CHAPTER 5

GIBBERELLA FUJIKUROI MATING POPULATION E ASSOCIATED WITH MAIZE AND TEOSINTE SPECIES

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ABSTRACT

Fungal strains identified as *Fusarium subglutinans* form part of the so-called *Gibberella fujikuroi* species complex. *F. subglutinans* is an anamorphic species that is associated with three mating populations (designated B, E and H), as well as a variety of plant hosts. These different *F. subglutinans* strains are generally considered indistinguishable using conventional morphological characters. However, molecular tools have made it possible to show that the *F. subglutinans* strains making up mating populations B, E and H, as well as those associated with the different plant hosts, represent separate species. Recently an additional putative mating population has been reported on the wild teosinte relatives of maize. These isolates are apparently closely related to the pitch canker fungus, *F. subglutinans* f. sp. *pini* (= *F. circinatum*; *G. fujikuroi* mating population H). The aim of the current study was to determine whether the population of *F. subglutinans* from teosinte constitutes a new or an existing lineage within the *G. fujikuroi* complex. For this purpose, portions of the mitochondrial small subunit ribosomal DNA, calmodulin and β-tubulin genes from the fungi were sequenced. Phylogenetic analyses and comparison with sequences from public domain databases indicated that the *F. subglutinans* isolates from teosinte are most closely related to strains in *G. fujikuroi* mating population E. These results were confirmed using sexual compatibility studies. The putative mating population from the wild relatives of maize, therefore, forms part of the existing *G. fujikuroi* mating population E and does not constitute a new lineage in this species complex.

INTRODUCTION

Fusarium subglutinans (Wollew. & Reinking) Nelson, Toussoun & Marasas is an anamorphic species that forms part of the *Gibberella fujikuroi* complex (11, 13). *Fusarium subglutinans* anamorphs are associated with a wide variety of plant hosts such as pine, mango, maize and many more (1, 7, 10, 11, 15, 19, 20, 28). Some of these strains also cause serious diseases and *formae speciales* have consequently been established for strains causing disease on pine and pineapple (3, 28). This anamorphic species is also associated with at least three genetically isolated populations also known as biological species or mating populations B, E and H (1, 7, 10, 11). Mating population B is associated with a variety of hosts including sugarcane (10, 11, 17), whereas mating populations E and H are associated with maize and pine, respectively (1, 10-12, 17, 29).

In recent years, the taxonomy of *F. subglutinans* has been the subject of considerable debate. Much of this is directly linked to the fact that strains associated with the different plants, mating populations and diseases are virtually indistinguishable using morphological characteristics (13-15, 21). The use of molecular tools has clarified many of the taxonomic questions and has provided valuable insight regarding the phylogeny of this anamorphic species (16, 17, 19, 22, 24, Chapters 2 and 4 of this thesis). These DNA based methods have shown that most strains associated with the different plants, *formae speciales* and mating populations represent separate evolutionary lineages. These lineages thus constitute discrete species. Many of these species have subsequently been re-evaluated by using both molecular tools and morphological characters. Hence, *F. subglutinans* strains belonging to mating population H that cause pitch canker of pine was assigned the name *F. circinatum* Nirenberg et O'Donnell (= *F. subglutinans* f. sp. *pini*) (15, 17). Those strains associated with disease of pineapple were named *F. guttiforme* Nirenberg et O'Donnell (= *F. subglutinans* f. sp. *ananas*) (15, 17). *Fusarium* strains representing the B-mating population are currently designated as *F. sacchari* (Butler) W. Gams, whereas those in mating population E are designated *F. subglutinans sensu stricto* (15, 17). In addition to these described species, several other monophyletic lineages displaying morphological characters that are typical of *F. subglutinans*, remain within the *G. fujikuroi* complex (17, 19, 22, Chapter 4 of this thesis).

Recently, a genetically isolated population of *F. subglutinans* isolates has been described by Desjardins et al. (4). This population of isolates originated from domestic maize (*Zea mays* ssp. *mays*) and its wild teosinte relatives (*Zea* spp.) in Mexico and Central America. Isolates of this population were interfertile, but none could mate with the mating type tester strains for mating populations E, B or H. As a result Desjardins et al. (4) suggested that this population might constitute a fourth distinct

mating population associated with *F. subglutinans* anamorphs. However, the description of the fourth mating population was not formalized. The reason was that one strain from this putative population showed a marginal degree of interfertility with a single strain from mating population H. The interfertility of these strains suggested that the population associated with teosinte might be similar or closely related to mating population H. Correct identification of this putative population is important, since isolates belonging to mating population H are serious pathogens of *Pinus* spp. Desjardins et al. (4) also speculated that teosinte and maize could represent sources of inoculum for pitch canker. Elucidation of the relationship between these two groups of fungi is therefore relevant, not only from a taxonomic standpoint but also from a quarantine perspective.

The objective of this study was to characterize a subset of isolates from maize and teosinte that represent the putative new mating population, using cultural and molecular traits. We particularly wished to clarify whether these strains form part of mating population H or another mating population in the *G. fujikuroi* complex. Our aim was four-fold: (i) to determine whether the isolates from teosinte are similar to those associated with pitch canker of pine using the histone *H3* PCR-restriction fragment length (RFLP) method described by Steenkamp et al. (24, Chapter 2 of this thesis); (ii) to identify possible candidates with which the strains isolated from teosinte are conspecific by comparing a portion of their mitochondrial small subunit (mtSSU) ribosomal DNAs to previously published sequences in the National Center for Biotechnology Information (NCBI) database; (iii) to determine the identity of isolates from the putative mating population using phylogenetic analyses of β-tubulin, and calmodulin gene sequences from the identified candidates and *F. sublglutinans* isolates associated with maize and teosinte; and (iv) to challenge our hypothesis using sexual compatibility studies.

MATERIAL AND METHODS

Fungal isolates. We included four *F. subglutinans* isolates associated with teosinte (*Zea* spp.) in Mexico (Table 1). Three *F. subglutinans* isolates, previously shown to belong to *G. fujikuroi* mating population E that were isolated from domestic maize (*Z. mays* ssp. *mays*) in South Africa (Table 1), were also included. The single isolate from mating population H (Fsp 34), previously found to be interfertile with an isolate collected from teosinte (Fst 51) (4), was also included. In addition, we included the mating type tester strains for *G. fujikuroi* mating populations E and H.

DNA isolation. DNA was isolated using a CTAB (*N*-cetyl-*N,N,N*-trimethyl-ammonium bromide) extraction method described previously (24, Chapter 2 of this thesis).

Histone *H3* PCR-RFLP. To determine whether the *F. subglutinans* isolates from teosinte are similar to those associated with pitch canker, we used the *H3* PCR-RFLP technique described by Steenkamp et al. (24, Chapter 2 of this thesis). All of the *F. subglutinans* isolates included in this study were subjected to RFLP-analyses of the amplified portion of their histone *H3* sequences as described by these authors.

PCR amplification and sequencing. Portions of the mtSSU, calmodulin and β-tubulin genes were amplified from all the isolates. The primers used for amplification of a portion of the calmodulin gene were CAL-228F (5'-gagttcaaggaggccttctccc-3') and CAL-737R (5'-catctttctggccatcatgg-3') (2). To amplify part of the β-tubulin gene the primers Bt1-a (5'-ttcccccgtctccacttcttcatg-3') and Bt1-b (5'-gacgagatcgttcatgttgaactc-3') (6) were used. The primers MS1 (5'-cagcagtcaagaatattagtcaatg-3') and MS2 (5'-gcggattatcgaattaaataac-3') (30) were used for amplification of part of the mtSSU. PCR reaction and cycling conditions were similar to those described previously (2, 6, 30). After PCR, the products were purified with a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions with the respective primers mentioned. 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, United Kingdom). Sequences were analyzed with Sequence Navigator version 1.0.1.™ (Perkin Elmer Applied BioSystems, Inc., Foster City, CA).

Identification of possible conspecific candidates. To identify *Fusarium* strains with which the isolates from teosinte are possibly conspecific, we used the internet-based programme BLAST (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/). This programme was used to compare the mtSSU sequences for the isolates from teosinte to those for other *Fusarium* spp. in the in NCBI nucleotide database. The reason for using mtSSU sequences, was that this database contains a considerable

number of *Fusarium*-related mtSSU entries. We also sequenced a larger portion of this gene [~680 base pairs (bp)] than for the β-tubulin and calmodulin genes. From the analyses using BLAST, all *Fusarium* strains with mtSSU sequences displaying more than 98% homology to those for the isolates from teosinte, were identified. These were considered possible candidates with which the isolates from teosinte are conspecific.

Phylogenetic analyses. Phylogenetic analyses were performed using the calmodulin and β-tubulin gene sequences for all the isolates included in this study. We also included calmodulin and β-tubulin gene sequences (obtained from GenBank) for the six candidates that are potentially conspecific with the *Fusarium* strains isolated from teosinte. In addition, we included the sequences for the remaining species in the so-called 'American Clade' of the *G. fujikuroi* complex (17). They were *F. guttiforme*, *F. circinatum*, *F. bulbicola*, *F. anthophilum*, *F. succisae* and *F. begoniae*. For comparative purposes the calmodulin and β-tubulin gene sequences for the A-, C-, D-, F- and G-mating populations of the *G. fujikuroi* complex were also included [see O'Donnell et al. (19) for GenBank accession numbers]. All sequences were aligned manually by inserting gaps (see Appendix 4 for aligned sequences). Phylogenetic analyses using parsimony were performed with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b1 (26). Gaps were treated as missing characters in heuristic searches, with tree-bisection-reconnection branch swapping and MULTREES (saving of all optimal trees) effective. Partition homogeneity tests were performed as previously described (18, 19). Bootstrap analyses were based on 1,000 replications.

Sexual compatibility tests and identification of mating types. All the *F. subglutinans* isolates from maize in South Africa and teosinte in Mexico were crossed with one another. We also attempted to repeat the previously reported (4) sexual interaction between an isolate from the H-mating population (Fsp 34) and an individual (Fst 51) from teosinte. For this purpose isolate Fsp 34 were crossed to all the isolates from maize and teosinte, as well as the mating type tester strains for mating populations E and H. To simplify these sexual compatibility tests, the mating types of the different *F. subglutinans* isolates were determined using the PCR-based technique reported by Steenkamp et al. (25, Chapter 3 of this thesis). Isolates with the *MAT-1* mating type were only crossed to those with *MAT-2* mating types and vice versa. Matings were done using previously published techniques (1, 8). Furthermore, because of the relatively low degree of female fertility among the isolates from Mexico (4), these isolates were used only as males in the crosses performed here. Crosses were scored as positives when viable ascospores were produced (1).

RESULTS

Histone *H3* PCR-RFLP. All of the *F. subglutinans* isolates associated with maize and teosinte displayed similar RFLP-profiles. These were similar to those of the E-mating type tester strains. The single isolate associated with pine (Fsp 34) that was previously found to be interfertile with an isolate associated with teosinte, displayed an RFLP-profile similar to those generated for the H-mating type tester strains (Fig. 1).

PCR amplification and sequencing. We amplified and sequenced ~680 bp fragments of the mtSSU, ~400 bp of the calmodulin and ~300 bp of the β-tubulin genes. The mtSSU sequences for the four different *F. subglutinans* isolates associated with teosinte were identical. They were also identical to those of the isolates from maize, as well as the mating type tester strains for mating population E. The sequenced portions of the calmodulin and β-tubulin genes of these fungi displayed some nucleotide differences, but were almost identical (>99.7% and >99.3% similarity, respectively). The calmodulin and β-tubulin sequences for the isolates from teosinte and maize were also very similar to those representing mating population H (>98%).

Identification of possible conspecific candidates. Six *Fusarium* strains in the NCBI nucleotide database displayed mtSSU sequences that were more than 98% homologous to those for the isolates from teosinte. The six strains were considered candidates that are possibly conspecific with the isolates from teosinte. They included two *F. subglutinans* strains (NRRL22016 and NRRL25622) and *F. bactridioides*. The remaining three *Fusarium* strains were NRRL29123, NRRL29124 and NRRL25623. The GenBank accession numbers for the mtSSU sequences of these strains are FSU34501, AF158292, FBU34518, AF158300, AF158301 and AF158291, respectively. The *F. subglutinans* strains NRRL22016 and NRRL25622 belong to *G. fujikuroi* mating population E and were isolated from maize in the United States and South Africa, respectively (19, 24, Chapter 2 of this thesis). Strain NRRL22016 is further one of the E-mating type testers (MRC6512) included in this study (17). *Fusarium* strain NRRL25623 was collected from mango in South Africa (19, 22, 23, Chapter 4 of this thesis). *Fusarium* strains NRRL29123 and NRRL29124 were both isolated from *Bidens pilosa* in the United States (19).

Phylogenetic analyses. Gene trees were generated from both the calmodulin and β-tubulin data sets. Their topologies were similar and the partition homogeneity test indicated that these genes evolve at comparable rates, thus representing homogenous partitions (P = 1.0). For this reason the calmodulin and β-tubulin data sets were combined to produce 12 most parsimonious trees (Fig. 2).

These trees were congruent with those generated from the single-gene data sets.

Sexual compatibility tests. The PCR-based method for distinguishing between the two possible mating types of these isolates revealed that MRC 1084, MRC 756, Fst 40 and Fsp 34 were *MAT-1*, whereas MRC 714, Fst 51, Fst 26 and Fst 10 were *MAT-2*. The three *MAT-2* strains collected from teosinte (Fst 51, Fst 26 and Fst 10) were sexually compatible with the *MAT-1* strain MRC 1084 collected from maize in South Africa. The only other positive interaction was between MRC 756 and Fst 51. The *F. subglutinans* strain (Fsp 34) from pine was sexually compatible only with the H-mating type tester MRC 6213. All crosses were highly fertile and numerous perithecia with oozing ascospores were produced. Viability of ascospores ranged from 90 to 96%.

DISCUSSION

The primary goal of this study was to determine whether the genetically isolated *F. subglutinans* population from maize and its teosinte relatives collected in Mexico (4), represents a previously undescribed lineage. Alternatively, whether these isolates form part of an existing lineage in the *G. fujikuroi* complex. By using a public domain nucleotide database we were able to identify candidate *Fusarium* strains or species that are closely related to or conspecific with the strains isolated from teosinte in Mexico. Furthermore, by generating phylogenies for two unlinked genes, we were able to identify the lineage to which they most probably belong. We were also able to confirm the identity of the lineage associated with Mexican teosinte, using conventional sexual compatibility studies.

Application of the histone *H3* PCR-RFLP technique indicated the isolates from teosinte are different from those associated with pine (Fig. 1). This is because distinctly different histone *H3* PCR-RFLP profiles were obtained for the isolates associated with pine and *Zea* spp. The fact that similar profiles were generated for the isolates associated with maize, teosinte and mating population E, further indicated a close relationship between these fungi. This relationship was also reflected in their mtSSU sequences, which were identical. However, the isolates from teosinte could not be positively assigned to *G. fujikuroi* mating population E (*F. subglutinans sensu stricto*), based on mtSSU sequence data alone. This was because two or more distinct *Fusarium* species can have identical mtSSU sequences (19). We, therefore, proceeded to identify other possible candidate isolates that could be conspecific with the isolates from teosinte.

The six candidate *Fusarium* strains with which the isolates from teosinte are possibly conspecific, included two described species and four undescribed *Fusarium* strains. As expected one of the described species was *F. subglutinans sensu stricto*. The other species was *F. bactridioides*, which is morphologically distinct from *F. subglutinans* and was previously classified in the *Fusarium* Section *Discolor* (13). The four undescribed candidates all display morphological characters typical of *F. subglutinans* (19, 22, 24, Chapters 2 and 4 of this thesis). Three of these undescribed fungi were previously shown to represent discrete species in the *G. fujikuroi* complex (19, 22, Chapter 4 of this thesis). The fourth undescribed *Fusarium* candidate was previously shown to belong to the E-mating population (24, Chapter 2 of this thesis).

In an attempt to determine which of the six possible candidates were the most closely related to or conspecific with the isolates associated with teosinte, phylogenetic analyses were performed. The

analyses were based on only the sequences for calmodulin and β-tubulin. The mtSSU sequences were excluded from these analyses, since they lack sufficient shared (synapomorphic) and unique derived (autapomorphic) characters (19). The phylogenies that were reconstructed using the calmodulin and β-tubulin genes (single or combined sequence data sets) indicated that the isolates from teosinte are closely related to the isolates from maize and mating population E, as well as *F. bactridioides* (Fig. 2). The sequenced portions of the *F. bactridioides* calmodulin and β-tubulin genes are, however, 4 to 6 bp different from those for the isolates associated with teosinte and maize. The isolates associated with maize, teosinte and mating population E never differ by more than 3 bp, suggesting that they are more closely related to one another than to *F. bactridioides*. We confirmed this hypothesis using sexual compatibility studies.

The results presented in this study strongly support the conspecificity of the isolates from teosinte in Mexico, maize in South Africa and the members of *G. fujikuroi* mating population E (*F. subglutinans senso stricto*). Three lines of molecular evidence suggested this relationship. Firstly, identical *H3* PCR-RFLP profiles are generated for these isolates. Secondly, the sequenced portions for their mtSSU are identical. Thirdly, phylogenetic analyses group these isolates together. The conspecificty of these isolates was also confirmed using conventional sexual compatibility studies. Two South African isolates from maize, that were previously shown to belong to the E-mating population (24, Chapter 2 of this thesis), were interfertile with three of the Mexican isolates from teosinte. The population from teosinte in Mexico and Central America is, therefore, part of the *G. fujikuroi* mating population E and not unique, as previously suggested (4).

The *F. subglutinans* isolates associated with maize, teosinte and mating population E appear to be subdivided into al least two phylogenetic groups (Fig. 2). This subdivision does not appear to be related to host or geographic origin, since isolates from teosinte in Mexico are found in both clusters, which is also true for the isolates from maize in South Africa. Although the bootstrap support for these groups are weak, their existence is confirmed by previous studies (19, 24, Chapter 2 of this thesis). Steenkamp et al. (24, Chapter 2 of this thesis) and O'Donnnell et al. (19) both showed the separation of mating population E into subgroups. However, as reported here and elsewhere, individuals from both groups are sexually compatible with individuals from the other group (4, 24, Chapter 2 of this thesis). Since the present study and those of Steenkamp et al. (24, Chapter 2 of this thesis) and O'Donnnell et al. (19) used different sets of isolates, no clear conclusions can be drawn regarding the relationships among these fungi. Application of phylogenetic tools to address questions on the population biology (5, 9, 27) of this group of fungi might reveal that they are diverging into discrete lineages, yet sufficiently similar to allow genetic exchange via sexual reproduction. If this is the case, the

significance of the term 'mating population' and its relatedness to aspects such as pathology, ecology and taxonomy needs to be re-evaluated.

Our data (Figs. 1 and 2) did not allow us to substantiate the hypothesis that the population sampled from teosinte and that from *G. fujikuroi* mating population H, share an unusually close relationship (4). Although both these populations form part of the so-called 'American Clade' proposed by O'Donnell et al. (17), this clade also includes other species that are phylogenetically closely associated with mating populations E and H (Fig. 1). We were further unable to reproduce the unusual sexual interaction between a single isolate (Fst 51 and Fsp 34) from each of these populations, reported by Desjardins et al. (4). We believe that this cross represents a hybrid interaction forced by favorable conditions. Whether such interactions also occur in nature requires further investigation. However, this type of interaction would not be impossible if one takes into account the fact that both these species probably co-evolved with their respective hosts, which have overlapping geographic ranges. If mating populations E and H also share a recent common ancestor, some individuals in both species might have retained sufficient 'common' genetic background to allow sexual recombination. Irrespective of whether these mating populations share a common origin or not, various researchers have shown that they are specific to their respective hosts and that there is no reciprocal pathogenicity between them (3, 11, 29). We, therefore, conclude that it is highly unlikely that the pitch canker disease of *Pinus* spp. can be caused by the *F. subglutinans* strains from mating population E that are found on maize and teosinte.

This and other studies have clearly shown that distinguishing the different *F. subglutinans* lineages that are associated with the different plant hosts and mating populations is troublesome (22, 23, Chapter 4 of this thesis). Traits such as morphology and host often result in ambiguous identifications. Although sexual compatibility studies can, to some extent, help in the identification process, this study clearly illustrated how the biological species concept can obscure relationships. We conclude that fungi displaying morphological characters typical of *F. subglutinans* can only be unequivocally identified using DNA sequence analyses. We further emphasize the need for formal description of these *Fusarium* lineages as species. This would greatly assist plant pathologists and mycologists to successfully distinguish between the species representing *F. subglutinans sensu lato*.

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Table 1. Hosts, geographic origins and sources of the isolates associated with maize, teosinte and pine used in this study.

Isolate [1]	Host	Geographic origin	Source
(E) MRC 6512; KSU 2192	*Z. mays* ssp. *mays*	Illinois, United States	J. F. Leslie
(E) MRC 6483; KSU 990	*Z. mays* ssp. *mays*	Illinois, United States	J. F. Leslie
(E) MRC 1084	*Z. mays* ssp. *mays*	Eastern Cape, South Africa	W.F.O. Marasas
(E) MRC 756	*Z. mays* ssp. *mays*	Mpumalanga, South Africa	W.F.O. Marasas
(E) MRC 714	*Z. mays* ssp. *mays*	Northern Province, South Africa	W.F.O. Marasas
Fst 10	*Z. diploperennis*	Jalisco, Mexico	A. E. Desjardins
Fst 26	*Z. mays* ssp. *mexicana*	Michoacan, Mexico	A. E. Desjardins
Fst 40	*Z. mays* ssp. *parviglumis*	Guerrero, Mexico	A. E. Desjardins
Fst 51	*Z. mays* ssp. *mexicana*	Texcococ, Mexico	A. E. Desjardins
Fsp 34	*Pinus* spp.	California, United States	T. R. Gordon
(H) MRC 6213	*Pinus* spp.	Mpumalanga, South Africa	W.F.O. Marasas
(H) MRC 7488	*Pinus* spp.	Mpumalanga, South Africa	W.F.O. Marasas

[1] Culture collections: MRC = W. F. O. Marasas, Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, Tygerberg, South Africa; KSU = J. F. Leslie, Department of Plant Pathology, Kansas State University, Manhattan Kansas; Fst = A. E. Desjardins, National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, Illinois, Fsp = T. R. Gordon, Department of Plant Pathology, University of California, Davis, California. *Gibberella fujikuroi* mating population E and H are indicated in parentheses.

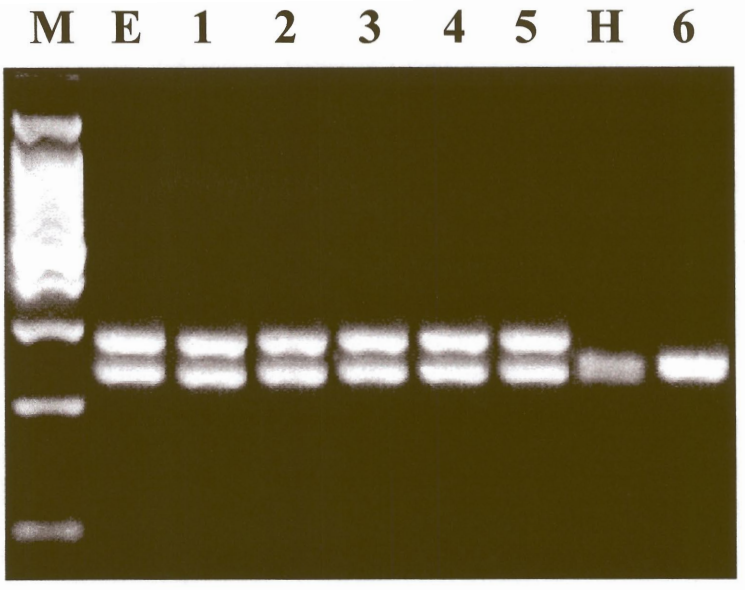

Figure 1. PCR-RFLP profiles generated by digesting amplified histone *H3* fragments from the mating type tester strains for mating populations E and H, as well as the isolates from teosinte and Fsp 34, with the restriction enzymes *Dde*1 and *Cfo*1 according to Steenkamp et al. (24, Chapter 2 of this thesis). Lane M, 100-bp ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp); Lane E, mating population E; Lane 1, Fst 10; Lane 2, Fst 26; Lane 3, Fst 40; Lane 4, Fst 51; Lane 5, MRC 1084; Lane H, mating population H; Lane 6, Fsp 34.

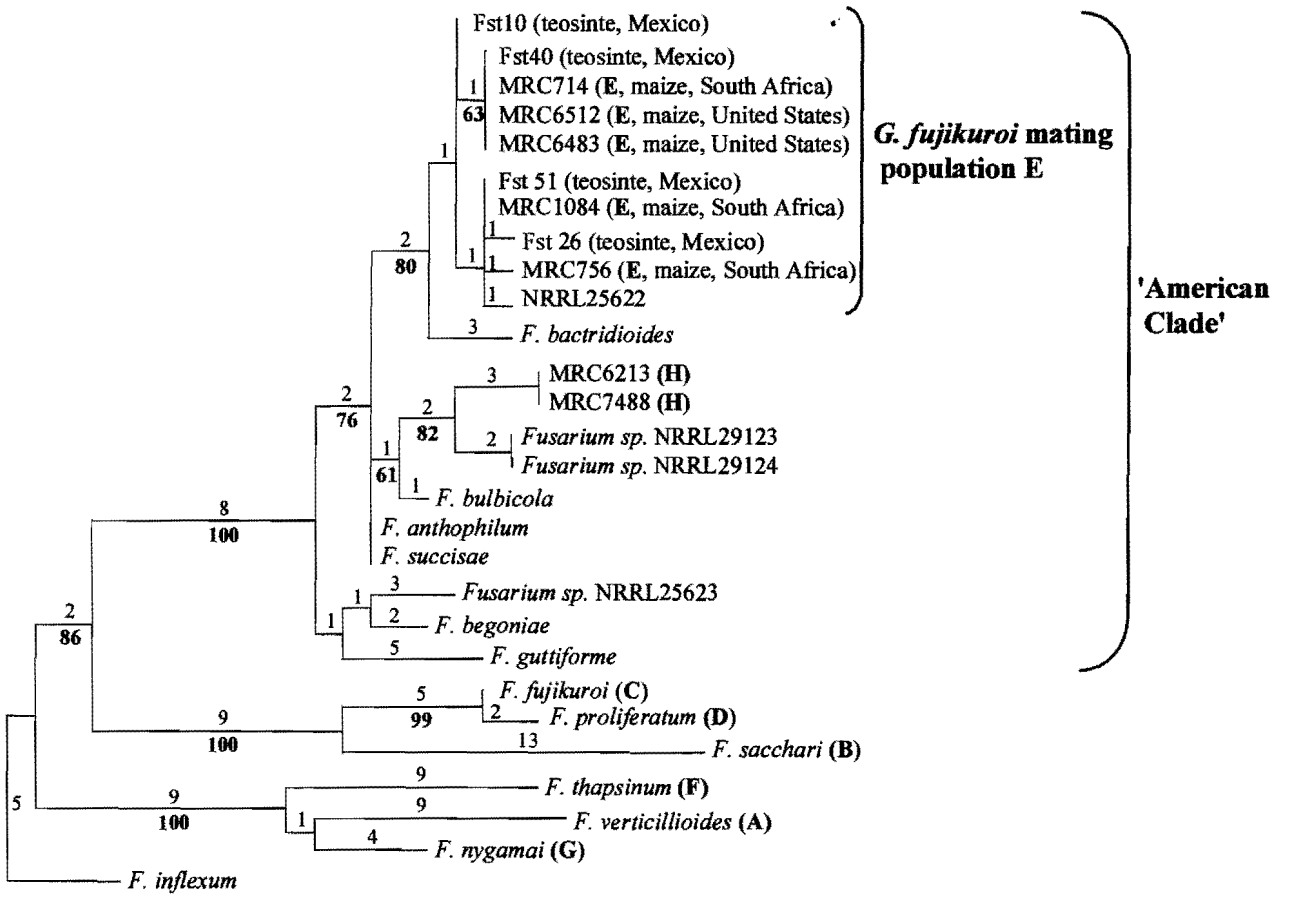

Figure 2. Phylogenetic tree reconstructed from the combined calmodulin and β-tubulin datasets obtained from the isolates included in this study, as well as those obtained from GenBank. The geographic origin and host for the *F. subglutinans* isolates are indicated in parentheses. The *G. fujikuroi* mating populations A to H are indicated in bold letters in parentheses. Branch lengths are indicated above the branches and bootstrap values are indicated in bold digits below the internodes. The tree is rooted to *F. inflexum*.