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# Molecular characterization of *Fusarium subglutinans* associated with mango malformation

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## SUMMARY

Mango malformation is a serious disease of *Mangifera indica* in tropical and subtropical regions of the world. This disease is caused by *Fusarium subglutinans*, which is also associated with diseases on many other hosts, such as pineapple, pine, maize and sugarcane. The *F. subglutinans* strains associated with different hosts are virtually indistinguishable using morphological characters, but can be easily differentiated using histone and  $\beta$ -tubulin gene sequencing, and some have subsequently been assigned to distinct species. The aim of this study was to characterize *F. subglutinans* isolates associated with mango malformation using histone *H3* gene sequencing and to compare them with other isolates in the *Gibberella fujikuroi* complex. Analysis of histone sequence data revealed the presence of two phylogenetically distinct groups of *F. subglutinans* isolates associated with mango malformation. We also considered the identity of the two groups of isolates associated with mango malformation and determined their relatedness to other *Fusarium* spp. For this purpose, portions of the  $\beta$ -tubulin gene were sequenced and compared with the  $\beta$ -tubulin sequences deposited in the National Center for Biotechnology Information (NCBI) nucleotide database. This comparison to the NCBI database indicated that one group of *F. subglutinans* strains isolated from mango constitutes a unique lineage in the *G. fujikuroi* complex. Based on  $\beta$ -tubulin and histone *H3* sequencing, the second group of isolates is conspecific with the *F. subglutinans* strains previously reported to be the causal agent of mango malformation.

## INTRODUCTION

Mango (*Mangifera indica* L.) is a fruit tree grown commercially in many tropical and subtropical areas of the world (Kumar

*et al.*, 1993; Ploetz, 1994a). The mango industries of many countries are threatened by the fungal disease known as mango malformation (Kumar *et al.*, 1993). The most prominent symptom of this disease is the deformation of flowers (Kumar *et al.*, 1993). This floral malformation is characterized by thick, fleshy and profusely branched panicles that are crowded by enlarged flowers (Kumar *et al.*, 1993). These malformed panicles generally do not bear fruit because they remain very small or are aborted prematurely (Kumar *et al.*, 1993; Varma *et al.*, 1974). The second important symptom of this disease is the deformation of mature trees (Kumar *et al.*, 1993). Nursery seedlings can also be infected, which leads to the spread of mango malformation to new areas (Kumar *et al.*, 1993; Ploetz, 1994a).

*Fusarium subglutinans* (Wollew. & Reinking) Nelson, Tousoun & Marasas is recognized as the causal agent of mango malformation and has been consistently isolated from diseased mango tissue (Fletcher *et al.*, 1970; Freeman *et al.*, 1999; Malo and McMillan, 1972; Manicom, 1989; Summanwar *et al.*, 1966; Varma *et al.*, 1972, 1974). This species forms part of the *Gibberella fujikuroi* (Sawada) Wollenw. complex and is associated with at least three biological species or mating populations (B, E and H) and many different hosts, such as pine, maize, sugarcane and pineapple (Britz *et al.*, 1999; Hsieh *et al.*, 1977; Kuhlman, 1982; Leslie, 1995; Rohrbach and Pfeiffer, 1976; Ventura *et al.*, 1993). Although some of these *F. subglutinans* collections have recently been elevated to species level (Nirenberg and O'Donnell, 1998; O'Donnell *et al.*, 1998), it is difficult and sometimes even impossible to distinguish *F. subglutinans* strains associated with different hosts. It is therefore impractical to use only morphological characters to differentiate *F. subglutinans* associated with mango from strains representing the other host-specific groups of this anamorph species.

Leslie (1995) showed that *F. subglutinans* isolated from mango and *F. subglutinans* mating population B (= *F. sacchari*) share similar isozyme profiles. Recently, Steenkamp *et al.* (1999a) reported on a PCR-RFLP (polymerase chain reaction-restriction

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**Table 1** Isolate information, source, vegetative compatibility group (VCG) and mating type of the *Fusarium subglutinans* strains associated with mango malformation used in this study.

Isolates*	Origin, orchard†, cultivar‡ and date of isolation	Source	VCG§	Mating type¶	GENBANK accession number
MRC 7559	Israel, Volcani, N/A, 1993	S. Freeman	VCG1	MAT-2	AF236779
MRC 7560	Israel, Ginosar, Kent, 1998	S. Freeman	VCG1	MAT-2	AF236780
MRC 7561	Israel, Sde Nitzan, Tomy, 1998	S. Freeman	N/D	MAT-2	AF236781
MRC 7562	Israel, Bene Dror, Keitt, 1998	S. Freeman	N/D	MAT-2	AF236782
MRC 7034	USA (Ploetz, 1994b)	R. Ploetz	VCG2	MAT-2	AF150864
MRC 7035	USA (Ploetz, 1994b)	R. Ploetz	VCG2	MAT-2	AF150866
MRC 7037	USA (Ploetz, 1994b)	R. Ploetz	VCG2	MAT-2	AF150863
MRC 7038	USA (Ploetz, 1994b)	R. Ploetz	VCG2	MAT-2	AF150862
MRC 2730	South Africa, Nelspruit, N/A, 1982	F. Wehner	VCG3	MAT-2	AF150865
MRC 3477	South Africa, Kwa-Zulu/Natal, N/A, 1984	C. Crookes	VCG3	MAT-2	AF150868
MRC 3478	South Africa, Kwa-Zulu/Natal, N/A, 1984	C. Crookes	VCG3	MAT-2	AF150869
MRC 3479	South Africa, Kwa-Zulu/Natal, N/A, 1984	C. Crookes	VCG3	MAT-2	AF150867
MRC 2802	South Africa, Tzaneen, N/A, 1982	J. Darvas	VCG4	MAT-1	AF236778
MRC 7605	South Africa, Tzaneen, Keitt, 1982	H. Britz	VCG4	MAT-1	AF236773
MRC 7873	South Africa, Tzaneen, Keitt, 1982	H. Britz	VCG4	MAT-1	AF236774
MRC 7875	South Africa, Tzaneen, Keitt, 1982	H. Britz	VCG4	MAT-1	AF236775
MRC 7876	South Africa, Tzaneen, Keitt, 1982	H. Britz	VCG4	MAT-1	AF236776
MRC 7877	South Africa, Tzaneen, Keitt, 1982	H. Britz	VCG4	MAT-1	AF236777

\*MRC, Collection of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, Tygerberg, South Africa.

†The exact locations of isolation of strains in Kwa-Zulu/Natal, South Africa, are not known.

‡N/A, the *M. indica* cultivar from which strains were isolated is not available.

§VCG phenotypes were determined as described by Correll *et al.* (1987). N/D, not done.

¶Mating types were assigned using the PCR-based techniques described by Steenkamp *et al.* (1999b) and Kerényi *et al.* (1999).

length polymorphism)-based method to differentiate between the *F. subglutinans* isolates associated with pine, maize, pineapple, sugarcane and mango. Their results and those of O'Donnell *et al.* (1998) indicated that the *F. subglutinans* isolates associated with mango malformation are closely related to, but distinct from, *F. subglutinans* mating population B. Both of these DNA-based methods showed that *F. subglutinans* associated with mango malformation cluster with isolates residing in mating populations B, C and D (O'Donnell *et al.*, 1998; Steenkamp *et al.*, 1999a).

In a recent survey of mango malformation in South Africa, *F. subglutinans* was consistently isolated from diseased mango trees. In contrast to the findings of Steenkamp *et al.* (1999a), histone *H3* PCR-RFLPs revealed not one, but two, distinct groups of *F. subglutinans* associated with this disease. Our aim was therefore to characterize the existing and new group of *F. subglutinans* isolates from mango using histone *H3* gene sequencing and to compare them with other *Fusarium* isolates in the *G. fujikuroi* species complex. We also attempted to determine the possible identities of the two groups of *F. subglutinans* isolates associated with mango malformation by sequencing two portions of the  $\beta$ -tubulin gene from selected individuals. These sequences were compared to the previously published sequences in the National Center for Biotechnology Information (NCBI) database.

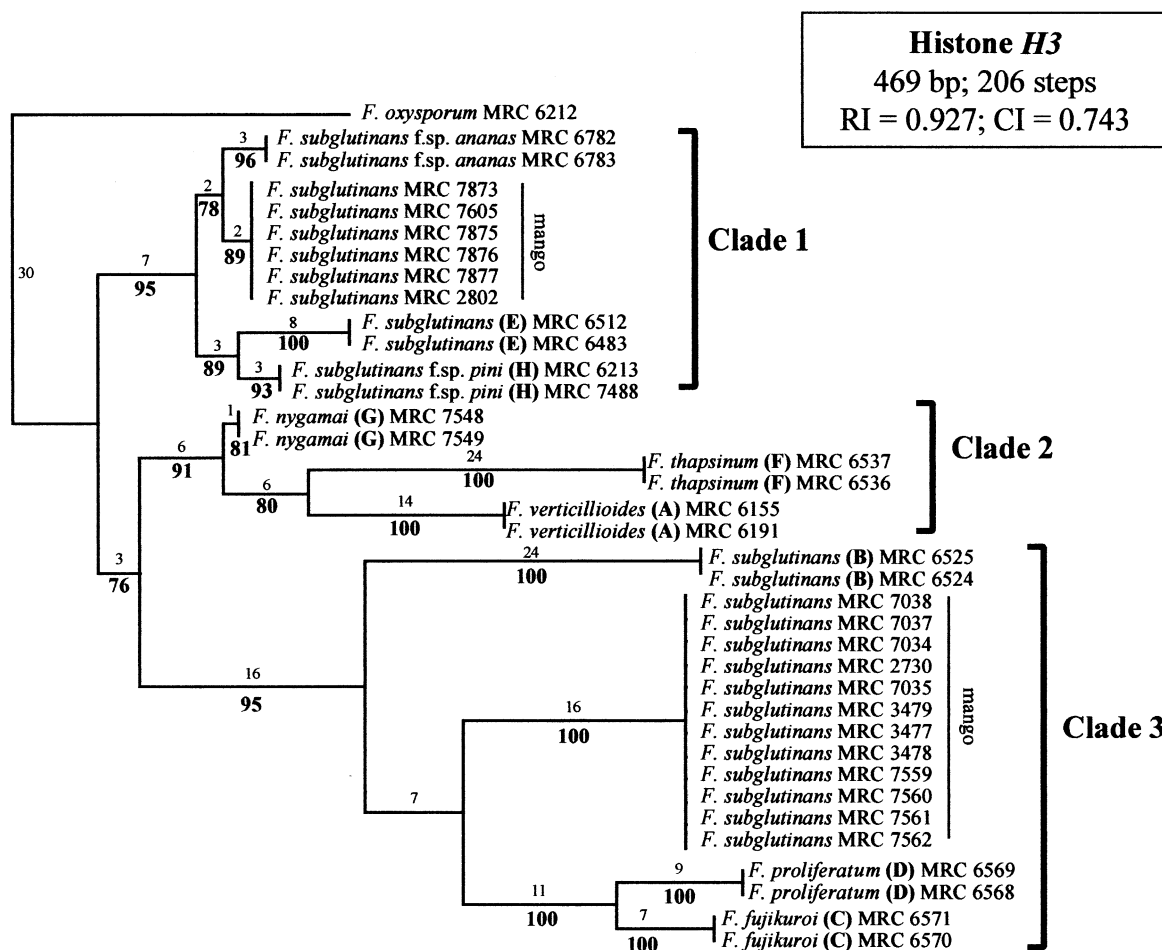
## RESULTS

### Fungal isolates

All 18 of the isolates associated with mango malformation included in this study produced abundant microconidia that were formed on polyphialides. These were always in false heads and never in chains, which is a characteristic feature of *F. subglutinans*. Macroconidia were also abundant and straight to slightly sickle-shaped. Chlamydospores were absent, which is another typical feature of *F. subglutinans*.

### Vegetative compatibility

The *F. subglutinans* strains associated with mango malformation included in this study could be divided into four distinct vegetative compatibility groups (VCGs) (Table 1). The two Israeli isolates were in one VCG (VCG1) and those from the USA were also in a single VCG (VCG2). Three of the South African isolates from the Kwa-Zulu/Natal area (MRC 3477, MRC 3478 and MRC 3479) and one from the Nelspruit area (MRC 2730) were in VCG3. The remaining six South African strains from the Tzaneen area (MRC 2802, MRC 7605, MRC 7873, MRC 7875, MRC 7876 and MRC 7877) were in VCG4.



**Fig. 1** Phylogenetic relationships inferred from histone *H3* gene sequencing data for the *Fusarium* isolates from the *G. fujikuroi* species complex included in this study. This tree is rooted to *F. oxysporum* and the bold letters in parentheses refer to the *G. fujikuroi* mating populations A–H. Branch lengths are indicated above the internodes and bootstrap values are indicated as percentages in bold digits below the internodes.

### ***H3* gene sequencing**

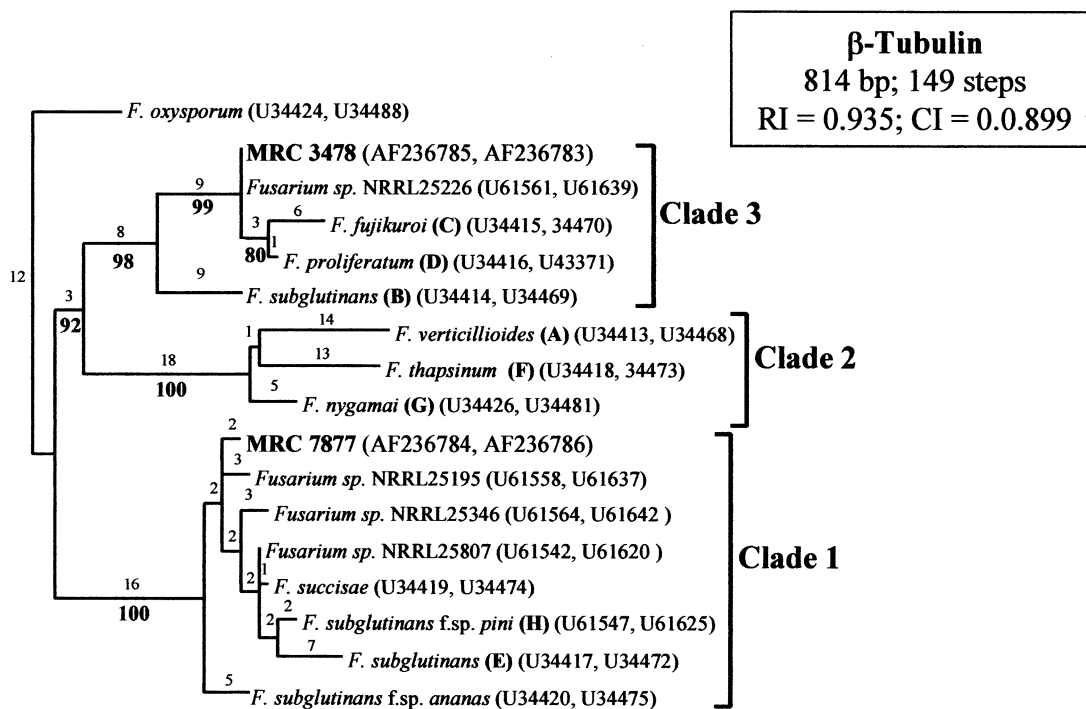
In this study, 455-bp sequences were used to determine the relationships between the *F. subglutinans* strains associated with mango malformation and species of the *G. fujikuroi* complex. Phylogenetic analysis using PAUP version 4.0b1 generated two most parsimonious trees that were similar except for the length of some of the minor branches. Both of these dendrograms consisted of three distinct clades, labelled Clade 1, 2 and 3 (Fig. 1). Clade 1 included the *G. fujikuroi* mating populations E and H, as well as *F. subglutinans* f.sp. *ananas* (Fig. 1). This clade also contained six of the South African *F. subglutinans* strains (Table 1) isolated from mango. *F. subglutinans* f.sp. *ananas*, together with the isolates from mango, formed a sister group to the one containing mating populations E and H. Clade 3 included mating populations B, C and D, as well as the 12 remaining *F. subglutinans* isolates (Table 1) associated with mango malformation in South Africa, Israel and the United

States (Fig. 1). Clade 2 included the *G. fujikuroi* mating populations A, F and G.

### **$\beta$ -Tubulin gene sequencing**

Using the  $\beta$ -tubulin primer sets T1/T21 and T121/T22, 337 bp and 466 bp, respectively, of DNA sequence were generated (O'Donnell and Cigelnik, 1997). Comparison of these sequences to those in the NCBI database indicated that both  $\beta$ -tubulin portions from the isolate MRC 3478 were 100% homologous to those of *Fusarium* strain NRRL 25226 (see Fig. 2 for GENBANK accession numbers). *Fusarium* strain NRRL 25226 was collected in India from mango (O'Donnell *et al.*, 1998).

Both of the sequenced portions of the  $\beta$ -tubulin gene from isolate MRC 7877 were 99% similar to those of four other *Fusarium* strains in the NCBI database (see Fig. 2 for GENBANK accession numbers). For the sequence generated with primers T1 and T21, isolate MRC 7877 shared 462 bp out of 466 bp



**Fig. 2** Phylogenetic relationships inferred from  $\beta$ -tubulin gene sequencing data for the *F. subglutinans* isolates MRC 3478 and MRC 7877 associated with mango malformation, as well as the *G. fujikuroi* mating populations. Also included are *F. subglutinans* f.sp. *ananas*, *F. succisae* and *Fusarium* strains NRRL 25226, 25195, 25346 and 25807. This tree is rooted to *F. oxysporum* and the bold letters in parentheses refer to the *G. fujikuroi* mating populations A–H. Branch lengths are indicated above the internodes and bootstrap values are indicated as percentages in bold below the internodes. The GenBank accession numbers for all the *Fusarium* strains are indicated in parentheses.

homology with *Fusarium* strains NRRL 25346, NRRL 25807 and *F. succisae*, and 460 bp out of 462 bp homology with the strain NRRL 25195. For the sequence generated with the second primer set, isolate MRC 7877 shared 335 bp out of 337 bp homology with the NRRL 25807 strain, 334 bp out of 337 bp with *F. succisae* and the NRRL 25195 strain, and 332 bp out of 335 bp homology with the NRRL 25346 strain. The *Fusarium* strains NRRL 25195, 25807 and 25346 were isolated from wood in Venezuela, *Pennisetum typhoides* in Namibia and *Ipomoea batatas* in Peru, respectively (O'Donnell *et al.*, 1998).

Phylogenetic analysis using these sequenced portions of the  $\beta$ -tubulin gene showed that the *F. subglutinans* isolate MRC 3478 grouped together with the *Fusarium* strain NRRL 25226 in Clade 3 (Fig. 2). The second *F. subglutinans* isolate from mango (MRC 7877) clustered with mating populations E, H and *F. subglutinans* f.sp. *ananas* in Clade 1 (Fig. 2). The *Fusarium* strains NRRL 25195, 25807 and 25346 and *F. succisae* also reside in this clade.

### Sexual compatibility and identification of mating types

None of the *F. subglutinans* isolates associated with mango malformation were sexually compatible with any of the *G.*

*fujikuroi* mating population tester strains. These isolates from mango also did not cross with one another. Six of the South African isolates associated with mango malformation were of mating type *MAT-1*, whereas the remaining 12 isolates associated with mango malformation had the *MAT-2* mating type (Table 1).

### DISCUSSION

We analysed 18 isolates of *F. subglutinans* associated with mango malformation (Table 1) and, based on *H3* sequence data, these isolates could be placed into two distinct groups (Fig. 1). The first group includes 12 *F. subglutinans* strains from South Africa, the United States and Israel (Table 1). Based on DNA sequence data, this group resides in Clade 3, together with the *G. fujikuroi* mating populations B, C and D (Fig. 1). The second group includes six *F. subglutinans* strains from South Africa and resides in Clade 1, together with the *G. fujikuroi* mating populations E and H (Table 1, Fig. 1). Mango malformation in South Africa is therefore associated with two phylogenetically distinct groups of *F. subglutinans* isolates, which are morphologically indistinguishable, but that occupy the same ecological niche.

In the Kwa-Zulu/Natal area of South Africa, only *F. subglutinans* strains belonging to Clade 3 were isolated. An additional strain belonging to this clade was also collected from the Nelspruit area. All of these isolates were, however, from a single vegetative compatibility group, VCG3 (Table 1). In the Tzaneen area, only *F. subglutinans* strains belonging to Clade 1 were isolated. Similar to the isolates included in Clade 3, all the isolates belonging to Clade 1 also belonged to a single VCG, VCG4 (Table 1). This study does not attempt to address any issues regarding the population structure of these two groups of fungi, since too few individuals were available. Our results do, however, suggest that the *F. subglutinans* strains associated with mango malformation in Clades 1 and 3 are clonal. The mating type data for the different South African strains isolated from malformed mango flowers (Table 1) further suggest that the isolates found in Clades 1 and 3 are clonal.

The position of the set of *F. subglutinans* isolates associated with mango malformation in Clade 3 (Fig. 1) suggests that this group of isolates is equivalent to the single *Fusarium* isolate (NRRL 25226) included by O'Donnell *et al.* (1998). This strain, isolated from mango in India, was further found to be conspecific with the *F. subglutinans* strain MRC 7559, which has previously been shown to be a causal agent of mango malformation (Freeman *et al.*, 1999). Comparison of the sequenced portion of the  $\beta$ -tubulin gene from the South African isolate MRC 3478 with the sequence in the NCBI nucleotide database indicates that this isolate is also conspecific with the Indian isolate (NRRL 25226) studied by O'Donnell *et al.* (1998) (Fig. 2). The fact that the  $\beta$ -tubulin sequence for the *F. subglutinans* strain MRC 3478 and *Fusarium* strain NRRL 25226 was identical to that of MRC 7559 suggests strongly that this group of isolates found in Clade 3 (Figs 1 and 2) is responsible for causing mango malformation.

The second group of isolates associated with mango malformation in South Africa, residing in Clade 1 (Fig. 1), is distinct from all the *G. fujikuroi* mating populations, but most closely related to *F. subglutinans* f.sp. *ananas*, based on histone gene sequencing. When compared with *F. subglutinans* f.sp. *ananas*, this group of isolates, however, has several differences in its histone gene sequence and appears to represent a discrete taxon. Comparison of the  $\beta$ -tubulin sequence for isolate MRC 7877 with the sequence in the NCBI database showed 99% similarity to several *Fusarium* strains. The sequence for the *F. subglutinans* isolate MRC 7877, however, differed by 5–7 bp from *F. succisae* and the *Fusarium* strains NRRL 25195, 25807 and 25346, which were all isolated from hosts other than mango. Based on histone and  $\beta$ -tubulin DNA sequencing, this group of *F. subglutinans* strains isolated from mango constitutes a distinct lineage within the *G. fujikuroi* species complex (Fig. 2). This lineage is consistently associated with mango malformation, but it remains to be shown via pathogenicity tests whether these isolates are able to cause mango malformation.

In South Africa, malformation of mango flowers is associated with *F. subglutinans* strains from the phylogenetic Clades 1 and 3. Interestingly, Clades 1 and 3 correspond to what O'Donnell *et al.* (1998) refer to as the 'American' and 'Asian' clades, respectively. These designations were based on the geographical origins and hosts of the species included in their study. It is therefore reasonable to expect a species from the so-called 'Asian' clade (Clade 3) to occur on mango, since the tree is native to India. The fact that a fungus from the so-called 'American' clade (Clade 1) was collected in Africa and occurs on an Asian host is less consistent with the terminology based on geographical regions. It is possible that an American species could have been introduced into Africa and has now established a niche on an Asian host. However, the assignment of *Fusarium* spp. in *Fusarium* subgroups linked to geographical regions should not be viewed as absolute (O'Donnell *et al.*, 1998), and the hypothesis that groups of species have discrete geographical origins needs further study.

The results of this study are congruent with those of Steenkamp *et al.* (1999a) and Leslie (1995), which also showed that the *F. subglutinans* strains associated with mango in Clade 3 are closely related to mating population B. It was also reported that the isolates used in these studies were not cross-fertile with one another or any of the mating tester strains for the mating populations in the *G. fujikuroi* complex (Leslie, 1995). Leslie (1995) concluded that these isolates either constitute a new mating population, or that they are sterile members of the B mating population. Our results show that the first hypothesis is at least partially correct, since the *Fusarium* isolates causing disease on mango are closely related to, but distinctly different from mating population B (Figs 1 and 2).

In this study, we set out to test the hypothesis that the isolates associated with mango constitute a new mating population, using sexual compatibility studies. None of the strains isolated from mango were, however, cross-fertile. This is partially explained by the fact that the isolates from Clades 1 and 3 are phylogenetically distinct and represent separate species (Fig. 1). The fact that isolates from one clade were sexually incompatible with other isolates from the same clade could only be explained after examining the mating types of the isolates studied. All of the isolates from mango in Clade 3 have one mating type, whereas isolates from Clade 1 have the opposite mating type (Table 1). All isolates were thus incapable of sexual reproduction, since individuals of opposite mating type and from the same phylogenetic group or species were never sampled in this study.

The presence of only one mating type in both Clades 1 and 3 has important implications regarding the taxonomy of the two groups of *F. subglutinans* isolates associated with mango malformation. Species descriptions in the *G. fujikuroi* complex

rely on the production of teleomorphs (Nelson *et al.*, 1983; Nirenberg and O'Donnell, 1998). It is, however, currently impossible to produce teleomorphs from the isolates associated with mango malformation. The possibility that isolates with the opposite mating type exist cannot be excluded, but until such time as these are found, an anamorph species will need to be described. The fact that one of these anamorphic groups of *F. subglutinans* associated with mango is known to be the causal agent of disease on an economically important crop necessitates description of at least the latter taxon. The descriptions must, however, be combined with a thorough characterization of these groups of fungi and populations in terms of morphology and pathogenicity.

## EXPERIMENTAL PROCEDURES

### Fungal isolates

We examined 18 *F. subglutinans* strains associated with mango malformation in South Africa, the United States and Israel (Table 1). The South African strains were isolated by surface sterilizing mango inflorescence clusters with 70% ethanol, after which single infected flowers were removed and placed on *Fusarium*-selective media (Nash and Snyder, 1965). Isolates obtained from this treatment were then morphologically characterized according to Nelson *et al.* (1983). For comparative purposes, we also included isolates of *F. subglutinans* f.sp. *pini* (*F. subglutinans* mating population H; strains MRC 6213 and MRC 7488), *F. subglutinans* mating population B (MRC 6524 and MRC 6525), *F. subglutinans* mating population E (MRC 6483 and MRC 6512) and *F. subglutinans* f.sp. *ananas* (MRC 6782 and MRC 6783). The *G. fujikuroi* mating tester strains for mating populations A (= *F. verticillioide*s; strains MRC 6191 and MRC 6155), C (= *F. fujikuroi*; strains MRC 6570 and MRC 6571), D (= *F. proliferatum*; strains MRC 6568 and MRC 6569), F (= *F. thapsinum*; strains MRC 6536 and MRC 6537) and G (= *F. nygamai*; strains MRC 7548 and MRC 7549) were also included. All isolates are available from the Collection of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, Tygerberg, South Africa.

### Vegetative compatibility

*F. subglutinans* isolates associated with mango malformation were examined for vegetative compatibility using nitrate non-utilizing (*nit*) mutants (Correll *et al.*, 1987).

### DNA isolation

DNA was isolated using an *N*-cetyl-*N,N,N*-trimethylammonium bromide (CTAB) extraction method (Steenkamp *et al.*, 1999a).

### H3 gene amplification and sequencing

The *H3* histone gene from all the isolates from mango was amplified using the primers H3-1a (5'-ACTAAGCAGACCGCCCGCAGG-3') and H3-1b (5'-GCGGGCGAGCTGGATGTCCTT-3') (Glass and Donaldson, 1995). PCR and cycling conditions were similar to those described previously (Steenkamp *et al.*, 1999a). After PCR, products were purified with a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions with the primers H3-1a and H3-1b. Reactions were performed on an ABI PRISM™ 377 automated DNA sequencer, with an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Warrington, UK). Sequences were analysed with Sequence Navigator version 1.0.1.™ (Perkin Elmer Applied Biosystems, Inc., Foster City, CA) and aligned manually by inserting gaps. Phylogenetic analyses were performed with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b1 (Swofford, 1998). Each gap was treated as a fifth character (newstate) in heuristic searches, with tree-bisection-reconnection (TBR) branch swapping and MULTEES (saving of all optimal trees) effective. Bootstrap analyses were based on 1000 replications. The histone *H3* gene sequence for the mating tester strains and *F. subglutinans* f.sp. *ananas* were obtained from GENBANK and were also included in the phylogenetic analysis (GENBANK accession numbers AF150833, AF150834, AF150844, AF150845, AF150849, AF150854, AF150855, AF150856, AF150857, AF150858, AF150859, AF150860, AF150861, AF150870, AF150871, AF150872, AF150873, AF238478). *Fusarium oxysporum* (GENBANK accession number AF150832) *H3* histone gene sequence was used as an outgroup in the analysis.

### β-Tubulin gene amplification and sequencing

The β-tubulin gene was amplified from two *F. subglutinans* isolates (MRC 7877 and MRC 3478) associated with mango malformation, using the primers T1 (5'-AACATGCGTGAGATTGTAAGT-3') and T22 (5'-TCTGGATGTTGTTGGGAATCC-3') (O'Donnell and Cigelnik, 1997). PCR and cycling conditions were similar to those described by these authors. PCR products were purified and cycle sequenced as described above. The first intron-rich region of the β-tubulin gene was sequenced using the forward primer T1 and the nested reverse primer T21 (5'-GGTTTGCCAGAAAGCAGCACC-3') (O'Donnell and Cigelnik, 1997). The last intron and the adjacent coding β-tubulin regions were sequenced using the nested forward primer T121 (5'-CCACCTGTCTCCGTTTCCCCG-3') and the reverse primer T22 (O'Donnell and Cigelnik, 1997).

Sequences were analysed with Sequence Navigator and then compared against the NCBI nucleotide database using BLAST (HTTP://HTTP://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/). This was done to determine the possible identity of the *F. subglutinans* isolates associated with mango malformation. For the possible

candidates, corresponding  $\beta$ -tubulin sequences were obtained from GENBANK. The sequences for the *G. fujikuroi* mating populations A–H and *F. subglutinans* f.sp. *ananas* were also obtained from GENBANK. These sequences, together with the  $\beta$ -tubulin sequences for isolates MRC 7877 and MRC 3478, were manually aligned and subjected to phylogenetic analyses as described above. The *F. oxysporum*  $\beta$ -tubulin sequence obtained from GENBANK was used as an outgroup in the analyses.

### Sexual compatibility tests and identification of mating types

The *F. subglutinans* isolates associated with mango malformation included in this study were crossed with the mating tester strains for the *G. fujikuroi* mating populations A–F and *F. subglutinans* f.sp. *ananas*, as well as with one another in all possible pairwise combinations using previously published techniques (Britz *et al.*, 1999; Klittich and Leslie, 1988). Crosses were scored as positive when ascospores exuded from perithecia. Additionally, the mating types of all the *F. subglutinans* isolates associated with mango malformation were determined using the PCR-based techniques reported by Kerényi *et al.* (1999) and Steenkamp *et al.* (1999b).

### REFERENCES

- Britz, H., Coutinho, T.A., Wingfield, M.J., Marasas, W.F.O., Gordon, T.R. and Leslie, J.F. (1999) *Fusarium subglutinans* f.sp. *pini* represents a distinct mating population in the *Gibberella fujikuroi* species complex. *Appl. Environ. Microbiol.* **65**, 1198–1201.
- Correll, J.C., Klittich, C.J.R. and Leslie, J.F. (1987) Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* **77**, 1640–1646.
- Fletcher, C.H.W., Kimati, H., Madcalf, J.C. and Ferrer, J. (1970) Preliminary observations on mango inflorescence malformation and the fungus, insects and mites, associated with it. *Anais E.S.A. 'Luis Queiroz'* **27**, 281–285.
- Freeman, S., Maimon, M. and Pinkas, Y. (1999) Use of GUS transformants of *Fusarium subglutinans* for determining etiology of mango malformation disease. *Phytopathology* **89**, 456–461.
- Glass, N.L. and Donaldson, G.C. (1995) Development of primer sets for use in PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* **61**, 1323–1330.
- Hsieh, W.H., Smith, S.N. and Snyder, W.C. (1977) Mating groups in *Fusarium moniliforme*. *Phytopathology* **67**, 1041–1043.
- Kerényi, Z., Zeller, K., Hornok, L. and Leslie, J.F.L. (1999) Molecular standardization of mating type terminology in the *Gibberella fujikuroi* species complex. *Appl. Environ. Microbiol.* **65**, 4071–4076.
- Klittich, C.J.R. and Leslie, J.F. (1988) Nitrate reduction mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*). *Genetics* **118**, 417–423.
- Kuhlman, E.G. (1982) Varieties of *Gibberella fujikuroi* with anamorphs in *Fusarium* section *Liseola*. *Mycologia* **74**, 756–768.
- Kumar, J., Singh, U.S. and Beniwal, S.P.S. (1993) Mango malformation: one hundred years of research. *Annu. Rev. Phytopathol.* **31**, 217–232.
- Leslie, J.F. (1995) *Gibberella fujikuroi*: available populations and variable traits. *Can. J. Bot.* **73**, S282–S291.
- Malo, S.E. and McMillan, R.T. (1972) A disease of *Mangifera indica* L. in Florida similar to mango malformation. *Proc. Fla. State Hort. Soc.* **85**, 264–268.
- Manicom, B.Q. (1989) Blossom malformation of mango. *S. Afr. Mango Grower's Assoc. Yearb.* **10**, 11–12.
- Nash, S.M. and Snyder, W.C. (1965) Quantitative and qualitative comparisons of *Fusarium* populations in cultivated fields and non-cultivated parent soils. *Can. J. Bot.* **43**, 939–945.
- Nelson, P.E., Toussoun, T.A. and Marasas, W.F.O. (1983) *Fusarium Species: An Illustrated Manual for Identification*. Pennsylvania: Pennsylvania State University, 183pp.
- Nirenberg, H.I. and O'Donnell, K. (1998) New species and combinations within the *Gibberella fujikuroi* species complex. *Mycologia* **90**, 434–458.
- O'Donnell, K. and Cigelnik, E. (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* **7**, 103–106.
- O'Donnell, K., Cigelnik, E. and Nirenberg, H.I. (1998) Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* **90**, 465–493.
- Ploetz, R.C. (1994a) Part III. Mango. In *Compendium of Tropical Fruit Diseases* (Ploetz, R.C., Zentmyer, G.A., Nishijima, W.T., Rohrbach, K.G. and Ohr, H.D., eds), pp. 33–44. St. Paul, MN: American Pathological Society.
- Ploetz, R.C. (1994b) Distribution and prevalence of *Fusarium subglutinans* in mango trees affected by malformation. *Can. J. Bot.* **72**, 7–9.
- Rohrbach, K.G. and Pfeiffer, J.B. (1976) Susceptibility of pineapple cultivars to fruit disease incited by *Penicillium funiculosum* and *Fusarium moniliforme*. *Phytopathology* **66**, 1386–1390.
- Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Wingfield, M.J. and Marasas, W.F.O. (1999a) Differentiation of *Fusarium subglutinans* f.sp. *pini* by histone gene sequence data. *Appl. Environ. Microbiol.* **65**, 3401–3406.
- Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Wingfield, M.J., Marasas, W.F.O. and Leslie, J.F. (1999b) PCR-based differentiation of MAT-1 and MAT-2 isolates from *Gibberella fujikuroi* mating population H. *Phytopathology* **89**, S75.
- Summanwar, A.S., Raychoudhuri, S.P. and Pathak, S.C. (1966) Association of the fungus *Fusarium* sp. Sheld. with the malformation in mango. *Indian Phytopathol.* **19**, 227–228.
- Swofford, D.L. (1998) *PAUP\*. Phylogenetic Analysis Using Parsimony (\*and Other Methods), Version 4.0\**. Sunderland, MA: Sinauer Associates.
- Varma, A., Lele, V.C., Raychoudhuri, S.P., Ram, A. and Sang, A. (1974) Mango malformation: a fungal disease. *Phytopathol. Z.* **79**, 254–257.
- Varma, A., Raychoudhuri, S.P., Lele, V.C. and Ram, A. (1972) Towards the understanding of the problem of malformation of mango (*Mangifera indica* L.). *Proc. Indian Natl. Sci. Acad.* **37**, 291–300.
- Ventura, J.A., Zambolom, L. and Gilbertson, R.L. (1993) Pathogenicity of *Fusarium subglutinans* to pineapples. *Fitopatol. Bras. Supl.* **18**, 280.



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