Characterization of the pitch canker fungus, *Fusarium circinatum*, from Chile

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***Fusarium circinatum* is the causal agent of the pine disease commonly referred to as pitch canker. During 2001, a *Fusarium* species was isolated from dying *Pinus radiata* clonal hedges in various forestry nurseries in Chile and was subsequently identified as *F. circinatum*. The aim of the study reported here was to provide a detailed characterization of Chilean isolates of the fungus. Morphological characters included microconidia carried on false heads and produced on polyphialides. Sterile coils and conidiophores on erect aerial mycelium were evident on synthetic, low nutrient agar. Furthermore, perithecia exuding viable ascospores were produced when isolates were crossed in all possible combinations with the mating tester strains representing the H mating population of *Gibberella fujikuroi* species complex. PCR–RFLP analysis of the histone H3 gene region, routinely used to distinguish between members of the *G. fujikuroi* complex, further confirmed the identification of the isolates as *F. circinatum*. DNA sequence data obtained for the same gene region placed the isolates within a well-characterized *G. circinata* clade. These studies provide unequivocal evidence that the pitch canker pathogen is well established on pines in Chilean nurseries.**

Introduction

The *Fusarium subglutinans sensu lato* complex accommodates several serious plant pathogens, including those of mango, pineapple and pine. These species are characterized by the typical *Fusarium subglutinans* (Wollenw. and Reinking) Nelson, Tousson and Marasas morphology that includes microconidia in false heads, absence of chlamydospores, and microconidia that are formed on mono- and polyphialides. Among these fungi, *Fusarium circinatum* Nirenberg and O'Donnell [*Fusarium subglutinans* (Wollenw. and Reinking) P.E. Nelson, Toussoun and Marasas f. sp. *pini* Correll *et al.*] is characterized by the presence of sterile coils and the formation of conidiophores on erect aerial mycelium.[1]

Fusarium circinatum causes a disease known as pitch canker that was first discovered in the southeastern United States.[2,3] Since then, the disease has spread to many parts of the world and has been reported from Japan, Mexico, Spain and South Africa.[4–7] *Fusarium circinatum* causes serious disease both in nurseries and on mature trees. Symptoms of infection in nurseries include damping-off and wilting of seedlings. On mature trees, pitch canker is characterized by branch die-back, stem cankers, copious pitch formation and mortality.[8–10] In South Africa, the fungus is found in nurseries[11,12] and the manifestation of pitch canker as it is known on mature trees was only recently observed.[13]

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Fusarium species residing in the *F. subglutinans* complex are morphologically similar and identification typically requires several techniques. *Fusarium circinatum* can be distinguished from other *Fusarium* species in the complex using reproductive compatibility, isozyme profiles and comparisons of DNA sequence data of various genes.[14–20] Isozyme profiles were used to distinguish mating populations of *G. fujikuroi* species complex A to G and placed *F. circinatum* in the B mating population.[18] Sexual crossing of isolates showed that *F. circinatum* represents a distinct mating population known as the H mating population in the section Liseola.[17] Sequence data obtained for the histone H3 gene, β-tubulin, mtSSU, and 28S rDNA provided unequivocal evidence that this species is distinct from the other species in the *F. subglutinans sensu lato* complex.[19,21]

In 2001, symptoms similar to those associated with *F. circinatum* infection in South African pine nurseries were observed on *P. radiata* clonal hedges in Chile.[22] These symptoms included tip die-back, and root and collar disease. The fungus responsible for the disease was identified as *F. circinatum* in a preliminary report. The aim of the study reported here was to provide a more comprehensive and unequivocal characterization of the fungus from Chile.

Materials and methods

Collection of isolates

One hundred symptomatic *P. radiata* seedlings were sampled from four different pine nurseries in Chile. Primary isolations from the diseased material were done by placing a small piece (3 mm) of pitch-soaked wood on carnation leaf agar (CLA)[23] and *Fusarium* selective medium (20.0 g agar, 15.0 g peptone, 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 1 g PCNB, 20 ml streptomycin sulphate in 1 litre of water),[24] respectively. Cultures were incubated at 25°C under cool-white fluorescent illumination. The plates were inspected regularly for fungal growth and all the colonies with typical *Fusarium* morphology were transferred to half-strength potato dextrose agar (PDA) (Merck, Germany). Single conidial isolates were stored at –70°C in 15% glycerol. Six isolates were randomly selected for further characterization.

Morphology

Characterization of the morphology of the six selected isolates (FCC 2915, 2916, 2917, 2918, 2919, 2920) was done on 10-day-old cultures grown on synthetic low-nutrient agar (SNA)[1] and CLA. Morphological features observed were compared with those characteristic of *F. circinatum*[1,5] as well as those reported in the ex-type culture of this species (MRC 7541, NRRL 25331).

Sexual compatibility

An isolate of the female tester strain of the H mating population of the *G. fujikuroi* species complex (MRC 6213, MAT1-1) was grown on carrot agar (CA),[14] while the male tester strain (MRC 7488, MAT1-2) was grown on complete medium (CM).[25] These isolates were incubated at 25°C under cool-white and dark-fluorescent light (12 hour cycles) for 7 days. Conidial suspensions of the six Chilean strains serving as males were spread over the surface of the CA plates with a glass rod. Reciprocal crosses, where the male parent was used as the female, were also made. All the crosses were repeated at least once. The crosses were examined daily for the formation of perithecia and the ascospores exuding from perithecia were collected. Ascospores from one perithecium per cross were plated on water agar (WA) and incubated for 24 h at 25°C, after which the percentage viability was determined by counting the number of germinated ascospores.

PCR amplification of the MAT-1 and MAT-2 loci, as described by Steenkamp *et al.*,[26] was used to confirm the mating types of the six isolates. The MAT idiomorphs were amplified with the primer sets GFmat1a (5′-GTTCATCAAAGGGCAAGCG-3′), GFmat1b (5′-TAAGCG CCTCTTAACGCCTTC-3′), GFmat2c (5′-AGCGTCATTATTCGATC AAG-3′) and GFmat2d (5′-CTACGTTGAGAGCTGTACAG-3′).[26] The following PCR reaction mixture was used: 1 × PCR buffer with $MgCl_2$, 200 μM of each dNTP, 0.1 μM of each primer, 25 ng template DNA and

0.5 U Roche *Taq* polymerase (Roche Pharmaceuticals, Germany). The PCR reaction conditions were an initial denaturation at 92°C for 1 min, followed by 35 cycles of denaturation at 92°C for 30 s, annealing at 63°C for 30 s and elongation at 72°C for 30 s. A final elongation step was taken at 72°C for 5 min. The products were resolved on a 1% agarose gel, containing ethidium bromide (0.2 μg/ml) and visualized under UV light.

DNA sequence comparisons

The six isolates were selected after the morphological evaluation for PCR–RFLP analysis. These isolates were grown in complete medium broth (CMB) at 25°C in the dark for 7 days. DNA was extracted using a modification of the method of Raeder and Broda.[27] Mycelium was placed in Eppendorf tubes and ground with *c.* 10 μg sterile, chemically treated sand in 500 μl DNA extraction buffer [DEB: 200 mM Tris-HCl pH 8, 150 mM NaCl, 25 mM EDTA, 0.59% SDS]. An additional 500 μl phenol and 300 μl chloroform was added, mixed and centrifuged for 30 min at 10 000 rpm. The phenol/chloroform step was repeated until the interface was clean. The supernatant was transferred to a new tube and double the volume of 100% ethanol was added and mixed. The DNA was allowed to precipitate at 4°C overnight and then pelleted by centrifugation for 30 min at 11 000 rpm. Pellets were washed with 300 μl 70% ethanol, dried and resuspended in 50 μl sterile distilled water and 3 μl RNAse (2.5 μM).

The histone H3 gene was amplified using the primer set H3-1a (5′-ACTAAGCAGACCGCCCGCAGG-3′) and H3-1b (5′-GCGGGC GAGC–TGGATGTCCTT-3′).[28] The PCR reaction mixture included 1 × PCR buffer with $MgCl_2$, 200 μM of each dNTP, 5 μM of each primer, 25 ng template DNA and 1 U Roche *Taq* polymerase (Roche Pharmaceuticals). The PCR reaction conditions were an initial denaturation at 92°C for 1 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 63°C for 1 min and elongation at 72°C for 1 min. A final elongation step was done at 72°C for 5 min.

Histone H3 PCR products obtained for the six isolates were digested with restriction enzymes *Cfo*I and *Dde*I (Roche Pharmaceuticals). Consecutive enzymatic digestion was performed by addition of 5 U *Cfo*I to 15 μl of the unpurified PCR product followed by incubation at 37°C for 3 h. Subsequently, the sodium chloride concentration was adjusted to 100 mM and 5 U of *Dde*I was added to the reaction mixture. This was followed by further incubation at 37°C for 5 h. The digested amplicons were resolved on a 2% agarose gel, containing ethidium bromide (0.2 μg/ml) and visualized under UV light.

DNA sequences of the histone amplicon were determined using an ABI 377 automated sequencer. The ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK), was used to sequence the DNA fragments. Both strands were sequenced using primers H3-1a and H3-1b. DNA sequences were manually aligned by inserting gaps, which were treated as missing data in the analysis. Analysis was done using the heuristic search option of PAUP 4.0* (Phylogenetic Analysis Using Parsimony* and Other Methods version 4.30b2a, ref. 29). The branch-swapping and tree bisection–reconnection algorithm (TBR) was used to find the most parsimonious tree. Bootstrap analysis was performed to determine the confidence levels (1000 replicates) of the branch nodes. Sequence data for closely related species, including *F. guttiforme* (MRC 6783, AF150833; MRC 6782, AF150834; MRC 6785, AF150835; MRC 6784, AF150836), *F. subglutinans* (MRC 1077, AF150837; MRC 1084, AF150838; MRC 756, AF150839; MRC 837, AF150840; MRC 714, AF150841; MRC 620, AF150842; MRC 115, AF150843; MRC 6512, AF150844; MRC 6483, AF150845), *F. circinatum* (MRC 7488, AF238478; MRC 7439, AF150852; MRC 7438, AF150851), *F. mangiferae* (MRC 7559, AF236779; MRC 3477, AF150868; MRC 7034, AF150864) and *G. fujikuroi* (MRC 6155, AF150858) were taken from previous studies and included in the analysis.

Pathogenicity

An initial trial using two of the six Chilean isolates was performed under confined laboratory conditions. Each of the two isolates was inoculated onto the stems of 20 *P. radiata* seedlings. The results of this preliminary trial showed that both isolates were pathogenic and that they did not differ significantly in their levels of virulence. Inoculations were then performed on three-year-old *P. radiata* and two-year-old *P. patula* seedlings using a single isolate (FCC 2916). These pathogenicity trials were conducted in a phytotron with daytime temperatures of approximately 25°C and night temperatures of 20°C.

Twenty trees of each species were inoculated after removing the bark with a cork borer. Mycelial plugs (6 mm in diameter) taken from actively growing fungal margins from PDA plates were placed in the wound and sealed with laboratory film (Parafilm, USA). Ten trees of each species were inoculated as controls with sterile plugs of PDA.

Trees were maintained in the phytotron for 4 weeks. Lesion lengths were measured and the statistical analysis was done by means of the *t*-test: Two-Sample Assuming Unequal Variances.[30] Re-isolations were made from all inoculation points on all plants.

Results

Isolations

All colonies that displayed typical *Fusarium* morphology on *Fusarium*-selective medium were placed on PDA. Six of these isolates were randomly selected for characterization. These selected isolates were designated the numbers FCC 2915 (PREM 58666), 2916 (PREM 58667), 2917 (PREM 58668), 2918 (PREM 58669), 2919 (PREM 58670) and 2920 (PREM 58671).

Morphological comparison

Morphological characteristics typical for *F. circinatum* were readily observed in the cultures growing on SNA medium. These characteristics included erect aerial mycelium (Fig. 1a) fusiform, 3-septate macroconidia (Fig. 1d), and microconidia (Fig. 1e) accumulating in false heads (Fig. 1b). Sterile coils (Fig. 1c) and polyphialides were also observed in cultures grown on CLA.

Sexual compatibility

After four to six weeks, perithecia (Fig. 1f) were observed in crosses between the individual tester strains of the H mating population and the six selected Chilean isolates. Five of the Chilean isolates were hermaphrodites and one isolate (FCC 2917) was female sterile. Only FCC 2917 crossed with MRC 6213 (MAT1-2) (PREM 58673), whereas the remaining five isolates crossed with MRC 7488 (MAT1-1) (PREM 58675, 58672, 58677, 58674, 58676). Ascospores from perithecia resulting from all six test isolates displayed an average of 98% viability.

The presence of both mating idiomorphs from the Chile isolates was confirmed by amplification of the MAT loci. FCC 2917 was the only isolate in which the MAT-1 idiomorph was amplified, while the MAT-2 idiomorph was amplified in isolates FCC 2915, 2916, 2918, 2919 and FCC 2920 (Fig. 2). These results confirmed the outcome of the sexual compatibility tests.

PCR–RFLP

The histone H3 gene was amplified in five of the Chilean isolates and a product of 515 bp was generated. The PCR–RFLP profiles obtained for the isolates were similar to those previously shown by Steenkamp *et al.*[21] for *F. circinatum,* and the profile obtained for the tester strain defining the H mating population (MRC 6213) (Fig. 3).

Phylogenetic analysis

Three of the Chilean isolates for which the PCR–RFLP profiles were obtained, were included in the DNA sequence analysis (DQ 364695, DQ 364696, DQ 364697). Alignment of the DNA sequences for the histone H3 gene resulted in a total of 58 parsimony-informative characters and one most parsimonious tree was generated (Fig. 4).

In the phylogenetic tree, the three Chilean isolates grouped together with the *F. circinatum* isolates in the same clade. The *F. guttiforme* and *F. mangiferae* isolates formed separate

clades. The *F. subglutinans sensu lato* isolates from maize grouped closely to, but separate from, the *G. circinata* isolates.

Pathogenicity

Lesions formed on both the *P. patula* and *P. radiata* seedlings after inoculation with isolate FCC 2916. Only very small lesions were observed on the control plants of either species**.** The lesions on the *P. patula* seedlings were smaller than those on *P. radiata.* The lesions on *P. patula* seedlings were an average of 29.9 mm (s.d. 4.5 mm) (s.e. 1.0 mm, $n = 20$) in length. The *P. radiata* seedlings had lesions of an average 109.2 mm (s.d. 36.1 mm) (s.e. 8.1 mm, $n = 20$) in length. The lesions on the control plants were an average of 0.5 mm (s.d. 0.6 mm) (s.e. 0.19 mm, $n = 10$). *Fusarium circinatum* was re-isolated from all inoculated plants and not from the plants inoculated as controls.

Discussion

This study has provided unequivocal evidence confirming the presence of the pitch canker fungus on dying *P. radiata* clonal hedge plants in Chile. A sub-set of isolates from diseased plants showed morphological characteristics typical of *F. circinatum.* The Chilean isolates were also sexually compatible with mating tester strains of *F. circinatum.* Five of the Chilean isolates considered in this study were shown to have MAT-2 mating type idiomorph, whereas one of the isolates contained the MAT-1 mating type idiomorph. Isolates from Chile also had PCR–RFLP profiles and histone H3 gene DNA sequences typical of *F. circinatum.* Furthermore, the isolates were highly pathogenic to *P. radiata* and *P. patula* seedlings, whereas control plants showed no symptoms.

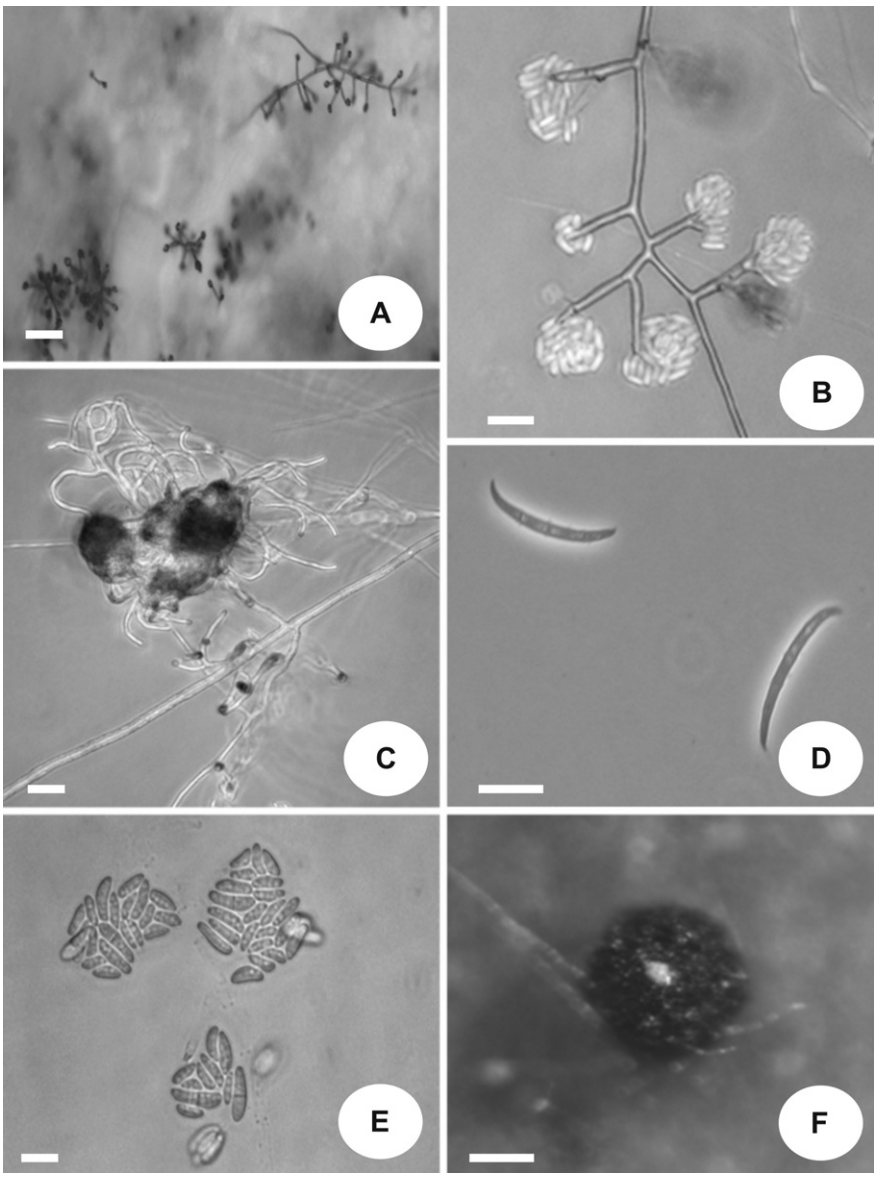

Fig. 1. Morphological characteristics of Chilean isolates used in this study. **a**, Erect conidiophores; **b**, microconidia accumulated in false heads; **c**, sterile coils; **d**, macroconidia; **e**, microconidia. Scale bars for a–e = 20 µm. **f**, Perithechium. Scale bar = 10 µm.

Several morphological characters were typical of the Chilean isolates. The presence of conidiophores on the erect aerial mycelium, polyphialides and sterile coils were the most important distinguishing features. The erect conidiophores distinguish the Chilean species from *F. subglutinans sensu stricto,* which is characterized by the formation of prostrate conidiophores on the aerial mycelium.[1,5] The Chilean isolates also formed sterile coils, which is consistent with the morphology of *F. circinatum. Fusarium pseudocircinatum* also produces sterile coils but carries its conidia in false chains.[1] No false chains were observed in *F. circinatum* isolates from Chile.

Sexual compatibility studies established that the Chilean isolates belong to the H mating population of the *G. fujikuroi* species complex. Fertile progeny were obtained when the isolates from Chile were crossed with the opposite mating tester strains. We further established that both mating types of the fungus are present in Chile. This was confirmed through amplification of the different mating idiomorphs. The presence of both MAT idiomorphs indicates that sexual reproduction could be taking place where the fungus occurs in Chile. This has

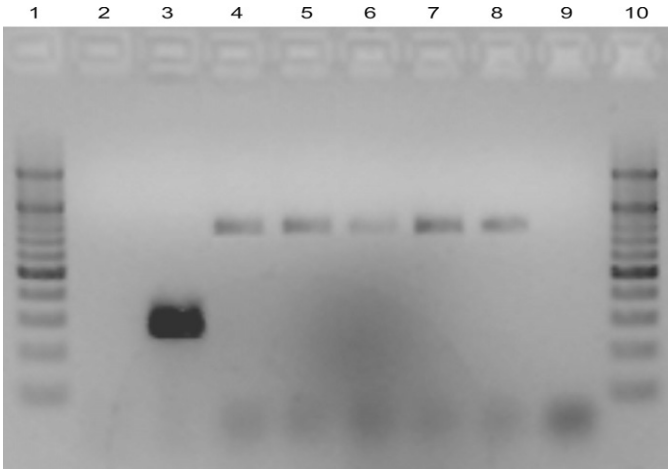

Fig. 2. Agarose gel (1%) showing the MAT-1 idiomorph amplicons from the Chilean isolates. Key to lanes: 1, 100-bp marker; 2, negative control for MAT-1 idiomorph; 3, FCC2917; 4, FCC2915; 5, FCC2916; 6, FCC2918; 7, FCC2919; 8, FCC 2920; 9, negative control for MAT-2 idiomorph; 10, 100-bp marker.

potential consequences for tree breeding programmes and the management of this pathogen. Further studies are under way to estimate the relative occurrence of the two mating types in a larger collection of Chilean isolates.

The PCR–RFLP profiles obtained for the five isolates were similar to those obtained for the tester isolate of the H mating population used in this study and illustrated by Steenkamp *et al.*[21] Using this technique, it was also possible to distinguish the Chilean isolates from *F. oxysporum,* which is a fungus commonly encountered in pine nurseries The PCR–RFLP technique can be used easily for routine confirmation of the presence of *F. circinatum.*

DNA sequence data for the histone H3 gene provided additional confirmation of the identity of the isolates obtained from diseased pine plants in Chile. In the phylogenetic analysis, the Chilean isolates grouped firmly with other *F. circinatum* isolates. Furthermore, preliminary pathogenicity tests showed clearly that *F. circinatum* isolates from Chile are highly pathogenic on *P. radiata* and *P. patula* trees. The lesions on *P. radiata* were also longer than those on *P. patula,* which is consistent with results of previous studies comparing susceptibility in these two *Pinus* species.[31]

In this study, a wide range of techniques were used to confirm the identity of *F. circinatum* isolates from Chilean nurseries. The first DNA sequence data for these isolates are presented here and we show for the first time that both mating types of the fungus are present in Chile. Furthermore, pathogenicity tests have revealed that the fungus in Chile is highly pathogenic as is the fungus elsewhere in the world. There is little doubt that it is the cause of the death of *P. radiata* clonal hedge plants and seedlings in Chile and is a pathogen that has the potential to greatly damage forestry in that country.

The presence of *F. circinatum* in Chilean forestry nurseries reflects the situation in South Africa, where the fungus was restricted to nurseries for several years after its first discovery.[11] This fungus is one of the most serious pathogens of *P. radiata* and it has the potential to damage plantations of this tree. Whether pitch canker will emerge on mature trees in Chile, as has happened recently in South Africa,[13] is open to speculation.[11,12] However, *F. circinatum*s presence in Chile, where *P. radiata* is the basis of a substantial forestry industry, is a matter for concern. Research on the disease is warranted and programmes to ensure the availability of planting stock resistant to *F. circinatum* should be encouraged.

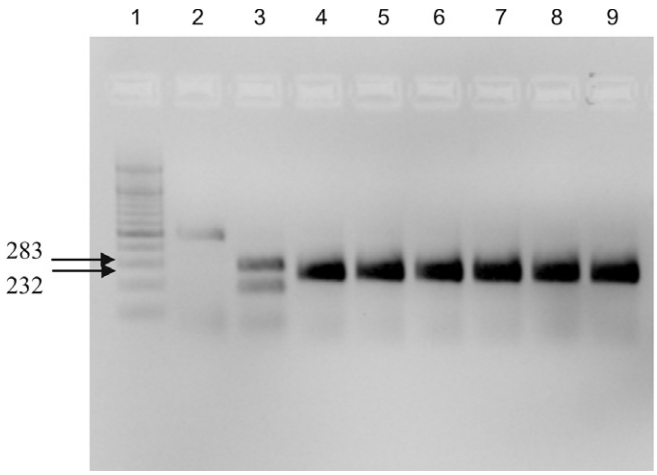

Fig. 3. Agarose gel (2%) PCR-RFLP profiles obtained for the Chilean isolates after digestion of the PCR amplicon with the restriction enzymes C*fo*I and D*de*I. The 515-bp product contains two restriction sites, yielding three fragments of 250, 232, and 33 bp. The 33-bp fragment is not visible, and the two other fragments are similar in size so as to appear as a single band in an agarose gel. The Chilean profiles are similar to that of the H mating population ex-type strain (MRC 6213). They are, however, different from the profile obtained for *F. oxysporum*. Key to lanes: 1, 100-bp marker; 2, uncut PCR amplicon; 3, *F. oxysporum*; 4, FCC2915; 5, FCC2916; 6, FCC2917; 7, FCC2918; 8, FCC 2919; 9, FCC 2920.

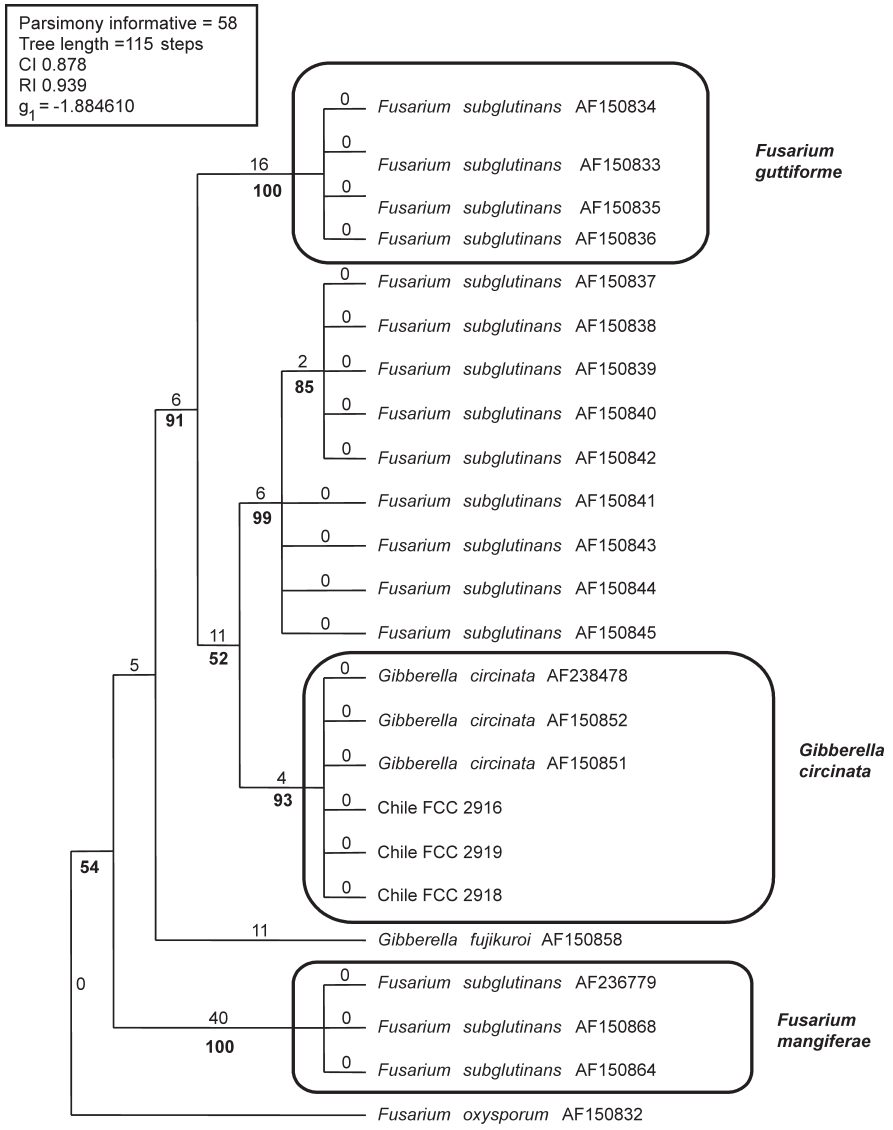

Fig. 4. Phylogenetic tree produced by PAUP heuristic option for the histone H3 gene with *Fusarium oxysporum* as the outgroup taxon. Bootstrap values above 50% (percentages of 1000 bootstrap replicates) are indicated in bold below the branches of the tree, whereas lengths are indicated above the branches.

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