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Genetic Diversity of Human Pathogenic Members of the *Fusarium oxysporum* Complex Inferred from Multilocus DNA Sequence Data and Amplified Fragment Length Polymorphism Analyses: Evidence for the Recent Dispersion of a Geographically Widespread Clonal Lineage and Nosocomial Origin

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Fusarium oxysporum **is a phylogenetically diverse monophyletic complex of filamentous ascomycetous fungi that are responsible for localized and disseminated life-threatening opportunistic infections in immunocompetent and severely neutropenic patients, respectively. Although members of this complex were isolated from patients during a pseudoepidemic in San Antonio, Tex., and from patients and the water system in a Houston, Tex., hospital during the 1990s, little is known about their genetic relatedness and population structure. This study was conducted to investigate the global genetic diversity and population biology of a comprehensive set of clinically important members of the *F. oxysporum* complex, focusing on the 33 isolates from patients at the San Antonio hospital and on strains isolated in the United States from the water systems of geographically distant hospitals in Texas, Maryland, and Washington, which were suspected as reservoirs of nosocomial fusariosis. In all, 18 environmental isolates and 88 isolates from patients spanning four continents were genotyped. The major finding of this study, based on concordant results from phylogenetic analyses of multilocus DNA sequence data and amplified fragment length polymorphisms, is that a recently dispersed, geographically widespread clonal lineage is responsible for over 70% of all clinical isolates investigated, including all of those associated with the pseudoepidemic in San Antonio. Moreover, strains of the clonal lineage recovered from patients were conclusively shown to genetically match those isolated from the hospital water systems of three U.S. hospitals, providing support for the hypothesis that hospitals may serve as a reservoir for nosocomial fusarial infections.**

Members of the phylogenetically diverse monophyletic *Fusarium oxysporum* complex (FOC) are best known as cosmopolitan soilborne plant pathogens that are responsible for economically devastating vascular wilts of an enormous range of agronomically important plant hosts (6). Members of the FOC are also frequently isolated from nonplant sources, particularly from the soil but also from air and animals. Over the past 2 decades, however, fusaria have emerged as opportunistic pathogens causing life-threatening disseminated infections in immunocompromised patients (3). In patients who are persistently neutropenic, deeply invasive fusarial infections cause 100% mortality (18). Most localized and disseminated cases of fusariosis are caused by members of the *Fusarium solani* species complex, followed by members of the FOC (1). Fortunately, the recent development of one strain of *F. oxysporum* as a model system will greatly facilitate the molecular genetic dissection of fungal virulence determinants during plant and animal pathogenesis (24).

Although molecular epidemiological studies have been completed for nosocomial fusariosis (1, 25), most of the analyses were conducted on members of the *F. solani* species complex.

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Nevertheless, a relationship between an environmental isolate and a patient isolate was determined in one case of infection due to a member of the FOC (1). Little is known about the molecular epidemiology of clinically important members of the FOC, even though they were isolated from a San Antonio, Tex., hospital pseudoepidemic (i.e., a false epidemic due to contamination of clinical specimens) associated with bronchoscopy specimens in 1997 to 1998 (S. E. Sanche, D. A. Sutton, K. Magnon, R. Cox, S. Revankar, and M. G. Rinaldi, Abstr. 98th Gen. Meet. Am. Soc. Microbiol., abstr. F-102, 1998) (hereafter referred to as Texas hospital A) and as part of a 1996 environmental survey of a Houston, Tex., hospital water system suspected of serving as a reservoir of nosocomial fusariosis (1, 14) (hereafter referred to as Texas hospital B). Phylogenetic analyses of the FOC have been limited to phytopathogens (2, 21, 26). Results of these genetic diversity studies using multilocus DNA sequence typing (MLST) and amplified fragment length polymorphisms (AFLPs) have shown that some plant host-specific pathogens, called formae speciales, have polyphyletic evolutionary origins (2, 21, 26) and that this complex appears to consist of a large number of predominately or exclusively clonal lineages distributed among at least three clades. The latter finding was not unexpected, because no member of the FOC has been shown to undergo sexual reproduction, even though the few strains tested were shown to possess apparently functional mating-type (*MAT*) genes that are expressed and processed correctly (38).

The objectives of this study were to (i) investigate the genetic relatedness and population structure of a comprehensive set of isolates of the FOC from patients and the hospital environment spanning four continents, focusing on those recovered from the Texas hospital A pseudoepidemic; (ii) evaluate the hypothesis that hospital water systems might serve as reservoirs for nosocomial fusariosis by comparing FOC strains recovered from the environment in hospitals in Texas (14), Maryland, and Washington with isolates derived from patients; and (iii) investigate the phylogenetic diversity and evolutionary origins of human- and hospital environment-derived isolates by comparing them with phytopathogenic strains chosen to represent the known pathogenic and phylogenetic diversity of the FOC. To achieve these objectives, we have developed an initial set of MLST- and AFLP-based molecular markers that can be incorporated into long-range epidemiological studies. Results of the MLST and AFLP analyses reported here provide independent support for the validity of a clinically important, widespread clonal lineage within the *F. oxysporum* complex.

MATERIALS AND METHODS

Fungal strains. Isolates of the *F. oxysporum* species complex (FOC) from patients, the environment, and other plant and animal sources were assembled from several national and international culture collections, with most of the isolates supplied by The University of Texas Health Science Center, San Antonio, Tex. (Table 1). All strains are stored cryogenically in the Agricultural Research Service (NRRL) Culture Collection, National Center for Agricultural Utilization Research, Peoria, Ill., for future reference.

DNA isolation, amplification, and sequencing. Liquid cultures were grown in yeast-malt broth, and total genomic DNA was extracted from freeze-dried mycelia by the hexadecyltrimethyl-ammonium bromide (Sigma, St. Louis, Mo.) protocol described by O'Donnell et al. (20). All PCR and sequencing primers are listed in Table 2. The total reaction volume of all PCR mixtures was 50 μl and included approximately 5 ng of total genomic DNA and $MgCl_2$ at a final concentration of 25 mM. Amplification of a portion of the translation elongation factor (1α) gene and the mitochondrial small-subunit (mtSSU) ribosomal DNA (rDNA) was accomplished by using the EF-1–EF-2 (21) and MS1-MS2 (35) PCR primer pairs, respectively, and AmpliTaq (Applied Biosystems [ABI], Foster City, Calif.) in a 9700 thermocycler and the following cycling parameters: 1 cycle of 30 s at 94°C; 40 cycles of 30 s at 94°C, 30 s at 52°C, and 90 s at 72°C; and then 10 min at 72°C and a 4°C soak. The entire nuclear ribosomal intergenic spacer (IGS) region (~2.5 kb) was amplified with the NL11-CNS1 primer pair (Table 2), using Platinum *Taq* DNA polymerase Hi-Fi (Invitrogen Life Technologies, Carlsbad, Calif.) in an ABI 9700 thermocycler and the following cycling parameters: 1 cycle of 90 s at 94°C; 40 cycles of 30 s at 94°C, 30 s at 52°C, and 3 min at 68°C; and then 1 cycle of 5 min at 68°C and a 4°C soak.

The sequences with GenBank accession numbers AB011379 and AB011378 were used to design PCR primers to amplify the *MAT1-1* and *MAT1-2* idiomorphs, respectively (38). The *MAT1-1* idiomorph was amplified as two overlapping segments by using the FOM132-FOM123 and FOM122-FOM111 primer pairs (Table 2), using the Platinum *Taq* PCR protocol described above. PCR primers FOM211 and FOM212 were used to amplify the *MAT1-2* idiomorph, using the AmpliTaq PCR protocol described above. A multiplex PCR, employing the FOM111-FOM112 primer pair for the *MAT1-1-2* gene and the FOM211-FOM212 primer pair for the *MAT1-2-1* gene, was used to screen all of the strains included in this study for *MAT* idiomorph (i.e., *MAT1-1* or *MAT1-2*), using the AmpliTaq PCR protocol outlined above.

PCR products were purified by using Montage PCR_{96} Cleanup filter plates (Millipore Corp., Billerica, Mass.) and then sequenced by using ABI BigDye chemistry version 3.0 in a 9700 thermocycler with the following cycling parameters: 1 cycle of 15 s at 96°C; 40 cycles of 15 s at 96°C, 10 s at 50°C, and 4 min at 60°C; and then a 4°C soak. Sequencing reaction mixtures were purified via ethanol precipitation and then run on an ABI 3100 or 3730 genetic analyzer. Sequences were edited and aligned by using Sequencher version 4.1.2 (Gene Codes, Ann Arbor, Mich.), after which the alignments were improved manually.

AFLP analysis. All genomic DNA samples included in the AFLP analysis were first treated with 2 μl of RNase A (10 μg/μl) (Sigma) per 200-μl total genomic DNA sample for 30 min at 65°C, after which they were subjected to a lithium chloride (Sigma) cleanup protocol. Briefly this protocol consisted of adding an equal volume of ice-cold 5 M LiCl to each genomic DNA, icing for 15 min, and then centrifuging at 13,000 × *g* for 15 min. After the supernatant was removed to a fresh tube, 1/16 volume of 5 M NaCl was added to each sample, followed by 2 volumes of ice-cold 95% ethanol. The samples were then placed in a −80°C freezer for 20 min to precipitate the DNA. Once the samples were removed from the freezer and thawed, DNAs were pelleted in a microcentrifuge at 13,000 × *g* for 10 min, followed by a 70% ethanol wash, and they were then resuspended in 50 μl double-distilled water (ddH_2O). DNA quantification of all genomic DNA samples was done by running them into a 1.5% agarose gel together with a known concentration of a HindIII (A↓AGCTT) digest of λ DNA (New England Biolabs [NEB], Beverly, Mass.). Restriction-ligation was conducted at 37°C overnight in an ABI 9700 thermocycler in a total volume of 10 μl by combining ~100 ng of total genomic DNA in 4.5 μl of ddH_2O with 5.5 μl of the following master mix: 1 μl of 10× T4 ligase buffer (NEB) (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 μg of bovine serum albumin [BSA] per ml), 1 μl of NaCl (0.5 M), 0.5 μl of 10× BSA (1 μg/μl), 1 μl of MseI adapter mix (50 pmol/μl), 1 μl of EcoRI (G↓AATTC) adapter mix (5 pmol/μl), and 1 μl of an enzyme master mix (for each set of 10 reactions) consisting of 1 μl of 10× T4 DNA ligase buffer (NEB), 1 μl of NaCl (0.5 M), 0.5 μl of 10× BSA (1 μg/μl), 0.5 μl of EcoRI (NEB) (100 U/μl), 0.2 μl of MseI (T↓TAA) (NEB) (50 U/μl), 0.33 μl of T4 DNA ligase (NEB) (2,000 U/μl), and 6.5 μl of ddH_2O. All adapters and primers used for the AFLP analysis are listed in Table 2. Once completed, the restriction-ligation mix was diluted 1:2 in ddH_2O and stored at −20°C when not in use.

The preselective amplification was performed in a total volume of 10 μl by first aliquoting 8 μl of a master mix consisting of 1 μl of 10× Invitrogen PCR buffer, 1 μl of 2 mM deoxynucleoside triphosphates, 0.5 μl of 50 mM $MgCl_2$, 1 μl of EcoRI nonselective primer (1 pmol/μl), 1 μl of MseI nonselective primer (1 pmol/μl), 0.065 μl of *Taq* polymerase (Invitrogen), and 3.5 μl of ddH_2O into each reaction tube, to which 2 μl of a diluted restriction-ligation mix was added. Amplifications were performed in an ABI 9700 thermocycler programmed as follows: 2 min at 72°C followed by 5 min at 94°C; 20 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C; and 1 cycle of 5 min at 72°C followed by a 4°C soak. Several amplification product mixtures were checked for a smear of DNA in the range of 100 to 800 bp, which is indicative of a successful preselective amplification, by electrophoreses of 5 μl of the reaction products into a 1.5% agarose gel, followed by staining with ethidium bromide and visualization over a UV transilluminator. Preselective amplicons were diluted 1:11 with ddH_2O, after which they were vortexed briefly and then stored at −20°C when not in use.

TABLE 1. Strains of the *F. oxysporum* complex and outgroups included in this study

NRRL no.	Other designation[a]	Yr	Isolate source[b]	Geographic origin	Hospital or laboratory data
22533	CBS 244.61	1961	*Aechmea fasciata*	Germany	
22548	CBS 744.79	1979	*Zygocactus truncatus*	Germany	
22550	CBS 794.70	1970	*Albizzia julibrissin*	Iran	
22555	CBS 797.70	1970	*Solanum tuberosum*	Iran	
22903[c]	IMI 375363	1987	*Pseudotsuga menziesii*	Oregon	
25184[c]	CBS 573.94	1994	Peat	Germany	
25375	IMI 169612	1973	Human	South Pacific	
25378	IMI 214661	1977	Human	Oklahoma	
25387	ATCC 26225	1971	Toenail	New Zealand	
25420	BBA 66843		*Gossypium* sp.	United States	
25433	BBA 69050		*Gossypium* sp.	China	
25509	FRC O-1853	1995	Cyclamen, nonpathogen	The Netherlands	
25512	FRC O-1858	1995	Cyclamen, nonpathogen	The Netherlands	
25594	ATCC 16415		*Ipomoea batatas*	South Carolina	
25598	ATCC 18774		Soybean	South Carolina	
25603	Ploetz A2		Banana	Australia	
25728	CBS 463.91	1991	Human	Germany	
25749	ATCC 64530	1985	Foot ulcer	Belgium	
26022	Ploetz JLT44		Banana	France	
26024	Ploetz STB2		Banana	Honduras	
26033	Kistler CL58	1996	Tomato	Florida	
26035	Kistler Guil2	1987	*Phoenix canariensis*	Tenerife, Canary Islands	
26178	Gordon B9-9S		*Cucumis melo*	Maryland	
26180	Gordon CR-III	1989	Soil	California	
26203	Kistler 73		Tomato	Italy	
26360	FRC O-755	1975	Eye	Tennessee	
26361	FRC O-783	1976	Human	Tennessee	
26362	FRC O-784	1976	Human	South Carolina	
26363	FRC O-1168	1982	Peritoneal fluid	Rhode Island	
26365	FRC O-1562	1987	Brain autopsy	New York	
26367	FRC O-1591	1987	Human	Maryland	
26368	FRC O-1723	1989	Amputated toe	California	
26370	FRC O-1732	1989	Foot	Louisiana	
26372	FRC O-1746	1990	Catheter, leukemic	New York	
26373	FRC O-1750	1992	Lung	Chile	
26374	FRC O-1777	1993	Leg ulcer	California	
26376	FRC O-1683	1988	Blood	New York	
26381	Kistler CL-57	1996	Tomato	Florida	
26383	Kistler GD-40	1996	Tomato	Florida	
26386	UTHSC 97–95	1997	Lung, unknown fever	San Antonio, Tex.	Hospital A
26387	UTHSC 96–2463	1996	Sputum, intestinal hemorrhage	San Antonio, Tex.	Hospital A
26388	UTHSC 96–2063	1996	Peritoneal dialysate	New York	
26389	UTHSC 96–1960	1991	Nail	Connecticut	
26390	UTHSC 96–1867	1996	Bronchial wash, lump in chest	San Antonio, Tex.	Hospital A
26391	UTHSC 96–1804	1996	Bronchial wash, respiratory neoplasm	San Antonio, Tex.	Hospital A
26392	UTHSC 96–1710	1996	Bronchial wash, bronchitis	San Antonio, Tex.	Hospital A
26393	UTHSC 96–1087	1996	Nail	Connecticut	
26394	UTHSC 96–840	1996	Leg ulcer	California	
26395	UTHSC 95–2234	1995	Whale blowhole	Ohio	
26397	UTHSC 95–1193	1995	Hand abscess	San Antonio, Tex.	Hospital A
26398	UTHSC 95–1173	1995	Foot wound	Florida	
26399	UTHSC 95–1058	1995	Bronchial wash, respiratory failure	San Antonio, Tex.	Hospital A
26400	UTHSC 95–727	1995	Throat	California	
26401	UTHSC 95–404	1995	Ointment contaminate	San Antonio, Tex.	Private industrial laboratory A
26402	UTHSC 95–315	1995	Ointment contaminate	San Antonio, Tex.	Private industrial laboratory A
26403	UTHSC 95–144	1995	Left calf	Minnesota	
26406	Gordon K419	1989	*Cucumis melo*	Jalisco, Mexico	
26409	ATCC 10913		Tobacco	Maryland	
26442	IMI 141108		*Lilium* sp.	South Carolina	
26551	UAMH 5692	1987	Leg ulcer	Saskatchewan, Canada	
26677	FRL F7325	1987	Nail	Sydney, Australia	
26679	FRL F7463	1987	Gum abscess	Sydney, Australia	
26680	FRL F8433	1989	Leukemic	Sydney, Australia	
28013	CDC B-5736	1996	Blood, leukemic	Delaware	
28031	CDC B-3882	1983	Toe nail	South Carolina	
28244	BBA 70516	1998	Greenhouse irrigation water	Finland	
28245	BBA 70517	1998	Greenhouse irrigation water	Finland	
28670	UWash 97–25989	1997	Sink drain	Seattle, Wash.	Hospital
28678	UWash 97–25459	1997	Mouthwash	Seattle, Wash.	Hospital, patient B
28680	UWash 97–9417–2	1997	Lung	Seattle, Wash.	Hospital, patient K
28683	UWash 98–1455	1998	Mouthwash	Seattle, Wash.	Hospital, patient F

Continued on following page

TABLE 1—*Continued*

NRRL no.	Other designation[a]	Yr	Isolate source[b]	Geographic origin	Hospital or laboratory data
28684	UWash 97–11584	1997	Mouthwash	Seattle, Wash.	Hospital, patient L
28685	UWash 93–11340	1993	Enteric stool screen	Seattle, Wash.	Hospital, patient P
28686	UWash 95–6570	1995	Sinus wash	Seattle, Wash.	Hospital, patient O
28687	UWash 93–8070	1993	Kidney autopsy	Seattle, Wash.	Hospital, patient E
31166	Hospital B#9	2000	Renal renal cancer	Houston, Tex.	Hospital B
32176	Hospital B#F15	2000	Lung cancer, lung autopsy	Houston, Tex.	Hospital B
32377	Hospital B#B	2000	Sputum, leukemic	Houston, Tex.	Hospital B
32507	FRC O-1885	1996	Sink faucet	Houston, Tex.	Hospital B
32509	FRC O-1887	1996	Shower drain	Houston, Tex.	Hospital B
32511	FRC O-1895	1996	Cancer patient	Houston, Tex.	Hospital B
32512	FRC O-1896	1996	Cancer patient	Houston, Tex.	Hospital B
32513	FRC O-1908	1996	Cold-water filter	Houston, Tex.	Hospital B
32514	FRC O-1909	1997	Cold-water filter	Houston, Tex.	Hospital B
32515	FRC O-1910	1997	Cold-water filter	Houston, Tex.	Hospital B
32516	FRC O-1911	1997	Cold-water filter	Houston, Tex.	Hospital B
32517	FRC O-1912	1997	Cold-water filter	Houston, Tex.	Hospital B
32525	PD 22005805–2	2002	Tomato seed	The Netherlands	
32527	PD 22005805–4	2002	Tomato seed	The Netherlands	
32914	UTHSC 01–1015	2001	BAL	California	
32915	UTHSC 01–1482	2001	Leg	Colorado	
32916	UTHSC 01–1998	2001	Blood CVC, esophageal cancer	Houston, Tex.	Hospital B
32917	UTHSC 01–2476	2001	Foot, cellulitis	San Antonio, Tex.	Hospital A
32920	UTHSC 02–1874	2002	Periesophageal	Washington, D.C.	
32921	UTHSC 02–1980	2002	Water supply	Baltimore, Md.	Hospital
32922	UTHSC 00–2402	2000	Environmental	San Antonio, Tex.	Private industrial laboratory B
32925	UTHSC 00–1311	2000	Food	San Antonio, Tex.	Private industrial laboratory B
32927	UTHSC 00–221	2000	Meatus	San Antonio, Tex.	Commercial laboratory
32929	UTHSC 99–1742	1999	BAL	San Antonio, Tex.	Hospital A
32930	UTHSC 99–1135	1999	BAL, AIDS	San Antonio, Tex.	Hospital A
32931	UTHSC 99–853	1999	Blood	Massachusetts	
32932	UTHSC 98–2469	1998	Blood	Pennsylvania	
32933	UTHSC 98–2404	1998	BAL, pneumonia	San Antonio, Tex.	Hospital A
32935	UTHSC 98–2189	1998	Blood	Maine	
32938	UTHSC 98–1748	1998	BAL, dysphagia	San Antonio, Tex.	Hospital A
32939	UTHSC 98–1747	1998	BAL	San Antonio, Tex.	Hospital A
32940	UTHSC 98–1741	1998	BAL, malignant neoplasm	San Antonio, Tex.	Hospital A
32941	UTHSC 98–1670	1998	Spleen, splenomegaly	San Antonio, Tex.	Hospital A
32942	UTHSC 98–1645	1998	BAL	San Antonio, Tex.	Hospital A
32943	UTHSC 98–835	1998	BAL, pneumonia	San Antonio, Tex.	Hospital A
32944	UTHSC 98–834	1998	BAL	San Antonio, Tex.	Hospital A
32945	UTHSC 98–718	1998	Lung, unspecified neoplasm	San Antonio, Tex.	Hospital A
32946	UTHSC 98–709	1998	Nail	Connecticut	
32947	UTHSC 98–361	1998	BAL	Colorado	
32948	UTHSC 97–2184	1997	BAL, nodular goiter	San Antonio, Tex.	Hospital A
32949	UTHSC 97–2090	1997	BAL, mass in chest	San Antonio, Tex.	Hospital A
32950	UTHSC 97–1922	1997	BAL, asthma	San Antonio, Tex.	Hospital A
32951	UTHSC 97–1684	1997	BAL, malignant neoplasm	San Antonio, Tex.	Hospital A
32952	UTHSC 97–1055	1997	Toe bone	Wisconsin	
32953	UTHSC 97–1476	1997	Open leg wound	San Antonio, Tex.	Hospital A
32954	UTHSC 97–1466	1997	BAL	San Antonio, Tex.	Hospital A
32955	UTHSC 97–1465	1997	BAL, bronchitis	San Antonio, Tex.	Hospital A
32956	UTHSC 97–1184	1997	BAL, voice disturbance	San Antonio, Tex.	Hospital A
32957	UTHSC 97–891	1997	BAL, bronchitis	San Antonio, Tex.	Hospital A
32958	UTHSC 97–476	1997	Lung wash, hemoptysis	San Antonio, Tex.	Hospital A
32960	UTHSC 97–357	1997	BAL, pneumonia	San Antonio, Tex.	Hospital A
32961	UTHSC 97–291	1997	BAL	San Antonio, Tex.	Hospital A
32962	UTHSC 97–290	1997	BAL, CVA	San Antonio, Tex.	Hospital A
32999	UTHSC 97–2110	1997	Sputum, respiratory failure	San Antonio, Tex.	Hospital A
34936	Di Pietro 4287	1984	Tomato	Spain	
36064	FRC O-1747	1991	Human cancer	Houston, Tex.	Hospital B

[a] ATCC, American Type Culture Collection, Manassas, Va.; BBA, Biologische Bundesanstalt für Land-und Forstwirtschaft, Institute für Mikrobiologie, Berlin, Germany; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; Di Pietro, Antonio Di Pietro, Universidad de Córdoba, Córdoba, Spain; FRC, Fusarium Research Center, The Pennsylvania State University, State College; FRL, Fusarium Research Laboratory, University of Sydney, Sydney, Australia; Gordon, Thomas Gordon, University of California, Davis; Hospital B, Hospital B, Houston, Tex. (13); IMI, CABI Biosciences, Egham, Surrey, England; PD, Plantenziektenkundige Dienst, Wageningen, The Netherlands; Ploetz, Randy Ploetz, University of Florida, Homestead; UAMH, Microfungus Collection, University of Alberta, Edmonton, Alberta, Canada; UTHSC, University of Texas Health Science Center, San Antonio; UWash, University of Washington, Seattle.

[b] With the exception of strain NRRL 26395 from a whale blowhole, all other clinical isolates are from humans. BAL, bronchoalveolar lavage; CVA, cough variant asthma; CVC = central venous catheter.

[c] Sequences of *Fusarium commune* NRRL 22903 (27) and *Fusarium* sp. strain NRRL 25184 were used to root the phylogram in Fig. 4.

TABLE 2. Primers used for PCR, DNA sequencing, and AFLP genotyping

Primer	Locus	Sequence (5′ to 3′)[a]
EF-1	EF-1α	ATGGGTAAGGARGACAAGAC
EF-11ta	EF-1α	GTGGGGCATTTACCCCGCC
EF-22t	EF-1α	AGGAACCCTTACCGAGCTC
EF-2	EF-1α	GGARGTACCAGTSATCATG
MS1	mtSSU rDNA	CAGCAGTCAAGAATATTAGTCAATG
MS2	mtSSU rDNA	GCGGATTATCGAATTAAATAAC
MS21	mtSSU rDNA	CTCTCCTCCTCAAGTACTGC
GFM136	*MAT1-1*	ATGGTCTACAGCCAGTCGCA
FOM132	*MAT1-1*	GGTAGTGTTGTTTGTGGTTG
FOM122	*MAT1-1*	TCCATGCCAAGATCCTCAGC
FOM123	*MAT1-1*	AAGGCAGAGTCAGAAATCCA
FOM112	*MAT1-1*	GCTGCTGCATCTTGGATTGC
FOM111	*MAT1-1*	GCTTGATCTGTTCGGTCATG
FOM211	*MAT1-2*	ACATATCGATAGCATCTACC
FOM212	*MAT1-2*	AGGCGGTAATCTGCTGTGTA
NL11	IGS rDNA	CTGAACGCCTCTAAGTCAG
OCNL13	IGS rDNA	TGTGATGTATGCGGTCCTAGG
ONL13B	IGS rDNA	GGTTCGAGGATCGATTCGAGG
OCNS3C	IGS rDNA	GCAAGATCTGATACTGAGAGG
CNS1	IGS rDNA	GAGACAAGCATATGACTAC
EcoRI-a	Adapter	CTCGTAGACTGCGTACC
EcoRI-b	Adapter	AATTGGTACGCAGTCTAC
MseI-a	Adapter	GACGATGAGTCCTGAG
MseI-b	Adapter	TACTCAGGACTCAT
EcoRI-ns	Nonselective	GACTGCGTACCAATTC
MseI-ns	Nonselective	GATGAGTCCTGAGTAA
EcoRI-CC	Selective	6-FAM-GACTGCGTACCAATTCCC
EcoRI-TC	Selective	6-FAM-GACTGCGTACCAATTCTC
MseI-G	Selective	GATGAGTCCTGAGTAAG

[a] R, AG; S, CG.

The selective amplification was performed in a total volume of 10 μl by first dispensing 8-μl aliquots of a master mix consisting of 1 μl of 10× Invitrogen PCR buffer, 1 μl of 2 mM deoxynucleoside triphosphates, 0.5 μl of 50 mM $MgCl_2$, 1 μl of either EcoRI-CC or EcoRI-TC 6-FAM-labeled selective primer (Qiagen, Alameda, Calif.) (Table 2), 1 μl of MseI-G selective primer (3 pmol/μl), 0.065 μl of Invitrogen *Taq* polymerase, and 3.5 μl of ddH_2O, to which 2 μl of a diluted preselective amplicon was then added. Selective amplifications were performed in an ABI 9700 thermocycler programmed as follows: 3 min at 94°C; 9 cycles of 30 s at 94°C, 30 s at 65°C, and then ramping down 1°C/cycle from 65 to 57°C, followed by 60 s at 72°C; 40 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C; and then 1 cycle of 5 min at 72°C followed by a 4°C soak. Amplifications were checked by running 5 μl of several amplicons into a 1.5% agarose gel as described above. Selective amplification mixtures were diluted 1:10 or 1:20 in ddH_2O, after which 1.5 μl of each mixture was added to a master mix consisting of 9.7 μl of HighDye formamide (ABI) and 0.3 μl of the GeneScan ROX 500 (ABI) size standard. All samples were run on an ABI 3100 genetic analyzer, using the GeneScan version 3.7 analysis software default run module for data collection and GenoTyper version 3.7 for scoring fragments in the range of 50 to 500 bp relative to the ROX 500 size standard. Only electropherogram peaks above 100 fluorescent units were scored for the presence or absence of bands of the same size. Reproducibility of the AFLP data was assessed by running two exemplars of each unique AFLP haplotype through the entire protocol, starting from the beginning with the isolation of total genomic DNA (15). Only bands detected in the duplicate AFLP analyses (90%) were included in the phylogenetic analyses.

Phylogenetic analysis. The MLST and AFLP matrices analyzed in the present study are available at http://www.ncaur.usda.gov/MGB/MGB-O'Donnell.htm. All phylogenetic analyses were conducted with PAUP* version 4.0b10 (28). Searches for the most-parsimonious trees (MPTs) used a heuristic search with 1,000 random addition replicates and tree bisection with reconnection branch swapping, after excluding ambiguously aligned nucleotide positions. The Templeton Wilcoxon signed rank (WS-R) test implemented in PAUP* was used to assess whether the various partitions could be combined, using 70% bootstrap majority rule trees from each partition as constraints. Results of the WS-R tests ($P = 1.0$) indicated that all of the partitions could be combined. Clade stability was assessed via parsimony bootstrapping in PAUP*, using a heuristic search with 1,000 pseudoreplications of the data and 10 random addition sequences per replicate and tree bisection with reconnection branch swapping. Constraints forcing the monophyly of all of the clinical isolates and all of the clinical isolates within clade 3 were compared with the MPTs by using the Kishino-Hasegawa test in PAUP*. Phylograms were either midpoint rooted or outgroup rooted based on more inclusive phylogenetic analyses (2, 21, 27).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under accession numbers AY527415 to AY527732.

RESULTS

The genetic relatedness and diversity of FOC isolates from patients associated with a pseudoepidemic at a San Antonio, Tex., hospital that peaked in 1997 to 1998 (hospital A) were investigated together with those of isolates collected from the hospital water systems of three U.S. hospitals by comparing them with a geographically diverse set of 88 clinical and 18 environmental isolates from the FOC (Table 1). Aligned partial DNA sequences of translation elongation factor (EF-1α, 655 bp) and the entire nuclear ribosomal intergenic spacer region (IGS rDNA, 2,510 bp) were analyzed phylogenetically, using sequences of two strains of the banana pathogen *F. oxysporum* f. sp. *cubense* (NRRL 25603 and 26024) to root the trees based on a more inclusive phylogenetic analysis (21). Results of the Templeton WS-R combinability test ($P = 1.0$) implemented in PAUP* (28), using 70% bootstrap consensus trees as constraints, indicated that the EF-1α and IGS rDNA partitions could be analyzed as a combined data set. Maximum-parsimony analysis of the combined data set resolved 21 unique EF-1α–IGS rDNA haplotypes among the 88 clinical isolates (Fig. 1), including one widespread clonal lineage which accounted for 63 (72%) of the clinical isolates and 17 out of 18 of the nonclinical isolates. Of the 82 strains of the clonal lineage sequenced, 80 shared an identical EF-1α–IGS rDNA haplotype, while the other two strains, which were isolated as saprophytes of cyclamen in The Netherlands (36), differed from other members of the clonal lineage by only a single base pair mutation within the IGS rDNA (Fig. 1B). All 33 isolates from the Texas hospital A pseudoepidemic are members of the clonal lineage. By comparison, 16 of the 21 EF-1α–IGS haplotypes associated with patients were represented by singletons, with the second most common haplotype being represented by only three strains (Fig. 1A). Eight of the 17 nonclinical isolates of the clonal lineage were recovered from the water systems of geographically distant hospitals in Houston, Tex., in 1996 to 1997 (1, 14); Seattle, Wash., in 1997; and Baltimore, Md., in 2002. Strains of the clonal lineage were also recovered from clinical cases in Canada, Belgium, and Germany; from a greenhouse irrigation system in Finland where tomatoes were being grown; and from tomato seed and cyclamen in The Netherlands.

To further characterize their population biology, all of the strains were subjected to a mating type (*MAT*) idiomorph test, using primer pairs FOM111-FOM112 and FOM211-FOM212 for *MAT1-1* and *MAT1-2*, respectively. This assay revealed that all 82 strains of the clonal lineage and strains of the four most closely related haplotypes possess a *MAT1-1* idiomorph (Fig. 1B). In addition, strains representing 14 of the 21 unique EF-1α–IGS haplotypes from patients were *MAT1-1*, compared

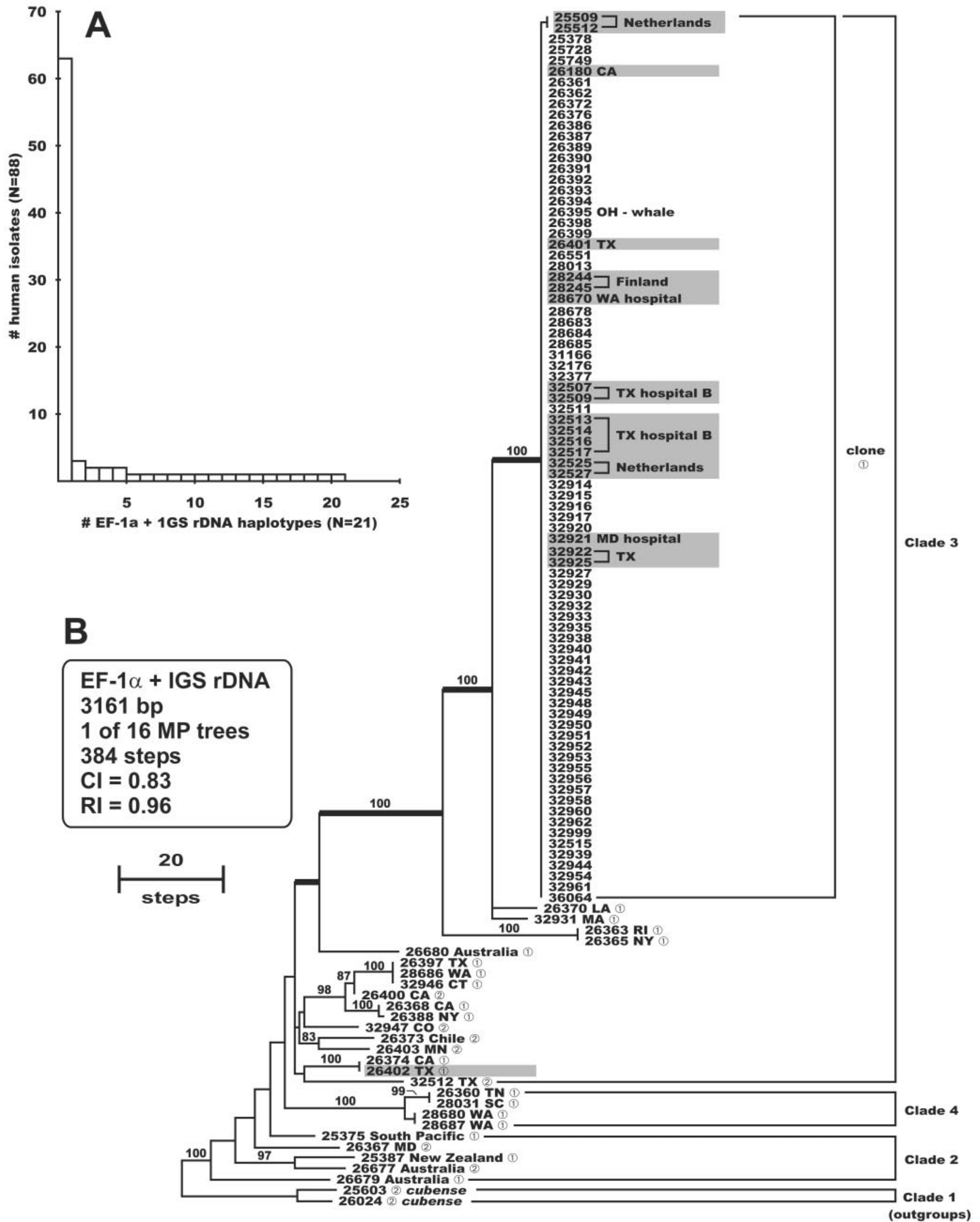

FIG. 1. (A) Distribution of the 88 human isolates among the 21 EF-1α–IGS rDNA sequence haplotypes. (B) One of 16 most-parsimonious phylograms inferred from the combined EF-1α–IGS rDNA sequence data rooted with sequences of *F. oxysporum* f. sp. *cubense* NRRL 25603 and 26024 from clade 1 of the FOC (21). All ingroup strains are from humans, except for strain NRRL 26395 from a whale and the 18 environmental

with only 7 *MAT1-2* strains, which were all associated with singleton haplotypes (Fig. 1B).

Further MLST characterization of 74 strains of the clonal lineage from clinical and environmental sources was conducted, together with that of strains representing six different EF-1α–IGS rDNA haplotypes, using partial EF-1α (651 bp) and mtSSU rDNA (677 bp) sequences and the entire IGS rDNA (2,506 bp). Maximum-parsimony analysis of the combined data set, totaling 3.8 kb (Table 1), revealed that 74 strains of the clonal lineage included in this analysis had an identical MLST (Fig. 2A). In an effort to obtain a finer level of genetic discrimination, these strains were subjected to AFLP genotyping, using two combinations of EcoRI plus 2-bp 6-FAM-labeled selective primers together with an MseI plus 1-bp selective primer (Table 2). Parsimony analysis of the AFLP matrix, consisting of 173 binary characters (i.e., restriction fragments ranging from 50 to 500 bp were coded as 1 for present and 0 for absent), yielded >64,000 MPTs of 128 steps in length (Fig. 2B) (consistency index [CI] = 0.84; retention index [RI] = 0.88) in which the clonal lineage formed a highly similar exclusive group consisting of only seven unique AFLP genotypes (AG1 to -7) (Fig. 2C). AFLP genotyping of the two saprobic strains isolated from cyclamen in The Netherlands (NRRL 25509 and NRRL 25512) revealed that they possess the AG2 AFLP genotype (data not shown). When the AFLP data for 74 strains of the clonal lineage were analyzed separately, the MPTs were only seven steps in length (CI = 0.86; RI = 0.98) as 167 of the 173 characters were identical across all strains (five polymorphic sites were synapomorphic and one was autapomorphic). Of the 28 clinical strains from hospital A in San Antonio, Tex. (where the pseudoepidemic was reported) that were subjected to AFLP fingerprinting, representatives of the three most common AFLP genotypes were recovered between 1996 and 2001 (AG1, $n = 19$; AG2, $n = 7$; and AG3, $n = 2$). Similarly, five AFLP genotypes of the clonal lineage (i.e., AG1 to -5) were recovered during the same time frame from hospital B in Houston, Tex., including a hospital environmental isolate that shared the identical AG2 genotype with an isolate from a patient (NRRL 32507 and NRRL 32511, both isolated in 1996). Matched patient-environment isolates of the AG2 genotype (NRRL 28670 and NRRL 28678) were also recovered in 1997 from a Seattle, Wash., hospital.

Genetic relationships among the AFLP genotypes were investigated further by PCR amplification and sequencing of the entire *MAT1-1* idiomorph from one or more strains representing the six most common AFLP genotypes (i.e., AG1 to -6) of the clonal lineage and strains of five near relatives (Fig. 3), using PCR primers described by Yun et al. (38) (Table 2). Parsimony analysis of the 4,017 aligned *MAT1-1* nucleotide characters yielded a single MPT of 41 steps (Fig. 3B) (CI = 1.0) in which exemplars of the six AFLP genotypes were identical (e.g., NRRL 26370) or nearly identical (e.g., NRRL 32931) to the two closest known relatives of the clonal lineage (Fig. 1B), reflecting the high conservation of the *MAT* genes, which have been shown to be under strong purifying selection (23). Hypothetical translations of the three *MAT1-1* genes suggest that they all encode functional proteins (GenBank accession numbers AY527415 to AY527427).

Finally, to address whether isolates from patients have monophyletic or multiple independent evolutionary origins within the FOC, parsimony analyses were conducted on aligned partial EF-1α (655 bp) and mtSSU rDNA (694 bp) sequences representing 17 strains from patients and 21 phytopathogenic strains chosen to represent the known pathogenic diversity of the FOC (2, 21). Parsimony analysis of the combined data set (1,349 bp) yielded 18 MPTs of 134 steps in length (CI = 0.90; RI = 0.94) with human isolates nested within three of the four clades (Fig. 4). However, most of the human isolates, including those of the widespread clonal lineage, were nested in clade 3, the most phylogenetically diverse clade. Constraints forcing the monophyly of the human isolates within clade 3 and all of the human isolates within the FOC in separate analyses were 11 and 33 steps longer, respectively, and significantly less parsimonious than the MPTs (Kishino-Hasegawa test, $P = 0.009$ and $P < 0.0001$, respectively). Results of the MAT idiomorph test revealed that *MAT1-1* and *MAT1-2* strains are represented in all four clades.

A summary of the tree statistics is given in Table 3.

DISCUSSION

This study describes the first MLST- and AFLP-based molecular markers for genotyping clinically important members of the FOC. These tools were used in a molecular epidemiological investigation of a pseudoepidemic in hospital A in San Antonio, Tex., that peaked in 1997 to 1998 (S. E. Sanche, D. A. Sutton, K. Magnon, R. Cox, S. Revankar, and M. G. Rinaldi, Abstr. 98th Gen. Meet. Am. Soc. Microbiol., abstr. F-102, 1998) and in a survey of the water systems of three U.S. hospitals suspected as being reservoirs of nosocomial fusariosis. The major finding of this study, based on concordant results from phylogenetic analyses of multilocus DNA sequence data and AFLPs, is that a geographically widespread clonal lineage comprises >70% of all FOC clinical isolates, including all of the strains recovered from the hospital A pseudoepidemic and from the water systems of a hospital in Houston, Tex. (hospital B) (1, 14) and of hospitals in Baltimore, Md., and Seattle, Wash. This clonal lineage consisted of only seven highly similar AFLP genotypes, and all of its members shared identical or nearly identical EF-1α and IGS rDNA sequences and possessed only *MAT1-1* idiomorphs, indicating that they were of clonal origin. To date, molecular epidemiological studies that have identified fungal pathogens with a highly clonal population structure are restricted to a relatively small number of clinically (8, 9), zoologically (17), and agriculturally (4, 5, 11) important species, including members of the FOC (13, 29). However, most clinically important fungi investigated to date

isolates indicated by shading. Note that 82 of the ingroup strains are members of a widespread clonal lineage. The number 1 or 2 following the five-digit NRRL culture collection number indicates that the strain was typed by the mating-type (*MAT*) idiomorph PCR assay as *MAT1-1* or *MAT1-2*, respectively. All strains of the clonal lineage and the five most closely related strains are *MAT1-1* (identified by boldface internodes). Internodes supported by bootstrap values of ≥70% are indicated.

FIG. 2. (A) One of four most-parsimonious midpoint rooted phylograms inferred from the combined EF-1α–IGS rDNA–mtSSU rDNA sequence data for the 80-taxon matrix. (B) One of >64,000 most-parsimonious phylograms inferred from the AFLP data, indicating the seven AFLP genotypes (AG1 to -7) for 74 strains of the widespread clonal lineage. Geographic origin and year isolated are indicated. A, San Antonio, Tex., hospital A, reporting the pseudoepidemic; B, Houston, Tex., hospital B, reporting the water system as a potential reservoir of nosocomial fusariosis (1, 14). Internodes supported by bootstrap values of ≥70% are indicated. (C) Distribution of 74 clinical and environmental strains of the widespread clonal lineage among the seven AFLP genotypes.

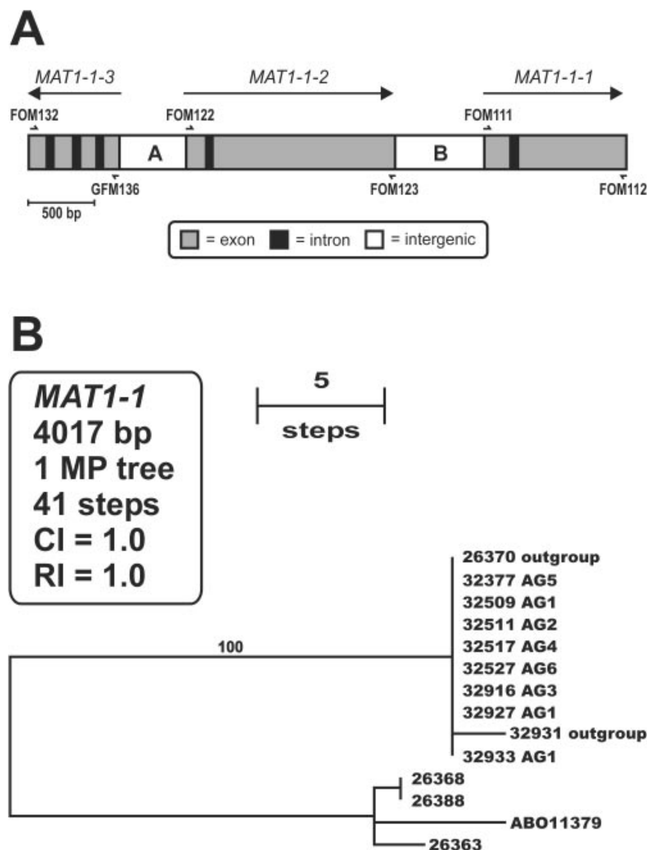

FIG. 3. (A) *MAT1-1* idiomorph showing coding and noncoding regions and directions of transcription of the three *MAT* genes. PCR and sequencing primers are indicated by half-arrows (Table 2). The two intergenic regions are arbitrarily designated A and B. (B) Single most-parsimonious midpoint rooted phylogram inferred from the *MAT1-1* nucleotide sequence data. Note that strains representing six AFLP genotypes (AG1 to -6) of the clonal lineage are identical to one another and to outgroup strain NRRL 26370 (100% bootstrap support). The AB011379 *MAT1-1* sequence was obtained from GenBank.

exhibit both clonality and recombination (reviewed in references 31 and 37).

Lacking the ability to satisfy Koch's postulates regarding FOC isolates from patients, we cannot distinguish isolates capable of infecting humans from other isolates, including potential secondary invaders or superficial environmental isolates not involved in infection. The frequent reoccurrence of the same MLST and AFLP genotypes from patients in different geographic regions, however, strongly suggests that these isolates are the etiological agents of these infections. Further study comparing the pathogenic potentials of different members of the FOC by utilizing animal and other models of pathogenicity (6, 24) is needed to shed light on this issue. However, if differences in pathogenic potential exist among the 74 members of the major clonal lineage associated with patients, they are not reflected in the extremely low level of genetic diversity observed.

The results of the present study support the findings of Anaissie et al. (1), who reported a possible molecular match between strains of *F. oxysporum* from a patient and the environment from Houston, Tex., hospital B, where the initial environmental survey for nosocomial fusaria was conducted (14). The two isolates had similar banding patterns based on random amplified polymorphic DNA (RAPD), interrepeat PCR, and restriction fragment-length polymorphism analyses conducted at the National Cancer Institute, Bethesda, Md. However, the authors of that study were conservative in not classifying this patient-environment isolate pair as a match because a RAPD analysis conducted at a second laboratory yielded discordant results. Because this pair of isolates was conclusively shown to be a member of the FOC widespread clonal lineage in the present study (Fig. 1B) (NRRL 32507 and NRRL 36064), it is clear that the RAPD results from the second laboratory in Houston represent a false negative. In all, 13 of the 14 FOC strains (i.e., 92.8%) that were isolated at Houston hospital B from cancer patients and the environment from 1991 through 2001 were conclusively shown to be members of the FOC clonal lineage via MLST and AFLP analyses (Table 1). The 13 isolates of the clonal lineage from hospital B included 6 of the 7 isolates from cancer patients and all 7 isolates isolated from the hospital water system by Kuchar (14) in 1996 and 1997.

Paradoxically, a separate molecular epidemiological study, also conducted at Houston, Tex., hospital B in 1996 and 1997, reported a complete mismatch between 15 environmental fusaria isolated by Kuchar (14) and 10 clinical isolates compared with them by means of RAPD data (25). Two nonexclusive scenarios are offered to explain the discordant results of Anaissie et al. (1) and Raad et al. (25). First, because most of the fusaria isolated at hospital B were members of the *F. solani* species complex (1, 14), it is possible that strains of the FOC clonal lineage may not have been included in the latter study, because isolates were not identified by species names. Second, reproducibility of the RAPD data may have been an issue in the study by Raad et al. (25). Due to problems of reproducibility and portability from laboratory to laboratory, RAPD and other forms of nondiscrete DNA data are rapidly being replaced with electronically portable MLST schemes (30).

Because most members of the FOC clonal lineage from San Antonio, Tex., hospital A were recovered from bronchoalveolar lavage specimens, as in numerous other nosocomial outbreaks and pseudoepidemics (http://www.umdnj.edu/rspthweb/bibs/fob_infc.htm), contaminated bronchoscopes or inadequate bronchoscope sterilization was suspected, but not proven, as the source of the contamination. Our molecular markers provided conclusive evidence that the AG2 genotype of the clonal lineage recovered from the water systems of hospital B in Houston, Tex., in 1996 and a Seattle, Wash., hospital in 1997 was a precise molecular match with strains recovered from cancer patients at these hospitals during the same years, suggesting potential nosocomiality. Similarly, AFLP markers have shown that some waterborne environmental isolates of *Aspergillus fumigatus* are genetically identical to those from hospital patients with invasive aspergillosis (34). The FOC clonal lineage may be widespread in hospital water systems within the United States, because four virtually identical AFLP genotypes of it were recovered from the three hospitals surveyed, including environmental isolates of the AG1, AG2, and AG4 genotypes from hospital B in 1996 (14). Not surprisingly, water also appears to serve as a reservoir for

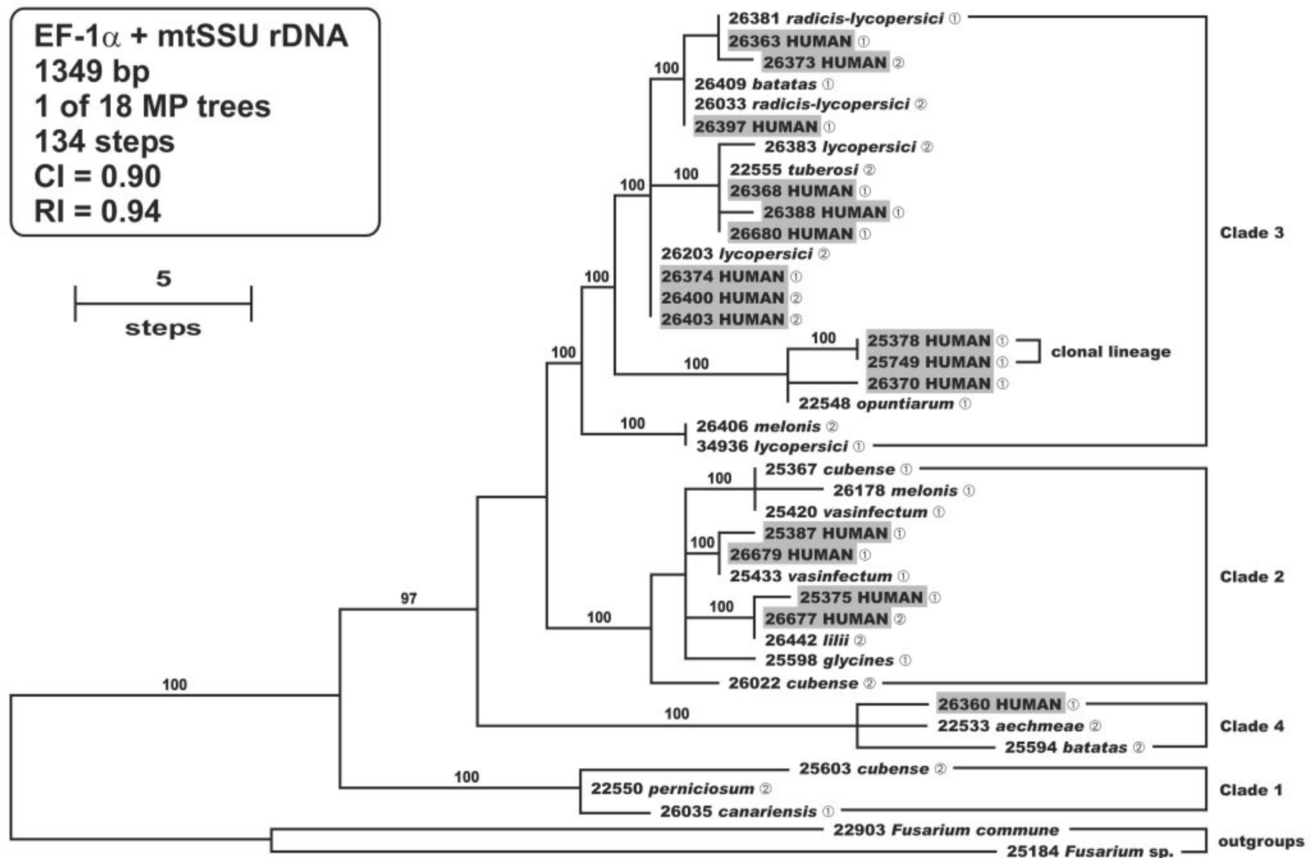

FIG. 4. Phylogenetic diversity of human isolates within the *F. oxysporum* complex inferred from parsimony analysis of the combined EF-1α–mtSSU rDNA sequence data. The human isolates exhibit a polyphyletic distribution among three of the four clades. The widespread clonal lineage is nested within clade 3. Internodes supported by bootstrap values of ≥70% are indicated. Sequences of *Fusarium commune* NRRL 22903 and *Fusarium* sp. strain NRRL 25184 were used as outgroups to root the phylogram.

nonhospital environmental isolates of the FOC clonal lineage, because the AG2 genotype was isolated from a greenhouse irrigation system in Finland where tomatoes were being grown and from the blowhole of a whale at a marine park in Ohio. Other potential environmental sources of the clonal lineage include agricultural soils (AG3, San Joaquin, Calif.) and industrial laboratories (AG1 and AG3, San Antonio, Tex.). Based on these observations, we hypothesize that any water source within and outside a hospital may be a potential reservoir for the FOC clonal lineage, and we suggest careful screening of all water sources that come in contact with immunocompromised patients.

Intercontinental distributions of the AG1, AG2, and AG3 genotypes in Europe and North America suggest recent dispersion that may have resulted from the relatively recent global trade of horticultural and agricultural plants and plant products. This scenario is consistent with the fact that members of the FOC are ubiquitous inhabitants of plants (e.g., NRRL 25509 and NRRL 25512 were isolated as nonpathogens of cyclamen in The Netherlands) (36). Vigilant surveillance of the clonal lineage within U.S. hospitals seems warranted, because it has been recovered in 16 different states, including from hospital water systems in Texas, Maryland, and Washington (Table 1). One surprise of this study is that the clonal lineage is phylogenetically distinct from all of the plant pathogens in our MLST database, which includes partial EF-1α–mtSSU rDNA sequences from over 700 FOC strains. Although this finding does not preclude the possibility that the clonal lineage is a plant pathogen, it suggests that it may not be economically significant, because most described phytopathogens within the FOC are represented in our database. The present study extends our current knowledge of FOC phylogeny (2, 21) through the discovery of a fourth clade containing the only strain isolated from a human eye infection (Fig. 4). Another surprise to emerge from the present study is the extreme rarity of eye infections caused by members of the FOC (i.e., only 1 of 88 among the human isolates), especially when compared with the *F. solani* species complex, where 43.5% of the clinical isolates subjected to MLST genotyping (i.e., 121 of 278) were recovered from ocular mycoses (N. Zhang et al., unpublished data). Because fusaria are opportunistic pathogens of humans, it was not surprising to discover the clinical isolates exhibit independent evolutionary origins within three of the FOC clades as well as support for a polyphyletic origin of human isolates within clade 3. This finding parallels studies of several plant pathogens within the FOC that appear to have evolved host specificity polyphyletically (2, 21, 26).

Although no member of the FOC has been shown to un-

TABLE 3. Tree statistics and summary sequence

No. of taxa	Data set	No. of:				Tree length (steps)	CI	RI	P (WS-R)[c]
		Characters	PIC[a]	Aut[b]	MPTs				
109	EF-1α	651	44	9	4	56	1.0	1.0	
109	IGS rDNA	2,510	144	107	63	312	0.84	0.96	
109	EF-1α + IGS rDNA (Fig. 1B)	3,161	188	116	16	384	0.83	0.96	1.0
80	EF-1α	651	6	6	2	12	1.0	1.0	
80	IGS rDNA	2,506	61	54	2	118	0.99	0.99	
80	mtSSU rDNA	677	4	1	1	5	1.0	1.0	
80	EF-1α + IGS rDNA	3,157	67	60	4	132	0.98	0.98	1.0
80	EF-1α + mtSSU rDNA	1,328	10	7	2	17	1.0	1.0	1.0
80	IGS rDNA + mtSSU rDNA	3,183	65	55	2	127	0.96	0.97	1.0
80	EF-1α + IGS + mtSSU (Fig. 2A)	3,834	71	61	4	141	0.95	0.96	1.0
80	AFLP D	87	37	23	>100	68	0.88	0.93	
80	AFLP E	86	29	18	>100	57	0.82	0.86	
80	AFLP D + E (Fig. 2B)	173	66	41	>64,000	128	0.84	0.88	1.0
	MAT1-1 idiomorph (Fig. 3B)	4,017	32	9	1	41	1.0	1.0	
41	mtSSU rDNA	694	16	19	>100	46	0.91	0.95	
41	EF-1α	655	42	31	9	84	0.93	0.96	
41	mtSSU rDNA + EF-1α (Fig. 4)	1,349	58	50	18	134	0.90	0.94	1.0

[a] PIC, parsimony informative characters (i.e., synapomorphies).
[b] Aut, autapomorphies.
[c] Probability, using the W-SR test, of getting a more extreme *T* value, with the null hypothesis being no difference between the two trees.

dergo a sexual cycle, our mating-type multiplex PCR assay demonstrated that all of the FOC strains included in this study possess either a *MAT1-1* or a *MAT1-2* idiomorph but not both. Three lines of evidence suggest that members of the FOC may undergo a cryptic sexual cycle: (i) *MAT1-1* and *MAT1-2* mating-type genes are expressed and processed correctly, and translations of the *MAT* genes that we sequenced suggest that they encode functional proteins (38; C. Waalwijk, K. Venema, P. Dyer, and G. Kema, Program 20th Fungal Genet. Conf., abstr. 187, 1999); (ii) *MAT1-1* and *MAT1-2* strains are represented in all four clades of the FOC, which indicates that *MAT* genes have been maintained within this complex on an evolutionary time scale that spans multiple speciation and cladogenic events; and (iii) *MAT* genes appear to be under strong purifying selection (23). Alternatively, two nonexclusive explanations of the long-term maintenance of the *MAT* locus within the FOC are that (i) sexual reproduction may have been lost recently and independently throughout this complex and/or (ii) *MAT* genes may function in processes other than sexual reproduction. However, our working hypothesis is that *MAT1-2* strains that are sexually compatible with the FOC clonal lineage may exist, but only *MAT1-1* strains of this species have come in direct contact with humans, most likely through global trade in horticultural and agricultural commodities.

Consistent with prior genetic analyses of phytopathogenic members of the FOC (2) and human pathogenic fungi (15, 34), AFLPs appear to have identified greater genetic variation than our MLST data, with the exception of the two cyclamen-associated strains that possess a unique MLST haplotype. The clinical relevance of the AFLP genotypes of the clonal lineage, if any, remains to be determined, because they are currently not associated with a phenotype. Even though the present AFLP analyses were semiautomated, we strongly prefer MLST for epidemiological purposes because it provides a more direct estimate of nucleotide diversity by using electronically portable discrete DNA sequence data and because it is much less labor-intensive. The results of the present study also highlight the importance of identifying MLST loci that resolve species limits. Although partial sequences of the nuclear ribosomal large-subunit (28S) rDNA were recently purported to differentiate medically important fusaria (10), our MLST data clearly show that DNA sequences from the 28S rDNA and other commonly used loci, such as the nuclear ribosomal internal transcribed spacer (ITS) region and the mtSSU rDNA, lack sufficient phylogenetic signal to resolve species boundaries among virtually all fusaria (19–22). As discovered for other clinically important fungi (reviewed in reference 32), we have found that single-copy nuclear genes interrupted by large and/or numerous introns such as EF-1α (7) are essential for developing a robust MLST typing scheme. Future development of high-resolution MLST genotyping of all medically and agriculturally important fusaria will be greatly accelerated by the availability of expressed-sequence tag and whole-genome sequence data (http://www.broad.mit.edu/annotation/fungi/fusarium/), thereby facilitating global epidemiology via the Internet (7, 12, 16, 33).

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