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).

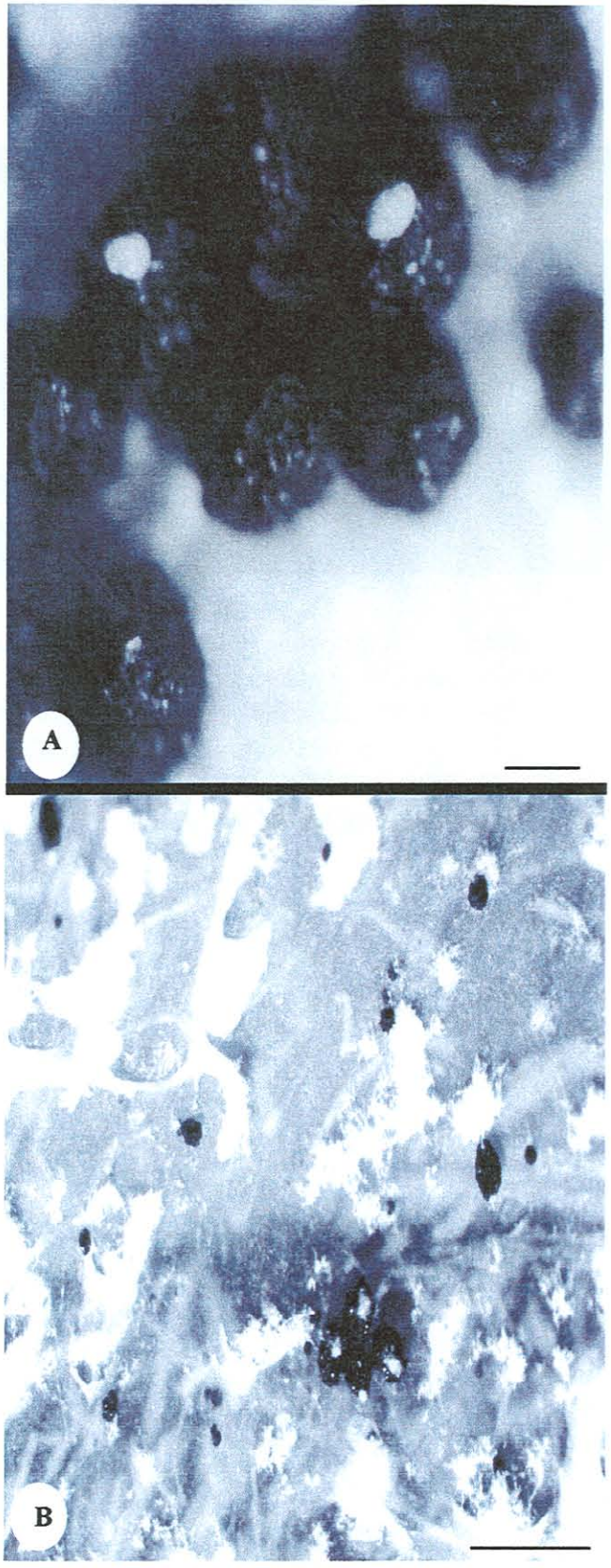

Fig. 1 (A) Opposite mating types of *Fusarium circinatum* produced fertile perithecia with ascospores when crossed on carrot agar. Scale bar = 100µm. **(B)** Isolates of *Fusarium oxysporum* f. sp. *cubense* produced dark purple to black perithecia-like structures without ascopsores. Scale bar = 1000µm

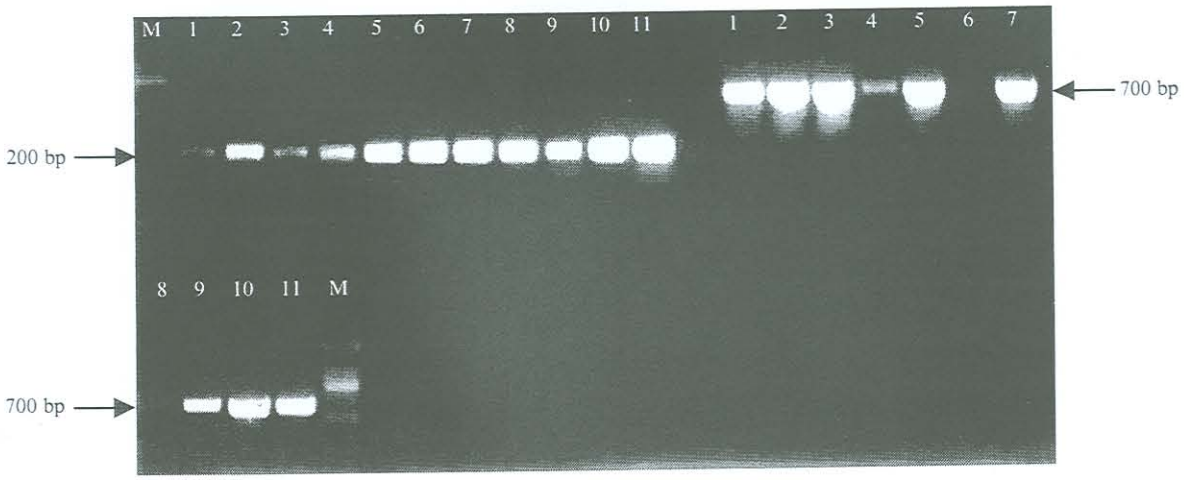

Fig. 2 A 1.5% agarose gel showing PCR amplicons of the *MAT*-2 idiomorph of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) using *MAT*-2 primer sets. The primer sets (Gfmat2c and Gfmat2d, Gfmat2c and FF1) resulted in PCR amplicon sizes of 200 bp and 700 bp respectively. Lanes 1-11 are *Foc* 4, *Foc* 8, *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.

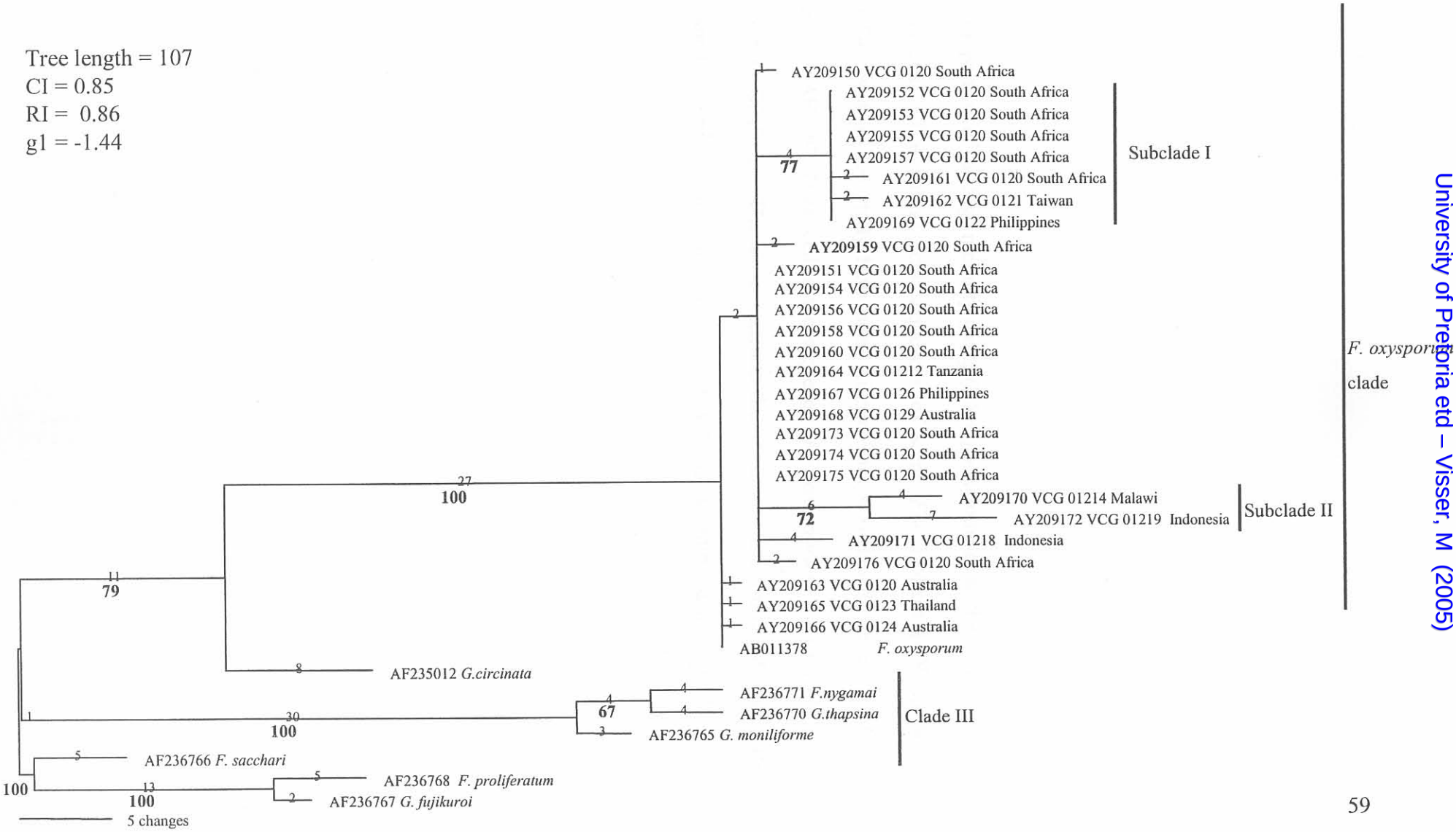

Fig. 5 Comparison of the high mobility group (HMG) box domains of the fungal *MAT*-2 gene products. The amino acid sequences of some *Foc* isolates (AY209150 – AY209174) used in this study have been aligned with sexual and asexual relatives (AF235012 – AF236767) as well as the sequence of *F. oxysporum* 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.