Members of the *Fusarium solani* Species Complex That Cause Infections in Both Humans and Plants Are Common in the Environment†

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Members of the *Fusarium solani* species complex (FSSC) are increasingly implicated as the causative agents of human mycoses, particularly in the expanding immunocompromised and immunosuppressed patient populations. Best known as ubiquitous plant pathogens and saprotrophs, the FSSC comprises over 45 phylogenetically distinct species distributed among three major clades. To identify which species are associated with human infections, we generated multilocus haplotypes based on four partial gene sequences from 471 isolates. Of these, 278 were from human patients, 21 were from hospital environments, and 172 were from other sources. Phylogenetic trees inferred from an ergosterol biosynthesis gene (*erg-3*) were highly discordant with those inferred from the three other partial gene sequences; therefore, this partition was analyzed separately. Multilocus analysis showed that isolates from humans were restricted to but spread throughout clade 3 of the FSSC phylogeny, comprising at least 18 phylogenetically distinct species. The majority (74.5%) of the clinical isolates, however, were associated with four major lineages, designated groups 1 to 4. Groups 1 and 2 were strongly supported as phylogenetic species, whereas groups 3 and 4 were not. Although isolates from ocular infections were found in all four groups, they had a significant tendency to belong to group 3 ($P < 0.001$). Human clinical isolates shared identical multilocus haplotypes with isolates from plants, other animals, and from hospital environments, suggesting potential nosocomiality. The major finding of this study is that FSSC-associated mycoses of humans and other animals have origins in a broad phylogenetic spectrum, indicating widespread ability to cause infection in this diverse species complex.

Fusarium solani is one of the most frequently isolated fungi from soil and plant debris and is also associated with serious invasive mycoses in immunocompromised and immunosuppressed patients (3, 18). This species, as defined based on morphology, is actually a diverse complex of over 45 phylogenetic and/or biological species (13 and this study), termed the *Fusarium solani* species complex (FSSC). These morphologically similar species are generally identified broadly under the name *F. solani* (12). They are ubiquitous in soil and decaying plant material, where they act as decomposers, but they are also host-specific pathogens of a number of agriculturally important plants, including pea, cucurbits, and sweet potato. Moreover, they are increasingly associated with opportunistic infections of humans and other animals, causing systemic infections with a high mortality rate (8), as well as localized infections in the skin and other body parts (5, 6). In immunocompetent patients, FSSC isolates are mainly known from my-

cotic keratitis subsequent to traumatic introduction of inoculum. Neutropenic patients, a category of particularly strongly immunocompromised patients, are susceptible to dissemination of infection from superficial or subcutaneous initiation; such infections are usually fatal (5, 6, 9).

The FSSC has been studied primarily with a focus on plant pathogenic isolates. Seven biological species, designated mating populations I to VII, each associated with disease on certain plant hosts, were defined based on mating experiments (10). A phylogenetic study based on DNA sequences of three genes from 35 isolates indicated a remarkable degree of phylogenetic diversity within this complex (13). Three major clades (clades 1, 2, and 3) consisting of 26 phylogenetically distinct species were resolved. Each of the seven biological species showed a one-to-one correlation with a different phylogenetic species. However, no isolates from clinical sources were included in this study.

A recent study (19) showed that clinical isolates of the FSSC are genetically heterogeneous, but this research relied on a single gene genealogy and did not place the isolates in the full known phylogenetic spectrum of this complex. While individual biological species of the FSSC are associated primarily with host-specific plant diseases (10), it is unknown whether isolates can infect both plants and humans, and the relationship between human infection and isolates in the environment has not been elucidated.

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Invasive mycoses are a major cause of death in the rapidly increasing populations of patients undergoing immunosuppressive therapy in association with cancer treatment and organ and tissue transplantation. Most opportunistic fungal infections are associated with one or a few well-defined fungal species, including *Aspergillus fumigatus*, *A. flavus*, *A. terreus*, *Penicillium marneffei*, *Coccidioides immitis*, *C. posadasii*, *Sporothrix schenckii*, the serotypes of *Cryptococcus neoformans*, and species of *Candida* (7). The majority of *Fusarium* species associated with human infections are associated with two species complexes that are typically referred to as single species, *F. oxysporum* and *F. solani* (15). Within the *F. oxysporum* complex, isolates associated with human infections were found to represent a few clones that are common in the environment, with the majority belonging to a single clonal lineage (15). In the present study of the FSSC, a species complex that comprises approximately two thirds of fusarial mycoses of humans and other animals, phylogenetic analyses were performed to identify evolutionary lineages corresponding to human pathogenic isolates and those from other sources. The following questions were addressed. (i) Do clinical isolates share a common ancestry? (ii) How are the clinical isolates related to plant pathogens and isolates from other environmental sources, including air, water, and soil? To this end, we generated three-locus 1,726-nucleotide DNA sequence haplotypes from 471 members of the FSSC, including 278 human clinical isolates. Analysis of sequences from a fourth locus recovered a gene tree that was highly discordant with the phylogeny inferred from the other three loci; therefore, it was analyzed separately. The goal of this study was to provide a better understanding of the potential environmental sources for *Fusarium* infections and to assist in the development of appropriate models for their study in the laboratory.

MATERIALS AND METHODS

DNA amplification and sequencing. The following two nuclear protein-coding and two nuclear rRNA genes were amplified and sequenced for the FSSC isolates: (i) a portion of the translation elongation factor 1- α gene (TEF; 736 bp) (14), (ii) a portion of the sterol C-14 reductase gene *erg-3* (ERG; 579 bp), (iii) the internal transcribed spacer region of the nuclear rRNA gene repeat (ITS; 506 bp) (22), and (iv) the D1/D2 region of the nuclear large subunit rRNA gene (LSU; 484 bp) (22). Amplification and sequencing of the TEF and ribosomal genes were performed as described previously (14). The ERG region, which contains three introns, was amplified and sequenced using primers ERG3-1 (CAGCATGGCTACGAGTTC) and ERG3-4 (CTCCTTGTTCCCGGCTT). Sequences were aligned with CLUSTAL X (21) and optimized visually.

Fungal isolates. Initially, 921 isolates of the FSSC, including the 35 isolates analyzed by O'Donnell (13), were screened using partial TEF sequences. All isolates from human infections were found to be members of clade 3 as described by O'Donnell (13), so all further analyses were limited to clade 3. A subset of 471 clade 3 isolates was chosen for further sequencing of the other three loci. In addition to including 278 clinical isolates, additional ones were chosen to represent the breadth of the TEF phylogeny and the diversity of host/substrate and geographical distribution. Clinical isolates came from 22 countries covering six continents (see the supplemental material for strain histories). Human isolates were recovered from ocular infections ($n = 121$), skin or nail infections ($n = 27$), and other body parts, or from systemic infections from cancer, transplantation, AIDS, and diabetic patients ($n = 130$). The twenty-one hospital environmental isolates were from various surfaces (e.g., wall, sink drain, and shower drain) sampled in two U.S. hospitals approximately 2,500 km apart. The remaining isolates represented a diverse set of geographic and environmental sources, including plant and animal lesions, vegetative debris, and soil. Cultures were deposited at the *Fusarium* Research Center, The Pennsylvania State University, and/or the Agricultural Research Service (NRRL) Culture Collection, National Center for Agricultural Utilization Research, Peoria, Ill. Most of the clinical

isolates were supplied by The University of Texas Health Science Center, San Antonio (see the supplemental material).

Data analyses. Two hundred-eighteen three-locus haplotypes (TEF, ITS, and LSU) were identified from the 471 isolates (see Results and Discussion) by using COLLAPSE v.1.1 (http://inbio.byu.edu/Faculty/kac/crandall_lab/Computer.html). For the 218 haplotypes, parsimony analyses were performed using PAUPRAT (17), a ratchet search program implemented in the phylogenetics software package PAUP*4.0b10 (20). For the complete 921-isolate TEF data set, neighbor joining was used with an uncorrected p-based distance estimate, where p represents the average number of nucleotide differences per site between two sequences. Genealogical concordance of the three individual data sets was evaluated using the Wilcoxon signed-ranks (WS-R) Templeton test implemented in PAUP*4.0b10 (20), with 90% bootstrap consensus trees used as constraints. Kishino-Hasegawa and Shimodaira-Hasegawa tests were applied to test the monophyly of haplotypes associated with humans. A chi-squared permutation test was used to determine whether the ocular isolates were distributed randomly in the phylogeny (http://inbio.byu.edu/Faculty/kac/crandall_lab/Computer.html).

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ236343 to DQ236687, DQ236689 to DQ236813, DQ094301 to DQ094645, DQ094647 to DQ094771, DQ246834 to DQ247710, DQ236814 to DQ237158, DQ237160 to DQ237284, and DQ275598). The alignments have been deposited in TreeBASE (accession no. SN2518).

RESULTS AND DISCUSSION

Human infections are associated exclusively with clade 3 of the FSSC, the most commonly encountered clade. The three-clade topology inferred by O'Donnell (13) was recovered in a neighbor-joining analysis of the TEF gene that included all 921 isolates, with all isolates from humans nesting within clade 3. We selected a subset of 471 isolates and sequenced the ITS and LSU regions. Phylogenies of all three genes clearly placed all human, animal, and hospital isolates in clade 3 as well. Clades 1 and 2 appear more limited in their geographic distribution than the far more frequently encountered clade 3 (13). Members of clades 1 and 2 are known exclusively from diseased or dead plants, whereas members of clade 3 are frequently isolated from soil and as saprotrophs in other environments. Isolates from clade 3 tend to have faster growth rates than those from clade 2, and they also tend to produce much greater quantities of small asexual propagules called microconidia (2). These factors may aid in the aggressiveness of clade 3 species and facilitate their entry into and dissemination within the host.

Clinical isolates are polyphyletic. The Wilcoxon signed-ranks (WS-R) test results indicated that the three sequence datasets were congruent with P values greater than 0.08. Two hundred-eighteen three-locus haplotypes were assembled from the 471 clinical and nonclinical clade 3 isolates. The 278 isolates from humans were represented in 99 of these haplotypes. Although parsimony analysis of the combined data set indicated a number of independent origins of the clinical isolates, there was a strong tendency for them to be nested within one of four specific groups (Fig. 1). These results suggest that phylogenetically diverse members of clade 3 of the FSSC can cause life-threatening, opportunistic infections in humans.

Kishino-Hasegawa and Shimodaira-Hasegawa maximum likelihood tests, comparing a hypothetical tree with all clinical isolates constrained to be monophyletic to the unconstrained most parsimonious tree, showed that the constrained tree was significantly less likely ($P < 0.0001$ in both tests), strongly indicating that clinical members of the FSSC do not share a common evolutionary origin.

FIG. 1. One of 1,270 most parsimonious (MP) trees showing the distribution of different sources among the 218 three-locus haplotypes together with the four major clinical groups, designated groups 1 to 4. The size of each pie chart is proportional to the number of isolates within each of the four groups. Mosaics indicate that an identical multilocus haplotype was shared by isolates from more than one source. Branches with black squares received $\geq 85\%$ bootstrap support. CI, consistency index; RI, retention index.

Haplotype frequency and association with infection. The majority of haplotypes (156/218) were represented by single isolates (singletons) (Fig. 2). From the 218 haplotypes, 33 haplotype classes were identified based on their sizes (number of members) and host/substrate association (isolates from human, nonhuman, and hospital environmental sources). The distribution of clinical isolates in haplotypes of various sizes was strongly tailed. Of 156 singleton haplotypes, 47 were of human origin and 109 were nonhuman. The following two observations indicate that FSSC human infections may be frequently acquired nosocomially: (i) the three most prevalent haplotypes were recovered from both human patients and environmental sources from the same hospital, and (ii) all but one isolate from the hospital environment shared an identical multilocus haplotype with a human isolate. These results strongly suggest that hospitals contain a resident inoculum for

infection, supporting the results of other studies on nosocomial fusarial infections of humans (1). This result points to the need for special scrutiny of hospital wards (such as frequently monitoring the water system) where immunosuppressed and immunocompromised patients are housed.

The most prevalent haplotypes are widespread geographically. The two most frequent haplotypes contained isolates recovered from human infections, the hospital environment, and other nonhuman sources, including plants. The most prevalent haplotype was shared by 29 isolates from various locations and hosts/substrates: cucumber and other cucurbits in the United States and Canada, *Pelargonium* in South Africa, a whale in the United States, a hospital sink and shower drain and other locations within two U.S. hospitals, and human patients in Italy, Brazil, Japan, and various locations in the United States.

FIG. 2. Histogram showing the tailed distribution of isolates from human, hospital environment, and other sources in each haplotype class defined by number of isolates per multilocus haplotype. The number above 12 of the haplotype classes identifies those classes represented by between 2 and 108 multilocus haplotypes.

Isolates from human infections are associated with four major groups. A total of 74.5% of the clinical isolates were resolved as four discrete groups in the multilocus phylogeny (Fig. 1). Group 1 contained 38 clinical and 27 nonclinical isolates, including the two most prevalent haplotypes. The main plant host associated with this group was cucurbits. Group 2 was represented by 40 clinical and 24 nonclinical isolates, including isolates from diverse marine animals (i.e., shark, shrimp, and sea turtle). Group 3 was the largest, containing 92 isolates from humans and 28 from other sources. Group 4 was composed of 37 clinical and 10 nonclinical isolates. Groups 1 and 2 received 100% and 85% bootstrap support, respectively, whereas groups 3 and 4 had very short internodes with poor bootstrap support.

Our selection of isolates is clearly biased toward isolates from human sources, given that 278/471 isolates analyzed in the multilocus study were recovered from human infections. Therefore, the observed frequencies of association with particular groups should not be inferred to reflect frequencies in the environment but rather as connections between isolates from humans and other sources.

Association of ocular infections with one of the four major groups. Eighty-seven of the 121 (71.9%) ocular isolates were members of the four major clinical groups; however, a large number nested within group 3 ($n = 56$) (Fig. 1). Although isolates derived from ocular infections were found in all four major groups and elsewhere within clade 3 of the FSSC phylogeny, a chi-squared permutation test indicated that the dis-

tribution of the ocular isolates in the four major clinical groups was significantly nonrandom ($P < 0.001$). Among the four groups, ocular infections were most frequently associated with group 3 and least frequently with group 1. Because all ocular isolates from southern Asia (17 from India and 1 from Indonesia) were associated with group 3, we suspected a geographic sampling bias. However, when the Indian isolates were removed from the analysis, the chi-squared permutation test still yielded a significant association between ocular isolates and group 3 ($P < 0.001$).

Discordance of ERG and TEF data. The WS-R test results indicated significant incongruence ($P < 0.003$ in WS-R) between the ERG and TEF datasets. In some cases, putatively nonspecific isolates sharing an identical TEF, ITS, and LSU rRNA gene multilocus haplotype possessed highly divergent ERG alleles (data not shown). The *erg-3* gene product catalyzes the reduction of the C-14 in ergosterol, the sterol component in the fungal cell membrane, and is the target of a variety of polyene, allylamine, and azole antifungal agents (4). Selection conferred by the exposure of FSSC isolates to these compounds in nature may have facilitated an unorthodox mode of evolution in this gene, including the possible maintenance of horizontally transferred alleles.

Association of clinical isolate groups with phylogenetic and biological species. The three-locus phylogeny (Fig. 1) resolved approximately 36 phylogenetic species within clade 3 of the FSSC, 18 of which were represented by clinical isolates. Groups 1 and 2 are both diagnosable phylogenetic species deserving formal recognition. Group 1, which received 100% bootstrap support, contains isolates known previously as members of mating population V, which is also known as forma specialis *cucurbitae* race 2 because of its specific ability to cause disease on cucurbits (10, 16). The two most prevalent haplotypes identified in this study were members of group 1, both of which contained isolates from human and cucurbit sources, as well as from the hospital environment. These isolates are the best candidates for future animal pathogenesis studies. Although an isolate of *Fusarium oxysporum* pathogenic to tomato has been developed as an animal model system (16), there are no reports of this isolate naturally causing infections in humans and other animals. Mostert et al. 2005 (11) also showed a common ability among phylogenetic species of *Phaeoacremonium* to cause both human and plant infections, but such connections are exceptional.

Group 2, which received 85% bootstrap support, contained isolates from diverse origins but were mostly from humans, other animals, and environmental sources. Only a single isolate from a plant (cyclamen) was associated with this group. Prevalent in group 2, however, were 11 of the 28 isolates from marine animal sources, including shrimp, shark, and sea turtle. A second phylogenetic species, exclusive of the four major human-associated groups, showed an even stronger association with marine animals, in that 11 of its 12 members were recovered from marine animal sources while the 12th isolate was from a human infection. This finding indicates that there are at least two distinct species in the FSSC specifically adapted to the marine environment that are capable of causing infections in marine animals and humans.

Groups 3 and 4 were poorly resolved, with low bootstrap values and short internodes. These groups may be better de-

limited by the analysis of additional data, but alternatively they may represent complexes of radiating clones that might be extremely difficult or impossible to resolve as phylogenetic species. Collectively, these two groups comprise 176 (37.3%) of the isolates analyzed in the present study and appear to represent the haplotypes most commonly isolated in the environment, including from soil.

In summary, the major finding of this study is that the ability to cause human infections spans the phylogenetic breadth of FSSC clade 3, with approximately 75% of the clinical isolates nesting within one of four discrete groups. Given that isolates from human infections often shared multilocus haplotypes with isolates from diverse environmental sources, including hospitals, we conclude that infections likely occur due to chance encounters between susceptible patients and the most prevalent members of the FSSC in their environment.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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