

Novel Multilocus Sequence Typing Scheme Reveals High Genetic Diversity of Human Pathogenic Members of the *Fusarium incarnatum*-*F. equiseti* and *F. chlamyosporum* Species Complexes within the United States[∇]

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Species limits within the clinically important *Fusarium incarnatum*-*F. equiseti* and *F. chlamyosporum* species complexes (FIESC and FCSC, respectively) were investigated using multilocus DNA sequence data. Maximum-parsimony and maximum-likelihood analyses of aligned DNA sequences from four loci resolved 28 species within the FIESC, within which the species were evenly divided among two clades designated Incarnatum and Equiseti, and four species within the FCSC. Sequence data from a fifth locus, β -tubulin, was excluded from the study due to the presence of highly divergent paralogs or xenologs. The multilocus haplotype nomenclature adopted in a previous study (K. O'Donnell, D. A. Sutton, A. Fothergill, D. McCarthy, M. G. Rinaldi, M. E. Brandt, N. Zhang, and D. M. Geiser, *J. Clin. Microbiol.* 46:2477–2490, 2008) was expanded to all of the species within the FIESC and FCSC to provide the first DNA sequence-based typing schemes for these fusaria, thereby facilitating future epidemiological investigations. Multilocus DNA typing identified sixty-two sequence types (STs) among 88 FIESC isolates and 20 STs among 26 FCSC isolates. This result corresponds to indices of discrimination of 0.985 and 0.966, respectively, for the FIESC and FCSC four-locus typing scheme using Simpson's index of discrimination. Lastly, four human and two veterinary isolates, received as members of the FIESC or FCSC, were resolved as five phylogenetically distinct species nested outside these species complexes. To our knowledge, these five species heretofore have not been reported to cause mycotic infections (i.e., *F. armeniacum*, *F. brachygibbosum*, *F. flocciferum*, and two unnamed *Fusarium* species within the *F. tricinctum* species complex).

Fusarium species are hyaline filamentous molds (*Hypocreales*, *Ascomycota*) that can cause superficial infections, such as onychomycoses and keratitis in immunocompetent individuals, or deeply invasive and hematogenously disseminated infections with high mortality in persistently and severely neutropenic patients (11). Despite a poor response, liposomal amphotericin B remains the antifungal of choice for the treatment of fusarioses (41). Unfortunately, most fusaria exhibit broad resistance to the spectrum of antifungals currently available, including amphotericin B, azoles, echinocandins, and terbinafine, which typically show high MICs in vitro (1, 2, 35, 41, 43).

Recent multilocus molecular phylogenetic studies have revealed that the most commonly reported fusaria causing infections in humans and other animals, such as *Fusarium solani*, *F. oxysporum*, and *F. moniliforme* (*F. verticillioides* pro parte), harbor multiple species, several of which are morphologically cryptic (30, 31, 36). To date, detailed molecular evolutionary studies have been published on clinically important members of the *F. solani* species complex (FSSC) (2, 30, 35, 51), *F.*

oxysporum species complex (FOSC) (32, 36), *Gibberella* (*Fusarium*) *fujikuroi* species complex (GFSC) (31, 33), and *F. dimmerum* species complex (FDSC) (44). Species within these four complexes account for approximately 85% of all fusarioses within the United States. Members of these complexes are estimated to cause infections at the following frequencies: FSSC 60%; FOSC, 10%; GFSC, 10%; and FDSC, 5%. Results of the present study indicate that the remaining approximately 15% of clinically relevant fusaria from the United States are mostly nested within two closely related lineages, the *F. chlamyosporum* species complex (FCSC) and *F. incarnatum*-*F. equiseti* species complex (FIESC). Although several members of the FIESC included in the Centers for Disease Control and Prevention (CDC) *Fusarium* keratitis investigation in 2005 to 2006 were analyzed phylogenetically (8), species limits within this complex and the FCSC have never been critically examined employing genealogical concordance phylogenetic species recognition (GCPSR) (49) using multilocus DNA sequence data.

Chang et al. (8) first introduced a multilocus haplotype nomenclature for members of the FSSC and FOSC involved in CDC's *Fusarium* keratitis investigation, which elucidated their epidemiology and population structure and facilitated accurate communication of their genetic diversity within the public health community. The multilocus species/haplotype nomenclature developed for these fusaria is important for accurately

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reporting on pathogen identity and their genetic diversity, primarily because several molecular phylogenetic studies have revealed that most fusaria pathogenic to humans and other animals lack Latin binomials (5, 34, 35, 44, 51). In the present study, species limits and evolutionary relationships within the FCSC and FIESC were investigated via GCPSR for the first time using DNA sequence data from portions of four loci. In addition, we report on five *Fusarium* species that to our knowledge have not been reported previously to cause infections of humans or other animals.

MATERIALS AND METHODS

Fungal isolates. Eighty-eight of the 120 isolates included in this study (Tables 1 and 2) were cultured from human or veterinary sources. The remaining 32 isolates were chosen to represent the phylogenetic breadth of the FIESC and FCSC represented within the culture collections of the Centraalbureau voor Schimmelcultures (CBS) Biodiversity Center (Utrecht, The Netherlands) and the *Fusarium* Research Center (FRC, Pennsylvania State University, State College, PA). With the exception of six clinical or veterinary isolates and the outgroup sequences of *Fusarium concolor* NRRL 13459 (received as the ex-type strain of *F. polyphialidicum*, which is a later synonym of *F. concolor*), the remaining isolates were members of the FCSC or FIESC. The 26 FCSC and 88 FIESC isolates were identified as members of these two species complexes via morphological analysis at the respective culture collections (Tables 1 and 2) and subsequent molecular phylogenetic analyses of aligned partial sequences of the RNA polymerase second largest subunit (*RPB2*) (34). All isolates are stored cryogenically in liquid nitrogen vapor (-175°C) in the Agricultural Research Service (NRRL) Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, where they are available upon request.

DNA manipulations for multilocus DNA sequencing. Mycelium was grown in yeast extract-malt broth (20 g of dextrose, 5 g of peptone, 3 g of yeast extract, and 3 g of malt extract per liter; Difco, Detroit, MI) on a rotary shaker at 100 rpm for 2 to 3 days and freeze dried, and then total genomic DNA was extracted using a hexadecyltrimethyl-ammonium bromide (Sigma, St. Louis, MO) protocol as previously described (31). Portions of five nuclear gene fragments were selected for multilocus sequence typing (MLST) based on previous analyses (33–35): translation elongation factor (*EF-1 α*), *RPB2*, the internal transcribed spacer (ITS) region, domains D1 and D2 of the nuclear large-subunit (LSU) rRNA, calmodulin (*CAM*), and β -tubulin. Data obtained from this last locus, however, was excluded from the study due to the presence of highly divergent paralogs (homologs evolved by gene duplication) or xenologs (homologs evolved by lateral gene transfer among different species) that complicated phylogenetic reconstruction. PCR and sequencing primers for the MLST scheme have been published previously (34, 35). All PCRs employed Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) and identical cycling parameters in an Applied Biosystems 9700 Thermocycler (Emeryville, CA), as previously reported (31). Applied Biosystems BigDye, version 3.1, Terminator reaction mixture was used in all DNA sequencing reactions (31).

Chromatograms were edited and aligned with Sequencher, version 4.1.2 (Gene Codes, Ann Arbor, MI), prior to manual improvement of the alignments to establish positional homology.

Phylogenetic analysis. Maximum-parsimony (MP) analyses implemented in PAUP*, version 4.0b10 (47), and maximum likelihood (ML) employing GARLI (52) were conducted as previously described (35), except that nonparametric ML bootstrapping was conducted with a 2.6-GHz MacBook Pro. The Akaike information criterion in MrModeltest, version 2.2 (29), was used to identify the best-fit model of nucleotide substitution for the ML analyses. Multilocus haplotypes or sequence types (STs) were identified using COLLAPSE, version 1.1 (http://inbio.byu.edu/Faculty/kac/crandall_lab/Computer.html).

Nucleotide sequence accession numbers. The DNA sequences determined in this study have been deposited in the GenBank under accession numbers GQ505373 to GQ505852.

RESULTS

Phylogenetic diversity of FIESC clinical isolates. Evolutionary relationships and species limits of 52 human or veterinary isolates, together with 36 nonclinical isolates within the FIESC, were inferred using multilocus DNA sequence data from four

loci. All of the clinically relevant isolates were from the United States except for one from an endocarditic patient from Brazil and the ex-type strain of *F. lacertarum*, which was isolated from lizard skin in India (45) (Table 1). Tree statistics and summary sequence for the individual and combined data sets are provided in Table 3. The combined data set comprised portions of the *EF-1 α* gene (717 bp), the ITS plus LSU (ITS+LSU) 28S rRNA gene (1,135 bp), *RPB2* (1,766 bp), and *CAM* (704 bp), totaling 4,322 bp of aligned nucleotide sequence data from each isolate. Analyses of the individual partitions revealed that the *EF-1 α* and ITS+LSU 28S rRNA genes were the most and least phylogenetically informative loci, respectively, based on parsimony-informative characters per bp and number of species resolved as monophyletic by MP bootstrapping of the four individual data sets (Table 3). Results of these analyses resolved the 21 FIESC species represented by two or more isolates as reciprocally monophyletic in the majority of the bootstrapped individual genealogies, thereby fulfilling a stringent interpretation of species recognition under GCPSR. The remaining seven putatively phylogenetically distinct FIESC species were each represented by a single highly divergent isolate, and therefore additional sampling is required to fully assess their species limits.

To determine whether DNA sequence data from the various gene partitions could be concatenated into a single data set, an MP bootstrap value of $\geq 70\%$ was used as a threshold for identifying topological incongruence. Results of these analyses indicated that the individual data sets could be combined and analyzed phylogenetically using MP in PAUP* (47) and ML in GARLI (52). DNA sequence data from a fifth locus, β -tubulin, was excluded from the study due to the widespread presence of highly divergent paralogs or xenologs. MP and ML phylogenetic analyses of the combined data set recovered trees that were highly concordant topologically (Fig. 1; only the MP tree is shown) and in which there was a deep basal split between two early diverging lineages, here informally designated the Equiseti and Incarnatum clades. The 12 most-parsimonious trees were 4,322 steps in length; the ML tree with the best negative log-likelihood score was $-15,737.46164$ based on 10 independent heuristic analyses, using the general time-reversible (GTR) model of nucleotide substitution with a proportion of invariant (I) sites and gamma-distributed (G) rate heterogeneity (i.e., GTR+I+G) in GARLI (52). Only relatively minor differences were observed between the MP and ML topologies. These differences were restricted to five internodes along the backbone of the phylogeny within the Incarnatum clade; however, the MP and ML bootstrap values differed by only 6 to 11% (Fig. 1). Analyses of the individual and combined partitions support the recognition of 14 phylogenetically distinct species within each clade. Of the 28 species within the FIESC, 9 species within the Equiseti clade and 11 within the Incarnatum clade were recovered from mycotic infections, and these spanned the phylogenetic breadth of each clade (Fig. 1). Latin binomials, however, can be applied with confidence to only three of the species within the Equiseti clade, namely, *F. lacertarum* (FIESC 4) (45), *F. scirpi* (FIESC 9) (7), and *F. equiseti* (FIESC 14), and none of the 14 species within the Incarnatum clade. *F. scirpi* is broadly circumscribed here to include two STs from Australia (FIESC 2-a and 2-b) and the highly divergent NRRL 26922 (FIESC 9-c) from France, which

TABLE 1. FIESC isolates subjected to DNA MLST

NRRL no.	FIESC MLST (species) ^a	Equivalent no(s). ^b	Isolate source	Origin
3020	10-a	FRC R-6053, 7.12 MRC	Unknown	Unknown
3214	10-a	FRC R-6054, 7.13 MRC	Unknown	Unknown
5537	8-a	ATCC 28805	Fescue hay	Missouri
6548	12-a	IMI 112503	Wheat	Germany
13335	21-a	FRC R-2138	Alfalfa	Australia
13379	23-b	FRC R-5198, BBA 62200	<i>Oryza sativa</i>	India
13402	9-b (<i>F. scirpi</i>)	FRC R-6363	Pine soil	Australia
13459	(<i>F. concolor</i>) ^c	CBS 691.87	Plant debris	South Africa
20423	4-a (<i>F. lacertarum</i>)	IMI 300797	Lizard skin	India
20697	14-b (<i>F. equiseti</i>)	CBS 245.61	Beet	Chile
20722	27-a	IMI 190455	<i>Chrysanthemum</i> sp.	Kenya
22244	25-a	H.-K. Chen F64	Rice	China
25795	5-c	CBS 394.93, BBA 64265	<i>Disphyma crassifolium</i> seed	Germany
26417	26-a	CBS 544.96	Leaf litter	Cuba
26419	14-a (<i>F. equiseti</i>)	CBS 307.94, BBA 68556 (neotype)	Soil	Germany
26921	12-a	CBS 731.87	Wheat	Germany
26922	9-c (<i>F. scirpi</i>)	CBS 610.95	Soil	France
28029	3-b	CDC B-3335	Human eye	California
28577	28-a	CBS 430.81	Grave stone	Romania
28714	26-b	ATCC 74289	<i>Acacia</i> sp. branch	Costa Rica
29134	9-a (<i>F. scirpi</i>)	CBS 448.84	Pasture soil	Australia
31011	12-a	BBA 69079	<i>Thuja</i> sp.	Germany
31160	15-c	MDA 3	Human lung	Texas
31167	18-a	MDA 10	Human sputum	Texas
32175	15-a	MDA F10	Human sputum	Texas
32181	15-c	MDA F20	Human blood	Oklahoma
32182	15-b	MDA F22	Human blood	Texas
32522	18-b	Loyola W-14182	Human diabetic cellulitis	Illinois
32864	17-a	FRC R-7245	Human	Texas
32865	21-b	FRC R-8480	Human endocarditis	Brazil
32866	23-a	FRC R-8822	Human cancer patient	Texas
32867	23-a	FRC R-8837	Human	Texas
32868	25-c	FRC R-8880	Human blood	Texas
32869	15-c	FRC R-9445	Human cancer patient	Texas
32871	5-a	FRC R-9561	Human abscess	Texas
32993	25-b	UTHSC 00-755	Human nasal tissue	Texas
32994	15-c	UTHSC 00-494	Human ethmoid sinus	Texas
32995	15-c	UTHSC 99-1964	Human sinus	Texas
32996	15-c	UTHSC 99-1741	Human leg wound	Texas
32997	7-a	UTHSC 99-423	Human toenail	Colorado
34001	15-e	UTHSC 95-1945	Human foot wound	Texas
34002	22-a	UTHSC 95-1545	Human ethmoid sinus	Texas
34003	20-a	UTHSC 95-28	Human sputum	Texas
34004	16-a	UTHSC 94-2581	Human BAL	Texas
34005	24-a	UTHSC 94-2471	Human intravitreal fluid	Minnesota
34006	15-a	UTHSC 93-2692	Human eye	Texas
34007	15-a	UTHSC 93-933	Human sputum	Texas
34008	15-d	UTHSC 92-1955	Human lung	Texas
34010	15-c	UTHSC 02-1698	Human maxillary sinus	Texas
34011	15-a	UTHSC 02-2060	Human sputum	Texas
34032	5-a	UTHSC 98-2172	Human abscess	Texas
34034	1-c	UTHSC 94-1167	Human leg	Arizona
34035	5-d	UTHSC 91-569	Human sinus	Colorado
34037	5-b	UTHSC 02-966	Human abscess	Colorado
34039	1-b	UTHSC 96-1394	Human	Connecticut
34056	16-b	Loyola M54234	Human bronchial wash	Illinois
34059	16-c	Loyola S8158	Human blood	Illinois
34070	17-c	Loyola W37591	Tortoise	Illinois
36123	4-b	CBS 102300, BBA 70843	Unknown	Unknown
36136	14-a (<i>F. equiseti</i>)	CBS 107.07, IMI 091982	Unknown	Unknown
36269	12-b	CBS 162.57	<i>Pinus nigra</i> seedling	Croatia
36318	3-a	CBS 185.31	Unknown	Unknown
36321	14-a (<i>F. equiseti</i>)	CBS 185.34	Soil	Netherlands
36323	3-a	CBS 186.31	Cotton yarn	England
36372	11-a	CBS 235.79	Air	Netherlands Antilles
36392	12-c	CBS 259.54	Seedling	Germany
36401	2-a	CBS 264.50	Cotton	Mozambique

Continued on following page

TABLE 1—Continued

NRRL no.	FIESC MLST (species) ^a	Equivalent no(s). ^b	Isolate source	Origin
36448	2-b	CBS 384.92	<i>Phaseolus vulgaris</i> seed	Sudan
36466	14-a (<i>F. equiseti</i>)	CBS 414.86	Potato peel	Denmark
36478	9-a (<i>F. scirpi</i>)	CBS 447.84	Pasture soil	Australia
36548	17-b	CBS 190.60	Banana	Congo
36575	20-b	CBS 976.97	<i>Juniperus chinensis</i> leaf	Hawaii
43297	24-b	W. Elmer 22	<i>Spartina</i> rhizomes	Connecticut
43498	8-b	CDC 2006743466	Human eye	Pennsylvania
43619	15-a	UTHSC 05-2847	Human finger	Texas
43622	15-c	UTHSC 03-2501	Human lung	Texas
43623	5-e	UTHSC 03-59	Human maxillary sinus	Colorado
43635	13-a	UTHSC 06-638	Horse	Nebraska
43636	14-c (<i>F. equiseti</i>)	UTHSC 06-170	Dog	Texas
43637	1-a	UTHSC 05-1729	Dog	Pennsylvania
43638	6-a	UTHSC R-3500	Manatee	Florida
43639	19-a	UTHSC 04-135	Manatee	Florida
43640	1-a	UTHSC 04-123	Dog nose	Texas
43694	6-a	CDC 2006743607	Human eye	Texas
43730	16-c	CDC 2006743605	Contact lens	Mississippi
45995	5-b	UTHSC 02-966	Human abscess	Colorado
45996	1-a	UTHSC 03-3101	Human sinus	New York
45997	5-f	UTHSC 04-1902	Human sinus	Colorado
45998	6-b	UTHSC 06-2315	Human toe	Texas

^a Arabic numerals identify species, and lowercase roman letters identify unique haplotypes within each species.

^b Sequences of *F. concolor* NRRL 13459 were used to root the phylogeny (Fig. 1).

^c ATCC, American Type Culture Collection, Manassas, VA; BBA, Biologische Bundesanstalt für Land-und Forstwirtschaft, Institute für Mikrobiologie, Berlin, Germany; CBS, CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands; CDC, Centers for Disease Control and Prevention, Atlanta, GA; Loyola, Loyola University, Maywood, IL; IMI, CABI Biosciences, Egham, Surrey, England; MDA, M. D. Anderson Cancer Center, Houston, TX; WE, Wade Elmer, Connecticut Agricultural Experiment Station, New Haven, CT.

suggests that additional sampling may reveal that FIESC 9-c represents a phylogenetically distinct species.

In the absence of our ability to confidently apply Latin binomials to 25 of the 28 species within the FIESC, the species and haplotype nomenclature previously adopted within the medically important clade 3 of the FSSC (35, 51) has been extended herein to all of the species within the species-rich FIESC. Results of the typing scheme revealed that, with the exception of FIESC 10 whose two isolates shared the same ST, the 20 other species represented by two or more isolates possessed between two and six STs with unique combinations of alleles. FIESC 15-a ($n = 5$) and FIESC 15-c ($n = 8$), which were restricted to Texas and Oklahoma, represented the most commonly sampled clinically relevant STs in the present study. Although six species exhibited intercontinental distributions (i.e., FIESC 3, 4, 9, 14, 23, and 25), FIESC 1-a represented the only ST out of the 62 unique haplotypes typed in the present study isolated on separate continents. In addition, FIESC 1-a and FIESC 6-a were the only two STs within this complex recovered from humans and other animals (Table 1). Employing Simpson's index of diversity, the four-locus FIESC typing scheme achieved a 0.985 index of discrimination (18).

Phylogenetic diversity of FCSC clinical isolates. Twenty of the 26 isolates analyzed phylogenetically within the FCSC were isolated within the United States from mycotic infections, and they included 19 from humans and a single isolate from a horse eye. Summary sequence and tree statistics of the four loci sampled are presented in Table 4. Based on parsimony informative characters (PIC) per bp (Table 4), *CAM* and the ITS+LSU 28S rRNA genes were the most and least phylogenetically informative loci, respectively, with 0.26 and 0.06 PIC/

bp, respectively. An ambiguously aligned 37-bp indel-containing region within *CAM* was excluded from all phylogenetic analyses. The same conditional combination approach employed above for the FIESC indicated that trees inferred from the four FCSC loci sampled represented the same underlying phylogeny, and therefore the data were analyzed as a combined data set using MP and ML. As noted above, the homoplastic distribution of highly divergent β -tubulin paralogs or xenologs precluded the use of this locus for phylogeny reconstruction. The 12 most-parsimonious trees were 4,299 steps in length; the ML tree with the best negative log-likelihood score, using the GTR+I+G model of nucleotide substitution, was $-14,131.69216$ based on 10 independent analyses in GARLI (52). MP and ML phylogenies resolved *F. nelsonii* as the earliest diverging lineage within the FCSC, forming a basal sister to the remaining members of this complex (Fig. 2; only the MP tree is shown). Analyses of the individual and combined data sets support the recognition of four genealogically exclusive, phylogenetically distinct species within this complex (Fig. 2). Three of the four FCSC species were associated with mycotic infections. However, because it is unclear which of these species, if any, represents *F. chlamydosporum*, herein these three species are designated FCSC 1, 2, and 3. High allelic diversity was observed, with 12 STs among 13 isolates within FCSC 1 and four STs among 9 isolates within FCSC 2. The latter species contained the most common ST sampled, FCSC 2-a, with human isolates from Texas and Pennsylvania and a soil isolate from Australia (Table 2). Lastly, the DNA typing results revealed that three of the four FCSC species exhibited transoceanic distributions (FCSC 1, 2, and 4 [equivalent, *F. nelsonii*]). Overall the FCSC four-locus typing scheme

TABLE 2. FCSC and novel pathogenic isolates subjected to DNA MLST

NRRL no.	MLST and/or species ^a	Equivalent no. ^c	Isolate source	Origin
13338	FCSC 4-a (<i>F. nelsonii</i>)	FRC R-2181	Soil	Australia
13444	FCSC 2-a	FRC T-550	Corn soil	Australia
13459	<i>F. concolor</i> ^b	CBS 691.87	Plant debris	South Africa
28505	FCSC 4-b (<i>F. nelsonii</i>)	FRC R-8670	Soil debris	South Africa
28578	FCSC 1-a	CBS 615.87	<i>Colocasia esculenta</i> leaf	Cuba
32521	FCSC 1-e	FRC T-0852	Human cancer patient	Texas
34012	FCSC 1-g	UTHSC 01-451	Human toe	Texas
34013	FCSC 2-a	UTHSC 01-2416	Human toenail	Pennsylvania
34014	FCSC 1-l	UTHSC 01-2668	Human maxillary sinus	Illinois
34015	FCSC 2-b	UTHSC 02-1276	Horse eye	Alabama
34016	FCSC 2-a	UTHSC 98-2537	Human leg	Texas
34017	FCSC 1-h	UTHSC 97-59	Human maxillary sinus	Texas
34018	FCSC 1-m	UTHSC 97-17	Human arm	Florida
34019	FCSC 1-c	UTHSC 96-2036	Human eye	Texas
34021	FCSC 2-a	UTHSC 95-1488	Human upper lobe wash	Texas
34022	FCSC 1-j	UTHSC 93-2120	Human paranasal sinus	Georgia
34023	FCSC 2-c	UTHSC 93-1353	Human finger	Texas
34033	<i>F. brachygibbosum</i>	UTHSC 97-99	Human foot cellulites	Texas
34036	<i>Fusarium</i> sp. strain 1, FTSC	UTHSC 01-1965	Human ethmoid sinus	Colorado
36147	<i>Fusarium</i> sp. strain 2, FTSC	CBS 109232	Human bronchial secretion	Unknown
36495	FCSC 2-b	CBS 491.77	Soil	Kuwait
36539	FCSC 1-k	CBS 677.77	Cultivated soil	Solomon Islands
43627	FCSC 2-d	UTHSC 05-3559	Human bronchial lavage	Texas
43628	FCSC 1-i	UTHSC 05-3396	Human finger	Florida
43629	FCSC 1-b	UTHSC 05-3200	Human blood	Utah
43630	FCSC 2-a	UTHSC 05-2743	Human sputum	Texas
43631	FCSC 3-a	UTHSC 05-2441	Human leg	Texas
43632	FCSC 1-d	UTHSC 05-1260	Human eye	Florida
43633	FCSC 1-f	UTHSC 03-3472	Human maxillary sinus	Tennessee
43641	<i>F. armeniacum</i>	UTHSC 06-1377	Horse eye	Missouri
45992	FCSC 1-j	UTHSC 06-3823	Human leg	South Carolina
45994	<i>Fusarium</i> sp. strain 2, FTSC	UTHSC 06-2616	Cloaca	Texas
45999	<i>F. flocciferum</i>	UTHSC 06-3449	Human scalp	California

^a MLST within FCSC. Arabic numerals designate species, and lowercase roman letters identify unique haplotypes within each species.
^b Sequences of *F. concolor* NRRL 13459 were used to root the phylogeny (Fig. 2).
^c CBS, CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands; CDC, Centers for Disease Control and Prevention, Atlanta, GA.

achieved an index of discrimination of 0.966 employing Simpson's index of diversity (18).

Five novel *Fusarium* species causing mycotic infections. Six isolates tentatively identified morphologically as members of

the FIESC or FCSC were resolved as five phylogenetically distinct species nested outside these complexes, based on comparisons of partial *EF-1α* sequences with the FUSARIUM-ID database (12) and partial *RPB2* gene sequences from a more

TABLE 3. FIESC tree statistics for the individual and combined partitions

Locus	Size (bp)	No. of PICs	No. of PICs/bp	AUT ^a	MPT length (no. of steps) ^b	CI ^c	RI ^d	No. of haplotypes ^e	No. of FIESC species supported as monophyletic ^f
<i>EF-1-α</i>	717	162	0.23	95	517	0.61	0.92	51	20
LSU+ITS rRNA genes	1,135	11	0.01	35	58	0.57	0.92	21	1
<i>RPB2</i>	1,766	220	0.12	184	629	0.58	0.92	46	19
<i>CAM</i>	704	133	0.19	109	385	0.64	0.94	45	14
<i>EF-1-α</i> plus rRNA genes	1,852	173	0.09	130	602	0.57	0.91	59	—
<i>EF-1-α</i> plus <i>RPB2</i>	2,483	382	0.15	279	1,181	0.57	0.91	57	—
<i>EF-1-α</i> plus <i>CAM</i>	1,421	295	0.21	204	934	0.59	0.92	54	—
rRNA genes plus <i>RPB2</i>	2,901	231	0.08	219	714	0.55	0.91	54	—
rRNA genes plus <i>CAM</i>	1,839	144	0.08	144	469	0.58	0.93	56	—
<i>RPB2</i> plus <i>CAM</i>	2,470	353	0.14	293	1,062	0.69	0.92	53	—
Combined	4,322	526	0.12	423	1,695	0.67	0.91	62	21

^a AUT, autapomorphy or a derived character.
^b MPT, most parsimonious tree.
^c CI, consistency index.
^d RI, retention index.
^e Number of multilocus haplotypes or STs determined using COLLAPSE, version 1.1.
^f Based on maximum parsimony bootstrap support. —, comparison not made.

EF-1 α + rDNA + RPB2 + CAM
 4322 bp
 526 PIC
 1 of 12 MP trees
 1695 steps
 CI = 0.67
 RI = 0.91

25
 steps

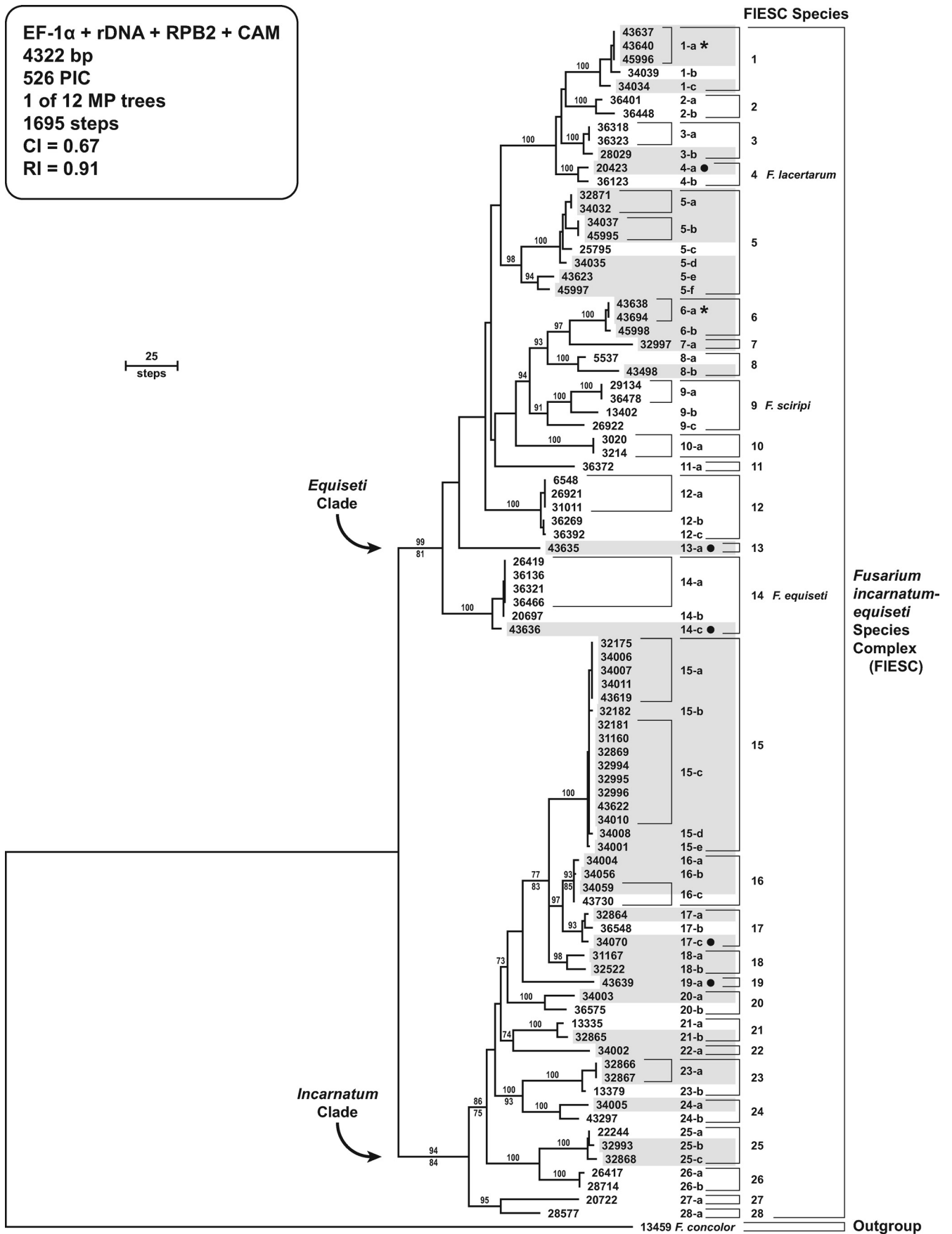


TABLE 4. FCSC tree statistics for the individual and combined partitions

Locus	Size (bp)	No. of PICs	No. of PICs/bp	AUT ^a	MPT length (no. of steps) ^b	CI ^c	RI ^d	No. of haplotypes ^e	No. of FCSC species supported as monophyletic ^f
<i>EF-1-α</i>	710	159	0.22	74	379	0.81	0.89	17 (7)	3
LSU+ITS rRNA genes	1,153	69	0.06	45	156	0.85	0.9	14 (6)	2
<i>RPB2</i>	1,766	335	0.19	111	697	0.71	0.88	17 (7)	3
<i>CAM</i>	670	177	0.26	59	364	0.82	0.88	12 (5)	2
<i>EF-1-α</i> plus rRNA genes	1,863	228	0.12	119	551	0.73	0.87	24 (7)	—
<i>EF-1-α</i> plus <i>RPB2</i>	2,476	494	0.2	185	1,084	0.72	0.88	23 (7)	—
<i>EF-1-α</i> plus <i>CAM</i>	1,380	336	0.24	133	745	0.77	0.88	21 (7)	—
rRNA genes plus <i>RPB2</i>	2,919	404	0.14	156	872	0.71	0.87	21 (7)	—
rRNA genes plus <i>CAM</i>	1,823	246	0.13	104	540	0.8	0.86	20 (6)	—
<i>RPB2</i> plus <i>CAM</i>	2,436	512	0.21	170	1,065	0.78	0.88	21 (7)	—
Combined	4,299	740	0.17	289	1,627	0.78	0.87	27 (7)	3

^a AUT, autapomorphy or a derived character.

^b MPT, most parsimonious tree.

^c CI, consistency index.

^d RI, retention index.

^e Number of multilocus haplotypes or STs determined using COLLAPSE, version 1.1. Note that the number in parentheses refers to the number of STs for the non-FCSC isolates within the ingroup.

^f Based on maximum parsimony bootstrap support. —, comparison not made.

inclusive data set (K. O'Donnell, unpublished data). Results of these analyses identified three isolates as described species: NRRL 34033 (from human foot cellulites; Texas) was identified as *F. brachygibbosum* by comparison with the ex-type strain NRRL 20954 (= BBA 64691); NRRL 43641 (from horse eye; Missouri) as *F. armeniacum* by comparison with the ex-type strain NRRL 26908 (CBS 485.94 and FRC R-9372); and NRRL 45999 (from human scalp; California) formed a genealogically exclusive group with *F. flocciferum* isolates NRRL 25471 (CBS 792.70 and BBA 11141) and NRRL 25473 (BBA 64346). The first two species are members of the trichothecene toxin-producing clade of fusaria (21). NRRL 45999 *F. flocciferum*, together with the remaining two clinical species, represented by NRRL 34036 *Fusarium* sp. strain 1 (from human ethmoid sinus; Colorado) and NRRL 36147 *Fusarium* sp. strain 2 (from human bronchial secretion; geographic origin unknown), and NRRL 45994 *Fusarium* sp. strain 2 (from cloaca; Texas), are members of a clade designated herein as the *F. tricinctum* species complex (FTSC) (21). NRRL 34036 *Fusarium* sp. strain 1 (from human ethmoid sinus; Colorado) and *Fusarium* sp. strain 2, represented by NRRL 36147 strain 1 (from human bronchial secretion; geographic origin unknown) and NRRL 45994 strain 2 (from cloaca; Texas), appear to represent two undescribed phylogenetically distinct species. To our knowledge, the current study represents the first report implicating these five species in causing mycotic infections of humans and other animals.

DISCUSSION

Species limits and evolutionary relationships within two closely related fusaria lineages, the FIESC and FCSC, together with five clinically novel *Fusarium* species that are important in medical and veterinary contexts, were investigated for the first time employing multilocus GCPSR (49). The major finding of the present study is that the FCSC and FIESC appear to comprise, respectively, 4 and 28 phylogenetically distinct species; that over 70% of the species within these two complexes are represented by isolates recovered from infections of humans or other animals; and that they comprise approximately 15% of all fusarial infections within the United States. The 3 species within the FCSC and all 21 species within the FIESC represented by two or more isolates fulfilled the highly conservative requirements of GCPSR as applied here in that they were resolved as genealogically exclusive in the majority of the four bootstrapped single-locus genealogies (37, 40). In addition, bootstrapping revealed that none of the individual genealogies contradicted the monophyly of these species (i.e., genealogical nondiscordance sensu Dettman et al.) (10). Additional sampling of more isolates is needed to assess the monophyly of one putative species within the FCSC and seven within the FIESC, given that they were each represented by a single genetically divergent isolate in the present study.

Phylogenetic diversity of FIESC clinical isolates. This study describes the first MLST typing scheme for species and hap-

FIG. 1. One of 12 most-parsimonious trees inferred from MP analysis of the combined four-locus data set for 88 isolates within the FIESC. The phylogram is rooted by the outgroup method using sequences of *F. concolor* NRRL 13459. Arabic numbers and lowercase Roman letters identify species and their multilocus STs, respectively. Human and veterinary isolates are distinguished from nonclinical isolates by shading. Veterinary isolates are distinguished from those from humans by a solid dot to the right of the NRRL number/ST. In addition, a star to the right of ST 6-a indicates that one isolate was from a veterinary source and the other from a human. Note that Latin binomials can be applied confidently to only 3 of 14 species within the Equiseti clade and to none of the species within the Incarnatum clade. MP bootstrap values based on 1,000 pseudoreplicates of the data are indicated above internodes. ML bootstrap values are indicated below internodes only when they differed by $\geq 5\%$ of the MP value.

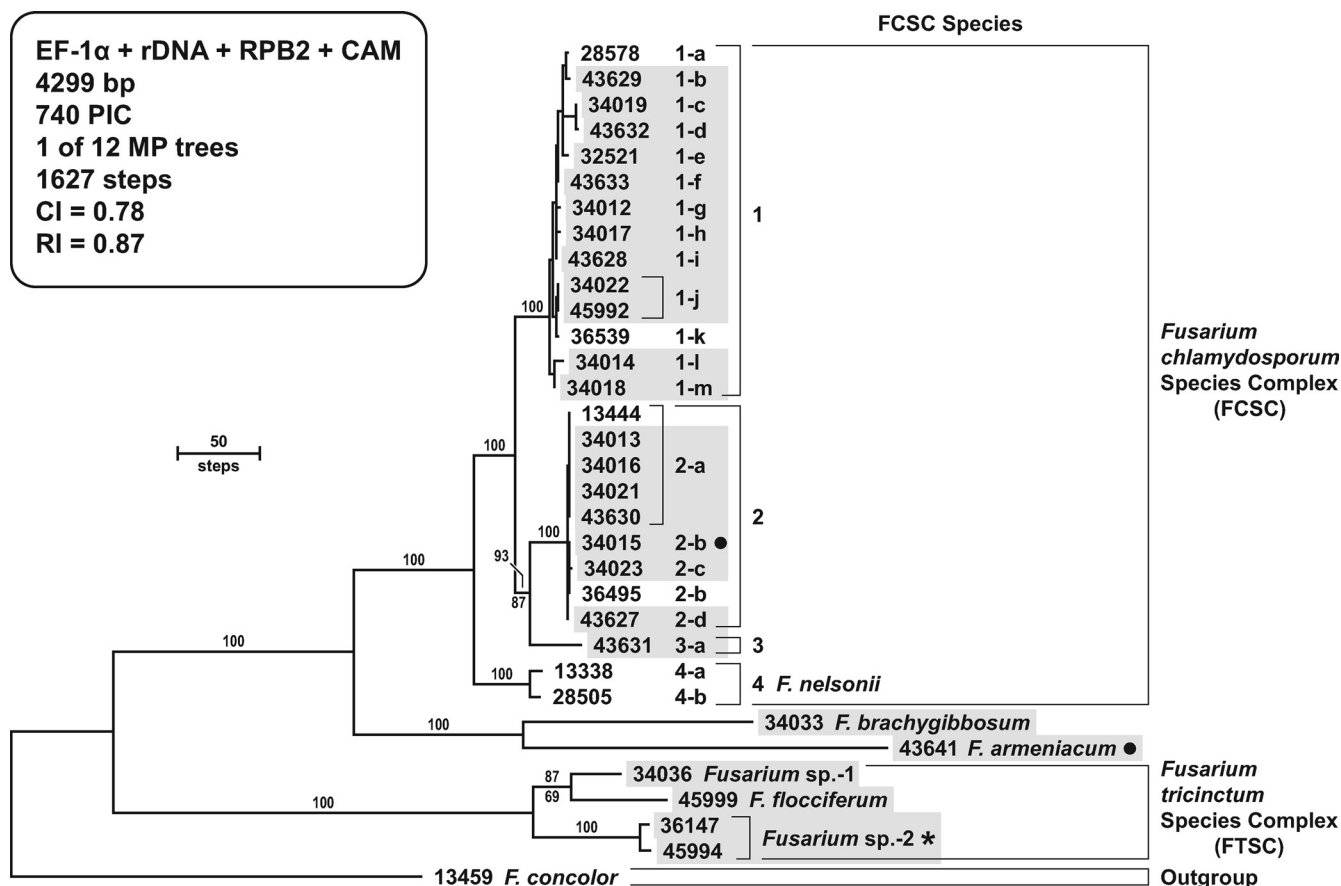


FIG. 2. One of 12 most-parsimonious MP phylograms inferred from the combined data from four loci for the FCSC and related clinically relevant fusaria rooted with sequences of *F. concolor* NRRL 13459. Arabic numbers and lowercase Roman letters are used to identify four species and their multilocus STs within the FCSC. Dark shading identifies isolates from human clinical and veterinary sources, the latter of which are further distinguished by a solid dot to the right of the NRRL number/ST. A star to the right of *Fusarium* sp. strain 2 indicates that it was represented by one human and one veterinary isolate. It is unclear which of the three unnamed species within the FCSC represents *F. chlamydosporum*, if any. Numbers above nodes represent the frequency (percent) with which they were recovered from 1,000 MP bootstrap pseudoreplicates of the data. ML bootstrap values are indicated only below those internodes that differed by $\geq 5\%$ of the MP value. The present study represents the first report of the five species basal to the FCSC causing mycotic infections in humans and other animals. *Fusarium brachygibbosum* and *F. armeniacum* are members of the trichothecene toxin-producing clade, whereas the other non-FCSC ingroup species are members of the FTSC.

lotypes based on nucleotide polymorphism within portions of four nuclear genes among members of the FIESC that are important in both clinical and veterinary contexts. Previous molecular phylogenetic studies of the FIESC have not focused on identifying GCPSR-based species limits among clinically relevant or mycotoxigenic isolates, given that their genetic diversity was assessed only via partial DNA sequence data from single nuclear genes such as *EF-1 α* (21, 42), *RPB2* (34), β -tubulin (3), 28S rDNA (15), or restriction fragment polymorphisms from the nuclear ribosomal intergenic spacer region (rDNA) (20). Based on the results of the present study, *EF-1 α* was the most phylogenetically informative gene and the ITS+LSU 28S rDNA was the least informative. Even though the latter locus possessed relatively little phylogenetic signal, the typing schemes benefited from its inclusion by an increase of six STs within the FIESC and three STs within the FCSC.

One of the most surprising results to emerge from the present study is that the species-rich FIESC comprises at least 20 mycoses-associated species among the 28 reciprocally monophyletic lineages resolved by the multilocus molecular

phylogenetics. What makes this finding all the more remarkable is that only one of the 52 clinical isolates was recovered from outside the United States, revealing that phylogenetically diverse human-opportunistic members of this complex are well represented in North America. Moreover, phenotypically based taxonomic treatments of the genus have underestimated species diversity within the FIESC by close to 1 order of magnitude (13, 22, 28). Similar GCPSR-based studies within other clinically important clades within *Fusarium*, such as the FSSC (30, 35, 51), GFSC (31, 33), FDSC (44), and FO SC (36) have revealed similar levels of cryptic speciation.

The result is that Latin binomials can be applied with confidence to only 3 of the 28 species within the FIESC (Fig. 1). This is due primarily to the discovery of the large number of phylogenetically distinct but morphologically cryptic species reported herein and also to unresolved taxonomic and nomenclatural problems associated with applying validly published names such as *F. incarnatum* and *F. pallidoroseum* and their varieties (19, 46), which might represent phylogenetically distinct species, to members of the FIESC. Although the name *F.*

semitectum has been used in the literature more than any other species within the Incarnatum clade, study of the type collection surprisingly revealed that this binomial has been misapplied because it is a later synonym of *Colletotrichum musae* (6). These systematic problems are exacerbated by the dearth of and homoplasious morphological characters within the FIESC and because type specimens, where known, are too old for DNA typing using the present four-locus MLST scheme.

In the absence of binomials for most of the species within the FIESC, one of the primary objectives of the present study was to extend the standardized multilocus species/haplotype nomenclature, first proposed in the CDC's keratitis outbreak investigation (8), to each member of the FIESC to facilitate communication of epidemiologically relevant data within the public health, phytopathological, and mycotoxin research communities. In this connection, it is worth mentioning that 59% of the FIESC isolates typed represented unique STs, with FIESC 15's 16 isolates and five STs from Texas or Oklahoma being the most common species sampled. This finding, however, undoubtedly represents a sampling bias given that close to two-thirds of the clinically relevant isolates we typed were obtained from the University of Texas Health Science Center's (UTHSC) Fungus Testing Laboratory in San Antonio, TX. Clearly, future studies are needed to elucidate how clinically relevant STs are distributed throughout North America and on other continents with the aim of identifying their environmental reservoirs. Surveys of the FIESC in nature indicate that they are common on phylogenetically diverse plants and plant debris and in soil in both hemispheres (13). Moreover, members of the Incarnatum clade are especially prevalent in the tropics and subtropics (13). Because members of the FIESC have been reported to produce type A and B trichothecene mycotoxins (14, 17, 25), which can alter immune function (39) and inhibit eukaryotic protein synthesis (50), as well as cytotoxic enniatins (16, 24) and estrogenic mycotoxins (14, 17, 20, 25), studies are needed to evaluate whether any of these toxins function as virulence factors in animal pathogenesis. In addition, ongoing studies are directed at investigating their mycotoxin potential in vitro using the phylogenetic framework developed in the present study.

Phylogenetic diversity of FCSC clinical isolates. Herein, we report on the first MLST scheme for members of the FCSC. This scheme was used to type 20 relevant isolates from clinical or veterinary sources from the United States, employing portions of the same four loci used for the FIESC. The discovery of highly divergent β -tubulin paralogs or xenologs, as in the FIESC and FSSC (30), precluded the use of this locus for phylogeny reconstruction in the present study. Even so, Azor et al. (3) recently reported that phylogenetic analysis of a 378-bp portion of the β -tubulin gene from eight isolates identified as *F. chlamydosporum* formed a weakly supported clade (61% bootstrap), which suggests that orthologous alleles were sampled. The only other published phylogenetic analysis of the FCSC, which strongly supported its monophyly (100% bootstrap), was conducted using a 1.8-kb portion of *RPB2* (34), but only four isolates were studied. Results of the present study represent the first GCPSR-based assessment of species limits within the FCSC. Species were recognized only if they were reciprocally monophyletic in at least half of the individual partitions and no genealogical discordance was observed (37,

40). Using these ranking criteria, three phylogenetically distinct, clinically relevant species were resolved within the morphotaxon *F. chlamydosporum*.

Given that the type specimen of this species was isolated from banana in Honduras (13) and that isolates from this host and/or geographic location were unavailable for study, it is unclear which of the three FCSC species, if any, corresponds to *F. chlamydosporum*. Because the type specimen of this species was collected in 1925 and an ex-type strain does not exist, study of isolates collected from banana at the type locality may help resolve this taxonomic problem. The species/haplotype nomenclature originally developed by Chang et al. (8) for the fusaria keratitis outbreak adopted in the present study, using Arabic numbers for species and lowercase roman letters for each unique ST, obviates these taxonomic issues and promotes precise communication of the MLST data within the scientific community. Results of the present study, which show that the two most common STs, FCSC 2-a and 2-b, exhibited intercontinental distributions and were represented by soil isolates, is consistent with reports that the broadly defined morphospecies *F. chlamydosporum* is common in soils and the rhizosphere of numerous vascular plants worldwide (13). Isolates of the fourth species within the FCSC, *F. nelsonii*, have not been reported to cause mycotic infections, possibly because they have been recovered only from remote or sparsely populated regions in South Africa (26) and Australia (this study) or possibly because they may have been misidentified as *F. chlamydosporum* or *F. incarnatum*. Given that the FCSC isolates from humans and other animals included in the present study were all from the United States, future studies are needed to elucidate the global distribution of clinically important species/STs, which should help identify their environmental reservoirs and identify widespread clones or clonal lineages (36). Further, because members of the FCSC are able to elaborate several mycotoxins, including trichothecenes and moniliformin (25), the phylogenetic framework developed in the present study will be used to evaluate species/ST mycotoxin potential to better understand the risk these strains pose to food safety and human health (38).

Five novel *Fusarium* species causing mycotic infections. The extremely homoplasious morphological characters within the FIESC and FCSC contributed significantly to the initial phenotypic misidentifications of the five novel fusaria causing infections of humans and other animals as *F. equiseti*, *F. incarnatum*, or *F. chlamydosporum*. Fortunately, accurate molecular identifications were easily obtained by simply comparing partial *EF-1 α* sequences with those in the FUSARIUM-ID database (12) and/or molecular phylogenetic analysis of a comprehensive data set of partial *RPB2* sequences for human pathogenic and phytopathogenic fusaria (O'Donnell, unpublished). Significant advantages of the molecular approach, based on results of the present study, include that it can provide accurate identifications of rare and novel mycotic agents that are named (i.e., *F. brachygibbosum*, *F. flocciferum*, and *F. armeniacum*) as well as those that apparently lack Latin binomials (i.e., *Fusarium* sp. strain 1 and *Fusarium* sp. strain 2). It is worth mentioning that a third isolate of *Fusarium* sp. strain 2, NRRL 28032, was received from the CDC as B-4271 in 1998, isolated from a toenail infection from a patient in Colorado.

Conclusions and future directions. It is important that the MLST schemes developed for the FIESC and FCSC in the present study, in contrast to those available via the Internet for some of the most important human pathogenic species (4, 23), focused primarily on identifying species limits within these closely related species complexes. Nevertheless, the four-locus typing schemes for the FIESC and FCSC achieved indices of discrimination of 0.985 and 0.966, respectively, using Simpson's index of diversity (18). Should the necessity arise, identification of additional phylogenetically informative loci for the MLST schemes will be greatly facilitated by four phylogenetically diverse fusarial genomes that are available online, one representing the FSSC from the Joint Genome Institute (<http://www.jgi.doe.gov>) and three from the Broad Institute of Massachusetts Institute of Technology and Harvard representing the FOOSC, GFSC, and the trichothecene toxin-producing fusaria (9; <http://www.broad.mit.edu/annotation/fungi/fgi/>). With the development of GCPSR-based MLST schemes for the six most important human-pathogenic species complexes within *Fusarium* (i.e., FSSC, FOOSC, GFSC, FDSC, FIESC, and FCSC) (see Fig. 1 in reference 34), which collectively comprise close to 100% of all medically important isolates, a uniform finding that has emerged from these studies is the dramatic discrepancy between species identifications using morphology alone versus molecular phylogenetics. Results of the present study and those published previously (34, 35, 51) have revealed that only 30% of clinically relevant fusaria (i.e., 20 of 65) have Latin binomials that can be applied with confidence. This is largely due to high levels of cryptic speciation and the concomitant extreme morphological homoplasy, especially within the FIESC and FSSC (30, 35, 51), as reflected by the fact that only 3 of the 21 FIESC and 3 of the 20 FSSC mycoses-associated species have known scientific names. In the absence for morphological apomorphies, the MLST schemes provide the only means by which isolates can be identified to species/haplotype with confidence and be accurately reported on in the scientific literature. Because species limits were delimited within the FIESC and FCSC for the first time in the present study, it is possible for us to recommend using a partial *EF-1 α* gene sequence for identifying species within these two complexes. With the present set of isolates, sequence data from this locus was used to identify all 28 species within the FIESC and all 4 species within the FCSC. However, as putatively novel species are detected, GCPSR-based studies will be required to fully assess their genealogical exclusivity. It is worth mentioning that matrix-assisted laser desorption ionization–time of flight analysis appears to provide a potential avenue for rapidly identifying clinical fusaria to the level of species complex (27) or in some cases to species level, assuming their boundaries have been defined previously by GCPSR. In this preliminary study, 35 of the 62 isolates analyzed were identified to one of three species complexes, with only isolates of *F. verticillioides* and *F. proliferatum* being identified to species. Even though these results are encouraging, it remains to be determined whether matrix-assisted laser desorption ionization–time of flight analysis can be used to identify most or all of the approximately 65 clinically relevant fusaria to the species level.

To further promote identification of pathogenic fusaria, Internet-accessible standardized MLST databases of clinically relevant fusaria will be made available at the CBS and the

FUSARIUM-ID website (<http://fcgp.fusariumdb.org/>) at Pennsylvania State University. These databases will be updated regularly as new species/STs are discovered, contingent on the deposit of associated chromatograms, which are essential to ensure that sequences are error free, and of cultures in an international, publicly accessible culture collection to promote further study by the scientific community. The MLST databases should be viewed as a work in progress (48), providing a novel baseline for understanding *Fusarium* population biology and potential changes in the spectrum of clinically relevant fusaria within a robust phylogenetic framework.

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The mention of trade products or firm names does not imply that they are recommended by the U.S. Department of Agriculture over similar products or other firms not mentioned.

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