CHAPTER 3

VALIDATION OF THE DESCRIPTION OF *GIBBERELLA CIRCINATA* AND MORPHOLOGICAL DIFFERENTIATION OF THE ANAMORPH *FUSARIUM CIRCINATUM*

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ABSTRACT

Fusarium subglutinans sensu lato is the causal agent of several diseases on a wide variety of host plants, including maize, mango, pine, pineapple and sugarcane. Pitch canker is an important disease caused by host specific *F. subglutinans* isolates from pine. Previously, *F. subglutinans* isolates occurring on different host plants could be distinguished from one another based only on pathogenicity and sexual compatibility. However, β-tubulin and histone *H3* gene sequences have recently been used to separate *F. subglutinans* isolates into distinct phylogenetic and morphological species. The pitch canker fungus was described as *F. circinatum* based on four strains. The teleomorph, *Gibberella circinata* was described based on a single cross between two of these strains. The objectives of the present study were to provide additional information and isolates to validate the description of *G. circinata* and to test the efficacy of distinguishing *F. circinatum* from *F. subglutinans sensu lato* using morphological characteristics. The single cross used in the description of *G. circinata* was repeated and vegetative compatibility tests and mating type segregation confirmed that these isolates were heterothallic. Morphological characteristics of *F. circinatum* and *G. circinata* were consistent with those given in the original description of the species with minor differences in the dimensions of the perithecia. Perithecial dimensions in this study were 332-453 µm high and 288-358 µm wide. The description of *G. circinata* was validated by providing information regarding the holotype specimen, names of collectors of isolates, date of collection and designation of the holotype specimen. Morphological criteria such as conidial morphology, type of conidiophore branching and presence of sterile coiled hyphae distinguished *F. circinatum* from *F. subglutinans sensu lato* isolates.

INTRODUCTION

Fusarium subglutinans (Wollenweber & Reinking) Nelson *et al.* is a successful plant pathogen with a cosmopolitan distribution and is responsible for several important plant diseases. Pitch canker of pines is one of the most significant of these diseases and accurate identification is therefore important. Correll *et al.* (1991; 1992) gave isolates of *F. subglutinans* pathogenic to pines *forma specialis* status, based on host specificity and restriction fragment patterns of mtDNA indicating that pine isolates differed from non-pine isolates. *F. subglutinans* f. sp. *pini* Correll *et al.* could be

distinguished from other host specific *F. subglutinans* isolates based only on pathogenicity and sexual compatibility (Correll *et al.*, 1991;1992; Viljoen *et al.*, 1997; Britz *et al.*, 1999). Sexual compatibility between *F. subglutinans* f. sp. *pini* isolates revealed that fertile *F. subglutinans* f. sp. *pini* were reproductively isolated and represented a distinct mating population (mating population H, MP-H) in the *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura complex (Britz *et al.*, 1999).

O'Donnell *et al.* (1998; 2000) recognized 44 *Fusarium* phylogenetic lineages in the *G. fujikuroi* complex (*Liseola* and related sections) based on β-tubulin gene and translation elongation factor 1α (EF-1α) gene sequences. Nirenberg & O'Donnell (1998) re-evaluated morphological characteristics and described morphological species that supported the phylogenetic lineages. Twelve of the 44 *Fusarium* taxa in the *G. fujikuroi* complex (residing in *Liseola* and related sections) were newly described (Nirenberg & O'Donnell, 1998; O'Donnell *et al.*, 1998; 2000). The pitch canker fungus, *F. subglutinans* f. sp. *pini* was named *F. circinatum* Nirenberg & O'Donnell (teleomorph, *G. circinata* Nirenberg & O'Donnell). Other *Fusarium* spp. that are morphologically similar to *F. subglutinans* (= *F. subglutinans sensu lato*) described by Nirenberg & O'Donnell (1998), include *F. begoniae* Nirenberg & O'Donnell ex *Begonia elatior* hybrid, *F. bulbicola* Nirenberg & O'Donnell ex *Nerine bowdenii, Vallota* and *Haemanthus* sp., *F. concentricum* Nirenberg & O'Donnell ex *Musa sapientum* (banana), *F. guttiforme* Nirenberg & O'Donnell ex *Ananas comosum* (pineapple) and *F. pseudocircinatum* O'Donnell & Nirenberg ex *Solanum* sp., *Pinus kesiya* and *Heteropsylla incisa.* According to the Index of Fungi (1999: vol. 6: 980), the new name *G. circinata* is invalid according to Article 37.3 of the International Code of Botanical Nomenclature (ICBN, Greuter *et al.*, 1994). Nirenberg & O'Donnell (1998) did not provide sufficient information to characterize unequivocally the type specimen in their description of *G. circinata* (John C. David, Editor of Index of Fungi, personal communication). The new names, *F. circinatum,* as well as *F. begoniae, F. concentricum F. guttiforme* and *F. pseudocircinatum* are, however, not invalid in terms of Article 37.3, because the host from which these isolates were derived are provided (John C. David, Editor of Index of Fungi, personal communication).

The description of the pitch canker fungus, *F. circinatum* by Nirenberg & O'Donnell (1998) was based on only four isolates, while the description of *G. circinata* relied upon a single cross between two *F. circinatum* isolates (BBA 69720 and BBA 69722). Thus, virtually no consideration of variability in the teleomorph characteristics was possible for this important plant pathogen. These authors also did not consider the fertility or the thallism of the *F. circinatum* isolates. Furthermore, even though an extensive, global collection of *F. circinatum* isolates exists, variability amongst isolates of *F. circinatum* was not considered.

The objective of this study was to provide additional information as well as isolates to support the description of *G. circinata,* which is validated by fulfilling the requirements of Article 37 (Greuter *et al.*, 1994). The previously selected mating testers for MP-H are confirmed to be the most appropriate strains to identify pitch canker isolates and the morphological differentiation of *F. circinatum* from *F. subglutinans sensu lato* isolates is reported.

MATERIALS AND METHODS

Isolates

Fusarium circinatum (= *F. subglutinans* f. sp. *pini*) isolates from different geographical areas, tester strains of *F. subglutinans* (MP-E) from maize (*Zea mays*) and *F. sacchari* (Butler) W. Gams (MP-B) from sugarcane (*Saccharum officinarum*) as well as ex-type *Fusarium* spp. in the *G. fujikuroi* complex that were previously identified as *F. subglutinans sensu lato* were studied (Table 1). All strains were single-spored, preserved by lyophilization and suspensions in 15% glycerol at –70°C and are available from the culture collection of the Medical Research Council (MRC), Tygerberg, South Africa as well as from the *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. *F. circinatum* isolates from California (FSP strain numbers) and Florida (FL strain numbers) were provided by T. R. Gordon, Department of Plant Pathology, University of California, California, USA. *F. sacchari* and *F. subglutinans* strains are also deposited in culture collections of FRC (Culture collection of the *Fusarium* Research Center, Department of Plant Pathology, Pennsylvania State University, University Park) and KSU (Collection of J. F. Leslie,

Kansas State University, Department of Plant Pathology, Kansas State University). All ex-type cultures used in this study are deposited in BBA (Biologische Bundesanstalt für Land – und Forstwirtschaft, Berlin, Germany) and NRRL (Northern Regional Research Laboratory, NCAUR, Peoria, IL, USA) culture collections.

Sexual compatibility

The holotype herbarium specimen of *G. circinata* (BPI 74609) was examined in detail (Fig. 1a). The cross between isolates BBA 69720 and BBA 69722 (Nirenberg & O'Donnell, 1998), was repeated by inoculating these two isolates on carrot agar, 2 cm from the center of Petri dishes opposite one another, as observed on the herbarium material. The cross was incubated at the lower temperature of 17°C as suggested by Covert *et al.* (1999) under near-ultraviolet and cool-white light (12 h photoperiod) until fertile perithecia were produced after 3 to 6 weeks. All crosses in this study were made on carrot agar as described by Klittich & Leslie (1988) except that we used 300 g of fresh carrots per liter of medium. Strains acting as females in the sexual cross were inoculated on carrot agar and those acting as males were inoculated on complete media (Correll *et al.*, 1987) slants. The carrot agar plates and complete media slants were incubated for 8 days at 25°C. A spore suspension of the male parent was prepared in 2.5% (v/v) TWEEN 60 and spread over the surface of the female parent plate. Fertilized plates were incubated upright, in a single layer under near-ultraviolet light and cool-white light (12 h photoperiod) at 17°C. Female fertility of the strains was tested by reversing the roles of the two strains in the cross. Strains that were fertile only as males were designated as female-sterile, while strains serving as either the male or female parent were designated hermaphrodites. All the crosses were examined weekly for fertile crosses (perithecia with exuding ascospores). All crosses were repeated at least once and those that were fertile were repeated again. The mating type designation (*MAT*-1 or *MAT*-2) of all isolates was based on their fertility with one of the tester strains of MP-H (MRC 6213 and MRC 7488) (Steenkamp *et al.*, 2000). Ascospores exuding from perithecia were randomly examined for viability by streaking them on 1.5% water agar and assessing germination after 24 h.

Thallism determination

The mating type segregation and vegetative compatibility groups (VCGs) of BBA 69720 and BBA 69722 as well as progeny from the cross were determined to establish the thallism of the cross between strains that has been used to typify *G. circinata*. The mating type segregation was determined by amplifying mating type idiomorphs using PCR primers and reaction conditions described by Steenkamp *et al.* (2000). Primers MatA and MatB amplify a product of approximately 300 base pairs (bp) in size of *MAT*-1 and primers MatC and MatD amplify a product of approximately 800 bp in size of *MAT*-2 (Steenkamp *et al.*, 2000). The VCGs of BBA 69720, BAA 69722 and progeny were determined as previously described by Britz *et al.* (1999).

Morphological description

Morphological characteristics such as perithecial dimensions and ascospore characteristics of *G. circinata* obtained from ten fertile crosses between *F. circinatum* isolates (BBA 69720 x BBA 69722, MRC 6213 x MRC 7488, BBA 69722 x MRC 7488, MRC 6208 x MRC 7488, MRC 7452 x MRC 6213, MRC 7505 x MRC 6213, MRC 7508 x MRC 7488, MRC 7509 x MRC 6213, MRC 7513 X MRC 6213 and MRC 7568 x MRC 6213) on carrot agar were compared with characteristics of *G. circinata* described by Nirenberg & O'Donnell (1998). The morphological characteristics, including: the shape of the conidia, type of conidiophore branching, origin of the conidiophore from the substrate, presence of chlamydospores and presence of sterile coiled hyphae (Nirenberg & O'Donnell, 1998), of *F. subglutinans sensu lato* isolates were also considered in detail (Table 1).

To stimulate culture and conidial development, isolates were transferred to carnation leaf agar (CLA, Fisher *et al.*, 1982) and potassium chloride (KCl) agar (Nelson *et al.*, 1983). Cultures were incubated at 25°C under near-ultraviolet light and cool-white light with a 12 h photoperiod and examined after 10 to 14 days, using a Zeiss Axioskop microscope. Secondary characteristics such as growth rate and colony color were determined on potato dextrose agar (PDA) after incubation at 25°C in the dark (Nelson *et al.*, 1983). Photomicrographs were taken of cultures grown on CLA. Fifty micro- and macroconidia, perithecia and ascospores were measured for each isolate and results represent minimum, mean and maximum values with the standard deviations in parentheses.

RESULTS

Sexual compatibility

Nirenberg & O'Donnell (1998) did not describe details of how they made the cross between BBA 69720 and BBA 69722, but it was clear from herbarium material (Fig. 1a) that isolates had been paired alongside each other. We repeated the cross in the same way and, although this is not an ideal technique, perithecia were produced. Mature perithecia exuding ascospores (Fig. 1b) also were produced when this cross was repeated using the standard method (Klittich & Leslie, 1988). The standard method was considerably more effective than when isolates were placed alongside each other. BBA 69720 and BBA 69722 were shown to be hermaphrodites and can, therefore, serve as effective tester strains. The fertility of the cross between BBA 69720 and BBA 69722 was compared with the fertility between the MP-H tester strains, MRC 6312 and MRC 7488. The fertile cross between these tester strains produced approximately three times more perithecia exuding ascospores (Fig. 1c) than the cross between BBA 69720 and BBA 69722 (Fig. 1d). All the fertile crosses recorded in this study were repeated successfully in at least two different tests. The percentage germination of random ascospores was higher than 80% in all cases.

The hermaphroditic strains MRC 6213 and MRC 7488 (Britz *et al.*, 1999) were identified as *MATH*-2 and *MATH*-1, respectively as has also been shown by Steenkamp *et al.* (2000). The mating type designation of all the other *F. circinatum* isolates was of the opposite mating type to the tester strains (MRC 6213 or MRC 7488) with which a fertile cross was produced (Table 1).

Thallism determination

Strain BBA 69720 amplified a PCR product of approximately 800 bp in size and BBA 69722 amplified a PCR product of approximately 300 bp in size. Five of the ascospore progeny produced a PCR product of approximately 800 bp in size and the other five produced PCR products of approximately 300 bp in size. Vegetative compatibility tests determined that the parent strains and 10 ascospore progeny all belonged to different VCGs.

Morphological description and validation

Gibberella circinata Nirenberg & O'Donnell Mycologia 90: 440 (1998).

Immersed and superficial perithecia formed on carrot agar. Ovoidal to obpyriform, dark purple to black perithecia (329)–332–396–453(–463) µm high and 288–337–358(–386) µm wide (Fig. 2a). Within 3-6 weeks after fertilization the perithecia exude pale brown, ellipsoidal 1–septate ascospores in cirrhi from perithecia (Fig. 1c; 2b). Anamorphic state, *F. circinatum,* characterized by sterile coiled hyphae (Fig. 2d), sympodially branched conidiophores bearing polyphialides (Fig. 2c) and conidiophores that originate directly from the substrate hyphae (erect). Macroconidia 3-septate, slender and cylindrical (lunate) (Fig. 2e). Microconidia non-septate, obovoid (Fig. 2f), occasionally oval to allantoid. The conidiophores of *F. circinatum* examined in this study had 2-5 openings and hyphal swellings occurred in some isolates.

HOLOTYPE: BPI 74609 = Dried preserved culture on 5% carrot agar of cross between isolates BBA 69720 and BBA 69722. *F. circinatum* BBA 69720 (ex-holotype culture) is *MAT*-1 hermaphrodite collected by T. R. Gordon from a symptomatic *Pinus radiata* branch in San Lorenzo, Almeda County, California, USA in 1988. Holotype deposited as a dried specimen in herbarium of Botanischer Garten und Botanisches Museum, Berlin-Dahlem, Germany (B), no accession number available (B. Hein, Curator of fungi, personal communication). Strain BBA 69722 is *MAT*-2 hermaphrodite collected by A. Viljoen from *P. patula* seedlings in the Ngodwana nursery, Mpumalanga, South Africa during July 1990 (Viljoen *et al.*, 1994). The isotype of *G. circinata* is deposited in herbarium B with no accession number.

The morphological characteristics of *G. circinata* produced in 10 crosses on carrot agar between members of MP-H of the *G. fujikuroi* complex were compared with those in the original description of *G. circinata*. In general, the perithecia and ascospore characteristics were consistent with those described by Nirenberg & O'Donnell (1998). However, perithecia in this study were 332–396–453 µm high and 288–337–358 µm wide, in contrast with the smaller perithecia (ca 325 µm high and 230 µm wide) reported by Nirenberg & O'Donnell (1998).

Isolates examined: Twenty-two *F. circinatum* isolates and 10 laboratory crosses between *F. circinatum* were examined (Table 1).

Morphological differentiation

Morphological characteristics of ex-type *Fusarium* spp. previously identified as *F. subglutinans* (Table 1) were examined to establish whether *F. circinatum* isolates could be distinguished from *F. subglutinans sensu lato*. All the *F. subglutinans sensu lato* isolates had obovoid microconidia. Oval to allantoid or fusoid microconidia are present in all *F. subglutinans sensu lato* isolates except in *F. guttiforme,* which only produces obovoid microconidia. Sterile coiled hyphae are only produced by *F. circinatum* and *F. pseudocircinatum* isolates. *F. circinatum* (MP-H), *F. pseudocircinatum* and *F. sacchari* (MP-B) produce sympodial conidiophores (defined by Nirenberg & O'Donnell (1998: 455) as "proliferating conidiophores–conidiophores with intercalary phialides often created by sympodially proliferating growth of the conidiophores"). Most of the *F. subglutinans sensu lato* isolates produce phialides with three and more conidiogenous openings, except for *F. begoniae, F. bulbicola* and *F. subglutinans* that produce three and fewer conidiogenous openings on the phialides. *F. guttiforme* (BBA 69661) produced sporodochia and 3-septate, falcate macroconidia with basal cell. Macroconidia with 3-5 septa are produced by *F. bulbicola, F. concentricum* and *F. subglutinans* whereas *F. begoniae, F. circinatum, F. guttiforme, F. pseudocircinatum* and *F. sacchari* produce 3-septate macroconidia. No short false chains were observed in *F. pseudocircinatum* (BBA 69636) (Nirenberg & O'Donnell, 1998) on CLA incubated under continuous black light.

DISCUSSION

Our study has provided sufficient information regarding the collector, date of collection and direct designation of the holotype specimen as specified by Article 37.3 of the ICBN (Greuter *et al.*, 1994) to validate the description of *G. circinata*. Results showed that the single cross used for the description for *G. circinata* is heterothallic, which is important because sexual compatibility tests are used to distinguish different mating populations in the *G. fujikuroi* complex. The variability of the morphological characteristics amongst a large collection of *F. circinatum* and *G. circinata* was consistent with those given in the original description of the species, except for minor

differences in the perithecial dimensions. Morphological characteristics such as conidial morphology, type of conidiophore branching and presence of sterile coiled hyphae were used to differentiate *F. circinatum* from *F. subglutinans sensu lato* isolates.

The cross between *F. circinatum* strains BBA 69720 (ex-type) and BBA 69722 used to describe *G. circinata* (Nirenberg & O'Donnell, 1998) was shown to be heterothallic. This was indicated by the 1:1 (*MAT*-1:*MAT*-2) mating type segregation of the ascospore progeny as well as the recombination indicated by vegetative compatibility tests, where progeny belonged to different VCGs than parent strains (BBA 67920 and BBA 69722). This removed any concern that the single cross might have been homothallic, an event previously observed in the related species, *F. sacchari (= F. subglutinans,* MP-B) (Leslie *et al.*, 1986; Britz *et al.*, 1999).

Fusarium circinatum is reproductively isolated from other species in the *G. fujikuroi* complex and resides in MP-H of the *G. fujikuroi* complex (Britz *et al.*, 1999). Sexual compatibility can be used as an alternative method to differentiate *Fusarium* spp. residing in different mating populations of the *G. fujikuroi* complex (Leslie, 1995). To accomplish the definition of mating populations, suitable tester strains are required. Suitable tester strains are hermaphrodites of opposite mating type that produce a fertile cross (Leslie, 1995). The strains BBA 69720 and BBA 69722 were identified as *MAT*-1 and *MAT*-2, respectively and were hermaphrodites. However, MRC 6213 and MRC 7488 have previously been identified as mating tester strains of MP-H (Britz *et al.*, 1999). MRC 6213 and MRC 7488 should preferably be used as the standard tester strains of MP-H of the *G. fujikuroi* complex, because they produce more perithecia exuding ascospores than crosses between BBA 69720 and BBA 69722.

Hyphal swellings were observed in some *F. circinatum* isolates. These swellings should, however, not be confused with chlamydospores, which could result in incorrect identification of this fungus. Nirenberg & O'Donnell (1998) based the description of the teleomorph on a single cross and consequently could not consider variability in the morphological characteristics. The observation of 3-septate, falcate macroconidia produced by *F. guttiforme* isolate (BBA 69661) facilitated the

differentiation of *F. circinatum* from *F. subglutinans sensu lato*. In the original description by Nirenberg & O'Donnell (1998) only 'conidia borne in aerial mycelium, obovoid, mostly 0-septate, occasionally 1-septate' were described for *F. guttiforme*. This illustrates the need to include a relatively large set of isolates displaying relevant characteristics, when describing new species of *Fusarium* (Burgess *et al.*, 1982).

Newly described *Fusarium* spp. that are morphologically similar to *F. subglutinans* were examined to identify differentiating morphological characteristics. *F. subglutinans sensu lato* isolates can be differentiated using conidial morphology, type of conidiophore branching, origin of the conidiophore from the substrate and presence of sterile coiled hyphae. In this study, we referred to "conidiophores formed by sympodial branching" (van Wyk *et al.*, 1991) as sympodial conidiophores, rather than proliferating conidiophores (Nirenberg & O'Donnell, 1998). Only *F. circinatum* and *F. pseudocircinatum* produce sterile coiled hyphae, but *F. pseudocircinatum* produces short false chains under continuous black light (Nirenberg & O'Donnell, 1998). This characteristic was not observed in our study in the ex-type culture of *F. pseudocircinatum,* under the same conditions. However, *F. circinatum* has erect conidiophores, whereas *F. pseudocircinatum* has prostrate conidiophores.

The use of morphological characteristics to identify fungi, that are very similar to each other, such as *Fusarium* spp., is time-consuming and difficult. This is particularly true for researchers not familiar with the taxonomy of this group of fungi. Sexual compatibility tests have been used to distinguish morphologically similar *Fusarium* spp., in the *G. fujikuroi* complex in the past. However, O'Donnell *et al.* (1998; 2000) and Steenkamp *et al.* (1999) have identified a number of conserved genes that can be used to distinguish *Fusarium* spp. in the *G. fujikuroi* complex. Rapid identification of closely related *Fusarium* spp. in the *G. fujikuroi* complex is possible with a PCR-RFLP technique based on histone *H3* gene sequences (Steenkamp *et al.*, 1999). This technique is reliable and provides the non-taxonomist with a valuable test for identification. *F. circinatum* can be differentiated from *F. subglutinans sensu lato* using morphological, genetic and molecular characteristics.

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Table 1. Origin and mating type of *Fusarium* strains in *Liseola* and related sections.

MRC no*	Other strain no§	Fertility‡	Mating type
F. circinatum South African isolates			
6208⊕		FS	*MAT*-1
6209	BBA 69854, NRRL 25621	FS	*MAT*-2
6213⊕		H	*MAT*-2
7448		H	*MAT*-2
7452		H	*MAT*-1
7454⊕	BBA 69722, NRRL 25333	H	*MAT*-2
7488e		H	*MAT*-1
F. circinatum California isolates			
7869	SL-1⊕, BBA 69720, NRRL 25331	H	*MAT*-1
7504	FSP 14	FS	*MAT*-1
7505⊕	FSP 48	FS	*MAT*-2
7506	FSP 52	H	*MAT*-2
7507	FSP 75	H	*MAT*-2
7508⊕	FSP 90	FS	*MAT*-1
F. circinatum Florida isolates			
7509⊕	FL 3	FS	*MAT*-1
7510	FL 17	H	*MAT*-1
7511	FL 19	FS	*MAT*-1
7512	FL 27	FS	*MAT*-2
7513⊕	FL 58	FS	*MAT*-1
F. circinatum Mexican isolates			
7568⊕	A1	FS	*MAT*-1
7570	A2	FS	*MAT*-1
7572	A3	-	-
7572	A5	FS	*MAT*-1
F. sacchari (MP-B) from sugarcane			
6524	KSU 3852, FRC-M6865	H	*MATB*-1
6525	KSU 3853, FRC-M6866	H	*MATB*-2

MRC no*	Other strain no§	Fertility‡	Mating type
F. subglutinans (MP-E) from maize			
6483	KSU 0990, FRC-M3696	H	*MATE*-2
6512	KSU 2192, FRC-M3693	H	*MATE*-1
F. begoniae			
7542	BBA 67781, NRRL 25300	-	-
F. bulbicola			
7534	BBA 13618, NRRL 63628	-	-
F. concentricum from banana			
7540	BBA 64354, NRRL 64354	-	-
F. guttiforme from pineapple			
7539	BBA 69661, NRRL 25295	-	-
F. pseudocircinatum			
7536	BBA 69636, NRRL 22946	-	-

* MRC, Medical Research Council culture collection, PROMEC, Tygerberg, South Africa.

§ BBA, NRRL, FSP, FL, FRC and KSU culture collection abbreviations explained in text. A = Original number of isolates collected in Mexico deposited in TPCP culture collection, FABI, University of Pretoria, Pretoria, South Africa.

‡ H= Hermaphrodite and FS = Female sterile.

⊕ Morphological characteristics examined of isolates in fertile crosses.

Fig. 1. (a) Holotype of *Gibberella circinata* (BPI 746094) of a cross between BBA 69720 and BBA 69722 on 5% carrot agar on a 90 mm Petri dish by Nirenberg & O'Donnell (1998) (bar = 12mm). (b) Cross of BBA 69720 and BBA 69722 produced on 5% carrot agar on a 65 mm Petri dish (bar = 12mm). (c) Perithecia with exuding ascospores produced in a cross between BBA 69720 and BBA 69722 on carrot agar (bar = 200µm). (d) Perithecia with exuding ascospores produced between mating testers, MRC 6213 and MRC 7488, on carrot agar (bar = 200µm).

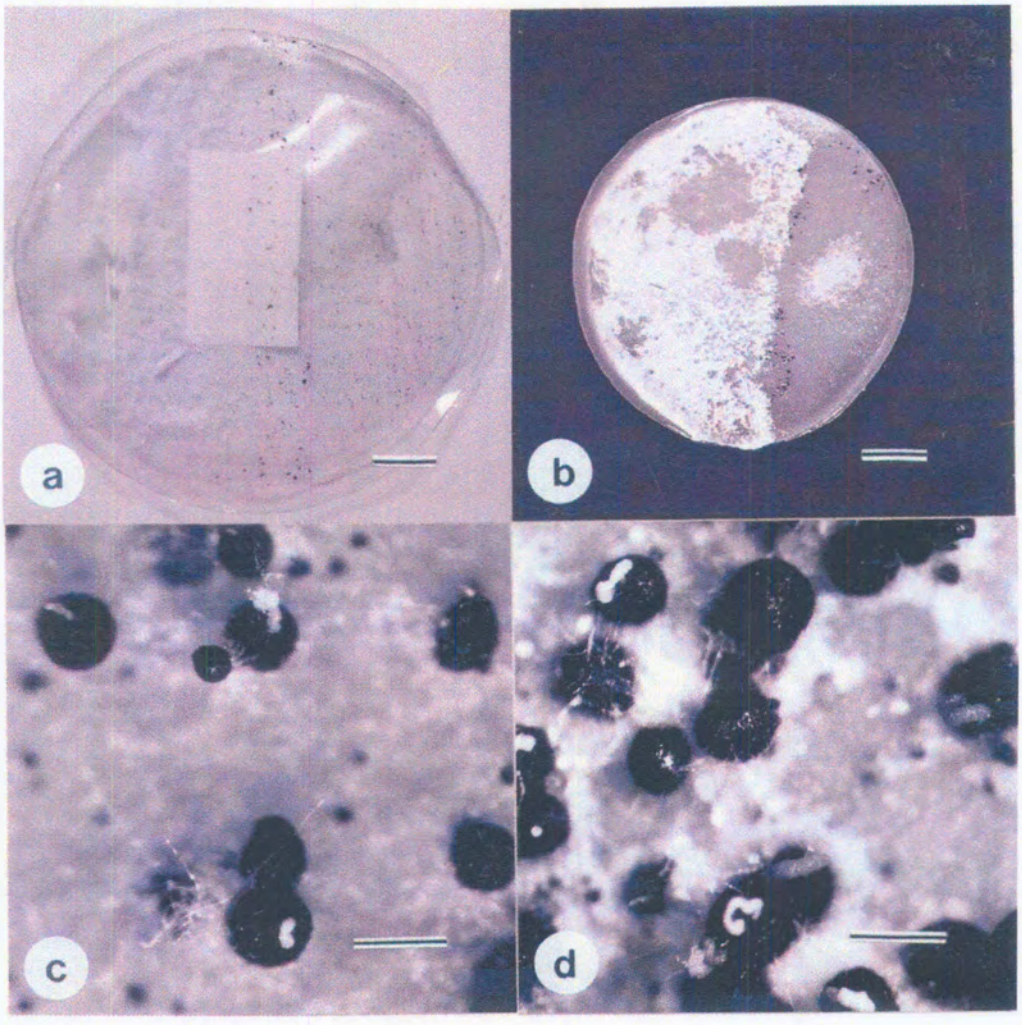
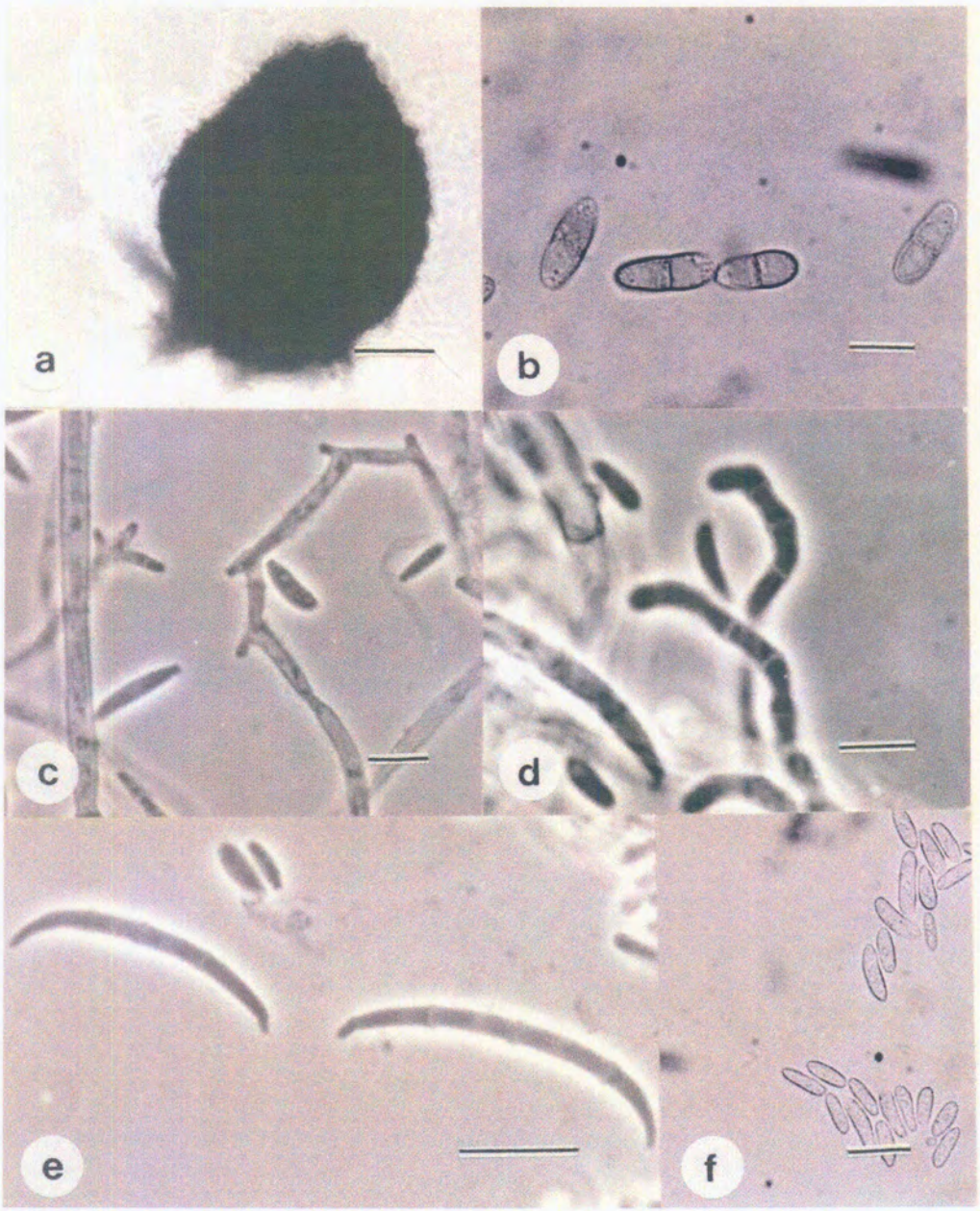


Fig. 2. Morphological characteristics of *G. circinata* and *F. circinatum* (CMW 6213). (a) Mature perithecia (bar = 100µm). (b) Septate ascospores (bar = 10µm). (c) Branched conidiophores (bar = 10µm). (d) Sterile coiled hyphae (bar = 10µm). (e) Slender and cylindrical macroconidia (bar = 10µm). (f) Obovoid microconidia (bar = 10µm).



CHAPTER 4

RELATEDNESS OF PITCH CANKER PATHOGEN POPULATIONS ASSESSED USING SEQUENCE CHARACTERIZED AMPLIFIED POLYMORPHISMS

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ABSTRACT

Fusarium circinatum is the causal agent of pitch canker disease of pines. This is one of the most important diseases of pine with various new epidemics having emerged in various parts of the world. Very little is known regarding the origin or population biology of *F. circinatum*. The aim of this study was, therefore, to develop co-dominant polymorphic molecular markers for *F. circinatum* and to determine the genetic relationships among populations of the fungus from California, Florida, Mexico and South Africa. The internal short sequence repeats (ISSR) PCR technique was used to develop the markers. Nine markers containing short simple repeats (SSR) sequences were developed. Alleles segregated in simple Mendelian ratios and tested loci were unlinked. Genetic diversity determined using these markers revealed the highest diversity in the Florida population while the lowest diversity was observed in the South African population. Furthermore, the Californian and Florida populations share lineages, which is evident from the close genetic distance between the populations. The origin of *F. circinatum* could not be determined with certainty although there is some evidence to suggest that the South African *F. circinatum* population originated in Mexico.

INTRODUCTION

The causal agent of pitch canker disease of pines, *Fusarium circinatum* Nirenberg & O'Donnell is morphologically similar to other *Fusarium* spp. associated with the *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura species complex. Sexual compatibility tests have shown some of these morphologically similar species to represent distinct biological species or mating populations (Leslie, 1991; 1995). Eight distinct mating populations (MP) in the *G. fujikuroi* complex, designated mating population A to H, have been identified (Leslie, 1991; Klaasen & Nelson, 1996; Klittich *et al.*, 1996; Britz *et al.*, 1999). *F. circinatum* and its teleomorph, *G. circinata* Nirenberg & O'Donnell (Nirenberg & O'Donnell, 1998), resides in MP-H of the *G. fujikuroi* complex (Britz *et al.*, 1999). Various molecular markers including RAPDs (Viljoen *et al.*, 1997a), isozymes (Huss *et al.*, 1996), β-tubulin gene (O'Donnell *et al.*, 1998), translation elongation factor 1α gene (EF-1α; O'Donnell *et al.*, 2000) and histone *H3* gene (Steenkamp *et al.*, 1999; 2000) have been used to distinguish *Fusarium* spp. in the *G. fujikuroi* complex from one another.

Fusarium circinatum was first reported causing pitch canker in the southeastern United States in 1945 and the disease spread rapidly throughout this region (Hepting & Roth, 1946; Dwinell *et al.*, 1985). In 1986, pitch canker was identified in landscape settings in California, predominately on *Pinus radiata* (Monterey pine) (McCain *et al.*, 1987). Subsequently, pitch canker has reached epidemic proportions in California where it is now causing extensive damage to Monterey pines in native forests (Storer *et al.*, 1994). Pitch canker has also been reported in Japan on native *P. luchuensis* (Muramoto *et al.*, 1988; Kobayashi & Muramoto, 1989) and it occurs on native pines in Mexico (Santos & Tovar, 1991; Guerra-Santos, 1999). In South Africa, the fungus has been confined to forest nurseries (Viljoen *et al.*, 1994) and is likely to have been recently introduced into South Africa (Viljoen *et al.*, 1997b; Britz *et al.*, 1998; 1999; Wingfield *et al.*, 1999).

Vegetative compatibility group (VCG) diversity in the Florida population, where *F. circinatum* has been established since the mid 1970's (Dwinell *et al.*, 1985), was reported to be high, with 45 VCGs among 117 isolates (Correll *et al.*, 1992). Correll *et al.* (1992) and Gordon *et al.* (1996) found the VCG diversity in the California population to be limited, which is consistent with a recently introduced pathogen and the absence, or rare occurrence of sexual reproduction. A relatively high VCG diversity in the South African population could be attributed to a fairly high level of sexual reproduction (Viljoen *et al.*, 1997b; Britz *et al.*, 1998).

Recently, Wikler & Gordon (2000) assessed the genetic relationship of *F. circinatum* populations from different areas, using eight polymorphic molecular markers and VCG tests. Those authors found a single VCG among five *F. circinatum* isolates from Japan. This single VCG was also found in the Californian population suggesting that the origin of the pitch canker in Japan may be California. The molecular markers also showed that the California and Japanese populations of *F. circinatum* share lineages with the Florida population (Wikler & Gordon, 2000). The Mexican population had the highest genetic diversity and seven VCGs were present among 10 *F. circinatum* isolates tested (Wikler & Gordon, 2000). These results supports the suggestions of Guerra-Santos (1999) that the VCG diversity would be high in Mexico since this is thought to be the origin of the pathogen (Wikler & Gordon, 1999; 2000).

PCR-based genetic markers, such as sequence characterized amplified regions (SCARs) (Paran & Michelmore, 1993) and simple sequenced repeats (SSRs, also known as microsatellites) (Tautz, 1989), are increasingly being used in population genetic studies. This is because these markers are polymorphic and exhibit codominance (Engel *et al.*, 1996; Moon *et al.*, 1999). Several techniques have been developed to exploit the highly polymorphic SSRs, which are short DNA repeats occurring throughout the genome (Cifarelli *et al.*, 1995; Rafalski *et al.*, 1996; Ender *et al.*, 1996). The internal short sequence repeat (ISSR)-PCR method was developed where ISSR fragments were sequenced and primers were designed to flank SSR motifs in the sequence (Dusabenyagasani *et al.*, 1998; van der Nest *et al.*, 2000; Burgess *et al.*, 2001). The polymorphisms in both these SCAR and SSR markers, could then be detected using length variation of the PCR product.

In this study, we have developed polymorphic markers for *F. circinatum* using the ISSR-PCR technique (van der Nest *et al.*, 2000; Burgess *et al.*, 2001). The markers were tested for their ability to differentiate amongst *F. circinatum* isolates from various geographical areas. Mendelian inheritance of the progeny was confirmed from crosses between *F. circinatum* isolates. Interspecies amplification was tested using tester strains for the eight different mating populations of the *G. fujikuroi* complex. Allelic differences at these polymorphic loci were, furthermore, used to measure the relationships between populations of *F. circinatum* in California, Florida (southern United States), Mexico and South Africa.

MATERIALS & METHODS

Isolates

Seventy-two *F. circinatum* isolates were included in this study (Table 1). These included 12 isolates from Mexico, 21 from California and 19 from Florida that were from collections described previously (Correll *et al.*, 1992; Gordon *et al.*, 1996; Wikler & Gordon, 2000; Britz *et al.*, 2001). Twenty South African isolates from the original nursery outbreak in 1990-1992 (Viljoen *et al.*, 1994) were also included (Table 1). Sample sizes and sampling methods varied due to the fact that different collaborators in different locations supplied isolates. In some cases, isolates were

collected under reasonably difficult circumstances and large collections could not be obtained.

Tester strains of MP-A (*F. vercillioides,* MRC 6191), MP-B (*F. sacchari,* MRC 6525), MP-C (*F. fujikuroi,* MRC 6571), MP-D (*F. proliferatum,* MRC 6568), MP-E (*F. subglutinans,* MRC 6512), MP-F (*F. thapsinum,* MRC 6536) and MP-G (*F. nygamai*, MRC 7548) of the *G. fujikuroi* complex were used in this study (Leslie, 1991; Klaasen & Nelson, 1996; Klittich *et al.,* 1997; Britz *et al.,* 1999). All the isolates are stored in 15% glycerol at –70°C in the *Fusarium* culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, Pretoria, South Africa. Representatives of the *F. circinatum* isolates used in this study have also been deposited in the culture collection of the Medical Research Council (MRC) at Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), P. O. Box 19070, Tygerberg, South Africa.

DNA extraction

Isolates (Table 1) were grown in Eppendorf™ tubes containing 0.5 ml complete media broth (CM) (Correll *et al.,* 1987) for five days at 25°C. After centrifugation, the broth was discarded and the harvested mycelium was freeze-dried. The mycelium was kept frozen with liquid nitrogen and ground to a fine powder using a pestle designed to fit into the Eppendorf tubes. The powdered mycelium was dispersed in 400 µl of CTAB buffer containing 1.4 mM NaCl, 20 mM EDTA, 10 mM Tris-HCl (pH 8.0), 1% 2–Mercaptoethanol (v/v), 1 % PVP (w/v) and 5% hexacyltrimethylammonium bromide (w/v) and placed in a 65°C water bath for 60 min. Equal volumes of phenol:chloroform (1:1, v/v) were added to the supernatant, vortexed and centrifuged for 60 min at 13 000 rpm. The aqueous phase was further purified by chloroform extraction. Subsequently, 2 volumes of absolute alcohol and 0.1 volumes of 3M sodium acetate (pH 5.4) were added to the aqueous phase to precipitate the nucleic acid. The resulting pellets were washed with 70% ethanol. The DNA pellets were dried and resuspended in sterile deionized water and stored at –20°C.

Marker development and screening

ISSR-PCR fragments from *F. circinatum* isolates, MRC 7601 (Mexico) and MRC 7484 (South Africa), were produced using primers 5'BDB$(ACA)_5$, 5'DDB$(CCA)_5$, 5'DHB$(CGA)_5$, 5'YHY$(GT)_5$G, HVH$(GTG)_5$, 5'NDV$(CT)_8$, and 5'HBDB$(GACA)_4$ (Lieckfeldt *et al.*, 1993; Buscot *et al.*, 1996; Hantula *et al.*, 1996) as previously described by Burgess *et al.* (2001). Amplified products were visualized on a 2% agarose gel containing ethidium bromide (0.1 µg/ml).

The ISSR fragments amplified with individual primers were purified using the High Pure™ PCR product purification kit (Roche Diagnostics, Germany). The purified products were ligated overnight at 4°C into the pGEM®-T vector using the pGEM®-T Easy Vector System (Promega, USA). Ligation products were transformed into competent *Escherichia coli* JM109 cells (Promega, USA) and screened on LB medium containing 80 µg ml^{-1} X-Gal, 0.5 mM IPTG and 100 µg ml^{-1} ampicillin. White colonies were selected and grown in LB broth containing 100 µg ml^{-1} ampicillin. The plasmids were extracted from individual colonies using the alkaline lysis method (Sambrook *et al.*, 1989) and cut with restriction enzyme, *Eco*R1 to determine insert size. Plasmids containing inserts in the size range of 300 -1500 base pairs (bp) were sequenced. Both strands were sequenced using universal primers, T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-TATTTAGGTGACACT ATAG-3') using the BigDye terminator cycle sequencing kit (Perkin Elmer Applied Biosystems). The sequenced products were analyzed using an ABI 377 DNA sequencer (Perkin Elmer Applied Biosystems). Resulting electropherograms were analyzed using the Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems).

Tandem repeats ($n \geq 2$) were identified in the DNA sequences. Although these tandem repeats were not simple microsatellite repeats, they provided a basis for selecting primer sequences most likely to amplify microsatellite-like regions. Primer pairs flanking tandem repeats, amplifying 100 – 350 bp product with a T_m (annealing temperature) between 55 and 61°C were selected with the aid of the computer program, Primer 3.1 (Table 2).

The same PCR conditions described (above) were used on eight *F. circinatum* isolates (MRC 7460, MRC 7484; MRC 7598, MRC 7601, MRC 7689, FCC 2500, FCC 2501 and FCC 2513) to amplify sequence characterized amplified regions with the designed primer pairs (Table 2). Polymorphisms between the amplified PCR products were identified by separating the PCR products using polyacylamide gel electrophoresis (PAGE) (6% polyacylamide in 50 mM TBE buffer for 7 h at 140 V) and visualizing the product by silver staining (Bassam & Cactano-Annollés, 1993). Nine primer pairs (Table 2) amplifying PCR fragments revealing polymorphisms, when using DNA isolated from the eight *F. circinatum* isolates, were selected for further study. The forward primer of each set was 5'-end labeled with a phosphoramidite fluorescent dye (Life technologies), to facilitate analysis of the *F. circinatum* populations using an ABI 377 sequencer.

PCR reactions using the nine primer pairs (Table 2) were performed in 15 µl volumes, containing PCR buffer (10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl), 50 µM of each dNTP, 0.2 µM of forward primer labeled with a phosphoramidite fluorescent dye, 0.2 µM of unlabeled reverse primer, 0.5 U *Taq* polymerase and 1 µl DNA. The reaction conditions were: 5 min initial denaturing at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C with a final extension of 72°C for 5 min.

Fluorescent labeled PCR products (0.5 µl of a 1/15 dilution) were added to 2 µl of sequencing buffer (0.5 volumes of formamide to 0.1 volumes of blue dextran) and 0.5 µl of GS-500 TAMRA (Perkin-Elmer Corp.). A 2 µl portion of the mixture was separated by PAGE on an ABI Prism 377 DNA sequencer. The sizes of the alleles amplified with the nine selected primers (Table 2), were measured in base pairs from the electrophoretic mobility through the gel, relative to the internal size standard (GS-500 TAMRA) as indicated by GenScan 2.1 program (Perkin Elmer Corp.).

Evaluation of Sequence characterized polymorphic loci

The amplified alleles were tested for Mendelian segregation. Progeny from crosses between tester strains of MP-H of the *G. fujikuroi* complex (MRC 6213 and MRC 7488) and *F. circinatum* strains MRC 7689 and MRC 7454, were used. These crosses were made on carrot agar as previously described (Klittich & Leslie, 1988; Britz *et*

al., 1998) at 17°C. Ten progeny were collected from each fertile cross. The parents and progeny of the cross between MRC 6213 X MRC 7488 were studied using six of the *F. circinatum* polymorphic loci (FC-2, 4, 5, 6, 7 and 9). The parents and progeny strains from the cross between MRC 7689 and MRC 7454 were studied using five of the loci (FC-2, 4, 5, 6 and 9).

Primer sets HB 12 and 13 (locus FC-3) and HB 14 and 15 (locus FC-4) were found to amplify different size alleles in *F. circinatum* isolates, MRC 6213, MRC 7689 and FCC 2513. The amplicons of these isolates using both primer pairs were sequenced to determine the nature of the polymorphism in each case. The sequence characterized polymorphisms of the desired loci, were then sequenced using the respective forward primer and analyzed using the Sequence Navigator version 1.0.1™ (Perkin Elmer Corp.).

Nucleotide sequence accession numbers:

The sequences for each of the loci (FC1-9) have been deposited in the Genbank database with accession numbers AF430131-AF430139 (sequences in Appendix 1).

Genetic diversity and population analyses

The allele frequency of each locus was calculated for each geographical region using the program Microsat (Microsatellite distance program, http://human.stanford.edu/microsat). The total genetic diversity was established using the equations of Nei & Chesser (1983): These included (i) the total genetic diversity within each sampling region: $H_{TR} = [(n)/(n\text{-}1)](1\text{-}\Sigma p_i^2)$, where n is the sample size and (ii) the total genetic diversity of all the sampling areas: $H_T = [(\tilde{n})/(\tilde{n}\text{ -}1)](1\text{-}\Sigma p_i^2)$, where $\tilde{n}$ is the mean of the regional sample size.

Genetic distance between populations was estimated by using two different algorithms calculated as defined in Microsat (http://hpgl.Stanford.EDU/ microsat/distance.html): (i) The genetic distance measure *Fst,* as estimated by Reynolds *et al.* (1983) and (ii) *Ds* as defined by Nei (1978) (Table 4). The program Migrate version 9.0 (http://evolution.genetics.washington.edu/lamarc.html) was used to calculate maximum likelihood estimates for migration rate and effective population

sizes of two populations using the infinite allele model (Table 4). In the Migrate program calculations was made using the following: (i) populations exchanging migrants with rate m_i per generation with the effective population size, N_e; (ii) parameters are scaled by mutation rate μ, which is calculated with allele data per site per generation; (iii) estimated parameters are, therefore: $\Theta_i = 2\ N_e^{(i)}\ \mu$ and $M_i = m_i/\mu$, the migration estimate is expressed as $2\ Nm\ =\ \Theta\ M$ (http://evolution.genetics.washington.edu/lamarc.html) (Table 4).

RESULTS

Marker development and screening

All of the primers amplified more than five ISSR fragments using DNA isolated from *F. circinatum* isolates, MRC 7601 and MRC 7488. Fifty-two clones containing putative ISSR-PCR inserts were obtained and sequenced. No perfect tandem repeats of longer than six repeats were identified from the sequenced clones (see Appendix 1). Twenty-three primer pairs (Table 2) were designed from the 52 DNA sequences.

The primer pairs were tested for polymorphisms using DNA from eight *F. circinatum* isolates (MRC 7460, MRC 7484; MRC 7598, MRC 7601, MRC 7689, FCC 2500, FCC 2501 and FCC 2513). The fragments sizes in base pairs were determined using PAGE. No DNA fragments were amplified for three of the primer pairs when using the eight *F. circinatum* isolates (Table 2). Seven of the primer pairs produced PCR amplicons, which were monomorphic using all eight *F. circinatum* isolates and five of the primer pairs resulted in the production of multiple bands (Table 2). The remaining nine primer pairs, all of which produced polymorphic DNA fragments when using DNA from the eight representative *F. circinatum* isolates (Table 2), were appropriate for use in this study.

Primer pairs amplifying loci FC-5 and FC-6 produced null alleles for some isolates. In every case where no PCR product was obtained, a PCR reaction where the annealing temperature was lowered by 2°C and the $MgCl_2$ concentration was increased to 3 mM were done. The samples containing the 'null allele' products were also separated, undiluted by PAGE on an ABI Prism 377 DNA sequencer. Not even

faint products were observed thus confirming that no product was amplified and a null allele was recorded.

Evaluation of Sequence characterized amplified polymorphisms

The parent and progeny strains produced in the cross MRC 6312 and MRC 7488 had the same size allele for loci FC-4, FC-6 and FC-7. DNA from these parent strains resulted in the amplification of different size alleles at loci FC-2, FC-5 and FC-9. Each allele from one of the parents appeared in approximately 50% of the progeny (1:1 ratio). No linkage was detected between any of the loci tested.

The parent and progeny strains produced in the cross between MRC 7689 and MRC 7454 had the same size allele for locus FC- 2 and FC-6. DNA from parent strains resulted in the amplification of different size alleles at loci FC-4, FC-5 and FC-9. Each allele was passed onto the progeny in approximately 50% of the progeny (1:1 ratio) and no linkage was detected between any of the segregating loci.

Interspecific differences were observed amongst the *G. fujikuroi* tester strains (Table 1). Alleles at loci FC-1, FC-3 and FC-7 were polymorphic between *G. fujikuroi* tester strains. Primer pairs amplifying loci FC-1 and FC-3 could thus be used to distinguish between the tester strains of all the mating populations used in this study.

Sequence differences between PCR fragments for loci FC-3 and FC-4 were determined for three *F. circinatum* isolates. Locus FC-3 contained perfect $(CAACACCT)_2$ and $(CA)_4$ repeats and an imperfect $(CCT)_3$ repeat (Genbank accession number AF430133). Locus FC-4 contained imperfect $(CT)_3$ and $(GT)_3$ repeats using isolate MRC 7484 (Genbank accession number AF430134). The sequence at locus FC-3 was identical for isolates MRC 6213 and MRC 7689. The sequence at locus FC-3 for isolate FCC 2513 had single mutations and random insertions and deletions over the length of the allele, when compared with the sequences of the same locus in isolates MRC 6213 and MRC 7689. The sequence at locus FC-4 was identical for isolates FCC 2513 and MRC 6213. However, the DNA product amplified from the same locus of isolate MRC 7689 had single mutations and random insertions and deletions. These consisted of one to five bases, over the length

of the allele when compared with the sequence for the same locus in isolates FCC 2513 and MRC 6213.

Allele frequencies

The allele frequencies at each of the nine selected markers estimated in the different geographical populations of *F. circinatum* revealed intraspecific differences (Table 3). There were 32 putative alleles amongst the nine sequence characterized amplified polymorphic markers (Table 3). The number of alleles at each locus ranged from two to five, with an average 3.56 alleles per locus. Amongst the 72 *F. circinatum* isolates, there were 15 unique alleles found only at a single location (referred to as private alleles by Goodwin *et al.*, 1992). Six unique alleles were found in the South African isolates. Two unique alleles were found in Mexican isolates, whereas isolates from both the Florida and Californian populations had four and three unique alleles, respectively (Table 3).

Allelic and genetic diversity

Without adjustment for sample size, the genetic diversity within each geographical population ranged from 0.151 in Mexico to 0.212 in Florida (Table 4). With adjustment for sample size, the allelic diversity within each geographical population (H_{TR}) ranged from 0.165 in Mexico to 0.212 in Florida (Table 4). Mexican isolates had the lowest allelic diversity followed by isolates from South Africa and California. Florida isolates had the highest allelic diversity. The total genetic diversity, H_T, was 0.180.

Genetic distance and migration

The values of *Fst* ranged from 0.060 to 0.439 and those of *Ds* ranged from 0.009 to 0.118 (Table 4). Based on both these measures of genetic distance, isolates from California and Florida are most closely related. In contrast, isolates from Florida and California were most distantly related to isolates from South African. The isolates from Mexico were most closely related to those from South Africa.

The migration parameter ($2Nm$) indicates the migration from one population to another population and it also reflects the direction of migration. The values of the migration parameter for the various populations studied ranged from 1.362 to 10.460

(Table 4). The highest migration from one population to another was from the Californian population into Mexico (*2Nm* = 10.460). The migration parameter also show migration from the Mexican into the South African population (*2Nm* = 7.031).

DISCUSSION

Nine sequence characterized amplified polymorphic markers were developed for analysis of population diversity in *F. circinatum*. Testing of the markers on progeny from two *F. circinatum* crosses indicated the tested loci are inherited in a simple co-dominate Mendelian fashion. Thus, these polymorphic co-dominant markers could subsequently be used to determine the genetic relationships of *F. circinatum* populations available to us and collected in different parts of the world.

In the present study, we used the ISSR-PCR technique (van der Nest *et al.*, 2000; Burgess *et al.*, 2001) that targets only those regions of the genome that are rich in SSR motifs. Sequenced ISSR-PCR fragments generated from amplified *F. circinatum* DNA had no more than six repeats and the polymorphism among *F. circinatum* loci was predominantly due to single mutations and random insertions and deletions. This is similar to polymorphisms generated with RAPDs and reflect point mutations, insertions or deletions of sequences rather than SSRs (Paran & Michelmore, 1993). These results might indicate that SSR motifs are not as large or abundant in fungal genomes as they are in animal and plant genomes. However, the large number of ISSR-PCR fragments amplified with each primer set suggests SSR motifs are not uncommon in the fungal genome.

Only two of the nine polymorphic markers for *F. circinatum* produced null alleles. Null alleles indicate the presence of polymorphism in the sequence flanking the sequence characterized amplified polymorphic regions resulting in the primer(s) no longer binding or a fragment too large to be amplified. High incidences (30%) of null alleles have been found in various eukaryotes (Callen *et al.*, 1993; Lehmann *et al.*, 1996) and the presence of null alleles (frequency of 22%) in this study is not without precedent.

The polymorphic markers developed in this study revealed interspecies differences amongst the mating populations of the *G. fujikuroi* complex. However, only single

strains representing each mating population were tested and further testing on a larger set of isolates would be required to determine the extent of the interspecies differentiation. Nonetheless, the markers showed polymorphisms both between mating populations of *G. fujikuroi* and within *F. circinatum.* They could, therefore, be applied as a diagnostic tool to distinguish closely related *Fusarium* spp. in the *G. fujikuroi* complex. This is in addition to their value in analyzing the genetic composition of *F. circinatum* populations.

Allelic diversity in this study indicates the *F. circinatum* isolates in Florida are highly diverse with the lowest diversity in the South African population. This is consistent with the results of Correll *et al.* (1992) who reported a high VCG diversity in the Florida population. This high allelic and VCG diversity in the Florida population supports the view that pitch canker is well established in that area (Dwinell *et al.*, 1985). The low allelic diversity in the South African population found in the present study is consistent with a recently introduced pathogen (Viljoen *et al.*, 1997b; Britz *et al.*, 1998; Wingfield *et al.*, 1999). However, Viljoen *et al.* (1997b) found a relatively high VCG diversity in the South African population. This led them to believe sexual reproduction is occurring which would result in the segregation of multiple vegetative incompatible loci. Even though a relatively larger number of VCG's are found in the South African population, it appears from the genetic analysis in this study that they are closely related. It thus confirms the notion of a recent introduction of the pathogen in South Africa (Viljoen *et al.*, 1997b; Britz *et al.*, 1998; 1999)

Mexico is thought to represent the area of origin of *F. circinatum* (Wikler & Gordon, 1999; 2000). The low level of allelic diversity for the Mexican isolates in this study is in contrast with high level of genetic diversity reported for Mexican isolates by Wikler & Gordon (2000). In this regard, our results should be viewed with caution. This is because small number of isolates were included and also because many of the isolates were collected from the same tree (Britz *et al.*, 2001). It has been valuable to include Mexican isolates, because results of our study indicated that Mexico represents a center of origin for *F. circinatum.* Furthermore, migration parameters in our study showed a high level of immigration from California into Mexico (*Nm* = 10.460). For the same reason given above, this is probably not an accurate reflection of the situation.

Various authors have hypothesized that pitch canker was introduced in South Africa from Mexico (Wikler & Gordon, 1999; 2000). Results of our study showing a relative close genetic distance between South Africa and Mexico support this view. Furthermore, the fact that isolates from both areas share an allele (locus FC-9, allele 242), that is present only in these populations, supports this hypothesis. The migration parameter used in this study indicates a high level of *F. circinatum* immigrants from Mexico into the South African populations ($2Nm$ = 7.031). This statistic also indicates the direction of *F. circinatum* migration to be from Mexico to South Africa. This is consistent with the view that *F. circinatum* was introduced into South Africa from Mexico (Wikler & Gordon, 1999; 2000).

In the present study, the closest genetic distance observed between populations was between those from California and Florida. These two populations also shared two alleles (Table 3) that were not present in isolates from other regions. These findings verify those of Wikler & Gordon (2000) who showed a connection between California, Japanese and southeastern United States (including Florida) populations of *F. circinatum*. The migration parameters calculated in the present study also reflects the close relationship between California and Florida populations, which is consistent with the findings of Wikler & Gordon (2000).

Sequence characterized amplified polymorphic markers developed in this study could be used to analyze the population diversity and distance within and between different geographical populations of *F. circinatum*. In general, our developed markers can be used as genetic tools that should be valuable to other researchers interested in analyzing genetic diversity of *F. circinatum* populations. The markers should also be useful to further test hypotheses relating to the origin of pitch canker.

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Table 1. Allele size in base pairs at nine loci of *Fusarium* isolates from different geographical areas.

Isolates		Locus[c]								
FCC[a]	Other no[b]	FC-1	FC-2	FC-3	FC-4	FC-5	FC-6	FC-7	FC-8	FC-9
South African population										
0566	MRC 6213	314	185	285	263	237	222	257	139	244
0521	MRC 7445	314	185	285	263	237	222	257	139	244
0516	MRC 7472	314	185	285	263	237	222	257	139	244
0887	MRC 7481	314	185	285	263	237	222	257	139	244
0159	MRC 7484	314	185	285	263	237	222	257	139	242
0119	MRC 7488	314	183	285	263	235	222	257	139	242
0854	MRC 7479	314	185	285	263	237	222	257	139	244
0309	MRC 7491	314	185	285	263	237	222	257	139	242
0563	MRC 7493	314	185	285	263	237	222	257	139	242
0306	MRC 7492	312	185	285	269	237	220	257	139	244
0523	MRC 7494	314	183	285	263	237	222	257	139	244
0127	MRC 7454	314	185	285	263	237	222	257	139	244
0124	MRC 7460	314	185	285	263	237	222	259	139	244
0514	MRC 7461	312	185	285	263	235	222	257	139	244
0424	MRC 7468	314	183	285	263	237	222	259	139	244
0507	MRC 7447	314	185	285	263	237	222	257	139	242
1031	MRC 7451	314	185	285	263	237	222	257	139	248
0479	MRC 7470	314	185	285	263	237	222	257	139	244
0500	MRC 7455	314	185	285	263	237	222	257	139	244
0340	MRC 7482	314	185	285	263	237	222	257	139	244
Florida population										
2480	MRC, FL102	314	185	285	263	237	222	257	139	246
0132	MRC6229, FRC-M3824	314	185	285	263	237	222	257	139	246
2476	MRC 7509, FL3	314	178	285	263	237	222	257	139	246
2472	FL11	314	185	285	263	237	222	257	139	246
554	FL107	314	185	285	263	237	222	257	139	246
2474	MRC 7439, FL15	314	183	285	263	239	222	257	139	246
2494	MRC 7510, FL17	314	185	285	263	237	222	257	139	246
2469	MRC 7511, FL19	314	183	285	263	237	222	257	139	246
2507	FL20	314	185	285	263	237	222	257	139	246
2509	MRC 7512, FL27	314	185	285	263	239	222	257	139	246
2479	FL88	314	185	285	263	239	222	257	139	246

Isolates		Locus[c]								
FCC[a]	Other no[b]	FC-1	FC-2	FC-3	FC-4	FC-5	FC-6	FC-7	FC-8	FC-9
Florida population										
2485	FL119	314	185	285	263	237	222	257	139	246
2500	FL57	314	191	279	263	237	229	257	123	246
2501	MRC 7513, FL58	314	191	292	270	237	229	257	139	246
2506	FL49	314	191	292	270	237	229	257	139	246
2493	FL72	314	185	285	263	233	222	257	139	246
2481	MRC 7437, FL103	314	185	285	263	233	222	257	139	246
2478	FL93	314	185	285	263	237	222	257	139	246
2482	FL105	314	185	285	263	233	222	257	139	246
Mexican population										
2248	MRC 7568	314	185	285	263	237	216	257	139	246
2253	MRC 7573	318	185	285	263	237	222	257	139	246
2257	MRC 7577	314	185	285	263	237	222	257	139	246
2271	MRC 7589	314	183	285	263	237	222	257	139	242
2288	MRC 7601	314	185	285	263	237	222	257	139	242
2276	MRC 7587	314	185	285	263	237	222	257	139	246
2249	MRC 7569	314	185	285	263	237	222	257	139	246
2282	MRC 7598	318	185	285	263	237	222	257	139	246
2272	MRC 7590	314	183	285	263	239	222	257	139	242
2270	MRC 7588	314	185	285	263	237	222	257	139	246
2274	MRC 7592	314	185	285	263	239	222	257	139	246
2279	MRC 7596	314	185	285	263	237	222	257	139	246
Californian population										
2519	CMW 3195	314	185	281	263	Null	222	257	139	246
2526	CMW 3197	314	183	281	263	237	222	257	139	246
2513	CMW 3193	314	183	281	263	237	222	257	139	246
2510	CMW 3194	314	183	281	263	237	Null	257	139	246
2514	CMW 3196	314	185	281	263	237	222	257	139	246
2511	CMW 3198	314	185	285	263	237	Null	257	139	246
2518	CMW 3199	314	185	285	263	237	222	257	139	246
0158	MRC 7689, SL-1	314	185	285	260	235	222	257	139	246
0986	FSP34	314	185	285	263	237	222	257	139	246
2204	CMW3380, FSP75	314	185	285	260	237	222	257	139	246
2220	CMW3384, FSP62	314	185	285	260	237	222	257	139	246
2229	CMW3382, FSP121	314	185	285	263	235	222	257	139	246
2228	CMW 3373, FSP9	314	185	285	263	237	222	257	139	246
2229	CMW 3377, FSP48	314	185	285	263	235	222	257	139	246

Isolates		Locus[c]								
FCC[a]	Other no[b]	FC-1	FC-2	FC-3	FC-4	FC-5	FC-6	FC-7	FC-8	FC-9
Californian population										
2222	CMW 3374, FSP14	314	185	285	263	237	Null	257	139	246
2201	CMW 3376, FSP32	314	185	285	263	237	222	257	139	246
2243	CMW3385, FSP44	314	185	292	270	237	229	257	139	246
2227	CMW3386, FSP84	314	185	285	263	237	222	257	139	246
2226	CMW3388, FSP117	314	185	285	270	237	222	257	139	246
2471	CA462	314	185	285	263	237	Null	257	139	246
2473	CA461	314	185	285	263	237	222	257	139	246
Gibberella fujikuroi mating populations										
2423	MRC 6191	314	185	298	263	237	222	273	139	244
2441	MRC 6525	301	185	298	263	237	222	257	139	244
2431	MRC 6571	314	185	296	263	237	222	257	139	244
2437	MRC 6568	314	185	292	263	237	222	257	139	244
2444	MRC 6512	314	185	287	263	237	222	248	139	244
2446	MRC 6536	314	185	296	263	237	222	255	139	244
	MRC 7548	314	185	301	263	237	222	244	139	244

[a] FCC = *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

[b] MRC = Medical Research Council culture collection, Tygerberg, South Africa; FRC = *Fusarium* Resource Center, Pennsylvania State University, USA; CMW = Culture collection of the TPCP, FABI, University of Pretoria, Pretoria, South Africa; FSP, CA and FL = *F. circinatum* isolates from T. R. Gordon, Department of Plant Pathology, University of California, USA.

[c] The allele size in base pairs at nine different loci (indicated as FC (= *F. circinatum*) 1 to 9).

Table 2. Nucleotide sequences of primers designed from sequence characterized amplified regions.

Primer (HB)	Primer sequence (5'→3')	T_m	PCR product	Product size (bp)[a]	Isolate (MRC)[b]	Dye label[c]
1	CATGGTGATCTTGCTGCTG	59.7	Multiple bands	199	7601	-
2	CGCACACTCTTGGTCCTAC	59.8				
3	CGATACGTCGTCCTAAAGGC	60.0	Single band	144	7601	-
4	CTTCAGCGAACACAAGGTCA	60.0				
5	CACTCTAGGCATCCTTTGGG	59.7	Single band	207	7484	-
6	CCATATCGTTGAAGAGCCG	60.1				
7	CGTCCATAAGCAACTCCGAT	60.1	Single band	314	7601	6-FAM
8	ATAAAGGTCGCGGAAGGTCT	60.1				
9	TCAATACCCCTCGCCTAGAA	59.7	HB 9 and HB10: Single band	185	7601	-
10	GACCACAGCCTCGAGAACAT	60.1				
11	CCACACTGCATTCTAGCCAA	59.7	HB10 & HB 11: Single band	251	7601	6-FAM
12	TCAATGAAAAGCAGCACGTC	55.3	Single band	285	7484	TET
13	TTTAGCTTGATGGCGAGTCC	55.3				
14	TTCCACCATGAGAGGAAACCC	57.3	Single band	263	7484	HEX
15	CCATTGCCAATCTTGATCCT	57.3				
16	ATAAGTTGACAACCGCCGTC	57.3	Single band	303	7484	-
17	GTAGCAGGAGCTTCCTGTGG	58.4				
18	ATATTCTGACGGGTCCACCA	57.3	Single band	237	7601	6-FAM
19	ACGGTCTCACAATGGCTTTC	57.3				
20	GGTGAGGAAAACAAGAGCCA	57.3	Single band	222	7601	TET
21	CCTCAGCTAGCCCATAACGA	57.3				
22	ACATGTAGACGACGCTGCAC	57.9	No amplification	207	7484	-
23	GCTGTCCTTGACATTGCAGA	57.9				
24	AATGACCCTTGATTTGCGAC	57.8	No amplification	103	7484	-
25	TGATCGATATCTTCCTCCGC	58.7				
26	ACGGTCTCACAATGGCTTTC	57.3	Single band	257	7484	6-FAM
27	GGAACCAGCCATACACGATT	57.3				
28	GAGAAGAGTGGCAGGGACTG	61.4	Single band	139	7484	HEX
29	GGGCTAATAGAACAGCAGCG	61.4				
30	AGGAAGCATGTCTGGCTGAT	57.8	Multiple bands	141	7484	-
31	ATTCCCTGGAACCTGCCTAT	57.7				
32	GACAGACATGATGATGG	50.4	Single band	226	7484	-
33	ACACTGTACGAATGCCCCTC	57.7				

Primer (HB)	Primer sequence (5'→3')	T_m	PCR product	Product size (bp)[a]	Isolate (MRC)[b]	Dye label[c]
34	TGAAGAGATGGAAGCTTCAGA	55.3	Single band	242	7484	6-FAM
35	GGTTTCCTCTCATGGTGGAA	57.3				
36	GTGGATGTCAACCATGCATG	57.3	Single band	278	7484	-
37	CACTGTTGGGAATGCTCTGA	57.3				
38	CGATACAAGCTTGACGCAAT	55.3	Multiple bands	110	7484	-
39	AATTTCATCATCACAGAATG	49.1				
40	ACTGGCTTGTGCCTCTGTAG	59.4	Multiple bands	174	7484	-
41	ACGACGAAAATGTGAGACCC	57.3				
42	AAGTTTTTGCTGGGGTGCGC	55.3	Single band	176	7484	-
43	TATCCTCGACACTGCAGGC	58.8				
44	GCGGGAATTCGATTCCCGTG	61.4	Multiple bands	225	7484	-
45	GAGGCTGTTGCCGATGTTAT	57.3				
46	CTCCCTCTGTGGTTCCCTCT	61.4	No amplification	138	7484	-
47	AGCTAGACGCAATCGGGATA	57.3				

[a] Product size for isolate indicated in next column.

[b] MRC = Medical Research Council culture collection.

[c] Forward primer 5' end labeled with a phosphoramidite fluorescent dye.

Table 3. Sequence characterized amplified polymorphic allele frequencies in different geographical populations of *Fusarium circinatum* at nine loci[a].

Locus[b]	Allele size (bp)	South Africa	Florida	Mexico	California
FC-1	312	0.100	-	-	-
	314	0.900	1.000	0.833	1.000
	318	-	-	0.167	-
FC-2	178	-	0.053	-	-
	183	0.150	0.105	0.167	0.143
	185	0.850	0.684	0.833	0.857
	191	-	0.158	-	-
FC-3	279	-	0.053	-	-
	281	-	-	-	0.238
	285	1.000	0.842	1.000	0.714
	291	-	0.105	-	0.048
FC-4	260	-	-	-	0.143
	263	0.950	0.895	1.000	0.762
	268	0.050	-	-	-
	270	-	0.105	-	0.095
FC-5	235	0.010	-	-	0.143
	237	0.900	0.684	0.833	0.810
	239	-	0.158	0.167	-
	NULL	-	0.158	-	0.048
FC-6	217	-	-	0.083	-
	220	0.050	-	-	-
	222	0.950	0.842	0.917	0.762
	229	-	0.158	-	0.048
	NULL	-	-	-	0.190
FC-7	257	0.900	1.000	1.000	1.000
	259	0.100	-	-	-
FC-8	122	-	0.053	-	-
	139	1.000	0.947	1.000	1.000
FC-9	242	0.250	-	0.250	-
	244	0.700	-	-	-
	246	-	1.000	0.750	1.000
	248	0.050	-	-	-

[a] Allele frequency calculated with program Microsat (http://human.stanford.edu/microsat).

[b] Each locus, FC= *F. circinatum* , are indicated by different numerical (1-9).

Table 4. Allelic and genetic diversities as well as genetic distances and migration parameters of *Fusarium circinatum* within sampled regions.

Geographical regions	Allelic diversity[a]	H_{TR}[b]	Genetic distance[c]				Migration parameter, $2Nm$ [d]			
							Receiving populations			
			SA	FL	MEX	CA	SA	FL	MEX	CA
South Africa	0.159	0.167	-	0.115	0.065	0.118	-	2.489	5.503	2.661
Florida	0.201	0.212	0.429		0.011	0.009	3.228	-	2.177	3.777
Mexico	0.151	0.165	0.316	0.077	-	0.021	7.031	1.362	-	5.236
California	0.197	0.207	0.439	0.060	0.118	-	3.804	3.428	10.460	-

[a] Allelic frequency = $(1-\Sigma p_i^2)$, where p_i is the frequency of the ith allele (Nei, 1973) was calculated using the Microsat program.

[b] H_{TR} is the genetic diversity within each region adjusted for sample size (equation in text).

[c] Values for *Fst* listed below the diagonal and values for D_S listed above the diagonal.

[d] The migration parameter $2Nm$ values indicate migrants from horizontally listed population into vertically listed (receiving) populations. The direction of migration can be established from the migration parameter values.