

**NATIVE *FUSARIUM* SPECIES FROM
INDIGENOUS FYNBOS SOILS OF THE
WESTERN CAPE**

By

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DECLARATION

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SUMMARY

The genus *Fusarium* contains members that are phytopathogens of a number of agricultural commodities causing severe diseases such as wilts and rots. *Fusarium* species also secrete mycotoxins that have devastating effects on humans and animals. The ability of *Fusarium* species to change their genetic makeup in response to their immediate environment allows these fungi to exist in diverse habitats. Due to the ubiquitous nature of *Fusarium*, it forms part of the fungal communities in both agricultural and native soils. Fynbos is the major vegetation type of the Cape Floristic Region (CFR), which is a region that is renowned for its high plant species diversity and endemism. In this study, the occurrence and distribution of *Fusarium* species in indigenous fynbos soils and associated plant debris is investigated. In addition, the phylogenetic relationships between *Fusarium* species occurring in this particular habitat are evaluated.

Fusarium isolates were recovered from soils and associated plant debris, and identified based on morphological characteristics. The morphological identification of isolates was confirmed using Polymerase Chain Reaction (PCR) based restriction fragment length polymorphism (RFLP) analyses of the translation elongation factor 1 alpha (TEF-1 α) and internal transcribed spacer (ITS) regions. Furthermore, phylogenetic relationships between *Fusarium* species were based on the TEF-1 α , ITS and β -tubulin gene regions.

One-hundred-and-twenty-two (122) *Fusarium* strains were isolated from the fynbos soils in the Cape Peninsula area (Western Cape). Based on both morphological and molecular identification, the most prevalent *Fusarium* species in the fynbos soils

were *F. oxysporum* Schlecht. emend. Snyder and Hans., *F. solani* (Martius) Appel and Wollenw. emend. Snyder and Hans., *F. equiseti* (Corda) Sacc. and an undescribed *Fusarium* species. *Fusarium oxysporum* was the dominant species in fynbos soils and strains of this species displayed significant genetic variability. Some strains of both *F. oxysporum* and *F. solani* showed close phylogenetic affinities to *formae speciales* (strains pathogenic to specific plant hosts) in the phylogenetic analyses. However, no diseased plants were observed in and within the vicinity of our sampling sites.

In the third chapter, the undescribed *Fusarium* strains are described as *Fusarium peninsulae* prov. nom. Morphologically these strains are characterized by falcate macroconidia produced from brown sporodochia. The macroconidia are pedicellate, falcate to curved with hooked apical cells. Also, this fungus produces apedicellate mesoconidia on polyphialides in the aerial mycelium and forms microconidia sparsely. Chlamydospores are formed abundantly on aerial mycelium and submerged hyphae. All these morphological characteristics closely relate this fungus to *F. camptoceras* species complex in *Fusarium* section *Arthrosporiella*. However, phylogenetic analysis based on the ITS sequences differentiate these strains from *F. camptoceras* and other related species in section *Arthrosporiella*.

Considering the fact that both as phytopathogens and saprophytic fungi, *Fusarium* species secrete a variety of cell wall degrading enzymes such as cellulases and xylanases. These enzymes allow the fungi to degrade the plant cell wall components to obtain nutrients. In *Fusarium*, notably endoxylanases play a role in phytopathogenesis of these fungi. Endoxylanase enzymes from *F. oxysporum* f. sp. *lycopersici*, *F. verticillioides* and *F. graminearum* have been characterized. In this final chapter, the use of the

endoxylanase encoding gene, as a molecular marker in phylogenetic analysis was evaluated using *F. graminearum* (*Fg*) clade species as model. Degenerated primers were designed and the endoxylanase region amplified by PCR, cloned and sequenced. PAUP-generated neighbour-joining analysis of the endoxylanase (XYL) region enabled all species to be distinguished and was as informative as the analysis generated with UTP-ammonia ligase (URA), phosphate permease (PHO), reductase (RED) and trichothecene 3-*O*-acetyltransferase (TRI101). Furthermore, the results of the phylogenetic analysis of XYL showed better species resolution in comparison to the analysis of the structural genes (TEF-1 α and histone H3). Overall, the results demonstrated that phylogenetic analysis of XYL combined with other functional genes (URA, PHO, RED and TRI101) clearly distinguished between the *Fg* clade species far better than the analysis of structural genes (TEF-1 α and histone H3).

OPSOMMING

Die genus *Fusarium* bestaan uit spesies wat fitopatogene is van 'n aantal landbou produkte en veroorsaak erge siektes soos verwelking en verrotting. *Fusarium* spesies skei ook mikotoksiene af wat 'n verwoestende uitwerking het op mense en diere. Die vermoë van *Fusarium* spesies om hulle genetiese samestelling te verander na gelang van hulle onmiddellike omgewing, stel hierdie fungi in staat om te voorleef in verskeie omgewings. As gevolg van die alomteenwoordige aard van *Fusarium* spesies, vorm dit deel van fungus gemeenskappe in beide landbou sowel as natuurlike gronde. Fynbos is die hoof plant-tipe wat voorkom in die Kaap Floristiese Streek (KFS), wat bekend is vir hoë plant diversiteit en endemisme. In hierdie studie word die voorkoms en verspreiding van *Fusarium* spesies in inheemse fynbos grond en die geassosieerde plant oorblyfsels ondersoek. Bykomend is die filogenetiese verwantskappe tussen verskillende *Fusarium* spesies wat in hierdie spesifieke habitat voorkom geëvalueer.

Fusarium isolate is herwin vanuit die grond en geassosieerde plant oorblyfsels, en geïdentifiseer op grond van morfologiese eienskappe. Die morfologiese identifikasie van die isolate is bevestig deur gebruik te maak van die Polimerase Ketting Reaksie (PKR) gebaseerde beperkingsfragment lengte polimorfisme ("restriction fragment length polymorphism"; RFLP) analise van die TEF-1 α geen en die ITS area van die ribosomale gene. Verder is filogenetiese verwantskappe tussen *Fusarium* spesies bepaal op grond van die TEF-1 α , ITS en β -tubulin geen areas.

Een-honderd-twee-en-twintig (122) *Fusarium* stamme is geïsoleer vanuit die fynbos grond van die Kaapse Skiereiland (Wes-Kaap). Gebaseer op beide morfologiese en molekulêre identifikasies, is die mees algemene *Fusarium* spesies in die fynbos grond

F. oxysporum Schlecht. emend. Snyd. & Hans., *F. solani* (Martius) Appel & Wollenw. emend. Snyd. & Hans., *F. equiseti* (Corda) Sacc. en 'n onbeskryfde *Fusarium* spesie. *Fusarium oxysporum* is die mees dominante spesie in fynbos grond en stamme van hierdie spesie vertoon beduidende genetiese variasie. Sommige stamme van beide *F. oxysporum* en *F. solani* toon nabyverwante filogenetiese verwantskappe met *formae speciales* (stamme patogenies op 'n spesifieke plant gasheer) in die analise. Alhoewel, geen siek plante waargeneem is in of naby enige van die ondersoek areas nie.

In die derde hoofstuk word die onbeskryfde *Fusarium* stamme beskryf as *Fusarium peninsulae* prov. nom. Morfologies word hierdie stamme deur gebuigde makrokonidia wat in bruin sporodochia gevorm word gekarakteriseer. Die makrokonidia is pediselaat, gebuig tot gekrom met sekelvormige apikale selle. Ook produseer hierdie fungus apediselate mesokonidia vanuit polifialide op die lug miselia en vorm min mikrokonidia. Chlamydospore word mildelik gevorm op lug miselia sowel as hifes onder die medium. Al hierdie morfologies eienskappe dui daarop dat hierdie fungus verwant is aan die *F. camptoceras* spesies kompleks in *Fusarium* seksie *Arthrosporiella*. Filogenetiese analise, gebaseer op die ITS basispaaropeenvolgings, onderskei hierdie stamme van *F. camptoceras* en ander verwante spesies in die seksie *Arthrosporiella*.

In ag genome dat hierdie fungus beide fitopatogenies en saprotitieses kan wees, skei *Fusarium* spesies 'n verskeidenheid van selwand-afbrekende ensieme soos sellulase en xylanase af. Die ensieme laat hierdie fungi toe om plant selwande af te breek om voedingstowwe te verkry. In *Fusarium*, speel veral die endoxylanases 'n rol in die fitopatogenesiteit van hierdie fungi. Die endoxylanase ensieme van *F. oxysporum* f. sp. *lycopersici*, *F. verticillioides* and *F. graminearum* is alreeds gekarakteriseer. In die finale

hoofstuk, word die endoxylanase geen as 'n molekulêre merker geëvalueer deur gebruik te maak van die *F. graminearum* (*Fg*) spesies-kompleks as model. Gedegenererde inleiers is ontwerp en die endoxylanase geen is geamplifiseer deur PCR, gekloneer en die basispaaropeenvolgings bepaal. PAUP-gegeneerde afstand-analise van die endoxylanase (XYL) geen toon dat alle spesies onderskei kan word en is gelykstaande aan die analise wat gegeneer word deur analise van 'n gekombineerde UTP-ammonia ligase (URA), phosphate permase (PHO), reductase (RED) en trichothecene 3-*O*-acetyltransferase (TRI101) geen datastel. Verder dui die resultate van die filogenetiese analise van die XYL geen daarop dat beter spesie resolusie verkry is in vergelyking met analise van strukturele gene (TEF-1 α en histone H3). In die geheel wys die resultate daarop dat filogenetiese analise van die XYL, gekombineer met ander funksionele gene (URA, PHO, RED and TRI101) duideliker kan onderskei tussen die *F. graminearum* kompleks, as analise van die strukturele gene (TEF-1 α en histone H3).

MOTIVATION

The term “fynbos” defines a vegetation type characteristic of more than 80 % of plant species occurring in the Cape Floral Region (CFR). This is a region located in the south-western part of South Africa and is one of the six floral kingdoms of the world. The CFR is the smallest in land area of the six floral kingdoms, covering about 90 000 km² in size and containing over 9 000 plant species, most of which are endemic (only found in this region) (Cowling and Holmes, 1992; Vandecasteele and Godard, 2008). Due to its size and plant species diversity index, the CFR is regarded as one of the world’s floral hot-spots (Goldblatt and Manning, 2000; Vandecasteele and Godard, 2008). At the south-western tip of the CFR, there is an area of about 470 km² in size, the Cape Peninsula (Cowling *et al.*, 1996), which is considered a floral hot-spot within the CFR (Cowling *et al.*, 1996; Picker and Samways, 1996; Simmons and Cowling, 1996). This area experiences wet, cool winters and hot, dry summers (mediterranean-type climate) (Taylor, 1978; Kruger and Taylor, 1979) and the fynbos biome is characterized by sandy, acidic and nutrient-poor soils (Bond and Goldblatt, 1984; Cowling *et al.*, 1996; Richards *et al.*, 1997; Goldblatt and Manning, 2000). Fire is a critical ecological factor in the fynbos biome and a great majority of the vegetation is adapted to periodic fire (Cowling, 1987). Also, fire within this biome is associated with the rejuvenation of plants (le Maitre and Midgley, 1992).

Plant species richness and endemism in the Cape Peninsula and the Cape Floristic Region as a whole have been extensively explored (Cowling, 1992; Simmons and Cowling, 1996; Trinder-Smith *et al.*, 1996; Picker and Samways, 1996), while botanists have well documented the diverse plants found in this region (Kidd, 1950; Lighton, 1973;

Vandecasteele and Godard, 2008). A number of plants in the fynbos region are also popular due to their medicinal properties (Van Wyk *et al.*, 1997) and these include *Agathosma betulina* (Berg.) also known as Buchu (Lis-Bachin *et al.*, 2001) and herbal teas such as *Aspalathus linearis* (rooibos tea) and *Cyclopia genistoides* (honeybush tea), which are endemic to the CFR (Vandecasteele and Godard, 2008). The combination of climate, floral and fauna diversity and oligotrophic soils of the fynbos biome, therefore, render this area an ecologically complex environment.

The first aim of this study was thus to investigate the occurrence and distribution in the native fynbos soils and associated plant debris, of one of the most adaptable fungal genera, namely *Fusarium*. Also, phylogenetic relationships between *Fusarium* species occurring in this particular niche were investigated.

Fusarium species occur in diverse soils types, in close association with different types of plants as pathogens causing diseases (Smith *et al.*, 1981; Burgess *et al.*, 1981; Britz *et al.*, 2002). Also, *Fusarium* species secrete a variety of mycotoxins that are implicated in a number of human and animal toxicoses (Marasas *et al.*, 1984; Zhang *et al.*, 2006; O'Donnell *et al.*, 2007). Furthermore, these fungi occur in close associations with plants as saprophytes degrading dead plant material or as endophytes living parts of their life cycles or entire life cycles within plant tissues without causing any damage to the plant tissues they colonize (Saikkonen *et al.*, 1998; Zeller *et al.*, 2003; Phan *et al.*, 2004).

Since the cell wall of all plants is made up of differential complex polysaccharides, both plant pathogenic and saprophytic fungi alike require a variety of cell wall degrading enzymes (CWDEs) to be able to breakdown cell walls and extract the

essential nutrients they require (Walton, 1994; Roncero *et al.*, 2003). Some of the cell wall degrading enzymes secreted by fungal species include pectinases, cutinases, cellulases and xylanases (Walton, 1994). In phytopathogenic fungi such as *Fusarium*, hydrolytic enzymes, notably cellulases and xylanases act as virulence factors and this means that they play a role in the phytopathogenesis of these fungi (Knogge, 1996; Beliën *et al.*, 2006). Phytopathogenic *Fusarium* species such as *F. verticillioides* (Saha, 2001), *F. oxysporum* f. sp. *lycopersici* (Christakopoulos *et al.*, 1996; Ruíz *et al.*, 1997; Ruíz-Roldán *et al.*, 1999; Gómez-Gómez *et al.*, 2001, 2002) and *F. graminearum* (Beliën *et al.*, 2005) secrete a number of endoxylanases.

Therefore, the second aim of this study was to evaluate, through phylogenetic analysis, the use of an endoxylanase encoding gene region as a molecular marker for *Fusarium* species identification using the *F. graminearum* complex as model group. In addition, the phylogenetic value of functional genes versus structural genes to discriminate between closely related *Fusarium* species were further evaluated.

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1. INTRODUCTION

The genus *Fusarium* Link was discovered and described by Link in 1809, with *Fusarium roseum* as the type species (Booth, 1971). However, after taxonomic revision of the type species of this genus, *Fusarium sambucinum* Fückel *sensu stricto* was the recognized type species (Gams *et al.*, 1997) of *Fusarium*. *Fusarium* species are defined as ascomycetous and filamentous fungi in the order *Hypocreales* (Guarro *et al.*, 1999). Members of *Fusarium* are characterized by the production of three types of asexual conidia, namely, macroconidia, microconidia and chlamydoconidia. The macroconidia of *Fusarium* are distinctively lunar or falcate shaped; multinucleate and have several transverse septa. They have a hooked apical cell and a distinctive foot-shaped basal cell, and are produced in cushion-like structures called sporodochia (Booth, 1971; Nelson *et al.*, 1983). The microconidia are smaller in size than the macroconidia and have variable shapes ranging from oval, reniform to obovoid shape. These conidia are formed in the aerial mycelium on phialidic or blastic conidiogenous cells. Chlamydoconidia are accessory conidia that mostly serve as survival structures in the environment (Booth, 1971; Nelson *et al.*, 1983; Guarro *et al.*, 1999; Nelson *et al.*, 1994).

Another group of filamentous fungi, the Coelomycetes, reproduce asexually by forming conidia in fruiting bodies and these conidia are similar in morphology to the macroconidia produced by *Fusarium*. Several of these Coelomycete genera include *Botryocrea* Petr., *Heteropatella* Fückel, *Cylindrocarpon* Wollenweber and *Pycnofusarium* Punith (Seifert, 2001). However, the genus *Cylindrocarpon* has the closest morphological resemblance to *Fusarium* (Seifert and Gams, 2001). Since much emphasis is placed on the shape of the macroconidia when identifying *Fusarium* species, it is important to

differentiate macroconidia produced by these fungal species from those produced by other genera such as *Cylindrocarpon*. Therefore, the foot-shaped basal cell of the *Fusarium* macroconidia is the most important feature that separates *Cylindrocarpon* from *Fusarium* (Seifert, 2001; Seifert and Gams, 2001).

The application of a species concept is very important in fungal taxonomy as this clearly defines the criteria used in a defining species (Summerell *et al.*, 2003). The three species concepts employed in defining *Fusarium* species are the morphological-, biological- and phylogenetic species concepts (Summerell *et al.*, 2003; Leslie *et al.*, 2006). A number of taxonomic systems have been proposed for *Fusarium*, with some recognizing as many as 65 species within the genus (Wollenweber and Reinking, 1935), while others recognized as few as nine species (Snyder and Hansen, 1940, 1941). Teleomorphs of *Fusarium* species, when present, occur mostly in the genus *Gibberella* Saccardo (Samuels *et al.*, 2001) with a few accommodated in *Calonectria* De Not. and *Haematonectria* Samuels and Nirenberg (Rossman *et al.*, 1999).

Fusarium species have been isolated from a variety of substrates but in nature they are found in different types of soils (Burgess, 1981), closely associated with plant debris (Marasas *et al.*, 1988a; Rheeder *et al.*, 1990), as saprophytes or endophytes (Zeller *et al.*, 2003), or as plant pathogens (Burgess *et al.*, 1981; Smith *et al.*, 1981). *Fusarium* species are well-known for their ability to infect and cause vascular wilts, as well as root and stem rots on a number of important agricultural commodities (MacHardy and Beckham, 1981; Hennequin *et al.*, 1999; Thrane and Seifert, 2000). Some of the diseases caused by these fungi that have resulted in significant economical losses on agricultural crops worldwide include *Fusarium* head blight (scab) of wheat (Burgess *et al.*, 1987), the

bakanae disease of rice (Sun and Snyder, 1981; Hoffmann-Benning and Kende, 1992; Desjardins *et al.*, 2000), pokkah-boeng disease of sugar cane (Burgess *et al.*, 1981; Singh *et al.*, 2006), and the Panama disease of banana (Burgess *et al.*, 1981; Daly and Walduck, 2006). *Fusarium* species can cause destructive wilts or rots on ornamental plants such as carnations (Baayen and Gams, 1988), and pitch-cankers on woody plants such as *Pinus* species (Viljoen *et al.*, 1995; Viljoen *et al.*, 1997; Britz *et al.*, 2001; Wingfield *et al.*, 2002; Jacobs *et al.*, 2006), *Acacia mearnsii* and *Eucalyptus grandis* (Roux *et al.*, 2001) have also been reported.

In addition to their phytopathogenicity, *Fusarium* species secrete a wide variety of mycotoxins, which have adverse effects on humans and animals that consume agricultural commodities infected by these fungi (Gelderblom *et al.*, 1988; Marasas *et al.*, 1988b; Luo *et al.*, 1990; Ross *et al.*, 1990; Thiel *et al.*, 1991; Nelson *et al.*, 1993; Plattner and Nelson, 1994; Gelderblom *et al.*, 2001; Marasas *et al.*, 2001a, b; Bennett and Klich, 2003). In the past couple of years *Fusarium* species have also emerged as opportunistic pathogens that cause serious infections, especially in immuno-compromised individuals (Nelson *et al.*, 1994; Guarro *et al.*, 2000; Walsh *et al.*, 2004; O'Donnell *et al.*, 2007).

2. HISTORY OF THE TAXONOMIC SYSTEMS OF THE GENUS *FUSARIUM*

Following Link's diagnosis of the genus *Fusarium* in 1809, many researchers were concerned with diagnosis and identification of *Fusarium* species that caused diseases on plant hosts. At one point, following Link's treatment, more than 1000 *Fusarium* species were recognized which were mostly isolated from diseased plants and because there were no guidelines and regulations applied in naming these isolates, the taxonomy of the genus

Fusarium was in disarray (Leslie and Summerell, 2006). In 1821 Fries validated the genus *Fusarium* according to the terms of the International Botanical Code and included it in his order *Tuberculariae* (Booth, 1971). However, the breakthrough that brought some order in the taxonomy of *Fusarium* was the publication of “Die Fusarien” by Wollenweber and Reinking (1935).

2.1. Wollenweber and Reinking (1935)

In the 1930's Wollenweber and Reinking formulated a taxonomic system that grouped *Fusarium* species within sections (Wollenweber and Reinking, 1935). The separation within sections was based on variable cultural characters. The characteristics used to separate sections were: (i) the presence or absence of microconidia, (ii) the shape of the microconidia, (iii) the presence or absence of chlamydospores, (iv) the location of the chlamydospores (v) the shape of the macroconidia, and (vi) the shape of the basal or foot cells on the macroconidia. Taxa within the sections were divided into species, varieties and forms on the basis of: (i) the colour of the stroma, (ii) the presence or absence of sclerotia, (iii) the number of septations in the macroconidia and (iv) the length and width of the macroconidia (Wollenweber and Reinking, 1935). Species in each section were grouped based on shared morphological features. The work of Wollenweber and Reinking is the foundation of most modern taxonomic systems.

Their taxonomic system described 65 *Fusarium* species and 77 subspecific varieties and forms within 16 sections. The sections that they recognized were *Macroconia*, *Submicrocera*, *Pseudomicrocera*, *Discolor*, *Roseum*, *Elegans*, *Liseola*, *Sporotrichiella*, *Gibbosum*, *Martiella*, *Ventricosum*, *Arachnites*, *Arthrosporiella*,

Eupionnotes, *Lateritium* and *Spicarioides* (Wollenweber and Reinking, 1935; Joffe, 1986; Windels, 1992).

2.2. Snyder and Hansen

In the 1940's Snyder and Hansen protested against the species distinctions of the Wollenweber and Reinking (1935) system. They argued that *Fusarium* cultures not initiated from single spores can display variable morphological features. Therefore, according to Snyder and Hansen (1940, 1941, 1945), the large morphological variations emphasized by Wollenweber and Reinking's system were due to cultures not initiated from single spores. Hence they regarded the morphological variations of the Wollenweber and Reinking's system as having no taxonomic value. This prompted them to reduce the number of *Fusarium* species described by Wollenweber and Reinking (1935) to nine species (Nelson *et al.*, 1983). They did not follow the grouping of species into sections and the nine species they recognized within the genus *Fusarium* corresponding to the Wollenweber and Reinking (1935) sections were *F. oxysporum* (section *Elegans*); *F. solani* (sections *Martiella* and *Ventricosum*); *F. moniliforme* (section *Liseola*); *F. roseum* (sections *Roseum*, *Arthrosporiella*, *Gibbosum* and *Discolor*); *F. lateritium* (section *Lateritium*); *F. tricinctum* (section *Sporotrichiella*); *F. nivale* (section *Arachnites*), *F. rigidiuscula* (section *Spicarioides*) and *F. episphaeria* (sections *Eupionnotes* and *Macroconia*) (Nelson *et al.*, 1983). Since taxa within the Wollenweber and Reinking (1935) sections were polyphyletic, the Snyder and Hansen (1940; 1941) system led to huge losses of information on a number of *Fusarium* species previously

described (Booth, 1971; Domsch *et al*, 1980; Messiaen and Cassini, 1981; Joffe, 1986; Windels, 1992; Leslie and Summerell, 2006).

For a number of years, different researchers would follow one or the other system. Some *Fusarium* researchers strictly followed the Wollenweber and Reinking's system or the Snyder and Hansen's system while others combined the two in their own taxonomic systems. Examples of such combined taxonomic systems include that of Gordon (1952), Messiaen and Cassini (1968), Booth (1971), Gerlach and Nirenberg (1982) and Nelson *et al.* (1983).

2.3. Gordon (1952)

In the 1950's Gordon first adopted the system of Snyder and Hansen (1940, 1941, 1945) but later based his work on that of Wollenweber and Reinking (1935). He combined some of Wollenweber and Reinking's species after observing variability displayed in some isolates. Gordon classified 26 *Fusarium* species within 14 sections and also included 5 varieties and 69 forms of *F. oxysporum*. He kept the four Wollenweber and Reinking's sections (*Discolor*, *Roseum*, *Arthrosporiella* and *Gibbosum*) instead of replacing them as *F. roseum*, as Snyder and Hansen had done. He also considered the sexual phases of the species in his taxonomic descriptions (Gordon, 1952; Domsch *et al*, 1980; Joffe, 1986; Leslie and Summerell, 2006), an aspect not addressed in the Wollenweber and Reinking and Snyder and Hansen's systems.

2.4. Messiaen and Cassini (1968)

Messiaen and Cassini based their taxonomic system on that of Snyder and Hansen (1940, 1941). The only difference in their work was that they adopted the use of botanical varieties instead of cultivars at subspecies level in *F. roseum*. Species *F. sambucinum*, *F. culmorum*, *F. graminearum* and *F. avenaceum* were all made varieties of *F. roseum* (Messiaen and Cassini, 1968; Booth, 1971).

2.5. Booth (1971)

In his work Booth recognized 51 *Fusarium* species within twelve sections namely *Arachnites* (*Submicrocera*), *Martiella* (*Ventricosum*), *Episphaeria* (*Eupionnotes* and *Macroconia*), *Sporotrichiella*, *Spicarioides*, *Arthrosporiella* (*Roseum*), *Coccophilum* (*Pseudomicrocera* and *Macroconia*), *Lateritium*, *Liseola*, *Elegans*, *Gibbosum* and *Discolor*. He also introduced the use of the morphology of the conidiogenous cells as an additional species-level diagnostic character. He used this character to distinguish within some of the species in sections *Liseola* and *Sporotrichiella*. Booth's taxonomic system was identical to that of Gordon (1952) (Booth, 1971; Domsch *et al.*, 1980; Joffe, 1986; Windels, 1992; Leslie and Summerell, 2006).

2.6. Gerlach and Nirenberg (1982)

In 1982 Gerlach and Nirenberg published an atlas that recognized 78 *Fusarium* species and 55 varieties within the 16 sections recognized by Wollenweber and Reinking (1935).

Their system is considered as an update of Wollenweber and Reinking (1935) but is also very similar to that of Booth (1971) (Gerlach and Nirenberg, 1982; Joffe, 1986).

2.7. Nelson, Toussoun and Marasas (1983)

Shortly after the publication of Gerlach and Nirenberg (1982), Nelson *et al.* (1983) published a taxonomic system that combined the best features of the taxonomic systems of Wollenweber and Reinking (1935), Snyder and Hansen (1940), Joffe (1986), Messiaen and Cassini (1968), Gerlach (1981) and Booth (1971). In their species descriptions they did not recognize the presence of polyblastic conidiogenous cells as a diagnostic character as done by Booth (1971). They recognized 30 *Fusarium* species within 12 sections namely section *Eupionnotes*, *Spicarioides*, *Arachnites*, *Sporotrichiella*, *Roseum*, *Arthrosporiella*, *Gibbosum*, *Discolor*, *Lateritium*, *Liseola*, *Elegans* and *Martiella-Ventricosum*. There were about 16 additional species that had insufficient descriptions and illustrations that they documented in their publication (Nelson *et al.*, 1983; Leslie and Summerell, 2006).

In summary, there are currently more than 100 species recognized within the genus *Fusarium*. However, due to taxonomic revisions of the morphological species concept, which was initially employed in *Fusarium* species identification, this number is expected to rise (Leslie and Summerell, 2006). Also, as much as there are a number of taxonomic systems that have been proposed for *Fusarium*, some systems have shown to be more popular than others amongst the *Fusarium* research communities. This is at least true for those researches that still employ morphological characters as the basis for species identification. The taxonomic system of Nelson *et al.* (1983) (Marasas *et al.*,

1985, 1986, 1987, 1988; Rheeder *et al.*, 1990, 1996; Viljoen *et al.*, 1997; Marasas *et al.*, 1998; Rheeder *et al.*, 1998; Marasas *et al.*, 2001c ; Roux *et al.*, 2001) is one example of such a system.

2.7.1. Morphological and cultural criteria used in the Nelson et al. (1983) taxonomic system

All the major *Fusarium* taxonomic systems (Wollenweber and Reinking, 1935; Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983) are based on morphological and cultural criteria. In the Nelson and co-workers (1983) taxonomic system, the grouping of species into sections was based on cultural characteristics such as the growth rate, colony morphology and pigmentation. The morphology of the macroconidia from sporodochia, microconidia from aerial mycelium, conidiophores and chlamyospores were also characters used to group species into sections (Table 1). Cultural characteristics are observed on Potato Dextrose Agar (PDA) and the morphology of the macroconidia, microconidia, conidiophores and chlamyospores should be done on cultures grown on Carnation Leaf Agar (CLA). Proper growth conditions are important in *Fusarium* species identification, therefore, cultures are usually grown in an alternating temperature of 25°C day/ 20°C night and incubated in diffuse daylight or in light from fluorescent tubes (Nelson *et al.*, 1983).

PDA is a medium considered to be rich in nutrients such as carbohydrates and because of its high available carbohydrate content, it promotes mycelial growth rather than sporulation in fungal cultures. Furthermore, PDA is mostly used in *Fusarium* cultures identification to induce pigmentation and to observe cultural growth rates, which

are important secondary characteristics in species identification (Nelson *et al.*, 1983; Summerell *et al.*, 2003). No complete identification of a *Fusarium* species can be made based on cultures grown on PDA since the high nutrient concentration of the medium induces morphological mutations in *Fusarium* isolates (Leslie and Summerell, 2006).

CLA on the other hand is a medium that is low in nutrients compared to PDA and promotes sporulation rather than mycelial growth in *Fusarium* cultures. Under correct growth conditions, CLA results in consistent morphological characters produced by *Fusarium* species (Burgess *et al.*, 1991). CLA gained its popularity in *Fusarium* species identification after Fisher *et al.* (1982) showed it to be a good medium to grow and preserve *Fusarium* cultures over long periods of time. The ability of *Fusarium* species to sporulate so very well in CLA could be attributed to its low available carbohydrate content and the presence of carnation leaves, which provide the same complex natural substances as in the natural environment (Leslie and Summerell, 2006).

3. TELEOMORPHS OF THE GENUS *FUSARIUM*

The known teleomorphs (sexual phases) of *Fusarium* species occur in the order *Hypocreales* in the Ascomycetes (Samuels *et al.*, 2001). Booth (1981) described the teleomorphs of *Fusarium* within four genera namely *Gibberella*, *Calonectria*, *Nectria* Samuels and *Monographella* Petr. (*Plectosphaerella*). Recent studies based on molecular characters, however, showed that the only anamorph in the genus *Calonectria*, *F. decemcellulare* Wollenweber should be accommodated in the genus *Albonectria* Rossman and Samuels (Rossman *et al.*, 1999). Similarly, *F. solani*, which had a teleomorph in *Nectria* showed affinities to *Haematonectria* instead of *Nectria* (Rossman

et al., 1999). Following the revision of the teleomorphs of *Fusarium* species based on molecular markers, no *Fusarium* teleomorphs have been found and defined under the genus *Monographella* as previously done by Booth (1981). The three *Fusarium* sections, *Arthrosporiella*, *Elegans* and *Sporotrichiella* have no known teleomorphs (Samuels *et al.*, 2001). Currently, the known teleomorphs of *Fusarium* species occur in the genera *Gibberella*, *Haematonectria* Samuels and Nirenberg and *Albonectria* (Table 2).

The genus *Gibberella* forms perithecia that are dark purple but appear black when growing on substrates (Samuels *et al.*, 2001). They are obovoid and subglobose shaped and have a rough outer appearance. The asci are relatively narrow and clavate, and the apical discharge mechanism is usually absent. The ascospores are fusoid shaped, three-or-more-septate and are straight or slightly curved. They are initially hyaline but become a light brown when discharged. The perithecia become red when stained with 3% Potassium hydroxide (KOH) and yellow in the presence of lactic acid (Rossman *et al.*, 1999; Samuels *et al.*, 2001). Teleomorphs that are accommodated in *Gibberella* include those of all the other *Fusarium* sections species, with the exception of *Fusarium* section *Martiella-Ventricosum* species (Table 2) (Rossman *et al.*, 1999; Samuels *et al.*, 2001; Leslie and Summerell, 2006).

Perithecia of *Haematonectria* species are yellow to red with globose to pyriform shape and are usually not embedded on the substrate. The asci are clavate and contain striated, ellipsoid one-septate ascospores. The perithecia turn darker when KOH is added onto them (Rossman *et al.*, 1999). *Fusarium* section *Martiella-Ventricosum* species is the only one accommodated in *Haematonectria* (Table 2).

Perithecia of *Albonectria* species are white to pale in colour, subglobose, globose to ellipsoid in shape. The asci contain four to eight ascospores and they are ellipsoid to long-ellipsoid and three septate. The perithecia do not react with KOH (Windels, 1992; Guarro *et al.*, 1999; Rossman *et al.*, 1999; Kerényi *et al.*, 2004; Leslie and Summerell, 2006). *Fusarium* section *Eupionnotes* species are the only species accommodated in *Albonectria* (Table 2).

4. SPECIES CONCEPTS APPLIED IN THE GENUS *FUSARIUM*

The problems and complexities encountered when dealing with the taxonomy of *Fusarium* originate from the fact that over the years the application of species concepts was different amongst the *Fusarium* research communities. An obvious example is in the taxonomic systems of Wollenweber and Reinking (1935) and Snyder and Hansen (1940, 1941, 1945), which recognize 65 species and 9 species, respectively. It is generally accepted that each species concept applied in species identification has criteria through which species can be identified and differentiated from each other (Taylor *et al.*, 2000; Summerell *et al.*, 2003). In *Fusarium* the three species concepts that are used in species identification are the morphological-, biological- and the phylogenetic species concepts (Taylor *et al.*, 2001; Leslie *et al.*, 2001; Summerell *et al.*, 2003).

4.1. Morphological species concept

The morphological species concept is the most dominant species concept in fungal taxonomy (Taylor *et al.*, 2000). It is based on the similarity of observable morphological

characters which can be both physical and physiological (Leslie *et al.*, 2001). The physical characters include the shape and size of the conidia, while physiological characters include growth rates (Taylor *et al.*, 2000; Leslie *et al.*, 2001) and secreted secondary metabolites such as mycotoxins (Desjardins *et al.*, 1992; Nelson *et al.*, 1993; Torp and Langseth, 1999; Thrane, 2001; Rheeder *et al.*, 2002). In the taxonomy of *Fusarium*, the shape of the macroconidia is the first character that is considered when defining a species (Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983; Summerell *et al.*, 2003; Leslie and Summerell, 2006). Other morphological characters such as the microconidia, conidiophores and chlamydoconidia are also important in the application of the morphological species definition (Booth, 1971; Gerlach and Nirenberg 1982; Nelson *et al.*, 1983).

The greatest advantage of this species concept in general is that it has been widely applied so that comparisons can be made among existing taxa and between new and existing taxa (Rheeder *et al.*, 1996; Klittich *et al.*, 1997; Marasas *et al.*, 1998; Taylor *et al.*, 2000). The taxonomic systems of Gerlach and Nirenberg (1982) and Nelson *et al.* (1983) are both morphological species concepts and currently act as the basis from which the biological- and phylogenetic species concepts are being constructed or formulated (Nirenberg and O'Donnell, 1998; Taylor *et al.*, 2000). However, the morphological species concept has disadvantages, especially in the genus *Fusarium*. Firstly, the similarities and differences observed in macroconidia of *Fusarium* species are dependent on growth conditions and can be difficult to discern without prior experience (Thrane and Seifert, 2000; Summerell *et al.*, 2003; Leslie and Summerell, 2006). Secondly, because of the growth dependent morphological characters, isolates that should be grouped as

different species tend to be grouped as a single species. Examples of this misrepresented heterogeneity are observed in the *Gibberella fujikuroi* (Sawada) Wollenweber (section *Liseola*) (Snyder and Hansen, 1945); *F. oxysporum* Schlecht. (section *Elegans*) and *F. solani* (Mart.) Sacc. (section *Martiella-Ventricosum*) species complexes (Snyder and Hansen, 1940, 1941).

In addition, the ability of *Fusarium* species to produce a variety of morphological characters has meant that in some species, the traditional morphological characters outlined in most taxonomic systems are not sufficient to make a complete species identification (Leslie and Summerell, 2006). This is evident in species such as *F. nygamai* Burgess and Trimboli (Burgess and Trimboli, 1986), *F. napiforme* Marasas, Nelson and Rabie (Marasas *et al.*, 1987), *F. beomiforme* Nelson, Toussoun and Burgess (Nelson *et al.*, 1987) and *F. dlamini* Marasas, Nelson and Toussoun (Marasas *et al.*, 1985), which are morphologically similar to taxa within both sections *Liseola* and *Elegans* (Nelson *et al.*, 1990). All four species form chlamydospores and this excludes them from belonging within section *Liseola* based on the monograph of Nelson *et al.* (1983). Also, the nature and mode of formation of microconidia in *F. nygamai*, *F. napiforme*, *F. beomiforme* and *F. dlamini* excludes them from belonging within section *Elegans* (Nelson *et al.*, 1990). In these species the morphology of the microconidia, microconidial conidiogenous cells and the presence or absence of chlamydospores are the most important morphological features used in their identification. Kwasna (1991) proposed an additional section *Dlaminia* to the already known Nelson *et al.* (1983) sections to accommodate these four *Liseola*-like

chlamydosporous species namely, *F. nygamai*, *F. napiforme*, *F. beomiforme* and *F. dlamini*.

4.2. Biological species concept

According to Hawksworth (1996) the biological species concept defines an “actually or potentially interbreeding population which is reproductively isolated from other such groups, whether or not they are distinguishable morphologically”. The offspring of a sexual cross has to be both viable and fertile (Summerell *et al.*, 2003) for the parents to be considered a biological species. Interfertility has been used in fungal taxonomy to identify groups of mating compatible individuals or mating populations (MPs) within a species (Leslie *et al.*, 2001). Members of the same mating population are considered to be members of the same biological species because they can be cross-fertile but are not cross-fertile with members of other MPs (van Etten and Kistler, 1988).

In *Fusarium*, the *G. fujikuroi* species complex (section *Liseola*) (Leslie, 1991, 1995; Leslie *et al.*, 2001) and *Haematonectria haematococca* (= *Nectria haematococca*) (Matuo and Snyder, 1973) species complex consists of taxa that have been grouped into mating populations. There are nine mating populations within the *G. fujikuroi* species complex namely, *F. verticillioides* (Saccardo) Nirenberg (MP A) (Gerlach and Nirenberg, 1982), *F. sacchari* (Butler) Gams (MP B) (Leslie *et al.*, 2005), *F. fujikuroi* Nirenberg (MP C) (Nirenberg, 1976), *F. proliferatum* (Matsushima) Nirenberg (MP D) (Gerlach and Nirenberg, 1982), *F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas (MP E) (Nelson *et al.*, 1983), *F. thapsinum* Klittich, Leslie, Nelson and Marasas (MP F) (Klittich *et al.*, 1997), *F. nygamai* (MP G) (Klaasen and Nelson, 1996),

F. circinatum Nirenberg and O'Donnell emend. Britz, Coutinho, Wingfield and Marasas (MP H) (Nirenberg and O'Donnell, 1998) and *F. konzum* Zeller, Summerell and Leslie (MP I) (Zeller *et al.*, 2003) while Matuo and Snyder (1973) described seven mating populations (MPI to MPVII) in the *H. haematococca* species complex.

The limitation in the application of the biological species concept is that it can only be applied to sexually reproducing species. In *Fusarium* this species concept cannot be applied to species of sections *Sporotrichiella*, *Elegans* and *Arthrosporiella* as they have no known teleomorphs (Samuels *et al.*, 2001). Another limitation is that even in sexually reproducing species, the presence of meiospores (spores resulting from meiosis) is not sufficient to infer mating (Taylor *et al.*, 2000). The relative frequencies of the mating type alleles, MAT-1 and MAT-2 determine (Leslie *et al.*, 2001) if mating actually takes place or not within species. Female fertility in field populations often is low (Mansuetus *et al.*, 1997; Britz *et al.*, 1998; Leslie *et al.*, 2001) and this also affects sexual crosses within members of these populations. The identification of mating-type allele specific PCR primers for some *Fusarium* species (Steenkamp *et al.*, 2000) has led to mating crosses only being made with the tester strain with which a fertile cross is expected (Leslie and Summerell, 2006). This saves researchers time as mating crosses require long time periods to complete and analyze (Leslie and Summerell, 2006).

4.3. Phylogenetic species concept

In the phylogenetic species concept, DNA sequences are used to generate characters that are assessed by cladistic analysis to form phylogenies (Hawksworth, 1996; Summerell *et al.*, 2003). Although DNA sequences are the most favourable characters used in

identifying and defining phylogenetic species, both morphological and physiological characters can be used, provided that they are sufficiently informative (Leslie *et al.*, 2001). The advantage of DNA sequences in comparison to other characters is that once evolutionary changes occur in the progeny, such changes can be recognized in gene sequences first before they are recognizable in mating behaviour or morphology of that progeny (Taylor *et al.*, 2000).

One of the negative aspects of the phylogenetic species concept is that individual strains may group in well-resolved clades but the decision on species boundaries is still subjective (Taylor *et al.*, 2000). This has been resolved by applying the concordance of more than one gene genealogy which has brought the term Genealogical Concordance Phylogenetic Species Recognition (GCPSR) into fungal taxonomy (Taylor *et al.*, 2000). This species concept has been applied in ascomycetous genera such as *Neurospora*, *Aspergillus* (Samson *et al.*, 2007), *Penicillium* (Seifert and Lévesque, 2004), *Lasiosphaeria* (Miller and Huhndorf, 2004) and *Fusarium* (O'Donnell *et al.*, 2000).

In *Fusarium*, GCPSR has been applied in phylogenetic species diagnosis of the *G. fujikuroi* species complex (O'Donnell *et al.*, 1998). O'Donnell *et al.* (1998) used nucleotide sequences of three genes namely, the β -tubulin, ITS2 region and mtSSU rDNA to analyze 45 species from this species complex. Twenty-six species of the 45 were resolved as new or re-discovered species based on the combined nucleotide sequences of the selected genes.

In summary, in a genus such as *Fusarium*, the application of the morphological species concept has been shown to have limitations due to inconsistencies in recognizable morphological characters produced by these fungi. These inconsistencies can be

attributed to the fact that *Fusarium* species have a high mutation frequency influenced by their immediate environment (Summerell *et al.*, 2003; Kerényi *et al.*, 2004). All these factors have led to conflicting taxonomic systems of the genus *Fusarium* such as that of Wollenweber and Reinking (1935) and Snyder and Hansen (1940) which inspired most of the recent taxonomic systems. Also, due to previous groupings of numerous taxa as a single species (Snyder and Hansen, 1940, 1941, 1945), certain *Fusarium* sections such as *Liseola*, *Elegans* and *Martiella-Ventricosum* are now referred to as species complexes. Furthermore, there is undeniable evidence that the morphological characters and physiological characters applied in the diagnosis of *Fusarium* species tend to overlap between taxa of different sections. Examples of such cases are sections *Liseola* and *Elegans* (Burgess and Trimboli, 1986; Marasas *et al.*, 1987; Nelson *et al.*, 1987), and *Discolor* and *Sporotrichiella* (Yli-Mattila *et al.*, 2004). Similarly, the biological species concept, which has been used in resolving taxa within some species complexes in *Fusarium*, has its own limitations. It can only be applied in classification of *Fusarium* species with known teleomorph phases (Taylor *et al.*, 2001). The advent of molecular techniques such as PCR (Paterson, 1996; Guarro *et al.*, 1999) and the development of universal oligonucleotide primers (White *et al.*, 1990) have led to the use of molecular characters such as gene sequences as supplementary tools in fungal taxonomy. Molecular characters are used to explore evolutionary relationships between microorganisms and hence the origin of the phylogenetic species concept in fungal taxonomy (Samuels and Seifert, 1995; Taylor *et al.*, 2000; Thornton and DeSalle, 2000; Hibbett *et al.*, 2007).

5. MOLECULAR CHARACTERS AND TECHNIQUES USED IN TAXONOMY OF *FUSARIUM* SPECIES

The application of the phylogenetic species concept in the genus *Fusarium* gained popularity with the advancement in molecular techniques such as the PCR (Guarro *et al.*, 1999). The two most commonly used methods in molecular taxonomy of *Fusarium* include genotyping (Klittich *et al.*, 1997; Guarro *et al.*, 1999; Savelkoul *et al.*, 2001) and DNA sequencing (Schilling *et al.*, 1996; Kristensen *et al.*, 2004)

5.1. Genotyping

Genotyping or DNA fingerprinting techniques have been employed to investigate genetic variability within fungal populations especially the phytopathogenic taxa. There are a number of genotyping techniques that are applicable in genetic diversity analysis but the most popular, especially in *Fusarium* are the random amplified polymorphic DNA- (RAPD) (Guarro *et al.*, 1999; Taylor *et al.*, 1999), amplified-fragment length polymorphism- (AFLP) (Vos *et al.*, 1995; Savelkoul *et al.*, 1999, Taylor *et al.*, 1999; Groenewald *et al.*, 2006) and restriction fragment length polymorphism- (RFLP) analyses (Guarro *et al.*, 1999; Taylor *et al.*, 1999). All three techniques have different principles with regards to their operational aspects but the banding patterns produced by each technique visualized on agarose gel are used to distinguish between taxa. Also, technically these three techniques differ in their robustness, reliability and reproducibility (Savelkoul *et al.*, 2001).

5.1.1. RAPD analysis

Randomly amplified polymorphic DNA analysis is based on the use of an arbitrary designed primer of 10 bp that binds on an unknown site on genomic DNA to produce complex amplicons. When these complex amplicons are separated on an agarose gel by electrophoresis, they result in banding patterns which are used to differentiate within studied taxa (Welsh *et al.*, 1990).

Ouellet and Seifert (1993) used RAPD and restriction analysis of amplified fragments (PCR-RFLP) to characterize strains of *F. graminearum*. They observed low genetic diversity between the tested strains but recommended the use of this method and its specific PCR profiles for tracking strains of *F. graminearum* in field environments. Genetic diversity in *F. oxysporum* f. sp. *vasinfectum*, the pathogen causing vascular wilt in cotton cultivars (*Gossypium species*) was investigated using RAPD analysis and pathogenicity tests (Assigbetse *et al.*, 1994). The pathogenicity tests differentiated 3 races (A, 3 and 4) within the tested strains of this pathogen while the RAPD profiles grouped the tested strains into three groups corresponding to their pathological reactions. This study showed that RAPD analysis could be a quick and reliable alternative to pathogenicity tests for *F. oxysporum* f. sp. *vasinfectum*. Random amplified polymorphic DNA analyses have also been used in conjunction with restriction analysis of PCR amplified ribosomal DNA (rDNA) (Talbot *et al.*, 1996) and intergenic spacer (IGS) regions (Carter *et al.*, 2000). However, the application of the RAPD technique has become less popular in studying genetic variability in fungal populations because of its poor reproducibility (Guarro *et al.*, 1999).

5.1.2. AFLP analysis

Amplified fragment length polymorphism analysis is a technique that is based on the detection of genomic restriction fragments by PCR amplification (Vos *et al.*, 1995; Savelkoul *et al.*, 2001). This technique involves restriction of DNA with two restriction enzymes, one with a low cutting frequency and another with a high cutting frequency; ligation of oligonucleotide adapters to reduce the restriction sites; selective PCR amplification of the restriction fragments; electrophoresis and visualization on a gel (Vos *et al.*, 1995; Savelkoul *et al.*, 2001). This technique is useful in both the evaluation of genetic variation within fungal populations and construction of genetic maps (Jurgenson *et al.*, 2002a, b; Leslie and Summerell, 2006).

The two published genetic maps in the genus *Fusarium* are that of *G. zaeae* (*F. graminearum*) and *G. moniliformis* (*F. verticillioides*) (Jurgenson *et al.*, 2002a, b) and were both constructed using AFLP and RFLP markers. In the construction of the genetic linkage map of *G. zaeae*, complementary nitrate-non utilizing (*nit*) mutants of *G. zaeae* strains R-5470 (from Japan) and Z-3639 (from Kansas) were crossed and 99 nitrate-utilizing (recombinant) progeny were selected and AFLP analysis performed using 34 pairs of two-base selective AFLP primers. One thousand and forty-eight polymorphic markers that mapped to 468 unique loci on nine linkage groups were identified and the total map length was ~ 1300 cM with an average interval of 2.8 map units between loci (Jurgenson *et al.*, 2002a). The genetic linkage map of *G. moniliformis* was constructed from an already existing RFLP-based map (Xu and Leslie, 1996) using AFLP markers. According to Jurgenson *et al.* (2002b) the already existing RFLP-based map of *G. moniliformis* contained significant gaps that made it difficult to routinely locate

biologically important genes such as those involved in pathogenicity and mycotoxin production by this pathogen. They used AFLP-markers to saturate the RFLP-based map, which added 486 AFLP markers to the ~ 150 markers of the existing map. The resulting map had an average marker interval of 3.9 map units and an average ~21 kp/map units.

AFLPs have been used in conjunction with RAPD analysis and were found to be more informative than RAPD analysis in studying genetic variation in the chickpea wilt pathogen *F. oxysporum* f. sp. *ciceri* (Sivaramakrishnan *et al.*, 2002). Also, evolutionary relationships have been investigated using AFLPs and gene genealogies between *F. graminearum* and *F. pseudograminearum* (Monds *et al.*, 2005). Isolates of the banana pathogen *F. oxysporum* f. sp. *cubense* of different vegetative compatibility groups (VCGs) and races were found to be polyphyletic when assessed by AFLP analysis (Groenewald *et al.*, 2006). The stringent PCR conditions involved in this technique make it highly discriminatory, more reproducible and reliable (Savelkoul *et al.*, 2001; Abdel-Satar *et al.*, 2003) compared to other techniques such as the RAPD analysis.

5.1.3. RFLP analysis

This technique is based on the use of a selected group of restriction enzymes to partially or completely digest DNA templates. After separation of the digests by electrophoresis, genetic variations are evaluated based on the produced patterns (Guarro *et al.*, 1999). Restriction fragment polymorphic markers can also be used in constructing genetic maps (Xu and Leslie, 1996).

Restriction analysis of mitochondrial DNA (mtDNA) was successful in determining genetic diversity in some *Fusarium* species (Kim *et al.*, 1992), but restriction analysis of PCR-amplified DNA sequences has been shown to be more popular (Steenkamp *et al.*, 1999; Konstantinova and Yli-Mattila, 2004). Hinojo *et al.* (2004) used a panel of five restriction enzymes *HhaI*, *EcoRI*, *AluI*, *PstI* and *XhoI* to generate RFLP profiles from a PCR-amplified IGS region to characterize morphologically identified *G. fujikuroi* isolates from different geographical regions. The generated RFLPs permitted discrimination between *G. fujikuroi* isolates from different hosts and with different toxigenic profiles.

Bogale *et al.* (2007) applied RFLP analyses of PCR-amplified translation elongation factor 1 alpha (TEF-1 α) to identify and distinguish between *F. redolens* and members of the three phylogenetic clades of *F. oxysporum*. There were three TEF1 α -RFLP patterns among *formae speciales* of *F. oxysporum* and these patterns corresponded with the three clades. The internal transcribed (ITS) regions have also been used in the application of RFLP-PCR methods (Lee *et al.*, 2000). The restriction fragment length polymorphism technique is a simple and inexpensive technique that is highly applicable in genotyping *Fusarium* species (Manicom *et al.*, 1990; Benyon *et al.*, 2000; Kosiak *et al.*, 2005; Llorens *et al.*, 2006) and other soil fungi (Viaud *et al.*, 2000).

5.2. DNA sequencing

DNA sequencing of genes such as the ribosomal DNA (rDNA), ITS regions, actin, β -tubulin, translation elongation factor 1-alpha (TEF-1 α), partial sequence of the intergenic spacer (IGS) region, mating-type (MAT1/ MAT2) and histone H3 genes are

popular in analyses of phylogenetic relationships between *Fusarium* species (O'Donnell, 1992; Duggal *et al.*, 1997; Hennequin *et al.*, 1999; Steenkamp *et al.*, 1999; Rakeman *et al.*, 2005). As much as these are the most preferred gene sequences in phylogenetic analyses not all of them are equally informative for species in all portions of the genus *Fusarium* (O'Donnell, 1992; Waalwijk *et al.*, 1996; Leslie and Summerell, 2006).

Sequencing of one or two genes can be used to characterize unknown *Fusarium* species. The DNA sequences are usually used together with morphological features to give a full description of a particular *Fusarium* isolate. *Fusarium commune* Skovgaard, Rosendahl, O'Donnell and Nirenberg was identified based on morphological characters combined with molecular characters (Skovgaard *et al.*, 2003). *Fusarium commune* morphologically differs slightly from its sister taxon *F. oxysporum* complex by having long, slender monophialides and polyphialides when cultured in the dark. Phylogenetic analyses of the combined dataset of the TEF-1 α and mitochondrial small subunit (mtSSU) rDNA genes showed *F. commune* to be a strongly supported clade that is closely related to *F. oxysporum* but independent of this and the *G. fujikuroi* species complexes (Skovgaard *et al.*, 2003). Genetic variability has also been evaluated in *F. verticillioides* species isolated from diverse hosts and geographic origins, using the IGS region and TEF-1 α gene sequences. ITS regions revealed a high genetic variability amongst the tested strains compared to TEF-1 α phylogenetic analysis (Mirete *et al.*, 2004).

In cases of human, animal or plant disease outbreaks caused by fungal isolates, characterization of the causative agent(s) based on morphological characters can be a long process. Sequencing of genes instead of morphological identification has therefore

proven to be efficient. Hennequin *et al.* (1999) sequenced the large subunit 28S ribosomal RNA (rRNA) gene for rapid identification of *Fusarium* species associated with human infections.

Roux *et al.* (2001) identified isolates of an unknown, nonsporulating fungus from diseased *Acacia mearnsii* and *Eucalyptus grandis* in South Africa using histone H3 and β -tubulin gene sequences and identified the unknown isolates as *F. graminearum* Schwabe species. Coutinho *et al.* (2007) identified a fungus causing pitch canker in a South African pine plantation as *F. circinatum* based on morphological features and the sequences of TEF-1 α and β -tubulin genes. Also, Steenkamp *et al.* (2000) identified a *Fusarium* species associated with mango malformation disease (MMD) as *F. subglutinans* based on the sequences of the histone H3 and β -tubulin genes. Furthermore, cryptic speciation in *F. subglutinans* was discovered based on phylogenetic analyses of the calmodulin, histone H3, β -tubulin, HB9, HB14 and HB26 gene sequences (Steenkamp *et al.*, 2002).

In addition, the use of more than one gene (genealogical concordance) to evaluate taxonomic relationships between *Fusarium* species (O'Donnell *et al.*, 2004; Yli-Mattila *et al.*, 2004) has led to the discovery that species diagnosed through the morphological species concept often encompass more than one species than when diagnosed by the biological and phylogenetic species concepts (Taylor *et al.*, 2000).

O'Donnell *et al.* (1998) used DNA sequences of nuclear TEF-1 α and mtSSU rRNA genes to investigate whether lineages of the Panama disease pathogen, *F. oxysporum* f. sp. *cubense* have a monophyletic origin. Phylogenetic trees inferred from the combined dataset resolved five lineages corresponding to "*F. oxysporum* f. sp.

cubense” with a large dichotomy between two taxa represented by strains commonly isolated from bananas with Panama disease. The result revealed that Panama disease of banana is caused by *Fusarium* with independent evolutionary origins (O’Donnell *et al.*, 1998).

Six gene genealogies of UTP-ammonia ligase (URA), 3-O-acetyltransferase (TRI101), phosphate permease (PHO), putative reductase (RED), β -tubulin (TUB) and TEF-1 α were used to test whether *F. graminearum* is panmictic throughout its range (O’Donnell *et al.*, 2000). With an exception of one hybrid strain, all six genealogies recovered the same seven biogeographically structured lineages in the *F. graminearum* (*Fg*) clade. The results suggested that the seven lineages represent phylogenetically distinct species among which gene flow has been very limited during their evolutionary history. Parsimony analysis of the combined dataset resolved most relationships among the lineages of the *Fg* clade (O’Donnell *et al.*, 2000). In another study, O’Donnell *et al.* (2004) used genealogical concordance between the mating type locus and seven nuclear genes to support formal recognition of nine phylogenetically distinct species within the *Fg* clade.

The taxonomy of a newly discovered species *F. langsethiae* Torp and Nirenberg in the *Fusarium* section *Sporotrichiella* has been difficult based on morphological characters alone (Torp and Nirenberg, 2004). *Fusarium langsethiae* resembles *F. poae* (Peck) Wollenweber in several morphological features but is similar to *F. sporotrichioides* Sherbakoff when toxin patterns are compared (Yli-Mattila *et al.*, 2004). Schmidt *et al.* (2004) resolved the taxonomic position of the species *F. langsethiae* in section *Sporotrichiella* by phylogenetic analyses of TEF-1 α , β -tubulin,

partial sequence of the IGS region and the ITS1 and ITS2 regions. The results strongly showed that *F. langsethiae* is closely related to *F. sporotrichioides*.

Fusarium pseudograminearum Aoki and O'Donnell, the causative agent of crown rot in wheat in Australia was resolved to be a single phylogenetic species based on analysis by maximum parsimony of four genealogies including the TEF-1 α , phosphate permase (PHO), putative reductase (RED) and β -tubulin (TUB) genes. The result also showed that *F. pseudograminearum* is a single phylogenetic species without consistent lineage development across genes (Scott *et al.*, 2006).

O'Donnell *et al.* (2007) used partial sequences of the RNA polymerase II second largest subunit (RPB2), TEF-1 α and nuclear rRNA genes to understand the phylogenetic diversity of human pathogenic *Fusarium* species implicated in keratitis outbreaks in the United States of America and Puerto Rico during 2005 and 2006. The results showed that the phylogenetic diversity represented among the corneal isolates is consistent with multiple sources of contamination.

6. ECOLOGY OF *FUSARIUM* SPECIES

Members of the genus *Fusarium* have a worldwide distribution. They occur in diverse soil types, in close association with plants as pathogens causing diseases (Smith *et al.*, 1981; Burgess *et al.*, 1981; González *et al.*, 1997; Britz *et al.*, 2002). Their pathogenicity is not only limited to agricultural crops but covers a wide spectrum of plant hosts including woody plants (Roux *et al.*, 2001; Jacobs *et al.*, 2006; Roux *et al.*, 2007; Wingfield *et al.*, 2002) and ornamental plants (Baayen and Gams, 1988; Eken *et al.*,

2004). They also associate with plant hosts as endophytes or with dead plant material as saprophytes (Burgess, 1981; Rheeder *et al.*, 1990; Rheeder and Marasas, 1998; James and Perez, 2000; Wang *et al.*, 2007). The mycotoxins produced by these fungi are detrimental to human and animal health (Marasas *et al.*, 1979; Marasas *et al.*, 1984; Marasas *et al.*, 1988a; Marasas *et al.*, 1988b; Marasas *et al.*, 2001a, b; Fandohan *et al.*, 2003). With the increase in number of immuno-suppressed individuals worldwide, *Fusarium* species have emerged as opportunistic pathogens causing serious infections in such individuals (Nelson *et al.*, 1994; Guarro *et al.*, 2000; Guarro *et al.*, 2003; Walsh *et al.*, 2004).

6.1. *Phytopathogenic Fusarium species*

Fusarium species are plant pathogens of important agricultural commodities worldwide (Thrane and Seifert, 2000) and they cause a variety of diseases in the plants they infect (Table 3). Infections on plants by *Fusarium* species are not limited to crops only but wilts and rots in ornamental plants and woody plants due to *Fusarium* species have also been observed (Louvet and Toutain, 1981; Roux *et al.*, 2001). Some of the diseases caused by *Fusarium* species that have led to significant economic losses in a number of countries worldwide include *Fusarium* head blight (FHB) or scab, the bakanae disease of rice and Panama disease of bananas (Burgess, 1981; Hoffmann-Benning and Kende, 1992; Desjardins *et al.*, 2000; Daly and Walduck, 2006).

Scab is a crown rot of wheat and is mainly caused by *F. graminearum* (Burgess *et al.*, 1983; Marasas *et al.*, 1988c). The symptoms of this disease on infected wheat include necrotic lesions on the roots and dark brown to black discolouration of the leaves at the crown. The infectious agent forms sporodochia on an infected spikelet and conidia from

these can be dispersed to the rest of the uninfected spikelets. The infected wheat plants stop growing and die prematurely (Burgess *et al.*, 1983).

Bakanae disease of rice is caused by *F. moniliforme* (= *F. verticillioides*, *G. fujikuroi*, MP A) (Ploetz, 2001). The symptoms of this disease are plants that are taller than normal plants with tillers that bear white and empty panicles. The fungus causing this disease usually occurs on the lower parts of the plants. The taller than normal rice plants can be attributed to the gibberellins produced by the pathogen. Gibberellin (GA) has been shown to be a plant growth promoter (Hoffman-Bening and Kende, 1992; Ploetz, 2001).

The Panama disease of bananas is caused by the *F. oxysporum* f. sp. *cabense* pathogen. This pathogen causes disease on a variety of banana cultivars but mostly on the *Musa* species of the banana plant. Discolouration of the stems of infected plants and brown leaves are the main symptoms of the progress of this disease in an infected plant (Burgess *et al.*, 1981; Moore *et al.*, 2001).

6.2. *Fusarium* species toxigenic to humans and animals

Fusaria diseases in both humans and animals are caused by ingesting or coming into contact with food or feed that is infected with mycotoxins secreted by *Fusarium* species. Some of the severe abnormalities caused by toxigenic *Fusarium* species in humans include alimentary toxic aleukia (ATA), Urov or Kashin-Beck disease and Akakabi-byo (scabby grain intoxication) (Marasas *et al.*, 1984; Nelson *et al.*, 1984) and esophageal cancer (Marasas *et al.*, 1979; Marasas *et al.*, 1981). In animals, some of the diseases

caused by toxigenic *Fusarium* species include equine leukoencephalomalacia (ELEM) in horses and porcine pulmonary edema (PPE) in swine (Table 4). *Fusarium* species have also emerged as opportunistic pathogens especially in immuno-compromised individuals (Guarro *et al.*, 2000; Summerbell *et al.*, 2002; Guarro *et al.*, 2003; Walsh *et al.*, 2004; Zhang *et al.*, 2006; O'Donnell *et al.*, 2007).

6.3. Mycotoxins produced by Fusarium species

Mycotoxins are low-molecular weight compounds produced as secondary metabolites by filamentous fungi. These compounds have been shown to have no contribution in the bioenergetics of the producing fungi (Bennett and Klich, 2003). The genus *Fusarium* contains well-known mycotoxin producing species and the major mycotoxins produced by *Fusarium* species are fumonisins, moniliformin, trichothecenes, zearalenone and fusarochromanones (Table 4). Other secondary metabolites produced by *Fusarium* species include cyclohexadepsipeptides such as enniatins and beauvericins. These two compounds have been shown to have cytotoxic, antibiotic, insecticidal and ionophoric properties but their effect on human and animal health has not been well established (Munkvold *et al.*, 1998; Fotso *et al.*, 2002; Logrieco *et al.*, 2002; Ivanova *et al.*, 2006; Jeschke *et al.*, 2007).

6.4. Saprophytic and endophytic Fusarium species

Saprophytic fungi colonize and degrade dead plant material and in that way facilitate in the recycling of nutrients in the soil ecosystem (Knogge, 1996). Saprophytic fungi require

diverse hydrolytic enzymes to be able to utilize dead plant material efficiently. Some of these enzymes include cutinases, cellulases and proteases, which hydrolyze the different host plant structures (Knogge, 1996). An endophyte, on the other hand, is defined as a fungus that lives a part of its life cycle or its entire life cycle within plant tissue without causing severe damage to the plant (Saikkonen *et al.*, 1998). Mutualistic symbiotic interactions between some fungal species such as *Epichloë* species (asexual *Neotyphodium*) and grass species such as *Festuca arundinaceae* and *Lolium perenne* species have been greatly explored (Schardl *et al.*, 1997; Müller *et al.*, 2005, Spiering *et al.*, 2005). These interactions have been shown to benefit the plant because the fungi produce mycotoxins that prevent herbivorous animals from devouring the plant host and in return the fungi obtain their nutrients and shelter from the plant (Saikkonen *et al.*, 1998).

Much attention by *Fusarium* researchers has been directed into phytopathogenic *Fusarium* species and little is known about the associations of *Fusarium* species with plants as endophytes although some species have been shown to occur in close association with native prairie grasses (Zeller *et al.*, 2003) and tropical grasses *Heteropogon triticeus* (Phan *et al.*, 2004). *Fusarium* species in both instances were recognized as endophytes or latent pathogens associated with the grasses.

7. APPLICATIONS OF *FUSARIUM* SPECIES

Fusarium species have gained popularity mostly as phytopathogenic and opportunistic pathogens of humans and animals; however these fungi also have positive applications in the food, biotechnology and agricultural industries. Some of the products synthesized by

Fusarium species which are available commercially as food or additives in food products include Quorn™ (Wiebe, 2004) and Phospholipase-A (Clausen, 2001). Quorn™ is a well developed product that is produced for human consumption as a secondary protein source. This mycoprotein is synthesized by growing *F. venenatum* A3/5 (ATCC PTA-2684), originally identified as a *F. graminearum* strain, in continuous flow culture to produce the mycoprotein. There are many products that have this mycoprotein incorporated in them and are available in a number of countries worldwide (Wiebe, 2004). Also, in the process of making vegetable oils from rape seeds, soy beans and sunflower seeds, the oil requires a refining step called oil-degumming. This is done to remove impurities that can affect the taste of the oil, smell, appearance and its shelf-life. One of the most crucial enzymes in the whole process, Phospholipase-A is produced by a *F. oxysporum* strain. The enzyme is commercially available as Lecitase® Novo (Clausen, 2001).

Furthermore, *Fusarium* species such as *F. oxysporum* can convert glucose to ethanol by simultaneous saccharification and fermentation (SSF) of cellulose. However, the conversion rate of the whole process is low and acetic acid is produced as an end-product (Panagiotou *et al.*, 2005). Another *F. oxysporum* 841 strain has been shown to convert glucose, xylose and cellulose to ethanol and acetic acid as end-products (Kumar *et al.*, 1991; Singh *et al.*, 1992). The ability of *F. oxysporum* strains to produce a broad range of cellulases and xylanases makes it possible for this fungus to be used in the process of lignocellulosic raw material hydrolysis (Christakopoulos *et al.*, 1996; Ruiz *et al.* 1997).

In the agricultural industry, *Fusarium* species can be used as biological control agents. Examples are *F. oxysporum* strains that have been applied as mycoparasites (fungi infecting other fungi), and parasites of weed and unwanted plant hosts (Gupta *et al.*, 1971; Benhamou *et al.*, 2002).

Table 1. Summary of the morphological characteristics used by Nelson *et al.* (1983) to group *Fusarium* species into sections.

		<i>Eupionnotes</i>	<i>Spicarioides</i>	<i>Arachnites</i>	<i>Sporotrichiella</i>	<i>Roseum</i>	<i>Arthrosporiella</i>
Cultural characteristics	Rate of growth	Very slow, Less than 2 to 3 cm in diameter in 10 days	Rapid or moderately slow, not more than 7 cm in diameter in 10 days	Moderately slow, not more than 7 cm in diameter in 10 days	Rapid	Rapid	Rapid
	Aerial mycelium	Absent, surface has slimy yeast-like appearance	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent
	Colour of aerial mycelium	N/A	White	White	White or tan	White	White
	Colour of colony	Absent but if present, Orange, tan, brown, or light purple	Shades of carmine red	Absent but if present, orange, tan, brown, or light purple	Absent but if present, orange, tan, brown or light purple or shades of carmine red	Absent but if present, orange, tan, brown or light purple or shades of carmine red	Absent but if present, orange, tan, brown or light purple or shades of carmine red
	Colour of spore masses	Orange, yellow to tan	Orange, yellow to tan	Orange, yellow to tan	Orange, yellow to tan or reddish-brown	Orange, yellow to tan or reddish-brown	Orange, yellow to tan
Macroconidia from sporodochia	Size	Occasionally short, generally 1- 2 septate or medium long, 3-7 septate	Very long, generally 8-9 septate	Short, generally 1-2 septate	Medium long, generally 3-7 septate	Medium long, generally 3-7 septate	Medium long, generally 3-7 septate
	Shape	Dorsi-ventral curvature, sides often unequally curved, very thin walls, needle-like	No marked dorsi-ventral curvature, sides relatively straight and parallel for most of spore length	Dorsi-ventral curvature, sides often unequally curved	Dorsi-ventral curvature, sides often unequally curved	Dorsi-ventral curvature, sides often unequally curved, very thin walls, needle-like	Marked dorsi-ventral curvature, sides often relatively straight and parallel for most of the spore length, some are spindle shape
	Shape of Apical & basal cells	Basal cell not distinct or papillate	Basal cell distinctly foot-shaped or notched	Basal cell not distinct or papillate	Basal cell not distinct or papillate or foot-shaped or notched	Basal cell distinctly foot-shaped or notched	Basal cell distinctly foot-shaped or notched

Table 1 (continued)

		<i>Eupionnotes</i>	<i>Spicarioides</i>	<i>Arachnites</i>	<i>Sporotrichiella</i>	<i>Roseum</i>	<i>Arthrosporiella</i>
Microconidia from aerial mycelium	Present or absent	Absent or sparse	Present and abundant	Absent or sparse	Present and abundant	Absent or sparse	Absent or sparse
	Chains or False heads	N/A	In chains and false heads	N/A	In false heads only	N/A	N/A
	Shape	N/A	Oval , ovoid, reniform to fusiform	N/A	Oval , ovoid, reniform to fusiform or globose (<i>F. poae</i>)	N/A	N/A
Conidiophores	Type	Monophialides only (bearing either microconidia or macroconidia)	Monophialides only (bearing either microconidia or macroconidia)	Monophialides only (bearing either microconidia or macroconidia)	Monophialides only (bearing either microconidia or macroconidia); polyphialides	Monophialides only (bearing either microconidia or macroconidia)	Monophialides only (bearing either microconidia or macroconidia); polyphialides
Chlamydospores	Present or absent	Can be present or absent	Absent	Absent	Present	Absent	Present
	Arrangement	Single or in pairs	N/A	N/A	Single, pairs, long chains or large lumps of more than three cells	N/A	Long chains or large lumps of more than three cells

N/A – not applicable

Table 1 (continued)

	<i>Gibbosum</i>	<i>Discolor</i>	<i>Lateritium</i>	<i>Liseola</i>	<i>Elegans</i>	<i>Martiella-Ventricosum</i>	
Cultural characteristics	Rate of growth	Rapid	Rapid or moderately slow, not more than 7 cm in diameter in 10 days (<i>F. reticulatum</i>)	Moderately slow, not more than 7 cm in diameter in 10 days	Rapid or moderately slow ≤ 7 cm in diameter in 10 days	Rapid	Rapid
	Aerial mycelium	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent	Present, sparse to felt-like or abundant; spore masses (sporodochia) present or absent
	Colour of aerial mycelium	White	White, tan	White	White or light purple	White or light purple	White
	Colour of colony (below)	Absent but if present, orange, tan, brown or light purple or shades of carmine red	Absent but if present, orange, tan, brown or light purple or shades of carmine red	Shades of carmine red	Absent but if present, orange, tan, brown or light purple or shades of carmine red or strong purple pigment	Absent but if present, orange, tan, brown or light purple or strong purple pigment	Absent but if present, orange, tan, brown or light purple or strong purple pigment
	Colour of spore masses	Orange, yellow to tan	Orange, yellow to tan or reddish-brown	Orange, yellow to tan	Orange, yellow to tan	Cream, orange, yellow to tan	Cream, blue-green to blue
Macroconidia from sporodochia	Size	Medium long, generally 3-7 septate	Medium long, generally 3-7 septate	Medium long, generally 3-7 septate	Medium long, generally 3-7 septate	Medium long, generally 3-7 septate	Medium long, generally 3-7 septate
	Shape	dorsi-ventral curvature, sides often unequally curved	Stout; with or without marked dorsi-ventral curvature, sides relatively straight and parallel for most of spore length	No marked dorsi-ventral curvature, sides relatively straight and parallel for most of spore length or spindle shaped	Dorsi-ventral curvature, sides relatively straight and parallel for most of spore length, very thin walls, needle-like	With or without dorsi-ventral curvature, sides often unequally curved or relatively straight and parallel	No marked dorsi-ventral curvature, sides relatively straight and parallel for most of spore length
	Shape of Apical & basal cells	Basal cell distinctly foot-shaped or notched, apical cell extended and whip-like	Basal cell distinctly foot-shaped or notched	Basal cell distinctly foot-shaped or notched	Basal cell distinctly foot-shaped or notched	Basal cell distinctly foot-shaped or notched	Basal cell distinctly foot-shaped or notched

Table 1 (continued)

		<i>Gibbosum</i>	<i>Discolor</i>	<i>Lateritium</i>	<i>Liseola</i>	<i>Elegans</i>	<i>Martiella-Ventricosum</i>
Microconidia from aerial mycelium	Present or absent	Present and abundant (<i>F. scirpi</i>) or absent or sparse	Present and abundant (<i>F. bactridioides</i>) or absent or sparse	Present and abundant or absent or sparse	Present and abundant	Present and abundant	Present and abundant
	Chains or False heads	N/A	N/A	N/A	In chains or false heads	In chains or false heads	In chains or false heads
	Shape	Oval, ovoid, reniform to fusiform	Oval, ovoid, reniform to fusiform (<i>F. bactridioides</i>)	N/A	Oval, ovoid, reniform to fusiform and globose (<i>F. anthophilum</i>)	Oval, ovoid, reniform to fusiform	Oval, ovoid, reniform to fusiform
Conidiophores	Type	Monophialides only (bearing either microconidia or macroconidia)	Monophialides only (bearing either microconidia or macroconidia)	Monophialides only (bearing either microconidia or macroconidia)	Monophialides only (bearing either microconidia or macroconidia); polyphialides	Monophialides only (bearing either microconidia or macroconidia)	Monophialides only (bearing either microconidia or macroconidia)
Chlamydospores	Present or absent	Present	Present	Present	Absent	Present	Present
	Arrangement	Long chains or large lumps of more than three cells	Long chains or large lumps of more than three cells	Single or in pairs	N/A	Single or in pairs	Single or in pairs

N/A – not applicable

Table 2. Teleomorph names for species with a *Fusarium* anamorph (adapted from Leslie and Summerell, 2006).

Teleomorph	<i>Fusarium</i> species
<i>Albonectria</i> species	
<i>A. rigidiuscula</i>	<i>F. decemcellulare</i>
<i>Gibberella</i> species	
<i>G. acuminata</i>	<i>F. acuminatum</i>
<i>G. avenacea</i>	<i>F. avenaceum</i>
<i>G. baccata</i>	<i>F. lateritium</i>
<i>G. buxi</i>	<i>F. lateritium</i> var. <i>buxi</i>
<i>G. circinata</i>	<i>F. circinatum</i>
<i>G. coronicola</i>	<i>F. pseudograminearum</i>
<i>G. cynae</i> (= <i>G. gordonii</i> ?)	<i>F. reticulatum</i> ^a
<i>G. fujikuroi</i>	<i>F. fujikuroi</i>
<i>G. heterochroma</i>	<i>F. flocciferum</i> ^a
<i>G. indica</i>	<i>F. udum</i>
<i>G. intermedia</i>	<i>F. proliferatum</i>
<i>G. intricans</i>	<i>F. bullatum</i>
<i>G. konza</i>	<i>F. konzum</i>
<i>G. moniliformis</i>	<i>F. verticillioides</i>
<i>G. nygamai</i>	<i>F. nygamai</i>
<i>G. pseudopulicaris</i>	<i>F. sarcochroum</i> ^a
<i>G. pulicaris</i>	<i>F. sambucinum</i>
<i>G. pulicaris</i> var. <i>minor</i>	<i>F. torulosum</i>
<i>G. sacchari</i>	<i>F. sacchari</i>
<i>G. stilboides</i>	<i>F. stilboides</i> ^a
<i>G. subglutinans</i>	<i>F. subglutinans</i>
<i>G. thapsina</i>	<i>F. thapsinum</i>
<i>G. tricincta</i>	<i>F. tricinctum</i>
<i>G. tumida</i>	<i>F. tumidum</i>
<i>G. xylarioides</i>	<i>F. xylarioides</i>
<i>G. zaeae</i>	<i>F. graminearum</i>
<i>Haematonectria</i> species	
<i>H. haematococca</i>	<i>F. solani</i>

^a Relationship between anamorph and teleomorph presumed but not proven.

Table 3. Some of the diseases caused by *Fusarium* species on important agricultural commodities.

Disease	Agricultural commodity	Causative agent(s)	Reference
<i>Fusarium</i> Head Blight or Scab	Wheat, barley, oats, rye, sorghum	<i>F. graminearum</i> , <i>F. avenaceum</i> , <i>F. culmorum</i>	O'Donnell <i>et al.</i> (2004); Cassini (1981)
Stem and tuber rots	Potato	<i>F. oxysporum</i> f. sp. <i>tuberosa</i>	Jones and Woltz (1981)
<i>Fusarium</i> rot	Beans	<i>F. solani</i> f. sp. <i>Phaseoli</i>	Kraft <i>et al.</i> (1981)
Seedling, root and stalk rot	Corn	<i>F. verticillioides</i> (syn. <i>F. moniliforme</i>)	Fuchs <i>et al.</i> (2004)
<i>Fusarium</i> rot	Muskmelon	<i>F. oxysporum</i> f. sp. <i>melonis</i>	Mas <i>et al.</i> (1981)
<i>Fusarium</i> wilt	Peas	<i>F. solani</i> f. sp. <i>Pisi</i>	Kraft <i>et al.</i> (1981)
<i>Fusarium</i> wilt	Lentils	<i>F. solani</i> f. sp. <i>Lentils</i>	Kraft <i>et al.</i> (1981)
Sudden death syndrome	Soybean	<i>Fusarium solani</i> f. sp. <i>glycines</i>	Li <i>et al.</i> (2000)
Bakanae disease	Rice	<i>F. moniliforme</i>	Sun and Snyder (1981)
<i>Fusarium</i> wilt	Tomato	<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	Jones and Woltz (1981)
Panama disease	Banana	<i>F. oxysporum</i>	Booth (1971)
<i>Fusarium</i> wilt	Cotton	<i>F. oxysporum</i> f. sp. <i>vasinfectum</i>	Dong <i>et al.</i> (2005)
Storey's bark disease	Coffee	<i>F. lateritium</i>	Burgess (1981)

Table 4. Important mycotoxins produced by *Fusarium* species and their effects in humans and animals.

Mycotoxin	Effects in humans/animals	Secretor agent(s)	Reference(s)
Type A trichothecenes: T-2 toxin, HT-2 toxin, Diacetoxyscirpenol (DAS), Neosolaniol	Alimentary toxic aleukia (ATA) in humans; Gastroenteritis, dermo-mucal necrosis, haemorrhage, reduced weight gain in swine, poultry and cattle	<i>F. sporotrichioides</i> , <i>F. poae</i> , <i>F. acuminatum</i> , <i>F. equiseti</i> , <i>F. sambucinum</i>	Joffe, 1986; Pettersson, 2004
Type B trichothecenes: Deoxynivalenol (DON) (syn. Vomitoxin), Nivalenol (NIV), Fusarenone-X (FUS)	Vomiting and feed refusal in pigs,	<i>F. graminearum</i> , <i>F. cerealis</i> , <i>F. culmorum</i>	Marasas <i>et al.</i> , 1984 Leslie and Summerell, 2006
Zearalenone (ZEA)	Vulvovaginitis, enlargements of mammary glands, fertility disorder in pigs, swine and sheep	<i>F. cerealis</i> , <i>F. equiseti</i> , <i>F. culmorum</i> , <i>F. graminearum</i> , <i>F. semitectum</i>	Alldrick <i>et al.</i> , 2004; Pettersson, 2004
Fumonisin (FB₁ and FB₂)	Equine leukoencephalomalacia (LEM) in horses and mules, Porcine pulmonary edema (PPE) in swine	<i>F. verticillioides</i> , <i>F. nygamai</i> , <i>F. proliferatum</i>	Marasas <i>et al.</i> , 1984; Ross <i>et al.</i> , 1990 Leslie and Summerell, 2006
Moniliformin	Equine leukoencephalomalacia (LEM), cardiotoxic,	<i>F. moniliforme</i> var. <i>subglutinans</i> , <i>F. proliferatum</i> , <i>F. avenaceum</i>	Marasas <i>et al.</i> , 1984 ; Marasas and Nelson, 1987
Fusarochromanone	Tibial dyschondroplasia (leg weakness) in poultry	<i>F. equiseti</i>	Pettersson, 2004

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CHAPTER 2

**Native *Fusarium* species occurring in indigenous fynbos soils of
the Western Cape Province, South Africa**

1. ABSTRACT

Fynbos is the major vegetation type of the Cape Floristic Region (CFR), which is renowned for its high plant species diversity and endemism. The occurrence and distribution of *Fusarium* spp. in indigenous fynbos soils were investigated. *Fusarium* spp. are soilborne fungi that are both phytopathogenic and saprophytic. One-hundred-and-twenty one (122) *Fusarium* strains were isolated from the fynbos soils in the Cape Peninsula area (Western Cape). *Fusarium* isolates were identified based on morphological characteristics and RFLP analyses of the TEF-1 α and ITS region gene fragments. Morphological identification of isolates was confirmed by sequence analyses of TEF-1 α , β -tubulin and ITS region genes. Based on morphology and gene sequencing, surprisingly few species were present and these were *F. oxysporum*, *F. solani* and *F. equiseti* and an undescribed species, *Fusarium* 'fynbos'. Most isolates, however, were found to belong to *F. oxysporum* (section *Elegans*). Also, no diseased plants were observed from the sampling sites, although *F. oxysporum* and *F. solani* are pathogens of a number of plant species.

2. INTRODUCTION

The term “fynbos” defines a vegetation type characteristic of more than 80% of plant species occurring in the Cape Floral Region (CFR). This is a region located in the south-western part of South Africa and is one of the six floral kingdoms of the world. The CFR is the smallest in land area of the six floral kingdoms, covering about 90 000 km² in size and containing over 9 000 plant species, most of which are endemic (only found in this region) (Cowling and Holmes, 1992; Manning, 2004; Vandecasteele and Godard, 2008). Due to its size and plant species diversity index, the CFR is regarded as one of the world’s floral hot-spots (Goldblatt and Manning, 2000; Vandecasteele and Godard, 2008). At the south-western tip of the CFR, there is an area of about 470 km² in size, the Cape Peninsula (Cowling *et al.*, 1996), which is considered a floral hot-spot within the CFR (Cowling *et al.*, 1996; Picker and Samways, 1996; Simmons and Cowling, 1996). This area experiences wet, cool winters and hot, dry summers (mediterranean-type climate) (Taylor, 1978; Kruger and Taylor, 1979) and the fynbos biome is characterized by sandy, acidic and nutrient-poor soils (Bond and Goldblatt, 1984; Cowling, *et al.*, 1996; Richards *et al.*, 1997; Goldblatt and Manning, 2000). Fire is a critical ecological factor in the fynbos biome and a great majority of the vegetation is adapted to periodic fire (Cowling, 1987). Also, fire within this biome is associated with the rejuvenation of plants (le Maitre and Midgley, 1992).

Plant species richness and endemism in the Cape Peninsula and the Cape Floristic Region as a whole have been extensively explored (Cowling *et al.*, 1992; Simmons and Cowling, 1996; Trinder-Smith *et al.*, 1996; Picker and Samways, 1996), while botanists have well documented the diverse plants found in this region (Kidd, 1950; Lighton, 1973;

Vandecasteele and Godard, 2008). A number of plants in the fynbos region are also popular due to their medicinal properties (Van Wyk *et al.*, 1997) and these include *Agathosma betulina* (Berg.) also known as Buchu (Lis-Bachin *et al.*, 2001) and herbal teas such as *Aspalathus linearis* (rooibos tea) and *Cyclopia genistoides* (honeybush tea), which are endemic to the CFR (Vandecasteele and Godard, 2008). The combination of climate, floral and fauna diversity and oligotrophic soils of the fynbos biome, therefore, render this area an ecologically complex environment. The objectives of this study were thus to investigate the occurrence and distribution in the native fynbos soils, of one of the most adaptable fungal genera, namely *Fusarium*. Furthermore, the phylogenetic relationships of *Fusarium* species within this niche were explored.

Fusarium include phytopathogenic fungi that infect a number of agricultural crops (Burgess *et al.*, 1981) and can produce mycotoxins, which have devastating effects on the health of humans and animals (Marasas *et al.*, 1984; Gelderblom *et al.*, 2001, 2002). These fungi can easily change their genetic makeup in response to their immediate environment and this gives them an ability to adapt to diverse environments (Burgess, 1981). Since *Fusarium* species are phytopathogenic mainly to agricultural crops, much attention from researchers has been directed to species from agricultural ecosystems with limited attention directed to species that occur in native uncultivated (virgin) soils (Brown, 1958; Wicklow, 1973). However, the ubiquitous nature of *Fusarium* makes it part of the fungal communities of native uncultivated soils and the plant hosts associated with them (England and Rice, 1957; Baird and Carling, 1998). Two recently identified *Fusarium* species, *Gibberella konzum* (*Fusarium konzum*) Zeller, Summerell and Leslie isolated from prairie grasses of tall grass prairie ecosystem (Kansas, U.S.A) and

G. gaditjirii (*F. gaditjirii*) Phan, Burgess and Summerell isolated from northern Australia both originated from native grasses (Zeller *et al.*, 2003; Phan *et al.*, 2004). Based on surveys of *Fusarium* species in South African soils, these fungi occur in plant debris of both cultivated and uncultivated soils (Marasas *et al.*, 1988; Rheeder and Marasas, 1998).

The taxonomy of *Fusarium* has always been in a disorganized state due to different researchers formulating taxonomic systems that contradict each other such as that of Wollenweber and Reinking (1935) and Snyder and Hansen (1940), which formed the basis of all modern taxonomic systems of the genus *Fusarium* (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983). Also, all these taxonomic systems were based on morphological characteristics, which are greatly influenced by the immediate environment where these fungi occur. As expected, different observations were made in different laboratories and hence different taxonomic systems for the same genus were formulated (Wollenweber and Reinking, 1935; Snyder and Hansen, 1940, 1941, 1945; Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983).

Molecular techniques such as the development of universal primers for Polymerase Chain Reaction (PCR) of different parts of fungal genomes (Glass and Donaldson, 1995), genotyping based on polymorphic regions in the genome and DNA sequence analyses (Samuels and Seifert, 1995; Taylor *et al.*, 2000) have all contributed to the identification of a number of fungal genera. In *Fusarium*, combining molecular markers such as genotyping (Xu and Leslie, 1996; Nelson *et al.*, 1997; Britz *et al.*, 2002) and DNA sequence analyses (O'Donnell *et al.*, 1998, 2000; Kristensen *et al.*, 2005) with traditional morphological characteristics used in species identification has allowed for

revision of the morphological species concept (Nirenberg and O'Donnell, 1998; O'Donnell *et al.*, 1998), which has greatly dominated the taxonomy of this genus.

Therefore, in this study, both micro- and macromorphological characteristics were used as the basis for identification of *Fusarium* isolates. In addition, restriction fragment length polymorphisms of PCR-amplified translation elongation factor 1 alpha (TEF-1 α) and the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) were used to confirm the morphological groupings of the isolates. Furthermore, sequence analyses of three genes namely TEF-1 α , the ITS region and β -tubulin were used in the phylogenetic analyses of the *Fusarium* isolates obtained in this study.

3. MATERIALS AND METHODS

3.1. Study sites and sampling period

Six sites in the pristine fynbos were chosen for sampling. These were Silvermine Nature Reserve (S 34° 06.650' E 18° 24.367); Tokai forest (S 34° 03.271' E 18° 24.656); Trappieskop (S 34° 07.557' E 18° 26.734); Boyes Drive (S 34° 05.859' E O18° 27.900) and two sites at Cape Point Nature Reserve (S 34° 15.757' E 18° 23.101), Olifantsbos A and B. Boyes Drive and Olifantsbos B were both burnt three months before sampling had commenced. Soil samples were collected in April, June, September and November 2006. In general, the abundance of fungal propagules in soil decreases with soil depth, with samples from depths more than 30 cm from the soils surface yielding relatively few fungal propagules (Leslie and Summerell, 2006). Therefore, at each sampling site a 5 m \times 5 m grid was demarcated and soil samples were collected at a depth of 20 cm using

an auger (0.075 m diameter). Each soil sample was a composite of six sub-samples taken randomly within the demarcated grid, which were collected into sterile, sealable polyethylene bags and transported to the laboratory (Department of Microbiology, University of Stellenbosch, South Africa) to be stored at 4°C until further analysis.

3.2. Handling of soil samples and plant debris

Soil samples were first sieved through a nest of two sieves (2 mm and 0.5 mm mesh), under aseptic conditions to separate plant debris which included roots, leaves and wooden material. Manually homogenized soils were placed in sterile sealable plastic containers. The plant debris separated from soil samples was handled according to Nelson *et al.* (1983). A tap was fitted with a spray nozzle to produce a fine spray, which was used to remove soil particles from the plant debris. Depending on the amount of plant debris that was present from each sample, the duration of washing was 30 to 60 min for 1 to 15 g of plant debris. Washed plant debris was placed on sterile paper towels and air-dried overnight in a laminar flow bench. Recovery of *Fusarium* isolates from soils and plant debris was carried out within five days of sampling (Nelson *et al.*, 1983).

3.3. Isolates recovery and subculturing

Fusarium was recovered from fynbos soils using the soil dilution technique. Soil dilutions were prepared by suspending 10 g of soil in 100 ml 0.05% Water Agar (WA) in 500 ml shott bottles. A ten-fold series of dilutions for each soil sample was made and 1 ml from each dilution series was plated out in triplicate on peptone-PCNB agar (15 g

Peptone; 1 g KH₂PO₄; 0.5 g MgSO₄·7H₂O; 750 mg Pentachloronitrobenzene in 1 L distilled water) (Burgess *et al.*, 1991).

Fusarium was recovered from the plant debris by placing five to ten pieces of plant material on the surface of peptone-PCNB agar (90 mm diameter) plates. The plates were incubated in the laboratory under natural day-night rhythm near a window for diffused light at ± 22°C for 7 days. Subculturing of *Fusarium* isolates recovered from both soils and plant debris was done by mass transfer of aerial mycelium of *Fusarium*-like colonies onto Synthetischer Nährstoffarmer agar (SNA) (Leslie and Summerell, 2006). All plates were incubated under the same conditions as previously mentioned.

To obtain single-conidial cultures of all the isolates, spore-dilutions were prepared from 7 day old SNA cultures. A culture was placed under a stereomicroscope to observe a single sporodochium, and with the tip of a sterile needle a mass of conidia was scooped and suspended in 10 ml sterile dH₂O. The suspension was mixed by vortexing and poured into 2% water agar (WA) but excess spore suspension was immediately discarded to avoid excess conidia settling onto the agar surface. The 2% WA plate containing the conidia was incubated one-side elevated overnight in the laminar flow bench to allow conidial germination (Leslie and Summerell, 2006). A single germinating conidium was excised using a sterile needle and plated on Carnation leaf Agar (CLA) (Fisher *et al.*, 1982) to induce constant morphological characteristics and Potato Dextrose Agar (PDA, Biolab, Johannesburg, South Africa) to induce cultural pigmentation (Nelson *et al.*, 1983). These were incubated under the same conditions as above.

3.4. Morphology

Morphological characteristics such as conidia type, shape and size were observed on CLA, while cultural characteristics such as colony morphology, growth rates and pigmentation were observed on PDA. Growth rates of cultures were determined on PDA under three different incubation conditions. These were incubation under natural day-night rhythm, near a window in the laboratory for diffused light ($\pm 22^{\circ}\text{C}$ for 3 days); incubation at 25°C for 3 days in an incubator; and thirdly incubation at 30°C in complete darkness for 3 days. Growth rates were measured in triplicate and an average of the three measurements was taken. The Methuen handbook of colour (Kornerup and Wanscher, 1978) was used for colony pigmentation. Observation of morphological characters was made from material mounted in 30% lactic acid. Fifty measurements were taken for each of the morphological relevant structures and the averages as well as standard deviations were calculated. All *Fusarium* isolates were identified to *Fusarium* section level (Nelson *et al.*, 1983) based on morphological and cultural characteristics of single-conidial strains (Leslie and Summerell, 2006).

3.5. Molecular identification

3.5.1. DNA extraction and PCR amplification

DNA was extracted using a modified method of Möller *et al.* (1992). Approximately, 50 mg mycelium was scraped directly from the surface of a 10 day PDA culture using a sterile scalpel and transferred to a clean 2 ml Eppendorf tube. The mycelium was immediately frozen in liquid nitrogen, ground into a fine powder using a sterile plastic micro-pestle. Subsequently, the powder was re-suspended in 500 μl TES lysis buffer (100

mM Tris pH8; 10 mM EDTA; pH8; 2% (w/v) SDS) and 5 μ l (100 ng/ml) and Proteinase K was added. The suspension was mixed by inverting the tubes twice, followed by incubation at 60°C for 60 min in a water bath. Subsequently, 140 μ l 5 M NaCl and 65 μ l pre-warmed (60°C) 10% (w/v) CTAB were added and the suspension incubated at 65°C for 10 min. Proteins were denatured by adding an equal volume of SEVAG [chloroform: isoamylalcohol (24:1, v/v)] and incubating on ice for 15 min. The suspension was centrifuged at 13000 \times g for 10 min in a micro-centrifuge at 4°C. The aqueous supernatant was transferred to a clean 1.5 ml Eppendorf tube, and the DNA precipitated by addition of 0.55 volumes cold isopropanol and placing the tube on ice for 15 min. The DNA was pelleted by centrifugation at 13000 \times g for 10 min in a micro-centrifuge at 4°C. The supernatant was discarded and the pellet washed twice with cold 70% (v/v) ethanol. The pellet was air-dried in a laminar flow bench and DNA re-suspended in 50 μ l MilliQ water. Genomic DNA concentrations were determined using the NanoDrop[®] ND-100 spectrophotometer (Central Analytical Facilities, University of Stellenbosch, South Africa). Extracted DNA was stored at -20°C until used.

All PCR were performed in a GeneAmp[®] PCR System 9700 thermal cycler (Applied Biosystems, Drive Foster City, CA, USA). The TEF-1 α gene fragments were amplified using *Fusarium* specific primers (Table 1) (Inqaba Biotechnical Industries (Pty) Ltd., South Africa). An amplification reaction was 25 μ l containing: 1 μ l DNA template, 0.5 μ l (10 μ M) of each primer and 23 μ l (2 \times) *KapaTaq* Ready Mix (Kapa Biosystems, Cape Town, South Africa). Cycling conditions in the thermal cycler were as follows: An initial denaturing step at 94°C for 5 min, followed by 34 cycles consisting of

94°C for 30 s, primers annealing at temperature (Table 1) for 45 s, chain elongation at 72°C for 1 min and an additional chain elongation step at 72°C for 7 min.

A similar procedure was followed when preparing reaction mixtures for the amplification of the β -tubulin and the ITS region fragments, where the respective primer sets (Table 1) were added. Cycling conditions in the thermal cycler were as follows: An initial denaturing step at 94°C for 5 min, followed by 34 cycles consisting of 94°C for 30 s, primers annealing at respective temperatures (Table 1) for 45 s, chain elongation at 72°C for 1 min and an additional chain elongation step at 72°C for 7 min. All control reactions contained the same components; however, an equal volume of MilliQ water was added to the reaction mixtures instead of the template DNA. The PCR products were visualized on 1% (w/v) agarose gels stained with ethidium bromide (1 μ g/ml) under UV illumination and DNA concentrations were determined using the NanoDrop[®] ND-100 spectrophotometer (Central Analytical Facilities, University of Stellenbosch, South Africa).

3.5.2. RFLP analyses of PCR-based *TEF-1 α* and ITS region

The gene fragments that were employed in the RFLP-analyses were the *TEF-1 α* and ITS region. The *TEF-1 α* PCR products were digested with restriction endonucleases *AluI*, *AvaI* and *RsaI*. The digestion reaction of each restriction endonuclease was 25 μ l containing: 0.1 μ l (1 U) restriction endonuclease, 2.5 μ l (10 \times) Buffer (Fermentas Pty (Ltd), Cape Town, South Africa), 6 μ l of PCR product and 16.6 μ l dH₂O.

The same procedure was followed when preparing the digestion reactions of the PCR products of the ITS region, except that restriction endonucleases *EcoRI* and *MspI*

(Fermentas Pty (Ltd), Cape Town, South Africa) were used. All digestion reactions were incubated at 37°C for 2 h in a water bath to ensure complete digestion. The digestion products were separated electrophoretically on 2% (w/v) agarose gels stained with ethidium bromide (2 µg/ml) at 60 mV for 2 h and gels were visualized under UV illumination.

3.5.3. Sequencing and Phylogenetic analyses

All amplified PCR products of TEF-1 α , β -tubulin and ITS region were purified using the Invitex DNA cleaning kit (Gesellschaft für Biotechnik & Biodesign mbH, Germany) according to the manufacturer's instructions. Sequencing was performed in a GeneAmp[®] PCR System 9700 thermal cycler using the same primers (Table 1) as in the PCR amplifications. The ABI PRISM[™] Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) was used for sequencing and sequence analyses were performed on a Perkin Elmer ABI3100 genetic analyzer (Analytical Facilities, University of Stellenbosch, South Africa).

The nucleotide sequences obtained in this study were edited using DNASTar SeqMan[™] II (DNA-STAR Inc., WI, USA). The β -tubulin and ITS region sequences were compared with other available *Fusarium* sequences on nucleotide Basic Local Alignment Search Tools (n-BLAST) software (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/). The TEF-1 α sequences were compared with other *Fusarium* TEF-1 α sequences on the FUSARIUM-ID v.1.0 database (www.fusarium.cbio.psu.edu) (Geiser *et al.*, 2004).

For each gene (TEF-1 α , β -tubulin and ITS region), a sequence dataset was created by downloading all sequences that showed high identity scores from Genbank. Edited sequences of the *Fusarium* isolates in this study were also added into each gene sequence database.

Sequence alignments were performed in CLUSTALX version 1.8 (Thompson *et al.*, 1997) and phylogenetic relationships between taxa were determined using distance analysis in PAUP version 4.0b10 (Swofford, 2001). Characters were treated as unweighted in the analysis and gaps were treated as missing data. The confidence levels of the nodes of distance analysis trees were evaluated by performing a bootstrap analysis of 1000 random replicates.

4. RESULTS

4.1. Morphology

In total, one hundred and twenty two (122) *Fusarium* isolates were recovered from fynbos soils and plant debris during this study (Table 2). The frequency of *Fusarium* isolates recovered differed between the sampling sites, the source of recovery and the sampling months (Appendix A). Boyes Drive sampling site had a significantly higher number (48/122) of *Fusarium* isolates compared to the other sites [Silvermine, Trappieskop, Tokai and Olifantsbos (burnt and unburnt)], which did not differ significantly. This was also true for all the months of recovery as Boyes Drive was the site with most isolates obtained (Table 2). Furthermore, more *Fusarium* isolates were

recovered from plant debris than soil and August was the month with the most *Fusarium* isolates recovered.

The identification of *Fusarium* isolates based on morphological characteristics resulted in the placement of most of the isolates into three *Fusarium* sections, namely, section *Elegans*, *Martiella-Ventricosum* and *Gibbosum* (Table 3). However, there was an additional morphological group comprised of nine strains, which could not be confidently placed under any *Fusarium* section (Nelson *et al.*, 1983). These strains were considered to represent an undescribed species and are collectively referred to as *Fusarium* ‘fynbos’ for the rest of this chapter.

One hundred and two (102) *Fusarium* isolates were grouped under *Fusarium* section *Elegans* and this was the dominant morphological group. Morphologically, all isolates within this group were typical section *Elegans* isolates (Table 3). However, there were considerable variations in their cultural characteristics. Variations were mainly observed in the colony pigmentations, the chlamydospore formation and disposition.

There were nine and two isolates respectively belonging to section *Martiella-Ventricosum* and section *Gibbosum*. Morphological characteristics, displayed by isolates within these two sections, were fairly constant (Table 3) and this can, however, be due to the small sample size analyzed. The *Fusarium* ‘fynbos’ group comprised of nine isolates formed distinctive morphological characteristics that were constant in all the strains (Table 3).

4.2. RFLP analyses of PCR-based TEF-1 α and ITS regions

PCR-based RFLP analyses were performed to confirm the validity of the morphological groups or sections in which the isolates were placed. In sections *Elegans* and *Martiella-Ventricosum*, the TEF-1 α -RFLP (Appendix B) analyses were more informative compared to the ITS-RFLP analyses, hence they were chosen for handling isolates in these two groups. No PCR products for the TEF-1 α gene fragment were obtained from *Fusarium* ‘fynbos’ isolates, however, the amplification of the ITS region was successful. Therefore, this group was treated together with section *Gibbosum* isolates using ITS-RFLP analysis.

Amplification with TEF-1 α primers (Table 1) produced a fragment of approximately 650 bp (base pairs), which was consistent in all isolates. Of the three restriction endonucleases (*AluI*, *AvaI* and *RsaI*) used in TEF-1 α -RFLP, *RsaI* and *AluI* produced consistent digests, while *AvaI* generated irreproducible results and was consequently excluded from this study.

Due to a large number of isolates placed in section *Elegans* (Table 3), and the inconsistency in morphological characteristics displayed by these isolates, representative subsets of twenty isolates (D3; D122; C135; C168; D177; D180; E184; E186; C198; C140; C152; C12; D4; A194; B170; B5; B203; E187; A2 and F117) were analyzed. Digestion of TEF-1 α fragments of the representative isolates with endonuclease *RsaI* generated three RFLP patterns (Fig. 1), while *AluI* generated three RFLP patterns (Fig. 2) within these isolates. The RFLP patterns revealed substantial genetic variability in the isolates of section *Elegans*.

Furthermore, there were nine isolates (D20; D23; F165; C169; F183; F200; C206; A209; C214) grouped under section *Martiella-Ventricosum* and digestion of the TEF-1 α fragments of these isolates with *RsaI* generated two RFLP patterns (Fig. 3), while digestion with *AluI* generated only one pattern (Fig. 4). There was not much genetic variability detected from the TEF-1 α -RFLP analysis of isolates within this group and this may possibly be due to the small number of isolates analyzed.

In addition, the amplification of the ITS region with primers (Table 1) resulted in approximately 550-570 bp fragments consistent in all isolates within section *Gibbosum* and *Fusarium* 'fynbos'. Digestion of the ITS-PCR products of section *Gibbosum* isolates (D216 and D232), as well as *Fusarium* 'fynbos' representative isolates (D175; D192; F179; E182; D218; D219 and D227) using *EcoRI*, produced two RFLP patterns in both morphological groups (Fig. 5). Section *Gibbosum* isolate, D232, had an identical RFLP pattern to *Fusarium* 'fynbos' isolates D175, D192 and D227. Furthermore, the ITS region of all the isolates within section *Gibbosum* as well as representative isolates of *Fusarium* 'fynbos' had no *MspI* restriction sites (Fig. 6). The observed ITS-RFLP results of section *Gibbosum* and *Fusarium* 'fynbos' isolates contradict the morphological distinctness of these two groups from each other.

4.3. Sequencing and Phylogenetic analyses

For both sections *Elegans* (*F. oxysporum* Schlecht. emend. Snyder and Hans.) and *Martiella-Ventricosum* (*F. solani* (Martius) Appel and Wollenw. emend. Snyder and Hans.), phylogenetic analyses were based on the TEF-1 α and β -tubulin gene sequences. For section *Gibbosum* (*F. equiseti* (Corda) Sacc.), phylogenetic analyses were based on

the ITS region and β -tubulin gene sequences, while only the ITS region sequences were employed in the phylogenetic analysis of the *Fusarium* ‘fynbos’ isolates.

Based on the neighbour-joining tree of the TEF-1 α (Fig. 7), the 26 representative isolates of *F. oxysporum* (C140; A2; A194; B5; D161; D225; D233; F117; D180; E184; D122; C152; D177; C168; D4; B170; B203; D3; D234; F205; F189; C12; C198; C135; E186 and E187) were interspersed within different clades in the *F. oxysporum* species complex. Also, there was no clear pattern of clustering of isolates based on their geographic origin.

The neighbour-joining analysis tree of β -tubulin (Fig. 8) conducted with the same representative isolates of *F. oxysporum* also had similar results as the TEF-1 α phylogenetic analysis. The representative isolates showed to be interspersed within different clades of the *F. oxysporum* species complex and there was no clear pattern of clustering between isolates based on geographic origin. In general, the neighbour-joining analyses of both TEF-1 α and β -tubulin revealed that there was substantial genetic variability in the representative isolates of *F. oxysporum* and this indicated that strains occurring in this particular habitat may not be propagating clonally. Furthermore, some isolates showed to be closely related to *formae speciales* (strains pathogenic to particular plant hosts) within the *F. oxysporum* species complex. Isolates that showed to maintain similar clustering patterns in neighbour-joining analyses of both genes are colour-coded (Figs. 7–8).

Phylogenetic analysis of *F. solani* isolates based on the neighbour-joining tree of TEF-1 α (Fig. 9) revealed two isolates (D23 and F200) to be interspersed within different clades of the tree. Isolate D23 formed a monophyletic group with *F. solani* strains with a

cosmopolitan origin, while isolate F200 grouped into a monophyletic clade with *F. solani* strains originating from soils worldwide. Interestingly, the seven remaining strains (A209; C214; F165; C206; D20; F183 and C169) formed a monophyletic clade closely related to *F. solani* strains of the soil origin but distantly related to those with a cosmopolitan origin.

Based on the results of the neighbour-joining analysis of β -tubulin (Fig. 10), the pattern of clustering of *F. solani* isolates was not significantly different from that observed in the phylogenetic analysis of TEF-1 α . Isolates D23 and D20 were interspersed within different clades distantly related to each other and the rest of the other isolates. The rest of the isolates (F200; C214; C169; C206; A209; F165 and F183) clustered in a monophyletic group closely related to isolate D20. In general, the neighbour-joining analyses of both TEF-1 α and β -tubulin of *F. solani* isolates revealed potential genetic variability within this group but the size of the sample analyzed was too small to validate the results. In addition, there was no correlation between the clustering pattern of these isolates and their geographic origin. Isolates that showed to maintain similar clustering patterns in neighbour-joining analyses of both genes are colour-coded (Figs. 9–10).

The neighbour-joining tree of the ITS region (Fig. 11) for both *F. equiseti* and *Fusarium* ‘fynbos’ resulted in clustering of the two *F. equiseti* isolates (D216 and D232) in a monophyletic clade with other *F. equiseti* strains. The *F. equiseti* isolates clearly resolved into a separate clade from *Fusarium* ‘fynbos’ isolates (D175; D192; D218; D219; E173; D227; F179; E182; E195). *Fusarium equiseti* isolates appeared to be closely related to *F. chlamydosporum* (section *Sporotrichiella*). However, *Fusarium* ‘fynbos’ isolates formed a distinctive clade that was closely related to *F. lateritium*. Based on the

β -tubulin neighbour-joining analysis (Fig. 12), the two *F. equiseti* isolates formed a separate clade from *F. subglutinans*, *F. oxysporum* f. sp. *lilii/ lycopersici/ radicis-lycopersici* and *F. lateritium* strains.

5. DISCUSSION

Acidic soils such as fynbos soils support the growth of primarily fungal populations and the genus *Fusarium* is one of the fungal genera reported to be associated with fynbos plant root systems (Allsopp *et al.*, 1987). In this study, *F. oxysporum*, *F. solani* and *F. equiseti* were shown to be prevalent in the native pristine, low nutrient fynbos soil. These are all cosmopolitan soilborne species that have been isolated in all types of soils and substrates (Burgess, 1981; Stoner, 1981). Also, a group of undescribed strains (*Fusarium* 'fynbos') were recovered and are described in chapter 3 of this thesis. Interestingly, these fungi showed more prevalence in the fynbos soil compared to *F. equiseti*, which had only two taxa recovered from fynbos soil. One reason for this might be the difference in the chlamydospores forming capabilities between these two fungi. Furthermore, the recovery frequency of *F. oxysporum*, *F. solani* and *F. equiseti* from the fynbos soil and plant debris differed significantly. However, the observed distribution of these three species in the fynbos soil generally resembles that reported for species associated with plant debris of uncultivated and cultivated South African soils (Doidge, 1938; 1950; Marasas *et al.*, 1988; Jeschke *et al.*, 1990; Rheeder and Marasas, 1998). In one study, Marasas *et al.* (1988) used the Table Mountain plateau (a fynbos area) as one of their sampling sites. No *F. oxysporum*, *F. solani* or *F. equiseti* species were recovered from this area. Therefore, to the best of our knowledge this study is the

first to report on the prevalence of these three *Fusarium* species in uncultivated fynbos soil within the Cape Peninsula area.

In addition, surveys conducted on *Fusarium* species in South African soils (Marasas *et al.*, 1988; Rheeder and Marasas, 1998) have only focused on those species that are associated with plant debris. However, in this study we expanded the spectrum of species that can be recovered by including the soil using the soil dilution technique. Our findings in this regard were in agreement with those of Marasas *et al.* (1988) and Rheeder and Marasas (1998) as significantly more of the fynbos *Fusarium* species were recovered from plant debris than the soil dilutions. This could indicate that *Fusarium* species occurring in the fynbos soils are closely associated with plant material mostly as saprophytes that play part in plant material decomposition.

Even though this study was not an intense survey of *Fusarium* species in the fynbos soils, the number of recovered *Fusarium* species was still very low. These results, however, came as no surprise since it is well accepted that the climate and even local variations in weather can limit the range of *Fusarium* species observed from a particular habitat (Leslie and Summerell, 2006). This in turn influences their relative frequency of recovery. Our results in this regard correlated well with the survey (Marasas *et al.*, 1988), in which two undisturbed indigenous sites, Table Mountain (plateau) and Knysna forest had low recovery frequencies and only two *Fusarium* species were recovered from each site. Also, *Fusarium* isolates recovered from this study displayed substantial genetic variability. This was no surprise since the fynbos area chosen for sampling is both a native and non-agricultural niche, which means that the *Fusarium* populations obtained

are expected to sustain more genetic variation than populations from an agricultural ecosystem (Leslie and Summerell, 2006).

Based on both morphology (Nelson *et al.*, 1983) and molecular markers, *F. oxysporum* was found to be the most dominant species in the fynbos soils. This fungus is pathogenic to a number of plant hosts and the pathogenicity of some strains is host specific (*formae speciales*) (Nelson *et al.*, 1981). The dominance of *F. oxysporum* in all the sampling sites is indicative of the pioneering status of these fungi in the fynbos soils. *Fusarium oxysporum* strains are good primary colonizers and display competitive saprophytic abilities compared to both *F. solani* and *F. equiseti* (Stoner, 1981; Leslie and Summerell, 2006), which were isolated in very low numbers during this study. The morphological variability displayed by *F. oxysporum* isolates in this study was expected since this is one of the nine species of Snyder and Hansen (1940), which consists of variable taxa with uncertain taxonomic status (Leslie and Summerell, 2006). Also, there was significant genetic variability in *F. oxysporum* obtained from the native fynbos soils and this correlates with the findings of Gordon *et al.* (1992), in which mitochondrial DNA (mtDNA) haplotypes revealed genetic diversity in native soil populations of this species.

Fusarium solani is also one of the nine species of Snyder and Hansen (1941), which consists of a number of variable taxa. This fungus, which is similar to *F. oxysporum*, is a soilborne fungus that occurs in native soils as well (Marasas *et al.*, 1988). *Fusarium solani* infects a number of plant hosts, causing destructive wilts and rots (Burgess, 1981). *Fusarium equiseti* is also a soil inhabiting fungus that has been recovered from very dry areas (Joffe and Palti, 1977; Mandeel, 1996). The pathogenicity

of this species has not been conclusively proven but it is regarded to occur as a secondary colonizer in diseased plant tissues (Leslie and Summerell, 2006).

6. CONCLUSION

The current study has provided information on the occurrence and distribution of *Fusarium* species in uncultivated fynbos soils. The most dominant species, *F. oxysporum*, displayed significant morphological and genetic variability. This was expected as non-pathogenic and saprophytic strains of this species occurring in undisturbed soils display these traits. Both *F. oxysporum* and *F. solani* are pathogenic to a number of plant hosts and some of the isolates from these two species showed close affinities with *formae speciales* strains. No pathogenicity tests have yet been carried out to ascertain the pathogenicity of the strains isolated during this study. However, no diseased plants were observed in and within the vicinity of our sampling sites. Regarding the potential pathogenicity of *F. oxysporum* and *F. solani* strains in the fynbos soils, we speculate that even if there are pathogenic strains of these species capable of causing diseases on fynbos plants within these areas, their propagation could be suppressed by the presence of the saprophytic *F. oxysporum* and *F. solani* strains. Thus this study has showed the potential of the fynbos area to serve as a reservoir of pathogenic *Fusarium* species and an environment harbouring unknown native *Fusarium* species such as *Fusarium* ‘fynbos’ that was recovered in this study.

Table 1. PCR primers used for species identification and gene sequencing of *Fusarium* isolates from fynbos soils and plant debris.

Morphological group	Target gene(s)	Primer name	Primer sequence	Annealing temperature (°C)
section <i>Elegans</i> ;	Translation elongation factor 1-alpha (TEF-1 α)	EF1 ^a	5'-ATG GGT AAG GAG GAC AAG AC-3'	62
section <i>Martiella-Ventricosum</i>		EF2 ^a	5'-GGA AGT ACC AGT GAT CAT GTT-3'	
section <i>Elegans</i> ;	β - tubulin (tub-2)	T1 ^a	5'-AAC ATG CGT GAG ATT GTA AGT-3'	61
section <i>Martiella-Ventricosum</i> ; section <i>Gibbosum</i>		T2 ^a	5'-TAG TGA CCC TTG GCC CAG TTG-3'	
section <i>Gibbosum</i>	Internal transcribed spacer (ITS-1 and ITS-2) regions	ITS1 ^b	5'-TCC GTA GGT GAA CCT GCG G-3'	56
<i>Fusarium</i> 'fynbos'		ITS4 ^b	5'-TCC TCC GCT TAT TGA TAT GC-3'	

Primer sequence references: a = O'Donnell *et al.*, 1998; b = White *et al.*, 1990

Table 2. The number of *Fusarium* species recovered from soil and plant debris during this study.

<i>Fusarium</i> species	Sampling site						Total isolates
	A	B	C	D	E	F	
<i>F. oxysporum</i> (section <i>Elegans</i>)	18	16	13	39	6	10	102
<i>F. solani</i> (section Martiella-Ventricosum)	1	0	3	2	0	3	9
<i>F. equiseti</i> (section <i>Gibbosum</i>)	0	0	0	2	0	0	2
<i>Fusarium</i> 'fynbos'	0	0	0	5	3	1	9
							122

A= Silvermine; B= Tokai; C = Trappieskop; D = Boyes Drive; E = Olifantsbos (unburnt); F = Olifantsbos (burnt)

Table 3. A summary of morphological characters dominant in isolates within sections *Elegans*, *Martiella-Ventricosum*, *Gibbosum* and morphological group *Fusarium* ‘fynbos’ (this study).

Section	Macroconidia from sporodochia	Microconidia	Chlamydoconidia	Growth rate (25°C)	Colony colour (PDA)
<i>Elegans</i>	Medium ($\approx 30\text{-}35\mu\text{m}$); 3 to 6 septate; slender and dorsiventrally curved; hooked to papillate apical cells; foot-shaped basal cells	Few to abundant; small to medium in size ($\approx 5\text{-}10\mu\text{m}$); oval and reniform shaped; borne on short monophialides	Abundant, took 3-4 weeks to form in some isolates; in pairs or short chains; terminal and intercalary	Rapid; $\approx 40\text{mm}$ after 3 days	tan; white tinged with violet; light to dark purple and magenta
<i>Martiella-Ventricosum</i>	Medium ($\approx 25\text{-}30\mu\text{m}$); 3 to 4 septate; stout and cylindrically shaped; blunt apical cells; distinctively notched to barely notched basal cells	Few to abundant; small ($\approx 10\mu\text{m}$); oval to ellipsoid shaped; borne on long monophialides	Present but few; in pairs; terminal and intercalary	Slow; $\approx 25\text{mm}$ after 3 days	Light brown or beige
<i>Gibbosum</i>	Large ($\approx 40\text{-}45\mu\text{m}$); 3-4 septate; dorsi-ventrally curved; tapering apical cells; pronounced foot-shaped basal cells	Absent	Abundant; chains or clumps	Rapid; $\approx 40\text{mm}$ after 3 days	Reddish brown
<i>Fusarium</i> ‘fynbos’	Medium ($\approx 25\text{-}30\mu\text{m}$); 3 to 6 septate; dorsi-ventrally curved; hooked apical cells; distinctively notched basal cells	Sparse to few; small ($\approx 5\mu\text{m}$); oval shaped	Abundant, chains; terminal and intercalary	Rapid; $\approx 32\text{mm}$ after 3 days	Dark brown

Fusarium ‘fynbos’: morphological group comprised of nine taxa not placed under any *Fusarium* section based on Nelson *et al.* (1983)

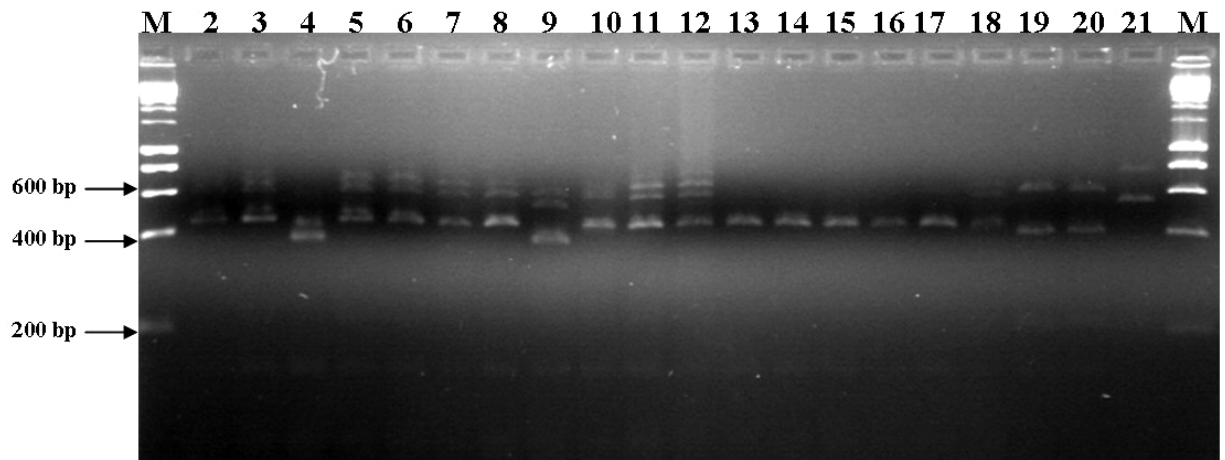


Figure 1. Restriction patterns of PCR-amplified translation elongation factor 1 alpha of section *Elegans* isolates digested with *RsaI*. Restriction patterns were analyzed in 2% agarose gel stained with ethidium bromide. **M** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-21** (section *Elegans* isolates): D3; D122; C135; C168; D177; D180; E184; E186; C198; C140; C152; C12; D4; A194; B170; B5; B203; E187; A2; F117.

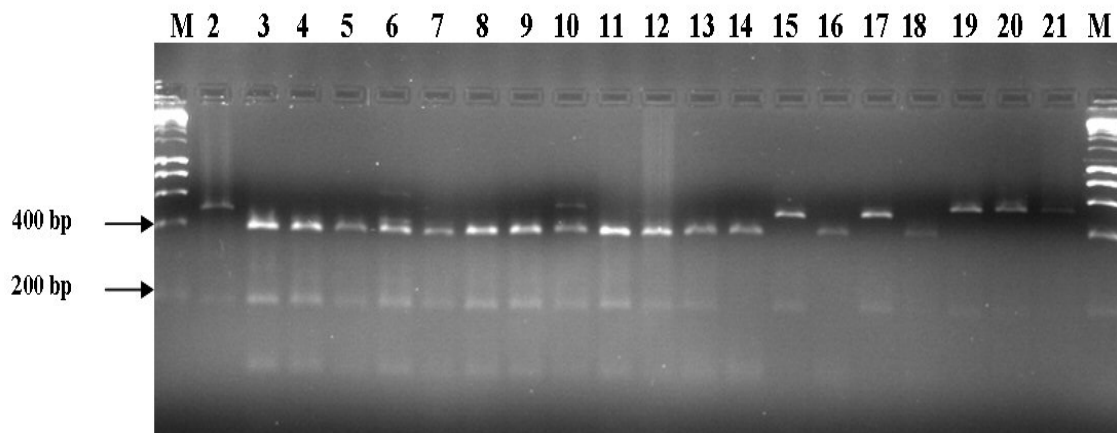


Figure 2. Restriction patterns of PCR-amplified translation elongation factor 1 alpha of section *Elegans* isolates digested with *AluI*. Restriction patterns were analyzed in 2% agarose gel stained with ethidium bromide. **M** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-21** (section *Elegans* isolates): D3; D122; C135; C168; D177; D180; E184; E186; C198; C140; C152; C12; D4; A194; B170; B5; B203; E187; A2; F117.

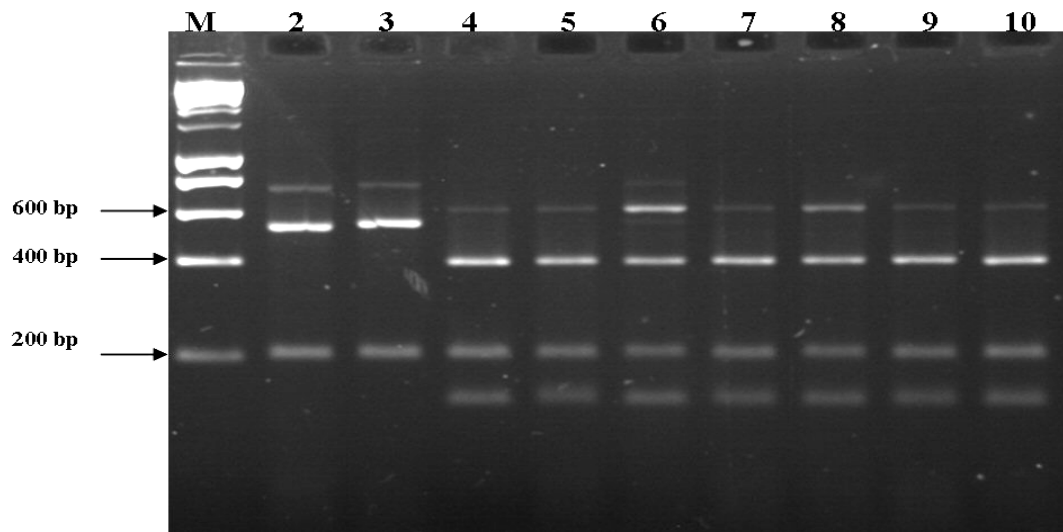


Figure 3. Restriction patterns of PCR-amplified translation elongation factor 1 alpha of section *Martiella-Ventricosum* isolates digested with *RsaI*. Restriction patterns were analyzed in 2% agarose gel stained with ethidium bromide. **M** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-10** (section *Martiella-Ventricosum* isolates): D20; D23; F165; C169; F183; F200; C206; A209; C214.

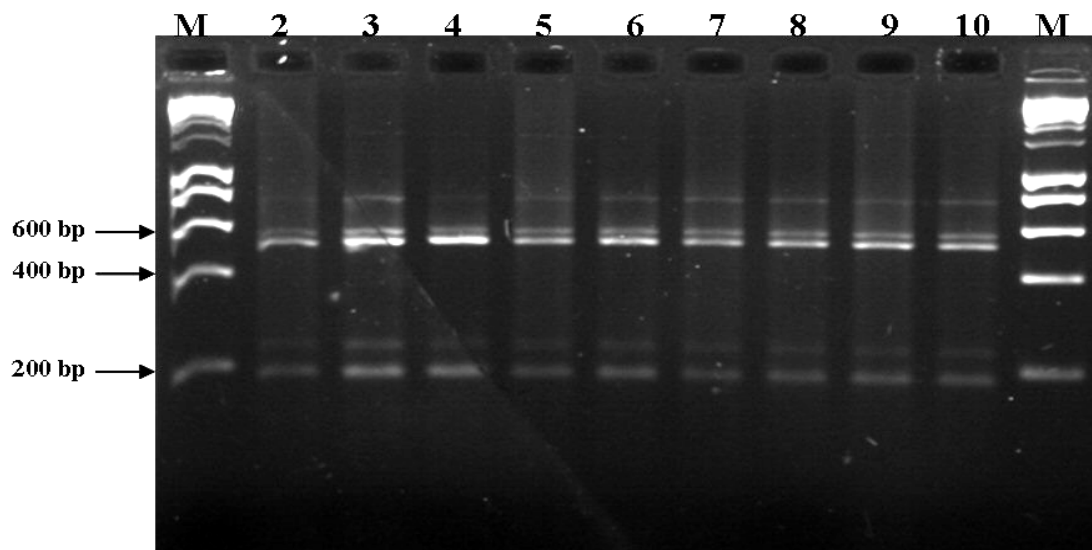


Figure 4. Restriction patterns of PCR-amplified translation elongation factor 1 alpha of section *Martiella-Ventricosum* isolates digested with *AluI*. Restriction patterns were analyzed in 2% agarose gel stained with ethidium bromide. **M** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-10** (section *Martiella-Ventricosum* isolates): D20; D23; F165; C169; F183; F200; C206; A209; C214.

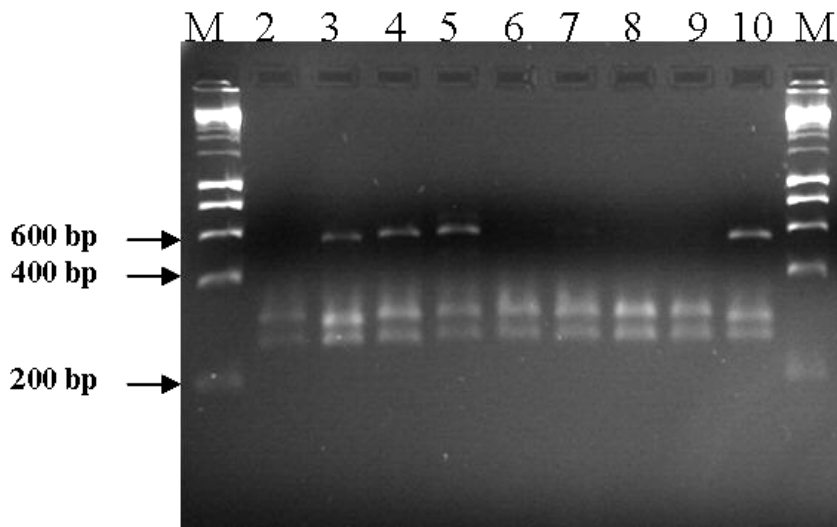


Figure 5. Restriction patterns of PCR-amplified internal transcribed spacer regions of section *Gibbosum* and *Fusarium* ‘fynbos’ digested with *EcoRI*. Restriction patterns were analyzed in 2% agarose gel stained with ethidium bromide. **M** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-5** (section *Gibbosum*): D216; D232; **lanes 6-10** (*Fusarium* ‘fynbos’): D175; D192; F179; E182; D218; D219; D227.

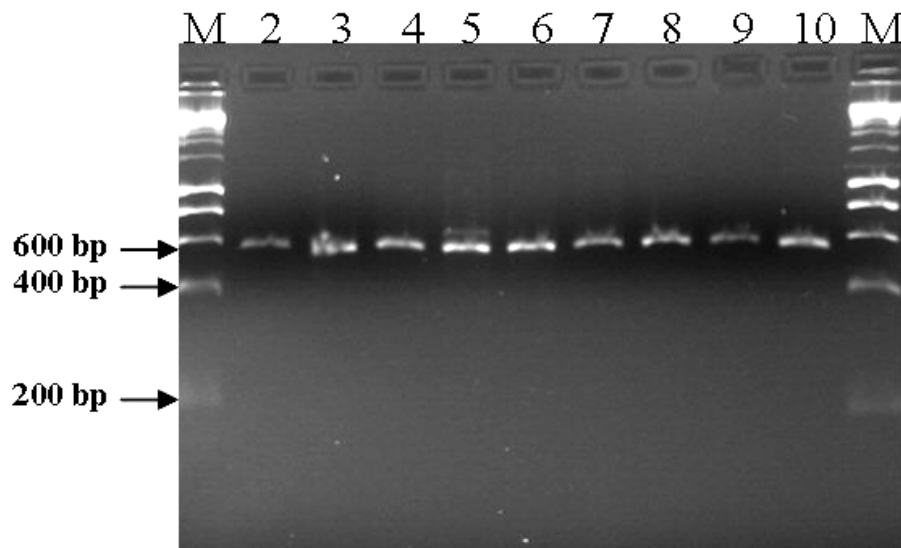


Figure 6. Restriction patterns of PCR-amplified internal transcribed spacer regions of section *Gibbosum* isolates and *Fusarium* ‘fynbos’ digested with *MspI*. Restriction patterns were analyzed in 2% agarose gel stained with ethidium bromide. **M** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-3** (section *Gibbosum*): D216; D232; **lanes 4-10** (*Fusarium* ‘fynbos’): D175; D192; F179; E182; D218; D219; D227.

NJ

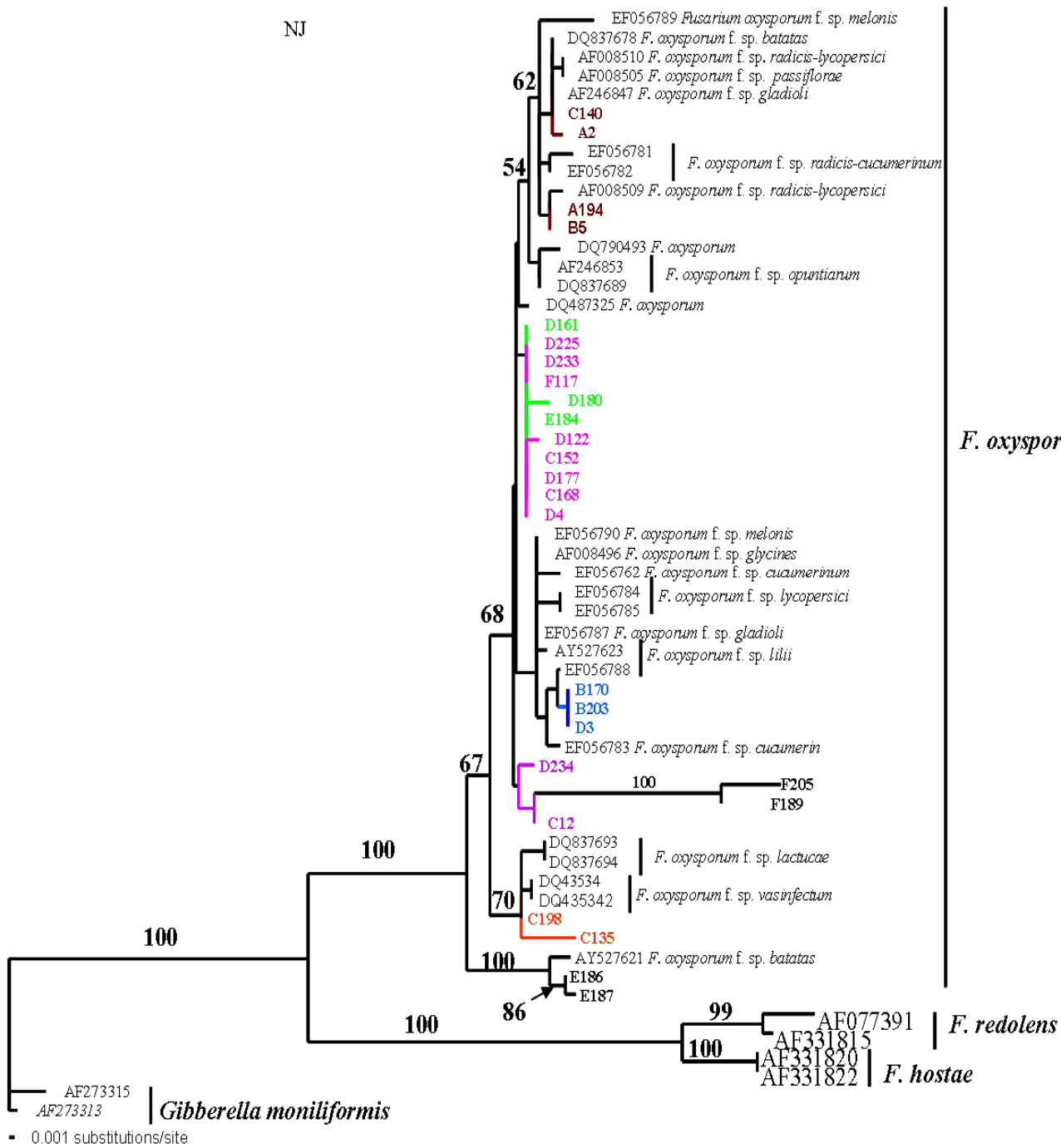


Figure 7. Neighbour-joining analysis of the TEF-1 α gene sequences of *F. oxysporum* isolates. Values above branch nodes indicate bootstrap values from 1000 replicates. Strains of *Gibberella moniliformis* (AF273315; AF273313) were used as outgroup.

NJ

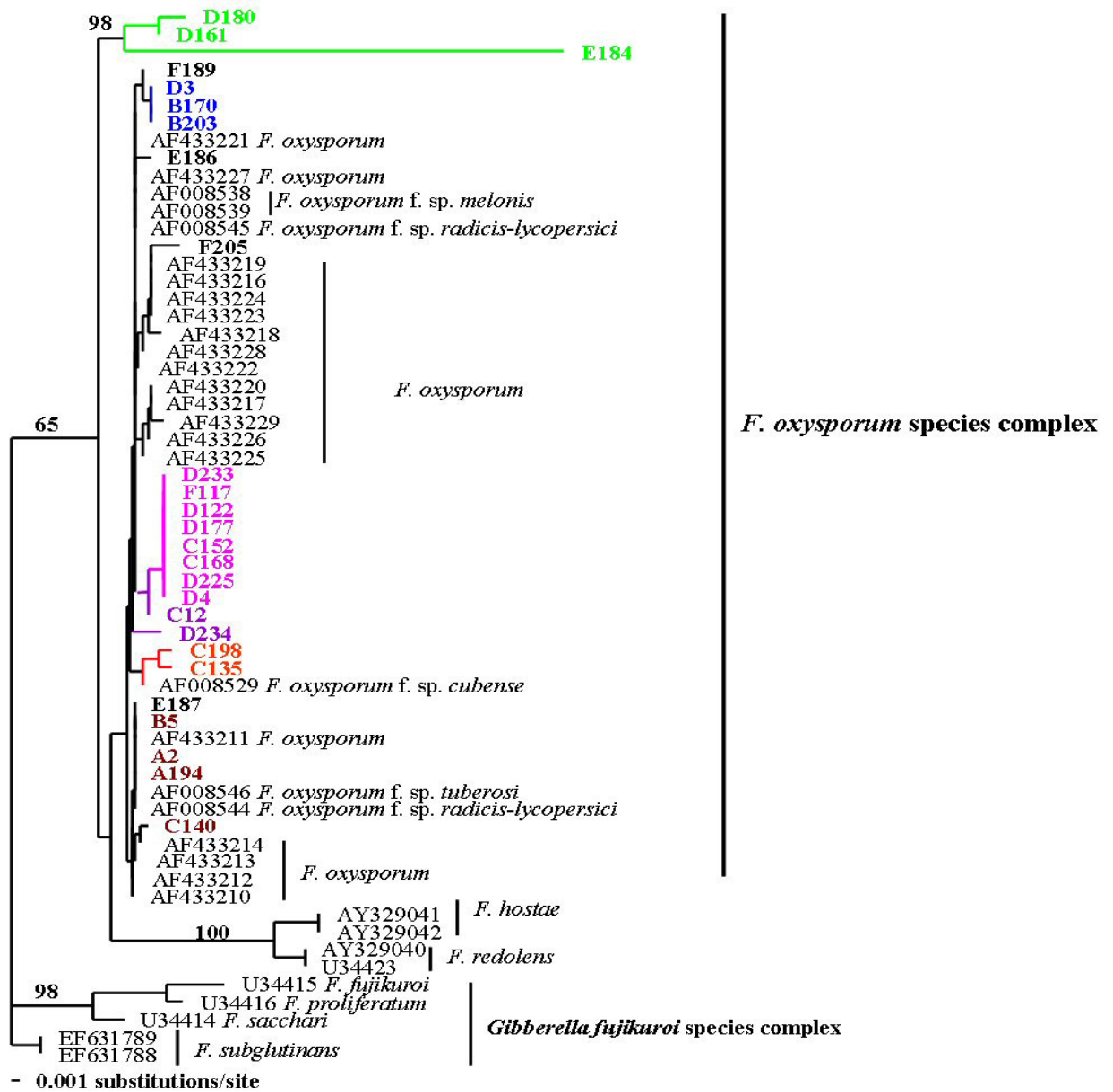


Figure 8. Neighbour-joining analysis of the β -tubulin gene sequences of *F. oxysporum* isolates. Values above branch nodes indicate bootstrap values from 1000 replicates. Strains of the *G. fujikuroi* species complex, *F. subglutinans* (EF631789; EF631788); *F. sacchari* (U34414); *F. proliferatum* (U34416); *F. fujikuroi* (U34415) were used as outgroup.

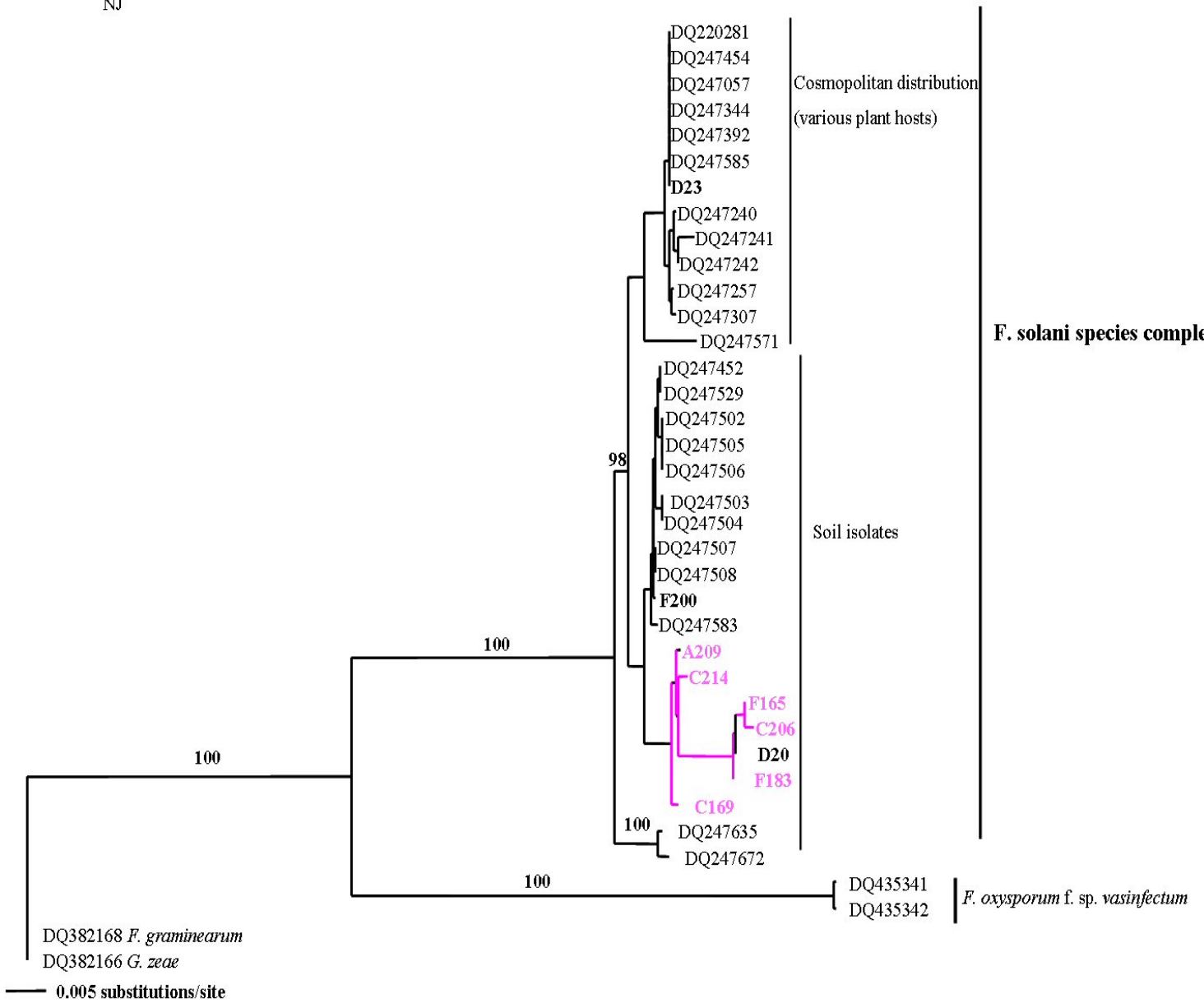


Figure 9. Neighbour-joining analysis of the TEF-1 α gene sequences of *F. solani* isolates. Values above branch nodes indicate bootstrap values from 1000 replicates. Strains of *F. graminearum* (DQ382168) and *G. zeae* (DQ382166) were used as outgroup.

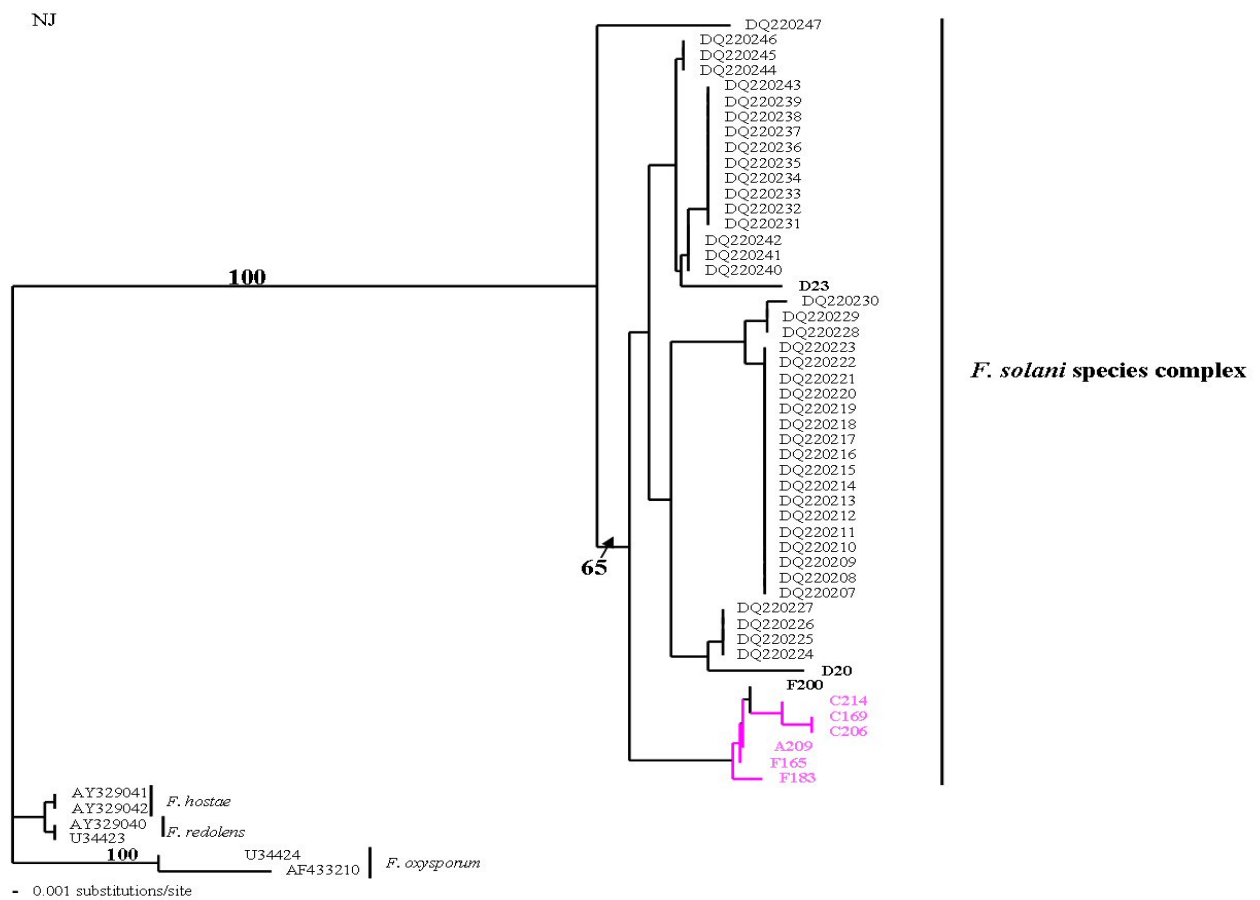


Figure 10. Neighbour-joining analysis of the β -tubulin sequences of *F. solani* isolates. Values above branch nodes indicate bootstrap values from 1000 replicates. Strains of *F. hostae* (AY329041; AY329042); *F. redolens* (AY322940; U34423); and *F. oxysporum* (U34424; AF433210) were used as outgroup.

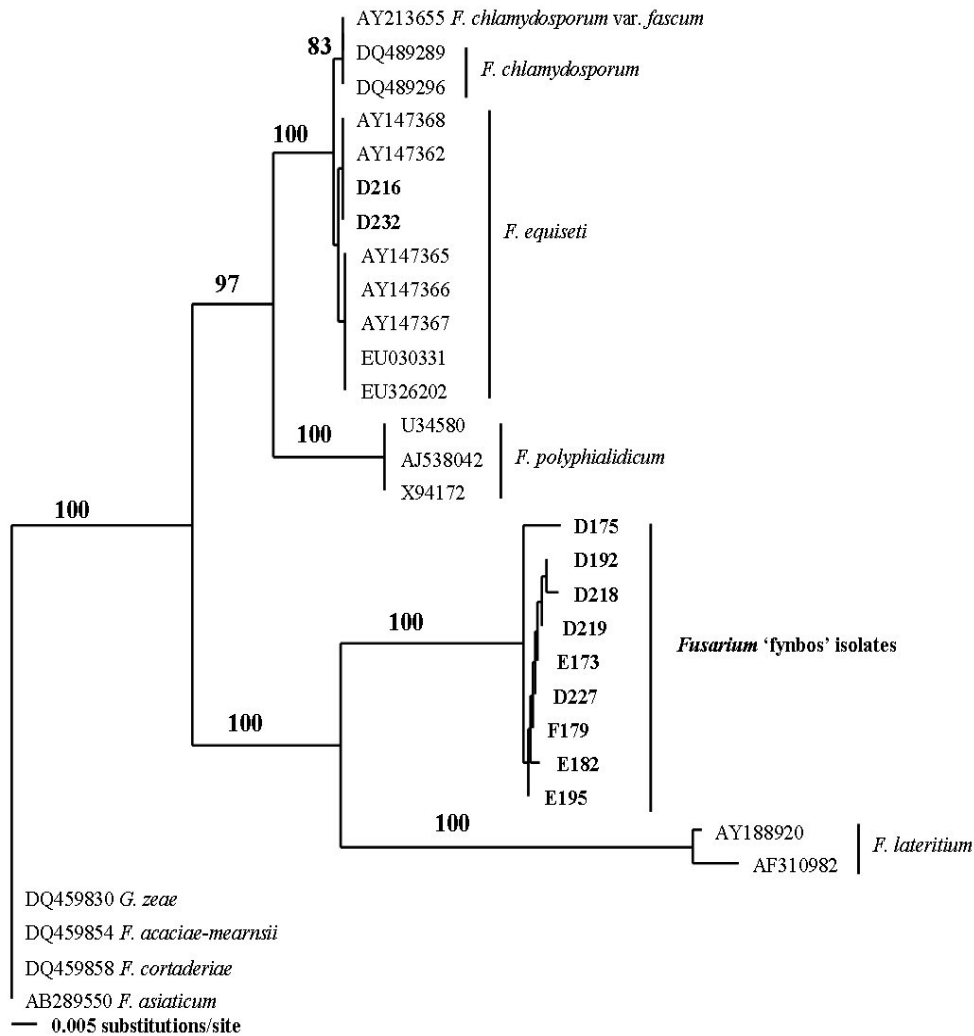


Figure 11. Neighbour-joining analysis of the ITS1-5.8SS-ITS2 region sequences of *F. equiseti* and *Fusarium* 'fynbos' isolates. Values above branch nodes indicate bootstrap values from 1000 replicates. Strains of *G. zeae* (DQ459830); *F. acaciae-mearnsii* (DQ459854); *F. cortaderiae* (DQ459858) and *F. asiaticum* (AB289550) were used as outgroup.

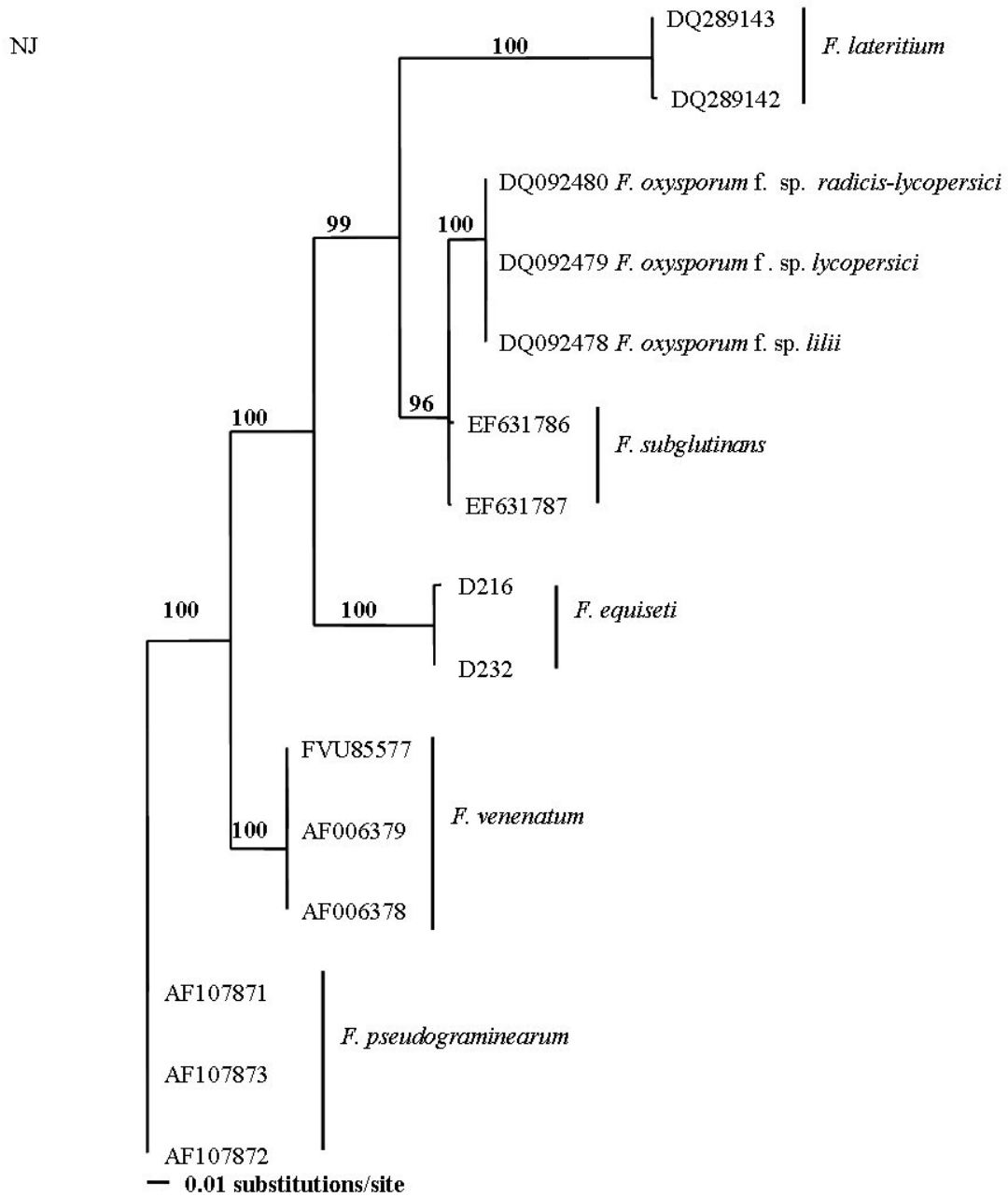


Figure 12. Neighbour-joining analysis of the β -tubulin gene sequences of *F. equiseti* isolates. Values above branch nodes indicate bootstrap values from 1000 replicates. Strains of *F. pseudograminearum* (AF107871; AF107873; AF107872) were used as outgroup.

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CHAPTER 3

Fusarium peninsulae prov. nom., a new species from fynbos soils of the Western Cape Province, South Africa

1. ABSTRACT

The genus *Fusarium* is well-known as a phytopathogen of a number of agricultural crops of the industrialized world, mycotoxin producers and opportunistic pathogens. This genus is ubiquitous and can be easily isolated from most plant substrates and soils. A recent survey into the dominant genera of the fynbos soil environment has led to the isolation of a group of unique strains that belong to the genus *Fusarium*. Morphologically these strains are characterized by falcate macroconidia produced from brown sporodochia. The macroconidia are pedicellate, falcate to curved with hooked apical cells. Also, these *Fusarium* isolates produce apedicellate mesoconidia on polyphialides in the aerial mycelium and forms microconidia sparsely. Chlamydospores are formed abundantly on aerial mycelium and submerged hyphae. All these morphological characteristics closely relate this fungus to *F. camptoceras* species complex in *Fusarium* section *Arthrosporiella*. However, ITS sequences differentiate these strains from *F. camptoceras* and other related species in section *Arthrosporiella*. Based on all the evidence, these strains are describe here as *Fusarium peninsulae* prov. nom.

2. INTRODUCTION

The genus *Fusarium* is ubiquitous in nature and is renowned for its phytopathogenesis to a number of agricultural crops worldwide (Thrane and Seifert, 2000). *Fusarium* species have also recently emerged as opportunistic pathogens, especially in immunocompromised individuals (Nelson *et al.*, 1994; O'Donnell *et al.*, 2007). The mycotoxins produced by many of the species have been extensively studied (Gelderblom *et al.*, 1988; Nelson *et al.*, 1993) and have contributed to the notoriety of this group of fungi.

A number of conflicting taxonomic systems have been formulated for *Fusarium* (Wollenweber and Reinking, 1935; Snyder and Hansen, 1940; 1941) and the reason for this conflict might have resulted from the ability of these fungi to morphologically and physiologically change in response to their environment. Identification of *Fusarium* isolates relies heavily on morphological characters, which are influenced by a number of factors such as the content of the media and growth conditions of the cultures (Wollenweber and Reinking, 1935; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983; Summerell *et al.*, 2003). However, even when using the best taxonomic systems available, identification of an unknown *Fusarium* species, based on morphology alone, can be challenging. Thus, morphological characteristics, in conjunction with molecular markers, have been used in the identification and characterization of a number of *Fusarium* species (Skovgaard *et al.*, 2003; Schmidt *et al.*, 2004).

Molecular identification of *Fusarium* species includes genotyping using differential Polymerase Chain Reaction (PCR)-based techniques such as randomly amplified polymorphic DNA (RAPD) analysis (Mitter *et al.*, 2001), amplified fragment length polymorphism (AFLP) analysis (Marasas *et al.*, 2001) and restriction

fragment length polymorphism (RFLP) analysis (Llorens *et al.*, 2006). More commonly these days, DNA sequencing of a number of genes is used as an additional molecular tool to facilitate in *Fusarium* species identification. In phylogenetic analyses of a number of fungal species including *Fusarium*, the ITS1 and ITS2 region gene sequences are amongst the favoured gene sequences used in species identification and phylogenetic analyses (Schilling *et al.*, 1996; Waalwijk *et al.*, 1996; Lee *et al.*, 2000; van Elsas *et al.*, 2000; Viaud *et al.*, 2000; Bao *et al.*, 2002; Mach *et al.*, 2004; Rakeman *et al.*, 2005). However, gene sequences such as the mitochondrial small subunit ribosomal DNA (mtSSU rDNA), partial translation elongation factor 1-alpha (TEF-1 α), β -tubulin (O'Donnell *et al.*, 1998; O'Donnell *et al.*, 2000; Knutsen *et al.*, 2004), intergenic spacer (IGS) region, actin, histone (H3) (Steenkamp *et al.*, 1999), and calmodulin (Mulè *et al.*, 2004) have also been successfully used to infer phylogenetic relationships between *Fusarium* species.

During a study of native *Fusarium* species from indigenous fynbos soils of the Cape Peninsula (Western Cape Province, South Africa) in 2006, nine isolates of an undescribed *Fusarium* species were isolated. Morphological characters of the strains placed this fungus in *Fusarium* section *Arthrosporiella*. However, closer examination revealed that the morphology of these strains did not correspond to those reported for other species in this section. The aim of this study was, therefore, to characterize the strains from fynbos soil based on morphology and molecular characters and compare them with closely related species within section *Arthrosporiella*.

3. MATERIALS AND METHODS

3.1. Morphology

Fusarium strains were recovered from fynbos soils using the soil dilution technique. Ten grams of soil was suspended in 100 ml 0.05% water agar (WA) (Bacterial Agar, BioLab, Johannesburg, South Africa), a ten-fold series of dilutions was prepared and 1 ml from each dilution series was plated out in triplicate on peptone-PCNB agar (Leslie and Summerell, 2006). Recovery of *Fusarium* isolates from plant debris associated with the soils was done by placing pieces of plant debris on peptone-PCNB agar. All plates were incubated in the laboratory under natural day-night rhythm near a window for diffused light at $\pm 22^{\circ}\text{C}$ for 7 days. Sequential subculturing was done, firstly by mass transfer of aerial mycelium from peptone-PCNB agar cultures onto Synthetischer Nährstoffarmer agar (SNA) (Leslie and Summerell, 2006) and secondly by single-conidial culturing (Nelson *et al.*, 1983) onto Carnation Leaf agar (CLA) (Fisher *et al.*, 1982).

Morphological characteristics such as conidial type, shape and size were observed from single-conidial cultures grown on CLA, while cultural characteristics such as colony morphology, growth rates and pigmentation were observed on PDA (39 g/L potato dextrose agar, BioLab, Johannesburg, South Africa). Growth rates of cultures were determined on PDA under three different incubation conditions. These were: incubation under natural day-night rhythm, near a window in the laboratory for diffused light ($\pm 22^{\circ}\text{C}$ for 3 days); incubation at 25°C for 3 days in an incubator; and thirdly incubation at 30°C in complete darkness for 3 days. Growth rates were measured in triplicate and an average of the three measurements was taken. Colony pigmentations were determined according to Kornerup and Wanscher (1978). All morphological characters were observed from material mounted in 30% lactic acid.

Fifty measurements were taken for each of the morphological relevant structures and the averages as well as standard deviations were calculated. Three strains, MRC 4570 (*Fusarium nelsonii*); MRC 4816 (*F. camptoceras*) and MRC 6312 (*F. camptoceras*), obtained from the Programme on Mycotoxins and Experimental Carcinogenesis, Medical Research Council, Tygerberg, South Africa (MRC) were used for morphological and molecular comparisons of *F. peninsulae*. These strains were chosen since they are part of the *F. camptoceras* species complex.

3.2. Molecular identification

3.2.1. DNA extraction and PCR amplification

Single-conidial cultures were grown on PDA (39 g/L potato dextrose agar, Biolab, Johannesburg, South Africa) at 25°C for 7 days. DNA was extracted using a modified method of Möller *et al.* (1992). Approximately, 50 mg mycelium was aseptically scraped from the surface of 7 day PDA cultures and transferred to 2 ml Eppendorf tubes. Mycelia was immediately frozen in liquid nitrogen, ground to powder with a sterile plastic micro-pestle and 500 µl TES lysis buffer (100 mM Tris pH8; 10 mM EDTA; pH8; 2% (w/v) SDS) and 5 µl (100 ng/ml) Proteinase K added. The tubes were inverted twice to mix the suspension, followed by incubation at 60°C for 60 min. Subsequently, 140 µl of 5 M NaCl, and 65 µl pre-warmed (60°C) 10% (w/v) CTAB were added and tubes incubated at 65°C for 10 min. Proteins were precipitated by adding an equal volume of SEVAG [chloroform: isoamylalcohol (24:1)] and incubating on ice for 15 min, followed by centrifugation at maximum speed (13 000 rpm, 10 min, 4°C). Supernatants were transferred to sterile 1.5 ml eppendorf tubes and DNA precipitated by adding 0.55 volumes cold isopropanol, followed by

centrifugation (13 000 rpm, 10 min, 4°C). The supernatants were discarded and the pellets washed twice with cold 70% ethanol. Pellets were air-dried and DNA re-suspended in 50 µl MilliQ water and stored at -20°C until used.

PCR amplifications were performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Drive Foster City, CA, USA). For amplification of the ITS-1-5.8S-ITS2 region, primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.*, 1990) were used. Genomic DNA concentrations were determined using the NanoDrop® ND-100 spectrophotometer (Central Analytical Facilities, University of Stellenbosch, South Africa) and were diluted accordingly to a final concentration of 10-20 ng/µl. Amplification reactions were performed in 25 µl volume containing: 23 µl (2×) *KapaTaq* Ready Mix (Kapa Biosystems, Cape Town, South Africa), 1 µl template and 0.5 µl (10 µM) of each primer (ITS1 and ITS4). The cycling conditions in the thermal cycler were as follows: an initial denaturing step at 94°C for 5 min, followed by 34 cycles consisting of 94°C for 30 s, primers annealing at 56°C for 45 s, chain elongation at 72°C for 1 min and an additional elongation step at 72°C for 7 min. The PCR products were visualized in 1% agarose gels stained with ethidium bromide under UV illumination.

3.2.2. Sequencing and Phylogenetic analysis

PCR products were purified using Invitex DNA cleaning kit (Gesellschaft für Biotechnik & Biodesign mbH, Germany) according to the manufacture's instructions. PCR products were sequenced in both directions with the same primer set that was used in DNA amplification. Sequencing was performed in a GeneAmp® PCR System

9700 thermal cycler using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) and sequence analysis performed on a Perkin Elmer ABI3100 genetic analyzer (Sequencing Facility, University of Stellenbosch, South Africa).

The obtained nucleotide sequences were analyzed and edited using DNASTar SeqMan™ II (DNA-STAR Inc., WI, USA). These were compared with other existing *Fusarium* ITS sequences available on Basic Local Alignment Search Tools (BLAST) software (National Center for Biotechnology Information, (www.ncbi.nlm.nih.gov/blast)). A dataset of all ITS sequences that showed high identity scores after BLASTn was compiled and edited. ITS sequences of the new isolates were also added into this dataset. Sequence alignments were performed in CLUSTALX version 1.8 (Thompson *et al.*, 1997) and phylogenetic relationships between taxa were determined using distance analysis in PAUP version 4.0b10 (Swofford, 2001). Characters were treated as unweighted in the analysis and gaps were treated as missing data. The confidence levels of the nodes of distance analysis trees were evaluated by performing a bootstrap analysis of 1000 random replicates.

4. RESULTS

4.1. Morphology

Cultural and morphological characteristics of the undescribed *Fusarium* strains from the fynbos soils (Table 1) placed them in *Fusarium* section *Arthrosporiella* (Wollenweber and Reinking, 1935) with a close resemblance to *F. semitectum* (= *F. incarnatum*) Berkeley and Ravenel and the *F. camptoceras* species complex (Marasas *et al.*, 1998). The strains from fynbos soils formed pedicellate macroconidia (from

sporodochia) that were slightly curved to falcate with papillate to slightly curved apical cells. Distinctive brown sporodochia were formed profusely on carnation pieces of CLA and on aerial mycelium of fresh cultures. Also, apedicellate mesoconidia were formed on aerial mycelium but microconidia were formed sparsely. Chlamydospores formed abundantly and colony pigmentation was dark brown on reverse (backside) of PDA plates in these strains (Nelson *et al.*, 1983).

4.2. Sequencing and Phylogenetic analysis

Amplification of the ITS region in undescribed taxa resulted in fragments of approximately 550 base pairs (bp). Neighbour-joining analysis of the ITS data revealed a strongly supported monophyletic group (bootstrap = 100%) of all nine isolates (Fig. 1) closely related to an unknown *Fusarium* strain DQ885388, which has been identified as an “uncultivated soil fungus”. There was no clear clustering based on geographic origin within undescribed *Fusarium* taxa. Also, the undescribed *Fusarium* strains formed a sister group to MRC 4570 (*F. nelsonii*). Furthermore, the undescribed *Fusarium* strains showed a closer affinity to *F. lateritium* than to *F. camptoceras* and *F. semitectum*. This is surprising since morphologically the undescribed *Fusarium* strains have a closer resemblance to *F. camptoceras* and *F. semitectum* than they do with *F. lateritium*. Based on the analysis of the ITS region, the strains from fynbos soils formed a strongly supported monophyletic group distinct from other species within section *Arthrosporiella*. Therefore, based on both morphological characteristics observed and the results of phylogenetic analysis, we describe these strains as follows.

5. TAXONOMY

Fusarium peninsulae Bushula, van Zyl, Marasas & Jacobs, **prov. nom.**

Figures 2–12

Mycobank MB512431

Etymology: epithet based on the location where the isolates were recovered, Cape Peninsula area in the Western Cape, South Africa.

Colonia in PDA post 3 dies in tenebris in 25°C (2.9-) 3.0–3.4 (-3.4) cm, 30°C (2.5-) 2.8–3.5 (-3.5) cm diametro, floccosa vel pulverea, supra laete brunnea, subtus atrobrunnea. Cellulae coniriogenae mono-et polyphialidicae. Macroconidia in sporodochiis in monophialidibus facta, curvata falcata cellulis basalibus pedicellatis, cellulis apicalibus attenuatis papillatis vel curvatis, 3–5 septatis, (23-) 26–32 (-35) × (3.7-) 4.2–5.1 (-5.5) μm (Figs. 2–3; 9). Mesoconidia in cellulis conidiogenis polyblastis in mycelio aereo, hyalina, fusiformia vel lanceolata, recta vel subcurvata, 3–5 septata, (14-) 22–33 (-35) × (3.2-) 4.6–6.7 (-7.6) μm (Figs. 4–5; 12). Sporodochia brunnea, in mycelio aereo et in fragmentis dianthi profusa, guttis parvis mucosis brunneis vel atrobrunneis in CLA integro. Microconidia fusiformia reniformia in cellulis conidiogenis mono- et polyphialidicis (Figs. 5; 10–11). Chlamydosporae celeriter (post hebdomadis 2–4) factis, 7–10 μm diametro (Figs. 6–7; 13–14).

Colony diameter of single-conidial cultures on PDA after dark incubation for 3 days (2.9-) 3.0–3.4 (-3.4) cm at 25°C and (2.5-) 2.8–3.5 (-3.5) cm at 30°C. Colonies floccose to powdery, light brown. Reverse of colonies on PDA dark brown.

Conidiogenous cells monophialidic and polyphialidic. *Mesoconidiophores* arising laterally from aerial hyphae, simple or branched. *Macroconidia* in sporodochia produced on monophialides, curved, falcate with a pedicellate basal cell and an attenuated, papillate to curved apical cell, 3–5-septate, mostly 5-septate, (23) 26–32 (-35) × (3.7-) 4.2–5.1 (-5.5) μm (Figs. 2–3; 9). *Mesoconidia* produced on polyblastic conidiogenous cells in the aerial mycelium on CLA, hyaline, fusiform or lanceolate, straight or slightly curved, 3–5 septate, (14-) 22–33 (-35) × (3.2-) 4.6–6.7 (-7.6) μm (Figs. 4–5; 12). *Sporodochia* on CLA, brown, produced profusely in aerial mycelium and carnation pieces, appearing as small, slimy brown to dark brown droplets on entire CLA plate's surface. *Microconidia* are sparse, fusiform and reniform borne on both monophialidic and polyphialidic conidiogenous cells (Figs. 5; 10–11) *Chlamydospores* produced rapidly (after 2–4 weeks) and abundantly in both aerial and submerged hyphae, terminal and intercalary, hyaline becoming brown, rough-walled, in chains or clumps, subglobose or globose, 7–10 μm in diameter (Figs. 6–7; 13–14).

Teleomorph: unknown.

Habitat: In fynbos soils and associated plant debris.

Known distribution: Olifantsbos (Western Cape Province, South Africa); Boyes Drive (Western Cape Province, South Africa).

Isolates examined. SOUTH AFRICA. WESTERN CAPE PROVINCE. CAPE PENINSULA: Olifantsbos, isolated from plant debris, August 2006, *V. S. Bushula*, **holotype** PREM 60027, culture ex-type E173; Olifantsbos, isolated from plant debris, August 2006, *V. S. Bushula*, **paratype** PREM 60032, culture ex-paratype E182; Olifantsbos, isolated from plant debris, August 2006, *V. S. Bushula*, **paratype** PREM

60031, culture ex-paratype E195; Boyes Drive, isolated from soil, November 2006, *V. S. Bushula*, **paratype** PREM 60028, culture ex-paratype D218); Boyes Drive, isolated from soil, November 2006, *V. S. Bushula*, **paratype** PREM 60029, culture ex-paratype D219; Boyes Drive, isolated from soil, November 2006, *V. S. Bushula*, **paratype** PREM 60030, culture ex-paratype D227. **Additional isolates:** Boyes Drive, isolated from plant debris, August 2006, *V. S. Bushula*, (culture ex-paratype = D175); Olifantsbos, isolated from plant debris, August 2006, *V. S. Bushula*, (culture ex-paratype = F179); Boyes Drive, isolated from plant debris, August 2006, *V. S. Bushula*, (culture ex-paratype = D192).

6. DISCUSSION

Fusarium peninsulae is characterized by its relatively fast growth rate on PDA, production of dark brown sporodochia on entire CLA surface area and the profuse formation of chlamydospores. The ability to form chlamydospores complements the type of environment this species was obtained from, the fynbos soils, which are nutrient-poor sandy soils. The overall micro- and macromorphological characteristics of *F. peninsulae* place this fungus in *Fusarium* section *Arthrosporiella* (Wollenweber and Reinking, 1935). Species in this section include *F. semitectum* (Nelson *et al.*, 1983); *F. polyphialidicum* (Marasas *et al.*, 1986), and *F. camptoceras* species complex, which consist of three species, namely *F. camptoceras*, *F. nelsonii* and *F. musarum* (Marasas *et al.*, 1998).

Fusarium peninsulae has a close morphological resemblance to both *F. semitectum* and *F. camptoceras* species. Macroconidial formation is not common in *F. semitectum*, and when these are formed they occur in orange sporodochia and

slightly differ in shape and size from those formed by *F. peninsulae*. Chlamydospore formation is also not a common feature of *F. semitectum* (Leslie and Summerell, 2006). The formation of brown pigmentation on reverse PDA and the presence of mesoconidia are, therefore, the only shared morphological characteristics between *F. semitectum* and *F. peninsulae* (Leslie and Summerell, 2006).

Marasas *et al.* (1998) emended the description of *F. camptoceras* and included the presence of pedicellate macroconidia (from sporodochia) and mesoconidia (produced on polyblastic conidiogenous cells in aerial mycelium) as additional morphological characteristics displayed by this species. Both *F. peninsulae* and *F. camptoceras* produce mesoconidia from aerial mycelium and macroconidia from sporodochia. Based on conidial (macroconidia and mesoconidia) measurements, *F. peninsulae* appears to produce conidia types that are shorter than those of *F. camptoceras* (Table 1). In comparison to the other two species in the *F. camptoceras* species complex, *F. nelsonii* and *F. musarum*, *F. peninsulae* can be distinguished based on pigmentation on agar and the presence or absence of macroconidia from sporodochia (Marasas *et al.*, 1998). *Fusarium nelsonii* and *F. musarum* produce red pigmentation on reverse PDA versus dark brown pigmentation of *F. peninsulae*. When compared to *F. nelsonii*, *F. peninsulae* produces macroconidia that are shorter than those of *F. nelsonii*, however, mesoconidia of *F. peninsulae* are longer than those of *F. nelsonii* (Marasas *et al.*, 1998). *Fusarium musarum* produces neither macroconidia nor sporodochia hence no direct morphological comparison can be made between *F. peninsulae* and *F. musarum*.

Phylogenetic analysis of the ITS region shows *F. peninsulae* to be closely related to *Fusarium* strain DQ885388, which was isolated from *Opium poppy* (*Papaver somniferum* L.) (Landa *et al.*, 2007), but no further taxonomic, pathological

and ecological information could be obtained on this particular strain. The phylogenetic relationship inferred by the ITS data between *F. peninsulae* and the *F. nelsonii* strain is not fully reflected by the morphological data. However, both *F. peninsulae* and *F. nelsonii* have been recovered from plant debris in uncultivated soils. Also, the fact that *F. peninsulae* is closer related to *F. lateritium* than to *F. camptoceras*, *F. semitectum* and *F. polyphialidicum* is surprising, as there are not many morphological similarities between *F. peninsulae* strains and *F. lateritium* (Wollenweber and Reinking, 1935).

Some of the major differences between *F. peninsulae* and *F. lateritium* include the absence of mesoconidia from aerial mycelium, the absence of dark brown pigmentation on reverse PDA and the absence of brown sporodochia in *F. lateritium* compared to *F. peninsulae*. However, *F. lateritium* is considered to be a species complex that contains numerous taxa that have unresolved taxonomic status (Leslie and Summerell, 2006).

The geographic range of *F. peninsulae* is not yet clearly defined but this fungus occurs in native fynbos soils and the associated plant debris as a saprophyte. It is evident that even though morphological characters formed by *F. peninsulae* show morphological affinity between this species and *F. semitectum* and *F. camptoceras*, phylogenetic analysis of the ITS region indicated that *F. peninsulae* is a distinct species from both these two species and other related species of section *Arthrosporiella*. The actual position of *F. peninsulae* within section *Arthrosporiella* still needs to be further explored using multigene phylogeny.

Table 1. Comparison of cultural and morphological characteristics of *Fusarium peninsulae*, *F. camptoceras* and *F. nelsonii*.

Characters		<i>F. peninsulae</i>	<i>F. camptoceras</i> ^a	<i>F. nelsonii</i> ^a
Colony diameter (cm) ^b	25 C	2.9–3.4 (mean = 3.2)	2.3–2.9 (mean = 2.6)	2.4–3.9 (mean = 3.0)
	30 C	2.5–3.5 (mean = 3.0)	2.0–2.9 (mean = 2.3)	2.6–4.1 (mean = 3.3)
Pigment on PDA in the dark		Dark brown	Brown	Red
Mesoconidia	Shape	Straight or curved	Straight or curved	Straight or curved
	Septa	3–5, mostly 5	0–7, mostly 3–4	0–3, mostly 3
	μm ^c	23–35 × 4–8 (27 × 5.6)	15–51 × 4–7 (28 × 4.8)	8–32 × 3–6 (18 × 3.5)
Sporodochia		Present	Present	Present
Macroconidia ^d	Shape	Curved, falcate	Curved, falcate	Straight or curved
	Septa	3–6, mostly 5	3–6, mostly 5	3–5, mostly 3
	μm ^c	24–35 × 4–5 (29 × 4.7)	30–60 × 4–6 (42 × 4.7)	20–42 × 4–6 (29 × 3.5)
Chlamydospores		Produced rapidly (2–4 weeks) and abundantly, mostly intercalary, in short chains or clusters, terminal pairs	Produced slowly and sparsely, mostly intercalary, single or in short chains, never in terminal pairs	Produced rapidly and abundantly, intercalary and terminal, typically in terminal pairs

^aMorphological characteristics adapted from Marasas *et al.* (1998).

^b Colony diameter of single-conidial cultures on PDA plates, 9 cm × 15 mm plastic petri dishes, incubated in the dark for 72 h.

^c Mean conidial size in parentheses

^d These are pedicellate macroconidia

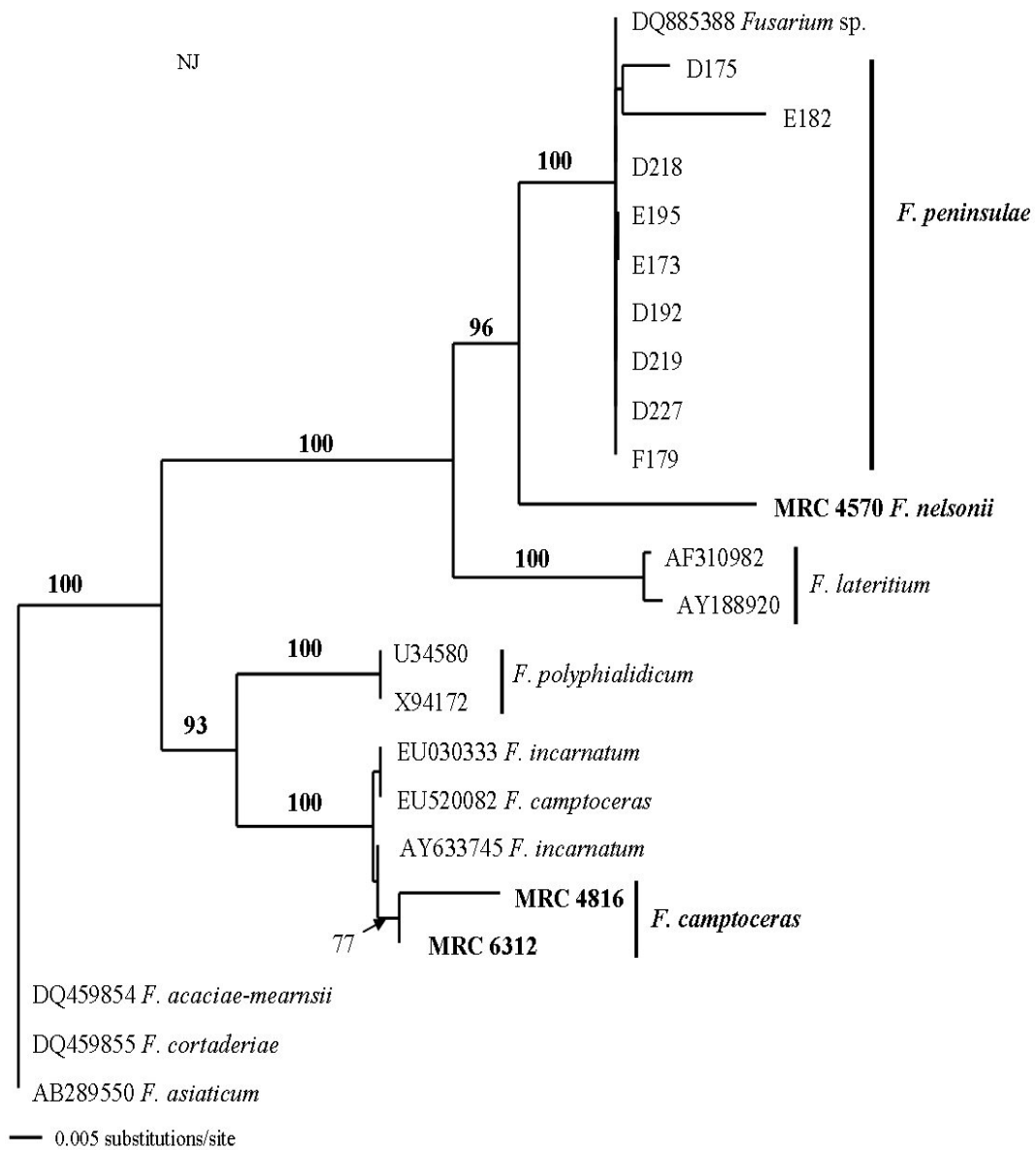
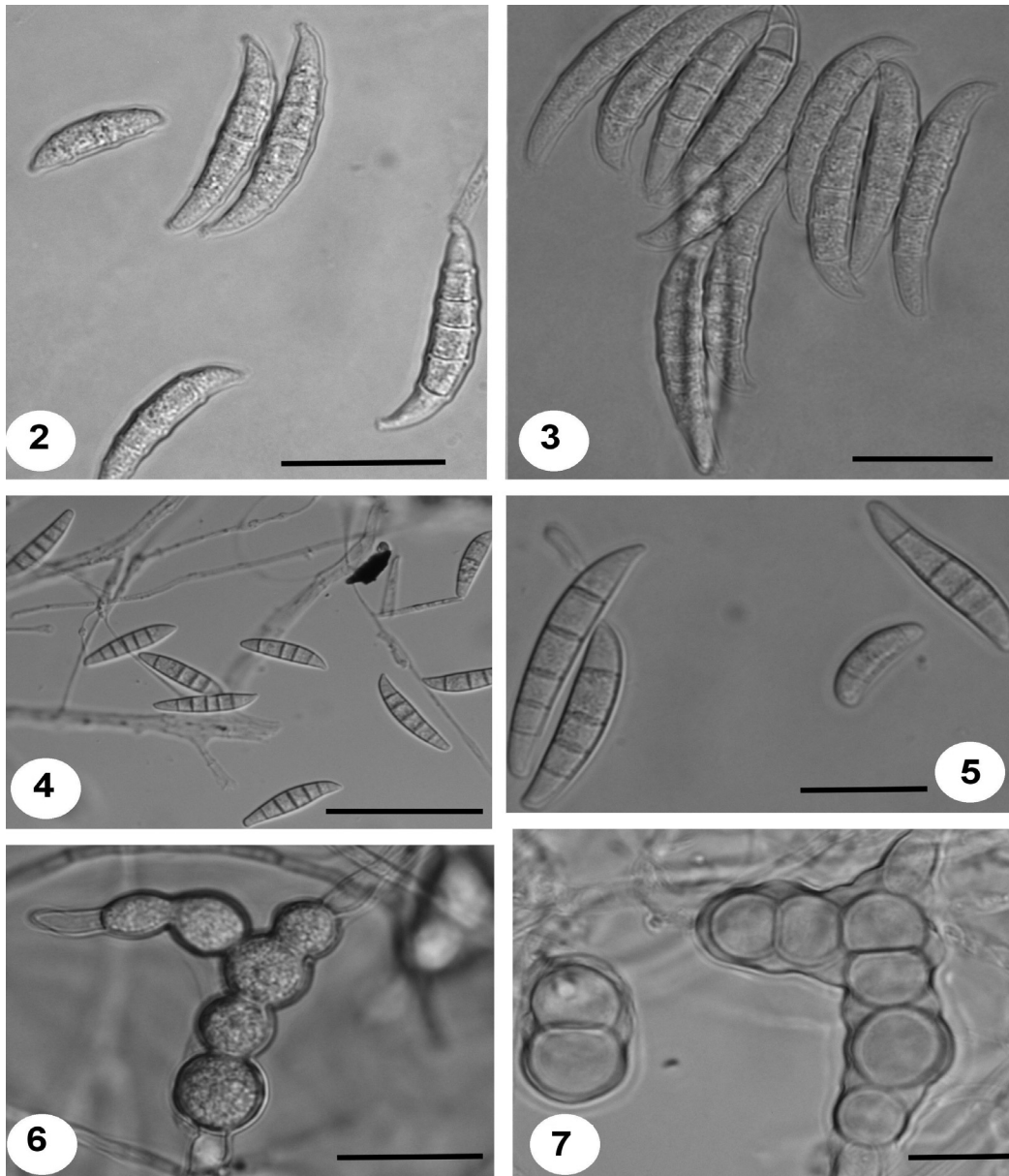
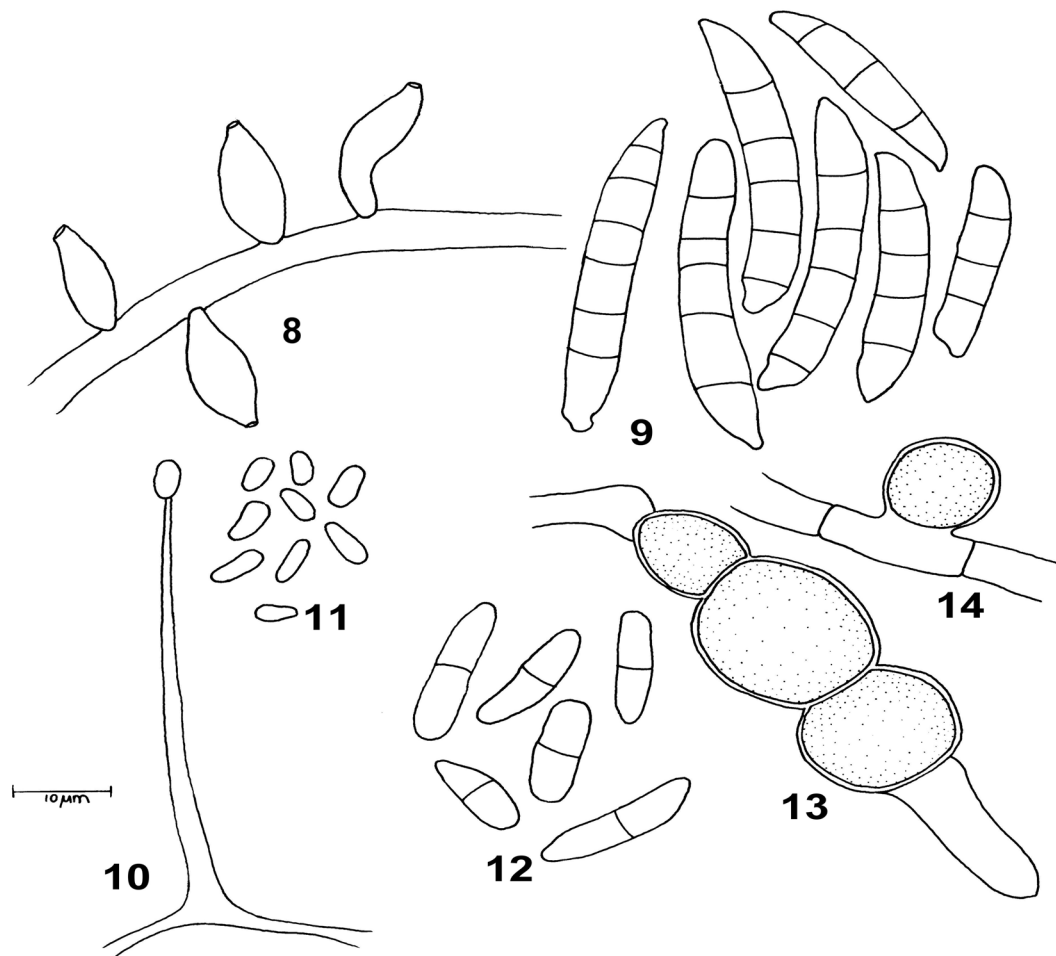


Figure 1. Neighbour-joining analysis of the ITS1-5.8S-ITS2 region sequences of *F. peninsulae* isolates. Values above branch nodes indicate bootstrap values from 1000 replicates. Strains of *F. acaciae-mearnsii* (DQ459854); *F. cortaderiae* (DQ459858); and *F. asiaticum* (AB289550) were used as outgroup.



Figures 2–7: Light micrographs of *F. peninsulae*. **Figs. 2-3.** Pedicellate macroconidia (3–5-septate) produced in sporodochia. **Fig. 4.** Apedicellate mesoconidia (3–5-septate). **Fig. 5.** Apedicellate mesoconidia and microconidia. **Figs. 6-7.** Intercalary cluster of chlamydospores. Bars: 2-7 = 10 μ m.



Figures 8–14: Line drawings of the morphological characters of *F. peninsulae* (MB512431). **Fig. 8.** Monophialidic conidiogenous cells. **Fig. 9.** Falcate, 3–5-septate pedicellate macroconidia. **Fig. 10.** Monophialide bearing a microconidium. **Fig. 11.** Fusiform microconidia. **Fig. 12.** Spindle-shaped apedicellate mesoconidia. **Figs. 13–14.** Intercalary chlamydoconidia, single intercalary chlamydoconidium.

7. LITERATURE CITED

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Chapter 4

Partial endoxylanase gene as a molecular marker for phylogenetic analysis and identification of *Fusarium* species

1. ABSTRACT

Plant cell walls are made up of various components, which consist of differential polymers. Fungal phytopathogens, like many other microbial pathogens secrete hydrolytic enzymes such as endoxylanases, which facilitate in the degradation of the plant cell walls. The xylan degrading endoxylanases of three *Fusarium* spp., *F. oxysporum* f. sp. *lycopersici*, *F. verticillioides* and *F. graminearum* have been characterized. The aim of this study was to evaluate, through phylogenetic analysis, the use of the endoxylanase encoding gene as a molecular marker for *Fusarium* species identification using the *F. graminearum* species complex as model. Degenerated primers resulted in PCR amplified fragments of approximately 600 bp of endoxylanase (XYL), which were cloned and sequenced. PAUP-generated neighbour-joining analysis of XYL enabled all species to be distinguished and was as informative as the analysis generated with UTP-ammonia ligase (URA), phosphate permase (PHO), reductase (RED) and trichothecene 3-*O*-acetyltransferase (TRI101). Also, a combination of the five data sets improved the accuracy of the analysis. Furthermore, the results of the phylogenetic analysis of XYL showed better species resolution in comparison to the analysis of the structural genes (TEF-1 α and histone H3). The overall results demonstrated that phylogenetic analysis of the functional gene (URA, PHO, RED, TRI101 and XYL) data sets clearly distinguished between the *F. graminearum* (*Fg*) clade species far better than the analysis of structural gene (TEF-1 α and histone H3) data sets. These results are paving a new way for the use of hydrolytic enzyme encoding genes as molecular markers in fungal species identification.

2. INTRODUCTION

The survival of soilborne microorganisms, such as bacteria, nematodes and fungi, are highly dependent on the availability of growth substrates in their immediate environment. These growth substrates are mainly available as dead plant material in the soil habitat (Beliën *et al.*, 2005). Generally, plant cell walls are made up of a number of structural components such as cellulose, hemicellulose and lignin (Bestawde, 1992). These plant structural components occur in varying degrees in different plant types and are each made up of differential polymers (Beliën *et al.*, 2005). Cellulose is the major constituent of plant cell wall polymers and is made up of cellobiose sugar units (Brett and Waldron, 1996). Cellulases such as endo- β -1,4-glucanase, β -glucosidase and cellobiohydrolase are the enzymes that act as a consortium to completely hydrolyze the cellulose complex (Walton, 1994).

Hemicellulose is a major constituent in cell walls of both monocots and hardwoods. It consists of xylan, mannan, galactan and arabinan as main heteropolymers (Beliën *et al.*, 2005). Xylan is the major component of hemicellulose and it varies in composition and structure according to the plant source (Bestawde, 1992). Endo- β -1,4-xylanase and β -xylosidase are two key enzymes necessary to hydrolyze the xylan backbone into simple sugars (Walton, 1994).

The cell wall is the first barrier of defense for a plant that is colonized by microorganisms (Knogge, 1996). The complex polysaccharides that make up every plant cell wall require complex enzymes from microorganisms that break this down into simple sugars. These enzymes include pectinases, cutinases, cellulases and xylanases (Walton, 1994). Therefore, both saprophytic and plant pathogenic fungi alike secrete a variety of these cell wall degrading enzymes (CWDEs) to be able to invade and extract essential nutrients from plant hosts (Walton, 1994; Roncero *et al.*, 2003). Furthermore, hydrolytic enzymes, notably cellulases and xylanases are implicated in playing a role in phytopathogenesis of fungal species (Knogge, 1996; Beliën *et al.*, 2006).

Xylanases are classified into glycosyl hydrolases based on the amino acid sequence similarities (Henrissat, 1991; Henrissat and Bairoch, 1993). Endoxylanases (endo- β -1,4-xylanases; EC 3.2.1.8) are classified into two families of glycosyl hydrolases, family 10 and 11 (Collins *et al.*, 2002; Beliën *et al.*, 2006). Furthermore, *F. verticillioides* (Saha, 2001), *F. oxysporum* f. sp. *lycopersici* (Christakopoulos *et al.*, 1996; Ruiz *et al.*, 1997; Ruiz-Roldán *et al.*, 1999; Gómez-Gómez *et al.*, 2001, 2002) and *F. graminearum* (Beliën *et al.*, 2005), which are all phytopathogens that secrete a number of endoxylanases.

The genus *Fusarium* is among the most economically important fungal groups. *Fusarium* species such as *F. oxysporum*, *F. verticillioides* and *F. graminearum* cause significant economic losses worldwide due to the destructive diseases they cause on agricultural commodities (Nelson *et al.*, 1981; Burgess *et al.*, 2001). Diseases caused by these species include wilts and rots, of which *F. oxysporum* is the main problem (Nelson *et al.*, 1981; Eken *et al.*, 2004). *Fusarium* head blight (scab) of wheat and barley, in turn is caused by *F. graminearum* Schwabe (Marasas *et al.*, 1988; Burgess, *et al.*, 1987; Marasas *et al.*, 1988) and occurs worldwide on a variety of hosts (Burgess *et al.*, 2001). Furthermore, the mycotoxins produced by these fungi also affect the seed quality of plants they infect and this further increases the financial scourge that these phytopathogens can cause (Burgess *et al.*, 2001).

In the identification of *Fusarium*, the application of morphological characteristics alone proves to be insufficient to resolve species boundaries (Torp and Nirenberg, 2004). Sequencing of a number of gene regions has therefore been applied in many cases to resolve species complexes that are indistinguishable based on morphological characters alone (O'Donnell *et al.*, 1998). Gene regions such as the nuclear large subunit (28S); internal transcribed spacer (ITS) regions; mitochondrial small subunit (mtSSU); β -tubulin; translation elongation factor 1 alpha (TEF-1 α) and calmodulin have been used in species complexes such as the *Gibberella fujikuroi* complex (O'Donnell *et al.*, 2000b). Also, O'Donnell *et al.* (2000a,

2004) used the gene regions of UTP-ammonia ligase (URA); phosphate permase (PHO), trichothecene 3-*O*-acetyltransferase (TRI101); putative reductase (RED), TEF-1 α and histone H3 in conjunction with morphological characteristics to resolve the *F. graminearum* species complex into nine phylogenetic distinct species. The majority of the gene regions used for inferring phylogenetic relationships between *Fusarium* species is nuclear protein-encoding genes.

Fusarium graminearum was initially regarded as two taxa, *F. graminearum* Group 1 and *F. graminearum* Group 2 based on etiological and pathological differences (Francis and Burgess, 1977). Diseases of the crowns of plants were regarded to be caused by members of Group 1, whereas diseases of aerial parts of plants were associated with members of Group 2 (Francis and Burgess, 1977). Aoki and O'Donnell (1999) used both morphological and molecular characteristics to re-evaluate the taxonomy of *F. graminearum* Groups 1 and 2. This led to Group 1 being described as *F. pseudograminearum* and Group 2 retaining the original *F. graminearum* name. The *F. graminearum* (Fg) clade is considered a species complex that consists of nine phylogenetically distinct species namely, *F. austroamericanum* Aoki, Kistler, Geiser and O'Donnell; *F. meridionale* Aoki, Kistler, Geiser and O'Donnell; *F. boothii* O'Donnell, Aoki, Kistler and Geiser; *F. mesoamericanum* Aoki, Kistler, Geiser and O'Donnell; *F. acaciae-mearnsii* Aoki, Kistler, Geiser and O'Donnell; *F. asiaticum* O'Donnell, Aoki, Kistler and Geiser; *F. graminearum*; *F. cortaderiae* O'Donnell, Aoki, Kistler and Geiser and *F. brasiliicum* Aoki, Kistler, Geiser and O'Donnell (O'Donnell *et al.*, 2000a, 2004; Ward *et al.*, 2002). *Fusarium graminearum* species secrete mycotoxins and have been implicated in a number of animal and human toxicoses (Marasas *et al.*, 1984).

With the above as background, the aim of this study was thus to evaluate, through phylogenetic analysis, the use of the endoxylanase encoding gene as a molecular marker for *Fusarium* species identification using *F. graminearum* species complex as model group. In

addition, the phylogenetic value of functional genes versus structural genes to discriminate between closely related *Fusarium* species was further evaluated.

3. MATERIALS AND METHODS

3.1. Fungal strains and growth conditions

Fusarium strains used in this study (Table 1) all belong to the *F. graminearum* (*Fg*) clade (O'Donnell *et al.*, 2000, 2004) and were obtained from the Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research (NCAUR), Peoria, Illinois. An agar plug from a pure culture of each strain was plated onto PDA (39 g/L potato dextrose agar, Difco, South Africa) and the plates were incubated in the laboratory under natural day-night rhythm, near a window for diffused light, at $\pm 25^{\circ}\text{C}$ for 7 days.

3.2. Genomic DNA extraction

Total genomic DNA was extracted using a modified method of Möller *et al.* (1992). Approximately, 50 mg mycelium was scraped directly from the surface of a 10 day PDA culture using a sterile scalpel and transferred to a clean 2 ml Eppendorf tube. The mycelium was immediately frozen in liquid nitrogen, ground into a fine powder using a sterile plastic micro-pestle. Subsequently, the powder was re-suspended in 500 μl TES lysis buffer (100 mM Tris pH 8; 10 mM EDTA; pH 8; 2% (w/v) SDS) and 5 μl (100 ng/ml) and Proteinase K was added. The suspension was mixed by inverting the tubes twice, followed by incubation at 60°C for 60 min in a water bath. Hundred-and-forty (140) μl 5 M NaCl and 65 μl pre-warmed (60°C) 10% (w/v) CTAB were added and the suspension incubated at 65°C for 10 min. Proteins were denatured by adding an equal volume of SEVAG [chloroform: isoamylalcohol (24:1, vol/vol)] and incubated on ice for 15 min. The suspension was centrifuged at $13000 \times g$ for 10 min in a micro-centrifuge at 4°C . The supernatant was transferred to a clean 1.5 ml

Eppendorf tube, and the DNA precipitated by addition of 0.55 volumes cold isopropanol and placed on ice for 15 min. The DNA was precipitated by centrifugation at $13000 \times g$ for 10 min in a micro-centrifuge at 4°C. The supernatant was discarded and the pellet washed twice with cold 70% (vol/vol) ethanol. The pellet was air-dried in a laminar flow bench and DNA re-suspended in 50 µl MilliQ water. Genomic DNA concentrations were determined using the NanoDrop[®] ND-100 spectrophotometer (Central Analytical Facilities, University of Stellenbosch, South Africa). Extracted DNA was stored at -20°C until used.

3.3. Primer design and PCR amplification

Endo-β-1,4-xylanase gene sequences of *Gibberella zeae* (AY289919) and *F. oxysporum* f. sp. *lycopersici* (AF052583, AF052582, AF246831, AF246830) obtained from Genbank were aligned in CLUSTALX version 1.8 (Thompson *et al.*, 1997) and the following degenerated primers were designed: XYLF1 (5'-GGY AAC TTT GTY GGT GGA AAG G-3') forward primer and XYLR1 (5'-CCR CTG CTC TGG TAA CCY TC-3') reverse primer.

All PCR amplifications were performed in a GeneAmp[®] PCR System 9700 thermal cycler (Applied Biosystems, Drive Foster City, CA, USA). The endo-β-1,4-xylanase gene fragments were amplified using degenerated primers XYLF1 and XYLR1 (Inqaba Biotechnical Industries (Pty) Ltd., South Africa). The reaction volume was 25 µl containing: 1 µl (90 ng) DNA template, 0.5 µl (10 µM) of each primer and 23 µl (2×) *KapaTaq* Ready Mix (Kapa Biosystems, Cape Town, South Africa). Two negative control reactions were prepared and contained the same components. However, 1 µl (90 ng) DNA template from a *Penicillium expansum* CV432 strain (Department of Microbiology, University of Stellenbosch, South Africa) was added to the reaction mixture for the one negative control reaction and an equal volume of MilliQ water was added instead of the template DNA in the other. Cycling conditions in the thermal cycler were as follows: An initial denaturing step at 94°C for 5 min, followed by 34 cycles consisting of 94°C for 30 s, primers annealing at 60°C for 45 s, chain

elongation at 72°C for 1 min and an additional chain elongation step at 72°C for 7 min. PCR products were visualized in 1% agarose gels stained with ethidium bromide under UV illumination.

3.4. Cloning and sequencing

Before cloning, PCR products were purified using the Invitex DNA cleaning kit (Gesellschaft für Biotechnik & Biodesign mbH, Germany). The purified PCR products were cloned into pTZ57R/T vector of the InsTAclone™ PCR Cloning Kit (Fermentas Pty (Ltd), South Africa) following the manufacture's instructions. Cell transformations were performed using EZ Chemically competent *Escherichia coli* (XL1-blue) cells (Lucigen Co., Johannesburg, South Africa) according to the manufacture's instructions. Successfully transformed cells were selected and recombinant plasmid DNA isolated using the High Pure Plasmid Isolation Kit (Roche Applied Science, South Africa). The presence of the PCR fragment insert was confirmed by digesting the plasmid DNA with *Bam*HI and *Eco*RI (Fermentas Pty (Ltd), Cape Town, South Africa). Purified plasmid DNA was submitted for sequencing and sequence analyses on a Perkin Elmer ABI3100 genetic analyzer (Analytical Facilities, University of Stellenbosch, South Africa) and primers M13 and T7 were used for the sequencing reactions.

The obtained nucleotide sequences were analyzed and edited using the software program DNASTar SeqMan™ II (DNA-STAR Inc., WI, USA). To verify whether the obtained sequences were endoxylanase sequences, a nucleotide search on Basic Local Alignment Search Tools (n-BLAST) software (National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) for each sequence was performed.

3.5. Phylogenetic analyses

Sequence alignments were performed in CLUSTALX version 1.8 and were manually adjusted in PAUP version 4.0b10 (Swofford, 2001). For all the strains (Table 1), four data sets were analyzed as follows: (i) an alignment of endoxylanase partial gene sequences (this study); (ii) an alignment of functional gene sequences of UTP-ammonia ligase (URA); phosphate permase (PHO), trichothecene 3-*O*-acetyltransferase (TRI101); putative reductase (RED) (O'Donnell *et al.*, 2000, 2004) and endoxylanase partial gene (this study); (iii) an alignment of structural gene sequences of translation elongation factor 1 alpha (TEF-1 α) and histone H3 (H-3) and (iv) an alignment of the combined structural and functional gene sequences.

To evaluate whether the different data sets could be combined, a Templeton nonparametric Wilcoxon Signed Rank (WS-R) test (Kellogg *et al.*, 1996) and a Kishino-Hasegawa (K-H) test implemented in PAUP version 4.0b10 (Swofford 2001) were conducted for the different combinations.

Phylogenetic relationships for each data set were determined using distance analysis by neighbour-joining in PAUP*. *Fusarium pseudograminearum* NRRL28062 sequences were selected for rooting all distance analysis trees for all datasets as it has shown to be distantly related to the tester strains used in this study (O'Donnell *et al.*, 2000, 2004). In all distance analyses, characters were treated as unweighted and gaps were treated as missing data. The confidence levels of the nodes of distance analysis trees were evaluated by performing a bootstrap analysis of 1000 random replicates.

4. RESULTS

4.1. Cloning and sequencing

PCR amplification of DNA from the strains (Table 1) using degenerated primers XYLF1 and XYLR1 designed in this study resulted in an approximately 600 bp fragment in all strains.

PCR products of only nine of the ten strains (Table 1) are illustrated (Fig. 1). The partial endoxylanase (hereafter referred to as XYL) sequences obtained in this study were compared to those available on the Genbank database. Endoxylanase sequences obtained in this study were homologous to endo- β -1,4-xylanases sequences of *Gibberella zeae* (AY289919) and *F. oxysporum* f. sp. *lycopersici* (AF246831).

Visual inspection of the aligned XYL partial gene sequences readily identified great variability in the intron regions of the sequences and these regions were excluded in the phylogenetic analyses (Appendix C).

4.2. Phylogenetic analyses

The WS-R and K-H tests (Appendix D), indicated that in the functional gene data sets, both RED and TRI101 data sets, individually could not be combined with the URA data set. Based on the low $P =$ values ($P = 0.0041$ and $P = 0.0002$, respectively), it is speculated that some degree of homoplasy (backward and parallel substitutions) in the two data sets, RED and TRI101, caused failure of both data sets to be successfully combined with the URA data set. Also, the WS-R and K-H tests showed that data sets of PHO and XYL ($P = 0.0001$) could not be combined. However, when the URA, PHO, RED, TRI101 and XYL data sets were evaluated, both the WS-R and K-H tests showed that the data sets could be combined in different combinations. The WS-R and K-H tests showed that TEF-1 α and H-3 could be combined (data not shown) and also the combined functional and structural gene data sets could be combined (data not shown).

The aligned data set of partial XYL consisted of 381 characters (introns excluded) of which 319 were constant. Based on the neighbour-joining analysis of partial XYL, strains in this study were resolved into three clades (Fig. 2). Clade 1 consisted of *F. acaciae-mearnsii* (NRRL26754), *F. meridionale* (NRRL28436), *F. austroamericanum* (NRRL2903) and *F. culmorum* (NRRL3288). Three of these strains, *F. acaciae-mearnsii*, *F. meridionale* and

F. austroamericanum, were previously recognized as lineages 5, 2 and 1, respectively, while *F. culmorum* had not been assigned a lineage number within the *F. graminearum* (*Fg*) clade (O'Donnell *et al.*, 2000, 2004). Clade 2 consisted of *F. asiaticum* (NRRL13818) and *F. graminearum* (NRRL28439), former lineage 6 and 7, respectively. A third clade consisted of *F. boothii* (NRRL29020), *F. mesoamericanum* (NRRL29148) and *F. cerealis* (NRRL25491). Both *F. boothii* and *F. mesoamericanum* are former lineages 3 and 4 within the *Fg* clade, while *F. cerealis* had no lineage number assigned to it (O'Donnell *et al.*, 2000, 2004). Internal branches within clade 1 were not strongly supported and bootstrap values (indicated bold) ranged from 65–87%. However, the grouping of strains in clade 2 was strongly supported by a 100% bootstrap value. Furthermore, grouping of clade 3 strains was not strongly supported (75% bootstrap value), but *F. boothii* and *F. mesoamericanum* grouped together within the clade with a 100% bootstrap value.

The aligned functional gene data sets consisted of 4164 characters of which 3734 characters were constant. Distance analysis of the combined data sets resolved all strains into three clades (Fig. 3). Clade 1 consisted of *F. acaciae-mearnsii*, *F. asiaticum* and *F. graminearum*. Interestingly, these strains are former lineages 5, 6 and 7 of the *Fg* clade, respectively. *Fusarium austroamericanum*, *F. meridionale*, *F. boothii* and *F. mesoamericanum* all grouped together into clade 2, also in an order of former lineage 1, 2, 3 and 4, respectively. A third clade of the tree consisted of *F. cerealis* and *F. culmorum*, which were both never assigned lineage numbers in the *Fg* clade (O'Donnell *et al.*, 2000, 2004). To evaluate whether the XYL data set influenced the topology of the tree, a second distance analysis tree of the combined functional gene data sets was generated with the XYL data set excluded from the analysis. The resulting tree had the same topology as the combined functional genes data tree with the XYL data set included (data not shown), however, the strength of the branches in four nodes showed a slight decrease. These new bootstrap values were indicated in parentheses above bootstrap values of the combined data sets (Fig. 3).

The aligned combined structural gene data sets consisted of 1096 characters, of which 970 characters were constant. Based on the distance analysis tree of these data sets (Fig. 4), the strains grouped into two distinct clades. Clade 1 consisted of *F. acaciae-mearnsii*, *F. asiaticum*, *F. austroamericanum*, *F. meridionale*, *F. mesoamericanum*, *F. boothii* and *F. graminearum*, former lineages 5, 6, 1, 2, 4, 3, 7, respectively. There was no clear resolution of species in this tree as observed in the distance analyses of both XYL and functional gene data sets. Clade 2 consisting of *F. cerealis* and *F. culmorum* in the tree was however identical to clade 3 of the combined functional gene data sets.

The aligned combined functional and structural gene data sets consisted of 5260 characters, of which 4710 were constant. The topology of the distance analysis tree for these data sets was identical to that of the functional gene data sets (Fig. 5) with slight decrease in the bootstrap values of the *F. acaciae-mearnsii* branch and *F. asiaticum* and *F. graminearum* branches in clade 1, respectively. The bootstrap value of clade 3 however increased from 72% (functional gene data sets analysis) to 95% for combined functional and structural data sets analysis (Fig. 3).

5. DISCUSSION AND CONCLUSION

The nine recognized phylogenetic species within the *Fg* clade were resolved by genealogical concordance of 11 nuclear gene regions (O'Donnell *et al.*, 2000, 2004). Firstly, O'Donnell *et al.* (2000) combined six gene loci, PHO, TEF-1 α , β -tubulin (TUB), URA, TRI101 and RED to reveal seven biogeographically structured lineages within the *Fg* clade using parsimony analysis. In another study (O'Donnell *et al.*, 2004) an additional five genes (scaffolding 2–histone H3 and scaffold 5–*MAT1-1-3*; *MAT1-1-2*, *MAT1-1-1* and *MAT1-2-1*) were included to the O'Donnell *et al.* (2000) genes to make a sum total of 11 genes. Maximum parsimony analyses performed on the combined data sets revealed that the seven previously recognized lineages plus two new ones (8 and 9) evidently represented nine

phylogenetically distinct species within the *Fg* clade (O'Donnell *et al.*, 2004). In this study, the distance analyses of the XYL, functional gene, structural gene and combined data sets resulted in tree topologies that were similar to those of O'Donnell *et al.* (2000, 2004). Clustering according to lineages (O'Donnell *et al.*, 2000, 2004) was mostly evident in the analyses of the XYL, functional gene and combined data sets.

In the phylogenetic analysis tree of the XYL data set, *F. acaciae-mearnsii* (former lineage 5) was shown to be distantly related to both *F. boothii* and *F. mesoamericanum*. This is in disagreement with the results of O'Donnell *et al.* (2004), which show *F. acaciae-mearnsii* to be closely related to *F. boothii* and *F. mesoamericanum*. Also, the positions of *F. culmorum* and *F. cerealis* in the XYL tree are different from those in the other trees in this study. *Fusarium culmorum* is shown to be closely related to *F. acaciae-mearnsii*, *F. meridionale* and *F. austroamericanum*, while *F. cerealis* is closely related to *F. boothii* and *F. mesoamericanum*. Our results in this regard are in disagreement with those of O'Donnell *et al.* (2000, 2004), which show these two species to be closely related. However, when the XYL data set is analyzed in combination with other functional gene data sets, then the clustering of the two species, *F. culmorum* and *F. cerealis* resembles the results of O'Donnell *et al.* (2000, 2004). In addition, when the XYL data set was excluded in distance analysis of the functional gene data sets, there was noticeable decrease in branch confidence values of four nodes. This indirectly reflects the input the XYL database has in species resolution within the functional gene data sets.

Furthermore, in comparison to the XYL and functional gene data sets, distance analysis of the structural gene data sets had a lesser discriminatory power between the species in the *Fg* clade. This could have been the result of using few genes, which affects the number of phylogenetic informative characters that can be available for phylogenetic analysis. Rokas and Carroll (2005) have shown that increasing gene number has a significant positive effect on phylogenetic accuracy in analyses.

The main objective of this study was to evaluate the use of an endoxylanase gene, a functional gene, as a molecular marker in phylogenetic analysis of *Fusarium* species identification using *Fg* clade species as model. Our results demonstrate that an endoxylanase gene region can resolve phylogenetically the 10 species of the *Fg* clade. The fact that this gene region resulted in phylogenetic grouping of species that resembled those of O'Donnell *et al.* (2000, 2004) was very interesting. Even though the XYL region used in the present study was a partial region, it nonetheless clearly resolved the different species within the *Fg* clade. This, to the best of our knowledge is the first report on the use of an endoxylanase gene region in phylogenetic analysis of fungal species in the genus *Fusarium*.

The results of the present study provide a starting point in exploring the use of genes encoding hydrolytic enzymes as molecular markers in phylogenetic analysis of fungal pathogens. However, the fact that endoxylanases are multicopy genes remains a challenge in the application of these genes as molecular markers. Future research on the endoxylanase gene region will therefore include using the obtained endo- β -1,4-xylanase sequences to design PCR species-specific primers, designating the primer annealing location within the xylanase gene and evaluating whether the primers anneal to coding or non-coding regions of this gene.

Table 1. Isolates, their geographic origin and gene sequences used in this study.

Isolate	Species	Geographic origin	Source	Genbank accession no.					
				URA	PHO	TRI101	RED	TEF-1 α	H-3
NRRL26754	<i>F. acaciae-mearnsii</i>	South Africa	NCBI	AF212632	AF212485	AF212595	AF212559	AF212448	AY452841
NRRL13818	<i>F. asiaticum</i>	Japan	NCBI	AF212635	AF212488	AF212598	AF212562	AF212451	AY452821
NRRL28439	<i>F. graminearum</i>	Netherlands	NCBI	AF212644	AF212497	AF212607	AF212571	AF212460	AY452839
NRRL2903	<i>F. austroamericanum</i>	Brazil	NCBI	AF212622	AF212475	AF212585	AF212549	AF212438	AY452822
NRRL28436	<i>F. meridionale</i>	New Caledonia	NCBI	AF212619	AF212472	AF212582	AF212546	AF212435	AY452824
NRRL29020	<i>F. boothii</i>	South Africa	NCBI	AF212627	AF212480	AF212590	AF212554	AF212443	AY452829
NRRL29148	<i>F. mesoamericanum</i>	Pennsylvania,USA	NCBI	AF212626	AF212479	AF212589	AF212553	AF212442	AY452847
NRRL25491	<i>F. cerealis</i>	Netherlands	NCBI	AF212649	AF212502	AF212612	AF212576	AF212465	AY452831
NRRL28062	<i>F. pseudograminearum</i>	Australia	NCBI	AF212652	AF212505	AF212615	AF212578	AF212468	AY452848
NRRL3288	<i>F. culmorum</i>	–	NCBI	AF212646	AF212499	AF212609	AF212573	AF212462	AY452833

NCBI = Isolates sequenced by O'Donnell *et al.* (2000) and sequences obtained from NCBI database (<http://www.ncbi.nlm.nih.gov/>).

URA = UTP-ammonia ligase; PHO = phosphate permase; TRI101 = trichothecene 3-*O*-acetyltransferase; RED = putative reductase; TEF-1 α = translation elongation factor 1 alpha; H-3 = histone H3.

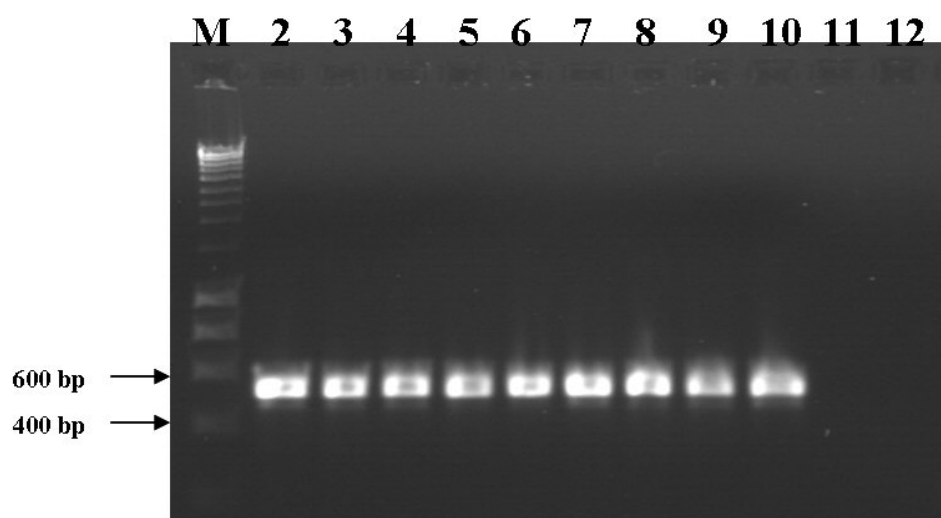


Figure 1. 1% Agarose gel of PCR-amplified endoxylanase gene fragments using degenerated primers XYLF1 and XYLR1. PCR products of nine of the ten tester strains are presented in the gel. **M** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-10:** NRRL26754, NRRL13818, NRRL28439, NRRL2903, NRRL28436, NRRL29020, NRRL29148, NRRL25491, NRRL28062; **lanes 11-12:** *P. expansum* CV432, negative control (without template DNA).

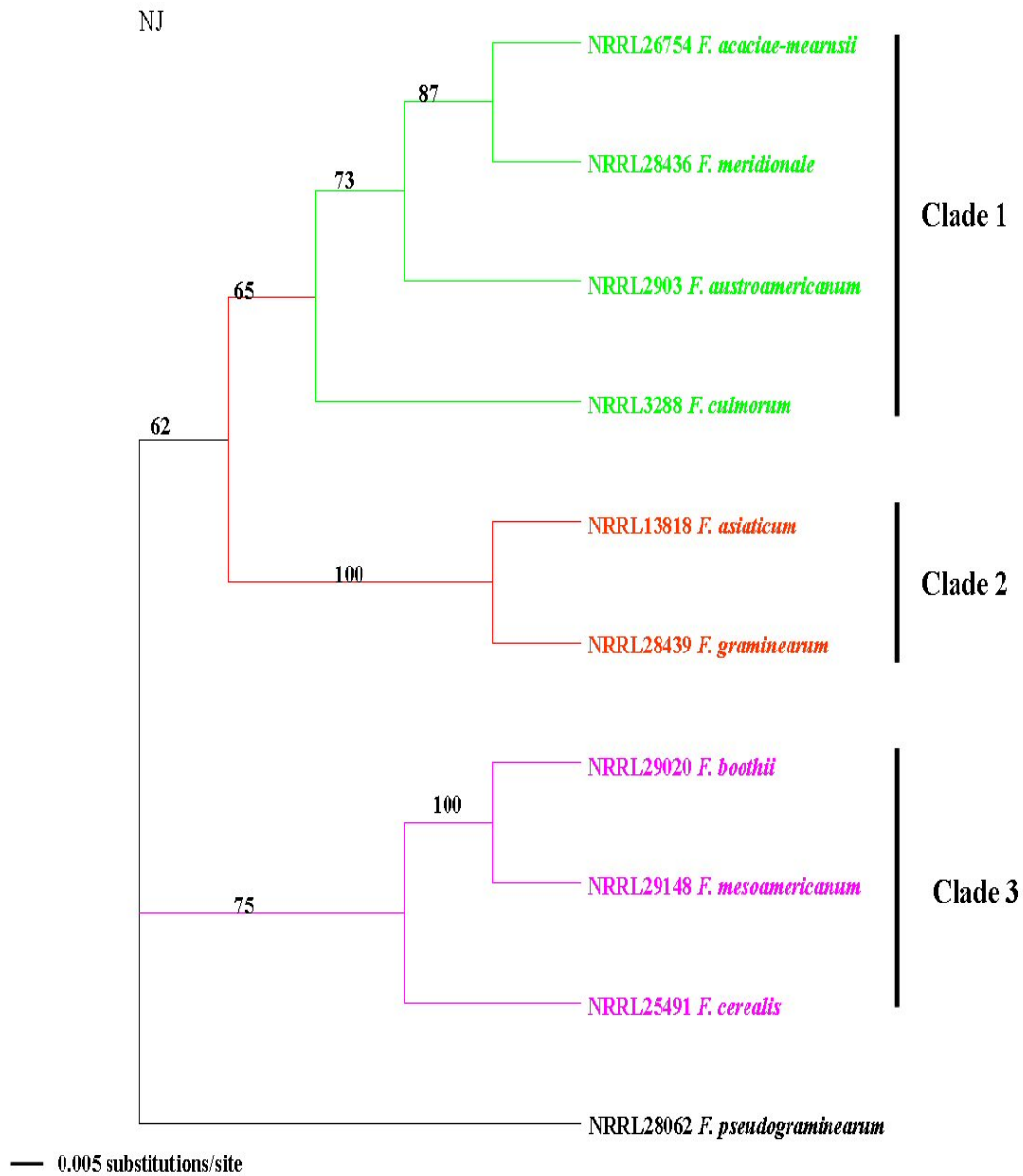


Figure 2. Cladogram of neighbour-joining analysis of partial endoxylanase (XYL) data set. Values above branch nodes indicate bootstrap values from 1000 replicates. *Fusarium pseudograminearum* NRRL28062 strain is used as outgroup.

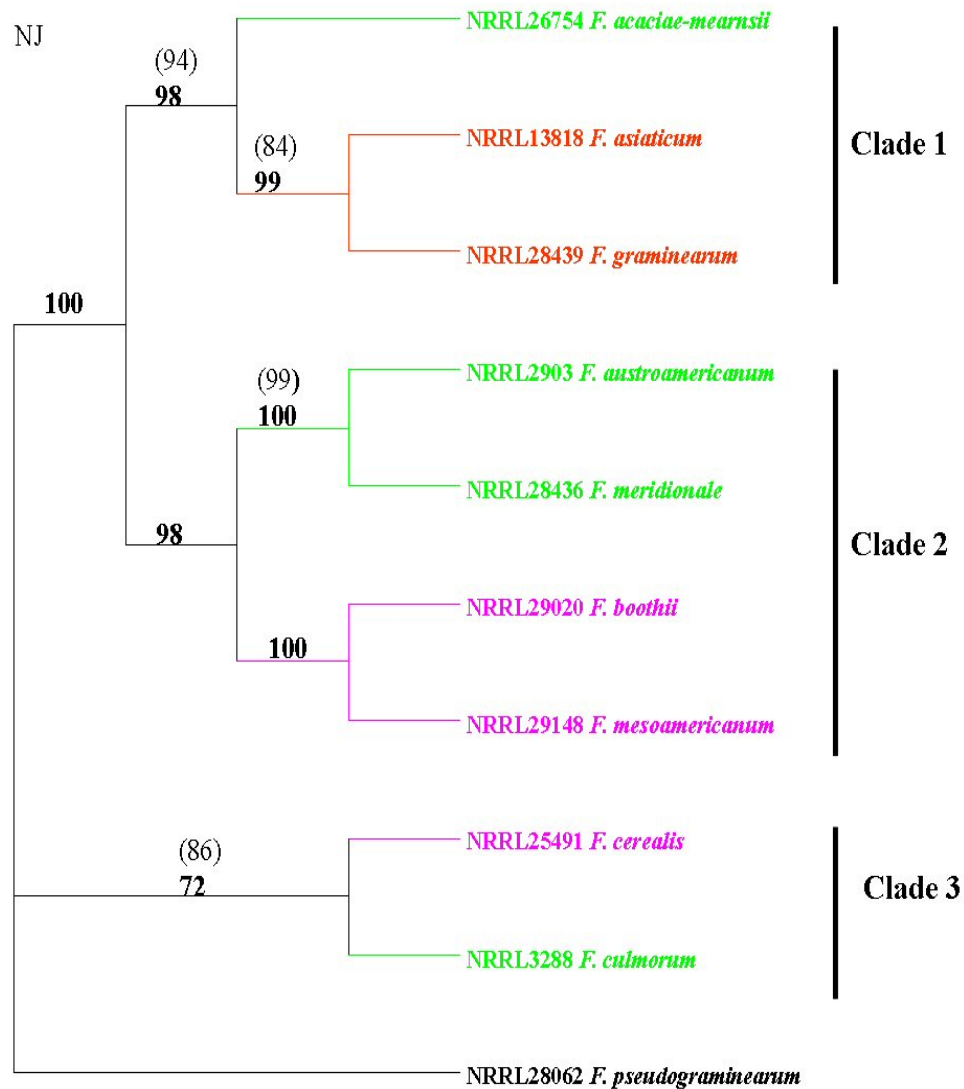


Figure 3. Cladogram of neighbour-joining analysis of functional gene (ARU-PHO-RED-TRI101-XYL) data sets. Values above branch nodes indicate bootstrap values from 1000 replicates. *Fusarium pseudograminearum* NRRL28062 strain is used as outgroup. Values in parentheses above the nodes represent bootstrap support after excluding the XYL data set from the functional gene data sets. The strains are colour-coded based on clustering as shown in Fig. 2.

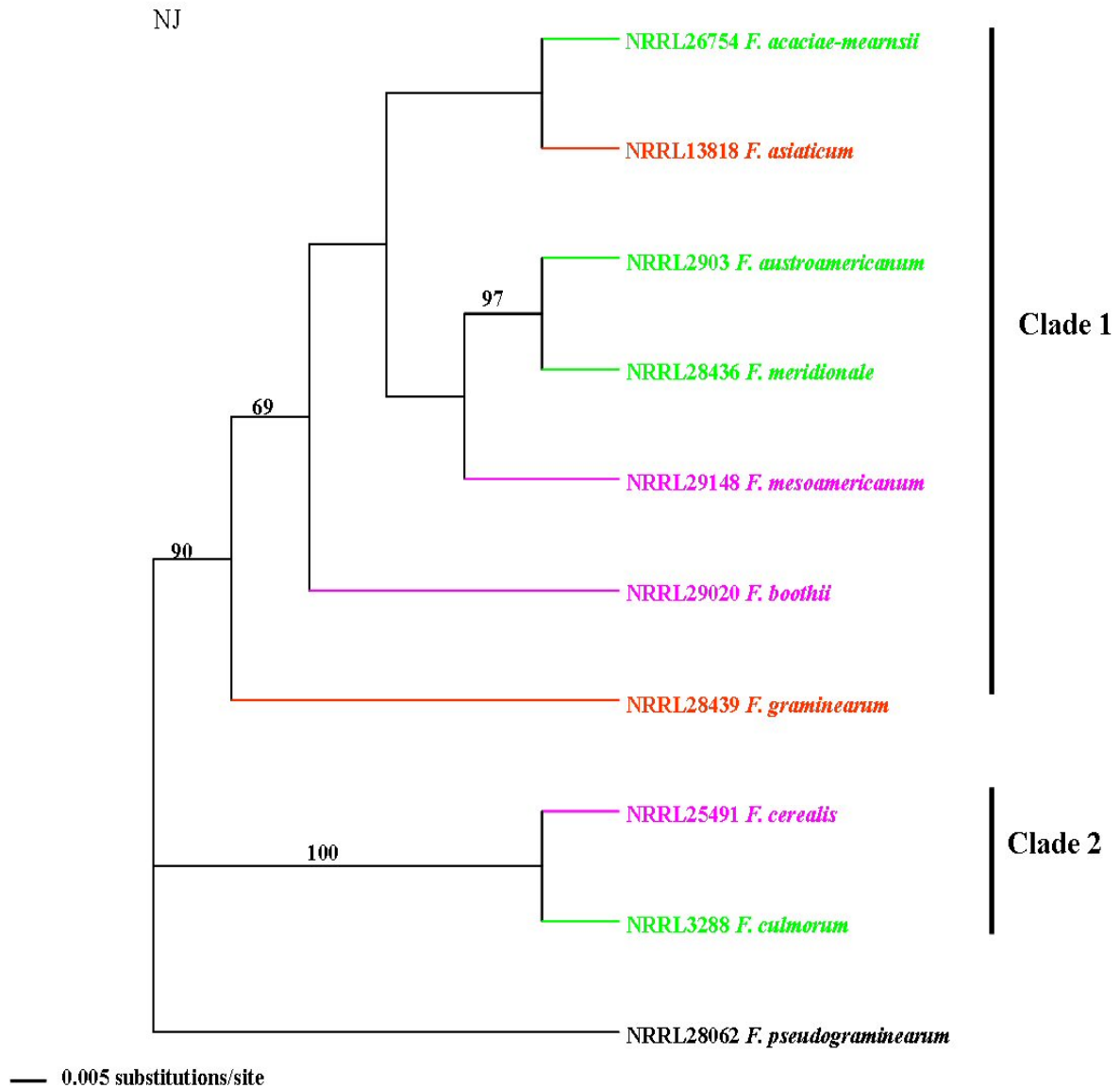


Figure 4. Cladogram of neighbour-joining analysis of structural gene (TEF-1 α and H-3) data set. Values above branch nodes indicate bootstrap values from 1000 replicates. *Fusarium pseudograminearum* NRRL28062 strain is used as outgroup. The strains are colour-coded based on clustering as shown in Fig. 2.

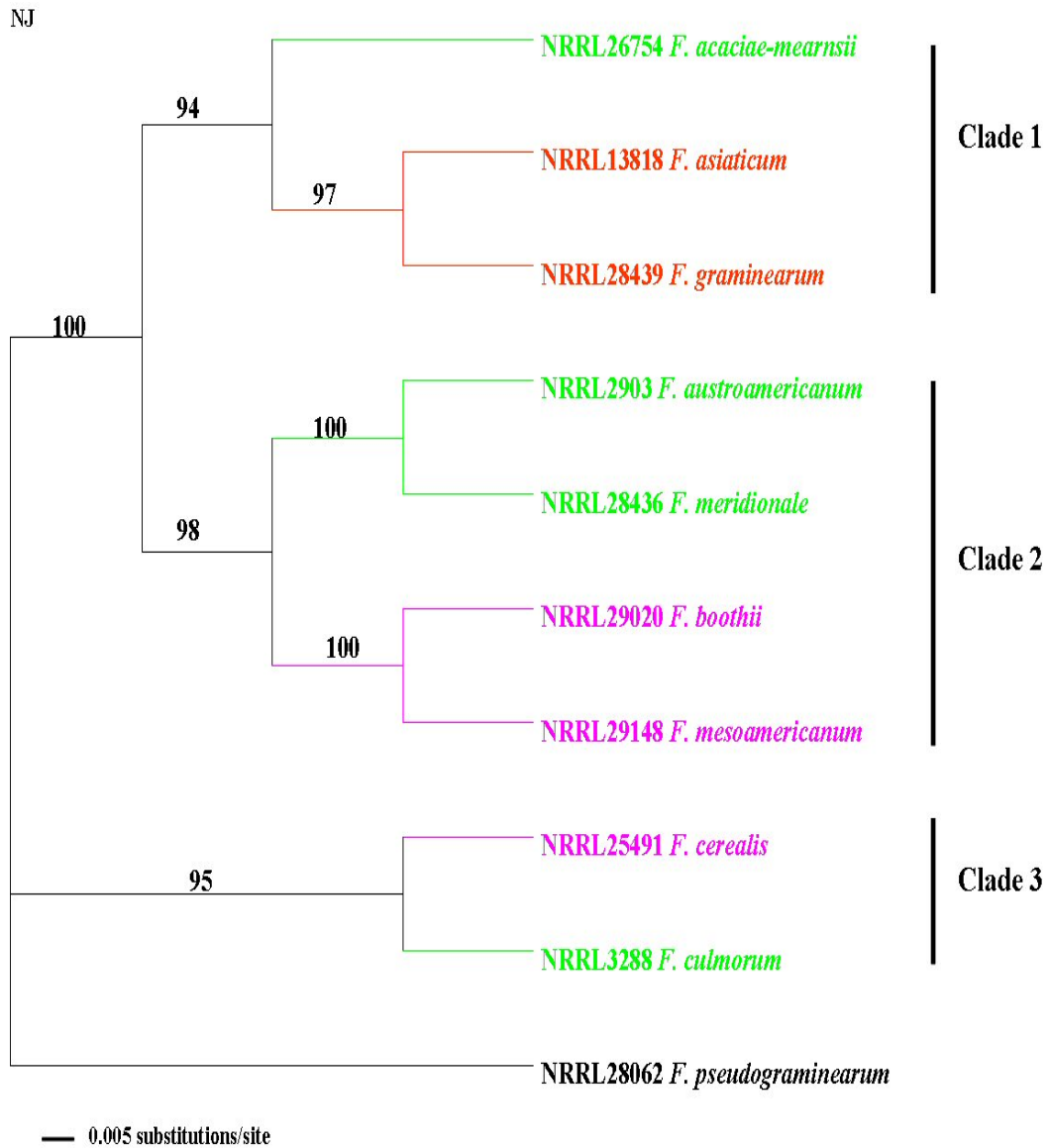


Figure 5. Cladogram of neighbour-joining analysis of combined functional gene and structural gene data sets. Values above branch nodes indicate bootstrap values from 1000 replicates. *Fusarium pseudograminearum* NRRL28062 strain is used as outgroup. The strains are colour-coded based on clustering as shown in Fig. 2.

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APPENDIX A

Appendix A: *Fusarium* isolates recovered from fynbos soils and plant debris (this study)

Sampling site	Isolate number	Source	Recovery month	<i>Fusarium</i> Species	Sampling site	Isolate number	Source	Recovery month	<i>Fusarium</i> Species
Silvermine	A2	plant debris	Jun.	<i>F. oxysporum</i>	Tokai	B5	plant debris	Apr.	<i>F. oxysporum</i>
	A37	plant debris	Apr.	<i>F. oxysporum</i>		B10	plant debris	Jun.	<i>F. oxysporum</i>
	A80	soil	Jun.	<i>F. oxysporum</i>		B14	plant debris	Jun.	<i>F. oxysporum</i>
	A84	soil	Apr.	<i>F. oxysporum</i>		B32	plant debris	Apr.	<i>F. oxysporum</i>
	A86	soil	Jun.	<i>F. oxysporum</i>		B33	plant debris	Jun.	<i>F. oxysporum</i>
	A146	soil	Jun.	<i>F. oxysporum</i>		B38	plant debris	Jun.	<i>F. oxysporum</i>
	A149	soil	Jun.	<i>F. oxysporum</i>		B41	plant debris	Apr.	<i>F. oxysporum</i>
	A151	soil	Jun.	<i>F. oxysporum</i>		B44	plant debris	Apr.	<i>F. oxysporum</i>
	A162	soil	Aug.	<i>F. oxysporum</i>		B50	plant debris	Apr.	<i>F. oxysporum</i>
	A185	plant debris	Aug.	<i>F. oxysporum</i>		B52	plant debris	Apr.	<i>F. oxysporum</i>
	A193	plant debris	Aug.	<i>F. oxysporum</i>		B60	plant debris	Apr.	<i>F. oxysporum</i>
	A194	plant debris	Aug.	<i>F. oxysporum</i>		B170	plant debris	Aug.	<i>F. oxysporum</i>
	A209	plant debris	Aug.	<i>F. solani</i>		B190	plant debris	Aug.	<i>F. oxysporum</i>
	A211	plant debris	Aug.	<i>F. oxysporum</i>		B197	plant debris	Aug.	<i>F. oxysporum</i>
	A212	plant debris	Aug.	<i>F. oxysporum</i>		B202	plant debris	Aug.	<i>F. oxysporum</i>
	A220	plant debris	Nov.	<i>F. oxysporum</i>		B203	plant debris	Aug.	<i>F. oxysporum</i>
A221	plant debris	Nov.	<i>F. oxysporum</i>						
A222	plant debris	Nov.	<i>F. oxysporum</i>						
A223	soil	Nov.	<i>F. oxysporum</i>						

* *F.* ‘fynbos’ = are the fynbos isolates that were grouped under the morphological group referred to as *Fusarium* ‘fynbos’

Appendix A (continued)

Sampling site	Isolate number	Source	Recovery month	<i>Fusarium</i> Species	Sampling site	Isolate number	Source	Recovery month	<i>Fusarium</i> Species
Trappieskop	C7	plant debris	Jun.	<i>F. oxysporum</i>	Boyes Drive	D21	plant debris	Apr.	<i>F. oxysporum</i>
	C12	plant debris	Aug	<i>F. oxysporum</i>		D23	plant debris	Apr.	<i>F. solani</i>
	C26	plant debris	Jun.	<i>F. oxysporum</i>		D35	plant debris	Jun.	<i>F. oxysporum</i>
	C30	plant debris	Jun.	<i>F. oxysporum</i>		D40	plant debris	Apr.	<i>F. oxysporum</i>
	C48	plant debris	Jun.	<i>F. oxysporum</i>		D47	plant debris	Apr.	<i>F. oxysporum</i>
	C54	plant debris	Apr	<i>F. oxysporum</i>		D55	plant debris	Apr.	<i>F. oxysporum</i>
	C135	plant debris	Aug	<i>F. oxysporum</i>		D56	plant debris	Apr.	<i>F. oxysporum</i>
	C140	soil	Jun.	<i>F. oxysporum</i>		D122	soil	Apr.	<i>F. oxysporum</i>
	C152	soil	Jun.	<i>F. oxysporum</i>		D123	soil	Apr.	<i>F. oxysporum</i>
	C168	plant debris	Aug.	<i>F. oxysporum</i>		D131	soil	Apr.	<i>F. oxysporum</i>
	C169	plant debris	Aug.	<i>F. solani</i>		D136	plant debris	Jun.	<i>F. oxysporum</i>
	C171	plant debris	Aug.	<i>F. oxysporum</i>		D141	soil	Jun.	<i>F. oxysporum</i>
	C198	plant debris	Aug.	<i>F. oxysporum</i>		D143	soil	Jun.	<i>F. oxysporum</i>
	C206	plant debris	Aug.	<i>F. solani</i>		D148	soil	Aug	<i>F. oxysporum</i>
	C208	plant debris	Aug.	<i>F. oxysporum</i>		D156	soil	Jun.	<i>F. oxysporum</i>
C214	plant debris	Aug.	<i>F. solani</i>	D159	soil	Jun.	<i>F. oxysporum</i>		
Boyes Drive	D3	plant debris	Jun.	<i>F. oxysporum</i>	D161	soil	Jun.	<i>F. oxysporum</i>	
	D4	plant debris	Jun.	<i>F. oxysporum</i>	D174	plant debris	Aug.	<i>F. equiseti</i>	
	D11	plant debris	Apr.	<i>F. oxysporum</i>	D175	plant debris	Aug.	* <i>F. 'fynbos'</i>	
	D13	plant debris	Apr.	<i>F. oxysporum</i>	D177	plant debris	Aug.	<i>F. oxysporum</i>	
	D17	plant debris	Apr.	<i>F. oxysporum</i>	D180	plant debris	Aug.	<i>F. oxysporum</i>	
	D20	plant debris	Apr.	<i>F. solani</i>	D191	plant debris	Aug.	<i>F. oxysporum</i>	
					D192	plant debris	Aug.		

* *F. 'fynbos'* = are the fynbos isolates that were grouped under the morphological group referred to as *Fusarium 'fynbos'*

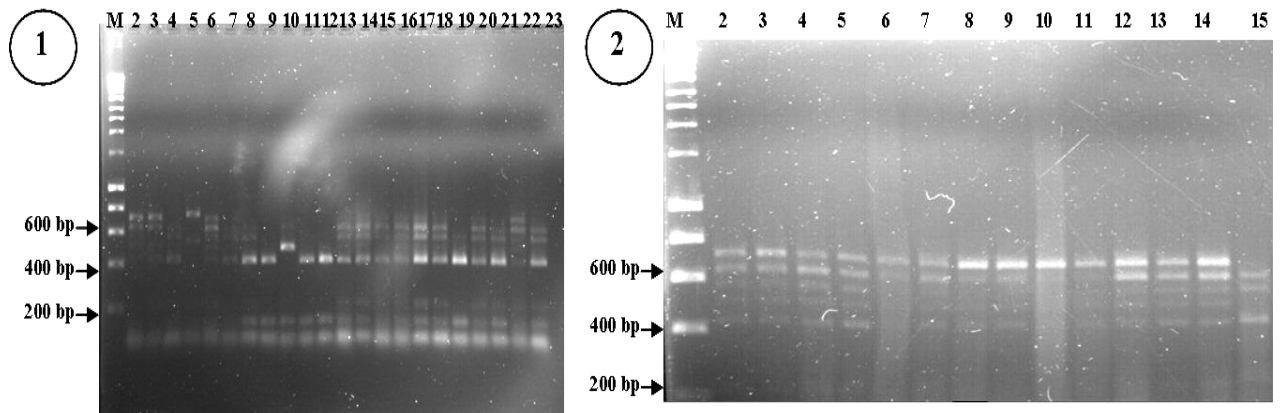
Appendix A (continued)

Sampling site	Isolate number	Source	Recovery month	<i>Fusarium</i> Species	Sampling site	Isolate number	Source	Recovery month	<i>Fusarium</i> Species
Boyes Drive	D201	plant debris	Aug.	<i>F. oxysporum</i>	Olifantsbos (unburnt)	E173	plant debris	Aug.	* <i>F. 'fynbos'</i>
	D207	plant debris	Aug.	<i>F. oxysporum</i>		E178	plant debris	Aug.	<i>F. oxysporum</i>
	D215	plant debris	Nov.	<i>F. oxysporum</i>		E182	plant debris	Aug.	* <i>F. 'fynbos'</i>
	D216	plant debris	Nov.	<i>F. equiseti</i>		E184	plant debris	Aug.	<i>F. oxysporum</i>
	D217	soil	Nov.	<i>F. oxysporum</i>		E186	plant debris	Aug.	<i>F. oxysporum</i>
	D218	soil	Nov.	* <i>F. 'fynbos'</i>		E187	plant debris	Aug.	<i>F. oxysporum</i>
	D219	soil	Nov.	* <i>F. 'fynbos'</i>		E195	plant debris	Aug.	* <i>F. 'fynbos'</i>
	D225	plant debris	Nov.	<i>F. oxysporum</i>		E199	plant debris	Aug.	<i>F. oxysporum</i>
	D226	plant debris	Nov.	<i>F. oxysporum</i>		E224	plant debris	Nov.	<i>F. oxysporum</i>
	D227	soil	Nov.	* <i>F. 'fynbos'</i>					
	D228	soil	Nov.	<i>F. oxysporum</i>	F117	Soil	Apr.	<i>F. oxysporum</i>	
	D229	soil	Nov.	<i>F. oxysporum</i>	F118	Soil	Apr.	<i>F. oxysporum</i>	
	D230	soil	Nov.	<i>F. oxysporum</i>	F165	Soil	Aug.	<i>F. solani</i>	
	D232	soil	Nov.	<i>F. equiseti</i>	F172	plant debris	Aug.	<i>F. oxysporum</i>	
	D233	soil	Nov.	<i>F. oxysporum</i>	F176	plant debris	Aug.	<i>F. oxysporum</i>	
	D234	soil	Nov.	<i>F. oxysporum</i>	F179	plant debris	Aug.	* <i>F. 'fynbos'</i>	
	D235	soil	Nov.	<i>F. oxysporum</i>	F181	plant debris	Aug.	<i>F. oxysporum</i>	
	D236	soil	Nov.	<i>F. oxysporum</i>	F183	plant debris	Aug.	<i>F. solani</i>	
	D237	soil	Nov.	<i>F. oxysporum</i>	F189	plant debris	Aug.	<i>F. oxysporum</i>	
				F196	plant debris	Aug.	<i>F. oxysporum</i>		
				F200	plant debris	Aug.	<i>F. solani</i>		
				F205	plant debris	Aug.	<i>F. oxysporum</i>		
				F213	plant debris	Aug.	<i>F. oxysporum</i>		
				F238	plant debris	Nov.	<i>F. oxysporum</i>		

* *F. 'fynbos'* = are the fynbos isolates that were grouped under the morphological group referred to as *Fusarium* 'fynbos'

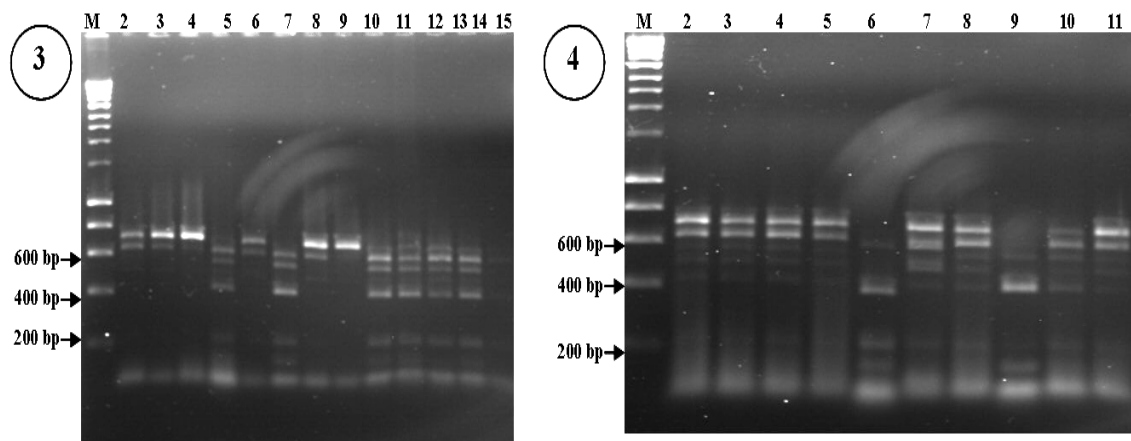
APPENDIX B

Appendix B



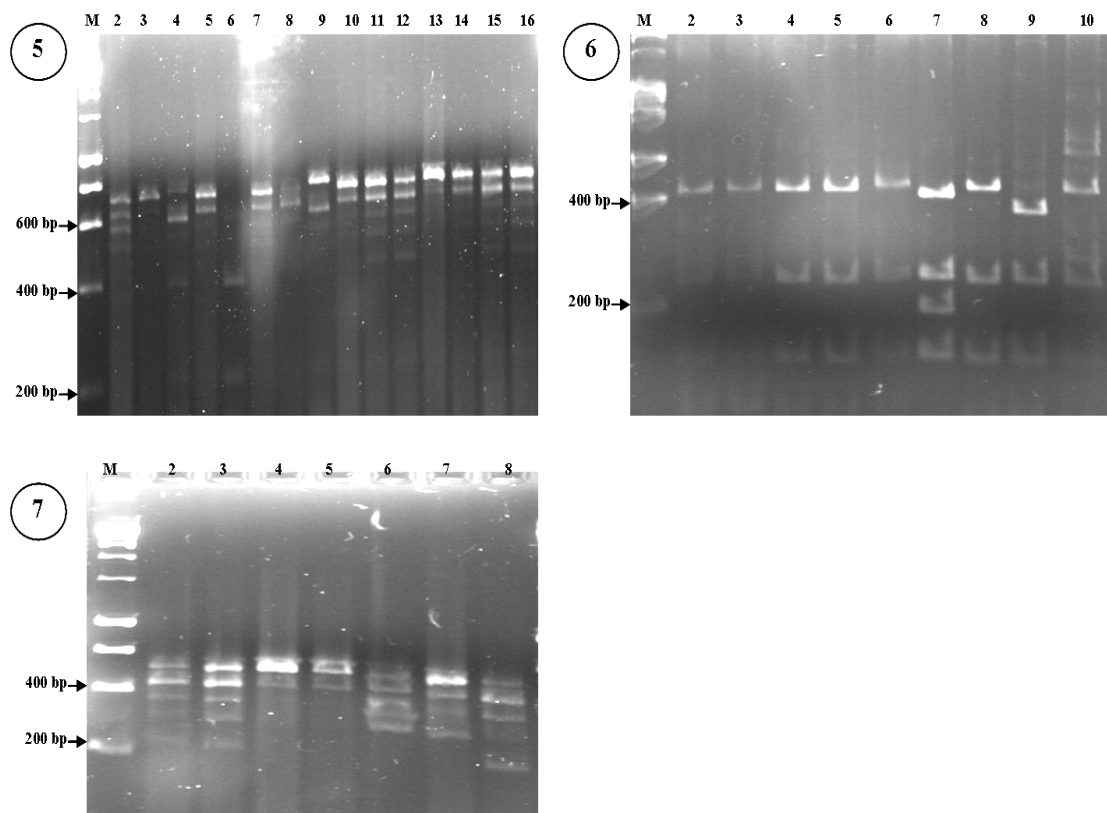
Appendix B: Restriction patterns of PCR-amplified translation elongation factor 1 alpha of *Fusarium* isolates digested with *RsaI*. Restriction patterns were analyzed in 2% agarose gel stained with ethidium bromide.

1. **M:** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-23:** B5, D17, D21, D23, C30, A2, D35, B38, D40, B41, B44, D47, C48, B52, C54, D55, D56, A80, D4, D11, C12, D13
2. **M:** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-15:** A84, A86, D123, D131, D136, C140, D141, D143, A149, C152, D156, D159, D161, A162.



Appendix B (continued): Restriction patterns of PCR-amplified translation elongation factor 1 alpha of *Fusarium* isolates digested with *RsaI*. Restriction patterns were analyzed in 2% agarose gel stained with ethidium bromide.

3. **M:** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-15:** A2, A37, B10, B14, B33, C7, C26, D3, D4, D11, D13, E182.
4. **M:** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-11:** F172, F176, E178, D180, F183, A185, E186, B190, A194, F196.



Appendix B (continued): Restriction patterns of PCR-amplified translation elongation factor 1 alpha of *Fusarium* isolates digested with *RsaI*. Restriction patterns were analyzed in 2% agarose gel stained with ethidium bromide.

5. **M:** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-16:** C198, E199, F200, B203, C206, D207, C208, A209, A211, A212, F213, D215, A220, A221, A222.
6. **M:** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-10:** D232, D192, A2, A37, A84, F181, E186, C198, F213.
7. **M:** (molecular weight marker): Hyper Ladder1 (Fermentas Pty (Ltd), Cape Town, South Africa); **lanes 2-8:** A223, E224, D225, D233, D234, D235, F118.

APPENDIX C

Appendix C: The endoxylanase (XYL) sequences (intron regions excluded) of the *F. graminearum* species (this study).

NRRL26754 *Fusarium acaciae-mearnsii*

GGTGGAAGGGATGGAACCCTGGTACTGGCCGGTAAGTTCTCTTAGATATAT
TATCTTACAAATCATTGACTTACTATGCAGAACCATCAACTACGGAGGTT
CCTTCAACCCTCAGGGTAACGGATACCTTTGCGTTTACGGATGGACCCGCGGT
CCCCTCGTCGAGTACTACGTAAGTGACTCTAACCTCAAACCTCCTATACCTCG
GTACTAACAATTATCCAGGTCATCGAGAGTTACGGTTCTTACAACCCCGGCA
GCCAGGCTCAGCACCGAGGTACCGTCTACACCGACGGTGACACCTACGATCT
CTACATGTCTACCCGTGTCCAGCAGCCTTCCATCGATGGTGTTTCAGACATTCA
ACCAGTACTGGTCCATCCGCCGCAACAAGCGCACCGGCTCCGTCAACAT
GCAGAACCACTTCAATGCTTGGAGATCTGCTGGCATGAACCTCGGCAACCAC
TACCACCAGATC

NRRL13818 *F. asiaticum*

GGTGGAAGGGATGGAACCCTGGTACTGGCCGGTAAGTTCTCTCAGACATAT
TATCTTACAAATCATTGACTTACTATGCAGAACCATCAACTACGGAGGTT
CCTTCAACCCTCAGGGTAACGGATACCTTTGCGTTTACGGATGGACCCGCGGT
CCCCTCGTCGAGTACTACGTAAGTGAATCTAACCTCAAACCTCCTATACCTCG
GTACTAACAATTATCCAGGTCATCGAGAGTTACGGTTCTTACAACCCCGGCA
GCCAGGCTCAGCACCGAGGTACCGTCTACACCGACGGTGACACCTACGATCT
CTATATGTCCACCCGTTACCAACAGCCTTCGATCGACGGTGTTTCAGACCTTCA
ACCAGTACTGGTCCATCCGCCGCAACAAGCGTACCAGCGGCTCCGTCAACAT
GCAGAACCACTTCAATGCTTGGAGATCTGCTGGCATGAACCTCGGAAACCAC
TACTACCAGATT

NRRL2903 *F. austroamericanum*

GGAAAGGGCTGGAACCCTGGTACTGGCCGGTAAGTTCTCTCACACATATTAT
CTTACAAATCATTTGACTTACACTATGTGCAGAACCATCAACTACGGAGGTTC
CTTCAACCCTCAGGGTAACGGATACCTTTGCGTTTACGGATGGACCCGCGGTC
CCCTCGTCGAGTACTACGCAAGTGACTCTAACCTCAAACCTCCTATACTCGG
TACTAACAATTATCCAGGTCATCGAGAGTTACGGTTCTTACAACCCCGGCAGC
CAGGCTCAGCACCGAGGTACCGTCTACACCGACGGTGACACCTACGATCTCT
ACATGTCTACCCGTGTCCAGCAGCCTTCCATCGATGGTGTTCAGACATTCAAC
CAGTAGTGGTCCATCCGCCGCAACAAGCGCACCAGCGGCTCCGTCAACATGC
AGAACCAATTCAATGCTTGGAGATCTGCTGGCATGAACCTCGGCAACCACTA
CTACCAGATG

NRRL 29020 *F. boothii*

GGAGGAAAGGGATGGAACCCTGGTACCGGCCGGTAAGTCTTCGCAGATATTG
ATTATCTCACAAATCGTTTGACTTACACTATGTGCAGAACCATCAACTACGGA
GGTTCCTTTAACCCTCAGGGCAACGGATACCTTTGCGTTTATGGATGGACTCG
CGGGCCTCTCGTCGAGTACTACGTAAGTGACACCAACATCAAACCTTTCTATAT
CTCGGTAATAACCATAATCTAGGTCATCGAGAGCTACGGCTCTTACAACCCCG
GTAGCCAGGCTCAGCACCGAGGTACCGTCTACACTGACGGTGACACCTACGA
TCTCTACTTGTCTACCCGTGTCCAGCAGCCTTCGATCGACGGTGTCCAGACTT
TCAACCAGTACTGGTCCATCCGCCGCAACAAGCGCACCAGCGGCTCCGTCAA
CATGCAGAACCACTTCAATGCTTGGAGGTCTGCTGGCATGAACCTCGGCAAC
CACTACTACCAGATC

NRRL25491 *F. cerealis*

GTGCGAAAGGGATGGAACCCTGGTACTGGCCGGTAAGTTCTCTCAGGCAGAT
TATCTTACAAATCATTTGACTTACACTATGCAGAACCATCAACTATGGAGGTT
CATTCAACCCTCAGGGTAACGGATACCTTTGCGTTTACGGATGGACACGCGG
TCCCCTCGTCGAGTACTACGTATGTGACTCCGCCATCCGAATTCTTGTATCAC
TACTAACGATTCTTTAGGTCATCGAGAGCTACGGCTCTTACAACCCCGGTAGC
CAGGCTCAGCACCGAGGTACCGTCTACACCGACGGTGATACCTACGATCTCT

ACATGTCCACACGTGTCCAGCAGCCTTCCATCGACGGTGTCCAGACATTCAAC
CAGTACTGGTCCATCCGCCGCAACAAGCGCACCAGCGGCTCCGTCAACATGC
AGAACCCTTCAATGCTTGGAGATCTGCTGGCATGAACCTCGGCAACCACTA
CTACCAGATC

NRRL3288 *F. culmorum*

GGTGGAAAGGGATGGAACCCTGGTACCGGCCGGTAAGTCTTCGCAGATATTG
ATTATCTCACGAATCGTTTGACTTACACTATTTTCAGAACCATCAACTACGGA
GGTTCCTTCAACCCTCAGGGTAACGGATACCTTTGCGTTTACGGATGGACCCG
CGGTCCCCTCGTCGAGTACTACGTAAGTGACTCTAACCTCAAACCTCCTATAC
CTCGGTACTAACAATTATCCAGGTCATCGAGAGTTACGGTTCTTACAACCCCG
GCAGCCAGGCTCAGCACCGAGGTACCGTCTACACCGACGGTGACACCTACGA
TCTCTACATGTCTACCCGTGTCCAGCAGCCTTCCATCGATGGTGTTCAGACAT
TCAACCAGTACTGGTCCATCCGCCGCAACAAGCGCACCAGCGGCTCCGTCAA
CATGCAGAACCCTTCAATGCTTGGAGATCTGCTGGCATGAACCTTGGAAAC
CACTACTACCAGATT

NRRL28439 *F. graminearum*

CGGGAAGTTCTCTCAGACATATTATATTACAAATCATTGACTTACACTATGC
AGAACCATCAACTACGGAGGTTCTTCAACCCTCAGGGTAACGGATACCTTT
GCGTTTACGGATGGACCCGCGGTCCCCTCGTCGAGCACTACGTAAGTGGCTC
AAACCTCACTTCTATACCTCGGTACTAACTATTTTCCTGCCATCGGAGTTA
CGGATCTTACCTCCCCGGCGGCCAGGCTCATCACCGAGGTACCGTCTACACC
GATGGTGACACCTACGATCTCTATATGTCCACCCGTTACCAACAGCCCACGAT
CGACGGTGTTCAAAGCTTCAACCAGTACTGGCACATCCGCCGCAAC

NRRL28436 *F. meridionale*

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TATCTTACAAATCATTGACTTACACTATGTGCAGAACCATCAACTACGGAGG
TTCCTTCAACCCTCAGGGTAACGGATACCTTTGCGTTTACGGATGGACCCGCG
GTCCCCTCGTCGAGTACTACGTAAGTGACTCTAACCTCAAACCTTCTTATACCT
CGGTACTAACAATTATCCAGGTCATCGAGAGTTACGGTTCTTACAACCCCGGC
AGCCAGGCTCAGCACCGAGGTACCGTCTACACCGACGGTGACACCTACGATC
TCTACATGTCTACCCGTGTCCAGCAGCCTTCCATCGATGGTGTTCAGACATTC
AACCAGTACTGGTCCATCCGCCGCAACAAGCGCACCAGCGGCTCCGTCAACA
TGCAGAACCCTTCAATGCTTGGAGATCTGCTGGCATGAACCTCGGCAACCA
CTACTACCAGATC

NRRL29148 *F. mesoamericanum*

GGTGGAAGGGATGGAACCCTGGTACCGGCCGGTAAGTCTTCGCAGATATTG
ATTATCTCACAATCGTTTGACTTACACTATGTGCAGAACCATCAACTACGGA
GGTTCCTTTAACCCTCAGGGCAACGGATACCTTTGCGTTTATGGATGGACTCG
CGGGCCTCTCGTCGAGTACTACGTAAGTGACACCAACATCAAACCTTTCTATAT
CTCGGTACTAACCATAATCTAGGTCATCGAGAGCTACGGCTCTTACAACCCCG
GTAGCCAGGCTCAGCACCGAGGTACCGTCTACACTGACGGTGACACCTACGA
TCTCTACTTGTCTACCCGTGTCCAGCAGCCTTCGATCGACGGTGTCCAGACTT
TCAACCAGTACTGGTCCATCCGCCGCAACAAGCGCGCCAGCGGCTCCGTCAA
CATGCGGAACCCTTCAATGCTTGGAGGTCTGCTGGCATGAACCTCGGCAAC
CACTACTACCAGATC

NRRL28062 *F. pseudograminearum*

GGTGGAAGGGATGGAACCCTGGTACTGGCCGGTAAGTTCTCTCAGACATTG
ATTATCTTACGGATTGTTTGACTTACATTGCGTGTAGAACCATCAACTACGGA
GGTTCCTTCAACCCTCAGGGCAACGGATACCTTTGTGTCTACGGTTGGACCCG
CGGTCCCCTCGTCGAGTACTACGTAAGTGACTACAACCTCAAACCTTGCTATAC
ACCGTTACTAACAATTATCCAGGTCATCGAGAGCTACGGTTCTTACAACCCCG
GTAGCCAGGCTCAGCACCGAGGTACCGTCTACACCGACGGTGACACCTACGA

TCTCTACATGTCCACCCGTGTCCAACAGCCTTCCATCGACGGTGTTTCAGACAT
TCAACCAGTACTGATCCATCCGCCGCAACAAGCGCACCAGCGGCTCCGTCAA
CATGCAGAACCACTTCAATGCTTGGAGATCTGCTGGCATGAACCTCGGCAAC
CACTACTACCAGATT

APPENDIX D