# Multilocus phylogenetics show high levels of endemic fusaria inhabiting Sardinian soils (Tyrrhenian Islands)

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***Abstract:*** The Mediterranean island of Sardinia is well known for high levels of vascular plant diversity and endemism, but little is known about its microbial diversity. Under the hypothesis that *Fusarium* species would show similarly high diversity, we estimated variability in *Fusarium* species composition among 10 sites around the island. Markers previously adopted for multilocus sequence typing (MLST) were used to determine multilocus DNA sequence haplotypes for 263 *Fusarium* isolates. In addition portions of the translation elongation factor 1-alpha and second largest RNA polymerase subunit genes were sequenced for all isolates. The intergenic spacer (IGS) region of the nuclear ribosomal RNA gene repeat was sequenced for members of the *F. oxysporum* species complex (FOSC), and a portion of the nuclear ribosomal RNA gene repeat comprising the internal transcribed spacer (ITS) and part of the large nuclear ribosomal RNA subunit was sequenced for members of the *F. solani* species complex (FSSC). Seventy-three multilocus haplotypes were identified among the 263 isolates typed, of which 48 represented FOSC and FSSC. Thirty-seven of 48 FOSC two-locus and FSSC three-locus haplotypes had not been observed previously. The 38 non-FOSC/FSSC fusaria comprised 25 haplotypes distributed among 10 species, five of which appear to represent novel, phylogenetically distinct species. In general newly discovered haplotypes were restricted to one or a few sites. All FSSC isolates represented new haplotypes in phylogenetic species FSSC 5 and 9, which differ from the phylogenetic species dominant in soils worldwide. No obvious correlations were found between haplotype diversity and geospatial or habitat distribution. Overall these results indicate a high degree of *Fusarium* genetic diversity on multiple geographic scales within Sardinia. These results contrast with recent work showing that common, cosmopolitan species dominate Sardinia's *Trichoderma* biodiversity. All data are available for access and viewing from the FUSARIUM-ID database.

***Key words:*** biogeography, island biodiversity, molecular ecology, mycoflora, soil fungi

## INTRODUCTION

Genus *Fusarium* Link (Nectriaceae, Ascomycota) has a cosmopolitan distribution and may be found in almost any biome, including forest, grassland, desert, littoral, agricultural and alpine zones, as well as in aquatic and manmade environments (Leslie and Summerell 2006). The genus is best known for its negative effects on agriculture, forestry (Windels 2000, Ploetz 1990, Wingfield et al. 2008), and human and animal health (Marasas et al. 1984). Moreover *Fusarium* species have emerged as clinically relevant filamentous fungi, causing localized and often fatal invasive infections in humans and other animals (Nelson et al. 1994; Dignani and Anaissie 2004; O'Donnell et al. 2004a, 2007, 2008, 2009a, b; Chang et al. 2006; Zhang et al. 2006; Schroers et al. 2009). Certain *Fusarium* spp. also produce one or more toxins, such as trichothecenes (i.e. deoxynivalenol, nivalenol, T-2 toxin, HT-2 toxin), fumonisins, moniliformin, zearalenone and enniatins (Marasas et al. 1984, Nelson et al. 1993, Placinta et al. 1999, Bottalico and Perrone 2002, Logrieco et al. 2002) that affect food safety. In contrast fusaria may function as beneficial saprophytes in soil and plant debris and grow endophytically where they may contribute to the overall fitness of the host.

As is the case in all micro-organisms, the community structure of *Fusarium* spp. in noncultivated and

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cultivated soils depends on a number of factors, including capacity to colonize different plant hosts and to compete saprophytically with the resident microbial community. In addition it depends on their ability to decompose organic material, to produce resistant structures, particularly chlamydospores, which let them survive desiccation, high and low temperatures and other harsh conditions (Leslie and Summerell 2006). Preliminary data suggests that different soil and environmental factors, such as organic content, pH, temperature and moisture, may influence the presence and distribution of individual *Fusarium* spp. (Stoner 1981, Jeschke et al. 1990, Marasas et al. 1988, Burgess and Summerell 1992).

Some broadly defined morphological species of *Fusarium* known to harbor multiple cryptic species, such as *F. oxysporum* Schltdl. emend. W.C. Snyder and H.N. Hansen, *F. equiseti* (Corda) Sacc., and *F. solani* (Mart.) Appel and Wollenw. emend. W.C. Snyder and H.N. Hansen, have been reported as cosmopolitan and seemingly independent of climatic factors, while other species, such as *F. sambucinum* Fuckel, *F. acuminatum* Ellis and Everh, *F. compactum* (Wollenw.) Gordon, and *F. longipes* Wollenw. and Reinking, were reported to exhibit a limited distribution correlated with certain environmental factors (Backhouse and Burgess 1995, Sangalang et al. 1995). It is important to note that this geospatial structure of *Fusarium* spp. has not been evaluated critically in the light of recent discoveries of more complex species diversity based on phylogenetics. Numerous molecular phylogenetics studies have demonstrated that morphologically defined *Fusarium* species often comprise multiple phylogenetically diagnosable species (O'Donnell et al. 1998b, 2000, 2004b, 2008, 2009b). The morphological species most commonly recovered from soil, *F. oxysporum*, *F. solani* and *F. equiseti*, now are known to represent species complexes (O'Donnell et al. 1998a, 2000, 2009b).

Within the *F. solani* species complex (FSSC), isolates from soil and plant debris collected worldwide appear to be dominated by one or perhaps two phylogenetic species, designated FSSC 3 + 4 (Zhang et al. 2006, O'Donnell et al. 2008). Studies have used the translation elongation factor 1-alpha gene to identify soil fusaria on a finer scale, showing high diversity in Spain (Maciá-Vicente et al. 2008) and Ethiopia (Bogale et al. 2009). Results of these studies highlight the advantages of developing multilocus DNA sequence typing schemes for assessing the diversity of soil fusaria in light of new systematic insights based on phylogenetic inference.

Sardinia (Italy) is an island located in the center of the western Mediterranean Sea with a surface area of 24 089 km$^2$. It is ~570 000 000 y old and has been separated from mainland Europe near the current location of Spain and France ~35 000 000 y. In the early Miocene, Sardinia and Corsica became an isolated block. Sardinia's long history of separation from the mainland has provided ample opportunity for the evolution of novel biodiversity. On the other hand all island ecosystems are vulnerable and prone to widespread species extinction due to a variety of factors, including relaxed selection pressure due to reduced competition and in more recent millennia anthropogenic factors (IUCN 2002). The Mediterranean region, including its islands, has been occupied by humans at least 8000 y. The greatest effects of human civilization have been major changes in ecosystems due to deforestation, intensive grazing and fires, and infrastructure development, especially on the coasts. Historically Mediterranean forests were burned to create agricultural lands, an effect particularly evident in Sardinia where nearly one-half of the island consists of grasslands used for grazing of sheep and goats. The agricultural lands, pastures, evergreen woodlands and maquis habitats that dominate Sardinia today are mostly the result of these anthropogenic disturbances. Habitat fragmentation is a serious problem for Sardinian biodiversity because the original vegetation remains in scattered patches; today a mere 5% of the original extent of the hotspot contains relatively intact vegetation, placing the Mediterranean Basin among the four most significantly altered hotspots on Earth included in European Union and International Union for Conservation of Nature conservation policies (Médail and Quézal 1999, Grill et al. 2006).

Because of its history of isolation and status as a biodiversity hotspot we hypothesized that Sardinia might possess unique fungal biodiversity. Migheli et al. (2009) analyzed the biodiversity of fungi in genus *Trichoderma* from diverse sites around Sardinia and found that common cosmopolitan species dominated, although one putative new species and a species known otherwise only from Australia also were found. In this study we turned to genus *Fusarium*, which provides molecular tools that allow discrimination at a much finer level. We characterized species and haplotype diversity in *Fusarium* species among 10 sites around the island, representing diverse ecological and climatic zones. To this aim 263 *Fusarium* isolates were identified from uncultivated soils and multilocus DNA sequence haplotypes were determined for each isolate with markers previously adopted for multilocus sequence typing (MLST) of fusaria. Species diversity was compared among sites to determine whether ecological or climatic factors

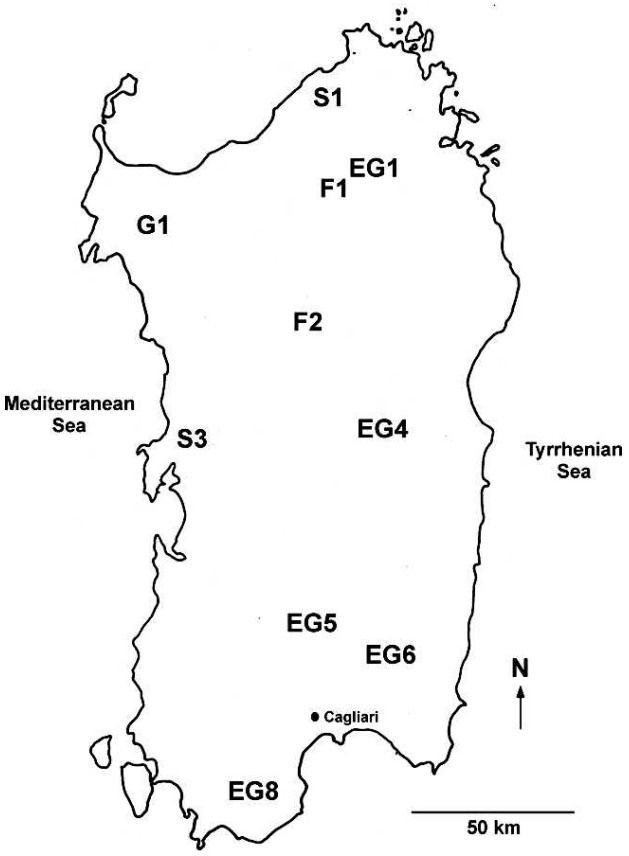


FIG. 1. Sardinia with *Fusarium* collection sites (TABLE I) indicated. EG = extensively grazed grassland, F = forest, G = grassland/savannah, S = shrubland/chapparal.

correlate with species composition. DNA sequence data are accessible from the FUSARIUM-ID database, which should help promote similar molecular ecological studies.

## MATERIALS AND METHODS

*Soil sampling and procedure.*—Soil samples were collected 2003–2006 at 10 sites in Sardinia, Italy. Different ecosystem types were sampled: forest (F, two sites), shrubland/chapparal (known locally as ''macchia mediterranea'') (S, two sites), grassland-savannas (G, one site) and extensively grazed grassland (EG, five sites) (FIG. 1). Details on prevalent vegetation were reported by Migheli et al. (2009). We provided environmental characteristics of the sampling sites, including global positioning system (GPS) coordinates, altitude, average yearly rainfall and mean temperature, soil type and pH, and percentage of organic matter (SOM) and organic carbon (C) (TABLE I).

At each site 5 ~ 200 g soil samples were taken with a weeding hoe from a depth of 0–15 cm, four located at the ends of two 20 m perpendicular transects and one at the intersecting point (Burgess et al. 1988, Summerell et al. 1993). The samples from each site were pooled and split in two parts, one used for physico-chemical analyses as described by Migheli et al. (2009) and the other sieved, air-dried, mixed thoroughly and analyzed for microbial composition.

*Fungal isolation and storage conditions.*—*Fusarium* cultures were isolated with a soil dilution method. One gram soil was suspended in 100 mL 0.05% water agar from which two serial 1 : 100 dilutions were prepared, 1 mL of which was spread on a 90 mm Petri dish containing peptone pentachloronitrobenzene (PCNB or modified Nash/Snyder) agar, a selective medium for *Fusarium* (Nash and Snyder 1962, Burgess et al. 1994) and incubated 7 d at 25 C under near ultraviolet light with a 12 h photoperiod. Each dilution was plated on three Petri dishes, and the dilution producing fewer than 30 colonies per plate was selected for subculturing. Single colonies of *Fusarium* were transferred to carnation leaf agar (CLA) and incubated under the same conditions 2–3 wk. *Fusarium* colonies were subcultured via single-spore transfers onto new plates of CLA and potato dextrose agar (PDA).

TABLE I. Global Positioning System (GPS) coordinates, altitude, climatic conditions (average yearly rainfall minimal, maximal and annual average temperatures [T]) and soil properties (texture, pH in water, percentage of soil organic carbon [C] and of soil organic matter [SOM] by dry weight) of the 10 collection sites.[a]

| Soil code | GPS coordinates N | GPS coordinates E | Altitude (m) | Yearly rainfall (mm) | T min | T max | T ave | Sand (%) | Silt (%) | Clay (%) | pH | C (%) | SOM (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EG1 | 41°00′29″ | 9°15′23″ | 246 | 877 | 6.2 | 22.6 | 13.8 | 75.2 | 13.3 | 11.5 | 5.2 | 6.2 | 10.7 |
| EG4 | 40°07′16″ | 9°15′55″ | 958 | 970 | 8 | 25.1 | 15.7 | 72.8 | 15 | 12.2 | 5 | 9.3 | 16.1 |
| EG5 | 39°36′07″ | 9°06′06″ | 314 | 741 | 7.1 | 23.8 | 14.7 | 41.7 | 22.1 | 36.2 | 7 | 1.6 | 2.7 |
| EG6 | 39°29′12″ | 9°14′11″ | 605 | 638 | 8 | 26.2 | 16.2 | 49.7 | 30.6 | 19.7 | 4.8 | 6.7 | 11.6 |
| EG8 | 38°53′34″ | 8°50′40″ | 38 | 642 | 10.7 | 25.1 | 17.3 | 80.4 | 9.7 | 9.9 | 6 | 3.1 | 5.3 |
| F1 | 40°51′05″ | 9°08′59″ | 1080 | 800 | 5.3 | 22.3 | 13.1 | 83.2 | 8.6 | 8.2 | 4.5 | 6 | 10.5 |
| F2 | 40°21′24″ | 8°55′13″ | 1019 | 738 | 4.8 | 22.4 | 12.8 | 67.8 | 20 | 12.2 | 5 | 15.3 | 26.3 |
| G1 | 40°37′14″ | 8°23′14″ | 128 | 622 | 6.5 | 22.7 | 14 | 55.6 | 25.2 | 19.2 | 5.4 | 4.1 | 7 |
| S1 | 41°06′26″ | 9°05′11″ | 69 | 747 | 8.3 | 24.4 | 15.4 | 67.5 | 16.5 | 16 | 5.5 | 6.3 | 10.9 |
| S3 | 40°04′06″ | 8°32′15″ | 239 | 574 | 9.9 | 24.5 | 16.8 | 43.1 | 28.9 | 28.1 | 5.4 | 4.7 | 8.2 |

[a] Site abbreviations correspond to these ecotypes: EG = extensively grazed grassland, F = forest, G = grassland/savannah, S = shrubland/chaparral.

Each isolate was identified provisionally to broadly defined morphospecies according to Nelson et al. (1983) and Leslie and Summerell (2006) before being subjected to multilocus DNA sequence typing schemes. Cultures were stored in 15% glycerol at −80 C in the collections of the Centro per la Conservazione e la Valorizzazione della Biodiversità Vegetale, University of Sassari, Italy, and in the ARS culture collection (NRRL) in Peoria, Illinois, where they are available on request.

*DNA isolation and sequencing.*—Total genomic DNA was extracted with a modified CTAB protocol, as described by O'Donnell et al. 1998a). A ~700-bp portion of the translation elongation factor 1-alpha gene (*EF-1α*) and two contiguous portions of the second largest RNA polymerase (*RPB2*) subunit gene totalling ~1900 bp were amplified and sequenced in all isolates as a primary identification tool, as described by Geiser et al. (2004) and O'Donnell et al. (2007). Additional gene regions also were sequenced depending on the species complex. Three-locus haplotypes were obtained for 23 isolates within the FSSC by sequencing portions of *EF-1α*, *RPB2* and the nuclear rDNA region comprising the internal transcribed spacer (ITS) region and the 5′ end of the large ribosomal RNA subunit gene (LSU) as described by O'Donnell (2000, O'Donnell et al. 2008). Similarly two-locus haplotypes were obtained for 202 members of the FOSC by sequencing a portion of *EF-1α* and a ~2000 bp portion of the intergeneric spacer (IGS) region of the nuclear ribosomal RNA gene repeat as described by O'Donnell et al. (2009a).

After sequence alignments were trimmed to exclude missing data, multilocus sequences were collapsed into haplotypes with COLLAPSE 1.1. software (http://inbio.byu.edu/Faculty/kac/crandall_lab /Computer.html). Phylogenetic analyses were performed on the collapsed multilocus datasets with PAUP* 4.1.11 software package (Swofford 2003). Maximum parsimony trees were inferred with heuristic searches performed with random sequence addition (10 replicates) and bootstrapping. Multilocus haplotypes were identified by comparison to datasets of FOSC (256 haplotypes, O'Donnell et al. 2009a) and FSSC (181 haplotypes; Zhang et al. 2006; O'Donnell et al. 2008), FIESC and FCSC (O'Donnell et al. 2009b), sequences available in the FUSARIUM-ID database (Geiser et al. 2004) and data from the clade that includes most species known to produce trichothecene mycotoxins (T.J. Ward and K. O'Donnell unpubl). Sequences were deposited in GenBank (accession numbers GU250537–GU250732) and in the FUSARIUM-ID database.

*Statistical analysis.*—Comparisons of haplotypes and species among sites were performed with the Bray-Curtis similarity measure cluster ordination on fourth root-transformed data. Formal significance tests for differences among ecosystem types were performed with the one-way ANOSIM permutation/randomization test (Clarke and Warwick 2001). To assess the contribution of a set of best matching environmental variables to dissimilarities among samples the Spearman rank correlation coefficient was calculated by correlating Euclidean distance similarity matrices of environmental variables with Bray-Curtis similarity matrices from biological data. All multivariate analyses were done with the PRIMER 5.2 package (Clarke and Warwick 2001).

## RESULTS

*Morphological and molecular identification of* Fusarium *species.*—A total of 263 fusaria were identified initially as eight morphospecies and an undescribed species (Nelson et al. 1983, Burgess et al. 1994, Leslie and Summerell 2006). It is important to note that four of these have been demonstrated to harbor multiple cryptic phylogenetic species and they are bracketed by apostrophes in the following sentence (O'Donnell et al. 2008, 2009a, b; K. O'Donnell and D.M. Geiser unpubl). In order of frequency they were '*F. oxysporum*', '*F. solani*', '*F. equiseti*' and/or '*F. compactum*', *F. polyphialidicum* Marasas, Nelson, Toussoun & van Wyk, *F. redolens* Wollenweber, *Fusarium* sp., *F. culmorum* (W.G. Smith) Sacc., *F. scirpi* Lambotte & Fautrey emend. Burgess, Nelson & Toussoun, and *F. chlamydosporum* Wollenweber & Reinking. *F. polyphialidicum* is a later synonym of *F. concolor* Reinking (O'Donnell et al. 2007, T. Gräfenhan and K. Seifert pers comm) and will be referred to hereafter by the latter name.

Molecular identification assigned most isolates to three different species complexes: FOSC (accounting for 76.8% of isolates), FSSC (8.8%) and *F. incarnatum-equiseti* species complex (FIESC; 5.3%). A total of 12 phylogenetically diagnosable species outside FOSC were represented; phylogenetic species within FOSC remain beyond diagnosis at this time. Non-FOSC isolates were represented by two phylogenetically distinct species within the FSSC, four within the FIESC including *F. equiseti* and *F. scirpi*, *F. concolor*, *F. redolens*, *F. culmorum*, two undescribed species (*F.* sp. 1 and *F.* sp. 2, FIG. 2) related to *F. brachygibbosum* within the trichothecene toxin-producing clade, and another undescribed species within the *F. chlamydosporum* species complex (FCSC; O'Donnell et al. 2007, 2009b). A total of 73 multilocus haplotypes were identified across all species and species complexes.

*Members of the* F. oxysporum *species complex.*—Isolates of FOSC were recovered from all sites. Of the 202 FOSC isolates, 37 two-locus haplotypes were identified (FIG. 2A). Eleven of 37 haplotypes were previously known, including seven that shared the same two-locus haplotype with isolates from these formae speciales: *apii* (haplotype 6), *raphani* (haplotype 19), *tulipae* (haplotype 22), *dianthi* (two haplotypes, 90 and 158) *gladioli* (haplotype 94) and *cucumerinum* (haplotype 191). Four haplotypes were previously known but possessed sequence types with no known connection to a forma specialis (haplotypes

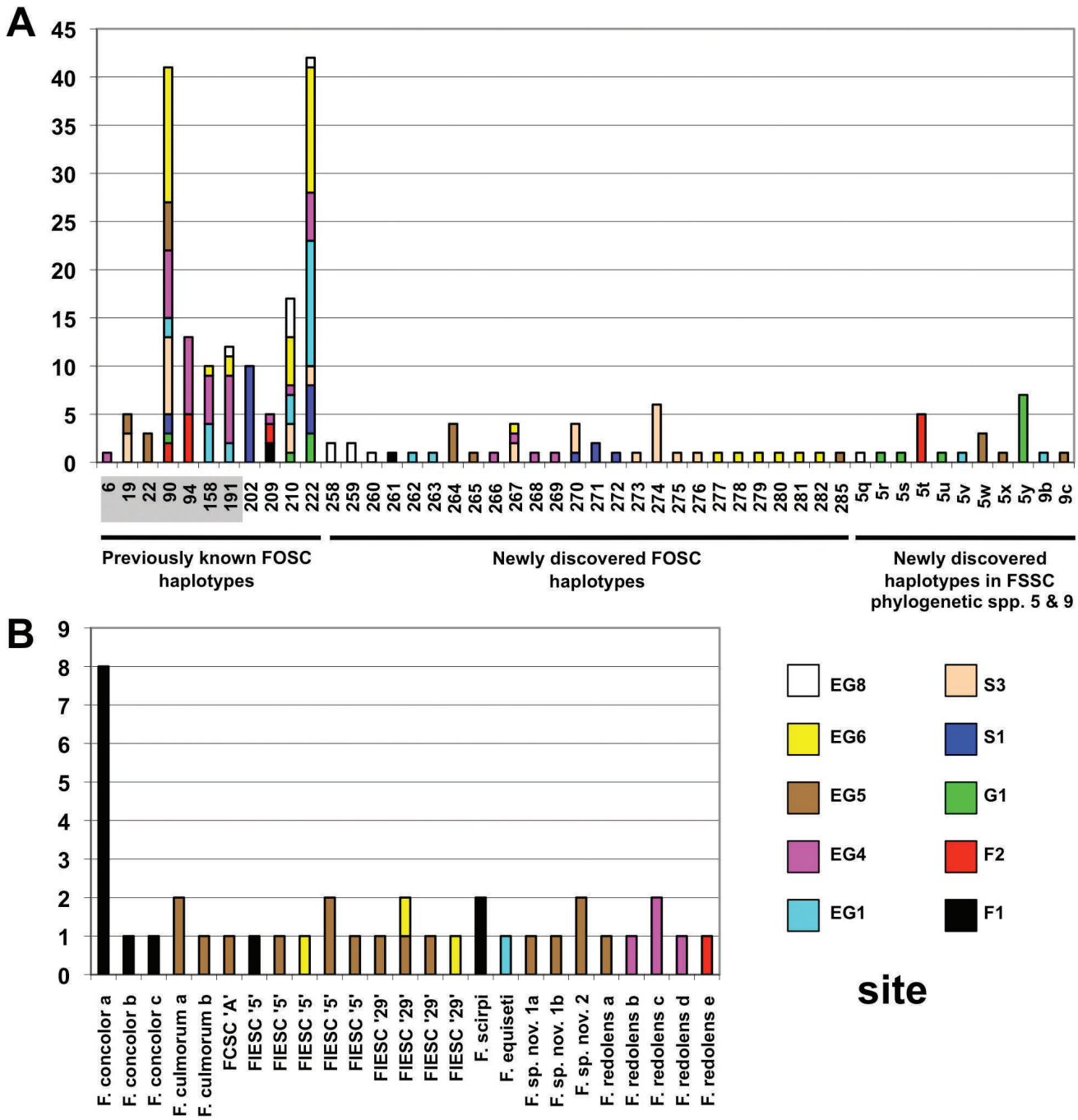


FIG. 2. A. Numbers of isolates per FOSC and FSSC haplotype (indicated by column height) observed at different sites. Colors represent the site from which individual isolates were collected. Shaded FOSC haplotypes share the same two-locus haplotype with known *formae speciales* (see RESULTS). B. Frequencies of species and haplotypes among non-FOSC/FSSC fusaria observed. Note that non-FOSC/FSSC fusaria are identified by species, not haplotype. Novel phylogenetic species within FIESC were given provisional designations '5' and '29' by O'Donnell et al. (2009b) with no haplotype designations because they are not comparable with the existing haplotype nomenclatural schemes. The novel phylogenetic species in FCSC does not correspond to any of the phylogenetic species recognized by O'Donnell et al. (2009b) and is given the provisional designation "FCSC 'A'".

201, 209, 210, 222). The remaining 26 haplotypes had not been detected previously. The most common and abundant were haplotypes 90 (41 isolates found in 8/10 sites) and 222 (42 isolates found in 7/10 sites). Twenty-four of the 26 newly identified haplotypes were found only at a single site, and of these 19 were represented by a single isolate (FIG. 2A). Only two novel haplotypes were found in more than one site, averaging 1.08 sites per haplotype, whereas eight out of the 11 previously known haplotypes were found in more than one site, with previously known haplotypes averaging 3.45 sites per sample. All sites produced more than one FOSC haplotype, with a range of 2–12 haplotypes/site. Forest samples yielded the fewest (two sites averaging 2.5 haplotypes per site) and grazed grasslands yielded the most (five sites averaging 8.6 haplotypes per site). All sites except two (F2 and G1) produced at least one novel haplotype, with site EG6 yielding seven.

*Members of the* F. solani *species complex.*—In contrast to the omnipresence of FOSC in Sardinian soils, only five of the 10 soil samples (three grazed grasslands, one native grassland, one forest) yielded isolates of FSSC (FIG. 2A). Eleven three-locus haplotypes were recovered, and they belonged to two unnamed phylogenetic species, designated FSSC 5 and FSSC 9 (O'Donnell et al. 2008). All 11 three-locus FSSC haplotypes were previously unknown, and all were unique to single-soil samples.

*Other* Fusarium *groups.*—Of the remaining 10 species five were known and five appeared to represent undescribed members of the main clade of fusaria associated with the *Gibberella* sexual stage ('*Gibberella* clade') (FIG. 2B). The known species included *F. concolor*, *F. culmorum*, *F. redolens*, *F. equiseti* and *F. scirpi*, and the unknown species included two putatively undescribed phylogenetic species in the *F. incarnatum-equiseti* species complex (referred to provisionally as FIESC '5' and FIESC '29'; O'Donnell et al. 2009b, FIG. 2B), two undescribed genealogically exclusive species phylogenetically related to *F. brachygibbosum* (*Fusarium* sp. nov. 1 and *Fusarium* sp. nov. 2; FIG. 2B), and one novel phylogenetic species within the FCSC (FCSC 'A'; FIG. 2B). Collectively 25 haplotypes were represented among the 38 non-FOSC/FSSC isolates. Seven of the 11 non-FOSC/FSSC species were represented by two or more haplotypes. Noteworthy among these was FIESC with nine haplotypes/11 isolates and *F. redolens* with five haplotypes/6 isolates. Across all 25 non-FOSC/FSSC haplotypes, only a single member of FIESC '29' was found in more than one site. All but one of the non-FOSC/FSSC haplotypes were found in EG grassland sites; a single haplotype of *F. redolens* was found in the savannah site (S3). Site EG5, which had the least organic matter and a pH of 7.0, had the greatest diversity of *Fusarium* species: six FOSC haplotypes, three FSSC haplotypes from two species and seven other species in the *Gibberella* clade.

*Statistical analysis of association between genotype and habitat.*—No statistically supported groupings of soil samples were observed in the cluster analysis, as evidenced by the lack of separation of groups composed of samples taken from similar ecosystem types (not shown). In addition no significant differences in haplotype and species composition were found among ecosystem types as shown by the one-way ANOSIM test ($R = 0.439$, $P = 4.8\%$), suggesting that haplotype and species composition does not depend on soil type. Moreover the weighted Spearman coefficient ($r_w$) failed to find any agreement between distribution of haplotypes and species and environmental variables considered (no combination of environmental variables provided a higher value than $r = -0.27$ and $P = 95.2\%$). Finally no significant linear correlation between biodiversity index (Simpson) with soil organic matter (SOM) and carbon (C) was observed.

## DISCUSSION

Analyses of fusaria from 10 ecologically diverse sites spanning the length and width of Sardinia yielded surprisingly high genetic diversity, including six putatively novel, phylogenetically distinct species and many new haplotypes of FOSC and FSSC. All previously described species were chlamydospore producers known to inhabit soils, possibly reflecting the harsh selective method used to isolate *Fusarium* spp. and the fact that plant debris was discarded.

*FOSC fusaria.*—Among 202 FOSC isolates 37 haplotypes were identified, 26 of which were new in comparison to a database of 256 FOSC haplotypes (O'Donnell et al. 2009a). Seven of the 11 previously known haplotypes exactly matched a haplotype containing a forma specialis. However such genotypic matches do not necessarily suggest common pathogenicity, and no obvious connection could be made between these formae speciales and the habitats from which they were isolated. While a connection between f. sp. *dianthi* and haplotypes 90 and 158 was made, a f. sp.-specific PCR assay (Chiocchetti et al. 1999) failed to amplify with these isolates (data not shown). Previously known haplotypes in FOSC were found frequently in more than one site (averaging 3.45 sites per haplotype), while novel haplotypes were found rarely in more than one site (averaging 1.08 sites per

haplotype), suggesting that novel haplotypes might represent endemic individuals.

*FSSC fusaria.*—Eleven haplotypes from two phylogenetic species were identified among the 23 FSSC isolates. To our surprise these two phylogenetic species, designated FSSC 5 and FSSC 9, were different from the group currently recognized as the most prevalent member of the FSSC in soils worldwide, FSSC 3 + 4 (Zhang et al. 2006, O'Donnell et al. 2008, Bogale et al. 2009, F.A. Nalim unpubl). FSSC 5 is a cosmopolitan species that commonly is isolated from human infections, as well as from a variety of plant hosts (lisianthus, potato, chickpea, corn, coffee, narcissus, wheat, onion). Only two isolates from soil of this species were previously cultured, one from Australia (FRC S-368) and one from China (FRC S-869) (Zhang et al. 2006). However in a recent study of FSSC from Ethiopian agricultural soils 13/43 isolates were from FSSC 5, as inferred from translation elongation factor 1-alpha sequences (denoted as lineage 4 in that study; Bogale et al. 2009). We cannot determine whether the Ethiopian FSSC 5 isolates match the haplotypes of the Sardinian isolates because data from only a single gene is comparable between the two studies. In contrast to our results and consistent with known FSSC diversity in soils worldwide FSSC 3 + 4 was the most commonly isolated phylogenetic species in the Ethiopian study (18/43 isolates, denoted as lineage 1 by Bogale et al. 2009). In addition a study of root-colonizing fungi from plants collected in Alicante province of southeastern Spain uncovered diverse FSSC isolates (Maciá-Vicente et al. 2008), but none in FSSC 3 + 4 or the two phylogenetic species found in Sardinia. In contrast only a few isolates of FSSC 9 were found previously, including isolates from human infections and isolates from corn roots (Illinois, USA) and from soil (Greece) (Zhang et al. 2006, D. Geiser unpubl.). Differences in species and haplotype diversity observed in different studies might be attributable to differences in isolation technique or habitat type. Nevertheless it is striking that all FSSC haplotypes in both phylogenetic species isolated in Sardinia were novel.

*Presence of clinically important species.*—FSSC 5 and 9 have been associated with infections of humans. FSSC 5 is commonly associated with both deeply invasive and subcutaneous infections, as well as infections of the cornea (Zhang et al. 2006; O'Donnell et al. 2008). It is unknown whether species such as FSSC 5 are specifically adapted to infecting humans in comparison to other members of FSSC or whether its higher association with human infections is due more to opportunities to infect because of frequent encounters with susceptible hosts. FSSC 9 is more rarely encountered, but a member of this phylogenetic species was associated with a keratitis case in the 2006 contact lens solution-associated keratitis outbreak (O'Donnell et al. 2007), and a second isolate is known that came from a neck lesion on a sea turtle (Zhang et al. 2006, O'Donnell et al. 2008). Such infections likely are caused by *Fusarium* haplotypes that are common in the patient's indoor or outdoor environment (Zhang et al. 2006, Chang et al. 2006). Because several fusaria that are capable of causing rare infections are common in the environment worldwide the widespread presence of FSSC 5 in Sardinian soils is not cause for alarm. Indeed FSSC 3 + 4, common in soils worldwide, also is frequently isolated from human infections. FOSC haplotype 202 was the only Sardinian isolate from this complex matching a known clinical isolate (NRRL 43454 *ex cornea* MA-USA).

*Non-FOSC/FSSC fusaria.*—Because our databases of sequences from species outside FOSC and FSSC are far less comprehensive the focus of our molecular phylogenetic analyses was directed at identifying known and novel phylogenetically distinct species instead of novel multilocus haplotypes. The results of these analyses indicate high endemism of Sardinian fusaria, given that five putatively novel phylogenetically distinct species were identified, coupled with the fact that 25 haplotypes were identified among the 38 non-FOSC/FSSC isolates. All previously described non-FOSC/FSSC species isolated (i.e. *F. concolor*, *F. culmorum*, *F. redolens*, *F. scirpi* and *F. equiseti*) were known to inhabit soils. Although the latter species was represented only by a single strain (NRRL 46628 forms a genealogically exclusive group with the *F. equiseti* neotype strain NRRL 26419 = BBA 68556), two putatively undescribed *F. equiseti*-like species within the FIESC (FIESC '5' and '29') were represented by 11 isolates and *F. scirpi* by two (NRRL 44910 and 44916). The remaining non-FOSC/FSSC isolates comprised three additional, phylogenetically distinct species, including two *F. brachygibbosum*-like species within the trichothecene clade (F. sp. nov. 1 represented by NRRL 46646 and 46648 and F. sp. nov. 2 represented by NRRL 46662 and 46663) and a *F. chlamydosporum*-like species (FCSC ''A'') represented by NRRL 46670.

*Endemism in Sardinian fusaria.*—Our analyzes revealed that the diversity of fusaria in Sardinian soils was not correlated with habitats. However the small number of sites sampled, half of which were of a single category (extensively grazed grasslands), confounds such an inference, as well as the high degree of endemism and fine geographic scale. Sixty-three of

73 haplotypes identified in this study were found only in a single site. Within FOSC previously known haplotypes showed a strong tendency to be distributed across more than one site, whereas new haplotypes were rarely found at more than one site. One hypothesis to explain this is that the previously described haplotypes were introduced in a widespread manner on a background of highly endemic local populations. In summary our results support the prediction that the geographically isolated island of Sardinia appears to possess an unusual array of endemic fusaria. Future studies are needed to determine whether high endemism observed in Sardinia also are observable elsewhere in the western and central Mediterranean, including its biogeographic neighbor, the island of Corsica, and Mediterranean islands influenced by or originating from volcanism such as Sicily and Malta.

These results contrast with observations of *Trichoderma* spp. from the same soils (Migheli et al. 2009). Among *Trichoderma* isolates previously known, cosmopolitan types dominated, with exceptions being the discovery of one novel species and another that previously had not been observed in Eurasia. Both experimental and biological factors might have contributed to the differences in these two studies. In the *Trichoderma* study molecular markers were used that did not permit the same degree of intraspecific discrimination as the multilocus sequence typing performed here. In the previous study randomly amplified polymorphic DNA (RAPD) markers were used as a preliminary screen to identify putative genetic individuals that were subjected to analysis with the internal transcribed spacer (ITS) region and occasionally part of the translation elongation factor 1-alpha gene. Biological differences between *Trichoderma* and *Fusarium* also might play important roles, including their dispersal biology, host associations and ability to form stable populations at a particular site.

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## LITERATURE CITED

Backhouse D, Burgess LW. 1995. Mycogeography of *Fusarium*: climatic analysis of the distribution within Australia of *Fusarium* species in section *Gibbosum*. Mycol Res 99:1218–1224.

Bogale M, Steenkamp ET, Wingfield MJ, Wingfield BD. 2009. Diverse *Fusarium solani* isolates colonise agricultural environments in Ethiopia. Eur J Plant Pathol 124: 369–378.

Bottalico A, Perrone G. 2002. Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe. Eur J Plant Pathol 108:611–624.

Burgess LW, Nelson PE, Toussoun TA, Forbes GA. 1988. Distribution of *Fusarium* species in sections *Roseum*, *Arthrosporiella*, *Gibbosum* and *Discolor* recovered from grassland, pasture and pine nursery soils of eastern Australia. Mycologia 80:815–824.

———, Summerell BA. 1992. Mycogeography of *Fusarium*: survey of *Fusarium* species in subtropical and semi-arid grassland soils from Queensland, Australia. Mycol Res 96:780–784.

———, ———, Bullock S, Gott KP, Backhouse D. 1994. Laboratory Manual for Fusarium Research. 3rd ed. Sydney: Fusarium Research Laboratory, Department of Crop Sciences, University of Sydney and The Royal Botanic Gardens. 133 p.

Chang DC, Grant GB, O'Donnell K, Wannemuehler KA, Noble-Wang, Rao CY, Jacobson LM, Crowell CS, Sneed RS, Lewis FMT, Schaffzin JK, Kainer MA, Genese CA, Alfonso EC, Jones DB, Srinivasan A, Fridkin SK, Park BJ. 2006. Multistate outbreak of *Fusarium* keratitis associated with use of a contact lens solution. J Am Med Assoc 296:953–963.

Chiocchetti A, Bernardo I, Daboussi M-J, Garibaldi A, Gullino ML, Langin T, Migheli Q. 1999. Detection of *Fusarium oxysporum* f. sp. *dianthi* in carnation tissue by PCR amplification of transposon insertions. Phytopathology 89:1169–1175.

Clarke KR, Warwick RM. 2001. Changes in marine communities: an approach to statistical analysis and interpretation. 2nd ed. PRIMER-E. Plymouth UK. 172 p.

Dignani MC, Anaissie EJ. 2004. Human fusariosis. Clin Microbiol Infect 10:67–75.

Geiser DM, Jiménez-Gasco MD, Kang S, Makalowska I, Veeraraghavan N, Ward TJ, Zhang N, Kuldau GA, O'Donnell K. 2004. FUSARIUM-ID v.1.0: a DNA sequence database for identifying *Fusarium*. Eur J Plant Pathol 110:473–479.

Grill A, Casula P, Lecis R, Menken SBJ. 2006. Endemism in Sardinia. In: Weiss S, Ferrand N, eds. Phylogeography of southern European refugia. Dordrecht, Netherlands: Springer-Verlag. p 273–296.

IUCN. 2002. IUCN and Mediterranean islands: opportunities for biodiversity conservation and sustainable use.

In: Liz, Hopkins, ed. Campanillas (Málaga), Spain: IUCN Center for Mediterranean Cooperation. 63 p.

Jeschke N, Nelson PE, Marasas WFO. 1990. *Fusarium* species isolated from soil samples collected at different altitudes in the Transkei, southern Africa. Mycologia 82:727–733.

Leslie JF, Summerell BA. 2006. The *Fusarium* laboratory manual. Oxford, UK: Blackwell Publishing. 388 p.

Logrieco A, Mulè G, Moretti A, Bottalico A. 2002. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. Eur J Plant Pathol 108:597–609.

Maciá-Vicente JG, Jansson HB, Abdullah SK, Descals E, Solinas J, Lopez-Llorca LV. 2008. Fungal root endophytes from natural vegetation in Mediterranean environments with special reference to *Fusarium* spp. FEMS Microbiol Ecol 64:90–105.

Marasas WFO, Burgess LW, Anelich RY, Lamprecht SC, van Schalkwyk DJ. 1988. Survey of *Fusarium* species associated with plant debris in South African soils. S Afr J Bot 54:63–71.

———, Nelson PE, Toussoun TA. 1984. Toxigenic *Fusarium* species: identity and mycotoxicology. University Park: Pennsylvania State Univ Press. 328 p.

Médail F, Quézal P. 1999. Biodiversity hotspots in the Mediterranean Basin: setting global conservation priorities. Conserv Biol 13:1510–1513.

Migheli Q, Balmas V, Komoń-Zelazowska M, Scherm B, Fiori S, Kopchinskiy AG, Kubicek CP, Druzhinina I. 2009. Soils of a Mediterranean hot spot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European, invasive species of *Hypocrea/Trichoderma*. Environ Microbiol 11:35–46.

Nash SM, Snyder WC. 1962. Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soils. Phytopathology 52:567–572.

Nelson PE, Desjardins AE, Plattner RD. 1993. Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry and significance. Annu Rev Phytopathol 31: 233–252.

———, Dignani MC, Anaissie EJ. 1994. Taxonomy, biology and clinical aspects of *Fusarium* species. Clin Microbiol Rev 7:479–504.

———, Tousson TA, Marasas WFO. 1983. *Fusarium* species: an illustrated manual for identification. University Park: Pennsylvania State Univ Press. 193 p.

O'Donnell K. 2000. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. Mycologia 92:919–938.

———, Cigelnik E, Nirenberg HI. 1998b. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90:465–493.

———, Gueidan C, Sink S, Johnston PR, Crous P, Glenn A, Riley R, Zitomer N, Colyer P, Waalwijk C, van der Lee T, Moretti A, Kang S, Kim H-S, Geiser DM, Juba J, Baayen RP, Cromey MG, Bithel S, Sutton DA, Skovgaard K, Ploetz R, Kistler HC, Elliott M, Davis M, Sarver BAJ. 2009a. A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex. Fungal Genet Biol 46: 936–948.

———, Kistler HC, Cigelnik E, Ploetz RC. 1998a. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc Natl Acad Sci USA 95:2044–2049.

———, ———, Tacke BK, Casper HH. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineage of *Fusarium graminearum*, the fungus causing wheat scab. Proc Natl Acad Sci USA 97:7905–7910.

———, Sarver BAJ, Brandt M, Chang DC, Noble-Wang J, Park BJ, Sutton DA, Benjamin L, Lindsley M, Padhye A, Geiser DM, Ward TJ. 2007. Phylogenetic diversity and microsphere array-based genotyping of human pathogenic fusaria, including isolates from the multistate contact lens-associated US keratitis outbreaks of 2005 and 2006. J Clin Microbiol 45:2235–2248.

———, Sutton DA, Fothergill A, McCarthy D, Rinaldi MG, Brandt ME, Zhang N, Geiser DM. 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. J Clin Microbiol 46: 2477–2490.

———, ———, Rinaldi MG, Gueidan C, Crous PW, Geiser DM. 2009b. A novel MLST scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-equiseti* and *F. chlamydosporum* species complexes within the US. J Clin Microbiol 47: 3851–3861.

———, ———, ———, Magnon KC, Cox PA, Revankar SG, Sanche S, Geiser DM, Juba JH, van Burik JAH, Padhye A, Anaissie EJ, Francesconi A, Walsh TJ, Robinson JS. 2004a. 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 geographically widespread clonal lineage and nosocomial origin. J Clin Microbiol 42:5109–5120.

———, Ward TJ, Geiser DM, Kistler HC, Aoki T. 2004b. Genealogical concordance between mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genet Biol 41:600–623.

Placinta CM, D'Mello JP, Macdonald AMC. 1999. A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. Anim Feed Sci Tech 78:21–37.

Ploetz RC, ed. 1990. Fusarium wilt of Banana. St Paul, Minnesota: APS Press. 146 p.

Sangalang AE, Burgess LW, Backhouse D, Duff J, Wurst M. 1995. Mycogeography of *Fusarium* species in soils from tropical, arid and Mediterranean regions of Australia. Mycol Res 99:523–528.

Schroers HJ, O'Donnell K, Lamprecht SC, Kammeyer PL, Sutton DA, Rinaldi MG, Geiser DM, Summerbell RC. 2009. Taxonomy and phylogeny of the *Fusarium dimerum* species group. Mycologia 101:44–70.

Stoner MF. 1981. Ecology of *Fusarium* in noncultivated soil. In: Nelson PE, Toussoun TA, Cook RJ, eds. Fusarium:

diseases, biology and taxonomy. University Park: Pennsylvania State Univ Press. p 276–286.

Summerell BA, Rugg CA, Burgess LW. 1993. Mycogeography of *Fusarium*: survey of *Fusarium* species associated with forest and woodland communities in north Queensland, Australia. Mycol Res 97:1015–1019.

Swofford DL. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Wingfield MJ, Hammerbacker A, Ganley RJ, Steenkamp ET, Gordon TR, Wingfield BD, Countinho TA. 2008. Pitch canker caused by *Fusarium circinatum*—a growing threat to pine plantations and forests worldwide. Eur J Plant Pathol 37:319–334.

Windels CE. 2000. Economic and social impacts of *Fusarium* head blight: changing farms and rural communities in northern Great Plains. Phytopathology 90:17–21.

Zhang N, O'Donnell K, Sutton DA, Nalim FA, Summerbell RC, Padhye AA, Geiser DM. 2006. Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. J Clin Microbiol 44:2186–2190.