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PREFACE

Fusarium subglutinans (section *Liseola*) has been described as a cosmopolitan fungus residing in three mating populations of the *Gibberella fujikuroi* complex. Morphological characters did not differentiate *F. subglutinans* strains from different hosts (=*F. subglutinans sensu lato*). Pathogenicity tests and sexual compatibility were, therefore, used to differentiate among *F. subglutinans sensu lato*. More recently, many *F. subglutinans sensu lato* isolates residing in mating populations in the *G. fujikuroi* complex have been elevated to species that were justified by molecular phylogeny and supported by associated morphological characters.

As a result of recent taxonomic studies, the pitch canker fungus, previously known as *F. subglutinans* f. sp. *pini,* has been described as *F. circinatum* (teleomorph *G. circinata*). However, the taxonomy of the *F. subglutinans sensu lato* strains causing mango malformation remains undefined. The major goal of studies in this dissertation has been to characterize *F. subglutinans sensu lato* isolates from mango and pine based on molecular techniques, morphological characteristics and sexual compatibility. Furthermore, the genetic diversity of South African populations of these two pathogens has also been investigated using various techniques. Each of the chapters in this thesis thus deals with different approaches to investigate the taxonomy and genetics of the pitch canker and mango malformation fungi.

The first chapter provides an overview of the literature published on the morphology, biological species and phylogenetic studies of *Fusarium* spp. in the *G. fujikuroi* complex. Studies leading to the most recent taxonomic system, where the morphological classification supports the mating populations, pathogenicity and phylogenetic species classification are treated. Emphasis in this chapter is placed on the taxonomy and population genetics of *F. subglutinans sensu lato* strains causing pitch canker and mango malformation.

Fusarium circinatum, the causal agent of pitch canker, has a world-wide distribution. However, very little is known about the occurrence of this fungus in Mexico. In chapter two, the identity of *Fusarium* isolates collected in Mexico was determined. Techniques used included morphological characteristics such as conidial morphology, origin of conidiophore from substrate, type of conidiophore branching and the presence of sterile coiled hyphae. Pathogenicity tests, sexual compatibility studies and histone *H3*-RFLPs were also used to characterize these isolates.

The name of the teleomorph of *F. circinatum, G. circinata* has been declared invalid, because insufficient information was provided to characterize the type specimen in the description. The aim of the study presented in chapter three was to provide information regarding the holotype specimen, names of collectors of isolates, date of collection and designation of the holotype specimen. Morphological criteria proposed in the recent classification were critically examined in order to determine whether *F. circinatum* could be differentiated from other *F. subglutinans sensu lato* isolates based on these characteristics.

Fusarium circinatum causes significant losses to the forestry industry and various strategies are being implemented to reduce them. An understanding of the population biology of the pathogen will lead to durable disease control. Population genetic analysis requires the identification of markers that are unambiguous and polymorphic. In chapter four, molecular markers that are co-dominant and polymorphic were developed. These markers were then used to analyze the genetic relationships among populations of *F. circinatum* from California, Florida, Mexico and South Africa.

Population genetics of *F. circinatum* is influenced by asexual and sexual reproduction. Clonal propagation is the result of the asexual cycle, while new genotypes are produced by the sexual cycle. The contribution of the sexual and asexual cycle to the *F. circinatum* population structure was investigated by comparing isolates from the initial *F. circinatum* outbreak, with isolates from recent outbreaks. Techniques used in this chapter included sexual compatibility, vegetative compatibility tests and molecular markers.

Two distinct phylogenetic species associated with mango malformation were previously recognized based on DNA sequences of histone *H3* and β-tubulin genes. In chapter six, these two new species have been formally described and differentiated from other species based on morphological characteristics.

Chapter seven reports on the distribution of the two *Fusarium* spp. described in chapter 6 from mango malformation symptoms in South Africa. A rapid molecular technique was used to differentiate these two species from one another. The genotypic diversity of both species was also determined using vegetative compatibility tests.

Each chapter this dissertation has been treated as an independent entity. Thus, redundancy between chapters could not be avoided. It is my hope that the results of these studies on two of the most important pathogens of tree crops in South Africa will contribute to a better understanding of the diseases that they cause. Also that these studies will form the basis of detailed and future investigations.

CHAPTER 1

LITERATURE REVIEW:

TAXONOMY AND POPULATION GENETICS OF *FUSARIUM SUBGLUTINANS SENSU LATO* ON PINE AND MANGO

1. INTRODUCTION

The genus *Fusarium* was first described by Link in 1809 (Wollenweber & Reinking, 1925). To simplify identification of *Fusarium* spp., Wollenweber & Reinking (1935) divided the genus into sections based on the presence or absence of microconidia and chlamydospores, as well as the shape of micro- and macroconidia. Thin-walled, falcate macroconidia and the absence of chlamydospores distinguished section *Liseola* from other sections in the genus (Wollenweber & Reinking, 1935; Booth, 1971; Nelson *et al.,* 1983). However, the taxonomy of *Fusarium* has continued to be problematic and the subject of considerable debate (Wollenweber & Reinking, 1935; Booth, 1971; Gerlach & Nirenberg, 1982; Nelson *et al.,* 1983; Nirenberg & O'Donnell, 1998). There is disagreement with respect to the name and number of species in the section *Liseola,* the morphological criteria that are appropriate to distinguish these species (Nelson *et al.,* 1983; Nirenberg & O'Donnell, 1998), as well as the treatment of the section *Liseola* as a monophyletic lineage (O'Donnell *et al.,* 1998; 2000). *F. subglutinans* Wollenweber & Reinking is one of the species that resides in the section *Liseola* (Nelson *et al.,* 1983). The foremost interest in this species, lies in its important role as a plant pathogen.

Fusarium subglutinans is the causal agent of economical important diseases on a wide variety of plants (Hepting & Roth, 1946; Booth, 1971; Hsieh *et al.,* 1977; Rohrbach & Pfeiffer, 1976; Kuhlman, 1982; Ventura *et al.,* 1993). Previously, it was not possible to use morphology to distinguish *F. subglutinans* strains, from different hosts. Pathogenicity tests and mating studies were thus used to make these distinctions (Hsieh *et al.,* 1977; Correll *et al.,* 1991; Elmer & Ferrandino, 1992; Elmer, 1995; Leslie, 1995; Viljoen *et al.,* 1997a). Recently, *F. subglutinans* isolates associated with different host plants have been elevated to species level based on molecular evidence (O'Donnell *et al.,* 1998) and morphological characteristics (Nirenberg & O'Donnell, 1998). However, for the purpose of this review *Fusarium* spp. morphologically similar to *F. subglutinans* will be referred to as *F. subglutinans sensu lato.*

Pitch canker is one of the most important diseases caused by *F. subglutinans* (Hepting & Roth, 1946). This disease is destructive on pines and was first observed in the southeastern United States (Hepting & Roth, 1946). More recently, the pathogen has

been reported from California, Japan, Mexico and South Africa (McCain *et al.*, 1987; Kobayashi & Muramoto, 1989; Santos & Tovar, 1991; Viljoen *et al.*, 1994). Pitch canker has thus become a pathogen of international importance that threatens pine plantations, orchards, natural stands and nurseries, globally (Blakeslee *et al.*, 1980; Dwinell & Barrows-Broaddus, 1981; Viljoen *et al.*, 1994).

Another economically important disease caused by *F. subglutinans* is mango malformation. This disease occurs in mango producing countries throughout the world (Kumar *et al.*, 1993). The causal agent has been characterized using sequences of the β-tubulin and histone *H3* genes (Steenkamp *et al.*, 2000a). However, the taxonomy of the fungi involved in this disease, has yet to be defined.

In this review I will discuss various aspects pertaining to the taxonomy of *F. subglutinans* (section *Liseola*). More specifically, the various characteristics used in the taxonomic classifications for the genus *Fusarium* will be considered. Mating populations have been defined for species in the section *Liseola* and the emerging biological species will also be considered. Additionally, studies have been undertaken to distinguish *Fusarium* spp. in *Liseola* and related sections as phylogenetic species based on multilocus sequences, due to the inability to distinguish these morphologically similar species. Due to the economic importance of *Fusarium* spp. in the section *Liseola,* the population genetics of several of these fungi have been studied. The various techniques used to study the population genetics will be treated. Two important diseases caused by *F. subglutinans sensu lato,* namely pitch canker and mango malformation will also be discussed in detail. Special emphasis is placed on, the influence of the various classification systems (morphology, phylogeny and biological species) used for the identification of these pathogens. Furthermore, the geographical distribution, spread, and molecular characterization of strains influencing the genetic and population diversity of the pitch canker and mango malformation pathogens, will be considered.

2. TAXONOMY

2.1 *Morphology*

Wollenweber & Reinking (1935) first recognized *F. moniliforme* Sheldon as the primary representative of the section *Liseola*. These authors later proposed variety status for *F. moniliforme* (Wollenweber & Reinking, 1935). The variety, *F. moniliforme* var. *subglutinans* Wollenweber & Reinking was the first of these varieties to be described and was first observed on bananas in Honduras (Wollenweber & Reinking, 1925) (Table 1). *F. subglutinans* var. *subglutinans* was distinguished from *F. moniliforme* by the absence of microconidia in chains and the presence of polyphialides in *F. moniliforme* var. *subglutinans* (Wollenweber & Reinking, 1935; Booth, 1971).

In 1955, Bilai proposed a new classification system for *Fusarium* (Bilai, 1955). In this classification, the sections *Liseola* and *Elegans* were combined as one section *Elegans* (Bilai, 1955; 1970). *F. moniliforme* and *F. moniliforme* var. *subglutinans* were, therefore, included in the section *Elegans*. *F. neoceras* Wollenweber & Reinking was included as a synonym of *F. moniliforme* var. *subglutinans* (Table 1).

Booth (1971) transferred *F. moniliforme* and *F. moniliforme* var. *subglutinans* back to the section *Liseola*. This decision was based on the fact that Booth (1971) used morphological characteristics such as colony colour, spore morphology, presence and absence of chlamydospores, as well as mono- and/or polyphialides to divide the genus into different sections. Species in the section *Liseola* were characterized by the absence of chlamydospores, presence of mono- and polyphialides, whereas species in the section *Elegans* produced chlamydospores and monophialides. Conidiophores and conidiogenous cells as well as spore morphology and colony morphology were used to distinguish *F. moniliforme* var. *moniliforme* and *F. moniliforme* var. *subglutinans* in the section *Liseola*.

F. moniliforme var. *subglutinans* was reported as the causal agent of seedling blight, root stalk and kernel rot of maize (*Zea mays* L.), as well as pokkah boeng disease of sugarcane (*Saccharum officinarum* L.) (Booth, 1971). However, Gerlach & Nirenberg (1982) described *F. moniliforme* var. *subglutinans* strains that were

associated with pokkah boeng disease of sugarcane as *F. sacchari* (Butler) Gams var. *sacchari*. *F. moniliforme* var. *subglutinans* strains causing stalk and cob rot of maize were described as *F. sacchari* var. *subglutinans* (teleomorph *G. fujikuroi* var. *subglutinans*) (Gerlach & Nirenberg, 1982). Gerlach & Nirenberg (1982) proposed that narrower microconidia and rarely produced sporodochia observed in *F. sacchari* var. *sacchari* strains, distinguished them from those of *F. sacchari* var. *subglutinans*. *F. moniliforme* var. *moniliforme* that was not pathogenic to sugarcane or maize was described as *F. sacchari* var. *elongatum* Nirenberg by Gerlach & Nirenberg (1982). Furthermore, *F. tricinctum* var. *anthophilum* (=*F. moniliforme* var. *anthophilum*), that was previously included as a synonym of *F. moniliforme* var. *subglutinans* (Booth, 1971), was described as *F. anthophilum* (Braun) Wollenweber (Gerlach & Nirenberg, 1982) based on its pyriform microconidia. These authors also included *F. neoceras* as a synonym of *F. sacchari* (Gerlach & Nirenberg, 1982) (Table 1).

The classification system of Nelson *et al.* (1983), was based on the work of Wollenweber & Reinking (1935). Nelson *et al.* (1983) distinguish *Fusarium* spp. based on colony colour on potato dextrose agar (PDA) and morphological characteristics such as shape and size of macroconidia, presence or absence of microconidia and chlamydospores as well as the type of conidiophores on carnation leave agar (CLA). *F. subglutinans, F. proliferatum* (Matsushima) Nirenberg, *F. moniliforme* and *F. anthophilum* were the species included in the section *Liseola*.

2.2 *Pathogenicity*

Fusarium subglutinans occurs on a wide variety of hosts causing diseases on economically important plants, such as mangos (*Mangifera indica* L.), pineapples (*Ananas comosus* L.), maize (*Z. mays*), sugarcane (*S. officinarum* L.) and sorghum (*Sorghum caffrorum* Beauv.) (Singh *et al.*, 1961; Booth, 1971; Hsieh *et al.*, 1977; Gerlach & Nirenberg, 1983; Ventura *et al.*, 1993) and the pitch canker complex on pine trees (*Pinus* spp.) (Hepting & Roth, 1946; Correll *et al.* 1991). A significant problem has been that *F. subglutinans* associated with specific hosts cannot be distinguished easily based on morphology. Pathogenicity was, therefore, used to distinguish host specific *F. subglutinans* strains. This led to the establishment of *formae speciales* status for some species.

Of the pathogenic strains of *F. subglutinans sensu lato,* only two *formae speciales* have been proposed. These include *F. subglutinans* f. sp. *ananas* Ventura *et al.* (=*F. guttiforme* Nirenberg & O'Donnell), which is pathogenic to pineapples (Ventura *et al.,* 1993) and *F. subglutinans* f. sp. *pini* Correll *et al.* (=*F. circinatum*), which is pathogenic to pines (Hsieh *et al.,* 1977; Correll *et al.,* 1991; Viljoen *et al.,* 1997a).

2.3 *Phylogeny*

Recently, 44 *Fusarium* spp. in the section *Liseola* and related sections (= *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura complex) were shown to represent distinct phylogenetic species using multilocus sequences (O'Donnell *et al.,* 1998; 2000). Nirenberg & O'Donnell (1998) re-evaluated the morphological characteristics of species in this complex, including *F. subglutinans*, and described morphological species that supported their phylogenetic species concept. *Fusarium* spp. in the *G. fujikuroi* complex were distinguished based on conidial shape, production of chlamydospores, origin of conidiophores from the substrate, degree of conidiophore branching and production of mono and/or polyphialides (Nirenberg & O'Donnell, 1998). Twelve of the 44 *Fusarium* taxa in *Liseola,* previously identified as *F. subglutinans sensu lato* were described as distinct species (Nirenberg & O'Donnell, 1998). The pitch canker fungus, *F. subglutinans* f. sp. *pini* was given the name *F. circinatum* Nirenberg & O'Donnell (teleomorph, *G. circinata* Nirenberg & O'Donnell). Other *F. subglutinans sensu lato* species 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. sacchari* var. *elongatum*), *F. concentricum* Nirenberg & O'Donnell ex *Musa sapientum* (banana), *F. guttiforme* ex *Ananas comosus* (pineapple) (= *F. subglutinans* f. sp. *ananas*) and *F. pseudocircinatum* O'Donnell & Nirenberg ex *Solanum* sp., *Pinus kesiya* and *Heteropsylla incisa* (Table 1).

The classification systems proposed for *Fusarium* differ from each other and thus recognize varying numbers and names of species in their various manifestations of the section *Liseola.* The morphological criteria used to distinguish these species also differ in all the proposed systems. Sexual compatibility (mating studies) and phylogenetic analysis have thus been promoted in recent years to differentiate

morphologically similar species in the *G. fujikuroi* complex. The following sections deal with each of these characteristics in more detail.

3. BIOLOGICAL SPECIES (MATING POPULATIONS)

An alternative approach to morphology, when attempting to differentiate *Fusarium* spp. in the section *Liseola* is sexual compatibility. Species in this section are heterothallic and, therefore, governed by two alleles, designated *MAT*-1 and *MAT*-2, at a single mating type locus (Fincham *et al.*, 1979; Yoder *et al.*, 1986). Strains of opposite mating type that belong to the same mating population are potentially sexually fertile, leading to the formation of sexual structures (perithecia). Traditionally, the mating type alleles have been arbitrarily designated as '+' and '-' after a cross has been attempted with each of two tester isolates, which are known to represent different mating types (Leslie, 1991; 1995; 1996).

Eight mating populations (MP) have been identified in the *G. fujikuroi* complex (indicated by the letters A to H) using sexual compatibility. Sexual compatibility in this complex is determined in the laboratory on artificial media under specific conditions. Several factors, including light, medium and temperature influence sexual compatibility (Nelson, 1996). Exposure to near ultra violet and fluorescent light following fertilization, has been reported to be essential for the development of perithecia (Kuhlman *et al.*, 1978). V-8 agar is generally considered to be the best medium for the formation of the sexual stage in MP-A and B, but was unfavorable for perithecium production in MP-C (Hsieh *et al.*, 1977; Kuhlman *et al.*, 1978). A method established by Leslie (1991) resolved this problem by using carrot agar. A lower incubation temperature has been reported to increase the fertility of sexual crosses between isolates of MP-H in the laboratory (Covert *et al.*, 1999).

Each of the eight mating populations residing in the *G. fujikuroi* complex is associated with a single *Fusarium* sp. These species are *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme*), *F. sacchari* (= *F. subglutinans*), *F. proliferatum* (Matsushima) Nirenberg, *F. fujikuroi* Nirenberg (= *F. proliferatum*), *F. subglutinans sensu stricto* and *F. thapsinum* Klittich *et al.*, *F. nygamai* Burgess & Trimboli and *F. circinatum* (*F. subglutinans* f. sp. *pini*) (Hsieh *et al.*, 1977; Burgess & Trimboli, 1986; Leslie, 1991, 1995; Klaasen & Nelson, 1996; Klittich *et al.*, 1997; Britz *et al.*,

1999). All but *F. nygamai* belong in the section *Liseola*. This *Liseola*-like species produces chlamydospores (Burgess & Trimboli, 1986), and could, therefore, not be included in the section *Liseola*. A new section *Dlaminia* was proposed in 1991 to accommodate *Liseola*-like species producing chlamydospores like *F. nygamai* (Waalwijk *et al.*, 1996; O'Donnell *et al.*, 1998; 2000).

The differentiation of the mating populations in the *G. fujikuroi* complex was changed with the introduction of the various taxonomic schemes for this group. At first, *F. moniliforme* was identified as the anamorph of MP-A, B and C of *G. fujikuroi* associated with maize, sugarcane and rice, respectively (Hsieh *et al.*, 1977). Kuhlman (1982) later included additional isolates from a variety of hosts and described four varieties in *G. fujikuroi* complex. The varieties included *G. fujikuroi* var. *fujikuroi* Wollenweber from rice, *G. fujikuroi* var. *moniliformis* (Wineland) Kuhlman from maize, *G. fujikuroi* var. *subglutinans* Edwards primarily from sugarcane and *G. fujikuroi* var. *intermedia* Kuhlman, primarily from maize.

In 1991, Leslie reported the presence of six biological species in the *G. fujikuroi* complex, that could not be clearly differentiated based on morphology (Leslie, 1991). Members of MP-A and F shared the *F. moniliforme* anamorph, members of the MP-B and E shared the *F. subglutinans* anamorph and members of the MP-C and D mating population shared the *F. proliferatum* anamorph (Leslie, 1991; Xu *et al.*, 1995). MP-F, known as the second mating population with a *F. moniliforme* anamorph (Klittich & Leslie, 1992), was later described as a distinct species, *F. thapsinum* (*G. thapsina* Klittich *et al.*) (Klittich *et al.*, 1997). Two other mating populations have also been described. MP-G that is based on *F. nygamai*, has been formally described as *G. nygamai* Klaasen & Nelson (Leslie & Mansuetus, 1995; Klaasen & Nelson, 1996). *F. subglutinans* f. sp. *pini* (= *F. circinatum*), the causal agent of pitch canker disease represents a distinct mating population in *G. fujikuroi*, known as MP-H (Viljoen *et al.*, 1997a; Britz *et al.*, 1999).

The defined mating populations of *G. fujikuroi* can be distinguished based on host specificity (Leslie, 1991; Leslie & Mansuetus, 1995), mycotoxin production (Leslie & Mansuetus, 1995) and a range of molecular techniques (DuTeau & Leslie, 1991; Xu *et al.*, 1995; Voigt *et al.*, 1995; Huss *et al.*, 1996; Amoah *et al.*, 1996; O'Donnell *et*

al., 1998; Steenkamp *et al.,* 1999; O'Donnell *et al.,* 2000). Members of the MP-A (*F. verticillioides* anamorph) usually occur on maize and members of the MP-F (*F. thapsinum* anamorph) are generally from sorghum (Klittich & Leslie, 1992). Strains belonging to these two mating populations can also be separated based on their ability to produce the mycotoxin fumonisin B_1. Strains belonging to the A population produce high levels and strains belonging to the F population produce low levels of this mycotoxin (Leslie *et al.,* 1992). Isolates residing in MP-C are primarily isolated from rice (Booth, 1971; Sun & Snyder, 1981), produce high levels of gibberellic acid (Kuhlman 1982; Hsieh *et al.,* 1977; Leslie, 1996) and have a *F. fujikuroi* (= *F. proliferatum*) anamorph (Leslie, 1996) (Table 2). Isolates residing in MP-D occur on various host plants, including asparagus (Elmer & Ferrandino, 1992; Elmer, 1995; Leslie, 1996) that produce the mycotoxin fumonisin (Leslie *et al.,* 1992) and have a *F. proliferatum* anamorph (Table 2).

Isolates residing in MP-B, E, and H represent distinct biological species with *F. subglutinans* anamorphs. These can be distinguished based on their isolated sexual outcrossing (Leslie, 1991, 1995, 1996; Viljoen *et al.,* 1997a; Britz *et al.,* 1999). MP-B strains have been reported to occur primarily on sugarcane, MP-E strains occur on maize and MP-H strains occur on *Pinus* spp. (Kuhlman, 1982; Leslie, 1991; 1995, Viljoen *et al.,* 1997a; Britz *et al.,* 1999) (Table 2). One isolate of *F. circinatum* (=*F. subglutinans* f. sp. *pini*) (ATCC 38479) has been reported to be sexually fertile with mating tester strains of MP-B (Kuhlman, 1982), but Correll *et al.* (1992) and Viljoen *et al.* (1997a) were not able to repeat this cross and queried the validity of the fertile cross. Britz *et al.* (1999) provided an explanation for these results. These authors showed that homothallism exists in MP-B of *G. fujikuroi* complex (Leslie *et al.,* 1986; Britz *et al.,* 1999), leading to the inaccurate conclusion that mating populations with *F. subglutinans* anamorphs are cross fertile with one another (Kuhlman, 1982).

Molecular tests enable designation of mating type alleles as *MAT*-1 and *MAT*-2 using PCR amplification. The *MAT*-2 idiomorph (Covert *et al.,* 1999; Kerenyi *et al.,* 1999; Steenkamp *et al.,* 2000b) and both *MAT*-1 and *MAT*-2 (Steenkamp *et al.,* 2000b; Wallace & Covert, 2000) can be amplified. The PCR technique developed by Steenkamp *et al.* (2000b) can be used to determine the mating type of *Fusarium* strains in the whole *G. fujikuroi* complex. The PCR technique developed by Wallace

& Covert (2000) specifically focuses on mating studies of *F. circinatum* in the *G. fujikuroi* complex.

Morphology, pathogenicity and mating studies indicate that *F. subglutinans sensu lato* strains residing in MP-B, E and H represent separate species (Nirenberg & O'Donnell, 1998; Viljoen *et al.*, 1997a; Leslie, 1995; Britz *et al.*, 1999). However, Desjardins *et al.* (2000) described a genetically isolated population of isolates identified as *F. subglutinans* from domestic maize (*Z. mays* ssp. *mays*) and its teosinte relatives (*Zea* spp.) in Mexico and Central America. These isolates, from the two closely related hosts, were cross fertile. One *F. subglutinans* isolate was, however, cross fertile with a strain of MP-H. This finding prohibited Desjardins *et al.* (2000) from describing isolates identified as *F. subglutinans* from maize and teosinte as a separate mating population of the *G. fujikuroi* complex. Sexual compatibility and phylogenetic analysis of isolates identified as *F. subglutinans sensu lato* from teosinte, maize (MP-E) and pine (MP-H) indicated that isolates from teosinte reside in the existing MP-E (Steenkamp, 2000).

In the past, sexual compatibility was used to differentiate morphologically similar species in the section *Liseola*. The recent classification system proposed by Nirenberg & O'Donnell (1998) has led to consistency between proposed morphological species and mating populations in the *G. fujikuroi* complex. Morphology and sexual compatibility can thus both be used to differentiate *Fusarium* spp. in the section *Liseola*.

4. PHYLOGENETIC SPECIES CONCEPT

More accurate and rapid techniques to discriminate among mating populations and morphological species in the *G. fujikuroi* complex, have been actively sought in recent years. This is mainly due to the importance of these fungi as pathogens and the need for diagnostic tools. A study conducted by Xu *et al.* (1995) determined that strains of each mating population could be distinguished using electrophoretic karyotyping, but this technique has been considered unduly cumbersome for use in routine diagnostics. Electrophoretic karyotypes can only be useful as a supplementary tool that provides insight into the genome organization and evolution of these fungi.

Huss *et al.* (1996) used isozyme analysis on seven mating populations of *G. fujikuroi* (MP A to G). The C and D mating populations were found to be the most similar of all the mating populations. Furthermore, the distinction of MP-B from the other six mating populations is difficult with isozyme analysis. Isozyme analyses can, thus, not be used to resolve all the morphological species in the *G. fujikuroi* complex (Huss *et al.*, 1996).

Random amplified polymorphic DNA (RAPD) (DuTeau & Leslie, 1991; Voigt *et al.*, 1995; Amoah *et al.*, 1996; Viljoen *et al.*, 1997a) and restriction fragment length polymorphisms (RFLPs) (Leslie & Mansuetus, 1995; Steenkamp *et al.*, 1999) have been used as a relatively rapid method to discriminate the mating populations from one another. RFLPs are technically complicated and did not give adequate results using mitochondrial DNA and the internal transcribed spacer (ITS) regions of the ribosomal DNA operon. However, histone-RFLPs can be used as a diagnostic tool in the *G. fujikuroi* complex (Steenkamp *et al.*, 1999). RAPDs have been used to distinguish the different mating populations from one another (Voigt *et al.*, 1995; Amoah *et al.*, 1996; Viljoen *et al.*, 1997a). Unfortunately, RAPDs have low repeatability. RAPDs, mtDNA-RFLP and ITS-RFLP are thus unsuitable for diagnostic purposes.

The ITS region (ITS1 and ITS2) has been used extensively in molecular systematic studies of the genus *Fusarium* (O'Donnell, 1992; Waalwijk *et al.*, 1996). O'Donnell & Cigelnik (1997) found that most *Fusarium* species have two highly divergent ITS2 sequences. These have been designated ITS2 type I and II (Waalwijk *et al.*, 1996). The ITS2 amplifies with conserved ITS primers (White *et al.*, 1990) and has been defined as the major type. The opposite type known as the minor ITS2 type, can be amplified with type-specific forward PCR primers (O'Donnell & Cigelnik, 1997). The gene consensus of ITS2 types I and II data is misleading and inadequate for parsimony analyses (O'Donnell & Cigelnik, 1997; O'Donnell *et al.*, 1998). Phylogenetic relationships should, therefore, not be attempted using only the ITS regions (O'Donnell *et al.*, 1998).

Recently, β-tubulin, calmodulin and translation elongation factor 1α (EF-1α) gene

sequences have been used in phylogenetic analyses of *Fusarium* spp. in the *G. fujikuroi* complex (O'Donnell *et al.*, 1998; 2000). These authors recognized 44 *Fusarium* taxa (phylogenetic lineages) in the *G. fujikuroi* complex. Of the 44 *Fusarium* lineages recognized in the *G. fujikuroi* complex, more than 30 lineages have no known teleomorphs. O'Donnell *et al.* (1998; 2000) referred to the 44 phylogenetically distinct species as *Fusarium* spp. in the *G. fujikuroi* complex, despite the fact that the genus *Gibberella* was established to encompass only *Fusarium* spp. that produce perithecia (teleomorph) with septate ascospores (Booth, 1971). Phylogenetic analyses of DNA sequences from multiple loci of the 44 *Fusarium* lineages in the *G. fujikuroi* complex, reside in the sections *Liseola* and *Dlaminia*. The *G. fujikuroi* complex was proposed as a monophyletic lineage, because the 44 *Fusarium* lineages reside in polyphyletic sections (O'Donnell *et al.*, 1998; 2000).

The 44 *Fusarium* lineages in the *G. fujikuroi* complex are separated into three distinct groups, which O'Donnell *et al.* (1998) referred to as 'African', 'American' and 'Asian' clades (O'Donnell *et al.*, 1998; 2000). O'Donnell *et al.* (1998) proposed a phylogeographic hypothesis for these fungi based on this phylogenetic clustering pattern and the geographic origins of the hosts. The hypothesis suggests that the evolutionary histories of *Fusarium* lineages in this complex are consistent with the species origins in Africa, South America and Asia respectively, following the fragmentation of the ancient Gondwanaland super-continent (O'Donnell *et al.*, 1998). However, the phylogenetic placement of some fungi in this scheme is open to criticism and requires further investigation (Steenkamp *et al.*, 1999; 2000a).

Steenkamp *et al.* (1999; 2000a) successfully used histone *H3* gene sequences to distinguish *Fusarium* spp. in the *G. fujikuroi* complex from one another. Phylogenetic analyses with *Fusarium* histone *H3* gene sequence data generated a phylogram similar to those of O'Donnell *et al.* (1999; 2000). The results of the studies of Steenkamp *et al.* (1999, 2000a) and O'Donnell *et al.* (1998; 2000) have thus identified conserved genes that are useful for phylogenetic and taxonomic studies for species of *Fusarium* in the *G. fujikuroi* complex. *Fusarium* spp. previously considered unduly closely related, can now be separated based on histone, EF-1α or β-tubulin gene sequence.

Phylogenetic trees generated from partial *MAT*-1 and *MAT*-2 sequences of eight mating populations of the *G. fujikuroi* complex gave similar phylogenetic clustering patterns, as those generated by O'Donnell *et al.* (1998) and Steenkamp *et al.* (1999) using β-tubulin and histone *H3* gene sequences. However, the *MAT*-2 tester of MP-B grouped with the C and D mating populations as seen with the β-tubulin and histone *H3* gene sequences. The *MAT*-1 sequences indicated that the MP-B grouped equidistant from the other seven mating populations.

5. POPULATION GENETICS

Various evolutionary processes influence the population genetic structure of pathogenic fungi. Genetic variability depends on the mode of reproduction, and thus, the relative contributions of sexual and asexual reproduction, outcrossing and selfing mechanisms and hyphal anastomosis (Brasier, 1992; Glass & Kuldau, 1992; Leslie, 1993; Milgroom, 1996). Many factors other than mating systems contribute to genetic evolution within fungal populations such as as gene flow (migration), population size and selection (McDonald & McDermott, 1993; Burdon & Silk, 1997; McDonald, 1997).

Vegetative compatibility has been broadly applied to provide insight into the genetic structure of fungal populations (Glass & Kuldau, 1992). Individuals of the same fungal species are vegetatively compatible when they fuse asexually to form a stable heterokaryon (Glass & Kuldau, 1992; Leslie, 1993). Vegetative compatibility is controlled by vegetative incompatibility (*vic*) loci and strains that are identical at all *vic* loci are able to form a stable heterokaryon and belong to the same vegetative compatibility group (VCG) (Glass & Kuldau, 1992; Leslie, 1993). Heterokaryon formation between anastomosing nitrate non-utilizing (*nit*) mutants are used to determine the VCGs (Puhalla, 1985; Glass & Kuldau, 1992; Leslie, 1993). Unfortunately, VCGs are not useful for inferences pertaining to evolutionary processes affecting population genetics (McDonalds, 1997). However, the emergence of molecular markers has made it possible to investigate evolutionary processes, similarities and differentiation in various fungal populations. (Williams *et al.*, 1990; McDonald *et al.*, 1989; Levy *et al.*, 1991; Fry *et al.*, 1992; Drenth *et al.*, 1993;

Mitchell & Brasier, 1994; Milgroom, 1995; Milgroom *et al.*, 1996; Bonfante *et al.*, 1997; McDonald, 1997; Moon *et al.*, 1999; Burgess *et al.*, 2001). The following markers have been extensively used in population genetics of several pathogenic fungi:

RAPDs: These are short arbitrary oligonucleotide primers targeting unknown sequences in the genome and low annealing temperatures are used in RAPD-PCR to amplify PCR products consisting of different size (Williams *et al.*, 1990). Often, size polymorphisms are identified within species, which are the result of mutations at specific binding sites (Welsh & McClelland, 1990). RAPD is a fast and relatively economical method for screening large numbers of isolates. RAPDs have been used to distinguish *Fusarium* spp. in the *G. fujikuroi* complex from one another (Voigt *et al.*, 1995; Amoah *et al.*, 1996; Viljoen *et al.*, 1997a). Viljoen *et al.* (1997a) could distinguish *F. subglutinans sensu lato* from one another by including isolates identified as *F. subglutinans* from maize, mango, pine, pineapple and sugarcane. Interestingly, he found that two isolates of *F. subglutinans* from mango (MRC 2802 and MRC 2370) grouped into two separate groups (Viljoen *et al.*, 1997a). However, the low temperatures used for amplification can cause problems with reproducibility, which together with the dominant nature of RAPDs (two alleles for each amplicon locus), limit the use of RAPDs as molecular markers in population studies (McDonald & McDermott, 1993; McDonald, 1997).

Isozymes: Isozyme analyses have been extensively used in the past for population studies of a variety of fungal pathogens (Old *et al.*, 1984, Burdon & Roelfs, 1985; McDermott *et al.*, 1989; Fry *et al.*, 1992; Goodwin *et al.*, 1992; Huss *et al.*, 1996). Isozyme analyses are inexpensive, selectively neutral, codominant and easy to assay (McDonald & McDermott, 1993). Huss *et al.* (1996) used isozymes to distinguish seven mating populations (MP-A to G) from one another. In this study, *F. sacchari* isolates (MP-B) could not be distinguished from the six other mating populations in the *G. fujikuroi* complex and isozymes cannot be used as a diagnostic tool to distinguish species in the *G. fujikuroi* complex. Furthermore, the quantification of isozyme markers is difficult and time-consuming (McDonald & McDermott, 1993; McDonald, 1997).

RFLPs: RFLPs of mitochondrial and nuclear DNA have provided useful genetic markers in various fungal population genetic studies (McDonald *et al.*, 1989; McDonald & Martinez, 1990; 1991; Gordon & Okamoto, 1991; Levy *et al.*, 1991; Kohn *et al.*, 1991; Drenth *et al.*, 1993; Milgroom *et al.*, 1996). The increased variability and number of loci obtained with RFLPs has the advantage of being more useful than isozyme markers (Engel *et al.*, 1996; McDonald, 1997). Furthermore, RFLPs have the additional advantage of being neutral and codominant. However, the number of enzymes available, limit the number of loci that can be examined. Another constraint of this technique is that large quantities of DNA are required. However, RFLPs of PCR products eliminate these problems. Histone *H3*-RFLPs have been used to distinguish *Fusarium* spp. residing in the *G. fujikuroi* complex from one another (Steenkamp *et al.*, 1999).

Simple sequence repeats (SSR) / microsatellites: SSRs, also known as microsatellite repeats, are short DNA repeats that are inherited in a single locus, codominant manner (Tautz, 1989; Weber & May, 1989). SSRs are highly polymorphic between individuals due to slippage of the DNA polymerase during synthesis and DNA repair (Tautz & Renz, 1984; Levinson & Gutman, 1987; Engel *et al.*, 1996; Rafalski *et al.*, 1996; Moon *et al.*, 1999). SSRs can, therefore, be used as a powerful tool for genetic analysis of population structure. SSR markers have been developed using either probing of genomic libraries with di- or trinucleotide repeats (Ostrander *et al.*, 1992; Smith & Devey, 1994; Tenzer *et al.*, 1999), sequencing of fragments amplified by RAPD-PCR (Burt *et al.*, 1994; Desmarais *et al.*, 1998; Dusabenyagasani *et al.*, 1998) or internal short sequence repeats (ISSR)-PCR (Brady *et al.*, 1996; Dusabenyagasani *et al.*, 1998; van der Nest, 2000; Burgess *et al.*, 2001). In different fungal studies, SSR markers have been used in genotypic assessment (Geistlinger *et al.*, 1997; Groppe & Boller, 1997; Longato & Bonfante, 1997; Neu *et al.*, 1999) and as polymorphic markers (Bonfante *et al.*, 1997; Moon *et al.*, 1999; Burgess *et al.*, 2001). Microsatellites can thus be used as ideal markers for fungal populations studies, because these markers are easy to analyze and highly polymorphic. They are, however, somewhat laborious and expensive to produce (Rafalski *et al.*, 1996).

6. PITCH CANKER AND MANGO MALFORMATION

Two diseases caused by *F. subglutinans sensu lato* are pitch canker and mango malformation. The taxonomic history, spread, geographical distributions as well as the sexual compatibility, molecular characteristics and population genetics of each associated pathogen will be discussed in the following sections.

6.1 *Pitch canker*

6.1.1 *Taxonomic history*

As discussed earlier, *F. subglutinans sensu lato* strains are specific to particular host plants and cause a variety of plant diseases (Singh *et al.*, 1961; Booth, 1971; Hsieh *et al.*, 1977; Gerlach & Nirenberg, 1982; Correll *et al.*, 1992; Ventura *et al.*, 1993). Pitch canker of pines is one of the most significant of these diseases. Pitch canker was first reported in 1946 on *Pinus virginiana* Mill. (Virginia pine) in North Carolina, USA (Hepting & Roth, 1946). The causal agent of the disease was recognized as an unidentified *Fusarium* sp. (Hepting & Roth, 1946). Despite interest in this fungus, there was very little known about its identity. Snyder *et al.* (1949) identified the pitch canker fungus as *F. lateritium* Nees. These authors found that isolates of *F. lateritium* recovered from pines, were pathogenic only to pines. A *forma specialis* was proposed for isolates pathogenic to pines and designated *F. lateritium* (Nees) emend. Snyder & Hanson f. sp. *pini* Hepting (Snyder *et al.*, 1949). The subsequent spread and epidemic nature of this pathogen, therefore, renewed interest and highlighted the importance of its taxonomy.

Dwinell (1978) and Kuhlman *et al.* (1978) identified the pitch canker fungus as *F. moniliforme* var. *subglutinans,* rather than *F. lateritium* f. sp. *pini.* However, when Nelson *et al.* (1983) reviewed the taxonomy of the genus *Fusarium*, they elevated *F. moniliforme* var. *subglutinans* to species level as *F. subglutinans.* Correll *et al.* (1991) proposed that the pitch canker pathogen should be recognized as a *forma specialis, F. subglutinans* f. sp. *pini,* based on host specificity and restriction fragment patterns of mtDNA, indicating that pine isolates differed from non-pine isolates. Viljoen *et al.* (1997a) found that isolates of *F. subglutinans* f. sp. *pini* were reproductively isolated from other mating populations of the *G. fujikuroi* complex and confirmed that this fungus has specific pathogenicity to pine seedlings.

DNA sequences of conserved genes distinguished *F. subglutinans* from different hosts as unique phylogenetic lineages (O'Donnell *et al.*, 1998). Morphological species were described that supported the phylogenetic lineages (Nirenberg & O'Donnell, 1998). The pitch canker fungus, was thus assigned the name *F. circinatum* (teleomorph: *G. circinata*) (Nirenberg & O'Donnell, 1998). A serious problem with this description by Nirenberg & O'Donnell (1998) was that it was based on only four isolates for the anamorph and the teleomorph description relied on a single cross between two *F. circinatum* isolate. There was thus virtually no consideration of variability in the teleomorph characteristics, and the availability of a well defined population of isolates was ignored (Correll *et al.*, 1991; 1992; Viljoen *et al.*, 1994; Gordon *et al.*, 1996; 1997a, b). Subsequently, the Index of Fungi (1999: vol. 6: 980) reported the new name *G. circinata* invalid according to Article 37.3 of the International Code of Botanical Nomenclature (ICBN, Greuter *et al.*, 1994).

6.1.2 *Geographical distribution*

Subsequent to the first report of the pitch canker fungus in North Carolina, the fungus was found throughout the southeastern United States where it has caused significant losses on a wide variety of pine species (Hepting & Roth, 1946; Schmidt, 1976; Dwinell & Phelps, 1977; Dwinell, 1978; Dwinell *et al.*, 1985). Pitch canker was also reported on *P. occidentalis* Swartz (Western Indian pine) in Haiti (Hepting & Roth, 1953). However, Dwinell *et al.* (1985) suggested that pitch canker is endemic to the southeastern United States, due to the epidemic nature of the disease in this area.

In 1986, pitch canker was found for the first time on *P. radiata* D. Don (Monterey pine) in Santa Cruz county, California (McCain *et al.*, 1987). The disease is now found throughout California and affects many other pine species as well as Douglas-fir (*Pseusotsuga menziesii* (Mirb.) Franco) (Storer *et al.*, 1994), although *P. radiata* is the most seriously affected (Gordon *et al.*, 1997). Subsequent to 1992, pitch canker was recognized as a threat to native *P. radiata* stands in California (Storer *et al.*, 1994; 1997).

Although pitch canker is most serious and of greatest concern in California, it is also in Japan on native *P. luchuensis* Mayr. (Ryukyu pines) (Muramoto *et al.*, 1988;

Kobayashi & Kawabe, 1992) and in Mexico, where several pine species are native (Santos & Tovar, 1991). In South Africa, the fungus was first reported from a single forestry nursery, where it resulted in serious losses to *P. patula* Schlechtend & Cham. (Mexican weeping pine) (Viljoen *et al.*, 1994). Stem cankers on larger trees such as those found in the United States (Hepting & Roth, 1946; Dwinell *et al.*, 1977) have, thus far, not been seen in South Africa (Viljoen *et al.*, 1995a, b; Wingfield *et al.*, 1999), although damage in nurseries has become increasing more serious in recent years.

6.1.3 *Symptoms*

The pitch canker pathogen is responsible for a complex of symptoms on vegetative and reproductive structures, at different stages of maturity on pines (Dwinell *et al.*, 1985). The vegetative symptoms caused by pitch canker include resin flow from infected branches, shoots, cones and/or the bole of pine trees. This resinous cankers and pitch- soaked wood (Hepting & Roth, 1946; Dwinell *et al.*, 1977; Dwinell & Phelps, 1977) (Dwinell & Phelps, 1977; Dwinell *et al.*, 1985; McCain *et al.*, 1987), often penetrating through to the pith (Dwinell & Phelps, 1977). In severe cases, these cankers girdle trees leading to shoot die-back and to tree mortality (Dwinell & Phelps, 1977; Dwinell *et al.*, 1977; 1981). Infection of the reproductive structures, such as female strobili and mature cones, results in mortality of strobili and poor germination of seeds (Dwinell *et al.*, 1977; Miller & Bramlett, 1978; Miller *et al.*, 1984; Dwinell *et al.*, 1985; Barrows-Broaddus, 1986). Cones on infected branches often abort before reaching full size and remain closed (Miller & Bramlet, 1978; Dwinell & Barrows-Broaddus, 1981; Miller *et al.*, 1984).

The pitch canker fungus has been reported to be a primary pathogen associated with seedlings in nurseries causing lesions on the root collar or upper root and the cotyledonary node region of the seedlings (Barnard & Blakeslee, 1980; Dwinell *et al.*, 1985; Viljoen *et al.*, 1994). Tip die-back, damping-off, chlorotic or reddish brown needle discoloration and wilting are observed on diseased seedlings (Barnard & Blakeslee, 1980; Rowan, 1982; Dwinell *et al.*, 1985; Huang & Kuhlman, 1990). *F. circinatum* is the only *Fusarium* spp. that is pathogenic to pine seedlings as well as mature trees (Viljoen *et al.*, 1995a, b).

6.1.4 *Spread*

Infection of pine trees by *F. circinatum* occurs through wounds (Hepting & Roth, 1946; Dwinell & Phelps, 1977; Dwinell & Barrows-Broaddus, 1981; Dwinell *et al.,* 1985; Storer *et al.,* 1997). Wounds are caused by mechanical damage (Dwinell & Phelps, 1977; Dwinell *et al.,* 1977; Blakeslee *et al.,* 1980; Dwinell & Barrows-Broaddus, 1981; Dwinell *et al.,* 1985; Barrow-Broaddus, 1986), weather-related injuries (Kelly & Williams, 1982; Dwinell & Barrows-Broaddus, 1983) or insects (McGraw *et al.,* 1976; Blakeslee *et al.,* 1980; Dwinell *et al.,* 1985; Fox *et al.,* 1991; Storer *et al.,* 1994; Dallara *et al.,* 1995; Storer *et al.,* 1997).

The deodar weevil (*Pissodes nemorensis* Germar) has been implicated in disease incidences on slash pines (*P. elliottii* Engelm. var. *elliottii*) in Florida (Dwinell & Phelps, 1977; Blakeslee *et al.,* 1980; Dwinell *et al.,* 1985). The tip moth (*Rhyacionia subtropica* Miller) may create wounds that can be infected by air-borne conidia of *F. circinatum* (McGraw *et al.,* 1976; Dwinell & Phelps, 1977; Blakeslee *et al.,* 1980). In California, infection of the pitch canker fungus appears to result from feeding activities of twig beetles (*Pityophthorus* sp.), cone beetles (*Conophthhorus radiatae* Hopkins), spittlebug (*Aphrophora canadensis* Walley) and engraver beetles (*Ips* spp.) (Fox *et al.,* 1991; Correll *et al.,* 1991; Storer *et al.,* 1994; Dallara *et al.,* 1995; Hoover *et al.,* 1996; Storer & Wood, 1998). The fungus has been isolated from *Ips* spp. emerging from pitch canker infected *P. radiata* trees (Fox *et al.,* 1991; Storer *et al.,* 1994; 1997). The association of the pitch canker pathogen with native and introduced insects can potentially have a dramatic impact on the spread of this pathogen.

Fusarium circinatum is thought to have been introduced into new areas on infected seed. In the past, *F. circinatum* has been isolated from both disinfested and non-disinfested seed (Barrows-Broaddus, 1987; Runion & Bruck, 1988) and pitch canker disease symptoms have also been initiated from infected seed (Huang & Kuhlman, 1990). Storer *et al.* (1998) isolated *F. circinatum* from pine seedlings originating from seed collected from cones on diseased as well as asymptomatic branches. They found that *F. circinatum* can be carried inside seeds and may remain dormant until germination, which increases the possibility of seedling infections. Infected plant material can also lead to the spread of the fungus into new areas (Gordon *et al.,* 1996).

6.1.5 *Sexual compatibility*

Before morphological and molecular characteristics were available, the most reliable technique for distinguishing *F. circinatum* from closely related *Fusarium* spp., was sexual compatibility (Correll *et al.*, 1992; Viljoen *et al.*, 1997a). Sexual cross-fertility was not observed between *F. circinatum* isolates from California and Florida (Correll *et al.*, 1992). However, Viljoen *et al.* (1997a) reported sexual compatibility between *F. circinatum* isolates and suggested that the pitch canker fungus represented a distinct biological species. This was verified with fertile crosses reported amongst *F. circinatum* strains from South Africa, California and Florida (Britz *et al.*, 1999). *F. circinatum* was thus shown to represent a unique mating population (MP-H) in the *G. fujikuroi* complex (Coutinho *et al.*, 1995, Viljoen *et al.*, 1997a, Britz *et al.*, 1998; 1999).

6.1.6 *Molecular characteristics*

Distinguishing *F. circinatum* from *Fusarium* spp. in the *G. fujikuroi* complex initially required pathogenicity or sexual compatibility tests (Correll *et al.*, 1991; 1992; Viljoen *et al.*, 1997a; Britz *et al.*, 1999). These tests are time-consuming, labour-intensive and not always reliable. Molecular techniques such as random amplification of polymorphic DNA (RAPD) (DuTeau & Leslie, 1991; Voigt *et al.*, 1995; Viljoen *et al.*, 1997a), mitochondrial restriction fragment length polymorphisms (RFLP) (Correll *et al.*, 1991), and ribosomal DNA (rDNA) internal transcribed spacer (ITS1 and ITS2) sequences (Waalwijk *et al.*, 1996; O'Donnell & Cigelnik, 1997) have been tested for their efficacy in differentiating *F. circinatum* isolates from those in the great *F. subglutinans sensu lato* group. However, these techniques had various problems such as being slow, non-repeatable and some did not differentiate all *F. subglutinans sensu lato* isolates from one another. Sequences of the histone (Steenkamp *et al.*, 1999), β-tubulin (O'Donnell *et al.*, 1998) and EF-1α (O'Donnell *et al.*, 2000) genes separate *F. subglutinans sensu lato* into distinct groups. A PCR-RFLP technique was thus developed from the histone *H3* gene sequence and is used by some groups as a diagnostic tool to distinguish *F. circinatum*, from other *Fusarium* spp. residing in *G. fujikuroi* complex (Steenkamp *et al.*, 1999).

6.1.7 *Population genetics*

Vegetative compatibility has been widely used to provide insight into the genetic structure of *F. circinatum* in Florida, California, South Africa, Japan and to a lesser extend in Mexico. Before the start of the pitch canker epidemic in the 1970s in the southern United States, the disease occurred sporadically (Schmidt *et al.*, 1976; Dwinell & Phelps, 1977). The disease has been well established for at least 27 years in Florida. This was verified by the high VCGs diversity in the Florida population with 45 VCGs amongst 117 *F. circinatum* isolates tested (Correll *et al.*, 1992). In contrast, the Californian population consisted of only 5 VCGs among 209 isolates, with one VCG accounting for 70% of the population collected between 1987 and 1989 (Correll *et al.*, 1992). After a later collection between 1993 and 1995 (Gordon *et al.*, 1996) eight VCGs were identified in the Californian population. This limited diversity is consistent with a recent introduction of the pathogen and the absence or rare occurrence of sexual reproduction in the population (Gordon *et al.*, 1996; Wikler & Gordon, 1999). However, a recent study conducted by Wikler *et al.* (2000) has shown that outcrossing is possible with Californian isolates in the laboratory on artificial media. This might indicate that conditions in California probably support asexual rather than sexual reproduction.

The genotypic diversity of the initial *F. circinatum* population in South Africa was determined using vegetative compatibility tests (Viljoen *et al.*, 1997b). A high genotypic diversity was found in this population where 23 VCGs were identified among 69 *F. circinatum* isolates. This high level of genotypic diversity led Viljoen *et al.* (1997b) to suggest that sexual reproduction was occurring within the South African population. Britz *et al.* (1998) considered the effective population number (N_e) (Wright, 1931; Leslie & Klein, 1996) that estimates the relative contribution of the sexual and asexual cycle to the population. The initial *F. circinatum* population in South Africa had a low frequency of hermaphrodite strains (Britz *et al.*, 1998). If the number of hermaphrodites continues to fall, the *F. circinatum* population in South Africa should ultimately become asexual (Britz *et al.*, 1998).

The Japanese population, where *F. circinatum* is affecting native *P. luchuensis* (Ryukyu pine), and the Californian population have a common VCG (C7) (Wikler & Gordon, 2000). This VCG was the only genotype found among five *F. circinatum*

isolates collected in Japan. The Mexican population, which is thought to represent the origin of pitch canker, had seven VCGs among 10 isolates indicating the population to be diverse (Viljoen *et al.,* 1995b; Wikler & Gordon, 2000).

Wikler & Gordon (2000) estimated the genetic relationships among populations of *F. circinatum* in different geographical areas, using eight polymorphic markers. These authors showed that the California and Japanese populations of *F. circinatum* share lineages with the Florida population. The Mexican population had the highest genetic diversity using eight polymorphic markers (Wikler & Gordon, 2000).

6.2 Mango malformation

Mango trees are grown commercially for their fruit, in the tropical and subtropical areas of the world. A fungal disease known as mango malformation threatens the mango industry. Despite the economic impact of mango malformation, various aspects such as taxonomy, spread, pathogenicity and genetic diversity needs further investigation.

6.2.1 *Taxonomic history*

In 1891, it was postulated that mango malformation was caused by excessive moisture in the soil (Kumar *et al.,* 1993). This disease was, therefore, initially believed to be a physiological disorder. Later, altered C/N ratios in shoots bearing mango malformation, reduced availability of copper and zinc in subsurface soil layers, poor soil and water management were thought to cause malformation (Kumar *et al.,* 1993).

In 1946, Sattar suggested that mango malformation might be of viral origin (Sattar, 1946), because of the virus-like symptoms of the disease, the association of some insects with symptomatic tissue and failure to isolate any obvious pathogenic agent from diseased tissue (Kumar *et al.,* 1993). However, electron microscopy, transmission, cultural and serological studies disproved the involvement of a virus in the disease (Kishtah *et al.,* 1985; Kumar *et al.,* 1993).

Mite infestations were amongst the suspected causal agents of mango malformation in 1946 (Varma, 1983). *Aceria* (*Eriophyes*) *mangiferae* Sayed, also known as the mango bud mite, has been observed to be associated with vegetative and floral

malformation. The cause of the disease was initially attributed to the feeding injuries of this mango bug mite (Narasimhan, 1954). A correlation was observed between mite populations and disease incidence, however, several other studies have shown no correlation between mite populations and disease incidences (Prasad *et al.*, 1965; Bindra & Bakhetia, 1971). It thus appears that *A. mangiferae* might play a role in the malformation disease cycle, but it is certainly not the sole cause of the disease (Wahba *et al.*, 1986).

Previous failures to isolate a pathogenic agent from malformed mango tissue have confused researchers. However, Summanwar *et al.* (1966) showed that the fungus identified as *F. moniliforme* (= *F. subglutinans*) was commonly associated with disease mango tissue. Isolates identified as *F. subglutinans* from malformed mango tissue were used to complete Koch's postulates to induce mango malformation symptoms (Summanwar *et al.*, 1966; Varma *et al.*, 1974; Chakrabarti & Ghosal, 1989; Manicom, 1989; Ploetz & Gregory, 1993). However, controversy regarding the inoculation methods used for induction of mango malformation symptoms remained (Kumar *et al.*, 1993). Freeman *et al.* (1999) eliminated any uncertainty regarding the cause of the disease by inducing typical mango malformation symptoms on mango trees with an isolate originally collected from malformed mango tissue in Israel and identified as the pathogen *F. subglutinans* (MRC 7559 = 506/2).

O'Donnell *et al.* (1998) indicated that a *Fusarium* isolate from mango malformation in India represented a unique lineage in the *G. fujikuroi* complex. However, these authors did not describe the species formally. Two phylogenetically distinct groups of isolates, currently referred to as *F. subglutinans* are associated with mango malformation in South Africa (Steenkamp *et al.*, 1999; 2000a). The one group of isolates represents an undescribed lineage in the *G. fujikuroi* complex and the second group of isolates is conspecific with *F. subglutinans* strains that were previously reported to be the causal agent of mango malformation (Steenkamp *et al.*, 2000a). The precise taxonomic classification of the mango malformation fungi remains undefined.

6.2.2 *Symptoms*

Mango malformation is a disease that affects both vegetative and floral tissue of mango (Varma, 1983; Kumar *et al.*, 1993). The malformation symptoms on mango have been referred to as abnormal inflorescence, bunchy top, die-back and blossom blight (Varma, 1983; Chakrabarti & Ghosal, 1989; Kumar *et al.*, 1993; Ploetz, 1994a). However, the two distinct symptoms of mango malformation are blossom (floral) and vegetative malformation. This disease causes a reduction in yield, because affected panicles are sterile (Kumar *et al.*, 1993; Ploetz, 1994a). Vegetative malformation was first described in 1953 (Kumar *et al.*, 1993) and is more pronounced on young seedlings. Typical symptoms are the swelling of the vegetative buds at the tip and the seedlings then produce small shootlets bearing scaly leaves with a bunch-like appearance. Symptoms of floral or blossom malformation appear with the emergence of inflorescences. Floral malformation is characterized by thick, fleshy branched panicles that are crowded by enlarged flowers (Varma, 1983; Kumar *et al.*, 1993; Ploetz, 1994a).

Darvas (1987) indicated that the mango malformation pathogen is not uniformly or widely distributed in affected trees in South Africa. This author recovered *F. subglutinans* most frequently from malformed panicles and less frequently from non-malformed blossoms. *F. subglutinans* was not recovered from the remaining parts of the affected mango trees (Darvas, 1987).

6.2.3 *Geographical distribution*

Mango malformation was first observed in India (Varma, 1983; Kumar *et al.*, 1993). Since this first report, the disease has been found in many mango producing countries throughout the world. These include America, Bangladesh, Brazil, Cuba, Egypt, Israel, Mexico, Pakistan, South Africa, Sudan, USA and the United Arab Emirates (Varma, 1983; Kumar *et al.*, 1993; Noriega-Cantú *et al.*, 1999; Ploetz, 1994a). Mango malformation was first reported in Mexico in 1958 (Noriega-Cantú *et al.*, 1999). Vegetative malformation is the dominant symptom observed in this country. In Florida, malformation was first reported in 1972 and was not regarded as an important disease, despite increases in incidences and severity of mango malformation (Ploetz & Gregory, 1993). A survey in South Africa, showed that the

disease is present on 73% of South African mango farms and that the severity varies between 1-70% of the trees being affected (Crookes & Rijkenberg, 1985).

6.2.4 *Spread*

Mango malformation is systemic and also spreads slowly from infected seedlings and trees to healthy plants in orchards and nurseries (Varma, 1983). The disease symptoms have been reproduced on mango plants by transferring the mango bug mite (*A. mangiferae*), which is commonly associated with mango plants and carries *F. subglutinans* on its body parts (Varma, 1983; Summanwar & Raychaudhuri, 1968). However, the small percentage of mites carrying the fungus has indicated that other factors are involved in the spread of this disease. One of the most important factors is the distribution of malformed plants into new areas, resulting in the distribution of this disease (Varma *et al.*, 1971; Varma, 1983).

6.2.5 *Sexual compatibility*

No teleomorph has been reported for isolates identified as *F. subglutinans* from mango (Steenkamp *et al.*, 2000a).

6.2.6 *Molecular characteristics*

O'Donnell *et al.* (1998) distinguished a single malformation isolate (NRRL 25226) from India in a unique phylogenetic lineage in the section *Liseola* based on β-tubulin gene sequences. In South Africa, mango malformation is associated with two phylogenetically distinct groups of isolates using β-tubulin and histone gene sequences (Steenkamp *et al.*, 1999; 2000a). The one group of these isolates represents an undescribed lineage in the *G. fujikuroi* complex. The second group of isolates is conspecific with strains identified as *F. subglutinans,* that were previously reported to be the causal agent of mango malformation (Steenkamp *et al.*, 2000a).

As previously mentioned (section 3 of this Chapter), O'Donnell & Cigelnik (1997) found two highly divergent ITS2 sequences in *Fusarium* spp. in the *G. fujikuroi* complex. The mango isolate (NRRL 25226) from India has an ITS2 type II sequence (O'Donnell *et al.*, 1998) and mango isolate (MRC 2802 = NRRL 25623) from South Africa has an ITS2 type I sequence (O'Donnell *et al.*, 2000). This further indicated that two groups of isolates are associated with mango malformation.

6.2.7 *Population genetics*

Very little is known regarding the population genetics of the mango malformation pathogen, globally. Ploetz (1994b) found one VCG to be dominant amongst isolates collected in Florida. Vegetative compatibility analysis of mango malformation populations collected in Egypt indicated that 4 VCGs are present and the most dominant VCG was also found in India (Zheng & Ploetz, 2001). Because mango originated in India, it seems probable that mango malformation was introduced into Egypt from India.

7. CONCLUSIONS

Fusarium subglutinans sensu lato are specific to particular plants and cause a variety of diseases. Morphology provides a very poor system to distinguish *F. subglutinans sensu lato* strains from each another. However, some of these strains can be distinguished based on pathogenicity and sexual compatibility.

Distinct phylogenetic lineages have been described for *Fusarium* spp. in the *G. fujikuroi* complex, which includes *F. subglutinans sensu lato*. This has led to a re-evaluation of morphological characteristics used to identify *Fusarium* spp. in the *G. fujikuroi* complex. Thus, morphological species have been described that are consistent with the phylogenetic species.

The pitch canker fungus has been described as *F. circinatum* (teleomorph: *G. circinata*) based on morphology and molecular phylogeny. However, the name *G. circinata* was declared invalid because insufficient information was supplied for the type specimen in the description. The name *G. circinata,* therefore, requires validation. A more detailed description, particularly encompassing the understanding that species are represented by populations of strains and not single isolates would also be desirable.

Isolates identified as *F. subglutinans* from malformed mango tissue have unequivocally been shown to cause mango malformation. Two phylogenetically distinct groups, defined by β-tubulin and histone H3 sequences, have also been shown to be associated with mango malformation. However, the taxonomic position within

the genus *Fusarium* and distinguishing morphological features of these fungi has yet to be defined.

Co-dominant molecular markers are increasingly being used to determine the genetic relationship of fungal populations. The pitch canker pathogen has been reported in various counties including the southern United States, California, Mexico, Japan and South Africa. Development of molecular markers for *F. circinatum* would be extremely useful as they could be used to determine relationships within and between different geographical populations of this important pathogen.

The species representing the two distinct, phylogenetic lineages associated with mango malformation in South Africa urgently require characterization. The distribution of these two species also needs to be investigated in South Africa. The determination of the vegetative compatibility diversity as well as the distribution of these two lineages will contribute towards the understanding of the biology of mango malformation in South Africa.

Morphological, pathogenicity, sexual compatibility and molecular studies have, in the last decade, contributed significantly towards our knowledge of *Fusarium* spp. in the section *Liseola*. Isolates previously identified as *F. subglutinans* can be identified as different species based on pathogenicity, mating studies (MP-B, E and H), morphology and molecular techniques. However, many questions remain regarding the taxonomy and population genetics of the *F. subglutinans sensu lato* from pine and mango. The aim of this thesis is to present studies to address some of these questions, particularly relating to these pathogens in South Africa.

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Table 1. A representation of various classifications that have been used for *Fusarium subglutinans*.

Wollenweber & Reinking (1935)	Bilai (1955)	Booth (1971)	Gerlach & Nirenberg (1982)	Nelson *et al.* 1983	Nirenberg & O'Donnell (1998)
F. neoceras			*F. neoceras*		
			F. sacchari var. *sacchari*		*F. sacchari*
				F. subglutinans f. sp. *pini*	*F. circinatum*
	F. moniliforme var. *subglutinans*			*F. subglutinans* f. sp. *ananas*	*F. guttiforme*
			F. sacchari var. *subglutinans*	*F. subglutinans*	*F. subglutinans*
F. moniliforme var. *subglutinans*		*F. moniliforme* var. *subglutinans*			
			F. sacchari var. *elongatum*		*F. bulbicola*
	F. moniliforme var. *anthophilum*		*F. anthophilum*	*F. anthophilum*	*F. anthophilum*

Table 2. Mating populations in the *Gibberella fujikuroi* complex

MP	*Fusarium* species	Synonym	Host	References
A	*F. verticillioides*	*F. moniliforme*	Maize	Booth, 1971
		G. fujikuroi var. *moniliformis*		Kuhlman, 1982
B	*F. sacchari*	*F. moniliforme* var.	Sugarcane	Booth, 1971
		subglutinans		Gerlach & Nirenberg, 1982
		F. sacchari var. *sacchari*		Kuhlman, 1982
		G. fujikuroi var. *subglutinans*		Nelson *et al.*, 1983
		F. subglutinans		
C	*F. fujikuroi*	*G. fujikuroi* var. *fujikuroi*	Rice	Booth, 1971
		F. proliferatum		Gerlach & Nirenberg, 1982
				Kuhlman, 1982
				Nelson *et al.*, 1983
D	*F. proliferatum*	*G. fujikuroi* var. *intermedium*	Asparagus	Booth, 1971
				Gerlach & Nirenberg, 1982
				Kuhlman, 1982
				Nelson *et al.*, 1983

MP	*Fusarium* species	Synonym	Host	References
E	*F. subglutinans*	*F. moniliforme* var. *subglutinans*	Maize	Booth, 1971 Gerlach & Nirenberg, 1982
		F. sacchari var. *subglutinans*		Kuhlman, 1982
F	*F. thapsinum*	*F. moniliforme*	Sorghum	Booth, 1971 Nelson *et al.*, 1983 Klittich *et al.*, 1997
G	*F. nygamai*	-	Sorghum	Burgess & Trimboli, 1986
H	*F. circinatum*	*F. moniliforme* var. *subglutinans*	Pine	Booth, 1971 Kuhlman, 1982
		F. subglutinans f. sp. *pini*		Nelson *et al.*, 1983 Correll *et al.*, 1991 Nirenberg & O'Donnell, 1998 Britz *et al.*, 1999

CHAPTER 2

CHARACTERIZATION OF THE PITCH CANKER FUNGUS, *FUSARIUM CIRCINATUM* FROM MEXICO

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ABSTRACT

Fusarium circinatum (=*F. subglutinans* f. sp. *pini*) is the causal agent of pitch canker of pines. This fungus occurs in the United States, Japan, Mexico and South Africa and it can be introduced into new areas on seed and infected plant material. Its presence in cones from symptomless trees is of concern, particularly with respect to seed transmission. In this study, isolates of *Fusarium* spp. were collected from *Pinus patula, P. greggii, P. teocote* and *P. leiophylla* trees in Mexico, showing typical symptoms of pitch canker, as well as from cones from apparently healthy trees. Morphological characteristics of the pitch canker fungus and isolates of *F. subglutinans* from other hosts are very similar. Therefore, pathogenicity tests, sexual compatibility studies and histone *H3*-RFLPs were used to characterize isolates. Isolates collected from *Pinus* spp. from Mexico were identified as *F. circinatum.* In this study we have thus confirmed that *F. circinatum* occurs on pines in Mexico and that the affected trees can be asymptomatic.

INTRODUCTION

Pitch canker, caused by *Fusarium circinatum* Nirenberg & O'Donnell (=*F. subglutinans* (Wollenweber & Reinking) Nelson *et al.* f. sp. *pini* Correll *et al.*), was first reported in the southeastern United States (Hepting & Roth, 1946). *F. circinatum* is now found throughout this region where it has caused significant losses on a wide variety of pine species. This led to the suggestion that pitch canker is endemic to the area (Dwinell *et al.*, 1985). More recently, pitch canker was identified and reported in California (McCain *et al.*, 1987), predominately on *Pinus radiata* planted in landscape settings (Correll *et al.*, 1991). Since 1992, it has been recognized as a threat to native *P. radiata* stands in California (Storer *et al.*, 1994; Storer *et al.*, 1998). *F. circinatum* is also found in Japan (Muramoto *et al.*, 1988; Kobayashi & Kawabe, 1992) and South Africa (Viljoen *et al.*, 1994; Viljoen *et al.*, 1997).

In South Africa, the fungus was reported from forestry nurseries where it has resulted in serious losses of *P. patula* (Viljoen *et al.*, 1994), *P. elliottii, P. greggii* and *P. radiata* seedlings. Stem cankers on larger trees such as those found in the United States (Hepting & Roth, 1946; Dwinell *et al.*, 1977) have not been seen in South

Africa (Wingfield *et al.*, 1999). Pitch canker has been reported in Mexico on a variety of native pine species (Santos & Tovar, 1991; Guerra-Santos, 1999) and this is thought to be the origin of the pathogen, for areas such as South Africa where Mexican pines are commonly propagated.

Fusarium circinatum has been isolated from stems and branches of trees (Hepting & Roth, 1946; Dwinell *et al.*, 1977), root collars of pine seedlings (Barnard & Blakeslee, 1980; Viljoen *et al.*, 1994), female strobili, mature cones and seeds (Dwinell *et al.*, 1977; Miller & Bramlett, 1978; Dwinell *et al.*, 1985; Barrows-Broaddus, 1986; 1987; Storer *et al.*, 1998; Dwinell, 1999). Recently, Storer *et al.* (1998) isolated *F. circinatum* from pine seedlings originating from seeds collected from cones on diseased as well as asymptomatic branches. Those authors hypothesized that *F. circinatum* can be carried inside seeds and may remain dormant until germination, which increases the possibility of seedling infections. The implication of seed transmission is serious, since current treatments may be ineffective in eliminating the pathogen. This would increase the possibility of introducing the pathogen into uninfested areas (Barrows-Broaddus & Dwinell, 1985; Storer *et al.*, 1998).

Fusarium subglutinans sensu lato includes species occurring on a wide variety of hosts, including pineapple, maize, mango, pine and sugarcane (Booth, 1971). Correll *et al.* (1991) distinguished pine and non-pine *F. subglutinans* isolates based on pathogenicity to pines. Those authors proposed that *F. subglutinans* from pine should be designated as *F. subglutinans* f. sp. *pini* based on its exclusive pathogenicity to pine trees (Correll *et al.*, 1991). Restriction fragment patterns of the mtDNA and random amplified polymorphic DNA (RAPD) also indicated that pine isolates differed from non-pine isolates (Correll *et al.*, 1992; Viljoen *et al.*, 1997).

Sexual compatibility among isolates causing pitch canker on pines confirmed that this group corresponded to a distinct biological species (Viljoen *et al.*, 1997). O'Donnell *et al.* (1998) showed pine isolates to be phylogenetically distinct and Nirenberg & O'Donnell (1998) thus proposed the name, *F. circinatum,* for it. Steenkamp *et al.*

(1999) could, furthermore, distinguish *F. circinatum* from other species in *F. subglutinans sensu lato* using histone *H3* gene sequences.

Until recently, the most reliable technique to distinguish *F. circinatum* from closely related *Fusarium* spp. has been sexual compatibility. A molecular technique based on RFLP profiles of the histone *H3* gene, reliably and rapidly distinguishes *F. circinatum* from other similar *Fusarium* spp. in the *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura complex (Steenkamp *et al.*, 1999). Sexual compatibility as well as histone *H3*-RFLPs can, therefore, be used to separate the eight different mating populations (biological species), designated by the letters A to H, in this complex. Heterothallic *F. circinatum* isolates reside in mating population H of the *G. fujikuroi* complex and tester strains representing opposite mating types have been selected and designated (Coutinho *et al.* 1995; Britz *et al.*, 1998; 1999). Sexual compatibility of field isolates with tester strains of mating population H (MRC 6213 and MRC 7488) provides a firm basis for the identification of field isolates as *F. circinatum.*

In this study, isolations from pine trees in Mexico showing typical canker symptoms were made. The possible association of *F. circinatum* with asymptomatic cones was also investigated. The identity of these isolates was verified using morphology (Nelson *et al.*, 1983; Nirenberg & O'Donnell, 1998) as well as pathogenicity, sexual compatibility and histone *H3*-RFLP comparisons.

MATERIALS AND METHODS

Isolates, cultural and morphological characteristics

Fusarium circinatum strains (MRC 7568-7587) were collected in Laguna Atezca and Hidalgo, Mexico, from cankers occurring on branches of native stands of *P. patula* and *P. greggii* (Table 1). Strains MRC 7568-7569 were isolated from apparently healthy cones collected from asymptomatic *P. patula* trees and strains MRC 7570-7587 were obtained from branches showing pitch canker symptoms. Strains MRC 7570–7572, MRC 7573-7576, 7577-7579, 7580-7582 and 7583-7585 were isolated from five trees. Strains MRC 7588-7601 were isolated from cankers on branches of

P. teocote and *P. leiophylla* in northern Michoacan, Mexico (Table 1). Single conidial isolates of all cultures were prepared and are maintained in 15% glycerol at –70°C in the *Fusarium* collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Isolates have also been deposited in the culture collection of the Medical Research Council (MRC), P. O. Box 19070, Tygerberg, South Africa.

For isolations, pine cones were immersed in 70% ethanol for 2 min. Small pieces (approximately 5 mm^2) of the asymptomatic cone scale tissue and wood pieces (2 mm^2) from the infected tree branches were removed and plated on 2% malt extract agar (MEA). Fungi were allowed to grow for seven days at room temperature. Small agar pieces (approximately 5 mm^2) from the edges of the colonies were transferred to 90 mm plastic Petri dishes containing carnation leaf agar (CLA) (Fisher *et al.*, 1982). Cultures were incubated at 23°C under near-ultraviolet and cool-white light with a 12 h photoperiod to stimulate culture and conidium development. Isolates were identified and morphological features were compared with *F. circinatum* tester strains (MRC 6213 and MRC 7488) after 10 to 14 days, using a light microscope. Diagnostic characteristics used were those specified by Nirenberg & O'Donnell (1998) and Nelson *et al.* (1983).

All isolates obtained from Mexico (Table 1), as well as, *F. circinatum* tester strains (MRC 6213 and MRC 7488) from South Africa were used in sexual compatibility tests and for comparison based on histone *H3*-RFLPs. *F. sacchari* (Butler) W. Gams (= *F. subglutinans*) and *F. subglutinans* standard tester strains of the B (MRC 6524 and MRC 6525) and E (MRC 6483 and MRC 6512) mating populations of the *G. fujikuroi* complex were also included. Tester strains of the B mating population are progeny from a fertile cross between two isolates from sugarcane (*Saccharum officinarum*) from Hsingying, Taiwan and tester strains of the E mating population were isolated from maize (*Zea mays*) in St. Elmo, Illinois, USA (Leslie, 1991). *F. subglutinans* isolates from maize (MRC 1077) and mango (*Mangifera indica*) (MRC

7035) were included in the histone *H3*-RFLP comparisons as representatives of host specific isolates of *F. subglutinans* (Table 1).

RFLPs of Histone H3 gene

DNA was extracted from cultures (Table 1) grown for 10 days in complete medium broth (CM) (Correll *et al.*, 1987) using the protocol described by Steenkamp *et al.* (1999). PCR reactions were performed using primers (H3-1a and H3-1b) for amplification of the Histone *H3* gene PCR product as described by Glass & Donaldson (1995). The same conditions as those described by Steenkamp *et al.* (1999) were used except Boehringer Mannheim polymerase and reaction buffers (Boehringer Mannheim South Africa Pty. Ltd.) were used. The restriction enzyme, *Dde* I, which distinguishes *F. circinatum* isolates from other similar *Fusarium* spp. (Steenkamp *et al.* 1999), was used in this study. Digests were performed using this restriction enzyme in a total reaction volume of 20 µl containing 5 U of the enzyme. Sodium chloride was added to the reactions with the enzyme *Dde* I to a final concentration of 100 mM. All digestion reactions were incubated at 37°C for 7 h.

Restriction fragments were separated using agarose gels (Promega Corporation, Madison, Wisconsin, USA) in the presence of ethidium bromide (0.1 µg/ml). RFLP fragments were electrophoresed on 3% (w/v) agarose gels and visualized using an ultraviolet transilluminator (Ultra-Violet Product). The visualized RFLP fragments were photographed using a gel documentation system (Microsoft Corporation) and evaluated using the methods described by Steenkamp *et al.* (1999).

Sexual compatibility

Crosses to determine sexual compatibility were made on carrot agar as described by Klittich & Leslie (1988), except that 300 g fresh carrots were used rather than the recommended 400 g. All crosses were also done on V8-agar (325 ml canned V8-juice per liter and 2% agar, pH 5.8-6.2). Hermaphrodite tester strains of the B, E and H mating populations of *G. fujikuroi* were crossed with each another (MRC 6524 x MRC 6525; MRC 6483 x MRC 6512 and MRC 6213 x MRC 7488). Isolates from Mexico were crossed with standard tester strains from the B (MRC 6524 and MRC

6525), E (MRC 6483 and MRC 6512) and H (MRC 6213 and MRC 7488) mating populations of the *G. fujikuroi* complex. All field isolates of *F. circinatum* from Mexico were crossed with each other in all possible combinations. The isolates in this study were also crossed against themselves as a negative control, i.e. where no mature perithecia should be produced. Reciprocal crosses, where the isolates corresponded to the male and female parents were reversed, were done for all crosses.

All the crosses recorded as positive were repeated at least once. Crosses were examined weekly and scored as positive when ascospores were observed, either by their exudation from perithecia or after crushing these structures. The viability of the ascospores was determined by streaking a portion of the ascospore cirrhus onto the surface of 2% water agar plates and estimating the percentage germination after 24 h.

Pathogenicity tests

Due to quarantine constraints in South Africa, pathogenicity tests of *F. circinatum* isolates from Mexico were conducted in greenhouse facilities of the Department of Plant Pathology, University of California. Tests to confirm pathogenicity were performed at approximately 25°C during the day and 18°C at night with a 12 h day/night cycle. Tests were performed on *P. radiata* seedlings, 3-4 years of age using 19 *F. circinatum* isolates collected in Mexico (Table 1), as well as, two isolates of *F. circinatum* (FSP 24 and FSP 34), known to be pathogenic to pines in California. All *F. circinatum* isolates were grown on potato dextrose agar (PDA) at 25°C for 7–10 days. Inoculations were performed by making a small wound in the seedling stems and placing a spore suspension (approximately 500 spores in distilled water) into each wound. Each isolate was inoculated into two *P. radiata* seedlings. The lesion lengths under the bark of the inoculated *P. radiata* plants were measured 41 days after inoculation.

RESULTS

Isolates, cultural and morphological characteristics

Isolates obtained from diseased pine branches, cankers and asymptomatic cones were identified as *F. subglutinans* based on morphological characteristics described by Nelson *et al.* (1983). These isolates could also be identified as *F. circinatum* based on the characteristics proposed by Nirenberg & O'Donnell (1998). Branched and proliferating conidiophores were observed and the polyphialides had 2-5 conidiogenous openings (Fig. 1c). Sterile coiled hyphae (Fig. 1d) and lunate macroconidia (Fig. 1e), reported by Nirenberg & O'Donnell (1998) to distinguish *F. circinatum* from similar *Fusarium* spp. in *Liseola* and related sections, were observed.

RFLPs of Histone H3 gene

The PCR products obtained using the primers H3-1a and H3-1b were approximately 500 base pairs (bp) in size. None of the PCR products from isolates belonging to the E mating population (MRC 6483 and MRC 6512) of the *G. fujikuroi* complex and the *F. subglutinans* isolate from maize (MRC 1077) were cut by *Dde* I, whereas the *F. subglutinans* isolate from mango had three RFLP fragments of approximately 110, 170 and 220 bp. All the isolates identified as *F. circinatum* based on morphology had the same banding pattern as the *F. circinatum* tester strains (MRC 6213 and MRC 7488), where two fragments of approximately 230 and 270 bp were evident (Fig. 2). Two histone *H3*-RFLP fragments of approximately 190 and 310 bp could be visualized for isolates in the B mating population (Fig. 2).

Sexual compatibility

Using sexual compatibility tests, we were able to verify that isolates from pine in Mexico belong to mating population H and, therefore, *F. circinatum* (Fig. 1). Perithecia with exuding ascospores (Fig. 1a, b) were produced four weeks after fertilization of the control crosses between tester strains of the B, E and H mating populations (MRC 6524 x MRC 6525; MRC 6483 x MRC 6512 and MRC 6213 x MRC 7488). The isolates from Mexico did not produce perithecia when crossed with

tester strains of either the B (MRC 6524 and MRC 6525) or the E (MRC 6483 and MRC 6512) mating populations. Mexican isolates MRC 7568, 7569, 7572, 7591, 7592, 7593, 7595, 7597, 7599 and 7600 produced perithecia with viable ascospores when crossed with the mating population H tester strain, MRC 6213. These isolates only produced fertile crosses when MRC 6213 was used as the female parent and are thus female-sterile. No mature perithecia with viable ascospores resulted from crosses amongst *F. circinatum* isolates from Mexico.

All the fertile crosses recorded in this study were repeated and identical results were obtained in at least two different tests. The same results were also obtained on both carrot and V8-agar. The percentage germination of ascospores in this study varied between 85-98%. None of the isolates in this study produced perithecia when crossed with themselves as negative controls.

Pathogenicity tests

The 21 selected *F. circinatum* isolates gave lesions after 41 days that varied in length between 10 to 90 mm. These isolates were, thus, pathogenic to *P. radiata* seedlings in the glasshouse. The pathogenicity of the *F. circinatum* isolates from California (FSP 24 and FSP 34) was consistent with that reported previously (Correll *et al.*, 1991; Gordon *et al.*, 1996).

DISCUSSION

In this study, we were able to identify *F. circinatum* from branches, cankers and asymptomatic cones from Mexico, based on a wide range of criteria. The association of the pitch canker fungus with asymptomatic cones demonstrates the possibility of spread on apparently healthy seeds such as reported by Storer *et al.* (1998). *F. circinatum* isolates considered in this study were collected from *P. patula, P. greggii, P. teocote* and *P. leiophylla* in Mexico. *P. patula, P. elliottii,* and *P. radiata* are the most important, commercially planted species in South Africa (Hinze, 1993). More recently, *P. greggii* has also become important to the forestry industry in South Africa (Malan, 1994). The isolation of *F. circinatum* from *P. patula* and *P. greggii* in native stands in Mexico, indicates that the pitch canker fungus could have been introduced

into South Africa from Mexico. This would most likely have occurred through seed importation. These findings emphasize the importance of screening seed for *F. circinatum* infection before it is exported. Seed treatment with fungicides would also reduce the chances of new introductions occurring, although it might not effectively eliminate internal infections (Storer *et al.*, 1998; 1999). Small seed lots, where the risk can be reduced through propagation under controlled conditions, may be an acceptable practice. The importation of large collections of seed for commercial planting cannot be managed effectively, and should be avoided.

In this study, only a small number (less than 28%) of *F. circinatum* isolates from Mexico were able to cross with the single *F. circinatum* tester (MRC 6213), from South Africa. It is possible that the isolates from Mexico that did not cross with MRC 6213 all belonged to the same mating type as this South African tester. Alternatively, low fertility or sterility (Perkins, 1994) could explain why none of these isolates cross with the tester strain of the opposite mating type. Female-sterility (Leslie & Klein, 1996) could also have contributed to the lack of sexual compatibility seen between Mexican *F. circinatum* isolates. The low level of sexual compatibility might also suggest that the population of *F. circinatum* in Mexico is evolving towards an asexual life history (Leslie & Klein, 1996). This is indicated by the high percentage of female-sterile isolates found in the sexual compatibility study. Despite the low level of sexual compatibility, sexual crosses confirmed that some of the Mexican isolates belonged to mating population H of *G. fujikuroi*.

Steenkamp *et al.* (1999) showed that *F. subglutinans* isolates from various plant hosts can be distinguished from one another with histone *H3*-RFLPs. Those authors concluded that this technique could be used for routine identification of *F. circinatum*. In this study, the histone *H3*-RFLP technique was critical for positive identification of *F. circinatum*. This was particularly due to the sometimes inconclusive and time-consuming pathogenicity tests and the low fertility among the *F. circinatum* isolates from Mexico.

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Table 1. List of *Fusarium* cultures used in this study.

Fusarium species	Host, substrate and origin	Isolates[a]
F. circinatum	*P. patula* cones, Hidalgo, Mexico	MRC 7568*, 7569*
F. circinatum	*P. patula* branches, Hidalgo, Mexico	MRC 7570*, 7571*, 7572*
F. circinatum	*P. greggii* branches, Laguna Atezca, Mexico	MRC 7573*, 7574*, 7575*, 7576*, 7577*, 7578*, 7579*, 7580*, 7581*, 7582*, 7583*, 7584*, 7585*, 7586*, 7587
F. circinatum	*P. teocote*, Northeastern Michoacan, Mexico	MRC 7588, 7589, 7590, 7591, 7592, 7593, 7594, 7595, 7596
F. circinatum	*P. leiophylla,* North-central Michoacan, Mexico	MRC 7597, 7598, 7599, 7600, 7601
F. circinatum	*P. radiata,* California	FSP 24*, 34*
F. circinatum	*P. patula* seedling, Ngodwana, South Africa	MRC 6213, 7488
F. sacchari	*Saccharum officinarum,* Taiwan	MRC 6524, 6525
F. subglutinans	*Zea mays*, South Africa	MRC 1077
F. subglutinans	*Z. mays,* Illinois, USA	MRC 6483, 6512
Fusarium sp.	*Mangifera indica*, Florida, USA	MRC 7035

[a] MRC refers to the culture collection of the Medical Research Council, P.O. Box 19070, Tygerberg, South Africa and FSP = *F. circinatum,* Department of Plant Pathology, University of California, Davis, California 95616. * Isolates marked are those used in pathogenicity tests.

Fig. 1. Morphological characteristics of *Fusarium circinatum*. (a) Mature perithecia (bar = 100µm). (b) Single septate ascospores (bar = 10µm). (c) Branched conidiophores bearing polyphialides with conidiogenous openings indicated by arrows (bar = 10µm). (d) Coiled sterile hyphae (bar = 10µm). (e) Lunate macroconidia (bar = 10µm).

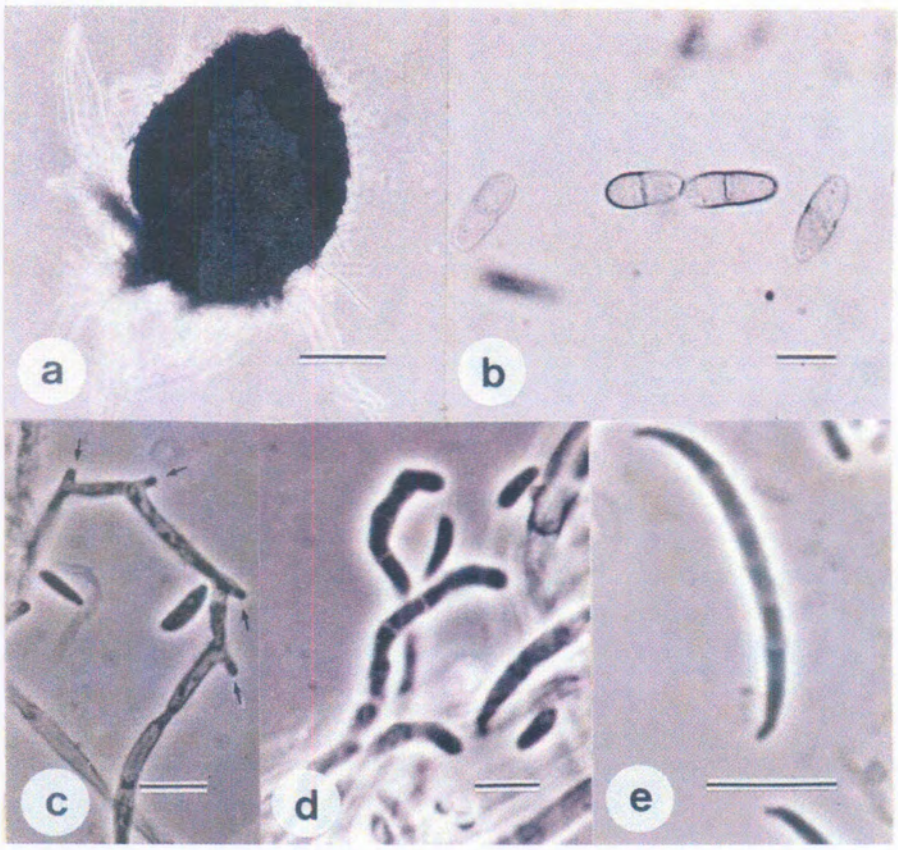
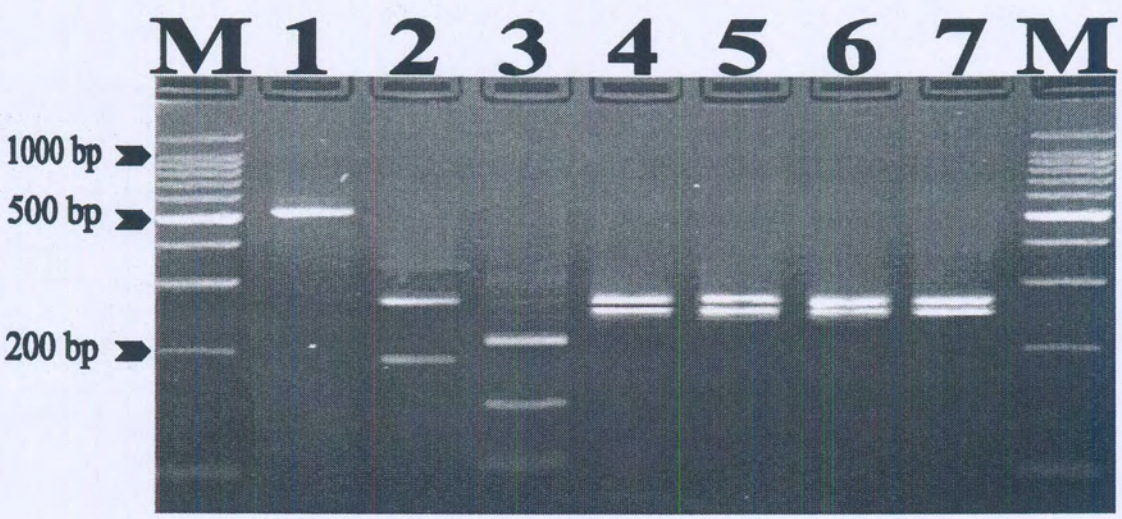


Fig. 2. *Dde* I RFLP profiles of the digestion of the histone *H3* gene amplification products on a 3% agarose gel. Lane marked as M = 100 bp ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp). Lane 1 = *F. subglutinans* (MRC 6512) from the E-mating population. Lane 2 = *F. sacchari* (MRC 6525) from the B-mating population. Lane 3 = *F. subglutinans* isolate (MCR 7035) from mango. Lane 4-6 = digestion products of *F. circinatum* (MRC 7568, 7573, 7587, 7589, 7596 and 7598) from Mexico. Lane 7 = digestion product of *F. circinatum* tester strain (MRC 6213) from mating population H.



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).

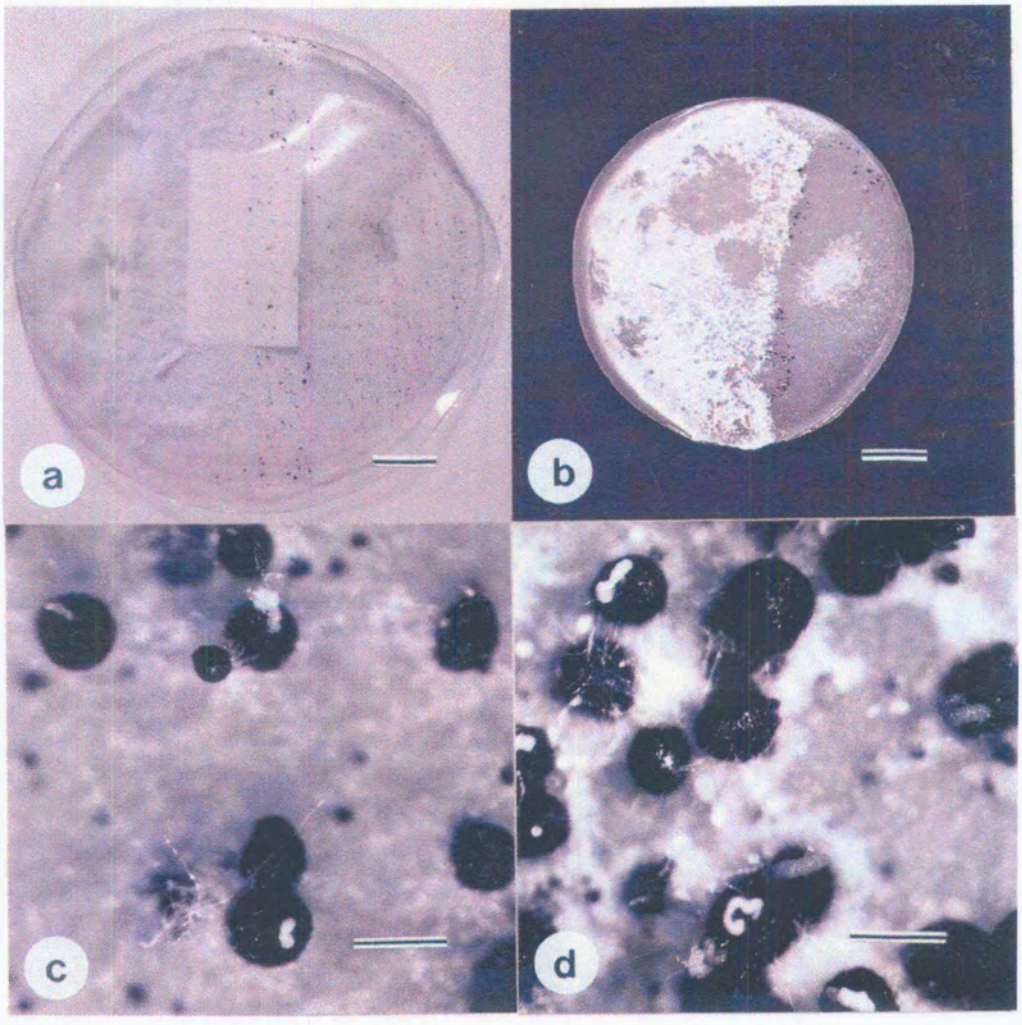
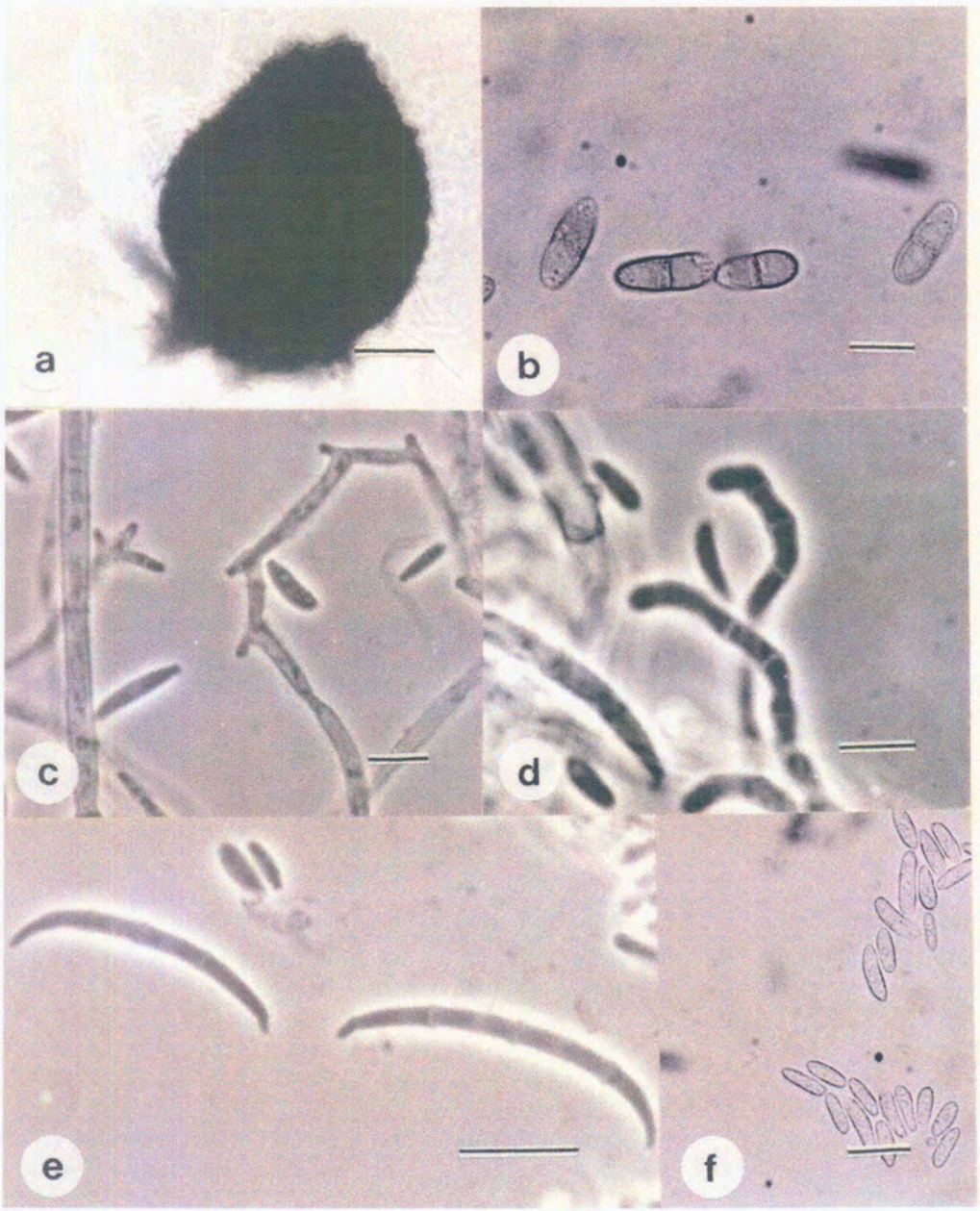


Fig. 2. Morphological characteristics of *G. circinata* and *F. circinatum* (CMW 6213). (a) Mature perithecia (bar = 100µm). (b) Septate ascospores (bar = 10µm). (c) Branched conidiophores (bar = 10µm). (d) Sterile coiled hyphae (bar = 10µm). (e) Slender and cylindrical macroconidia (bar = 10µm). (f)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

CHAPTER 5

INFLUENCE OF SEXUAL REPRODUCTION ON THE *FUSARIUM CIRCINATUM* POPULATION IN SOUTH AFRICA

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ABSTRACT

Fusarium circinatum (=*F. subglutinans* f. sp. *pini*) is an important pathogen in pine nurseries in South Africa. The initial outbreak of pitch canker disease caused by this pathogen was limited to a single nursery. Subsequently, several other pine nurseries in South Africa have become infected. A high level of genotypic diversity was observed in the initial South African *F. circinatum* population, suggesting that sexual reproduction occurs in the fungus. Sexual crosses performed in the laboratory indicated a low frequency of hermaphrodites in the initial population, suggesting that the fungus reproduces asexually rather than sexually. The aim of this study was, therefore, to determine the influence of sexual reproduction on the *F. circinatum* population in South Africa by comparing *F. circinatum* populations collected from initial *F. circinatum* outbreak and from recent outbreaks. This was done by determining the effective population number, N_e that estimates the contribution of the sexual and asexual cycle on the population structure. The allelic diversity determined with nine polymorphic sequence characterized amplified markers and vegetative compatibility group (VCG) diversity were also used to assess the two populations. The N_e of the initial population based on the number of male and hermaphrodites was lower (N_e = 80.6-86.6%) than in the recent outbreaks (N_e = 93.0-96.0%). The allelic diversity of the initial population was also lower (0.154) than the recent population (0.231) and six new VCGs have emerged since the initial outbreak of *F. circinatum* in South Africa 10 years ago. The increase in N_e, allelic diversity and number of VCGs over the last 10 years indicated that sexual reproduction is occurring relatively frequently, even though signs of this have not been observed in the field.

INTRODUCTION

The pitch canker pathogen, *Fusarium circinatum* Nirenberg & O'Donnell (=*F. subglutinans* (Wollenweber & Reinking) Nelson *et al.* f. sp. *pini* Correll *et al.*) causes a destructive disease of pines. *F. circinatum* first appeared in South Africa in a single forestry nursery in 1990, causing a root disease of *Pinus patula* seedlings (Viljoen *et al.*, 1994). Since this initial outbreak, *F. circinatum* has spread to several other forestry nurseries causing serious root and collar rot of various *Pinus* spp. Stem cankers on mature trees typical of those found elsewhere have, however, not been observed in South Africa (Viljoen *et al.*, 1997a; Wingfield *et al.*, 1999).

The genotypic diversity of the initial *F. circinatum* population in South Africa have been determined using vegetative compatibility groups (VCGs) (Viljoen *et al.*, 1997b) and allelic diversity using nine sequence characterized polymorphic markers (Britz *et al.*, submitted (chapter 4 of this thesis)). A high level of VCG diversity (23 VCGs among 69 isolates) led Viljoen *et al.* (1997b) to suggest that sexual reproduction is occurring naturally within the South African population. However, the allelic diversity determined using sequence characterized amplified polymorphic markers indicated a relative low allelic diversity in the initial *F. circinatum* population (Britz *et al.*, submitted). Therefore, the VCG and allelic diversity data (Viljoen *et al.*, 1997b; Britz *et al.*, submitted) indicates that sexual reproduction is occurring in South Africa among closely related isolates in the South African population. This also indicate that the pathogen in South Africa has been recent introduced (Viljoen *et al.*, 1997a, b).

Fusarium circinatum and other related *Fusarium* spp. residing in distinct mating populations of *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura complex (Leslie, 1995; Klaasen & Nelson, 1996; Klittich *et al.*, 1997; Britz *et al.*, 1999) is heterothallic and mating is controlled by two alleles at a single mating type locus. *F. circinatum* resides in mating population H of the *G. fujikuroi* complex (Britz *et al.*, 1999). *F. circinatum* strains of opposite mating type can recombine, leading to the formation of the sexual structures (Britz *et al.*, 1998; Viljoen *et al.*, 1997a). The name, *G. circinata* Nirenberg & O'Donnell has recently been applied to the teleomorph (Nirenberg & O'Donnell, 1998).

Fusarium circinatum can reproduce both asexually and sexually and each of these cycles affects the population structure differently (Leslie & Klein, 1996; Britz *et al.*, 1998). The asexual cycle results in clonal propagation, whereas the sexual cycle results in recombination leading to new genotypes (Leslie & Klein, 1996). The effective population number (N_e) estimates the relative contribution of each cycle towards the population (Leslie & Klein, 1996). However, the numbers of strains that function as female parents usually limits the N_e (Leslie & Klein, 1996; Britz *et al.*, 1998). The initial *F. circinatum* population in South Africa had a low frequency (27%) of hermaphrodites (Britz *et al.*, 1998). If the number of hermaphrodites in the *F. circinatum* population decreases then the population in South Africa could become asexual (Britz *et al.*, 1999; 1999).

Several new outbreaks have occurred in South Africa since the initial outbreak of *F. circinatum* in 1990 (Viljoen *et al.*, 1994). The opportunity has, therefore, arisen to be able to compare the N_e of the initial *F. circinatum* population with a recently collected *F. circinatum* population. Thus enabling the determination of the influence of sexual reproduction on the population over 10 years. However, the N_e was determined at a lower incubation temperature, because Covert *et al.* (1999) found lower temperatures increase the fertility of sexual crosses of *F. circinatum* in the laboratory. Furthermore, the VCG and allelic diversity in the *F. circinatum* population collected six years after the initial outbreak of the disease in South Africa were also determined and compared with previous studies on the initial population.

MATERIALS AND METHODS

Sampling

Fusarium circinatum isolates were collected from six different forestry nurseries in South Africa (Table 1). The initial *F. circinatum* population was from a single nursery (Ngodwana) and isolates representing the recent *F. circinatum* population were collected from nurseries in the Piet Retief, Karatara, Sutherland, Tweefontein and Klipkraal areas. The initial *F. circinatum* population represents 85 *F. circinatum* isolates collected between 1990 and 1992 (Viljoen *et al.*, 1994). The recent *F. circinatum* populations consist of 74 isolates that were obtained from outbreaks between 1996 and 1998. A total of 159 *F. circinatum* isolates were examined in this study (Table 1).

Roots, root collars and stems of pine seedlings showing disease symptoms, e.g. tip die-back and needle discoloration, were immersed in 70% ethanol for 2 min. Small pieces (approximately 5 mm long) of the infected tissue were removed and plated on *Fusarium* selective medium (Nash & Snyder, 1962). Cultures were allowed to grow for 5 days at 25°C. Small agar pieces (approximately 5 mm^2) from the edges of the colonies were transferred to 90 mm diameter Petri dishes containing carnation leaf agar (CLA) (Fisher *et al.*, 1982). Cultures were incubated at 23°C under near-ultraviolet and cool-white light with a 12 h photoperiod to stimulate culture and conidium development. Isolates identified as *F. circinatum* on CLA were purified as single conidial cultures and stored

as conidial suspensions in 15% glycerol at –70°C and are available 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. A large number of the strains also have been deposited in the culture collection of the Medical Research Council (MRC), P. O. Box 19070, Tygerberg, South Africa.

Sexual compatibility and mating type determination

All the *F. circinatum* isolates were crossed with the mating population H tester strains, MRC 6213 and MRC 7488 (Britz *et al.*, 1998; 1999). The tester strains were crossed with each other as a positive control. Crosses were made on carrot agar as described by Klittich & Leslie (1988) at 17°C. All the crosses were examined weekly and designated sterile, female-sterile or hermaphrodite (Table 1). All fertile crosses were repeated at least once. The tester strains, MRC 7488 and MRC 6213, were designated *MAT*-1 and *MAT*-2, respectively (Steenkamp *et al.*, 2000). Isolates that produced perithecia with exuding ascospores, when crossed with the tester strains were assigned to the mating type opposite to that of the tester strains (Table 1).

The effect of mating type allele frequency and female fertility on the South African *F. circinatum* population was assessed by determining the effective population number for the mating type ($N_{e(mt)}$), effective population number ($N_{e(f)}$), the average number of asexual generations per sexual generation using the equations described in Leslie & Klein (1996).

Vegetative compatibility (VC) tests

Nitrate non-utilizing (*nit*) mutants were generated on 1.5 – 2.5% chlorate agar and the *nit* mutants were identified as *nit1*, *nit3* or *nit*M based on the utilization of different nitrogen sources (Puhalla & Spieth, 1983; 1985; Correll *et al.*, 1987). Incompatibility was determined by pairing isolates in all possible combinations on minimal media for 14 days at 25°C. Heterokaryon self-incompatibility (HSI) of isolates (Correll *et al.*, 1989) was determined by pairing a *nit1* or *nit3* mutant with a *nitM* mutant of the same isolate. All parings were repeated at least once.

Each VCG was assigned a number (SA1-SA29). The most common VCGs in the *F. circinatum* population were determined by identifying the number of representatives for

each VCG. The diversity of the VC groups for isolates representing the initial and recent *F. circinatum* outbreaks was determined in two ways. First, the number of VCGs was divided by the sample size in each population (S/N). Secondly, the Shannon diversity index (H′), which provides an indication of the population structure was calculated using the equation:

$$H' = -\Sigma p_i \ln p_i$$

where p_i is the observed frequency of the VC group (genotype). If all the individuals have the same genotype then $H' = 0$, and when they all belong to a different genotype, then H' will have the highest value (Sheldon, 1969).

Allelic diversity

The allele frequency and diversity values of the initial *F. circinatum* population (determined with sequence characterized amplified polymorphic markers) were obtained from Britz *et al.* (submitted) and values of the recent population were determined in the present study (Table 3). In the present study, the allelic frequency and diversity of 32 isolates representing recent *F. circinatum* outbreaks were calculated using data of nine sequence characterized amplified polymorphic markers. The DNA extraction procedures, PCR conditions, the method of determining allele size for the 32 isolates as well as the analysis were done as described by Britz *et al.* (submitted).

Multi-locus diversity was calculated using genotype data of each isolate (at all nine sequence characterized amplified polymorphic loci) from the initial (Britz *et al.*, submitted) and recent *F. circinatum* populations, $H_M = -\Sigma g_i \ln g_i$, where g_i is the frequency of the ith multi-locus genotype and $H_{EM} = H_M / \ln S$, where S is the sample size (Sheldon, 1969; Goodwin *et al.*, 1993) (Table 3).

RESULTS

Sexual compatibility and mating type distribution

Some *F. circinatum* isolates did not produce perithecia when crossed with the mating tester strains. The percentage of sterile strains collected from the different nurseries ranged from 11-17% with the exception of 29% sterile isolates found among those collected from Piet Retief nursery. No sterile *F. circinatum* isolates were collected from

the Tweefontein and Sutherland nurseries, and the single isolate collected from Karatara nursery was also sterile. Sterile isolates were excluded from the calculation of $N_{e(mt)}$ and $N_{e(f)}$. However, the $N_{e(f)}$ was also calculated for each nursery by including the sterile strains in the calculation, as female-sterile strains (Table 1).

Reassessment of initial F. circinatum population

Of the 85 *F. circinatum* isolates collected from the initial outbreak at the Ngodwana nursery, 71 were cross fertile with one of the mating type testers (MRC 6213 and MRC 7488). The mating type distribution of the initial population had a 1:1 ratio giving a $N_{e(mt)}$ of 99%. Among the 71 fertile isolates, 33 were hermaphrodites and 38 female-sterile (Table 1). The percentage of the hermaphrodites ranged from 21 - 69% (observed value 47%), when only the fertile strains were used. The average number of asexual generations per sexual generation was estimated to range from 20-79. This variation depends on the values for the mutation rate (μ) to female sterility and for selection (θ) against hermaphrodites during the asexual generation of the life cycle which were $0.98 > \mu(1-\theta) > 0.99$. When all the strains were included, then the percentage of hermaphrodites ranged from 12 - 63% (observed value 39%) with the average number of asexual generations per sexual generation ranging from 24 to 117 depending on the values of μ and θ.

Recent F. circinatum population

The recently collected *F. circinatum* population included isolates from nurseries other than the Ngodwana nursery, where the initial population was studied. In the recently collected population consisting of 74 isolates, 42 hermaphrodites were present amongst 72 fertile strains collected from five different nurseries (Table 2). The recently collected *F. circinatum* population had a 1:2 (*MAT-1*:*MAT-2*) mating type ratio, giving a $N_{e(mt)}$ of 86.7%. The hermaphrodite frequency varied from 45 – 82% (observed value 67%) with the estimated average asexual generations ranging from 10 – 39 per sexual generation, if only fertile isolates were considered. If all strains (fertile and sterile *F. circinatum* isolates from recent outbreaks) are considered, the hermaphrodite frequency ranged from 36 – 76% (observed value 58%) with the average number of asexual generations per sexual generation ranging between 13 – 51 ($0.98 < \mu(1-\theta) < 0.99$).

Combined F. circinatum population

Of the combined population of 157 *F. circinatum* isolates (initial and recent outbreaks), 134 were cross fertile with one of the tester strains (MRC 6213 or MRC 7488). These isolates included 75 hermaphrodites and 59 female-sterile strains. The hermaphrodite frequency ranged from 32 – 75% (observed value 56%) with the average asexual generations per sexual generation ranging from 15 – 56, if only fertile isolates are considered. If all strains (fertile and sterile) were included, the hermaphrodite frequency ranges from 23 – 69% (observed value 48%) with the average asexual generations per sexual generation ranging from 18 – 73 ($0.98 < \mu(1-\theta) < 0.99$).

Vegetative compatibility tests

Chlorate-resistant *nit* mutants were produced for 125 *F. circinatum* isolates. Twenty-nine VCGs were identified amongst these isolates. Of the 29 VCGs, 23 were the same as those identified from the initial *F. circinatum* outbreak occurring in 1990 (Viljoen *et al.*, 1997b). Six new VCGs were identified from the recent *F. circinatum* outbreaks. The six new VCGs were from the Klipkraal and Sutherland nurseries (Table 2). A single sterile heterokaryon self-incompatible *F. circinatum* isolate was identified.

Three dominant VCGs, SA-8, SA-12 and SA-14, were present in the initial outbreak of *F. circinatum* and represented 43% of the isolates (Viljoen *et al.*, 1997b). In the recent outbreaks, VCG SA-2 and SA-4 were dominant and represented 43% of the isolates. All VCG SA-2 isolates were collected from the Ngodwana (initial outbreak) and Klipkraal nurseries. VCG SA-4 isolates were present in all nurseries except Ngodwana and Sutherland.

The diversity of VCGs varied among the nurseries (Table 2). The number of VCGs divided by the sample size in the initial, recent and combined population (S/N) was 0.32, 0.30 and 0.24, respectively (Table 2). In the Ngodwana nursery, most isolates belonged to a different VCG, H'=2.89. The H' value for the recent outbreaks was 2.25 and the H' value when all the outbreaks were considered was 3.02 (Table 2). The H' value becomes inaccurate as the number of isolates collected in each nursery decreases (N<7). With normalization, H_{EM} (Sheldon, 1969; Goodwin *et al.*, 1993), the recent population was 0.564 and the initial population was 0.678 (Table 2). However, most of

the H_{EM} values of populations collected in recent outbreaks were higher than the H_{EM} value of the initial outbreak. The H_{EM} value for the population collected in Klipkraal in April 1998 from *P. elliottii* was the lowest, because most of the isolates belonged to a single dominant VCG.

Allelic diversity

The allele frequency using nine polymorphic markers for the initial *F. circinatum* population was 0.154 without adjusting for sample size (Britz *et al.*, submitted, Table 3). In this study, the allele frequency for isolates representing the recent population is 0.231 without adjusting for sample size. With adjustment for sample size, the allelic diversity (H_{TR}) for the initial population is 0.163 (Britz *et al.*, submitted) while the recent population is 0.331 (Table 3). The combined allelic diversity, H_T, for all isolates thus far studied in South Africa was 0.216. There were 25 putative alleles amongst the nine polymorphic markers in the combined South Africa populations. The number of alleles at each locus ranged from two to four, with an average 3.78 alleles per locus.

The initial *F. circinatum* population had nine multi-locus genotypes and the recent population had 18 multi-locus genotypes. The combined population had 25 multi-locus genotypes. The multi-locus diversity measured using the Shannon statistic H_M, was 1.730 for the initial population and 2.680 in the recent population (Table 3). The diversity values were normalized by maximizing the possible diversity in view of the sample size in each location (Sheldon, 1969; Goodwin *et al.*, 1993). With the normalization, H_{EM}, the diversity value of the initial South African population was 0.578 and that of the recent population was 0.773 (Table 3).

DISCUSSION

The first outbreak of *F. circinatum* in South Africa was reported in a single nursery (Viljoen *et al.*, 1994). Since this time, the disease has spread to several other pine nurseries in the country. A high level of VCG diversity was reported in the initial South African population, indicating that sexual recombination is occurring (Viljoen *et al.*, 1997b). However, the sexual structures of *F. circinatum* have never been found in the field. The primary objective of this study was to consider changes in the *F. circinatum* population in South Africa since the first outbreak. In this study, the N_e and number asexual generations per sexual generation of the recent *F. circinatum* population

indicates that sexual reproduction probably occurs more frequently than was obvious when the initial population was studied. This is evident from the increase in allelic diversity and the fact that new VCGs are present in the recently collected *F. circinatum* population. All indications are that active sexual reproduction is the origin of the higher level of genotypic diversity in the recently collected populations of *F. circinatum* in South Africa.

The fact that sexual structures representing *G. circinata*, have not been observed in the field is intriguing. This could be attributed to the fact that sexual reproduction does not occur at the time that the fungus is most commonly collected during disease outbreaks. This would be consistent with the fact that sexual reproduction often is coupled to overwintering, whereas asexual reproduction is coupled to the epidemic phase of disease (Linders, 1996). In the laboratory, *F. circinatum* also requires low temperatures to undergo sexual recombination (Covert *et al.*, 1999) and it seems likely that our sampling strategy has not co-incided with the appearance of sexual structures as most of the disease out breaks have occurred during the warmer months.

The high $N_{e(f)}$ in this study indicates that sexual reproduction in the South African *F. circinatum* population is more frequent for recent outbreaks ($N_{e(f)}$ = 93.1-96.0) than it was at the time of the initial outbreak ($N_{e(f)}$ = 80.6-86.6). The allelic diversity and multi-locus diversity also support the view that sexual reproduction is more frequently associated with recent outbreaks of *F. circinatum*. However, the Shannon diversity index obtained from vegetative compatibility tests on isolates representing the initial *F. circinatum* outbreak was higher than that for the more recent outbreaks. This is despite the fact that six new VCGs were identified for recent outbreaks. This might be due to the fact that a larger amount of isolates was available for the initial disease outbreak in a single nursery than for the recent outbreaks in various nurseries.

Sexual crosses performed among isolates collected from the initial outbreak of *F. circinatum* indicated a low hermaphrodite frequency (Britz *et al.*, 1998). These authors hypothesized that the number of hermaphrodites would continue to fall due to the high number of female-sterile strains and that the population would evolve towards asexuality. However, in the present study, a lower incubation temperature was used due to the fact that it is known to promote sexual crossing in cultures of *F. circinatum*

(Covert *et al.*, 1999). Female fertility and mating type distribution for the *F. circinatum* population from the initial disease outbreak was previously determined at 22°C (Britz *et al.*, 1998). The hermaphrodite frequency and effective population number were, therefore, reassessed in this study at 17°C. In the present study, we have shown that the hypothesis of Britz *et al.* (1998) was unfounded, because female fertility was inadvertently not assessed at optimal conditions. Using optimized conditions, we have now shown that a high frequency of hermaphrodites was present in the initial *F. circinatum* population (47%). This indicates that sexual reproduction occurs frequently in this population and that the relative number of hermaphrodites is increasing as proposed by Leslie & Klein (1996).

After the initial outbreak of *F. circinatum* in a single nursery in 1990, the fungus has spread to other major pine nurseries in South Africa. The fact that the same VCGs were found in the recent outbreak situations, suggests that the fungus has been introduced to these nurseries. It is known that the fungus can move to new areas with infected seedlings (Gordon *et al.*, 1996). However, nurseries in South Africa are not known to exchange plant material. It is more likely that the fungus has moved through air or seed-borne inoculum. *F. circinatum* now appears to be well established and widely distributed in pine nurseries in South Africa. It is an aggressive pathogen and management strategies will be needed to reduce losses.

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Table 1. Mating type and fertility characteristics of the *Fusarium circinatum* isolates collected in various South African forestry nurseries.

Nurseries[a]	Host	Collection Date	Characteristics of isolates[b]									
			n	*MAT-1*	*MAT-2*	N	FS	H	S	$N_{e(mt)}$	$N_{e(f)}$	$N_{e(f)}$[c]
Ngodwana (NG)[b]	*P. patula*	Jun 1990 – Jul 1992	71	35	36	85	38	33	14	99.9	86.6	80.6
Piet Retief (PR)	*P. patula*	1996-1997	5	3	2	7	5	0	2			
Karatara (KA)	*P. radiata*	1996	-	-	-	1	-	-	1	Data not determined due		
Sutherland (SU)	*P. patula*	Sept 1998	8	0	8	8	0	8	0	to small sample size		
Tweefontein (TF)	*P. patula*	Feb 1996 & Sept 1998	2	2	0	2	2	0	0			
Klipkraal (KK1)	*P. greggii*	Mrt 1997	11	3	8	11	2	9	0	79.3	99.0	99.0
Klipkraal (KK2)	*P. patula*	Apr 1998	16	6	10	18	8	8	2	93.8	88.9	85.5
Klipkraal (KK3)	*P. elliottii*	Apr 1998	21	6	15	25	4	17	4	81.6	98.9	96.4
Total Klipkraal (KK)	-	-	48	15	33	54	14	34	6	85.9	97.1	94.8
Total recent	-	1996-1998	63	20	43	72	21	42	9	86.7	96.0	93.1
Combined populations	-	1990-1998	134	55	79	157	59	75	23	96.8	92.0	87.5

[a] Forestry nurseries with abbreviations for each. Ngodwana represents the initial *F. circinatum* population,

[b] Characteristics of *F. circinatum* isolates: n = Total number of fertile isolates as determined in crosses; *MAT-1* and *MAT-2* indicates the number of isolates belonging to either mating type; N = Total number of isolates sampled in each area; FS = Female-sterile isolates; H = Hermaphrodite isolates; S = Sterile isolates; $N_{e(mt)}$ = Effective population number based on mating type expressed as a percentage of the total amount of fertile isolates in the population; $N_{e(f)}$ = Inbreeding effective number based on number of male and hermaphrodites and expressed as a percentage of the fertile isolates in each population.

[c] Inbreeding effective number based on number of male and hermaphrodites and expressed as a percentage of the fertile and sterile isolates in each population.

Table 2. *Fusarium circinatum* vegetative compatibility groups in South Africa nurseries.

South African Nurseries[a]	VCGs[c]	N[d]	VCG diversity		
			S/N[e]	H'[f]	H_{EM}[g]
NG (Initial)	23	71	0.32	2.89	0.678
PR	5	7	0.71	1.55	0.797
KA	1	1	1.00	0	0
SU	1	8	0.13	0	0
TF	1	1	1.00	0	0
KK1	7	10	0.70	1.89	0.821
KK2	7	11	0.64	1.76	0.734
KK3	4	16	0.25	0.95	0.343
KK	13	37	0.35	2.10	0.582
Recent[b]	16	54	0.30	2.25	0.564
Combined	29	125	0.23	3.02	0.625

[a] Abbreviations for nurseries are listed in Table 1, with NG being the initial population and the combined population includes the initial and recent populations.

[b] Recent population collected from outbreaks since 1996-1998 in PR, KA, SU, TF and KK nurseries.

[c] Number of VCGs present in each nursery.

[d] Sample size in each nursery.

[e] Number of VCGs found in each nursery.

[f] Shannon diversity index (equation in text).

[g] Sheldons's index, $H_{EM}=H'/\ln S$, where S=sample size (Sheldon, 1969).

Table 3. Allelic, genetic and multi-locus diversity of South African *F. circinatum* populations.

South Africa population	Allelic diversity[b]	H_{TR}[c]	Multi-locus diversity		
			Genotypes	H_M[d]	H_{EM}[e]
Initial[a]	0.154	0.162	9	1.730	0.578
Recent	0.231	0.331	18	2.680	0.773
Combined	0.212	0.216	25	1.868	0.473

[a] Data obtained from Britz *et al.* (submitted (chapter 4 of this thesis)).

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

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

[d] The equation of Shannon statistic information, H_M (referred to as *H* by Sheldon (1969) and *M* by Goodwin *et al.* (1993)), is in the text.

[e] Sheldon's index, H_{EM} (equation in text).

CHAPTER 6

TWO NEW SPECIES OF *FUSARIUM* SECTION *LISEOLA* ASSOCIATED WITH MANGO MALFORMATION

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ABSTRACT

Mango malformation is an economically important disease of *Mangifera indica* globally. A recent DNA-based study indicated that two distinct, phylogenetic lineages previously identified as *Fusarium subglutinans* are associated with this disease in South Africa. The objective of this study was to characterize *Fusarium* isolates associated with mango malformation, including the two different *F. subglutinans* groups, based on morphological characteristics. For this purpose we examined *Fusarium* strains isolated from diseased mango inflorescences from diverse geographical origins. We also used sexual compatibility tests to determine whether sexual reproduction among the strains was possible. The morphological characters considered were; shape of the conidia, presence of mono- and/or polyphialides, origin of the conidiophores from the substrate, presence of chlamydospores and the presence of sterile coiled hyphae. Three unique *Fusarium* spp. were identified. In this paper, we provide formal descriptions for the two new taxa in the section *Liseola* that we have named *F. mangiferae* and *F. sterilihyphosum*. *F. mangiferae* is conspecific with strains that were previously identified as *F. subglutinans* and reported to be the causal agent of malformation in mango growing areas throughout the world. *F. sterilihyphosum,* on the other hand, has been isolated only from malformed mango tissue in South Africa.

INTRODUCTION

Mango (*Mangifera indica* L.) malformation is an economically important disease in mango-growing areas of the world including India, Pakistan, Egypt, South Africa, Brazil, Israel, Florida and Mexico (Kumar *et al.,* 1993; Freeman *et al.,* 1999). This disease causes abnormal development of vegetative shoots and inflorescences (Kumar *et al.,* 1993). Floral malformation is the most prominent symptom and is characterized by abnormal, thick and fleshy panicles (Varma, 1983; Kumar *et al.,* 1993). Affected panicles bear no fruit, resulting in significant economic losses (Varma *et al.,* 1974; Varma, 1983; Kumar *et al.,* 1993).

The etiology of mango malformation disease is controversial. Physiological abnormality, virus infections, mite infestations and fungal pathogens have been reported as the causal agents of this disease (Kumar *et al.,* 1993). Summanwar *et al.*

(1966) identified the fungal pathogen commonly associated with the disease as *Fusarium subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas (= *F. moniliforme* Sheldon var. *subglutinans* Wollenweber & Reinking), residing in section *Liseola.* Freeman *et al.* (1999) recently demonstrated that isolates identified as *F. subglutinans* induced typical mango malformation symptoms on mango trees using the isolate MRC 7559 (506/2) originally collected from mango inflorescences in Israel.

Fusarium subglutinans forms part of the *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura species complex (Leslie, 1995; Britz *et al.,* 1999). *F. subglutinans* is, however, a polyphyletic taxon (= *F. subglutinans sensu lato*) that has been associated with various plant hosts, each of which represents a distinct lineage in the *G. fujikuroi* complex (Leslie, 1995; O'Donnell *et al.,* 1998; Britz *et al.,* 1999; Steenkamp *et al.,* 1999; O'Donnell *et al.,* 2000; Steenkamp *et al.,* 2000a). These lineages are difficult to distinguish using conventional morphological characters such as those proposed by Nelson *et al.* (1983). Until relatively recently, these fungi were distinguished from each other using pathogenicity and mating studies (Leslie, 1995). The different lineages representing *F. subglutinans sensu lato* are, however, readily distinguishable using DNA sequences of genes for β-tubulin, translation elongation factor EF-1α, histone *H3* and calmodulin (O'Donnell *et al.,* 1998; Steenkamp *et al.,* 1999; O'Donnell *et al.,* 2000; Steenkamp *et al.,* 2000a).

Mango malformation in South Africa is associated with two phylogenetically distinct groups of isolates until recently referred to as *F. subglutinans* (Steenkamp *et al.,* 1999; 2000a). Based on the histone *H3* and β-tubulin gene sequences, one group of isolates represents a previously undescribed lineage in the *G. fujikuroi* complex. The second group of isolates is conspecific with isolates that were previously reported to be the causal agent of mango malformation (Steenkamp *et al.,* 2000a). The results presented by these authors also confirmed those of Viljoen *et al.* (1997), O'Donnell *et al.* (2000) and Leslie (personal communication), who have shown using random amplified polymorphic DNAs (RAPDs), DNA sequence of several genes and isozymes, respectively, that mango malformation is associated with two distinct species, both with morphological characters typical of *F. subglutinans.*

The aim of this study was to characterize *Fusarium* spp. isolated from malformation mango tissue, from diverse geographical origins using morphology. For this purpose the morphological characteristics proposed by Nirenberg & O'Donnell (1998) were used. Sexual compatibility tests were also used to verify the identity of some of these *Fusarium* spp.

MATERIALS AND METHODS

Morphological and cultural studies

Fusarium spp. associated with mango malformation in South Africa were isolated from mango trees in Tzaneen (Northern Province), which included the areas Letsitele (LS) and Deer Park (DP). Isolates were also collected from Nelspruit (NS), Fredenheim (FH), Malelane (ML) and Hazyview (HZ) (Mpumalanga). Other isolates used in this study were isolated from malformed mango tissue by other collectors in Florida, Egypt, Israel, Malaysia and South Africa (Table 1). Mating tester strains (MRC 6213 and MRC 7488) for *F. circinatum* Nirenberg & O'Donnell (mating population H of the *G. fujikuroi* complex) were used in sexual compatibility tests (Britz *et al.*, 1999). All the isolates were stored in 15% glycerol at –70°C in the *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and the culture collection of the Medical Research Council (MRC), P. O. Box 19070, Tygerberg, South Africa.

Mango inflorescence clusters, collected in South Africa, were surface sterilized with 70% ethanol for 2 min and washed with sterile deionized water for 1 min. Single malformed flowers were removed from the sterilized cluster and plated onto a *Fusarium* selective medium (Nash & Snyder, 1962). After incubation for 5 days at 25°C, small agar pieces overgrown with mycelium were taken from the edges of the colonies and transferred to 90 mm diameter Petri dishes containing carnation leaf agar (CLA) (Fisher *et al.*, 1982). After incubation on CLA at 25°C for 7 days single conidial isolates were prepared and stored in 15 % glycerol at –70°C.

To stimulate culture and conidial development, *Fusarium* isolates (Table 1) were transferred to CLA (Fisher *et al.*, 1982) and KCl agar (Nelson *et al.*, 1983). Cultures were incubated at 23°C under fluorescent and cool-white light with a 12 h photoperiod. After 10 to 14 days of incubation, the following morphological characters were examined: shape of the conidia, presence of mono- and/or polyphialides, origin of the conidiophores from the substrate, presence of chlamydospores and sterile coiled hyphae (Nirenberg & O'Donnell, 1998). Secondary characteristics such as growth rate and colony color (Rayner, 1970) were determined on potato dextrose agar (PDA) after incubation at 25°C in the dark (Nelson *et al.*, 1983) for the two newly described species. Each isolate was plated onto three different PDA plates and the growth rate was determined over a period of 10 days. This entire procedure was repeated once more. A one way ANOVA was done to determine whether growth rate differed significantly for the different isolates. Colony color was determined after 14 days using the colour coding system of Rayner (1970). Fifty measurements were made of all diagnostic morphological characters. The measurements are indicated as minimum, mean and maximum with the standard deviations in parentheses.

Mating type and sexual compatibility tests

The mating type (*MAT*-1 or *MAT*-2) of all the isolates included in this study were determined using the PCR-based method described by Steenkamp *et al.* (2000b). Only isolates with opposite mating types were crossed within each species and between species using the method described by Klittich & Leslie (1988) with some modifications (Britz *et al.*, 1999). Covert *et al.* (1999) found that a lower incubation temperature increased sexual fertility among isolates of the *G. fujikuroi* complex (mating population H). We, therefore, used an incubation temperature of 17°C for our crosses. Sexual crosses have already been performed within and between isolates of the two new species in a previous study (Steenkamp *et al.*, 2000a), and the present study served to confirm those results using a more clearly defined collection of isolates. Since the morphological characteristics of some isolates were similar to those of *F. circinatum*, all isolates (Table 1) were crossed with the standard tester strains (MRC 6213 and MRC 7488) for *G. circinata* Nirenberg & O'Donnell (anamorph: *F. circinatum*).

RESULTS

Morphological and cultural studies

Fusarium isolates from trees suffering from mango malformation in Malaysia, Egypt, Israel, South Africa, and Florida were separated into three different groups based on morphological characters defined by Nirenberg & O'Donnell (1998). Based on the morphological characters that were used, each of these groups represented new species in the *G. fujikuroi* complex. Of the three groups, two are clearly discrete taxa based both on morphological characteristics and sequencing data (Steenkamp *et al.*, 2000a). These two taxa are represented by an extensive group of isolates and we elect to describe them as new species in *Fusarium* section *Liseola*.

Fusarium mangiferae Britz, Wingfield et Marasas sp. nov. Figs. 1-4

Coloniae in agaro PDA apud 25°C 3.4 mm per diem crescentes. Mycelium aerium floccosum, album, infra roseolo-luteum ad atropurpureum. Conidiophora in agaro CLA erecta vel prostrata, simplicia vel ramosa, cellulae conidiogenae mono- et polyphialides, usque ad 30 x 3 µm. Hyphae steriles absentes. Microconidia in capitulis falsis, hyalina, plerumque obovoidea, subinde ovata vel allantoidea, plerumque 0-septata, subinde 1-septata, (2.5–)4.3–9.0–18.4 x (1.5–)1.7–2.4–3.3 µm. Sporodochia praesentia, alba ad aurantiaca. Macroconidia hyalina, falcata, gracilia, leniter curvata, tenuitunicata, cellula basali pedicellata, cellula apicali leniter curvata, 3–5-septata, (39.9–)43.1–51.8–61.4(–63.7) x (1.2–)1.9–2.3–3.4 µm. Clamydosporae absentes.

HOLOTYPUS: Cultura exsiccata in agaro CLA ex MRC 7559, sejuncta a inflorescentis malformatis *Mangifera indica,* Volcani Center, Bet Dagan, Israel, 1993, S. Freeman (PREM 57299).

Colonies on PDA with average growth rate of 3.4 mm/d at 25°C. Aerial mycelium white, floccose. Reverse of colonies sometimes rosy buff (17"f) to dark purple (65k). Conidiophores on aerial mycelium originating erect and prostrate from substrate. Conidiophores sympodially branched bearing mono- and polyphialides (Fig. 1, 2). Polyphialides have 2–5 conidiogenous openings (Fig. 1, 2). Phialides on the aerial

conidiophores mono- and polyphialidic, up to 30.0 µm long and 3 µm wide. Sterile hyphae absent. Microconidia variable in shape, obovoid conidia the most abundant type, oval to allantoid conidia occurring occasionally (Fig. 3). Microconidia mostly 0-septate with 1-septate conidia occurring less abundantly, 0-sepate: (2.5–)4.3–9.0–14.4 x (1.5–)1.7–2.4–3.3 µm. Sporodochia present, cream (19'f) and orange (15b). Macroconidia long and slender, usually 3–5 septate (Fig. 4): (39.9–)43.1–51.8–61.4(–63.7) x (1.2–) 1.9–2.3–3.4 µm. Chlamydospores absent.

Etymology: *Mangiferae* (L. gen) indicating the species association with the genus *Mangifera* L.

Specimens examined: ISRAEL: Bet Dagan, Volcani center plantation. Mango malformation inflorescence on *M. indica*, 1993, *S. Freeman* 506/2 (PREM 57299, HOLOTYPE; MRC 7559, ex-holotype); Ginosar. Inflorescence malformation of *M. indica* cultivar Kent, 1998, *S. Freeman* 34 (MRC 7560); Sde Nitzar. Inflorescence malformation of *M. indica*, 1998, *S. Freeman* 41 (MRC 7561); Bene Dror. Inflorescence malformation of *M. indica* cultivar Keitt, 1998, S. *Freeman* 86 (MRC 7562). SOUTH AFRICA. MPUMALANGA: Nelspruit. Inflorescence malformation of *M. indica,* 1982, *F. Wehner* MRC 2730 (PREM 57300, PARATYPE; KSU 3873, ex-paratype); inflorescence malformation of *M. indica*, 1998, *H. Britz* NS1-1 (MRC 8080); Inflorescence malformation of *M. indica*, 1998, *H. Britz* NS1-9 (MRC 8081); Nelspruit, Fredenheim. Inflorescence malformation of *M. indica*, 1998, *H. Britz* FH1-6 (MRC 8085); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1537, FH1-8 (MRC 8078); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1542, FH1-16 (MRC 8079); inflorescence malformation of *M. indica*, 1998, *H. Britz* FH1-73 (MRC 8084). MPUMALANGA: Malelane. Inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1551 = ML3-1 (MRC 8077); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1547 = ML1-9 (MRC 8082); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1545 = ML1-6 (MRC 8086); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1546 = ML1-8 (MRC 8087); inflorescence malformation of *M. indica*, 1998, *H. Britz* FCC 1548 = ML2-1 (MRC 8083); KWAZULU-NATAL: Inflorescence malformation of *M. indica,* 1984, *C. Crookes* MRC 3477 (PREM 57301, PARATYPE; KSU-X3875, ex-paratype); inflorescence malformation of *M. indica,* 1984, *C. Crookes* MRC 3478

(KSU-X 3876); inflorescence malformation of *M. indica,* 1984, *C. Crookes* MRC 3479 (KSU-X 3877). EGYPT: Inflorescence malformation of *M. indica, Ibrahim Mausour,* KSU-X4706 (MRC 8089); inflorescence malformation of *M. indica, Ibrahim Mausour* KSU-X4702 (MRC 8090); inflorescence malformation of *M. indica, Ibrahim Mausour* KSU-X4700 (MRC 8091). USA. FLORIDA: Miami, Dade County. Inflorescence malformation of *M. indica* cultivar Keitt, 1994, *R. Ploetz* FS16 (MRC 7034); inflorescence malformation of *M. indica* cultivar Keitt, 1994, *R. Ploetz* FS23 (MRC 7035); inflorescence malformation of *M. indica* cultivar Keitt, 1994, *R. Ploetz* FS55 (MRC 7038); inflorescence malformation of *M. indica* cultivar Keitt, 1994, *R. Ploetz* MRC 7039; inflorescence malformation of *M. indica*, *R. Ploetz* KSU-X4079 = FRC-M3622 (MRC 8088). MALAYSIA: Inflorescence malformation of *M. indica*, *Baharuddin Salleh* KSU-X4382 (MRC 8092); inflorescence malformation of *M. indica, Baharuddin Salleh* KSU-X4384 (MRC 8093).

Commentary: A dried culture to serve as holotype has been deposited at the Plant Protection Research Institute, Pretoria, South Africa (PREM 57299). Ex-holotype cultures have been deposited in the culture collection of the South African Medical Research Council, Tygerberg, South Africa (MRC 7559) and the department of Plant Pathology, Kansas State University, Manhattan, Kansas, USA (KSU 11781). Ex-paratype cultures have been deposited as MRC 2730 (KSU-X3873) and MRC 3477 (KSU-X3875).

Fusarium sterilihyphosum Britz, Marasas & Wingfield, sp. nov. Figs. 5-8

Coloniae in agaro PDA apud 25°C 4.8 mm per diem crescentes. Mycelium aerium floccosum, subalbum, infra giseo-roseum ad pallido- purpureum. Conidiophora in agaro CLA erecta vel prostrata, simplicia vel ramosa, cellulae conidiogenae mono- et polyphialides, usque ad 30 x 3 µm. Hyphae steriles circinatae praesentes. Microconidia in capitulis falsis, hyalina, plerumque obovoidea, subinde ovata vel allantoidea, plerumque 0-septata, subinde 1-septata, (2.3–)4.5–8.8–24.2 x (1.4–)1.6–2.6–3.5(–3.8) µm. Sporodochia raro praesentia. Sporodochia raro praesentia, alba ad aurantiaca. Macroconidia hyalina, falcata, gracilia, leniter curvata, tenuitunicata, cellula basali pedicellata, cellula apicali leniter curvata, 3-5-septata, (24.5–)28.4–37.1–47.1(–49.7) x (2.3–)2.4–3.2–4.1 µm. Clamydosporae absentes.

HOLOTYPUS: Cultura exsiccata in agaro CLA ex MRC 2802, sejuncta a inflorescentis malformatis *Mangifera indica,* Letsitele, Tzaneen, Northern Province, South Africa, 1982, J. M. Darvas (PREM 57302).

Colonies on PDA with average growth rate of 4.8 mm/d at 25°C. Aerial mycelium almost white ('1). Reverse of colonies straw to grayish rose (3"f) and light purple (63i). Conidiophores on aerial mycelium erect, occasionally prostrate. Conidiophores sympodially branched bearing mono- and polyphialides (Fig. 5). Phialides on aerial conidiophores mono- and polyphialidic, up to 30.0 µm long and 3 µm wide. Sterile hyphae present (Fig. 6). Microconidia obovoid, oval to allantoid, 0-septate conidia abundant, 1-septate conidia less common (Fig. 7, 8): 0-septate: 4.5–8.8–14.2 x 1.6–2.6–3.5 µm. Sporodochia seldom present, cream (19'f) to orange (15b). Macroconidia slightly beaked apical cells, a footlike basal cell, 3-5 septate (Fig. 8), 28.4–37.1–47.1 x 2.4–3.2–4.1 µm. Chlamydospores absent.

Etymology: *Sterilihyphosum* (L. adj) refers to the presence of sterile hyphae in mycelium.

Specimens examined: SOUTH AFRICA. NORTHERN PROVINCE: Tzaneen, Letsitele area. Mango malformation inflorescence on *M. indica,* 1982, *J. M. Darvas* MRC 2802 = NRRL 25623 (PREM 57302, HOLOTYPE; KSU-X3874, ex-holotype); mango malformation of *M. indica,* 1997, *H. Britz* A33-1 (MRC 7606); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1315 = A40-1 (PREM 57303, PARATYPE; MRC 8095, ex-paratype); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1367 = A1-2 (MRC 7602); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1398 = A20-1 (MRC 7605); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1286 = A26-1 (PREM 57304, PARATYPE; MRC 8101, ex-paratype); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1478 = B12-1 (MRC 8102); mango malformation of *M. indica,* 1997, *H. Britz* C6-1 (MRC 8103); mango malformation of *M. indica,* 1997, *H. Britz* D2-1 (MRC 8094); mango malformation of *M. indica,* 1997, *H. Britz* FCC 1146 = E6-1 (MRC 8096); Deerpark. Mango malformation of *M. indica,* 1998, *H. Britz* DP3-5 (MRC 8100); mango malformation of *M. indica,* 1998, *H. Britz* DP3-7 (MRC 8106); mango malformation of *M. indica,*

1998, *H. Britz* FCC 1632 = DP3-9 (MRC 8107). MPUMALANGA: Hazyview. Mango malformation of *M. indica,* 1998, *H. Britz* FCC 1563 = HZ1-9 (MRC 8099); mango malformation of *M. indica,* 1998, *H. Britz* FCC 1555 = HZ1-1 (MRC 8104); mango malformation of *M. indica,* 1998, *H. Britz* FCC 1557 = HZ1-3 (MRC 8108); Malelane. Mango malformation of *M. indica,* 1998, *H. Britz* ML2-10 (MRC 8105).

Commentary: A dried culture to serve as holotype has been deposited at the Plant Protection Research Institute, Pretoria, South Africa (PREM 57302). Ex-holotype culture specimens have been deposited in the culture collection of the South African Medical Research Council, Tygerberg, South Africa (MRC 2802) and the department of Plant Pathology, Kansas State University, Manhattan, Kansas, USA (KSU-X3874). Ex-paratype cultures have been deposited as MRC 8095 (KSU 11783) and MRC 8101 (KSU 11782).

Of the three *Fusarium* species found associated with mango malformation, two have been described as new in this study. The third group included three isolates (KSU-X4379, KSU-X4381 and KSU-X2330) that were collected from malformed mango tissue in Malaysia. These isolates have sparse aerial mycelium. Their aerial mycelial conidiophores emerge directly from the substrate hyphae (referred to as erect). The polyphialides of these isolates have fewer than 3 conidiogenous openings. Microconidia are borne in false heads and are obovoid in shape, predominantly without septa but with 1-septate conidia occurring occasionally. Macroconidia are short 19.3–24.8–29.5 x 1.3–2.0–3.0 µm and 3-5 septate. Chlamydospores and sterile coiled hyphae are absent (Table 2). We believe that this fungus also represents a new taxon, but the collections are insufficient in number to justify a formal description at this stage.

The newly described species, *F. mangiferae* and *F. sterilihyphosum* had different growth rates on PDA at 25°C. *F. mangiferae* had a slower growth rate than *F. sterilihyphosum*, but variation in growth rate among isolates of both species was observed. The one way ANOVA indicated that the growth rate did not differ significantly when the growth rate of all the isolates of both species were analyzed ($P>0.001$). Colony color of the two species was the same.

Mating type and sexual compatibility tests

All isolates of the undescribed *Fusarium* species represented by only 3 isolates were of the *MAT*-1 mating type. Most of the *F. mangiferae* isolates were *MAT*-2 except for two isolates from Malaysia (MRC 8092 and MRC 8093) that were *MAT*-1. Both mating types were identified amongst *F. sterilihyphosum* isolate. The majority of these isolates were *MAT*-1 and isolates MRC 8101, MRC 8104 and MRC 8105 were *MAT*-2.

Isolates of *F. mangiferae* and *F. sterilihyphosum* of opposite mating type were sexually incompatible when crossed within each species and between the two species. None of the other *Fusarium* isolates (Table 1) were sexually compatible with the tester strains of mating population H.

DISCUSSION

In this study we have shown that at least two distinct *Fusarium* spp. are associated with mango malformation symptoms, namely *F. mangiferae* and *F. sterilihyphosum*. *F. mangiferae* was previously shown to be the causal agent of mango malformation and *F. sterilihyphosum* is associated with similar disease symptoms in South Africa. A third taxon was also identified, but our collections are insufficient in number to justify describing the fungus. Furthermore, the fungus does not occur in South Africa, and has not been a primary focus of our investigation.

The results of this study, together with those of Steenkamp *et al.* (2000a), have shown that mango malformation in South Africa is associated with two distinct species, *F. mangiferae* and *F. sterilihyphosum*. *F. sterilihyphosum* has only been isolated from malformed mango tissue in South Africa. The histone *H3* and β-tubulin gene sequences for isolates of *F. mangiferae* are similar to those of *F. subglutinans* strains NRRL 25226 and MRC 7559 (Steenkamp *et al.*, 2000a), which were previously reported to be the causal agent of mango malformation (Freeman *et al.*, 1999).

Fusarium mangiferae has been isolated from mango malformation symptoms in various geographical areas, such as South Africa, Florida, Egypt, India, Israel and Malaysia. *Fusarium mangiferae* is morphologically most similar to *F. concentricum*

Nirenberg & O'Donnell and *F. guttiforme* Nirenberg & O'Donnell. *F. concentricum* has long slender 3–4 septate macroconidia similar to those produced by *F. mangiferae,* which has sympodially branched conidiophores in contrast to the branched conidiophores of *F. concentricum.* *Fusarium guttiforme* can be distinguished from *F. mangiferae* based on the presence of the uniformly obovoid microconidia and 3-septate macroconidia that are shorter in length than those of *F. mangiferae.* The occasional production of 3-septate macroconidia in *F. guttiforme* isolates (MRC 7539, MRC 6784 and MRC 6785) found in the present study was also observed by Viljoen *et al.* (1997). Nirenberg & O'Donnell (1998) did not refer to the macroconidial characteristics in their description of *F. guttiforme.*

Fusarium sterilihyphosum has been isolated only from South Africa. This species is morphologically similar to *F. mangiferae,* but can be distinguished from *F. mangiferae. F. sterilihyphosum* has shorter 3–5 septate macroconidia, faster growth rate on PDA at 25°C than *F. mangiferae* and produces sterile coiled hyphae. *F. sterilihyphosum* is most closely related to *F. guttiforme* based on histone gene sequence (Steenkamp *et al.,* 2000a). Morphologically, *F. sterilihyphosum* resembles *F. circinatum* and *F. pseudocircinatum* O'Donnell & Nirenberg. These three species all produce sterile coiled hyphae. However, macroconidia are long, slender and 3–5 septate in *F. sterilihyphosum,* while shorter 3-sepate macroconidia are produced in both *F. circinatum* and *F. pseudocircinatum.*

Both *F. sterilihyphosum* and *F. mangiferae* are morphologically distinct from species belonging to *F. subglutinans sensu lato* occurring on various host plants, including *F. begoniae* Nirenberg & O'Donnell, *F. bulbicola* Nirenberg & O'Donnell, *F. circinatum* (MP-H), *F. guttiforme, F. concentricum, F. pseudocircinatum, F. sacchari* (Butler) W. Gams (MP-B) and *F. subglutinans sensu stricto* (MP-E) (Table 2). *Fusarium mangiferae* and *F. sterilihyphosum* can also be distinguished from each other based on morphological characteristics. Sterile coiled hyphae and shorter 3–5 septate macroconidia produced by *F. sterilihyphosum* distinguish it from *F. mangiferae. Fusarium mangiferae* had a slower growth rate than *F. sterilihyphosum* on PDA at 25°C. However, growth rate is a secondary morphological characteristic and no significant difference ($P>0.001$) among isolates of both species was observed. Furthermore, secondary characteristics are generally not used in species descriptions

in view of the variability within populations and / or the instability of these characters (Gerlach & Nirenberg, 1982; Nelson *et al.*, 1983; Nirenberg & O'Donnell, 1998).

Both mating types (*MAT*-1 and *MAT*-2) were identified in *F. mangiferae* and *F. sterilihyphosum* isolates. This is in contrast to the Steenkamp *et al.* (2000a) study, where isolates of each of these species included only a single mating type. In the present study, isolates of the two species having opposite mating type were sexually incompatible.

Likewise, isolates of *F. mangiferae* and *F. sterilihyphosum* of different mating types failed to cross with each other. This failure to produce sexual crosses could be explained by sterility, female-sterility of isolates or unfavorable conditions for crosses to occur (Perkins, 1994; Leslie, 1995). At this stage, there is thus no evidence to suggest that sexual outcrossing is occurring within or between these two fungi from mango.

Mango is native to Asia, eastern India, Burma, and the Andaman Islands, and mango malformation was first reported over a century ago in India (Kumar *et al.*, 1993). *F. mangiferae* isolates from South Africa, United States, Israel, Malaysia and Egypt grouped into the so-called 'Asian clade' of O'Donnell *et al.* (1998) based on histone *H3* and β-tubulin gene sequences (O'Donnell *et al.*, 1998; O'Donnell *et al.*, 2000; Steenkamp *et al.*, 2000a). *F. mangiferae* from different geographical areas was most probably introduced from India (Zheng & Ploetz, 2002), which would explain the presence of *F. mangiferae* isolates grouping in the 'Asian clade'. *F. sterilihyphosum* isolates from mango malformation symptoms in South Africa grouped into the so-called 'American clade' (O'Donnell *et al.*, 2000; Steenkamp *et al.*, 2000a). O'Donnell *et al.* (2000) speculated that *F. sterilihyphosum* (MRC 2802 = NRRL 25623) originated from mango that was imported into South Africa from South America, although the basis for this supposition is not known to us. However, vegetative malformation has been reported in Mexico (Noriega-Cantu *et al.*, 1999). These Mexican isolates produce sterile coiled hyphae and grouped also in the 'American clade' based on β-tubulin gene sequences, like *F. sterilihyphosum* isolates from South Africa (David M. Geiser, personal communication). Clearly, further

investigations with strains from South America would be required to test the hypothesis that *F. sterilihyphosum* isolates in South Africa originated in South America.

The fact that three distinct taxa are found associated with mango malformation symptoms emphasizes a serious problem regarding the etiology of mango malformation disease. *Fusarium mangiferae* has been unequivocally indicated as the causal agent of mango malformation (Freeman *et al.*, 1999). It is, however, not known whether *F. sterilihyphosum* or the undescribed *Fusarium* sp. are also able to cause diseases on mango trees. Their role in the etiology of mango malformation disease clearly requires further intensive study.

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Table 1. *Fusarium* species isolated from malformed mango tissue.

Species	Origin	MRC no[a]	Other no[b]	Original Source[c]
Fusarium sp.	Malaysia	8079	KSU-X4379	B. Salleh
	Malaysia	8070	KSU-X4381	B. Salleh
	Malaysia	8071	KSU-X2330	B. Salleh
F. mangiferae	South Africa (ex-paratype)	2730	KSU-X3873	F. Wehner
	South Africa (ex-paratype)	3477	KSU-X3875	C. Crookes
	South Africa	3478	KSU-X3876	C. Crookes
	South Africa	3479	KSU-X3877	C. Crookes
	South Africa	8077	FCC 1551, ML3-1	H. Britz
	South Africa	8078	FCC 1537, FH1-8	H. Britz
	South Africa	8079	FCC 1542, FH1-16	H. Britz
	South Africa	8080	NS1-1	H. Britz
	South Africa	8081	NS1-9	H. Britz
	South Africa	8082	FCC 1547, ML1-9	H. Britz
	South Africa	8083	FCC 1548, ML2-1	H. Britz
	South Africa	8084	FH1-73	H. Britz
	South Africa	8085	FH1-6	H. Britz
	South Africa	8086	FCC 1545, ML1-6	H. Britz
	South Africa	8087	FCC 1546, ML1-8	H. Britz
	Florida, USA	7034		R. Ploetz
	Florida, USA	7035		R. Ploetz
	Florida, USA	7038		R. Ploetz
	Florida, USA	7039		R. Ploetz
	Florida, USA	8088	KSU-X4079, FRC-M3622	R. Ploetz
	Egypt	8091	KSU-X4700	I. Mausour
	Egypt	8090	KSU-X4702	I. Mausour
	Egypt	8089	KSU-X4706	I. Mausour

Species	Origin	MRC no[a]	Other no[b]	Original Source[c]
F. mangiferae	Israel (ex-holotype)	7559	FCC 73, KSU 11781, 506/2	S. Freeman
	Israel	7560	FCC 74	S. Freeman
	Israel	7561	FCC 80	S. Freeman
	Israel	7562	FCC 81	S. Freeman
	Malaysia	8092	KSU-X4382	B. Salleh
	Malaysia	8093	KSU-X4384	B. Salleh
F. sterilihyphosum	South Africa (ex-holotype)	2802	KSU-X3874, NRRL 25623	J. Darvas
	South Africa	7602	FCC 1367, A1-2	
	South Africa	7605	FCC 1398, A20-1	H. Britz
	South Africa	7606	A33-1	H. Britz
	South Africa	8094	D2-1	H. Britz
	South Africa (ex-paratype)	8095	FCC 1315, KSU 11783, A40-1	H. Britz
	South Africa	8096	FCC 1143, E6-1	H. Britz
	South Africa	8099	FCC 1563, HZ1-9	H. Britz
	South Africa	8100	DP3-5	H. Britz
	South Africa (ex-paratype)	8101	FCC 1286, KSU 11782, A26-1	H. Britz
	South Africa	8102	FCC 1478, B12-1	H. Britz
	South Africa	8103	C6-1	H. Britz
	South Africa	8104	FCC 1555, HZ1-1	H. Britz
	South Africa	8105	ML2-10	H. Britz
	South Africa	8106	DP3-7	H. Britz
	South Africa	8107	FCC 1632, DP3-9	H. Britz
	South Africa	8108	FCC 1557, HZ1-3	H. Britz

[a] MRC = Culture collection of the Medical Research Council, Tygerberg, South Africa.

[b] KSU-X= Kansas State University culture collection, Department of Plant Pathology, Kansas State University, Manhattan. Kansas, USA, FRC = *Fusarium* Research

Center, Pennsylvania State University, USA, NRRL = Northern Regional Research Laboratory, Peoria, Illinois, USA, FCC = *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa with original numbers indicated as follows: A, B, C, D, E = Isolates from different orchards from Letsitele, South Africa, HZ = Isolates from Hazyview, South Africa, ML = Isolates from Malelane, South Africa, DP = Isolates from Deer Park, South Africa, FH = Isolates from Fredenheim farm, Nelspruit, South Africa, NS = Isolates from Nelspruit, South Africa.

[c] B. Salleh collected isolates KSU-X2330, 4379, 4381, 4382 and 4384 in Malaysia; Ibrahim Mausour collected KSU-X4079, 4700, 4702 and 4706 in Egypt; C. Crookes collected isolates MRC 3477–3479 in KwaZulu-Natal, South Africa; S. Freeman collected MRC 7559–7562 in Israel; R. Ploetz collected isolates MRC 7034–7035, 7038–7039 in Florida, USA.

Table 2. Distinguishing characteristics described by Nirenberg and O'Donnell (1998) of isolates of *Fusarium subglutinans sensu lato* as well as characteristics observed for isolates in this study.

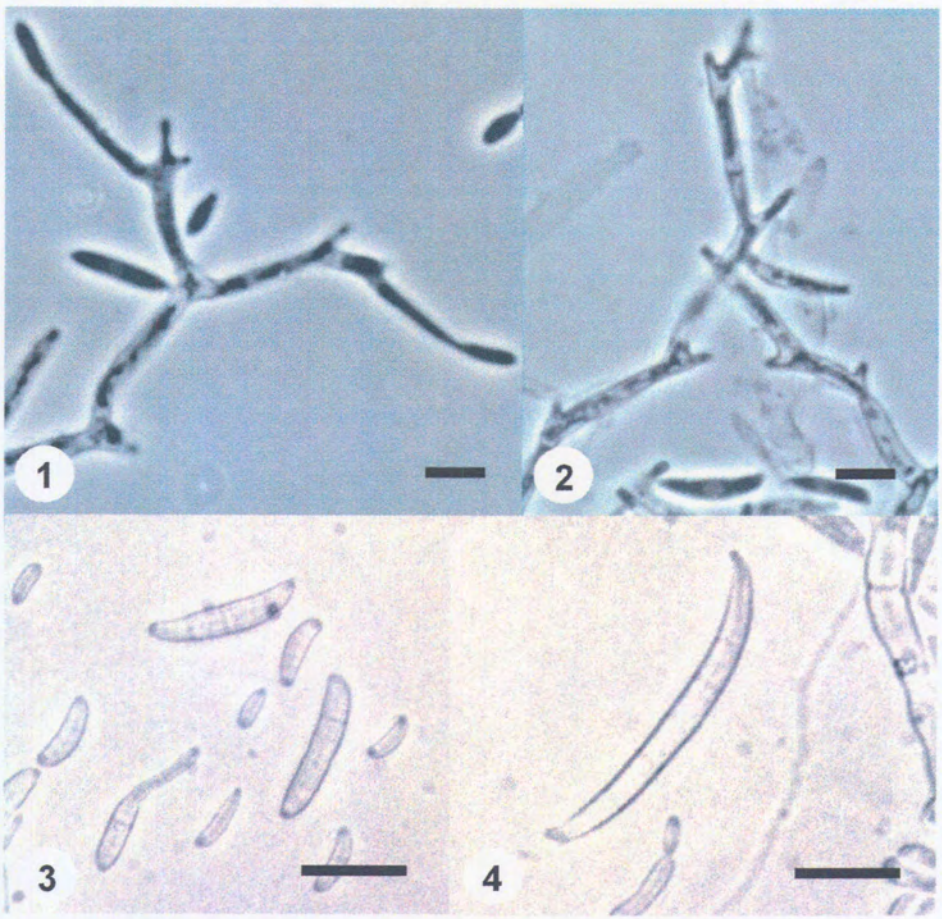
	F. subglutinans sensu lato								New species		
Morphological characteristics[a]	*F. begoniae*	*F. bulbicola*	*F. circinatum*	*F. concentricum*	*F. guttiforme*	*pseudocircinatu*	*F. sacchari*	*F. subglutinans*	*F. mangiferae*	*sterilihyphosum*	Fusarium sp.
Microconidia obovoid	+	+	+	+	+	+	-	-	+	+	+
Microconidia oval to allantoid / or fusoid	+	+	(+)	+	-	+	+	+	+	+	+
Sterile coiled hyphae	-	-	+	-	-	+	-	-	-	+	-
Conidiophore originated erect	-	-	+	+	+	?	-[b]	+[b]	+	+	+
Conidiophore originated prostrate	+	+	-	-	+	+	+[b]	+[b]	+	+	-
Conidiogenous openings: ≤3	+	+	-	-	-	-	-[b]	+	-	-	+
Conidiogenous openings: ≥3	-	-	+	+	+	+	+[b]	-	+	+	-
Microconidia 0-septate, occasionally 1-septate	+	+	+	+	+	+	+[c]	-[c]	+	(+)	+
Microconidia 0-3 septate	-	-	-	-	-	-	-[c]	+[c]	-	-	+
Macroconidia 3-septate	+	-	+	-	+	+	+[c]	-[c]	-	-	-
Macroconidia 3-5 septate	-	+	-	+	-	-	-[c]	+[c]	+	+	+
Host specific	+	+	+	?	+	?	+	+	?	?	?

[a] + indicates the presence of the characteristic, (+) indicates that the character is not present in all isolates of the species, - indicates the absence of the characteristic and ? indicates that the characteristic has not been reported.

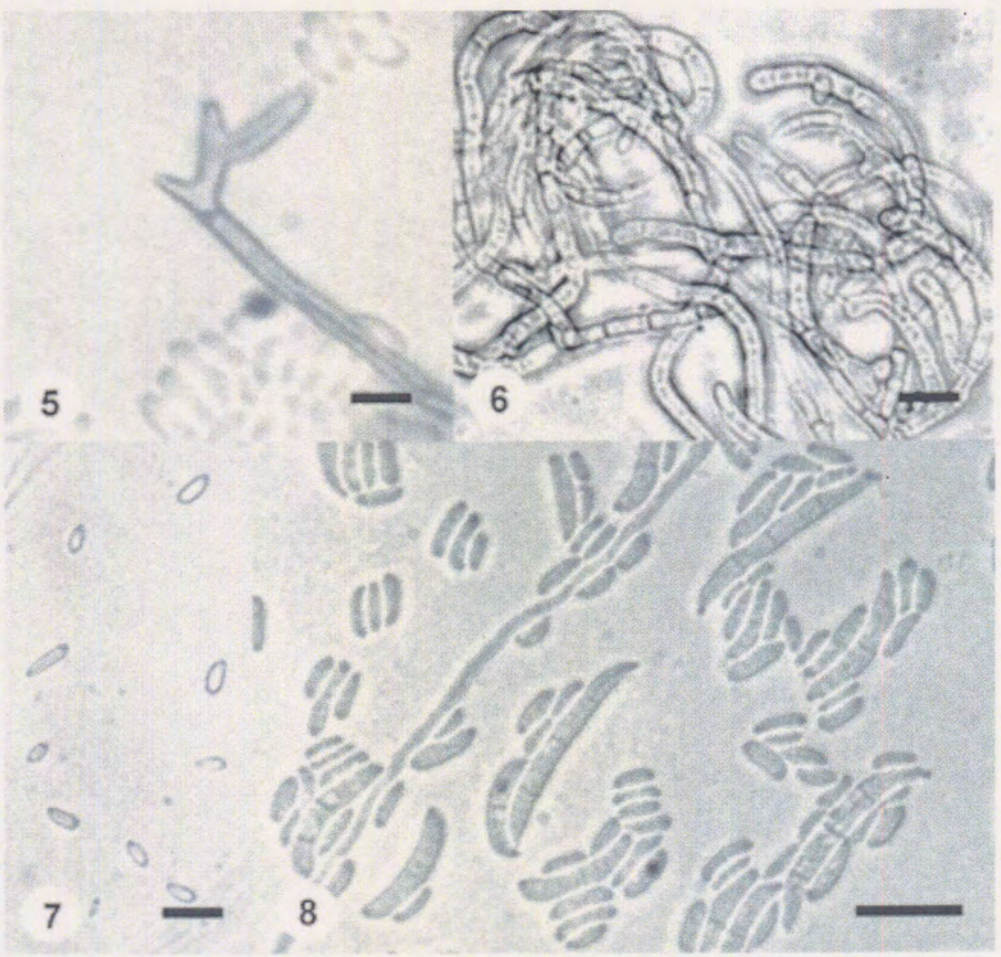
[b] Morphological characteristic identified from *F. sacchari* isolates (FRC-M941, 943) from sugarcane in India (MP-B) and *F. subglutinans* isolates (BBA 11157 from Iran and FRC-M3696 from St. Elmo, Illinois).

[c] Morphological characteristics obtained from Gerlach & Nirenberg (1982).

Fig. 1–4. *Fusarium mangiferae.* 1. Branched conidiophores bearing polyphialides with 3 conidiogenous openings (bar = 5µm). 2. Branched conidiophores bearing mono- and polyphialides (bar = 5µm). 3. Microconidia (bar = 15µm). 4. Macrocondium (bar = 15µm).



FIGS. 5–8. *Fusarium sterilihyphosum.* 5. Conidiophores bearing polyphialides with 3 conidiogenous openings (bar = 5µm). 6. Sterile coiled hyphae (bar = 10µm). 7. Microconidia (bar = 10µm). 8. Microconidia with 0–1 septa and 3-septate macroconidia (bar = 20µm).



CHAPTER 7

VEGETATIVE COMPATIBILITY AND DISTRIBUTION OF TWO *FUSARIUM* SPECIES ASSOCIATED WITH MANGO MALFORMATION IN SOUTH AFRICA

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ABSTRACT

Mango malformation is an economically important disease of mango in many parts of the tropics and sub-tropics. The causal agent of this disease has recently been described as *Fusarium mangiferae* (=*F. subglutinans sensu lato*). In South Africa, *F. mangiferae* and a second species, known as *F. sterilihyphosum,* are associated with malformed mango tissue. In this study, the distribution and vegetative compatibility of *F. mangiferae* and *F. sterilihyphosum* isolates collected in South Africa were determined. In South Africa, *F. sterilihyphosum* and *F. mangiferae* were found to have distinct distributions with the former species primarily occurring in the Northern Province and the latter in Mpumalanga and KwaZulu-Natal. Based on vegetative compatibility, isolates of both species represent a single clone. From these results, it is evident that both species are associated with mango malformation symptoms and that they represent single genets in each of the areas where they occur.

INTRODUCTION

Mango malformation is one of the most important diseases affecting mango (*Mangifera indica* L.) production in the tropics and sub-tropics. The disease has been reported from all major mango-growing areas including Egypt, Brazil, Florida, India, Israel, Mexico Pakistan and South Africa (Varma, 1983; Kumar *et al.*, 1993; Ploetz, 1994a). Mango malformation induces abnormal development of vegetative shoots and inflorescences. Of these, inflorescence malformation is the most dominant symptom causing abnormal thick fleshy blossoms on profusely branched panicles (Varma, 1983; Kumar *et al.,* 1993; Ploetz, 1994a).

The fungal pathogen identified as *Fusarium subglutinans* (Wollenweber & Reinking) Nelson *et al.* (= *F. moniliforme* Sheldon var. *subglutinans* Wollenweber & Reinking) has been shown to be associated with mango malformation (Summanwar *et al.*, 1966). There has been considerable dispute regarding the cause of mango malformation (Varma, 1983; Kumar *et al.,* 1993). However, recent pathogenicity tests, where typical mango malformation symptoms were induced with an isolate identified as *F. subglutinans,* have dispelled doubt regarding the cause of the disease (Varma *et al.,* 1974; Manicom, 1989; Ploetz & Gregory, 1993; Freeman *et al.,* 1999).

The fungus primarily responsible for mango malformation in most parts of the world has recently been described as *F. mangiferae* Britz, Wingfield & Marasas (Britz *et al.*, 2002). The justification for this description arises from histone *H3* and β-tubulin gene sequences (Steenkamp *et al.*, 2000) and distinct morphological characteristics (Britz *et al.*, 2002). The latter authors have also shown that a second species in the *F. subglutinans sensu lato* complex is commonly associated with malformation in South Africa. This fungus has been described as *F. sterilihyphosum* Britz, Wingfield & Marasas, although its role as a causal agent of malformation is not yet known.

Vegetative compatibility groups (VCGs) are relatively easy to detect and can provide useful insights into the genetic diversity of different *Fusarium* (section *Liseola*) populations (Leslie, 1995; Leslie & Mansuetus, 1995; Gordon *et al.*, 1996). Despite this, very little attention has been given to the population structure of the mango malformation pathogen, *F. mangiferae,* in various parts of the world. Previous studies in Florida (Ploetz & Gregory, 1993; Ploetz, 1994b) and recently in Egypt, Israel, South Africa and USA (Zheng & Ploetz, 2002) have shown that populations of *F. mangiferae* are homogeneous in these countries, where most of the isolates belonged to a single dominant VCG.

The aim of this study was to determine the distribution as well as the VCG diversity of the two *Fusarium* spp. associated with mango malformation in South Africa. To achieve this goal, we collected inflorescence tissue from mango-growing areas in South Africa. We then used morphology and ITS-RFLPs, which were developed as part of the present study, to distinguish isolates of *F. sterilihyphosum* and *F. mangiferae* from each another. The distribution of the two fungi and the genetic diversity of their populations in South Africa was then determined.

MATERIALS & METHODS

Morphological and cultural studies

In South Africa, *Fusarium* spp. were isolated from malformed blossoms collected in 1997 and 1998 in Letsitele (23'50''S 30'09''E), Trichardtsdal and Deerpark (Northern Province). Isolates were also collected from Nelspruit (25'28''S 30'58''E),

Fredenheim farm near Nelspruit, Malelane (25'30''S 31'28''E) and Hazyview (Mpumalanga) (Fig. 1). An isolate associated with mango malformation in Florida (MRC 7035) was received from R. Ploetz. The South African isolates received from the Medical Research Council (MRC) culture collection included: isolate MRC 2802 (NRRL 25623) collected by J. Darvas in 1982 in the Letsitele area, Tzaneen, Northern Province, isolate MRC 2730 collected in 1982 by F. Wehner in Nelspruit, Mpumalanga and isolates MRC3477-3479 collected by C. Crookes in 1984 in KwaZulu-Natal (Britz *et al.*, 2002). All isolates were stored in 15% glycerol at –70 °C in the *Fusarium* culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and representative isolates of *F. mangiferae* and *F. sterilihyphosum* (Table 1) have been deposited in the culture collection of the Medical Research Council (MRC), South Africa.

Fusarium spp. were isolated in South Africa from mango inflorescence clusters showing typical symptoms of blossom malformation. A selective medium (Nash & Snyder, 1962) was used to facilitate isolation. Single conidial isolates were prepared and stored in 15 % glycerol at –70 °C.

For morphological observations, all isolates were grown on CLA (Fisher *et al.*, 1982) and KCl agar (Nelson *et al.*, 1983) for 10 to 14 days at 23°C under near-ultraviolet light and cool-white light with a 12-hour photoperiod. *Fusarium* spp. were identified using morphological characteristics described by Nirenberg & O'Donnell (1998) and Britz *et al.* (2002).

ITS-RFLP

Previous studies have shown that a *F. sterilihyphosum* (MRC 2802 = NRRL 25623) isolate and *F. mangiferae* (NRRL 25226, and 506/2 = MRC 7559) isolates have different major ITS2 types (Freeman *et al.*, 1999; O'Donnell *et al.* 1998; 2000). We, therefore, used this difference as the basis to develop a restriction fragment length polymorphism (RFLP) technique to rapidly confirm the identity of *F. mangiferae* and *F. sterilihyphosum* isolates. The ITS regions of additional isolates of both species were sequenced in order to develop this technique.

DNA extraction

Fusarium mangiferae and *F. sterilihyphosum* isolates of were grown on complete medium agar (CMA) (Correll *et al.,* 1987) for 10 days. Mycelium was scraped from the CMA plates, frozen in liquid nitrogen and crushed to a fine powder with a mortar and pestle. The powdered mycelium was transferred to 1.5 ml Eppendorf™ tubes and DNA was extracted as described by Raeder & Broda (1985).

PCR amplification and sequencing

Amplification of the ITS1, 5.8S and ITS2 rDNA regions was achieved using primers ITS 1 and 4 (White *et al.,* 1990). PCR reactions were performed in 50 µl containing 1 µM of both primers, 0.1 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, and 1U of *Taq* DNA polymerase (Roche Diagnostics, Germany). PCR reactions were performed using an Omnigene thermocycler (Hybaid, Middlesex, United Kingdom) with an initial denaturing step of 1 min at 94°C. This step was followed by 35 cycles of 1 min at 92°C, 1 min at 56°C and 1 min at 72°C. A final extension was performed at 72°C for 5 min.

PCR products were purified with the High Pure™ PCR product purification kit (Roche Diagnostics, Germany). Both strands of the ITS1, 5.8S and ITS2 regions of isolates MRC 2802, MRC 7602, MRC 7035 and MRC 3477 were sequenced. Reactions were performed on an ABI Prism 377 DNA sequencer using the BigDye terminator cycle sequencing kit (Perkin Elmer Applied BioSystems). DNA sequences from isolates MRC 3477, MRC 7035 and MRC 7602 that were sequenced in this study were deposited in the Genbank database with accession numbers AF430128, AF430129 and AF430130, respectively. These sequences were also aligned manually with sequences AF158305 (MRC2802 = NRRL 25623) and U61691 (NRRL 25226) obtained from the Genbank database (aligned sequences in Appendix 2). The ITS2 sequences of isolates MRC 2802, MRC 7035 and MRC 3477 were identified as either ITS2 type I or type II by comparing the sequences with those in the Genbank database (O'Donnell *et al.*, 1998; 2000).

Restriction digests

Restriction sites that distinguished between ITS2 type I and type II were determined with Webcutter 1.0 (RE analysis). Digests were performed on 63 isolates from South Africa using the restriction enzyme, *Alu* I, in a total reaction volume of 20 µl that contained 15 µl of PCR product and 5 U of the enzyme. All digestion reactions were incubated at 37°C for 6 h. Digested PCR fragments were separated using 2% (w/v) agarose gels (Promega Corporation, Madison, Wisconsin, USA) containing ethidium bromide (0.1 µg/ml) and visualized using an ultraviolet transilluminator (Ultra-Violet Product).

Vegetative compatibility tests

Nitrate non-utilizing (*nit*) mutants were generated on 1.5 – 2.5% chlorate agar and the *nit* mutants were identified as *nit1*, *nit3* or nitM based on the utilization of different nitrogen sources (Puhalla & Spieth, 1983; 1985; Correll *et al.*, 1987). Incompatibility was determined by pairing isolates in all possible combinations on minimal media for 14 days at 25°C. Heterokaryon self-incompatibility (HSI) of isolates (Correll *et al.*, 1989) was determined by crossing a *nit1* or *nit3* mutant with a nitM mutant of the same isolate. All parings were repeated at least once.

RESULTS

Morphology

Fifty-eight *Fusarium* isolates collected from malformed mango tissue in South Africa during 1997 and 1998 were identified based on morphology. In addition, *Fusarium* isolates associated with mango malformation received from colleagues from Florida (1 isolate) and South Africa (5 isolates) were identified using morphology. Thus, 18 of the 58 South African isolates were identified as *F. mangiferae* and the remaining 40 isolates represented *F. sterilihyphosum.* All isolates received from Florida (MRC 7035) and South Africa (MRC 2730, MRC 3477, MRC 3478 and MRC 3479) were identified as *F. mangiferae,* except for a South African isolate MRC 2802 that represents *F. sterilihyphosum* as was shown by Britz *et al.* (2002).

ITS-RFLP

Amplication of the ITS1, 5.8S and ITS2 region of the rDNA operon of *Fusarium* isolates (Table 1) from South Africa resulted in a 450 bp product when amplified with primers ITS1 and ITS 4. The PCR products of three isolates, MRC 2802, MRC 7602, MRC 7035and MRC 3477 were sequenced and aligned with published sequences of both species (O'Donnell *et al.,* 1998; 2000). The sequence of isolate MRC 2802 studied in our laboratory was identical to the that published by O'Donnell *et al.* (2000) in Genbank (AF158305). The restriction enzyme, *Alu* I, was identified from sequence data as being useful to distinguish major ITS2 type I and II isolates from one another. These sequence data showed that *F. sterilihyphosum* had the ITS2 type I, including two *Alu* I restriction sites and *F. mangiferae* has the ITS2 type II, without *Alu* I restriction sites. Forty-one of the 63 isolates (Table 1) had the ITS2 type I PCR-RFLP profile producing three RFLP fragments of approximately 380, 50 and 30 bp when digested with *Alu* I (Fig. 2). However, the two smaller fragments (50 and 30 bp) were not visible on a 2% agarose gel. The remaining 22 had the ITS2 type II PCR-RFLP profile producing a single band of approximately 450 bp (Fig. 2).

Vegetative incompatibility tests

Nit mutants were generated for 22 *F. mangiferae* isolates including those from South Africa. Vegetative compatibility tests showed that South African *F. mangiferae* isolates included 2 VCGs. *Nit* mutants were also generated for the 41 *F. sterilihyphosum* isolates (Table 1), which belonged to a single VCG. Two isolates, FCC 1370 and 1525, were heterokaryon self-incompatible (Table 1).

Distribution of Fusarium spp. in South Africa

Isolates collected in the Mpumalanga and Kwazulu-Natal provinces of South Africa were identified as *F. mangiferae*. *F. sterilihyphosum* was isolated from the Northern Province in a 100 km radius around the Letsitele area near Tzaneen (Fig. 1). This species was also isolated from Mpumalanga in the Hazyview area that lies on the border of Mpumalanga and Northern Province (Fig. 1). A single *F. sterilihyphosum* isolate was collected from an orchard in the Malelane area in Mpumalanga.

DISCUSSION

Results of this study have shown that the morphologically distinct *F. mangiferae* and *F. sterilihyphosum* could easily be identified using RFLP profiles from their respective ITS regions. Although this was not a primary objective of the study, this approach will be useful in future studies where cultures originating from mango malformation symptoms can easily be identified.

In this study we have shown that *F. sterilihyphosum* isolates belong to a single VCG and *F. mangiferae* isolates from South Africa belong to two VCGs. Zheng & Ploetz (2002) have suggested that *F. mangiferae* has spread as a clonal population from India to Egypt as well as from Florida to South Africa. It is, therefore, possible that mango malformation caused by *F. mangiferae* in South Africa was introduced from Florida. This view is supported by the fact that several Florida *M. indica* cultivars are grown in South Africa (Zheng & Ploetz, 2002). The limited number of clones of these fungi in South Africa also supports the view that *F. mangiferae* and *F. sterilihyphosum* are exotic pathogens in the country.

Fusarium mangiferae has a wide distribution globally and the fact that it causes mango malformation is now well established (Freeman *et al.*, 1999). *F. sterilihyphosum* is the only species isolated from malformed mango tissue in the Northern Province of South Africa. Furthermore, its consistent association with malformation symptoms suggests strongly that it causes the disease in these areas. Despite this strong anecdotal evidence suggesting that *F. sterilihyphosum* is able to cause mango malformation, pathogenicity remains to be proven.

In South Africa, *F. sterilihyphosum* isolates were found only in the Northern Province. *F. mangiferae* was primarily isolated from Mpumalanga in the Malelane area, Nelspruit area, Fredenheim farm near Nelspruit as well as from Kwazulus-Natal. These findings suggest that *F. mangiferae* and *F. sterilihyphosum* have discrete distributions in South Africa. Although the origin of *F. sterilihyphosum* is not known, it is assumed that it was introduced into South Africa and the distribution of the two fungi is presumably related to movement of mango planting stock in the country.

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Table 1. Origin and vegetative compatibility groups of *Fusarium* isolates associated with mango malformation.

Origin	Strain no.[a]	VCG[b]
F. mangiferae		
South Africa, Nelspruit	MRC 2730	SA1
South Africa, Kwazulu-Natal	MRC 3477, MRC 3478, MRC 3479	SA1
South Africa, Fredenheim	MRC 8078, MRC 8079, MRC 8084, MRC 8085, FCC 1533, FCC 1534, FCC 1538, FCC 1539	SA2
South Africa, Nelspruit	MRC 8080, FCC 1526, FCC 1530, FCC 1532	SA2
South Africa, Malelane	MRC 8077, MRC 8081, MRC 8082, MRC 8083, MRC 8086, MRC 8087	SA2
Florida	MRC 7035	-
F. sterilihyphosum		
South Africa, Letsitele	MRC 2802, MRC 7602, MRC 7606, MRC 7605, MRC 7875, MRC 7877, MRC 8094, MRC 8095, MRC 8101, MRC 8102, MRC 8103	FS1
South Africa, Letsitele	FCC 1370, FCC 1525	HSI
South Africa, Deerpark	MRC 8100, MRC 8106, MRC 8107, FCC 1598, FCC 1604, FCC 1620, FCC 1625, FCC 1629	FS1
South Africa, Hazyview	MRC 8099, MRC 8104, MRC 8108, FCC 1233, FCC 1568, FCC 1570, FCC 1574, FCC 1584, FCC 1585, FCC 1592, FCC 1593, FCC 1594	FS1
South Africa, Trichardtsdal	FCC 1217, FCC 1227, FCC 1228, FCC 1231, FCC 1232, FCC 1233, FCC 1245	FS1
South Africa, Malelane	MRC 8105	FS1

[a] MRC = Culture collection of the Medical Research Council (MRC), Tygerberg, South Africa; KSU-X= Kansas State University culture collection, Department of Plant Pathology, Kansas State University, Manhattan. Kansas; 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] VCG = Vegetative compatibility group, HSI = Heterokaryon self-incompatible.

Fig. 1. Detailed map of sampled area in Mpumalanga and Northern Province.

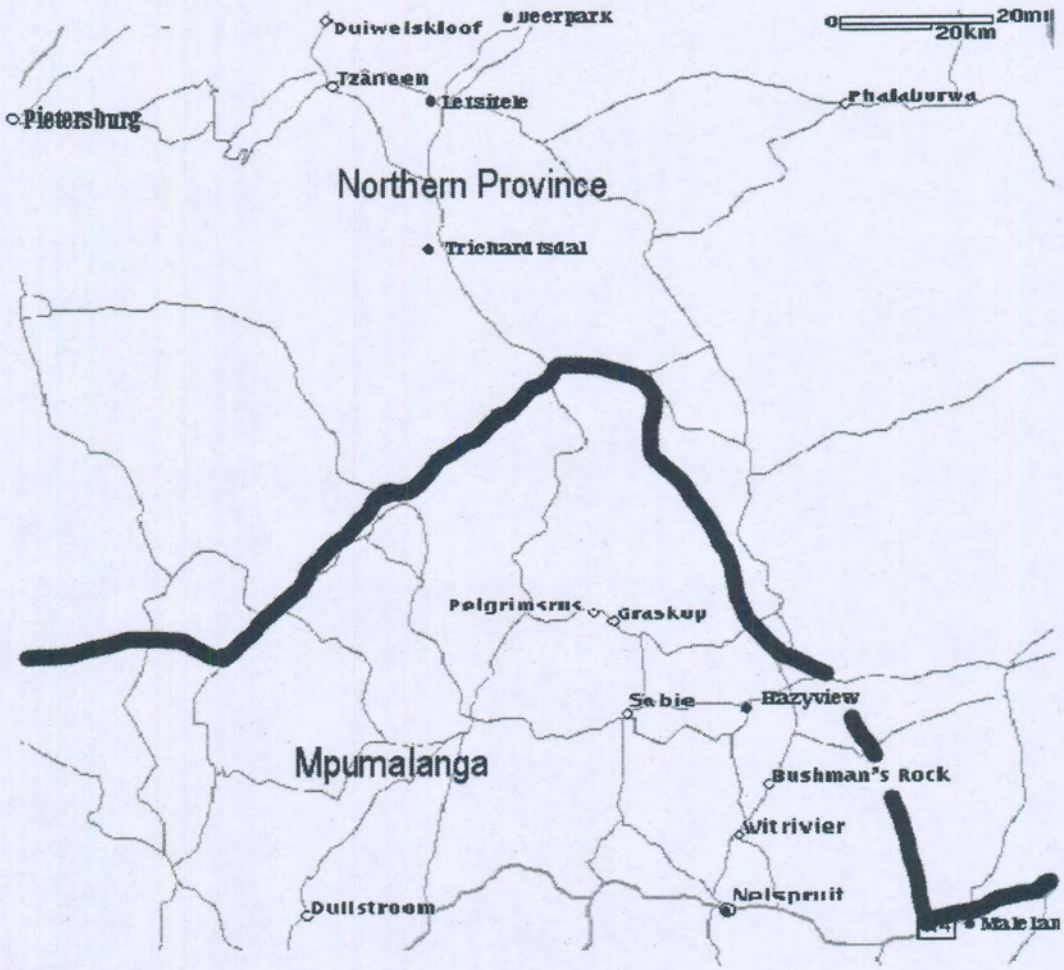
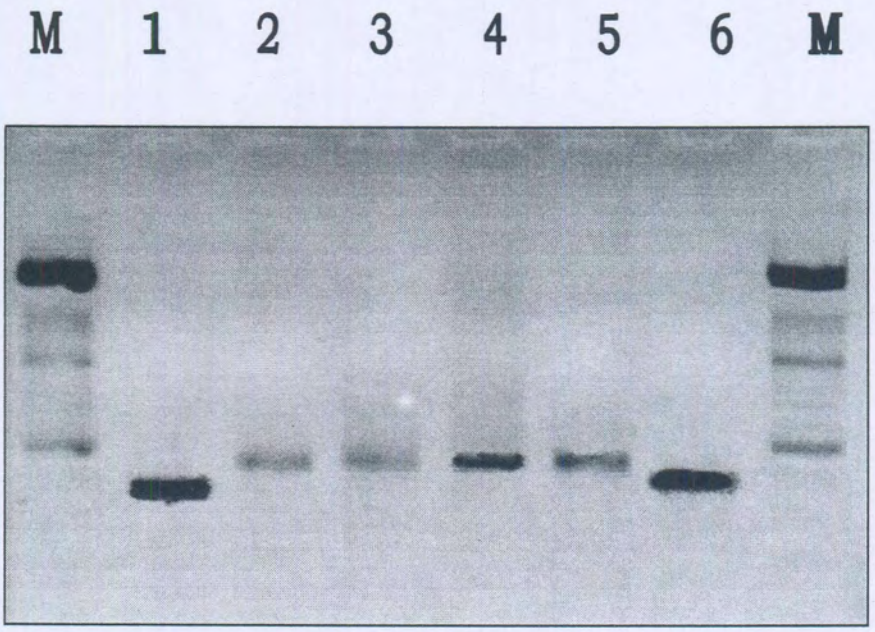


Fig. 2. ITS-RFLP profiled generated by digestion of ITS1, 5.8S and ITS2 rDNA gene with *Alu* I. Lane M, 100 bp ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp); Lane 1 and 6, *F. sterilihyphosum* isolates MRC 8106 and MRC 8101; Lane 2-5, *F. mangiferae* isolates MRC 7569, MRC 8081, MRC 8085 and MRC 8086.



APPENDIX 1

DNA sequences of sequence characterized amplified polymorphic loci of *Fusarium circinatum*. Genbank accession numbers, underlined primer binding sites and the amounts of nucleotide base pairs are also indicated.

FC-1 (AF430131): 314bp

CGTCCATAAGCAACTCCGATGCAATGTCCACCTTCAGGAAGCTCCCCAGAAAAGTACATT
ATATAGAGTCATCTTCCAGTTTCCGCACGTCAGGGCCCAATAATTTCTGCCACTCGTCCAT
CCATCTCCAGGGAGGGCATCTTGCTGGAGATGAGTCCACTCGCCAAAGACATCATCTGCC
ACAGCGACCTGGATGTGGTGACCACCGCCGCTTGTTGCAAAAGACACCCAGTGGTCGTCT
TGGTCTTGAACAAGGCATGGGTCGGGGAAGTTGATATCTAGTCGTGGAGAGAAAGACCTT
CCGCGACCTTTAT

FC-2 (AF430132): 185bp
GACCACAGCCTCGAGAACATTACATGAATCAATACTTCTCAGGTGAGCTGCGCTATACTA
GACAACACCTGGACAGTAAACAAGACAAAAAACTCCTGTAGGTCCGTTCCCAGCTTCCCA
TGCGCTGATCCCATTGAGAGAGGTCTGCTCAAAAATAATAATGTGTTGGCTAGAATGCAG
TGTGG

FC-3 (AF430133): 285bp
TCAATGAAAAGCAGCACGTCCTGTCCTTCTTCGTCTCTGAACTACTCAGCTACAGTCAAAC
TGTCGAGATATTGCGTTAGCTGGCGTCCAAGAGTCGTAAACAGAACATCATGATATAGTA
TGAGTATCAAAGCATGTGTGTGGTGTATGGTGTTGAGGTGTTGCAACAGGAAGGGAAGGC
ACTATCTGTCAACTTACCCGGTAAGGGCGACACGGGCACGAGCTCAGGGGGCTCGTTCAT
CTGACCGAAGACCAGGGCGACCTTGGACTCGCCATCAAGCTAAA

FC-4 (AF430134): 263bp
TTCCACCATGAGAGGAAACCTCCTCTTCGATTCACGTGCCAGCGGAATTACCCCAAGAGG
ACAAGGAGCGGGAACTTGATGTGAGGCACGAAATAAGCTAATCCAACAGATCCAATGGG
AGCTCTCAAAGCGACACGATGCATGGAATAGAACAAGCACAGACCACTCATTTCACGCCC
GATGCCGGATCCCGGTGTAAGCGCTCTCGAGAAGACAGTGAAAGTGAGGTTAAGGCGAA
GAAGGAGGATCAAGATTGGCAATGG

FC-5 (AF430135): 237bp

ATATTCTGACGGGTCCACCACAGTCAGTCCATTGATCATAAGAGGACTAGCTTCTCGCTCA
TGGGGATGCAAGTCTTATTACCTTGACGATGACTGGAACAACGGCAATCCGATAAGCTTC
CATTTCTCAACGGGACACCACACGGCTAAGTGCAACATGGTTGACCTGCATTATCTAGAA
ACAGAACCAGCCATACACGATTCAGATATGCCGTGTGAAAGCCATTGTGAGACCGT

FC-6 (AF430136): 222bp

GGTGAGGAAAACAAGAGCCACGTGAGTAACACTACGTAGCGCACGGAGTGTAGAGGTAG
CAGTCGTCACAGCACGCACAGTACGTGTATAGTACTGGAGAGACAGCTGGTGCGCCTTAT
CAGCAGCTGCAGAGTCGATGGTTGGCTGCGTGAAGGCGTTGACGGCACCGCCTAGGGCAC
AGCACGTCTCACGGACAGCGGCATCGTTATGGGCTAGCTGAGG

FC-7 (AF430137): 257bp

ACGGTCTCACAATGGCTTTCTTGATACCTTGAAAGCTTCGGTCTTAGTGGGAGCTTACAGT
TAGATCAGTCTCCGGCACAGAAGACGATATGATGAGCGCAGTGGCGTGCTAGACATCTGT
GGTGTACTATACCTACTGCACAGCAAGGTAGGTACTGTGTANGTAGTAGTTAGGTAAGTT
ACCTACCTTACCTTAGTACCTACCTAGGCTTTTACTTTCTGACACTGGCATATCTGAATCGT
GTATGGCTGGTTCC

FC-8 (AF430138): 139bp

GAGAAGAGTGGCAGGGACTGTGATACCTCTGGGAGCAGATTGGGAAGCAACCGGTTATG
CCCGGTTCAAGTTCAATCATCTACTATCACATCTTCCATGATTGAGACTCTTTTGTGGCGCG
CTGCTGTTCTATTAGCCC

FC-9 (AF430139): 241bp

TGAAGAGATGGAAGCTTCAGACGAACGAAAGCAACAACAATACGGTTATTCTGTTGTCAA
TTGCTTGTCAACCATTCTTTTATTCTTTTCATCGTTTATAGCACAAATCAAAATGCTAGCCT
GACTATGAATGTCAAAGAGGTCCAGCAATGTATTCATGGCGCATAGACAGTGTCATCACA
TACAAAAGCAAAAGATGAAAAGTCGAGGTCGAGCTAAAATTCCACCATGAGAGGAAACC

APPENDIX 2

Aligned ITS1, 5.8S and ITS2 sequences of *F. mangiferae* isolates NRRL 25226 (Genbank database accession number U61691), MRC 3477 (Genbank database accession number AF430128) and MRC 7035 (Genbank database accession number AF430129) as well as sequences of *F. sterilihyphosum* isolates MRC 2802 = NRRL 25623 (Genbank database accession number AF158305), MRC 7602 (Genbank database accession number AF430130).

```
                1                                                        60
NRRL 25226      CGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCAATTGTTGCC
MRC 3477        ............................................................
MRC 7035        ............................................................
MRC 2802        ............................................................
MRC 7602        ............................................................

               61                                                       120
NRRL 25226      TCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCC-TAAACTCT
MRC 3477        ...........................................................C........
MRC 7035        ...........................................................C........
MRC 2802        ...........................................................C........
MRC 7602        ...........................................................C........

               121                                                      180
NRRL 25226      GTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATC
MRC 3477        ............................................................
MRC 7035        ............................................................
MRC 2802        ............................................................
MRC 7602        ............................................................

               181                                                      240
NRRL 25226      TCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGA
MRC 3477        ............................................................
MRC 7035        ............................................................
MRC 2802        ............................................................
MRC 7602        ............................................................
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                241                                                                300
NRRL 25226      ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCAT
MRC 3477        ····------------------------------------------------------------
MRC 7035        ····------------------------------------------------------------
MRC 2802        ····------------------------------------------------------------
MRC 7602        ····------------------------------------------------------------

                301                                                                360
NRRL 25226      GCCTGTTCGAGCGTCATTTCAACCCTCAGGCCCCCGGGTTTGGTGTTGGGGATCGGCGAG
MRC 3477        ····------------------------------------------------------------
MRC 7035        ····------------------------------------------------------------
MRC 2802        ····------------------------A·····---A·C···········AC·····-····
MRC 7602        ····------------------------A·····---A·C···········AC·····-····

                361                                                                420
NRRL 25226      CCCTTGCGGCAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGCAGCTTCCATTGCGTA
MRC 3477        ····------------------------------------------------------------
MRC 7035        ····------------------------------------------------------------
MRC 2802        -------AAATC··GTT·····-····TG·T·····A···-·CG·····A·····
MRC 7602        -------AAATC··GTT·····-····TG·T·····A···-·CG·····A·····
```