posited for the widespread *Fusarium oxysporum* species complex 3-a clonal lineage (38), global transposition associated with world trade may help explain why some of the 17 STs within FSSC clade 3 presently exhibit transoceanic distributions.

The whole-genome sequencing project for NRRL 45880 FSSC 11-c, informally known as *Fusarium solani* f. sp. *pisi*, at the DOE Joint Genome Institute (JGI; http://www.jgi.doe.gov/), and the availability of the whole genome sequence of three other phylogenetically diverse fusaria at the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/fungi/fgi/), should provide a wealth of molecular markers for additional species- and population-level studies of clinically important STs within the FSSC and other species complexes within *Fusarium*.

Because sequences of the nuclear LSU region resolved only one ST that was not differentiated by the ITS rRNA genes, the LSU could be excluded from the nuclear rRNA gene partition in future MLST studies of the FSSC without significantly diminishing the discriminatory power of the three-locus typing scheme. A similar three-locus system that differs by using β-tubulin sequences rather than RPB2 was recently used to differentiate 28 STs within a collection of 50 FSSC isolates mostly from human infections (6). Our MLST analyses of 26 of these isolates identified eight phylogenetically distinct species, two of which were novel (FSSC 34-a and FSSC 35-a), and 24 three-locus STs, including 8 novel clinically relevant multilocus haplotypes (Table 1). Consistent with the findings of Zhang et al. (63), all of the human pathogenic species were nested within clade 3. It is noteworthy that strain FMR 8340 (NRRL 46706 FSSC 1-a) in the aforementioned study was reported to possess a “subclade” III β-tubulin allele but “subclade” IV EF-Iα and ITS rRNA gene alleles, which suggests that the β-tubulin allele in this isolate may be paralogous. Given that this interpretation is consistent with a report that highly divergent β-tubulin paralogs appear to be distributed throughout the FSSC (35), we conclude that the use of β-tubulin gene sequences for phylogeny reconstruction within the FSSC could be problematic unless their orthology has been firmly established.

**FSSC antifungal susceptibility testing in vitro.** Consistent with the findings of Azor et al. (6) and others (5, 15, 26, 41, 44, 48, 49), the 10 antifungals tested in the present study showed very poor in vitro activities against 19 phylogenetically diverse members of the FSSC. The most significant finding from the susceptibility testing is that the polypeptide AMB showed the highest activity against members of the FSSC (Table 4); however, the MICs for AMB were highly varied. The in vitro data obtained in this study are concordant with other antifungal susceptibility studies and in vivo results, which indicate that AMB is the drug of choice for invasive fusarioses (43). In addition, the highly varied MIC results we observed for AMB (range of 0.5 to >16 μg/ml) are consistent with other studies that have reported varied efficacies of this drug in vitro (6, 26, 48) and in vivo (43). Even though the triazole VORI exhibited poor in vitro activity against phylogenetically diverse members of the FSSC in the present and prior studies (6, 15), VORI has been used successfully to treat some patients where AMB therapy failed to be efficacious (42). Because FSSC isolates have been reported to exhibit greater resistance to antifungals than other fusaria in some studies (15, 41, 49) but not others (26, 44), future in vitro susceptibility tests need to evaluate these findings by use of sufficient numbers of isolates that have been characterized phylogenetically via MLST (37) so that statistically significant conclusions can be drawn. In addition, studies are needed to evaluate whether and to what extent species- and strain-specific differences in antifungal susceptibility exist within *Fusarium* (26). Toward this end, future in vitro studies of antifungal susceptibility within *Fusarium* should benefit from adopting the haplotype nomenclature described herein.
and elsewhere (11, 37) to facilitate interlaboratory comparisons of the results. Results of the present study emphasize this point, given that members of the FSSC represent at least 20 clinically relevant phylogenetically distinct species (reference 63 and the present study) and account for approximately two-thirds of all invasive fusarial infections (37).

Because most of the antifungals currently available exhibit such poor in vitro activity against fusaria, and because fusarioses involving hematogenous dissemination virtually always result in 100% mortality (12), invasive fusarial infections have resulted in major outbreaks that have resulted in 100% mortality (12), invasive fusarial infections have emerged over the past 2 decades as a significant threat to the growing population of neutropenic patients, including soft and hard organ transplant patients, who are at risk for life-threatening invasive nosocomial infections (2).

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We thank Alison Strom and Jean Juba for excellent technical assistance, Don Fraser for preparation of the figures, Jennifer Steele for typing the results. Results of the present study emphasize this point, given that members of the FSSC represent at least 20 clinically relevant phylogenetically distinct species (reference 63 and the present study) and account for approximately two-thirds of all invasive fusarial infections (37).

REFERENCES


HUMAN PATHOGENIC MEMBERS OF FSSC 2489


