MOLECULAR PLANT PATHOLOGY (2001) **2**(4), 215–221

Gibberella fujikuroi mating population E is associated with maize and teosinte

E. T. STEENKAMP[1,*], T. A. COUTINHO[1], A. E. DESJARDINS[2], B. D. WINGFIELD[1], W. F. O. MARASAS[3] AND M. J. WINGFIELD[1]

[1]*Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, (FABI), Departments of Genetics, Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, South Africa;*
[2]*Mycotoxin Research Unit, National Center for Agricultural Utilization of Research, USDA, University Street, Preoria, Illinois 61604, USA;*
[3]*PROMEC, Medical Research Council (MRC), PO Box 19070, Tygerberg 7505, South Africa*

SUMMARY

Isolates of *Fusarium subglutinans* mating population E are usually found on maize. This fungus forms part of the so-called *Gibberella fujikuroi* species complex. Previously, *F. subglutinans* has been associated with two additional mating populations (B and H) and a variety of plant hosts. This was mainly due to a lack of diagnostic morphological characters, but the use of DNA sequence information showed that the strains making up mating populations B, E and H, as well as those associated with the different plant hosts, represent separate species. Recently, another putative mating population has been reported on the wild teosinte relatives of maize. Based on sexual compatibility studies, these isolates were 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 of *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 E-mating population and does not constitute a new lineage in the *G. fujikuroi* species complex.

INTRODUCTION

Isolates of *Fusarium subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas are usually isolated from maize (*Zea mays* ssp. *mays*). Based on sexual compatibility studies they represent mating population E of the *Gibberella fujikuroi* (Sawada) Wollenw. species complex (Leslie, 1995; Nelson *et al*., 1983; Nirenberg and O'Donnell, 1998). Previously, *F. subglutinans* (*sensu lato*) has also been associated with two additional mating populations (B and H), as well as several other plant hosts (Britz *et al*., 1999; Correll *et al*., 1991; Hsieh *et al*., 1977; Kuhlman, 1982; Nirenberg and O'Donnell, 1998; O'Donnell *et al*., 2000; Rohrbach and Pfeiffer, 1976; Ventura *et al*., 1993). The use of DNA sequence information has, however, shown that the isolates constituting mating populations B, E and H, as well as those associated with the different plant hosts, represent separate species (O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998, 2000; Steenkamp *et al*., 1999, 2000a).

Much of the confusion regarding the taxonomy of *F. subglutinans sensu lato* was directly linked to the fact that strains associated with the different plants and mating populations are morphologically very similar (Nelson *et al*., 1983; Nirenberg and O'Donnell, 1998; Nirenberg, 1989; Snyder and Hansen, 1945). For example, all these strains are characterized by microconidia that are formed in false heads, macroconidia that are straight to slightly sickle-shaped and the absence of chlamydospores. Identification of additional diagnostic morphological characters, which were brought about by the DNA-based studies, led to formal description of many of the species comprising *F. subglutinans sensu lato* (Nirenberg and O'Donnell, 1998; O'Donnell *et al*., 1998). Strains isolated from maize that belong to mating population E were designated *F. subglutinans sensu stricto* (Nirenberg and O'Donnell, 1998; O'Donnell *et al*., 1998). Those isolated from pine that belong to mating population H were designated as *F. circinatum* Nirenberg et O'Donnell (= *F. subglutinans* f. sp. *pini* Correll *et al*.) (Britz *et al*., 1999; Nirenberg and O'Donnell, 1998; O'Donnell *et al*., 1998; Viljoen *et al*., 1997). Those isolated from sugarcane that represent mating population B were designated as *F. sacchari* (Butler) W. Gams (Kuhlman, 1982; Leslie, 1995; O'Donnell *et al*., 1998 Nirenberg and O'Donnell, 1998), and those associated with disease of pineapple were designated as

***** *Correspondence*: Department of Biology, University of York, PO Box 373, York, YO10 5YW, UK. E-mail: es21@york.ac.uk

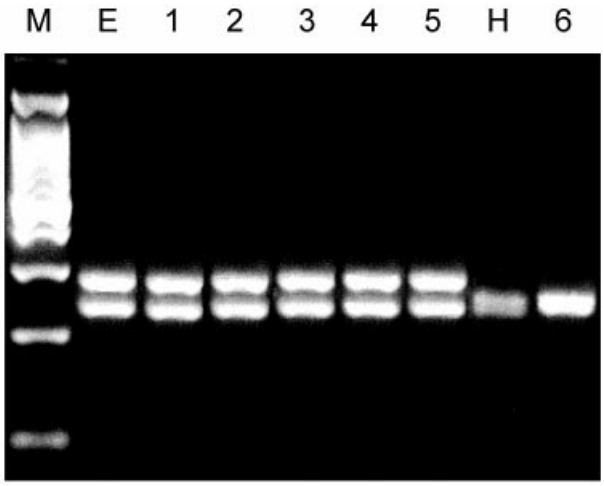

Fig. 1 PCR-RFLP profiles generated by digesting amplified histone *H3* fragments from the tester strains for mating populations E and H, as well as the isolates from teosinte and MRC7870, with the restriction enzymes *Dde*1 and *Cfo*1 according to Steenkamp *et al.* (2000). Lane M, 100-bp ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp); Lane E, mating population E (*F. subglutinans sensu stricto*); Lane 1–4, Mexican teosinte isolates MRC7787, MRC7803, MRC7818 and MRC7828; Lane 5, E-mating population strain MRC1084 from South African maize; Lane H, mating population H (*F. circinatum*); Lane 6, H-mating population strain MRC7870.

F. guttiforme Nirenberg et O'Donnell (= *F. subglutinans* f. sp. *ananas* Ventura Zambolim and Gilb.) (Nirenberg and O'Donnell, 1998; O'Donnell *et al.*, 1998). In addition to these species, several other undescribed monophyletic lineages displaying morphological characters that are typical of *F. subglutinans sensu lato*, remain within the *G. fujikuroi* complex (O'Donnell *et al.*, 1998, 2000; Steenkamp *et al.*, 2000a).

Recently, a genetically isolated population of *F. subglutinans* strains has been described by Desjardins *et al.* (2000). 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.* (2000) suggested that this population might constitute a fourth distinct mating population associated with *F. subglutinans* anamorphs, but a description of this 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. This suggested that the population associated with teosinte might be similar or closely related to mating population H (*F. circinatum*). A correct identification of this putative population is important, since isolates belonging to mating population H are serious pathogens of *Pinus* species. Desjardins *et al.* (2000) 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 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 fourfold: (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.* (1999); (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. subglutinans* isolates associated with teosinte; and (iv) to challenge our hypothesis using sexual compatibility studies.

RESULTS

Histone *H3* PCR-RFLP

All of the *F. subglutinans* isolates associated with mating population E (*F. subglutinans sensu stricto*) and teosinte displayed similar RFLP-profiles. The single isolate from pine (MRC7870) that was previously found to be interfertile with an isolate from teosinte, displayed an RFLP-profile similar to that generated for the *F. circinatum* H-mating type tester strains (Fig. 1).

PCR amplification and sequencing

We amplified and sequenced ≈ 680 base pair (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 mating population E (*F. subglutinans sensu stricto*) isolates from maize. The sequenced portions of the calmodulin and β-tubulin genes of these fungi displayed some nucleotide differences, but were almost identical (< 0.3% and < 0.7% difference, respectively). The calmodulin and β-tubulin sequences for the isolates from teosinte and mating population E were also very similar to those representing mating population H (< 2% difference).

Identification of possible conspecific candidates

Six *Fusarium* strains in the NCBI nucleotide database displayed mtSSU sequences that were more than 98% homologous to

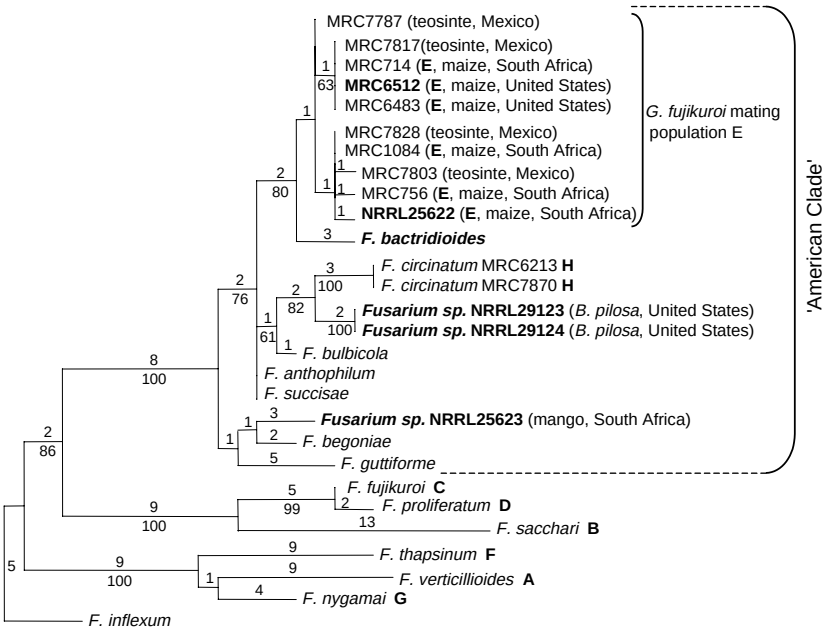

Fig. 2 One of 12 most parsimonous trees reconstructed from the combined calmodulin and β-tubulin datasets obtained for the isolates included in this study, as well as those obtained from GenBank. The six candidates identified by comparison of mtSSU sequences to those in the NCBI database, with which the isolates from teosinte were possibly conspecific, are indicated in bold. The geographical origin and host for the *F. subglutinans* isolates associated with teosinte, as well as those for the conspecific candidates, are indicated in parentheses. The *G. fujikuroi* mating populations A to H are shown in capital letters. Branch lengths are indicated above the branches and bootstrap values are indicated below the internodes. The tree is rooted to *F. inflexum* Schneider.

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 sensu stricto* strains (NRRL22016 and NRRL25622) and *F. bactridioides* Wollenw. 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 sensu stricto* (*G. fujikuroi* mating population E) strains NRRL22016 and NRRL25622 were isolated from maize in the USA and South Africa, respectively (O'Donnell *et al.*, 2000; Steenkamp *et al.*, 1999). Strain NRRL22016 is a further one of the E-mating type testers (MRC6512) included in this study (O'Donnell *et al.*, 1998). *Fusarium* strain NRRL25623 was collected from mango in South Africa (O'Donnell *et al.*, 2000; Steenkamp *et al.*, 1998, 2000a). *Fusarium* strains NRRL29123 and NRRL29124 were both isolated from *Bidens pilosa* L. in the USA (O'Donnell *et al.*, 2000).

Phylogenetic analyses

Gene trees were generated from both the calmodulin and β-tubulin datasets. Parsimony analysis of the β-tubulin dataset generated 30 most parsimonious (MP) trees that were 41 steps in length. These trees were based on 296 characters, of which 20 were parsimony informative. The consistency (CI) and retention (RI) indexes for these trees were 0.85 and 0.90, respectively. Parsimony analysis of the calmodulin sequences generated two MP trees that were 70 steps in length. The calmodulin trees were based on 404 characters, of which 34 were informative, and their CI and RI were 0.93 and 0.95, respectively. The calmodulin and β-tubulin datasets represented homogenous partitions, resulting in a partition homogeneity test *P*-value of 1.0. Thus, all the test summed tree lengths were shorter than the length of the actual tree. This was true, irrespective of whether the parsimony informative sites were included or excluded in the analyses. The calmodulin and β-tubulin datasets were thus combined to produce 12 MP trees (Fig. 2). These trees were 111 steps in length and were based on a total of 700 characters, 54 of which were parsimony informative. The CI and RI for the trees generated from the combined dataset were 0.90 and 0.93, respectively.

The topologies for the 12 MP trees generated from the combined datasets were highly similar. In all 12 cases, the strains belonging to the so-called 'American Clade' of O'Donnell *et al.* (1998) grouped together (100% bootstrap support; Fig. 2). The same was true for the species belonging to the 'African' (*F. thapsinum*, *F. verticillioides* and *F. nygamai*) and 'Asian' (*F. proliferatum*, *F. fujikuroi* and *F. sacchari*) clades. In all the MP trees the E-mating population strains and those isolated from teosinte clustered together (80% bootstrap support) within the 'American Clade'. Most of the differences in topology between the 12 MP trees could, however, be attributed to phylogenetic 'instability' within the E-mating population cluster. Similar results were also obtained for the two calmodulin MP trees and 30 β-tubulin MP trees, although the phylogenetic 'instability' was extended to the entire 'American Clade', in the case of the β-tubulin trees.

Sexual compatibility tests

The PCR-based method for distinguishing between the two possible mating types of the isolates revealed that MRC1084,

MRC756 and MRC7817 were *MAT-1*, whereas MRC714, MRC7828, MRC7803 and MRC7787 were *MAT-2*. The three *MAT-2* strains collected from teosinte (MRC7828, MRC7803 and MRC7787) were sexually compatible with the mating population E *MAT-1* strain MRC1084 that was collected from maize in South Africa. The only other positive interaction was between the South African E-mating population strain MRC756 and the Mexican teosinte strain MRC7828. The mating type for the isolate (MRC7870) from pine was *MAT-1*, and was sexually compatible only with the *MAT-2 F. circinatum* H-mating type tester MRC6213. All the positive 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 (Desjardins *et al*., 2000), 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 (Steenkamp *et al*., 1999) indicated that the isolates from teosinte are different from those associated with pine (Fig. 1). The fact that similar profiles were generated for the isolates associated with teosinte and mating population E, further indicated a close relationship between these fungi. This relationship was reflected in their mtSSU sequences that 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 and *H3* PCR-RFLP profiles alone. This is because two or more distinct *Fusarium* species can have identical mtSSU sequences (O'Donnell *et al*., 2000) and the *H3* PCR-RFLP was developed and tested for diagnosing the pitch canker fungus and not strains belonging to mating population E (Steenkamp *et al*., 1999). 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 were possibly conspecific, included two described species and three 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, but phylogenetically closely related to *F. subglutinans* (Nelson *et al*., 1983; O'Donnell *et al*., 1998). The three undescribed fungi were previously shown to represent discrete species in the *G. fujikuroi* complex (O'Donnell *et al*., 2000; Steenkamp *et al*., 2000a).

In an attempt to determine which of the six possible candidates were 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 variation for this purpose, as illustrated here and by O'Donnell *et al*. (2000). The phylogenies that were reconstructed using the calmodulin and β-tubulin genes (single and combined sequence datasets) indicated that the isolates from teosinte were distinctly different from the three undescribed species (Fig. 2). The *F. subglutinans* strains isolated from Mexican teosinte were most closely related to isolates of *F. bactridioides* and mating population E (Fig. 2). Inspection revealed that the *F. bactridioides* sequences are 5–7 bp different from those of the teosinte and mating population E isolates, while those from the latter never differ by more than 3 bp. This suggests that the isolates from teosinte and mating population E are more closely related to one another than to *F. bactridioides*.

The results presented here strongly support the conspecificity of the isolates from teosinte in Mexico and the members of *G. fujikuroi* mating population E (*F. subglutinans sensu stricto*). Firstly, identical *H3* PCR-RFLP profiles were generated for these isolates (Fig. 1). Secondly, the sequenced portions for their mtSSU were identical and thirdly, phylogenetic analyses group them together (Fig. 2). Even though the conventional *E*-tester strains did not appear to be interfertile with the Mexican teosinte isolates (Desjardins *et al*., 2000), some of the South African E-mating population isolates from maize were interfertile with some of the Mexican teosinte isolates. The population from teosinte in Mexico and Central America is therefore part of *G. fujikuroi* mating population E and not unique, as previously suggested (Desjardins *et al*., 2000). The reason for the lack of sexual compatibility between the conventional *E*-tester strains and the isolates associated with teosinte is unclear, but low fertility may play a role.

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 as suggested by Desjardins *et al*. (2000). Although both these populations form part of the so-called 'American Clade' proposed by O'Donnell *et al*. (1998), 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 sexual interaction between the isolates MRC7828 and MRC7870 from each of these populations, reported by Desjardins *et al*. (2000). This was despite the fact that the cross has, subsequently, been repeated several times by these

authors (Desjardins *et al.* unpublished data). 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 geographical 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. Nevertheless, various researchers have shown that both species are specific to their respective hosts and that there is no reciprocal pathogenicity between them (Correll *et al.*, 1991; Leslie, 1995; Viljoen *et al.*, 1997). We therefore conclude that it is highly unlikely that the pitch canker disease of *Pinus* species can be caused by the *F. subglutinans* strains from mating population E that are found on maize and teosinte.

The *F. subglutinans* isolates associated with teosinte and mating population E are subdivided into smaller groups (Fig. 2). This subdivision does not appear to be related to host or geographical origin, because isolates from Mexican teosinte and South African maize appear to be interspersed in all of the subgroups. The bootstrap support for these groups is weak, but their existence is confirmed by previous studies (O'Donnell *et al.*, 2000; Steenkamp *et al.*, 1999). However, the present study and those of Steenkamp *et al.* (1999) and O'Donnell *et al.* (2000) used different sets of isolates and no clear conclusions can be drawn on the relationships among these fungi. The application of phylogenetic tools (Geiser *et al.*, 1998; Koufopanou *et al.*, 1997; Taylor *et al.*, 1999) to address questions on the population biology 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.

This and other studies have clearly shown that distinguishing the described and undescribed species representing *F. subglutinans sensu lato* is troublesome (Steenkamp *et al.*, 1999, 2000a). As reported by several authors, traits such as morphology, host range and sexual compatibility often result in ambiguous identifications (Brazier, 1997; Harrington and Rizzo, 1999; Taylor *et al.*, 2000). Conversely, DNA sequence analyses facilitate unequivocal diagnoses of *Fusarium* species and the undescribed lineages. We also emphasize the need for a formal description of these undescribed lineages as species. This would greatly assist plant pathologists and mycologists in successfully distinguishing between the species representing *F. subglutinans sensu lato*.

EXPERIMENTAL PROCEDURES

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 (MRC7870), previously found to be interfertile with an isolate collected from teosinte (MRC7828) (Desjardins *et al.*, 2000), was also included. In addition, we included the

Table 1 Host, geographical origin and source of the isolates associated with maize, teosinte and pine used in this study.

Isolate*	Host	Geographic origin	Source	GenBank accession number
(E) MRC6512; KSU2192	*Z. mays* L. ssp. *mays*	Illinois, USA	J.F. Leslie	AF366525; AF366535; AF366551
(E) MRC6483; KSU990	*Z. mays* ssp. *mays*	Illinois, USA	J.F. Leslie	AF366522; AF366536; AF366552
(E) MRC1084	*Z. mays* ssp. *mays*	Eastern Cape, South Africa	W.F.O. Marasas	AF366521; AF366537; AF366550
(E) MRC756	*Z. mays* ssp. *mays*	Mpumalanga, South Africa	W.F.O. Marasas	AF366527; AF366538; AF366549
(E) MRC714	*Z. mays* ssp. *mays*	Northern Province, South Africa	W.F.O. Marasas	AF366520; AF366539; AF366548
MRC7787; Fst10	*Z. diploperennis* Iltis, Doebley and Guzman	Jalisco, Mexico	A.E. Desjardins	AF366523; AF366540; AF366544
MRC7803; Fst26	*Z. mays* ssp. *mexicana* (Schrader) Iltis	Michoacan, Mexico	A.E. Desjardins	AF366526; AF366541; AF366545
MRC7817; Fst40	*Z. mays* ssp. *parviglumis* Iltis and Doebley	Guerrero, Mexico	A.E. Desjardins	AF366524; AF366542; AF366546
MRC7828; Fst51	*Z. mays* ssp. *mexicana*	Texcoco, Mexico	A.E. Desjardins	AF366529; AF366543; AF366547
(H) MRC7870; Fsp34	*Pinus* L. spp.	California, USA	T.R. Gordon	AF366528; AF366534; AF366555
(H) MRC6213	*Pinus* spp.	Mpumalanga, South Africa	W.F.O. Marasas	AF366531; AF366533; AF366553
(H) MRC7488	*Pinus* spp.	Mpumalanga, South Africa	W.F.O. Marasas	AF366530; AF366532; AF366554

*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, US Department of Agriculture, Peoria, Illinois, Fsp = T.R. Gordon, Department of Plant Pathology, University of California, Davis, California. Strains belonging to *Gibberella fujikuroi* mating populations E and H are indicated.

tester strains for *G. fujikuroi* mating population E, MRC6483 and MRC6512, and mating population H, MRC6213 and MRC7488 (Table 1).

DNA isolation

DNA was isolated using the CTAB (*N*-cetyl-*N,N,N*-trimethyl-ammonium bromide) extraction method described previously (Steenkamp *et al*., 1999).

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*. (1999). 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′) (Carbone and Kohn, 1999). To amplify part of the β-tubulin gene the primers Bt1-a (5′-ttcccccgtctccacttcttcatg-3′) and Bt1-b (5′-gacgagatcgttcatgttgaactc-3′) (Glass and Donaldson, 1995) were used. The primers MS1 (5′-cagcagtcaagaatattagtcaatg-3′) and MS2 (5′-gcggattatcgaattaaataac-3′) (White *et al*., 1990) were used for amplifying part of the mtSSU. PCR reaction and cycling conditions were similar to those described previously (Carbone and Kohn, 1999; Glass and Donaldson, 1995; White *et al*., 1990). 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. For this purpose an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Warrington, United Kingdom) and an ABI PRISM™ 377 automated DNA sequencer were used. Sequences were analysed with Sequence Navigator version 1.0.1.™ (Perkin Elmer Applied BioSystems Inc., Foster City, California).

Identification of possible conspecific candidates

To identify *Fusarium* strains with which the isolates from teosinte are possibly conspecific, we used the internet-based program Blast (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/). This program 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 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 (O'Donnell *et al*., 1998). They were *F. guttiforme*, *F. circinatum*, *F. bulbicola* Nirenberg et O'Donnell, *F. anthophilum* (A. Braun) Wollenw., *F. succisae* (Schröter) Sacc. and *F. begoniae* Nirenberg et O'Donnell. 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*. (2000) for GenBank accession numbers]. All sequences were aligned manually by inserting gaps. Phylogenetic analyses using parsimony were performed with Paup (Phylogenetic Analysis Using Parsimony) version 4.0b1 (Swofford, 1998). Gaps were treated as missing characters in heuristic searches, with tree-bisection-reconnection branch swapping and MulTrees (saving of all optimal trees) effective. Bootstrap analyses were based on 1000 replications. To test combinability if the calmodulin and β-tubulin datasets, the partition homogeneity test was performed using Paup. This was done with tree-bisection-reconnection branch swapping, simple addition sequence, MaxTrees set to 500 and 1000 repartitions. The test was performed twice, once by excluding the parsimony uninformative characters and again by including all characters.

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 (Desjardins *et al*., 2000) sexual interaction between an isolate from the H-mating population (MRC7870) and an individual (MRC7828) from teosinte. For this purpose, isolate MRC7870 was crossed with all the isolates from maize and teosinte, as well as the mating type tester strains for mating populations E and H. To simplify the tests, mating types of the different *F. subglutinans* isolates were determined using the PCR-based technique reported by

Steenkamp *et al.* (2000b). Isolates with the *MAT-1* mating type were only crossed with *MAT-2* isolates and vice versa. Matings were carried out using previously published techniques (Britz *et al.*, 1999; Klittich and Leslie, 1988). Furthermore, because of the relatively low degree of female fertility among the isolates from Mexico (Desjardins *et al.*, 2000), these isolates were used only as males in the crosses performed here. Crosses were scored as positives when viable ascospores were produced (Britz *et al.*, 1999).

REFERENCES

Brazier, C.M. (1997) Fungal species in practice: identifying species units in fungi. In: *Species: the Units of Biodiversity* (Claridge, M.F., Dawah, M.R. and Wilson, M.R., eds). London: Chapman & Hall, pp. 135–170.

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.

Carbone, I. and Kohn, L.M. (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia*, **91**, 553–556.

Correll, J.C., Gordon, T.R., McCain, A.H., Fox, J.W., Koehler, C.S., Wood, D.L. and Schultz, M.E. (1991) Pitch canker disease in California: pathogenicity, distribution and canker development on Monterey pine (*Pinus radiata*). *Plant Dis.* **75**, 676–682.

Desjardins, A.E., Plattner, R.D. and Gordon, T.R. (2000) *Gibberella fujikuroi* mating population A and *Fusarium subglutinans* from teosinte species and maize from Mexico and Central America. *Mycol. Res.* **104**, 865–872.

Geiser, D.M., Pitt, J.I. and Taylor, J.W. (1998) Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proc. Natl Acad. Sci. USA*, **95**, 388–393.

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.

Harrington, T.C. and Rizzo, D.M. (1999) Defining species in the fungi. In: *Structure and Dynamics of Fungal Populations* (Worrall, J.J., ed.). Boston, MA: Kluwer Academic Press, pp. 43–70.

Hsieh, W.H., Smith, S.N. and Snyder, W.C. (1977) Mating groups in *Fusarium moniliforme*. *Phytopathology*, **67**, 1041–1043.

Klittich, C.J.R. and Leslie, J.F. (1988) Nitrate reduction mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*). *Genetics*, **118**, 417–423.

Koufopanou, V., Burt, A. and Taylor, W.J. (1997) Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitis*. *Proc. Natl Acad. Sci. USA*, **94**, 5478–5482.

Kuhlman, E.G. (1982) Varieties of *Gibberella fujikuroi* with anamorphs in *Fusarium* section *Liseola*. *Mycologia*, **74**, 756–768.

Leslie, J.F. (1995) *Gibberella fujikuroi*: Available populations and variable traits. *Can. J. Bot.* **73**, S282–S291.

Nelson, P.E., Toussoun, T.A. and Marasas, W.F.O. (1983) *Fusarium Species: an Illustrated Manual for Identification*. Pennsylvania: Pennsylvania State University.

Nirenberg, H.I. (1989) Identification of *Fusaria* occurring in Europe on cereals and potatoes. In: *Fusarium: Mycotoxins, Taxonomy and Pathogenicity* (Chelkowski, J., ed.). New York: Elsevier, pp. 179–193.

Nirenberg, H.I. and O'Donnell, K. (1998) New *Fusarium* 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**, 103–116.

O'Donnell, K., Cigelnik, E. and Nirenberg, H.I. (1998) Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia*, **90**, 465–493.

O'Donnell, K., Nirenberg, H.I., Aoki, T. and Cigelnik, E. (2000) A multigene phylogeny of the *Gibberella fujikuroi* species complex: Detection of additional phylogenetically distinct species. *Mycoscience*, **41**, 61–78.

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.

Snyder, W.C. and Hansen, H.N. (1945) The species concept in *Fusarium* with reference to *Discolor* and other sections. *Am. J. Bot.* **32**, 657–666.

Steenkamp, E., Britz, H., Coutinho, T., Wingfield, B., Marasas, W. and Wingfield. M. (2000a) Molecular characterization of *Fusarium subglutinans* associated with mango malformation. *Mol. Plant Pathol.* **1**, 187–193.

Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Marasas, W.F.O. and Wingfield, M.J. (1998) Histone gene sequence used to distinguish the host-specific groups of *Fusarium subglutinans*. *Proceedings of the 7th International Congress of Plant Pathology*, p. 2.2.81.

Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Wingfield, M.J. and Marasas, W.F.O. (1999) 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., Zeller, K.A., Wingfield, M.J., Marasas, W.F.O. and Leslie, J.F. (2000b) PCR-based identification of *MAT-1* and *MAT-2* in the *Gibberella fujikuroi* species complex. *Appl. Environ. Microbiol.* **66**, 4378–4382.

Swofford, D.L. (1998) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4.0*. Sunderland, MA: Sinauer Associates.

Taylor, J.W., Geiser, D.M., Burt, A. and Koufopanou, V. (1999) The evolutionary biology and population genetics underlying fungal strain typing. *Clin. Microbiol. Rev.* **12**, 126–146.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S. and Fisher, C. (2000) Phylogenetic species recognition and species concepts in fungi. *Fungal Genet. Biol.* **31**, 21–32.

Ventura, J.A., Zambolim, L. and Gilbertson, R.L. (1993) Proposição de nova forma speciales em *Fusarium subglutinans* no abacaxizeiro. *Fitopatol. Bras. Supl.* **18**, 280.

Viljoen, A., Wingfield, M.J. and Marasas, W.F.O. (1997) Characterization of *Fusarium subglutinans* f. sp. *pini* causing root disease of *Pinus patula* seedlings in South Africa. *Mycol. Res.* **101**, 437–445.

White, T.J., Bruns, T., Lee, S. and Taylor, J.W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols. A Guide to Methods and Applications* (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., eds). London: Academic Press, pp. 315–322.