Molecular biological studies of the Fusarium wilt pathogen of banana in South Africa.

BY

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Declaration

I, the undersigned, declare that the work contained in this thesis is my own and original work and that it has not previously in its entirety or part submitted for a degree to any other university.

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PREFACE

Fusarium wilts, induced by special forms of the soilborne fungus *Fusarium oxysporum,* are considered to be among the most severe plant diseases in the world. Possibly the most devastating of these wilts is Fusarium wilt of banana, caused by *F. oxysporum* f. sp. *cubense* (*Foc*). Historically this disease became notorious for destroying many thousands of hectares of prime banana fields in Central America, but it is still considered a major constraint to banana production and expansion in many countries including South Africa.

Effective management of Fusarium wilt of banana requires a good knowledge of *Foc* in order to design the necessary control measures. The occurrence of variants of the pathogen has inspired scientists to extensively study this fungus in the last 20 years. These studies used both phenotypic and genotypic techniques such as VCGs and DNA fingerprinting techniques, and have resulted in useful information on pathogen diversity, origin, distribution, phylogeny and dispersal. Studies in this thesis were aimed at making deductions on the phylogeny, reproductive potential, host pathogen interactions and understanding the genetic diversity at the loci level. It shed light on pathogen diversity using several different molecular techniques. Each chapter in this thesis is presented as an independent entity with redundancy between chapters being unavoidable.

Chapter 1 presents the history of Fusarium wilt of banana, and provides the reader with a general review on the taxonomy, origin and spread of *Foc* in the world. The chapter gives a broad overview of techniques currently used to study the population diversity of the pathogen. The application of molecular techniques such as sequencing of the different gene genealogies used to subdivide *Foc* populations and understand evolutionary patterns of origin and distribution are discussed. The review concludes by emphasizing shortcomings in current research on this pathogen.

Knowing the reproductive capability of *Foc* is important in order to predict the ability of the fungus to diversify and overcome disease management strategies. Knowledge of the population structure of the pathogen in South Africa could further provide an indication as to its diversity. In **Chapter 2** the presence of mating type genes and *Foc*'s ability to reproduce sexually have been investigated. The *MAT*-2 gene region was sequenced and the phylogenetic relationships of the South African isolates compared to those of representative genotypes from different geographical regions.

Phylogenetic analysis of DNA characters from two loci was used to help resolve the genetic relationships among *Foc* isolates from diverse geographic origins in **Chapter 3**. These two regions represent a nuclear region and a highly polymorphic region of mitochondrial DNA respectively. The results strongly indicate the presence of clonal lineages among isolates of *Foc*.

Little is known about the interaction between banana plants and *Foc*. With the new knowledge available on the diversity in pathogenic populations of the fungus, studies on host-pathogen interactions are becoming increasingly more important. In **Chapter 4** the stable transformation of *Foc* with the green fluorescent protein (GFP) protein is described. GFP provides a powerful tool to study early stages of fungal infection and to quantify resistance responses in bananas.

A study of the population structure of *Foc* using both VCGs and molecular markers was needed to provide insights into the diversity of the pathogen in South Africa. This required the development of molecular markers that are highly reproducible and codominant. Microsatellite assays have rapidly become established as a powerful tool for the analysis of population structure, reproductive mode and genetic isolation of fungal populations. **Chapters 5** and **6**

deal with the development, testing and the use of these polymorphic microsatellite markers on a worldwide collection of *Foc* isolates. Developing microsatellite markers would be essential for solutions to the current questions still pertaining to diversity in *Foc*. The abilities of pathogen populations to overcome control measures such as resistant cultivars and chemical control can be deducted from such studies.

CHAPTER 1

Fusarium wilt of banana caused by *Fusarium oxysporum* f. sp *cubense*: A literature review with special reference to the taxonomy and population biology of the pathogen

INTRODUCTION

Soilborne plant pathogens have had an important economic impact on our lives and have significantly influenced agricultural practices. Management of these diseases poses a constant challenge to farmers and plant pathologists. An interesting example is provided by *Fusarium* species that are economically important as pathogens on most agricultural crops grown worldwide.

The soilborne fungal species *Fusarium oxysporum* Schlechtend.: Fr. has a worldwide distribution (Booth 1971). The species has been described based on the morphology of its asexual reproductive structures, such as the shape of the macroconidia, the structure of the microconidiophores, and the formation of chlamydospores (Snyder & Hansen 1940, Booth 1971, Nelson, Toussoun & Marasas 1983). The pathogenic members residing in *F. oxysporum* include those causing highly destructive vascular wilt diseases of many commercially important plant crops (Booth 1984). These pathogens are treated as special forms (*formae speciales*) based on the host species that they infect. At least 120 *formae speciales* exist for *F. oxysporum* (Hawksworth 1995), and these exclude isolates considered as non-pathogenic members of the species. Races within *formae speciales* are determined based on virulence of individual isolates on differential host cultivars (Snyder & Hansen 1940, Armstrong & Armstrong 1981, Booth 1984).

One of the most destructive *formae speciales* of *F. oxysporum* is *F. oxysporum* f. sp. *cubense* (E. F. Smith) Snyder and Hansen (*Foc*), the causal agent of Fusarium wilt (Panama disease) of banana (Stover 1986, Ploetz 1990, Pegg, Moore & Bentley 1996). The damage caused by this pathogen in export banana plantations in Central America between 1900-1960 has made Fusarium wilt one of the most catastrophic plant diseases in the recorded history of

agriculture (Stover 1986, Ploetz 1990, Pegg *et al.* 1996, Ploetz & Pegg 1999). Only the replacement of the highly prized Gros Michel cultivar with resistant Cavendish cultivars at the time of the most severe outbreaks saved the banana export industry from complete collapse (Stover 1962a, Ploetz 1990).

Two of the important traits that need to be considered when managing fungal pathogens are their genetic diversity and reproductive strategy (McDonald & McDermott 1993, Kistler 1997, McDonald 1997, Taylor, Jacobson & Fisher 1999). *Fusarium oxysporum* is an asexually reproducing fungus that includes a number of genetically homogeneous sub-populations (clonal lineages) (Booth 1971). Pathogens including only a few clonal lineages can be controlled more easily than those with a diverse population structure, since diverse fungal populations can relatively easily overcome control strategies such as the use of fungicides or disease resistant plants (McDonald & McDermott 1993, Burdon & Silk 1997). Diversity in fungi is primarily derived through sexual reproduction. The aim of this review is to provide a background to banana cultivation, the importance of Fusarium wilt disease of banana, and to treat the topic of diversity in *Foc*, how it arises, and means whereby it can be determined.

ORIGIN AND HISTORY OF BANANAS

Bananas belong to the genus *Musa* and family *Musaceae*, in the order *Zingiberales* (Simmonds 1962). The genus *Musa* includes five sections, divided into 40 species. *Eumusa* is the largest and best known section and includes *M. acuminata* Colla and *M. balbisiana* Colla, which are the principal progenitors of most edible banana cultivars (Stover 1962a, Waite 1963, Ploetz & Pegg 1999). Inedible, seed-bearing diploids that are still found in Southeast Asia and the western Pacific regions are the ancestors of modern-day bananas and

plantains (Stover 1986, Ploetz & Pegg 1997). Over time, evolution has resulted in the many inedible diploids crossing naturally and resulting in inter – and intraspecific hybrids (Buddenhagen 1990, Robinson 1996).

Musa acuminata crosses were selected, cultivated, propagated and distributed locally in Southeast Asia. In India and the Philippines, wild seeded diploids of *M. balbisiana* grew naturally many years ago (Simmonds 1962, Stover 1962a). Early breeders apparently made crosses to produce hybrids between *M. acuminata* and *M. balbisiana*. In this way, seedless, edible diploid, triploid and tetraploid dessert bananas, cooking bananas and plantains were produced. Dessert bananas mainly contain sets of chromosomes from *M. acuminata*, and include the AA and AAA types. Cooking bananas contain sets of chromosomes from both *M. acuminata* and *M. balbisiana*, for example the AAB and AABB bananas and plaintains (Buddenhagen 1990).

Humans have been responsible for moving vegetative banana planting material (suckers) outside Asia and around the world. The mode and time of introduction of bananas to Africa is believed to be from India around the 1400's. They were then spread across the continent from east to west (Simmonds 1959, Robinson 1996). The Portuguese carried the plant to the Canary Islands some time after 1402 and from that area to the New World (Simmonds 1959). Dessert and cooking varieties were introduced into the Americas from Southeast Asia before 1750 (Wardlaw 1961). Gros Michel was first introduced into Panama before 1866, and with the expanding export industry at the time, was distributed throughout Central America (Stover 1962a). The Silk (ABB) variety was introduced into Australia before 1876 and the Gros Michel cultivar was introduced only around 1910 (Stover 1962a). This set the stage for the cultivation of bananas as a dessert and as a staple crop, around the world.

Fusarium wilt of banana, caused by the soilborne fungus *Foc*, is a classical fungal wilt disease of agricultural crops (Beckman *et al.* 1961, Beckman, Halmos & Mace 1962). *Foc* infects plants through the roots and once in the xylem, progresses by means of conidia carried in the vascular system (Waite & Stover 1960, Beckman *et al.* 1961, 1962). It systematically blocks the xylem of susceptible banana plants and results in a lethal vascular wilt (Stover 1959, 1962a, 1986, 1990, Ploetz & Pegg 1999). Characteristic internal symptoms become visible as reddish to dark brown discolouration of infected roots. These symptoms progress to the rhizome and are most pronounced as a yellow-brown discoloration where the stele joins the cortex (Stover 1962a). Eventually the fungus colonizes the pseudostem, and symptoms are evident as faint brown streaks. Vascular discoloration may extend throughout the pseudostem and into the fruit stalk (Stover 1962a, Beckman *et al.* 1962, Pegg & Langdon 1987). The first external symptoms of Fusarium wilt are yellowing of the oldest leaves, which eventually turn brown, wilt, and die as the disease progresses to the youngest leaves (Beckman *et al.* 1961, Stover 1962a). The pseudostem eventually decays, and its base may split. When the affected parts collapse, the fungus grows out of the xylem into adjacent tissues and produces chlamydospores that are released back into the soil. Chlamydospores can survive under different environmental conditions for long periods and render further production of bananas in infected fields impossible (Stover 1962a).

Chemical, biological and cultural control methods are ineffective in the management of Fusarium wilt of bananas (Jones 1999). The only effective control strategy for the disease is by means of genetic resistance of the plant. Such resistance can be introduced into susceptible cultivars either by classical or non-conventional breeding methods. Since edible bananas are mostly parthenocarpic (seedless), they can be propagated only as suckers or tissue

culture-derived plants (Stover & Buddenhagen 1986, Buddenhagen 1987, Robinson 1996). Fusarium wilt can spread from one field to another by means of non-symptomatic, yet infested suckers. Tissue culture plants eliminate the possibility of introducing *Foc* into non-infested fields because they are produced under sterile laboratory conditions. The disadvantage, however, is that tissue cultured plants are more prone to Fusarium wilt than those generated from suckers (Smith *et al.* 1998).

ORIGIN AND TAXONOMIC HISTORY OF *FOC*

It has been suggested that *Foc* co-evolved with banana in Asia in the distant past (Ploetz 1990). The first discovery of Fusarium wilt, however, was made in Australia in 1874 (Bancroft 1876). This was followed by reports of the disease from tropical America (Costa Rica and Panama in 1890) during the time when Gros Michel was widely planted in the area (Stover 1962a, 1990). The first report of the disease in Africa was approximately around 1924 (Stover 1962a, 1990). Fusarium wilt was first reported from Mexico in 1932, and has more recently been found in Florida, United States (Ploetz & Shephard 1989, Ploetz & Pegg 1999). Stover (1962a) speculated that the movement of the disease in Asia, Africa and Australia has been closely linked to the dissemination of susceptible clones into these areas, since the pathogen is often introduced into new areas on infected rhizomes that are free of visual symptoms (Stover 1962a, Pegg *et al.* 1996). Stover & Buddenhagen (1986) believed that *Foc* might have been introduced into Central and South America and the West Indies with the Silk (AAB) variety that came from South India. Fusarium wilt has now been reported from all countries where bananas are grown, except those bordering the Mediterranean islands in the South Pacific, and Somalia (Stover & Simmonds 1987, Ploetz 1994).

The Fusarium wilt pathogen of bananas was first isolated from Cuban banana plant material and named *Fusarium cubense* (Smith 1910). Ashby (1913) presented the first full description of symptoms and field pathology, while Brandes (1919) made the first extensive studies, proving conclusively that *Foc* was the cause of this disease (Waite & Stover 1960). Reinking (1926) confirmed Brandes's (1919) findings that *Foc* is the cause of banana wilt (Waite & Stover 1960). Between 1926 and 1959, however, no significant research was conducted on the pathogen. The export trade using the Gros Michel variety in South America was well established and successful, but suffered huge losses due to the rapid spread of *Foc* and the susceptibility of Gros Michel. Research efforts then focussed on the importance of the pathogen, when the susceptible Gros Michel was replaced with Cavendish cultivars during the 1960s. Cavendish cultivars, however, were found to be susceptible to Fusarium wilt in the subtropics. Damage to Cavendish clones was first observed in the Canary Islands in the 1920s (Ashby 1926) and, thereafter, from other countries with sub-tropical growing areas such as South Africa (1940s), Australia (1950s), and Taiwan (1970s) (Su, Hwang & Ko 1986). This was followed by reports of a race of the fungus attacking Cavendish varieties in the tropical north of Australia, Indonesia and Malaysia (Pegg, Moore & Sorensen 1994, Ploetz 1994, Bentley *et al.* 1998). The discovery that Cavendish cultivars were susceptible to Fusarium wilt in the subtropics and more recently in some parts of the tropics, has led to greatly increased research efforts on the pathogen.

Various authors have studied the dissemination of *Foc*, and strongly support the theory of its co-evolution with banana in Asia (Stover 1962b, 1990, Vakili 1965, Stover & Buddenhagen 1986). Ploetz & Pegg (1997) conducted an extensive study and suggested that *Foc* evolved to the east of Wallace's line. Wallace, a naturalist, distinguished the two landmasses after studying the fauna and flora found on either side. Evidence points towards an Asian origin for the pathogen (Ploetz & Pegg 1997). Several other studies on the genetic diversity and

ancestry of the pathogen have also confirmed a Southeast Asian origin for *Foc* (Pegg, Moore & Sorensen 1993, Koenig, Ploetz & Kistler 1997, O'Donnell *et al.* 1998). Higher pathogen diversity found within Southeast Asian populations compared to populations in other banana growing regions is further proof of to the pathogen's origin in this region (Ploetz & Pegg 1997, Bentley *et al.* 1998). Most isolates of the pathogen from outside Asia are related to the Asian population (Koenig *et al.* 1997). However, independent evolution could also have occurred outside this centre of origin (Ploetz & Pegg 1997). A variant of the fungus has been isolated in Malawi, which is limited to a specific geographical area and that is genetically distinct from other isolates of *Foc* (Ploetz 1994). Thus, it is suggested that the ability to cause disease on the banana host could have originated within as well as outside the centre of origin of banana (Ploetz 1994, Ploetz & Pegg 1997, Bentley *et al.* 1998).

DEVELOPMENT OF DIVERSITY IN *FOC*

The genus *Fusarium* includes many species that reproduce both sexually and asexually. The section *Elegans*, which includes *F. oxysporum*, has no known teleomorph or sexual stage. There is some potential for non-sexual recombination among isolates that are very similar (Buxton 1962, Tinline & Macneil 1969). Genetic variation that occurs in this species is, therefore, assumed to arise through neutral mutations (Ploetz 1993, Brasier 1995) as well as by parasexuality and heterokaryosis (Buxton 1956, 1962, Parameter, Snyder & Reichle 1963, Tinline & Macneil 1969, Taylor, Jacobson & Fisher 1999).

Parasexuality is a non-sexual mode of genetic exchange, without meiosis or the development of sexual structures, and is unique to some fungi (Buxton 1956, 1962, Parameter *et al.* 1963, Leslie 1993). Heterokaryosis is a form of genetic exchange initiated by fusion of vegetative hyphae (anastomosis) between individuals with very similar genomes. Parasexuality and

heterokaryosis may occur in nature and are considered by some, to contribute towards variability (Buxton 1956, 1962, Parameter *et al.* 1963, Tinline & Macneil 1969). To prove that herokaryosis and parasexuality play an important role, many researchers have attempted to force anastomosis in *F. oxysporum* by pairing complementary auxotrophs (Buxton 1956, Garber, Wyttenbach & Dhillon 1961, Tuveson & Garber 1961). Buxton's (1956) work on heterokaryosis and parasexual recombination in *Foc* is not widely accepted, since he utilized nutritional auxotrophs, highly mutated by UV irradiation. Similar studies to those of Buxton (1956) have been conducted in the laboratory, but much speculation remains as to whether parasexuality has any significant contribution to diversity in *Foc* (Kuhn *et al.* 1995, D'Alessio, Cortes & Kuhn 1998). Ploetz (1993) has argued that the variation observed in *Foc* is rather due to mutation and age of the pathotype.

ANALYSING DIVERSITY IN *FOC*

The first taxonomic work on *F. oxysporum* focused on grouping morphologically similar isolates into specialised forms based on the host species they affect (Booth 1971, Armstrong & Armstrong 1981, Gordon & Martyn 1997, Kistler 1997). Considerable progress has been made over the years to analyse specialised forms making use of different phenotypic and genotypic markers (McDonald & McDermott 1993, Kistler 1997, McDonald 1997). The following section deals with the various phenotypic and genotypic characteristics that have been employed to distinguish and characterise different individuals in *Foc*.

Phenotypic characteristics:

Host and Cultivar specificity (race designation): Virulence to specific host plants has been useful in differentiating strains of *F. oxysporum* into *formae speciales*, although there are some inherent problems associated with characterising isolates based solely on pathogenicity (Armstrong & Armstrong 1981, Kistler 1997). Groupings based on host-pathogen interaction

(virulence) are dictated by the genetic make up of the differential hosts or cultivars used to distinguish strains (Correll 1991). The assumption is that isolates with a shared host range, and thus within the same *formae speciales*, are more similar genetically than isolates with other host specificities. The resulting interpretation from this assumption is that *formae speciales* are monophyletic and that isolates with a shared host range are likely derived from a single, particularly successful, pathogenic genotype (Gordon & Martyn 1997, Kistler 1997). This assumption could be misleading, as it has been shown in *Foc* that there is a polyphyletic rather than a monophyletic origin of the pathogen (O'Donnell *et al.* 1998).

Races in *Foc* are groups of isolates that are pathogenic to certain cultivars in the field. Three pathogenic races of *Foc* are recognised. Race 1 was responsible for the epidemics on Gros Michel (Pegg & Langdon 1987, Stover & Simmonds 1987) and race 2 affects Bluggoe (Stover & Buddenhagen 1986, Stover & Simmonds 1987). Race 4 affects clones that are susceptible to race 1 and race 2 in addition to the Cavendish cultivars (Su *et al.* 1986, Stover & Simmonds 1987, Ploetz *et al.* 1990, Ploetz & Pegg 1997). Race 4 is subdivided into tropical and subtropical race 4.

Foc subtropical race 4 attacks Cavendish bananas in countries such as South Africa, Australia, Taiwan, and the Canary Islands (Brake *et al.* 1990, Ploetz *et al.* 1990, Su *et al.* 1996, Gerlach *et al.* 2000) where cold winter temperatures may predispose Cavendish cultivars to infection (Stover 1962b, Stover and Simmonds 1987, Ploetz *et al.* 1990, Ploetz & Pegg 1997). The tropical strain of *Foc* race 4 attacks Cavendish bananas in the tropics in Southeast Asia and Australia (Pegg *et al.* 1993, Pegg *et al.* 1994, Ploetz 1994, Bentley *et al.* 1998). Race 3 represents *F. oxysporum* isolates that affect *Heliconia* spp., a distant relative of *Musa*. Isolates of *F. oxysporum* that were recovered from wilted heliconias still need additional

testing (using molecular techniques) to determine the genetic relatedness to *Foc* (Waite 1963, Stover & Malo 1972, Ploetz & Pegg 1999).

Cultural Characteristics: *Fusarium* species have a remarkable ability to adapt both their form and colour in response to environmental constraints (Follin & Laville 1966, Booth 1971, Nelson *et al.* 1983, Booth 1984). As a result of their capacity to change rapidly in culture, accurate identification has often been conflicting and confusing. Within *Foc*, isolates may differ in appearance *in vitro*. Based on these characteristics, *Foc* isolates in culture will also differ with respect to type of growth, colony colour, and presence of sclerotia (Waite & Stover 1960). Waite & Stover (1960) proposed that cultural characteristics to identify the different types of growth for *Foc* be divided into sprodochial, sclerotial, cottony, ropy and slimy pionnotal types. All these different forms, however, are simply part of an aging process in *F. oxysporum* on artificial media under laboratory conditions (Follin & Laville 1966). Some researchers have also suggested that races of *Foc* can be distinguished in culture. Sun & Su (1978), for example, found that race 4 of *Foc* can be distinguished from races 1 and 2 on modified synthetic Komada's medium, but not on potato dextrose agar.

Volatile production: Some isolates of *Foc,* when grown on steamed rice, produced volatile substances (Brandes 1919). Stover (1962a) used these volatile compounds to differentiate isolates of the pathogen into volatile and non-volatile groups. The same inexpensive cultural method to determine whether volatile substances are produced on steamed rice was used by Moore *et al.* (1991) and Pegg *et al.* (1993) to distinguish among races of Australian isolates of *Foc*. In that study all race 4 isolates produced volatile odours whereas race 1 and 2 isolates did not produce volatiles. Volatile production could thus be used in resource-poor laboratories to determine and differentiate culturally between races 1 and 2 and race 4.

Vegetative compatibility groups (VCGs): Vegetative compatibility has frequently been used in studies on the diversity and population biology of plant pathogenic fungi. Vegetative compatibility is a fundamental trait for recognising genetic entities in fungi (Puhalla 1985). In *F. oxysporum,* a phenotypic character such as heterokaryon formation between isolates is often used to recognise self and non-self (Leslie 1993). Isolates that have identical alleles at their vegetative compatibility (*vic*) loci will result in heterokaryon formation between them, which indicates the they belong to the same VCG.

Numerous researchers have studied vegetative compatibility in *Foc* (Correll & Leslie 1987, Ploetz & Correll 1988, Brake *et al.* 1990, Hernandez *et al.* 1993, Moore *et al.* 1993, Rutherford *et al.* 1998). *Foc* has 21 VCGs worldwide, of which only a few are common and widespread (Table 1). Of these, nine VCGs belong to race 4, eight VCGs to race 1 and four VCGs in race 2, while races associated with VCGs 01217, 01218, 01219, 01221 have not been determined. Two VCGs are found in tropical race 4, and seven are found in subtropical race 4. VCG 0120 and VCGs 0124-0125 have a worldwide occurrence, and can be found in most subtropical countries. Cross-compatibility between isolates in different VCGs has been demonstrated and described as VCG complexes (Table 1) (Ploetz 1990, Bentley *et al.* 1998).

Fifteen VCGs have been found in Asia, where the pathogen and host probably co-evolved (Pegg *et al.* 1994, Pegg *et al.* 1996). In comparison, few VCGs are found in Africa and the Americas. The small number of VCGs found in these regions suggests that *Foc* has been moved into these areas and that these introductions have been limited in number (Ploetz 1993). Furthermore, this observation suggests that VCG evolution outside Asia is uncommon. VCG analysis has confirmed that some strains of the Panama disease pathogen that occur in Asia have been introduced into Australia with banana planting material (Brake *et al.* 1990, Moore *et al.* 1993). In *Foc*, mutations could have given rise to reproductively

isolated populations through the development of new VCGs. New VCGs arising through mutations could have become independent evolutionary units (Brasier 1995, Kistler 1997, O'Donnell 1993, O'Donnell & Cigelnik 1997, O'Donnell *et al.* 1998).

Populations of *Foc* have been intensively analysed in most banana-growing countries in the world. These analyses, however, are based primarily on the races and VCGs that occur in a particular country. Although cultural growth characteristics and volatile production can give some indication of the physiological characteristics of *Foc* isolates, these tests give no indication of the genetic relatedness or diversity within or between groups of isolates. Since rapid changes can occur on synthetic media, separating races in culture is not always reliable.

Using race designation and VCG analysis as measures of diversity has limitations. Successful disease development relies on the intricate interaction between pathogen and plant genotypes, which, in the case of Fusarium wilt of banana, appear to be strongly influenced by environmental conditions (Moore *et al.* 1993). This has previously been demonstrated when *Foc* VCG 0120 caused disease to Cavendish bananas in the subtropics but not in the tropics (Su *et al.* 1986, Stover & Simmonds 1987). Since Cavendish banana cultivars serve as a differential for race identification, the same fungal phenotype will, therefore, be identified as race 4 in the subtropics and as race 1 in the tropics. Furthermore, tropical and subtropical strains of *Foc* race 4 have been shown to be genotypically different (Bentley *et al.* 1995, 1998). These inconsistencies occur primarily because the grouping of *Foc* isolates in races is loosely determined by their pathogenicity to a limited number of banana differentials, under field conditions. In all other *formae speciales* of *Fusarium oxysporum,* there is a defined genetic basis (cultivars containing certain resistance genes) for race designation. In *Fusarium oxysporum* f. sp. *melonis,* a pathogen of muskmelon, certain cultivars contain genes for resistance against Fusarium wilt and others do not (Punja, Parker & Elmhirst 2001). This is

also true for many other *F. oxysporum* species pathogenic on crucifers, sweet potato, and chickpeas (Bosland & Williams 1986, Clark, Hyun & Hoy 1998, Navas-Cortes, Hau & Jimenez-Diaz 2000). A better understanding of the genetic make-up of both pathogen and host, and the interaction between them under different environmental conditions, therefore, needs to be studied in order to effectively designate races in *Foc*.

VCGs provide a useful means for subdividing *Foc* into genetically isolated groups. They could provide information on pathogen diversity, point of introduction, distribution, phylogeny and dispersal. VCGs can, however, be misleading in terms of true genetic relatedness among isolates (Bentley *et al.* 1995). A study of the population structure of *Foc* using both VCGs and complementary molecular techniques would provide more valuable insights into true genetic relatedness or variation. Once VCGs are clearly defined, they have many applications. Firstly, they can be used to determine whether a new race has arisen from an existing pathogen or from a formerly non-pathogenic predecessor (Correll, Puhalla & Schneider 1986). Also, in isolates that are vegetatively compatible, horizontal transfer of deleterious cytoplasmic infectious agents (dsRNA) (Gobbi *et al.* 1990) and viruses (Anagnostakis & Waggoner 1981) can occur. In *Foc* this may provide an opportunity for biological control through hypovirlence of pathogens without the risk of infecting non-pathogenic strains of *F. oxysporum*.

Genotypic characteristics

DAFs and RAPDs: PCR methods based on the amplification of anonymous DNA fragments include randomly amplified polymorphic DNA analysis (RAPDs) (Williams *et al.* 1990) and DNA amplification fingerprinting (DAFs) (Caetano-Anollés, Bassam & Gresshoff 1991). Bentley, Pegg & Dale (1995) and Bentley & Bassam (1996) used RAPDs to study a worldwide population of *Foc*. They divided isolates of *Foc* into two major groups based on

RAPD fingerprinting. Group 1 contained all isolates in VCGs 0120, 0121, 0122, 0126, 01210, 01211 and 01212. Group 2 contained isolates in VCGs 0123, 0124, 0124/0125 and 0125. Bentley *et al.* (1998) used the DAF system to identify nine clonal lineages among 341 isolates of *Foc* based on DNA fingerprinting analysis. Similar or identical banding patterns were found for isolates within a VCG that was independent of their geographical and host origins. According to these authors, DNA fingerprinting patterns were VCG-specific, with each VCG representing a unique genotype.

Isolates divided into subgroups as defined by Bentley *et al.* (1995) differed only in host genotype. The VCG 0120-01215 complex originated exclusively from the *M. acuminata* banana hybrids and VCG complex 0124-0125-0128-01220 is derived from the *M. acuminata* X *M. balbisiana* hybrids (Pegg *et al.* 1993, Boehm *et al.* 1994, Ploetz & Kistler 1994). This observation led to the hypothesis that the two groups may have co-evolved in the respective centres of diversification of the intra- and interspecific hybrids.

Electrophoretic karyotyping and RFLPs: Based on their electrophoretic karyotype, isolates of *Foc* were divided into two broad groups (Boehm, Ploetz & Kistler 1994). Group 1 contained all isolates in VCGs 0124, 0125, 0124-0125, 01210 and 01214 and group 2 contained isolates in VCGs 0120, 0121, 0122, 0123, 0129, and 01213. Isolates in group 1 had a higher chromosome number and a larger genome size than group 2 isolates. Koenig *et al.* (1997) further differentiated these two major groups using anonymous, single-copy, restriction fragment length polymorphism (RFLP) loci. Seventy-two new haplotypes were identified and most of the isolates were identified by the five most common haplotypes. They found that isolates with identical haplotypes were geographically separated and all isolates within a VCG were found in the same clade and clonal lineage. Ten clonal lineages based on RFLP analysis were identified with the two largest lineages having pantropical distribution.

These authors conclude that the largest lineages, the unique lineage from Africa were genetically distinct and that the ability to be pathogenic on banana were acquired independently.

Foc phylogeny: O'Donnell *et al.* (1998) tested a monophyly hypothesis suggesting that the *formae specialis* concept in *F. oxysporum* is phylogenetically misleading. These authors compared DNA sequences of nuclear and mitochondrial genes of four *formae speciales* of *F. oxysporum*, including *Foc*. Concordant evidence from the respective gene genealogies revealed that *Foc* harbours at least five lineages with independent evolutionary origins. These lineages are as different from each other as they are from other *formae speciales* of *F. oxysporum*. Lineages II and I of *Foc* were genetically more similar to *F. oxysporum* f. sp. *niveum*, a pathogen of watermelon, than they were to each other, and as closely related to each other as they were to *F. oxysporum* f. sp. *lycopersici*, a pathogen of tomato. The five lineages in *Foc* can further be divided into two clades based on phylogenetic analysis (O'Donnell *et al.* 1998). The distant relationship between the two lineages suggests that the major populations of *Foc* evolved independently and provides significant evidence for the geographically separate development of isolates in these clades.

Several DNA-based techniques have been used to analyse the worldwide population of *Foc*, often with confusing and contradicting results. For example, a study by Bentley *et al.* (1995), (1998) divided isolates of *Foc* representing all VCGs into two major groups based on RAPD fingerprinting and nine clonal lineages based on DAF analysis, which in the absence of any sequence identity is reduced to a phenetic technique. RAPDs and DAFs require careful optimisation to ensure reproducibility between laboratories, and they suffer from a lack of portability between laboratories (Gillings & Holley 1997). O'Donnell *et al.* (1998) supported the division of VCGs in *Foc* into two major groups, but found only five clonal lineages after

sequencing of the nuclear and mitochondrial gene regions. Thirdly, Boehm *et al.* (1994) used electrophoretic karyotyping of *Foc* to divide the population into two broad groups containing slightly different VCGs to those described by Bentley *et al.* (1995) and O'Donnell *et al.* (1998). Koenig *et al.* (1997) found ten clonal lineages in *Foc* based on RFLP analysis. The RFLP study is the first significant phylogeny study by focussing on the resolution of subpopulations within *Foc* utilizing cDNA probes. The advantages and limitations of all these techniques can be debated but it is clear that a suitable molecular technique needs to be found to subdivide the worldwide population of *Foc* into appropriate subunits to determine the distribution and relationships of the fungus. I believe that the development of microsatellite markers for *Foc* will provide the best solution to this problem.

Microsatellite markers: Microsatellite markers have rapidly become established as a powerful tool for the analysis of population genetic structure, mating systems, reproductive mode and genetic isolation (Page & Holmes 1998). These markers have several advantages over RFLP or RAPD based techniques. The dominant nature and lack of reproducibility of RAPDs limit their use as routine molecular markers in population studies (McDonald & McDermott 1993, McDonald 1997). RFLPs represent neutral co-dominant markers that provide useful information in population genetic studies. This technique, however, requires large quantities of DNA and the number of enzymes available would ultimately limit the number of loci studied. The high mutation rate of microsatellites resulting in different allele sizes for individuals in the same population, simple pattern of Mendelian inheritance and co-dominance makes microsatellites a powerful molecular marker (Bruford & Wayne 1993, Page & Holmes 1998). Microsatellite markers can, therefore, be used to determine the genetic relationships among and within VCGs from geographically separate origins. The question of gene flow and genotypic diversity between populations of *Foc* from Southeast Asia,

Australia, and South Africa at the locus level could be determined with population genetic studies using microsatellite markers.

In asexually reproducing fungi such as *Foc*, DNA fingerprinting offers a powerful tool for identification of an individual and distinguishing among different clonal lineages in a population with a high degree of certainty. Bentley *et al.* (1998) were thus able to link specific fingerprints to some VCGs in *Foc*. However, a polymorphic marker unique to *Foc* has not been described. Analysing sequence data of nuclear and mitochondrial genes with different evolutionary rates or using microsatellite markers might lead to the development of such a marker for the different races.

Mating type: The capacity of plant pathogens to survive, reproduce, and extend their host range is of fundamental importance in the study and control of these pathogens. The genus *Fusarium* consists of species with a variety of complex reproductive lifestyles. The section *Elegans*, containing *F. oxysporum*, is assumed to reproduce asexually with some potential for nonsexual recombination among isolates that are very similar (Buxton 1962, Tinline & Macneil 1969). However, the relatively large number of VCGs and genetic groupings present in *F. oxysporum* in general, and in *Foc* specifically, justify an investigation into the question whether sexual reproduction has occurred at some stage in the distant past. Since genes coding for opposite mating types in fungi are highly conserved (Glass & Kuldau 1992, Kronstad & Staben 1997) evidence for an early sexual event in *F. oxysporum* should be investigated by searching for the presence of such genes. In a study on *F. oxysporum* f. sp. *lycopersici* Arie *et al.* (2001), the presence of both *MAT*-1 and *MAT*-2 idiomorphs (known to control mating type in ascomycetous fungi) was found in different strains of the pathogen. A study searching for a sexual event in *F. oxysporum*, however should not be limited to a single *formae speciales*, but should eventually be extended to both pathogenic and non-pathogenic

members of the species. Taylor *et al.* (1999) suggested that the sexual cycle might still be active in the *F. oxysporum* complex, although a teleomorph has not been observed.

Investigating the reproductive capabilities, of a worldwide population of *Foc* would result in useful information pertaining to genetic diversity and population structure of *Foc*. Taylor *et al.* (1999) argues that multiple VCGs and races within a given *formae specialis* could have independent origins, with pathogenicity and virulence evolving more than once through mutation or transposition, or spread to distantly related strains through parasexuality or horizontal gene transfer (Brasier 1995, Taylor *et al.* 1999). Baayen *et al.* (2000) suggested that population genetic studies on species in the *F. oxysporum* complex are mostly biased, because isolates investigated originate from agricultural crops. This results in an overestimation of clonality through human dissemination of infected propagative materials. Appel & Gordon (1996) support this view, based on their finding that clonality is less common in putatively non-pathogenic populations of *F. oxysporum*.

CONCLUSIONS

Understanding and measuring diversity and diversification in *Foc* should be an important component of research programmes on Fusarium wilt of banana that focus on disease management. Once the population structure of the pathogen has been analysed, appropriate control measures can be designed for a specific country or region. Measuring diversity in a clonally reproducing fungus such as *Foc*, however, is complicated, and requires a combination of phenotypic and genotypic tools. Whether current tools available are efficient for analysing populations of *Foc* is debatable. But the fact remains that there are several gaps in our knowledge on the worldwide population structure of the pathogen. The full range of

options for disease prevention and management will remain unclear until there is a better understanding of the molecular events controlling pathogen variation.

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Table 1. Vegetative compatibility groups, races and origins of strains of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Jones 1999).

VCG[1]	VCG complex[2]	Race	Origins
0120	0120-01215	1, 4	South Africa, Canary Islands, Australia, Brazil, Costa Rica, Honduras, Indonesia, Jamaica, Malaysia, Nigeria, Portugal, Spain, Taiwan, Florida (USA)
0121	none	4	Indonesia, Taiwan,
0122	none	4?[3]	Philippines
0123	none	1	Malaysia, Philippines, Taiwan, Thailand
0124	0124-0125-0128-01220	1, 2	Australia, Brazil, Burundi, China, Cuba, Democratic Republic of Congo, Haiti, Honduras, India, Jamaica, Malawi, Malaysia, Mexico, Nicaragua, Rwanda, Tanzania, Thailand, Uganda, Florida (USA)
0125	0124-0125-0128-01220	1, 2	Australia, Brazil, Burundi, China, Cuba, Democratic Republic of Congo, Haiti, Honduras, India, Jamaica, Malawi, Malaysia, Mexico, Nicaragua, Rwanda, Tanzania, Thailand, Uganda, Florida (USA)
0126	none	1	Honduras, Indonesia, Papua New Guinea, Philippines
0128	0124-0125-0128-01220	1, 2	Australia, Brazil, Burundi, China, Cuba, Democratic Republic of Congo, Haiti, Honduras, India, Jamaica, Malawi, Malaysia, Mexico, Nicaragua, Rwanda, Tanzania, Thailand, Uganda, Florida (USA)
0129	none	4	Australia
01210	none	1	Cayman Islands, Cuba, Florida (USA),
01211	none	4	Australia
01212	none	?[4]	Tanzania
01213	01213-01216	T4	Australia, Indonesia, Malaysia, Taiwan
01214	none	2	Malawi
01215	0120-01215	1, 4	Canary Islands, Australia, Brazil, Costa Rica, Honduras, Indonesia, Jamaica, Malaysia, Nigeria, Portugal, Spain, Taiwan, Florida (USA)
01216	01213-01216	T4[5]	Australia, Indonesia, Malaysia, Taiwan
01217	none	?	Malaysia
01218	none	?	Indonesia, Malaysia, Thailand
01219	none	?	Indonesia
01220	0124-0125-0128-01220	4?	Australia, Brazil, Burundi, China, Cuba, Democratic Republic of Congo, Haiti, Honduras, India, Jamaica, Malawi, Malaysia, Mexico, Nicaragua, Rwanda, Tanzania, Thailand, Uganda, Florida (USA)
01221	none	?	Thailand

[1]Vegetative compatibility groups (VCG) a phenotypic marker used to characterize fungal isolates based on heterokaryon formation (Puhalla 1985).
[2]isolates in a VCG are compatible with isolates in different VCG, forming a VCG complex.
[3]4? Possible race 4.
[4]? Race undetermined.
[5]T4 Tropical race 4.

Chapter 2

Mating type genes and the reproductive potential of *Fusarium oxysporum* f.sp. *cubense*

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ABSTRACT

Fusarium oxysporum f. sp. *cubense* (*Foc*) is an apparently asexually reproducing fungus that causes Fusarium wilt (Panama disease) of bananas. The fungus is believed to have originated in Southeast Asia, from where it has spread to virtually all areas where bananas are grown. Twenty-one different vegetative compatibility groups (VCGs) have been identified in *Foc*, reflecting a relatively high genetic diversity for an asexual fungus. The aim of this study was to consider the possible occurrence of sexual reproduction in the fungus. Forty-one isolates of *Foc*, representing all races and the dominant VCGs, were selected for this study. The presence of mating type genes was determined using *MAT*-1 and *MAT*-2 primers from conserved alpha and high mobility group (HMG) protein domains. The presence or absence of the *MAT*-1 and *MAT*-2 genes was further tested using Southern blot hybridization analysis. To determine whether *Foc* is capable of reproducing sexually, crosses between isolates of *Foc* were attempted using tester strains of the heterothallic *Gibberella circinata* (=*Fusarium circinatum*), as a positive control. PCR amplification analysis showed that the *MAT*-2 idiomorph is present in all isolates of *Foc* tested. Southern hybridization and PCR amplification using the *MAT*-1 idiomorph region as a probe was consistently negative in all isolates. Most isolates of *Foc* produced dark purple to black protoperithecia when crossed on carrot agar, but no ascospores were formed. The occurrence of only one mating type in the *Foc* population could explain the reported absence of sexual reproduction in the fungus, worldwide.

The fungal pathogen, *Fusarium oxysporum* f. sp. *cubense* (E. F. Smith) Snyder and Hansen (*Foc*) causes a highly destructive vascular wilt disease of bananas known as Fusarium wilt or Panama disease. The disease is widespread in banana growing regions of Africa, Australia, Asia, South Pacific and the tropical Americas (Stover & Simmonds 1987, Ploetz *et al.* 1990). Humans have played a major role in the global distribution of the disease. The pathogen has been introduced into new areas on infected rhizomes that are free of visual symptoms (Stover 1962).

Foc has a relatively diverse population structure for an apparently clonal fungus, which exists as three races (Stover & Buddenhagen 1986, Pegg & Langdon 1987, Stover & Simmonds 1987) and 21 vegetative compatibility groups (Ploetz & Pegg 1999). Mutations and parasexuality (Buxton 1956, 1962, Parameter, Snyder & Reichle 1963, Tinline & Macneil 1969) are considered to be the main basis for genetic variation in *F. oxysporum* including *Foc*.

Variation in *Foc* has been assessed using vegetative compatibility group (VCG) analysis (Ploetz & Correll 1988, Brake *et al.* 1990), PCR methods such as randomly amplified polymorphic DNA analysis (RAPDs) (Bentley & Bassam 1996), DNA amplification fingerprinting (DAF), (Bentley *et al.* 1998, Gerlach *et al.* 2000), RFLPs (Koenig, Ploetz & Kistler 1997) and the sequencing of the nuclear and mitochondrial gene regions (O'Donnell *et al.* 1998). These techniques have also been used to consider questions pertaining to the distribution and origin, phylogeny and dispersal of the pathogen (Ploetz & Pegg 1997, Bentley *et al.* 1998, O'Donnell *et al.* 1998).

The mode of reproduction and genetic variability of fungal populations is important for implementing management strategies to reduce disease impact (McDonald & McDermott 1993, Taylor *et al.* 1999). Sexual reproduction in heterothallic ascomycetous fungi is controlled by genes that reside in a genetic locus called the mating type or *MAT* locus (Metzenburg & Glass 1990, Kronstad & Staben 1997). Mating type is determined by the DNA sequence present at the mating type locus. The two alleles at the mating type loci are apparently unrelated, and have been described as idiomorphs (Metzenburg & Glass 1990, Glass & Kuldau 1992, Kronstad & Staben 1997). The lack of sequence similarity between the idiomorphs prevents recombination and they are thus inherited uniparentally (Glass *et al.* 1988).

A teleomorph for *F. oxysporum* has never been observed and the pathogen appears to rely solely on asexual reproduction. Southern blot hybridization and PCR amplification experiments have demonstrated that many asexual species have both *MAT*-idiomorphs (Arie *et al.* 1997, Arie *et al.* 2000). These include fungi such as, *Bipolaris sacchari* (E. Butler) Shoemaker and *Alternaria alternata* (Fries: Fries) von Keissler (Sharon *et al.* 1996, Arie *et al.* 1997). *MAT* idiomorphs for *Fusarium oxysporum* f. sp. *lycopersici* have also been described (Arie *et al.* 2000).

The aim of this study was to consider whether both mating type genes are present in *Foc* and further to consider whether sexual reproduction might occur in this fungus. This was achieved by crossing isolates of *Foc* using media known to allow sexual reproduction in other *Fusarium* spp. and under different temperature conditions as well as light and dark cycles. The presence or absence of *MAT* idiomorphs in the available isolates was also determined.

MATERIALS AND METHODS

Fungal isolates

Forty-one isolates representing the three pathogenic races and 11 VCGs of *Foc* were analysed (Table 1). In addition, isolates representing both mating types of *Gibberella circinata* (=*Fusarium circinatum*), mating population H, causal agent of pitch canker of pines, was included in the study to serve as a positive control.

Crosses between isolates

To determine whether *Foc* is capable of reproducing sexually, crosses were made according to the method described by Klittich & Leslie (1988). Isolates considered to be "male" were grown on potato dextrose agar (PDA), and those considered "female" were grown on carrot agar. These cultures were then incubated in growth chambers at a constant temperature of either 18°C, 20°C, or 25°C, with alternating white and black light. After 7-10 days, or when plates were fully-grown with mycelia covering the whole surface, the "female" strains were fertilised using conidia from the "male" cultures.

Conidial suspensions of the "male" isolate were prepared by pipetting 1.5ml of 2.5% Tween 60 onto the sporulating "female" mycelia. The conidia were then dislodged, and the suspension poured onto the surface of the recipient isolate. The "male" inoculum was spread equally over the entire "female" colony with a glass rod. Crosses were attempted between *Foc* 42 and *Foc* 1, *Foc* 61 and *Foc* 47, *Foc* 1 and *Foc* 46, *Foc* 51 and *Foc* 53, *Foc* 6 and *Foc* 46, and *Foc* 51 and *Foc* 4 (Table 1). As a positive control, a "male" and "female" isolate of *G. circinata*, mating population H (FSP 118 [nit M], and SK11 [nit1]) were crossed with each other. *Foc* isolates were also crossed with the known "male" and "female" parents of mating

population H. After crosses were completed, the plates were returned to the growth chamber and incubated at 18°C, 20°C, or 25°C with alternating white and black light for 6 weeks.

DNA Extraction

Conidia of *Foc* isolates grown on PDA were used to inoculate 100 ml of potato dextrose broth (PDB) medium in 250ml flasks. Isolates were grown in PDB without shaking for 10-14 days, harvested and freeze-dried. Mycelium was ground to a fine powder in liquid nitrogen. Total DNA from each isolate was extracted using the phenol-chloroform based extraction method described by Raeder & Broda (1985).

MAT primers

MAT-1 and *MAT*-2 specific primer pairs, designed by Steenkamp *et al.* (2000), were used to identify the *MAT* idiomorphs. Additional *MAT*-1 primers (FO-MAT-1-For 5'ACC GCC AGC CGT CGT GCA GTG 3'and FO-MAT-1-Rev 5'CTT GCG GGG GTA TGA GAA CGC 3') were designed based on *MAT*-1 idiomorph sequences in GenBank. An additional *MAT*-2 reverse primer specific for the HMG box was designed for *Foc* FF1 *Foc* 5' GTA TCT TCT GTC CAC CAC AG 3' and used with the forward primer Gfmat2c that was designed by Steenkamp *et al.* (2000).

PCR amplification of MAT-1 and MAT-2

To determine whether *MAT*-1 and *MAT*-2 genes were present in *Foc*, DNA of each of the isolates was amplified with the specific primers. For each isolate, a 25-µl PCR reaction cocktail was prepared that contained 0.4 mM of each deoxynucleoside triphosphates (dNTPs), 1 x PCR buffer, 1.0 pmole of each primer, 0.25 units Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals, Germany), 2 ng DNA, and sterile deionised water. PCR reactions were performed on a Hybaid TouchDown PCR machine (Hybaid Limited, United

Kingdom). Reaction conditions for the *MAT*-1 region were as follows: initial denaturation at 95°C for 2 min; followed by denaturation at 92°C for 30 s; primer annealing at 62°C for 40 s; elongation at 72°C for 2 min repeated for 35 cycles; and a final extension at 72°C for 7 min. Reaction conditions for the *MAT*-2 region were as follows: initial denaturation at 95°C for 2 min, followed by denaturation at 92°C for 30 s; primer annealing at 54°C for 40 s, elongation at 72°C for 2 min for 40 cycles, and a final extension at 72°C for 7 min. The amplified product was resolved on a 1.5% (w/v) agarose gel electrophoresis in TBE buffer (Tris Boric acid EDTA; pH 8.0), stained with ethidium bromide and visualized under UV illumination (Sambrook, Fritsch & Maniatis 1989). Size estimates of the PCR fragments were done using a molecular weight standard (100 bp ladder Promega, Madison, Wisconsin).

Southern blot hybridization

Genomic DNA of *Foc* isolates was digested using the restriction enzymes *Eco* RI and *Hind* III (Roche Molecular Biochemicals). The digested DNA samples were separated on a 1% agarose gel at 4 V/cm overnight. The DNA in the gels was depurinated, denatured, and then blotted onto a positively charged nylon membrane (Roche Molecular Biochemicals) by capillary action (Sambrook *et al.* 1989). The DNA was fixed onto the membrane for 5 min using UV irradiation. Hybridization reactions and subsequent stringency washes were carried out as recommended by the manufacturer (Roche Molecular Biochemicals).

Preparation of DIG-labelled DNA probes

A *MAT*-1 probe from *F. circinatum* and a *MAT*-2 probe from *Foc* were used for the hybridization of genomic DNA of *Foc*. The *MAT*-1 and *MAT*-2 probes were produced from PCR products using *MAT*-specific primers. This amplicon was purified using the High Pure Purification kit (Promega). For labelling, 16 µl of the PCR product was denatured at 98°C for 10 minutes. The denatured DNA was immediately transferred to ice, followed by the addition

of 4 µl of DIG-High Prime mixture (Roche Molecular Biochemicals). Random labelling was allowed to proceed overnight at 37°C, and the reaction stopped by inactivating the polymerase at 65 °C for 10 minutes. The probe was stored at –20°C until use. The rest of the procedure was carried out using the methods specified in the manual on non-radioactive DIG labelling (Promega).

DNA sequencing

For analysis of the *MAT*-2 region PCR amplified DNA of 35 isolates of *Foc* was purified with the QIAquick purification kit (QIAGEN, Germany). The partial *MAT*-2 idiomorph was sequenced using an ABI PRISM™ 377 automated DNA sequencer and an ABI PRISM™ Dye terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Warrington, United Kingdom). PCR products were sequenced in both directions with the primers that were used to amplify the fragments. DNA sequences were then aligned and compared with the *F. oxysporum* strain from GenBank (accession number AB011378) using the PILEUP program of the GCG Sequence Analysis Software Package (version 9.1; Genetics Computer Group, Madison, WI). All sequences from this study were deposited in GenBank and Treebase (Table1).

Phylogenetic analyses of the *MAT*-2 idiomorph DNA sequences were performed using Phylogenetic Analysis Using Parsimony (PAUP) 4.0b10* (Swofford 2000). Ambiguously aligned regions and parsimony-uninformative characters were excluded from the data sets for the analysis. The remaining characters were re-weighted according to the mean consistency index (CI). For parsimony analysis heuristic searches for the most parsimonious trees were conducted using closest step-wise addition by tree bi-section-reconnection (TBR). Gaps inserted for alignment were treated as missing data. For each analysis, 1000 bootstrap replicates were performed to assess the statistical support for branch points. The outgroup

fujikuroi (Sawada) Wollemw., *F. proliferatum* (Matsush) Nirenberg ex Gerlach & Nirenberg, *F. sacchari* (E. J. Butler & Hafiz Khan) W. Gams and *G. moniliforme* (Sawada) Wollemw. The CI and retention (RI) indexes were calculated for the datasets. The phylogenetic signal in the dataset (g1) was assessed by evaluating tree length distributions for 100 randomly generated trees (Hillis & Huelsenbeck 1992).

RESULTS

Crosses between isolates

Crosses between mating testers of *Fusarium circinatum* produced fertile perithecia 6 weeks after incubation at 20°C (Fig. 1a). The perithecia were dark purple to black in appearance and contained fertile ascospores. Crosses between *Foc* isolates gave rise to protoperithecia-like structures at 20°C and at 25°C. Protoperithecia were not produced at 18°C and where alternating cycles of cool white and black lights were applied. Some of the protoperithecia in *Foc* crosses were virtually indistinguishable from the perithecia produced by *F. circinatum* (Fig. 1b), while others were small and numerous. All of the protoperithecia produced in the *Foc* crosses were sterile, and no ascospores were observed.

PCR amplification of MAT-1 and MAT-2

Amplification of genomic DNA with *MAT*-2 specific primers (Gfmat2c and FF1 *Foc*; Gfmat2c and Gfmat2d) produced an amplicon in all the *Foc* isolates tested (Fig. 2). A PCR reaction with the primer pair Gfmat2c and FF1 amplified a 700bp fragment, and the primer pair Gfmat2c and Gfmat2d resulted in a 200 bp PCR product (Fig. 2). The 700 bp PCR product was then selected for further phylogenetic analyses. This PCR fragment included part of the conserved HGM box domain and the 3'flanking region. PCR using the *MAT*-1 primers, with genomic DNA as a template consistently failed to produce an amplicon.

Southern blot hybridization

Southern hybridization of *Foc* genomic DNA with a *MAT*-1 idiomorph specific probe showed complete absence of any sequence homologous to the *MAT*-1 idiomorph in the *Foc* population tested. Only the *MAT*-2 probe gave positive results in Southern hybridization (Fig. 3a, b).

DNA sequencing

Forty most parsimonious trees with a tree length of 107 were generated from *MAT*-2 sequence data (Fig. 4). The consistency index (CI), retention index (RI) and g1 values were 0.85, 0.86 and –1.44 respectively. The *Foc* isolates grouped with a strain of *F. oxysporum* for which sequence data were obtained from GenBank and this is considered to represent the *F. oxysporum* clade (Fig. 4). Two subclades were present within the *F. oxysporum* clade. Subclade I included South African isolates and two representative isolates from Taiwan and the Philippines. Subclade II included two isolates from Malawi and Indonesia respectively. Clade III represented the putative sister groups with known sexual and asexual mode of reproduction. The sexual *Gibberella thapsina* (Klittich & J. F. Leslie), *G. moniliforme*, *G. fujikuroi* and asexual *Fusarium nygamai*, (L. W. Burgess & Trimboli), *F. sacchari*, *F. proliferatum*, putative sister groups formed a sub-group separately and basal to the *F. oxysporum* clade.

The putative amino acid sequence for the *MAT*-2 gene product described by Arie *et al.* (1997, 2000) and the protein sequence for the *Foc* isolates in this study were very similar. A few amino acid changes were, however, observed between the *Foc* isolates and related species of *Fusarium*. All *Foc* isolates and related sister species (*G. circinatum*, *F. nygamai*, *G. thapsina*) had an intron at a conserved position within the HMG domain (Fig. 5).

DISCUSSION

Analysis of a representative population of isolates of *Foc* in this study provided strong evidence that sexual reproduction is absent in this fungus. This finding is of importance in the development of future management strategies for Fusarium wilt of banana, because phytopathogenic fungi that are able to undergo sexual reproduction may overcome disease resistance in plants more rapidly than asexual forms. This has also been true in banana where the sexually reproducing fungus responsible for black Sigatoka, *Mycosphaerella fijiensis* Morelet rapidly overcame resistance to fungicides (Jones 1999). However, where reproduction is limited to asexual reproduction, such as the case with *Foc*, control strategies appear to have remained relatively stable (McDonald & McDermott 1993). For example where Cavendish bananas have been used to replace the highly susceptible Gros Michel banana in Central America, the former variety has resisted infection by *Foc* race 1 for more than 40 years.

An interesting outcome of this study was the production of sterile protoperithecia when a large group of isolates of *Foc* was crossed in culture. To the best of our knowledge, this is the first time that such structures have been observed in *Foc*. The appearance of these structures suggests that sexual reproduction might be possible when both mating types were present in the fungus. Sharon *et al.* (1996) have found similar structures when crosses were made between *Bipolaris sacchari* and *Cochliobolus heterostrophus* Drechsler isolates. These authors suggested that *B. sacchari* is capable of producing sexual structures.

Only the *MAT*-2 idiomorph was found in our collection of *Foc,* which included isolates of diverse geographic origins. The absence of the *MAT*-1 idiomorph in the *Foc* population suggests that it is uncommon in agricultural situations, but it may occur at a low frequency or in areas we have not sampled. Both mating types have previously been reported from *F.*

oxysporum f. sp. *lycopersici* another *formae specialis* of *F. oxysporum* (Yoshida *et al.* 1998, Kerényi *et al.* 1999), but is restricted to only one mating type in a single isolate. Arie *et al.* (2000) confirmed that both mating types of the *F. oxysporum* occur but that they are specific to single individuals of the fungus.

The phylogenetic tree generated in this study from the partial *MAT*-2 sequence suggests that the South African population of *Foc* harbours isolates that are closely related to *Foc* isolates from other regions. The value of using these sequences in phylogenetic analyses has previously been demonstrated by Steenkamp *et al.* (2000), who considered the phylogenetic relatedness of *Fusarium* species residing in the section *Liseola*. Clearly, isolates of *Foc* are heterogeneous. This was also observed with studies of Koenig *et al.* (1997) and O'Donnell *et al.* (1998) where phylogenetic analyses resolved *Foc* populations into well-supported clades composed of genetically distant clonal lineages.

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Table 1. Isolates and sequence information of *Fusarium oxysporum* f. sp. *cubense* used in this study.

Foc number[1]	Other name[2]	Geographic origin	Host origin	Race	VCG[4] group	Donor or collector	GenBank Accession number
Foc 1		Kiepersol, South Africa	Williams	4	VCG 0120	E. Grimbeek	
Foc 6		..	..	..		..	
Foc 4		..	..	..	..	A. Viljoen	AY209150
Foc 8		..	Chinese Cavendish	..	..	..	AY209151
Foc 9		..	Williams	..	..	..	AY209152
Foc 10		..	..	..	..	..	AY209153
Foc 11		..	Israeli Grand Naine	..	..	..	AY209154
Foc 12		..	DC24R22	..	..	..	AY209155
Foc 16		Ramsgate, South Africa	Israeli Grand Naine	..	..	E. Grimbeek	AY209156
Foc 19		..	Williams	..	..	..	AY209157
Foc 20		Umzumbe, South Africa	..	..	..	..	AY209158
Foc 21		Port Edward, South Africa	..	..	..	..	AY209159
Foc 22		Munster, South Africa	..	..	..	..	AY209160
Foc 23		Port Edward, South Africa	..	..	..	..	AY209161
Foc 28	Taiwan 14	Taiwan		..	VCG 0121	R. Ploetz	AY209162
Foc 42		Ramsgate, South Africa	Williams	..	VCG 0120	A. Viljoen	
Foc 46	23486	Wamuran, Australia	Cavendish	..	VCG 0120	N. Moore	AY209163
Foc 47	STNP4	Tanzania	Ney Poovan	?[3]	VCG 01212	R. Ploetz	AY209164
Foc 48	Thail-2	Thailand	Kluai Namwa	1	VCG 0123	..	AY209165
Foc 50		Australia		..	VCG 0124	..	AY209166
Foc 51	Phil 6	Phillipines	Latundan	..	VCG 0126	..	AY209167
Foc 52		Australia	Cavendish	4	VCG 0129	..	AY209168
Foc 53	Indo 14	Indonesia	Pisang Ambon Putih	..	VCG 0120	I. Djatnika	
Foc 54	RPMW40	Phillipines		..	VCG 0122	R. Ploetz	AY209169
Foc 57		Malawi	Bluggoe	2	VCG 01214	R. Ploetz	AY209170
Foc 60		Indonesia	Pisang Siem	?	VCG 01218	..	AY209171
Foc 61		..	Pisang Ambon	?	VCG 01219	..	AY209172
Foc 109		Burgershall, South Africa	Israeli Grand Naine	4	VCG 0120	A. Viljoen	AY209173
Foc 144		Ramsgate, South Africa	Williams	..	..	E. Grimbeek	AY209174
Foc 147		Tzaneen, South Africa	Israeli Grand Naine	..	..	A. Viljoen	AY209175
Foc 148		Tzaneen, South Africa	Israeli Grand Naine	..	..	..	AY209176
F. oxysporum	SUF 959						AB011378
G. circinatum	UF2-B528						AF235012
G. moniliforme	MRC 6191						AF236765
F. sacchari	MRC 6524						AF236766
G. fujikuroi	MRC 6571						AF236767
F. proliferatum	MRC 6568						AF236768
G. thapsina	MRC 6537						AF236770
F. nygamai	MRC 7549						AF236771

[1] *Foc* isolates without accession numbers were used only in the mating crosses.
[2] Indicates names from original collection or donor.
[3] "?" races undetermined.
[4] VCG- Vegetative compatibility groups used to group *Foc* isolates based on heterokaryon formation using known testers (Puhalla 1985).

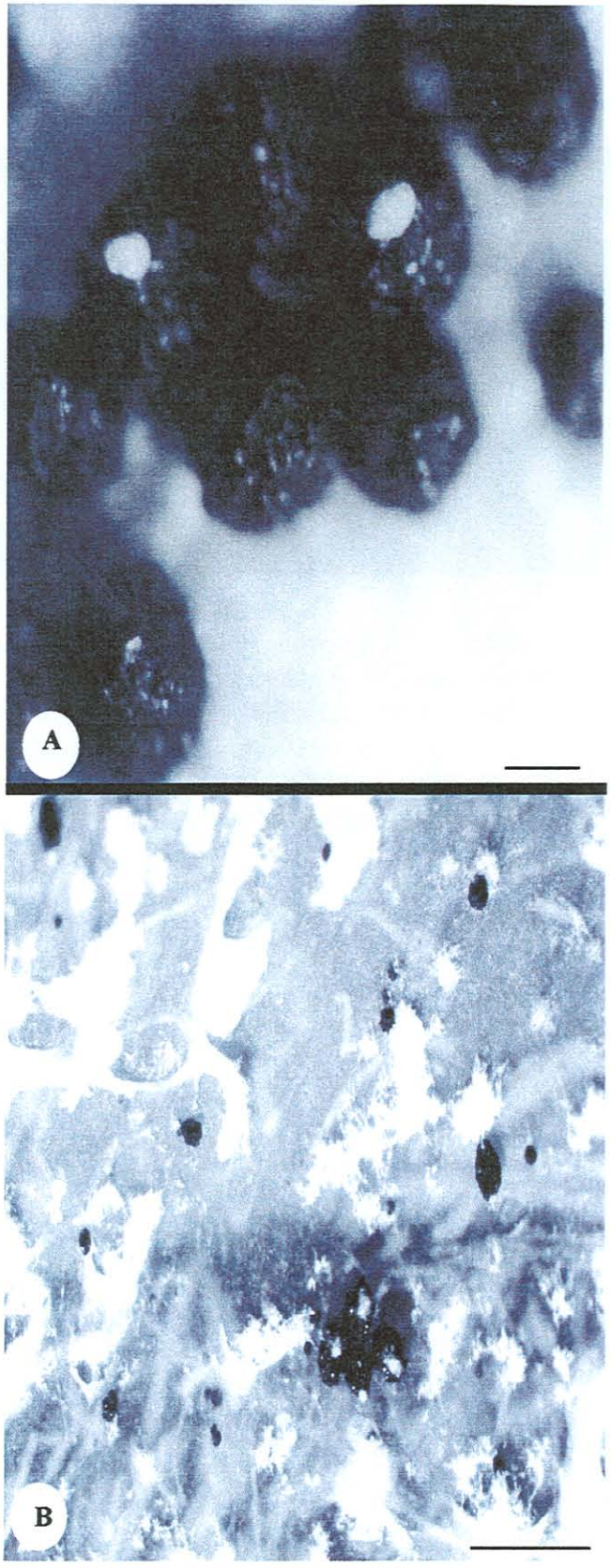


Fig. 1 (A) Opposite mating types of *Fusarium circinatum* produced fertile perithecia with ascospores when crossed on carrot agar. Scale bar = 100µm. **(B)** Isolates of *Fusarium oxysporum* f. sp. *cubense* produced dark purple to black perithecia-like structures without ascopsores. Scale bar = 1000µm

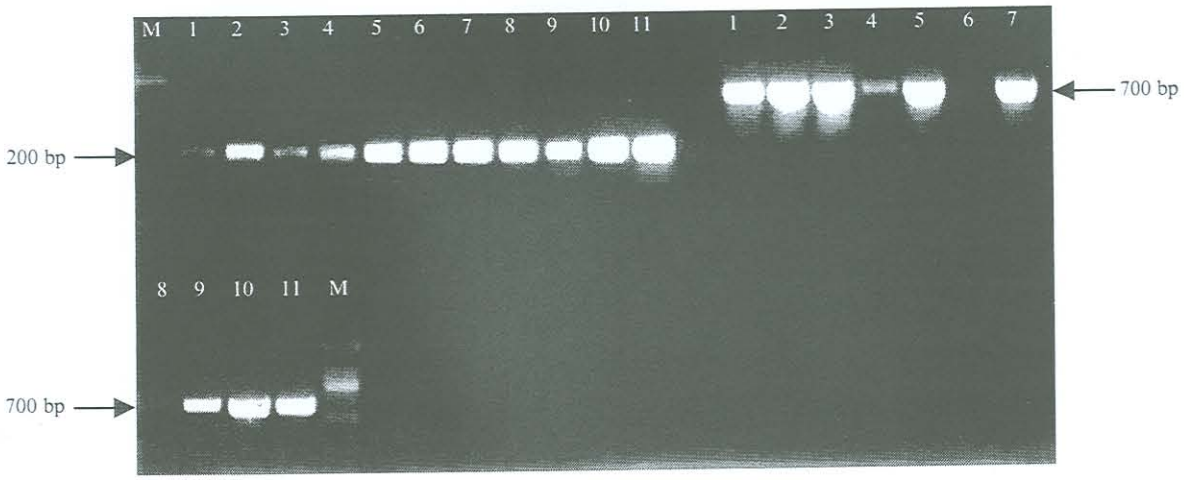


Fig. 2 A 1.5% agarose gel showing PCR amplicons of the *MAT*-2 idiomorph of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) using *MAT*-2 primer sets. The primer sets (Gfmat2c and Gfmat2d, Gfmat2c and FF1) resulted in PCR amplicon sizes of 200 bp and 700 bp respectively. Lanes 1-11 are *Foc* 4, *Foc* 8, *Foc* 10, *Foc* 12, *Foc* 16, *Foc* 19, *Foc 20, Foc* 21, *Foc* 28, *Foc* 46, *Foc* 47. A 100 bp ladder is indicated by the letter (M).

Fig. 4 One of 40 most parsimonious trees based on sequence data from the *MAT-2* idiomorph of *Fusarium* spp. The phylogram was obtained using the heuristic search option in PAUP. Branch lengths are indicated above the branches and bootstrap values below the branches.

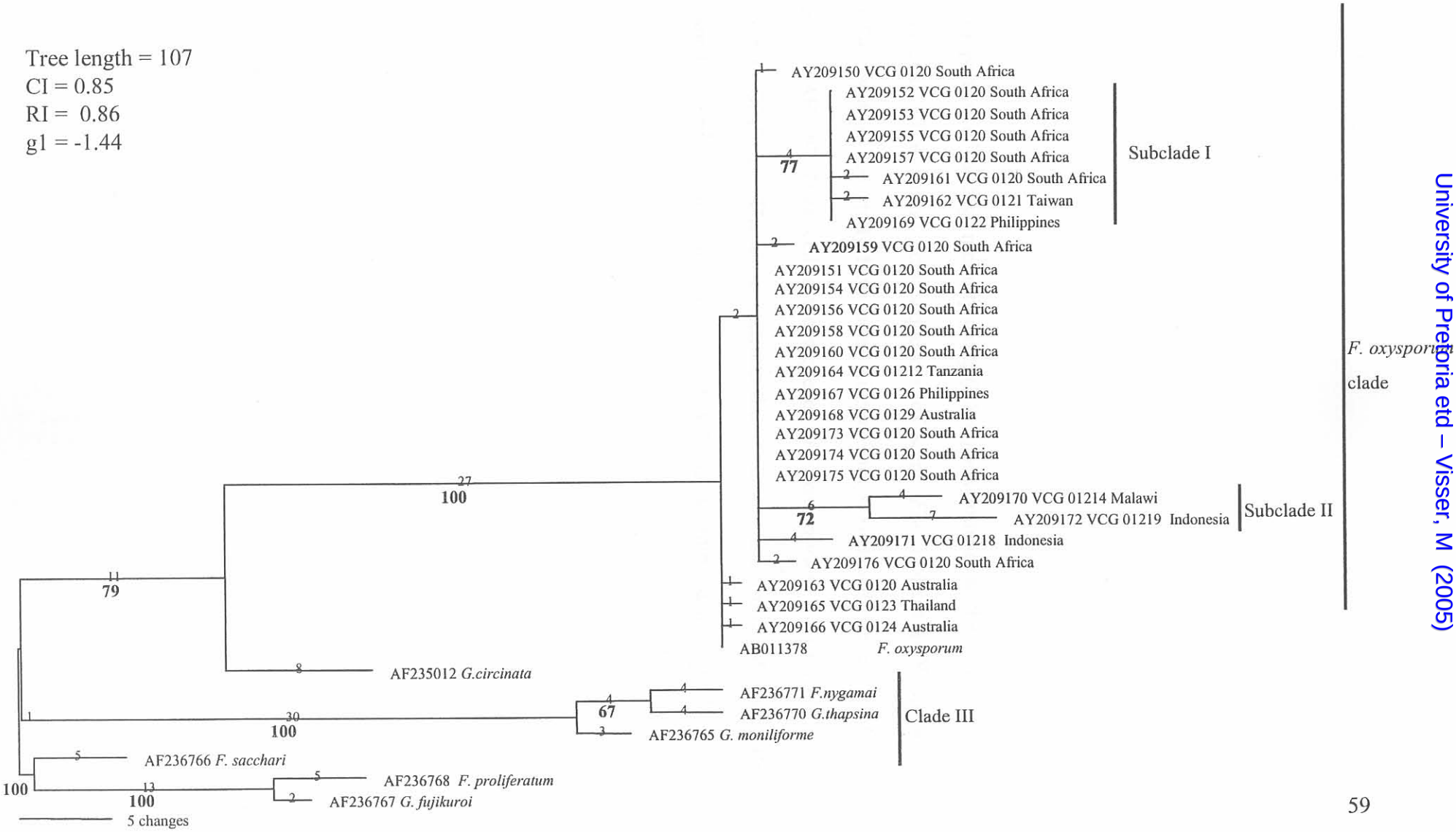
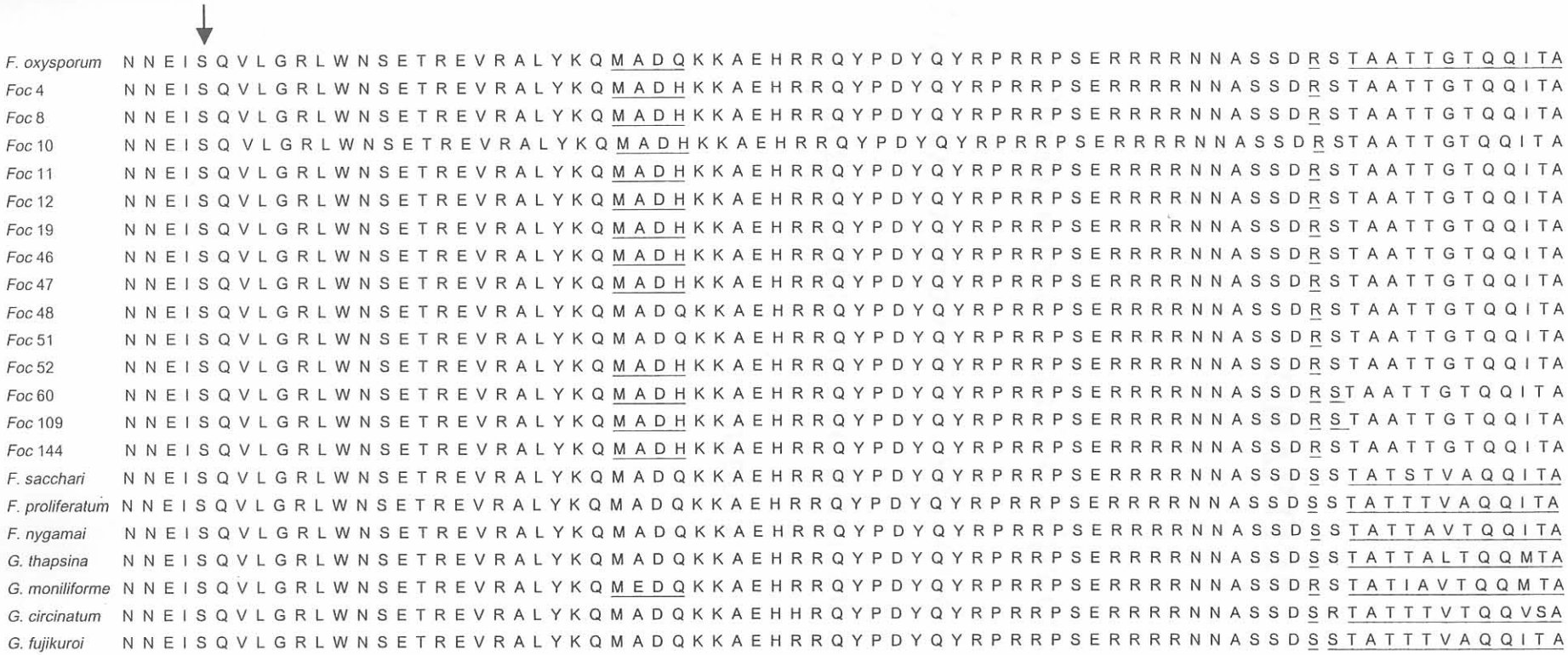


Fig. 5 Comparison of the high mobility group (HMG) box domains of the fungal *MAT*-2 gene products. The amino acid sequences of some *Foc* isolates (AY209150 – AY209174) used in this study have been aligned with sexual and asexual relatives (AF235012 – AF236767) as well as the sequence of *F. oxysporum* strain (AB011378) obtained from GenBank. The arrow indicates the position of the conserved intron site in this domain. Amino acids that differ from the AB011378 strain are underlined.



CHAPTER 3

Phylogenetic relationships of South African *Fusarium oxysporum* f. sp *cubense* isolates based on mitochondrial and nuclear gene sequence comparisons

ABSTRACT

Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is a serious vascular disease of bananas in most subtropical and tropical regions of the world. Isolates of *Foc* can be grouped into at least three pathogenic races and 21 vegetative compatibility groups (VCGs), loosely determined by their ability to cause disease on a small set of differential cultivars and heterokaryon compatibility on artificial media, respectively. Despite the importance of Fusarium wilt of bananas, relatively little is known regarding the phylogenetic relationships among isolates. The growing availability of DNA-based techniques provides great opportunity to gather such knowledge. In this study 33 isolates representing a collection of *Foc* from diverse geographic origins were selected for study. The majority of the isolates represented 'tropical' and 'subtropical' race 4 of VCG 0120. A phylogenetic analysis of the DNA sequences of the mitochondrial direct repeat region and the elongation factor (EF) 1-α gene was undertaken. Sequences for the mitochondrial region provided more variable characters than the EF 1-α region, which had a 96% similarity for all *Foc* isolates examined. Trees inferred from both gene regions had comparable topologies and highlighted the polyphyletic nature of *Foc* isolates. The data also supported the view that distinct clonal lineages have become established in geographically isolated regions.

INTRODUCTION

Fusarium wilt of banana, commonly known as Panama disease, is caused by the soil-borne fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (E. F. Smith) Snyder and Hansen (*Foc*). *Foc* is generally considered to be one of the most destructive *formae speciales* of *Fusarium oxysporum* (Stover 1962, Ploetz 1990). Fusarium wilt seriously hampers sustained banana production because the pathogen persists in infested soils for long periods of time, the difficulty with which the disease is managed and the lack of good sources of resistance (Stover & Buddenhagen 1986). Current control strategies include using tissue culture-derived plantlets, crop hygiene, replacement of susceptible with resistant cultivars, and planting in disease free soils (Stover & Simmonds 1987, Viljoen 2002).

Three pathogenic races (races 1, 2 and 4) of *Foc* are recognised, based on their virulence to a defined group of banana cultivars (Stover & Buddenhagen 1986, Stover & Simmonds 1987, Stover 1990). There is, however, no genetically defined basis for the assignment of races in this pathosystem (Ploetz 1990). Cavendish cultivars have resistance to *Foc* race 1, but are susceptible to race 4 isolates of the pathogen in subtropical countries such as South Africa, Australia and Taiwan (Stover & Simmonds 1987). Cavendish banana plants were recently reported to be susceptible to a new variant of the pathogen in the tropics (Pegg *et al.* 1994, Ploetz 1994, Bentley *et al.* 1998). Although predisposing factors, such as cold temperatures, are associated with damage in the subtropics, they are not involved in the tropics. To recognise these environmental effects and differences that exist between the populations of *Foc* that are involved, the pathogens are called respectively, subtropical and tropical race 4.

Previous studies conducted on worldwide populations of *Foc* have divided the fungus into two major groups, based on vegetative compatibility (VCGs), electrophoretic karyotyping, RFLP analyses and DNA fingerprinting (Boehm *et al.* 1994, Koenig *et al.* 1997, Bentley *et al.* 1995). Bentley *et al.* (1998) used DNA amplified fingerprinting (DAF) analysis to identify nine clonal lineages and 33 different genotypes among 341 isolates of *Foc*, of which 14 new genotypes did not belong to any of the previously described VCGs. DNA sequence analysis of several *formae speciales* of *F. oxysporum*, including isolates of *Foc,* further showed that the Fusarium wilt fungus represents two genetically distinct populations (O'Donnell *et al.* 1998b). Concordant evidence from the gene genealogies revealed that *Foc* harbours at least five clonal lineages with independent evolutionary origins (Ploetz & Pegg 1997, O'Donnell *et al.* 1998b).

In this study the phylogenetic relationships among isolates of *Foc* from different geographic origins was assessed by analysing sequence data for the mitochondrial DNA direct repeat and the elongation factor (EF) 1-α gene region. These two loci represent a nuclear and a mitochondrial locus, which would thus have different evolutionary histories (Bruns *et al.* 1991). Specific emphasis was placed on VCG 0120, the largest and most widely spread VCG of *Foc*, which includes both race 1 and 'subtropical' race 4 isolates and occurs in South Africa where this study was initiated.

MATERIALS AND METHODS

Fungal isolates

Thirty-three isolates of *Foc,* from different host genotypes and origins, were selected for study (Table 1). These isolates were primarily from South Africa and represented VCG 0120 and 'subtropical' race 4. Several 'tropical and 'subtropical' race 4 isolates as well as isolates

representing *Foc* race 1 and 2 were included. Single-spored cultures were produced from plant material and from cultures received from other collections and these were stored on filter paper at 4°C in the culture collection of the Forestry and Agriculture Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction

Fungal isolates were grown in potato dextrose broth (PDB) without shaking at room temperature for 7-10 days, after which the mycelium was harvested, freeze dried and stored at –20°C. For total DNA extraction, mycelial masses were ground to a fine powder in liquid nitrogen. DNA from each isolate was extracted using the rapid method described by Raeder & Broda (1985). The DNA concentration was estimated after agarose gel electrophoresis by comparing the intensity of ethidium bromide fluorescence of the DNA samples to known concentrations of lambda DNA marker (marker III) (Roche Molecular Biochemicals, Mannheim, Germany) on a UV transilluminator imaging system (UVP, Germany).

PCR amplification

Amplification of the mitochondrial region 7

Polymerase chain reaction (PCR) amplification of the mitochondrial region 7 for *Fusarium* was performed with reagents from Roche Molecular Biochemicals. A standard 25 µl PCR reaction mixture contained 1 x PCR buffer, 0.4 mM of each deoxynucleotide triphosphate (dNTPs), 1.5 pmol of each primer (U9 5'GTAACCTCTGACTCACCG, R117 5'GTCAACCAGGAGCAGACTG) (Kim *et al.* 1993), 2 ng of DNA template and 0.25 units of *Taq* DNA polymerase. The reactions were carried out in a HYBAID (United Kingdom) thermocycler programmed for an initial denaturation of 2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 40 s at 54°C, 1 min at 72°C and a final extension of 7 min at 72°C. The amplified products were verified using 1% agarose gel electrophoresis in Tris Borate EDTA

(TBE, pH 8.0) buffer. Gels were stained for 5-10 min in ethidium bromide and briefly destained before visualisation under UV light. The resulting PCR amplicons were purified using a QIAquick PCR Purification kit (QIAGEN, Germany) following the specifications of the manufacturer.

Amplification of the EF 1-α gene region

Part of the EF 1-α gene region was PCR amplified using primers EF-1 and EF-2, (O'Donnell *et al.* 1998b). The PCR reaction mixture was the same as that described for the mitochondrial amplification, but with EF 1-α primers. The reaction conditions were as follows: initial denaturation of 2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 40 s at 60°C, 1 min at 72°C and a final extension of 7 min at 72°C. Reactions were carried out in a HYBAID thermocycler. The resulting amplicons were electrophoresed in 1% TBE agarose gels. The DNA was visualized and purified as before.

DNA Sequencing

Sequencing reactions were performed directly on purified PCR products using the same set of primers for the mitochondrial region and specific sequencing primers (EF-11 (forward), and one of two reverse primers (EF-21 and EF-22) (O'Donnell *et al.* 1998b, Baayen *et al.* 2000) for the EF 1-α region. DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Foster City, California) and an ABI PRISM™ 377 automated sequencer. In order to obtain the full sequence for the EF 1-α region, an additional set of internal sequencing primers were also used (O'Donnell *et al.* 1998b). For comparative purposes five EF 1-α region DNA sequences that represent the five different clonal lineages and two clades of *Foc* reported by O'Donnell *et al.* (1998b), were obtained from GenBank and included in the analyses.

Sequence analysis

Alignments of the mitochondrial and EF 1-α data sets were performed manually and the gaps inserted for alignment were treated as fifth characters in the analyses. Ambiguously aligned regions and parsimony-uninformative characters were excluded from the datasets. The remaining characters were re-weighted according to the mean consistency index (CI). Concordance of the mitochondrial region and EF 1-α gene sequenced datasets was evaluated with the partition-homogeneity test implemented in PAUP with MAXTREES set to 1000. The Templeton Nonparametric Wilcoxon Signed Ranked test in PAUP (Farris *et al.* 1994) was also used to determine whether the datasets could be combined.

Phylogenetic analyses were performed using PAUP (Phylogenetic Analysis Using Parsimony*) (Swofford 1998) on DNA sequences of the mitochondrial and EF 1-α regions. Parsimony analysis was performed on the individual datasets, with the heuristic search option and random addition sequences (1000 replicates) with the MULPARS function active and with tree bisection-reconnection branch (TBR) swapping. Bootstrap confidence levels were assessed by 1000 parsimony replications. The CI and retention indexes (RI) were calculated for both datasets. The outgroup species selected for rooting the gene trees represent putative sister groups to *Foc*. The outgroup taxon used for the mitochondrial sequence dataset was the pitch canker pathogen *F. circinatum,* while *F. graminearum* was selected as an outgroup for the EF 1-α dataset.

RESULTS

Amplification of the mitochondrial DNA region 7 and EF 1-α

Amplification of the mitochondrial region yielded PCR fragments of 1200-1500 base pairs (bp) in length. The sizes of the PCR products were variable among isolates of the South

African population of *Foc*, as well as among isolates representing different countries (Fig. 1). The primer pair of EF-1 and EF-2 produced a 700 bp fragment in the global population of *Foc*, irrespective of geographic origin and VCG. This region appeared to be constant in all *Foc* isolates included in this study (Results not shown).

Sequencing and Data analysis

Aligned datasets were analysed separately after both the partition homogeneity test (PHT) ($P< 0.05$) and the Templeton test failed to support their combination. Manual alignment of the DNA sequences for the mitochondrial region 7 from the *Foc* isolates resulted in a data set of 964 characters. The ingroup dataset contained 33 taxa and characters in this dataset were reweighted using the maximum value of consistency indices. Heuristic searches on the dataset generated 10 most parsimonious trees with a consistency index (CI) of 0.800 and retention index (RI) of 0.929 (Fig. 2). Alignment of the EF 1-α sequences for 33 isolates resulted in a dataset with a total of 697 characters. Nine most parsimonious trees were retained with a tree length of 20, consistency index (CI) of 0.863 and retention index of 0.922 (Fig. 2). All sequences generated in this study have been deposited in GenBank and Treebase (Table 2).

The *Foc* isolates included in this study, are broadly separated into two statistically unsupported clades (clades A and B) based on their aligned mitochondrial sequences (Fig. 2). The first clade (clade A) comprised of *Foc* isolates representing eight different VCGs, namely, VCGs 0120, VCGs 0121, 01212, 01213, 0122, 0126, 0120/01215, 01216 and 01218. The South African population of *Foc* VCG 0120,'subtropical' race 4 grouped within this clade with isolates from the rest of Africa and Australasia (Fig. 2). This grouping as well as the relationships within it however, is not supported by a high bootstrap values. A separate and well-resolved subclade and supported by a bootstrap of 93%, consisted mainly of isolates

from Australia, Canary Island and Honduras. A further separate subclade supported by weak bootstrap value, consisting mainly of isolates from Asia was also present in clade A. This subclade included isolates from Malaysia (*Foc* 58) and Indonesia (*Foc* 60, *Foc* 232) representing VCG 01216, 01213 and 01218, respectively.

Clade B included isolates representing VCGs 0123, 0124, 0125, 01217, 01214 and 01219. This clade had 3 subclades made up of VCGs 0123 and 0127 from Southeast Asia, VCG 0124 and 0125 from Australia, and VCG 01214 and 01219 from Africa and Southeast Asia, respectively. Isolate VCG 01214 appeared to be distantly related to the rest of the *Foc* isolates in the group.

The tree emerging from analysis of the EF 1-α sequence data was divided into 4 clades supported by high bootstrap values, however relationships among clades are unsupported (Fig. 2). Clade A included isolates representing six VCGs (0120, 0122, 0126, 01213, 01216 and 01219) from Africa and Australasia. Sequences for the two isolates from GenBank, (NRRL 25605 and 26029) representing clonal lineages 2 and 4 as described by O'Donnell *et al.* (1998a), were closely related to isolates in clade A supported by a 76% bootstrap value. Isolates from the Indo-Malaysian region representing VCGs 01213 and 01216 (*Foc* 58, 229 and 232) appeared to be form a subclade within clade A supported by a bootstrap value of 67%.

The 3 subclades are non-monophyletic, i.e. not a true clade. Clade B contained isolates from Australia and Africa. These are isolates (VCGs 01212, 0124 and 0125) from Australia and Tanzania and was supported by a bootstrap of 60%. The GenBank isolate (NRRL 25607) representing clonal lineage 1 resided in this subclade. Clade C is not significantly supported and include VCG 01214 from Malawi and VCG 01218 from Indonesia, grouped together with

the Genbank isolate (NRRL 25367) O'Donnell' *et al.* (1998a) clonal lineage 5. Clade D consisted of isolates from Thailand (VCG 0123) and Malaysia (VCG 01217) and formed part of clonal lineage 3 (NRRL 26022) supported by a bootstrap value of 78%.

DISCUSSION

Phylogenetic analyses of sequence data for mitochondrial and nuclear gene regions in this study have highlighted relationships among isolates from various parts of the world. In general, the results are similar to those of previous studies where populations of *Foc* were analysed using RFLPs (Koenig *et al.* 1997), RAPDs and DAF (Bentley *et al.* 1995, 1998) and DNA sequencing for several *formae speciales* of *F. oxysporum* (O'Donnell *et al.* 1998b). The distinct clades within the banana pathogen suggest separate evolutionary origins in *Foc* as indicated by Koenig *et al.* (1997) and O'Donnell *et al.* (1998b) and they illustrate clearly the well-supported groups among all the isolates.

Individual isolates represented by discrete VCGs gave rise to phylogenetic trees, which were generally similar, although there were some exceptions. The Tanzanian (*Foc* 56; VCG 01212) and Indonesian (*Foc* 60; VCG 01218) isolates, for instance, grouped within clade A, based on the mitochondrial sequences and in clade B and C in the EF 1-α tree. Likewise, the Indonesian isolate *Foc* 61 (VCG 01219) grouped in clade B using mitochondrial sequences and in clade A in the EF 1-α tree. Isolate *Foc* 229 (VCG 01213) was more closely related to VCG 0120 isolates in the mitochondrial tree than to other "tropical" race 4 isolates (represented by VCGs 01213 and 01216) based on EF 1 –α sequences. These differences also resulted in a situation where the partition homogeneity and Templeton tests did not support combination of the datasets. Reasons for this apparent incongruence could be the fact that

neither tree is highly supported by bootstrap values. The different underlying evolutionary histories of the two genes may also play a role (Bruns *et al.* 1991).

Several clonal lineages appear to exist within *Foc*. Based on DNA sequence comparisons of the mitochondrial gene region, clade A can be divided into at least two lineages and clade B into three lineages. The first clonal lineage in clade A was almost entirely made up of isolates representing VCG 0120, which all represent *Foc* 'subtropical' race 4. The second clonal lineage in clade 1 mostly included isolates representing *Foc* 'tropical' race 4. Clade B consisted of three clonal lineages; all made up of isolates belonging to *Foc* races 1 and 2. The same clonal lineages were present in the phylogram based on sequence analysis of the EF 1- α region, although the placement of certain isolates in clades was inconsistent in the two phylograms. The five clonal lineages suggested in O'Donnell *et al.* (1998a) study by the EF 1- α region were also supported by our results and suggest that clonal lineage 2 and 4 are very closely related.

In *Fusarium oxysporum*, vegetative compatibility groups (VCGs) and DNA fingerprints often fail to give congruent lineages (Jacobson & Gordon 1990, Koenig *et al.* 1997). Our results support the findings of others that show that *Foc* is a special form of the pathogen with a polyphylectic origin (O'Donnell *et al.* 1998b). The global movement of plant material infested by a limited number of *Foc* clones and their subsequent movement and diversification in different geographical areas seem to have contributed to the current global structure of *Foc* populations. More important is the independent origins in Africa of VCG 01214, Southeast Asia of VCG 0120 and Asia, VCG 0124 based on different musaceous hosts species. Thus different clonal lineages could be the result of the pathogen infesting different hosts around the world. Geographical barriers, local selection pressures, and different environmental conditions, could result in unique clonal lineages (Brasier 1987). Some of

these clonal lineages could be phylogenetically distinct. In the case of *Foc,* different hosts in areas of introduction, and even hybridisation between lineages seem a more likely event because of the supporting geographic isolation evidence.

Phylogenetic analysis of phytopathogenic fungi can often be linked to geographical regions (Bruns *et al.* 1998, O'Donnell *et al.* 1998a). This is somewhat true for *Foc.* There is strong evidence that Southeast Asia is the centre of diversity of the banana pathogen (Stover 1962, Ploetz 1990). However, there is some evidence suggesting secondary centres of development, and that some genotypes are exclusive to specific countries. An example being VCG 01214 that is found only in Malawi and nowhere else, and by most analyses is quite distinct, genetically, from other *Foc* isolates. Most often, genotypes present in Southeast Asia can be found in Australia, Africa and the Americas. Our analysis indicates that genotypes residing in clade A are present in Southeast Asia, but also in Africa, Australia, Central America and the Canary Islands. Isolates from clade B, however, are restricted to Australasia and Africa. These results generally support those of Koenig *et al.* (1997), O'Donnell *et al.* (1998b).

The occurrence of two main VCG complexes and five clonal lineages in *Foc* should be considered when screening plants for resistance. The current race structure in the fungus is not well defined, and genotypic groups can aid substantially in redefining pathotypes in *Foc.* Variation in pathogen virulence has a great impact on disease management strategies when these are primarily based on host resistance. Any variation in virulence may result in a complete crop failure if the plants are not also resistant to the new strain of the pathogen (Woudt *et al.* 1995). Since different pathogenic lineages may be capable of causing disease epidemics on different host genotypes, breeding programs must consider different pathogen lineages when developing stable host resistance.

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Table 1. Isolates and sequence information of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) used in this study.

Foc number	Other name[1]	Geographic origin	Host origin	Race	VCG	Donor or collector	EF 1-α	Mitochondrial
Foc 009		Kiepersol, South Africa	Williams	4	VCG0120	E. Grimbeek	AY217170	AY217138
Foc 018		Ramsgate, South Africa	Israeli Grand Naine	4	VCG0120	..	AY217171	AY217139
Foc 019		Ramsgate, South Africa	Williams	4	VCG0120	..	AY217172	AY217140
Foc 020		Umzumbe, South Africa	Williams	4	VCG0120	..	AY217173	AY217141
Foc 046		Australia		4.	VCG0120	N. Moore	AY217174	AY217142
Foc 047	STNP4	Tanzania	Ney Poovan	[1]?	VCG 01212	R. Ploetz	AY217175	AY217143
Foc 48	Thai1-2	Thailand	Kluai Namwa	1	VCG 0123	N. Moore	AY217176	AY217144
Foc 049		Australia		1	VCG0124	..	AY217177	AY217145
Foc 050		Australia		2	VCG 0125	..	AY217178	AY217146
Foc 051	Phil 6	Philippines	Latundan	1	VCG 0126	..	AY217179	AY217147
Foc 054	Phil 36	Philippines		4	VCG 0122	..	AY217180	AY217148
Foc 057		Malawi	Bluggoe	2	VCG 01214	R. Ploetz	AY217181	AY217149
Foc 058		Malaysia		[2]T4	VCG 01216	N. Moore	AY217182	AY217150
Foc 059		Malaysia		?	VCG 01217	..	AY217183	AY217151
Foc 060	Indo 5	Indonesia	Pisang Siem	?	VCG 01218	..	AY217184	AY217152
Foc 061	Indo 25	Indonesia	Pisang Ambon	?	VCG 01219	..	AY217185	AY217153
Foc 137		Munster, South Africa	Williams	4	VCG0120	E. Grimbeek	AY217186	AY217154
Foc 140		Port Edward, South Africa	Williams	4	VCG0120	..	AY217187	AY217155
Foc 141		Port Edward, South Africa	Grand Naine	4	VCG0120	..	AY217188	AY217156
Foc 144		Ramsgate, South Africa	Williams	4	VCG0120	..	AY217189	AY217157
Foc 147		Tzaneen, South Africa	Israeli Grand Naine	4	VCG0120	..	AY217190	AY217158
Foc 148		Tzaneen, South Africa	Israeli Grand Naine	4	VCG0120	..	AY217192	AY217159
Foc 229	II 5	Central Sulawesi, Indonesia	Pisang Manurung	?	VCG 01213	R. Ploetz	AY217193	AY217160
Foc 230	NIG	Onne, Nigeria	Gros Michel	?	VCG0120/01215	..	AY217194	AY217161
Foc 231	ORT2	Laoratava, Canary Islands	Dwarf Cavendish	4	VCG 0120	..	AY217195	AY217162
Foc 232	DMI 8	Sulawesi, Indonesia	Pisang Capatu	?	VCG 01213	..	AY217196	AY217163
Foc 236	23486	Queensland, Australia	Cavendish	4	VCG 0120	..	AY217197	AY217164
Foc 242	22410	Queensland, Australia	Cavendish	4?	VCG 0120	..	AY217198	AY217165
Foc 244	22615	Byron Bay, NSW, Australia	Lady finger	4	VCG 0120	..	AY217199	AY217166
Foc 246	34661	Honduras	Highgate	1	VCG 0120	..	AY217200	AY217167
Foc 247	C1	Canary Islands	Cavendish	4?	VCG 0120	..	AY217201	AY217168
Foc 248	PD 14-1	Onne, Nigeria	Gros Michel	?	VCG 0120/01215	..	AY217202	AY217169
F.circinatum	*Fcc* 49	Ngdowana, South Africa				A. Viljoen		AY217170
F. graminearum	NRRL 29297						AY225885	

[1]Name from original collection or donor.
[2]Races undetermined.
[3]'Tropical' race 4.

Figure 1: PCR amplification products of the mitochondrial region 7 of a worldwide collection of *Fusarium oxysporum* f. sp. *cubense* (*Foc*). The 1.0% agarose gel was resolved in 1x TBE and stained in ethidium bromide. Sizes of the amplified products were estimated using a 1 kb molecular weight standard. Lanes 1: molecular weight standard, 2-30: *Foc* 46, *Foc* 49, *Foc* 50, *Foc* 60, *Foc* 61, *Foc* 231, *Foc* 51, *Foc* 54, *Foc* 57, *Foc* 58, *Foc* 59, *Foc* 47, *Foc* 48, *Foc* 229, *Foc* 244, *Foc* 246, *Foc* 232, *Foc* 236, *Foc* 9, *Foc* 18, *Foc* 19, *Foc* 20, *Foc* 137, *Foc* 140, *Foc* 141, *Foc* 144, *Foc* 147, *Foc* 148.

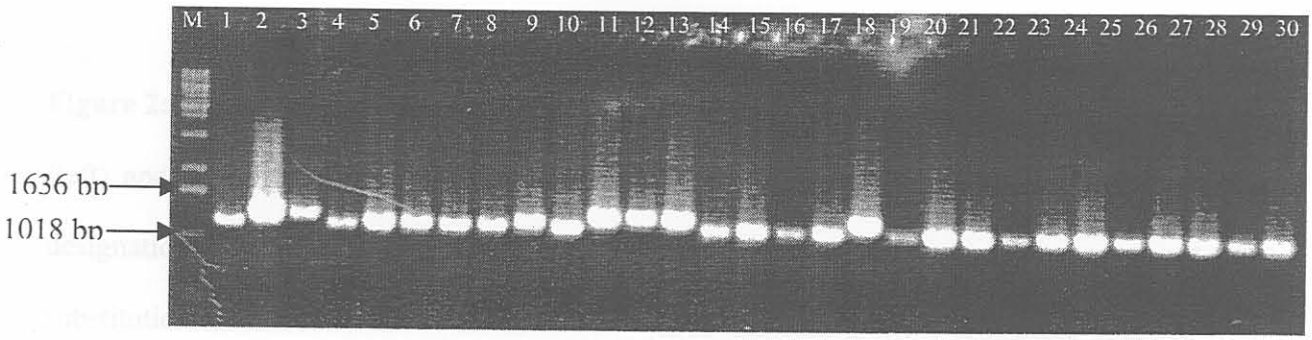
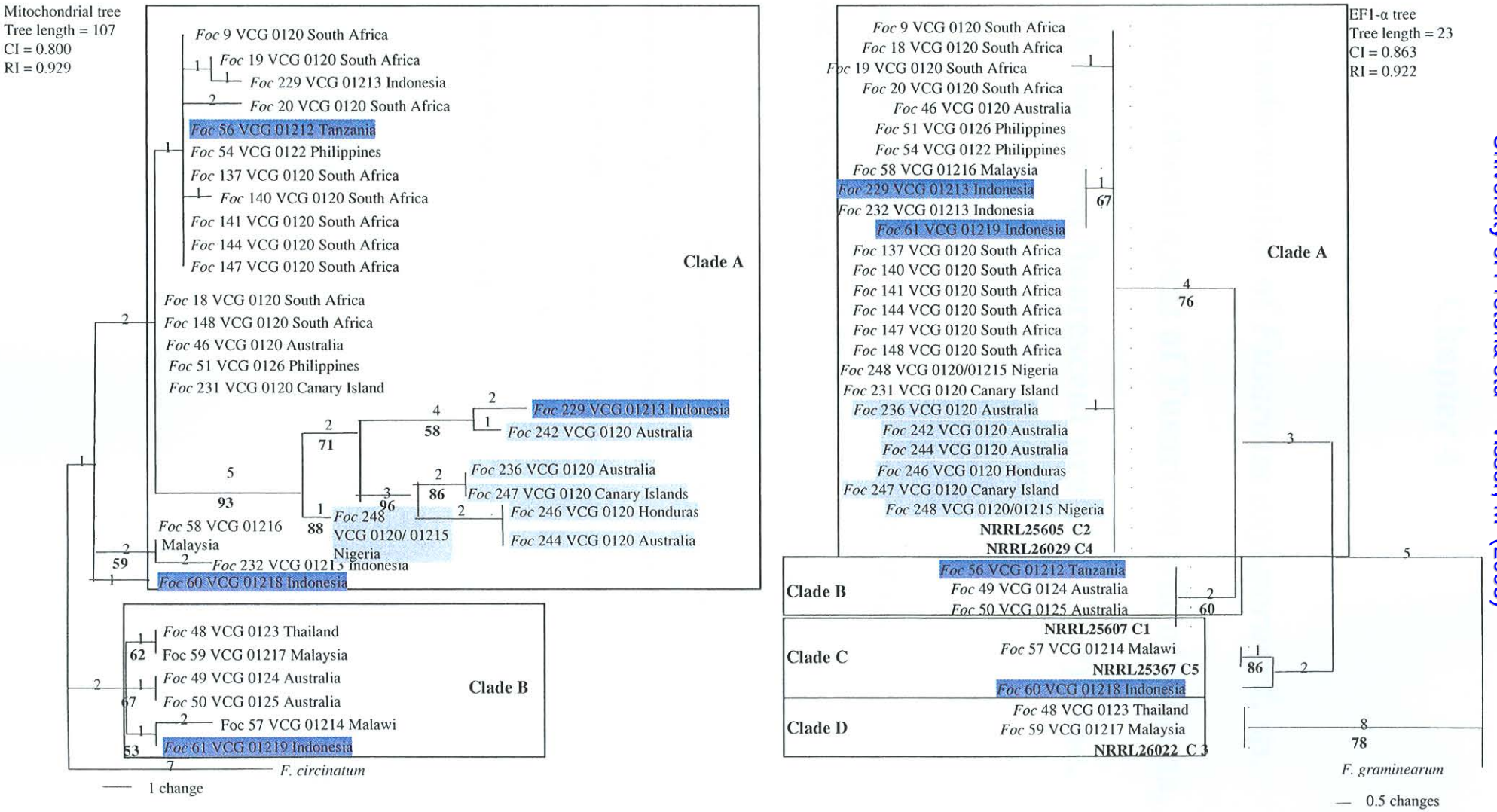

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
1636 bp
1018 bp

Figure 2: Phylogenetic analysis of 33 *Foc* isolates based on the mitochondrial DNA direct repeat (left) and 1- α EF region (right). Geographic origin is listed beside isolate codes and VCG designation. Bootstrap values are indicated below the branches while the number of base substitutions is indicated above the branches. NRRL numbers indicate sequences obtained from GenBank and represent the 5 clonal lineages of *Foc* previously reported by O'Donnell *et al* (1998b). The shading signifies the different placement of the same isolates in the respective trees.



Chapter 4

Transformation of *Fusarium oxysporum* f. sp. *cubense*, causal agent of Fusarium wilt of banana, with the green fluorescent protein (GFP) gene.

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ABSTRACT

Fusarium oxysporum f. sp. *cubense* (*Foc*) is the causal agent of Fusarium wilt (Panama disease) of bananas in most tropical and subtropical banana-producing regions of the world. The fungus infects through roots, colonizes the rhizomes and eventually blocks the vascular system of the pseudostems, resulting in plant death. The green fluorescent protein (GFP) emits green fluorescence when excited by blue light, making it a useful tool to study early stages of fungal infection. The objective of this study was to transform *Foc* isolates with the GFP gene. Isolates representing 'subtropical' race 4 of the fungus were transformed with the *sGFP* derivative using hygromycin as a selectable marker. Efficiency and transformation of spheroplasts depended on mycelium age, the choice of enzymes and the temperature and duration of incubation. The transformed isolates did not differ markedly from the wild type isolates in growth and morphological characteristics *in vitro*. Fluorescence microscopy showed expression of the green fluorescent protein in fungal structures. The presence of the GFP DNA in the fungal cells was confirmed by PCR using a GFP specific primer pair and southern blot analysis. Pathogenicity tests showed that the transformation process did not alter pathogenicity of *Foc* isolates. Fungal hyphae within tissues of infected plants fluoresced and the transformed fungus was re-isolated from artificially inoculated plants. Transformants of *Foc* will facilitate future infection studies with this pathogen on banana.

INTRODUCTION

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Shimomura) has been developed as a reporter for gene expression (Prasher 1992, Chalfie *et al.* 1994, Cubitt *et al.* 1995, Niedenthal *et al.* 1996, Spellig, Bottin & Kahmann 1996, Tsien 1998), a tracer for studying cellular dynamics of filamentous fungi (Suelmann & Fischer 1997, Suelmann & Fischer 2000) and a label to follow development of pathogens within their plant hosts (Sheen *et al.* 1995, Chalfie & Kain 1998, Bottin *et al.* 1999). GFP was first expressed in *Escherichia coli* and *Caenorhabditis elegans* (Chalfie *et al.* 1994).

Expression of the GFP has subsequently been successful in plants (Haseloff & Amos 1995, Sheen *et al.* 1995, Chiu *et al.* 1996) mammals (Pines 1995) and yeasts (Cormack *et al.* 1997). The GFP has also been expressed in numerous filamentous fungi such as *Ustilago maydis* (DC.) Corda (Spellig *et al.* 1996), *Podospora anserina* (Rabenh.) Niessl (Berteaux-Lecellier *et al.* 1998), *Magnaporthe grisea* (T. T. Hebert) M. E. Barr, Kershaw, (Walkey & Talbot 1998), *Cochliobolus heterostrophus* Drechsler (Maor *et al.* 1998), *Mycosphaerella graminicola* (Fuckel) J. Schröt. (Skinner *et al.* 1998), *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara, (Dumas *et al.* 1999), *Phytophthora parasitica* Dastur, (Bottin *et al.* 1999), *Aspergillus niger* Tiegh. (Du *et al.* 1999) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker, (Lagopodi *et al.* 2002).

The formation of the fluorescent chromophore is apparently not species dependent and this has resulted in success with expression in several heterologous systems. It requires only ultra violet (UV) or blue light and oxygen to fluoresce. The detection of GFP is non-invasive, and non-destructive. There is no need for staining with special dyes or fixing of the sample using heat. However, successful expression of GFP in filamentous fungi requires a GFP derivative that is efficiently translated in fungi, the development of a transformation system in the target

species, and a strong fungal promoter that drives strong constitutive expression of the GFP (Lorang *et al.* 2001). The *sGFP* derivative has been shown to be efficiently translated and expressed in most filamentous fungi tested (Lorang *et al.* 2001).

The fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (E. F. Smith) Snyder and Hansen (*Foc*) causes a highly destructive vascular wilt disease of banana plants (Stover 1972). *Foc* is a soilborne fungus that infects banana plants through their roots, then colonizes the rhizomes and eventually blocks the vascular vessels of the pseudostems (Stover 1972).

The objectives of this study were to transform isolates of *Foc* with a plasmid harbouring the s*GFP* derivative. Thereafter, we determined whether the transformed isolates retained their integrity and pathogenicity, and confirmed the expression of the GFP in infected plant material.

MATERIALS AND METHODS

Fungal isolates and culture conditions

Five virulent 'subtropical' race 4 isolates of *Foc* were selected for transformation with the GFP. These included isolates from South Africa (*Foc* 1, 6, 42), Taiwan (*Foc* 28), and Australia (*Foc* 46). All isolates are maintained on filter paper at 4°C in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Preparation of fungal spheroplasts

Spheroplasts of *Foc* were prepared using the method described by Vollmer & Yanofsky (1986) with minor modifications. Isolates were grown on potato dextrose agar (PDA) and the aerial mycelium was harvested after 9-10 days. Conidial suspensions (3.5×10^9 conidia/ml)

were prepared and inoculated into 500 ml potato dextrose broth (PDB) in 2 l Erlenmeyer flasks and placed on a shaker at 15.5 rcf for 18 hours. Cultures were grown overnight, vacuum filtered and then washed with 100 ml of 0.6 M magnesium sulphate ($MgSO_4.7\ H_2O$) solution. Fungal mycelium (1.5 g) was transferred to pre-weighed sterile 50 ml disposable centrifuge tubes and placed on ice. The cells were resuspended in osmotic medium (OM) (0.98 M $MgSO_4$, 8.4 mM Na_2HPO_4, 1.6 mM NaH_2PO_4) (10 ml/g mycelium) and the suspension was transferred to a sterile 250 ml flask on ice.

To ensure complete digestion of fungal cell walls, a modification of the enzyme mixture described by Churchill *et al.* (1990) was used. The enzyme mixture (2 ml/g mycelium) consisted of lysing enzyme (0.15 mg/ml) (Novo Industries 7367155), chitinase (10.4 mg/ml) (Fluka), and β-D-gluconase (21.6 mg/ml) (Sigma). Nine millilitre of enzyme mixture was added to each flask followed by helicase (0.3 ml/g mycelium) (Sigma) added separately, mixed gently, and incubated on ice for 5 min. Bovine serum albumin (BSA) (0.75 ml/g mycelium) (Sigma) was added after 5 min of incubation. This mixture was shaken at 6.7 rcf at 30°C for 3–4 hours. The yield of spheroplasts was checked microscopically to ensure complete digestion of the cell walls.

Five-millilitre-aliquots of the spheroplast solution were mixed with 5 ml of OM. This suspension was carefully overlaid with 10 ml of trapping buffer (0.4 M sorbitol in 100 mM Tris-HCl, pH 7) and centrifuged for 15 min at 3824 rcf, at 4°C to produce two separate phases. The spheroplasts were removed and pooled, placed on ice, and diluted with 2 volumes of 1 M sorbitol. The spheroplast solution was centrifuged in a bench top centrifuge at 2655 rcf at 4°C for 5 min. The pellet was washed with 500 µl of ice-cold sorbitol and centrifuged at 2655 rcf for 5 min at 4°C. The pellet was resuspended in 500 µl STC [1 M sorbitol, 50 mM Tris HCl (pH 8), 50 mM $CaCl_2$] and centrifuged at 2151 rcf for 5 min at 4°C.

The spheroplasts (at 3 x 10^8 cells/ml) were finally resuspended in a spheroplast storage buffer containing 4 parts STC and 1 part 60% polyethylene glycol (PEG) 6000 and 1% (v/v) dimethylsulfoxide (DMSO). Cells were used or immediately frozen in vials at –80°C for later use.

Transformation of fungal spheroplasts

A plasmid construct harbouring the s*GFP* derivative was used to express GFP in *Foc*. The construct also contained a hygromycin-B resistance gene, which was used as a selective marker by culturing transformants on a medium containing the antibiotic. The transformation vector (pCT74), which expresses s*GFP* from the ToxA promoter of *Pyrenophora tritici-repentis* (Died.) Drechsler was obtained from L. M. Ciuffetti (Freitag, Ciuffetti & Selker 2001, Lorang *et al.* 2001).

For the transformation, spheroplasts (2-3 x 10^8/ml in 100 µl) were mixed with 20 µl (0.1-1 µg) plasmid, suspended in 5 µl distilled water, 25 µl of 2 x STC, in a 15 ml screw cap tube. This was followed by the addition of 25 µl 60% PEG 4000 with gentle mixing and incubation at room temperature for 20 min. An additional 1.2 ml of 60% PEG 4000 was then added, mixed gently and the mixture incubated for exactly 5 min at room temperature. Cells clumped together at this stage. Four millilitres of 1 x STC was added and mixed.

Transformed spheroplasts were plated using 500 µl of protoplast suspension per 20 ml of molten regeneration agar precooled to 50°C. The regeneration medium was prepared by dissolving 24 g of PDB and 9 g of agar in 400 ml distilled water. In a separate bottle 273.84 g of sucrose was dissolved in 600 ml distilled water. The contents of both bottles were then autoclaved and mixed while the temperature was still above 50°C. The plates were incubated right side up, overnight. After 16-18 hours, each plate was overlaid with 10-12 ml of 1%

water agar containing hygromycin-B at a concentration of 150 µg/ml. A control plate was included which was not overlaid with hygromycin-B. This was done in order to check the viability of the spheroplasts. After the overlaid agar solidified, plates were incubated right side up at room temperature. Transformed isolates grew through the overlay in the presence of hygromycin-B within 2-7 days.

Morphological and cultural characteristics

Transformants were transferred to water agar, and PDA (supplemented with hygromycin-B) to examine the morphology and cultural characteristics of *Foc* transformants. Non-transformed cultures of the same isolates were used as controls. Slide preparations using a drop of sterile water and a strand of hyphae were studied under the microscope using white and UV light.

Detection of the GFP gene using GFP specific PCR primers

Transformed isolates were grown on PDA plates containing hygromycin-B for 7-10 days. Conidia from actively growing cultures on PDA were used to inoculate PDB medium in a total volume of 100 ml in 250 ml flasks. Fungal isolates were grown in PDB in still culture for 10-14 days, harvested and freeze dried. Mycelial masses were ground to a fine powder in liquid nitrogen. Total DNA from each isolate was extracted using the phenol-chloroform based extraction method described by Raeder & Broda (1985).

Specific primers designed by Lorang *et al.* (2001) were used to confirm the presence of the GFP in *Foc*. The 25 µl PCR reaction cocktail contained 0.4 mM of each deoxynucleoside triphosphates (dNTPs), 10 x PCR buffer, 10 pmole of each primer, 0.25 units Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals), and 2 ng of DNA. Both diluted (1:10) and undiluted DNA template was used in the optimisation of the PCR. The GFP

specific primers sequences were GFP1 5' TAG TGG ACT GAT TGG AAT GCA TGG AGG AGT 3'; GFP2 5' GAT AGA ACC CAT GGC CTA TAT TCA TTC 3'. PCR reactions were performed on a Hybaid TouchDown PCR machine (Hybaid Limited). Reaction conditions were initial denaturation at 96°C for 2 min, denaturation at 94°C for 30 s, primer annealing at 58°C for 45 s, elongation of 72°C for 45 s, for 30 cycles, and a final extension 72°C for 7 min. Amplified product was resolved on a 1.5% agarose gel in Tris Acetic acid EDTA (TAE) (242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA at pH 8.0) and stained in ethidium bromide and visualised under UV illumination. Sizes of the PCR fragments were estimated using a molecular weight standard (100 bp ladder, Promega).

Confirmation of integration of GFP using Southern blots

The method described by Sambrook, Fritsch & Maniatis (1989) was used for southern blot analysis. Total isolated DNA was digested with *Eco*RI and fragments were separated on a 1% agarose gel in 1 x TAE buffer and blotted onto a positively charged nylon membrane (Roche Molecular Biochemicals). For probe labelling, the Gene Image random prime labelling kit was used (Amersham Life Science). Labelled probes (GFP PCR amplified product) were allowed to hybridise to blotted DNA. This was followed by different stringency washes and incubation in a liquid blocking solution as recommended by the supplier of the kit. Membranes were then incubated with a 500-fold diluted anti-fluorescein-AP conjugate to obtain a fluorescence signal. After washing, fluorescence signals on the membrane were detected using the Gene Images CDP-Star detection kit (Amersham Life Science), which was followed by exposure to an X-ray film.

Pathogenicity tests

Five transformed isolates of *Foc* were used to test whether pathogenicity to bananas had been retained in these cultures. Transformants were grown on half-strength PDA supplemented with hygromycin-B. Conidia were harvested after 5 days and inoculated into Armstrong's

Fusarium medium (Booth 1971) to enhance sporulation. After 5 days, the conidia suspensions were passed through sterile cheesecloth to separate the mycelium from the conidia. The conidial suspension was adjusted to a final concentration of 5 x 10^6 conidia/ml.

Tissue culture-derived Cavendish banana plants were inoculated with the transformed isolates. A hydroponics system where plants were grown in 250 ml plastic cups in sterile distilled water for 7 days was used. The plants were fertilised with a hydroponic mixture of 0.6 g/l $Ca(NO_3)_2$ H_2O, 0.9 g/l agrasol 'O and 3 g/l Micromax. For inoculation, the plants were removed from plastic cups, the roots slightly wounded by crushing gently and the conidial suspension added to the medium to obtain a final concentration of 5 x 10^5 conidia/ml. Plants were returned to the cups and maintained in a greenhouse at 25°C. Five plants per inoculum were used for the pathogenicity trial. Control plants were treated in the same manner and maintained in sterile water. Disease severity was evaluated 6 weeks after addition of the conidial suspension. Plants were cut just above the roots and internal symptoms rated, using a standardized disease rating scale (Orjeda 1998) for Fusarium wilt of banana.

To determine whether banana tissue had been colonised with transformed *Foc*, thin sections (8–12 µm) of the rhizome and pseudostem were made using a microtome. Sections were prepared, mounted on slides in sterile distilled water and observed under white and UV light using fluorescent microscopy (Zeiss, Mannheim, Germany) equipped with filter blocks (Nikon standard fluorescence filter cubes 78648) with spectral properties matching those of GFP (480 nm excitation, 515 nm emission). Images were captured with an AxioCam HR camera (Carl Zeiss Ltd, Mannheim, Germany) and processed with Corel DRAW-Version 10.0 software (Corel Corporation Ltd. Ontario, Canada).

RESULTS

Preparation of spheroplasts and the transformation process

Approximately 4 x 10^7 uninucleate spheroplasts were generated for each of the *Foc* isolates used in this study. Mycelial age had a significant effect on the frequency of spheroplast regeneration. Younger mycelial networks resulted in positive and higher frequency of spheroplast regeneration. The type of osmotic stabiliser (sucrose, NaCl, and KCl) used in the regeneration medium also influenced the regeneration of spheroplasts. Sucrose was found to result in the optimal regeneration of spheroplasts.

One hundred hygromycin-B resistant transformants were obtained after the transformation procedure. Sixty percent of transformants were stable when transferred to fresh hygromycin containing medium. Thus, the efficiency of the transformation process with the plasmid containing the transformation vector (pCT74) that expresses *sGFP* from the *ToxA* promoter of *P. tritici-repentis* was sufficient for the purposes of this study.

Morphological and cultural characteristics

Strong constitutive expression of *sGFP* occurred and could be visualised in fungal hyphae, micro- and macroconidia as well as on the dying vascular tissue, pseudostem and rhizome (Fig. 1). All transformants generated from spheroplasts appeared bright green and fluoresced uniformly. No morphological changes in size and shape of vegetative structures were observed. Transformed and wild type isolates varied slightly in mycelial growth but not in sporulation and colony appearance. Transformed strains retained the colony morphology typical of the wildtype, including cottony growth of aerial mycelium and pink pigmentation (Fig. 2).

Detection of the GFP gene using GFP specific PCR primers

A 417 base pair PCR product was consistently amplified using GFP specific primers and the genomic DNA (diluted or undiluted) of transformed isolates as template. A primer annealing temperature of 58°C was optimal for the PCR conditions. All nontransformed isolates were consistently negative after the amplification reaction (Fig. 3).

Confirmation of the detection of GFP using Southern blots

The presence of the GFP gene in the fungal genome was confirmed with Southern blot analysis. Successful hybridisation occurred using the 417 bp PCR product as a probe against the DNA of the transformed isolates. The gene was not detected in the negative control (Fig. 4).

Inoculation experiments

In the pathogenicity tests, all *Foc* GFP transformants were as pathogenic as the isolates from which they were derived. After 7-14 days wilt symptoms became visible on banana plants inoculated with both transformed and wild type isolates of *Foc*. Severe yellowing and subsequent necrosis of the roots appeared 6 weeks after inoculation (Fig. 5). Characteristic internal symptoms were reddish to dark brown discolouration of infected roots and the vascular tissue. All control plants remained asymptomatic and the pathogen could not be isolated from them. *Foc* transformed with the GFP was observed in the vascular tissue of inoculated banana plants. The movement and colonisation of the fungus could be traced into the stem. Auto fluorescence of the plant did not prevent visualisation of GFP expression. Transformants were successfully re-isolated from diseased plant tissue.

DISCUSSION

This study reports on the first transformation of *Foc* with the gene coding the GFP. Similar to results obtained for other fungi transformed with the GFP (Freitag *et al.* 2001, Lorang *et al.* 2001), transformed isolates of *Foc* fluoresced uniformly and the integration of GFP was proved to be stable. This emphasizes the effectiveness of this gene as a marker. The slight variation in GFP intensity experienced in the current study can probably be explained by the integration of the plasmid into different chromosomal sites as has been suggested previously (Lorang *et al.* 2001, Lagopodi *et al.* 2002).

Fusarium species are known to change form and colour in response to environmental constraints due to small mutations (Follin & Laville 1966, Booth 1971, Nelson, Toussoun & Marasas 1983, Hawksworth *et al.* 1995). These changes are expressed in *Foc* by means of their growth, colony colour and presence of sclerotia on artificial media (Waite and Stover 1960). Transformation with the GFP in this study, however, did not affect the colony morphology, growth, pigmentation, or sporulation of *Foc*.

Similar to studies involving the GFP transformation of *C. heterostrophus*, (Maor *et al.* 1998), *U. maydis* (Spellig *et al.* 1996) and *Phytophthora palmivora* E. J. Butler (Van West *et al.* 1999), the transformed *Foc* retained its virulence. In their respective studies, Spellig *et al.* (1996) found that GFP expression does not significantly interfere with *U. maydis* development in planta. Maor *et al.* (1998) and Van West *et al.* (1999) found that transformed isolates were as virulent as the wild type isolates, and that high expression levels of GFP did not affect pathogenicity.

The ability to visualize *Foc* in banana tissue infested with GFP-transformed isolates will make it possible to study the infection processes of this fungus in greater detail than has previously

been possible. Transformation of other fungal pathogens has facilitated infection studies in the past (Spellig *et al.* 1996, Van West *et al.* 1999, Freitag *et al.* 2001). We, thus, expect to study details of the disease process, such as the exact mode of penetration and the growth of *Foc* within the host in future.

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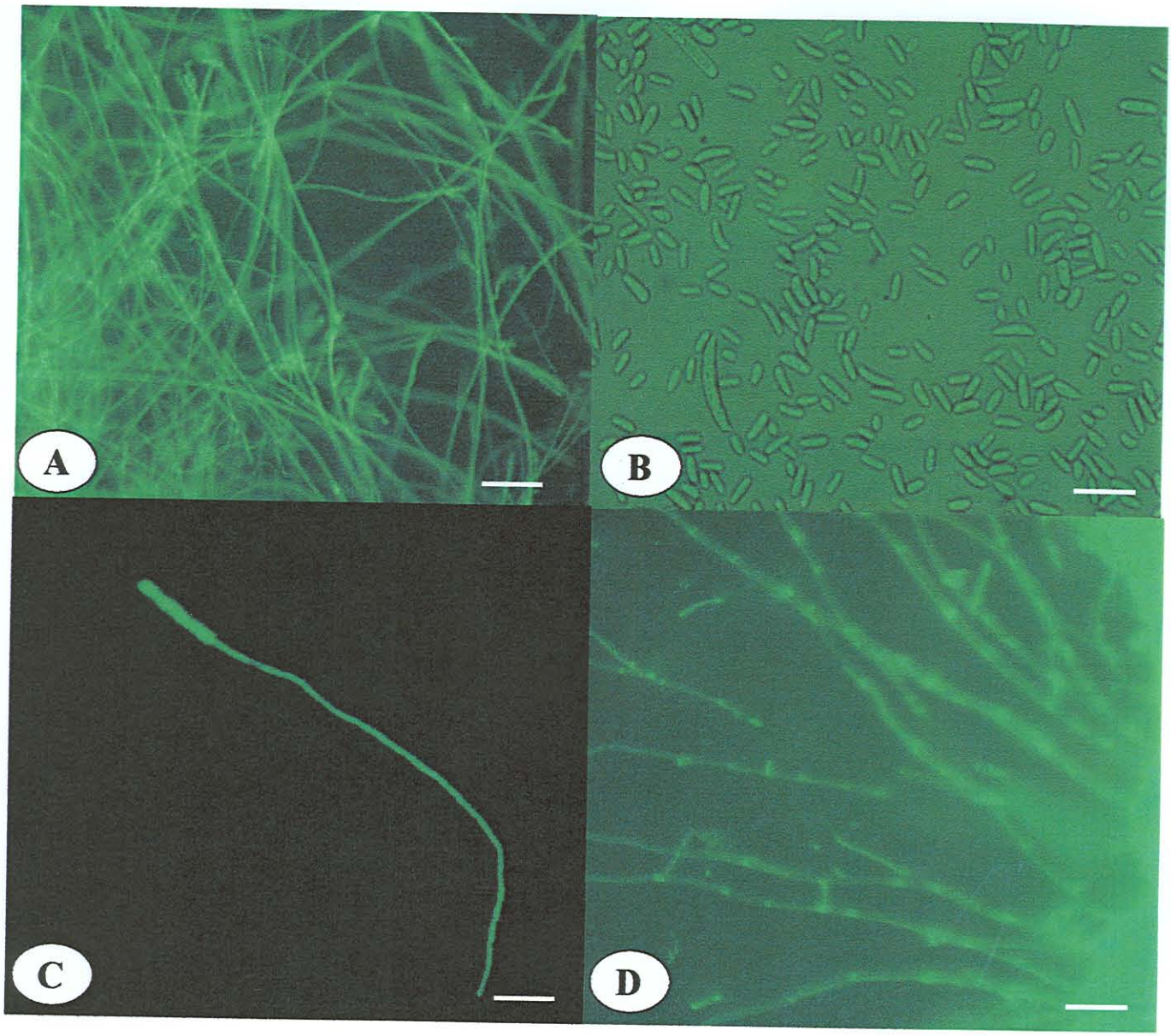

Fig. 1 Structures of transformed isolates of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) fluorescing bright green. **(A)** Fluorescing hyphal mass. **(B)** Typical size and shape of microconidia and macroconidia of *Foc*. **(C)** A germinating macroconidium on a glass slide confirming viability of the fungus. **(D)** Cross section of banana pseudostem showing network of hyphae of *Foc* through the plant tissue.
Scale bar: 10mm = 24 μm)

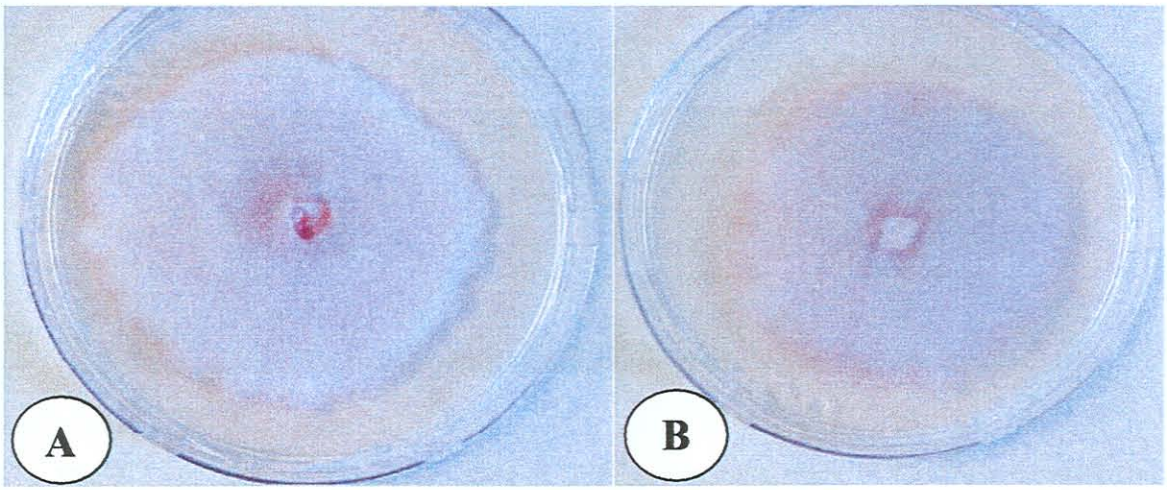

Fig. 2 Cultural characteristics of transformed and wildtype isolates of *Fusarium oxysporum* f. sp. *cubense* grown on PDA. **(A)** Typical cottony growth of wildtype. **(B)** Typical cottony growth of transformed isolate.

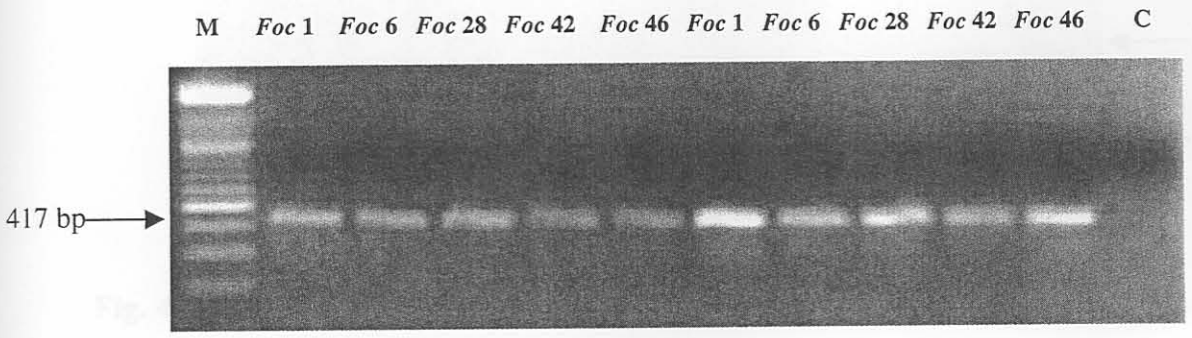

Fig. 3 Confirmation of transformation of GFP gene in the genome of *Fusarium oxysporum* f. sp. *cubense* by PCR using GFP specific primers. A 1.5% agarose gel stained in ethidium bromide and run in 1x TAE to resolve amplified product. Sizes of the amplified products were estimated using a 100 bp ladder. Lanes 1, molecular weight standard; 2-6 amplified product using undiluted template DNA; 7-11 amplified product using 1:10 dilution of template DNA; lane 12 negative control using genomic DNA of an nontransformed *Foc* 145 strain.

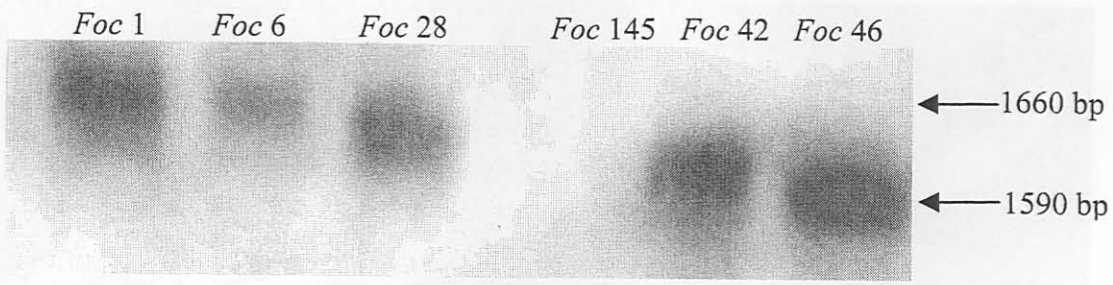

Fig. 4 Southern blot analysis of digested genomic DNA showing the presence of the GFP in the genome of *Fusarium oxysporum* f. sp. *cubense*. Lanes 1-3, *Foc* 1, *Foc* 6, *Foc* 28, lane 4 not loaded, lane 5 nontransformed *Foc* 145, lane 6-7, *Foc* 42, *Foc* 46.

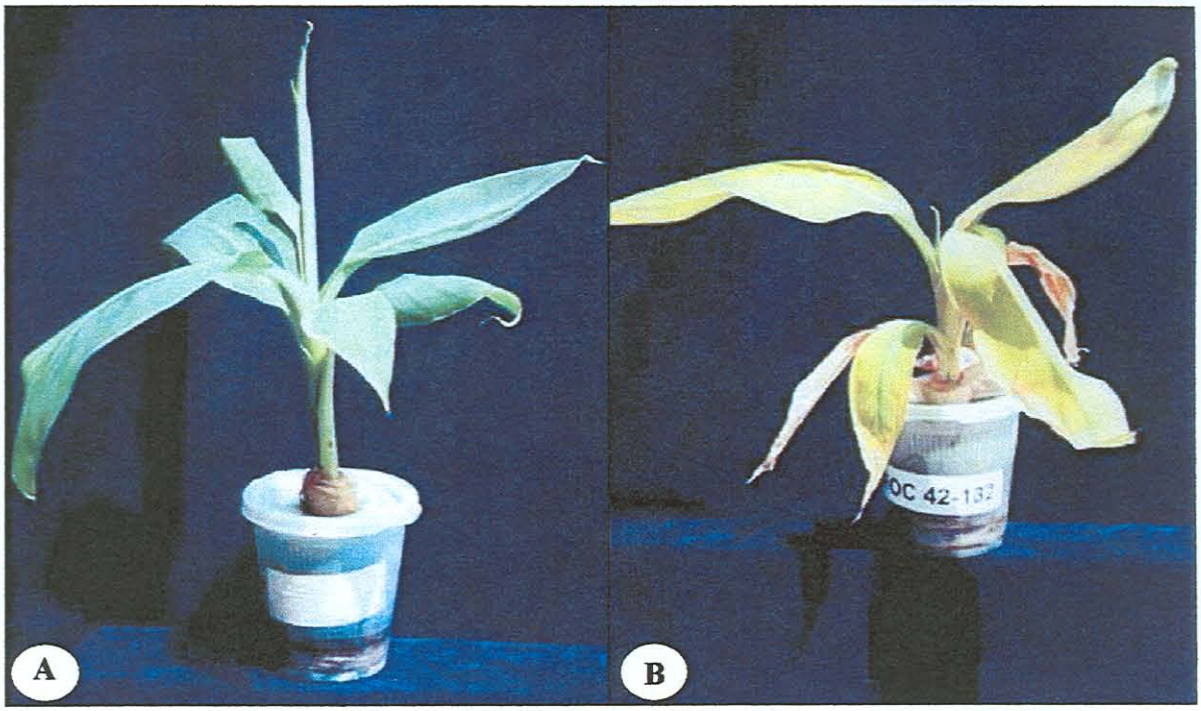

Fig. 5 **(A)** Control plant showing no disease symptoms. **(B)** Plant inoculated with transformed isolate of *Fusarium oxysporum* f. sp. *cubense* (*Foc* 42) showing symptoms of Fusarium wilt.

Chapter 5

Development of microsatellite markers for the banana wilt pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*).

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ABSTRACT

Fusarium oxysporum f. sp. *cubense* (*Foc*) is a serious fungal pathogen causing great losses to the banana industry worldwide. We describe the development of primers that target eight microsatellite markers to be used for population genetics studies on *Foc*. In this study the ISSR-PCR technique was used to develop the markers. The eight microsatellite loci amplified consistently well and were polymorphic among *Foc* isolates from different geographical origins. These polymorphic markers will be useful in large-scale studies to investigate the population structure, variation and movement of *Foc*.

INTRODUCTION

Fusarium oxysporum f. sp. *cubense* (*Foc*), the causal agent of Panama disease or Fusarium wilt, is one of the most serious problems affecting banana production in the world. In South Africa and other areas, the major commercial cultivar is Cavendish. This cultivar is highly susceptible to *Foc* race 4. The presence of this race in South Africa is therefore a serious threat to banana cultivation in the country (Stover 1962, Ploetz 1990).

The best defense against *Foc* infection at present is to prevent spread of the pathogen through education of farmers on appropriate sanitation practices (Robinson 1996). These include the use of disease-free planting material and cleaning of farming equipment to remove inoculum. Where Fusarium wilt occurs, the most effective and economical method for control is to grow genetically resistant cultivars. Because genetic resistance can be compromised by changes in the pathogen population, it is necessary to understand the genetic variability of the pathogen. One way to achieve this is to characterize isolates using polymorphic genetic markers such as microsatellites. To this end, the aim of this study was to develop microsatellite markers for *Foc* and to confirm their utility by examining *Foc* isolates representative of different geographical regions.

MATERIALS AND METHODS

Genomic DNA from the South African isolate *Foc* 135 (Table 1) was used in PCR reactions containing internal short sequence repeats (ISSR) primers: 5' NDV$(CT)_8$, DBB$(CAA)_5$, 5' DHB$(CGA)_5$, 5' YHY$(GT)_5$G, DBD$(CAC)_3$, 5'NDB$(CA)_7$C, 5' HVH$(GTG)_5$, 5' DDB$(CAT)_5$ and 5' HBDB$(GACA)_4$ (Lieckfeldt, Meyer & Borner 1993, Meyer & Mitchell 1995, Buscot *et al*. 1996). Each PCR reaction contained 0.4 mM of each dNTP, 1x PCR buffer containing

15mM $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA), 1.0 pmole of each primer (in combination), 0.25 units Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals), 2 ng genomic DNA and sterile, deionised water to a final volume of 25 µl. PCR reactions were performed on a Hybaid TouchDown PCR machine (Hybaid Limited, UK).

Reaction conditions were: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 s, primer annealing at 48°C for 45 s and elongation at 72°C for 2 min. A final extension at 72°C for 10 min was included to complete the reactions. Amplified products were resolved on a 1.5% or 2% (w/v) agarose gel in 1x Tris-Acetate-EDTA (TAE) buffer (Sambrook, Fritsch & Maniatis 1989), stained with ethidium bromide and visualised under UV illumination. Product sizes were estimated using a molecular weight standard (100 bp ladder Promega, Corp., Madison, WI). PCR products were purified using the Magic PCR Preps Purification System (Promega), according the manufacturer's instructions.

The PCR products from the ISSR primer combinations were cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions and propagated in *E. coli* JM109 cells. White bacterial colonies containing recombinant plasmids were selected and grown in 2 ml Luria-Bertani (LB) broth (Sambrook *et al.* 1989) supplemented with 100 µg/ml ampicillin (Sigma Chemicals Co, USA). Plasmid DNA containing cloned PCR amplicons was extracted using alkaline lysis (Sambrook *et al.* 1989). The DNA was treated with RNAse H (10 mg/ml, Roche Molecular Biochemicals) and digested with *Eco*RI (Roche Molecular Biochemicals) to excise inserts for sizing.

Plasmids containing inserts between 150-1500 base pairs (bp) in size were sequenced with universal M13 plasmid primers using the BigDye terminator cycle sequencing kit (Perkin-

Elmer Applied Biosystems) and ABI PRISM™ 377 and 3100 sequencer (Perkin-Elmer Applied Biosystems, Inc., Forster City, Calif). Sequences were analysed using the Sequence Navigator™ 1.0.1 computer program (Perkin-Elmer Applied Biosystems). Primer pairs were designed from sequences flanking microsatellite regions using Vector NTI (3.1). These primers were designed to have annealing temperatures between 56˚C and 68˚C and amplify different sized (120-480 bp) fragments. Inserts that contained microsatellite regions near the 3' or 5' ends required genome walking. This was performed as described by Siebert *et al.* (2001).

The primers were used in PCR reactions to test for amplification of the expected fragment. Genomic DNA of five *Foc* isolates originating from three different geographic regions (Table 1) was selected to test the primers. Each PCR reaction contained 0.4 mM of each dNTP, 1x PCR buffer containing 15 mM $MgCl_2$, (Roche Molecular Biochemicals) 1.0 pmole of each primer, 0.25 units Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals), 2 ng genomic DNA and sterile double distilled water to a final volume of 25 µl. Reaction conditions were: initial denaturation at 96°C for 2 min, 10 cycles of denaturation at 94°C for 20 s, specific primer annealing temperature for 45 s, elongation at 72°C for 45 s. Another 25 cycles were carried out with a 5 s extension step after each cycle with the annealing time of to 40 s. A final extension step of 10 min at 72°C was included. Amplified products were electrophoresed, stained and visualized as before.

PCR products were purified using the Magic PCR Preps Purification System (Promega). Only primer pairs, which resulted in the amplification of single amplicons for all five isolates, were tested further. These amplicons were cycle sequenced on an ABI PRISM™ 3100 Autosequencer (Perkin-Elmer Applied Biosystems) using the BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems). Sequences of the five

isolates were aligned manually using Sequence Navigator (Perkin-Elmer Applied Biosystems) and checked for polymorphisms. Where primer pairs yielded polymorphic products, one primer from a primer pair was 5' end-labelled with a fluorescent dye (Applied Biosystems) and used for subsequent analysis.

The polymorphic primer pairs were used to amplify PCR products from an additional nine (Table 1) geographically diverse *Foc* isolates. These reactions were performed in 15 µl volumes containing 1 x PCR buffer, 50 µM of each dNTP, 0.2 µM of each labeled and unlabeled primer, 0.25 units of *Taq* polymerase and 2 ng of DNA. The same cycling conditions were used as above. The annealing temperature for all primers was 58°C since all primers annealed sufficiently at this temperature. Fluorescently labelled PCR products were fractionated on an ABI Prism 3100 DNA sequencer. The sizes of the DNA fragments were determined using the Genescan® 2.1 and Genotyper® 3.0 analysis software packages (Perkin Elmer Corp.) with LIZ™ as an internal size standard (Applied Biosystems). A UPGMA (Unweighted Pair-Group Method with Arithmetic mean) dendrogram obtained using Nei's (1972) genetic distance matrix was constructed in POPGENE version 1.31. This was done to evaluate genetic distance between all the isolates from the three geographic origins.

RESULTS

From a total of fourteen primer pairs that were tested, eight primer pairs amplified polymorphic regions. Genescan analysis identified a total of 36 alleles across 8 polymorphic loci (Table 2). No null alleles were detected. The smallest number of alleles per locus was two and the largest was eight. Allele sizes for all loci combined, ranged from 148 to 467 bp in length.

DISCUSSION

The number of alleles detected in our sample was high despite the small sample size. *Foc* isolates from South Africa and Australia were more similar to each other than they were to the Asian isolates. The isolates from Asia displayed the highest size variability, reflecting the presence of many unique alleles. This finding is consistent with the view that *Foc* probably originated in Southeast Asia (Fig 1) (Ploetz & Pegg 1999).

The polymorphic microsatellite markers developed in this study will enable researchers to study bigger populations of *Foc* to gain more insights into the structure and diversity of geographically distinct populations. Better characterization of population structure may help to identify patterns of disease spread and the origin of new pathotypes.

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Table 1: Isolates of *Foc* used for the development and testing of microsatellite markers.

Isolate no[1]	Country of origin	VCG designation[2]	Donor
Foc 1	South Africa	VCG 0120	E. Grimbeek
Foc 135	..	..	..
Foc 145	..	..	..
Foc 147	..	..	A. Viljoen
Foc 46	Australia	..	N. Moore
Foc 49	..	VCG 0124	..
Foc 50	..	VCG 0125	..
Foc 52	..	VCG 0129	..
Foc 54	Philippines	VCG 0122	R. Ploetz
Foc 57	Malawi	VCG 01214	..
Foc 58	..	VCG 01216	N. Moore
Foc 60	Indonesia	VCG 01218	..
Foc 61	..	VCG 01219	..
Foc 229	Indonesia	VCG 01213/01216	..

[1]All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

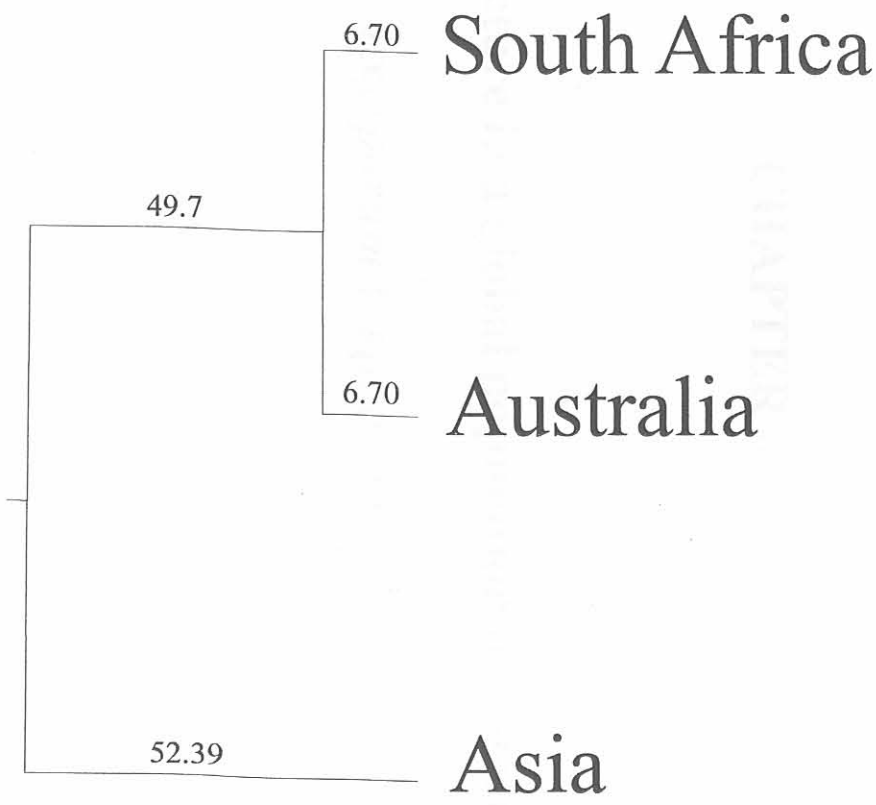
[2]Vegetative compatibility groups (VCGs) are a phenotypic character used to group fungal isolates based on heterokayon formation (Puhalla 1985).

Table 2: PCR primer information and the core microsatellites regions amplified.

SSR primer	Primer sequence	Tm (°C)[1]	Ta[2] (°C)[3]	Polymorphic fingerprints	[3]Core sequence	Fluorescent label	Allelic range
G1	F 5′CTC GTC CTT TGC GAA TGA CC	59.4	58	Yes	*GA_{19}*	6-FAM™	444,454,455,457,460,467
MV15/1	R 5′ GAC CAC CTC GGT GAT GGT GAG ACG G	69.5	58				
G2	F 5′ GAG CTG CTG GTC TCG ATG TGG	63.7	58	Yes			
G2/3	R 5′ GCA GCA TGT ACG TTA CTC AAT CTG GCG GC	69.5	58		*TGG_7*	PET™	342,343,344,345,346,347,348,353
G4	F 5′ CGT CCT CAA GAG CAG CGA C	61.0	58	Yes	*GAT_6*	NED™	386,387,389,390
G4/5	R 5′ GCG CCT GGC GTT ACT GGC AGT TTG G	69.5	58				
G16	F 5′ GAG AAC TGG GCG TAT TTG TTA GAT CAC	63.4	58	Yes	*$GGC*_4$ *GAC_3*	VIC™	276,277,280,281,282,285
G16/8	R 5′ GCT TAG GCC GAG GGA GGC AGA G	67.7	58				
G20	F 5′ GTG GAG CAA CAG AAT GTG GCC GAG CC	69.5	58	Yes			
G20/21	R 5′ GCG GCT CCA GGA GCT GCT CTG AAG TG	71.1	58		*$GTT_7GAT_2GTT_2GA_2$*	6-FAM™	284,285
G40	F 5′ CCA CGG CTT GTC CGA GCT AGT GGA G	74.3	58	Yes			
G40/41	R 5′ CAA GCC GCT CTC CAC GGC GAA GGC G	69.5	58		*$GGT_1GT_1GGT_7$*	NED™	210,211,215,219,222
G42	F 5′ GGA GGT AAT GTT ACG CAA GAG G	63.3	58	Yes	*CA_8*	PET™	203,205
G42/43	R 5′ CGA CAC TCA AGC AAG AGT GTG C	62.4	58				
M42	F 5′ CGC GTC TCC AAT CAA GCC TCA ACC	66.1	58	Yes	*$CAACAG_2CAA*_4$	VIC™	149,150,153
M43	R 5′ GGC GGT TCG GTT GAT AGG GCT CCC AG	71.1	58				

[1] calculated melting temperature
[2] actual optimum annealing temperatures for PCR primers
[3] * varying lengths of sequence on either side of the core sequence

Fig. 1 UPGMA dendrogram of *Foc* from South Africa, Australia and Asia obtained using Nei's (1973) genetic distance based on allele frequency. South Africa and Australia have the least genetic distance compared with Asia.



CHAPTER 6

Genetic structure in a global population of *Fusarium oxysporum* f. sp. *cubense*

ABSTRACT

Fusarium oxysporum f. sp. *cubense* (*Foc*) is a haploid, asexually reproducing ascomycete that causes Fusarium Wilt or Panama Disease of bananas. The fungus has been found in most banana producing areas of the world. For effective disease management knowledge of the population structure is becoming essential. In the present study, vegetative compatibility groups (VCGs) of *Foc* isolates from South Africa were determined and compared with those from other areas where Panama disease occurs. We also used eight polymorphic PCR-based microsatellite markers to characterize population structure and genetic diversity of *Foc* from diverse sources. This meta-population was divided into geographically defined sub-populations, namely those from Asia, Australia and South Africa. Genetic differentiation between the Asian and South African populations was high and gene flow minimal. Index of association (I_A) and linkage disequilibrium tests showed no evidence of recombination within the global population. This supports the view that *Foc* reproduces clonally wherever it occurs. Isolates from the three regions studied appeared to be closely related although there were groups consistent with origin.

INTRODUCTION

Fusarium oxysporum is an important causal agent of vascular wilt on many agricultural crops (Booth 1971). Pathogens belonging to this species are generally grouped according to *formae speciales* (Hawksworth 1995). Amongst these, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) cause of Panama disease devastates Cavendish bananas in the two regions of South Africa (Ploetz 1990, Viljoen 2002). The pathogen is soilborne, infecting bananas through the roots and moving up into the vascular system (Stover 1962). *Foc* thus blocks the xylem vessels preventing the movement of nutrients and water and ultimately, resulting in plant death. Races are poorly defined in *Foc* and are simply based on host pathogen interactions (Stover & Buddenhagen 1986). Isolates representing different populations of the pathogen also show variation in virulence due to differences in environmental conditions (Ploetz 1990). For example, Cavendish is susceptible to *Foc* 'subtropical' race 4, which occurs in South Africa but is resistant to race 1 of the pathogen occurring in the tropics (Ploetz *et al.* 1990).

Foc is believed to have been spread worldwide through infected planting material (Pegg & Langdon 1987, Stover 1962, Ploetz 1990). The route of entry of the pathogen into South Africa is unknown due to the incomplete records of banana production in the country. It is thought that Indian labourers, who worked on sugar cane plantations in KwaZulu-Natal during colonial times, could have introduced infected rhizomes into South Africa (Ploetz *et al.* 1990, Robinson 1996).

Previous studies considering diversity amongst *Foc* isolates have used both phenotypic and genotypic markers to characterize the pathogen. The phenotypic character most commonly used to subdivide *Foc* isolates is vegetative compatibility (Ploetz & Correll 1988, Brake *et al.* 1990, Moore *et al.* 1993). This character differentiates isolates that have identical alleles at

each of the loci that govern heterokaryon formation (Correll, Kliitich & Leslie 1987, Leslie 1993). *Foc* has 21 known vegetative compatibility groups (VCGs), of which only a few are common and widespread (Ploetz & Pegg 2000). Fifteen VCGs have been found in Asia, which is believed to be the centre of origin of bananas (Ploetz & Pegg 2000). Although vegetative compatibility is a useful means of subdividing *Foc* into genetically isolated groups, it does not measure genetic relatedness among isolates. In addition, VCGs are phenotypic markers that may be subject to selection (Glass & Kuldau 1992, Leslie 1993). Therefore, neutral DNA-based genetic markers would be more suitable for analysing genetic variation within and between *Foc* populations.

DNA-based genetic markers have had a significant impact on the study of population genetics of many organisms (McDonald 1997). PCR-based genetic markers, such as microsatellites that exhibit co-dominance, produce highly reproducible results that are ideal for population genetics studies (Bruford & Wayne 1993, Queller, Strassman & Hughes 1993, Taylor *et al.* 1999). Microsatellites are tandemly repeated units of 1-5 bp that are distributed throughout the eukaryotic genome (Queller *et al.* 1993). Microsatellites can vary substantially in repeat numbers between individuals (Bruford & Wayne 1993, Dayanandan, Rajora & Bawa 1998). Microsatellites are therefore popular markers in fungal population genetics.

The aim of this study was to consider the identity of *Foc* VCGs among isolates from the two primary banana-growing regions in South Africa. Secondly, we wanted to examine the genetic structure of *Foc* in South Africa and compared this with two populations of the fungus from other parts of the world. Finally we aimed to evaluate the potential of gene flow between these populations. The genetic variation within different populations was also determined, using microsatellite markers.

MATERIALS AND METHODS

Fungal isolates

All VCG tester isolates examined in this study were from the world-wide collection of *Foc* provided by Dr. Natalie Moore, ACDI Institute, Australia, Dr S. Bentley, University of Queensland and Dr R. Ploetz, University of Florida. These included a total of 75 isolates of which 16 were from Australia, 19 from Southeast Asia and 40 from South Africa. South African isolates were obtained from the main banana-growing regions in the country and included 21 from Mmpumalanga and 19 from Kwa-Zulu Natal. These isolates were stored on filter paper at 4°C and are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (Table 1).

Generation of nit mutants and VCG determination

Isolates were characterised based on vegetative compatibility using the technique of Puhalla (1985). This technique assigns isolates to VCGs based on heterokaryon formation between complementary nitrate-nonutilizing (*nit*) mutants. *Nit* mutants are produced on media supplemented with chlorate. Nit mutants were produced for all South African isolates as well as the known VCG tester strains from elsewhere in the world and these are stored in the culture collection of FABI. All *nit* 1 and *nit* 3 mutants were paired at least twice on minimal medium (MM) (Correll *et al.* 1987) with each of the Nit M mutants representing eleven tester strains for the known VCGs. Nit M, *nit* 3 and *nit* 1 mutants of the same isolate were also paired to test for self-compatibility. Complementary *nit* mutants formed dense, wild-type growth on MM as a result of heterokaryosis and were assigned to the same VCG. Vegetatively incompatible isolates were detected by their inability to form a heterokaryon when paired and grown on MM.

Microsatellite marker analysis

DNA extractions

All 75 isolates were grown in potato dextrose broth (PDB) without shaking at room temperature for 7-10 days. The mycelium was then harvested, freeze-dried and stored at –20°C. For total DNA extraction, mycelial masses were ground to a fine powder in liquid nitrogen using a mortar and pestle. Genomic DNA was extracted from each isolate using the phenol-chloroform based extraction method described by Raeder & Broda (1985).

PCR amplifications

Eight microsatellite primers designed for *Foc* (Chapter 5) were used in PCR reactions. Each 25-µl PCR reaction contained 0.4mM of each deoxynucleoside triphosphate (dNTP), 1x PCR buffer, 1.0 pmol/µl of each primer (one primer from a primer pair fluorescently labelled), 0.25 units Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals), and 2 ng of DNA. PCR reactions were performed on a Hybaid TouchDown PCR machine (Hybaid Limited, UK). PCR reaction conditions included an initial denaturation cycle at 96°C for 2 min followed by 10 cycles of denaturation at 94°C for 20s, primer annealing at 58°C for 45s and extension at 72°C for 45s. A further 25 cycles of denaturation at 94°C for 20s, annealing at 58°C for 40s and extension at 72°C for 50s was carried out. A final extension step at 72°C for 10 min was included. Amplified products were resolved on 1.5% or 2% (w/v) agarose gels in Tris Acetate EDTA (TAE) (pH 8.0) stained with ethidium bromide and visualised under UV illumination (Sambrook, Fritsch & Maniatis 1989).

Genescan analysis

Fluorescently labelled PCR products were combined with a 1:14 mixture of formamide and LIZ ™ standard. Samples were then separated on an ABI Prism 3100 DNA sequencer. The

sizes of DNA fragments were determined using the Genescan® 2.1 and Genotyper® 3.0 analysis software (Perkin Elmer Corp.).

Data sets were generated for the analysis of the three subpopulations based on different geographic origins (South Africa, Australia, Asia). Statistical analysis of amplified loci was performed using all data sets to determine allele frequency, genetic diversity, genotypic diversity, population differentiation and gene flow.

Population analysis

Gene diversity (H) at each locus was estimated by allele frequency for each sub-population using the formula provided by Nei (1973). A graph showing the genotypic diversity (G_{ST}) (Stoddart & Taylor 1988) against the number of loci (1000 resampling repetitions) was also plotted for all populations. This was done to determine whether the eight loci were sufficiently variable for population structure analysis for *Foc*.

Population differentiation and gene flow was calculated among subpopulations using Weir's theta (θ) (Wright's F_{ST}) for randomized datasets (Weir 1996) using the program Multilocus 1.2 (Agapow & Burt 2000). To test for the presence of partitions within the global data set, G_{st} was calculated using the equation provided by Nei (1973). Indirect estimation of gene flow (N_m) was also calculated from the estimate of G_{st}.

The mode of reproduction, i. e. the hypothesis of random association among loci, was tested using the index of association (I_A) statistic, generated with the program Multilocus. The I_A test was simulated by comparing the observed value of I_A to that expected under the null hypothesis of random mating using 1000 randomizations of the original data matrix (Agapow & Burt 2000).

The genetic distance for the three combined populations was calculated using absolute distances (D_{AD}). A distance matrix was constructed using the measure $\delta\mu^2$ (Goldstein, Pitt & Taylor *et al.* 1995). The program MICROSAT (http://human.stanford.edu/microsat) was used for this purpose. A UPGMA (Unweighted Pair-Group Method with Arithmetic mean) dendrogram based on the distance matrix was constructed in MEGA version 2.1 (Kumar *et al.* 2001). Each individual population was analysed using all the available isolates. A dendrogram for the three combined populations was also constructed, in order to evaluate genetic distance between them.

RESULTS

Generation of nit mutants and VCG determination

Nit mutants were generated for all the South African isolates and all the known testers used in this study. The proportion of *nit* 1 mutants was 90%, Nit M mutants 80% and *nit* 3 mutants 70%. Almost all isolates produced at least one Nit M mutant. Crossings of Nit M with *nit* 3 or *nit* 1 isolates produced a zone of wild-type growth where the two *nit* mutants anastomosed and produced a heterokaryon. Complementary *nit* mutants formed a dense, wild-type growth on MM and were subsequently placed in the same VCG as the tester strain. Isolates from the South African population only formed heterokaryons when they were paired with the Australian VCG 0120 tester strains. No positive reactions resulted from pairings with VCGs 0121, 0122, 0129, 01211, the other VCGs associated with race 4, or isolates representing other VCG complexes.

Population analysis

Isolates from Asia had the greatest number of alleles (35) followed by Australia (21) and South Africa (18), respectively. Asian isolates shared more alleles with Australian isolates than with isolates from South Africa (Table 2). All loci were polymorphic in the global and

than with isolates from South Africa (Table 2). All loci were polymorphic in the global and subpopulations of *Foc*, except for one (G20/G20-21), which was monomorphic for the South African population. Some alleles were unique to certain groups of isolates. South African isolates had 18 alleles of which one was unique, Australian isolates had 21 alleles and only one was unique, and those from Asia had 35 alleles of which 14 were unique (Table 2).

A total of 33 different genotypes from the world-wide population of *Foc* were identified. Only one genotype was shared across the three populations. The common genotype shared in all geographic subpopulations was genotype 1 and this was also the dominant genotype in South Africa. South African and Asian populations shared three genotypes (1, 7, and 10), while Australian and Asian populations shared three genotypes (5, 10, and 25). The highest level of genotypic diversity ($\hat{G}$ = 79%) was found in the Asian population while the Australian and South African populations had $\hat{G}$ = 50% and $\hat{G}$ = 6% respectively (Table 3). The eight markers were shown to be sufficiently variable for population structure analysis by calculating genotypic diversity (G_{ST}) and plotting this value against the number of loci using randomisation in the Multilocus program (Fig 1). Only the sample representing the South African population did not reach a plateau.

Gene diversity (*H*) values were 0.108, 0.351, and 0.501 for the South African, Australian and Asian populations, respectively (Table 3). G_{ST} between subpopulations (2.5, 8 and 15.04, respectively) could also be determined as a measure of genetic variation. The data set could be divided into partitions that correlated with the geographic origin of isolates between the subpopulations (Table 3).

Population differentiation (Weir 1996) in all possible combinations produced θ values of 0.416, 0.224, and 0.175 ($P < 0.001$) for the populations from South Africa, Australia and Asia

respectively (Table 4). The level of gene flow (N_m) was the highest between the Australian and Asian population ($\frac{1}{\theta}$= 5.69, N_m = 3.315). The amount of gene flow between the South African population and the Australian population was also high ($\frac{1}{\theta}$ = 4.46, N_m = 3.601) (Table 4). The lowest amount of gene flow was found between the South African and Asian population ($\frac{1}{\theta}$= 2.39, N_m = 1.642).

The observed values for the I_A in Australian and Asian populations fell beyond the distribution range for a recombining population (P<0.001). The analysis of the Australian and Asian populations indicated clonal patterns of reproduction. Only the South African population had non-significant P-values and the null hypothesis of random mating could not be rejected (Fig. 2).

The UPGMA dendrogram based on genetic distance between the three subpopulations grouped South African isolates together and separately from the other two geographical regions (Fig. 3). One South African isolate clustered with isolates from Southeast Asia, and two isolates from Indonesia were grouped with the South African population. The Australian isolates share several genotypes with those from Asia. The Australian and Asian isolates were interspersed in the dendrogram.

DISCUSSION

In this study, both VCGs and microsatellite markers were used to evaluate the gene diversity, genotypic diversity, mode of reproduction, gene flow and partitioning between subpopulations of *Foc* from different geographical origins. The South African population was more extensively sampled than the Australian and Asian populations.

All South African isolates were in VCG 0120, which also occurs in Australia, Southeast Asia and many other banana-producing areas worldwide. This indicates that the diversity within the South African population is low and reconfirms the idea that the fungus was introduced into this area. It also confirms the wide distribution of VCG 0120 and the success of this well-adapted clone in many parts of the world. VCG analysis also supports previous reports that strains of *Foc* have been introduced into Australia from Asia, probably with banana planting material (Brake *et al.* 1990, Moore *et al.* 1993). Thus, some genotypes from the Asian gene pool have been incorporated into the gene pool of the Australian and South African populations.

The highest gene diversity found in this study was for isolates from Asia. Gene diversity is defined as the probability of obtaining two different alleles at a locus within the population (Nei 1973). The mean of H ($\overline{H}$) was calculated to facilitate comparisons among the populations. This value is supported by a higher mean allelic diversity at the loci (Hs = 0.707) and a higher genotypic diversity $\hat{G}$ = 79%. More unique alleles and genotypes were found in the Asian population than in South Africa and Australia, providing further support for an Asian origin of the pathogen. The higher gene diversity could be the result of mutations accumulating over time (Burdon & Silk 1997). According to Fisher *et al.* (2000), unique alleles are evidence that the pathogen is native to a given region (McDonald & McDermott 1993), in this case, Asia. All living organisms including fungal populations tend to be more diverse at the centre of origin and more homogeneous in areas of introduction (Andrivon 1996). Southeast Asia is believed to be the centre of origin of both the Panama disease pathogen and its host (Ploetz & Pegg 1997). Our results support this hypothesis.

The low genotypic diversity amongst *Foc* isolates from South Africa shows that *Foc* represents a clonal lineage in this country. A limited number of genotypes are shared between

the ancestral Asian population, Australia and South Africa, where the pathogen is known to be introduced. The few identical genotypes among the three populations illustrate how *Foc* is dominated by clonal lineages, presumably reflecting the effects of clonal selection (Kumar *et al.* 1999). Asexual reproduction allows for the amplification of fit genotypes, which can occur at a high frequency within a population (Pringle & Taylor 2001, McDonald & Linde 2002). In addition, geographic isolation from populations in Southeast Asia could have resulted in genetic drift, which could contribute to differences between isolated populations over time (Brasier 1987, 1995). Selection, of genotypes favoured by the environmental conditions in areas of introduction could also have resulted in new genotypes (Lobuglio, Pitt & Taylor 1993). The I_A statistics for two of the three populations also indicate a clonal pattern of reproduction. However, the South African population showed a population structure indicative of recombination. This could be due to resampling of specific genotypes, or migration (Chen & McDonald 1996, Douhan, Peever & Murray 2002) between the different banana growing regions in South Africa.

Graphs plotting G_{ST} versus number of loci showed that the Asian and Australian populations reached a plateau. G_{ST} is defined as the probability that two individuals taken at random have different genotypes (Nei 1973). The South African isolates do not appear to represent all the variation that is present in this population. However, this population has been extensively sampled and represents the highest number of isolates in this study. The low level of genotypic diversity found in the South African population was to be expected given the fact that only a single VCG occurs in this area.

Populations of *Foc* resemble clones and differ in the distribution of genotypes. Only one genotype was most common in each of the geographical subpopulations and only one was dominant in the South African population. Greater ecological fitness or reproductive

dominant in the South African population. Greater ecological fitness or reproductive advantage over other genotypes (Pringle & Taylor 1999, Schilder *et al.* 1999) could explain the large geographical range of a specific genotype. The dominance of genotype 1 in the South African population of *Foc* could be due to genetic bottlenecks and founder effects (Gillespie 1998).

There was a good correlation between population differentiation (θ) and geographical distance between subpopulations of *Foc*. The higher gene flow and low population differentiation between the Asian and Australian populations is probably due to the close geographic proximity of the two regions. Low levels of gene flow between the South African and Asian populations could be due to a physical barrier, such as distance, that would have limited migration between the populations. Fisher *et al.* (2000) showed in their study that reduced gene flow due to geographic or reproductive isolation would result in populations with low genetic variation due to random drift. Our results showed that only three genotypes are shared between South African and Australian isolates and all other genotypes are unique to these regions. This provides evidence that bananas were introduced into new areas at different times. High levels of differentiation between populations might imply that sufficient time has elapsed for the onset of genetic drift, which can eliminate alleles from a population (Leung, Nelson & Leach 1993). High levels of population differentiation observed in this study are probably due to geographic and genetic isolation. Populations may become differentiated by isolation and the accumulation of different mutations in a population (Fry *et al.* 1992).

A UPGMA dendrogram based on genetic distances between the three *Foc* populations showed that specific groups within the *Foc* metapopulation. (A metapopulation is loosely defined as the set of individuals that inhabit a landscape). The South African isolates formed

a single group but this also included two isolates from Asia. The remainder of the Australian and Asian isolates formed a subgroup. This indicates that there is a certain degree of host specialisation or environmental adaptation influencing the evolution of *Foc* in the three geographically separated regions. It could also be that the South African population has resulted from a limited number of introductions.

Results of this study indicate that in most areas *Foc* propagates clonally. It appears that Asian populations have been extensively dispersed world-wide and only some Asian genotypes have become fixed in certain geographical areas. The extensive geographical range of certain genotypes confirms that clonal populations are well adapted to different environmental conditions. Thus, the widespread distribution of the VCG 0120, and the stability of certain genotypes within populations of *Foc,* emphasises the ability of this clonal pathogen to thrive.

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Table 1. Isolates of *Foc* from a worldwide population of the pathogen used in this study.

Isolate number[1]	Other name[2]	Origin	Cultivar	VCG[3]	Donor/ Collector
Foc 1		South Africa	Cavendish	0120	A. Viljoen
Foc 2		..		..	..
Foc 4		..	Williams	..	..
Foc 5		..		..	E. Grimbeek
Foc 6		..	Cavendish	..	..
Foc 7		..		..	..
Foc 8		..	Chinese Cavendish	..	E. Grimbeek
Foc 9		..	Williams	..	..
Foc 10		..		..	A. Viljoen
Foc 11		..	Israeli Grand Naine	..	..
Foc 12		..	DC24R22	..	..
Foc 14		..		..	..
Foc 17		..	Cavendish	..	..
Foc 18		..	Israeli Grand Naine	..	..
Foc 19		..	Williams	..	E. Grimbeek
Foc 20		..	Williams	..	..
Foc 21		..	Williams	..	..
Foc 22		..	Williams	..	..
Foc 23		..	Williams	..	..
Foc 24		..		..	A. Viljoen
Foc 27		..	Cavendish	..	..
Foc 28	Taiwan 14	Taiwan		0121	R. Ploetz
Foc 31		South Africa	Cavendish	0120	A. Viljoen
Foc 42		..	..	..	..
Foc 43		..	..	..	..
Foc 45		..	..	..	..
Foc 46	23486	Australia	Cavendish	..	N. Moore
Foc 48	Thai1-2	Thailand	Kluai Namwa	0123	..
Foc 49		Australia		0124	..
Foc 50		..		0125	..
Foc 51	Phil 6	Philippines	Latundan	0126	..
Foc 52		Australia		0129	..
Foc 53	Indo-14	Indonesia	Pisang Ambon Putih	0120	I. Djatnika
Foc 54		Philippines		0122	R. Ploetz
Foc 57	RPMW40	Malawi	Bluggoe	01214	..
Foc 58		..		01216	..
Foc 59		..		01217	..
Foc 60		Indonesia		01218	..
Foc 61		..		01219	..
Foc 109		South Africa	Cavendish	0120	E. Grimbeek
Foc 130		..	..		..
Foc 135		..	..		..
Foc 136		..	..		..
Foc 137		..	..		..
Foc 140		..	..		..
Foc 141		..	Grand Naine	..	..
Foc 144		..	Williams	..	..
Foc 145		..	..	..	..
Foc 147		..	Israeli Grand Naine	..	A. Viljoen
Foc 148		..	Israeli Grand Naine	..	..
Foc 149		..	Cavendish	..	..
Foc 150	RP 4	..	..	..	R. Ploetz
Foc 151	RP 6	..	..	..	..

Isolate number[1]	Other name[2]	Origin	Cultivar	VCG[3]	Donor/ Collector
Foc 152	RP 16	South Africa	Cavendish	0120	E. Grimbeek
Foc 153		Australia	..	..	N. Moore
Foc 229	II 5	Indonesia	Pisang Manurung	01213	R. Ploetz
Foc 231	CV-2	..	Valery	..	..
Foc 232	Indo 93	..	Cavendish	..	..
Foc 233	Indo 129	..	Kepok (BBB)	..	..
Foc 234	DMI 8	..	Pisang Capatu	..	R. Ploetz
Foc 235	22424	Australia	Ladyfinger	0120	K. Pegg, N. Moore
Foc 236	23486	..	Cavendish	..	..
Foc 237	23539	..		..	..
Foc 238	23987	..	Cavendish	..	..
Foc 239	N5631	..	Cavendish	..	..
Foc 240	W91307	..		..	..
Foc 241	W91 345	..	Lady Finger	..	..
Foc 242	22410	..	Cavendish	..	..
Foc 244	22615	..	Lady Finger	..	..
Foc 243	A2	..	Mons mari	..	R. Ploetz
Foc 245	O-1220	..	Mons	..	..
Foc 246	Jak 1	Indonesia	Pisang berangan	01213	..
Foc 247	DMI 4	..	Pisang Ambon	..	..
Foc 248	RPML1	Malaysia	Pisang mas	01213/01216	..
Foc 249	RPML24	..	Pisang awak	..	..

[1]Isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[2]Names as used in other culture collection from donor.
[3]Vegetative compatibility groups (VCGs) a phenotypic marker used to characterise fungal isolates based on heterokaryon formation (Puhalla 1985).

Table 2. Alleles, genotype configuration for each allele, allele frequencies and gene diversity for each isolate present at eight microsatellite loci.

Locus[1]	Allele size[2]	Genotype configuration[3]	Allele frequencies		
			South Africa	Australia	Asian
G40/G41	210	A	0.975	0.812	0.315
	211	B	0	0.062	0.052
	215	C	0	0	0.21
	219	D	0.025	0.125	0.105
	222	E	0	0	0.315
			H = 0.048	H = 0.321	H = 0.743
G4/G4-5	386	A	0	0	0.21
	387	B	0	0.062	0.421
	389	C	0.925	0.5	0.263
	390	D	0.075	0.437	0.105
			H = 0.138	H = 0.555	H = 0.698
M41/M42	149	A	0.1	0.375	0.105
	150	B	0.875	0.5	0.684
	153	C	0.025	0.125	0.21
			H = 0.223	H = 0.593	H = 0.477
G16/G6-8	276	A	0	0	0.052
	277	B	0	0.125	0.052
	280	C	0.05	0.125	0.052
	281	D	0.850	0.75	0.736
	282	E	0.1	0	0.052
	285	F	0	0	0.052
			H = 0.265	H = 0.406	H = 0.444
G42/G4	203	A	0.025	0.125	0.631
	205	B	0.975	0.875	0.368
			H = 0.0487	H = 0.218	H = 0.466

Locus[1]	Allele size[2]	Genotype configuration[3]	Allele frequencies		
			South Africa	Australia	Asian
G1/MV1-15	284	A	0.075	0.5	0.421
	285	B	0.925	0.5	0.578
			H = 0.138	H = 0.5	H = 0.488
G20/G20-21	444	A	0	0	0.105
	454	B	1	0.875	0.315
	455	C	0	0	0.421
	457	D	0	0	0.0526
	460	E	0	0.125	0
	467	G	0	0	0.105
			H = 0	H = 0.218	H = 0.698
G2/G2-3	342	A	0.025	0.125	0.052
	343	B	0	0	0.105
	344	C	0.1	0	0
	345	D	0.875	0.875	0.315
	346	E	0	0	0.052
	347	F	0	0	0.052
	348	G	0	0	0.368
	353	H	0	0	0.052
			H = 0.223	H = 0.218	H = 0.743
Population size			40	16	19
No. of alleles			18	21	35
No. of unique alleles			1	1	14
No. of polymorphic loci			7	8	8
Gene diversity			0.108	0.351	0.501

[1]The eight polymorphic loci as defined in Chapter 5.
[2]Allele size ranges.
[3]Each allele of the eight loci was assigned a different letter.

Table 3. Population parameters calculated for *Foc* populations from South Africa, Australia and Asia.

Parameter	South Africa	Australia	Asia
Population size (N)	40	16	19
Number of alleles	18	21	35
Number of unique alleles	1	1	14
Gene diversity ($\overline{H}$)	0.108	0.351	0.501
Genotypic diversity (G_{ST})	2.061	0.697	1.13
$\hat{G}$	6%	50%	79%
G_{st}	0.2838	0.3418	0.0665
Reproductive mode	recombining	clonal	clonal

$\hat{G}$ -Maximum percentage (%)of genotypic diversity (G_{ST}) using the formula $\hat{G} = G/N*100$, where N is the population size (McDonald *et al.* 1994).
G_{st} -To test for the presence of partitions within a population using the equation provided by Nei (1973)

Table 4. Population parameters calculated for *Foc* populations from South Africa, Australia and Asia.

	Asia	Australia	South Africa
Asia θ		$\theta = 0.175$ $P < 0.001$ $\left(\frac{1}{\theta}\right) = 5.69$	$\theta = 0.416$ $P < 0.001$ $\left(\frac{1}{\theta}\right) = 2.39$
Australia	$Gst = 0.1311$ $Nm = 7.018$ M = 3.315		$\theta = 0.224$ $P < 0.001$ $\left(\frac{1}{\theta}\right) = 4.46$
South Africa	$Gst = 0.2334$ $Nm = 1.9363$ M = 1.642	$Gst = 3.6011$ $Nm = 1.2618$ M = 3.601	

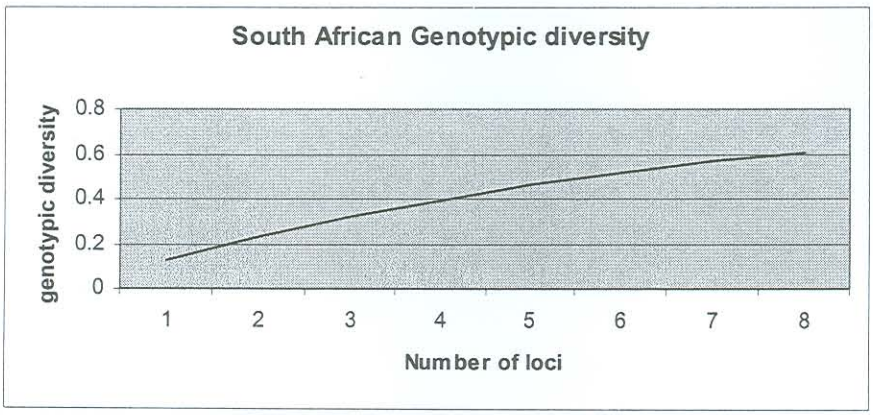
θ- theta value, population differentiation estimate Weir (1996)

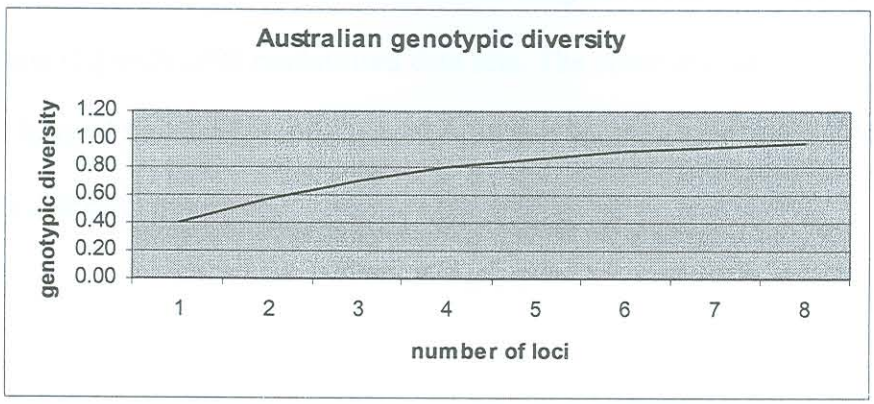
$\left(\frac{1}{\theta}\right)$- indirect estimation of gene flow from θ

Nm- indirect estimation of gene flow from *Gst* Nei (1973)

M- number of migrants from each population, an estimate from *Nm*

Fig. 1 Plot of genotypic diversity against the number of loci ($P<0.001$) for the global, VCG 0120, and three subpopulations based on geographical origin using 1000 sampling events.


South African Genotypic diversity
genotypic diversity
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
Number of loci


Australian genotypic diversity
genotypic diversity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1
2
3
4
5
6
7
8
number of loci

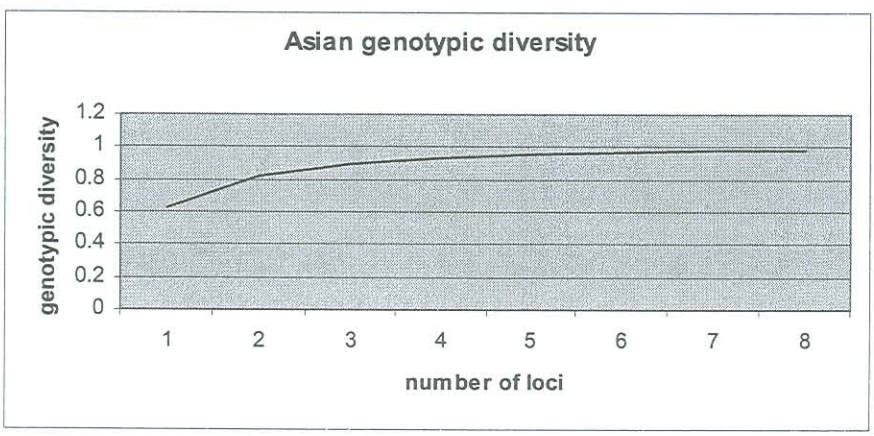
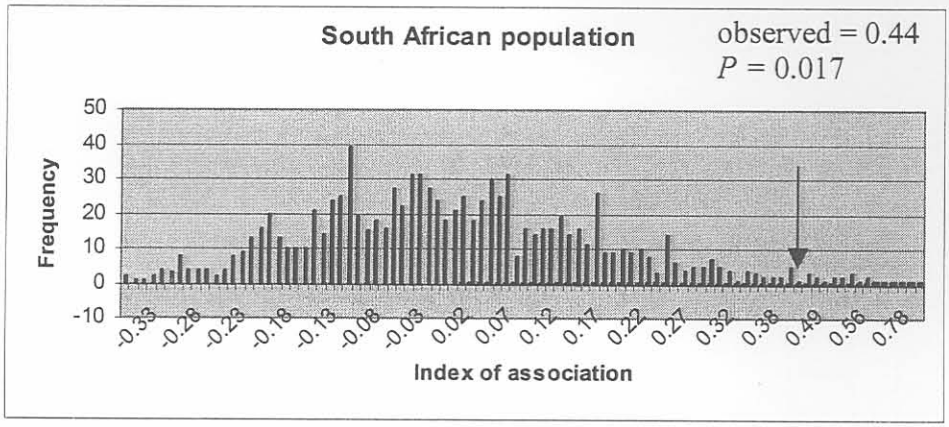
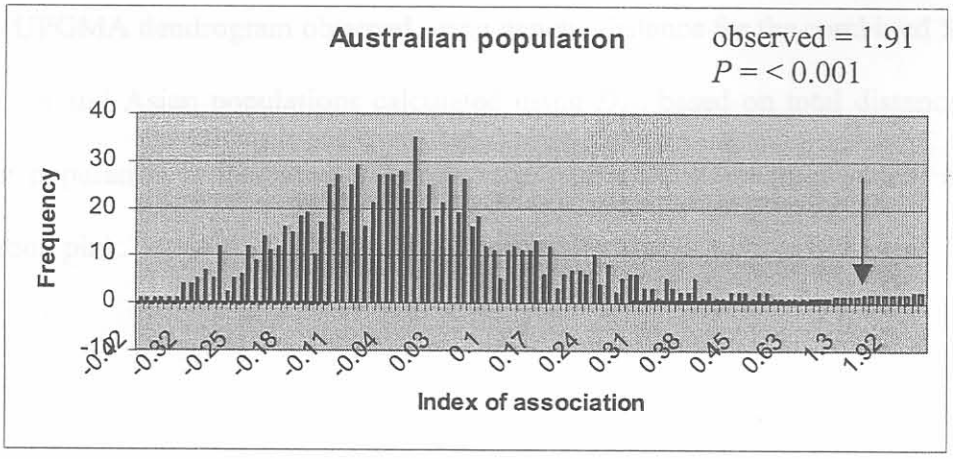

Asian genotypic diversity
genotypic diversity
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
number of loci

Fig. 2 Histograms representing the distribution range of clonal populations using the Index of Association (I_A) with 1000 randomised data sets. The observed values for I_A in all populations fall well beyond the distribution range for the recombining population (P<0.001) but not for the South African population.


South African population
observed = 0.44
P = 0.017
Frequency
50
40
30
20
10
0
-10
-0.33
-0.28
-0.23
-0.18
-0.13
-0.08
-0.03
0.02
0.07
0.12
0.17
0.22
0.27
0.32
0.38
0.49
0.56
0.78
Index of association


Australian population
observed = 1.91
P = < 0.001
Frequency
40
30
20
10
0
-0.32
-0.25
-0.18
-0.11
-0.04
0.03
0.1
0.17
0.24
0.31
0.38
0.45
0.63
1.3
1.92
Index of association

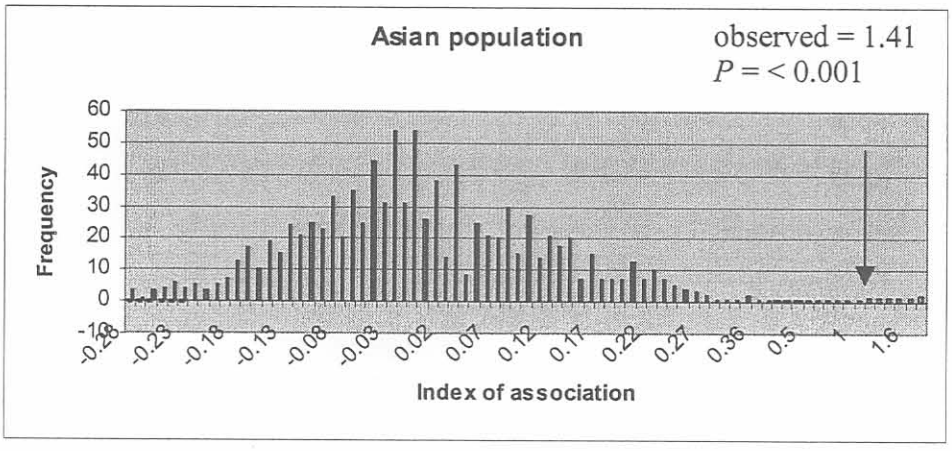
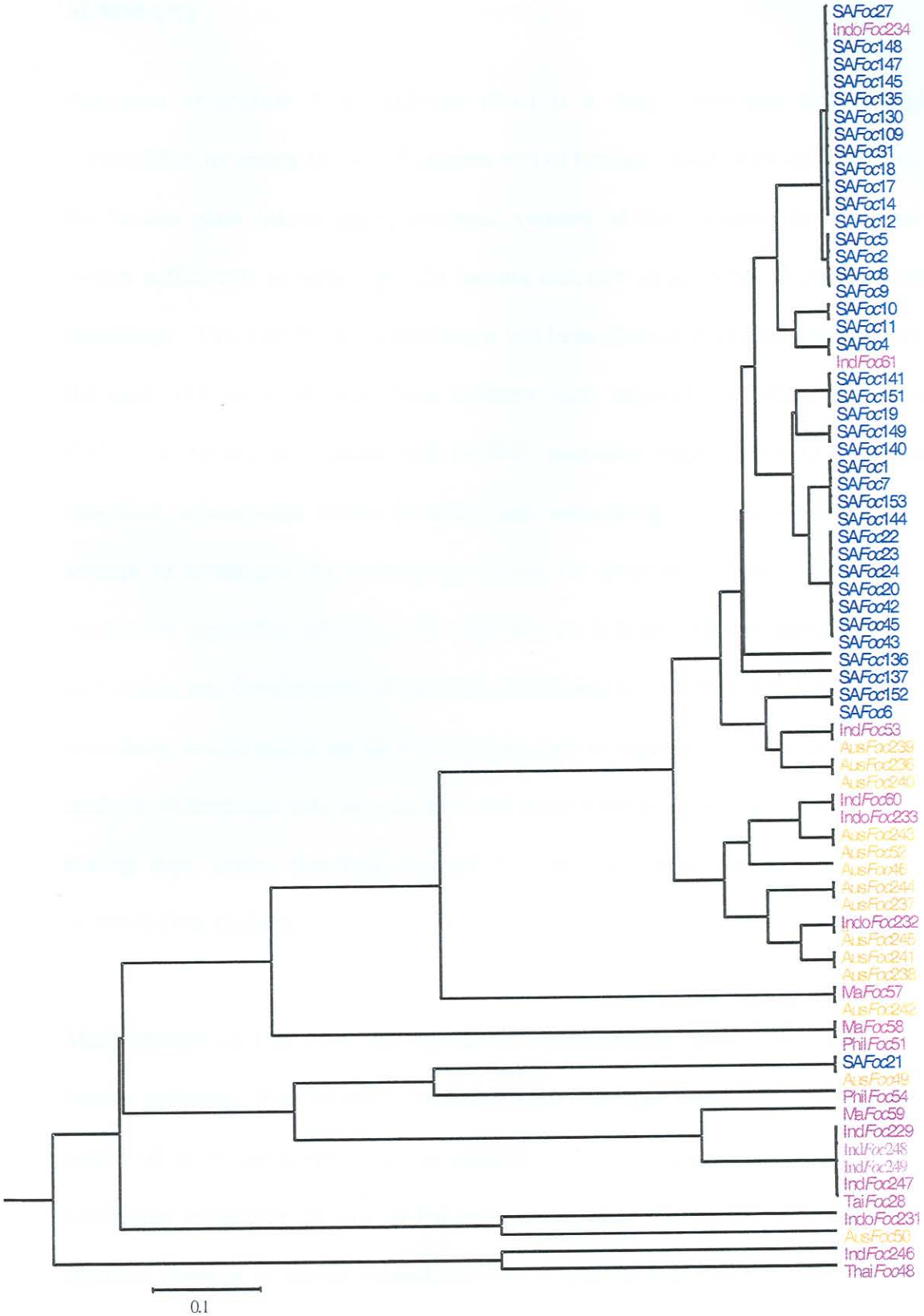

Asian population
observed = 1.41
P = < 0.001
Frequency
60
50
40
30
20
10
0
-0.28
-0.23
-0.18
-0.13
-0.08
-0.03
0.02
0.07
0.12
0.17
0.22
0.27
0.36
0.5
1
1.6
Index of association

Fig. 3 UPGMA dendrogram obtained using genetic distance for the combined South African, Australian and Asian populations calculated using D_{AD} based on total distance. The South African population is indicated with blue, the Australian population yellow and the Asian population pink.


SAFoc27
IndoFoc234
SAFoc148
SAFoc147
SAFoc145
SAFoc135
SAFoc130
SAFoc109
SAFoc31
SAFoc18
SAFoc17
SAFoc14
SAFoc12
SAFoc5
SAFoc2
SAFoc8
SAFoc9
SAFoc10
SAFoc11
SAFoc4
IndFoc61
SAFoc141
SAFoc151
SAFoc19
SAFoc149
SAFoc140
SAFoc1
SAFoc7
SAFoc153
SAFoc144
SAFoc22
SAFoc23
SAFoc24
SAFoc20
SAFoc42
SAFoc45
SAFoc43
SAFoc136
SAFoc137
SAFoc152
SAFoc6
IndFoc53
AusFoc239
AusFoc236
AusFoc240
IndFoc60
IndoFoc233
AusFoc243
AusFoc52
AusFoc46
AusFoc244
AusFoc237
IndoFoc232
AusFoc245
AusFoc241
AusFoc238
MaFoc57
AusFoc242
MaFoc58
PhilFoc51
SAFoc21
AusFoc49
PhilFoc54
MaFoc59
IndFoc229
IndFoc248
IndFoc249
IndFoc247
TaiFoc28
IndoFoc231
AusFoc50
IndFoc246
ThaiFoc48
0.1

SUMMARY

Fusarium oxysporum f. sp. *cubense* (*Foc*) is a fungal pathogen that is highly successful in its ability to cause Fusarium wilt of banana. Apart from its specificity to the banana plant (*Musa* spp.), genotypic variants of the pathogen have proven to evolve sufficiently to target specific banana cultivars under different environmental conditions. This variability in the fungus has been illustrated in numerous studies in the past, and involved phenotypic markers such vegetative compatibility groups (VCGs) and genotypic markers such as DNA amplified fingerprints (DAFs), random amplified polymorphic DNAs (RAPDs) and sequencing. This thesis has made an attempt to investigate the underlying reasons for diversity and diversification of a worldwide population of *Foc*, with emphasis on a South African population. The application and development of molecular biological tools for phylogenetic and host-specificity studies which included techniques such as vegetative compatibility studies, analysis of sequence data of a nuclear and mitochondrial gene region, searching for mating type genes, transformation of *Foc* and the development and testing of microsatellite markers.

Many studies on *Foc* have been useful in demonstrating the diverse nature of the banana pathogen. Few of these, however, have made any attempts to investigate the nature of its diversification. In an examination of the reproductive potential of a worldwide population of *Foc*, it has been proven that the fungus is clonal with an apparent absence of sexual recombination. It may be that *Foc* has become such a genetically isolated population and such an evolutionary advanced pathogen that no sexual stage will ever be found. Mutations therefore, are expected to be the primary mechanism responsible for genetic variation in *Foc*.

Sequence data of nuclear and mitochondrial gene regions with different evolutionary rates proved that *Foc* consists of several clonal lineages that maybe genetically distinct. These clonal lineages group into separate groups. The first of these groupings included the South African population while the second included isolates from the Australasian region. It is suggested that clonal lineages could be linked with distinct pathotypes of the fungus. All isolates of *Foc*, however, maintain one common link, their ability to cause disease on the banana host.

The relationship between *Foc* and the banana plant has been studied extensively many years ago on the Gros Michel cultivar. The successful transformation of *Foc* with the green fluorescent protein, however, now makes it possible to better understand the interactions between different fungal pathotypes and host genotypes. It will also substantially assist in understanding the process of fungal infection and host defence responses. Details of the interactions, especially between Cavendish varieties and the pathogen, needs to be investigated further in future.

A study of the population structure of *Foc*, using VCGs and microsatellite markers, provided valuable insights into the diversity of the South African population in relation to a worldwide collection of the fungus. Both markers showed that the South African population of *Foc* was remarkably homogeneous, strongly reminiscent of an introduced population. This was in clear contrast to the large genetic diversity apparent in populations from Southeast Asia, the reported centre of origin of the pathogen. VCGs are believed to represent clonal lineages within a species. They are useful but not ideal markers for defining diversity in a pathogen like *Foc* that has no defined genetic basis for race assignment. While VCGs are believed to represent

clonal lineages within a species, the assumption that isolates in the same VCG are genetically very similar can be misleading.

The development of microsatellite markers for *Foc* has provided an opportunity to study evolutionary relationships among populations and subpopulations of the Fusarium wilt pathogen. Differentiation between subpopulations was found to be high due to restricted gene flow and random drift. The high gene diversity revealed within the native *Foc* population is a clear indication that management of Fusarium wilt should involve the development of resistance cultivars.