CHAPTER 2

DIFFERENTIATION OF *FUSARIUM SUBGLUTINANS* F. SP. *PINI* BY HISTONE GENE SEQUENCE DATA

E.T. Steenkamp[1], T.A. Coutinho[1], B.D. Wingfield[1], M.J. Wingfield[1] and W.F.O. Marasas[2]

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[1];
PROMEC, Medical Research Council, P.O. Box 19070, Tygerberg 7505, South Africa[2].

Published: Steenkamp et al. *Applied and Environmental Microbiology* (1999) **65**(8), 3401-3406.

ABSTRACT

Fusarium subglutinans f. sp. *pini* (= *F. circinatum*) is a pathogen of pine and is one of eight mating populations (i.e., biological species) in the *Gibberella fujikuroi* species complex. This species complex includes *F. thapsinum*, *F. moniliforme*, (= *F. verticillioides*), *F. nygamai* and *F. proliferatum*, as well as *F. subglutinans* associated with sugarcane, maize, mango and pineapple. Differentiating these forms of *F. subglutinans* usually requires pathogenicity tests, which are often time-consuming and inconclusive. Our objective was to develop a technique to differentiate isolates of *F. subglutinans* f. sp. *pini* from other isolates identified as *F. subglutinans*. We sequenced the histone *H3* gene from a representative set of *Fusarium* isolates. The *H3* gene sequence was conserved and contained two introns in all the isolates studied. From both the intron and exon sequence data, we developed a PCR-RFLP technique that reliably distinguishes *F. subglutinans* f. sp. *pini* from strains associated with the other hosts, as well as the remaining biological species in the *G. fujikuroi* species complex.

INTRODUCTION

Fusarium subglutinans f. sp. *pini* is an important pathogen of pine that causes pitch canker in mature trees (6, 13) and root rot and damping off in seedlings (2, 34). This fungus can be spread by both infected seedlings and seed (1, 28). The management of *F. subglutinans* f. sp. *pini* would be greatly improved if a quick screening method were available for seed and nursery stock.

F. subglutinans f. sp. *pini* represents one of eight mating populations (i.e., biological species) in the *Gibberella fujikuroi* species complex (6, 23). Three of these mating populations, B, E and H (*F. subglutinans* f. sp. *pini*), have *F. subglutinans* anamorphs (5, 14, 19, 20). Strains of *Fusarium* isolated from pineapple (*F. subglutinans* f. sp. *ananas*) and mango, for which a teleomorph is not known, also have *F. subglutinans* anamorphs (27, 32, 33).

Distinguishing *F. subglutinans* f. sp. *pini* from the other species in the *G. fujikuroi* complex usually requires pathogenicity tests or sexual crosses with known mating tester strains (6, 7, 35). These assays are time-consuming, labor-intensive, and do not always yield clear-cut answers. Molecular tools such as random amplified polymorphic DNAs (RAPDs) (9, 35, 36), mitochondrial restriction fragment length polymorphisms (RFLPs) (7) and ribosomal DNA (rDNA) internal transcribed spacer (ITS1 and ITS2) sequences (25, 37) have been tested for their efficacy in differentiating *F. subglutinans* f. sp. *pini* from other isolates of *F. subglutinans*. Because of the technical difficulties associated with mitochondrial RFLPs and the low repeatability of RAPD data, we do not consider these techniques useful for diagnostic purposes. Furthermore, two different copies of the ITS2 region have been identified in the same isolate within some of the species in the *G. fujikuroi* complex (25, 37), and a reliable diagnostic technique based on these sequences could not be developed. Alternative regions such as the histone and β-tubulin genes might be used more effectively.

O'Donnell et al. (26) used DNA sequence of the nuclear rDNA large subunit, mitochondrial small subunit, and β-tubulin, to develop a phylogeny that includes 36 taxa in the *G. fujikuroi* species complex. These sequences may potentially be useful for diagnostics, but we began our study prior to publication of the O'Donnell et al. (26) phylogeny. We used an alternative region of the genome, the histone *H3* gene, to distinguish *F. subglutinans* f. sp. *pini* from other isolates of *F. subglutinans*.

Histone genes encode the histone proteins. These proteins are the major constituents of chromatin (16, 21) and four histone proteins make up the nucleosomal core, H2A, H2B, H3 and H4 (17). The gene encoding the H3 protein is well conserved, especially at the amino acid level (12, 31). The presence of introns enhances also its value in taxonomic and phylogenetic studies of closely related organisms (8, 38). Although the *H4* histone gene also has these characteristics, it is

generally too conserved to be suitable for evolutionary studies (30).

Our objectives in this study were (i) to sequence the histone *H3* gene from various strains in the *G. fujikuroi* species complex, (ii) to compare the relationships thus determined with those established using other sequences, and (iii) to develop a PCR-RFLP procedure, based on the histone *H3* gene sequences, for the routine identification of *F. subglutinans* f. sp. *pini*.

MATERIALS AND METHODS

Fungal isolates. All isolates were maintained on 2% (wt/vol) malt extract agar (Biolab Diagnostics Ltd., Fedlife Park, Midrand, South Africa) in the culture collections of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria, Pretoria, South Africa and the Medical Research Council at Tygerberg, South Africa. We examined 42 *Fusarium* isolates including *F. subglutinans* f. sp. *pini*, pathogenic to pine; *F. subglutinans* f. sp. *ananas*, pathogenic to pineapple; *F. subglutinans* isolates associated with maize and mango; and the mating type tester strains from all eight mating populations in the *G. fujikuroi* species complex (Table 1). To test the efficacy of the PCR-RFLP technique for use as a species diagnostic technique (see below), we tested 60 strains of the H-mating population identified by Britz et al. (5) and 80 strains representing populations A to F identified by Yan et al. (39). These strains were reassorted and then encoded so that the assays were done in a blind manner.

DNA isolation. Flasks containing 100 ml malt extract broth (2% [wt/vol]) (Biolab) were inoculated with 1-ml spore suspensions (>1,000 spores/ml). After 2 weeks of static incubation at room temperature (20 to 25°C), mycelium was harvested by filtration through Whatman no. 1 filter paper (Whatman BioSystems Ltd., Maidstone, Kent, United Kingdom). Harvested fungal tissue was ground to a powder in liquid nitrogen with a mortar and pestle and homogenized in extraction buffer, containing 5% (wt/vol) CTAB (*N*-cetyl-*N,N,N*-trimethyl-ammonium bromide), 1.4 M NaCl, 0.2% (vol/vol) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0) and 1% (wt/vol) polyvinylpyrrolidone. This homogenate was incubated at 60°C for 1 h and centrifuged (16,000 x *g*) at room temperature. We performed phenol-isoamyl alcohol-chloroform (25:1:24) extractions and removed residual phenol with an additional chloroform extraction. Nucleic acids were precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of 2-propanol, followed by incubation at 4°C overnight. Precipitated DNA was centrifuged (16,000 x *g*), washed with 70% ethanol and resuspended in deionized water. This protocol is a variation of the one developed by Murray and Thompson (22).

PCR amplification. PCR amplification was performed as described by Glass and Donaldson (12) with the primers H3-1a (5'-actaagcagaccgcccgcagg-3') and H3-1b (5'-gcgggcgagctggatgtcctt-3'). These primers were constructed to flank at least one intron and amplify approximately 450 bp of the *Neurospora crassa* histone *H3* gene. Each PCR reaction contained 1 mM deoxynucleotide triphosphates (0.25 mM of each), 2.5 mM $MgCl_2$, 0.2 μM H3-1a, 0.2 μM H3-1b, 0.25 ng/μl DNA, 0.05 U/μl Super-Therm DNA polymerase (Southern Cross Biotechnology (Pty) Ltd., Cape Town, South Africa) and 1 x Super-Therm reaction buffer. PCR reactions were overlaid with mineral oil and reactions were performed on an Omnigene thermocycler (Hybaid,

Middlesex, United Kingdom), with an initial denaturation step of 1 min at 92°C. This was followed by 30 cycles of denaturing at 92°C (1 min), annealing at 68°C (1 min), and elongation at 72°C (1 min). A final extension was performed at 72°C for 5 min.

DNA sequencing. PCR products were purified with a QIAquick PCR Purification Kit (Qiagen GmbH, Germany). Histone *H3* gene fragments from 42 *Fusarium* isolates included in this study, were sequenced (see Table 1 for GenBank accession numbers) 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, United Kingdom).

Sequence Navigator™ version 1.0.1. (Perkin Elmer, Applied BioSystems, Inc. Foster City, CA) was used for translation of DNA sequences to amino acid sequences. DNA sequences were aligned manually by inserting gaps (see Appendix 1 for aligned sequences) and phylogenetic analyses were performed with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b1 (29). Each gap was treated as a fifth character (newstate) in heuristic searches, with tree-bisection-reconnection branch swapping and MUL TREES (saving of all optimal trees) effective. Bootstrap analyses were based on 1,000 replications. *F. oxysporum* (MRC 6212) was used as an outgroup.

Sexual compatibility tests. The seven *F. subglutinans* isolates recovered from maize in South Africa (Table 1) were crossed with the mating population E tester strains and with one another in all possible pairwise combinations (5, 18). Crosses were scored as positive when ascospores were observed exuding from perithecia.

PCR-RFLP technique. Amplified DNA was digested with two restriction enzymes, *Cfo*1 and *Dde*1 (Boehringer Mannheim South Africa Pty. Ltd.). Digestions were performed consecutively by adding 5 units of *Cfo*1 to 15 µl of unpurified PCR product (3). After 3 h of incubation at 37°C, we added 5 units of *Dde*1 and adjusted the sodium chloride concentration to 100 mM. These digestion reaction mixtures were then incubated at 37°C for an additional 5 h. We resolved PCR-RFLP profiles on 3% (wt/vol) agarose gels (Promega Corporation, Madison, WI; Molecular Biology Grade agarose) containing ethidium bromide (0.2 µg/ml). Electrophoresis was performed at 3 V/cm (room temperature) with electrophoresis buffer, containing 4.5 mM Tris, 4.5 mM boric acid, and 1 mM EDTA (pH 8.0). Nucleic acids were visualized with an UV trans-illuminator (302 nm).

Verification of technique. To test the efficacy of the PCR-RFLP technique described here, histone *H3* gene PCR products from 60 strains representing mating population H and 80 strains representing the mating populations A to F were amplified, digested and electrophoresed as described above. We compared the resulting PCR-RFLP profiles to those generated from the representatives of the *G. fujikuroi* species complex.

RESULTS

DNA sequencing. The *Fusarium* histone *H3* gene fragment ranged from 519 to 527 bp in length and contained two introns (Intron 1 and Intron 2), the positions of which are conserved. Intron 1 was 83 bp in length for strains from mating population H, *F. oxysporum* and *F. subglutinans* f. sp. *ananas*; 81 bp long for mating populations C and D, as well as *F. subglutinans* isolates from mango; 85 bp long for mating populations A and G, 82 bp long for mating population E and F, as well as *F. subglutinans* isolated from maize; and 77 bp long for mating population B. Intron 2 was 57 bp long for all of the isolates, except for *F. oxysporum*, for which it was 58 bp long.

The coding regions of the *Fusarium H3* genes were highly conserved, and we observed no deletions or insertions. We detected no differences in amino acid sequence because variation within the coding sequence was generally limited to the third position within the codon. The *Fusarium* histone *H3* amino acid sequence differed from that of *N. crassa* (GenBank Accession number CAA25761) only at position 91 (A→L) (38), whereas that of *Aspergillus nidulans* (GenBank Accession number CAA39154) differed at two positions, 29 and 99 (both S→A) (10). Additionally, *N. crassa* has a single intron at the same position as the *Fusarium* Intron 2, but its sequence was quite different from that of Intron 2.

Phylogenetic analysis with PAUP 4.0b1 generated a single most parsimonious tree from 469 bp of aligned DNA sequence (Fig. 1). This tree was comprised of two distinct clades. Clade 1 included isolates from mating populations H and E, as well as isolates of *F. subglutinans* f. sp. *ananas* and *F. subglutinans* isolates from maize. The bootstrap value for this clade indicated 96% unity. Clade 2 included isolates from mating populations A, B, C, D, F and G, as well as *F. subglutinans* isolates from mango. The support for unity of this clade was 70%.

Two subgroups made up Clade 1 (Fig. 1). The first subgroup included *F. subglutinans* f. sp. *ananas*. The second subgroup included *F. subglutinans* f. sp. *pini* and isolates from mating population E, clustering together with 96% certainty. Clade 2 was subdivided into two smaller subgroups, one of which included isolates from mating populations B, C and D, as well as *F. subglutinans* isolates from mango, with 87% support. The second subgroup in Clade 2 contained isolates from mating populations A, F and G, with 71% support.

Sexual compatibility tests. Three of the *F. subglutinans* isolates associated with maize (MRC 1077, MRC 837 and MRC 714) were sexually compatible with one of the mating type tester strains for mating population E (MRC 6483). The remaining four isolates did not cross with one another or either of the tester strains.

PCR-RFLP technique. PCR-RFLP analysis of the amplified histone *H3* gene products with *Dde*1 and *Cfo*1 enabled us to distinguish *F. subglutinans* f. sp. *pini* from the rest of the isolates

included in this study (Fig. 2). Unique PCR-RFLP profiles were generated for each group included in this study, except for mating populations C and D, mating population G and *F. subglutinans* isolated from mango. From the restriction enzyme profiles we constructed restriction maps for all the host-specific groups of *F. subglutinans*, as well as *F. moniliforme*, *F. proliferatum*, *F. thapsinum* and *F. nygamai* (Fig. 3).

Verification of technique. All 60 of the H-mating population strains were positively identified as *F. subglutinans* f. sp. *pini* in a blind test of the PCR-RFLP technique. We identified none of the strains from the collection of Yan et al. (39) as *F. subglutinans* f. sp. *pini* and the expected profiles were generated for each of their representatives of mating populations A, B, E and F. The blind test on 140 samples was 100% successful, providing 95% confidence that the error rate for this test is less than 2%.

DISCUSSION

In this study, we were able to distinguish *F. subglutinans* f. sp. *pini* (mating population H) from *F. subglutinans* isolates associated with mango, maize (mating population E), sugarcane (mating population B), and pineapple, as well as *F. moniliforme* (mating populations A), *F. proliferatum* (mating populations C and D), *F. thapsinum* (mating population F) and *F. nygamai* (mating population G). The PCR-RFLP technique has been used successfully by the Tree Pathology Co-operative Programme diagnostic clinic, to identify isolates of *F. subglutinans* f. sp. *pini* for the last year. Seven outbreaks of root rot in South African nurseries have been correctly diagnosed as being caused by *F. subglutinans* f. sp. *pini* (4). We thus have confidence that this technique is robust and can be used with a high degree of certainty.

Phylogenetic analyses with the *Fusarium* histone *H3* gene sequence data generated a phylogram (Fig. 1) that was similar to those produced by O'Donnell et al. (26). The results presented here and those based on β-tubulin and mitochondrial small subunit DNA sequences (26) are similar to those obtained with isozymes (15) in two aspects. First, mating populations C and D form a closely related group in all cases. Second, mating population E is phylogenetically distinct from mating populations A, B, C, D, F, and G. There are, however, two major differences between the DNA based phylogenies and the one based on isozymes. With isozymes Huss et al. (15) showed mating populations C and D to be most closely related to mating population G. The DNA based phylogenies (26, this study), however, indicated that mating population G is most closely related to mating populations A and F, and that these three mating populations form a distinct cluster separate from both mating populations C and D. Also, in contrast with the results from the isozyme study (15), both DNA based phylogenies (26, this study) indicated that mating populations C and D are most closely related to mating population B.

F. subglutinans f. sp. *pini* has previously been reported to belong to mating population B (29), but our results and those presented by Britz et al. (5) and O'Donnell et al. (26) suggest otherwise. Nirenberg and O'Donnell (24) elevated this fungus to species level and provided the name *F. circinatum* (teleomorph = *G. circinata*) for it. Although our results are consistent with those of O'Donnell et al. (26) and support the placement of *F. subglutinans* f. sp. *pini* in a distinct taxon, the distinguishing morphological characters reported by Nirenberg and O'Donnell (24) appear to be inadequate to make definite identifications of the fungus (5).

Fusarium subglutinans f. sp. *pini*, *F. subglutinans* f. sp. *ananas*, mating population E, and *F. subglutinans* isolated from maize, are closely related to each other and are included in Clade 1. Although some of the *F. subglutinans* isolates from maize and those belonging to mating population E appeared in two separate but closely related groups, this separation is caused by only

two nucleotide base pair differences. Since some individuals from both of these groups could cross with one of the mating type E testers, we do not believe that the second cluster of isolates from maize represents a separate mating population. The overall appearance of Clade 1 corresponds to that of the so-called 'American Clade' described by O'Donnell et al. (26). This similarity suggests an equivalence of *F. subglutinans* f. sp. *pini* and *F. circinatum*, as well as *F. subglutinans* f. sp. *ananas* and *F. guttiforme*.

The two subgroups that constitute Clade 2 in our study, correspond to the 'African' and 'Asian' clades of O'Donnell et al. (26). The 'African' clade includes mating populations A, F, and G, whereas the 'Asian' clade includes mating populations B, C and D. The latter clade also includes the *F. subglutinans* isolates associated with mango, which are phylogenetically separate from *F. subglutinans* associated with maize, pineapple and pine, but phylogenetically more closely related to *F. subglutinans* from the B-mating population (Fig.1).

The results of this study and those of O'Donnell et al. (26) have identified a number of conserved genes that are useful for phylogenetic and taxonomic studies among species of *Fusarium*. The *H3* gene, as well as the β-tubulin gene, allows for a higher degree of resolution than the rDNA ITS1 and ITS2. Species, previously considered too closely related for separation into distinct groups, can now be separated based on histone or β-tubulin gene sequence. Moreover, rapid identification of fungi such as the pitch canker pathogen is now possible using a PCR-RFLP technique based on the histone *H3* gene sequence.

REFERENCES

1. **Anderson, R. L., E. Belcher, and T. Miller.** 1984. Occurrence of fungi inside slash pine seeds produced in seed orchards in the United States. Seed Sci. Technol. **12:**795-799.
2. **Barnard, E. L., and G. M. Blakeslee.** 1980. Pitch canker of slash pine seedlings: a new disease in forest tree nurseries. Plant Dis. **64:**695-696.
3. **Blanck, A., B. Glück, R. Wartbichler, S. Bender, M. Pöll, and A. Brandl.** 1997. Activity of restriction enzymes in a PCR mix. Biochemica. **3:**25.
4. **Britz, H.** Personal communication.
5. **Britz, H., T. A. Coutinho, M. J. Wingfield, W. F. O. Marasas, T. R. Gordon, and J. F. Leslie.** 1999. *Fusarium subglutinans* f. sp. *pini* represents a distinct mating population in the *Gibberella fujikuroi* species complex. Appl. Environ. Microbiol. **65:**1198-1201.
6. **Correll, J. C., T. R. Gordon, A. H. McCain, J. W. Fox, C. S. Koehler, D. L. Wood, and M. E. Schultz.** 1991. Pitch canker disease in California: pathogenicity, distribution and canker development on Monterey pine (*Pinus radiata*). Plant Dis. **75:**676-682.
7. **Correll, J. C., T. R. Gordon, and A. H. McCain.** 1992. Genetic diversity in California and Florida populations of the pitch canker fungus of *Fusarium subglutinans* f. sp. *pini*. Phytopathology. **82:**415-420.
8. **Donaldson, G. C., L. A. Ball, P. E. Axelrood, and N. L. Glass.** 1995. Primer sets developed to amplify conserved genes from filamentous ascomycetes are useful in differentiating *Fusarium* species associated with conifer. Appl. Environ. Microbiol. **61:**1331-1340.
9. **DuTeau, N. M., and J. F. Leslie.** 1991. RAPD markers for *Gibberella fujikuroi* (*Fusarium* section *Liseola*). Fungal Genet. Newsl. **38:**37.
10. **Ehinger, A., S. H. Denison, and G. S. May.** 1990. Sequence, organization and expression of the core histone genes of *Aspergillus nidulans*. Mol. Gen. Genet. **222:**416-424.
11. **Gerlach, W., and H. I. Nirenberg.** 1982. The genus *Fusarium* – A pictorial atlas. Mitt. Biol. Bundesanst. Land-Forstwirtsch. Berlin-Dahlem. **209:**1-406.
12. **Glass, N. L., and G. C. Donaldson.** 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. **61:**1323-1330.
13. **Hepting, G. H., and E. R. Roth.** 1946. Pitch canker, a new disease of some southern pines. J. Forest. **44:**742-744.
14. **Hsieh, W. H., S. N. Smith, and W. C. Snyder.** 1977. Mating groups in *Fusarium moniliforme*. Phytopathology. **67:**1041-1043.

15. **Huss, M. J., C. L. Campbell, D. B. Jennings, and J. F. Leslie.** 1996. Isozyme variation among biological species in the *Gibberella fujikuroi* species complex (*Fusarium* section *Liseola*). Appl. Environ. Microbiol. **62:**3750-3656.

16. **Igo-Kemenes, T., W. Horz, and H. G. Zachau.** 1982. Chromatin. Annu. Rev. Biochem. **51:**89-121.

17. **Isenberg, I.** 1979. Histones. Annu. Rev. Biochem. **48:**159-191.

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

19. **Kuhlman, E. G.** 1982. Varieties of *Gibberella fujikuroi* with anamorphs in *Fusarium* section *Liseola.* Mycologia. **74:**756-768.

20. **Leslie, J. F.** 1995. *Gibberella fujikuroi*: available populations and variable traits. Can. J. Bot. **73:**S282-S291.

21. **McGee, J. D., and G. Felsenfeld.** 1980. Nucleosome structure. Annu. Rev. Biochem. **49:**1115-1156.

22. **Murray, M. G., and W. F. Thompson.** 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8:**4321-4325.

23. **Nelson, P. E., T. A. Toussoun, and W. F. O. Marasas.** 1983. *Fusarium* species: An illustrated manual of identification. Pennsylvania State University Press, University Park.

24. **Nirenberg, H. I., and K. O'Donnell.** 1998. New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. Mycologia. **90:**434-458.

25. **O'Donnell, K., and E. Cigelnik.** 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. **7:**103-117.

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

27. **Rohrbach, K. G., and J. B. Pfeiffer.** 1976. Susceptibility of pineapple cultivars to fruit disease incited by *Penicillium funiculosum* and *Fusarium moniliforme*. Phytopathology **66:**1386-1390.

28. **Storer, A. J., T. R. Gordon, and S. L. Clark.** 1998. Association of the pitch canker fungus, *Fusarium subglutinans* f. sp. *pini*, with Monterey pine seeds and seedlings in California. Plant Pathol. **47:**649-656.

29. **Swofford, D. L.** 1998. PAUP Phylogenetic analysis using parsimony version 4.0b1. Sinauer Associates, Sunderland, Massachusetts.

30. **Tabata, T., S. Kimiko, and M. Iwabuchi.** 1983. The structural organization and DNA sequence of wheat H4. Nucleic Acids Res. **11:**5865-5875.

31. **Thatcher, T. H., J. MacGaffey, J. Bowen, S. Horowitz, D. L. Shapiro, and M. A. Gorovsky.** 1994. Independent evolutionary origin of histone H3.3-like variants of animals and *Tetrahymena*. Nucleic Acids Res. **22:**180-186.

32. **Varma, A., V. C. Lele, S. P. Raychaudhuri, A. Ram, and A. Sang.** 1974. Mango malformation: a fungal disease. Phytopathol. Z. **79:**254-257.

33. **Ventura, J. A., L. Zambolim, and R. L. Gilbertson.** 1993. Pathogenicity of *Fusarium subglutinans* to pineapples. Fitopatol. Brasil. Supl. **18:**280.

34. **Viljoen, A., M. J. Wingfield, and W. F. O. Marasas.** 1994. First report of *Fusarium subglutinans* f. sp. *pini* on pine seedlings in South Africa. Plant Dis. **78:**309-312.

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

36. **Voigt, K., S. Schleier, and B. Brückner.** 1995. Genetic variability in *Gibberella fujikuroi* and some related species of the genus *Fusarium* based on random amplification of polymorphic DNA (RAPD). Curr. Genet. **27:**528-535.

37. **Waalwijk, C., J. R. A. de Koning, R. P. Baayen, and W. Gams.** 1996. Discordant groupings of *Fusarium* spp. from the sections *Elegans*, *Liseola* and *Dlaminia* based on ribosomal ITS1 and ITS2 sequences. Mycologia. **88:**361-328.

38. **Woudt, L. P., A. Pastink, E. Kempers-Veenstra, A. E. M. Jansen, W. H. Mager, and R. J. Planta.** 1983. The gene encoding histone H3 and H4 in *Neurospora crassa* are unique and contain intervening sequences. Nucleic Acids Res. **11:**5347-5360.

39. **Yan, K, M. D. Dickman, J. R. Xu, and J. F. Leslie.** 1993. Sensitivity of field strains of *Gibberella fujikuroi* (*Fusarium* section *Liseola*) to benomyl and hygromycin B. Mycologia. **85:**206-213.

Table 1. Origin and host of the different *Fusarium* isolates from the *Gibberella fujikuroi* (Sawada) Wollenw. species complex used in this study.

Mating population	Species[1]	Isolate(s)[2]	Host and/or origin	Source	GenBank accession no.
A	*F. moniliforme* Sheldon	MRC 6191; KSU 0999; PEN M3703	Maize, United States	J. F. Leslie	AF150859
A	*F. moniliforme*	MRC 6155; KSU 0149; PEN M3125	Maize, United States	J. F. Leslie	AF150858
B	*F. subglutinans* (Wollenw. and Reinking) Nelson, Toussoun and Marasas	MRC 6524; KSU 3852; PEN M6865	Laboratory cross	J. F. Leslie	AF150861
B	*F. subglutinans*	MRC 6525; KSU 3853; PEN M6866	Laboratory cross	J. F. Leslie	AF150860
C	*F. proliferatum* (Matsushima) Nirenberg	MRC 6570; KSU 4921	Rice, Taiwan	J. F. Leslie	AF150873
C	*F. proliferatum*	MRC 6571; KSU 4922	Rice, Taiwan	J. F. Leslie	AF150872
D	*F. proliferatum*	MRC 6568; KSU 4853	Laboratory cross	J. F. Leslie	AF150871
D	*F. proliferatum*	MRC 6569; KSU 4854	Laboratory cross	J. F. Leslie	AF150870
E	*F. subglutinans*	MRC 6483; KSU 0990; PEN M3696	Maize, United States	J. F. Leslie	AF150845
E	*F. subglutinans*	MRC 6512; KSU 2192; PEN M3693	Maize, United States	J. F. Leslie	AF150844
F	*F. thapsinum* Klittich et al.	MRC 6536; KSU 4092;	Laboratory cross	J. F. Leslie	AF150857
F	*F. thapsinum*	MRC 6537; KSU 4093	Laboratory cross	J. F. Leslie	AF150856
G	*F. nygamai* Burgess and Trimboli	MRC 7548; KSU 5111	Laboratory cross	J. F. Leslie	AF150854
G	*F. nygamai*	MRC 7549; KSU 5112	Laboratory cross	J. F. Leslie	AF150855
H	*F. subglutinans* f. sp. *pini* Correll et al.	MRC 6209; BBA 69854	Pine, South Africa	A. Viljoen	AF150846
H	*F. subglutinans* f. sp. *pini*	MRC 6211	Pine, South Africa	A. Viljoen	AF150847
H	*F. subglutinans* f. sp. *pini*	MRC 6213	Pine, South Africa	A. Viljoen	AF150849
H	*F. subglutinans* f. sp. *pini*	MRC 6228; PEN M1290	Pine, United States	P. E. Nelson	AF150850
H	*F. subglutinans* f. sp. *pini*	MRC 7437; FL 103	Pine, United States	T. R. Gordon	AF150848
H	*F. subglutinans* f. sp. *pini*	MRC 7438	Pine, United States	A. Viljoen	AF150851
H	*F. subglutinans* f. sp. *pini*	MRC 7439; FL 15	Pine, United States	T. R. Gordon	AF150852
H	*F. subglutinans* f. sp. *pini*	MRC 7440; FSP 9	Pine, United States	T. R. Gordon	AF150853
	F. subglutinans	MRC 2730	Mango, South Africa	W. F. O. Marasas	AF150865
	F. subglutinans	MRC 3477	Mango, South Africa	W. F. O. Marasas	AF150868
	F. subglutinans	MRC 3478	Mango, South Africa	W. F. O. Marasas	AF150869
	F. subglutinans	MRC 3479	Mango, South Africa	W. F. O. Marasas	AF150867
	F. subglutinans	MRC 7034	Mango, United States	W. F. O. Marasas	AF150864
	F. subglutinans	MRC 7035	Mango, United States	W. F. O. Marasas	AF150866
	F. subglutinans	MRC 7037	Mango, United States	W. F. O. Marasas	AF150863
	F. subglutinans	MRC 7038	Mango, United States	W. F. O. Marasas	AF150862
E	*F. subglutinans*	MRC 115	Maize, South Africa	W. F. O. Marasas	AF150843
E	*F. subglutinans*	MRC 620	Maize, South Africa	W. F. O. Marasas	AF150842
E	*F. subglutinans*	MRC 714	Maize, South Africa	W. F. O. Marasas	AF150841
E	*F. subglutinans*	MRC 756	Maize, South Africa	W. F. O. Marasas	AF150839

Table 1. Continued.

Mating population	Species[1]	Isolate(s)[2]	Host and/or origin	Source	GenBank accession no.
E	*F. subglutinans*	MRC 837	Maize, South Africa	W. F. O. Marasas	AF150840
E	*F. subglutinans*	MRC 1077	Maize, South Africa	W. F. O. Marasas	AF150837
E	*F. subglutinans*	MRC 1084	Maize, South Africa	W. F. O. Marasas	AF150838
	F. subglutinans f. sp. *ananas* Ventura, Zambolim and Gilb.	MRC 6782	Pineapple, Brazil	J. A. Ventura	AF150834
	F. subglutinans f. sp. *ananas*	MRC 6783	Pineapple, Brazil	J. A. Ventura	AF150833
	F. subglutinans f. sp. *ananas*	MRC 6784	Pineapple, Brazil	J. A. Ventura	AF150836
	F. subglutinans f. sp. *ananas*	MRC 6785	Pineapple, Brazil	J. A. Ventura	AF150835
	F. oxysporum Schlecht. emend. Snyd. and Hans.	MRC 6212	Pine, South Africa	A. Viljoen	AF150832

[1] Synonyms for *F. moniliforme*, *F. subglutinans* f. sp. *pini* and *F. subglutinans* f. sp. *ananas* are *F. verticillioides* Gerlach and Nirenberg (11), *F. circinatum* Nirenberg and O'Donnell (24) and *F. guttiforme* Nirenberg and O'Donnell (24), respectively. The proposed synonyms for *F. subglutinans* from mating population B and *F. prolifertum* from mating population C are *F. sacchari* O'Donnell and Cigelnik (25) and *F. fujikuroi* Gerlach and Nirenberg (11), respectively.

[2] MRC = W. F. O. Marasas, Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, Tygerberg, South Africa; PEN = P. E. Nelson culture collection, Department of Plant Pathology, Pennsylvania State University, University Park; KSU = J. F. Leslie, Kansas State University, Department of Plant pathology, Manhattan; FL and FSP = T. R. Gordon, Department of Plant Pathology, University of California, Hutchison Hall, Davis; BBA = Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany.

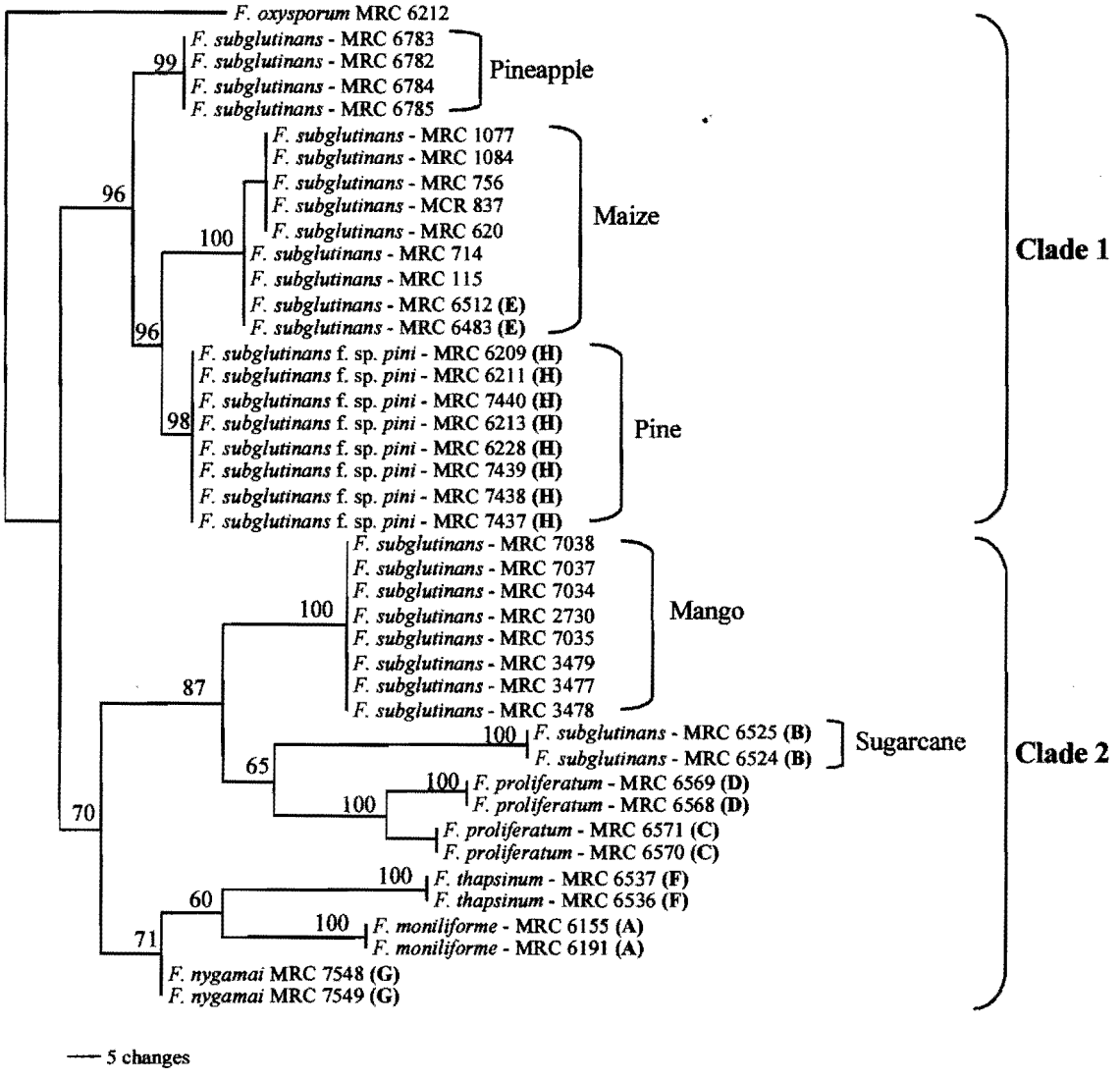

Figure 1. Phylogram generated with histone *H3* gene sequence data from the isolates included in this study using PAUP 4.01b. Bootstrap values based on 1,000 replications are indicated as percentages. Bold letters in parentheses refer to the *G. fujikuroi* mating populations. This dendogram is rooted to the *F. oxysporum* (MRC 6212). The length of the tree was 201 steps, and the values for the homoplasy index and retention index were 0.24 and 0.94, respectively.

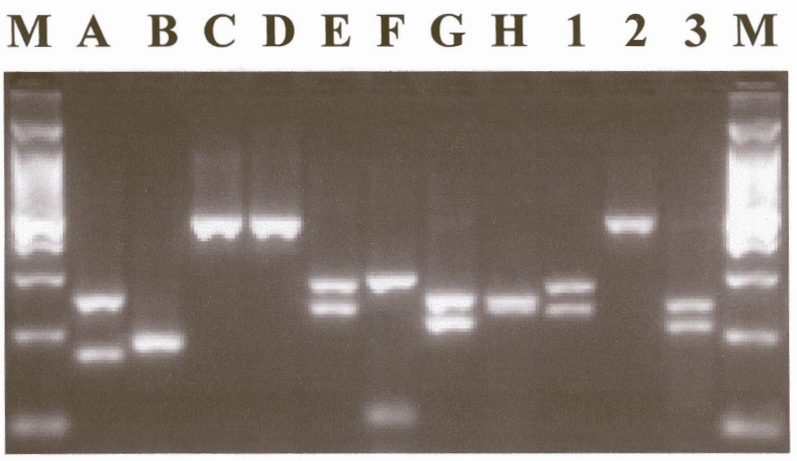

Figure 2. PCR-RFLP profiles generated by digesting the *Fusarium* histone *H3* gene amplification products with *Dde*1 and *Cfo*1. Electrophoresis was performed on 3% agarose gels at 3 V/cm. Lane M = 100-bp ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp); lane A, mating population A; lane B, mating population B (*F. subglutinans* associated with sugarcane); lane C, mating populations C; lane D, mating populations D, lane E, mating population E (*F. subglutinans* associated with maize); lane F, mating population F; lane G, mating population G; lane H, *F. subglutinans* f. sp. *pini* (mating population H); lane 1, *F. subglutinans* from maize; lane 2, *F. subglutinans* from pineapple (*F. subglutinans* f. sp. *ananas*); lane 3 = *F. subglutinans* from mango.

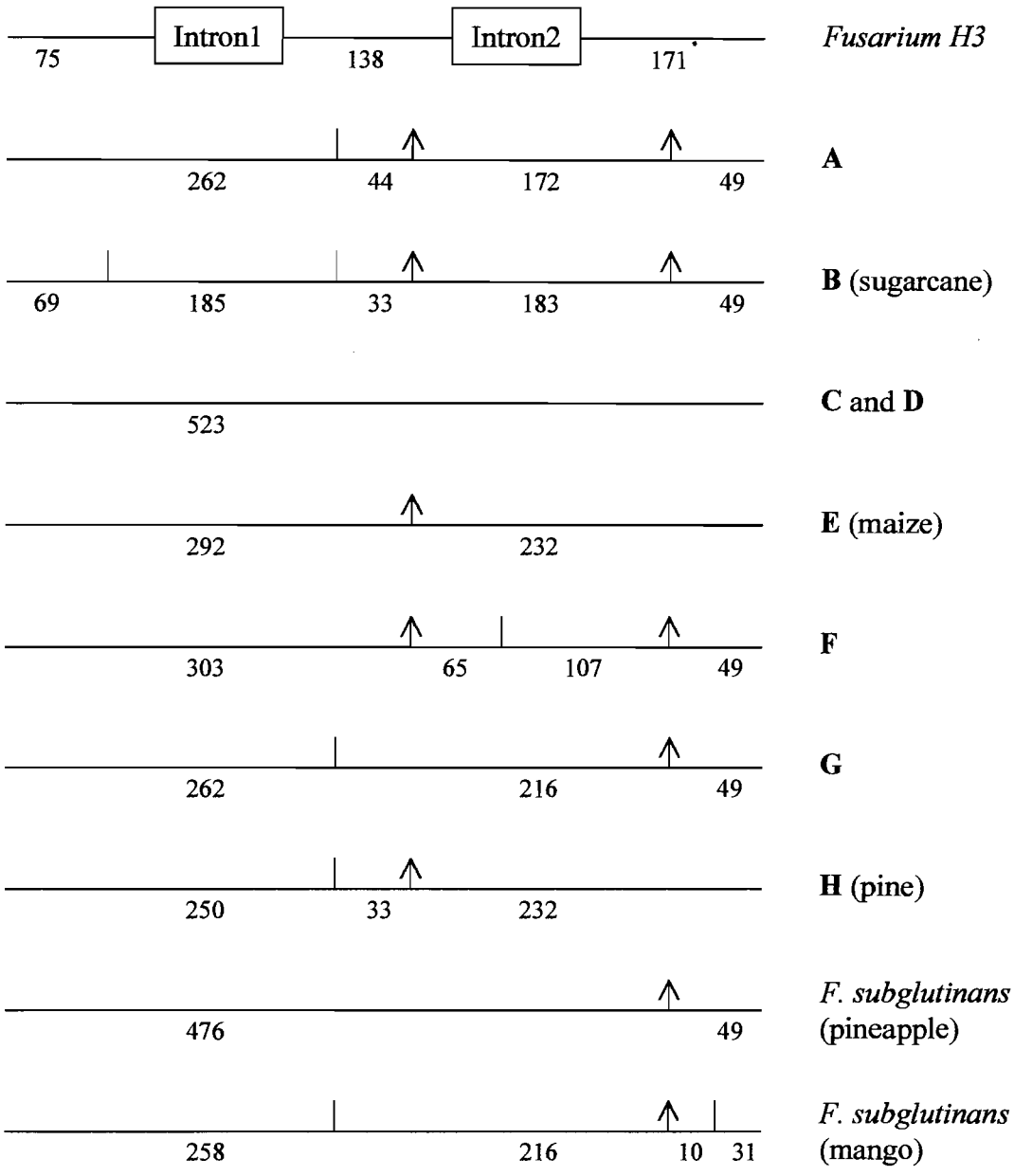

Figure 3. Restriction maps of the histone *H3* gene from the different isolates of *Fusarium*, generated with the restriction enzymes *Dde*1 and *Cfo*1. The *Fusarium* introns are indicated as boxes and the exons are indicated as lines. Bold letters refer to the *G. fujikuroi* mating populations. An arrow indicates a *Cfo*1 restriction site and a vertical line indicates a *Dde*1 restriction site. Exon and all fragment sizes are indicated as base pairs.