Taxonomy of species within the *Gibberella fujikuroi* complex

BY

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I, Adriaana Jacobs declare that the thesis, which I hereby submit for the degree of Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: **Date:** May 2010.

Dedicated to Adriaan Albertus Koen,
The man who inspired me to follow my dream

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Technical report

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Preface

Fusarium is a large genus of filamentous fungi with a cosmopolitan distribution and a well established association with a variety of plant species. Numerous of these plant hosts are economically important such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), pineapple (*Ananas comosus* (L.) Merr.) and banana (*Musa* spp.). Plant diseases caused by *Fusarium* spp. have thus had major impacts on the global economy. Some members of the genus produce mycotoxins in cereal crops. These toxins affect human and animal health when they enter the food chain. Amongst the more important groups of toxins produced by *Fusarium* species are fumonisins, moniliformin and beauverin.

The taxonomy of *Fusarium subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas and the Section *Liseola* Wollenw. & Reinking of which it is a member, has been the subject of extensive taxonomic revision in the last decade. When *F. subglutinans* was first described, it was recognised as a variety of *F. moniliforme* J. Sheld. but in 1955, *F. moniliforme* var. *subglutinans* Wollenw. & Reinking was synonymised with *F. neoceras* Wollenweber & Reinking, and included in a combined Section *Elegans* and *Liseola*. Lately, the results of DNA-based phylogenetic studies have emphasised the polyphyletic nature of this taxon. Of the distinct phylogenetic lineages in the *Gibberella fujikuroi* species complex, at least 13 are represented by isolates with morphology typical of *F. subglutinans*. They include the pathogens *F. circinatum* Nirenberg & O'Donnell (causal agent of pine pitch canker), *F. sacchari* (E.J. Butler & Hafiz Khan) W. Gams (causal agent of pokkah boeng disease of sugarcane), *F. guttiforme* Nirenberg & O'Donnell (associated with fusariosis of pineapple), and *F. mangiferae* Britz, M.J. Wingf. & Marasas (causal agent of mango malformation disease). Species with the *F. subglutinans*-type morphology have also been recovered from *Nerine bowdenii*, *Musa sapientum*, *Begonia* spp. and *Pinus radiata* (i.e. *F. bulbicola* Nirenberg & O'Donnell, *F. concentricum* Nirenberg & O'Donnell, *F. begoniae* Nirenberg & O'Donnell and *F. pseudocircinatum* O'Donnell & Nirenberg, respectively).

In the first chapter of this thesis a taxonomic overview of the species concepts used in the demarcation of *Fusarium* species are reviewed. The review includes the advantages and shortcomings of these concepts. The morphological species concept is based on the morphological differences amongst species while the biological species concept clusters species together based on their potential to produce fertile offspring after mating. The phylogenetic species concept distinguishes species based on their evolutionary relatedness based on sequence data of specific target genes.

Fusarium subglutinans sensu lato accommodates a number of morphologically and phylogenetically distinct species. The second chapter of this thesis encompasses the re-evaluation of *F. subglutinans sensu lato*. The evaluation is based on taxonomically morphologically informative

characters, such as the nature of the aerial mycelium, microconidial and macroconidial morphology, growth rates and the presence or absence of sterile coils. Furthermore, the species were distinguished based on sequence data generated for the translation elongation factor-1α (TEF-1α), β-tubulin (BT), calmodulin (cal) genes as well as the large subunit of the rDNA gene region. A dichotomous key for easy identification of species in *F. subglutinans sensu lato* was also established.

Many species of *Fusarium* have been described from economically important crops but far less is known about those that occur in native ecosystems. As native ecosystems are replaced with monoculture for agriculture or forestry the *Fusarium* spp. that occur in these ecosystems come in contact with new potential hosts. In the third chapter the taxonomic placement of a new *Fusarium* species from Poaceae, in South Africa is considered. Both morphological and molecular characters, based on the sequence data for various protein-coding genes were used to determine the relationship of the new species to other species of *F. subglutinans sensu lato*. More specifically with the two species, *F. subglutinans sensu stricto* and *F. circinatum*, with which it formed fertile offspring after sexual crosses were made.

In the fourth chapter the causal agent of pine decline in Chilean nurseries is characterised by means of sexual crosses, phylogenetics and morphological comparisons. The causal agent is identified as *F. circinatum*. This discovery led to the first report of this fungus in Chile.

Fusarium infection of *Ananas comosus* plants in South America nearly devastated the pineapple industries in many of these countries. In the fifth chapter the taxonomic placement of a new *Fusarium* species from *A. comosus* in South Africa is considered. Both morphological characters such as colony colour, the nature of the aerial mycelium and macro conidial morphology as well as sequence data generated for the Histone H3, BT and TEF-1α genes were used to determine the relationship of the new species to other species in *F. subglutinans sensu lato* and more specifically with *F. guttiforme* which is also associated with *A. comosus* in other parts of the world.

The overall objectives of the studies presented in this thesis were made to elucidate the taxonomic placement and demarcation of species in *F. subglutinans sensu lato*. This study has increased our understanding of this complex of species in this large and economically important genus.

CHAPTER 1

Species concepts in *Fusarium* with specific reference to species in *Fusarium subglutinans sensu lato*

Literature Review*:* **Species concepts in *Fusarium* with specific reference to species in *Fusarium subglutinans sensu lato***

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1. Introduction

The genus *Fusarium* was established by Link in 1809. At that time, this genus accommodated species with fusiform, non-septate spores borne on stromata (Booth, 1971). The three isolates described as the type, *F. roseum* Link, however, represented three distinct species. One of the three original specimens corresponded to *F. sambucinum* Wollenw, the second to *F. graminearum* Schwabe (Booth, 1971) and the third specimen (*F. roseum* from a gramineous plant) may have represented *F. graminum* Corda (Gerlach and Nirenberg, 1982). The genus was validated in 1821 by Fries. He included it in the order Tuberculariae (Booth, 1971) with *F. sambucinum* as the type species (Gams *et al*., 1997).

Fusarium is a large genus of filamentous fungi widely distributed in soil and associated with plants (Leslie and Summerell, 2006). Numerous of these plant species are economically important such as banana, maize, pineapple and wheat. Plant diseases caused by *Fusarium* spp. have had major impacts on the global economy. These include the near devastation of the banana and pineapple industries in South America and huge losses suffered by farmers in the USA due to *Fusarium* infection of cereals (Leslie and Summerell, 2006).

Some species of *Fusarium* produce mycotoxins in cereal crops. These affect human and animal health when they enter the food chain (Marasas *et al*., 1984; 2001; Bryden *et al*., 2001). Amongst the more important groups of toxins produced by *Fusarium* species are fumonisins (Marasas *et al*., 1984; 2001) and trichothecenes (Marasas *et al*., 1984).

The aim of this review is to provide a broad overview on speciation in *Fusarium*. It also treats the application of species concepts in the genus and more especially in the *Gibberella fujikuroi* species complex.

2. Species concepts used in the demarcation of *Fusarium* species

Species concepts can be divided into two groups. The ‘pattern based’ concepts include the taxonomic or morphological species concepts in which the taxonomist differentiates the species based on the shared morphological characters that distinguish a group of individuals from other groups. The phylogenetic species concept also forms part of the pattern based groups of species concepts. It defines a species as the smallest discernable cluster of individual organisms within which there is a parental pattern of ancestry and decent (Higgs and Attwood, 2005; Lowe *et al*., 2006).

The 'process-based species concepts' include species concepts based on the biology of the organism. These include the biological species, recognition and cohesive species concepts. The biological species concept is based on Mayr's (1940) definition of a species. He described a species as groups of 'actually or potentially interbreeding natural populations which are reproductively isolated from other such groups' (Rieger *et al*., 1991).

Three of the above mentioned species concepts are commonly followed for *Fusarium*. These are the pattern-based approaches using morphological characteristics and phylogenetic inference with specific emphasis on the genealogical concordance of species. In addition, the process-based biological species concept is also commonly applied to this group of fungi.

2.1. Taxonomic history

Fusarium has had a long and confused taxonomic history. The genus was established by Link in 1809 with *F. roseum* as the type species. At that time, the genus accommodated species with fusiform, non-septate spores borne on stromata in the order Tuberculariae (Booth, 1971).

The book 'Die Fusarien' by Wollenweber (1935) (as cited in Toussoun and Nelson, 1975; Leslie and Summerell, 2006) is generally regarded as the foundation for *Fusarium* taxonomy. The authors accepted 143 species grouped into 16 sections. Snyder and Hansen (1940) (as cited in Toussoun and Nelson, 1975; Leslie and Summerell, 2006) reduced the genus to 9 species with no varieties or forms. Booth (1971) recorded 44 species and 7 varieties, in 12 groups. In a later evaluation, Gerlach and Nirenberg (1982) expanded these to 90 species.

Classification based on the Snyder and Hansen (1940) (as cited in Toussoun and Nelson, 1975; Leslie and Summerell, 2006) system is characterised by a huge reduction in species numbers. They considered the shape of the macroconidia as the most stable characteristic to be used. Secondary characteristics included the presence or absence of chlamydospores or microconidia as well as the shape of the microconidia. The rationale behind the reduction in species was to provide plant pathologists of the time with a practical classification.

Bilai (1955) (as cited in Toussoun and Nelson, 1975; Leslie and Summerell, 2006) maintained the classification system of Wollenweber (1935) but based all her observations on single spore cultures. She concluded that the most fundamental characters used to delimit species were the curvature of the conidia and the shape of the top of the macroconidial apical cell. She considered culture characteristics such as pigmentation as well as mode of sporulation and absence and presence of sclerotia as unimportant.

Messiaen and Cassini (1968) (as cited in Toussoun and Nelson, 1975; Leslie and Summerell, 2006) followed the Snyder and Hansen (1940) system of classification for *Fusarium*, with some adjustment to accommodate plant pathologists, who at that time preferred to use the

taxonomic scheme of Snyder and Hansen (1940). The reasons for this preference were that they comprehended the Snyder and Hansen system as practical and easy to use. The major modification made by Messiaen and Cassini (1968) was to substitute botanical varieties for cultivars.

Booth (1971) based his classification of *Fusarium* on spore morphology and the morphology of the conidiogenous cells. The emphasis on conidiophore morphology and the mode of production of conidia is considered as Booth's most prominent contribution to *Fusarium* taxonomy. In this classification system, the Section *Liseola* contained one species and one variety differentiated on phialide morphology (Booth, 1971; 1977).

In their treatment of *Fusarium* spp., Nelson *et al*. (1983) accommodated 31 species and 17 insufficiently documented species in 13 sections. They relocated the non-chlamydospore producing *Fusarium* spp. in the Section *Elegans*, to the Section *Liseola*. They recognised only 4 taxa in the Section *Liseola*. These included *F. moniliforme*, *F. proliferatum* (Matsush.) Nirenberg, *F. subglutinans* and *F. anthophilum* (A. Braun) Wollenw., *F. annulatum* Bugnicourt and *F. succisae* Schört. were treated as insufficiently documented species (Ellis, 1988).

The Section *Liseola* was established by Wollenweber and Reinking (1935) to accommodate morphologically similar species. Within this section, the *Gibberella fujikuroi* (Sawada) Ito in Ito and K. Kimura species complex accommodates species that are characterised by the absence of chlamydospores and microconidia accumulating in false heads. The establishment of eleven mating populations within this complex broadly agrees with the 11 independent morphological species. However, *F. subglutinans* was shown to morphologically resemble three of the eleven mating populations. These are the B (*F. subglutinans* var. *sacchari*) (*F. sacchari*) (Leslie *et al*., 2005), E (*F. subglutinans*) (Leslie, 1991; 1995) and H (*F. subglutinans* f. sp. *pini*) (Britz *et al*., 1999) (*F. circinatum*) mating populations.

Nirenberg and O'Donnell (1998) elevated numerous *formae speciales* of *Fusarium* to species level. They used shape of the conidia, type of conidiophore branching, origin of the conidiophores from the substrate, presence of chlamydospores or sterile coiled hyphae to distinguish the new species that they described in the Section *Liseola*. The morphological data used for this taxonomic re-evaluation was supported by phylogenetic relationships. These relationships will be discussed in more detail later in the review.

In the latest overview of *Fusarium* taxonomy by Leslie and Summerell (2006), 70 species are recognised but they are not assigned to sections. This is the first system that incorporates the morphological, genetic and phylogenetic information for all the recognised species.

2.2 Morphological species concept in *Fusarium*

This species concept represents the most widely used system of fungal identification. It is based on the morphological characteristics displayed by the particular fungus on different media. Different growth conditions have been tested for their influence on the expression of stable unique morphological characters. These are discussed in more detail in the following sections.

2.2.1 The influence of culture media on the morphology of *Fusarium* species

The growth media used in studies evaluating the taxonomy of *Fusarium* spp. have changed throughout the history of studies on these fungi. In Booth's (1971; 1977) evaluation of *Fusarium* spp., he used potato dextrose agar (PDA) on which to cultivate the isolates before describing them. In contrast, Nirenberg (1976) used synthetic low nutrient agar (SNA), with a filter paper layered on top of the agar surface, in her evaluation of *Fusarium* spp.

Kuhlman (1982) reported that an isolate of *F. moniliforme* var. *anthophilum*, only forms false chains on V8 and potato dextrose agar (PDA), while Nelson *et al.* (1983) distinguished the four species in the Section *Liseola* as *F. moniliforme* (microconidia formed in chains on monophialides), *F. proliferatum* (microconidia formed in chains on polyphialides), *F. subglutinans* (microconidia formed in false heads on polyphialides) and *F. anthophilum* (microconidia including pyriform or globose microconidia formed in false heads on polyphialides) on carnation leaf agar (CLA). *Fusarium thapsinum* Klittich, J.F. Leslie, P.E. Nelson & Marasas is distinguished from *F. moniliforme* (*F. verticillioides*) by means of a yellow pigmentation that is evident only on PDA and complete medium, but not on CLA (Klittich *et al.*, 1997).

The carnation leaves used in CLA represent an undefined nutrient source. By providing nutrients, they promote growth, sporodochium formation and the production of uniform conidia (Fischer *et al.*, 1982). In contrast, the filter paper in SNA serves as a defined source of cellulose (Nirenberg, 1990), contributing to the standardization of growth conditions. Variability within a species can only be eliminated when the fungus is grown on a fully defined medium such as SNA compared to a complex medium such as CLA. Furthermore, SNA provides the additional advantage of serving as an isolation medium, with the filter paper omitted.

Burgess *et al.* (1991) evaluated CLA, low-nutrient agar (LNA) and dichloran-chloramphenicol-peptone agar (DCPA) for their usefulness in the identification of *Fusarium* species. Typical macroconidia, microconidia and chlamydospores were consistently produced on CLA, but on LNA few macroconidia in the aerial mycelium were formed and the sporodochia did not form. DCPA was not suitable for the reliable differentiation of species, with distorted morphology observed in the species. Nirenberg (1990) compared an isolate of *F. arthrosporioides* Sherb. grown on SNA, PDA, oatmeal agar (OA) and beer wort agar (BWA) for morphological

variation. After six weeks of growth, sporodochial conidia were scored as 100 % variants (morphology differed from standard) on BWA, 50 % variants on OA, 30 % on PDA and none on SNA. The results indicated that SNA was a consistent medium for use in morphological studies. A view shared by Leslie and Summerell (2006), although they stated that it is not suited for the evaluation of macroconidial morphology.

The presence of certain chemicals or other factors in the medium can influence the morphology of *Fusarium* strains. Hsieh *et al*., (1977) reported that the formation of false heads or chains by a species is influenced by the presence of potassium chloride (KCl) in the medium. In addition, available water favoured the formation of false heads, while less available water favoured the formation of false chains. Fisher *et al*. (1983) determined that not only does the concentration of KCl but also the age of the culture influence false chain formation in *F. proliferatum*, *F. moniliforme* and *F. annulatum* Bugnic. Marasas *et al*. (1987) used both KCl and CLA to evaluate chain formation in *F. napiforme* Marasas, P.E. Nelson & Rabie, a species isolated from *Pennisetum typhoides* and *Sorghum caffrorum*. These two media were also used in the description of *F. globosum* Rheeder, Marasas & P.E. Nelson (Rheeder *et al*., 1996). Gordon *et al*. (1996) also reported that 1.5 % KCl was required as an addition to media before a positive distinction between isolates of *F. proliferatum* and *F. subglutinans* could be made.

Degeneration as a result of repeated sub-culturing has been observed in *Fusarium* and other fungal species (Wellman and Blaisdell, 1941; Nelson *et al*., 1983, Burgess *et al*., 1988; Schoers *et al*., 2004). Reasons for degeneration of *Fusarium* isolates include the nature of culture mycelium (Brown, 1926 as cited by Kwasna and Bateman, 2006, Booth, 1971), the age of the cultures (Booth, 1975) and the methods by which the cultures are propagated (Fisher *et al*., 1982; Summerell *et al*., 2003; Leslie and Summerell, 2006). Pionnotal cultures are characterised by an abundance of sporodochia and a lack of mycelium, while mycelial cultures produce abundant mycelium and few if any sporodochia.

Methods to prevent degeneration of cultures include the sub-culturing of a single hyphal tip or single macroconidium (Nelson *et al*., 1983; Burgess *et al*., 1988). These techniques did not prevent degeneration of strains of *F. culmorum* (W.G. Sm.) Sacc. (Burgess *et al*., 1988). The single hyphal tip cultures did, however, degenerate one generation later than the single conidial culture techniques (Wing *et al*., 1995). Growth media have also been shown to play a role in that the cultures transferred on PDA degenerated faster than those on CLA (Wing *et al*., 1995).

Light conditions represent an important growth requirement for *Fusarium* spp. (Fisher *et al*., 1983; Aoki and Nirenberg, 1998). There is, however, no consensus amongst researchers as to the ideal light conditions for growth of this group of fungi. Nirenberg (1990) reported incubating cultures in a 12 h light and dark cycle under near ultraviolet light. However, in her later studies

(Nirenberg *et al*., 1998; Nirenberg and O'Donnell, 1998) she incubated the cultures in the dark. In the descriptions of *F. nelsonii* Marasas and Logrieco and *F. musarum* Logrieco and Marasas the isolates were incubated under 12 h light and dark cycles to stimulate sporulation but light was omitted when the cultures were prepared for growth studies or colony colour determination (Marasas *et al*., 1998). Aoki *et al.* (2001) incubated isolates of *F. concentricum* Nirenberg & O'Donnell under black light, while Skovgaard *et al.* (2003) observed morphologically distinctive characters after incubating the cultures under black light as well as in the dark. Leslie and Summerell (2006) stated that cultures used for their evaluation were grown in a 12 h light/dark cycle, but that chlamydospore formation on standard media should be similar whether the cultures are grown in the dark or light.

2.2.2 Conidial morphology and its use in *Fusarium* taxonomy

Hughes (1953) first proposed the circumscription of fungal genera based on their mode of conidiogenesis. This remains a taxonomically informative character for many fungi (Kohn, 1992). Two primary modes of ontogeny are recognised in fungi, namely phialidic and annelidic.

In the case of annelidic ontogeny, the first conidium is formed holoblastically. The first conidium secedes from the apex of the coindiogenous cell via percurrent proliferation. This is followed by the second conidium that is produced in basipetal succession via enteroblastic ontogeny. Development of the septum at the base of this conidium is critical to both secession and proliferation of the fertile cell (Cole and Kendrick, 1981).

Phialidic ontogeny is characterised by the absence of elongation of conidiogenous cells (Minter *et al*., 1983a). Periclinical thickening (Minter *et al*., 1983b), however, forms as a result of the outer walls of the conidiogenous cells remaining in the collarette after the formation of each conidium (Hughes, 1953; Minter *et al*., 1983b). The phialides are characterised by a fixed endogenous meristem (Minter *et al*., 1983a). Each conidium is consequently formed by a succession of enteroblastic proliferation, holoblastic ontogeny, conidial delimitation and secession. The accumulation of the proliferating cell walls may result in the total blockage of the phialide and end further production of conidia by phialides (Carroll and Carroll, 1974).

Conidial formation in *Fusarium* is phialidic. Booth (1971) noted that microconidiophore structure provides the distinction between *F. oxysporum* Schltdl. and *F. solani* (Mart.) Sacc. Analyses of the ontogeny in *F. crookwellense* Burgess, Nelson and Toussoun indicated that the first conidium can be produced enteroblastically following disintegration of the apex of the conidiogenous cell. Conidiogenous cell remnants may be present at the apex of the first conidium as a result of enteroblastic development (Van Wyk *et al*., 1988). Despite this generalization, mutants characterised by unique conidiogenesis lacking enteroblastic development and conidium

formation have been reported in *F. verticillioides* (Sacc.) Nirenberg (Glenn *et al*., 2004). In *F. chlamydosporum* each locus is blocked after a single conidium is produced holoblastically. Production of all subsequent conidia is preceded by enteroblastic sympodial proliferation. In *F. sporotrichioides* the first conidium is produced by means of holoblastic proliferation of the conidiogenous locus preceded by production of subsequent conidia from the same locus. Enteroblastic sympodial proliferation of the conidiogenous cell precedes production of a new conidiogenous locus (Van Wyk *et al*., 1991).

The conidia formed via phialidic ontogeny provide stable informative characters that are crucial in the correct identification of Fusaria. The presence or absence of macroconidia as well as the morphology of these conidia is the characteristic most commonly used to identify *Fusarium* spp. (Nelson *et al*., 1983). The presence of fusoid macroconidia with a foot cell bearing some kind of heel is accepted as the most definite character of species in the genus *Fusarium*. The foot cell separates the genera *Fusarium* and *Cylindrocarpon*. In *Fusarium* taxonomy the size, shape and morphology of the apical and foot cell of the macrocondium serve as morphological distinct characters to demarcate species (Leslie and Summerell, 2006).

Microconidia are not formed by all *Fusarium* species, so their presence alone is an important character. Their arrangement on and around the conidiogenous cell, their shape and the conidiogenous cells that produce them are also taxonomically informative. The microconidia are usually 0-1 septate but some may be 2-septate (Leslie and Summerell, 2006). The shape of the microconidium can vary dramatically between species and can be globose (as in *F. globosum*), napiform (as seen in *F. napiforme*) or oval (as seen in *F. decemcellulare*).

The influence of sample size on the observation of morphological characters in *F. circinatum* was clearly illustrated by Britz *et al*. (2002) in their evaluation of species in the *G. fujukuroi* complex. Small sample numbers may lead to the "Iceberg effect" (Tibayrenc, 1999) where a relatively small number of cultures are analyzed and differences are not noted because they are not observed within the small sample. If the sample size is increased, however, the diagnostic characters are observed. Thus a species description based on a relatively large set of strains collected at multiple times or locations are more reliable than one that is based on a relatively small collection. Although sample size is not a crucial variable in making diagnostic strain identifications, the reliability of the species description can be affected by this parameter (Summerell *et al*., 2003).

2.2.3 Biochemical and phenotypic characters

Biochemical, genetic and ecological characters serve as additional informative characters (Kohn, 1992) to supplement morphology, which differs amongst individuals. Biochemical characters are based on factors such as diagnostic reactions with chemicals (sensitivity to

fungicides) (Yan *et al*., 1993) and secondary metabolite production (Leslie, 1995; Thrane, 2001), while genetic characters include cytological karyotypes (Kohn, 1992). Ecological characters incorporate disease symptoms (Summerell *et al*., 2003) and host preference (Leslie, 1995).

Fungi display different reactions on contact with antibiotics and these differences have been utilised in various studies treating the identification of *Fusarium* spp. For example, isolates representing the mating populations in the *G. fujikuroi* species complex were subjected to the antibiotic hygromycin (Yan *et al*., 1993). An overlap was observed in the resistance/sensitivity to hygromycin by a few representatives of *G. thapsina* and *G. fujikuroi* (Klittich *et al*., 1997) but the mating populations A-F could be distinguished based on their sensitivity to hygromycin (Yan *et al*., 1993). Hygromycin is, however, not currently widely used as a species determining factor.

Cytological karyotypes such as the size of the chromosomes and the dispersed nature of chromatin during meiosis (Kohn, 1992), have been used to characterise *F. oxysporum* (Boehm *et al*., 1994) and six of the mating populations in the *G. fujikuroi* species complex (Xu *et al*., 1995). Results suggested that the karyotype of *F. oxysporum* is subject to a great deal of change. These changes should have less influence on a population of an asexual fungus such as *F. oxysporum* than in members of the mating populations in the *G. fujikuroi* complex, in which less karyotypic variation is observed, because it does not form part of the natural life cycle of the fungus.

Due to the fact that many *Fusarium* spp. are pathogens, host range and susceptibility of plants to infection has played an important part in the identification of *Fusarium* spp. For example, to accommodate the known pathogenic variants within *F. oxysporum*, Snyder and Hansen described 25 *formae speciales*. This designation was intended to describe the physiological potential of the fungi and was not a part of the formal taxonomic hierarchy. Within the *G. fujikuroi* species complex the *forma specialis* status has been applied to *F. subglutinans*. The first *forma specialis*, *F. subglutinans* f. sp. *pini* applied to isolates of *F. subglutinans* that specifically infected *Pinus* spp. Another *forma specialis* of *F. subglutinans*, *F. subglutinans* f. sp. *ananas* was applied to isolates that caused fusariosis of pineapples in South America (Ventura *et al*., 1993).

2.2.4 Toxins produced by species of *Fusarium*

Fusarium is one of the three major fungal genera producing toxins (Marasas *et al*., 1984). More than 50 mycotoxin compounds were recorded to be produced by species of *Fusarium*. Two of the most relevant groups of toxins are moniliformin and fumonisins (Nelson *et al*., 1993; Bryden *et al*., 2001; Rheeder *et al*., 2002).

The toxin profiles of *Fusarium* spp. can represent a taxonomically informative character. Leslie (1995), for example, determined that the 8 mating populations in the *G. fujikuroi* species complex could be distinguished based on their mycotoxin profiles. The toxin profiles of *F.*

moniliforme, *F. proliferatum* and *F. subglutinans* differed in their nature and in the levels of toxin produced (Jimenez *et al*., 1997). *Gibberella thapsina* produced no fumonisins but moniliformin, while *G. fujikuroi* produced no moniliformin but fumonisin (Klittich *et al*., 1997). When toxin production was used as a determining character, *F. verticillioides* and *F. thapsinum* could be distinguished from each other (Leslie *et al*., 1996). Kumar and Ram (1999) were, however, unable to distinguish isolates causing mango malformation using toxin profiles, but fifteen *Fusarium* species in the *G. fujikuroi* species complex could be distinguished based on their mycotoxin profiles by Fotso *et al*. (2002).

Fusarium subglutinans (mating population E) produces different toxins. It has been reported to produce moniliformin (Kriek *et al*., 1977), fusaproliferin (Logrieco *et al*., 1996) and fumonisin B (Visconti and Doko, 1994), although the latter compound was detected in small or insignificant amounts. Leslie *et al*. (2004a) reported that *F. subglutinans* strains from native prairie grasses produced fusaproliferin, beauverin but no fumonisins. This may indicate that the substrate on which *F. subglutinans* grows may influence the toxin production and thus the identification of the strain.

2.3 The Biological species concept

The biological species concept was established by Mayr in 1942 (as cited in Wang *et al*., 1998) and is based on Dobzansky's observation that the process responsible for species formation is the development of pre- and/or postzygotic reproductive isolating barriers. There are two means by which fungi and other organisms can transmit genes to the next generation, via clonal reproduction or via mating and recombination. In the case of clonality, each of the progeny would have one parent and its genome would be an exact mitotic copy of the parental genome. Clonality in fungi need not be mitotic and asexual because self-fertilizing or homothallic fungi produce spores via meiosis with identical parental and progeny genomes (Burnett, 1975; Taylor *et al*., 1999). Furthermore, pseudohomothallism may result in approximate clonality within a recombining population if the partners routinely develop from sibling meiospores. (Fincham and Day, 1963; Burnett, 1975; Taylor *et al*., 1999).

In heterothallic ascomycetes, individuals belonging to the same biological species may be sexually fertile i.e. they may belong to the same mating population. Most heterothallic filamentous ascomycetes have a dimitic or bipolar mating system with two alleles located at a single locus (Fincham and Day, 1963). The two alleles located at a single locus are referred to as idiomorphs because they have sequence similarity, but they occupy the same locus on the organism's chromosome. They are typically designated as *MAT-1* and *MAT-2*. The *MAT-2* idiomorph contains a single open reading frame (ORF) encoding a regulating protein with a DNA-binding domain of the high mobility group (HMG) type, while the *MAT-1* idiomorph contains an ORF encoding a

protein with a motif known as the alpha-box first studied in *Saccharomyces cerevisiae* (Gutz and Schmidt, 1990; Tóth *et al*., 2004). In most homothallic filamentous ascomycetes both mating types occur in a common cytoplasm and the heterokaryon formed is self-fertile (Fincham and Day, 1963).

In *Fusarium*, both homothallic and heterothallic species have been documented. At present no pseudohomothallic species has been reported but in many species, the life cycles have not been studied sufficiently to provide detail to distinguish homothallic and pseudohomothallic life cycles (Leslie and Summerell, 2006). Most *Fusarium* species are heterothallic (Leslie and Summerell, 2006). Heterothallic strains are usually morphologically indistinguishable but differ physiologically in that they have opposite mating types. The alleles may either be functional resulting in a fertile strain or non-functional resulting in a sterile strain (Leslie and Summerell, 2006).

2.3.1 Aspects pertaining to the implementation of the Biological species concept in *Fusarium*

Defining a *Fusarium* species as an interbreeding population avoids problems associated with morphology such as interspecies variation, alteration of morphology caused by mutation and variations due to the environment (Klittich *et al*., 1997). The incompatibility of *Fusarium* isolates does, however, pose a major constraint to the implementation of this concept (Kuhlman, 1982; Summerell *et al*., 2003, Leslie and Summerell, 2006).

Sexual crosses are laborious and time-consuming to achieve and it may take up to 6-8 weeks for perithecia to form in some *Fusarium* spp. Temperature is important for inducing perithecial formation (Samuels *et al*., 2001). However, the temperature for perithecial formation may or may not be the same as for ascospore discharge (Covert *et al*., 1999). Besides environmental conditions, mating tests in *Fusarium* may also be sensitive to naturally occurring mutations that may affect such traits as the number of spores per ascus, perithecial initiation and pigmentation, meiosis and ascus maturation (Leslie, 1991). Despite these disadvantages, sexual recombination even as a rare event under field conditions may provide useful diagnostics because it provides additional information that enables researchers to determine species identification. It also serves as a means by which the maximum level of genetic exchange can be determined and it can provide essential information for population genetic analyses (Leslie, 1995).

There are practical difficulties with applying a biological concept to *Fusarium* spp. These include high levels of asexual reproduction, unequal relative frequencies of the mating-type idiomorphs and often limited numbers of strains that are fertile as females. All of these limitations are known to occur in field populations (Leslie and Summerell, 2006).

2.3.2 Biological species in *Fusarium*

Fertility in heterothallic *Fusarium* species has led to the establishment of eleven biological or mating populations (A to K) in the *G. fujikuroi* species complex (Zeller *et al*., 2003, Desjardins, 2003). These mating populations include a number of species in the Section *Liseola*. They include *F. verticillioides (sacc.)* Nirenberg (A), *F. sacchari* (E.J. Butler & Hafiz Khan) W. Gams (B), *F. fujikuroi* Nirenberg (C), *F. proliferatum* (T. Matsushima) Nirenberg (D), *F. subglutinans* (Wollenw. & Reinking) (E) (Britz *et al*., 1999), *F. thapsinum (G. thapsina)* (F) (Klittich *et al*., 1997), *F nygamai* Burgess & Trimboli (G), *F. circinatum* Nirenberg (H), *F. konzum* Zeller, Summerell & J.F., Leslie (I) (Zeller *et al*., 2003), *F. gaditjirrii* Phan, L.W. Burgess & Summerell (J) (Phan *et al*., 2004) and *F. xylarioides* Steyaert (K) (Lepoint *et al*., 2005). *Fusarium subglutinans* as a genetically isolated species in this complex represents 3 of the 8 mating populations. These are the B, E and H mating populations, representing *F. sacchari* from sugarcane, *F. subglutinans* from maize and *F. circinatum* from pine, respectively (Leslie *et al*., 2005; Britz *et al*., 1999; 2002).

In *Fusarium*, fertility forms the basis of the biological species concept. However, fertility in this group represents a continuum with varying degrees of fertility separating clearly fertile from clearly infertile strains (Leslie *et al*., 2004b). This is evident from studies that have shown that viable ascospores did not ooze from the perithechia but that when they were crushed, the ascospores were present and viable (Aoki and O'Donnell, 1999). The application of the biological species concept should thus be done with caution and the limitations of the concept taken into consideration when the results of such experiments are analysed.

Exceptions to the biological species concept have been reported where *Fusarium* spp. cross over mating population boundaries (Xu *et al*., 1995; Desjardins *et al*., 2000; Leslie *et al*., 2004b). For example, the fertile cross between isolates from the C and D mating populations of *G. fujikuroi* occasionally produce a few perithechia containing a few viable ascospores (Xu *et al*., 1995; Leslie *et al*., 2004b), while the cross between the E and H population were also successfully repeated (Desjardins *et al*., 2000). This interspecific hybrid cross between *G. circinata* and *G. subglutinans* was used to compile a genetic linkage map by means of a total of 578 AFLP markers together with the mating type (*MAT-1* and *MAT-2*) genes and the histone (H3) gene (De Vos *et al*., 2007). The taxonomic placement of these post-zygotic species recognition events in the kingdom fungi is a controversial issue and encompasses the description of species that are not morphologically indistinguishable but belong to different evolutionary lineages (Schardl and Craven, 2003; Sáez and Lozano, 2005).

Post-zygotic species recognition events were described as cryptic species or sibling species by Mayr (1942) (as cited in Wang *et al*. 1998). These cryptic species were viewed as rare and of limited economic importance, and thus their identification was viewed as of academic importance

only. Yet some morphologically similar species in the Section *Liseola* such as *F. andiyazi* Marasas, Rheeder, Lamp., K.A. Zeller & J.F. Leslie (Marasas *et al*., 2001) and *F. thapsinum* (Klittich *et al*., 1997) were previously treated as variants of *F. moniliforme* until molecular data highlighted the genetic variation between them (Leslie, 1995). Phylogenetic tools were also used to address the issue of cryptic species in *F. subglutinans* (Steenkamp *et al*., 2002). The rationale being that incongruence among gene trees emerging from sequences for different loci indicate interbreeding among individuals as a result of recombination of genomes and unique evolutionary histories.

2.4 Phylogenetic species concept

The phylogenetic species concept defines species as monophyletic groups that consist of "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent" (Cracraft, 1983 as cited by Avise 2004). This species concept has both strengths and weaknesses. It provides the advantage of a rapid answer compared to the laborious processes needed to apply the biological species concept and increased accuracy compared to traditional morphological studies. The application of this concept is, however, hampered by the fact that molecular characters used to define species may not display sufficient variation or have a clear and independent basis (Higgs and Attwood, 2005).

A wide array of DNA-based molecular techniques has been applied in the quest to better understand the phylogenetic relationships amongst *Fusarium* species. In particular, to those species in the genus that are morphologically very similar and that are sexually incompatible with the known tester strains defining the biological populations. These molecular techniques include Restriction Fragment Length Polymorphisms (RFLP), Random Amplified Polymorphic DNA (RAPD) profiles and DNA sequence data. They are discussed briefly in the following section.

2.4.1 Some molecular based techniques used to distinguish *Fusarium* species

2.4.1.1 Restriction fragment length polymorphisms

Restriction Fragment Length Polymorphisms (RFLPs) are based on restriction enzymes that cleave DNA, which is then separated by agarose-gel electrophoresis. The resulting gels are then enhanced by means of Southern blotting. Each restriction enzyme recognises a specific nucleotide sequence and cuts the DNA specifically, every time the sequence occurs (Miller and Martin, 1988; Guarro *et al*., 1999).

Restriction fragment length polymorphisms were used to distinguish a number of *Fusarium* species, *formae speciales* and races (Manicom *et al*., 1987; Edel *et al*., 1996; Schilling *et al*., 1996). Later RFLP profiles were based on protein-coding genes (Donaldson *et al*., 1995) in stead of the conserved genomic sequences. These included the histone H3, H4, β-tubulin 1 and 2 regions and

compared these results with that obtained for the ITS region. Sequences of the internal transcribed spacers of the ribosomal DNA were used to compare 13 species from *Fusarium* Sections *Elegans*, *Liseola* and *Dlaminia*. The UPGMA dendrogram for ITS1 revealed a close similarity for most species, while species groupings based on the ITS2 sequences formed two main clusters that did not coincide with section boundaries. This indicated that the two ITS2 groupings do not accurately reflect the phylogeny of the included fungi (Waalwijk *et al*., 1996). The ITS data alone should thus be interpreted with considerable care since *Fusarium* species are known to have two forms of the ITS2 region (Waalwijk *et al*., 1996; Lieckfeldt and Siefert, 2000).

The rapid evolution and clonal inheritance of mitochondrial DNA (mtDNA), renders mtRFLPs suitable for analyses of divergence at the subspecies level (Avise, 1989). However, the transfer of mitochondrial variants between isolates through sexual recombination, vegetative fusion and simple hyphal contact (Collins and Saville, 1990; Kempken, 1995) may increase the observed diversity in haplotypes (Láday *et al*., 2004a). Genetic diversity in *F. proliferatum* was determined by means of mtDNA RFLPs (Láday *et al*., 2004a). Although one haplotype was dominant in the *Fusarium* isolates from maize no correlation between host specificity and the haplotypes was observed (Láday *et al*., 2004a). Similarly isolates representing the seven lineages of *F. graminearum* were also compared and high similarity was observed between isolates within the lineages, but there were significant differences between the various lineages (Ladáy *et al*., 2004b). No geographical separation was seen but there were indications of host-fungus co-evolution exemplified by the presence of haplotypes restricted to certain hosts. High levels of variability in isolates representing the *F. oxysporum* complex have also been identified using mtRFLPs (Attitalla *et al*., 2004). In contrast Correll *et al*. (1992) reported that all the *F. circinatum* isolates that they studied had the same unique mtDNA RFLP profile.

2.4.1.2 Cleaved amplified polymorphic sequences

A variation the RFLP technique is PCR-RFLP or CAPS (Cleaved amplified polymorphic sequences). As a diagnostic tool, it has been used for *Fusarium* species (Llorens *et al*., 2006), including the *G. fujikuroi* species complex (Steenkamp *et al*., 1999). The PCR product obtained for a specific gene is digested with a number of restriction enzymes and the resulting fragments are visualised on an agarose gel to observe genetic differences (Chawla, 2002). Bateman *et al*. (1996) distinguished 18 *Fusarium* haplotypes using the ITS1, 5.8S and ITS2 ribosomal gene region, while Edel *et al*. (1996) amplified a further 600 bp into the 5' end of the 28S rDNA gene, which included the variable D1 and D2 domains, and were able to distinguish a further five haplotypes. To distinguish the pine pathogen, *F. circinatum* from other important pathogens in the complex, Steenkamp *et al*. (1999) utilised the signal within the histone H3 gene. Similarly, Britz *et al*. (2001)

characterised the pitch canker fungus in Mexico, using this technique while Mirete *et al*. (2003) utilised the intergenic spacer (IGS) region to characterise *Fusarium* strains isolated from pine seeds.

2.4.1.3. Random amplified polymorphic DNA

Random amplified polymorphic DNA (RAPDs) are based on the PCR amplicons obtained with 10-mer primers. These are visualised on an agarose gel and size differences in the bands are scored as polymorphisms (Welsh and McClelland, 1990; Williams *et al*., 1990). Although, difficulties are recognised in transferring the RAPD profile markers between laboratories, this technique has been successfully used to compare *Fusarium* isolates within a laboratory (Quellet and Seifert 1993, Schilling *et al*., 1994, Schilling *et al*., 1996, Zheng and Ploetz, 2002, Nicholson *et al*., 2004, Schroers *et al*., 2004). Nicholson *et al*. (2004) utilised RAPD fragments that do not hybridise even to closely related species in Southern Blots to develop a single assay format to detect a large number of closely related species. Zheng *et al*. (2002) developed a 20-mer primer pair that only amplified the *Fusarium* mango isolates and a smaller band for *F. nygamai*. The recombination-based map of *G. fujikuroi* was based on 142 RFLP markers, two auxotrophic genes (arg1, nic1), mating type, female sterility, spore-killer and a gene governing the production of the mycotoxin, fumonisin B_1 (fum1) (Xu and Leslie, 1996). The number of primers used in their study may have influenced the genetic variability detected. Schilling *et al*., (1994) found high intra specific variation among isolates using 120 primer sets while Quellet and Seifert (1993) detected low levels of intra specific variation with 40 primer sets. PCR identification of *F. culmorum*, *F. graminearum* and *F. avenaceum* was based on species specific primers based on RAPD markers (Schilling *et al*., 1996). Schroers *et al*. (2004) distinguished *F. foetens*, *F. begoniae* and others based on the RAPD profiles obtained for 3 primer sets. The data were concordant with morphological and sequence comparisons for the TEF-1α and β-tubulin gene regions.

2.4.2 Species concept based on DNA sequence comparisons

Identification of the shortest gene trees represents the key to genealogical concordance phylogenetic species recognition (GCPSR) (Taylor *et al*., 1999; Fisher *et al*., 2002). Genealogical concordance phylogenetic species recognition is being used increasingly for both the meiosporic and mitosporic fungi (Fisher *et al*., 2002) and the *G. fujikuroi* species complex (O'Donnell *et al*., 1998).

There is no set number of sequence differences that indicates a species boundary for GCPSR (Taylor *et al*., 1999). Cummings *et al*. (1995) suggested that the phylogenetic relationships obtained for species based on neighbouring nucleotides might be less likely to display the true

phylogenetic relationships between species than samples composed of nucleotides drawn individually throughout the genome.

Homoplasy or alignment of sequence data may influence the topology of phylogenetic trees despite the phylogenetic analyses done afterwards. The most common way of dealing with homoplasy is the exclusion of these regions (Berbee and Taylor, 1993) but the subjectivity associated with this process can lead to different phylogenies, depending on the combination and/or exclusion of sites (Felsenstein, 2004). Furthermore, the exclusion of ambiguously aligned regions and the delimitation of these sites may lead to loss in resolution. To limit the detrimental effect of inserting gaps in an alignment, gaps have been treated as missing data. An alternative is to assign the gaps fifth character state. This can, however, result in over-weighting adjacent gaps by treating them as independent indels, while they are most likely part of a single genetic change (Lutzoni *et al*., 2000). A possible solution may be to code the indels, however, this also provides a very subjective perspective.

The statistical methods used to analyse sequence data generated for different loci has a significant influence on the topology of the phylogenetic trees. In parsimony analyses, the possible trees are compared and each is given a score that serves as a reflection of the minimum number of character state changes that would be required over evolutionary time to fit the sequences into the tree (Eisen, 1998; Felsenstein, 2004). The optimal tree is considered to be the one requiring the fewest changes for distance analyses. The optimal tree is generated by first calculating the estimated evolutionary distance between all pairs of sequences. Then these distances are used to generate a tree in which the branch patterns and lengths best represent the distance matrix (Eisen, 1998). Maximum likelihood is similar to parsimony methods in that possible trees are compared and given a score (Eisen, 1998; Felsenstein, 2004). The score is based on the likelihood that sequences have evolved in a particular tree given a model of nucleotide substitution probabilities. The optimal tree is considered to be the one that has the highest probability (Eisen, 1998; Felsenstein, 2004).

2.4.2.1 Gene regions utilised in *Fusarium* taxonomy

2.4.2.1.1 Ribosomal DNA

Ribosomal DNAs (rDNAs) serve as taxonomic indicators because they are highly conserved and are universally found in living cells (Van de Peer *et al*., 2000). The 28S-like molecules are compiled of well conserved domains as well as more rapidly evolving domains. This characteristic makes the comparison between closely and isolated species possible but does not provide enough evolutionary signal to distinguish between *formae speciales* or races in the case of *F. oxysporum* (Guadet *et al*., 1989; Waalwijk *et al*., 1996; O'Donnell and Cignelik, 1997).

The separation of the Sections, *Liseola*, *Elegans*, *Discolor* and *Spicarioides* in *Fusarium* were supported by 28S sequence data (Guadet *et al*., 1989). Guadet *et al*. (1989), however, stipulated that the division of species within these different sections, such as, *F. oxysporum*, *F. solani* and *F. graminearum*, is not possible based on 28S sequence data. Additionally Guadet *et al*. (1989) also recognised a correlation between 28S sequence data and teleomorph separation.

Species distinction within monophyletic sections proved to be paraphyletic based on the nuclear rDNA internal transcribed spacers (ITS) data (Waalwijk *et al*., 1996) and other gene sequences (O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998). The inclusion of *Cylindrocarpon lichenicola* and *Acremonium falciforme* in the *F. solani* complex by Summerbell and Schroers (2002) was based on ITS data. This resulted in the resurrection of *F. lichenicola* C.B. Massalongo and the description of *F. falciforme*. Discordance in the ITS2 gene tree for species in the *G. fujikuroi* species complex was attributed to paralogous or xenologous ITS2 sequences (Waalwijk *et al*., 1996). Analysis showed that every strain of the species tested in the *G. fujikuroi* complex possessed two highly divergent nonorthologous ITS2 types. They were designated as type 1 and 2. O'Donnell and Cignelik (1997) hypothesised that the major type of ITS exhibits a homoplastic pattern of evolution, thus obscuring true ancestral relationships. When the ITS region of more *Fusarium* spp. was examined, O'Donnell and Cignelik (1997) concluded that the sections currently recognised are artificial and needed revision. The phylogenetic groupings obtained did, however, support the perithecial morphology, habitat and mycotoxin profiles.

2.4.2.1.2 Protein-coding genes

rDNA sequences do not have sufficient evolutionary signal to distinguish between closely related *Fusarium* species. For this purpose, protein-coding genes were included to provide the additional signal needed (Appel and Gordon, 1996; O'Donnell *et al*., 1998; 2000; Roux *et al*., 2001; Baayen *et al*., 2000; 2001; Tóth *et al*., 2004; Mulé *et al*., 2004). One such gene, TEF-1α gene forms the basis for the FUSARIUM-ID v. 1.0 database (http://isolate.fusariumdb.org/index.php/2010). This is a publicly available database representing a selected portion of the diversity in the genus *Fusarium*. This database includes representatives of the type B trichothecene toxin producers, the *G. fujikuroi*, *F. oxysporum* and *F. solani* species complexes (Geiser *et al*., 2004). An advantage of the website is that it includes only authentic sequences attached to voucher strains. Other protein-coding genes used to distinguish *Fusarium* spp. are the β-tubulin, calmodulin, and histone genes (O' Donnell *et al*., 1998; 2000; Steenkamp *et al*., 2002; Leslie and Summerell, 2006).

In most phylogenetic analyses of *Fusarium* species, a combination of protein-coding genes is used. DNA sequence data for the mitochondrial small subunit (mtSSU) ribosomal RNA and

TEF-1α gene regions were combined for phylogenetic analysis of plant pathogenic *F. oxysporum* strains. The multiple evolutionary origins of some *formae speciales* in *F. oxysporum* versus the monophyletic origin of others were established using data from a combination of genes (Baayen *et al*., 2000). Furthermore, maximum parsimony analysis of combined data sets identified the *F. redolens-F. hostae* clade as a sister group to a phylogenetically diverse clade in which the *G. fujikuroi* species complex formed the most basal lineage (Baayen *et al*., 2001).

The phylogenetic relationships among species in the *G. fujikuroi* species complex have been studied based on the data obtained for numerous genes and gene regions. Mulé *et al*. (2004) distinguished *F. subglutinans*, *F. proliferatum* and *F. verticillioides* based on sequence differences in the calmodulin gene, while Steenkamp *et al*. (1999) used the histone H3 primers developed by Glass and Donaldson (1995) to distinguish species. The IGS region of the rDNA operon appears to present substantial variability at the intraspecific level in *Fusarium* (Appel and Gordon, 1996; Tóth *et al*., 2004). This is because it is subject to convergent evolution due the suppression of recombination (James, 2001).

3. Conclusions

Fusarium species are ubiquitous fungi occurring in soil as saprophytes and as important pathogens of many plant species. In addition, some species produce mycotoxins that have a seriously detrimental effect on both animal and human health if it enters the food chain. Accurate identification of these species is thus crucially important.

The *G. fujikuroi* species complex accommodates the monophyletic assemblage of species including the Section *Liseola*, together with certain members of the Sections *Dlaminia* and *Elegans*. Initially, species such as *F. subglutinans* and *F. proliferatum* were described based on morphological characters and the morphological species concept. Species in this complex are now demarcated using an integrated approach including the morphological, biological and phylogenetic species concepts. This approach ensures that the species are demarcated based on stable and constantly expressed characters that enable both taxonomists and applied scientists to identify taxa in a meaningful way.

In the case of *Fusarium*, morphological differences as the basis of species demarcation have served the scientific community well. However, this approach also exposed the greatest weaknesses linked to a taxonomy based on morphology. The morphological species concept as applied during the past 25 years requires a trained eye to distinguish species and has thus led to the incorrect identification of many strains that cause plant and animal diseases or that produce mycotoxins. A single species demarcation was based on a limited number of morphological characters with different evolutionary origins and thus resulted in subjective interpretation. This subjective

interpretation of morphological characters represents one of the biggest shortcomings of the system.

For the morphological species concept to fill its position as an important part of an integrated taxonomic approach, a paradigm shift was needed. The definition of the term 'informative character' also needed to be re-examined. Characters previously considered insignificant have now, with the aid of less subjective tools, been shown to be useful to separate species. Yet, even with reliable morphological characters at hand, it is necessary to examine large numbers of strains in order to ensure the constant expression of the character within a population i.e. the frequency of the character within the population as well as the occurrence of this character in populations of related species.

The biological species concept has played a vital role in the determination of anamorph-teleomorph connections in fungi. This has also been true for species in the *G. fujikuroi* species complex. The number of asexual species known in this group, however, limits the application of this approach. Furthermore, the biological species concept is laborious and time consuming and the results are frequently affected by environmental conditions. The results, however, are apparent and leave little room for subjective interpretation as is the case with morphology. This is also despite a dynamic debate surrounding the definition of a viable cross between strains of opposite mating type. Sexual crosses between strains residing in different phylogenetic lineages cause problems and the absence of the ability to use this approach for homothallic species present other challenges.

The third species concept addressed in this review, the phylogenetic species concept, enables taxonomists to demarcate species based on their evolutionary relatedness. Even so the choice of the gene regions used in the statistical analyses and the coding of data leave room for subjectivity in the interpretation of data. This concept provides the information needed to separate morphologically closely related species or species isolated from different hosts but that display the same morphology. This concept can even provide preliminary population structure information based on gene trees. It also provided the basis for whole genome comparisons that enabled the phenotypic and genotypic diversity within individual *Fusarium* species to be studied using the genome of sequenced isolates as a reference.

The strengths and weaknesses of the different species concepts applied to fungi, including those in the *G. fujikuroi* species complex should not imply that they are opposing approaches to defining species. Researchers should rather aim to determine the appropriateness of a specific system for the particular group of fungi with which they are concerned. Species concepts should be seen as complementary and should be used in conjunction with each other to make the demarcation of species scientifically sound and user friendly. In addition, it is important to remember that the purpose of taxonomy is twofold. The first is of scientific importance and concerns the delineating

and classifying of species. The second is that taxonomy should seek to provide a service to the community, including naming species and providing tools for their easy and accurate identification.

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CHAPTER 2

Morphological and molecular distinction of the different species of *Fusarium subglutinans sensu lato*

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Morphological and phylogenetic species concepts to distinguish between species in *Fusarium subglutinans sensu lato*

ABSTRACT

Fusarium subglutinans forms part of the *Gibberella fujikuroi* complex. Independent phylogenetic and morphological studies have shown that this is a polyphyletic taxon, which should be treated as *F. subglutinans sensu lato*. The aim of this study was to consider the value of morphological characters along with DNA sequence data to distinguish species in *F. subglutinans sensu lato*. Five morphological characters that have previously been reported as useful to identify species in this group were evaluated. These included the origin of conidiophores with respect to the hyphae on the substrate surface, conidiophore branching, the number of conidiogenous openings on the polyphialides, macroconidial septation and the presence or absence of sterile coiled hyphae. Using these characters, it was possible to separate most of the species from each other. For molecular comparison, the DNA sequences from the translation elongation factor-1α, β-tubulin and calmodulin genes as well as the large subunit of the rDNA gene region were considered. Both the phylogenetic and morphological data show that an integrated approach is needed for the unambiguous identification of species in *F. subglutinans sensu lato*.

Keywords: Translation elongation factor-1α, Morphology, Identification

Introduction

The taxonomy of *Fusarium* Section *Liseola* Wollenw. & Reinking has attracted substantial interest during the course of the last decade (O'Donnell *et al*., 1998a; 2000; Steenkamp *et al*., 2000a; 2002; Leslie *et al*., 2005; Leslie and Summerell 2006; Kvas *et al*., 2009). This is primarily because the group includes a large number of important plant pathogens and their identification has been substantially confused. The results of DNA-based phylogenetic studies (O'Donnell *et al*., 1998a; 2000; Steenkamp *et al*., 2000b; 2002) have highlighted the polyphyletic nature of members in this section, as well as for taxa that it accommodates. As a result, O'Donnell *et al*. (1998a) introduced the term "*Gibberella fujikuroi* (Sawada) Wollenweber species complex" (GFC) to refer to the monophyletic assemblage that includes Section *Liseola*, together with certain members of the Sections *Dlaminia* Kwasna and *Elegans* Wollenw.

When *F. subglutinans* was first described, it was recognised as a variety of *F. moniliforme* Sheldon (Wollenweber & Reinking 1925). In 1955, *F. moniliforme* var. *subglutinans* Reinking was synonymised with *F. neoceras* Wollenweber & Reinking and included in a combined Section *Liseola* (Bilay, 1955 as cited in Toussoun & Nelson, 1975). Based on the absence of microconidial chains and the production of macroconidia by means of polyphialides, Booth (1971; 1977) retained *F. moniliforme* and *F. moniliforme* var. *subglutinans* as a separate variety in the Section *Liseola*. These characters were later used to elevate the taxa to species level (Nelson *et al*., 1983).

Of the approximately 50 distinct phylogenetic lineages in GFC (O'Donnell *et al*., 2000; Aoki *et al*., 2001), at least 13 are represented by isolates with morphology typical of *F. subglutinans*. They include the pathogens *F. circinatum* (causal agent of pine pitch canker), *F. sacchari* (causal agent of pokkah boeng disease of sugarcane), *F. guttiforme* (associated with fusariosis of pineapple), and *F. mangiferae* (causal agent of mango malformation disease) (Nirenberg and O'Donnell, 1998; Britz *et al*., 2002a; Leslie *et al*., 2005). Species with a *F. subglutinans*-type morphology have also been recovered from symptoms on *Mangifera indica*, *Nerine bowdenii*, *Musa sapientum*, *Begonia* spp. and *Pinus radiata* and reside in *F. sterilihyphosum*, *F. bulbicola*, *F. concentricum*, *F. begoniae* and *F. pseudocircinatum*, respectively (Nirenberg and O'Donnell, 1998; Britz *et al*., 2002a; Leslie *et al*., 2005).

The aim of this study was to compare and evaluate the value of key morphological characters (Nelson *et al*., 1983; Nirenberg and O'Donnell, 1998; Britz *et al*., 2002b) in distinguishing the different species encompassed in *F. subglutinans sensu lato* from each other. We also compared the DNA sequences for the genes encoding translation elongation factor-1α (TEF-1α), β -tubulin (β-tub), calmodulin (Cal) and the large subunit of the ribosomal DNA (LSU) to determine their value in differentiating the species alongside the morphological characters that are typically most useful to plant disease diagnosticians.

Materials and Methods

Fungal isolates

The ex-holotype isolates, where applicable, as well as other isolates representing the species for all of the *F. subglutinans sensu lato* species were included in this study (Table 1). We also included the mating tester for *F. subglutinans sensu stricto* that was originally isolated from maize, as well as a representative member of the cryptic species associated with maize in South Africa and teosinte in Central America (Desjardins *et al*., 2000; Steenkamp *et al*., 2002). All isolates are maintained in the *Fusarium* culture collection of the Tree Protection Co-operative Programme at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and at the Medical Research Council (MRC) Culture Collection, Tygerberg, Cape Town, South Africa.

Morphology and culture comparisons

Isolates were grown on potato dextrose agar (PDA), carnation leaf agar (CLA) (Nelson *et al*., 1983), and synthetic low nutrient agar (SNA) (Nirenberg, 1976). Colony morphology was determined on PDA and colony colour was assigned using the colour charts of Rayner (1970). Morphological characters were described from structures produced on SNA. Growth rates were determined by placing a plug of actively growing mycelium or a single germinating macroconidium on a PDA plate and calculating the average growth in millimeter from the plug or macroconidium over 5 days (Burgess *et al*., 1988).

DNA extraction, amplification and sequencing

DNA was isolated using a modified version of the technique described by Jacobs *et al*. (2007) from isolates grown on complete medium (Correll *et al*., 1987) at 25 ºC for 7 d. Mycelium was scraped from the surface of the growth medium and homogenised in the presence of ca. 10 µg sterile chemically treated sand (Merck, Germany) and DNA extraction buffer containing 200 mM Tris-HCl (pH 8), 150 mM NaCl, 25 mM ethylenediamine tetra-acetic acid (EDTA, pH 8), and 0.59 % sodium dodecyl sulphate (SDS). Following phenol-chloroform extraction, the DNA in the aqueous phase was precipitated with ethanol and pelleted by centrifugation (Sambrook *et al*., 1989). After washing with 70 % ethanol, pellets were air- dried, resuspended in 50 µL sterile deionised water and subjected to an RNAse A (Roche Pharmaceuticals, Germany) treatment (Sambrook *et al*., 1989). The extracted DNA was then used as template for subsequent amplification using PCR.

To amplify specific regions of the genes encoding TEF-1α, β-tub, Cal and LSU, the primer sets EF1 and EF2 (O'Donnell *et al*., 1998b), T1 and T222 (O'Donnell and Cigelik 1997), 228F and 737R (Carbone and Kohn, 1999), and ITS 4 (White *et al*., 1990) and LR3 (Vilgalys and Hester, 1990) were used. All amplification reactions included template DNA (25 ng/µL), dNTPs (250 µM each), primers (0.2 µM each), Roche *Taq* polymerase (0.5 U) and Roche *Taq* Reaction buffer with

$MgCl_2$. The cycling conditions for all gene regions included an initial denaturation at 92 ºC for 1 min, followed by 30 cycles of denaturing at 92 ºC for 1 min, annealing at 54 ºC for 1 min and elongation at 72 ºC for 1 min, concluding with a final elongation step at 72 ºC for 5 min. The only exception was that the LSU PCR was performed using an annealing temperature of 48 ºC.

The resulting amplicons were purified with a QIAquick PCR Purification kit (QIAGEN, Germany) and subjected to automated DNA sequencing using the same primers that were used for the respective PCRs and the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, (Applied Biosystems, UK) and Applied Biosystems' 3730 DNA Analyzer. Raw sequence trace files were analyzed with Sequence Navigator version 1.0.1 (Applied BioSystems, UK). All of the sequences generated in this study have been deposited in GenBank (Table 2).

DNA-based comparisons

All sequences generated in this study were compared with those in GenBank (http://www.ncbi.nlm.nih.gov) using *BLASTN*. The TEF-1α sequences for the *F. subglutinans sensu lato* isolates examined, were also compared with those in the *Fusarium* ID database (http://fusarium.cbio.psu.edu/index.html/2004) (Geiser *et al*., 2004). Multiple sequence alignments for TEF-1α, β-tub, Cal and LSU were then generated for the *F. subglutinans sensu lato* sequences, as well as sequences obtained from GenBank for other known biological species of the GFC using BioEdit version 7.0.5.2 (Hall, 1999). These were submitted to TreeBase SN 4076.

Each of the *F. subglutinans sensu lato* sequence sets, were subjected to maximum parsimony (MP) analyses using PAUP* version 4b10 (Phylogenetic Analysis Using Parsimony* and Other Methods version 4, Swofford, 2002). In these analyses, alignment gaps were treated as missing data and phylogenetic trees were constructed using heuristic searches with a 1 000 rounds of random sequence addition, tree bisection-reconnection (TBR) branch swapping, and with MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal ($g1$) in the data sets was assessed by evaluating tree length distributions over 100 randomly generated trees (Hillis and Huelsenbeck, 1992). The consistency (CI) and retention indexes (RI) were determined for all data sets. For the extended TEF-1α dataset, we constructed trees based on MP, maximum likelihood (ML) and Bayesian Inference (BI) using PAUP*, PhyML version 2.4.3 (Guindon and Gascuel, 2003) and MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001), respectively. The MP analysis was performed as above and the ML analysis utilised the general time reversible (GTR) model (Tavaré, 1986) with eight gamma categories (G) and a proportion invariable sites (I) as indicated by Modeltest version 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). The BI analysis utilised Metropolis-coupled Monte Carlo Markov Chain analysis, 2 000 000 generations using one cold and three heated chains, a burnin of 4 000 and the GTR+I+G model as indicated by MrModeltest 2.2

(Nylander, 2004; Posada and Crandall, 1998). Bootstrap support values and Bayesian posterior probabilities were determined using 1 000 replications using the same parameters and settings as those used for inferring the ML, MP and BI trees. All trees were rooted with *F. oxysporum* as monophyletic sister outgroup to the rest of the taxa.

Results

Morphology and culture comparisons

All of the examined *F. subglutinans sensu lato* isolates displayed morphological characters typical of *F. subglutinans* as described by Nelson *et al.* (1983). These include the absence of chlamydospores and conidia that were produced via mono- or polyphialides and accumulating in false heads that were never in chains. Among the characteristics used (Fig 1) to distinguish the various *F. subglutinans sensu lato* species were those reported by Nirenberg and O'Donnell (1998), which included the nature of the conidiophores on the aerial mycelium, macroconidial morphology and the presence of sterile outgrowths.

The nature of the aerial mycelium bearing conidiophores was either erect or prostrate. Erect conidiophores were defined as conidiophores borne on aerial hyphae that arise directly from the substrate (Nirenberg and O'Donnell, 1998). In contrast, prostrate conidiophores arose from aerial hyphae that grew horizontally to the agar surface. *Fusarium begoniae*, *F. concentricum*, *F. pseudocircinatum*, the cryptic species of *F. subglutinans sensu lato*, *F. sterilihyphosum* and *Fusarium* species from Poaceae formed only prostrate aerial mycelium. *Fusarium bulbicola*, *F. circinatum*, *F. sacchari*, *Fusarium ananatum* and *F. subglutinans sensu stricto* had erect aerial mycelium. *Fusarium guttiforme* and *F. mangiferae* were the only species that have been reported to produce both types of conidiophores (Nirenberg and O'Donnell, 1998; Britz *et al.*, 2000b). However, in this study, only prostate aerial mycelium was observed in *F. guttiforme*, while *F. mangiferae* produced both types of aerial mycelium.

Fusarium subglutinans sensu lato conidia accumulate in false heads around conidiogenous openings and did not remain attached to the conidiogenous cell to produce true heads (Nirenberg and O'Donnell, 1998). The morphology of the conidia, both macro- and microconidia, was essential for the distinction of the species. Mostly 3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell were produced by *F. begoniae*, *Fusarium* species from Poaceae, *F. circinatum*, *F. concentricum*, *F. mangiferae*, *F. sterilihyphosum*, *F. sacchari* and *F. subglutinans sensu stricto* and its cryptic species. *Fusarium guttiforme* and *Fusarium ananatum* produced no sporodocial macroconidia, while *F. pseudocircinatum* produced only sporodochia (oval to obovoid) under continuous black light. The macroconidia formed by *F. bulbicola* were 3-5 septate with a slightly elongate apical and foot cell. In the aerial mycelium, *F. begoniae* produced allantoid and

obovoid, mostly non-septate microconidia, while *F. circinatum*, *F. mangiferae*, *F. sterilihyphosum* and *F. concentricum* produced obovoid, occasionally oval to allantoid, mostly non-septate microconidia. *Fusarium guttiforme*, *F. pseudocircinatum* and *Fusarium ananatum* formed obovoid non-septate microconidia only. *Fusarium bulbicola* and *Fusarium* species from Poaceae produced long oval to allantoid, non-septate microconidia. *Fusarium sacchari*, *F. subglutinans sensu stricto* and its cryptic species produced oval non-septate microconidia.

Sterile outgrowths are hyphae that bear no conidiophores and their presence or absence was useful in identifying four of the *F. subglutinans sensu lato* species, *F. circinatum*, *F. pseudocircinatum*, *Fusarium* species from Poaceae and *F. sterilihyphosum.*

Based on average growth rate at 25 and 30 °C we were able to distinguish all of the species examined, including the cryptic species of *F. subglutinans sensu lato* (Fig 2). Although some species displayed similar growth rates at a specific temperature, their growth rates were clearly different at the other temperature (e.g. *Fusarium* species from *A. comosus* and *F. bulbicola*), supporting the previous findings (Burgess *et al.*, 1988) that some species are indistinguishable if only one of the temperatures were used.

All of the species residing in the *F. subglutinans sensu lato* complex examined in this study could be distinguished from each other based on morphology, with the exception of the cryptic species (Table 3). It was thus possible to construct a dichotomous key to facilitate the identification of these taxa (Table 4). The observed colony colours were summarised in Table 5

DNA extraction, amplification and sequencing

Individual alignments for the TEF-1α, β-tub, Cal and LSU gene regions were respectively 640, 340, 509 and 900 characters in length. MP trees generated from these data displayed similar clustering patterns, although the LSU tree lacked resolution (results not shown) and different species did not necessarily have different LSU sequences. Overall, the TEF-1α data appeared more informative than those for any of the other regions studied based on number of parsimony informative sites (Table 6) and sequence comparisons using those in public domain databases.

DNA-based comparisons

The amplicons of the TEF-1α, β-tub, Cal and LSU gene regions were 540, 530, 413 and 420 base pairs (bp) in size, respectively. The phylogenetic signal for the respective datasets were analysed and is presented in Table 6.

The different gene regions were analysed separately to determine their usefulness in distinguishing the different species in this complex. The sizes of the regions used as well as the statistical analyses are presented in Table 6. All parsimony-uninformative and constant characters

were excluded. Heuristic searches on the data sets with the TBR option were performed. Phylogenetic trees obtained for the TEF-1α, β-tub and Cal genes are presented (Figs 3-5) Analysis of the LSU data gave a tree without definition and this is consequently not shown.

Bayesian analyses utilised the Metropolis-coupled Markov chain Monte Carlo search algorithm as implemented in the program MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001). All Bayesian analyses consisted of 1 000 000 generations running one cold and three heated chains, with Bayesian inference posterior probabilities (biPP) calculated after discarding a burnin were determined. BInt analyses utilised the K80+I substitution model for the Cal data, the HKY+G substitution model for the β-tub data and GTR+I+G substitution model with separate parameters for the TEF-1α gene.

The 13 species included in the study resided in three independent clades based on the β-tub data (Fig 3). *Fusarium pseudocircinatum* was the only species in the complex that is accommodated in clade A. *Fusarium sacchari*, *F. sterilihyphosum* and *F. concentricum* made up clade B, while *F. guttiforme*, *Fusarium ananatum*, *F. begoniae*. *F. bulbicola*, *F. circinatum* and *F. subglutinans sensu stricto*, the cryptic species of *F. subglutinans*, *Fusarium* sp. from Poaceae and *F. mangiferae* constituted clade C.

Two major clades were observed based on the Cal dataset (Fig 4). *Fusarium pseudocircinatum*, *F. sacchari*, *F. concentricum* and *F. sterilihyphosum* formed clade A, while *F. guttiforme*, *Fusarium* sp. from *A. comosus*, *F. bulbicola*, *F. circinatum*, *F. subglutinans sensu stricto*, the cryptic species of *F. subglutinans*, *Fusarium* sp. from Poaceae, *F. begoniae* and *F. mangiferae* were included in clade B.

Two clades were observed based on the TEF-1α, data (Fig 5). *Fusarium pseudocircinatum* was the only species in the complex accommodated in clade A, while the reminder of the species grouped into clade B with the exception of *F. concentricum* and *F. sterilihyphosum* that grouped outside these two clades. Trees with similar topologies were inferred using MP, ML and BI. Based on the TEF-1α ML tree (Fig 6) the taxa included were separated into two distinct clades (A and B), representing the so-called "Asian" and "American" clades defined by O'Donnell *et al.* (1998a). Typical of phylogenies based on TEF-1α alone, the third or so-called "African" clade in the GFC was not supported. *Fusarium pseudocircinatum* was the only *F. subglutinans sensu lato* species among the taxa that represented the "African" clade based on multigene phylogenies (Fig 6). The majority of the *F. subglutinans sensu lato* species (*F. guttiforme* and *Fusarium* sp. from Poaceae, *F. begoniae*, *F. bulbicola*, *F. circinatum* and *F. subglutinans sensu stricto* and its cryptic species) were situated in Clade B, while *F. mangiferae*, *F. concentricum* and *F. sacchari* were located in clade A (Fig 6).

As part of the Asian clade, *F. sacchari* and *F. concentricum* could be clearly separated with bootstrap values of 100 and 97 % respectively (Fig 6). The separation of *F. mangiferae* from *Fusarium* sp. NRRL 26427 was not supported. In the American clade, *F. bulbicola*, *F. subglutinans sensu stricto*, *Fusarium* sp. from Poaceae and *F. circinatum* could not be separated clearly from other species and bootstrap values were not higher than 70 % (Fig 6). *Fusarium begoniae* separated from *F. guttiforme* as a distinct species with a bootstrap value of 85 %. The cryptic species encompassed in *F. subglutinans sensu lato* and *F. sterilihyphosum* grouped together with a bootstrap value of 92 % (Fig 6). *Fusarium pseudocircinatum* grouped separately in the African clade but this separation had low (< 70 %) support. All the mating populations included in this complex, namely mating population B, *F. sacchari*, mating population E, *F. subglutinans sensu stricto* and mating population H, *F. circinatum* were accommodated in the different clades, with mating population H en E clustering together in clade B.

Discussion

The demarcation of species lies at the heart of all classification systems (Lowe *et al*., 2006). This concept has undergone much restructuring and today depends on the unbiased results obtained after using different tools and to a lesser extent, the subjective opinion of the scientist. We evaluated the criteria used in the identification and diagnosis of *Fusarium* species with morphology typical of *F. subglutinans*, i.e. those representing *F. subglutinans sensu lato*. For this purpose we applied the pattern-based morphological species concept and the process-based phylogenetic species concepts.

Species identification using morphology has been widely used for many years for *Fusarium* (Booth 1971; 1977; Gerlach and Nirenberg, 1982; Nelson *et al*., 1983; Nirenberg and O'Donnell, 1998, Leslie and Summerell, 2006) and includes characteristics such as growth rate, macro- and microconidia morphology, nature of aerial mycelium and presence of sterile hyphae, properties of chlamydospores if they are produced and microconidia produced in chains (Nelson *et al*., 1983; Nirenberg *et al*., 1998). However, none of the species represented by *F. subglutinans sensu lato* have ever been shown to produce chlamydospores, which is also true for the majority of the known species in the GFC. Also, unlike many other GFC species, those representing *F. subglutinans sensu lato* do not form microconidial chains (Nirenberg and O'Donnell, 1998) and thus did not form part of our evaluation.

The abundance and shape of the macroconidia (Kuhlman, 1982, Nelson *et al*., 1983) have been important informative characters for the classification of *Fusarium* species (Kuhlman, 1982; Nelson *et al*., 1983; Nirenberg and O'Donnell, 1998). In this study, we were able to distinguish all the species except the cryptic species of *F. subglutinans sensu stricto* based on macroconidial morphology. Microconidial morphology provided additional data to separate the *Fusarium* species

evaluated. The *F. begoniae* group produced obovoid, occasionally oval to allantoid, mostly non-septate microconidia while the *F. guttiforme* group formed obovoid non-septate microconidia only. *Fusarium bulbicola* and *Fusarium* sp. from Poaceae produce long oval to allantoid, non-septate microconidia, while *F. concentricum* produced oval to obovoid non-septate microconidia.

The increasing need for more informative morphological characters to distinguish species of *Fusarium* is illustrated by the increasing numbers of phylogenetic species with identical morphology. One such additional distinguishing character is the presence of sterile outgrowths that do not bear any conidiophores (Nirenberg and O'Donnell, 1998). Four members of *F. subglutinans sensu lato* (i.e. *F. circinatum*, *F. pseudocircinatum*, *F. sterilihyphosum* and *Fusarium* species from Poaceae) are characterised by these structures (Nirenberg and O'Donnell, 1998; Britz *et al*., 2000b). In *F. circinatum*, these outgrowths conjugate into coils, while in *Fusarium* sp. from Poaceae they terminate in serpentine-like hyphae. All four of these species could also be distinguished from each other based on other morphological characteristics.

The aerial mycelium bearing conidiophores can be either erect or prostrate. Based on this characteristic, we divided the species in this complex into three groups. The first group accommodated species with erect aerial mycelium such as *F. circinatum*, while the second group of species form prostrate aerial mycelium, such as the *Fusarium* sp. from Poaceae. The last group accommodates *F. mangiferae* which produces two types of aerial mycelium.

The morphological demarcation of species has a number of limitations. In the case of *Fusarium*, the ability to recognise morphological differences requires experience that may take years to acquire. Furthermore, environmental conditions alter observed morphology as different growth conditions may favour certain morphological characters as in the case of the false chain formation of microconidia in *F. pseudocircinatum*. Despite these limitations all the species in *F. subglutinans sensu lato* with the exception of the cryptic species could be distinguished.

Application of the phylogenetic species concept has been demonstrated to delineate all of the known species in the *F. subglutinans sensu lato* complex. For this purpose, phylogenetic analyses were based on the sequence information for the four unlinked regions TEF-1α, β-tub, Cal and LSU. Although the intron-rich portions of protein coding genes TEF-1α, β-tub and Cal proved useful for species delineation, the TEF-1α has become the preferred region for *Fusarium* diagnostics (Geiser *et al*., 2004). This is largely due to the fact that this region appears to be more informative at the species level and the fact that non-orthologous copies of TEF-1α have never been detected in *Fusarium*. As a result Geiser *et al*. (2004) introduced the publicly available *Fusarium* ID database containing TEF-1α sequences for all known species and lineages in the genus. We used the data generated for this gene region to compare the thirteen species in the *F. subglutinans sensu lato* complex to the database. All the species could be distinguished except the cryptic species.

Based on the sequence data generated for the three gene regions, all the species in the *F. subglutinans sensu lato* complex could be distinguished. In the separate analyses of the gene sequences the clades accommodating the thirteen species were well supported. The clustering of species in the analyses of the separate datasets was constant with the exception of the *F. circinatum* and *F. bulbicola* relationship. In the phylogenetic analyses of the TEF-1α, the two species do not group together, as is the case in the analyses based on the cal and β-tub genes.

Accurate identification of *Fusarium* species, clearly requires the use of DNA information in combination with other data such as morphology and ecology. Based on morphology, phylogeny and host specificity the species are separated into four clusters. The first cluster accommodates the species that occur on *A. comosus* and has both erect and prostate aerial mycelium. The second cluster accomodates *F. bulbicola*, *F. circinatum*, *F. subglutinans sensu stricto* and its cryptic species. These species are characterised by erect aerial mycelium and a growth rate of 15-30 mm per 5 d. All the host plants in this cluster originated from the Northern Hemisphere with the exception of *Nerine* spp. Most of the host species are monocotyledonous herbaceous plants. Species in both clusters 1 and 2 have the same macroconidial morphology but differ from that observed in cluster 3. Cluster 3 accommodates *F. begoniae*, *F. mangiferae* and the *Fusarium* sp. from Poaceae. All these species have prostate aerial mycelium and a growth rate of 10-25 mm per 5 d. All host plants in this cluster originated in Asia and are dicotyledonous plants with adaptation for pollination by insects, with the exception of the wind pollinated monocotyledonous grasses. Grasses, however, have a cosmopolitan distribution. The fourth cluster accommodates *F. sterilihyphosum*, *F. sacchari* and *F. concentricum*. These three species have similar microconidial morphology, but different aerial mycelia i.e *F. concentricum* has prostrate aerial mycelia, while *F. sacchari* and *F. sterilihyphosum* have erect aerial mycelia. All the hosts in this cluster originated in Asia.

Cryptic species have been viewed as evolutionary events of limited economical importance but recently cryptic species associated with economically important crops have been reported. The cryptic species of *F. subglutinans sensu stricto*, although clearly phylogenetically distant from *F. subglutinans sensu stricto* (O'Donnell *et al.*, 2000; Steenkamp *et al.*, 2002) could not be morphologically distinguished from *F. subglutinans sensu stricto*. This is in contrast with *Fusarium andiyazi* and *F. thapsinum*, previously accommodated in *F. moniliforme*, which could be distinguished from *F. moniliforme* based on a number of morphological characters (Marasas, *et al.*, 2001; Klittich, *et al.*, 1997).

Host-specificity appears to be a relatively useful and informative character for separation of the *F. subglutinans sensu lato* taxa. The association of certain strains with certain plant hosts has led to the establishment of *formae speciales* such as *F. subglutinans* f. sp *pini* and *F. subglutinans* f. sp. *ananas*, for the *F. subglutinans sensu lato* pathogens of *Pinus* species and *Ananas* species,

respectively (Hepting and Roth 1946; Ventura *et al*., 1993a/b). These have since been renamed *F. circinatum* and *F. guttiforme*. From this study we may conclude that division of species based on host could tentatively be applied. However, as more natural environments are surveyed more species may be described from the same host. For example, the plants *Ananas sp*., *M. indica* and *Zea mays* are each associated with two different *F. subglutinans sensu lato* species (Table 1). Host-specificity as a character on its own, therefore, may harbour problems (Benyon *et al*., 2000), but if it is used in conjunction with discrete morphological and phylogenetic characters as part of an integrated approach to speciation, it may prove very useful.

A variety of factors including reproductive and geographic isolation contribute to speciation (Barraclough and Nee, 2001; Orr, 2001; Rieseberg, 2001; Butlin, 2005). In some cases, the effect of such processes in *Fusarium* and many other organisms is reflected in morphology, host range and sexual compatibility. In other cases, the effect of speciation is apparently only visible at the DNA-level (e.g. *F. subglutinans sensu stricto* and its cryptic species). The biological species concept was not included in this evaluation of species in the *F. subglutinans sensu lato* complex due to its limited application. The teleomorphs of only a few species are known and thus the evaluation was only focused on the anamorph morphology. Integrated approaches for classification are, therefore, crucial for interpreting the genetic variation within entities previously treated as species (Klittich, *et al*., 1997; Marasas, *et al*., 2001; Steenkamp *et al*., 2002). Such approaches are also critically important for the development of taxonomic systems that both support the scientific knowledge accumulated as well as a practical application for the plant pathologists who have to use these systems (Summerell *et al*., 2003). In this study we have utilised two species concepts, the morphological and phylogenetic species concepts in an attempt to establish an integrated approach to the identification of closely related species in *F. subglutinans sensu lato*.

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Table 1: *Fusarium subglutinans sensu lato* strains included in the study.

Culture number	Species	Host	Geographic origin	GenBank accession numbers
MRC* 8165	*Fusarium ananatum*	*Ananas comosus*	South Africa	**Data generated in this study:**
				DQ282166 (Elongation factor-1α)
				DQ282173 (β-tubulin)
				HM204764 (Calmodulin)
				HM204755 (28S)
MRC 7541	*F. circinatum*	*Pinus radiata*	United States	**Existing data used:**
				AF333930 (Elongation factor-1α)
				AF333946 (β-tubulin)
				AF366531 (Calmodulin)
				AY249397 (28S)
MRC 7540	*F. concentricum*	*Musa sapientum*	Costa Rica	**Existing data used:**
				AF160282 (Elongation factor-1α)
				U61548 (β-tubulin)
				AF158335 (Calmodulin)
				U61652 (28S)
MRC 7542	*F. begoniae*	*Begonia* hybrid	Germany	**Existing data used:**
				AF160293 (Elongation factor-1α)
				U61543 (β-tubulin)
				AF158346 (Calmodulin)
				U61647 (28S)

MRC 7534	*F. bulbicola*	*Nerine bowdenii*	Germany	**Existing data used:** AF160294 (Elongation factor-1α) U61546 (β-tubulin) AF158347 (Calmodulin) U61650 (28S)
MRC 7539	*F. guttiforme*	*Ananas comosus*	Brazil	**Data generated in this study:** DQ282165 (Elongation factor-1α) DQ282172 (β-tubulin) HM204762(Calmodulin) HM204756 (28S)
MRC 7536	*F. pseudocircinatum*	*Solanum* sp.	Ghana	**Existing data used:** AF160271 (Elongation factor-1α) U34427 (β-tubulin) AF158324 (Calmodulin) U34540 (28S)
MRC 6524	*F. sacchari*	Sugarcane	Taiwan	**Existing data used:** AF160278 (Elongation factor-1α) U34414 (β-tubulin) AF158331 (Calmodulin) U34527 (28S)

MRC 6512	*F. subglutinans*	*Zea mays*	United States	**Data generated in this study:** HM135532 (Elongation factor-1α) HM135540 (β-tubulin) HM135536 (Calmodulin) HM204761 (28S)
MRC 1077	*Fusarium* sp. (Cryptic species)	*Zea mays*	United States	**Data generated in this study:** HM135533 (Elongation factor-1α) HM135541 (β-tubulin) HM135537 (Calmodulin) HM204759 (28S)
MRC 2802	*F. mangiferae*	*Mangifera indica*	South Africa	**Data generated in this study:** HM135531 (Elongation factor-1α) HM135539 (β-tubulin) HM135535 (Calmodulin) HM204757 (28S)
MRC 2730	*F. sterilihyphosum*	*Mangifera indica*	South Africa	**Data generated in this study:** HM135534 (Elongation factor-1α) HM135542 (β-tubulin) HM135538 (Calmodulin) HM204758 (28S)
MRC 6747	*Fusarium* sp.	Poaceae	South Africa	EU921238 (Elongation factor-1α) EU921247 (β-tubulin) HM204763 (Calmodulin) HM204760 (28S)

***** MRC refers to the culture collection of the Medical Research Council, Tygerberg, Cape Town, South Africa.

Table 2: Other *Fusarium* species included in extended translation elongation factor-1α dataset.

Culture number	Name	Elongation factor 1-α‡
*NRRL 13308	*F. acutatum*	AF160276
NRRL 25206	*F. anthophilum*	AF160264
NRRL 20476	*F. bactridioides*	AF160290
NRRL 25300	*F. begoniae*	AF160293
NRRL 25446	*F. brevicatenatum*	AF160265
NRRL 25302	*F. denticulatum*	AF160269
NRRL 13164	*F. dlamini*	AF160277
NRRL 43470	*F. fujikuroi*	DQ790494
NRRL 25200	*F. lactis*	AF160272
NRRL 13604	*F. napiforme*	AF160266
NRRL 13592	*F. nygamai*	AF160263
NRRL 43542	*F. oxysporum*	DQ790509
NRRL 13617	*F. phyllophilum*	AF160274
NRRL 22944	*F. proliferatum*	AF160280
NRRL 25206	*F. pseudoanthophilum*	AF160264
NRRL 13592	*F. pseudonygamai*	AF160263
NRRL 25208	*F. ramigenum*	AF160267
NRRL 13999	*F. sacchari*	AF160278
NRRL 43543	*F. sacchari*	EF452969
NRRL 26427	*Fusarium* sp.	AF160286
NRRL 25309	*Fusarium* sp.	AF160284
NRRL 25303	*Fusarium* sp.	AF160283
NRRL 26794	*Fusarium* sp.	AF160287
NRRL 26757	*Fusarium* sp.	AF160308
NRRL 25195	*Fusarium* sp.	AF160298
NRRL 25807	*Fusarium* sp.	AF160305
NRRL 25622	*Fusarium* sp.	AF160301
NRRL 29124	*Fusarium* sp.	AF160311
NRRL 25204	*Fusarium* sp.	AF160299

NRRL 25346	*Fusarium* sp.	AF160296
NRRL 26064	*Fusarium* sp.	AF160302
NRRL 25221	*Fusarium* sp.	AF160268
NRRL 26152	*Fusarium* sp.	AF160306
NRRL 26061	*Fusarium* sp.	AF160302
NRRL 25615	*Fusarium* sp.	AF160304
NRRL 26793	*Fusarium* sp.	AF160309
NRRL 13613	*F. succisae*	AF160291
NRRL 43693	*F. thapsinum*	EF453018
NRRL 22949	*F. udum*	AF160275
NRRL 43697	*F. verticillioides*	EF453022

‡Sequences adapted from O'Donnell *et al*., (1998a)

*NRRL refers to the Agricultural Research Service Culture Collection at the National Centre for Agricultural Utilization Research, Peoria, Illinois, USA.

Table 3: Summary of morphological characters distinguishing the species of *F. subglutinans sensu lato*.

Culture number	Species	Nature of aerial mycelium: Prostrate/Erect	Macroconial morphology	Microconial morphology	Sterile oputgrowths
MRC* 8165	*Fusarium ananatum*	Erect	No sporodochial macro conidia are formed	obovoid non-septate microconidia	Absent
MRC 7541	*F. circinatum*	Erect	3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell	obovoid, occasionally oval to allantoid, mostly non-septate microconidia	Present
MRC 7540	*F. concentricum*	Prostrate	3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell	obovoid, occasionally oval to allantoid, mostly non-septate microconidia	Absent
MRC 7542	*F. begoniae*	Prostrate	3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell	allantoid and obovoid, mostly non-septate microconidia	Absent

MRC 7534	*F. bulbicola*	Erect	3-5 septate, slightly elongated macroconidia with a distinct apical and foot cell	long oval to allantoid, non-septate microconidia	Absent
MRC 7539	*F. guttiforme*	Prostrate	No sporodochial macro conidia are formed	obovoid non-septate microconidia	Absent
MRC 7536	*F. pseudocircinatum*	Prostrate	Sporodochial macro conidia are no formed after incubation in the dark	obovoid non-septate microconidia	Present
MRC 6524	*F. sacchari*	Erect	3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell	oval non-septate microconidia	Absent
MRC 6512	*F. subglutinans*	Erect	3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell	oval non-septate microconidia	Absent

MRC 1077	*Fusarium* sp. (Cryptic species)	Prostrate	3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell	oval non-septate microconidia	Absent
MRC 2802	*F. mangiferae*	Both	3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell	obovoid, occasionally oval to allantoid, mostly non-septate microconidia	Absent
MRC 2730	*F. sterilihyphosum*	Prostrate	3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell	obovoid, occasionally oval to allantoid, mostly non-septate microconidia	Present
MRC 6747	*Fusarium* sp.	Prostrate	3-septate, long falcate, almost straight macroconidia with a distinct apical and foot cell	long oval to allantoid, non-septate microconidia	Present

Table 4: Key to the species of *F. subglutinans sensu lato*.

1	One type of aerial mycelium observed……..…….2
	Both types of aerial mycelium observed…..…….3
2	Erect aerial mycelium ……..…...……...4
	Prostrate aerial mycelium …….…….…….…….....5
3	No sporodochial macroconidia formed…….....…….....6
	Macroconidia formed…..……..…….……10
4	Sterile outgrowths present……..…....……7
	Sterile growths absent…….……*F. bulbicola*
5	Sterile outgrowths present…….……...…*F. pseudocircinatum*
	Sterile growths absent…….....…….……8
6	Salmon colony colour observed……..…*Fusarium ananatum*
	Salmon colony colour not observed…….……*F. guttiforme*
7	Conidiophores branched…….……..……...*Fusarium* species from Poaceae
	Conidiophores branched and proliferated…….....…..*F. circinatum*
8	Macroconidia 3-septate…….……..……9
	Macroconidia 3-5 septate…….……..……11
9	Growth rate at 25 ˚C is 23 mm after 5 days……..……..*F. begoniae*
	Growth rate at 25˚C is 9 mm after 5 days……...…….……*F. sacchari*
10	Macroconidia long and slender, usually 3-5 septate……...*F. mangiferae*
	Macroconidia with defined apical and foot cell……..……...…..*F. sterilihyphosum*
11	Microconidia oval shaped…….…….……....*F. subglutinans sensu lato*
	Microconidia oval to obovoid shaped……..……..*F. concentricum*

Fusarium subglutinans sensu stricto and its cryptic species could not be distinguished morphologically.

Table 5: Colony colours observed after 10 d growth on potato dextrose agar.

Isolate Number	Species	Top of plate on PDA	Rayner reference	Reverse of plate on PDA	Rayner reference
MRC 6524	*F. sacchari*	Rosy buff to pale vinaceous	13"f to 1"f	Buff to Luteous	19"f to 19b
MRC 2730	*F. mangiferae*	Pale vinaceous	1"f	Rosy buff to luteous	13"d to 19b
MRC 6512	*F. subglutinans*	Rosy vinaceous to pale vinaceous	7"d to 1"f	Rosy buff to ochreous	13"d to 15'b
MRC 7534	*F. bulbicola*	Rosy vinaceous to vinaceous	7"d to 1"d	Red	2
MRC 2802	*F. sterilihyphosum*	White to pale vinaceous	0- to 1"f	Buff to ochreous	19"f to 15'b
MRC 1077	Cryptic species	Rosy vinaceous	7"d	Rosy buff to ochreous	13"d to 15'b
MRC 7536	*F. pseudocircinatum*	Rosy buff to rosy vinaceous	13"d to 7"d	Rosy buff to ochreous	13"d to 15'b
MRC 7540	*F. concentricum*	Pale vinaceous	1"f	Buff to ochreous	19"f to 15'b
MRC 7539	*F. guttiforme*	Buff	19"f	Pale luteous	19b
MRC 7541	*F. circinatum*	Livid vinaceous	1'"b	Rosy buff to rosy vinaceous	13"d to 7"d
MRC 7542	*F. begoniae*	Saffron	7f	Buff to Luteous	19"f to 19b
MRC 6747	*Fusarium* species from Poaceae	Pale vinaceous to livid vinaceous	1"f to 1'"b	Livid vinaceous	1'"b
MRC 8165	*Fusarium ananatum*	Salmon	11'd	Salmon	11'd

Table 6: Summary of phylogenetic signal for data sets used in the study.

Locus	Number of Characters (bp)	Parsimony tree length	Number of trees after re-weighting with CI value	Number of informative characters	CI/RI	g_1
Translation elongation factor 1-α	540	155	9	58	0.875/0.803	-0.302319
Calmodulin	413	82	54	34	0.921/0.941	-0.668814
β-tubulin	530	89	4	43	0.949/0.968	-0.781253
Large subunit	418	10	4	3	0.9/0.667	-3.023029

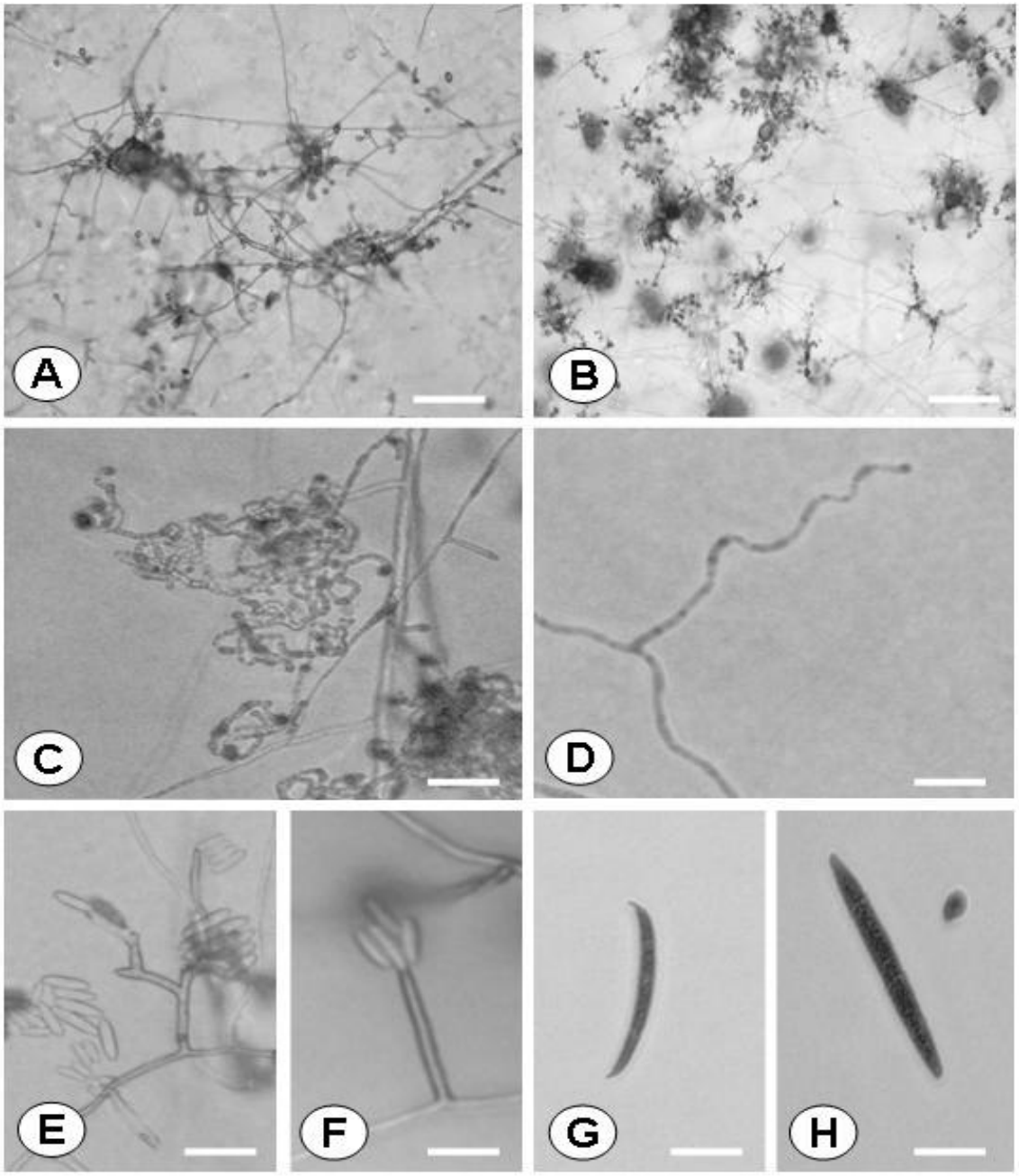

Fig 1 - Morphological characteristics of *F. subglutinans sensu lato*. A) Conidiophores on prostrate aerial mycelium. B). Conidiophores on erect aerial mycelium. C) Sterile coils formed on SNA. D) Serpentine hyphae formed on CLA. E) Polyphialide formed on CLA. F) Monophialide formed on CLA. G-H) Macroconidia formed on CLA. Bars (A) = 4 µm; (B) = 4 µm ; (C) = 0.3 µm; (D-E) = 0.5 µm; (F) = 1 µm; (G-H) = 0.5 µm.

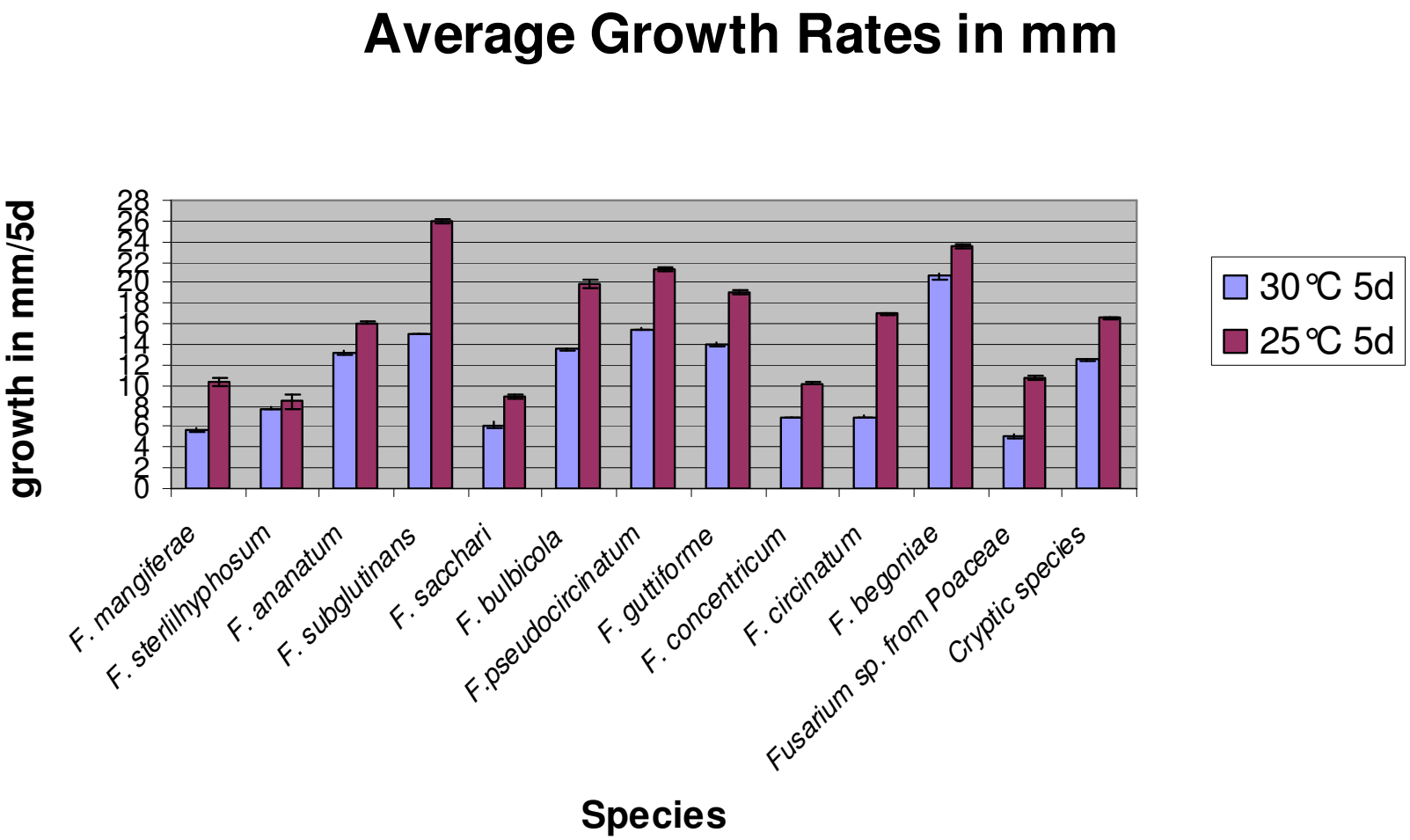

Fig 2: Average growth rate at 25°°C and 30°°C for *F. subglutinans sensu lato.*

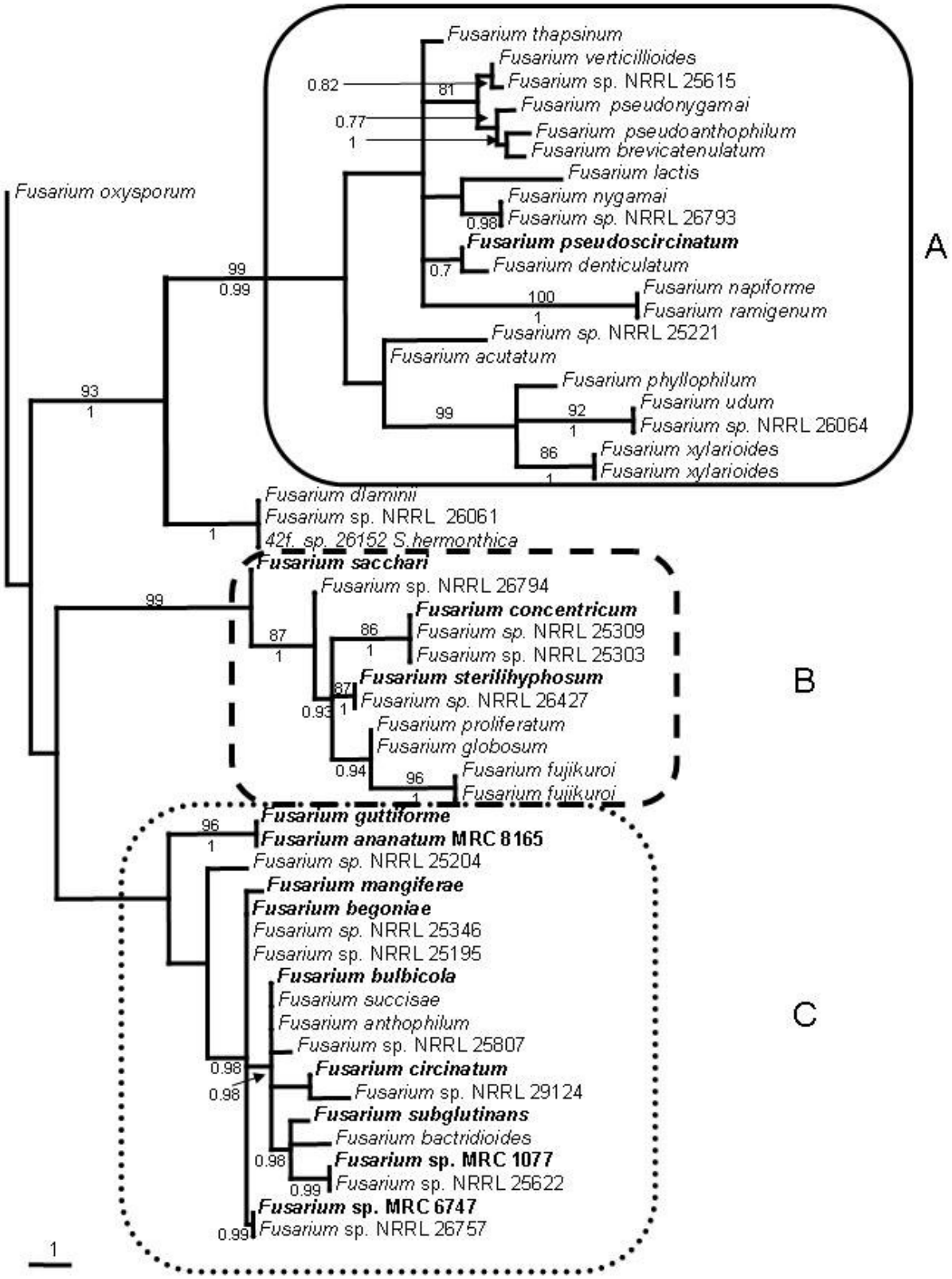

Fig 3: Phylogenetic tree of *Fusarium subglutinans sensu stricto* and related species produced using parsimony of the data of the β-tubulin gene, with *F. oxysporum* as outgroup. Parsimony-informative characters = 34; Tree length = 98 steps; CI = 0.9082; RI = 0.9400; g1 = -0.684116. Bootstrap values above 70 % (percentages of 1 000 bootstrap replicates) are indicated below the branches of the tree and the Bayesian Posterior probability values are indicated above the branches.

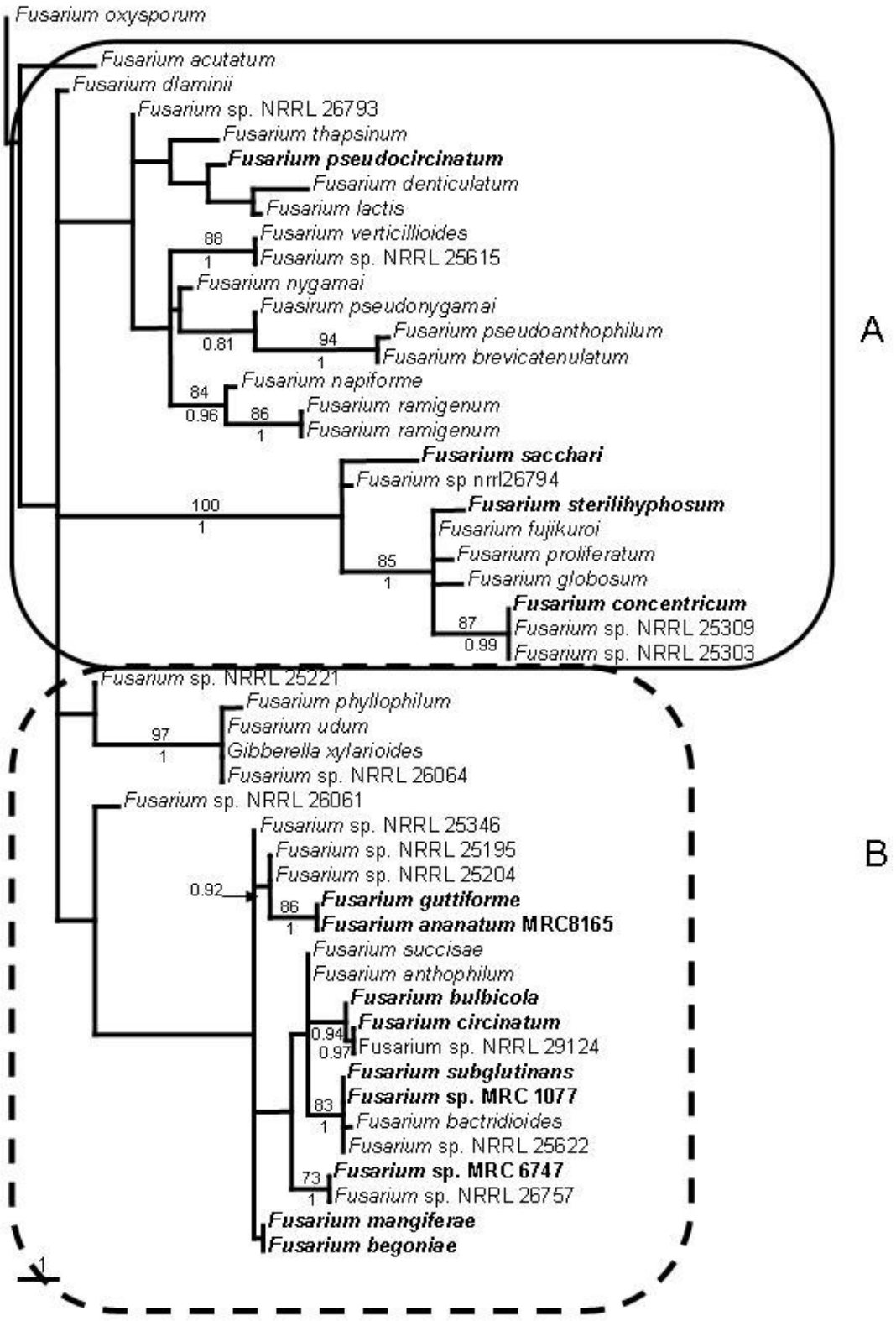

Fig 4: Phylogenetic tree of *F. subglutinans sensu stricto* and related species produced using parsimony of the data of the calmodulin gene, with *F. oxysporum* as outgroup. Parsimony-informative characters = 34; Tree length = 89 steps; CI = 0.8876; RI = 0.9174; g1 = -0.668814. Bootstrap values above 70 % (percentages of 1 000 bootstrap replicates) are indicated below the branches of the tree and the Bayesian Posterior probability values are indicated above the branches.

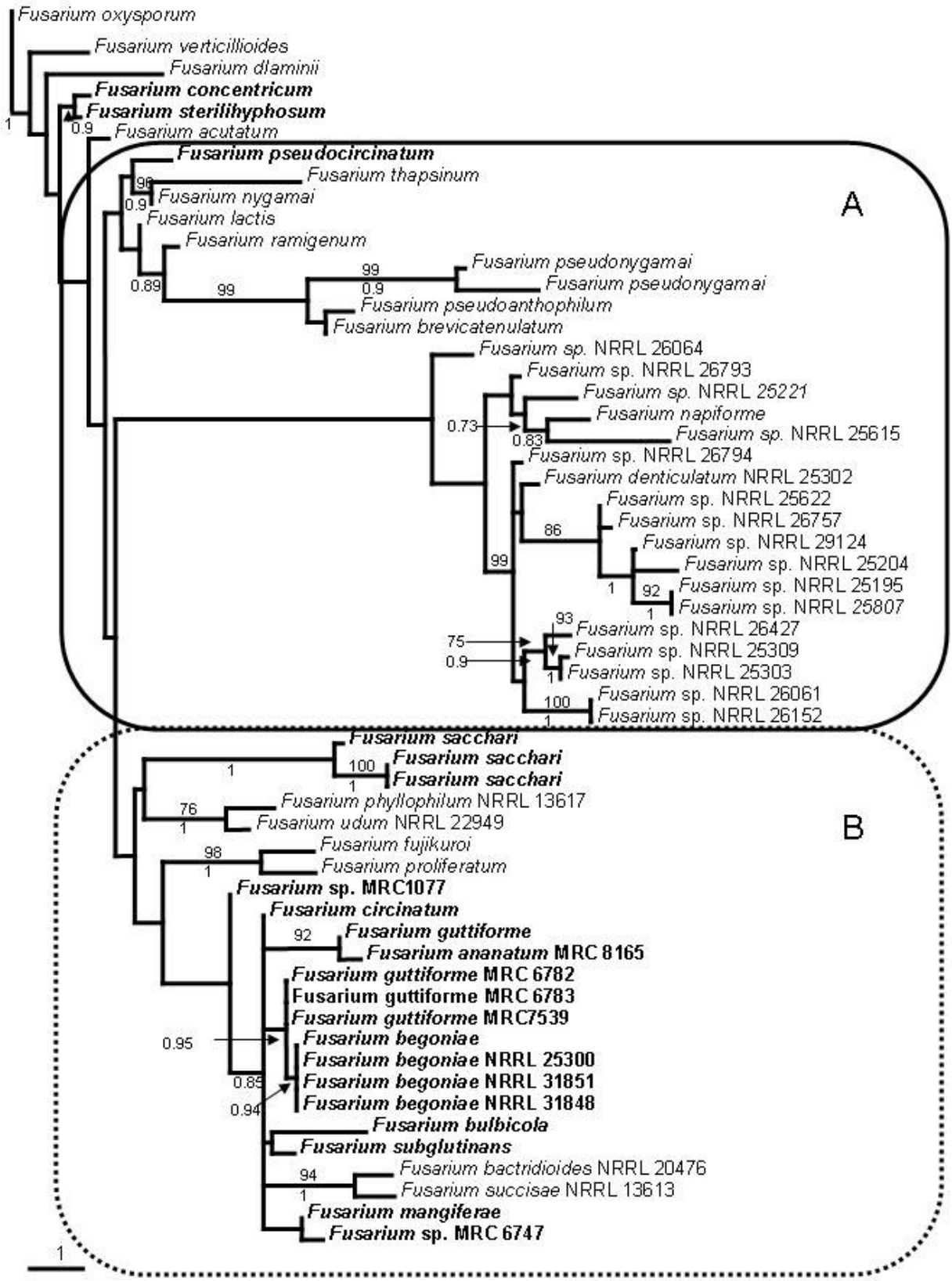

Fig 5: Phylogenetic tree of *F. subglutinans sensu stricto* and related species produced using parsimony of the data of the elongation factor 1α gene, with *F. oxysporum* as outgroup. Parsimony-informative characters = 146 steps; Tree length = 629; CI = 0.4092; RI = 0.6979; g1 = -0.302319. Bootstrap values above 70 % (percentages of 1 000 bootstrap replicates) are indicated below the branches of the tree and the Bayesian Posterior probability values are indicated above the branches.

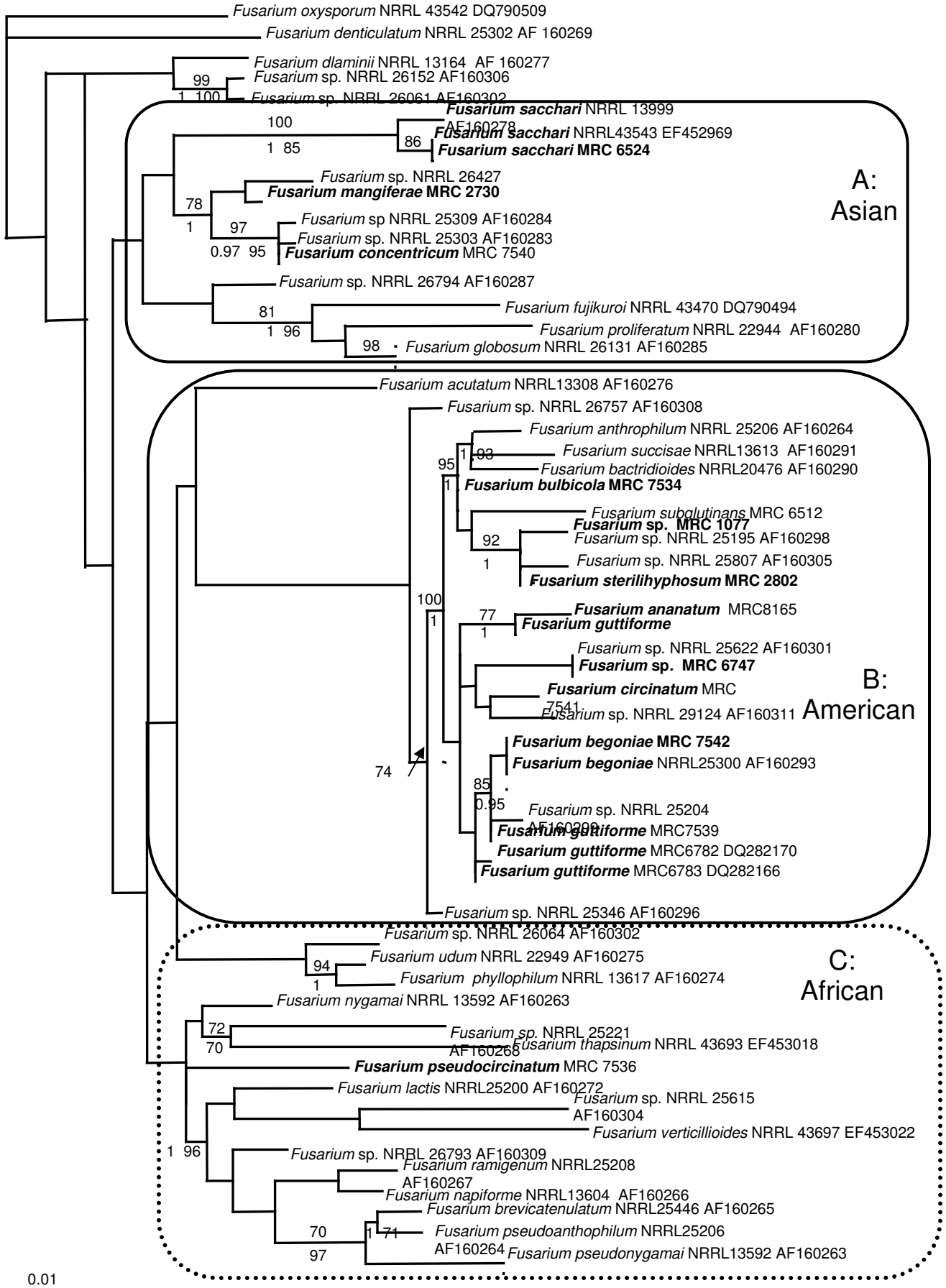

Fig 6: Maximum likelihood (ML) phylogeny of the GFC based on TEF-1α DNA sequence information. The MP values are indicated above the branches, ML and BI values are indicated below the branches. The tree is rooted to *F. oxysporum*.

CHAPTER 3

A new *Fusarium* species from Poaceae in South Africa

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A new *Fusarium* species from Poaceae in South Africa

ABSTRACT

During routine disease surveys, seven isolates representing *Fusarium subglutinans sensu lato* were obtained from diseased *Pinus patula* seedlings, as well as the grasses *Panicum maximum* and *Phragmites mauritianus*, growing in association with the pine seedlings in South Africa. The presence of curved sterile hyphae, produced on synthetic nutrient agar, and vinaceous gray colonies in young cultures, distinguished them from known species in this complex. The aim of this study was to characterise these isolates using phylogenetic analyses of partial sequences obtained for the ß-tubulin (BT) and translation elongation factor-1α (TEF-1α) genes. Phylogenetic analyses showed that the isolates from *Pa. maximum* and *Ph. mauritianus* group in a distinct clade from *F. begoniae and F. circinatum*. Morphologically, the latter two species are distinguished from each other based on the absence or presence of sterile coils, macroconidial morphology and the nature of the aerial mycelium. The unknown isolates were phylogenetically distinct from other species and mating tests showed that they were sexually compatible with the mating testers strains for *F. subglutinans* and *F. circinatum*. They were also not pathogenic to *P. patula* seedlings in contrast to *F. circinatum* that caused disease on these plants. The *Fusarium* isolates from the Poaceae are thus described as new and provided with the name *Fusarium ophiodes* sp. nov.

Keywords: Fungi, *Fusarium*, Phylogenetic species, Mating populations, Hybridization

Introduction

Species of fungi in the G*ibberella fujikuroi* (Sawada) Wollenw. complex include many important pathogens of agricultural and forestry crops. They include eleven biological species or mating populations (Leslie and Summerell, 2006). Three of these mating populations (B, E and H) form part of the so called *Fusarium subglutinans* morpho-species complex and are pathogens of *Saccharum* spp., *Zea mays* and *Pinus* spp., respectively (Leslie *et al*., 1992; 2005; Britz *et al*., 1999)

Species accommodated in *F. subglutinans sensu lato* are morphologically similar. Some of the morphological characters used by Nirenberg and O'Donnell (1998) in their evaluation of these species include the arrangement of conidiophores on the aerial mycelium, the number of conidiogenous openings on the polyphialides, the presence or absence of sterile coils, formation of false chains and macroconidial morphology. The phylogenetic relationships between these species has been determined based on sequence comparisons for the protein coding genes translation elongation factor-1α, β-tubulin, the mitochondrial small subunit, as well as the ITS2 region of the rDNA gene region (O'Donnell *et al*., 1998a; Aoki *et al*., 2001).

The pine pitch canker pathogen, *F. circinatum*, resides in the *F. subglutinans sensu lato* complex. This fungus has a cosmopolitan distribution and has been reported from the USA, Chile, Japan, Mexico, Spain, Italy and South Africa (Wingfield *et al*., 2008). In South Africa, the fungus was originally found only in pine nurseries (Wingfield *et al*., 1998) but pitch canker, as it is known on mature trees, has recently been reported on mature pine stands in the country (Coutinho *et al*., 2007).

After the first outbreak of *F. circinatum* in a major pine seedling producing nursery in South Africa (Viljoen *et al*., 1994), surveys were undertaken and plants growing in the area of the nursery were analysed for infection. These included the native grass, *Panicum maximum*, and the well-established, non-native reed, *Phragmites mauritianus*. Isolations from these grasses resulted in a suite of *Fusarium* isolates of unknown identity. The aim of this study was to identify the unknown *Fusarium* isolates.

Materials and methods

Isolations and isolates

Isolations were made from *Pa. maximum* (grass isolates) and *Ph.* (reed isolates) growing at a forestry nursery near Nelspruit, Mpumulanga, South Africa. Isolations were made by placing small pieces (3 mm) of root tissue onto *Fusarium* selective medium (20 g Agar, 15 g Peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_20$, 1 g PCNB, 20 mL Streptomycin sulphate in 1 L water) in Petri dishes (Nelson *et al*., 1983). Petri dishes were incubated at 25 °C under cool-white fluorescent light. The plates were checked

regularly and all the colonies with typical *Fusarium* morphology were transferred to half-strength potato dextrose agar (PDA) (Merck, Germany). Single conidial cultures were made and stored at -70 °C in 15 % glycerol.

All isolates used in this study are maintained in the *Fusarium* culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and in the culture collection of the Medical Research Council (MRC), Tygerberg, Cape Town, South Africa. A representative collection of isolates has also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

DNA extractions and PCR

Isolates were grown on complete medium (CM) (Correll *et al*., 1987) at 25 ºC for 7 d and mycelium was harvested by scraping it from the agar surface with a sterile blade. DNA was isolated using the technique described by Jacobs *et al*. (2007) where ca. 10 µg of sterile, chemically treated sand and 500 µL extraction buffer [DEB: 200 mM Tris-HCl pH 8, 150 mM NaCl, 25 mM EDTA, 0.59 % SDS] was added to a microcentrifuge tube half filled with fungal mycelium to break open the cell walls. An additional 500 µL of phenol and 300 µL chloroform was added, mixed and centrifuged for 30 min at 10 000 rpm. The phenol/chloroform step was repeated until the interface was clean. The supernatant was transferred to a new tube and double the volume of 100 % ethanol was added and mixed. The DNA was allowed to precipitate at 4 °C overnight and then pelleted by centrifugation for 30 min at 11 000 rpm. Pellets were washed with 300 µL 70 % ethanol, dried and resuspended in 50 µL sterile, deionised water and 3 µL of RNAse (2.5 µM).

Extracted DNA was used as template in PCR reactions to amplify the β-tubulin (BT) and translation elongation factor -1α (TEF-1α) genes. The TEF-1α gene region was amplified using the primer set EF1 (5'-CGAATCTTTGAACGCACATTG-3') and EF2 (5'-CCGTGTTTCAAGACGGG-3') (O'Donnell *et al*., 1998b). The β-tubulin gene region was amplified using the primer set T1 (5'-TTCCCCCGTCTCCACTTCTTCATG-3') and T222 (5'-GACCG-GGGAAACGGAGACAGG-3') (O'Donnell and Cigelik, 1997).

The PCR reactions consisted of 1x Roche *Taq* Reaction buffer with $MgCl_2$, dNTPs (200 µM each), primers (0.2 µM each), template DNA (25 ng) and Roche *Taq* polymerase (0.5 U) (Roche Pharmaceuticals, Germany). The PCR reaction conditions included an initial denaturation at 94 ºC for 2 min. This was followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 54 ºC for 1 min

and elongation at 72 ºC for 1 min, with a final elongation step at 72 ºC for 5 min. The resulting PCR amplicons were purified using a QIAquick PCR Purification kit (QIAGEN, Germany).

DNA sequencing and sequence comparisons

DNA sequences were determined from PCR amplicons using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK), using the same primers as those used in the PCR reactions. All of the sequences generated in this study were deposited in GenBank (Table 1).

DNA sequences were aligned in BioEdit. Gaps were treated as missing data and thus discarded from the subsequent analysis. Phylogenetic analysis was based on parsimony using PAUP* version 4b10 (Phylogenetic Analysis Using Parsimony* and Other Methods version 4, Swofford, 2002). Heuristic searches were done with random addition of sequences (100 replicates), tree bisection-reconnection (TBR) branch swapping, and MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets ($g1$) was assessed by evaluating tree length distributions over 100 randomly generated trees (Hillis and Huelsenbeck, 1992). The consistency (CI) and retention indices (RI) were determined for all data sets. Phylogenetic trees were rooted to *F. oxysporum* as monophyletic sister outgroup to the rest of the taxa. Bootstrap analyses were performed to determine the confidence intervals (1 000 replicates) for the branching points for the most parsimonious trees generated from all the data sets. The combinability of the TEF-1α and BT datasets was tested using the partition homogeneity test in PAUP* version 4b10 (Farris *et al.*, 1994).

Bayesian analyses utilised the Metropolis-coupled Markov Chain Monte Carlo search algorithm as implemented in the program MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). All Bayesian analyses consisted of 1 000 000 generations running one cold and three hot chains, with Bayesian inference posterior probabilities (biPP) calculated after burnin had been determined. BInt analyses utilised the GTR+I+G substitution model with separate parameters for each gene (partition) and an eight-category gamma model.

Morphological comparisons

Fungal strains and culture conditions

Isolates from grass and reed were grown on synthetic low nutrient agar (SNA) (Nirenberg and O'Donnell, 1998) and carnation leaf agar (CLA) (Nelson *et al.*, 1983) for 7 d at 25 ºC, under near ultraviolet light. Fungal structures produced on these media were mounted on microscope slides and used to describe the morphology of the grass and reed isolates. Colony colour was assigned using the colour charts of Rayner (1970). Growth in culture was assessed by placing a single macroconidium

(Nelson *et al*., 1983) at the centre of five PDA plates and incubating these in temperature-controlled incubators at temperatures ranging from 5 to 30 ºC at five degree intervals. Colony diameters were measured after 5 days by means of an electronic ruler and the averages of all measurements for each temperature computed. The standard error was calculated for each isolate at every temperature considered.

Sexual compatibility

To determine the mating types of the seven isolates from grass and reed, the *MAT-1* and *MAT-2* loci were amplified using PCR, as described by Steenkamp *et al*. (2000). The MAT idiomorphs were amplified with the primer sets GFmat1a (5'-GTTCATCAAAGGGCAAGCG-3'), GFmat1b (5'-TAAGCGCCTCTTAACGCCTTC-3'), GFmat2c (5'-AGCGTCATTATTCGATCAAG-3') and GFmat2d (5'-CTACGTTGAGAGCTGTACAG-3') (Steenkamp *et al*., 2000). For the PCR reactions, 1 x Roche *Taq* Reaction buffer with $MgCl_2$, 200 µM of each dNTP, 0.1 µM of each primer, 25 ng template DNA and 0.5 U Roche *Taq* polymerase (Roche Pharmaceuticals, Germany) was used. The PCR reaction conditions were an initial denaturation at 92 °C for 1 min, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 63 °C for 30 s and elongation at 72 °C for 30 s. A final elongation step was done at 72 °C for 5 min. The products were resolved on a 1 % agarose gel, containing ethidium bromide (0.2 µg/mL) and visualised under UV light.

All seven isolates from grass and reed were used in sexual compatibility experiments. Single conidial isolates were crossed in all possible combinations with each other and with the mating testers defining the mating populations H and E of the *G. fujikuroi* species complex (Britz *et al*.,1999; Leslie *et al*., 1992) as well as isolates from teosinte (Desjardins *et al*., 2000). Female tester strains were grown on carrot agar (CA) as described by Klittich and Leslie (1988), while the male testers were grown on complete medium slants. The isolates were incubated at 25 ºC under cool-white and dark-fluorescent light for 7 days. All crosses were made on CA as described by Britz *et al*. (1999). Conidial suspensions of the strains serving as males were spread over the mycelium surface in the CA plates (strains serving as females) using a glass rod. Reciprocal crosses, where the male strain was used as the female parent, were done when fertile crosses were not obtained in the first instance. Plates containing mated isolates were incubated at 18 ºC and examined regularly for the formation of perithecia. The ascospores exuding from one perithecium per cross were plated on water agar (WA) and incubated for 24 h at 25 ºC, after which the percentage germination was determined.

Pathogenicity tests

Twenty *P. patula* saplings were inoculated with isolates FCC 49 (*F. circinatum*), FCC 1092 (from *Ph. mauritianus*) and FCC 1093 (from *Pa. maximum*), the latter two of which represented the unknown species treated in this study. *Fusarium circinatum* was included for comparative purposes and because it is the fungus most commonly found on *P. patula* seedlings in the nursery from which the unknown isolates originated.

Discs of agar (6 mm diam.) were removed from the margins of actively growing cultures and placed in wounds of equal size, exposing the cambium on the stems of plants. Inoculation sites were sealed with laboratory film (Parafilm, USA) to prevent desiccation and contamination. Ten trees were inoculated with plugs of sterile PDA to serve as controls. Trees were maintained in the greenhouse for 4 weeks after which lesion lengths were measured and averages determined.

Results

Isolations and isolates

Seven isolates from grass and reed were selected for this study and these included those from *Pa. maximum* (MRC 6742/FCC 2996), (MRC 6744/FCC 2979, 2980), (MRC 6745/FCC 2977, 2978), (MRC 6747/FCC 1093), (MRC 6750/FCC 2972, 2973), (MRC 6754/FCC 2974, 2975) and from *Ph. mauritianus* (MRC 6748/FCC 1092) (Table 1).

DNA sequence comparisons

Amplification of DNA for the TEF-1α and BT gene regions resulted in amplicons of 660 and 700 bp, respectively. Only the first ca 500 bp of the BT sequence was used in the analyses to ensure equal sequence lengths for all included species.

Results of the partition homogeneity test (P = 0.138) showed sufficient support to combine the sequence datasets for the TEF-1α and BT gene regions. Alignment by inserting gaps resulted in a total of 1 593 characters used in the comparison of the different species. All parsimony-uninformative and constant characters were excluded, resulting in 100 parsimony-informative and 1 075 parsimony-uninformative characters.

Heuristic analyses of the data set generated four most parsimonious trees of which one is presented (Fig 1). The grass and reed isolates grouped together in a distinct clade in this tree (Fig 1). The representative strain of the *F. subglutinans* cryptic species (MRC 1077) and the two teosinte isolates group together with *F. subglutinans sensu stricto* in a separate clade.

Morphological comparisons

Comparisons of the morphology of the isolates from grass and reed with those of the ex-type or where applicable, tester strains for all members of the *F. subglutinans sensu lato* complex, showed that they represent a distinct morphological entity. Key characteristics defining these isolates included vinaceous gray colonies but with dispersed aerial mycelium and prostrate conidiophores produced on the aerial hyphae (Fig 2). Although the vinaceous gray colour of the colonies is characteristic of all species in the Section *Liseola* it is observed even in young cultures. In contrast to the colony colour, the nature of the aerial mycelium distinguished the grass and reed isolates from phylogenetically closely related species in the complex including *F. begoniae*, *F. bulbicola*, *F. circinatum* and *F. subglutinans sensu stricto*. The grass and reed isolates as well as *F. concentricum* and *F. pseudocircinatum* have prostrate aerial mycelium, while *F. bulbicola*, *F. circinatum*, *F. sacchari* and *F. subglutinans sensu stricto* produced erect aerial mycelium.

The *Fusarium* spp. from grass and reed could, furthermore, be distinguished from other species in *Fusarium subglutinans sensu lato* on macroconidial morphology. The *Fusarium* spp. from grass and reed produce long falcate, almost straight macroconidia with distinct apical and foot cells, which are distinctly different to *F. guttiforme* and *F. sterilihyphosum* that produced no sporodocial macroconidia. Likewise, *F. pseudocircinatum* produced sporodochia only under continuous black light whereas the isolates from grass and reed produce sporodochia under near ultraviolet light. The macroconidia of *F. bulbicola* are 3-5 septate with a slightly elongate apical and foot cell, but *F. begoniae*, *F. circinatum*, *F. concentricum*, *F. mangiferae and F. subglutinans sensu stricto* all produced macroconidia identical to those of the species from grass and reed.

Fusarium circinatum could be distinguished from the *Fusarium* sp. from grass and reed by the lack of sterile coils in the latter species. In addition it is also possible to distinguish between *F. subglutinans sensu lato* which include the teosinte isolates and the isolates from grass and reed based on the serpentine hyphae in the latter species.

The average growth rate of the *Fusarium* spp. from grass and reed distinguished it from the known species in *F. subglutinans sensu lato*. *Fusarium* spp. from grass and reed displayed no growth at 5 ºC and 10 ºC, and had an average growth rate of 4 mm per day at 15 ºC (standard error = 0.1), 9 mm per day at 20 ºC (standard error = 0.23), 14 mm per day at 25 ºC (standard error = 0.25), 11 mm per day at 25 ºC (standard error = 0.13). Closely related species such as *F. begoniae*, *F. circinatum*, *F. concentricum* and *F. subglutinans sensu stricto* and the two teosinte isolates have growth rates of approximately 5, 3, 2, 5 and 5.2 mm per day at 25 ºC, respectively.

Sexual compatibility

Using the MAT PCR primer pairs, the *MAT-1* idiomorph was amplified in all seven South African isolates from grass and reeds (Fig 3). Thus all isolates represented the same mating type. Consistent with this finding, none of the crosses amongst these isolates gave rise to perithecia i.e. they are the same mating population. However, where they were crossed with the mating tester strains for the H (*G. circinata*) and E (*G. subglutinans*) mating populations in the *G. fujikuroi* species complex and two teosinte strains, they gave rise to fertile perithecia. Where the grass and reed isolates produced perithecia after crossing with the tester strains of the E and H mating populations and the teosinte strains, they generally produced few ascospores in some cases the perithecia were barren. All the grass and reed isolates produced fertile crosses with MRC 6213 (H mating population) (*MAT-2*), MRC 6483 (E mating population) (*MAT-2*), and MRC 7828 (the teosinte strain) (*MAT-2*). No perithechia were formed in the crosses between the grass and reed isolates and MRC 7488 (H mating population) (*MAT-1*), or MRC 6512 (E mating population) (*MAT-1*), or FCC 1005 (the teosinte strain) (*MAT-1*). In the cases where ascospores were produced, they had a germination percentage of 85 % in contrast to the 95% germination was observed in the control crosses.

Taxonomy

The *Fusarium* isolates from grass and reed in South Africa could easily be separated from the other species in *F. subglutinans sensu lato* based on DNA sequence comparisons for a number of different gene regions. This includes the cryptic species of *F. subglutinans* (Steenkamp *et al.*, 2002). A number of morphological characters were used in conjunction with phylogenetic inference to distinguish these isolates and those of other species in *F. subglutinans sensu lato*. These characters include the nature of the conidiophores, conidial morphology and the presence of serpentine hyphae on the agar surface.

Although crosses between the isolates of the unknown *Fusarium* sp. from grass and reeds and various other taxa in the *F. subglutinans* species complex gave positive results, this is most likely due to pre-zygotic recognition factors (Taylor, 1999; 2000; Kohn, 2005). The unknown *Fusarium* sp. from grass and reed is sufficiently different to any other species in *F. subglutinans sensu lato*, including the cryptic species of *F. subglutinans* to be considered as an undescribed taxon and it is thus provided with the following description:

Fusarium ophiodes Jacobs, Coutinho, Marasas **sp. nov**. Fig 2

MycoBank: MB 493475

Etym.: The specific epithet is from the Greek *ophis* that means snake and refers to the serpentine hyphae in this species.

Margo coloniae integer; coloniae in PDA crescunt mediocriter 3 mm per diem in 25°C. Mycelium aerium omnino in colonia, in PDA pallide vinosum (9’’’f), infra livide vinosum (69’’’’d). Sclerotia, sporodochia chlamydosporaeque absunt. Conidiophorae prostrates in superficie agari faciuntur. Hyphae in SNA 2 (-4)–3 (-5) µm latae. Conidia in pseudocapitulis cumulantes. Conidiophorae in mycelio aerio prostrates e substrato faciuntur, ramosae vel non. Phialides mycelii aerii cylindricae monophialidicae 6-22 (-29) x 1-2 µm et polyphialidicae 2-3 aperturis conidiogenis 3(-6)-12(-18) x 1-2 µm. Microconidia plerumque non septata, rarius uniseptata, 2-6 x 1-2 µm. Macroconidia solum in mycelio aerio, non in sporodochiis, facta, falcata vel subrecta, extremis curvatis, parietibus tenuibus (8-)12-24(-32) x 1 µm.

Colony margin entire. Colonies on PDA with average growth rate of 3 mm /d at 25 ºC. Aerial mycelium spread throughout the colony. Aerial mycelium on PDA pale vinaceous grey (9’’’f) in colour, reverse of colony vinaceous grey (69’’’’d) in colour. Sclerotia, sporodochia and chlamydospores absent. Hyphae on SNA 2(-4)-3(-5) µm wide. Conidia accumulating in false heads. Conidiophores on aerial mycelium originating prostrate from substrate, branched or unbranched. Phialides of the aerial mycelium, cylindrical**,** monophialidic, 6-22 (-29) x 1-2 µm and polyphialidic, with 2-3 condiogenous openings, 3(-6)-12(-18) x 1-2 µm. Microconidia mostly 0-septate with 1-septate conidia occurring less abundantly, 2-6 x 1-2 µm. Macroconidia produced only on aerial mycelium and not in sporodochia, sickle-shaped to almost straight, with ends curved; thin walled, (8-)12-24(-32) x 1 µm.

Material examined: **South Africa:** *Ngodwana*, Mpumulanga, *Panicum maximum*, November 1994, M.J. Wingfield, PREM 58894 (*Holotype;* dried down culture of MRC 6744, FCC 2979, 2980; CBS 118512). PREM 58891 (*paratype;* dried down culture of MRC 6747; FCC 1092; CBS 118510). PREM 58892 (*paratype*; dried down culture of MRC 6748; FCC 1093; CBS 118509). PREM 58893 (*paratype*; dried down culture of MRC 6754; FCC 2974, 2975; CBS 118515). **Additional material:** *Ngodwana*, Mpumulanga, *Panicum maximum*, November 1994, M.J. Wingfield, MRC 6742 (FCC 2996; CBS 118511; PREM 58889), MRC 6750 (FCC 1972, 2973; CBS 118514), MRC 6745 (FCC 2977, 2978; CBS 118513).

Pathogenicity

None of the three *F. ophiodes* isolates from grass and reed gave rise to lesions on the inoculated *P. patula* seedlings. Inoculation with the *F. circinatum* isolate gave rise to distinct lesions, an average of 89 mm long. Control inoculations with sterile agar plugs resulted in no lesion development.

Discussion

Results of this study have shown that *Fusarium* isolates from reed and grass growing in association with pine seedlings in a South African forestry nursery represents a new species in *F. subglutinans sensu lato*. Most importantly, the fungus is clearly distinct from *F. circinatum*, the causal agent of pitch canker, which is commonly isolated from *P. patula* in the nursery where *F. ophiodes* was found.

The morphological distinction of *F. ophiodes* is based on three prominent characters and as in the case of *F. andiyazi* these characters are supported by DNA sequence comparisons (Marasas *et al*., 2001). The defining morphological characteristics of *F. ophiodes* include the nature of the conidiophores on the aerial mycelium, the vinaceous gray colony colour of isolates and the serpentine hyphae formed on the agar surface. The closely related species *F. circinatum* can be distinguished from *F. ophiodes* by its sterile coiled hyphae (Nirenberg and O'Donnell, 1998). Another closely related species *F. begoniae*, has prostate aerial mycelium and macroconidia similar to *F. ophiodes*, but it can be distinguished based on microconidial morphology. *Fusarium begoniae* has allantoid and obovoid, mostly 0-septated microconidia, while *F. ophiodes* have long oval to allantoid, 0-septated microconidia. *Fusarium ophiodes* and *F. concentricum* can be distinguished from each other based on the presence of concentric circles of mycelium by the latter but not in the former species (Nirenberg and O'Donnell, 1998). *Fusarium mangiferae* produces both erect and prostrate aerial mycelium and *F. subglutinans sensu stricto* is distinguished by erect aerial mycelium and oval microconidia. These are in contrast to those of *F. ophiodes* that are long and oval to allantoid in shape.

Based on a biological species concept, *F. ophiodes* would not be considered distinct from *F. subglutinans* or *F. circinatum*. However, there is precedence for fertile crosses between different species in the *G. fujikuroi* species complex. For example, isolates representing the C and D mating populations in the *G. fujikuroi* species complex produced fertile perithechia (Leslie *et al*., 2004). These crosses were defined as partly fertile, in that fewer than the normal number of ascospores was produced per perithecium (Perkins, 1994). They were also defined as intermittently fertile in that some of the crosses gave rise to completely barren perithecia, while at other times, relatively numerous fertile perithecia were produced (Leslie *et al*., 2004). Results of this study were similar to those of *F. konzum*

(Leslie *et al*., 2004) where the perithecia obtained in the crosses between *F. ophiodes* and the tester strains produced few ascospores and in some cases only barren perithecia. Because isolates of *F. ophiodes* represent a distinct phylogenetic entity and they have unique morphological features, we have chosen to define them as representing a distinct species and suggest that where crosses did occur with the E and H mating testers, these were due to pre-zygotic recognition where two gametes recognise each other (Kohn, 2005; Le Gac *et al*., 2007).

The phylogenetic separation of *F. ophiodes* from all other *Fusarium* spp. in *F. subglutinans sensu lato* was established based on the sequence data for three gene regions. The cryptic species associated with *F. subglutinans* and *F. subglutinans sensu stricto* grouped into a separate clade. The separation between these species is based on the incongruence among gene trees emerging from sequences for different loci indicate interbreeding among individuals as a result of recombination of genomes and unique evolutionary histories. The phylogenetic closely related species to *F. ophiodes* were *F. begoniae*, *F. bulbicola* and *F. circinatum*.

The discovery of *F. ophiodes* emerged from studies of the pitch canker fungus *F. circinatum* in a South African nursery environment. Peripherally, the fungus is very similar to *F. circinatum*. Without DNA sequence and indepth morphological comparisons, its unique nature would not have been discovered. Although very little is known regarding the ecology of the fungus, it is clearly not a pine pathogen. It is also ecologically different to *F. circinatum*, which is specific to pine and has never been isolated from plants other than conifers, especially pines. It would be interesting to know more regarding the biology of *F. ophiodes* and such studies are planned for the future.

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Table 1: List of fungi included in this study

Culture Number	Alternative designation[b]	Name	Origin	Host	β-tubulin	TEF-1α	Large subunit of the rDNA operon	Reference
FCC 2977/2978	MRC 6742	*F. ophiodes*	South Africa	*Panicum maximum*	EU921251	EU921242	FJ59686	This study
FCC 2979/2980	MRC 6744	*F. ophiodes*	South Africa	*Panicum maximum*	EU921248	EU921239	FJ59683	This study
FCC 2996/2997	MRC 6745	*F. ophiodes*	South Africa	*Panicum maximum*	EU921249	EU921240	FJ59684	This study
FCC 1093	MRC 6747	*F. ophiodes*	South Africa	*Panicum maximum*	EU921247	EU921238	FJ59682	This study
FCC 1092	MRC 6748	*F. ophiodes*	South Africa	*Phragmites mauritianus*	EU921252	EU921243	FJ59687	This study
FCC 2972/2973	MRC 6750	*F. ophiodes*	South Africa	*Phragmites mauritianus*	EU921246	EU921237	FJ59681	This study
FCC 2974/2975	MRC 6754	*F. ophiodes*	South Africa	*Phragmites mauritianus*	EU921250	EU921241	FJ59685	This study

	NRRL 31851	*F. begoniae*	Germany	*Begonia* hybrid	AY329045	AY329036	AY249393	This study
FCC 984		*Fusarium* sp.	Nepal	*Teosinte* sp.				Desjardins *et al*., 2000
FCC 1005		*Fusarium* sp.	Nepal	*Teosinte* sp.				Desjardins *et al*., 2000
FCC 49		*F. circinatum*	South Africa	*Pinus radiata*				This study
	NRRL 13618	*F. bulbicola*	Germany	*Nerine bowdenii*	U61546	AF160294	AY249395	This study
	NRRL 25331	*F. circinatum*	USA	*Pinus radiata*	U61547	AF160295	U61651	This study
	NRRL 29944	*F. concentricum*	Costa Rica	*Musa sapientum*	AF333951	AF333935	AF333943	O'Donnell *et al*., 2000.
	MRC 7539	*F. guttiforme*	Brazil	*Ananas comosus*				O'Donnell *et al*., 2000.
	NRRL13488	*F. nygamai*	Australia	*Sorghum bicolor*	U34426	AF160273	AY898252	O'Donnell *et al*., 2000.
	NRRL 22944	*F. proliferatum*			U34416	AF160280	AY249383	O'Donnell *et al*., 2000.

	NRRL 22946	*F. pseudocircinatum*	U34453	AF160271	U34511	This study
	NRRL 13999	*F. sacchari*	U34414	AF160278	U34527	This study
	NRRL 22045	*F. thapsinum*	U34444	AF160270	U34531	This study
	NRRL 22172	*G. moniliformis*	U34413	AF160262	U34526	O'Donnell *et al.*, 2000.

FCC refers to the *Fusarium* culture collection of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. b) MRC refers to the culture collection of the Medical Research Council, Tygerberg, Cape Town, South Africa. NRRL refers to the the U.S. Department of Agriculture, Northern Regional Research Laboratories, housed within the Microbial Genomics and Bioprocessing Research Unit at the National Centre for Agricultural Utilization Research (NCAUR) in Peoria, Illinois, USA.

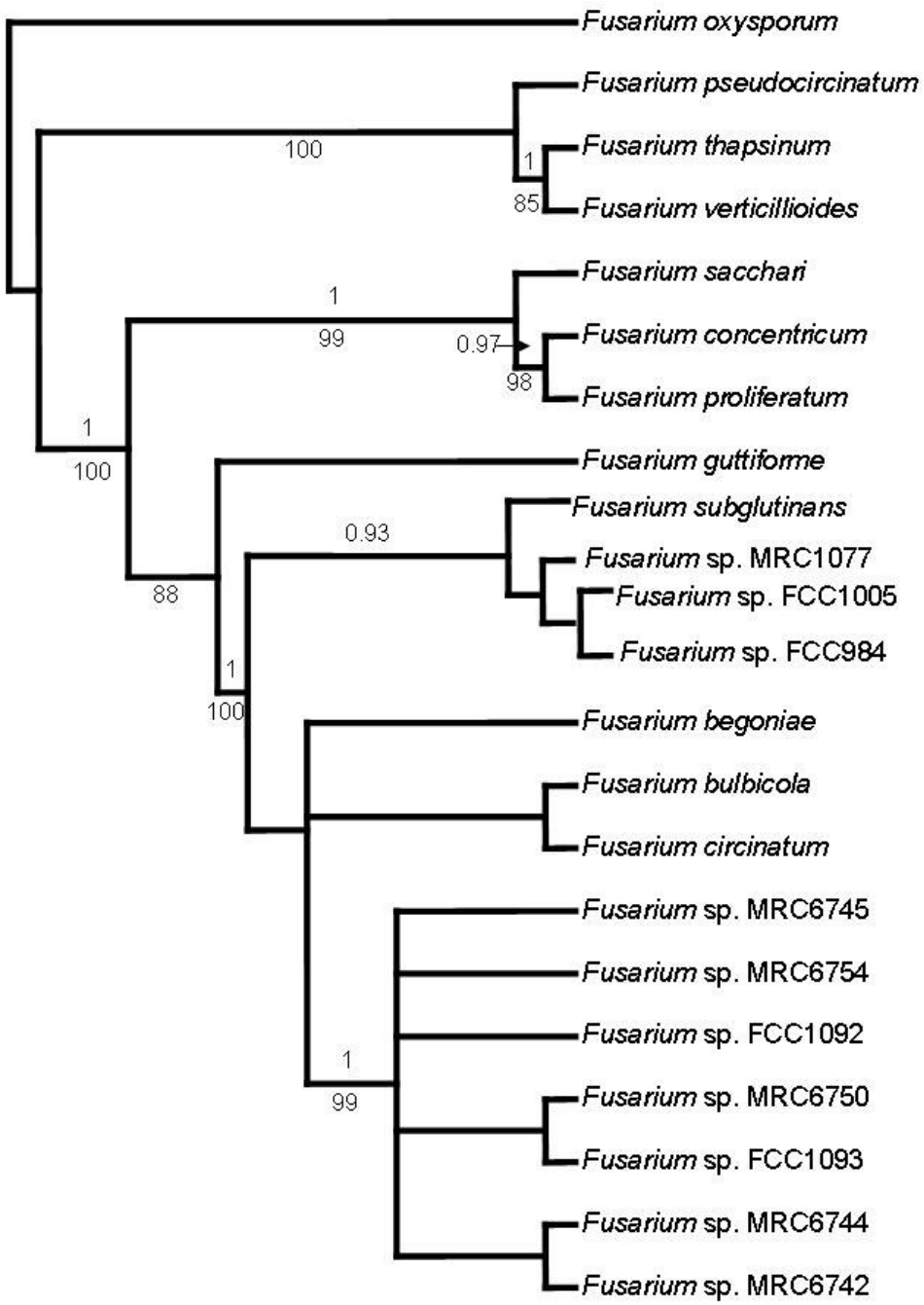

Fig 1: Phylogenetic tree produced using a parsimony analysis of sequences for the β-tubulin and TEF-1α genes and with *F. oxysporum* as the outgroup taxon. Bootstrap values above 70 % (percentages of 1 000 bootstrap replicates) are indicated below the branches of the tree and the Bayesian Posterior probability values are indicated above the branches. Parsimony informative characters = 100; Tree length = 99 steps; RI = 0.9605; CI = 0.9138; g1 = -0.895110.

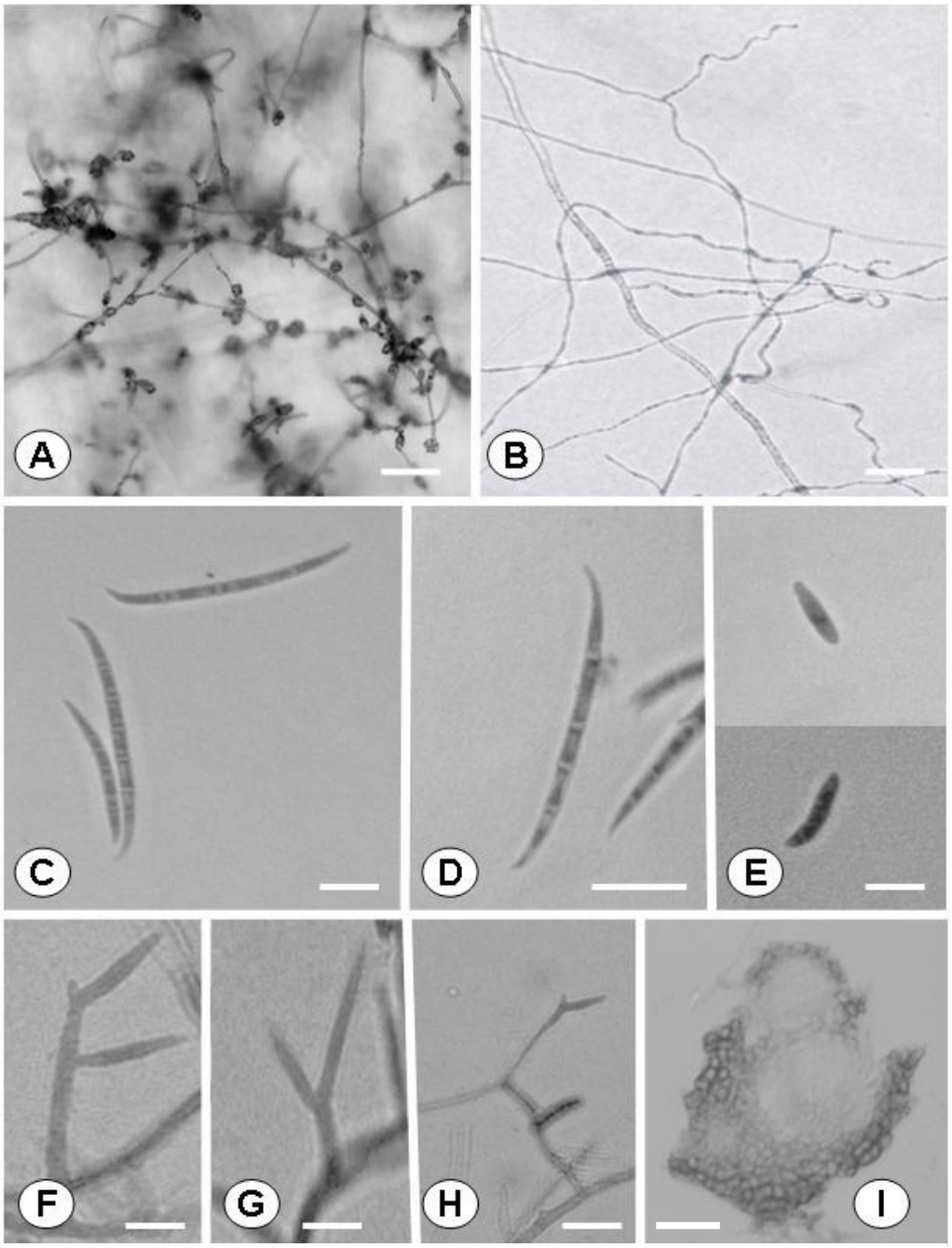

Fig 2: Morphological characteristics of *F. ophiodes*. A) Conidiophores on prostrate aerial mycelium. B). Serpentine-like hyphae produced on CLA. C) Macroconidia on CLA. D) Macroconidia on CLA. E) Microconidia on CLA. F-H) Polyphialides formed on CLA. I) Perithecium formed as result of a cross between *F. ophiodes* and *F. subglutinans*. Bars (A) = 4µm; (B) = 3 µm; (C) = 0.5 µm; (D) = 0.3 µm; (E) = 0.6 µm; (F-H) = 1 µm.; (I) = 0.5 µm.

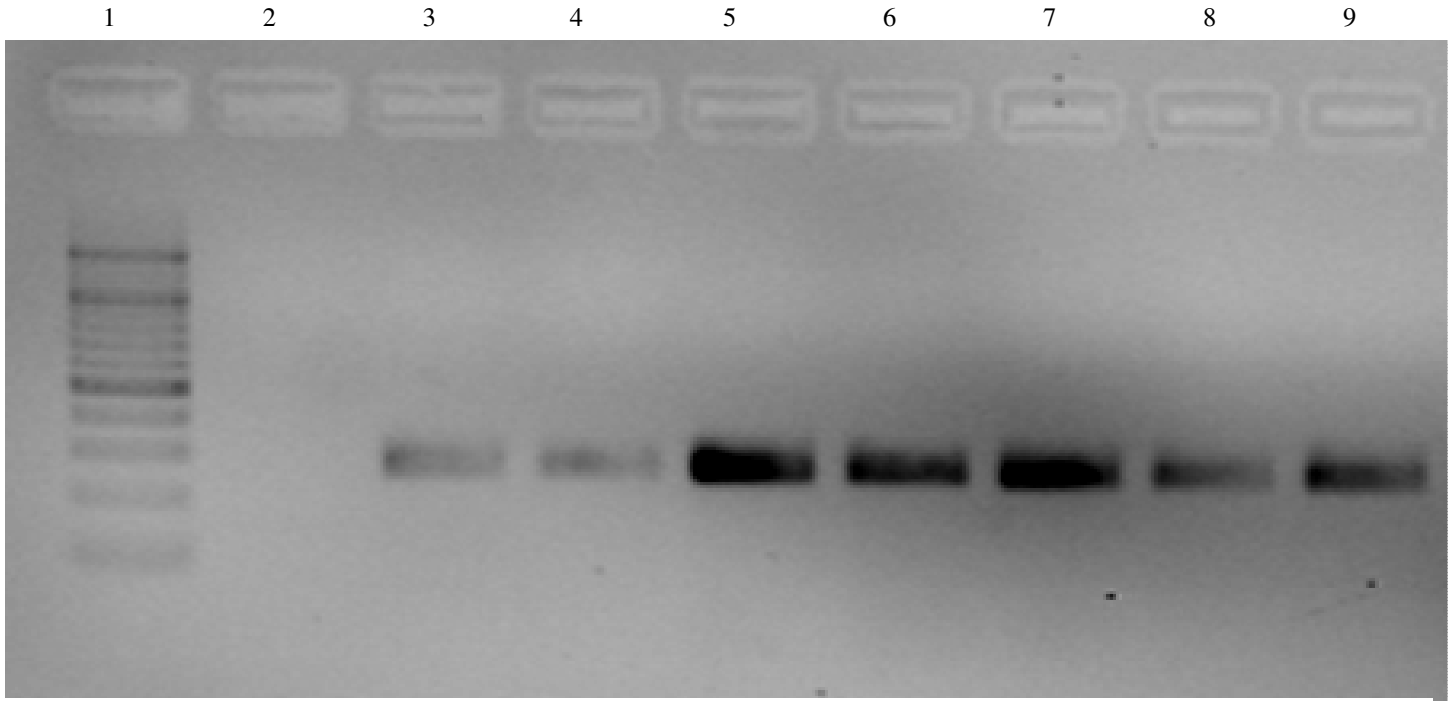

Fig 3: Agarose gel (3%) showing the *MAT-1* idiomorph amplicons from the *F. ophiodes* isolates. Lane 1 represents a 100 bp marker. Lane 2 is the negative control. Lanes 3-9 represent FCC 1092, 1093, 2996, 2972, 2974, 2977, 2979.

Chapter 4

Characterisation of the pitch canker fungus, *Fusarium circinatum*, from Chile

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Characterisation of the pitch canker fungus, *Fusarium circinatum*, from Chile

ABSTRACT

Fusarium circinatum is the causal agent of the pine disease commonly referred to as pitch canker. During 2001, a *Fusarium* sp. was isolated from dying *P. radiata* clonal hedges in various forestry nurseries in Chile and was subsequently identified as *F. circinatum*. The aim of this study was to provide a detailed characterisation of Chilean isolates of the fungus. Morphological characters included microconidia carried on false heads and produced on polyphialides. Sterile coils and conidiophores on erect aerial mycelium were evident on synthetic low nutrient agar. Furthermore, perithecia exuding viable ascospores were produced when isolates were crossed in all possible combinations with the mating tester strains representing the H mating population of *F. circinatum*. PCR-RFLP analysis of the histone H3 gene region, routinely used to distinguish between members of the *Gibberella fujikuroi* complex, further confirmed the identification of the isolates as *F. circinatum*. DNA sequence data obtained for the same gene region placed the isolates within a well-characterised *G. circinata* clade. Results of these studies provide unequivocal evidence that the pitch canker pathogen is well established on pines in Chilean nurseries.

Keywords: Fungal phylogeny, Mating studies, Pathogenicity.

Introduction

The *Fusarium subglutinans sensu lato* complex accommodates a number of serious plant pathogens, including those of mango, pineapple and pine. These species are characterised by the typical *F. subglutinans* (Wollenw. and Reinking) Nelson, Toussoun and Marasas morphology that includes microconidia in false heads, absence of chlamydospores and microconidia that are formed on mono- and polyphialides. Amongst these fungi, *F. circinatum* Nirenberg and O'Donnell (*F. subglutinans* f. sp. *pini* J.C. Correll, T.R. Gordon, McCain, J.W. Foz, C.S. Koehler, D.L. Wood and M.E. Schultz) is characterised by the presence of sterile coils and the formation of conidiophores on erect aerial mycelium (Nirenberg and O'Donnell, 1998).

Fusarium circinatum causes a disease known as pitch canker that was first discovered in the south-eastern United States (Hepting and Roth, 1946; Gordon *et al*., 2001). Since that time, the disease has spread to many parts of the world and has been reported from Japan, Mexico, Spain, Italy and South Africa (Britz *et al*., 2001; Carlucci *et al*., 2007; Landeras *et al*., 2005; Muramoto *et al*., 1988; Viljoen *et al*., 1994). *Fusarium circinatum* causes serious disease both in nurseries and on mature trees. Symptoms of infection in nurseries include damping–off and wilting of seedlings. On mature trees, pitch canker is characterised by branch die-back, stem cankers, copious pitch formation and mortality (Dwinell *et al*., 1977; 1981; McCain *et al*., 1987). In South Africa, the fungus is found in nurseries (Wingfield *et al*., 1998; 2002a) and the manifestation of pitch canker as it is known on mature trees was only recently observed (Coutinho *et al*., 2007).

Fusarium spp. residing in the *F. subglutinans* complex are morphologically similar and identification typically requires a number of techniques. *Fusarium circinatum* can be distinguished from other *Fusarium* species in the complex using reproductive compatibility, isozyme profiles and comparisons of DNA sequence data of various genes (Britz *et al*., 1999; Huss *et al*., 1996; Klittich *et al*., 1997; Leslie 1991; O'Donnell *et al*., 1998; 2000; Xu *et al*., 1995). Isozyme profiles were used to distinguish mating populations of *G. fujikuroi* species complex A to G and placed *F. circinatum* in the B mating population (Huss *et al*., 1996). However, sexual crossing of isolates showed that *F. circinatum* represents a distinct mating population, known as the H mating population in the Section *Liseola* (Britz *et al*., 1999). Sequence data obtained for the histone H3 gene, β-tubulin, mtSSU, and 28S rDNA provided unequivocal evidence that this species is distinct from the other species in the *F. subglutinans sensu lato* complex (O'Donnell *et al*., 1998; Steenkamp *et al*., 1999).

In 2001, symptoms similar to those associated with *F. circinatum* infection in South African pine nurseries were observed on *Pinus radiata* clonal hedges in Chile (Wingfield *et al*., 2002b). These symptoms included tip die-back, root and collar disease. The fungus responsible for the disease was identified as *F. circinatum* in a preliminary report. The aim of the present study is to

provide a more comprehensive and unequivocal characterisation of the fungus from Chile.

Materials and Methods

Collection of isolates

One hundred symptomatic *P. radiata* seedlings were sampled from four different pine nurseries in Chile. Primary isolations from the diseased material were done by placing a small piece (3 mm) of pitch-soaked wood on carnation leaf agar (CLA) (Nelson *et al*., 1983) and *Fusarium* selective medium (20 g Agar, 15 g Peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_20$, 1 g PCNB, 20 mL Streptomycin sulfate in 1 L water) (Nash and Snyder, 1964), respectively. Cultures were incubated at 25 ºC under cool-white fluorescent illumination. The plates were inspected regularly for fungal growth and all the colonies with typical *Fusarium* morphology were transferred to half-strength potato dextrose agar (PDA) (Merck, Germany). Single conidial isolates were stored at –70 ºC in 15 % glycerol. Six isolates were randomly selected for further characterization.

Morphology

Characterisation of the morphology of the six selected isolates (FCC 2915, 2916, 2917, 2918, 2919, 2920) was done on 10 d old cultures grown on synthetic low nutrient agar (SNA) (Nirenberg and O'Donnell, 1998) and CLA. Morphological characteristics observed were compared with those characteristic of *F. circinatum* (Britz *et al*., 2001; Nirenberg and O'Donnell, 1998) as well as those observed in the ex-type culture of this species (MRC 7541, NRRL 25331).

Sexual compatibility

An isolate of the female tester strain of the H mating population of *F. circinatum* (MRC 6213, *MAT-1*) was grown on carrot agar (CA) (Leslie, 1991), while the male tester strain (MRC 7488, *MAT-2*) was grown on complete medium (CM) (Correll *et al*., 1987). These isolates were incubated at 25 ºC under cool-white and dark-fluorescent light (12 hour cycles) for 7 d. Conidial suspensions of the six Chilean strains serving as males were spread over the surface of the CA plates with a glass rod. Reciprocal crosses, where the male parent was used as the female, were also made. All the crosses were repeated at least once. The crosses were examined daily for the formation of perithecia and the ascospores exuding from perithecia were collected. Ascospores from one perithecium per cross were plated on water agar (WA) and incubated for 24 h at 25 ºC, after which the percentage viability was determined by counting the number of germinated ascospores.

PCR amplification of the *MAT-1* and *MAT-2* loci, as described by Steenkamp *et al*. (2000), was used to confirm the mating types of the six isolates. The MAT idiomorphs were amplified with the primer sets GF mat1a (5'-GTTCATCAAAGGGCAAGCG-3'), GFmat1b (5'-TAAGCGCCTCTTAACGCCTTC-3'), GFmat2c (5'-AGCGTCATTATTCGATCAAG-3') and

GFmat2d (5'-CTACGTTGAGAGCTGTACAG-3') (Steenkamp *et al*., 2000). The following PCR reaction mixture was used: 1 x Roche *Taq* Reaction buffer with $MgCl_2$, 200 µM of each dNTP, 0.1 µM of each primer, 25 ng template DNA and 0.5 U Roche *Taq* polymerase (Roche Pharmaceuticals, Germany). The PCR reaction conditions were an initial denaturation at 92 °C for 1 min, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 63 °C for 30 s and elongation at 72 °C for 30 s. A final elongation step was done at 72 °C for 5 min. The products were resolved on a 1 % agarose gel, containing ethidium bromide (0.2 µg/mL) and visualised under UV light.

DNA sequence comparisons

The six isolates were selected after the morphological evaluation, for PCR-RFLP analysis. These isolates were grown in complete medium broth (CMB) (Correll *et al*., 1987) at 25 ºC in the dark for 7 d. DNA was extracted using a modification of the method of Raeder and Broda (1985). Mycelium was placed in Eppendorf tubes and ground with ca. 10 µg sterile, chemically-treated sand in 500 µL of DNA extraction buffer [DEB: 200 mM Tris-HCl pH 8, 150 mM NaCl, 25 mM EDTA, 0.59 % SDS]. An additional 500 µL of phenol and 300 µL chloroform was added, mixed and centrifuged for 30 min at 10 000 rpm. The phenol/chloroform step was repeated until the interface was clean. The supernatant was transferred to a new tube and double the volume of 100 % ethanol was added and mixed. The DNA was allowed to precipitate at 4 °C overnight and then pelleted by centrifugation for 30 min at 11 000 rpm. Pellets were washed with 300 µL 70 % ethanol, dried and resuspended in 50 µL sterile distilled water and 3 µL RNAse (2.5 µM).

The histone H3 gene was amplified using the primer set H3-1a (5'-ACTAAGCAGACCGCCCGCAGG-3') and H3-1b (5'-GCGGGCGAGCTGGATGTCCTT-3') (Glass and Donaldson, 1995). The PCR reaction mixture included 1 x Roche *Taq* Reaction buffer with $MgCl_2$, 200 µM of each dNTP, 5 µM of each primer, 25 ng template DNA and 1U Roche *Taq* polymerase (Roche Pharmaceuticals, Germany). The PCR reaction conditions were an initial denaturation at 92 °C for 1 min, followed by 30 cycles of denaturation at 92 °C for 1 min, annealing at 63 °C for 1 min and elongation at 72 °C for 1 min. A final elongation step was done at 72 °C for 5 min.

Histone H3 PCR products obtained for the six isolates were digested with restriction enzymes CfoI and DdeI (Roche Pharmaceuticals, Germany). Consecutive enzymatic digestion was performed by addition of 5 U CfoI to 15 µL of the unpurified PCR product followed by incubation at 37 °C for 3 h. Subsequently the sodium chloride concentration was adjusted to 100 mM and 5 U of DdeI was added to the reaction mixture. This was followed by further incubation at 37 °C for 5 h.

The digested amplicons were resolved on a 2 % agarose gel, containing ethidium bromide (0.2 µg/mL) and visualised under UV light.

DNA sequences of the histone amplicon were determined using an ABI 377 automated sequencer. The ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK), was used to sequence the DNA fragments. Both strands were sequenced using primers H3-1a and H3-1b. DNA sequences were manually aligned by inserting gaps, which were treated as missing data in the analysis. Analysis was done using the heuristic search option of PAUP* version 4b10 (Phylogenetic Analysis Using Parsimony* and Other Methods version 4.30b2a, Swofford, 2002). The branch-swapping and tree bisection-reconnection algorithm (TBR) was used to find the most parsimonious tree. Bootstrap analysis was performed to determine the confidence levels (1 000 replicates) of the branch nodes. Sequence data for closely related species, including *F. guttiforme* (MRC 6783, AF150833; MRC 6782, AF150834; MRC 6785, AF150835; MRC 6784, AF150836), *F. subglutinans* (MRC 1077, AF150837; MRC 1084, AF150838; MRC 756, AF150839; MRC 837, AF150840; MRC 714, AF150841; MRC 620, AF150842; MRC 115, AF150843; MRC 6512, AF150844; MRC 6483, AF150845), *F. circinatum* (MRC 7488, AF238478; MRC 7439, AF150852; MRC 7438, AF150851), *F. mangiferae* (MRC 7559, AF236779; MRC 3477, AF150868; MRC 7034, AF150864) and *G. fujikuroi* (MRC 6155, AF150858) were taken from previous studies and included in the analysis.

Pathogenicity

An initial trial using two of the six Chilean isolates was performed under confined laboratory conditions. Each of the two isolates was inoculated onto the stems of 20 *P. radiata* seedlings. The results of this preliminary trial showed that both isolates were pathogenic and that they did not differ significantly in their levels of pathogenicity. Inoculations were then performed on three-year-old *P. radiata* and two-year-old *P. patula* seedlings using a single isolate (FCC 2916). These pathogenicity trials were conducted in a phytotron with daytime temperatures of approximately 25 ºC and night temperatures of 20 ºC.

Twenty trees of each species were inoculated after removing the bark with a cork borer. Mycelial plugs (6 mm in diameter) taken from actively growing fungal margins from PDA plates were placed in the wound and sealed with laboratory film (Parafilm, USA). Ten trees of each species were inoculated as controls with sterile plugs of PDA.

Trees were maintained in the phytotron for 4 weeks. Lesion lengths were measured and the statistical analysis was done by means of the t-Test: Two-Sample Assuming Unequal Variances (Samuels and Witmer, 1995). Re-isolations were made from all inoculation points on all plants.

Results

Isolations

All colonies that displayed typical *Fusarium* morphology on *Fusarium* selective medium were isolated and placed on PDA. Six of these isolates were randomly selected for characterization. These selected isolates were designated the numbers FCC 2915 (PREM 58666), 2916 (PREM 58667), 2917 (PREM 58668), 2918 (PREM 58669), 2919 (PREM 58670) and 2920 (PREM 58671).

Morphological comparison

Morphological characteristics typical for *F. circinatum* were readily observed in the cultures growing on SNA medium. These characteristics included erect aerial mycerlium (Fig 1a) fusiform, 3-septate macroconidia (Fig 1d) and microconidia (Fig 1e) accumulating in false heads (Fig 1b). Sterile coils (Fig 1c) and polyphialides were also observed in cultures grown on CLA.

Sexual compatibility

After four to six weeks, perithecia (Fig 1e) were observed in crosses between the individual tester strains of the H mating population and the six selected Chilean isolates. Five of the Chilean isolates were hermaphrodites and one isolate (FCC 2915) was female sterile. Only FCC 2915 crossed with MRC 6213 (*MAT-2*) (PREM 58673), while the remaining five isolates crossed with MRC 7488 (*MAT-1*) (PREM 58675, 58672, 58677, 58674, 58676). Ascospores from perithecia resulting from all six test isolates displayed an average of 98 % viability.

The presence of both mating idiomorphs from the Chile isolates was confirmed by amplification of the MAT loci. FCC 2915 was the only isolate in which the *MAT-1* idiomorph was amplified, while the *MAT-2* idiomorph was amplified in isolates FCC 2916, 2917, 2918, 2919 and FCC 2920 (Fig 2). These results confirmed the outcome of the sexual compatibility tests.

PCR-RFLP

The histone H3 gene was amplified in five of the Chilean isolates and a product of 515bp was generated. The PCR-RFLP profiles obtained for the isolates were similar to those previously shown by Steenkamp *et al*. (1999) for *F. circinatum*, and the profile obtained for the tester strain defining the H mating population (MRC 6213) (Fig 3).

Phylogenetic analysis

Three of the Chilean isolates for which the PCR-RFLP profiles were obtained, were included in the DNA sequence analysis (DQ 364695, DQ 364696, DQ 364697). Alignment of the DNA sequences for the histone H3 gene resulted in a total of 58 parsimony-informative characters and one most parsimonious tree was generated (Fig 4).

In the phylogenetic tree, the three Chilean isolates grouped together with the *F. circinatum* isolates in the same clade. The *F. guttiforme* and *F. mangiferae* isolates formed separate clades. The

F. subglutinans sensu lato isolates from maize grouped closely to, but separate from the *G. circinata* isolates.

Pathogenicity

Lesions formed on both the *P. patula* and *P. radiata* seedlings after inoculation with isolate FCC 2916. Only very small lesions were observed on the control plants of either species**.** The lesions on the *P. patula* seedlings were smaller than those on *P. radiata*. The lesions on *P. patula* seedlings were an average of 29.9 mm (SD = 4.49 mm) (SE = 1.004 mm, n = 20) in size. The *P. radiata* seedlings had lesions of an average 109.15 mm (SD = 36.13 mm) (SE = 8.079 mm, n = 20) in size. The lesions on the control plants were an average of 0.5 mm (SD = 0.6 mm) (SE = 0.190 mm, n = 10). *Fusarium circinatum* was re-isolated from all inoculated plants and not from the plants inoculated as controls.

Discussion

Results of this study have provided unequivocal evidence confirming the presence of the pitch canker fungus on dying *P. radiata* clonal hedge plants in Chile. A sub-set of isolates from diseased plants showed morphological characteristics typical of *F. circinatum*. The Chilean isolates were also sexually compatible with mating tester strains of *F. circinatum*. Five of the Chilean isolates considered in this study were shown to have the *MAT-2* mating type idiomorph while one of the isolates contained the *MAT-1* mating type idiomorph. Isolates from Chile also had PCR-RFLP profiles and histone H3 gene DNA sequences typical of *F. circinatum*. Furthermore, the isolates were highly pathogenic to *P. radiata* and *P. patula* seedlings, as compared to control plants that showed no symptoms.

Several morphological characters were typical of the Chilean isolates. The presence of conidiophores on the erect aerial mycelium, polyphialides and sterile coils were the most important distinguishing characteristics. The erect conidiophores distinguish the Chilean species from *F. subglutinans sensu stricto*, which is characterised by the formation of prostrate conidiophores on the aerial mycelium (Britz *et al.*, 2001; Nirenberg and O'Donnell, 1998). The Chilean isolates also formed sterile coils, which is consistent with the morphology of *F. circinatum. Fusarium pseudocircinatum* also produces sterile coils but carries its conidia in false chains (Nirenberg and O'Donnell, 1998). No false chains were observed in *F. circinatum* isolates from Chile.

Sexual compatibility studies established that the Chilean isolates belong to the H mating population of the *G. fujikuroi* species complex. Fertile progeny were obtained when the isolates from Chile were crossed with the opposite mating tester strains. It was further established that both mating types of the fungus are present in Chile. This was confirmed through amplification of the

different mating idiomorphs. The presence of both MAT idiomorphs indicates that sexual reproduction could be taking place where the fungus occurs in Chile. This has potential consequences for tree breeding programmes and the management of this pathogen. Further studies are underway to estimate the relative occurrence of the two mating types in a larger collection of Chilean isolates.

The PCR-RFLP profiles obtained for the five isolates were similar to those obtained for the tester isolate of the H mating population used in this study and illustrated by Steenkamp *et al.* (1999). Using this technique, it was also possible to distinguish the Chilean isolates from *F. oxysporum*, which is a fungus commonly encountered in pine nurseries. The PCR-RFLP technique can be used easily for routine confirmation of the presence of *F. circinatum*.

DNA sequence data for the histone H3 gene provided additional confirmation of the identity of the isolates obtained from diseased pine plants in Chile. In the phylogenetic analysis, the Chilean isolates grouped firmly with other *F. circinatum* isolates. Furthermore, preliminary pathogenicity tests showed clearly that *F. circinatum* isolates from Chile are highly pathogenic on *P. radiata* and *P. patula* trees. The lesions on *P. radiata* were also longer than those on *P. patula*, which is consistent with results of previous studies comparing susceptibility in these two *Pinus* species (Viljoen *et al.*, 1995).

In this study, a wide range of techniques were used to confirm the identity of *F. circinatum* isolates from Chilean nurseries. The first DNA sequence data for these isolates are presented and it was shown for the first time that both mating types of the fungus are present in Chile. Furthermore, pathogenicity tests have shown that the fungus in Chile is highly pathogenic, which is consistent with the fungus elsewhere in the world. There is little question that it is the causal agent of the death of *P. radiata* clonal hedge plants and seedlings in Chile and it is a pathogen that has the potential to seriously damage forestry in that country.

The presence of *F. circinatum* in Chilean forestry nurseries represents a situation similar to that in South Africa, where the fungus was restricted to the nursery environment for numerous years after its first discovery (Wingfield *et al.*, 1998). This fungus is one of the most serious pathogens of *P. radiata* and it has the potential to seriously damage plantations of this tree. Whether pitch canker will emerge on mature trees in Chile as it has recently done in South Africa (Coutinho *et al.*, 2007) is open to substantial speculation (Wingfield *et al.*, 1998; 2002a). However, *F. circinatum*'s presence in Chile, where plantation forestry based on *P. radiata* represents a very substantial industry, is a matter for concern. Extensive research on the disease is warranted and programmes to ensure the availability of planting stock resistant to *F. circinatum* should be encouraged.

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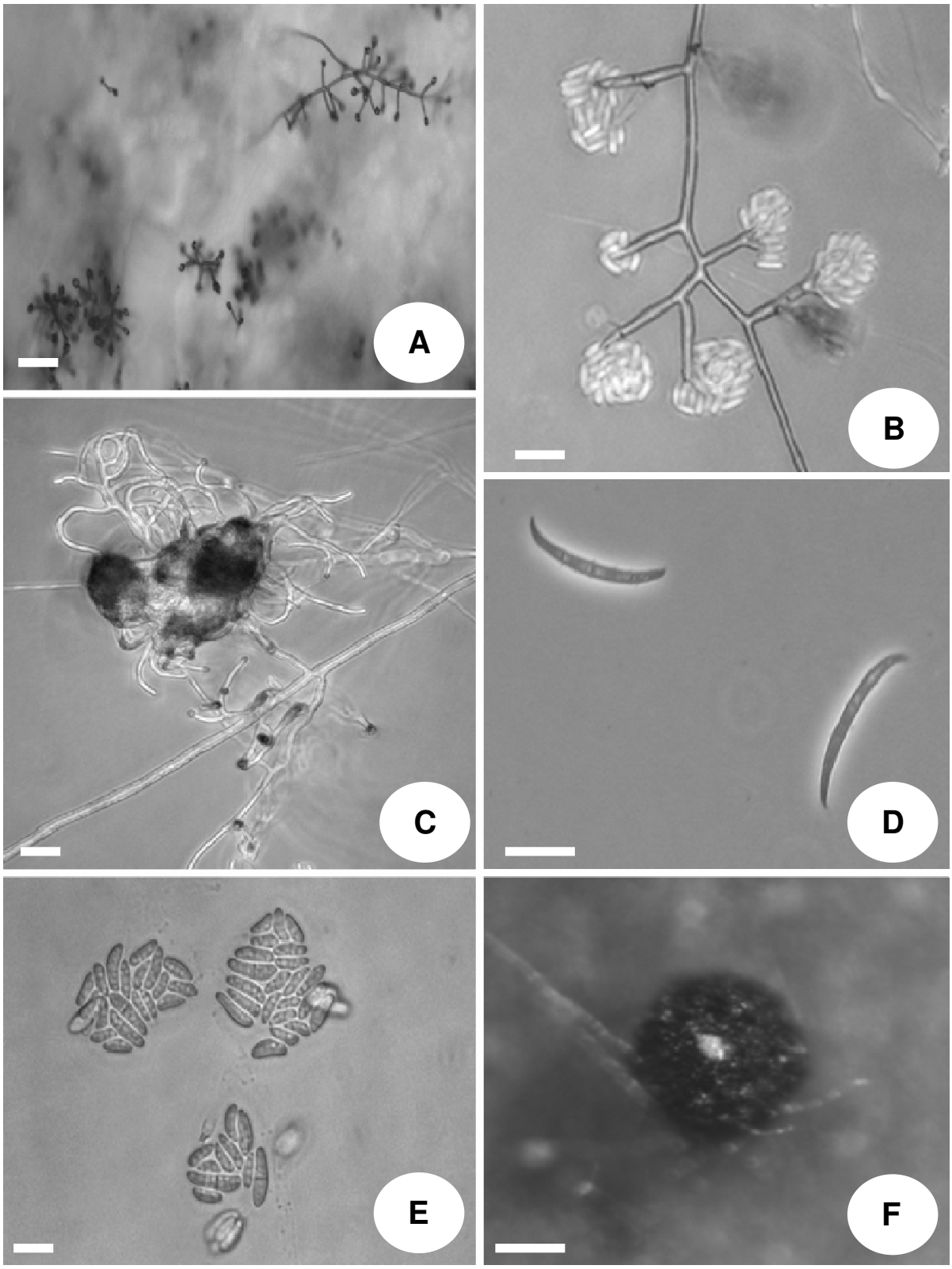

Fig 1: Morphological characteristics of Chilean isolates used in this study. A) Erect conidiophores B) Microconidia accumulated in false heads C) Sterile coils D) Macroconidia E) Microconidia. F) Perithechium. Bars (A-E) = 20 µm; (F) = 10 µm.

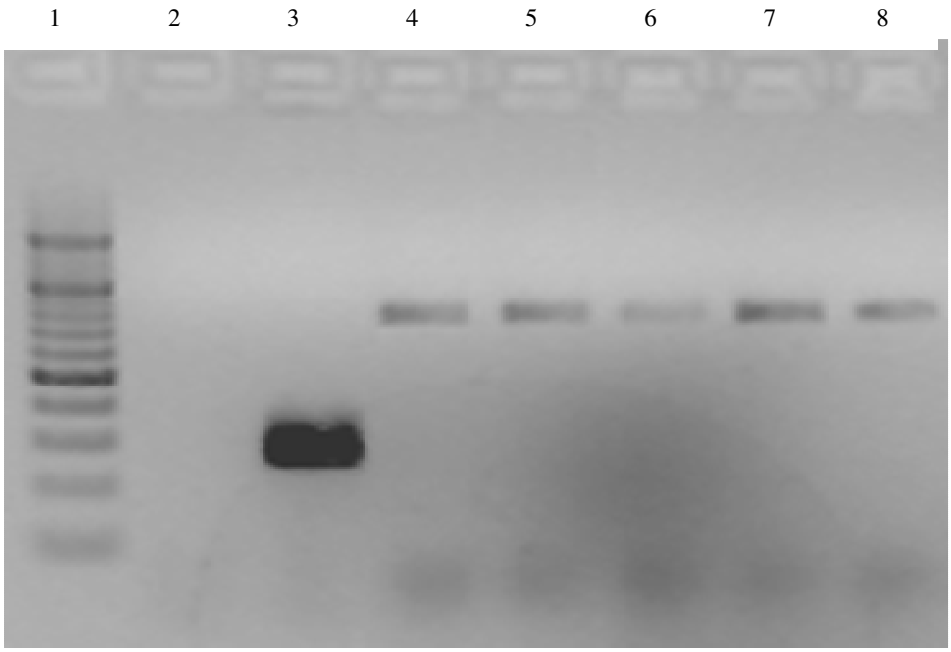

Fig 2: Agarose gel (3 %) showing the *MAT-2* idiomorph amplicons from the Chilean isolates. Lanes 1 represents a 100 bp marker. Lane 2 is the negative control. Lanes 3-8 represent FCC 2915-2920. Five of the six selected isolates are *MAT-2* idiomorphs and one isolate, FCC 2915, is *MAT-1*.

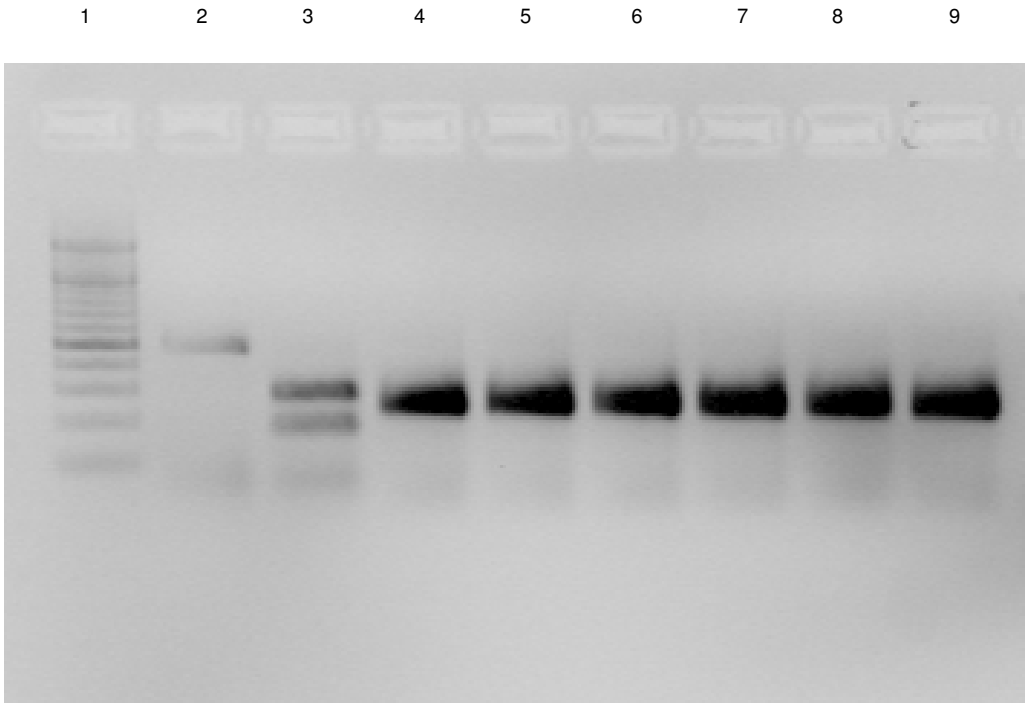

Fig 3: Agarose gel (2 %) PCR-RFLP profiles obtained for the Chilean isolates after digestion of the PCR amplicon with the restriction enzymes CfoI and DdeI. The 515 bp product contains two restriction sites, yielding three fragments of 250, 232, and 33 bp. The 33 bp fragment is not visible, and the other two fragments are similar in size as to appear as a single band in an agarose gel. The Chilean profiles are similar to that of the H mating population ex-type strain (MRC 6213). However, they are different from the profile obtained for *F. oxysporum* that yielded two fragments of 200 and 300 bp.
Lane 1= 100 bp marker; Lane 2= undigested PCR product; Lane 3= *F. oxysporum* digest profile; Lanes 4-9 represent FCC 2915-2920.

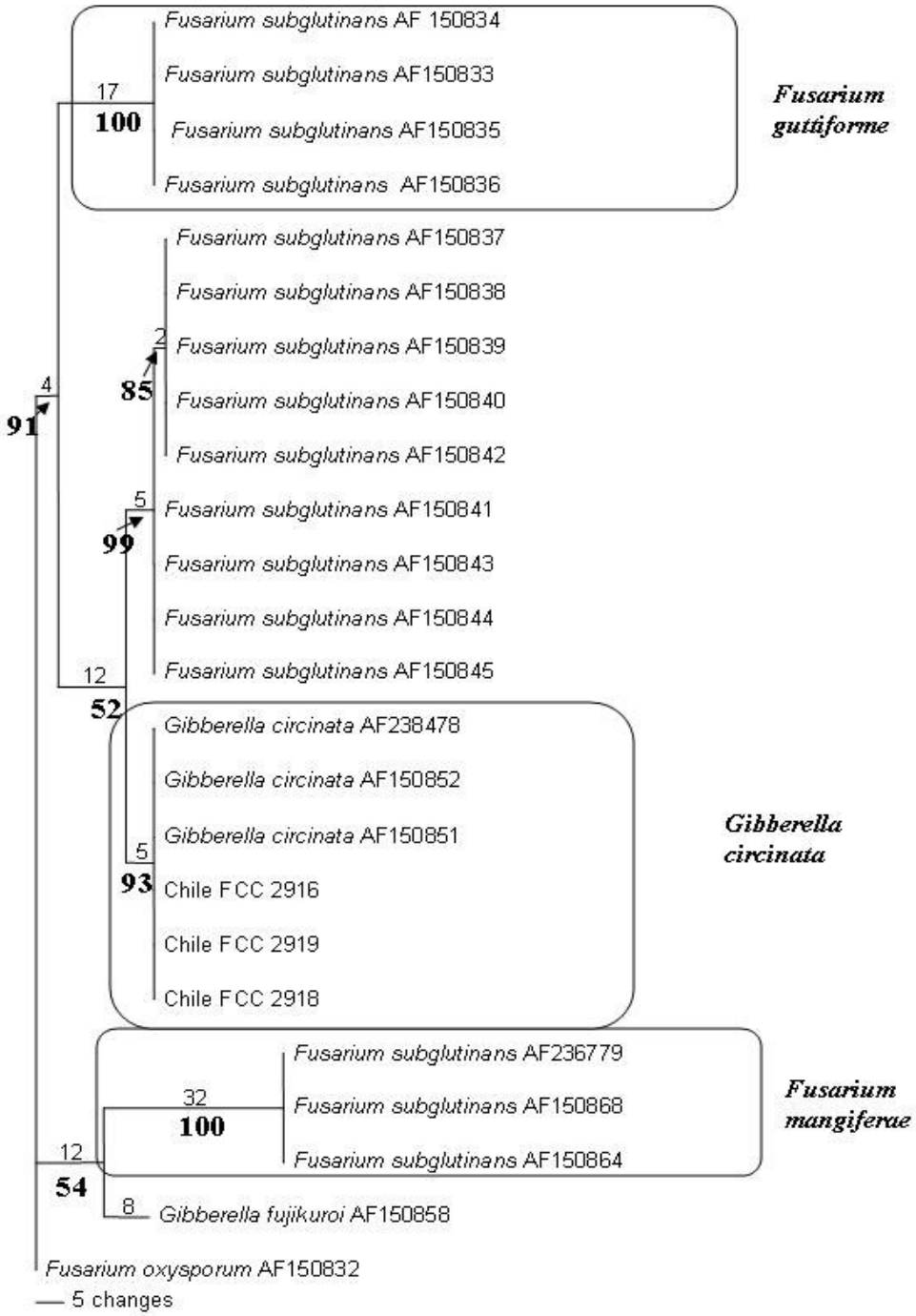

Fig 4 - Phylogenetic tree produced by PAUP heuristic option for the histone H3 gene with *F. oxysporum* as the outgroup taxon. Bootstrap values above 50 % (percentages of 1 000 bootstrap replicates) are indicated in bold below the branches of the tree, while branch lengths are indicated above the branches. Parsimony-informative characters = 58; Tree length = 115 steps; CI = 0.878; RI =0.939; g1 = -1.884610.

Chapter 5

Fusarium ananatum sp. nov. in the *Gibberella fujikuroi* species complex from pineapples with fruit rot in South Africa

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Fusarium ananatum sp. nov. in the *Gibberella fujikuroi* species complex from pineapples with fruit rot in South Africa

ABSTRACT

Pineapple (*Ananas comosus*) is native to South America and widely planted as a fruit crop in the tropics and sub-tropics. This plant is susceptible to a number of fungal diseases of which the most severe is fusariosis. The disease is caused by *Fusarium guttiforme* and occurs only in South and Central America. The occurrence of a similar disease on pineapples in South Africa has prompted a re-evaluation of the *Fusarium* spp. associated with pineapple fruit rot. Phylogenetic relationships of isolates from pineapples collected in Brazil and South Africa were assessed based on sequence data for the translation elongation factor-1α, histone H3 and β-tubulin gene regions. Analyses showed that the South African isolates represent a species distinct from Brazilian isolates. The South African isolates are characterised by a concentration of aerial mycelium at the centres of the colonies, different to the Brazilian isolates that have an even distribution of aerial mycelium. Both phylogenetic and morphological data show that the disease on pineapple in South Africa is caused by a new *Fusarium* species described here as *F. ananatum* sp. nov.

Keywords: DNA sequence comparisons, Fungi, Fusariosis, Phylogenetic analyses, Pineapple diseases

Introduction

Pineapple [*Ananas comosus* (L.) Merr.] is native to South America and is the fourth most important crop planted in the tropics (Ploetz, 2001). The first report of a serious disease caused by a *Fusarium* species and known as fusariosis of pineapple was from Argentina in 1954 (Rohrbach, 1994). Ten years later, this disease was reported from Brazil, where pineapple is an economically important crop. Pineapple fusariosis was so serious at that time that Brazil lost its position as the world's leading pineapple producer (Ventura *et al*., 1993a; Ploetz, 2001).

All pineapple plant parts can be infected with the causal agent of fusariosis, but symptoms are most conspicuous on the fruit. Symptoms include bent or dead stem apices, shortened stems, disrupted phyllotaxy, general stunting and chlorosis (Pires de Matos, 1995; Ploetz, 2001). Fruit symptoms include an initial discolouration of the infected area exemplified by light to dark brown fruitlet septa, which may extend into the fruitlet core. The diseased areas become sunken and profuse pink fungal sporulation and gum exudation is evident (Pires de Matos, 1995; Rohrbach, 1994). Infection can occur from the start of flowering through all stages of fruit development. However, it is most severe when plants are infected during the early stages of flowering (Ploetz, 2001).

Previously, the fungus causing fusariosis in pineapple was identified as *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas (basionym: *F. moniliforme* J. Sheld. var. *subglutinans* Wollenw. & Reinking). The species is specific to pineapple, and causes fruitlet core rot and fusariosis (Ploetz, 2001). Based on its host specificity, Ventura *et al*. (1993b) proposed a new *forma specialis*, *F. subglutinans* f. sp. *ananas* for the fungus. Nirenberg and O'Donnell (1998) described this fungus as *F. guttiforme* Nirenberg and O'Donnell.

Fusarium guttiforme forms part of the *G. fujikuroi* complex. Species in this complex can be distinguished from each other based on morphological characteristics and DNA sequence comparisons (Nirenberg and O'Donnell, 1998; O'Donnell *et al*., 1998a). The morphological characteristics used to distinguish between species in this group include conidiophore arrangement on the aerial mycelium, the number of conidiogenous openings on the polyphialides, the presence or absence of sterile coils or curved hyphae, and macroconidial morphology (Aoki *et al*., 2001; Nirenberg and O'Donnell, 1998). DNA based characterisation most commonly considers sequence data for the β-tubulin, translation elongation factor-1α and mitochondrial small subunit (mtSSU) genes (Aoki *et al*., 2001; O'Donnell *et al*., 1998a).

Pineapple fusariosis has been reported only from South America (Ploetz, 2001). In Cuba, a disease called 'fusariose' caused by *F. subglutinans* has been reported (Borras *et al*., 2001; Hildalgo *et al*., 1999), but it is not clear whether it is the same disease as fusariosis in South America. A

similar disease known as fruitlet core rot occurs in Hawaii (Rohrbach and Pfeiffer, 1976), and the associated '*F. moniliforme*' strains have been incorrectly referred to as *F. guttiforme* (Nirenberg and O'Donnell 1998; Rohrbach and Schmitt, 2003). Recently, a *Fusarium* species was isolated from pineapples with a fruit rot disease in South Africa and there was concern that this might reflect a first report of fusariosis in the country. Alternatively, the disease might have been the same as the one known as 'black spot' and associated with *Penicillium funiculosum* and '*F. moniliforme*' in South Africa (Edmonstone-Sammons, 1958). The aim of this study was to compare South African isolates from diseased pineapples with those from Brazil, including the ex-type isolate of *F. guttiforme*. Isolates were compared based on DNA sequences for the translation elongation factor-1α (TEF-1α), the partial β-tubulin gene (BT), and the histone H3 (His) gene as well as on their morphology.

Materials and methods

Symptoms and isolations

Diseased pineapple fruit were obtained from Hluhluwe, Kwazulu Natal. The symptoms on these fruit included an initial off-colour appearance, followed by the tissue becoming sunken with characteristic V-shaped lesions appearing on the outside of the fruit. This extends to the internal tissue and ultimately manifests itself as a core rot.

Primary isolations from the diseased material were done by placing small pieces (3 mm) of diseased tissue onto *Fusarium* selective medium (Nelson *et al*., 1983) in Petri dishes. These Petri dishes were incubated at 25 ºC under cool-white fluorescent illumination. The plates were checked routinely and all the colonies with typical *Fusarium* morphology were transferred to half-strength potato dextrose agar (PDA) (Merck, Germany). Single conidial cultures were stored using a cryopreservation method at –70 ºC in 15 % glycerol aqueous solution.

Fungal isolates

All isolates used in this study (Table 1) are maintained in the *Fusarium* culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and in the Medical Research Council (MRC) Culture Collection, Tygerberg, Cape Town, South Africa. Representative isolates have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Morphological comparisons

Isolates were grown on synthetic low nutrient agar (SNA) (Nirenberg, 1976) and carnation leaf agar (CLA) (Nelson *et al*., 1983) for 7 d at 25 ºC, under near ultraviolet light. Fungal structures produced on these media were mounted on microscope slides in lactophenol with cotton blue and

used in the morphological comparison of the South African and Brazilian groups of isolates. Colony colour was assigned using the colour charts of Rayner (1970) for isolates grown on PDA 7 d at 25 ºC, under near ultraviolet light. Growth rates were determined by placing a single macroconidium (Nelson *et al*., 1983) on a PDA plate and calculating the average growth in millimeter over 5 days. The standard errors were determined for data representing each isolate at all temperatures. The presented measurements are the average of 50 measurements per morphological structure.

DNA extraction and amplification

Isolates were grown in complete medium (CM) (Correll *et al*., 1987) at 25 ºC for 7 d. DNA was isolated using a modified version of the technique described by Raeder and Broda (1985). Mycelium was placed in Eppendorf tubes and ground with ca. 10 µg sterile, chemically treated sand in 500 µL of DNA extraction buffer [DEB: 200 mM Tris-HCl pH 8, 150 mM NaCl, 25 mM EDTA, 0.59 % SDS]. Thereafter, 500 µL of phenol and 300 µL chloroform were added, mixed and centrifuged for 30 min at 10 000 rpm. The phenol/chloroform step was repeated until the interface was clean. The supernatant was transferred to a new tube and double the volume of 100 % ethanol was added and mixed. The DNA was allowed to precipitate at 4 °C overnight and then pelleted by centrifugation for 30 min at 11 000 rpm. Pellets were washed with 300 µL 70 % ethanol, dried and resuspended in 50 µL sterile distilled water and 3 µL RNAse (2.5 µM) (Roche Pharmaceuticals, Switzerland).

Extracted DNA was used as template in PCR reactions to amplify regions of the histone H3, β-tubulin (BT) and translation elongation factor-1α (TEF-1α) genes. The histone H3 gene region was amplified using primer sets H3-1a (5'-ACTAAGCAGACCGCCCGCAG-3') and H3-1b (5'-GCGGGCGAGCTGGATGTCCTT-3') (Glass and Donaldson, 1995). Part of the TEF-1α was amplified using the primer set EF1 (5'-CGAATCTTTGAACGCACATTG-3') and EF2 (5'-CCGTGTTTCAAGACGGG-3') (O'Donnell *et al*., 1998b). The BT gene region was amplified using the primer set T1 (5'-AACATGCGTGAGATTGTAAGT-3') and T222 (5'-GACCGGGGAAACGGAGACAGG-3') (O'Donnell *et al*., 2000). The PCR reaction consisted of 1x Roche *Taq* Reaction buffer with $MgCl_2$, dNTPs (250 µM each), primers (0.2 µM each), template DNA (25 ng) and Roche *Taq* polymerase (0.5 U) (Roche Pharmaceuticals, Switzerland). The PCR reaction conditions, for the amplification of the histone H3 gene, were an initial denaturation at 92 ºC for 1 min. This was followed by 30 cycles of denaturing at 92 ºC for 1 min, annealing at 63 ºC for 1 min and elongation at 72 ºC for 1 min, followed by a final elongation step at 72 ºC for 5 min. The TEF-1α and BT gene regions were amplified by initial denaturation at 94 ºC for 2 min. This was followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 54 ºC for 1 min and

elongation at 72 ºC for 1 min, with a final elongation step at 72 ºC for 5 min. The resulting PCR amplicons were purified using a QIAquick PCR Purification kit (QIAGEN, Germany).

DNA sequencing and phylogenetic analyses

DNA sequences were determined from PCR amplicons using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, (Applied Biosystems, UK) using the primers H3-1a, H3-1b, EF1, EF2, T1, and T222. Sequences generated in this study have been deposited in GenBank (Table 1).

DNA sequences were manually aligned by inserting gaps. Gaps were treated as new state in the subsequent analysis. Phylogenetic analysis was based on parsimony using PAUP* version 4b10 (Phylogenetic Analysis Using Parsimony* and Other Methods version 4b10; Swofford 2002). Heuristic searches were done with random addition of sequences (100 replicates), tree bisection-reconnection (TBR) branch swapping, and MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets (*g*1) was assessed by evaluating tree length distributions over 100 randomly generated trees (Hillis and Huelsenbeck, 1992). The consistency (CI) and retention (RI) indices were determined for all data sets. Phylogenetic trees were rooted with *F. oxysporum* as monophyletic sister outgroup to the rest of the taxa. Bootstrap analyses were performed to determine branching point confidence intervals (1 000 replicates) for the most parsimonious trees generated for the TEF-1α, BT and histone H3 data sets. The combinability of the TEF-1α and BT datasets was tested using the partition homogeneity test in PAUP* version 4b10 (Farris *et al*., 1994).

Bayesian analyses utilised the Metropolis-coupled Markov Chain Monte Carlo search algorithm as implemented in the program MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). All Bayesian analyses consisted of 1 000 000 generations running one cold and three hot chains, with Bayesian inference posterior probabilities (biPP) calculated after a burnin was determined. BInt analyses utilised the GTR+I substitution model with separate parameters for each gene (partition) and an eight-category gamma model. The data sets were deposited in Treebase 10424.

Mating studies and MAT genes

In order to determine the mating types of the seven isolates from pineapple in South Africa, the *MAT-1* and *MAT-2* loci were amplified using PCR, as described by Steenkamp *et al*. (2000). The MAT idiomorphs were amplified with the primer sets GFmat1a (5'-GTTCATCAAAGGGCAAGCG-3'), GFmat1b (5'-TAAGCGCCTCTTAACGCCTTC-3'), GFmat2c (5'-AGCGTCATTATTCGATCAAG-3') and GFmat2d (5'-CTACGTTGAGAGCTGTACAG-3') (Steenkamp *et al*., 2000). The following PCR reaction mixture was used: 1 x Roche *Taq* Reaction buffer with $MgCl_2$, dNTP (250 µM) each, primers (0.1

µM of each), template DNA (25 ng) and 0.5 U Roche *Taq* polymerase (Roche Pharmaceuticals, Germany). The PCR reaction conditions were an initial denaturation at 92 °C for 1 min, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 63 °C for 30 s and elongation at 72 °C for 30 s. A final elongation step was done at 72 °C for 5 min. The products were resolved on a 1 % agarose gel, containing ethidium bromide (0.2 µg/mL) and visualised under UV light. The presence of the MAT idiomorphs in the pineapple isolates was confirmed by amplification of the MAT loci.

Results

Symptoms and isolations

The external fruit symptoms on South African pineapples (Fig 1) were similar to but less severe than those reported for *F. guttiforme* infections on pineapples (Ploetz, 2001; Rohrbach, 1994). Isolations from these symptoms yielded cultures that resembled *F. guttiforme*. Seven of these isolates were used in this study.

Morphological comparisons

Based on the results of morphological comparisons of the South African isolates with the ex-type of *F. guttiforme* as well as other Brazilian isolates of the species showed that they could be distinguished based on a number of morphological characteristics. The Brazilian isolates were characterised by dark purple colonies on PDA but with dispersed aerial mycelium and where the conidiophores produced in these hyphae are prostrate. The South African isolates were characterised by saffron-coloured colonies on PDA (Fig 6). In older cultures, a dark purple colour appeared at the colony centres in the South African isolates. Furthermore, the aerial mycelium of these isolates was concentrated at the middle of the SNA plates.

Taxonomy

The *Fusarium* isolates from pineapple in South Africa could easily be separated from those of *F. guttiforme* from pineapple in Brazil based on DNA sequence comparisons for three different gene regions. These isolates were also phylogenetically distinct from those of all other species in the *G. fujikuroi* species complex. The isolates from pineapple in South Africa could also be distinguished from *F. guttiforme* based on distinct morphological characteristics such as the nature of the conidiophores and the colony colour. This species is, therefore, described as a new taxon as follows.

Fusarium ananatum A. Jacobs, Marasas & Van Wyk, **sp. nov.** Fig 7

MycoBank no.: MB 511686

*Etym.***:** The specific epithet refers to host, *Ananas comosus*, from which the species was isolated.

Margo coloniae integra. Coloniae crescunt circiter 3.2 mm/d in 25 ºC in PDA. Mycelium aerium in medio coloniae congregatum; in PDA incrementum conformatione orbium concentricorum signatum. Mycelium aerium in PDA croceum, infra bubalinum. Sclerotia, sporodochia chlamydosporaeque desunt. Hyphae in SNA (2-)3-5(-6) µm latae. Conidia in pseudocapitulis aggregata. Conidiophorae in mycelio aerio erectae e substrato orientes, ramosae vel non, centrum coloniae versus plurimae, (15-)22-40(-47) x (1-)2-3 µm. Conidiophorae sympodialiter ramosae, mono- et polyphialidibus instructae. Phialides mycelii aerii cylindricae, monophialidicae, 10-46(-56) x (1-)2-3 µm, ramis 3-17(-22) x (1-)2-3 µm, et polyphialidicae aperturis conidiogenis 2-4, 25-90(-115) x 1-2(-4) µm. Microconidia plerumque non septata raro uniseptata, (6-)8-16(-17) x 1-2 µm. Macroconidia in mycelio aerio facta subfalcata vel fere recta, superficiebus dorsalibus ventralibusque subparallelis, parietibus tenuibus, (14-)16-31(-32) x 3-6 µm.

Colony margin entire. Colonies on PDA with average growth rate of 3.2 mm/d at 25ºC. Aerial mycelium concentrated at the middle of the colony and growth on PDA characterised by the formation of concentric circles. Aerial mycelium on PDA saffron (7f) in colour, reverse of colony buff (19''f) in colour. Sclerotia, sporodochia and chlamydospores absent. Hyphae on SNA (2-)3-5(-6) µm wide. Conidia accumulating in false heads. Conidiophores on aerial mycelium originating erect from substrate, branched or unbranched, numerous towards centre of colony, (15-)22-40(-47) x (1-)2-3 µm. Conidiophores sympodially branched bearing mono- and polyphialides. Phialides of the aerial mycelium, cylindrical**,** monophialidic, 10-46(-56) x (1-)2-3 µm, with branches 3-17(-22) x (1-)2-3 µm, and polyphialidic, with 2-4 condiogenous openings, 25-90(-115) x 1-2(-4) µm. Microconidia mostly 0-septate with 1-septate conidia occurring less abundantly, (6-)8-16(-17) x 1-2 µm. Macroconidia produced only on aerial mycelium and not in sporodochia, slightly sickle-shaped to almost straight, with the dorsal and ventral surfaces almost parallel with thin walls, 3-4 septate, (14-)16-31(-32) x 3-6 µm.

Materials examined: **South Africa,** *Hluhluwe*, Kwazulu Natal, *Ananas comosus*, November 2001, P.S. Van Wyk, PREM 58713 (*holotype*; dried down culture of MRC 8165, FCC 2986, CMW 18685, CBS 118516). PREM 58714 (*paratype*; dried down culture of MRC 8166, FCC 2988, CMW 18686, CBS 118517); PREM 58715 (*paratype* dried down culture of MRC 8167, FCC 2990, CMW 18687, CBS 118518); PREM 58716 (*paratype* dried down culture of MRC 8168, FCC 2991, CMW 18688, CBS 118519).

Sequence comparisons and phylogenetic analyses on the TEF-1α, BT and histone H3 genes

The amplication of the TEF-1α, BT and histone H3 gene regions resulted in products of 640, 520 and 540bp, respectively.

Parsimony analysis of combined and separate data sets for the TEF-1α and BT gene regions were done to determine the phylogenetic placement of *F. guttiforme* isolates from Brazil in relation to the South Africa pineapple isolates in the *G. fujikuroi* species complex. The partition homogeneity test showed sufficient probability to accept the null hypothesis (P<0.5) to combine the datasets for the TEF-1α and BT gene regions. Alignment of combined data by inserting gaps resulted in a total of 1 059 characters used in the comparison of the different species in the combined data sets. All parsimony-uninformative and constant characters were excluded, resulting in 127 parsimony-informative characters. Heuristic searches on the data set generated one most parsimonious tree (Fig 2). Alignment of separate TEF-1α data set by inserting gaps resulted in a total of 528 characters used in the comparison of the different species. All parsimony-uninformative and constant characters were excluded, resulting in 53 parsimony-informative characters. Heuristic searches on the data set generated six most parsimonious trees of which one is presented (Fig 3). The separate BT data set consisted of 484 parsimony-uninformative and 46 parsimony-informative characters. Heuristic searches on the data set generated two most parsimonious trees of which one is presented (Fig 4).

In the combined data set, the Brazilian isolates from pineapple, grouped together with the ex-type isolate of *F. guttiforme* to form a distinct clade (Fig 2). The South African isolates from diseased pineapple formed a separate clade, with 99 % bootstrap support. The grouping of all *Fusarium* isolates associated with pineapples from both Brazil and South Africa was supported by a bootstrap value of 96 %. Some differences were found in the sequences of the South African pineapple isolates, most likely indicating genetic variation in this population.

In the separate TEF-1α data set (Fig 3), the Brazilian isolates from pineapple grouped together with the ex-type isolate of *F. guttiforme* to form a distinct clade, although not supported by a high bootstrap value. The South African isolates from diseased pineapple formed a separate clade, with 87 % bootstrap support. Some differences were found in the sequences of the South African pineapple isolates, most likely indicating genetic variation in this population. In the separate BT data set the Brazilian isolates from pineapple, grouped together with the ex-type isolate of *F. guttiforme* to form a distinct clade (Fig 4). The South African isolates from diseased pineapple formed a separate clade with 95 % bootstrap support.

Parsimony analysis on the histone H3 gene region was done separately to those of the other gene regions because data from representatives of the different mating populations of *G. fujikuroi*

were included to test the grouping of four South African and Brazilian isolates from pineapple. All parsimony-uninformative and constant characters were excluded, resulting in 85 parsimony-informative characters. Heuristic searches on the dataset generated one most parsimonious tree after reweighing of the characters based on the CI value (Fig 5). The topography of the tree was similar to that obtained for combined sequences of the TEF-1α and BT gene regions. The Brazilian isolates grouped together with the ex-type culture of *F. guttiforme* (MRC 7539) and they represent a discrete taxon. The South African isolates from diseased pineapple resided in a distinct clade supported by a bootstrap value of 88 %.

Mating studies and MAT genes

No perithecia were observed in any of the crosses between or amongst the pineapple isolates from Brazil and South Africa. There were also no positive results for crosses between the South African pineapple isolates and the mating tester strains for eight of the biological species in the *G. fujikuroi* species complex. The control crosses for all the tester strains produced fertile progeny showing that conditions for these tests were appropriate to stimulate sexual recombination. All of the seven South African isolates had the *MAT-2* idiomorph and were thus of the same mating type.

Discussion

Results of this study have shown that *Fusarium* isolates from diseased pineapple in South Africa represent a new species described here as *F. ananatum*. This species forms part of the *G. fujikuroi* species complex and can be distinguished from all other species in this group based on DNA sequence comparisons and morphology. *F. ananatum* isolates were supported as monophyletic with high bootstrap support for all three gene phylogenies studied. By contrast, similar results were not observed for *F. guttiforme*.

Although DNA sequence comparisons form the most important basis for distinguishing *F. ananatum* from other related species, it is possible to distinguish the South African fungus based on morphology. The defining morphological characteristics include the nature of the conidiophores on the aerial mycelium and the distribution of the aerial mycelium on the surface of isolates. The original description of the *F. guttiforme* suggested that this species is characterised by erect and prostrate conidiophores (Nirenberg and O'Donnell, 1998). After the re-evaluation of the ex-type material (MRC 7539) and the Brazilian isolates, we conclude that *F. guttiforme* has distinctly prostrate conidiophores. This is in contrast to *F. ananatum* that is characterised by erect conidiophores on the aerial mycelium. The colony morphology of these two groups on SNA is also distinct. *F. ananatum* is characterised by concentrations of aerial mycelium at the centres of plates. In contrast, *F. guttiforme* has aerial mycelium distributed evenly over the surface of the colonies.

Concentric circles of mycelial growth were also observed in *F. ananatum* cultures while these are absent in *F. guttiforme*. Furthermore, the distinct saffron colony colour of *F. ananatum* on PDA distinguishes it from *F. guttiforme*, which has dark purple coloured colonies on PDA.

Despite numerous attempts to cross isolates, no perithecia were obtained between the tester strains for eight mating populations in the *G. fujikuroi* species complex (Desjardins, 2003) and the South African isolates from pineapple. Crosses amongst isolates of *F. ananatum* also failed to produce perithecia. This clearly resulted from the fact that the seven isolates tested all had the same mating type idiomorph. It is thus possible that crosses amongst a larger number of isolates, assuming some have the *MAT-1* idiomorph, might produce a teleomorph.

Symptoms associated with both *F. guttiforme* and *F. ananatum* on fruit are similar but they are less severe in the case of the latter fungus. For *F. guttiforme*, these symptoms include a light to dark brown discolouration of the fruitlet septa that can extend to the fruitlet core (Rohrbach, 1994). The infected fruit area initially appears off-colour, then becomes sunken with profuse pink sporulation and exudation of gum (Rohrbach, 1994). Similar core discolouration occurs in fruit infected with *F. ananatum*. However, the infected fruit area is characterised by a V-shaped lesion that initially appears discoloured, and then becomes sunken. No exudation of gum was observed in the case of pineapples infected with *F. ananatum*, which appears to be a symptom typical of fusariosis.

In their re-evaluation of the *G. fujikuroi* species complex, O'Donnell *et al*. (1998a) included an isolate from *A. comosus* in England (NRRL 22945 = CBS 184.29 = IMI 375350 = DAOM 225144) as *F. guttiforme*. In the present study, this isolate grouped in the clade that accommodates the *F. ananatum* isolates (Figs 2-4). It is also the only isolate included in the morphological comparison by Nirenberg and O'Donnell (1998) that originates from the United Kingdom and not a South American country. This unusual grouping is further supported by the fact that South Africa exports pineapples to the United Kingdom and, thus, may have inadvertently sent fruit infected with *F. ananatum* to that country. This emphasises the importance of basing phylogenetic relationships employing ex-type strains and not on other isolates that might not represent the fungus intended for comparison.

At present, nothing is known regarding the pathogenicity of *F. ananatum*. The fungus is closely associated with rot of fruit and it most likely is the cause of this disease. However, pathogenicity tests will be needed to determine its relative importance as a pathogen.

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Table 1: List of the *Fusarium* strains included in the phylogenetic analyses, representing closely related species in the *Gibberella fujikuroi* species complex.

Strain number[a]	Species	Origin	Collector	GenBank accession number			Reference for DNA sequences
				β-tubulin	Elongation factor 1-α	Histone H3	
MRC 8165/FCC 2986/CMW 18685/CBS 118516, ex–type	*F. ananatum*	*Ananas comosus*, South Africa	PS Van Wyk	DQ282174	DQ282167	DQ282181	This study
MRC 8166/FCC 2988/CMW 18686/CBS 118517	*F. ananatum*	*Ananas comosus*, South Africa	PS Van Wyk	DQ282178	DQ282171	DQ282182	This study
MRC 8167/FCC 2990/CMW 18687/CBS 118518	*F. ananatum*	*Ananas comosus*, South Africa	PS Van Wyk	DQ282176	DQ282169	DQ282183	This study
MRC 8168/FCC 2991/CMW 18688/CBS 118519	*F. ananatum*	*Ananas comosus*, South Africa	PS Van Wyk	DQ282175	DQ282168	DQ282180	This study
FCC 4251/CMW 28597	*F. ananatum*	*Ananas comosus*, South Africa	A Jacobs	EU668309	EU668312		This study

FCC 4252/CMW 28598	*F. ananatum*	*Ananas comosus*, South Africa	A Jacobs	EU668310	EU668313		This study
FCC 4253/CMW 28599	*F. ananatum*	*Ananas comosus*, South Africa	A Jacobs	EU668311	EU668314		This study
NRRL 25300, ex-type	*F. begoniae*	*Begonia* hybrid, Germany	Unknown	AY329045	AY329036		O'Donnell *et al.*, 1998a
NRRL 13618, ex-type	*F. bulbicola*	*Nerine bowdenii*, Germany	Unknown	U61546	AF160294		O'Donnell *et al.*, 1998a
NRRL 25331, ex-type	*F. circinatum*	*Pinus radiata*, USA	Unknown	U61547	AF160295	AF150852	O'Donnell *et al.*, 1998a; Steenkamp *et al.*, 1999
MRC 6213/KSU 10850	*F. circinatum (MAT H-2)*[b]	*Pinus* spp., South Africa	A Viljoen			AF150844	Steenkamp *et al.*, 1999
MRC 7488/KSU 10847	*F. circinatum (MAT H-1)*	South Africa	A Viljoen			AF238478	Steenkamp *et al.*, 1999
NRRL 25181, ex-type	*F. concentricum*	*Musa sapientum*, Costa Rica	Unknown	AF333951	AF333935		O'Donnell *et al.*, 1998a
MRC 6570/KSU1993	*F. fujikuroi (MAT C-1)*	*Oryza sativa*, Taiwan	JF Leslie			AF150873	Steenkamp *et al.*, 1999
MRC 6571/KSU 1995	*F. fujikuroi (MAT C-2)*	*Oryza sativa*, Taiwan	JF Leslie			AF150872	Steenkamp *et al.*, 1999

MRC 6782/CMW 30032/CBS 124146	*F. guttiforme*	*Ananas comosus*, Brazil	JA Ventura	DQ282177	DQ282170	AF150834	This study
MRC 6783/CMW 30033/CBS 124145	*F. guttiforme*	*Ananas comosus*, Brazil	JA Ventura	DQ282173	DQ282166	AF150833	This study
MRC 7539, ex-type	*F. guttiforme*	*Ananas comosus*, Brazil	H Nirenberg	DQ282172	DQ282165	DQ282179	This study
NRRL 22945	*F. guttiforme*	*Ananas comosus*, England	Unknown	U34420	AF160297		O'Donnell *et al.*, 1998a
MRC 6784	*F. guttiforme*	*Ananas comosus*, Brazil	JA Ventura			AF150836	Steenkamp *et al.*, 1999
MRC 6785	*F. guttiforme*	*Ananas comosus*, Brazil	JA Ventura			AF150835	Steenkamp *et al.*, 1999
MRC 7559	*F. mangiferae*	*Mangifera indica*, South Africa	Unknown			AF236779	Steenkamp *et al.*, 1999
MRC 3477	*F. mangiferae*	*Mangifera indica*, South Africa	Unknown			AF150868	Steenkamp *et al.*, 1999
NRRL 13488, ex-type	*F. nygamai*	*Sorghum bicolour*, *Australia*	Unknown	U34481	AF160273		O'Donnell *et al.*, 1998a
MRC 7548/KSU 51[11]	*F. nygamai (MAT G-1)*	Lab cross	JF Leslie			AF150854	Steenkamp *et al.*, 1999

MRC 7549/KSU 5112	*F. nygamai (MAT G-2)*	Lab cross	JF Leslie			AF150855	Steenkamp *et al.*, 1999
MRC 6212	*F. oxysporum*	South Africa	A Viljoen			AF150832	Steenkamp *et al.*, 1999
NRRL 26374	*F. oxysporum*	Unknown	Unknown	AF008518	AF008483		O'Donnell *et al.*, 1998a
NRRL 22944	*F. proliferatum*	*Cattleya* hybrid, Germany	Unknown	U34471	AF160280		O'Donnell *et al.*, 1998a
NRRL 31071	*F. proliferatum*	Unknown	Unknown			AF291059	Steenkamp *et al.*, 1999
MRC 6568/KSU 4853	*F. proliferatum (MAT D-1)*	Lab cross	JF Leslie			AF150871	Steenkamp *et al.*, 1999
MRC 6569/KSU 4854	*F. proliferatum (MAT D-2)*	Lab cross	JF Leslie			AF150870	Steenkamp *et al.*, 1999
NRRL 22946, ex-type	*F. pseudocircinatum*	*Solanum* sp., Ghana	Unknown	U34482	AF160271		O'Donnell *et al.*, 1998a
NRRL 13999	*F. sacchari*	Unknown	Unknown	U34469	AF160278		O'Donnell *et al.*, 1998a
MRC 6524/KSU 3852	*F. sacchari (MAT B-1)*	Lab cross	JF Leslie			AF150861	Steenkamp *et al.*, 1999
MRC 6525/KSU 7853	*F. sacchari (MAT B-2)*	Lab cross	JF Leslie			AF150860	Steenkamp *et al.*, 1999
MRC 7873	*F. sterilihyphosum*	*Mangifera indica*, South Africa	Unknown			AF236774	Steenkamp *et al.*, 1999

MRC 7605	*F. sterilihyphosum*	*Mangifera indica*, South Africa	Unknown			AF236773	Steenkamp *et al.*, 1999
MRC 6512/KSU 2192	*F. subglutinans (MAT E-2)*	*Zea mays*, USA	JF Leslie	AF366552	AF160289	AF150844	O'Donnell *et al.*, 1998a; Steenkamp *et al.*, 1999
MRC 6483/KSU 990	*F. subglutinans (MAT E-1)*	*Zea mays*, USA	JF Leslie			AF150845	Steenkamp *et al.*, 1999
MRC 1077	*F. subglutinans*	*Zea mays*, South Africa	FC Wehner			AF150837	Steenkamp *et al.*, 1999
NRRL 22045	*F. thapsinum*	Unknown	Unknown	U34473	AF160270		O'Donnell *et al.*, 1998a
MRC 6536/KSU 4094	*F. thapsinum (MAT F-1)*	Lab cross	JF Leslie			AF150857	Steenkamp *et al.*, 1999
MRC 6537/KSU 4093	*F. thapsinum (MAT F-2)*	Lab cross	JF Leslie			AF150856	Steenkamp *et al.*, 1999
NRRL 22172	*F. verticillioides*	Unknown	Unknown	U34468	AF160262		O'Donnell *et al.*, 1998a
MRC 6155/KSU 149	*F. verticillioides (MAT A-1)*	*Sorghum bicolor*, USA	JF Leslie			AF150858	Steenkamp *et al.*, 1999
MRC 6191/KSU 999	*F. verticillioides (MAT A-2)*	*Zea mays*, USA	JF Leslie			AF150859	Steenkamp *et al.*, 1999

[a]MRC: Culture collection of the Medical Research Council, Tygerberg, Cape Town, South Africa. NRRL: Agricultural Research Service Culture Collection at the National Centre for Agricultural Utilization Research, Peoria, IL, USA. FCC: The *Fusarium* culture collection of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. CMW: The culture collection of Mike Wingfield housed at TPCP, FABI, University of Pretoria, Pretoria, South Africa. CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. [b]Mating type tester strains are indicated in brackets with their associated mating population represented by the letter and the number indicating the mating idiomorph.

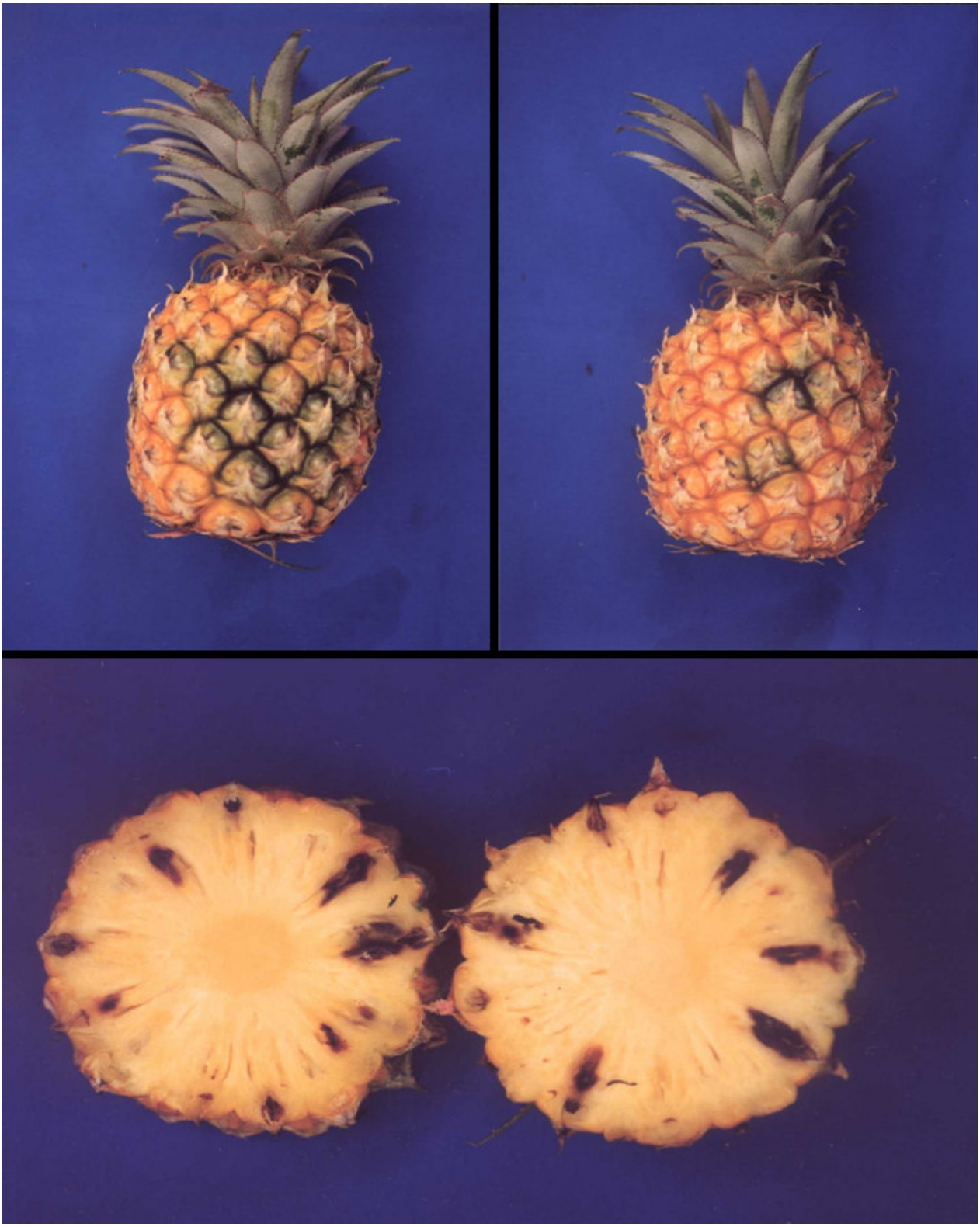

Fig 1: Internal and external lesions formed on susceptible pineapple fruits.

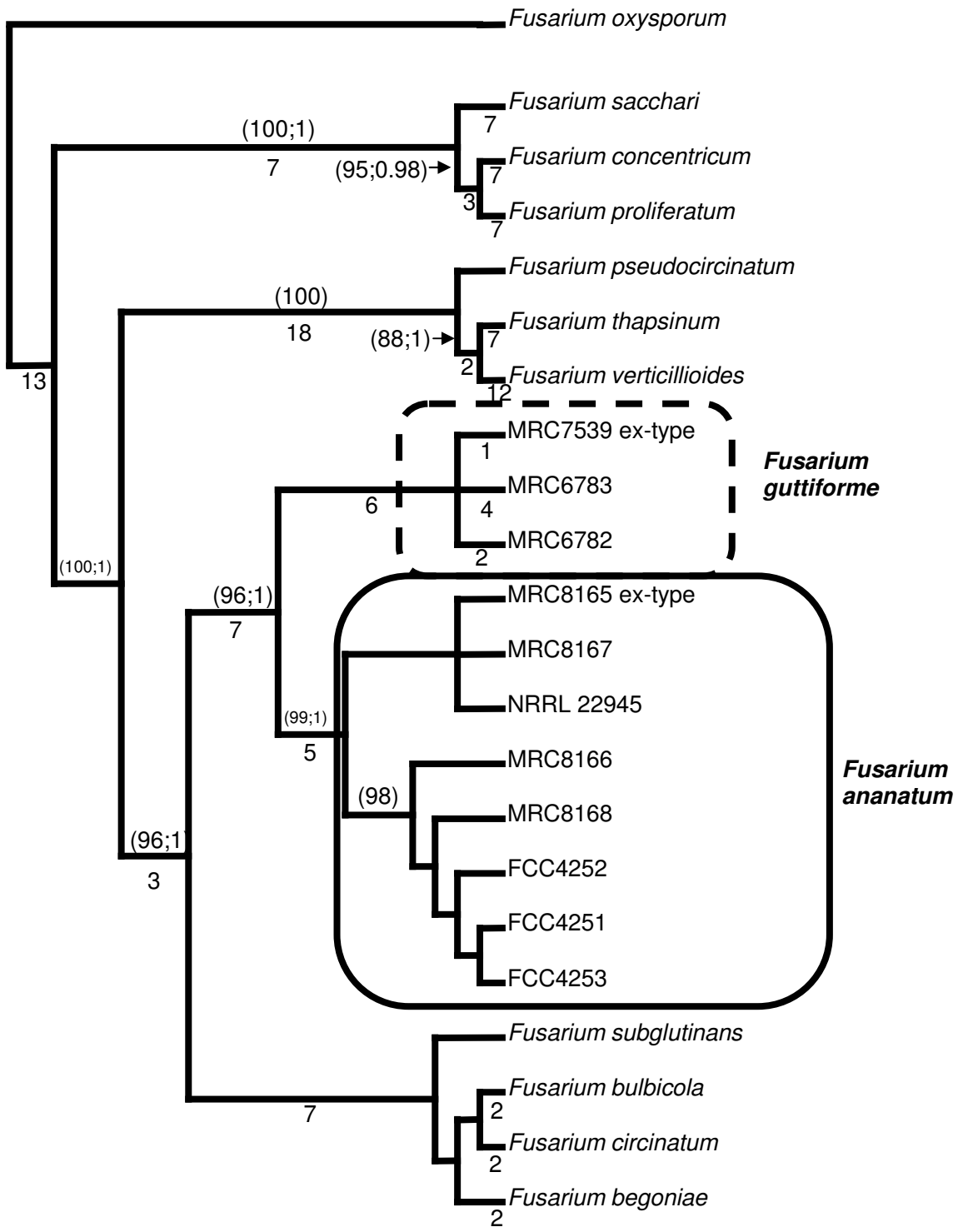

Fig 2: Phylogenetic tree of *F. ananatum* and related species produced using parsimony of the combined data of the translation elongation factor-1α and β-tubulin genes, with *F. oxysporum* as outgroup. Bootstrap values above 50% (percentages of 1000 bootstrap replicates) are indicated in brackets above the branches of the tree. Branch lengths are indicated below the branches. **Parsimony informative characters = 127; CI = 0.8593; RI = 0.9395; g1= -0.860858 TL = 223 steps.**

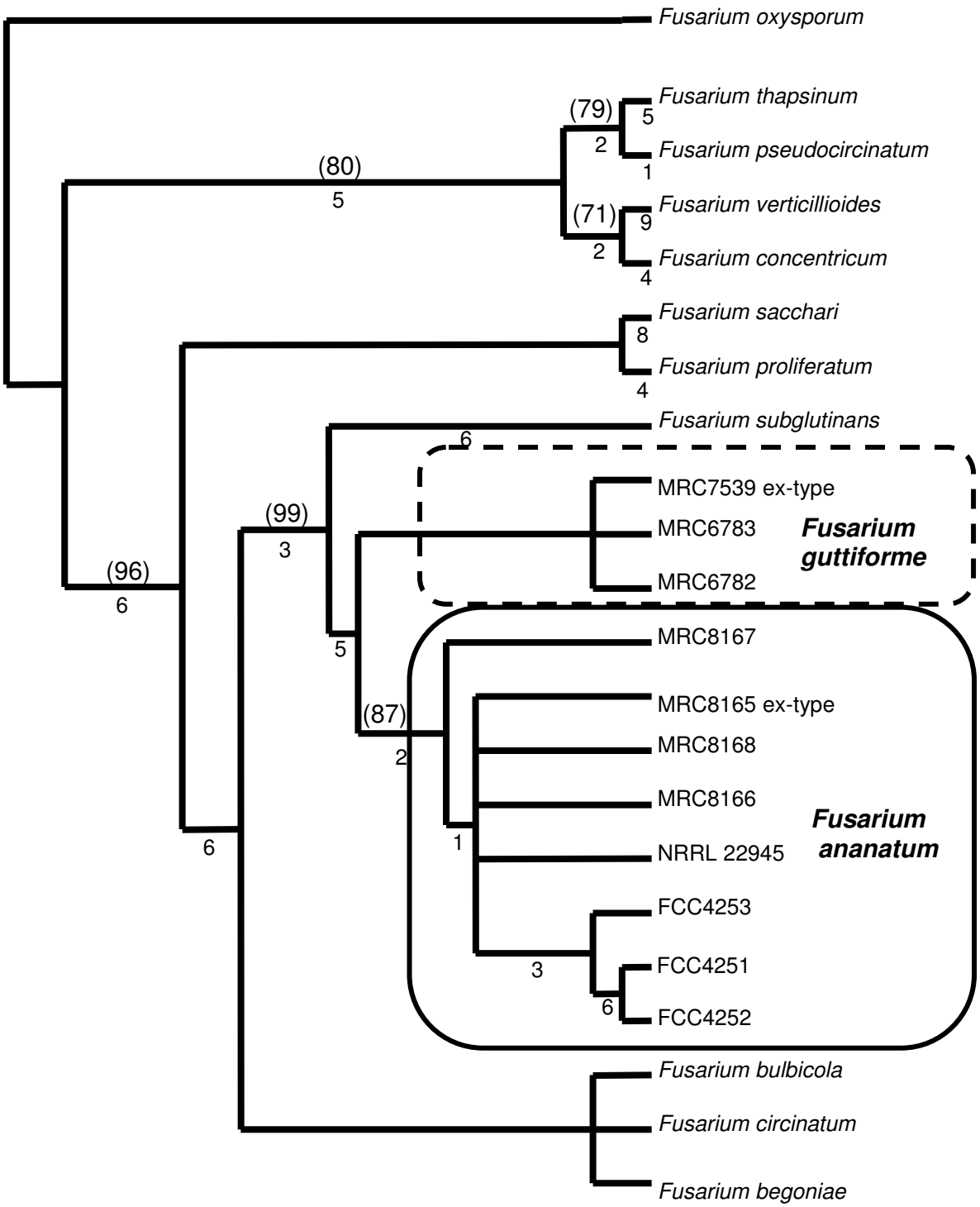

Fig 3: Phylogenetic tree of *F. ananatum* and related species produced using parsimony of the translation elongation factor-1α gene with *F. oxysporum* as outgroup. Bootstrap values above 50% (percentages of 1000 bootstrap replicates) are indicated in brackets above the branches of the tree. Branch lengths are indicated below the branches. **Parsimony informative characters = 53; CI = 0.6737; RI = 0.8510; g1 = -0.476216 TL = 50 steps.**

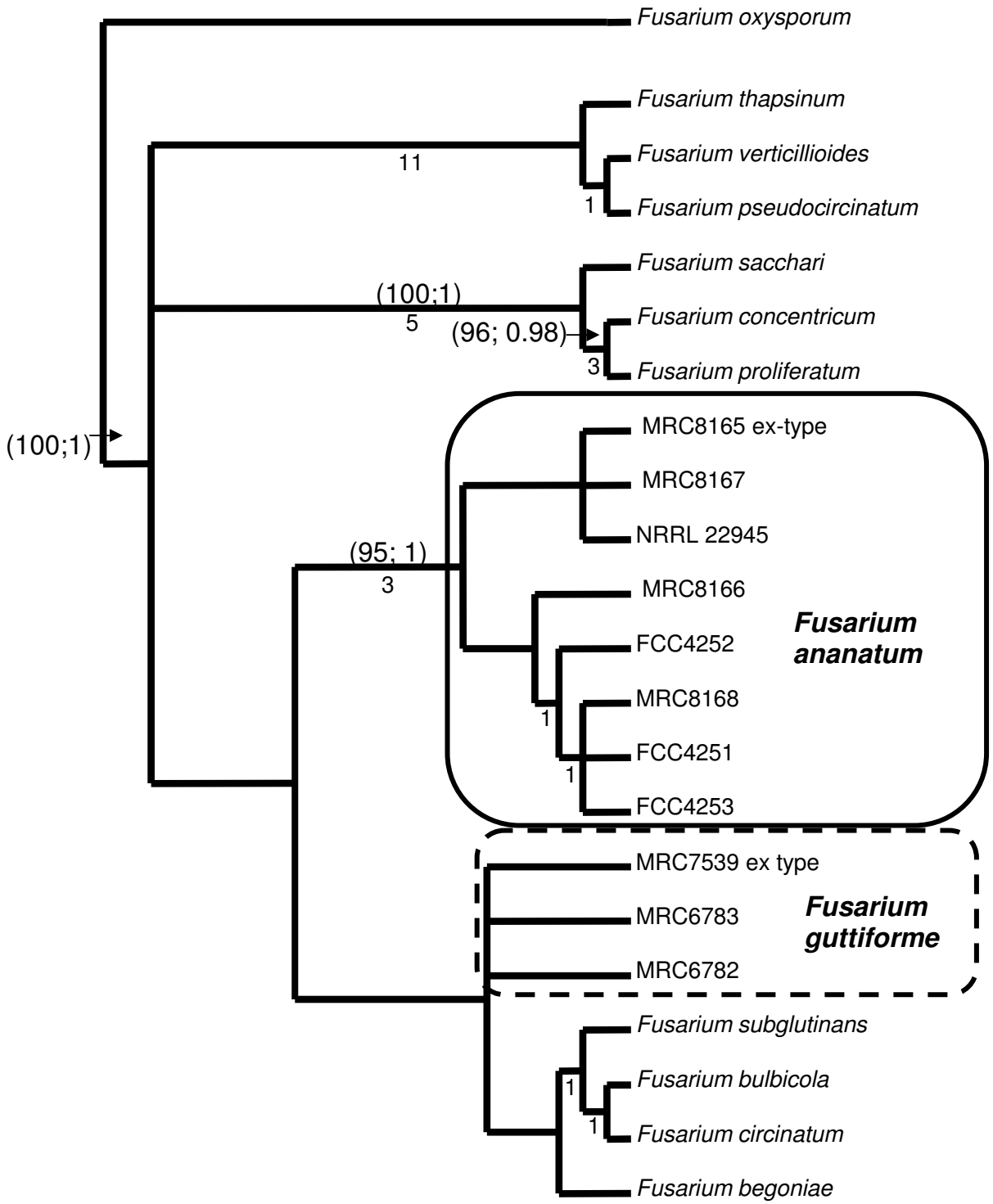

Fig 4: Phylogenetic tree of *F. ananatum* and related species produced using parsimony of the β-tubulin gene with *F. oxysporum* as outgroup. Bootstrap values above 50% (percentages of 1000 bootstrap replicates) are indicated in brackets above the branches of the tree. Branch lengths are indicated below the branches. **Parsimony informative characters = 46; CI = 0.8593; RI = 0.9395; g1 = -0.860858 TL = 43 steps.**

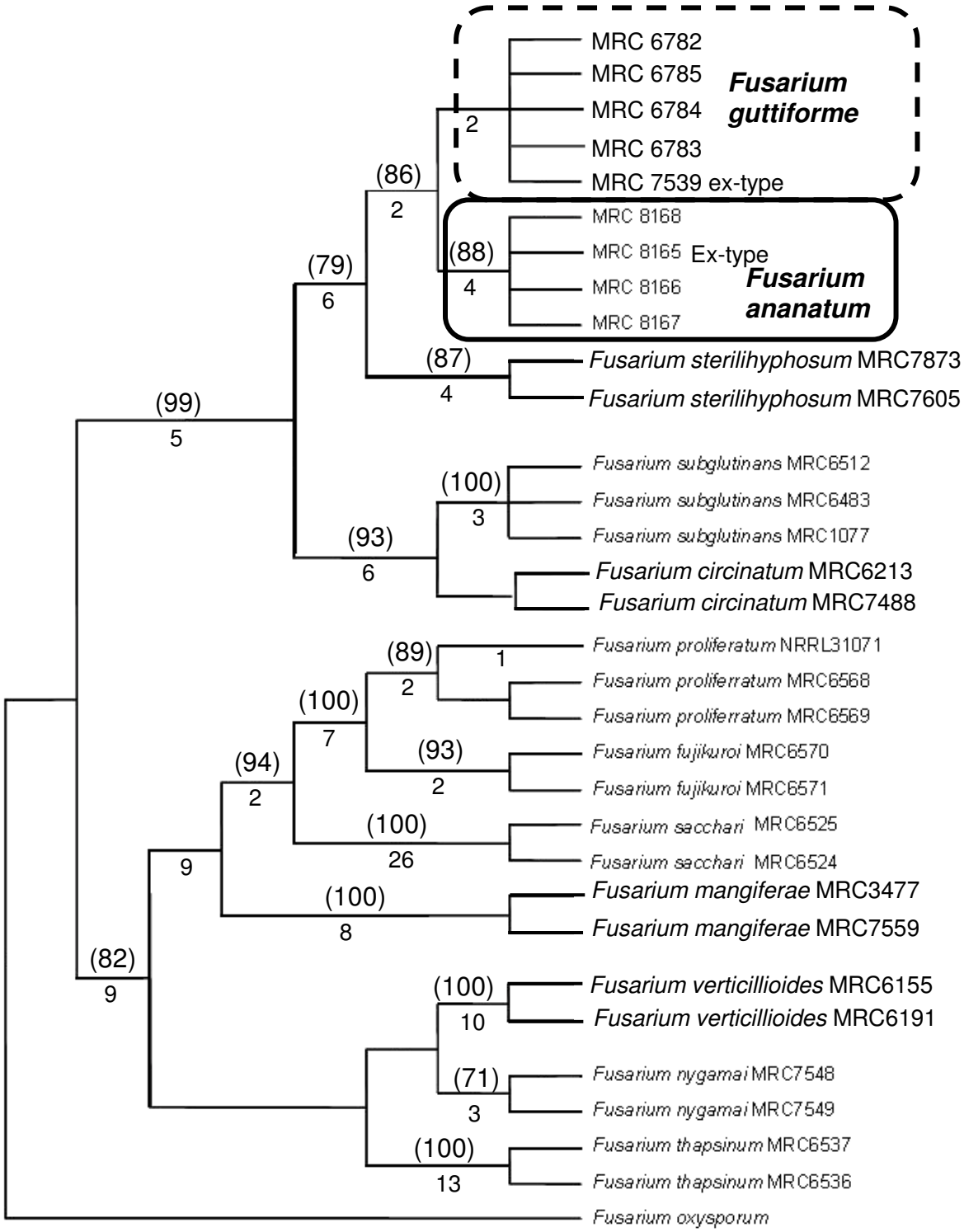

Fig 5: Phylogenetic tree of *Fusarium ananatum* and related species produced using parsimony of the Histone H3 gene with *F. oxysporum* as outgroup. Bootstrap values above 50% (percentages of 1000 bootstrap replicates) are indicated in brackets above the branches of the tree. Branch lengths are indicated below the branches. **Parsimony informative characters = 53; CI = 0.6737; RI = 0.8510; g1 = -0.476216; TL = 115 steps.**

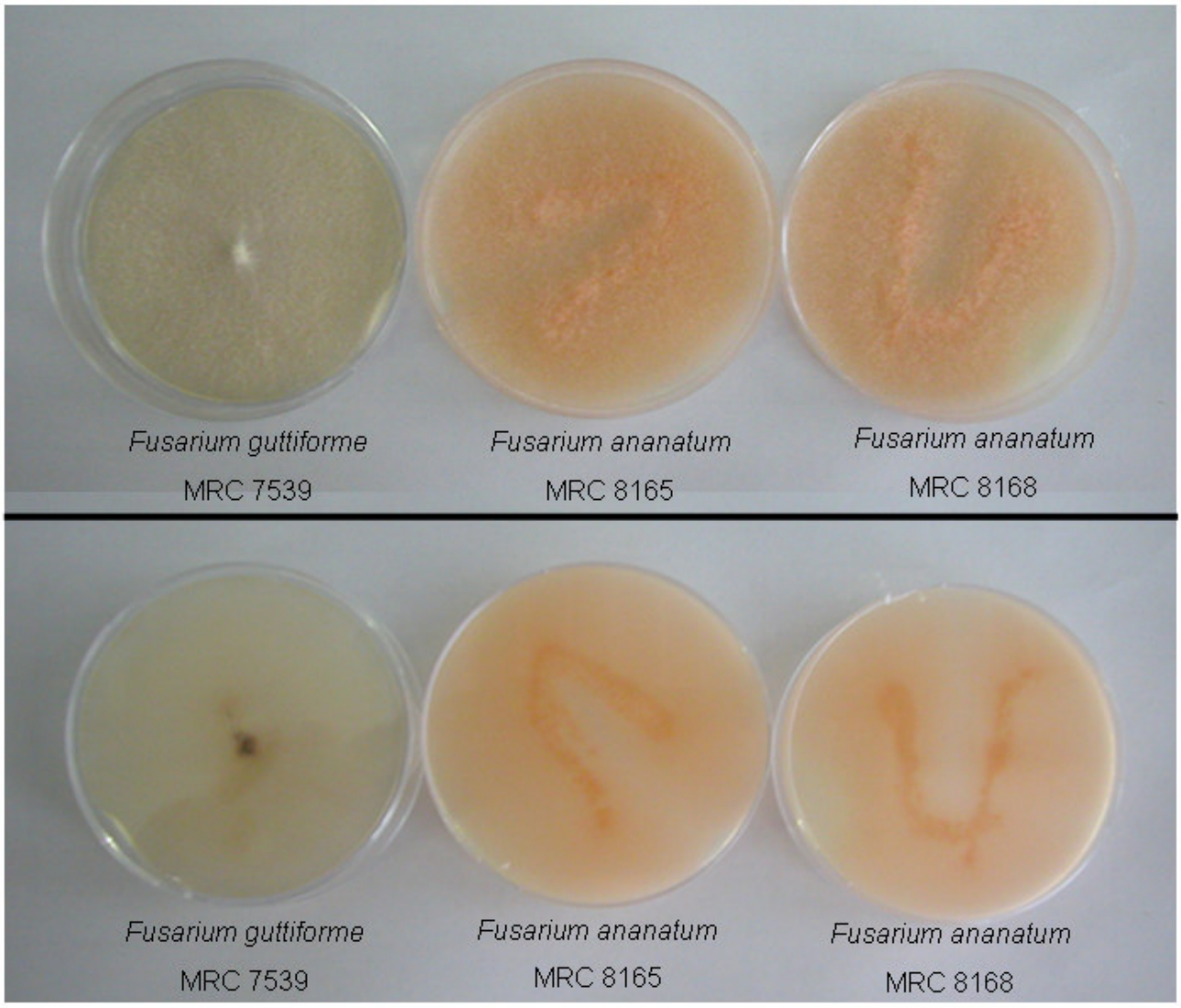

Fig 6: Colony morphology of *Fusarium ananatum* and *F. guttiforme* on PDA at 25 ℃ after 5d. First set of plates represent the top of the colonies and the second row of plates represent the reverse of the colonies.

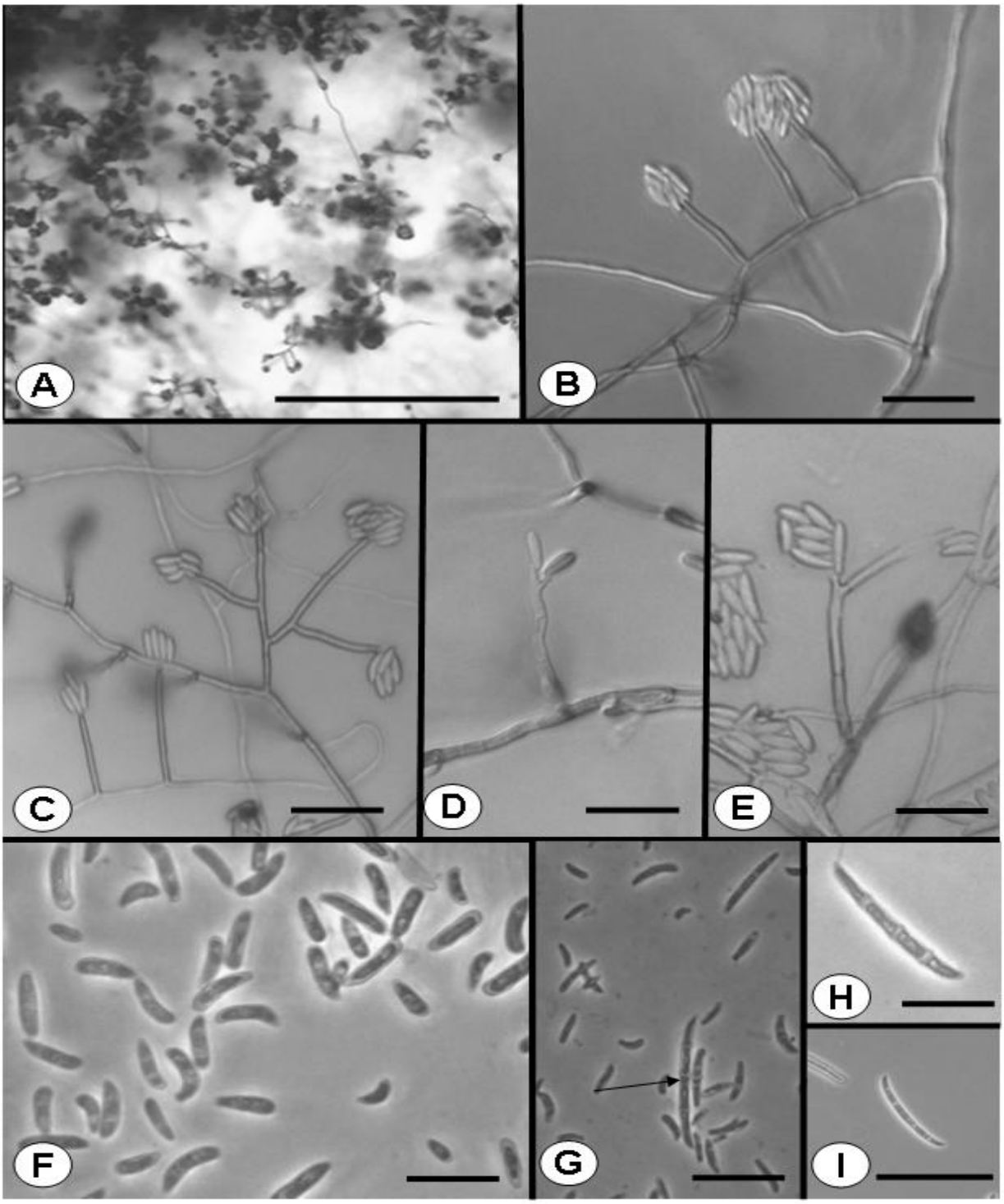

Fig 7: Morphological characteristics of *Fusarium ananatum*. (A) Erect conidiophores on aerial mycelium on SNA. (B) False conidial heads on SNA. (C) Branched and unbranched monophialides on CLA. (D) Polyphialdes on SNA. (E) Polyphialides on CLA. (F) Microconidia on CLA. (G-I) Macroconidia on CLA. Bars (A) = 130 µm; (B, C, E-H) = 15 µm; (D) = 30 µm; (I) = 20 µm.

Taxonomy of the species within the *Gibberella fujikuroi* complex

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PhD degree

Summary

Fusarium was established to accommodate phialidic fungi with fusiform macroconidia borne on poly- or monophialides. They are characterised by significant variation in morphological characters. These morphological characteristics have been used as basis to demarcate species within the genus with a wide host range and cosmopolitan distribution. In the first chapter these morphological characters and other criteria used to demarcate species in the genus *Fusarium* are reviewed. Furthermore the predominant three species concepts applied to *Fusarium* are discussed.

The second chapter of the thesis deals with polyphyletic nature pertaining to *F. subglutinans sensu lato*. This study was based on informative morphological characters as well as sequence data obtained from the translation elongation factor 1α, β-tubulin and 28S regions of the rDNA gene region. The results indicate that *F. subglutinans sensu lato* represents at least thirteen independent species associated with a variety of hosts. This manuscript was submitted for peer-viewing.

In the third chapter the taxonomic placement of a new *Fusarium* species from Poaceae, in South Africa is considered. Both morphological and molecular characters were used to determine the relationship of the new species to other species in the *F. subglutinans sensu lato* species complex and more specifically the two species, *F. subglutinans sensu stricto* and *F. circinatum*, with which it formed fertile sexual crosses. The *Fusarium* sp. isolated from Poaceae was described as *Fusarium ophiodes* sp. nov. This manuscript was submitted for peer-viewed.

In the fourth chapter the causal agent of pine decline in Chilean nurseries are characterised as *Fusarium ciricinatum*. This was done by means of sexual crosses, phylogenetics and morphological comparisons. This led to the first report of the disease in Chile with different aspects published as two peer-reviewed articles.

The last chapter deals with the generic placement of a new *Fusarium* species associated with *Ananas comosus*. The species was described as *F. ananatum*. It displays salmon colony colour with conidia produced by means of both poly- and monophialides. The erect aerial mycelium distinguishes it from the closely related species, *F. guttiforme*. Based on the sequence data obtained from the translation elongation factor 1α, β-tubulin and histone 3 genes, this species represent a new species in *F. subglutinans sensu lato*. The study resulted in a peer-reviewed publication.