Characterisation of *Fusarium* species associated with floral malformation of *Syzygium cordatum*

by

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I dedicate this dissertation to the best mother in this world

To my mother Irena

Za moju mamu

Declaration

I, the undersigned, hereby declare that the dissertation submitted herewith for the degree *Magister Scientiae* to the University of Pretoria contains my own independent work. This work has hitherto not been submitted for any degree at any other University.

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PREFACE

Syzygium cordatum (Family Myrtaceae), commonly known as the waterberry, is an indigenous evergreen tree in Southern Africa. In rural areas, *S. cordatum* is used for timber, food and as traditional medicine. This tree is also planted widely as an ornamental in subtropical and warm-temperate climates. However, in many locations *S. cordatum* appears to be affected by an abnormal inflorescence development, reminiscent of the malformation disease of *Mangifera indica* (Family Anacardiaceae). For both, waterberry and mango, the inflorescence malformation is associated with fungi in the genus *Fusarium*.

Fusarium represents a large group of diverse, cosmopolitan filamentous fungi. Many species commonly occur in soil, while others are associated with plants and, to a lesser extent, animals. The phytopathogenic species are often responsible for huge economic losses due to the destructive diseases that they cause on a wide variety of agriculturally important plant species. Many *Fusarium* species are also of great medical and veterinary importance, as they produce numerous highly toxic secondary metabolites that contaminate human food and animal feed. Many of the important plant pathogenic and mycotoxin-producing *Fusarium* species are included in the *Gibberella fujikuroi* complex. This complex also includes several species associated with *M. indica* floral malformation, as well as a species that was isolated from malformed *S. cordatum* inflorescences. The overall aim of this dissertation was to explore and characterise the diversity of *Fusarium* species associated with the floral malformation of these two plant species.

The use of morphological traits for characterising *Fusarium* species has dominated the taxonomy of this genus since its establishment. However, the lack of suitable diagnostic characters to differentiate between species has complicated the taxonomy of this group. This has also led to underestimations of the true *Fusarium* diversity in particular environments. Resolution of these issues became possible with the advent of PCR-based molecular biology and the subsequent use of DNA sequence information for taxonomic purposes. A critical overview of the literature pertaining to the diversity and taxonomy of *Fusarium* species, especially those in the *G. fujikuroi* complex, is presented in the first chapter of this dissertation. This complex is also considered from an evolutionary point of view, where the relationships among species are compared based on morphology, host associations, mycotoxicology and phylogeny. **This**

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Chapter 2 provides a review of the literature on the biotic agents associated with inflorescence malformations of economically important plants, focussing specifically on the diversity of the microorganisms and small invertebrates. Current knowledge regarding the mango malformation disease is also reviewed briefly. The review is concluded by comparing *M. indica* and *S. cordatum* malformations in terms of the *Fusarium* and mite species with which they are associated.

Due to the global importance of *Fusarium* species, our understanding of their distribution and species richness is paramount. Unfortunately, most previous studies on the diversity of *Fusarium* species in particular environments are biased towards those associated with hosts and/or substrates of agricultural or medical importance. Very few studies evaluate the diversity of *Fusarium* in indigenous ecosystems. Therefore, the primary aim of the research presented in Chapter 3 was to develop an operational procedure for cataloguing the *Fusarium* diversity in a specific environment by making use of *S. cordatum* malformed inflorescences as a model system. To differentiate between *Fusarium* species, the taxonomic value of morphological characters and two genomic regions were evaluated. *Fusarium* species were then identified using DNA barcodes and inferred phylogenetic relationships.

Mango malformation is the most important floral disease of this tropical crop and is caused by at least three different *Fusarium* species. Effective management of the disease is thus dependent on knowledge regarding the specific taxonomic identity of the disease-causing species. The aim of the final chapter of this dissertation was to identify *Fusarium* species associated with mango malformation in the Sultanate of Oman, by making use of morphological characters, as well as comparisons and phylogenetic analyses of DNA sequence information. The correct identification of a causal agent in the agricultural environment, such as the one responsible for mango malformation in the Sultanate of Oman, will have a positive impact on the fruit industry as appropriate integrated disease management strategies can be implemented. **This chapter has been published as: Kvas, M., Steenkamp, E.T., Al Adawi, A.O., Deadman, M.L., Al Jahwari, A.A., Marasas, W.F.O., Wingfield, B.D., Ploetz, R.C. and**

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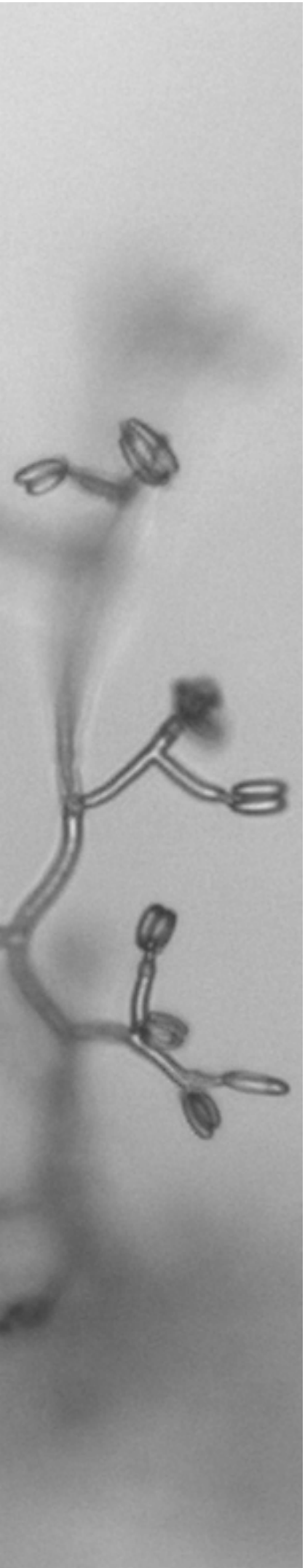
The work presented in this dissertation is focused on characterising *Fusarium* species in indigenous and agricultural environments. The exploration of *Fusarium* species associated with malformed *S. cordatum* inflorescences represents the most extensive study undertaken on these fungi in an indigenous South African niche. It is hoped that the results obtained in this study will represent a valuable milestone in studies on the diversity within this genus. Examination of other native ecological niches will yield additional novel taxa, which, together with those identified in this study will aid us in increasing our understanding of the evolution of this important group of fungi.



Chapter 1: Literature Review

Diversity and evolution of *Fusarium* species in the *Gibberella fujikuroi* complex

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Abstract

The *Gibberella fujikuroi* complex is a monophyletic taxon that includes an assemblage of *Fusarium* species with similar and overlapping morphological traits that complicates their differentiation. Most of the species in this complex are associated with devastating diseases of many economically important plants. They also produce a remarkably wide range of secondary metabolites or mycotoxins that contaminate food/feed worldwide and can subsequently cause a variety of diseases in humans and farm animals. Recent developments in molecular systematics have revealed that the *G. fujikuroi* complex includes at least 50 distinct species or phylogenetic lineages. Of these, 34 species have been formally described using morphological characters, 10 have been also described based on sexual fertility and at least 20 species produce one or more mycotoxins. Here, we review the most important criteria for recognising and defining *Fusarium* species in the *G. fujikuroi* complex. We also consider the diversity within this complex, specifically from an evolutionary point of view. We, therefore, discuss the morphological, biological and phylogenetic diversity in the *G. fujikuroi* complex, by reviewing these properties together with aspects such as their mycotoxicology, geographic distribution and host/substrate preference of the various *Fusarium* species with respect to their phylogeny.

Introduction

The genus *Fusarium* represents one of the most important groups of ascomycetous fungi. Its members are distributed across the globe where they are responsible for huge economic losses due to reductions in harvest yields and/or the quality of staple foods (Nelson *et al.*, 1983; Leslie and Summerell, 2006). At least 80% of all cultivated plants are associated with at least one disease caused by a *Fusarium* species (Leslie and Summerell, 2006). Various *Fusarium* species cause destructive diseases on cereal grains (White, 1980; Parry *et al*., 1995; Nyvall *et al.*, 1999; Goswami and Kistler, 2004), some are responsible for vascular wilts or root rots on many important vegetable, ornamental and field crops (Kraft *et al*., 1981; Linderman, 1981; Nelson *et al*., 1981), while others produce cankers on soft- and hardwood trees (Bloomberg, 1981; Dwinell *et al*., 1981, 2001; Wingfield *et al*., 2008). Recently, *Fusarium* species have emerged as human pathogens where they are associated with deeply invasive infections of immunocompromised patients (Nelson *et al*., 1994; Nucci and Anaissie, 2002; Summerbell, 2003; Dignani and Anaissie, 2004). *Fusarium* species are further notorious for producing mycotoxins that contaminate food/feed worldwide (reviewed by Marasas *et al.*, 1984; Joffe, 1986; Chelkowski, 1989; Desjardins, 2006), the consumption of which may lead to various serious human and animal diseases, reduced productivity in livestock and even death if prolonged exposure occurs (D'Mello *et al.*, 1999; Placinta *et al.*, 1999; Morgavi and Riley, 2007).

Since its establishment in 1809 by Link, the genus *Fusarium* has received much attention in the scientific literature. A significant portion of these studies dealt with taxonomic issues (see Leslie and Summerell, 2006), which for the most part have been dominated by the use of morphology to differentiate species and groups or sections. In the 1990s, with the increased utility of DNA-based methods, it rapidly became clear that the morphology-based classifications greatly underestimate the true diversity in the genus. The use of DNA sequence information for separating species has, therefore, revolutionised *Fusarium* taxonomy and it is now widely accepted that taxa previously thought to represent single sections or species are actually species complexes consisting of numerous distinct taxa (e.g. O'Donnell, 2000; O'Donnell *et al*., 2000a, 2004). Currently, one of the best-studied species complexes is the *Gibberella fujikuroi* complex (GFC), which includes numerous mycotoxigenic and/or phytopathogenic species. In this paper,

we review the taxonomy of the GFC and its species, as well as the various criteria used for defining species in the complex. We also consider the GFC from an evolutionary point of view, where we specifically address geographic distribution, interactions with plant hosts and mycotoxin production.

Taxonomy of the GFC

The term "*Gibberella fujikuroi* complex" refers to the monophyletic taxon that broadly corresponds to the Section *Liseola*, but that also accommodates certain species originally classified in other *Fusarium* sections (Fig. 1) (Nirenberg and O'Donnell, 1998; O'Donnell *et al*., 1998, 2000b; Geiser *et al*., 2005). Section *Liseola* was established by Wollenweber and Reinking (1935) based on the morphology of three species (*F. moniliforme*, *F. lactis*, *F. neoceras*) and their varieties that produce macroconidia in sporodochia or pionnotes, microconidia in false heads and/or chains and no chlamydospores (Snyder and Hansen, 1945; Leslie and Summerell, 2006). Later, Snyder and Hansen (1945) argued that the production of microconidia borne in chains is an unstable character that is inappropriate for reliably separating species and varieties in this section. As a result, they lumped all of Wollenweber and Reinking's species and recognised only *F. moniliforme* as a member of Section *Liseola*. Booth (1971) used the morphology of conidiogenous cells to separate *F. moniliforme* from its variety *F. moniliforme* var. *subglutinans*. In 1983, Nelson and colleagues introduced a system that bridged the existing *Fusarium* classification systems (Nelson *et al*., 1983; Leslie and Summerell, 2006) and recognised four species (*F. anthophilium*, *F. moniliforme*, *F. proliferatum* and *F. subglutinans*) in the Section *Liseola*. They differentiated these based on shape and production of microconidia in chains and/or false heads from polyphialides and/or monophialides. Following this system, and the use of some additional morphological traits (e.g. production of sterile coiled hyphae, pseudochlamydospores), as well as relying on molecular and biological traits, various other *Fusarium* species in the GFC were subsequently described (Rheeder *et al.*, 1996; Klittich *et al.*, 1997; Nirenberg and O'Donnell, 1998; Nirenberg *et al*., 1998; Aoki *et al*., 2001; Marasas *et al*., 2001; Britz *et al*., 2002b; Zeller *et al*., 2003).

In the 1990s, advances in technology have made the use of DNA sequence information more readily accessible for classification purposes and a range of DNA-based methods were

then applied to characterise the GFC species. These include genomic fingerprinting techniques such as electrophoretic karyotyping (Xu *et al*., 1995), random amplified polymorphic DNA (RAPD) (Voigt *et al*., 1995; Viljoen *et al*., 1997), polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis of specific genes (Steenkamp *et al*., 1999; Mirete *et al*., 2003) and amplified fragment length polymorphism (AFLP) analysis (Marasas *et al*., 2001; Zeller *et al*., 2003). For the purposes of direct sequence analysis, various genomic regions have been evaluated as taxonomic markers (O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998, 2000b; Steenkamp *et al*., 1999, 2000a; Schweigkofler *et al*., 2004), including the ribosomal RNA (rRNA) internal transcribed spacer (ITS) widely used for other fungi (Bruns *et al*., 1991; Seifert *et al*., 1995). However, this region has been proven ineffective for classifying *Fusarium* species, due to the presence of two divergent and non-orthologous copies of the ITS2 region in most *Fusarium* species examined (Waalwijk *et al*., 1996; O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998). The gene encoding the translation elongation factor 1-alpha (TEF) has become the marker of choice as it is a single-copy gene that is highly informative among closely related species (Geiser *et al*., 2004).

During the course of the last decade, application of DNA-based methods and phylogenetic analyses of multiple genomic regions have revealed the non-monophyletic nature of many of the *Fusarium* sections including the section *Liseola* (e.g. O'Donnell *et al*., 1998, 2000b). It is now widely accepted that the GFC includes species previously accommodated in other sections. For example, certain chlamydospore-forming species that were classified in the Section *Dlaminia* by some workers (Kwasna *et al*., 1991) are now accepted as part of the GFC (O'Donnell *et al*., 1998, 2000b). The GFC also includes a number of species that were previously classified in the *Discolor*, *Elegans* and *Lateritium* sections (Wollenweber, 1934; Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al*., 1983; O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998, 2000b; Geiser *et al*., 2005). However, a particular set of morphological synapomorphies (shared derived characters) has not yet been identified for the clade and its existence is still supported only by multigene phylogenies (O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998, 2000b; Geiser *et al*., 2005).

Recognising species in the GFC

To recognise and define species in the GFC, various operational species concepts have been applied. Although a variety of genetic, ecological and biological traits and properties may be used for this purpose (Rojas, 1992; Mayden, 1997) only Morphological Species Recognition, Biological Species Recognition and Phylogenetic Species Recognition (MSR, BSR and PSR, respectively; Taylor *et al*., 2000) have contributed significantly to the classification of *Fusarium* species in the GFC. Of these, the MSR was the most widely used and has dominated *Fusarium* taxonomy since its establishment in 1809. Based on this recognition system, species are primarily identified using shape and size of macroconidia and microconidia, while other characters such as the aerial arrangement of microconidia, morphology of conidiogenous cells and presence/absence of chlamydospores are also used (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983; Leslie and Summerell, 2006). The MSR also takes into account physiological characters such as growth rates at different temperatures, host associations, and secondary metabolite production (Nelson *et al.*, 1983). Based on the MSR, the GFC currently includes 34 morphospecies (O'Donnell *et al.*, 1998, 2000b; Nirenberg and O'Donnell, 1998; Nirenberg *et al.*, 1998; Aoki *et al.*, 2001; Marasas *et al.*, 2001; Britz *et al.*, 2002b; Zeller *et al.*, 2003; Geiser *et al.*, 2005). However, the overall shortage of diagnostic morphological characters complicates separation of similar species and description of new species. This is particularly true for the various species known to be part of the GFC that still await description (O'Donnell *et al.*, 1998; 2000b). Therefore, application of the MSR is typically associated with underestimation of true *Fusarium* diversity. Nevertheless, the MSR remains an integral part of all *Fusarium* species descriptions and allows the sorting of isolates for final species determination using BSR and PSR (Leslie and Summerell, 2006).

In terms of BSR, two fungi belong to the same species if they are sexually compatible and able to enter a teleomorph stage during which sexual fruiting structures bearing fertile progeny are produced (reviewed by Taylor *et al.*, 2000; Coyne and Orr, 2004). Sexually fertile strains of the GFC are heterothallic (reviewed by Desjardins, 2003), except for *F. sacchari* that is sometimes apparently homothallic or self-fertile (Britz *et al.*, 1999). All sexually fertile strains of the GFC produce a teleomorph in the genus *Gibberella*, which was first described by Fries in 1822 based on its blue/purple perithecia with hyaline/pale yellow ascospores that are straight or

curved with 1–3 septa (Samuels *et al.*, 2001; Desjardins, 2003). To facilitate the use of BSR for identifying GFC species, extensive population studies have been conducted for selecting fertile hermaphrodite or female-fertile strains of opposite mating type to be used as tester strains in diagnostic sexual crosses (Klittich and Leslie, 1992; Klaasen and Nelson, 1996; Britz *et al.*, 1998, 1999; Zeller *et al.*, 2003; Lepoint *et al.*, 2005). These tests have been simplified by the introduction of PCR-based techniques for scoring mating type (Covert *et al.*, 1999; Kerényi *et al.*, 1999; Steenkamp *et al.*, 2000b). Currently, the GFC includes ten well-characterised biological species or so-called mating populations (MP) that have been designated MP A – MP J (Kuhlman, 1982; Leslie, 1991, 1995; Klittich and Leslie, 1992; Klaasen and Nelson, 1996; Klittich *et al.*, 1997; Britz *et al.*, 1999, 2002a; Samuels *et al.*, 2001; Zeller *et al.*, 2003; Geiser *et al.*, 2005; Lepoint *et al.*, 2005; Leslie *et al.*, 2005a).

Application of BSR for GFC classification purposes has four major disadvantages. First, sexual fruiting structures are known for about 20% of the recognised species in this complex, rendering the BSR useless for diagnosing the vast majority of the known GFC species. Second, populations of many of the species in this complex are characterised by unequal relative frequencies of occurrence of the two mating types (Leslie and Summerell, 2006). For example, Britz *et al.*, (2002b) found that the majority of *F. mangiferae* and *F. sterilihyphosum* isolates they examined were of a single mating type and in such cases, extensive sampling is required in order to ensure that isolates of opposite mating type are identified. Third, the occurrence of hermaphrodite strains in many GFC populations is limited (Kuhlman, 1982; Leslie and Summerell, 2006), which can potentially result in the scoring of sexual crosses as incompatible, even though the interacting individuals may represent members of the same biological species. Finally, the results of sexual compatibility tests are not always clear-cut because different species of the GFC apparently have the ability to interbreed. Examples of these are *F. fujikuroi* and *F. proliferatum* (Leslie *et al.*, 2004b) and *F. circinatum* and *F. subglutinans* (Desjardins *et al.*, 2000b; Steenkamp *et al.*, 2001). Despite these shortcomings, the single most important advantage of using the BSR for classifying *Fusarium* species is that it provides a means of measuring the amount of variation associated with morphology, DNA sequence, and physiology within a well-defined species, thus allowing a better understanding of the boundaries between species, especially those thought to be reproducing mainly asexually.

The PSR determines the hierarchical evolutionary relationships between species based on DNA sequence information and interprets them in terms of classification systems (Davis, 1996). Of the known versions of the PSR (reviewed by Avise and Ball, 1990; Luckow, 1995; Davis, 1996, 1997; Mayden, 1997), a modified version of Nixon and Wheeler's (1990) diagnostic PSR (O'Donnell *et al.*, 1998) has been used most extensively in the GFC (O'Donnell *et al.*, 1998, 2000b; Aoki *et al.*, 2001; Steenkamp *et al.*, 1999, 2000a; Geiser *et al.*, 2005). According to this version of the PSR, species represent the smallest group of populations or lineages that can be diagnosed by an exclusive combination of fixed apomorphies or attributes that include information on their morphology, sexual behaviour and phylogenetic affinities (Nixon and Wheeler, 1990; O'Donnell *et al.*, 1998). To apply this version of the PSR, phylogenetic analyses that are based on the combined DNA sequence information for various gene regions (e.g. nuclear 28S rRNA, small subunit of the mitochondrial (mtSSU) rRNA, β-tubulin, histone *H3*, calmodulin and TEF) have been used (O'Donnell *et al.*, 1998, 2000b; Steenkamp *et al.*, 1999, 2000a; Aoki *et al.*, 2001; Geiser *et al.*, 2005). These studies showed that the GFC includes at least 50 distinct phylogenetic species or lineages (Fig. 1), which roughly correspond to those recognised using MSR and BSR. However, the resolving power of PSR far outweighs that of MSR and BSR. For example, PSR facilitates separation of biological species known to be capable of interbreeding in the laboratory (see above). Also, PSR allows identification of the various distinct phylogenetic species that make up individual morphospecies (Aoki *et al.*, 2001; Britz *et al.*, 2002b; Nirenberg and O'Donnell, 1998; Nirenberg *et al.*, 1998; O'Donnell *et al.*, 1998, 2000b; Steenkamp *et al.*, 1999, 2000a, 2002).

Although it is possible to recognise all known *Fusarium* species in the GFC by applying only PSR, the application of this operational concept alone is impractical and it may yield results that are not biologically meaningful (e.g. Coyne and Orr, 2004; Dayrat, 2005). Therefore, the PSR is generally used in combination with MSR and BSR, when recognising *Fusarium* species in the GFC (Klittich *et al.*, 1997; Zeller *et al.*, 2003). Typically in these situations, the PSR is based on the data for multiple unlinked genomic regions (O'Donnell *et al.*, 1998, 2000b; Taylor *et al.*, 2000), while additional morphological, physiological and ecological data for MSR and data on reproductive behaviour for BSR are also included and evaluated. The majority of the current GFC species definitions and descriptions are based on such polyphasic or integrative taxonomic approaches that incorporate various types of data (Klittich *et al.*, 1997; Marasas *et*

al., 2001; Zeller *et al.*, 2003; Dayrat, 2005; Will *et al.*, 2005). This integrative approach also extends to routine species identifications where applications of single diagnostic procedures such as the analysis of the TEF-barcoding region (Geiser *et al.*, 2004) or the examination of morphological structures are not sufficient for unambiguous diagnoses (Leslie and Summerell, 2006; Dayrat, 2005; Will *et al.*, 2005).

Phylogenetic clades of the GFC

Based on multigene phylogenies, the *Fusarium* species in the GFC (O'Donnell *et al.*, 1998, 2000b) can be separated into three large clades (Fig. 1). To explain the existence of these clades, the authors formulated a biogeographic hypothesis based on the origins of the plant hosts from which the respective *Fusarium* species included in their study were isolated. Accordingly, these clades were referred to as the "African", "American" and "Asian" clades (Fig. 1) (O'Donnell *et al.*, 1998). In the following section, the morphology, mycotoxicology and host/substrate associations for the known members of each clade are briefly reviewed.

The "African Clade"

The so-called "African Clade" is the largest of the three clades with 23 phylogenetic lineages, of which four represent biological species (Fig. 1). These include *F. verticillioides* (MP A), *F. thapsinum* (MP F), *F. nygamai* (MP G) and *F. xylarioides* (MP J), which are the anamorphs of *G. moniliformis*, *G. thapsina*, *G. nygamai* and *G. xylarioide*s, respectively (Klittich and Leslie, 1992; Leslie, 1995; Klaasen and Nelson, 1996; Klittich *et al.*, 1997; Geiser *et al.*, 2005; Lepoint *et al.*, 2005). *Gibberella indica*, the sexual state of *F. udum*, has been observed, but it has not been recognised as an MP due to the lack of sufficiently fertile female tester isolates (Rai and Upadhyay, 1982). *Fusarium pseudonygamai* has been mentioned by Leslie *et al.* (2007) as possibly representing a new MP. Of the 19 remaining "African Clade" phylogenetic lineages, 13 represent formally described *Fusarium* species for which only the anamorphs are known and 6 await description.

Amongst the agriculturally important pathogens (*F. verticillioides*, *F. denticulatum*, *F. thapsinum*, *F. nygamai*, *F. lactis*, *F. phyllophilum*, *F. udum*, *F. xylarioides*) included in the clade, *F. verticillioides* is the best known (Booth, 1971; Gerlach and Nirenberg, 1982; Burgess

and Trimboli, 1986; Michailides *et al.*, 1994, 1996; Clark *et al.*, 1995; Nelson *et al.*, 1995; Klittich *et al.*, 1997; Nirenberg and O'Donnell, 1998; Leslie and Summerell, 2006). This cosmopolitan pathogen of maize causes seedling blight as well as seed, root, stalk and ear rot (White, 1980; Kommedahl and Windels, 1981; Parry, 1995). It also produces various mycotoxins including fusarins (Marasas *et al.*, 1984; Faber and Scott, 1989), fusaric acid (Marasas *et al.*, 1984; Bacon *et al.*, 1996), trace levels of moniliformin (Marasas *et al.*, 1984, 1986; Leslie *et al.*, 1996) and beauvericin (Leslie *et al.*, 2004a). It is, however, most notorious for producing very high levels of fumonisins, specifically fumonisin B_1 (Gelderblom *et al.*, 1988; Thiel *et al.*, 1991; Nelson *et al.*, 1993; Marasas, 2001; Desjardins, 2006). This mycotoxin causes equine leukoencephalomalacia (Kellerman *et al.*, 1990), porcine pulmonary oedema (Harrison *et al.*, 1990), hepatocarcinoma in rodents (Gelderblom *et al.*, 1991) and has been implicated in the high incidences of human oesophageal cancer in various areas of the world (reviewed by Marasas, 2001). *Fusarium verticillioides* has also been identified as an opportunistic pathogen in human infections (Hennequin *et al.*, 1997; Guarro *et al.*, 2000).

Morphologically, *F. verticillioides* is characterised by oval to club shaped microconidia produced in long chains that are borne only on monophialides (Fig. 2; Nelson *et al.*, 1983). It is these monophialides that distinguishes it from species such as *F. proliferatum* and *F. fujikuroi*, which reside in the "Asian Clade" and that produce chains from polyphialides (Leslie and Summerell, 2006). Although, *F. verticillioides* and *F. thapsinum* have previously been considered as the same species, they are distinguishable using BSR (Klittich and Leslie, 1992), mycotoxin production (Fig. 2), host preference (Fig. 3) and DNA-based information (Xu *et al.*, 1995; O'Donnell and Cigelnik, 1997) amongst other methods (Klittich *et al.*, 1997). *Fusarium thapsinum* is a causal agent of stalk rot and kernel mould in sorghum, but it has also been associated with banana, maize, peanut (Klittich *et al.*, 1997) and native grasses in the USA (Leslie *et al.*, 2004a). Unlike *F. verticillioides*, it produces high levels of moniliformin and low levels of fumonisins (Klittich *et al.*, 1997; Leslie *et al.*, 2004a). *Fusarium verticillioides* and *F. thapsinum* are morphologically similar to *F. andiyazi*, but the production of unique pseudochlamydospores by the latter species distinguishes it from any other taxon (Marasas *et al.*, 2001). *Fusarium andiyazi*, a sorghum pathogen, produces fumonisins in trace levels, and otherwise little is known about its mycotoxin production capabilities (Rheeder *et al.*, 2002). Despite substantial similarity in their morphology, *F. verticillioides* and *F. thapsinum* are not

phylogenetically very closely related (Fig. 1), while the phylogenetic affinity of *F. andiyazi* remains to be determined.

The "African Clade" includes most of the GFC chlamydospore-formers, specifically *F. dlamini*, *F. napiforme*, *F. nygamai*, *F. acutatum*, *F. pseudoanthophilum*, *F. udum* and *F. xylarioides* (O'Donnell *et al.*, 1998, 2000b; Geiser *et al.*, 2005). *Fusarium dlamini* can easily be misidentified as *F. napiforme* or *F. anthophilum* (Fig. 2). This is due to the production of napiform microconidia by both species. However, *F. anthophilum* does not produce chlamydospores (Marasas *et al.*, 1985) while *F. napiforme* produces microconidia in chains (Marasas *et al.*, 1987). *Fusarium dlamini* is associated with plant debris in the soils of Southern Africa and it is characterised by allantoid to fusiform and napiform microconidia produced in false heads on monophialides (Marasas *et al.*, 1985). *Fusarium napiforme* was initially identified from millet and sorghum grains in Southern Africa (Marasas *et al.*, 1987; Onyike *et al.*, 1991, 1992) and more recently, it has been identified as a human pathogen (Melcher *et al.*, 1993). *Fusarium dlamini* and *F. napiforme* are both known to produce fumonisins (Nelson *et al.*, 1992) and moniliformin (Marasas *et al.*, 1991), while they respectively also produce beauvericin (Logrieco *et al.*, 1998; Moretti *et al.*, 2007) and fusaric acid (Bacon *et al.*, 1996) (Fig. 2).

Based on morphology, *F. nygamai* shares traits with *F. pseudonygamai*, *F. verticillioides*, *F. napiforme* and *F. thapsinum* (Fig. 2). *Fusarium nygamai* is, however, most similar to *F. pseudonygamai*, although the latter species produces swollen hyphal cells rather than chlamydospores (Nirenberg and O'Donnell, 1998). These two fungi are also associated with different plant hosts (Fig. 3), with *F. pseudonygamai* isolated from pearl millet, and *F. nygamai* associated with many hosts such as sorghum, millet, maize and broad bean to name just a few (Leslie and Summerell, 2006). *Fusarium nygamai* can be distinguished from *F. napiforme* by the production of polyphialides in the former species (Leslie and Summerell, 2006), although this character is difficult to detect and considered an unreliable morphological trait (Burgess and Trimboli, 1986). Chlamydospore production and microconidial arrangement in shorter chains distinguishes *F. nygamai* from *F. verticillioides* and *F. thapsinum* (Leslie and Summerell, 2006). *Fusarium nygamai* produces fumonisins (Thiel *et al.*, 1991; Nelson *et al.*, 1992; Leslie *et al.*, 2005b), beauvericin (Logrieco *et al.*, 1998; Moretti *et al.*, 2007), moniliformin (Leslie *et al.*, 2005b) and fusaric acid (Desjardins, 2006) (Fig. 2). Although

Marasas *et al.* (1991) reported that *F. nygamai* produces moniliformin, Leslie and Summerell (2006) suggest that the specific isolates probably represented *F. pseudonygamai*, which is known to produce moniliformin and fusaproliferin (Fotso *et al.*, 2002; Leslie *et al.*, 2005b).

Besides both being chlamydospore-formers, *F. xylarioides* and *F. udum* also cause serious vascular wilt diseases of coffee and pigeonpea, respectively (Booth, 1971). Booth (1971) reported *F. xylarioides* with "sex-linked morphological characters" and termed these "male and female" strains. His view was that "male" isolates produced curved, cylindrical, 5–7-septate macroconidia, while "female" isolates produced small, highly curved, 0–3-septate macroconidia. Using the PSR, Geiser *et al.* (2005) concluded that "male" strains belong to the *Lateritium* clade or section, while "female" strains represent authentic *F. xylarioides* isolates that form part of the GFC (Fig. 1). *Fusarium udum* is characterised by strongly curved, 1–3 occasionally 5 septate macroconidia and ovoid-fusoid or curved single celled microconidia (Booth, 1971). Morphologically, *F. udum* is most similar to *F. acutatum* that is also associated with pigeonpea disease, but the macroconidial acute apical cell of *F. acutatum* differentiates it from *F. udum* (Nirenberg and O'Donnell, 1998). Together, *F. xylarioides*, *F. udum* and *F. acutatum* appear to form a closely related group that also includes *F. phyllophilum* (Fig. 1). The latter species is a leaf pathogen of plants in the families Asphodelaceae and Dracaenaceae and it produces clavate conidia in false heads and chains from mono- and polyphialides (Gerlach and Nirenberg, 1982; Nirenberg and O'Donnell, 1998; Figs. 2 and 3). In terms of mycotoxins, *F. udum* produces fusaric acid (Booth, 1971) and low levels of fusaproliferin (Moretti *et al.*, 2007), *F. acutatum* produces trace or low levels of beauvericin, enniatins and fumonisins, while *F. phyllophilum* produces high levels of moniliformin, significant levels of beauvericin and low levels of fumonisins (Fotso *et al.*, 2002; Desjardins, 2006; Moretti *et al.*, 2007). *Fusarium xylarioides* has not yet been tested for mycotoxin production.

Based on phylogeny, the chlamydospore-former *F. pseudoanthophilum* is most closely related to *F. brevicatenulatum* (Fig. 1). *Fusarium pseudoanthophilum* is associated with maize cultivated in Zimbabwe, whereas *F. brevicatenulatum* was initially isolated from a parasitic weed of cereals in Madagascar (Nirenberg *et al.*, 1998). Both species are characterised by long-oval to obovoid microconidia produced in false heads and short chains on monophialides and less occasionally also polyphialides (Fig. 2) and the only morphological feature separating them are the chlamydospores and pyriform microconidia produced by *F. pseudoanthophilum*

(Nirenberg *et al.*, 1998). It is these pyriform microconidia that makes the species morphologically similar to *F. anthophilum* ("American Clade"; see below), but the production of very short chains and chlamydospores by the former differentiates them (Nirenberg *et al.*, 1998). Strains of *F. pseudoanthophilum* are known to produce beauvericin, while *F. brevicatenulatum* strains produce fumonisins (Fotso *et al.*, 2002).

Fusarium ramigenum is most closely related to *F. napiforme* based on phylogenetic data (Fig. 1), although it does not resemble this species morphologically (Fig. 2). *Fusarium ramigenum* is, however, similar to *F. lactis* (Nirenberg and O'Donnell, 1998; O'Donnell *et al.*, 1998, 2000b), which differs from *F. ramigenum* by its production of microconidia in geniculate chains (Nirenberg and O'Donnell, 1998). Both *F. ramigenum* and *F. lactis* have been isolated from figs in the USA (Nirenberg and O'Donnell, 1998), but only *F. lactis* has been proven to cause endosepsis (Michailides *et al.*, 1994, 1996). The mycotoxicology of these two species is also very similar, as both have been recognised as moniliformin producers (Fotso *et al.*, 2002), whilst Moretti *et al.* (2007) reported that a strain of *F. lactis* also produced beauvericin.

The results of phylogenetic analysis group the remaining two "African Clade" species *F. denticulatum* and *F. pseudocircinatum* with the microconidial chain-former *F. thapsinum* (Fig. 1). *Fusarium denticulatum* is a leaf pathogen of sweet potato that was previously misidentified as *F. lateritium* (Clark *et al.*, 1995; Nelson *et al.*, 1995). Nirenberg and O'Donnell (1998) described it as a new species, which is uniquely characterised by denticulate polyphialidic conidiogeneous openings. *Fusarium pseudocircinatum*, isolated from various substrates in pantropical regions (Nirenberg and O'Donnell, 1998), displays morphological characters typical of *F. subglutinans* in the "American Clade" (see below; Fig. 2), although they are distinguishable based on the production of coiled sterile hyphae in *F. pseudocircinatum* (Nirenberg and O'Donnell, 1998). This trait is, however, also observed in the "American Clade" species *F. circinatum* and *F. sterilihyphosum* (see below; Fig. 2), from which *F. pseudocircinatum* can be distinguished by its short microconidial chains (Nirenberg and O'Donnell, 1998) and the different numbers of cells in the macroconidia (Britz *et al.*, 2002b), respectively. Mycotoxigenically, *F. denticulatum* has been shown to produce enniatins and *F. pseudocircinatum* fusaproliferin and fumonisins, while both species are known to produce beauvericin and moniliformin (Fotso *et al.*, 2002; Desjardins, 2006; Moretti *et al.*, 2007).

The "American Clade"

The so-called "American Clade" contains 18 phylogenetic lineages, of which ten have been described using morphology (Wollenweber, 1934; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983; Nirenberg and O'Donnell, 1998; Britz *et al.*, 1999, 2002b). The species *F. subglutinans*, *F. circinatum* and *F. konzum* can also be diagnosed using BSR. Their respective teleomorphs are *G. subglutinans* (MP E), *G. circinata* (MP H) and *G. konza* (MP I) (Britz *et al.*, 1999; Samuels *et al.*, 2001; Zeller *et al.*, 2003). The majority of the described species in this clade display morphological traits typical of those described for *F. subglutinans* by Nelson *et al.* (1983) (Fig. 2). Application of the PSR has, however, facilitated the resolution of this taxon, which is now known to include the "American Clade" species *F. subglutinans sensu stricto*, *F. circinatum*, *F. begoniae*, *F. bulbicola*, *F. sterilihyphosum* and *F. guttiforme*, the "African Clade" species *F. pseudocircinatum* (discussed above) and the "Asian Clade" species (see below) *F. sacchari*, *F. concentricum* and *F. mangiferae* (O'Donnell and Cigelnik, 1997; Nirenberg and O'Donnell, 1998; O'Donnell *et al.*, 1998; Britz *et al.*, 2002b; Leslie *et al.*, 2005a).

The morphological characters that define *F. subglutinans sensu stricto* are single-celled oval microconidia produced only in false heads from mono- and polyphialides (Nelson *et al.*, 1983). This fungus is morphologically very similar to *F. anthophilum* and *F. succisae* (Fig. 2), although they can be distinguished by pyriform microconidia in *F. anthophilum* (Nelson *et al.*, 1983) and "U" shaped macroconidia in *F. succisae* (Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983). *Fusarium subglutinans* is a globally distributed pathogen of *Zea* species and it has also been associated with other plants such as banana, millet, sorghum and many others (Leslie and Summerell, 2006), as well as with human infections (Summerbell, 2003). *Fusarium succisae* causes flower rot of *Succisa pratensis* (Gerlach and Nirenberg, 1982; Nirenberg and O'Donnell, 1998) while *F. anthophilum* is associated with *Lolium* species and *Zizania palustris* (wild rice) but has never been shown to be a pathogen (Engels and Kramer, 1996; Nyvall *et al.*, 1999). *Fusarium subglutinans* and *F. anthophilum* both appear to include a number of cryptic lineages (Steenkamp *et al.*, 2002; Zeller *et al.*, 2003). Mycotoxigenically, *F. subglutinans* has been shown to produce trace levels of fumonisins (Nelson *et al.*, 1992), moniliformin, fusaric acid (Marasas *et al.*, 1984), beauvericin (Logrieco *et al.*, 1998) and fusaproliferin (Moretti *et al.*, 2007). *Fusarium anthophilum* produces moniliformin (Marasas *et al.*, 1986), fumonisins

(Nelson *et al.*, 1992) and together with *F. succisae* it is known to produce beauvericin and fusaproliferin (Moretti *et al.*, 2007).

Fusarium circinatum is the causal agent of pitch canker and had been known for many years as *F. subglutinans* forma specialis *pini* due to its host specificity to *Pinus* species (Correll *et al.*, 1991). Its current name refers to the coiled sterile hyphae, a character it shares with *F. sterilihyphosum* and the "African Clade" species *F. pseudocircinatum* (Nirenberg and O'Donnell, 1998; Britz *et al.*, 2002b). Morphologically, *F. circinatum* is differentiated from *F. pseudocircinatum* by the production of erect conidiophores and arrangement of microconidia only in false heads (Nirenberg and O'Donnell, 1998). *Fusarium sterilihyphosum* is associated with mango malformation in South Africa and it is differentiated from these two species by the morphology of its macroconidia (Britz *et al.*, 2002b). *Fusarium circinatum* has been reported to produce beauvericin and fusaproliferin (Fotso *et al.*, 2002; Desjardins, 2006; Moretti *et al.*, 2007), while the mycotoxicology of *F. sterilihyphosum* is unknown.

Another species in the *F. subglutinans sensu lato* group is *F. guttiforme* associated with rotten pineapple fruits (Nirenberg and O'Donnell, 1998). Aerial conidia of *F. guttiforme* are very similar to those produced by *F. circinatum* (Fig. 2), although *F. guttiforme* is most likely to be confused with *F. subglutinans* and the "Asian Clade" species *F. sacchari* (Nirenberg and O'Donnell, 1998; see below). *Fusarium guttiforme* can be differentiated from *F. sacchari* by the oval to allantoid or fusoid microconidia in the former species and from *F. circinatum* by the production of its sterile coiled hyphae. Furthermore, *F. subglutinans* can be distinguished from *F. guttiforme* by the latter's production of obovoid microconidia and more conidogeneous openings (Britz *et al.*, 2002b; Leslie and Summerell, 2006). *Fusarium guttiforme* has been reported to produce beauvericin and fusaproliferin (Fotso *et al.*, 2002; Desjardins, 2006; Moretti *et al.*, 2007).

Morphologically, *F. begoniae* and *F. bulbicola* are difficult to differentiate from each other and/or from other species in the *F. subglutinans sensu lato* group. These two species are, however, not closely related (Fig. 1). *Fusarium begoniae* is a pathogen of *Begonia* hybrids, while *F. bulbicola* is associated with bulb rot of various horticulturally important plants (Nirenberg and O'Donnell, 1998). Conidiophores of *F. begoniae* are prostrate and seldom branched, while the conidiophores of *F. bulbicola* are erect and regularly branched. *Fusarium begoniae* has been reported to produce moniliformin, fumonisins (Fotso *et al.*, 2002) and

fusaproliferin (Moretti *et al.*, 2007), while *F. bulbicola* produces fusaproliferin and beauvericin (Moretti *et al.*, 2007).

Fusarium konzum was isolated from native prairie grasses in Kansas State, USA (Zeller *et al.*, 2003). This species is characterised by oval, pyriform and napiform to globose microconidia that are borne on mono- and polyphialides and arranged singly or in small false heads in the aerial mycelium. *Fusarium konzum* is morphologically most similar to *F. anthophilum* due to its pyriform microconidia, but the longer monophialides and more swollen polyphialides found in *F. konzum* distinguish them (Zeller *et al.*, 2003). In terms of phylogeny, *F. konzum* is closely related to *F. sterilihyphosum* (Fig. 1) that produces coiled sterile hyphae. *Fusarium konzum* produces fumonisins, fusaproliferin and beauvericin (Leslie *et al.*, 2004a), while one strain has also been reported to produce gibberellin (Malonek *et al.*, 2005).

Fusarium bactridioides is the only species in the so-called "American Clade" that has been reported to produce chlamydospores (O'Donnell *et al.*, 1998). However, this trait needs to be verified, as the ex-holotype of this species does not produce chlamydospores (Nirenberg and O'Donnell, 1998). *Fusarium bactridioides* was isolated as a pathogen of the rust fungus *Cronartium conigenum* (Wollenweber, 1934). Due to its stout, slightly curved and thick-walled macroconidia, it was previously classified in Section *Discolor* by Wollenweber (1934) and Gerlach and Nirenberg (1982). Very little is known regarding its biology, pathology or mycotoxicology.

The "Asian Clade"

With its ten known phylogenetic lineages, the so-called "Asian Clade" is the smallest of the three GFC clades (Fig. 1). Only three of these lineages await characterisation and seven have been formally described using the MSR (Nelson *et al.*, 1983; Rheeder *et al.*, 1996; Aoki *et al.*, 2001; Britz *et al.*, 2002b; Leslie *et al.*, 2005a). Three of these morphospecies are known to produce sexual stages and are recognisable using the BSR. *Gibberella sacchari*, *G. fujikuroi* and *G. intermedia* are the teleomorphs of *F. sacchari*, *F. fujikuroi* and *F. proliferatum*, which correspond to MP B, C and D, respectively (Kuhlman, 1982; Leslie, 1991, 1995; Samuels *et al.*, 2001; Leslie *et al.*, 2005a).

In terms of morphology and biology *F. fujikuroi* and *F. proliferatum* are very similar (Leslie *et al.*, 2007), and the PSR seems to be the least time-consuming and most effective

method of separating them. Both species produce their microconidia from false heads and in chains from poly- and monophialides (Gerlach and Nirenberg, 1982). However, they are associated with different hosts (Fig. 3) as *F. fujikuroi* causes bakane disease of rice and *F. proliferatum* is associated with a vast number of agricultural hosts (see Leslie and Summerell, 2006). They can also be distinguished using the BSR (Kuhlman, 1982; Leslie, 1991, 1995; Samuels *et al.*, 2001), although the biological separation between the species is apparently incomplete as some isolates of both species are inter-fertile and able to produce hybrid progeny (Desjardins *et al.*, 1997; Leslie *et al.*, 2004b, 2007). The two species also differ mycotoxicologically, with *F. proliferatum* known to produce higher levels of fumonisins than *F. fujikuroi* (Rheeder *et al.*, 2002; Fandohan *et al.*, 2003; Desjardins, 2006). Certain strains of *F. proliferatum* are able to produce even higher levels of fumonisins than some strains of *F. verticillioides* (Rheeder *et al.*, 2002; Leslie *et al.*, 2004a). This is a great health concern as this cosmopolitan fungus is associated with many agricultural crops. *Fusarium proliferatum* also produces enniatins, fusaproliferin and fusarins (Fig. 2; Marasas *et al.*, 1986; Desjardins, 2006; Moretti *et al.*, 2007). Except for a single strain of *F. konzum*, *F. fujikuroi* is the only species in the GFC recognised to produce gibberellins that are plant hormones responsible for abnormal development of rice shoots (Sun and Snyder, 1981; Malonek *et al.*, 2005). Both *F. proliferatum* and *F. fujikuroi* are reported as beauvericin, fusaric acid and moniliformin producers (Marasas *et al.*, 1986; Bacon *et al.*, 1996; Logrieco *et al.*, 1998; Desjardins, 2006; Moretti *et al.*, 2007).

Based on phylogeny (Fig. 1), the four species *F. fujikuroi*, *F. proliferatum*, *F. globosum* and *F. fractiflexum* form a well-supported group. Within this group, *F. proliferatum* is most closely related to *F. globosum* that occurs in maize, while *F. fractiflexum* that is associated with a leaf disease of *Cymbidium* species in Japan (Aoki *et al.*, 2001) represents the basal taxon. Morphologically, *F. globosum* is quite similar to *F. proliferatum* and *F. fujikuroi*, but globose microconidia singly or in clusters distinguishes it from them (Rheeder *et al.*, 1996). *Fusarium fractiflexum* also shares morphological characters with *F. proliferatum* and *F. fujikuroi*, but the fact that it produces microconidia in geniculate chains on the aerial mycelium distinguishes it from them (Leslie and Summerell, 2006). Although the latter trait resembles *F. lactis* morphology, the two species can be differentiated based on colony colour, conidial length and host preference (Aoki *et al.*, 2001). From a mycotoxicological point of view, *F. globosum* also resembles *F. proliferatum* and *F. fujikuroi*, although it does not produce fusaric acid and

moniliformin. There is no information available regarding the mycotoxicology of *F. fractiflexum*.

The "Asian" *F. subglutinans sensu lato* species *F. sacchari*, *F. concentricum* and *F. mangiferae* are all associated with different plant hosts. *Fusarium sacchari* is well known as a causal agent of the pokkah boeng disease of sugar cane (Gerlach and Nirenberg, 1982). *Fusarium concentricum* has been associated with banana in Guatemala and Costa Rica and insects in South Korea (Nirenberg and O'Donnell, 1998). *Fusarium mangiferae* is a causal agent of mango inflorescence malformation (Freeman *et al.*, 1999; Steenkamp *et al.*, 2000a; Britz *et al.*, 2002b). Based on morphology, *F. concentricum* is most similar to the "American Clade" species *F. circinatum* and *F. guttiforme*, but *F. circinatum* produces coiled hyphae, while *F. guttiforme* produces greater numbers of polyphialides that are more strongly branched (Nirenberg and O'Donnell, 1998). Morphologically, *F. sacchari*, *F. mangiferae* and *F. subglutinans sensu stricto* are extremely difficult to distinguish from each other and their unambiguous separation requires information on host associations and application of the BSR and PSR (Kuhlman, 1982; Leslie, 1995; Steenkamp *et al.*, 2000a; Britz *et al.*, 2002b). Very little is known concerning the mycotoxicology of *F. sacchari* and *F. mangiferae* because they were all treated as *F. subglutinans* for many years. *Fusarium concentricum* has been reported to produce beauvericin, enniatins, fusaproliferin and moniliformin (Fotso *et al.*, 2002; Desjardins 2006; Moretti *et al.*, 2007).

Evolution of the GFC

According to the biogeographic hypothesis of O'Donnell *et al.* (1998), all of the species and lineages in the so-called "African", "American" and "Asian" clades should be associated with hosts that have evolved or that have centres of origin on the African, American and Asian continents, respectively. For the most part, this seems to be true as specific *Fusarium* species appear to have emerged with their host plants on the respective continents (Fig. 3). However, there are a number of exceptions where the species composition of the clades does not fit this hypothesis (Fig. 3). For example, *F. verticillioides* is a member of the "African Clade", although its maize and teosinte hosts are now widely accepted to have a Mexican origin (e.g. Sauer, 1993). Other "African Clade" species with non-African origins include *F. pseudoanthophilum*

and *F. denticulatum* (Sauer, 1993; Nirenberg *et al.*, 1998; Nirenberg and O'Donnell, 1998). In the "American Clade", species such as *F. succisae*, *F. bulbicola* and *F. sterilihyphosum* are associated with non-American host plants (Gerlach and Nirenberg, 1982; Sauer, 1993; Adams, 1995; Vorster and Spreeth, 1996; Nirenberg and O'Donnell, 1998; Britz *et al.*, 2002b). In the so-called "Asian Clade", the most notable exception is *F. globosum* that was originally isolated in South Africa (Rheeder *et al.*, 1996) and subsequently reported to be associated with wheat in Japan (Aoki and Nirenberg, 1999). These exceptions were attributed to anthropological dispersal of economically important plants and host jumps by the fungi (O'Donnell *et al.*, 1998). For example, it was postulated that following the introduction of American hosts such as *Zea mays* and *Ipomoea batatas* into Africa, species such as *F. verticillioides* and *F. pseudoanthophilum* established their associations with maize and *F. denticulatum* with sweet potato. Trans-oceanic dispersal without the influence of humans was also invoked to explain the occurrence of an "American Clade" fungus (*Fusarium* sp. NRRL 25807) in Australian forest soil (O'Donnell *et al.*, 1998). Therefore, despite the fact that the general species compositions of the GFC clades apparently support the vicariant biogeographic hypothesis, much additional research, especially in indigenous regions where human interference in biodiversity is limited, is required to completely explain the existence of these clades.

At first glance, the morphological characters applied in the taxonomy of the GFC appear not to be phylogentically informative (Fig. 2), because shared traits among different species do not seem to reflect ancestry (e.g. Steenkamp *et al.*, 1999, 2000a). This is particularly true for some species ability to produce sterile coiled hyphae, which is the case for the "American Clade" species *F. circinatum* and *F. sterilihyphosum* and the "African Clade" species *F. pseudocircinatum* (Fig. 2). The production of microconidia with distinctive shapes and from specific forms of conidiophores also does not reflect the evolutionary history of this complex (Fig. 2). However, the aerial arrangement of microconidia seems to be an informative character, as all the examined "American Clade" species produce their microconidia in false heads only and never in chains, while most of the "Asian Clade" species produce their microconidia in false heads and long chains. Among the "African Clade" species, all bear their microconidia in false heads, although the majority also produce mostly short microconidial chains (Fig. 2). Also, the "African Clade" includes all but one of the chlamydospore-formers (Fig. 2). If the "African Clade" indeed represents the ancestral GFC clade because of it is "phylogenetically diverse and

speciose" nature (O'Donnell *et al.*, 1998), the first members of the complex probably represented fungi able to produce chlamydospores and to form their microconidia in false heads and chains. Later, the ability to produce chlamydospores appears to have been lost in most lineages (Fig. 2). The ability to produce microconidia in chains also appears to have been lost early during the evolution of the "American Clade" as this trait has not been detected among any of the examined species that all primarily display a *F. subglutinans*-like morphology (Fig. 2).

Analysis of the distribution of mycotoxin production capabilities relative to the phylogeny of the GFC suggests that the ability to produce a specific mycotoxin is not evolutionary informative (Fig. 2). However, it does appear that the "African Clade" includes the largest number of mycotoxigenic species and that they produce the greatest diversity of secondary metabolites. Although these trends may be due to taxonomic sampling bias, they would also be consistent with the idea of an ancestral "African Clade" for the GFC. Nevertheless, very little information regarding the evolution of the genes and pathways involved in mycotoxin production is available for the GFC. So far only the fumonisin and gibberellin biosynthetic pathways, both encoded by large gene clusters (reviewed by Proctor *et al.*, 2004; Malonek *et al.*, 2005) have been analysed from an evolutionary point of view. Proctor *et al.* (2004) showed that the sporadic distribution of fumonisin biosynthetic genes across the GFC potentially explains the discontinuous distribution of the production of this mycotoxin (Fig. 2) by the members of the complex. With respect to gibberellins, non-production by specific GFC species was largely attributed to non-functional genes (Malonek and Tudzynski, 2003), because most of the examined GFC species have at least one gene or the entire gibberellin biosynthetic cluster (Malonek *et al.*, 2005). As these gene clusters act as "selfish" genetic elements, horizontal gene transfer is thought to play an important role in their evolution and distribution (e.g. Rosewich and Kistler, 2000; Walton, 2000). Indeed, it has been suggested that the evolution of the fumonisin and gibberellin production capabilities in the GFC is determined by the effects of both horizontal gene transfer and/or differential inheritance from a common ancestor (Proctor *et al.*, 2004; Seifert and Lévesque, 2004; Malonek *et al.*, 2005). Whether this would also be the case for the various other mycotoxins produced by species in the GFC, remains to be determined.

Future perspectives

The concurrent application of MSR, BSR and PSR has contributed significantly to resolving taxonomic confusion in the GFC and it has facilitated the recognition and description of all the species in this complex. Generally, species definitions based on such an integrative approach are extremely robust and have stood the test of time (Leslie and Summerell, 2006). In our opinion, the only noteworthy limitation associated with the current GFC taxonomy is the description of some species (e.g. *F. begoniae*, *F. bactridioides*, *F. phyllophilum*, *F. pseudonygamai*, *F. ramigenum*, *F. brevicatenulatum*, *F. pseudoanthophilum* and *F. fractiflexum*) (Nirenberg and O'Donnell, 1998; Nirenberg *et al.*, 1998; Aoki *et al.*, 2001) based on a very small number of strains. Although these species probably represent valid taxa, their polyphasic re-evaluation and definition using populations of isolates that more accurately represent them in nature (Leslie *et al.*, 2001) will substantially improve our perception of their biological relevance. This is also true for the various phylogenetic species or lineages of the GFC (O'Donnell *et al.*, 1998; 2000b) that still await formal description.

Full appreciation of the evolution of important GFC characters such as mycotoxin production and phytopathogenicity will depend, to a large extent, on our ability to resolve true phylogenetic history of this complex and its species. This in turn would be dependent on the reconstruction of a well-resolved phylogeny of the GFC, which will require inclusion of additional and previously unexploited genomic regions for phylogenetic analyses and suitably representative *Fusarium* isolates. Even though many species are already known in this complex, its current composition is strongly biased towards species that are of agricultural, medical or veterinary importance. Hardly, any information is available regarding the diversity of GFC species in unique niches and indigenous ecosystems. The exploration of such areas will certainly reveal numerous additional members of this complex and their inclusion in analyses will substantially enhance the phylogenetic resolution of the GFC. Combined with determining the possible ages of the GFC clades, such studies will allow modification or unequivocal acceptance of the vicariant biogeographic hypothesis (O'Donnell *et al.*, 1998) and help to clarify the role of the host in the evolution of these fungi. As the exploitation of full genome sequences becomes more feasible, comparative and phylogenomic approaches will facilitate elucidation of the

evolution of various morphological, reproductive and other biological properties such as mycotoxin biosynthesis in the *Fusarium* species of the GFC.

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Fig. 1. A maximum likelihood phylogeny of *Fusarium* species in the GFC (*Gibberella fujikuroi* complex) based on combined sequence information for the genes encoding translation elongation factor 1 alpha and beta-tubulin. All the members of the three well-established clades (O'Donnell *et al.*, 1998, 2000b) are included, with the exception of *F. andiyazi* as its phylogenetic affinity remains to be determined. Thick branches are supported by bootstrap values >70% as previously reported (O'Donnell *et al.*, 1998, 2000b; Geiser *et al.*, 2005). MP A–J indicates the mating populations in the complex and the tree is rooted with *F. oxysporum* and *F. inflexum*.

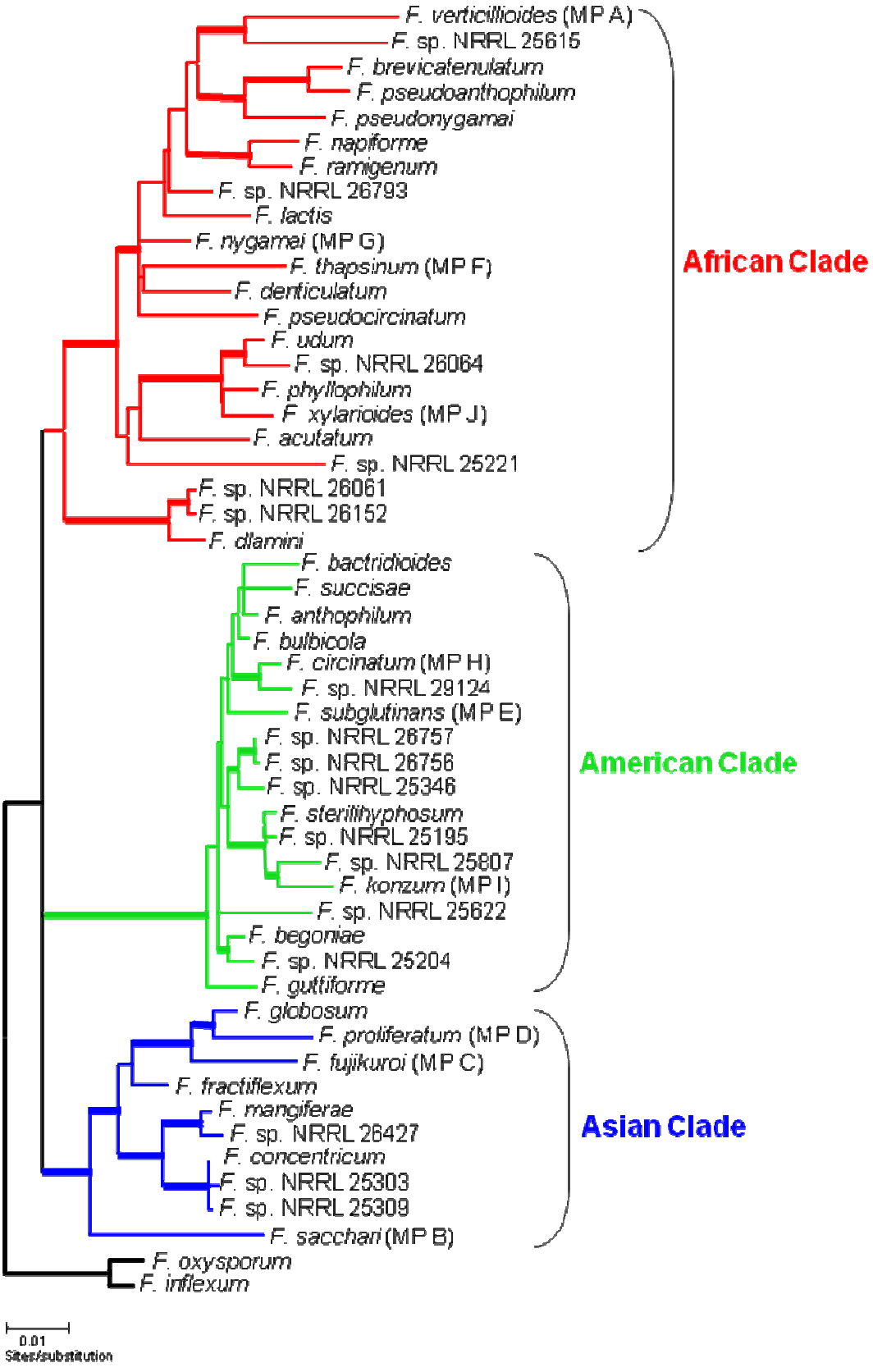

F. verticillioides (MP A)
F. sp. NRRL 25615
F. brevicatenulatum
F. pseudoanthophilum
F. pseudonygamai
F. napiforme
F. ramigenum
F. sp. NRRL 26793
F. lactis
F. nygamai (MP G)
F. thapsinum (MP F)
F. denticulatum
F. pseudocircinatum
F. udum
F. sp. NRRL 26064
F. phyllophilum
F. xylarioides (MP J)
F. acutatum
F. sp. NRRL 25221
F. sp. NRRL 26061
F. sp. NRRL 26152
F. dlamini
African Clade
F. bactridioides
F. succisae
F. anthophilum
F. bulbicola
F. circinatum (MP H)
F. sp. NRRL 29124
F. subglutinans (MP E)
F. sp. NRRL 26757
F. sp. NRRL 26756
F. sp. NRRL 25346
F. sterilihyphosum
F. sp. NRRL 25195
F. sp. NRRL 25807
F. konzum (MP I)
F. sp. NRRL 25622
F. begoniae
F. sp. NRRL 25204
F. guttiforme
American Clade
F. globosum
F. proliferatum (MP D)
F. fujikuroi (MP C)
F. fractiflexum
F. mangiferae
F. sp. NRRL 26427
F. concentricum
F. sp. NRRL 25303
F. sp. NRRL 25309
F. sacchari (MP B)
Asian Clade
F. oxysporum
F. inflexum
0.01
Sites/substitution

Fig. 2. Comparison of the morphological traits and mycotoxicological properties of *Fusarium* species in the GFC with respect to their phylogeny as indicated in Fig. 1. Morphological characters that define each species were reported by Booth (1971), Gerlach and Nirenberg (1982), Nelson *et al.* (1983), Nirenberg and O'Donnell (1998), Aoki *et al.* (2001) and Leslie and Summerell (2006). The presence of macroconidia typical of the GFC (slender, thin walled and almost straight with parallel dorsal and ventral surfaces) are indicated with "+" (Leslie and Summerell, 2006). Taxa that have not yet been formally described are indicated in parenthesis with "ND". For the major secondary metabolites, "++" represents the production of a significant level of the specific compound, "+" and "-" indicate trace and not detected amounts, respectively, while "?" signifies that the trait has not been examined in a specific species. References for the production of a specific mycotoxin by a certain species are as follows: 1. Marasas *et al.* (1984); 2. Marasas *et al.* (1986); 3. Bacon *et al.* (1996); 4. Shephard *et al.* (1999); 5. Leslie *et al.* (2004a); 6. Desjardins (2006); 7. Moretti *et al.* (2007); 8. Fotso *et al.* (2002); 9. Leslie *et al.* (2005b); 10. Marasas *et al.* (1991); 11. Nelson *et al.* (1992); 12. Thiel *et al.* (1991); 13. Logrieco *et al.* (1998); 14. Leslie and Summerell (2006); 15. Leslie *et al.* (1996); 16. Booth (1971); 17. Sydenham *et al.* (1997); 18. Desjardins *et al.* (2000a).

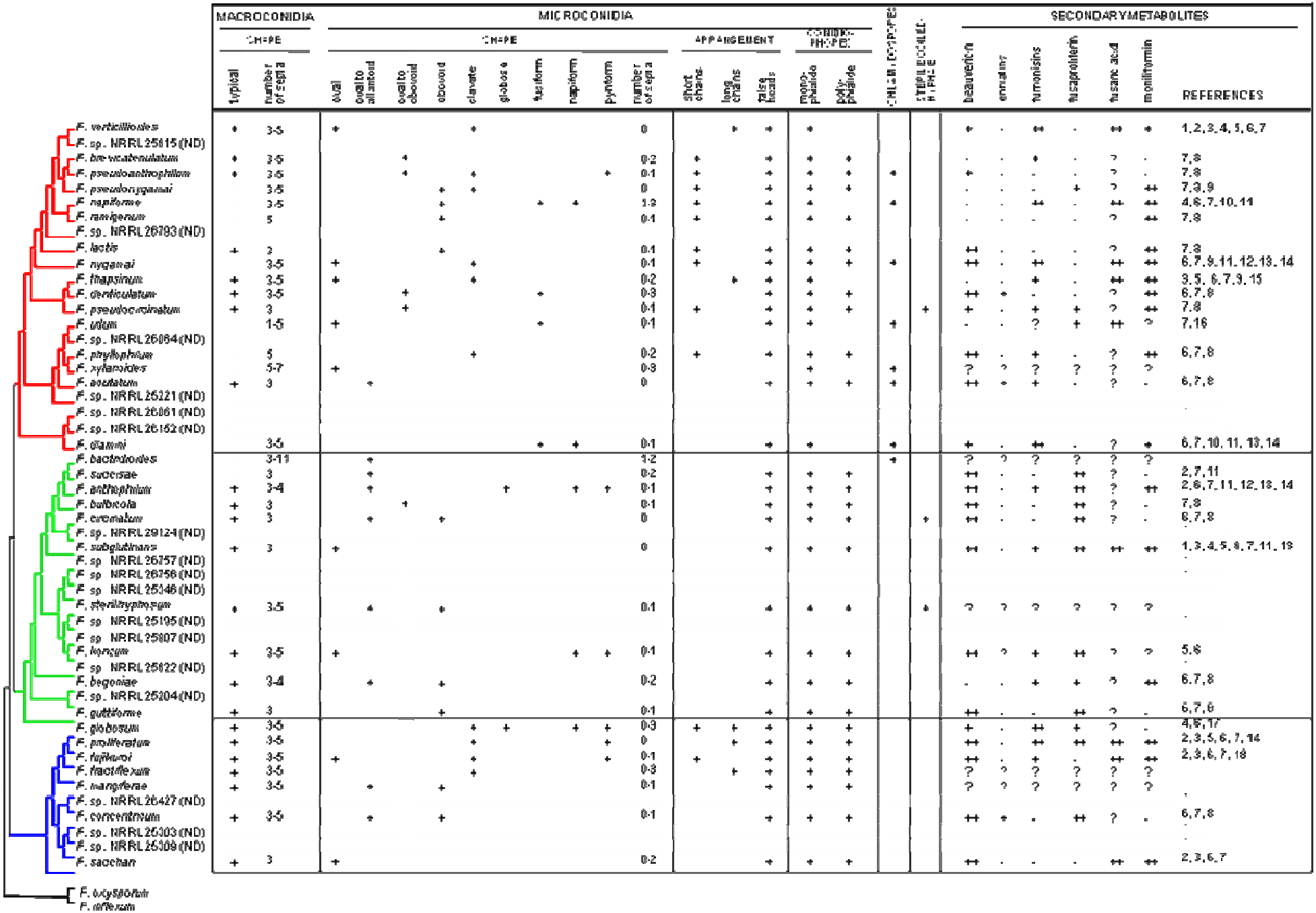
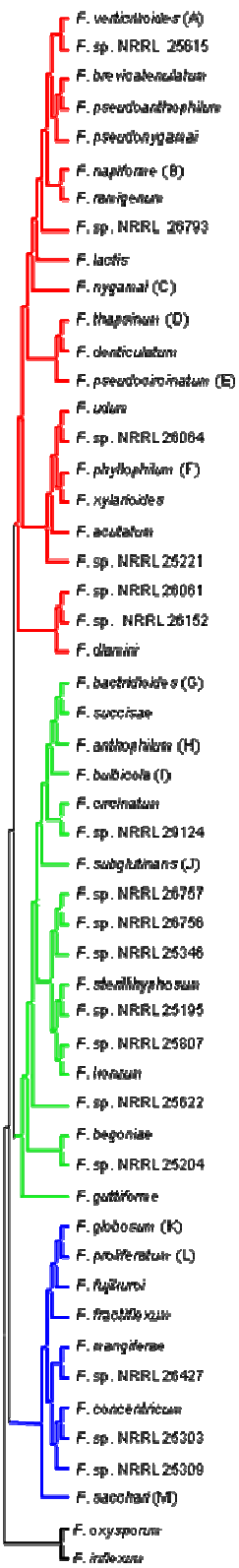
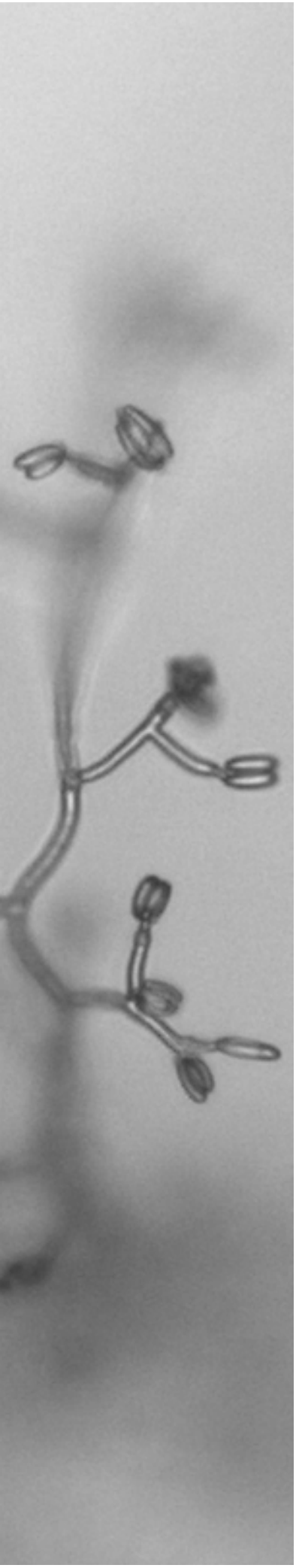

MACROCONIDIA
SHAPE
typical
number of septa
MICROCONIDIA
SHAPE
oval
oval to allantoid
oval to obovoid
obovoid
clavate
globose
fusiform
napiform
pyriform
number of septa
ARRANGEMENT
short chains
long chains
false heads
CONIDIOPHORE
mono-phialide
poly-phialide
CHLAMYDOSPORES
STERILE ECHINULATE HYPHAE
SECONDARY METABOLITES
beauvericin
enniatins
fumonisins
fusaproliferin
fusaric acid
moniliformin
REFERENCES
F. verticillioides
F. sp. NRRL 25615 (ND)
F. brevicatenulatum
F. pseudoanthophilum
F. pseudonygamai
F. napiforme
F. ramigenum
F. sp. NRRL 26793 (ND)
F. lactis
F. nygamai
F. thapsinum
F. denticulatum
F. pseudocircinatum
F. udum
F. sp. NRRL 26064 (ND)
F. phyllophilum
F. xylarioides
F. acutatum
F. sp. NRRL 25221 (ND)
F. sp. NRRL 26061 (ND)
F. sp. NRRL 26152 (ND)
F. dlamini
F. bactrinodes
F. succisae
F. anthophilum
F. bulbicola
F. croceum
F. sp. NRRL 29124 (ND)
F. subglutinans
F. sp. NRRL 26757 (ND)
F. sp. NRRL 26756 (ND)
F. sp. NRRL 25146 (ND)
F. sterilihyphosum
F. sp. NRRL 25195 (ND)
F. sp. NRRL 25807 (ND)
F. lyarum
F. sp. NRRL 25622 (ND)
F. begoniae
F. sp. NRRL 25204 (ND)
F. guttiforme
F. globosum
F. proliferatum
F. fujikuroi
F. fractiflexum
F. nanlingense
F. sp. NRRL 26427 (ND)
F. concentricum
F. sp. NRRL 25303 (ND)
F. sp. NRRL 25309 (ND)
F. sacchari
F. oxysporum
F. redolens

Fig. 3. Comparison of the host associations of *Fusarium* species in the GFC with respect to their phylogeny as indicated in Fig. 1. The geographic region where the specific plants presumably evolved or where they have been domesticated is indicated according to Sauer (1993) or as follows: 1. Mohamed *et al.* (2001); 2. Kislev *et al.* (2006); 3. van der Maesen (1990); 4. Smith and van Wyk (1991); 5. Adams (1995); 6. Vorster and Spreeth (1996); 7. Holm *et al.* (1977); 8. Du Puy and Cribb (1988). Where specific fungal strains were isolated from substrates other than plant tissue, the geographic region in which the samples were collected is indicated. For specific species/lineages that are associated with more than one host or substrate, alternatives hosts/substrates are indicted in parentheses with A–M as follows: A: Isolated from teosinte (*Zea* sp., Poaceae, Central America) (Desjardins *et al.*, 2000b), native prairie grasses in USA (Leslie *et al.*, 2004a), millet (*Pennisetum typhoides*, Poaceae, Northern Africa) and sorghum (*Sorghum bicolor*, Poaceae, Northern Africa) (Leslie *et al*., 2005b); B: Found on millet by Marasas *et al.* (1987), as well as in Australian soil by Burgess and Summerell (1992) and Sangalang *et al.* (1995); C: Also described from bean roots (*Phaseolus vulgaris*, Fabaceae, Central and South America) and soils from Australia, Thailand and Puerto Rico (Burgess and Trimboli, 1986); D: Also associated with banana (*Musa sapientum*, Musaceae, South Asia), maize (*Zea mays*, Poaceae, Central America), peanuts (*Arachis hypogaea*, Fabaceae, South America) (Klittich *et al.*, 1997) and native grasses in USA prairie (Leslie *et al*., 2004a); E: Reported also from *Pinus kesiya* (Pinaceae, Northeast Asia), textile and dead leaves (Nirenberg and O'Donnell, 1998); F: Also described from *Dracaena* and *Sansevieria* spp. (Dracaenaceae, Africa and Asia [Mwachala and Mbugua, 2007]); G: Discovered parasitizing *Cronartium conigenum*, a cone blister rust of *Pinus leiophylla* (Pinaceae, North and Central America) (Wollenweber, 1934); H: Also associated with *Lolium* spp. (Poaceae, Europe, Asia, Africa) (Engels and Kramer, 1996); I: Also described from *Vallota* and *Haemanthus* spp. (Nirenberg and O'Donnell, 1998) both belonging to South African Amaryllidaceae (Vorster and Spreeth, 1996); J: Also found on banana (Jiménez *et al.*, 1993), wild rice (*Zizania palustris*, Poaceae, North America) (Nyvall *et al.*, 1999), millet (Onyike *et al.*, 1991), sorghum (Onyike *et al.*, 1992), teosinte (Desjardins *et al.*, 2000b) and native prairie grasses (Leslie *et al.*, 2004a); K: Also described from wheat (*Triticum* sp., Poaceae, Middle East) (Aoki and Nirenberg, 1999); L: Colonises banana (Jiménez *et al*., 1993), sorghum (Leslie *et al*., 1990), maize (Logrieco *et al.*, 1995), rice (Desjardins *et al.*, 2000a), mango (*Mangifera indica*, Anacardiaceae, South Asia) (Marasas *et al.*, 2006) and native grasses in USA (Leslie *et al.*, 2004a); M: Also isolated from sorghum (Leslie *et al.*, 2005b).



	PLANT HOST			OTHER SUBSTRATE	REFERENCES
	LATIN NAME	FAMILY	GEOGRAPHICAL ORIGIN		
F. verticillioides (A)	*Zea mays*	Poaceae	Central America	soil - Israel	Gerlach and Nirenberg (1982); Joffe and Palti (1977)
F. sp. NRRL 25615	*Oryza sativa*	Poaceae	Southeast Asia		O'Donnell *et al.* (2000b)
F. brevicatenulatum	*Striga asiatica*	Scrophulariaceae	Africa		Nirenberg *et al.* (1998)
F. pseudoanthophilum	*Zea mays*	Poaceae	Central America		Nirenberg *et al.* (1998)
F. pseudobrygamai	*Pennisetum typhoides*	Poaceae	Northern Africa		Nirenberg and O'Donnell (1998)
F. napiforme (B)	*Sorghum bicolor*	Poaceae	Northern Africa	soil - Africa	Marasas *et al.* (1987); Jeschke *et al.* (1990)
F. ramigenum	*Ficus carica*	Moraceae	Middle East		Nirenberg and O'Donnell (1998)
F. sp. NRRL 26793	*Striga hermonthica*	Scrophulariaceae	Africa		O'Donnell *et al.* (1998)
F. lactis	*Ficus carica*	Moraceae	Middle East		Nirenberg and O'Donnell (1998)
F. nygamai (C)	*Sorghum bicolor*	Poaceae	Northern Africa	soil - South Africa	Burgess and Trimboli (1986)
F. thapsinum (D)	*Sorghum bicolor*	Poaceae	Northern Africa		Klittich *et al.* (1997)
F. denticulatum	*Ipomoea batatas*	Convolvulaceae	Central America		Nirenberg and O'Donnell (1998)
F. pseudocircinatum (E)	*Solanum* species	Solanaceae	South America	*Heteropsylla incisa* (Homoptera: Psyllidae)	Nirenberg and O'Donnell (1998)
F. udum	*Cajanus cajan*	Fabaceae	Asia or Eastern Africa		Booth (1971)
F. sp. NRRL 26064	*Sorghum bicolor*	Poaceae	Northern Africa		O'Donnell *et al.* (2000b)
F. phyllophilum (F)	*Gasteria* species	Asphodelaceae	South Africa		Nirenberg and O'Donnell (1998)
F. xylarioides	*Coffea* species	Rubiaceae	East Africa		Booth (1971); Geiser *et al.* (2005)
F. acutatum	*Cajanus cajan*	Fabaceae	Asia or Eastern Africa	wheat aphids	Nirenberg and O'Donnell (1998)
F. sp. NRRL 25221	*Zea mays*	Poaceae	Central America		O'Donnell *et al.* (1998)
F. sp. NRRL 26061	*Striga hermonthica*	Scrophulariaceae	Africa		O'Donnell *et al.* (2000b)
F. sp. NRRL 26152	*Striga hermonthica*	Scrophulariaceae	Africa		O'Donnell *et al.* (2000b)
F. dlamini	n/a	n/a	n/a	soil - South Africa	Marasas *et al.* (1985)
F. bactridioides (G)	n/a	n/a	n/a		Wollenweber (1934)
F. succisae	*Succisa pratensis*	Dipsacaceae	Europe, Africa		Gerlach and Nirenberg (1982)
F. anthophilum (H)	*Zizania palustris*	Poaceae	North America	soil - Australia	Nyvall *et al.* (1999); Sangalang *et al.* (1995)
F. bulbicola (I)	*Nerine bowdenii*	Amaryllidaceae	South Africa		Nirenberg and O'Donnell (1998)
F. circinatum	*Pinus* species	Pinaceae	Northern Hemisphere		Nirenberg and O'Donnell (1998)
F. sp. NRRL 29124	*Bidens pilosa*	Asteraceae	South America		O'Donnell *et al.* (2000b)
F. subglutinans (J)	*Zea mays*	Poaceae	Central America		Nelson *et al.* (1983)
F. sp. NRRL 26757	ornamental reed	n/a	South Africa		O'Donnell *et al.* (2000b)
F. sp. NRRL 26756	ornamental grass	n/a	South Africa		O'Donnell *et al.* (2000b)
F. sp. NRRL 25348	*Ipomoea batatas*	Convolvulaceae	Central America		O'Donnell *et al.* (1998)
F. stevilliyphosum	*Mangifera indica*	Anacardiaceae	Southeast Asia		Britz *et al.* (2002b)
F. sp. NRRL 25195	n/a	n/a	n/a		O'Donnell *et al.* (1998)
F. sp. NRRL 25807	n/a	n/a	n/a	soil - Australia	O'Donnell *et al.* (1998)
F. lunatum	*Andropogon gerardii*	Poaceae	North America		Zeller *et al.* (2003)
F. sp. NRRL 25622	*Zea mays*	Poaceae	Central America		O'Donnell *et al.* (2000b)
F. begoniae	*Begonia* hybrid	Begoniaceae	South America		Nirenberg and O'Donnell (1998)
F. sp. NRRL 25204	palm	n/a	n/a		O'Donnell *et al.* (1998)
F. guttiforme	*Ananas comosus*	Bromeliaceae	South America		Nirenberg and O'Donnell (1998)
F. globosum (K)	*Zea mays*	Poaceae	Central America		Rheeder *et al.* (1996)
F. proliferatum (L)	*Asparagus officinalis*	Asparagaceae	Europe, Asia, Africa		Elmer (2001)
F. fujikuroi	*Oryza sativa*	Poaceae	Southern Asia		Gerlach and Nirenberg (1982)
F. fractiflexum	*Cymbidium* species	Orchidaceae	Asia		Aoki *et al.* (2001)
F. mangiferae	*Mangifera indica*	Anacardiaceae	Southeast Asia		Britz *et al.* (2002b)
F. sp. NRRL 26427	n/a	n/a	n/a	soil - Papua New Guinea	O'Donnell *et al.* (2000b)
F. concentricum	*Musa sapientum*	Musaceae	South Asia	aphid	Nirenberg and O'Donnell (1998)
F. sp. NRRL 25303	*Oryza sativa*	Poaceae	Southern Asia		O'Donnell *et al.* (1998)
F. sp. NRRL 25309	*Triticum* species	Poaceae	Middle East		O'Donnell *et al.* (1998)
F. sacchari (M)	*Saccharum officinarum*	Poaceae	South Asia		Leslie *et al.* (2006a)
F. oxysporum					
F. redolens					



Chapter 2: Literature Review

Floral malformations with special reference to those occurring on mango and waterberry

Abstract

Malformation of the plant's floral parts can be associated with genetic predisposition, environmental factors, pests and the presence of various microorganisms. While floral malformation is well known on plants, knowledge as to its cause is often limited. This review seeks to summarise such knowledge, by firstly treating the diversity of insects, mites, nematodes, viruses, bacteria, fungi and oomycetes associated with this symptom. Each of these biotic groups is defined and specific examples of the inflorescence abnormality that they induce are provided. Following this general analysis, contemporary knowledge on *Mangifera indica* (mango) malformation, one of the best studied examples of floral malformation is interrogated, emphasising the diversity of *Fusarium* species and lineages associated with this disease. As mango malformation is strikingly similar to floral malformation on *Syzygium cordatum* (waterberry), a widely distributed Southern African indigenous tree, these two diseases are critically compared and their associations with *Fusarium* species and *Eriophyes* mite species are discussed.

Introduction

Human kind is almost entirely dependent on flowering plants for food, wood, fibre and various important medicinal, pharmaceutical and chemical compounds. Angiosperms are the most widespread group of terrestrial plants, with more than 250 000 described species divided into 17 000 genera (Groombridge, 1992). The floral parts that characterise angiosperms represent their reproductive organs, responsible for the development of fruit and formation of seeds (Fahn, 1990). For this reason, floral diseases or abnormalities strongly influence subsequent plant generations and their dispersal, survival and diversity. In the case of agriculturally important plants, floral abnormalities or diseases can also lead to harvest and economic losses, and in some cases, food insecurity.

Floral malformations and diseases can be due to heritable disorders, abiotic agents and/or biotic agents such as pests and pathogens (Meyer, 1966; Williams, 1994). For the purposes of this review, the focus is on the diversity of biotic agents associated with floral malformations. One of the best known forms of floral malformation is on mango (*Mangifera indica*, Anacardiaceae), an economically important crop. The disease is associated with and/or caused by various fungal species in the ascomycetous genus *Fusarium*. The malformation symptoms on mango are remarkably similar to those of the inflorescences of an indigenous Southern African tree, waterberry (*Syzygium cordatum*, Myrtaceae). This review is concluded by drawing an analogy between mango and waterberry malformations, by focusing particularly on their association with various *Fusarium* species.

Floral malformations

All flower organs are homologous and each flower develops from a mass of undifferentiated cells to eventually generate a structure in which each organ occupies a distinct position (Meyerowitz *et al*., 1989). Therefore, flower abnormalities usually involve the unusual development of bracts, sepals, petals, carpels, stamens and/or pistils in parts of the blossom or plant where they normally do not occur (Meyer, 1966). These malformations are referred to as phyllody (development of leaves within inflorescence), sepalody (metamorphosis of various floral parts into sepals), petalody (abnormal development of floral organs into petals),

carpellody (distortion of the ovule-bearing structure), staminody (metamorphosis of various floral organs into stamens) and their characteristics have been extensively reviewed by Meyer (1966). Swelling, thickening or distortions of floral parts can also occur and are usually the result of hypertrophy (increased cell size) or hyperplasia (increased cell number) (Preece and Hick, 1994). Virescence or the loss of normal flower pigments and development of green flowers is another common form of inflorescence abnormality (Meyer, 1966). Most of these abnormal inflorescence developments can lead to plant sterility (Meyer, 1966).

Floral malformations have been associated with a range of abiotic agents (e.g. temperature, humidity, soil moisture, light, air pollution and trace element availability) (Agrios, 2005). Environmental conditions can influence organ differentiation, production of stamens and pistils, sex reversal and fertility in flowers (Meyer, 1966). Deficiency in trace elements such as copper can cause reductions in head size and kernel quantity in wheat, while air pollutants such as ethylene may reduce the number of blossoms (Agrios, 2005). In addition to bringing about such physiological changes in the plant, climatic phenomena such as global warming, elevated carbon dioxide and ozone concentrations also potentially increase stress on the plant, thus enhancing plant susceptibility to infection by pathogens (Pritchard and Amthor, 2005). Therefore, floral malformations and other physiological disorders of plants, resulting from biotic factors can also lead to changes in the organisms associated with them. In this regard, global climate change caused by anthropogenic factors is likely to have dramatic effects on the ecology and evolution of plant hosts and their pathogens/pests (Burdon and Thrall, 2008).

Diverse organisms are associated with floral malformations

Most floral malformations are caused by biotic agents and in the following section suitable examples are discussed. These include microorganisms such as viruses, bacteria, fungi and oomycetes and various animals such as insects, mites and nematodes, among which the insects are by far the most abundant malformation inducing agents.

Insects

Insects (Class: Insecta) are one of the most diverse group of organisms on Earth and it has been suggested that if only species richness is considered, life on Earth would appear to

consist almost entirely of insects and microorganisms (Groombridge, 1992). There are over one million described species (Gullan and Cranston, 2004) with an estimated 13 000 known to have cecidogenous (gall inducing) effects on plants (Dreger-Jauffert and Shorthouse, 1992). Although, the leaves are most often affected, these insects can also infest flowers and other plant organs (Dreger-Jauffert and Shorthouse, 1992; Gullan and Cranston, 2004). Cecidogenous insects initiate gall formation by providing a stimulus to the plant and controlling the growth and differentiation of the structures by manipulating plant physiology to their advantage (Dreger-Jauffert and Shorthouse, 1992; Shorthouse *et al*., 2005). Gall inducing insects are classified into six orders (Thysanoptera, Hemiptera, Hymenoptera, Lepidoptera, Coleoptera and Diptera), many of which are host and organ specific (Dreger-Jauffert and Shorthouse, 1992; Gullan and Cranston, 2004).

Among the cecidogenous insects, those in the order Hemiptera (e.g. bugs, psyllids, aphids and scales) show the greatest diversity (Dreger-Jauffert and Shorthouse, 1992). An example of a hemipteran insect causing inflorescence malformation is *Livia juncorum* (Psyllidae), which produces virescence and witches' broom on *Juncus articulatus* (Juncaceae) (Meyer, 1987; Dreger-Jauffert and Shorthouse, 1992). Other examples from this order are *Copium* and *Paracopium* species (Tingidae) that induce hypertrophy of the corolla and inhibit development of stamens and pistils on *Teucrium* (Lamiaceae) and *Clerodendrum* (Verbenaceae) species, respectively (Meyer, 1987). Insect species in the order Thysanoptera are known to cause petal scarring and distortions (Powell and Lindquist, 1997), and this group includes the flower-feeding thrips in the genus *Haplothrips* (Mound, 1994). An example of a lepidopteran insect associated with inflorescence abnormalities is *Coleophora icterella* (Coleophoridae), which forms floral bud galls on *Polygonum convolvulus* and *P. dumetorum* (Polygonaceae) that subsequently serve as a case for the larvae (Meyer, 1987; Dreger-Jauffert and Shorthouse, 1992). An example of a coleopteran insect causing floral abnormalities is *Lixus punctiventris* (Curculionidae), which induces virescence and unusual proliferation of *Crepis biennis* (Asteraceae) flowers (Meyer, 1987; Dreger-Jauffert and Shorthouse, 1992). Examples from the orders Diptera and Hymenoptera include *Asphondylia scrophulariae* (Cecidomyiidae) that induces flower galls on *Scrophularia canina* (Scrophulariaceae), and *Aylax papaveris* (Cynipidae) that causes ovarian malformation of *Papaver dubium* (Papaveraceae), respectively (Meyer, 1987).

Mites

Mites (Class: Arachnida, Subclass: Acari) are microscopic spindle-like arthropods, with four pairs of legs in all life stages (Walter and Proctor, 1999). More than 40 000 species of Acari have been described (Groombridge, 1992) from a wide range of ecosystems. There are more than 5 000 phytophagous mites and those in superfamily Eriophyoidea (*ca*. 2 400 spp.) are the most important due to the damage they cause to agricultural crops (Westphal, 1992; Boczek and Griffiths, 1994; Walter and Proctor, 1999). Eriophyiod mites attack all aerial organs of plants and a vast majority of them are host specific (Walter and Proctor, 1999). They are known to be vectors of many plant viruses and bacteria (see Boczek and Griffiths, 1994), as they use stylet-like mouthparts to pierce plant cells in order to gain access to its nutrients (Walter and Proctor, 1999). Plant reactions to mite feeding vary, and depending on the eriophyoid species, symptoms such as russeting, shrivelling, leaf rolling, bud proliferation, witches' brooms, galls and discolorations can be caused (Boczek and Griffiths, 1994; Walter and Proctor, 1999).

In terms of floral malformation, mites cause virescence, phyllody and sterility (Westphal, 1992). Examples of mites causing virescence and sterility are *Eriophyes lycopersici* and *E. peucedani* on hosts such as *Solanum dulcamara* (Solanaceae) and *Pimpinella saxifrage* (Apiaceae), respectively (Meyer, 1987). Another example of eriophyiod causing malformation is *E. triradiatus* associated with twig elongation or bud proliferation on willows, which under heavy infestations may deform whole flower clusters (Boczek and Griffiths, 1994). Examples of mites causing witches' broom are *E. loewi* on lilac (Meyer, 1987) and *E. fraxinivorus* on ash (Westphal, 1992).

Nematodes

Nematodes (Phylum: Nematoda) are microscopic, worm-like, colourless invertebrates that include about 20 000 formally described species, of which 4 000 are plant parasites (Weischer and Brown, 2000). Plant parasitic nematodes feed by injecting their stylets into the cells of plants and extracting the nutrients (Weischer and Brown, 2000). Some nematodes have wide host ranges, and many are known as vectors of viruses (e.g. ringspot virus), bacteria (e.g. *Pseudomonas* and *Erwinia*) and fungi (e.g. *Fusarium* and *Botrytis*) (Weischer and Brown, 2000). Most nematodes infest plant roots where they cause root galls, lesions and excessive branching which are accompanied by aboveground symptoms such as yellowing, wilting and

reduced growth (Agrios, 2005). However, certain nematode species inhabit aerial plant organs where they cause galls, necrosis, leaf distortions and abnormal flower development (Agrios, 2005). The best known example of a nematode affecting flowers is *Anguina tritici* that causes seed galls on wheat (Weischer and Brown, 2000; Agrios, 2005; Bridge and Starr, 2007). Initially, the juveniles of *A. tritici* cause leaf and stem malformations, but subsequently they enter the floral primordial and produce seed galls from undifferentiated floral tissues (Agrios, 2005). The affected inflorescences are wider and shorter with transformed seed galls full of nematodes in an anhydrobiotic state (Bridge and Starr, 2007).

Viruses

Plant viruses are extremely small, non-cellular parasitic nucleoproteins that only replicate in a living cell (Bos, 1999). Viruses can enter the cell through any sort of mechanical wound such as those made during grafting (Agrios, 2005). They can spread to subsequent plant generations through vegetative propagation, pollination and seed production (Bos, 1999; Agrios, 2005). Many viruses are also transmitted by other biotic agents such as fungi, insects, mites and nematodes (Cooper, 1993; Bos, 1999; Agrios, 2005). Viral infections often cause stunted appearance, necrosis, wilting, colour deviations and malformations (Meyer, 1987; Bos, 1999). In terms of blossom infection, they can decrease flower numbers, cause pollen sterility and reduce fruit and seed set (Daughtrey *et al.*, 1995). Most viruses of angiosperms are positive-strand RNA viruses, which include 14 families with about 70 genera (Villarreal, 2005).

The oldest known example of a viral disease affecting flowers is colour breaking in tulips (Bos, 1999). Virus-associated flower breaking has also been reported for *Anemone* (Ranunculaceae), *Primula* (Primulaceae), *Begonia* (Begoniaceae) and *Pelargonium* (Geraniaceae) species (Daughtrey *et al*., 1995). The best known example of floral virescence and witches' broom caused by a virus is on *Salix alba* (Salicaceae) (Meyer, 1987). Virus-associated floral malformations have been reported in plant species such as *Fuchsia* and *Myrica* (Cooper, 1993). In banana, inflorescences may be infected with a nanovirus resulting in the banana bunchy-top disease, where the plants do not produce fruits as the inflorescences are prevented from forming (Dale, 1994). Other important food crop plants such as maize and rice are also affected by virus induced floral malformation diseases, and various viruses (e.g.

tenuiviruses) cause stunted growth, fewer or no panicles and absence of flowers or sterility (Agrios, 2005).

Bacteria

Bacteria are single-celled prokaryotic organisms with about 4 000 described species (Groombridge, 1992), of which those in 21 genera are plant pathogens (Agrios, 2005). Plant pathogenic bacteria may cause leaf spots and blights, soft rots, wilts, scabs and cankers (Billing, 1987; Agrios, 2005), and their transmission may be mediated by various abiotic (e.g. water and wind) and biotic (e.g. insects, animals and humans) agents. The most important plant pathogenic bacteria are rod-shaped, Gram-negative bacteria in the family Enterobacteriaceae and Pseudomonadaceae or Gram-positive coryneforms, with an exception of filamentous *Streptomyces* (Billing, 1987; Agrios, 2005). Although, some of these bacteria can parasitize blossoms (e.g. *Erwinia amylovora* that causes fire blights of fruits), the largest group of malformation inducing phytopathogenic bacteria are phytoplasmas.

Phytoplasmas are wall-less pleiomorphic, obligate bacteria in the class Mollicutes, which parasitize the phloem tissue of the plants (Lee *et al*., 2000). Phylogenetic studies have revealed that these bacteria represent a distinct monophyletic group closely related to another Mollicute genus, *Acholeplasma* (Lee *et al*., 2000; Hogenhout *et al*., 2008). Phytoplasmas may alter hormone levels in the plant, as well as phloem sap content and function, thus inducing visible symptoms such as yellowing, stunting, general decline and proliferations (Lee *et al.*, 2000; Christensen *et al*., 2005). Although they may be spread during vegetative propagation (Lee *et al.*, 2000), phytoplasmas are mainly transmitted from plant to plant by insect vectors, specifically sap-sucking insects of the Order Hemiptera (e.g. leafhoppers, planthoppers and psyllids [Weintraub and Beanland, 2006]). These obligate pathogens have a broad plant host range that are dependent on the plant feeding range of their insect vectors (Hogenhout *et al*., 2008), thus making phytoplasma disease outbreaks erratic and difficult to detect and control (Hogenhout *et al*., 2008).

Phytoplasmas are associated with numerous diseases of fruits, vegetables, ornamentals and forestry trees and are well known to cause devastating diseases such as coconut lethal yellowing, peach X-disease, ash and elm yellows, among others. (Lee *et al*., 2000). The most prominent floral abnormalities caused by phytoplasmas are phyllody, virescence and sterility

(Meyer 1987; Lee *et al*., 2000). Examples of phyllody are found in plant species such as clover, soybean and cotton, while virescence may be observed on gladiolus, onion, strawberry and others (Meyer, 1987; Lee *et al*., 2000). Perhaps, the best known example is aster yellows of numerous vegetables and ornamentals, where the disease is characterised by general chlorosis and decline, abnormal production of shoots and malformation of flowers and other organs (Lee *et al*., 2000; Agrios, 2005).

Oomycetes

The oomycetes are microscopic, eukaryotic organisms with glucans and cellulose in their cell walls (Hawksworth *et al.*, 1995). Although they share some morphological features with fungi, the oomycetes are not related to the Eumycota as they form part of the Chromalveolate clade that also includes brown algae and diatoms (Baldauf *et al*., 2000; Schlegel, 2003; Adl *et al.*, 2005). The oomycetes are widely recognised as being classified in the kingdom Chromista, phylum Oomycota, which includes approximately 700 described species (Kendrick, 2000; Carlile *et al*., 2001). As obligate parasites of vascular plants, they are commonly known as water moulds, white rusts, downy mildews and damping-off (Carlile *et al*., 2001). The best known examples of pathogens affecting inflorescences are those in the genetically diverse *Albugo candida* species complex (Meyer, 1987; Thines and Spring, 2005; Choi *et al*., 2006). Its members form white blister patches on all the hypertrophied organs of brassicaceous plants (e.g. *Brassica*, *Raphanus*, *Capsella*), but the disease is particularly evident on inflorescences, where the flowers are deformed, abnormally enlarged and virescent (Meyer, 1987; Thines and Spring, 2005).

Fungi

Fungi are eukaryotic, heterotrophic, spore-bearing organisms with chitin and glucans in their cell walls (Hawksworth *et al*., 1995). In total, about 100 000 fungal species are known (Hawksworth, 2004) of which more than 10 000 species are associated with plant diseases (Kendrick, 2000; Agrios, 2005). Fungi can be transmitted from plant to plant primarily by the distribution of their spores by wind, water, insects, animals and humans. They are associated with disease symptoms such as leaf spots and curls, cankers, dieback, damping-off, decline, rots, wilts, rusts, smuts, blights, scab, anthracnose, warts and galls (Agrios, 2005). In terms of

inflorescence diseases, fungi can cause hypertrophy or hyperplasia by either attacking specific organs of the flower or the whole inflorescence, which may prevent reproduction (Kendrick, 2000). A wide variety of these are caused by species in the dikaryomycotan phyla Ascomycota and Basidiomycota, although species in the phylum Zygomycota are better known for blossom rots (Agrios, 2005).

Ascomycota produce sexual ascospores inside sac-shaped structures known as asci (Hawksworth *et al*., 1995). There are more than 32 000 species in this Phylum, making them the most abundant fungal group (Kendrick, 2000; Carlile *et al*., 2001). The oldest known example of a fungal disease affecting inflorescences is ergot of rye caused by *Claviceps purpurea*. The ascospores of this species infect young flowers during blooming by penetrating into their ovaries, multiplying and replacing the whole grain with fungal sclerotia (van Dongen and van Groot, 1995; Agrios, 2005). A similar disease, namely, ergot of sorghum is caused by *Claviceps sorghi* (Bandyopadhyay *et al*., 1998). Several species of *Taphrina* may also attack blossoms to cause flower deformations, in addition to leaf distortions (Agrios, 2005). For example, floral malformation of *Alnus incana* (Betulaceae) is caused by *Taphrina alni-incanae* that results in the abnormal enlargement of the bracts and ovaries of female catkins (Meyer, 1987). Several species in this phylum (e.g. *Colletotrichum*, *Botrytis*, *Monilinia*, etc.) are widely known to cause blossom blights that subsequently cause devastating fruit rots and thus huge economical losses (Daughtrey *et al*., 1995; Powell and Lindquist, 1997; Agrios, 2005; Larena *et al*., 2005; Williamson *et al*., 2007).

The Basidiomycota include approximately 22 000 species, all of which form basidiospores, an outside specialised meiosporangia called basidia when they reproduce sexually (Hawksworth *et al*., 1995; Kendrick, 2000; Carlile *et al*., 2001). Besides being decomposers of woody and herbaceous plants, this group also contains very destructive phytopathogens known as rust and smut fungi, which are notorious for huge yield and economic losses (Agrios, 2005). Smut fungi (Ustilaginales), in particular are well known for deforming organs of various cereals. There are about 1 200 smut species infecting maize, rice, wheat, sorghum and other agricultural crops (Agrios, 2005). For example, *Ustilago maydis* infects actively growing auxiliary buds, individual flowers of the ears and tassels and the leaves and stalks of maize (Martínez-Espinoza *et al*., 2002; Agrios, 2005; Brefort *et al*., 2009). The fungus infects the plant's ovaries and stimulates the cells to produce hypertrophied galls to transform

the whole kernels into voluminous sacs filled with dark, dusty teliospores (Agrios, 2005). Other examples include *Tilletia* species that attack Poaceae seedlings, prior to head appearance, thus sterilising deformed flowers (Meyer, 1987), which produce slimmer heads with shorter and thicker kernels filled with teliospores (Agrios, 2005). Examples of species outside of the smut group that cause floral malformations are *Exobasidium camelliae* (Exobasidiales) and *Moniliophthora* (formally *Crinipellis*) *perniciosa* (Agaricales) (Meyer, 1987). The former is a pathogen of *Camellia japonica* (Theaceae), which causes large pink galls on the petals, sepals and leaves of this plant (Ing, 1994). The latter induces witches' brooms on cacao by infecting developing pods through the flower cushions, resulting in reduced yield and economical losses in Brazil and other parts of the world (Purdy and Schmidt, 1996).

Mango malformation

Mango is native to Southeast Asia and India (Sauer, 1993) and it is also cultivated in many other tropical regions where it is widely exploited for its nutritional purposes. In 2001, the global production of mango was estimated at more than 23 million tons (Jedele *et al*., 2003). In many regions mango is affected by a devastating disease known as mango malformation (Marasas *et al*., 2006), which represents the most important floral disease of this tropical crop (Kumar *et al*., 1993). Typically, the disease is spread by the movement of infected nursery seedlings and the propagation of asymptomatic but infected material (Kumar *et al*., 1993; Ploetz, 2001). Mango malformation disease (MMD) was first observed in India in 1891 and since then it has been reported from a number of countries in Africa, Southeast Asia, Middle East and Americas (Fig. 1; Ploetz, 1994, 2001; Kumar *et al*., 1993; Marasas *et al*., 2006; Kvas *et al*., 2008).

MMD affects both inflorescences and vegetative shoots of mango plants (Fig. 2; Kumar *et al*., 1993). Manifestation of the disease on inflorescences is characterised by abnormally enlarged panicles (Fig. 2b) that are excessively branched, shortened and thickened with sterile flowers that do not bear fruit which, subsequently, can cause great economic losses (Kumar *et al*., 1993; Ploetz, 1994, 2001; Marasas *et al*., 2006). Phyllody may also occur (Kumar *et al*., 1993; Marasas *et al*., 2006). Vegetative shoot deformation, due to MMD, is observed more frequently on immature trees, where auxiliary or apical buds produce swollen, misshapen shoots

with brittle, dwarfed leaves (Fig. 2c; Kumar *et al*., 1993). These distorted shoots tend to remain compact thus giving rise to a bunchy-top appearance (Kumar *et al*., 1993).

Epidemiology and aetiology of MMD has been attributed to various abiotic and biotic agents (Kumar *et al*., 1993). Initially, physiological disorders such as excessive moisture and reduced availability of particular elements in soil were considered to cause MMD (Kumar *et al*., 1993). In 1946, a virus was suspected to cause the disease, but subsequent studies showed that this was probably not the case (Kumar *et al*., 1993). MMD has also been associated with the eriophyiod mite, *Aceria mangiferae*, as the feeding injuries made by these mites are frequently found on both the floral and vegetative malformed organs (Kumar *et al*., 1993). Recently, it has been established that the mite is a vector of at least one *Fusarium* species (Gamliel-Atinsky *et al*., 2009).

MMD is associated with at least eight species of *Fusarium* (Leslie, 1995; Britz *et al*., 2002; Marasas *et al*., 2006; Lima *et al*., 2009). Initially, *F. moniliforme* was isolated and identified from malformed tissue (Summanwar *et al*., 1966). In 1974, Varma *et al*. used then called *F. moniliforme* var. *subglutinans* isolates in pathogenicity tests and induced both forms of the disease. Later, Nelson *et al.* (1983) renamed the fungus *F. subglutinans* and classified it in the section *Liseola*, which broadly corresponds to the so-called *Gibberella fujikuroi* complex (GFC) (O'Donnell *et al*., 1998). In 1999, Freeman *et al.* conclusively showed that *F. subglutinans* isolates associated with MMD can cause the disease and in 2002 these isolates were formally described as *F. mangiferae* (Britz *et al*., 2002). *Fusarium mangiferae* has been reported from India, Egypt, Israel, Malaysia, USA (Florida), South Africa and Oman as a causal agent of MMD (Freeman *et al*., 1999; Britz *et al*., 2002; Marasas *et al*., 2006; Kvas *et al*., 2008). Its occurrence in Pakistan (Iqbal *et al*., 2006) needs to be confirmed with phylogenetic analyses.

Ten years after establishing that MMD is caused by *F. mangiferae*, Lima *et al.* (2009) confirmed Koch's postulates for two additional *Fusarium* species. Both these species form part of the GFC and can cause the vegetative form of the disease. The one species is *F. sterilihyphosum*, which has been initially associated with diseased mangoes in South Africa (Britz *et al*., 2002), but was subsequently also discovered in Brazil (Marasas *et al*., 2006; Lima *et al*., 2009). The second species has not yet been described and is represented by *Fusarium* isolate CML 345 (Lima *et al*., 2009), which is closely related to *F. sterilihyphosum*. This new

species has been reported only from Brazil (Lima *et al*., 2009) and future population studies of these closely related fungi may shed light on their possible origins and spread.

Fusarium oxysporum and four additional *Fusarium* species in the GFC have been associated with MMD (see Ploetz, 2001; Marasas *et al*., 2006). The latter include *F. proliferatum* and as yet undescribed lineage from Malaysia (Leslie, 1995; Britz *et al*., 2002; Marasas *et al*., 2006), as well as *F. subglutinans* and *F. sacchari* in Brazil (see references in Lima *et al*., 2009). A *Fusarium* species different from *F. oxysporum* and *F. mangiferae* has been associated with the disease in Mexico (Rodríguez-Alvarado *et al*., 2008). As *F. subglutinans* and *F. sacchari* were identified based only on morphological characteristics, their identity and involvement in MMD must be re-evaluated following the polyphasic approach (Kvas *et al.*, 2009). The isolates of *F. oxysporum* that have been reported to cause MMD could also have been misidentified or they might represent a unique chlamydospore-producing species within either the *F. oxysporum* or other species complexes of *Fusarium* (Marasas *et al*., 2006). The role of any of these *Fusarium* taxa in the development of the disease has not been tested thus far.

Waterberry malformation

The evergreen waterberry tree, *S. cordatum*, is indigenous to Southern Africa where it occurs in the vicinity of streams from KwaZulu-Natal northwards to Mozambique, as well as Swaziland, the Caprivi Strip of Namibia, north Botswana and Zimbabwe (van Wyk and van Wyk, 1997). In rural areas, *S. cordatum* has many uses, for example, for timber and as medicine in the treatment of stomach and respiratory ailments (Pooley, 1993). In its native habitat and in urban areas, where this species is used as an ornamental plant, waterberry is often affected by abnormally developed inflorescences (Fig. 3). The symptoms have been noticed in the Gauteng, KwaZulu-Natal, Mpumalanga and Western Cape provinces of South Africa (M. Kvas, personal observation), in the Caprivi Strip of Namibia and in Zambia, (J. Roux, personal communication). Panicles of the affected waterberry inflorescences remarkably resemble those of mango affected by MMD. Such deformed inflorescences are unable to produce berries and subsequently seeds, which are essential in propagation of the species.

The aetiology of waterberry malformation disease (WMD) is unknown. A single study in 1985 identified *F. subglutinans* from malformed waterberry material (Crookes and Rijkenberg,

1985). At that time *F. subglutinans* was still considered a single species and its polyphyletic nature had not been revealed (Leslie, 1995; O'Donnell *et al.*, 1998; Kvas *et al*., 2009). DNA-based studies have shown that a variety of GFC species display morphological characters similar to *F. subglutinans* and malformed waterberry inflorescences may in fact be colonised by any of the known *F. subglutinans sensu lato* species/lineages or by another novel *Fusarium* species with *F. subglutinans* morphology. Further studies are needed to determine whether WMD is associated with a single fungal species or whether it is more complex like MMD and associated with a wide variety of *Fusarium* species.

Another striking similarity between WMD and MMD is the association of the affected floral tissues with eriophyiod mites. Examination of malformed waterberry inflorescences throughout South Africa (Fig. 4) has revealed that *Aceria* (*Eriophyes*) *afroensis* is present at all the locations sampled. This mite species has been previously reported from bracts in deformed inflorescences of *S. guineense* and *S. legatii* (Meyer and Ueckermann, 1989). For the WMD associated mite, however, the species identification is not yet conclusive, as high levels of variation in morphology were observed between specimens from one and/or different collections (C. Craemer, personal communication). Although this could reflect intraspecific variation, it might also suggest that sibling or morphologically closely related species occur in similar symptomatic tissues (C. Craemer, personal communication). Therefore, to confirm these identifications, the morphological variation in mite specimens collected from the symptoms on several *Syzygium* species should be studied and be accompanied by DNA sequence information.

Future perspectives

Eight *Fusarium* species have been associated with MMD. If we take into consideration that South Africa is a biodiversity hotspot, a number of *Fusarium* species might also be associated with WMD. Exploration of the rich South African mycoflora associated with indigenous vegetation (Crous *et al*., 2006) will likely reveal novel species, which could expand our taxonomic knowledge of *Fusarium* biodiversity. This could provide unique opportunities for studying the evolution of this genus. Furthermore, characterisation of *Fusarium* species associated with indigenous African flora would lead to the identification of species that originated and evolved in Africa and could further improve our understanding of the evolution

of this important group of fungi. Future studies could also directly evaluate the phylogeographic hypothesis that Africa represents the centre of origin of the GFC (O'Donnell *et al*., 1998; Kvas *et al*., 2009). By exploring the *Fusarium* diversity associated with these native ecosystems, we could also reconstruct the evolution of the GFC for testing hypotheses pertaining to their host/substrate preferences, geographic distribution, mycotoxicology, reproductive biology and pathology.

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Fig. 1. A map of the world indicating the countries in which mango malformation has been reported.

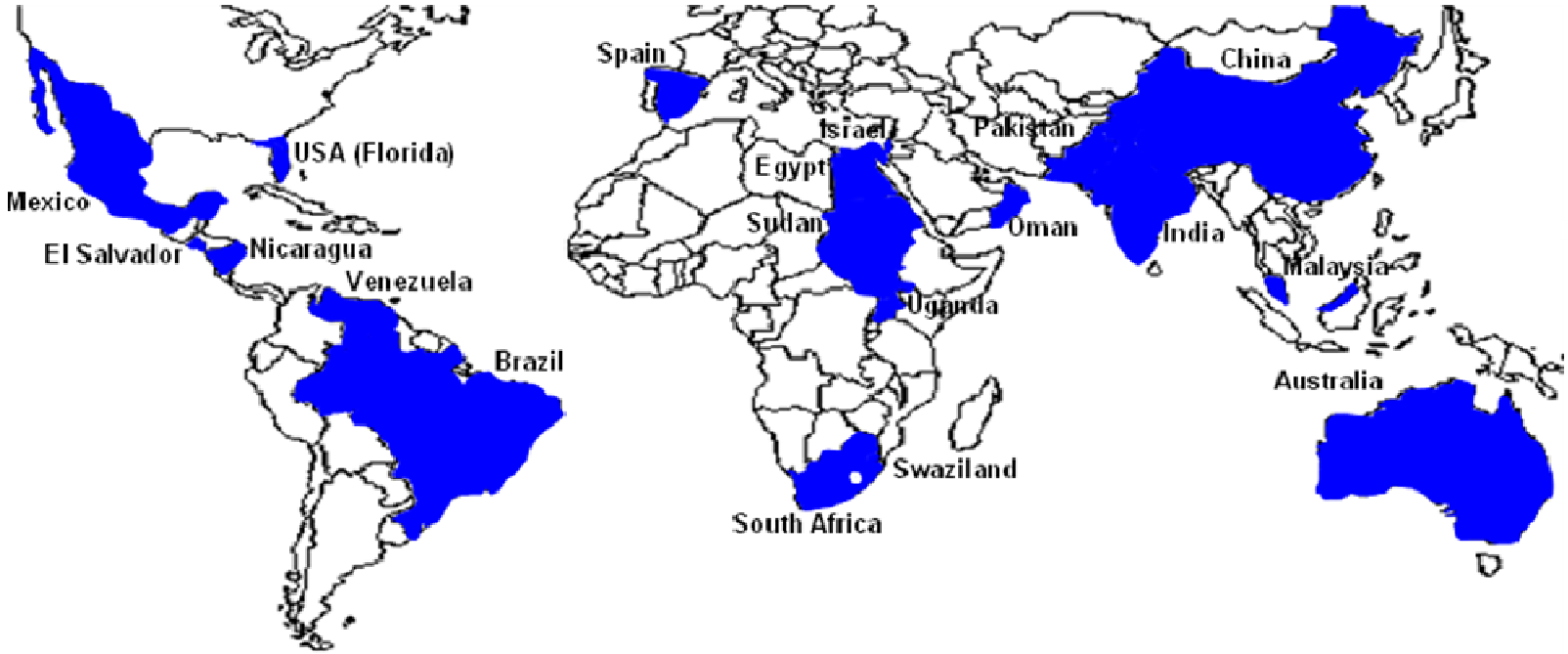
Spain
China
Israel
Pakistan
USA (Florida)
Egypt
Mexico
Sudan
Oman
India
El Salvador
Nicaragua
Malaysia
Venezuela
Uganda
Brazil
Australia
Swaziland
South Africa

Fig. 2. *Mangifera indica*. (a) healthy mango inflorescences; (b) malformed mango inflorescence with abnormally branched and thickened panicles that produce up to three times the normal number of flowers; (c) vegetative malformed seedling showing bunchy-top appearance of the abnormally developed auxiliary or apical buds. Photos by Dr. Ali Al Adawi.

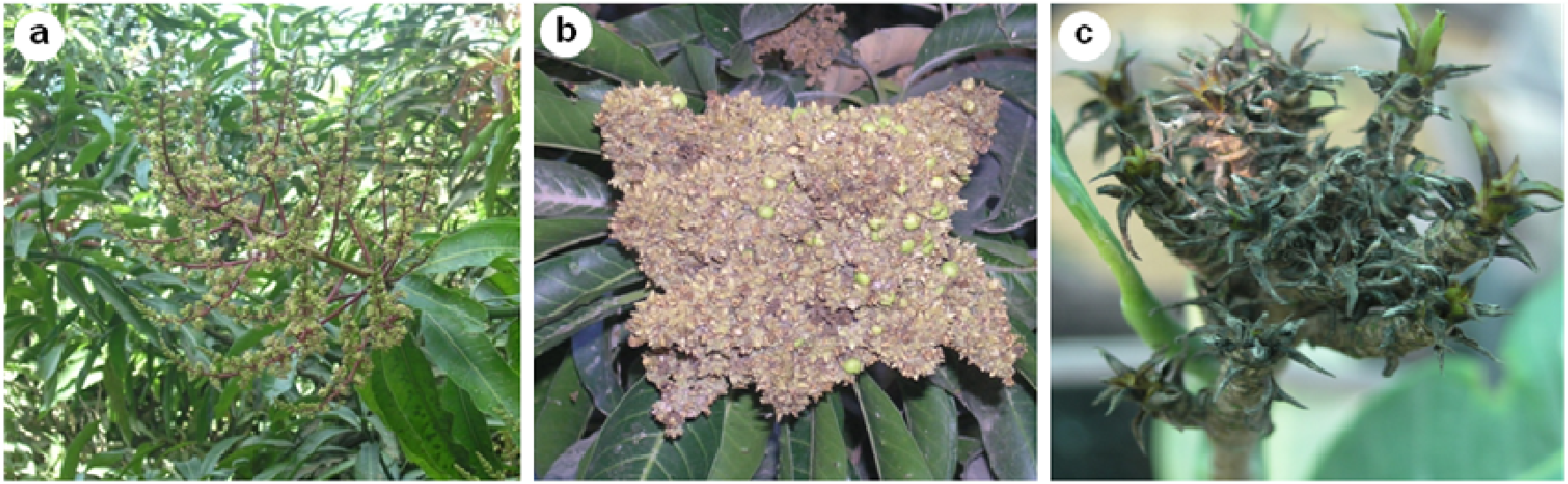
a
b
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Fig. 3. *Syzygium cordatum*. (a) healthy waterberry inflorescences; (b) (c) malformed panicles resembling mango malformation, become dried-out with time and are unable to form fruits.

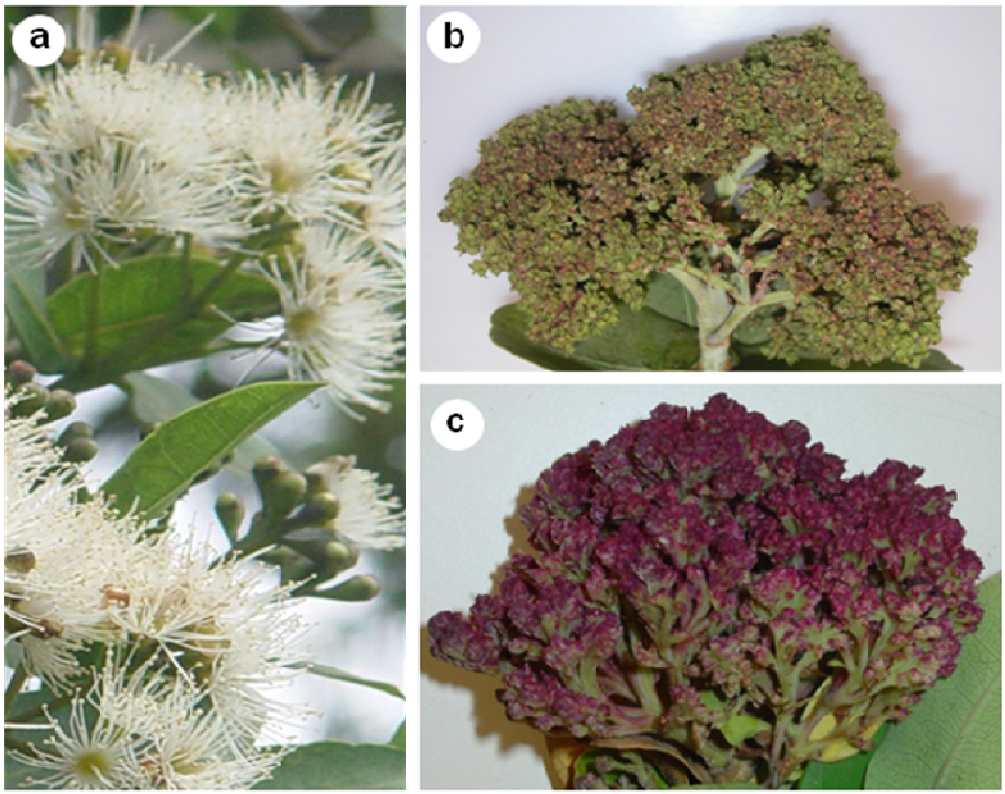
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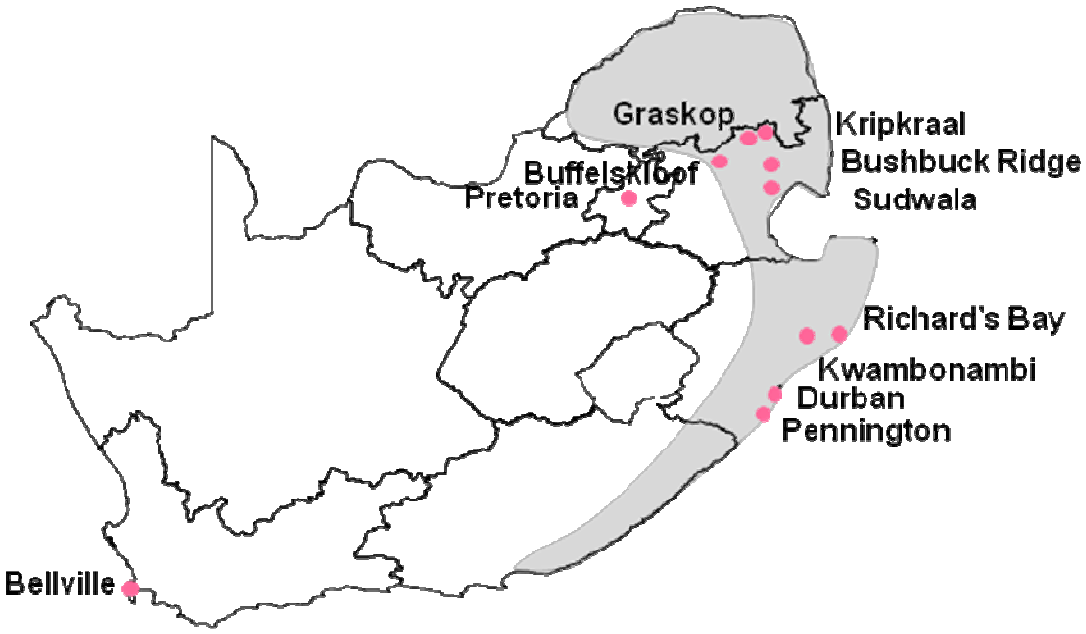
Fig. 4. A map of South Africa indicating distribution of *Aceria* (*Eriophyes*) *afroensis* on *Syzygium cordatum.* The natural distribution of *S. cordatum* is indicated in grey as according to van Wyk and van Wyk (1997).


Graskop
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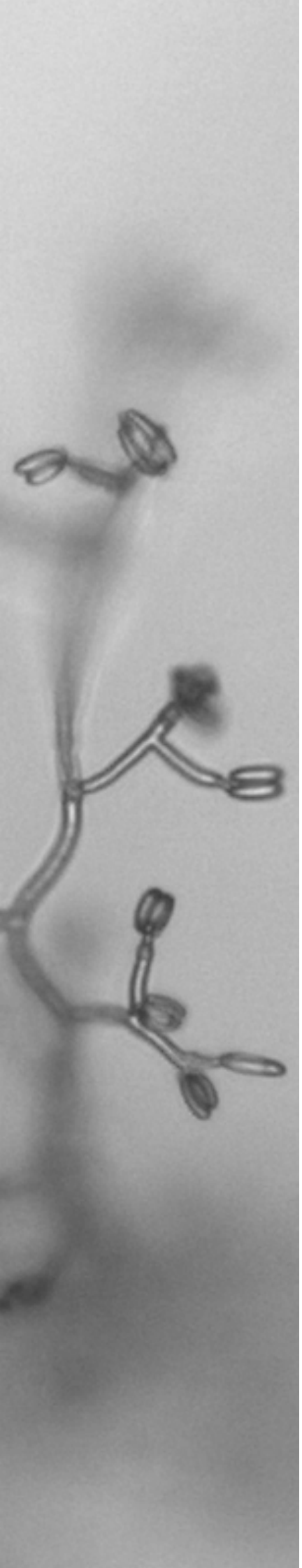
Bushbuck Ridge
Buffelskloof
Pretoria
Sudwala
Richard's Bay
Kwambonambi
Durban
Pennington
Bellville



Chapter 3

DNA barcoding reveals unexpected *Fusarium* biodiversity associated with *Syzygium cordatum* floral malformation in South Africa

Abstract

Indigenous ecosystems are usually associated with rich fungal diversity. In order to explore the diversity associated with a single ascomycetous genus, *Fusarium*, we developed an operational DNA-based procedure to facilitate the large-scale identification and cataloguing of the species in this genus. For this purpose, we used the inflorescences of the Southern African native plant *Syzygium cordatum* as a model substrate. Three DNA-based approaches were evaluated, namely PCR-RFLP of the rRNA intergenic spacer region, barcoding similarity-based analysis of a portion of the gene encoding translation elongation factor 1-alpha (TEF), and TEF-based phylogenetic analyses. Although all three approaches revealed that the inflorescences of *S. cordatum* are associated with an unexpectedly high *Fusarium* diversity, PCR-RFLP and barcoding analyses were not effective due to potentially high intraspecific variability, several instances of low interspecific variability, and most importantly, unavailability of suitable reference strains and taxon under-representation in the barcode database. Unambiguous identification of distinct taxa was possible only with phylogenetic analyses of well-represented TEF alignments. Our collection of 185 *Fusarium* isolates from *S. cordatum* inflorescences represented at least 52 species/lineages, of which 69% appears to be novel. Most isolates belonged to *F. incarnatum/F. equiseti*, *F. oxysporum* and *Gibberella fujikuroi* complexes, with others representing *F. chlamydosporum/F. nelsonii*, *F. compactum*, *F. tricinctum/F. avenaceum* and members of the *F. graminearum* complex. Our results, therefore, suggest that the generation of large-scale *Fusarium* species inventories can be done through sequencing the TEF barcode region followed by phylogenetic analyses.

Introduction

An estimated 1.5 million fungal species are thought to exist on the planet, yet, our knowledge of fungal diversity is limited to approximately 5% that have been described (Hawksworth, 1991, 2001, 2004). This gap in our understanding of Earth's mycological diversity is even more pronounced when regions known for their significant biodiversity are considered. For example, South Africa, which includes three of the 25 global hotspots (Myers *et al*., 2000; www.biodiversityhotspots.org), has been suggested to harbour an estimated 171 500 fungal species (Crous *et al*., 2006). Of this estimated diversity, only about 0.45% of the species have been described (Crous *et al*., 2006). Most of these species are unique to South African ecosystems (e.g. Crous *et al*., 1996; Janse van Rensburg *et al*., 2006; Kamgan *et al*., 2008) and/or are associated with indigenous plant species such as those in the families Proteaceae and Restionaceae (e.g. Taylor *et al*., 2001; Denman *et al*., 2003; Lee *et al*., 2004) and the woody host *Syzygium cordatum* (Family Myrtaceae) (Crous *et al*., 1993, 1995; Sutton and Crous, 1997; Crous, 1999; Heath *et al*., 2006; Nakabonge, *et al*., 2006; Pavlic *et al*., 2007).

Syzygium cordatum is a tree, indigenous to South Africa that commonly occurs along watercourses on forest margins and swampy areas (Pooley, 1993; van Wyk and van Wyk, 1997; Palgrave, 2002). This species is widely used in rural areas for timber, fuel, food and traditional medicine (Pooley, 1993; van Wyk and van Wyk, 1997; Palgrave, 2002). Throughout its native range, as well as in urbanised areas where the species is used as an ornamental, *S. cordatum* is affected by abnormally developed inflorescences, which are characterised by unusually enlarged, thick and branched panicles, which become dry with time and unable to bear fruits (Fig. 1). This condition is similar in appearance to the symptoms of the mango malformation disease, which is associated with a number of *Fusarium* species (Marasas *et al*., 2006; Lima *et al*., 2009). Apart from a single report in 1985 that identified *F. subglutinans* from malformed panicles (Crookes and Rijkenberg, 1985), nothing is known regarding the fungal diversity associated with *S. cordatum* malformation.

Fusarium species are well known, cosmopolitan and economically important pathogens of plants, humans and animals (Booth, 1971; Nelson *et al*., 1983; Summerbell, 2003; Dignani and Anaissie, 2004; Leslie and Summerell, 2006). Some species can also produce mycotoxins that contaminate staple food/feed of humans and animals, thus posing

considerable health risks (Marasas *et al*., 1984; Desjardins, 2006). Due to the importance of these fungi, their taxonomy has received much attention in scientific literature (e.g. Booth, 1971; Nelson *et al*., 1983; Leslie and Summerell, 2006). Initially, *Fusarium* species were recognised primarily based on their morphology, which can be extremely variable, thus complicating morphology-based identification (e.g. Booth, 1971; Nelson *et al*., 1983; Leslie and Summerell, 2006). However, the application of DNA-based approaches during the course of the last decade has revolutionised *Fusarium* taxonomy. Various DNA-based phylogenetic studies have shown the non-monophyletic nature of *Fusarium* sections (e.g. *Liseola* and *Dlaminia*; O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998a, 2000b) and species (e.g. *F. subglutinans sensu lato*; O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998a, 2000b; Steenkamp *et al*., 2000, 2001), while others have revealed cryptic species (e.g. Steenkamp *et al*., 2002) and/or numerous novel species and lineages (e.g. O'Donnell, 2000; O'Donnell *et al*., 1998a, 2000a, 2000b, 2004b, 2008; Steenkamp *et al*., 2000, 2001; Aoki *et al*., 2001; Britz *et al*., 2002; Zeller *et al*., 2003; Phan *et al*., 2004; Schroers *et al*., 2009). DNA-based approaches are, therefore, essential to identify *Fusarium* species (Leslie *et al*., 2001; Summerell *et al*., 2003; Kvas *et al*., 2009), as identifications based solely on morphology underestimate the diversity of *Fusarium* in any particular environment. This in turn hinders our understanding of their biological and ecological importance (Godfray, 2002; Wilson, 2003).

To expedite the process of cataloguing the Earth's biodiversity, Hebert *et al*. (2003a, 2003b) introduced a procedure known as DNA barcoding (see Marshall, 2005; Savolainen *et al*., 2005; Hajibabaei *et al*., 2007). This procedure is based on the application of a short, standardised gene region that is highly informative even among closely related species (Hebert *et al*., 2003a, 2003b). Although a 648 base pair (bp) region of the mitochondrial cytochrome *c* oxidase I (COI) gene appears effective for most animals (Hebert *et al*., 2004a, 2004b; Ward *et al*., 2005; Hajibabaei *et al*., 2006), algae (Saunders, 2005) and oomycetes (see Seifert, 2009), alternative barcoding regions have been proposed for amphibians (e.g. 16S ribosomal RNA [rRNA]; Vences *et al*., 2005a, 2005b), plants (e.g. *rbcL*, *matK*, *trnH-psbA*; Chase *et al*., 2005; Kress *et al*., 2005) and fungi (e.g. rRNA internal transcribed spacers [ITS]; Druzhinina *et al*., 2005; Seifert, 2009). For *Fusarium* species, however, the application of ITS and COI as barcodes are not feasible due to the presence of multiple non-orthologous copies of these regions in a single individual (Waalwijk *et al*., 1996; O'Donnell and Cigelnik, 1997; Gilmore *et al*., 2009), and/or an overall lack of polymorphism among many species in the case of ITS (O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998a).

Therefore, Geiser *et al*. (2004) proposed that a *ca*. 650 bp region of the gene encoding the eukaryotic translation elongation factor 1-alpha (TEF) be used for *Fusarium* barcoding. The informative value of this region at the species level and the fact that non-orthologous copies of TEF have not been detected, have contributed greatly to the fact that the TEF barcoding region has been widely used for *Fusarium* identifications (e.g. Vujanovic *et al*., 2006; Maciá-Vicente *et al*., 2008; Punja *et al*., 2008).

Studies of *Fusarium* diversity, biology and ecology usually focus on hosts and environments of medical, agricultural and/or veterinary importance (Kvas *et al*., 2009). In an attempt to understand how DNA-based procedures influence diversity estimates for species in this genus, and to develop an operational procedure for generating an inventory of *Fusarium* species in indigenous South African ecosystems, we concentrated on the malformed inflorescences of *S. cordatum*. The main aim of this study was, therefore, to isolate and catalogue the diversity of *Fusarium* species inhabiting this unique niche by making use of morphology and DNA-based approaches. For this purpose, we evaluated two DNA regions, namely the TEF barcode and rRNA intergenic spacer (IGS) regions, for the use in PCR-RFLP (restriction fragment length polymorphism) analyses to cluster the isolates in a cost effective manner. This clustering approach was compared with direct sequence analysis of the TEF barcoding region to allow the possible inference of species identities by database searches and phylogenetic analyses. Finally, we evaluated the effectiveness of these DNA-based approaches to consider the diversity of *Fusarium* in comparison to conventional morphology-based approaches.

Materials and Methods

Fungal isolates

The *Fusarium* isolates used in this study were obtained from symptomatic and asymptomatic inflorescences of *S. cordatum* collected throughout South Africa (Table 1; Fig. 2). Isolates were collected from 10 different geographical locations, sampling between one and eleven *S. cordatum* trees at each site. From each tree, 1-5 malformed inflorescences were collected, as well as a single healthy inflorescence and/or closed flower buds if present at the time of sampling. After dividing the inflorescences into manageable portions, they were surface-disinfested by submerging them into undiluted commercial bleach (3.5-5% available chlorine), and thereafter, into 75% ethanol for 2 min. The samples were then rinsed

in sterile distilled water, after which small flower pieces were plated onto *Fusarium* selective medium containing 20 g l^{-1} agar (Biolab Diagnostics, Wadeville, South Africa), 15 g l^{-1} peptone (Biolab Diagnostics), 1 g l^{-1} pentachloronitrobenzene (Sigma-Aldrich, Steinheim, Germany), 1 g l^{-1} KH_2PO_4 and 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$ (Leslie and Summerell, 2006). After incubation at 25°C for 7-14 days, fungi resembling *Fusarium* were transferred onto Petri dishes containing half strength potato dextrose agar (20 g l^{-1} PDA [Biolab Diagnostics], 5 g l^{-1} agar). Following incubation at 25°C for 7 days, spore suspension was plated onto water agar (20 g l^{-1} agar) and pure fungal cultures were obtained by transferring a single germinating conidium onto fresh half strength PDA. All isolates are stored and maintained in the *Fusarium* collection (CMWF) of the Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). An additional set of 59 *Fusarium* reference strains representing 36 species of well known plant pathogens and saprophytes were obtained from the Medical Research Council (MRC), Tygerberg, South Africa and used in this study (Table 2).

Isolation of genomic DNA

After 7 days of growth at 25°C on half strength PDA, approximately 50-100 mg of mycelium was scraped directly from the surface of the medium and homogenised in the presence of 500 µl extraction buffer (100 mM Tris-HCl [pH 8.0], 10 mM EDTA, 2% SDS, 0.2 µg μl^{-1} Proteinase K [Sigma-Aldrich]) and incubated for 1 h at 60°C. This homogenate was then mixed with 260 µl of a CTAB-NaCl (2.25:1) solution (10% CTAB [n-cetyl-N, N, N-trimethyl-ammonium bromide, Saarchem, Wadeville, South Africa], 5 M NaCl) and incubated at 65°C for 10 min. After the addition of 500 µl phenol-chloroform (1:1) solution, the mixture was vortexed and centrifuged at 20 817 *g* for 15 min. Thereafter, the aqueous phase was transferred to a fresh tube and the phenol-chloroform extractions were repeated until the organic-aqueous interface was clear. After a final chloroform extraction to remove residual phenol, the nucleic acids in the aqueous phase, were precipitated with 0.6 volumes of ice-cold isopropanol and overnight incubation at -20°C. The precipitated nucleic acids were then harvested by centrifugation (20 817 *g*) for 30 min, washed with 70% ice-cold ethanol, air-dried and re-suspended in 30-50 µl deionised nuclease-free water. The extracted nucleic acids were subjected to electrophoresis on 1% agarose (Whitehead Scientific, Johannesburg, South Africa) gels that contained 1 mg ml^{-1} ethidium bromide (Sambrook and Russell, 2001) and compared to the λDNA/*Eco*RI+*Hind*III standard size marker (Fermentas, Burlington, Canada) under UV light for evaluation of integrity and quantification.

DNA fingerprinting and cluster analyses

In order to group the isolates included in this study, we employed a PCR-RFLP procedure for which we evaluated the IGS and TEF barcoding region by making use of the 36 reference *Fusarium* species. For the TEF barcoding region, we used an *in silico* approach where the 59 DNA sequences (see below) for the *Fusarium* reference strains were subjected to the online analysis tool DistinctiEnz (http://www.bioinformatics.org/~docreza/cgi-bin/restriction/DistinctiEnz.pl) to generate restriction maps for the commonly available restriction enzymes *Alu*I, *Hae*III, *Hha*I, *Mbo*I, *Msp*I and *Rsa*I.

For the IGS region, the entire spacer was amplified using primers CNL12 (5'-ctgaacgcctctaagtcag-3') and CNS1 (5'-gagacaagcatatgactac-3') (Appel and Gordon, 1995). Each 25-µl PCR mixture contained 2-4 ng μl^{-1} DNA template, 2.5 mM $MgCl_2$, 200µM of each dNTP**,** 10 µM of each primer, 1 U μl^{-1} *Taq* DNA polymerase and reaction buffer (Supertherm, Southern Cross Biotechnologies, Cape Town, South Africa). Thermocycling was carried out on a BioRad iCycler (Hercules, California, USA) or Eppendorf (Eppendorf, Germany) with an initial denaturation at 95^{o}C for 3 min, followed by 25 cycles of 94^{o}C for 30 s, 50^{o}C for 45 s, and 72^{o}C for 1 min, with a final extension at 72°C for 10 min. The IGS-PCR products were subjected to digestion with the enzymes *Hae*III and *Hha*I, both of which have four-base recognition sites. The restriction digestion reactions with *Hae*III (Fermentas) consisted of 0.5 µl of endonuclease, 1 µl of buffer R (Fermentas) and 8.5 µl of the PCR product. Those for *Hha* I (New England BioLabs, Ipswich, USA) included 0.5 µl of endonuclease, 0.1 µl bovine serum albumin, 0.9 µl of buffer 4 (New England BioLabs) and 8.5 µl of the PCR product. The reaction mixtures were incubated at 37^{o}C for 4 h, after which the digested products were separated by electrophoresis at 3 V cm^{-1} on 3% agarose gels (containing 1 mg ml^{-1} ethidium bromide) in 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) (Sambrook and Russell, 2001). Three lanes on each gel included 100 bp DNA ladder (Promega, Madison, USA) as a molecular size marker.

All gel images were captured with an E-box gel documentation system (Vilber Lourmat, France). The RFLP profiles were compared and normalised according to electrophoretic mobilities of the marker fragments using BioNumerics v4 (Applied Maths, Kortrijk, Belgium). By making use of this software, the bands ranging from 100 to 900 bp were automatically scored for their presence or absence, followed by manual correction to achieve optimal detection. BioNumerics was then used to cluster the profiles using the unweighted pair group method with arithmetic mean (UPGMA; Sneath and Sokal, 1973) and

the Jaccard similarity coefficient (Kosman and Leonard, 2005). The band-matching tolerance was set at 2%, while an optimisation of 1% was applied to the analyses (Applied Maths).

TEF barcoding

TEF barcodes were generated for all the isolates included in this study by making use of primers EF1 (5'-atgggtaagga(a/g)gacaagac-3') and EF2 (5'-gga(g/a)gtaccagt(g/c)atcatgtt-3') (O'Donnell *et al*., 1998b). Each 25-µl TEF PCR mixture contained reaction buffer with KCl (Fermentas), 25 mM $MgCl_2$, 200 µM of each dNTP, 10 µM of each primer, 1 U μl^{-1} *Taq* polymerase (FABI, Pretoria, South Africa) and 2-4 ng μl^{-1} of DNA template. PCR conditions were the same as for the IGS PCR, except that 35 cycles and 54^{o}C annealing temperature were used. Amplicons were purified using G50 Sephadex columns (Sigma-Aldrich), and both strands were sequenced using the original PCR primers, an ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and an ABI PRISM 3100™ DNA sequencer (Applied Biosystems).

The resulting electropherograms were examined and manually corrected where necessary using Chromas Lite v2.0 (Technelysium, Australia) and BioEdit v7.0.5.2 (Hall, 1999). The TEF nucleotide sequences were aligned using MAFFT v6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) with the L-INS-i option effective (Katoh *et al*., 2002). To calculate the sequence similarity of the obtained sequences, the alignment was subjected to a distance-based analysis in MEGA v4 (Tamura *et al*., 2007) by making use of the "No. of nucleotide differences" and "pairwise deletion" options. All TEF sequences were also compared to those in the *Fusarium* Identification Database (FID; Geiser *et al*., 2004; http://isolate.fusariumdb.org/index.php) using the Basic Local Alignment Search Tool (BLAST, Altschul *et al*., 1990).

TEF phylogenetic analyses

For phylogenetic analyses, multiple alignments for the TEF nucleotide sequences were generated using MAFFT as described above. The alignment included the sequences generated in this study (Table 1), as well as the 59 sequences representing the 36 *Fusarium* reference species (Table 2) and an additional 206 sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/) and/or the FID. Separate alignments were also constructed for the *F. incarnatum/F. equiseti* complex (FIEC; O'Donnell *et al*., 2007), *F. oxysporum* complex (FOC, Fourie *et al*., 2009) and *Gibberella fujikuroi* complex (GFC, Geiser *et al*., 2005). Phylogenetic relationships were inferred using maximum likelihood (ML) and

Bayesian inference (BI) analyses with PHYML v2.2.4 (Guindon and Gascuel, 2003) and MrBayes v3.1.2 (Ronquist and Heuelsenbeck, 2003), respectively. All analyses were performed under the best-fit evolutionary models as determined with Modeltest v3.7 (Posada and Crandall, 1998; Swofford, 2003). The evolutionary model for the *Fusarium* dataset was the General Time Reversible (GTR) model (Rodríguez *et al*., 1990) with a proportion of invariable sites (I=1.0112) and gamma distribution to account for among site variation (α=0.1855). This model was also applied to the GFC (α=0.5939 and I=0.2469) dataset. The TrN model (Tamura and Nei, 1993) with gamma distribution (α = 0.6689) was used for the FIEC dataset, while the FOC dataset utilised the Hasegawa-Kishino-Yano (HKY) model (Hasegawa *et al*., 1985) with a transition/transversion ratio of 2.0693 and a gamma shape parameter of 0.7911. The ML branch supports were estimated using 1 000 bootstrap (mlB) replicates. For each dataset, the BI analysis employed the Metropolis-coupled Markov Chain Monte Carlo search algorithm, running one cold and three heated chains with 2 000 000 generations and saving trees every 100 generations. The BI posterior probabilities (biPP) were calculated after discarding a burnin of 2 500 generations past stationarity, leaving 34 977, 34 989, 34 900, 35 002 trees for *Fusarium*, FIEC, GFC and FOC datasets, respectively, from which consensus trees were calculated.

Morphology and culture characteristics

Based on the results of the TEF barcoding analysis, a subset of 63 *Fusarium* isolates was selected for morphological examination (Table 1). These were grown on full strength PDA (39 g l^{-1} PDA [Biolab Diagnostics]), carnation leaf agar (CLA; 20 g l^{-1} agar with 3-5 mm^2 gamma irradiated carnation leaves placed onto medium [Fischer *et al*., 1982]) and synthetic nutrient agar (SNA; 20 g l^{-1} agar, 1 g l^{-1} KH_2PO_4, 1 g l^{-1} KNO_3, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1} KCl, 0.2 g l^{-1} dextrose (Biolab Diagnostics), 0.2 g l^{-1} sucrose (Biolab Diagnostics), 600 µl l^{-1} 1N NaOH [Nirenberg and O'Donnell, 1998]) for 7-10 days at 25^{o}C under near ultraviolet light. Colony morphology and colour were determined from the PDA plates. Fungal structures produced on CLA and SNA were mounted onto microscope slides and examined using a light microscope (Carl Zeiss, Munich, Germany). The diagnostic traits examined were those proposed by Nelson *et al*. (1983) and Leslie and Summerell (2006). Digital photographs were made using the HRc Axiocam digital camera and AxioVision software (Carl Zeiss).

Results

Fungal isolates

A total of 185 fungi resembling *Fusarium* were isolated from malformed and healthy inflorescences of *S. cordatum* (Table 1). Isolates were collected in four different provinces of South Africa namely, KwaZulu-Natal (88 isolates), Western Cape (58 isolates), Gauteng (21 isolates) and Mpumalanga (18 isolates). The majority of the *Fusarium* isolates (82%) was obtained from malformed plant material, while only 18% were associated with healthy inflorescences. Since the *S. cordatum* trees were scattered and not easily accessible in rural areas, most of the *Fusarium* isolates (85%) were collected from trees in urban areas such as Kwambonambi (70 isolates), Bellville (54 isolates), Pretoria (21 isolates), Richard's bay (8 isolates) and Hartenbos (4 isolates), while 15% were from rural areas such as Pennington (10 isolates), Kripkraal (8 isolates), Buffelskloof (5 isolates), Sudwala (4 isolates) and Graskop (1 isolate).

DNA fingerprinting and cluster analyses

The results for the *in silico* PCR-RFLP analysis of the TEF barcoding region for the 59 *Fusarium* reference strains indicated the presence of one to six restriction sites for the various enzymes (Table 3). However, none of the evaluated restriction enzymes allowed for differentiation of the reference species using agarose electrophoresis. The reason was that the recognition sites within the target sequences were not sufficiently polymorphic and many different *Fusarium* species would have the same fingerprint. In such instance, *Fusarium* species would have been distinguishable only if combinations of several different endonucleases were utilised. Due to the cost and effort associated with this multiple enzyme TEF-PCR-RFLP approach, its application for generating large scale species inventories was considered not feasible. In contrast, the IGS-PCR-RFLP analysis of the 36 *Fusarium* reference species with the *Hae*III and *Hha*I restriction enzymes indicated the presence of an average of five restriction sites in every 2.5 kb IGS amplicon. Although there were a few exceptions for either one of the two RFLP data sets (e.g. identical profiles were generated for the two strains of *F. globosum* when digested with *Hae*III; for the two strains of *F. poae* digested with same enzyme; and for the *F. longipes* strains digested with *Hha*I), both enzymes clearly resolved all of the reference species included in this study (see Fig. 3 and 4).

Therefore, all of the 185 isolates obtained from the *S. cordatum* inflorescences were subjected to IGS-PCR-RFLP analysis with these two restriction enzymes.

Depending on the isolate, the digestion of the IGS amplicon with *Hae*III resulted in four to eight fragments of *ca*. 100-900 bp, while restriction with *Hha*I gave two to seven fragments in the same size range. Restriction fragments shorter than 100 bp were not taken into consideration as they were not clearly resolved by electrophoresis. UPGMA analysis of the two sets of profiles indicated that the *Hae*III fingerprints were more complex than those for *Hha*I. On average, more clusters representing groups of isolates with unique PCR-RFLP profiles or single-isolate unique taxa (118 in total) were found among the *Hae*III profiles (Fig. 3), while 100 of these clusters or single-isolate taxa were found among the *Hha*I patterns (Fig. 4). However, even though reference strains were included in these analyses, the isolates could not be identified based on IGS-PCR-RFLP analyses as the profiles of very few reference strains matched those of the isolates from *S. cordatum* (e.g. the *Hae*III profiles for isolates CMWF 990 and CMWF 1000 were identical to that of the *F. proliferatum* reference isolate MRC 8550).

TEF barcoding

A total number of 52 unique sequences for the TEF barcoding region were identified amongst the 185 isolates obtained from the *S. cordatum* inflorescences. Within the 810-nucleotide alignment for this region, the percentage similarity based on the number of nucleotides shared between pairs of isolates ranged from 20-100%. This corresponds to 165-0 nucleotide differences in the aligned TEF sequences of the 185 isolates associated with *S. cordatum* inflorescences.

BLAST comparisons of the TEF barcodes to those in the FID revealed sequence similarity values ranging from 89% to 100% (Table 1) to 21 different *Fusarium* species/lineages. The TEF sequences for only 21% of the isolates were 100% similar to those of known species/isolates in the FID, suggesting that the remaining 79% represent either intraspecific or novel diversity. Therefore, a large proportion of the isolates obtained from *S. cordatum* could not be identified unambiguously.

Based on the results of the FID BLAST searches, the majority of the isolates formed part of the FIEC (55%), followed by FOC (23%) and GFC (18%), with the remaining 4% being represented by various groups (Table 1; Fig. 5). Among the 185 isolates examined, most displayed 90-100% TEF sequence similarity to the FID entries of *F. oxysporum* (42 isolates), *F.* sp. cf. *equiseti* (38 isolates), *F. pallidoroseum* (30 isolates), *F.* sp. cf.

bullatum (28 isolates) and *F. proliferatum* (14 isolates). Although, the majority of the *Fusarium* isolates was obtained from malformed plant material, the isolates with TEF sequences similar to *F. pallidoroseum*, *F.* sp. cf. *bullatum*, *F.* sp. cf. *equiseti*, *F. proliferatum* and *F. fujikuroi* were also isolated from healthy inflorescences. Amongst our samples, only isolates with TEF sequence similarity to *F. pallidoroseum*, *F.* sp. cf. *bullatum* and *F. fujikuroi* were found in both urban and rural areas.

Putative taxonomic affinities were allocated to each IGS-PCR-RFLP type based on TEF sequence similarity. This revealed that multiple PCR-RFLP profiles were associated with a single TEF barcode entry. For example, 6 *Hae*III and 4 *Hha*I profiles were revealed for 14 putatively identified *F. proliferatum* isolates (Fig. 3 and 4). The opposite was also observed, for example, a single IGS-PCR-RFLP profile was associated with multiple putative species such as *F. pallidoroseum*, *F.* sp. cf. *bullatum*, *F.* sp. cf. *equiseti*. This was more prominent for the *Hha*I results (Fig. 4), which displayed 15 of these "mixed" terminal taxa, while *Hae*III had only 11. The fact that numerous TEF barcode entries were associated with multiple profiles thus suggested a high level of genetic diversity among the putative representatives of *F. pallidoroseum*, *F.* sp. cf. *bullatum*, *F.* sp. cf. *equiseti* and *F. oxysporum* (Fig. 3 and 4).

Phylogenetic analyses

The aligned TEF dataset for phylogenetic analyses of the genus *Fusarium* consisted of 185 *Fusarium* sequences obtained during this study, 59 sequences for the reference strains and an additional 22 sequences obtained from GenBank and/or the FID. The results of the analyses separated the *Fusarium* isolates associated with *S. cordatum* inflorescences into eight distinct groups, designated I to VIII (Fig. 6). These groups were represented by isolates related to species/lineages in the FIEC (I), GFC (VI), FOC (VII), the *F. graminearum* complex (II and III) and the *F. chlamydosporum*/*F. nelsonii* clade (V), while single isolates were closely related to *F. compactum* (IV) and a clade containing *F. negundis*, *F. tricinctum*, and *F. avenaceum* (VIII). To examine the relationships within these groups more closely, separate phylogenies were inferred for each clade. Within these groups (I, VI, VII), individual taxa that potentially correspond to distinct species were recognised as the smallest, mutually exclusive and well-supported (≥80 mlB and ≥0.95 biPP) groups of isolates.

Phylogenetic analyses of the FIEC indicated considerable variation among the isolates obtained from *S. cordatum* as they grouped into at least 34 mutually exclusive

clades. Of these, 28 (FIEC 1-28) probably represent novel species/lineages (Fig. 7). Known representatives of *F. equiseti* were included in seven clades of which four included isolates from *S. cordatum*. Isolates CMWF 971 and CMWF 988 formed a mutually exclusive well-supported group with the *F. equiseti* reference strain MRC 8421 (100% mlB; 1.00 biPP), while 15 other isolates grouped with the FID voucher strain NRRL 25795 and *F. equiseti* 45.1.2.1 obtained from GenBank (99% mlB; 1.00 biPP). Isolate CMWF 909 grouped with *F. equiseti* strain VI01087 (Fig. 7) that was isolated from wheat in Norway (Kristernsen *et al*., 2005), while two isolates grouped with *F. equiseti* strain 28/3.2.1 that was obtained from *Ammophila arenaria* ssp. *australis* (Maciá-Vicente *et al*., 2008). In addition, isolate CMWF 1154 was closely related to *F.* sp. cf. *scripi*, while isolate CMWF 1058 grouped with *F.* sp. cf. *camptoceras* NRRL 13381 (94% mlB; 1.00 biPP). None of the isolates from *S. cordatum* formed mutually exclusive groups with the representatives of *F. semitectum* (MRC 6715; MRC 3228), *F.* sp. cf. *bullatum*, *F. pallidoroseum* and *F. incarnatum*.

A high level of species richness among the isolates obtained from *S. cordatum* was also observed for the GFC (Fig. 8). Although monophyletic "African" and "Asian" clades (O'Donnell *et al*., 1998a, 2000b) were not recovered due to a lack of sufficient phylogenetic information in the TEF gene region alone (Geiser et *al*., 2005), the highest diversity was observed among the so-called "African clade" isolates. All of the nine "African clade" isolates (GFC-1 to GFC-5 and GFC-8) appeared to represent novel taxa. However, GFC-1 is closely related to the, as yet undescribed, GFC species/lineage represented by isolate NRRL 25615 that was isolated from rice seeds in Nigeria (O'Donnell *et al*., 2000b), GFC-2 is closely related to *F. verticillioides*, and GFC-8 is closely related to *F. dlamini*. GFC-3 includes isolate NRRL 26793 and GFC-8 includes isolates NRRL 26061 and NRRL 26152, all of which were isolated from *Striga hermontica* in various African countries (O'Donnell *et al*., 2000b). Similarly, the five so-called "American clade" isolates also probably represent novel taxa (GFC-6 and GFC-7), although GFC-6 includes the known isolates NRRL 26756 and NRRL 26757 that were obtained from ornamental grass and reed in South Africa (O'Donnell *et al*., 2000b). Most of the GFC isolates obtained from *S. cordatum* (19 isolates) formed part of the so-called "Asian clade" where they formed mutually exclusive groups with the representatives of *F. proliferatum* (99% mlB; 1.00 biPP), *F. fujikuroi* (89% mlB; 0.99 biPP) and *F. sacchari* (100% mlB; 1.00 biPP).

Phylogenetic analyses of the FOC data set revealed that the *F. oxysporum* isolates from *S. cordatum* are highly diverse (Fig. 9). However, resolution and significant statistical support for the four known clades in this species complex (O'Donnell *et al*., 2004a; Fourie *et*

al., 2009) were absent due to a lack of sufficient polymorphic sites in the TEF region alone (O'Donnell *et al.*, 2004a; Bogale *et al*., 2006; Fourie *et al*., 2009). Nevertheless, a total of 29 isolates from *S. cordatum* were associated with strains that are pathogenic to plants and humans and known to be part of Clade C of the FOC (O'Donnell *et al*., 2004a; Fourie *et al.*, 2009). Thirteen isolates were related to Clade B strains that are known to be pathogens of plants such as melon, banana and dianthus, as well as non-pathogenic strains isolated from South African and Ethiopian soils (Bogale *et al*., 2006; Fourie *et al*., 2009). None of the *F. oxysporum* isolates obtained from *S. cordatum* were related to the known strains in Clades A and D.

Collectively, the results of these phylogenetic analyses revealed a large number of putative species among the isolates obtained from healthy and malformed *S. cordatum* inflorescences. If each of the various clades and unique lineages identified here are considered, our collection of 185 isolates represents no less than 52 putative species. At least 34 putative species were identified in the FIEC (Fig. 7), eleven in the GFC (Fig. 8), two lineages in the FOC (Fig. 9) as well as at least one putative species related to each of the *F. chlamydosporum*/*F. nelsonii* clade, *F. compactum*, the *F. tricinctum*/*F. avenaceum* clade and two in the *F. graminearum* complex (Fig. 6). However, confirmation of these findings requires improved species representation and analyses of additional DNA sequence information.

Morphology and culture characteristics

Among the 63 isolates obtained from *S. cordatum*, that were selected for microscopic examination (Table 1), eight morphological groups were observed based on macro- and microconidial morphology, shape of conidiogenous cells and presence/absence of chlamydospores. The FIEC representatives were separated into three morphological groups, namely those that resemble *F. semitectum*, *F. equiseti* and *F. camptoceras* following the descriptions in Nelson *et al.* (1983). Although TEF barcoding did not reveal any *F. semitectum* entries among our isolates associated with *S. cordatum*, many of the examined isolates (CMWF 897, CMWF 901, CMWF 905, CMWF 931, CMWF 947, CMWF 961, CMWF 986, CMWF 1024, CMWF 1028 and CMWF 1054) displayed morphological characters distinctive of this species (Nelson *et al*., 1983). These traits were: light orange to brown pigment production; slightly curved macroconidia with 3–5 septa (Fig. 10a); the presence of mesoconidia in the aerial mycelium giving the typical "rabbit ears" appearance (Fig. 11a); the presence of mono- and polyphialides and the production of chlamydospores

in certain isolates (*i.e.*, CMWF 986 and CMWF 1028). Putative *F. equiseti* isolates (*i.e.*, CMWF 892, CMWF 951, CMWF 967, CMWF 991, CMWF 1032, CMWF 1033 and CMWF 1049) produced white to tan cultures. Their macroconidia were slender and long, with a whip-like apical cell and distinct foot cell (Fig. 10b). Chlamydospores were present only in isolate CMWF 967. Colonies of putative *F. camptoceras* isolates were cream in colour and their macroconidia were squat and falcate (Fig. 10c). While their microconidia were scarce in number, numerous mesoconidia were produced from polyphialides in aerial mycelia.

Cultures of the GFC displayed mycelium colours that varied from white to intense violet. The isolates could be separated into two major groups: those that produced microconidia from mono- and polyphialides (Fig. 11e, f) only in false heads (Fig. 11c), thus resembling *F. subglutinans* (*i.e.*, CMWF 894, CMWF 942, CMWF 954, CMWF 955, CMWF 956, CMWF 1010, CMWF 1029) and those that also produce chains (Fig. 11d) with morphological characters typical of *F. proliferatum* (*i.e.*, CMWF 924, CMWF 948, CMWF 958, CMWF 976, CMWF 980, CMWF 988, CMWF 992, CMWF 1000, CMWF 1133, CMWF 1182). Generally, macroconidia were scarce among these isolates, while oval and single-celled microconidia were observed in most of the isolates examined in this complex (Fig. 10e, f).

Isolates CMWF 1023 and CMWF 1051 displayed morphological traits of *F. chlamydosporum.* These strains produced a burgundy pigment in the agar. Their macroconidia were rare, moderately curved with 3–5 septa, while their microconidia were abundant (Fig. 10g), single-celled and borne on polyphialides in aerial mycelium (Fig. 11b). Large numbers of rough-walled chlamydospores were produced in chains in these isolates (Fig. 11h). In addition, examination of representatives of the *F. graminearum* complex revealed that isolates CMWF 933 and CMWF 1140 formed a carmine red pigment in PDA, while their mycelium was yellow to red-brown. Their macroconidia were thick-walled, slightly curved and 5–6-septate, while microconidia and chlamydospores were not observed. Finally, the FOC cultures (isolates CMWF 904, CMWF 921, CMWF 927, CMWF 968, CMWF 1002, CMWF 1014, CMWF 1021, CMWF 1030, CMWF1034, CMWF 1053 and CMWF 1057) produced white to pale violet mycelium with dark violet pigment. Their macroconidia were almost straight and usually 3-septate (Fig. 10d), microconidia were borne on short monophialides in false heads (Fig. 11g) and chlamydospores were abundant (Fig. 11i).

Discussion

Indigenous ecosystems generally harbour considerably greater fungal diversity than agricultural environments (e.g. Hawksworth and Rossman, 1997; Anderson *et al*., 2003; Leslie *et al*., 2004; Crous *et al.*, 2006; Kvas *et al*., 2009). This is also true for South Africa where indigenous systems are thought to contain the majority of the country's predicted fungal diversity. This suggests that an estimated 12% of the global fungal diversity is associated with a mere 10% of the world's plant diversity (Crous *et al*., 2006). Nevertheless, our knowledge of the diversity of *Fusarium* and other fungi in South Africa is largely based on agricultural crops and relatively little is known regarding fungal diversity associated with native plants in regions that have not been exploited by humans. Therefore, following the assumption that the native South African flora is rich in fungal diversity, this study focused on the *Fusarium* diversity associated with *S. cordatum* inflorescences. Interestingly, the results revealed that this unique niche harbours at least 52 putative species of *Fusarium*, of which about 36 (69%) are novel taxa. This estimate is relatively conservative, as previous studies on *Fusarium* have shown that the application of multigene genealogies with improved representation per putative species would undoubtedly reveal many additional cryptic species/lineages (e.g. O'Donnell, 2000; O'Donnell *et al*., 1998a, 2000a, 2000b, 2004b; Steenkamp *et al*., 2000, 2001, 2002).

In an attempt to develop a cost effective approach for clustering similar isolates on a large scale, we evaluated two PCR-RFLP procedures. *In silico* RFLP analyses of the TEF barcoding region for a set of reference species revealed that the conserved nature of this region limits its potential application to differentiate between species based on PCR-RFLP analysis, even when multiple restriction endonucleases are utilised. The more variable IGS locus was, therefore, used for this purpose, as it has been previously used in characterising *Fusarium* species either based on PCR-RFLP analyses (e.g. Edel *et al*., 2001; Mirete *et al*., 2003; Llorens *et al*., 2006) or using direct sequence analyses (e.g. O'Donnell *et al*., 2004a; Schweigkofler *et al*., 2004; Fourie *et al*., 2009). Results of the present study using IGS-PCR-RFLP and UPGMA cluster analyses revealed a high degree of genetic variation among the examined isolates from *S. cordatum*, with 118 *Hae*III-based and 100 *Hha*I-based unique profiles. However, despite the ability to cluster this unexpected diversity, most isolates could not be identified to the species level based solely on this technique. This was most likely due

to high levels of intraspecific variability, as well as an overall lack of appropriate reference strains to allow for meaningful comparisons.

DNA barcoding has recently emerged as a rapid and accurate means of identifying specimens. The identification accuracy using this approach is strongly dependent on well-populated and publicly available databases that contain reliable information for taxonomically correctly identified voucher specimens (Nilsson *et al*., 2006). However, few public domain databases satisfy both of these requirements. Databases such as GenBank (http://www.ncbi.nlm.nih.gov/nucleotide), DDBJ (DNA Data Bank of Japan; http://www.ddbj.nig.ac.jp) and EMBL (European Molecular Biology Laboratory; http://www.ebi.ac.uk/embl) include significant numbers of sequences that are linked to wrongly identified taxa (Nilsson *et al*., 2006), while quality and submission controlled databases such as the FID (*Fusarium* Identification Database; Geiser *et al*., 2004; http://isolate.fusariumdb.org/index.php), *Trich*OKey (Druzhinina *et al*., 2005; http://www.isth.info) and UNITE (Kõljalg *et al*., 2005; http://unite.ut.ee) are not yet well populated. Under-representation in the FID database impacted negatively on this study as the TEF barcode comparisons against this database allowed identification of only 37 isolates with high level of certainty (Table 1). Among all 185 isolates, these were the only isolates with TEF barcode sequences that were identical to those in the FID. Clearly, additional evidence is required for conclusive species diagnoses, as it is not possible to exclude the possibility that related but distinct species have identical TEF sequences. Therefore, until DNA barcoding databases are more commonly utilised and their taxon representation has been considerably improved, DNA barcoding alone will rarely provide unambiguous identifications for *Fusarium* species.

Of the three DNA-based identification approaches used in this study (*i.e.* IGS-PCR-RFLP analysis, FID-assisted TEF DNA barcode analysis and TEF-based phylogenetic analyses), phylogenetic analyses were most efficient for identifying taxa that potentially correspond to unique *Fusarium* species. Apart from the fact that the IGS-PCR-RFLP analyses revealed a great diversity among the isolates studied, these data were not particularly useful for species identifications. To some extent, this was also true for the FID-assisted TEF DNA barcode analysis. In fact, the latter approach could, in some cases, have resulted in incorrect identifications if the top BLAST results were interpreted as species identifications without consideration of the degree of sequence similarity. For example, the DNA barcode analysis of isolates CMWF 1165 and CMWF 892 showed that the TEF sequences of these isolates were only 90% similar to those of *F.* sp. cf. *equiseti* (Table 1),

while they actually represent a novel species (FIEC-26; Fig. 7) related to *F.* sp. cf. *camptoceras*. This is despite the fact that the FID includes a voucher *F.* sp. cf. *camptoceras* entry. Similar discrepancies were also apparent in cases where the sequences of the TEF barcodes were highly similar to those in the FID. For example, the top FID BLAST result for isolates CMWF 942 and CMWF 1038 were both *F. udum* (97.69% and 98.3% sequence similarity, respectively; Table 1), but based on the results of the phylogenetic analyses, both isolates probably represent novel species (GFC-4 and GFC-5; Fig. 8). Furthermore, the TEF sequences of some *Fusarium* species are not yet available in the FID. For example, isolates within *F. chlamydosporum*/*F. nelsonii* clade V (Fig. 6), were misidentified as *F. nelsonii* based on barcoding results due to the lack of *F. chlamydosporum* representatives in the FID. Although these isolates most probably represent novel species, they were morphologically similar to *F. chlamydosporum*. These results thus highlight an important caveat associated with sequence similarity-based interpretations of DNA barcode data (DeSalle *et al*., 2005; Will *et al*., 2005). Furthermore, they emphasise the fact that DNA barcoding studies should include analyses that take into account the actual phylogenetic information associated with the specific nucleotide differences (reviewed by Frézal and Leblois, 2008).

Other important considerations for interpretation of DNA barcoding data pertain to issues of species concepts and species recognition (e.g. Will *et al*., 2005; Elias *et al*., 2007; Frézal and Leblois, 2008). According to Coyne and Orr (2004), a species concept is a general idea that can be applied to delimit, identify and classify species. Of the numerous species concepts that have been proposed (reviewed by Mayden, 1997), *Fusarium* identifications generally rely on the morphological, biological and phylogenetic species concepts, of which the latter is most informative (reviewed by Kvas *et al*., 2009). Although the genealogical concordance phylogenetic species recognition approach, proposed by Taylor *et al*. (2000), has been used to identify and delineate some *Fusarium* species (O'Donnell *et al*., 2000a, 2004b, 2008; Steenkamp *et al*., 2002), many species have been characterised mainly in terms of Nixon and Wheeler's (1990) diagnostic approach of the phylogenetic species concept (O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998a, 2000b). In the current study, a similar approach was applied to identify the *Fusarium* species isolated from *S. cordatum* inflorescences, since a species was recognised as the smallest, mutually exclusive, monophyletic aggregation of isolates that can be diagnosed using a unique set of characters as reflected by well-supported branches (≥80 mlB and ≥0.95 biPP) in the TEF-based phylogenetic trees. However, the potential problems associated with using single gene data for inferring species relationships are well-documented (e.g. Maddison,

1997; Rosenberg, 2002; Sanderson and Shaffer, 2002). Further characterisation and formal description of the putative species identified here will, therefore, require the analyses of additional genomic loci.

Most previous studies aimed at understanding the diversity of *Fusarium* species associated with a particular niche have employed conventional morphology-based identifications (e.g. Burgess and Trimboli, 1986; Nelson *et al.*, 1987; Burgess *et al*., 1988; Jeschke *et al.*, 1990; Burgess and Summerell, 1992; Sangalang *et al*., 1995; James, 2004). For example, surveys of *Fusarium* species associated with plant debris (mostly agricultural) in South Africa revealed the presence of species such as *F. armeniacum*, *F. camptoceras*, *F. compactum*, and the then undescribed species *F. polyphialidicum*, *F. nelsonii* and *F. dlamini* (Marasas *et al*., 1985, 1987, 1988, 1998). However, due to the general lack of diagnostic morphological traits for species of this genus (Leslie and Summerell 2006), these previous studies are likely to have underestimated the true fungal diversity. In the current study, only about a third of the isolates were subjected to morphological examination. This was done as part of a "reverse taxonomy" procedure (Markmann and Tautz, 2005) in order to confirm that the morphological and cultural characteristics of the isolates correspond to their TEF barcode-based identities. These isolates represented 14 distinct FID TEF entries and formed part of 31 separate TEF-based phylogenetic clades potentially representing distinct species. As expected, examination of these isolates revealed only eight morphological groups using the Nelson *et al.* (1983) classification system. Thus, if the *Fusarium* isolates obtained in this study had been identified solely based on morphological traits, a huge diversity of *Fusarium* species would have been overlooked and the majority of the identifications would not have been correct.

A large proportion (55%) of the *Fusarium* isolates obtained from *S. cordatum* inflorescences formed part of the FIEC (Fig. 5). However, the taxonomy and phylogeny of this assemblage of species is still essentially unresolved and species boundaries have not been revised according to DNA-based information. For example, *F. pallidoroseum* and *F. incarnatum* are recognised as synonyms of *F. semitectum* (Leslie and Summerell, 2006), but based on the results of TEF-based phylogenetic analyses, these species are distinct (Fig. 7). Also, individual reference strains for *F. equiseti* do not group together and are separated into several non-related clades (Fig. 7). Nevertheless, the FIEC isolates obtained from *S. cordatum* probably represent at least 34 species and not 5 species as implied by the FID-assisted barcode analysis (Table 1; Fig. 7). Among these, only three mutually exclusive clades are likely to correspond to the known species, *i.e.*, *F. scirpi*, *F. camptoceras* and *F.*

equiseti. The known members of the FIEC are well known saprophytes, but are also occasionally known as weak or opportunistic plant pathogens (see Leslie and Summerell, 2006). The FIEC isolates included in this analysis could, therefore, be saprophytes that possibly invaded the tissue after the onset of malformation. However, certain isolates of FIEC-1, 4, 8, 13, 14 and 16 were obtained from healthy *S. cordatum* tissue, suggesting they could also be endophytes.

The second most common *Fusarium* group isolated from *S. cordatum* inflorescences was that representing the FOC (Fig. 5). This species complex includes numerous economically important plant pathogens with wide host and geographic ranges (Booth, 1971; Armstrong and Armstrong, 1981; Nelson *et al*., 1981; Gordon and Martyn, 1997, Kistler, 1997, 2000; O'Donnell and Cigelnik, 1999; Baayen *et al*., 2000; Leslie and Summerell, 2006). Although, *F. oxysporum* was considered a single species in the past (Snyder and Hansen, 1940; Booth, 1971; Nelson *et al*., 1983), contemporarily it is a genetically diverse complex composed of morphologically indistinguishable species (Waalwijk *et al*., 1996; O'Donnell and Cigelnik, 1997; Kistler, 1997; Baayen *et al*., 2000; Leslie and Summerell, 2006; Fourie *et al*., 2009) remains unresolved. Isolates representing this complex reside in four major clades (O'Donnell *et al*., 2004a; Fourie *et al*., 2009), each sub-divided into many distinct phylogenetic lineages (O'Donnell and Cigelnik, 1999; Baayen *et al*., 2000; O'Donnell *et al*., 2004a; Fourie *et al*., 2009). Diverse *F. oxysporum* isolates obtained from *S. cordatum* grouped with various known taxa from Clades B and C, where a significant number are associated with known pathogenic isolates or *formae speciales* (Fig. 9). Further studies should consider whether *F. oxysporum* isolates from *S. cordatum* are pathogenic to this host and whether they might have the capacity to induce floral malformation.

Several species/lineages of the GFC were recovered from malformed *S. cordatum* inflorescences. This species complex includes numerous plant pathogens and mycotoxin producers (reviewed by Kvas *et al*., 2009). In addition to the isolates that probably represent novel species/lineages, some isolates from *S. cordatum* are possibly conspecific with *F. proliferatum*, *F. fujikuroi* and *F. sacchari*, all of which are known phytopathogens. *Fusarium proliferatum* has a wide host range (see Leslie and Summerell, 2006), while *F. sacchari* causes the pokkah boeng disease on sugar cane (Gerlach and Nirenberg, 1982). Similarly, *F. fujikuroi* causes bakane disease of rice (Leslie and Summerell, 2006) and is known to produce the plant hormones, gibberellins, which are responsible for the abnormal development of rice shoots (Sun and Snyder, 1981). Such a compound could potentially be involved in the abnormal development of *S. cordatum* inflorescences, suggesting that these

putative *F. fujikuroi* isolates should be tested for their ability to induce malformation symptoms on *S. cordatum* inflorescences. Interestingly, both *F. proliferatum* and *F. sacchari* have been reported from malformed inflorescences of mango (Leslie, 1995; Britz *et al*., 2002; Marasas *et al*., 2006; Lima *et al*., 2009). Although *F. subglutinans* has been reported from malformed *S. cordatum* inflorescences (Crookes and Rijkenberg, 1985), none of the isolates examined in this study are conspecific with this species. However, because many species in the GFC resemble *F. subglutinans* morphologically, the isolates identified in 1985 by Crookes and Rijkenberg could represent any of the five species found in the current study *i.e.* GFC-1, GFC-3, GFC-6, GFC-7 and *F. sacchari*.

Based on the geographic origins of the host plants or substrates of species in the GFC, the three clades of this complex have been designated as the so-called "African", "Asian" and "American" clades (O'Donnell *et al*., 1998a, 2000b). To some extent, the results of the present study support this hypothesis as six of the putative species identified are part of "African" clade, while only two and three putative species were identified from the "American" and "Asian" clades, respectively. However, the occurrence of the "American" *Fusarium* species GFC-6, that includes the strains NRRL 26756 and NRRL 26757, and GFC-7, is inconsistent with this hypothesis. Interestingly, the strains were isolated from plants in South Africa (O'Donnell *et al*., 2000b) and have been recognised as an exception to the phylogeographic hypothesis for a decade. Most likely, further exploration of African indigenous ecosystems will reveal numerous fungi native to the continent, which will aid us to support or reject the biogeographic hypothesis for the GFC.

Based on morphology and the FID-assisted TEF barcode analysis, four isolates from *S. cordatum* were associated with *F. chlamydosporum*/*F. nelsonii*. The former species is a well known saprophyte and inhabitant of soils, but has also been reported to cause damping-off of South African indigenous rooibos plants (Engelbrecht *et al*., 1983) and blight of kangaroo paw (Satou *et al*., 2001). *Fusarium nelsonii* was isolated from plant debris in South African soil (Marasas *et al*., 1998). The FID-assisted TEF barcode analysis showed that the four isolates were similar to *F. nelsonii* (Table 1; Fig. 6), while they displayed morphological traits typical of *F. chlamydosporum*. However, the results of the TEF-based phylogenetic analysis indicated that they most likely represent a unique and novel species (Clade V; Fig. 6). Their sampling location in Pretoria was the only area where this species was encountered. *Syzygium cordatum* trees planted in this area are surrounded by many other ornamental herbaceous and woody hosts. It is possible that this fungus originated from plants growing nearby and was thus an opportunistic or secondary invader on *S. cordatum*.

Amongst the less commonly isolated *Fusarium* species encountered in this study, two isolates (*i.e.*, CMWF 933 and CMWF 1140) appeared to form part of the so-called *F. graminearum* complex (O'Donnell *et al*., 2000a, 2004b). Species/lineages of this complex (O'Donnell *et al*., 2000a, 2004b, 2008; Starkey *et al*., 2007) are known to occur worldwide on cereal grains and cause destructive head blight epidemics (Cook, 1981; Goswami and Kistler, 2004). It is thus possible that these isolates associated with *S. cordatum* inflorescences originate from the surrounding agricultural crops in the Pennington (South Africa) area, as has been proposed for the presence of *F. graminearum* on Canadian wild grasses (Inch and Gilbert, 2003). In addition, a single isolate obtained from *S. cordatum* (*i.e.*, CMWF 1178), was closely related to *F. compactum* (clade IV; Fig. 6), while isolate CMWF 1136 was closely related to a group of species that includes *F. avenaceum*, *F. tricinctum* and *F. negundis* (clade VIII; Fig. 6). *Fusarium compactum* is commonly isolated from grasslands and desert soils (Marasas *et al*., 1988; Burgess and Summerell, 1992) and has also been associated with some plant diseases, although it is generally considered as saprophyte (Leslie and Summerell, 2006). *Fusarium avenaceum* is known to cause plant diseases, *F. tricinctum* is typically a saprophyte and little is known regarding *F. negundis* (Leslie and Summerell, 2006). Whether the isolates from *S. cordatum* represent members of these existing species or new species remains to be determined.

In order to develop an operational procedure for generating inventories of *Fusarium* species in indigenous South African ecosystems, three DNA-based techniques were evaluated. Although all three approaches revealed that the isolates examined are highly diverse, only the TEF-based phylogenetic analyses generated unambiguous results. The most appropriate steps, following the sample collection and DNA isolation, for cataloguing the diversity of *Fusarium* species in a specific environment are: PCR amplification of the TEF barcoding region, FID-assisted analysis of the barcodes to determine the isolates' species complex/group affinity, and lastly phylogenetic analyses of TEF region, with appropriate reference sequences, to establish evolutionary relationships among the isolates. Each of the resulting mutually exclusive and well-supported phylogenetic groups of isolates would, in turn, represent feasible hypotheses to be tested in subsequent taxonomic studies aimed at characterising and describing the novel taxa. By following this approach, the exploration of indigenous South African ecosystems will undoubtedly lead to the discovery of numerous new *Fusarium* species. Ultimately, we should be able to improve our understanding of the boundaries between species and to develop strongly supported hypotheses regarding the

evolution of the genus as a whole, as well as the origins of important traits in specific groups or species.

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Table 1. Origin and putative identities of the *Fusarium* isolates from *Syzygium cordatum* used in this study

Culture No.[a]	FID-based identity[b]	Percentage sequence similarity[c]	Phylogeny-based identity[d]	Substratum[e]	Geographic origin[f]
CMWF 1140[c]	*F. brasilicum*	98.75% (636/644)	III	MI	Pennington, KZN
CMWF 962	*F. bulbicola*	98.25% (618/629)	GFC-7	MI (1)	Sudwala, MP
CMWF 902	*F. fujikuroi*	97.65% (625/640)	GFC- *F. fujikuroi*	HI	Kwambonambi, KZN
CMWF 924	*F. fujikuroi*	97.65% (625/640)	GFC- *F. fujikuroi*	MI (2)	Kwambonambi, KZN
CMWF 936	*F. fujikuroi*	97.65% (625/640)	GFC- *F. fujikuroi*	FB	Pennington, KZN
CMWF 933	*F. meridionale*	100.00% (644/644)	II	MI	Pennington, KZN
CMWF 1136	*F. negundis*	91.56% (597/652)	VIII	MI	Bellville, WCP
CMWF 974	*F. nelsonii*	96.13% (622/647)	V	MI (3)	Pretoria, GP
CMWF 975	*F. nelsonii*	96.41% (645/669)	V	MI	Pretoria, GP
CMWF 1023	*F. nelsonii*	96.41% (645/669)	V	MI	Pretoria, GP
CMWF 1051	*F. nelsonii*	96.41% (645/669)	V	MI (3)	Pretoria, GP
CMWF 1001	*F. oxysporum*	99.53% (646/649)	FOC- Clade C	MI (5)	Bellville, WCP
CMWF 1003	*F. oxysporum*	100.00% (650/650)	FOC- Clade C	PMI (6)	Bellville, WCP
CMWF 1012	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	PMI (6)	Bellville, WCP
CMWF 1013	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	PMI (6)	Bellville, WCP
CMWF 1014	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (5)	Bellville, WCP
CMWF 1030	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	PMI (7)	Bellville, WCP
CMWF 1034	*F. oxysporum*	99.84% (649/650)	FOC- Clade C	PMI (6)	Bellville, WCP
CMWF 1036	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	PMI (7)	Bellville, WCP
CMWF 1040	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (5)	Bellville, WCP
CMWF 1041	*F. oxysporum*	100.00% (650/650)	FOC- Clade C	MI (6)	Bellville, WCP
CMWF 1043	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (7)	Bellville, WCP
CMWF 1045	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	PMI (6)	Bellville, WCP
CMWF 1046	*F. oxysporum*	100.00% (649/649)	FOC- Clade B	PMI (6)	Bellville, WCP
CMWF 1048	*F. oxysporum*	100.00% (650/650)	FOC- Clade C	PMI (6)	Bellville, WCP
CMWF 1053	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	PMI (6)	Bellville, WCP
CMWF 1055	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (7)	Bellville, WCP
CMWF 1150	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (5)	Bellville, WCP
CMWF 1168	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (4)	Bellville, WCP
CMWF 1175	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (5)	Bellville, WCP
CMWF 1176	*F. oxysporum*	99.84% (648/649)	FOC- Clade C	MI	Bellville, WCP
CMWF 904	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (8)	Bellville, WCP
CMWF 907	*F. oxysporum*	100.00% (649/649)	FOC- Clade B	HI (9)	Bellville, WCP
CMWF 911	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (10)	Bellville, WCP

Table 1 (continued). Origin and putative identities of the *Fusarium* isolates from *Syzygium cordatum* used in this study

Culture No.[a]	FID-based identity[b]	Percentage sequence similarity[c]	Phylogeny-based identity[d]	Substratum[e]	Geographic origin[f]
CMWF 940	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI	Bellville, WCP
CMWF 979	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (7)	Bellville, WCP
CMWF 985	*F. oxysporum*	100.00% (649/649)	FOC- Clade C	MI (5)	Bellville, WCP
CMWF 1017	*F. oxysporum*	99.84% (648/649)	FOC- Clade B	MI (12)	Kwambonambi, KZN
CMWF 1019	*F. oxysporum*	99.53% (646/649)	FOC- Clade B	MI (13)	Kwambonambi, KZN
CMWF 1021	*F. oxysporum*	98.61% (640/649)	FOC- Clade B	MI (13)	Kwambonambi, KZN
CMWF 1057	*F. oxysporum*	100.00% (525/525)	FOC- Clade B	PMI (12)	Kwambonambi, KZN
CMWF 1162	*F. oxysporum*	99.84% (648/649)	FOC- Clade B	MI (11)	Kwambonambi, KZN
CMWF 921	*F. oxysporum*	100.00% (650/650)	FOC- Clade C	PMI (14)	Kwambonambi, KZN
CMWF 926	*F. oxysporum*	100.00% (650/650)	FOC- Clade C	MI (15)	Kwambonambi, KZN
CMWF 927	*F. oxysporum*	100.00% (650/650)	FOC- Clade C	MI (15)	Kwambonambi, KZN
CMWF 937	*F. oxysporum*	100.00% (649/649)	FOC- Clade B	MI	Kwambonambi, KZN
CMWF 938	*F. oxysporum*	98.48% (648/658)	FOC- Clade C	MI (16)	Kwambonambi, KZN
CMWF 957	*F. oxysporum*	99.69% (650/652)	FOC- Clade C	MI (17)	Kwambonambi, KZN
CMWF 981	*F. oxysporum*	98.72% (618/626)	FOC- Clade B	MI (13)	Kwambonambi, KZN
CMWF 983	*F. oxysporum*	99.68% (632/634)	FOC- Clade B	MI (13)	Kwambonambi, KZN
CMWF 1002	*F. oxysporum*	99.84% (648/649)	FOC- Clade B	MI (19)	Pretoria, GP
CMWF 968	*F. oxysporum*	100.00% (649/649)	FOC- Clade B	MI (18)	Pretoria, GP
CMWF 1181	*F. oxysporum*	100.00% (649/649)	FOC- Clade B	MI (20)	Richards bay, KZN
CMWF 1132	*F. pallidoroseum*	94.76% (615/649)	FIEC-6	MI (21)	Buffelskloof, MP
CMWF 1158	*F. pallidoroseum*	95.36% (597/626)	FIEC-3	MI (21)	Buffelskloof, MP
CMWF 959	*F. pallidoroseum*	93.51% (577/617)	FIEC-3	MI (22)	Buffelskloof, MP
CMWF 1028	*F. pallidoroseum*	96.29% (623/647)	FIEC-8	MI (23)	Hartenbos, WCP
CMWF 1031	*F. pallidoroseum*	95.04% (614/646)	FIEC-5	MI (23)	Hartenbos, WCP
CMWF 1054	*F. pallidoroseum*	96.23% (613/637)	FIEC-8	MI (23)	Hartenbos, WCP
CMWF 1148	*F. pallidoroseum*	96.29% (623/647)	FIEC-8	MI (23)	Hartenbos, WCP
CMWF 923	*F. pallidoroseum*	96.59% (624/646)	FIEC-10	MI	Kripkraal, MP
CMWF 945	*F. pallidoroseum*	95.72% (605/632)	FIEC-8	MI (24)	Kripkraal, MP
CMWF 1006	*F. pallidoroseum*	96.13% (622/647)	FIEC-9	MI (12)	Kwambonambi, KZN
CMWF 1007	*F. pallidoroseum*	95.98% (622/648)	FIEC-8	PMI (12)	Kwambonambi, KZN
CMWF 1042	*F. pallidoroseum*	95.00% (608/640)	FIEC-8	MI (12)	Kwambonambi, KZN
CMWF 1050	*F. pallidoroseum*	95.98% (622/648)	FIEC-8	MI (13)	Kwambonambi, KZN
CMWF 1141	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	MI (25)	Kwambonambi, KZN
CMWF 896	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	MI (25)	Kwambonambi, KZN

Table 1 (continued). Origin and putative identities of the *Fusarium* isolates from *Syzygium cordatum* used in this study

Culture No.[a]	FID-based identity[b]	Percentage sequence similarity[c]	Phylogeny-based identity[d]	Substratum[e]	Geographic origin[f]
CMWF 916	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	HI (26)	Kwambonambi, KZN
CMWF 918	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	MI (25)	Kwambonambi, KZN
CMWF 928	*F. pallidoroseum*	96.14% (623/648)	FIEC-8	MI (2)	Kwambonambi, KZN
CMWF 952	*F. pallidoroseum*	95.98% (622/648)	FIEC-8	MI (11)	Kwambonambi, KZN
CMWF 977	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	HI (27)	Kwambonambi, KZN
CMWF 984	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	PMI (12)	Kwambonambi, KZN
CMWF 986	*F. pallidoroseum*	95.99% (623/649)	FIEC-8	HI (27)	Kwambonambi, KZN
CMWF 999	*F. pallidoroseum*	95.84% (623/650)	FIEC-8	HI (27)	Kwambonambi, KZN
CMWF 935	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	FB	Pennington, KZN
CMWF 1129	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	MI (20)	Richards bay, KZN
CMWF 903	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	MI (20)	Richards bay, KZN
CMWF 943	*F. pallidoroseum*	95.04% (614/646)	FIEC-1	MI (20)	Richards bay, KZN
CMWF 947	*F. pallidoroseum*	96.14% (623/648)	FIEC-8	HI (28)	Richards bay, KZN
CMWF 1142	*F. pallidoroseum*	95.37% (618/648)	FIEC-3	MI (1)	Sudwala, MP
CMWF 899	*F. pallidoroseum*	95.98% (622/648)	FIEC-8	MI (1)	Sudwala, MP
CMWF 1182	*F. proliferatum*	98.91% (640/647)	GFC- *F. proliferatum*	HI (9)	Bellville, WCP
CMWF 1000	*F. proliferatum*	99.53% (643/646)	GFC- *F. proliferatum*	MI (12)	Kwambonambi, KZN
CMWF 1155	*F. proliferatum*	99.07% (640/646)	GFC- *F. proliferatum*	PMI (14)	Kwambonambi, KZN
CMWF 1161	*F. proliferatum*	99.07% (640/646)	GFC- *F. proliferatum*	MI (29)	Kwambonambi, KZN
CMWF 958	*F. proliferatum*	99.38% (643/647)	GFC- *F. proliferatum*	MI (17)	Kwambonambi, KZN
CMWF 965	*F. proliferatum*	98.91% (639/646)	GFC- *F. proliferatum*	PMI (14)	Kwambonambi, KZN
CMWF 976	*F. proliferatum*	99.53% (643/646)	GFC- *F. proliferatum*	PMI (13)	Kwambonambi, KZN
CMWF 980	*F. proliferatum*	99.38% (643/647)	GFC- *F. proliferatum*	HI (27)	Kwambonambi, KZN
CMWF 898	*F. proliferatum*	99.07% (640/646)	GFC- *F. proliferatum*	MI (18)	Pretoria, GP
CMWF 948	*F. proliferatum*	98.91% (639/646)	GFC- *F. proliferatum*	FB	Pretoria, GP
CMWF 988	*F. proliferatum*	99.07% (640/646)	GFC- *F. proliferatum*	HI (30)	Pretoria, GP
CMWF 990	*F. proliferatum*	99.04% (622/628)	GFC- *F. proliferatum*	HI (30)	Pretoria, GP
CMWF 992	*F. proliferatum*	95.20% (615/646)	GFC- *F. proliferatum*	HI (30)	Pretoria, GP
CMWF 1133	*F. proliferatum*	99.52% (635/638)	GFC- *F. proliferatum*	MI	Richards bay, KZN
CMWF 1009	*F. sacchari*	99.69% (644/646)	GFC- *F. sacchari*	MI (13)	Kwambonambi, KZN
CMWF 1029	*F. sacchari*	99.69% (644/646)	GFC- *F. sacchari*	MI (13)	Kwambonambi, KZN
CMWF 1166	*F.* sp. cf. *bullatum* NRRL 31005	95.24% (621/652)	FIEC-14	HI (9)	Bellville, WCP
CMWF 970	*F.* sp. cf. *bullatum* NRRL 31005	97.84% (635/649)	FIEC-11	MI (31)	Kripkraal, MP
CMWF 1144	*F.* sp. cf. *bullatum* NRRL 31005	95.54% (622/651)	FIEC-7	MI (31)	Kripkraal, MP

Table 1 (continued). Origin and putative identities of the *Fusarium* isolates from *Syzygium cordatum* used in this study

Culture No.[a]	FID-based identity[b]	Percentage sequence similarity[c]	Phylogeny-based identity[d]	Substratum[e]	Geographic origin[f]
CMWF 1145	*F.* sp. cf. *bullatum* NRRL 31005	95.54% (622/651)	FIEC-7	MI (31)	Kripkraal, MP
CMWF 1146	*F.* sp. cf. *bullatum* NRRL 31005	97.84% (635/649)	FIEC-11	MI (31)	Kripkraal, MP
CMWF 1016	*F.* sp. cf. *bullatum* NRRL 31005	95.07% (618/650)	FIEC-1	MI (12)	Kwambonambi, KZN
CMWF 1018	*F.* sp. cf. *bullatum* NRRL 31005	95.07% (618/650)	FIEC-1	HI (27)	Kwambonambi, KZN
CMWF 1024	*F.* sp. cf. *bullatum* NRRL 31005	94.76% (616/650)	FIEC-1	MI (12)	Kwambonambi, KZN
CMWF 1037	*F.* sp. cf. *bullatum* NRRL 31005	94.61% (615/650)	FIEC-1	MI (12)	Kwambonambi, KZN
CMWF 1039	*F.* sp. cf. *bullatum* NRRL 31005	94.42% (593/628)	FIEC-4	MI (12)	Kwambonambi, KZN
CMWF 1052	*F.* sp. cf. *bullatum* NRRL 31005	95.07% (618/650)	FIEC-1	PMI (12)	Kwambonambi, KZN
CMWF 1056	*F.* sp. cf. *bullatum* NRRL 31005	94.76% (616/650)	FIEC-1	MI (13)	Kwambonambi, KZN
CMWF 1157	*F.* sp. cf. *bullatum* NRRL 31005	96.11% (618/643)	FIEC-12	PMI (14)	Kwambonambi, KZN
CMWF 1159	*F.* sp. cf. *bullatum* NRRL 31005	94.76% (616/650)	FIEC-1	MI (17)	Kwambonambi, KZN
CMWF 1160	*F.* sp. cf. *bullatum* NRRL 31005	95.10% (564/593)	FIEC-1	MI (17)	Kwambonambi, KZN
CMWF 901	*F.* sp. cf. *bullatum* NRRL 31005	94.78% (618/652)	FIEC-13	HI	Kwambonambi, KZN
CMWF 905	*F.* sp. cf. *bullatum* NRRL 31005	95.23% (619/650)	FIEC-1	HI (26)	Kwambonambi, KZN
CMWF 913	*F.* sp. cf. *bullatum* NRRL 31005	94.78% (618/652)	FIEC-13	HI (26)	Kwambonambi, KZN
CMWF 914	*F.* sp. cf. *bullatum* NRRL 31005	94.62% (616/651)	FIEC-4	HI (26)	Kwambonambi, KZN
CMWF 922	*F.* sp. cf. *bullatum* NRRL 31005	94.92% (617/650)	FIEC-1	MI (25)	Kwambonambi, KZN
CMWF 939	*F.* sp. cf. *bullatum* NRRL 31005	95.99% (623/649)	FIEC-12	MI (29)	Kwambonambi, KZN
CMWF 941	*F.* sp. cf. *bullatum* NRRL 31005	93.99% (595/633)	FIEC-1	MI (17)	Kwambonambi, KZN
CMWF 949	*F.* sp. cf. *bullatum* NRRL 31005	94.48% (617/653)	FIEC-4	HI	Kwambonambi, KZN
CMWF 897	*F.* sp. cf. *bullatum* NRRL 31005	95.54% (622/651)	FIEC-2	FB (32)	Pennington, KZN
CMWF 931	*F.* sp. cf. *bullatum* NRRL 31005	93.67% (607/648)	FIEC-1	MI	Pennington, KZN
CMWF 934	*F.* sp. cf. *bullatum* NRRL 31005	94.76% (616/650)	FIEC-1	MI	Pennington, KZN
CMWF 1180	*F.* sp. cf. *bullatum* NRRL 31005	95.55% (581/608)	FIEC-1	HI (28)	Richards bay, KZN
CMWF 961	*F.* sp. cf. *bullatum* NRRL 31005	95.09% (620/652)	FIEC-15	MI (1)	Sudwala, MP
CMWF 1177	*F.* sp. cf. *camptoceras* NRRL 13381	91.76% (602/656)	FIEC-25	MI (22)	Buffelskloof, MP
CMWF 929	*F.* sp. cf. *camptoceras* NRRL 13381	91.76% (602/656)	FIEC-25	MI	Buffelskloof, MP
CMWF 944	*F.* sp. cf. *camptoceras* NRRL 13381	90.31% (597/661)	FIEC-24	MI (24)	Kripkraal, MP
CMWF 1011	*F.* sp. cf. *camptoceras* NRRL 13381	97.54% (637/653)	FIEC-27	MI (12)	Kwambonambi, KZN
CMWF 1058	*F.* sp. cf. *camptoceras* NRRL 13381	98.32% (645/656)	FIEC- *F. camptoceras* NRRL 13381	PMI (12)	Kwambonambi, KZN
CMWF 1032	*F.* sp. cf. *equiseti* NRRL 25795	94.15% (612/650)	FIEC- *F. equiseti* 28/3.2.1	HI	Bellville, WCP
CMWF 1044	*F.* sp. cf. *equiseti* NRRL 25795	99.53% (642/645)	FIEC- *F. equiseti* NRRL 25795	MI (6)	Bellville, WCP
CMWF 1137	*F.* sp. cf. *equiseti* NRRL 25795	99.49% (586/589)	FIEC- *F. equiseti* NRRL 25795	MI (10)	Bellville, WCP
CMWF 1149	*F.* sp. cf. *equiseti* NRRL 25795	99.37% (641/645)	FIEC- *F. equiseti* NRRL 25795	MI (5)	Bellville, WCP

Table 1 (continued). Origin and putative identities of the *Fusarium* isolates from *Syzygium cordatum* used in this study

Culture No.[a]	FID-based identity[b]	Percentage sequence similarity[c]	Phylogeny-based identity[d]	Substratum[e]	Geographic origin[f]
CMWF 1163	*F.* sp. cf. *equiseti* NRRL 25795	99.53% (642/645)	FIEC- *F. equiseti* NRRL 25795	MI (33)	Bellville, WCP
CMWF 1165	*F.* sp. cf. *equiseti* NRRL 25795	90.61% (589/650)	FIEC-26	MI (34)	Bellville, WCP
CMWF 1167	*F.* sp. cf. *equiseti* NRRL 25795	97.27% (606/623)	FIEC- *F. equiseti* NRRL 25795	MI (4)	Bellville, WCP
CMWF 1169	*F.* sp. cf. *equiseti* NRRL 25795	99.37% (641/645)	FIEC- *F. equiseti* NRRL 25795	MI (8)	Bellville, WCP
CMWF 1172	*F.* sp. cf. *equiseti* NRRL 25795	98.16% (641/653)	FIEC- *F. equiseti* NRRL 25795	MI (4)	Bellville, WCP
CMWF 1174	*F.* sp. cf. *equiseti* NRRL 25795	100.00% (615/615)	FIEC- *F. equiseti* NRRL 25795	MI	Bellville, WCP
CMWF 893	*F.* sp. cf. *equiseti* NRRL 25795	94.16% (613/651)	FIEC-17	MI (34)	Bellville, WCP
CMWF 906	*F.* sp. cf. *equiseti* NRRL 25795	90.56% (461/509)	FIEC-14	HI (9)	Bellville, WCP
CMWF 909	*F.* sp. cf. *equiseti* NRRL 25795	96.27% (621/645)	FIEC- *F. equiseti* VI01087	MI (34)	Bellville, WCP
CMWF 912	*F.* sp. cf. *equiseti* NRRL 25795	99.37% (641/645)	FIEC- *F. equiseti* NRRL 25795	MI (10)	Bellville, WCP
CMWF 919	*F.* sp. cf. *equiseti* NRRL 25795	100.00% (645/645)	FIEC- *F. equiseti* NRRL 25795	MI (4)	Bellville, WCP
CMWF 920	*F.* sp. cf. *equiseti* NRRL 25795	99.69% (645/647)	FIEC- *F. equiseti* NRRL 25795	MI	Bellville, WCP
CMWF 946	*F.* sp. cf. *equiseti* NRRL 25795	100.00% (645/645)	FIEC- *F. equiseti* NRRL 25795	HI (9)	Bellville, WCP
CMWF 950	*F.* sp. cf. *equiseti* NRRL 25795	99.69% (645/647)	FIEC- *F. equiseti* NRRL 25795	HI (9)	Bellville, WCP
CMWF 951	*F.* sp. cf. *equiseti* NRRL 25795	94.15% (612/650)	FIEC- *F. equiseti* 28/3.2.1	MI (33)	Bellville, WCP
CMWF 998	*F.* sp. cf. *equiseti* NRRL 25795	99.22% (640/645)	FIEC- *F. equiseti* NRRL 25795	MI (7)	Bellville, WCP
CMWF 1008	*F.* sp. cf. *equiseti* NRRL 25795	94.30% (613/650)	FIEC-18	PMI	Kwambonambi, KZN
CMWF 1033	*F.* sp. cf. *equiseti* NRRL 25795	93.96% (607/646)	FIEC-20	MI (13)	Kwambonambi, KZN
CMWF 1035	*F.* sp. cf. *equiseti* NRRL 25795	95.22% (559/587)	FIEC- 28	MI	Kwambonambi, KZN
CMWF 978	*F.* sp. cf. *equiseti* NRRL 25795	94.16% (613/651)	FIEC-18	PMI (13)	Kwambonambi, KZN
CMWF 1179	*F.* sp. cf. *equiseti* NRRL 25795	93.62% (587/627)	FIEC-16	FB	Pennington, KZN
CMWF 892	*F.* sp. cf. *equiseti* NRRL 25795	90.61% (589/650)	FIEC-26	MI	Pennington, KZN
CMWF 1049	*F.* sp. cf. *equiseti* NRRL 25795	93.07% (605/650)	FIEC-19	MI	Pretoria, GP
CMWF 1152	*F.* sp. cf. *equiseti* NRRL 25795	95.65% (617/645)	FIEC- *F. equiseti* MRC 8412	MI (18)	Pretoria, GP
CMWF 1153	*F.* sp. cf. *equiseti* NRRL 25795	95.96% (619/645)	FIEC-21	MI (18)	Pretoria, GP
CMWF 1170	*F.* sp. cf. *equiseti* NRRL 25795	95.96% (619/645)	FIEC-22	FB	Pretoria, GP
CMWF 967	*F.* sp. cf. *equiseti* NRRL 25795	95.82% (619/646)	FIEC-21	MI (18)	Pretoria, GP
CMWF 971	*F.* sp. cf. *equiseti* NRRL 25795	95.65% (617/645)	FIEC- *F. equiseti* MRC 8412	MI (19)	Pretoria, GP
CMWF 991	*F.* sp. cf. *equiseti* NRRL 25795	99.22% (640/645)	FIEC- *F. equiseti* NRRL 25795	HI (30)	Pretoria, GP
CMWF 993	*F.* sp. cf. *equiseti* NRRL 25795	99.22% (640/645)	FIEC- *F. equiseti* NRRL 25795	HI (30)	Pretoria, GP
CMWF 930	*F.* sp. cf. *equiseti* NRRL 25795	94.08% (589/626)	FIEC-16	HI (28)	Richards bay, KZN
CMWF 1047	*F.* sp. cf. *equiseti* NRRL 29128	94.78% (618/652)	FIEC-23	PMI (6)	Bellville, WCP
CMWF 1138	*F.* sp. cf. *equiseti* NRRL 29128	93.98% (531/565)	FIEC-23	MI	Bellville, WCP
CMWF 1164	*F.* sp. cf. *equiseti* NRRL 29128	94.93% (619/652)	FIEC-23	MI (33)	Bellville, WCP

Table 1 (continued). Origin and putative identities of the *Fusarium* isolates from *Syzygium cordatum* used in this study

Culture No.[a]	FID-based identity[b]	Percentage sequence similarity[c]	Phylogeny-based identity[d]	Substratum[e]	Geographic origin[f]
CMWF 1154	*F.* sp. cf. *scirpi* NRRL 29134	97.67% (631/646)	FIEC-*F. scirpi*	MI (18)	Pretoria, GP
CMWF 1156	*F.* sp. NRRL 25615	100.00% (641/641)	GFC-1	PMI (14)	Kwambonambi, KZN
CMWF 954	*F.* sp. NRRL 25615	98.29% (634/645)	GFC-1	MI (16)	Kwambonambi, KZN
CMWF 955	*F.* sp. NRRL 25615	98.14% (634/646)	GFC-1	MI (16)	Kwambonambi, KZN
CMWF 956	*F.* sp. NRRL 25615	98.29% (634/645)	GFC-1	MI	Kwambonambi, KZN
CMWF 1151	*F.* sp. NRRL 25615	98.29% (634/645)	GFC-1	MI	Pretoria, GP
CMWF 1131	*F.* sp. NRRL 26061	99.69% (645/647)	GFC-8	MI (29)	Kwambonambi, KZN
CMWF 1171	*F.* sp. NRRL 26756	100.00% (641/641)	GFC-6	MI (8)	Bellville, WCP
CMWF 1173	*F.* sp. NRRL 26756	100.00% (641/641)	GFC-6	PMI (14)	Kwambonambi, KZN
CMWF 894	*F.* sp. NRRL 26756	100.00% (641/641)	GFC-6	MI (14)	Kwambonambi, KZN
CMWF 1010	*F.* sp. NRRL 26793	99.07% (640/646)	GFC-3	MI (5)	Bellville, WCP
CMWF 1178	*F.* sp. NRRL 3299	89.18% (577/647)	IV	MI	Kripkraal, MP
CMWF 942	*F. udum*	98.30% (637/648)	GFC-4	MI	Graskop, MP
CMWF 1038	*F. udum*	97.69% (635/650)	GFC-5	MI (12)	Kwambonambi, KZN
CMWF 900	*F. verticillioides*	99.53% (643/646)	GFC-2	FB (32)	Pennington, MP

[a] CMWF = *Fusarium* Collection, Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa. All entries were isolated by M. Kvas. Isolates examined morphologically in this study are given in bold.

[b] These represent the top BLAST hit in the TEF sequence searches against the FID. Conspecificity can only be assumed with confidence where a specific TEF sequence for an isolate from *S. cordatum* was identical (*i.e.*, 100% sequence similarity) to those in this database.

[c] Number of sequence base pair matches/Total length of sequence are indicated in parentheses.

[d] These are based on phylogenetic relationships inferred from TEF region. *Fusarium* isolates are separated into 8 distinct clades/groups (I-VIII), of which FIEC, GFC and FOC represent *F. incarnatum/F. equiseti, G. fujikuroi* and *F. oxysporum* complexes, respectively. See Figs. 6-9.

[e] MI = malformed inflorescence; HI = healthy inflorescence; PMI= petiole of malformed inflorescence; FB = flower bud. Between one and eleven trees were sampled at each location. Number in parentheses indicates which isolates were obtained from the same plant.

[f] GP, KZN, MP, WCP refer, respectively, to the Gauteng, KwaZulu-Natal, Mpumalanga, and Western Cape Provinces of South Africa.

Table 2. Collection numbers and source of the *Fusarium* reference strains used in this study

Fusarium species	MRC No.[a]	Other No.[b]	Source
F. andiyazi	MRC 6122	KSU 4804; FRC M-8413	S. Lamprecht
F. andiyazi	MRC 8046	KSU 4647	T. Hussein
F. anthophilum	MRC 3236	FRC M-1238	L.W. Burgess
F. avenaceum	MRC 8381	KSU 11441; F12844	J.F. Leslie
F. chlamydosporum	MRC 8391	KSU 11396; F11782	J.F. Leslie
F. circinatum	MRC 7488	KSU 10847	H. Britz
F. circinatum	MRC 7541	KSU 10766; BBA 69720; NRRL 25331	H. Nirenberg
F. compactum	MRC 2800	FRC R-6837	W.F.O Marasas
F. compactum	MRC 6142	n/a	S. Lamprecht
F. crookwellense	MRC 2878	FRC R-4758	L.W. Burgess
F. crookwellense	MRC 8399	KSU 11453; F13169	J.F. Leslie
F. culmorum	MRC 1823	BBA 62190	W. Gerlach
F. culmorum	MRC 8403	KSU 11427; F12282	J.F. Leslie
F. decemcellulare	MRC 1795	BBA 62192	W. Gerlach
F. dimerum	MRC 1652	BBA 62195	W. Gerlach
F. dlamini	MRC 3023	KSU 5009; BBA 69026; FRC M-1557; NRRL 25442	W.F.O. Marasas
F. dlamini	MRC 3032	BBA 69859; FRC M-1637; NRRL 13164	W.F.O. Marasas
F. equiseti	MRC 8412	KSU 11437; F6510	J.F. Leslie
F. fujikuroi	MRC 1836	BBA 63873	W. Gerlach
F. fujikuroi	MRC 8534	KSU 1995; FRC M-1150	J.F. Leslie
F. globosum	MRC 6647	KSU 11555; FRC M-8014; NRRL 26131	J. P. Rheeder
F. globosum	MRC 6648	NRRL 26132	J. P. Rheeder
F. graminearum	MRC 4712	n/a	P.S. van Wyk
F. graminearum	MRC 4927	n/a	W.F.O Marasas
F. guttiforme	MRC 6784	BBA 69860; NRRL 25624	J. Ventura
F. konzum	MRC 8544	KSU 11615	J.F. Leslie
F. konzum	MRC 8545	KSU 11616	J.F. Leslie
F. lateritium	MRC 1845	BBA 62458	W. Gerlach
F. lateritium	MRC 2465	FRC L-86	L. Burgess
F. longipes	MRC 8429	KSU 11429; F5837	J.F. Leslie
F. longipes	MRC 8430	KSU 11431; F11993	J.F. Leslie
F. mangiferae	MRC 2730	KSU 3873	F. Wehner
F. mangiferae	MRC 8432	MRC 7559; KSU 11781	S. Freeman
F. napiforme	MRC 4144	BBA 69861; FRC M-3563; NRRL 13604	A Lübben
F. nelsonii	MRC 4570	ITEM 1229; FRC R-8670	S. Lamprecht

Table 2 (continued). Collection numbers and source of the *Fusarium* reference strains used in this study

Fusarium species	MRC No.[a]	Other No.[b]	Source
F. nygamai	MRC 8546	KSU 5111	J.F. Leslie
F. oxysporum	MRC 8435	KSU 11392; F6733	J.F. Leslie
F. oxysporum	MRC 8437	KSU 11395; F90270	J.F. Leslie
F. poae	MRC 8485	MRC 3295; KSU 11550; FRC T-342	J.F. Leslie
F. poae	MRC 8486	MRC 3225; KSU 11551	P.S. van Wyk
F. proliferatum	MRC 8549	KSU 4854	J.F. Leslie
F. proliferatum	MRC 8550	KSU 4853	J.F. Leslie
F. sacchari	MRC 8552	KSU 3853	J.F. Leslie
F. sambucinum	MRC 6971	FRC R-8575	A. Desjardins
F. sambucinum	MRC 6972	BBA 64995	J.F. Leslie
F. scirpi	MRC 3230	FRC R-6279	J.F. Leslie
F. semitectum	MRC 3228	KSU 11549; FRC R-4237	n/a
F. semitectum	MRC 6715	KSU 11548	A. Lübben
F. solani	MRC 3237	FRC S-714	S.N. Smith
F. solani	MRC 8454	KSU 11420; F90009	J.F. Leslie
F. sporotrichioides	MRC 43	FRC T-424; NRRL 3299	W. Snyder
F. sporotrichioides	MRC 4333	KSU 11552; DAOM 175513	G. Neish
F. subglutinans	MRC 115	n/a	W.F.O. Marasas
F. subglutinans	MRC 8554	KSU 2192	J.F. Leslie
F. thapsinum	MRC 8558	KSU 4094; BBA 70187; FRC M-6564	J.F. Leslie
F. tricinctum	MRC 1574	FRC T-399	El-Gholl
F. tricinctum	MRC 2623	FRC T-542	A. Ylimäki
F. verticilliodes	MRC 826	KSU 11556; FRC M-1325; NRRL 20960	W.F.O. Marasas
F. verticilliodes	MRC 8559	KSU 149	J.F. Leslie

[a]MRC = Medical Research Council, Tygerberg, South Africa.

[b]BBA = Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany; DAOM = National Mycological Herbarium, Department of Agriculture, Ottawa, Canada; F = *Fusarium* Research Laboratory, Department of Plant Pathology and Agricultural Entomology, University of Sydney, Sydney, Australia; FRC = *Fusarium* Research Center, Pennsylvania State University, University Park, Pennsylvania, USA; KSU + Kansas State University, Manhattan, Kansas, USA; NRRL = Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA.

Table 3. The fragment sizes revealed by the *in silico* PCR-RFLP analyses of the *Fusarium* reference strains using TEF sequences

***Fusarium* species**[a]	**Restriction enzymes**					
	***Alu*I**	***Hha*I**	***Hae*III**	***Mbo*I**	***Msp*I**	***Rsa*I**
F. andiyazi MRC 6122	44 - 45 - 158 - 381	38 - 125 - 209 - 256	52 - 151 - 167 - 258	70 - 132 - 426	11 - 123 - 494	26 - 178 - 424
F. andiyazi MRC 8046	42 - 65 - 142 - 381	38 - 125 - 230 - 237	13 - 60 - 135 - 167 - 255	70 - 153 - 407	144 - 486	47 - 162 - 421
F. anthophilium MRC 3236	42 - 65 - 158 - 378	36 - 126 - 228 - 253	13 - 151 - 479	70 - 151 - 422	11 - 142 - 490	35 - 47 - 143 - 418
F. avenaceum MRC 8381	42 - 159 - 389	2 - 38 - 134 - 167 - 249	590[b]	88 - 502	11 - 79 - 500	179 - 411
F. chlamydosporum MRC 8391	42 - 157 - 395	2 - 57 - 128 - 153 - 252	149 - 172 - 323	23 - 70 - 76 - 425	12 - 67 - 515	177 - 417
F. circinatum MRC 7541	42 - 65 - 158 - 378	36 - 127 - 227 - 253	13 - 151 - 479	70 - 80 - 423	11 - 141 - 491	35 - 47 - 143 - 418
F. circinatum MRC 7488	42 - 65 - 143 - 374	32 - 127 - 227 - 238	13 - 136 - 475	70 - 80 - 404	141 - 483	47 - 163 - 414
F. compactum MRC 2800	42 - 155 - 165 - 285	2 - 37 - 57 - 89 - 208 - 250	13 - 148 - 487	17 - 21 - 23 - 40 - 55 - 68 - 423	11 - 124 - 512	48 - 175 - 424
F. compactum MRC 6142	42 - 155 - 165 - 286	2 - 37 - 57 - 89 - 209 - 250	13 - 148 - 487	21 - 23 - 40 - 70 - 71 - 423	11 - 123 - 514	47 - 175 - 426
F. crookwellense MRC 2878	42 - 156 - 370	2 - 33 - 57 - 88 - 135 - 251	568[b]	58 - 70 - 440	11 - 49 - 508	176 - 392
F. crookwellense MRC 8399	42 - 155 - 443	2 - 33 - 57 - 88 - 208 - 250	13 - 627	70 - 131 - 439	11 - 122 - 507	47 - 175 - 418
F. culmorum MRC 1823	42 - 154 - 419	2 - 33 - 57 - 88 - 184 - 249	615[b]	70 - 107 - 438	11 - 98 - 506	22 - 174 - 419
F. culmorum MRC 8403	42 - 155 - 444	2 - 33 - 57 - 88 - 209 - 250	13 - 628	70 - 132 - 439	11 - 123 - 507	47 - 175 - 419
F. decemcellulare MRC 1795	42 - 163 - 487	2 - 58 - 135 - 235 - 262	8 - 13 - 69 - 139 - 148 - 315	35 - 60 - 62 - 71 - 464	11 - 148 - 183 - 350	47 - 183 - 462
F. dimerum MRC 1652	13 - 42 - 66 - 154 - 382	657[b]	13 - 147 - 199 - 298	62 - 97 - 153 - 345	11 - 144 - 502	174 - 207 - 276
F. dlamini MRC 3023	42 - 65 - 159 - 387	39 - 130 - 230 - 254	13 - 152 - 487	61 - 92 - 500	11 - 144 - 498	35 - 144 - 474
F. dlamini MRC 3032	42 - 65 - 159 - 386	39 - 129 - 230 - 254	13 - 152 - 488	61 - 92 - 499	11 - 144 - 497	35 - 47 - 144 - 426
F. equiseti MRC 8412	42 - 156 - 446	86 - 277 - 281	13 - 149 - 482	22 - 71 - 152 - 399	11 - 62 - 81 - 490	168 - 176 - 300
F. fujikuroi MRC 1836	42 - 65 - 156 - 377	36 - 129 - 230 - 245	149 - 491	12 - 61 - 70 - 80 - 417	11 - 144 - 485	47 - 176 - 417
F. fujikuroi MRC 8534	42 - 65 - 141 - 377	36 - 129 - 230	13 - 134 - 478	12 - 61 - 70 - 80 - 402	144 - 481	47 - 161 - 417
F. globosum MRC 6647	42 - 65 - 156 - 383	37 - 128 - 230 - 251	13 - 149 - 482	12 - 61 - 70 - 80 - 423	11 - 144 - 491	47 - 176 - 423
F. globosum MRC 6648	42 - 63 - 156 - 383	37 - 128 - 228 - 251	149 - 463	12 - 61 - 70 - 78 - 423	11 - 142 - 491	45 - 176 - 423
F. graminearum MRC 4712	42 - 155 - 447	2 - 57 - 123 - 210 - 252	13 - 630	70 - 133 - 441	11 - 124 - 509	47 - 175 - 422
F. graminearum MRC 4927	42 - 155 - 446	2 - 57 - 123 - 209 - 252	6 - 7 - 11 - 354	70 - 132 - 441	11 - 123 - 198 - 311	47 - 175 - 421
F. guttiforme MRC 6784	42 - 65 - 158 - 377	36 - 126 - 227 - 253	13 - 151 - 479	70 - 150 - 422	11 - 141 - 490	35 - 47 - 143 - 417
F. konzum MRC 8544	42 - 65 - 158 - 376	35 - 126 - 227 - 253	13 - 151 - 477	58 - 92 - 491	11 - 141 - 489	35 - 47 - 143 - 416
F. konzum MRC 8545	42 - 46 - 158 - 376	35 - 126 - 208 - 253	151 - 471	58 - 73 - 491	11 - 122 - 489	28 - 35 - 143 - 416
F. lateritium MRC 1845	100 - 112 - 172	2 - 53 - 118 - 211	13 - 669	71 - 133 - 180	57 - 67 - 260	22 - 54 - 308
F. lateritium MRC 2465	42 - 137 - 156 - 172 - 175	2 - 53 - 91 - 236 - 300	13 - 125 - 472	71 - 158 - 210 - 243	11 - 57 - 92 - 522	47 - 54 - 176 - 405
F. longipes MRC 8429	42 - 132 - 436	2 - 29 - 54 - 90 - 206 - 227	13 - 151 - 475	23 - 32 - 70 - 129 - 356	120 - 490	47 - 152 - 411
F. longipes MRC 8430	42 - 158 - 439	2 - 57 - 119 - 208 - 253	13 - 151 - 486	23 - 43 - 70 - 131 - 176 - 196	11 - 122 - 506	48 - 178 - 413

Table 3 (continued). The fragment sizes revealed by the *in silico* PCR-RFLP analyses of the *Fusarium* reference strains using TEF sequences

***Fusarium* species**[a]	**Restriction enzymes**					
	***Alu*I**	***Hha*I**	***Hae*III**	***Mbo*I**	***Msp*I**	***Rsa*I**
F. mangiferae MRC 2730	40 - 42 - 69 - 156 - 305	30 - 251 - 331	13 - 149 - 475	61 - 66 - 67 - 418	11 - 48 - 71 - 482	22 - 35 - 141 - 414
F. mangiferae MRC 8432	42 - 65 - 69 - 156 - 305	30 - 251 - 356	13 - 60 - 151 - 422	61 - 66 - 92 - 418	11 - 48 - 96 - 482	35 - 47 - 141 - 414
F. napiforme MRC 4144	42 - 65 - 158 - 381	38 - 126 - 229 - 253	13 - 149 - 484	70 - 152 - 424	11 - 143 - 492	47 - 178 - 192 - 229
F. nelsonii MRC 4570	42 - 154 - 165 - 287	2 - 39 - 146 - 208 - 251	147 - 172 - 329	43 - 70 - 94 - 441	8 - 126 - 514	28 - 174 - 446
F. nygamai MRC 8546	42 - 65 - 144 - 383	39 - 125 - 231 - 239	147 - 172 - 329	70 - 154 - 410	145 - 489	47 - 164 - 423
F. oxysporum MRC 8435	42 - 65 - 158 - 385	230 - 420	13 - 128 - 488	70 - 153 - 427	11 - 144 - 495	35 - 47 - 143 - 425
F. oxysporum MRC 8437	42 - 65 - 135 - 387	230 - 399	13 - 129 - 454	70 - 153 - 406	144 - 485	12 - 47 - 143 - 427
F. poae MRC 8485	42 - 136 - 418	2 - 153 - 208 - 233	13 - 152 - 454	23 - 70 - 131 - 372	122 - 474	47 - 156 - 393
F. poae MRC 8486	42 - 159 - 418	2 - 153 - 208 - 256	13 - 127 - 484	23 - 70 - 131 - 395	11 - 122 - 486	47 - 179 - 393
F. proliferatum MRC 8549	42 - 65 - 157 - 383	37 - 128 - 230 - 252	149 - 443	12 - 61 - 70 - 80 - 424	12 - 144 - 491	47 - 58 - 177 - 365
F. proliferatum MRC 8550	11 - 42 - 156 - 383	37 - 128 - 176 - 251	13 - 611	12 - 26 - 61 - 70 - 423	11 - 90 - 491	51 - 176 - 365
F. sacchari MRC 8552	42 - 65 - 156 - 383	37 - 128 - 230 - 251	151 - 477	70 - 153 - 423	11 - 144 - 491	35 - 47 - 141 - 193 - 230
F. sambucinum MRC 6971	42 - 62 - 155 - 365	2 - 163 - 207 - 250	13 - 609	23 - 70 - 130 - 165 - 236	11 - 121 - 492	175 - 449
F. sambucinum MRC 6972	42 - 62 - 153 - 365	2 - 163 - 207 - 248	13 - 149 - 493	23 - 70 - 130 - 165 - 234	9 - 121 - 492	173 - 449
F. scirpi MRC 3230	42 - 129 - 338	31 - 84 - 173 - 221	13 - 151 - 479	21 - 48 - 68 - 372	39 - 470	149 - 168 - 192
F. semitectum MRC 3228	42 - 156 - 457	120 - 248 - 287	149 - 450	22 - 71 - 153 - 409	11 - 144 - 182 - 318	47 - 173 - 176 - 259
F. semitectum MRC 6715	42 - 156 - 401	120 - 231 - 248	8 - 13 - 139 - 520	22 - 71 - 97 - 409	11 - 88 - 182 - 318	37 - 176 - 386
F. solani MRC 3237	42 - 51 - 154 - 430	2 - 58 - 133 - 234 - 250	8 - 13 - 139 - 510	61 - 69 - 97 - 450	11 - 149 - 517	111 - 174 - 392
F. solani MRC 8454	42 - 154 - 474	2 - 46 - 134 - 235 - 253	13 - 632	61 - 70 - 97 - 150 - 292	11 - 149 - 211 - 299	174 - 496
F. sporotrichioides MRC 43	41 - 155 - 449	2 - 35 - 57 - 90 - 210 - 249	13 - 633	70 - 133 - 442	11 - 124 - 510	48 - 175 - 422
F. sporotrichioides MRC 4333	42 - 155 - 449	2 - 35 - 57 - 91 - 209 - 250	13 - 632	70 - 132 - 444	11 - 123 - 512	47 - 175 - 424
F. subglutinans MRC 115	42 - 65 - 157 - 380	36 - 129 - 227 - 252	150 - 494	70 - 80 - 424	11 - 141 - 492	47 - 177 - 420
F. subglutinans MRC 8554	42 - 65 - 160 - 378	36 - 127 - 227 - 255	13 - 272 - 362	70 - 150 - 425	12 - 141 - 492	36 - 47 - 144 - 418
F. thapsinum MRC 8558	42 - 51 - 158 - 377	32 - 127 - 216 - 253	13 - 621	2 - 70 - 137 - 419	11 - 130 - 487	33 - 178 - 417
F. tricinctum MRC 1574	42 - 159 - 446	2 - 38 - 133 - 223 - 251	13 - 636	144 - 503	11 - 135 - 501	47 - 72 - 107 - 421
F. tricinctum MRC 2623	42 - 159 - 448	2 - 38 - 135 - 223 - 251	15 - 50 - 151 - 406	144 - 505	11 - 135 - 503	47 - 179 - 423
F. verticilliodes MRC 826	42 - 55 - 158 - 325	125 - 206 - 291	13 - 15 - 60 - 127 - 406	129 - 493	11 - 58 - 62 - 491	24 - 178 - 198 - 222
F. verticilliodes MRC 8559	42 - 55 - 65 - 134 - 325	125 - 229 - 267	13 - 15 - 60 - 151 - 407	152 - 469	58 - 85 - 478	47 - 154 - 198 - 222

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

[b]The restriction enzyme does not cut the sequence.

Fig. 1. Inflorescences of *Syzygium cordatum.* (a) healthy white flowers of *S. cordatum* with numerous fluffy stamens; (b) abnormally enlarged, malformed inflorescence with dried-out and barren flowers.

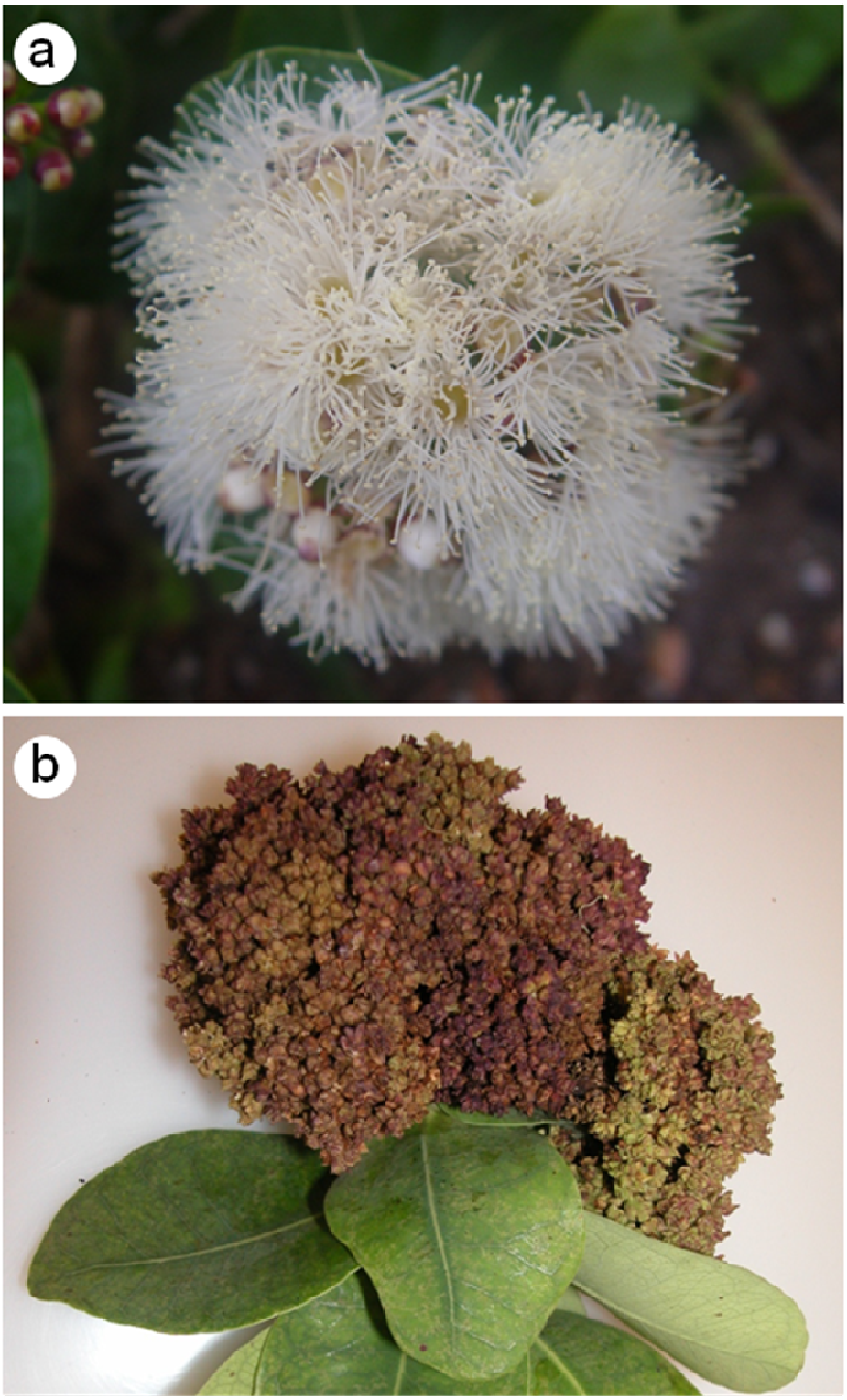

a
b

Fig. 2. A map of South Africa highlighting the natural distribution of *Syzygium cordatum* (van Wyk and van Wyk, 1997). Blue and green circles mark urban and rural sites, respectively, from where isolates of *Fusarium* identified in this study were collected.

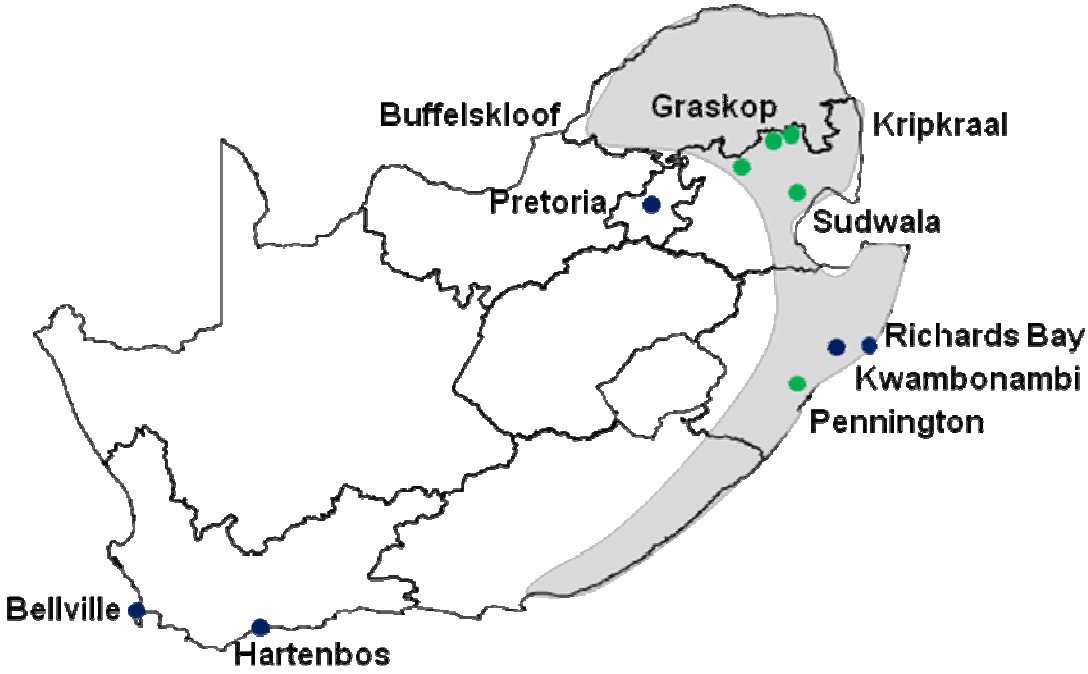
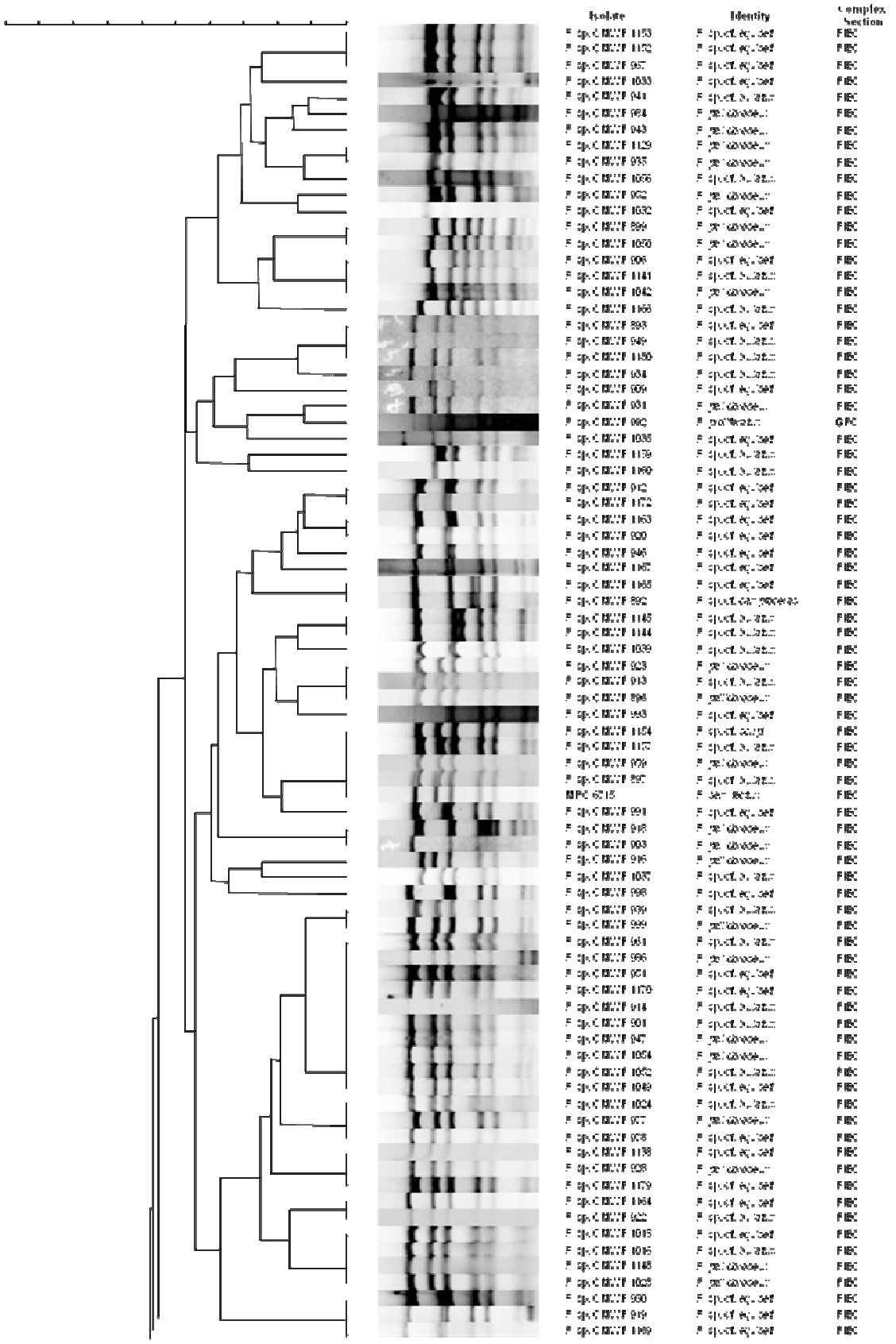
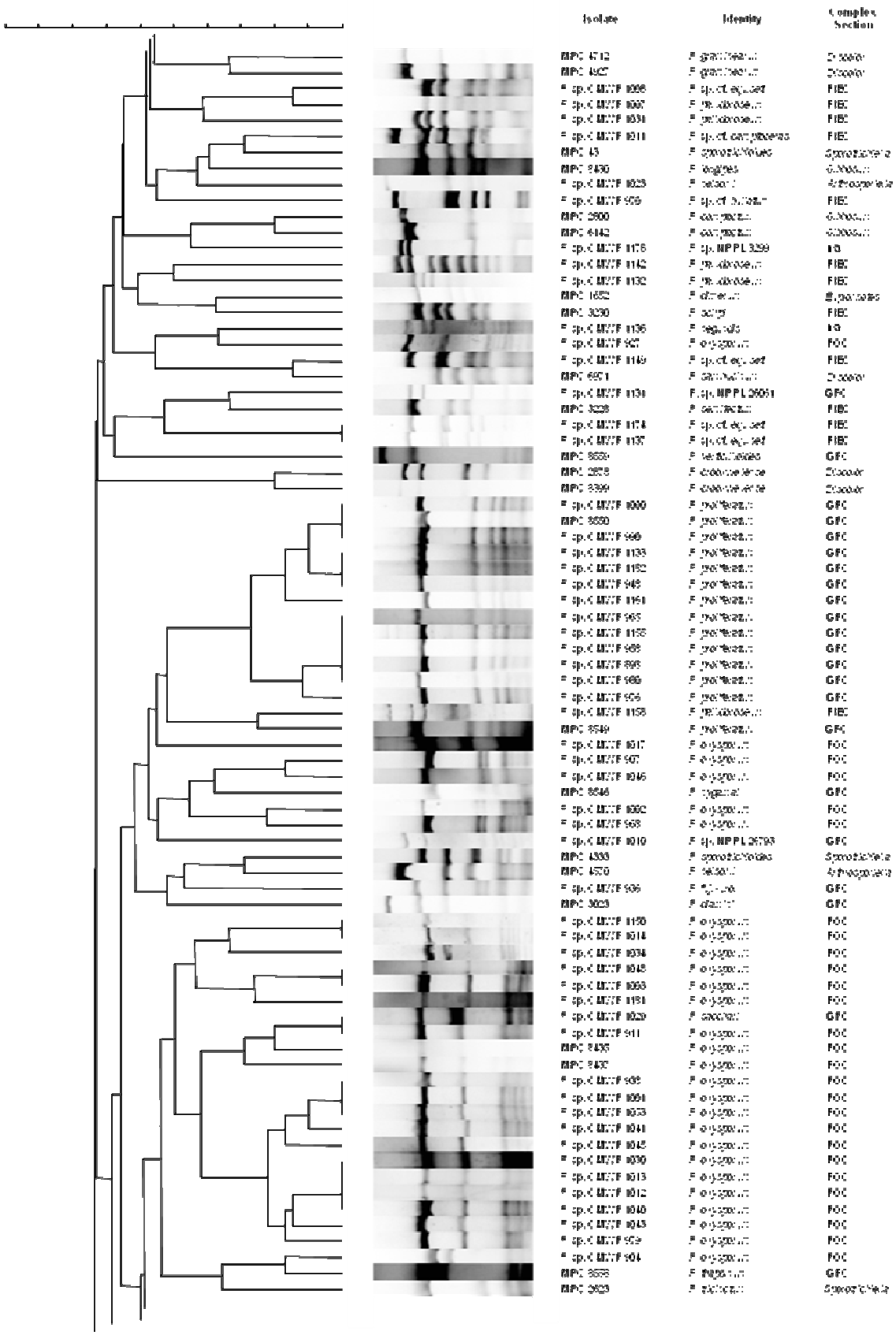

Buffelskloof
Graskop
Kripkraal
Pretoria
Sudwala
Richards Bay
Kwambonambi
Pennington
Bellville
Hartenbos

Fig. 3. Cluster analyses (UPGMA with the Jaccard coefficient) of PCR-RFLP fingerprints of *Fusarium* reference strains and isolates obtained from *Syzygium cordatum* inflorescences generated by *Hae*III restriction of IGS PCR products. The reference strains (MRC) are labelled and classified according to morphology (Nelson *et al.*, 1983; Leslie and Summerell, 2006), while identities of *S. cordatum* isolates are based on the TEF barcoding results (see Table 1). FIEC, GFC and FOC represent *F. incarnatum/F. equiseti*, *G. fujikuroi* and *F. oxysporum* complexes, respectively, while others are classified in sections according to Nelson *et al.* (1983). Scale shows percentage genetic similarity.


Isolate
Identity
Complex Section


Isolate
Identity
Complex Section

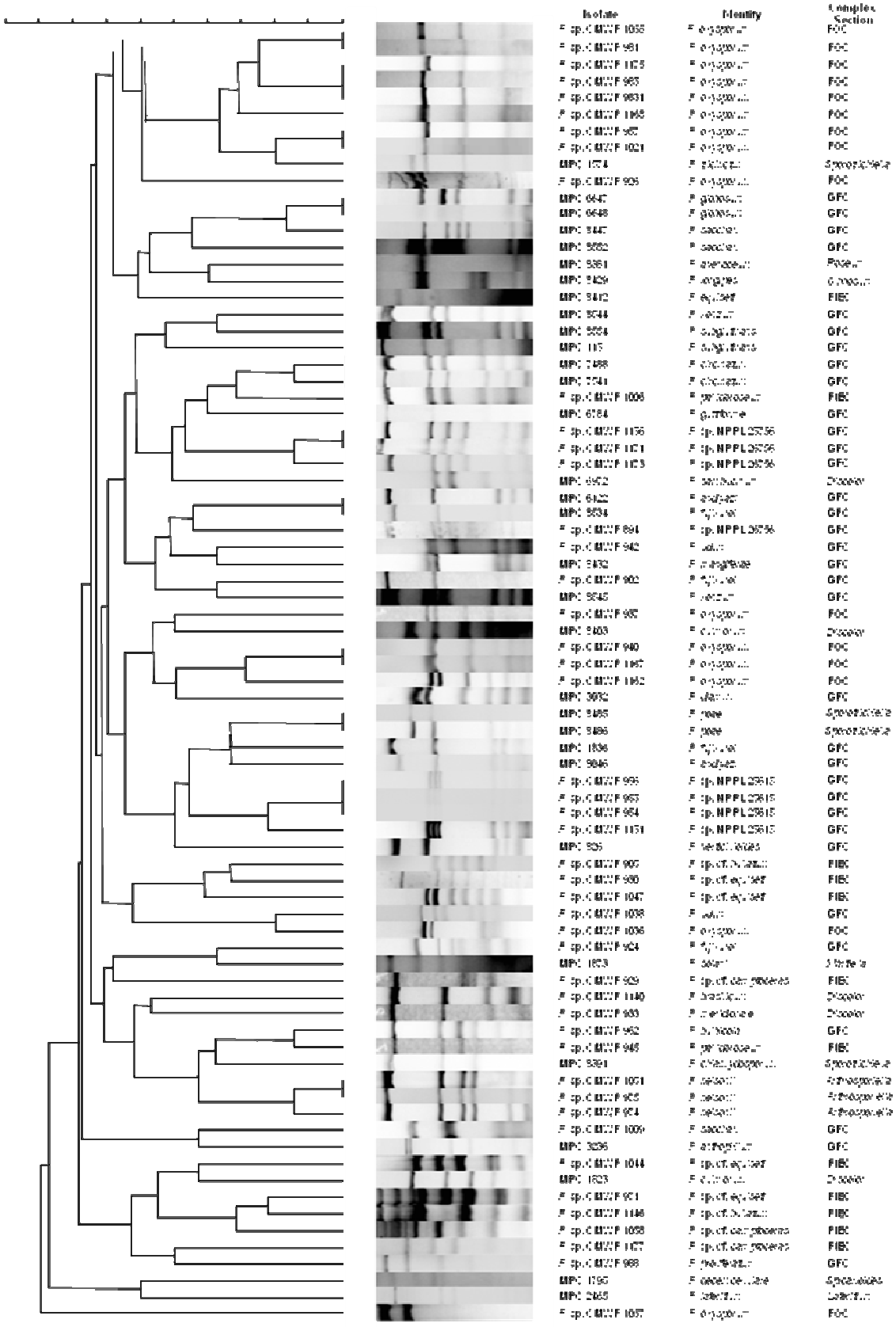
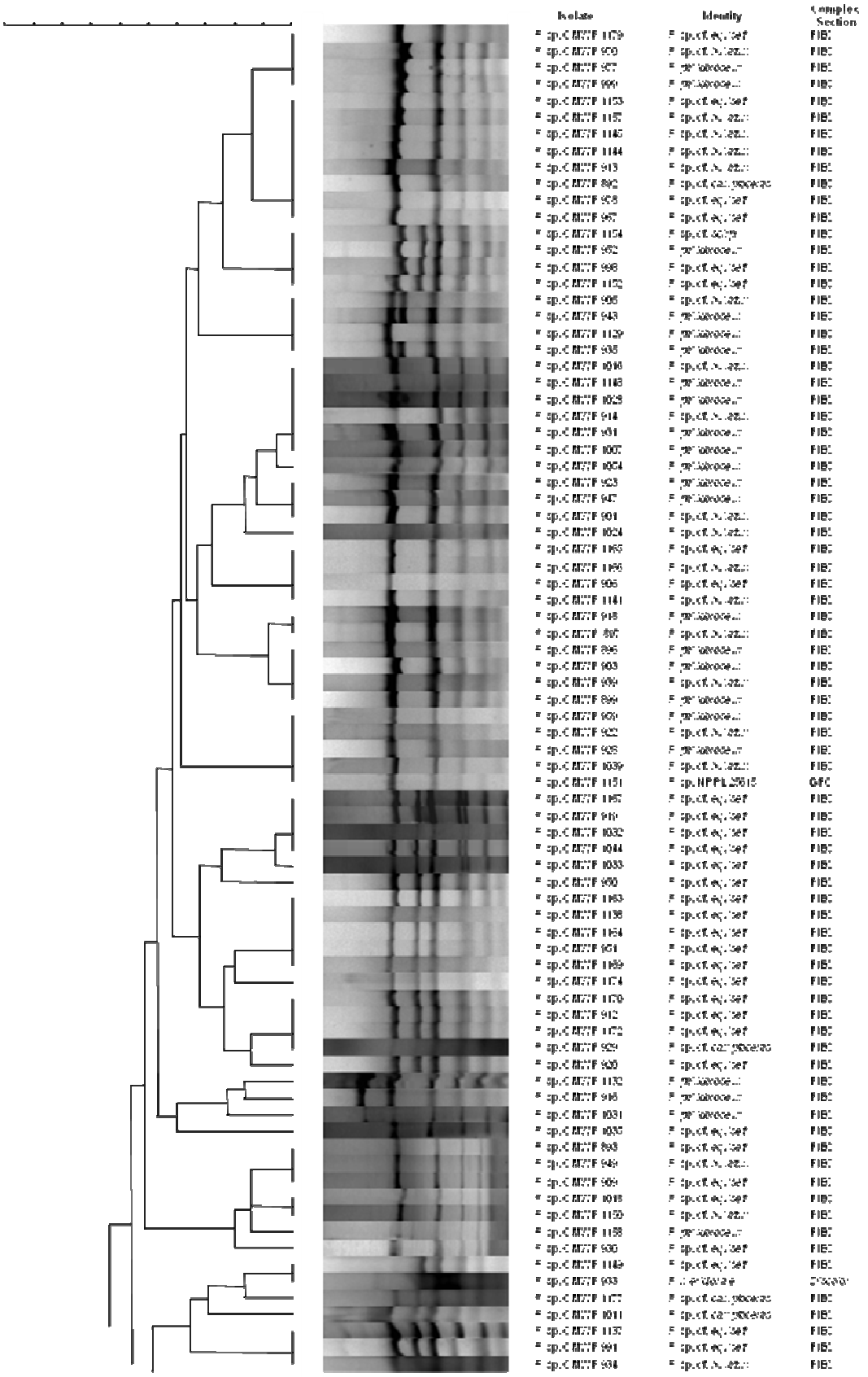
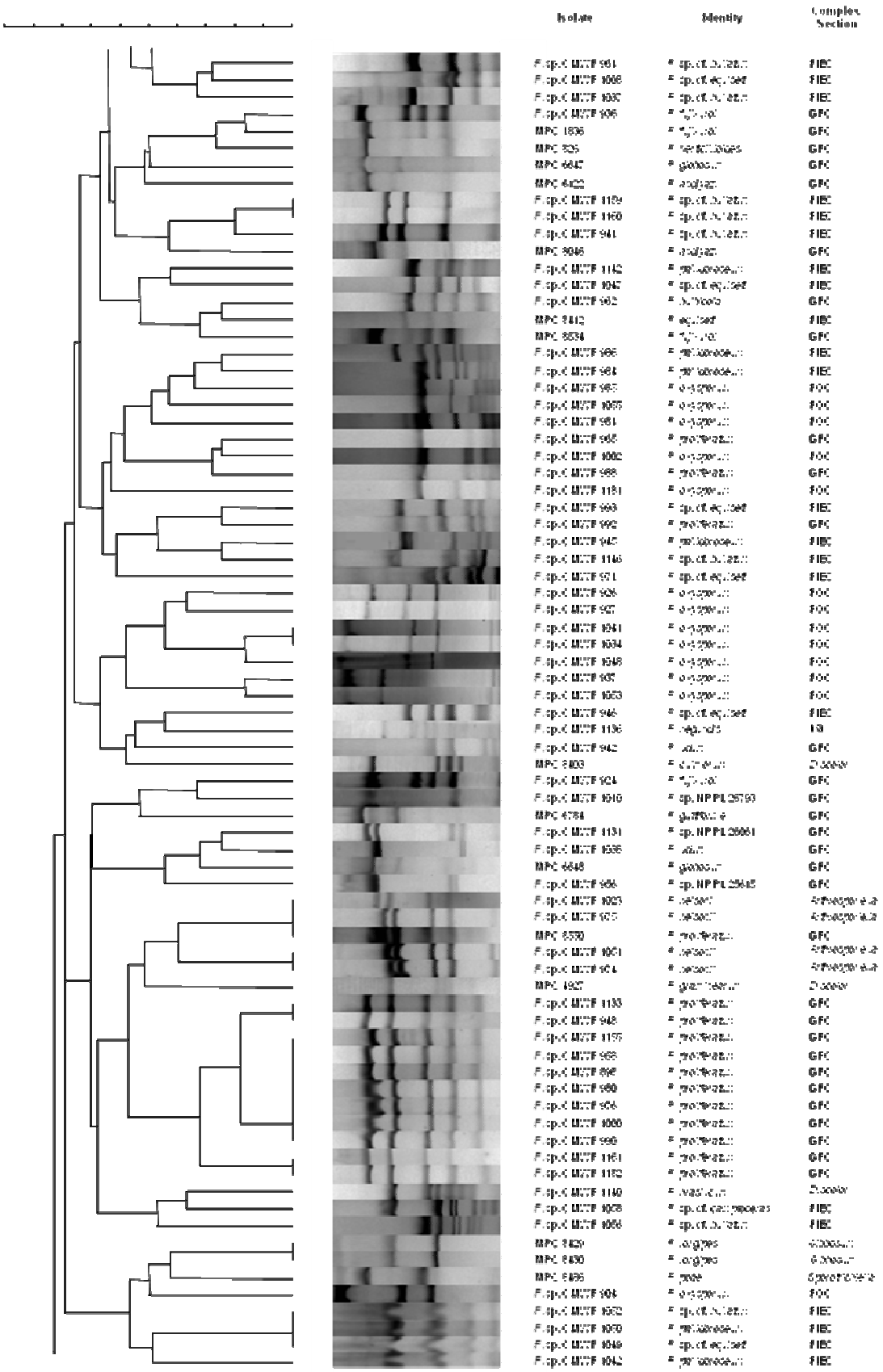

Isolate
Identity
Complex
Section

Fig. 4. Cluster analyses (UPGMA with the Jaccard coefficient) of PCR-RFLP fingerprints of *Fusarium* reference strains and isolates obtained from *Syzygium cordatum* inflorescences generated by *Hha*I restriction of IGS PCR products. The reference strains (MRC) are labelled and classified according to morphology (Nelson *et al.*, 1983; Leslie and Summerell, 2006), while identities of *S. cordatum* isolates are based on the TEF barcoding results (see Table 1). FIEC, GFC and FOC represent *F. incarnatum/F. equiseti*, *G. fujikuroi* and *F. oxysporum* complexes, respectively, while others are classified in sections as according to Nelson *et al.* (1983). Scale shows percentage genetic similarity.


Isolate
Identity
Complex Section


Isolate
Identity
Complex Section

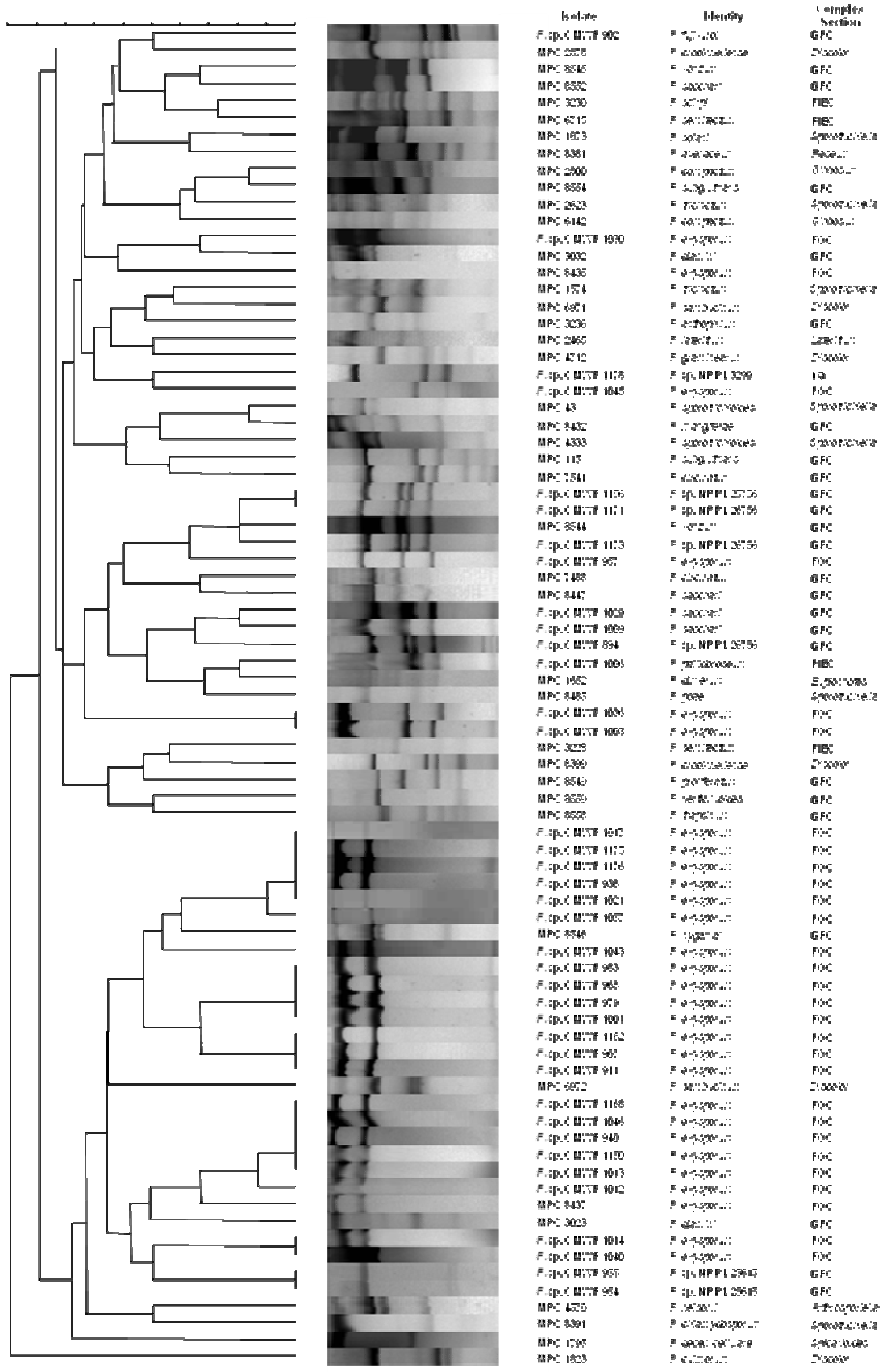

Isolate
Identity
Complex Section

Fig. 5. A pie chart indicating the percentage of the each *Fusarium* complex/group from the total number of isolates obtained from inflorescences of *Syzygium cordatum* in South Africa. FIEC represents the *F. incarnatum/F. equiseti* complex which includes *Fusarium* species in the *Arthrosporiella* and *Gibbosum* sections as defined by Nelson *et al.* (1983). GFC and FOC represent *G. fujikuroi* and *F. oxysporum* complexes, respectively.

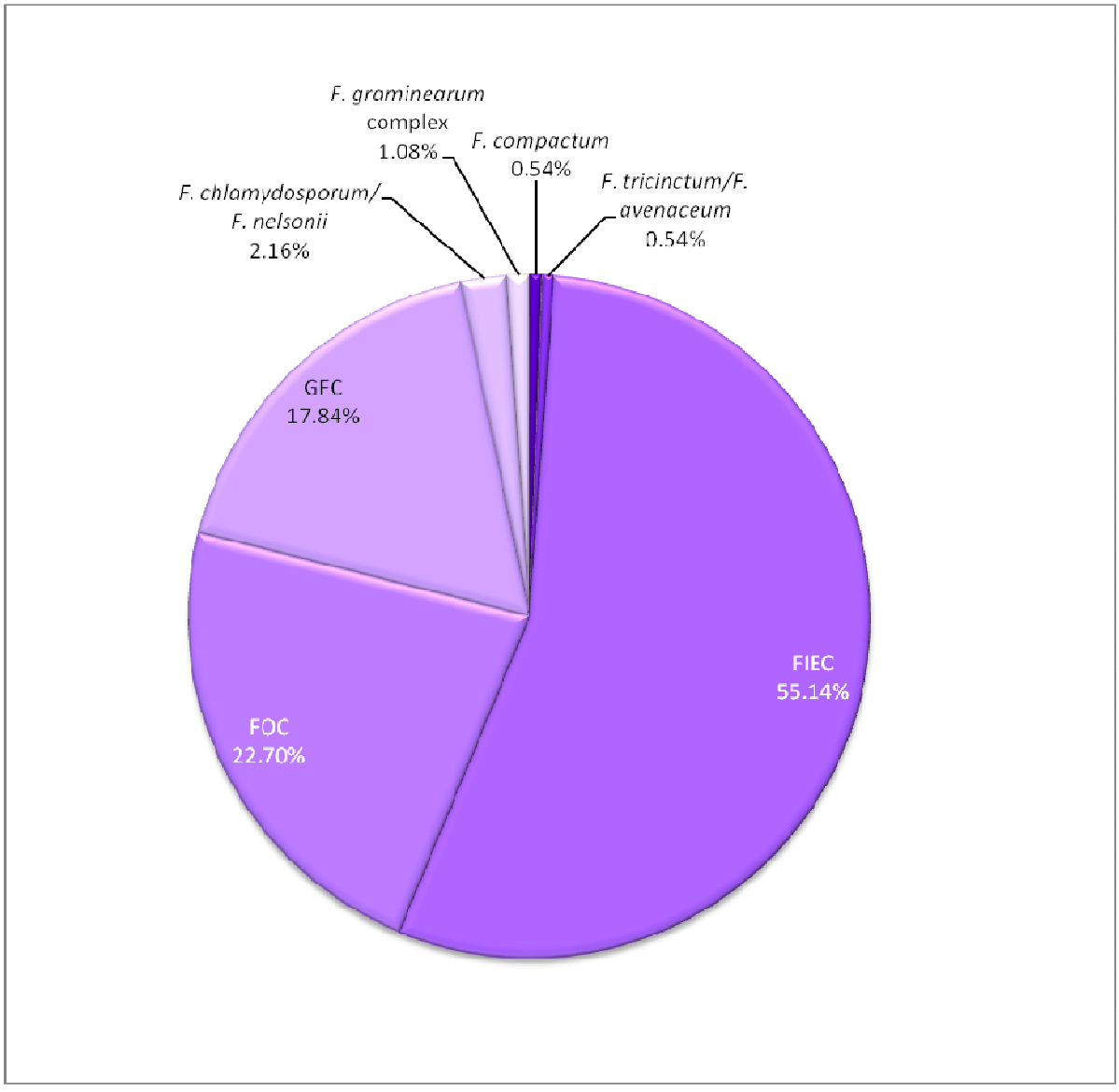

F. graminearum complex 1.08%
F. compactum 0.54%
F. tricinctum/F. avenaceum 0.54%
F. chlamydosporum/ F. nelsonii 2.16%
GFC 17.84%
FOC 22.70%
FIEC 55.14%

Fig. 6. The ML phylogeny for the genus *Fusarium* based on TEF sequence data. *Fusarium* isolates associated with *Syzygium cordatum* as well as *Fusarium* reference strains of the 36 well known species were included in the alignment. The 185 isolates obtained from *S. cordatum* inflorescences are separated into 8 distinct clades/groups (I-VIII). FIEC, GFC and FOC represent *F. incarnatum/F. equiseti*, *G. fujikuroi* and *F. oxysporum* complexes, respectively. The geographic origins of *Fusarium* isolates from *S. cordatum* are shown. Similar topology was generated using Bayesian inference (BI) analyses. ML bootstrap values (≥80%) and biPP (≥0.95) are indicated at the internodes in the order ML/BI. Branches with bootstrap support values <80% and/or <0.95 are indicated with "–". The tree is rooted with the outgroup taxa *Cylindrocarpon cylindroides* and *Neonectria ramulariae*.

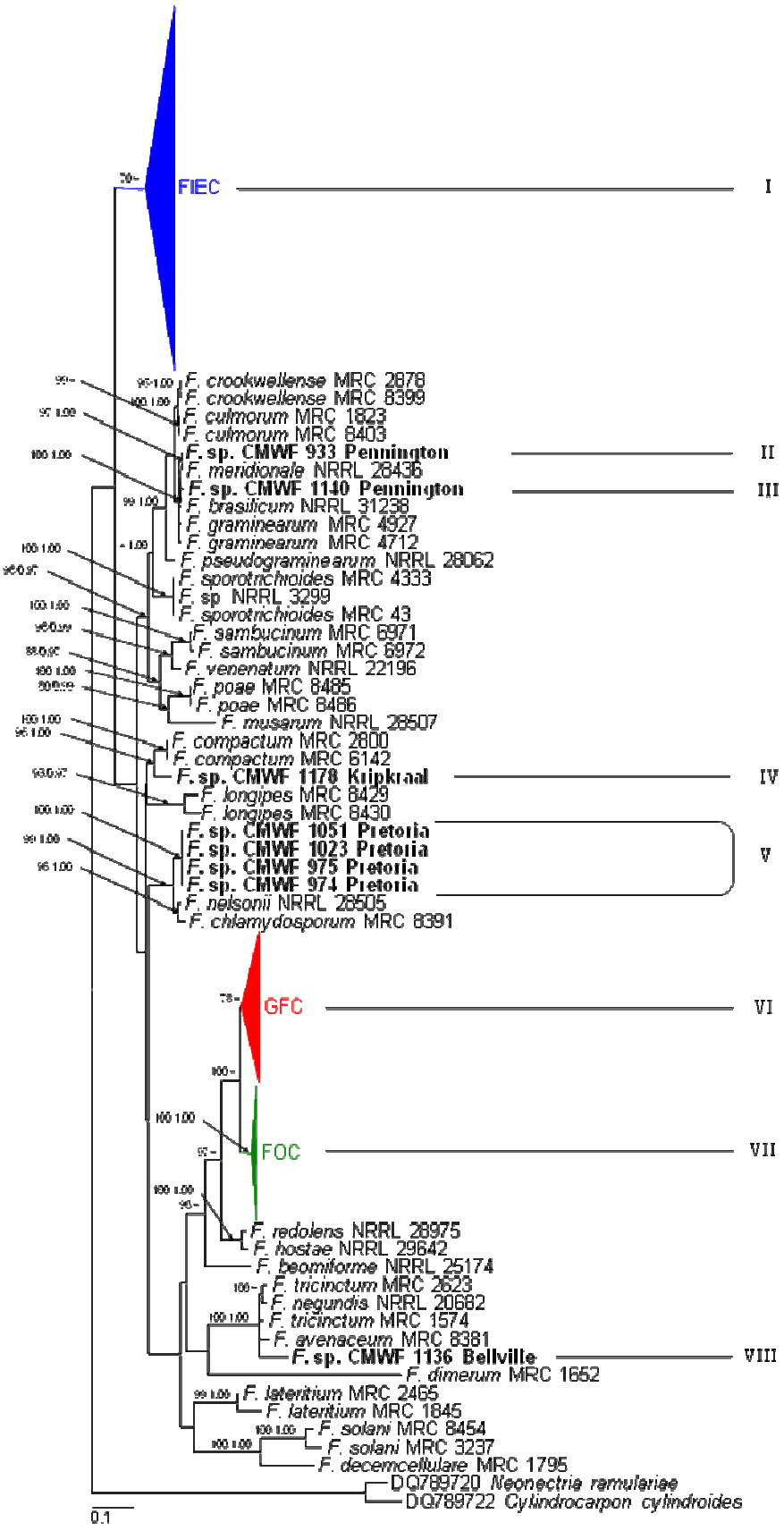

FIEC
I
F. crookwellense MRC 2878
F. crookwellense MRC 8399
F. culmorum MRC 1823
F. culmorum MRC 8403
F. sp. CMWF 933 Pennington
II
F. meridionale NRRL 26436
F. sp. CMWF 1140 Pennington
III
F. brasilicum NRRL 31238
F. graminearum MRC 4927
F. graminearum MRC 4712
F. pseudograminearum NRRL 28062
F. sporotrichioides MRC 4333
F. sp NRRL 3299
F. sporotrichioides MRC 43
F. sambucinum MRC 6971
F. sambucinum MRC 6972
F. venenatum NRRL 22196
F. poae MRC 8485
F. poae MRC 8486
F. musarum NRRL 26507
F. compactum MRC 2800
F. compactum MRC 6142
F. sp. CMWF 1178 Kuipkraal
IV
F. longipes MRC 8429
F. longipes MRC 8430
F. sp. CMWF 1051 Pretoria
F. sp. CMWF 1023 Pretoria
F. sp. CMWF 975 Pretoria
F. sp. CMWF 974 Pretoria
V
F. nelsonii NRRL 28505
F. chlamydosporum MRC 8391
GFC
VI
FOC
VII
F. redolens NRRL 28975
F. hostae NRRL 29642
F. beomiforme NRRL 25174
F. tricinctum MRC 2623
F. negundis NRRL 20682
F. tricinctum MRC 1574
F. avenaceum MRC 8381
F. sp. CMWF 1136 Bellville
VIII
F. dimerum MRC 1652
F. lateritium MRC 2465
F. lateritium MRC 1845
F. solani MRC 8454
F. solani MRC 3237
F. decemcellulare MRC 1795
DQ789720 Neonectria ramulariae
DQ789722 Cylindrocarpon cylindroides
0.1

Fig. 7. The ML phylogenetic tree of the *F. incarnatum/F. equiseti* complex (FIEC) inferred from TEF sequence data. The dataset consisted of 120 taxa, including sequences for the reference strains of *F. semitectum*, *F. pallidoroseum*, *F. incarnatum*, *F.* sp. cf. *bullatum*, *F.* sp. cf. *camptoceras*, *F. equiseti* and *F.* sp. cf. *scirpi*, which were obtained from GenBank or the FID. The *Fusarium* isolates from *Syzygium cordatum* and their geographic origins are indicated in bold. Putative species identities are indicated to the right of the tree. Bayesian inference analyses generated a consensus tree with similar topology. Bootstrap values (≥80%) for the ML and biPP (≥0.95) are indicated at the internodes in that order. Branches with bootstrap support lower than specified are indicated with "–". The tree is rooted with the outgroup taxon *F. chlamydosporum* MRC 8391.

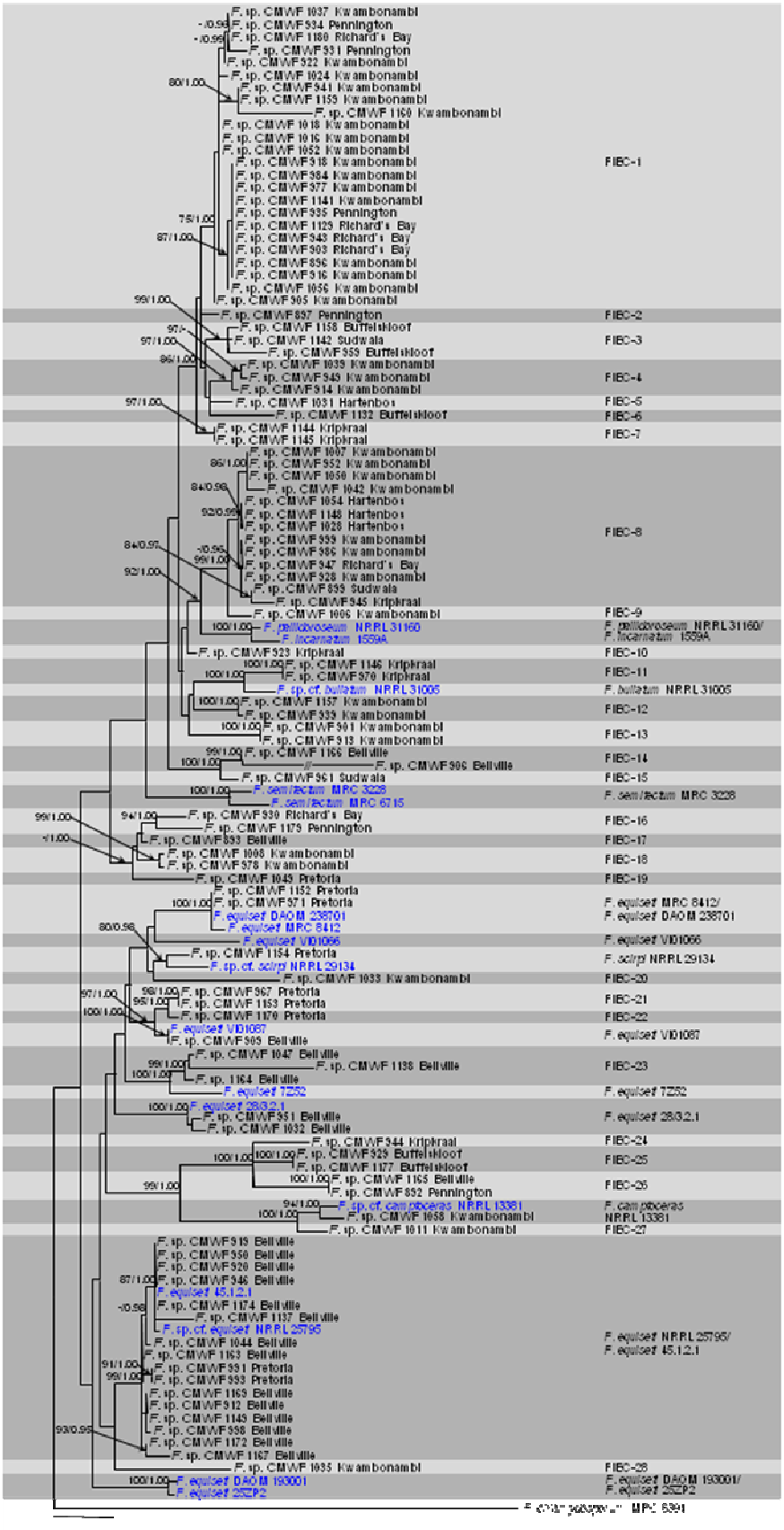

F. sp. CMWF 1037 Kwambonambi
F. sp. CMWF 534 Pennington
F. sp. CMWF 1180 Richard's Bay
F. sp. CMWF 531 Pennington
F. sp. CMWF 522 Kwambonambi
F. sp. CMWF 1024 Kwambonambi
F. sp. CMWF 941 Kwambonambi
F. sp. CMWF 1155 Kwambonambi
F. sp. CMWF 1150 Kwambonambi
F. sp. CMWF 1018 Kwambonambi
F. sp. CMWF 1016 Kwambonambi
F. sp. CMWF 1052 Kwambonambi
F. sp. CMWF 918 Kwambonambi
F. sp. CMWF 984 Kwambonambi
F. sp. CMWF 977 Kwambonambi
F. sp. CMWF 1141 Kwambonambi
F. sp. CMWF 935 Pennington
F. sp. CMWF 1129 Richard's Bay
F. sp. CMWF 943 Richard's Bay
F. sp. CMWF 903 Richard's Bay
F. sp. CMWF 896 Kwambonambi
F. sp. CMWF 916 Kwambonambi
F. sp. CMWF 1056 Kwambonambi
F. sp. CMWF 905 Kwambonambi
F. sp. CMWF 857 Pennington
F. sp. CMWF 1158 Buffelskloof
F. sp. CMWF 1142 Sudwala
F. sp. CMWF 953 Buffelskloof
F. sp. CMWF 1039 Kwambonambi
F. sp. CMWF 949 Kwambonambi
F. sp. CMWF 914 Kwambonambi
F. sp. CMWF 1031 Hartenbos
F. sp. CMWF 1132 Buffelskloof
F. sp. CMWF 1144 Kriphraal
F. sp. CMWF 1145 Kriphraal
F. sp. CMWF 1007 Kwambonambi
F. sp. CMWF 952 Kwambonambi
F. sp. CMWF 1050 Kwambonambi
F. sp. CMWF 1042 Kwambonambi
F. sp. CMWF 1054 Hartenbos
F. sp. CMWF 1148 Hartenbos
F. sp. CMWF 1028 Hartenbos
F. sp. CMWF 959 Kwambonambi
F. sp. CMWF 986 Kwambonambi
F. sp. CMWF 947 Richard's Bay
F. sp. CMWF 928 Kwambonambi
F. sp. CMWF 899 Sudwala
F. sp. CMWF 945 Kriphraal
F. sp. CMWF 1006 Kwambonambi
F. gamsbroseum NRRL 31160
F. incarnatum 1559A
F. sp. CMWF 923 Kriphraal
F. sp. CMWF 1146 Kriphraal
F. sp. CMWF 970 Kriphraal
F. sp. cf. bullatum NRRL 31005
F. sp. CMWF 1157 Kwambonambi
F. sp. CMWF 939 Kwambonambi
F. sp. CMWF 901 Kwambonambi
F. sp. CMWF 913 Kwambonambi
F. sp. CMWF 1166 Bellville
F. sp. CMWF 906 Bellville
F. sp. CMWF 961 Sudwala
F. semitectum MRC 3228
F. semitectum MRC 6715
F. sp. CMWF 930 Richard's Bay
F. sp. CMWF 1179 Pennington
F. sp. CMWF 893 Bellville
F. sp. CMWF 1006 Kwambonambi
F. sp. CMWF 978 Kwambonambi
F. sp. CMWF 1045 Pretoria
F. sp. CMWF 1152 Pretoria
F. sp. CMWF 971 Pretoria
F. equiseti DAOM 238701
F. equiseti MRC 8412
F. equiseti VI01066
F. sp. CMWF 1154 Pretoria
F. sp. cf. scirpi NRRL 29134
F. sp. CMWF 1033 Kwambonambi
F. sp. CMWF 967 Pretoria
F. sp. CMWF 1153 Pretoria
F. sp. CMWF 1179 Pretoria
F. equiseti VI01087
F. sp. CMWF 905 Bellville
F. sp. CMWF 1047 Bellville
F. sp. CMWF 1138 Bellville
F. sp. 1164 Bellville
F. equiseti 7252
F. equiseti 28/3.2.1
F. sp. CMWF 951 Bellville
F. sp. CMWF 1032 Bellville
F. sp. CMWF 944 Kriphraal
F. sp. CMWF 929 Buffelskloof
F. sp. CMWF 1127 Buffelskloof
F. sp. CMWF 1165 Bellville
F. sp. CMWF 852 Pennington
F. sp. cf. camptoceras NRRL 13381
F. sp. CMWF 1058 Kwambonambi
F. sp. CMWF 1011 Kwambonambi
F. sp. CMWF 915 Bellville
F. sp. CMWF 950 Bellville
F. sp. CMWF 920 Bellville
F. sp. CMWF 946 Bellville
F. equiseti 45.1.2.1
F. sp. CMWF 1174 Bellville
F. sp. CMWF 1137 Bellville
F. sp. cf. equiseti NRRL 25795
F. sp. CMWF 1044 Bellville
F. sp. CMWF 1163 Bellville
F. sp. CMWF 991 Pretoria
F. sp. CMWF 993 Pretoria
F. sp. CMWF 1169 Bellville
F. sp. CMWF 912 Bellville
F. sp. CMWF 1149 Bellville
F. sp. CMWF 998 Bellville
F. sp. CMWF 1172 Bellville
F. sp. CMWF 1167 Bellville
F. sp. CMWF 1035 Kwambonambi
F. equiseti DAOM 193001
F. equiseti 252P2
FIBC-1
FIBC-2
FIBC-3
FIBC-4
FIBC-5
FIBC-6
FIBC-7
FIBC-8
FIBC-9
F. gamsbroseum NRRL 31160/
F. incarnatum 1559A
FIBC-10
FIBC-11
F. bullatum NRRL 31005
FIBC-12
FIBC-13
FIBC-14
FIBC-15
F. semitectum MRC 3228
FIBC-16
FIBC-17
FIBC-18
FIBC-19
F. equiseti MRC 8412/
F. equiseti DAOM 238701
F. equiseti VI01066
F. scirpi NRRL 29134
FIBC-20
FIBC-21
FIBC-22
F. equiseti VI01087
FIBC-23
F. equiseti 7252
F. equiseti 28/3.2.1
FIBC-24
FIBC-25
FIBC-26
F. camptoceras
NRRL 13381
FIBC-27
F. equiseti NRRL 25795/
F. equiseti 45.1.2.1
FIBC-28
F. equiseti DAOM 193001/
F. equiseti 252P2

Fig. 8. The ML phylogeny of the *G. fujikuroi* complex based on TEF sequence data. The alignment for this group included 53 sequences of the well defined GFC species/lineages, as well as 33 sequences obtained in this study. The *Fusarium* isolates from *Syzygium cordatum* and their geographic origins are indicated in bold. Putative species identities are indicated to the right of the tree. Three so-called phylogeographic clades of the GFC (O'Donnell *et al.*, 1998a, 2000b) are specified in different colours (*i.e.*, the "African clade" is indicated in red, the "American clade" in green and the "Asian clade" in blue). Monophyletic "African" (red) and "Asian" (blue) clades (O'Donnell *et al.*, 1998a, 2000b) were not recovered, due to a lack of sufficient phylogenetic information in the TEF gene region (Geiser et *al.*, 2005). MP A-J represents the mating populations or biological species of this complex. Statistical support ≥80% and ≥0.95 for the ML and biPP, respectively are indicated at the internodes in the order ML/BI. Branches with bootstrap support values <80% and/or <0.95 are indicated with "–". The tree is rooted with the outgroup taxa *F. oxysporum* and *F. inflexum*.

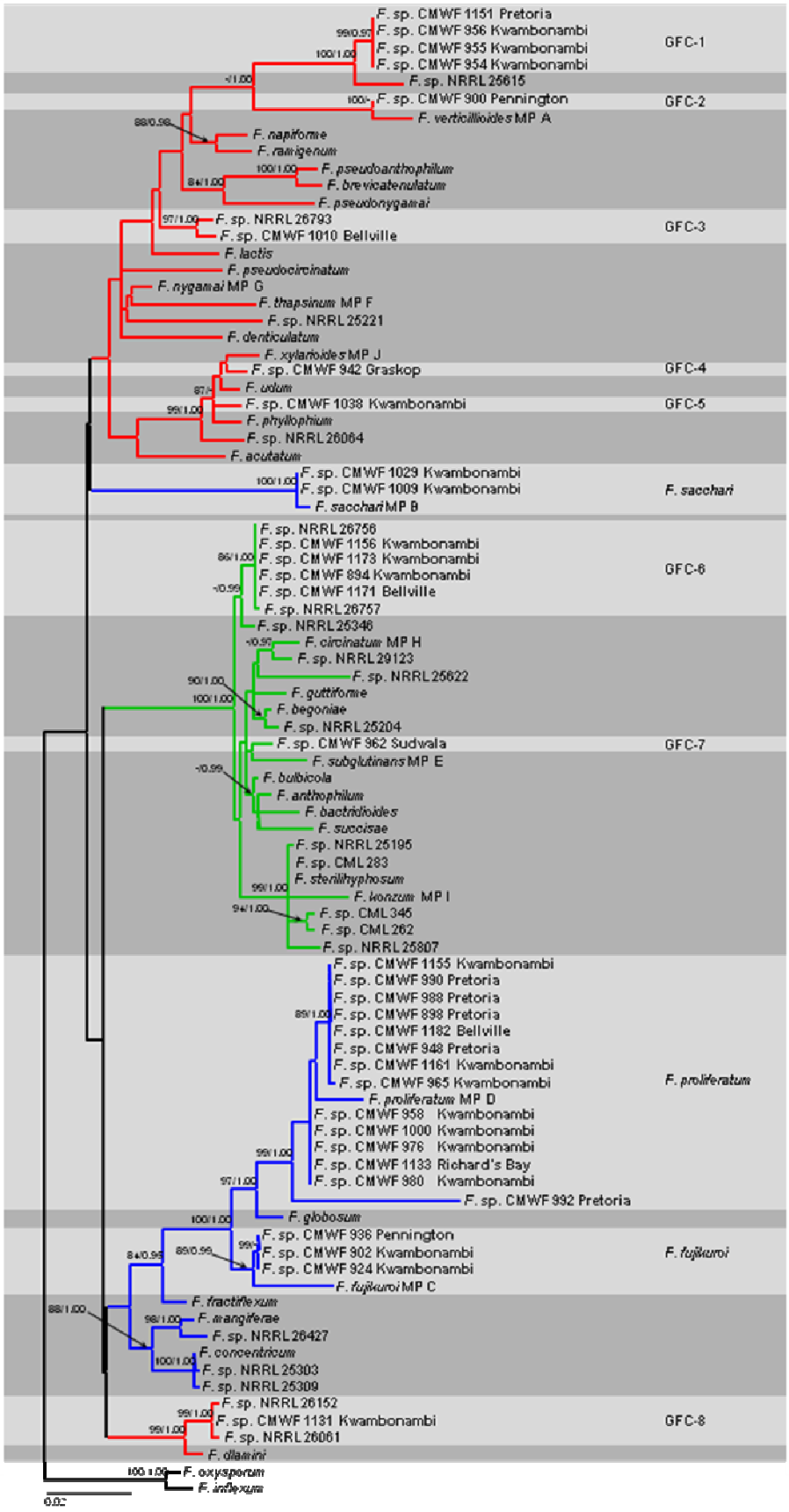

F. sp. CMWF 1151 Pretoria
F. sp. CMWF 956 Kwambonambi
F. sp. CMWF 955 Kwambonambi
F. sp. CMWF 954 Kwambonambi
F. sp. NRRL 25615
F. sp. CMWF 900 Pennington
F. verticillioides MP A
F. napiforme
F. ramigenum
F. pseudoanthophilum
F. brevicatenulatum
F. pseudonygamai
F. sp. NRRL 26793
F. sp. CMWF 1010 Bellville
F. lactis
F. pseudocircinatum
F. nygamai MP G
F. thapsinum MP F
F. sp. NRRL 25221
F. denticulatum
F. xylarioides MP J
F. sp. CMWF 942 Graskop
F. ulum
F. sp. CMWF 1038 Kwambonambi
F. phyllophilum
F. sp. NRRL 26064
F. acutatum
F. sp. CMWF 1029 Kwambonambi
F. sp. CMWF 1009 Kwambonambi
F. sacchari MP B
F. sp. NRRL 26758
F. sp. CMWF 1156 Kwambonambi
F. sp. CMWF 1173 Kwambonambi
F. sp. CMWF 894 Kwambonambi
F. sp. CMWF 1171 Bellville
F. sp. NRRL 26757
F. sp. NRRL 25346
F. circinatum MP H
F. sp. NRRL 29123
F. sp. NRRL 25622
F. guttiforme
F. begoniae
F. sp. NRRL 25204
F. sp. CMWF 962 Sudwala
F. subglutinans MP E
F. bulbicola
F. anthophilum
F. bactridioides
F. succisae
F. sp. NRRL 25195
F. sp. CML 283
F. sterilihyphosum
F. konzum MP I
F. sp. CML 345
F. sp. CML 262
F. sp. NRRL 25807
F. sp. CMWF 1155 Kwambonambi
F. sp. CMWF 990 Pretoria
F. sp. CMWF 988 Pretoria
F. sp. CMWF 898 Pretoria
F. sp. CMWF 1182 Bellville
F. sp. CMWF 948 Pretoria
F. sp. CMWF 1161 Kwambonambi
F. sp. CMWF 965 Kwambonambi
F. proliferatum MP D
F. sp. CMWF 958 Kwambonambi
F. sp. CMWF 1000 Kwambonambi
F. sp. CMWF 976 Kwambonambi
F. sp. CMWF 1133 Richard's Bay
F. sp. CMWF 980 Kwambonambi
F. sp. CMWF 992 Pretoria
F. globosum
F. sp. CMWF 936 Pennington
F. sp. CMWF 902 Kwambonambi
F. sp. CMWF 924 Kwambonambi
F. fujikuroi MP C
F. fractiflexum
F. sp. NRRL 26427
F. concentricum
F. sp. NRRL 25303
F. sp. NRRL 25309
F. sp. NRRL 26152
F. sp. CMWF 1131 Kwambonambi
F. sp. NRRL 26061
F. oxysporum
F. inflexum
GFC-1
GFC-2
GFC-3
GFC-4
GFC-5
F. sacchari
GFC-6
GFC-7
F. proliferatum
F. fujikuroi
GFC-8

Fig. 9. The TEF-based ML phylogeny of the *F. oxysporum* complex. Apart from the 42 isolates obtained during this study, the alignment contained the sequences for 131 strains of *F. oxysporum* (Skovgaard *et al.*, 2001; O'Donnell *et al.*, 2004a; Bogale *et al*., 2006; Fourie *et al.*, 2009). For each of the known isolates, strain number, geographic origin and pathogenic nature are indicated *(i.e*., nonpathogenic, specific *forma specialis* or human pathogen). The *Fusarium* isolates from *Syzygium cordatum* and their geographic origins are indicated in bold. Due to an overall lack of sufficient phylogenetic information in the TEF gene region, the four known clades of this complex were not recovered. However, the known clade affinities of the 131 previously characterised *F. oxysporum* strains (O'Donnell *et al.*, 2004a; Fourie *et al.*, 2009) are indicated according to colour (*i.e.*, strains known to form part of Clade A are indicated in blue, those in Clade B are indicated in green, Clade C in red and Clade D in purple). The BI analyses generated trees with similar topology. Bootstrap values (≥80%) for the ML and ≥0.95 biPP are indicated at the internodes in that order. Branches with lower statistical support are indicated with "–". The phylogram is rooted with the outgroup taxa *Fusarium* sp. strains NRRL 22903 and NRRL 25184.

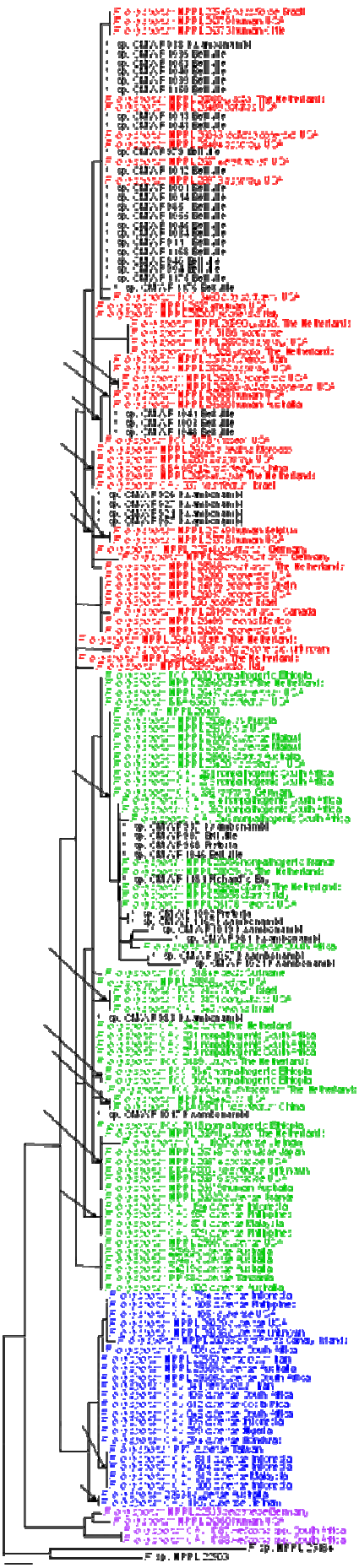


Fig. 10. Light micrographs of the conidial morphology of the seven *Fusarium* morphospecies isolated from *Syzygium cordatum* inflorescences in South Africa. (a) macroconidia of *F. pallidoroseum* CMWF 1054; (b) macroconidia of *F. equiseti* CMWF 967; (c) macroconidium of *F. camptoceras* CMWF 1011; (d) macroconidia of *F. oxysporum* CMWF 1034; (e) microconidia of *F. proliferatum* CMWF 976; (f) microconidia of *F. sacchari* CMWF 1029; (g) microconidia of *F. chlamydosporum* CMWF 1023. Scale bars = 10µm.

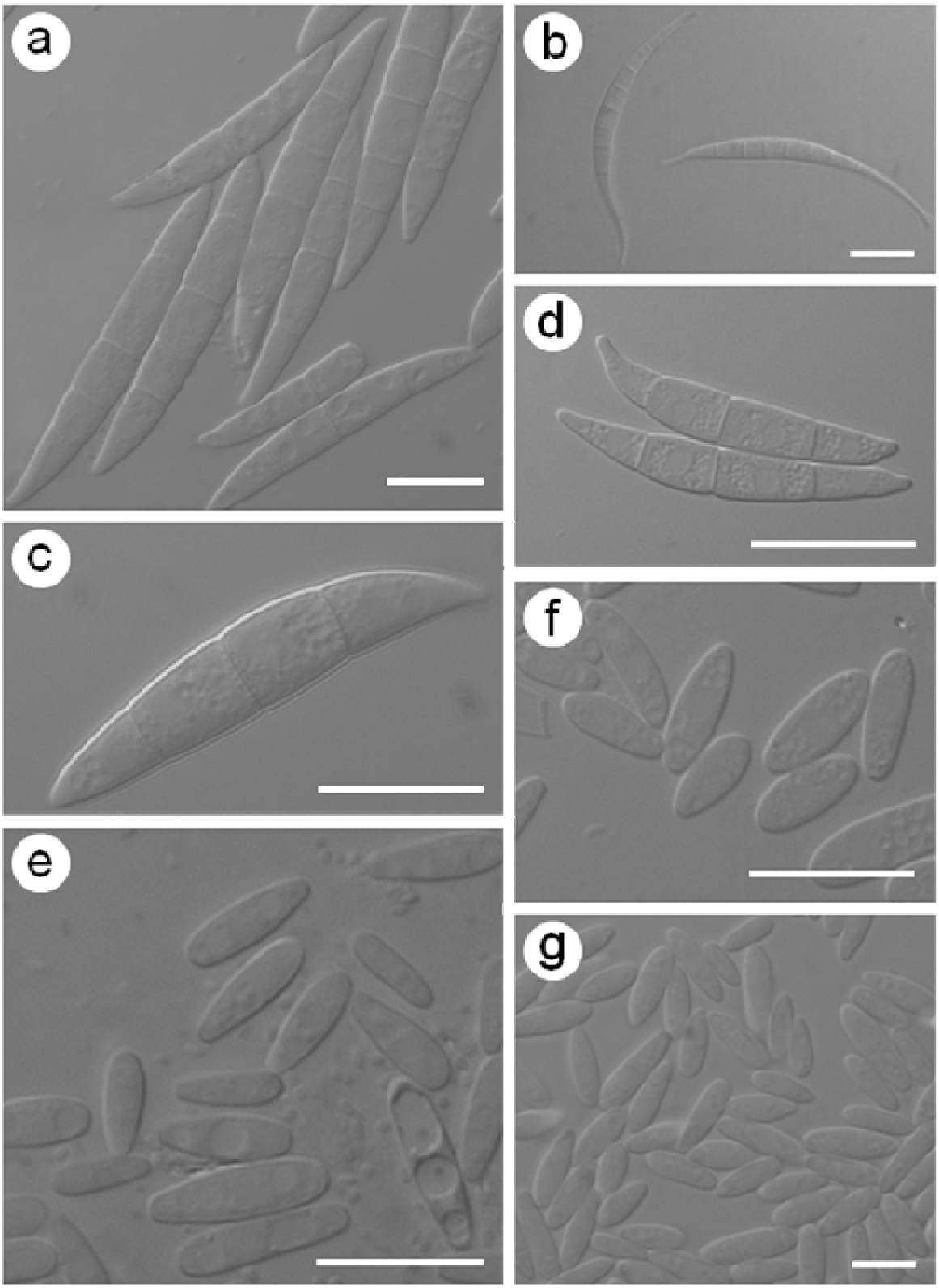
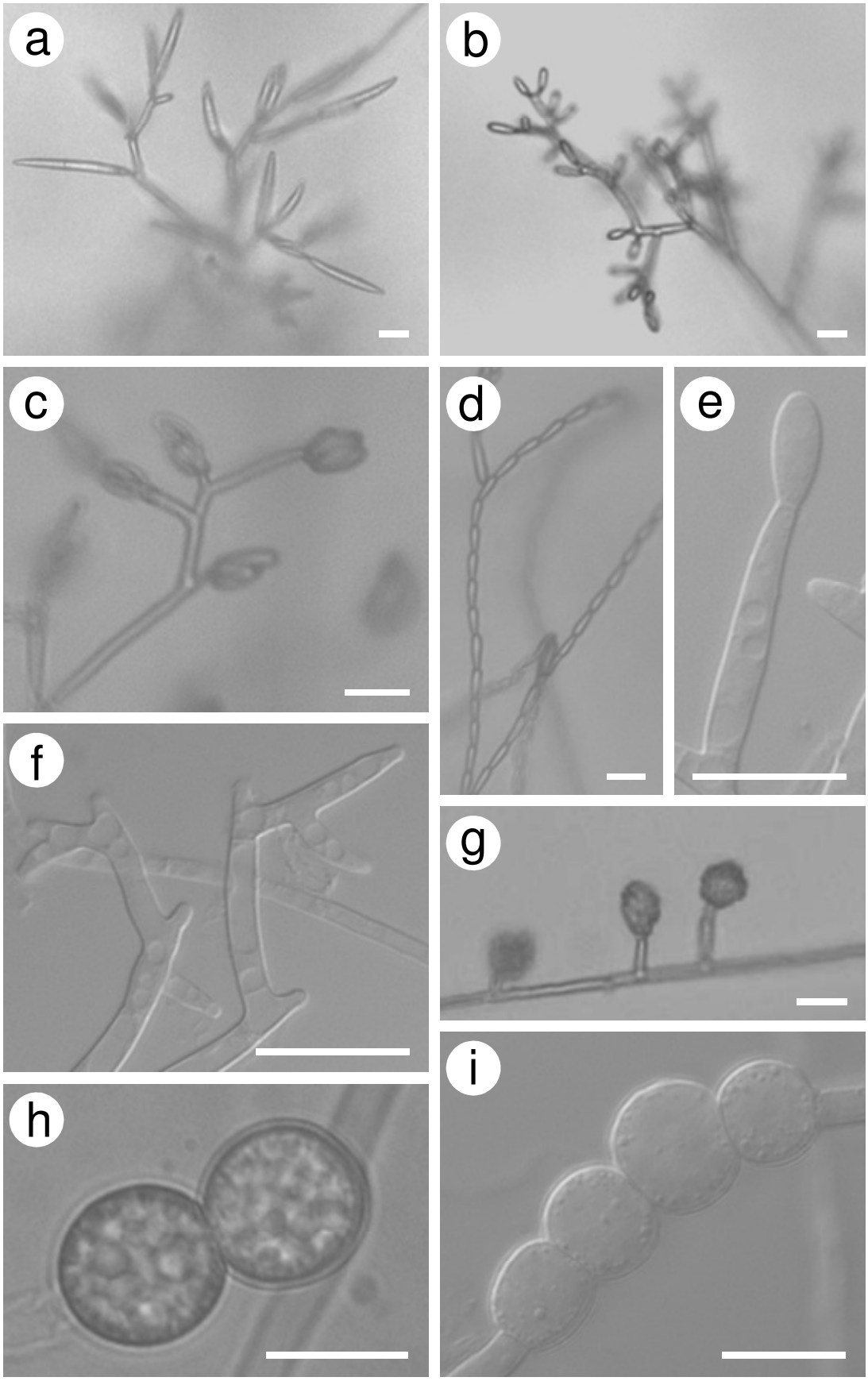

a
b
d
c
f
e
g

Fig. 11. Light micrographs of aerial mycelia, conidiogeneous cells and chlamydospores of various *Fusarium* morphospecies associated with *Syzygium cordatum* inflorescences in South Africa. (a) *F. pallidoroseum* CMWF 1028 mesoconidia *in situ*; (b) aerial mycelium of *F. chlamydosporum* CMWF 1023; (c) microconidia of *F. sacchari* CMWF 1029 in false heads borne on polyphialides (d) *F. proliferatum* CMWF 976 displaying microconidia in chains; (e) monophialide of *F. sacchari* CMWF 1029; (f) polyphialides of *F. sacchari* CMWF 1029; (g) microconidia and monophialides of *F. oxysporum* CMWF 927; (h) chlamydospores of *F. chlamydosporum* CMWF 1023; (i) chlamydospores of *F. oxysporum* CMWF 1034. Scale bars = 10µm.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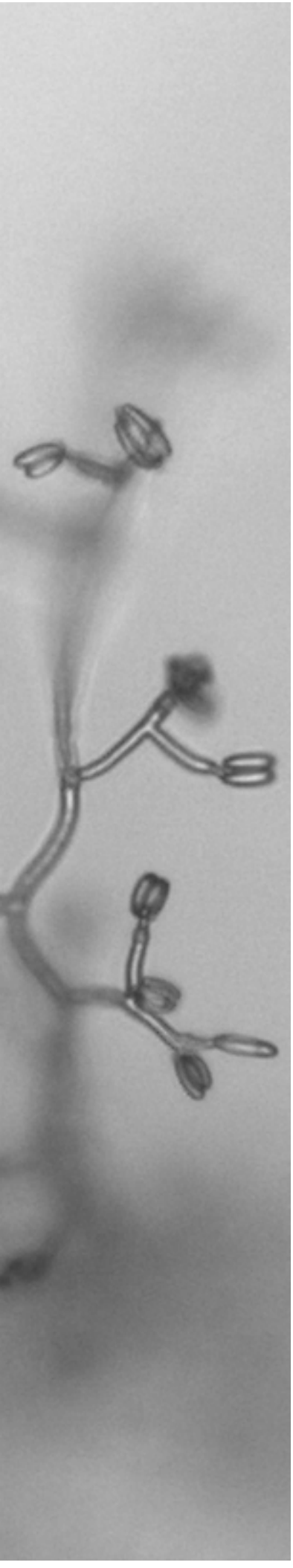
i



Chapter 4: Short Communication

Fusarium mangiferae associated with mango malformation in the Sultanate of Oman

Published as: Kvas, M., Steenkamp, E.T., Al Adawi, A.O., Deadman, M.L., Al Jahwari, A.A., Marasas, W.F.O., Wingfield, B.D, Ploetz, R.C. and Wingfield, M.J. (2008). *Fusarium mangiferae* associated with mango malformation in the Sultanate of Oman. European Journal of Plant Pathology 121: 195–199.

Abstract

Mango malformation, caused by *Fusarium mangiferae*, represents the most important floral disease of mango. The first symptoms of this disease were noticed in the beginning of 2005 in plantations at Sohar in the Sultanate of Oman. The affected inflorescences were abnormally enlarged and branched with heavy and dried-out panicles. Based on morphology and DNA-sequence data for the genes encoding translation elongation factor 1-alpha and β-tubulin the pathogen associated with these symptoms was identified as *F. mangiferae*.

Mango (*Mangifera indica*) is an important perennial crop in the Sultanate of Oman and in 2004 the production of local varieties of this fruit exceeded 8 600 tons (http://www.maf.gov.om/). In many mango-growing regions of the world, an infectious disease known as mango malformation has been reported to limit production and cause substantial economical loss (Kumar *et al*., 1993). The disease was first observed in India in 1891 and has since been reported from a number of countries in Asia, Africa and the Americas (Marasas *et al*., 2006).

Mango malformation is characterised by the abnormal development of vegetative shoots and inflorescences. The vegetative form of the disease is observed more frequently on young seedlings, where auxiliary or apical buds produce misshapen shoots, have shortened internodes and brittle leaves that are significantly smaller than those of healthy plants (Kumar *et al*., 1993). Malformed shoots tend to remain compact thus giving rise to a bunchy-top appearance (Kumar *et al*., 1993; Ploetz, 1994; Marasas *et al*., 2006). The major symptoms of inflorescence malformation include abnormally branched and thickened panicles that produce up to three times the normal number of flowers (Kumar *et al*., 1993). These flowers are unusually enlarged, sterile and do not bear fruit (Kumar *et al*., 1993; Ploetz, 1994; Marasas *et al*., 2006).

Historically, the aetiology of the disease has been poorly understood. Many factors such as physiological abnormalities, viral infection, mite (*Aceria mangiferae*) infestation and fungal infections have been suggested as possible causal agents of the disease (Kumar *et al*., 1993). Various *Fusarium* species have been associated with the disease (Marasas *et al*., 2006). Although there are unpublished reports of at least three different taxa in this genus causing symptoms of malformation on mango (C. Lima, personal communication; G. Rodríguez, personal communication), a fourth taxon, *F. mangiferae*, is the only one which has conclusively been shown to cause mango malformation (Freeman *et al*., 1999; Britz *et al*., 2002; Marasas *et al*., 2006). To date, the presence of *F. mangiferae* has been confirmed in Egypt, USA (Florida), Israel, Malaysia and South Africa (Britz *et al*., 2002; Marasas *et al*., 2006). Based on results with species-specific PCR primers, the pathogen may also exist in Spain (S. Freeman, personal communication). A recent report from Pakistan cannot be confirmed based on the diagnostic data it contained (Iqbal *et al*., 2006).

When *F. mangiferae* was first isolated from malformed tissue, Summanwar *et al*. (1966) recognised it as *F. moniliforme*. Later, Varma *et al*. (1974) used the name *F. moniliforme* var.

subglutinans and demonstrated its involvement in both the vegetative and floral forms of the disease. Nelson *et al*. (1983) recognised the fungus as *F. subglutinans* in the section *Liseola*, which broadly corresponds with the so-called *Gibberella fujikuroi* complex (GFC) (O'Donnell *et al*., 1998a). To accommodate morphologically and phylogenetically related isolates of *F. subglutinans* (Steenkamp *et al*., 2000) that had been shown previously to cause mango malformation (Freeman *et al*., 1999), Britz *et al*. (2002) established the taxon, *F. mangiferae*.

Early in 2005, typical symptoms of mango malformation were noticed on mango trees at Sohar, 250 km north-west of Oman's capital Muscat. These symptoms included abnormally enlarged inflorescences with thick, branched and heavy panicles (Fig. 1). To confirm the presence of mango malformation in Oman, we used DNA sequence comparisons and morphology to identify *F. mangiferae* in symptomatic tissue.

Samples of malformed inflorescences were collected from infected trees and surface-sterilised by submerging pieces of plant tissue in a sodium hypochlorite (1%) solution and then in 70% ethanol for one minute each. Samples were then rinsed in sterile distilled water and dried on sterile filter paper before plating small flower pieces onto 39 g l^{-1} potato dextrose agar (PDA, Biolab, Merck). Following incubation at 25^{o}C for 7 days, pure fungal cultures were obtained by single conidial spore transfers onto 20 g l^{-1} PDA medium. All isolates are stored and maintained in the *Fusarium* collection of the Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

To determine the identity of the fungus that was recovered from the diseased mango tissue, three representative isolates were characterised based on morphological characteristics and DNA sequence comparisons. In order to observe morphological features, isolates were grown on 39 g l^{-1} PDA, synthetic low nutrient agar (Nirenberg and O'Donnell, 1998) and carnation leaf agar (Fisher *et al*., 1982). After incubation at 25^{o}C for 10 days under near ultraviolet light, the isolates were examined using a light microscope and the diagnostic characters noted by Britz *et al*. (2002) and Nelson *et al*. (1983).

For the DNA comparisons, the first ~700 and ~500 bases of the genes encoding translation elongation factor 1-alpha (TEF) and β-tubulin, respectively, were sequenced. For this purpose, genomic DNA was extracted using the CTAB (N-cetyl-N, N, N-trimethyl-ammonium bromide) method (Steenkamp *et al*., 1999). The two gene regions were amplified with primer set EF1+EF2 [5'-atgggtaagga(a/g)gacaagac-3' and 5'-gga(g/a)gtaccagt(g/c)atcatgtt-3';

O'Donnell *et al*., 1998b] and T1+T2 [5'-aacatgcgtgagattgtaagt-3' and 5'-tagtgacccttggcccagttg-3'; O'Donnell and Cigelnik, 1997], respectively, using previously described PCR reaction and cycling conditions (Geiser *et al*., 2005). After purification with G50 Sephadex columns (Sigma, Steinheim, Germany), PCR products were sequenced in both directions using the original PCR primers, an ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and a 3730 DNA Analyzer (Applied Biosystems). The electropherograms were visualised and corrected where necessary with Chromas Lite v2.0 (Technelysium, Australia) and BioEdit v7.0.5.2 (Hall, 1999). All TEF nucleotide sequences were compared using the BLAST search tool (Altschul *et al*., 1990) to those in the *Fusarium* identification database (Geiser *et al*., 2004; http://fusarium.cbio.psu.edu/) and all β-tubulin nucleotide sequences were compared to those in the database of the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) to obtain preliminary identifications.

Nucleotide sequences were aligned using MAFFT v5.8 with the L-INS-i option effective (Katoh *et al*., 2002, 2005; http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). These alignments included the sequences generated in this study, as well as those for representatives of the recognised species in the GFC (O'Donnell *et al*., 1998a, 2000; Geiser *et al*., 2005) which were obtained from GenBank. Relevant sequences for all of the known unique phylogenetic lineages in the so-called "Asian Clade" of the GFC (O'Donnell *et al*., 1998a, 2000) were also included. The resulting aligned datasets were analysed separately as well as combined, because they were previously shown to represent homogenous partitions (O'Donnell *et al*., 1998a, 2000). PAUP* 4b10 (Swofford, 2003) was used to perform neighbour-joining distance (NJ) analyses and parsimony analyses using heuristic searches of 1 000 random addition replicates and tree bisection reconnection branch swapping analyses. Maximum likelihood (ML) analyses were performed with PHYML v2.1 (Guindon and Gascuel, 2003) and Bayesian analyses were performed with MrBayes v3.1 (Ronquist and Heuelsenbeck, 2003). The latter was based on the Metropolis-coupled Markov Chain Monte Carlo search algorithm with 1 000 000 generations, and calculation of Bayesian posterior probabilities after discarding a burnin of 500 generations. Modeltest v3.7 (Posada and Crandall, 1998) and MrModelTest v2.2 (Nylander, 2004; Posada and Crandall, 1998) were used to determine the appropriate evolutionary models for the NJ, ML and Bayesian analyses. The General Time Reversible (GTR) model (Rodríguez *et al*., 1990)

with gamma correction for rate variation was used for TEF, while the Hasegawa-Kishino-Yano (HKY) model (Hasegawa *et al*., 1985) with gamma correction was used for the β-tubulin dataset. Analyses of the combined dataset utilised the GTR model with proportion invariable sites and gamma correction. ML, NJ and parsimony branch supports were estimated using 1 000 bootstrap replicates.

Morphological examination revealed that all three isolates produced macroconidia with 3–5 septa and oval microconidia in false heads from mono- and polyphialides. None of the representative isolates produced chlamydospores under the conditions tested. These morphological characters are typical of *F. mangiferae*, as well as most fungi previously recognised as *F. subglutinans sensu lato* (Britz *et al*., 2002).

Results of the DNA sequence comparisons indicated that the fungi isolated from diseased Omani mango flowers, represent *F. mangiferae*. Similarity searches and sequence comparisons revealed that the TEF and β-tubulin sequences of the Oman isolates are identical to that of *F. mangiferae* NRRL 25226 (GenBank Accessions AF160281 and U61561; O'Donnell *et al*., 2000; Steenkamp *et al*., 2000; Britz *et al*., 2002). Their TEF sequences differed at two nucleotide positions from that of the ex-holotype isolate (FCC 4581; Britz *et al*., 2002). However, results of the phylogenetic analyses clearly showed that the Oman isolates form part of the so-called "Asian Clade" (O'Donnell *et al*., 1998a, 2000) of the GFC, where they are most closely associated with known *F. mangiferae* isolates (Fig. 2). These results conclusively demonstrate that the Oman isolates from malformed mango inflorescences represent *F. mangiferae*.

To the best of our knowledge, *F. mangiferae* and mango malformation have not previously been reported in the Sultanate of Oman. Mango malformation has the potential to have a significant negative impact on the mango industry in Oman and the Middle East as this disease significantly reduces yields. As a result, the spread of the disease must be halted by removing and burning affected trees, as well as by planting non-infected nursery supplies (Marasas *et al*., 2006). Research is underway to ascertain the distribution of the disease in Oman and the route by which the disease entered the country.

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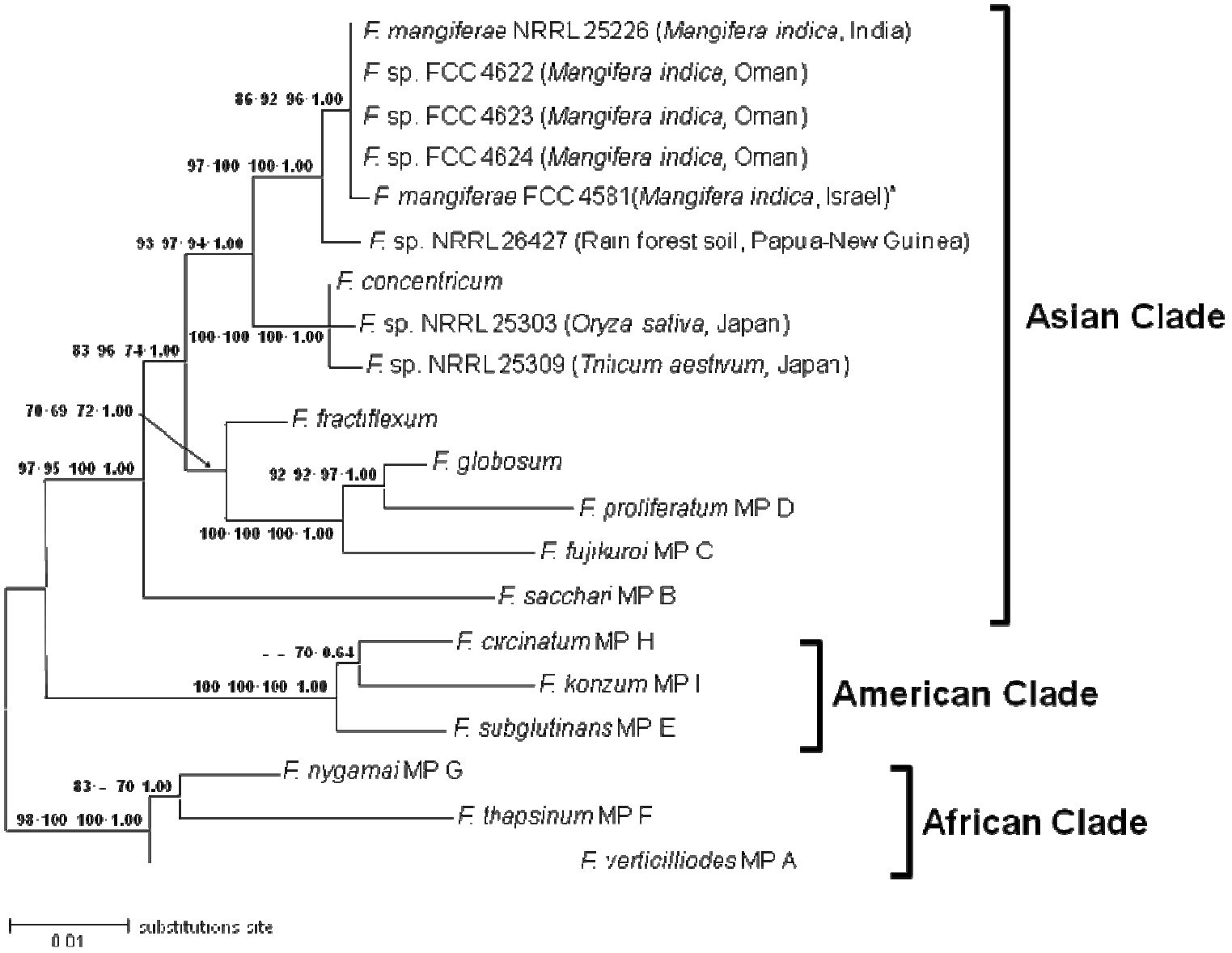
Fig. 1. Malformed inflorescence of mango collected at Sohar in the Sultanate of Oman. Photo by Dr. Ali Al Adawi.

Fig. 2. A maximum likelihood (ML) phylogeny of the *Gibberella fujikuroi* species complex based on combined translation elongation factor 1α and β-tubulin sequence data. The mating populations (MP) of this complex, as well as all of the known members of the so-called "Asian Clade" (O'Donnell *et al*., 1998a, 2000) are included. The ex-holotype of *F. mangiferae* is indicated with an asterisk and the tree is rooted with the *Fusarium* species in the so-called "African Clade". Neighbour joining (NJ) distance, parsimony and Bayesian analyses of the combined dataset generated trees with topologies similar to that of the ML tree. The topologies of trees generated from analyses of the individual gene datasets were also congruent with these trees. Bootstrap support values >60% based on 1 000 replications and Bayesian posterior probabilities are indicated at the branches in the order parsimony, NJ, ML and Bayesian. Branches with bootstrap support values <60% are indicated with "-".


F. mangiferae NRRL 25226 (Mangifera indica, India)
F. sp. FCC 4622 (Mangifera indica, Oman)
F. sp. FCC 4623 (Mangifera indica, Oman)
F. sp. FCC 4624 (Mangifera indica, Oman)
F. mangiferae FCC 4581(Mangifera indica, Israel)*
F. sp. NRRL 26427 (Rain forest soil, Papua-New Guinea)
F. concentricum
F. sp. NRRL 25303 (Oryza sativa, Japan)
F. sp. NRRL 25309 (Triticum aestivum, Japan)
F. fractiflexum
F. globosum
F. proliferatum MP D
F. fujikuroi MP C
F. sacchari MP B
F. circinatum MP H
F. konzum MP I
F. subglutinans MP E
F. nygamai MP G
F. thapsinum MP F
F. verticilliodes MP A
Asian Clade
American Clade
African Clade
substitutions site
0.01

SUMMARY

Inflorescence malformation in *Syzygium cordatum* and *Mangifera indica* (mango) is characterised by abnormally enlarged and excessively branched panicles, which are unable to bear fruits. In mango, the malformation disease is caused by at least three *Fusarium* species in the economically important group of fungi that are collectively known as the *Gibberella fujikuroi* complex. Previously, a single *Fusarium* species that also forms a part of this complex has been reported from the malformed inflorescences of *S. cordatum*. The primary aim of this dissertation was to identify *Fusarium* species associated with the floral malformation on native *S. cordatum* in South Africa and agriculturally important *M. indica* in the Sultanate of Oman.

In Chapter 1, a critical overview of the diversity, taxonomy and evolution of *Fusarium* species in the *G. fujikuroi* complex is presented. Currently, the complex includes at least 50 *Fusarium* species and phylogenetic lineages. Of these, 34 represent morphospecies and 10 are sexually fertile biological species that have been described formally. The taxonomic history of the complex and species concepts used to resolve taxonomic predicaments are discussed. From an evolutionary point of view, the species in the *G. fujikuroi* complex are grouped into three major clades, named after the geographic origins of their respective host plants. The biogeographic hypothesis appears to be true for most of the species, although each clade includes at least one species that does not fit this view. Apart from the aerial arrangement of microconidia, no other morphological character, host/substrate preference and/or mycotoxigenic property uniquely differentiates the members of the three clades from one another. Our understanding of the phylogeography and evolution of these fungi, therefore, will be dependent on the study of *Fusarium* species that were isolated from indigenous ecosystems.

Floral malformations are important diseases of angiosperms that can cause plant sterility. Chapter 2 of this dissertation focuses on the diversity of insects, mites, nematodes, viruses, bacteria, fungi and oomycetes associated with these diseases. Specific examples of inflorescence abnormalities that these biotic groups induce are provided. As the malformations of both *M. indica* and *S. cordatum* have been associated with *F. suglutinans sensu lato* and mites in the genus *Eriophyes*, special emphasis is given to the *Fusarium* species and mites that are known to colonise the inflorescences of these plants. Future studies should elucidate the role of these *Fusarium* taxa in the development of the malformation disease.

As part of the research presented in Chapter 3, a total of 52 *Fusarium* species/lineages were identified from malformed inflorescences of *S. cordatum* in South Africa. These taxa were characterised using PCR-RFLP fingerprinting, UPGMA cluster analyses, TEF barcoding, phylogenetic analyses and morphological examination. Although fingerprinting and UPGMA clustering allowed grouping of large numbers of isolates, identifications could not be performed due to a lack of appropriate reference strains. TEF sequence analysis, however, revealed a high level of interspecific variation among *Fusarium* species, thus confirming the reliability of the TEF gene region for use in barcoding. This was also evident in the results of the phylogenetic analyses, which allowed unambiguous identification of the *Fusarium* species associated with *S. cordatum*. *Fusarium* species/lineages colonising *S. cordatum* included members of the *F. incarnatum/F. equiseti*, *F. oxysporum*, *G. fujikuroi*, *F. graminearum* complexes, as well as close relatives of *F. chlamydosporum/F. nelsonii*, *F. compactum*, *F. tricinctum/F. avenaceum*. *Fusarium* isolates within *F. oxysporum* complex formed two distinct groups. At least 28 and 8 novel species/lineages were detected in the *F. incarnatum/F. equiseti* and *G. fujikuroi* complexes, respectively. Formal description of these novel species will require detailed analyses of their morphological traits and phylogenetic studies based on multiple gene regions.

Mango production represents an important component of the economy of the Sultanate of Oman. In 2005, mango malformation was recorded for the first time in this country when malformation symptoms were observed in plantations at Sohar. As the disease can be caused by a number of different *Fusarium* species, the aim of Chapter 4 was to identify the species associated with the disease in Oman. By making use of a polyphasic approach, it was shown conclusively that mango malformation in the Sultanate of Oman is associated with *F. mangiferae*. At present, the disease is not serious and every effort must be made to ensure that its severity does not increase.

In total eight *Fusarium* species are known to be associated with mango malformation. Apart from the three species that are known to induce the disease (i.e. *F. mangiferae*, *F. sterilihyphosum* and *F.* sp. CML 345), *F. proliferatum*, *F. subglutinans*, *F. sacchari* and *F. oxysporum* have also been reported. Except for *F. oxysporum*, all of these species form part of the *G. fujikuroi* complex. In comparison, 52 putative species were isolated from the malformed inflorescences of *S. cordatum*. Of these, two were conspecific to *F. proliferatum* and *F. sacchari* and several isolates represented *F. oxysporum.* Although *F. subglutinans* has been reported

previously from both of these hosts, none of the isolates obtained in the current study, from *S. cordatum* or in previous studies from *M. indica*, were conspecific to it. This is not surprising as at least ten distinct species (*F. subglutinans*, *F. circinatum*, *F. pseudocircinatum*, *F. begoniae*, *F. bulbicola*, *F. sterilihyphosum*, *F. guttiforme*, *F. sacchari*, *F. concentricum* and *F. mangiferae*) within the *G. fujikuroi* complex resemble *F. subglutinans sensu lato* at the morphological level. In the current study, at least five of the putative species identified have morphological features typical of these fungi. The initial reports of *F. subglutinans* associated with the malformed inflorescences of *S. cordatum* and *M. indica* could, therefore, have referred to any of a large number of *Fusarium* species with similar morphological traits.

The results presented in this study provide the first comprehensive information on *Fusarium* species associated with indigenous Myrtaceae in South Africa. Additional sampling in the native vegetation of the vast African continent is needed to further extend our knowledge on the diversity and evolution of *Fusarium* as many species still remain to be discovered. Additional gene genealogies and population studies on *Fusarium* species associated with these two and other hosts could provide us with more information on the distribution, movement and origin of *Fusarium* species. It is my hope that the research presented in this dissertation will contribute towards a better understanding of *Fusarium* species and will also elevate interest in discovering novel ones.