CHAPTER 3

PCR-BASED IDENTIFICATION OF *MAT-1* AND *MAT-2* IN THE *GIBBERELLA FUJIKUROI* SPECIES COMPLEX

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Published: Steenkamp et al. *Applied and Environmental Microbiology* (2000) **67**(10), 4378-4382.

ABSTRACT

All sexually fertile strains of the *Gibberella fujikuroi* species complex are heterothallic. The individual mating types for these strains are conferred by the broadly conserved ascomycete idiomorphs *MAT-1* and *MAT-2*. We sequenced both alleles from all the known mating populations or biological species. From these sequences we developed a multiplex PCR technique to distinguish the *MAT-1* and *MAT-2* idiomorphs. We also tested this technique on representative strains from the eight biological species and 22 species or phylogenetic lineages in the *G. fujikuroi* complex. In most cases, either an ~800 bp fragment from *MAT-2* or a ~200 bp fragment from *MAT-1* is amplified. The amplified fragments cosegregate with mating type, as defined by sexual cross-fertility, in a cross of *Fusarium moniliforme* (= *F. verticillioides*). Neither of the primer pairs amplify fragments from *Fusarium* species such as *F. graminearum, F. pseudograminearum,* and *F. culmorum*. These species have, or are expected to have, *Gibberella* sexual stages, but are thought to be relatively distant from the species in the *G. fujikuroi* species complex. Our results further suggest that *MAT* allele sequences are useful indicators of phylogenetic relatedness in these and other *Fusarium* species.

INTRODUCTION

Fusarium isolates in the *Gibberella fujikuroi* species complex include important plant pathogens and may be divided into eight different biological species or mating populations and 32 additional asexual species or phylogenetic lineages (4, 10, 13, 15, 16). All sexually fertile species in the *G. fujikuroi* complex are dimictic, i.e., two isolates are cross-fertile if they carry the different mating type idiomorphs *MAT-1* and *MAT-2* (4, 6, 9, 13). These idiomorphs share no sequence similarity with respect to either DNA sequence or the proteins encoded (5). The *MAT-2* idiomorphs thus far characterized have a conserved HMG (high-mobility-group) domain (3, 5, 6, 19), whereas the *MAT-1* idiomorphs have a conserved α-domain (5, 25).

Our objectives in this study were (i) to sequence conserved portions of the *MAT-1* and *MAT-2* alleles from the eight known *G. fujikuroi* mating populations, (ii) to develop a multiplex PCR reaction to be used for the identification of both mating type idiomorphs within the defined biological species of the *G. fujikuroi* complex, (iii) to determine the range of *Fusarium* species that have *Gibberella* teleomorphs to which this technique can be successfully applied, and (iv) to test the use of the *MAT* idiomorph sequences for phylogenetic analyses. This technique would ease the identification of strains to be used in crosses to identify new biological species and would also eliminate the need for sexual crosses to score this trait. *MAT* sequences might also provide an additional marker for testing phylogenetic robustness.

MATERIALS AND METHODS

Fungal isolates. We examined the 16 standard mating type testers (4, 10, 13) from the eight described mating populations in the *G. fujikuroi* species complex, 128 progeny from the mapping population described by Xu and Leslie (23), and all of the strains examined by Kérenyi et al. (9). We also used 29 additional species or phylogenetic lineages; names indicated with an * are invalid (1). These strains were [Species, strain number(s)] as follows: *F. acuminatum* MRC 7681, KSU X-05020, FRC R-6666; *F. acutatum* MRC 7544, KSU X-10679, BBA 69580; *F. annulatum* MRC 2577, KSU X-03831, FRC M-1220, BBA 63629; *F. anthophilum* MRC 2578, KSU X-03818, FRC M-0854, BBA 63270; *F. avenaceum* MRC 7680, KSU X-05017, FRC R-6550; *F. begonieae**, MRC 7542, KSU X-10767, BBA 67781; *F. beomiforme* MRC 4602, KSU X-05013, FRC M-1088; *F. brevicatenulatum** MRC 7531, KSU X-10756, BBA 69197; *F. bulbicola* MRC 7534, KSU X-10759, BBA 63628; *F. concentricum** MRC 7540, KSU X-10765, BBA 64354; *F. crookwellense* MRC 2878, KSU X-04833; *F. culmorum* MRC 7682, KSU X-06576, FRC R-5626; *F. denticulatum** MRC 7538, KSU X-10763, BBA 67772; *F. dlamini* MRC 3023, KSU X-05009, FRC M-1557; *F. graminearum* (*G. zeae*) MRC 7677, KSU Z-03639; *F. guttiforme** MRC 7539, KSU X-10764 , BBA 69661; *F. lactis* MRC 7532, KSU X-10757, BBA 68590; *F. napiforme* MRC 3105, KSU X-05015, FRC M-1646; *F. nisikadoi* MRC 7533, KSU X-10758, BBA 69015; *F. oxysporum* f. sp. *cubense* MRC 7671, KSU O-02332; *F. oxysporum* f. sp. *chrysanthemi* MRC 7672, KSU O-02523, FRC O-734; *F. oxysporum* f. sp. *niveum* MRC 7673, KSU O-02529, FRC O-1087; *F. oxysporum* f sp. *radicis-lycopersici* MRC 7674, KSU O-02530, FRC O-1090; *F. oxysporum* f. sp. *vasinfectum* MRC 7675, KSU O-02533, FRC O-1139; *F. phyllophilum* MRC 2576, KSU X-03829, FRC M-1218, BBA 62262, and MRC 7543, KSU X-10768, BBA 63625; *F. pseudoanthophilum** MRC 7530, KSU X-10755, BBA 69002; *F. pseudocircinatum** MRC 7678, KSU X-04379, and MRC 7536, KSU X-10761, BBA 69636; *F. pseudograminearum* (*G. coronicola*) MRC 7670, KSU X-00629, FRC R-5210; *F. pseudonygamai** MRC 7537, KSU X-10762, BBA 69552; *F. ramigenum** MRC 7535, KSU X-10760, BBA 68592; *F. solani* MRC 7676, KSU X-03198; *F. subglutinans* (mango) MRC 7679, KSU X-04706; *F. succisae* MRC 2579, KSU X-03832, FRC M-1221 and BBA 63627. Strains were from the Medical Research Council (MRC), Tygerberg, South Africa; Kansas State University (KSU), Manhattan, Kansas; the *Fusarium* Research Center (FRC), The Pennsylvania State University, University Park, Pennsylvania; and the Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Berlin, Germany.

DNA isolation and manipulation. DNA manipulations and general molecular biology protocols followed those of Sambrook et al. (19). Fungal tissue was harvested from liquid cultures

and ground to a powder in the presence of liquid nitrogen. DNA was isolated as previously described (9, 20), resuspended in deionized water or Tris-EDTA and stored at -20°C.

PCR amplification of *MAT-2*. We examined the conserved HMG-domain and 3'-idiomorph flank of *MAT-2*, including the variable sequence between these regions. We used a previously described degenerate primer pair, NcHMG1 and NcHMG2 (3), to amplify the HMG-domain from MRC 6213. This PCR reaction mixture contained 1 ng/µl of DNA, 1 mM deoxynucleoside triphosphates (dNTPs) (0.25 mM of each), 2.5 mM $MgCl_2$, 2 µM of each primer, and 0.05 U/µl Super-Therm DNA polymerase and reaction buffer (Southern Cross Biotechnology (Pty) Ltd, Cape Town, South Africa). Reaction mixtures were overlaid with mineral oil to prevent evaporation. The initial denaturation at 92°C for 1 min was followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. A final extension was performed at 72°C for 10 min. Fragments were resolved and sized on a 2% agarose gel in 0.5 × TBE. A 300 bp fragment was excised from the gel and purified with the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), after which it was cloned into the pCR-Script™ Amp SK (+) vector from Stratagene (Stratagene Cloning Systems, La Jolla, CA). Plasmids were harvested by alkaline lysis and inserts sequenced using M13 forward and reverse primers. Based on this sequence, we designed a *G. fujikuroi*-specific primer for the 5' end of the HMG-domain, GFmat2c (5'-agcgtcattattcgatcaag-3').

To amplify the HMG-domain and a portion of the conserved 3'-idiomorph flank, we performed PCR with the primers, GFmat2c and Fo14. Fo14 (25) is part of the conserved 3'-idiomorph flank from *F. oxysporum* (GenBank Accession number AB011378). PCR conditions were the same as those described for the degenerate PCR, except that 0.2 µM of primers GFmat2c and Fo14 were used. The ~900 bp PCR products from each of the eight *G. fujikuroi MAT-2* tester strains were sequenced, and these sequences were used to design a second *G. fujikuroi*-specific *MAT-2* primer GFmat2d (5'-ctacgttgagagctgtacag-3'). GFmat2c and GFmat2d can be used to amplify an ~800 bp fragment that includes part of the conserved HMG-domain and the 3'-idiomorph flank, as well as a variable sequence between these regions. We also analyzed some strains using the GfHMG1 and GfHMG2 primers and PCR amplification conditions of Kerényi et al. (9).

PCR amplification of *MAT-1*. We used the Falpha1 and Falpha2 degenerate primers (25) to PCR amplify the *MAT-1* α-domain from the eight mating type tester strains that were not *MAT-2*. The ~200 bp PCR products from each were sequenced. Based on these sequences we constructed two specific primers, GFmat1a (5'-gttcatcaaagggcaagcg-3') and GFmat1b (5'-taagcgccctcttaacgccttc-3') that can be used to amplify a ~200 bp portion of the relatively conserved *G. fujikuroi MAT-1* α-domain.

DNA sequencing. *MAT-1* and *MAT-2* fragments were sequenced in both directions using either primers GFmat2c and Fo14, or primers Falpha1 and Falpha2. PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced by using an ABI PRISM™ 377 automated DNA sequencer and an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Warrington, United Kingdom).

We translated DNA sequence and analyzed the inferred amino acid sequence with Sequence Navigator version 1.0.1. (Perkin-Elmer Applied BioSystems, Inc. Foster City, CA). DNA sequences were manually aligned by inserting gaps (see Appendix 2 for aligned sequences), then analyzed with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0*; Sinauer Associates, Sunderland, Mass. In these analyses gaps were treated as fifth characters (newstate) in heuristic searches, with tree-bisection-reconnection branch swapping. We also performed bootstrap analyses to estimate the confidence of branching points. Trees generated in this way were rooted to *F. oxysporum MAT-1* and *MAT-2* sequences (GenBank accession numbers AB011378 and AB011379).

Diagnostic multiplex PCR for *MAT-1* and *MAT-2*. The multiplex PCR included the four primers GFmat1a, GFmat1b, GFmat2c, and GFmat2d. We optimized the reaction conditions by varying Mg^{2+} concentrations (1.5, 2.0, 2.5, and 3.0 mM), *Taq* polymerase concentration (0.35, 0.40 and 0.45 units per reaction), target DNA concentration (~100 to 20 and ~10 to 2 ng/µl), the annealing temperatures (61, 63, 65 and 67°C), and annealing times (30 or 60 s). After optimization we used the following reaction conditions (10 µl, final volume): 1× PCR buffer (Sigma, St. Louis, MO), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1µM of each of the four primers, and 0.4 units of *Taq* DNA polymerase (Sigma). We amplified PCR products according to the following program: an initial denaturation at 94°C for 1 min, followed by 35 cycles of 30 s at 92°C, 30 s at 67°C, and 30 s at 72°C. After the last cycle there was a final elongation step for 5 min at 70°C.

Blind Test Verification of Diagnostic Multiplex PCR. We examined 60 strains *G. fujikuroi* mating population H (4), and 102 strains from *G. fujikuroi* mating populations A to F (24). To demonstrate Mendelian segregation and cosegregation of the molecular markers with their corresponding mating phenotype, we examined 128 progeny of a cross between two *F. verticillioides* isolates from the A mating population (23). Prior to these analyses, all isolates were renumbered and the tests were done blind.

RESULTS

Analysis of *MAT-2*. We amplified and sequenced the *MAT-2* HMG-domain, the 3'-idiomorph flank, and the variable sequence flanked by these conserved regions, from a mating type tester representing each of the eight described *G. fujikuroi* mating populations (GenBank accession numbers AF236765 to AF236772). The sequenced portion of the HMG-domain and 3'-idiomorph flank were highly similar (> 92% nucleotide sequence similarity), whereas the variable sequences flanked by these conserved regions were relatively heterogeneous (< 88% sequence similarity). *Neurospora crassa a* (GenBank accession number M54787), *Cochliobolus heterostrophus MAT-2* (GenBank accession number X68398), and *Podospora anserina mat*$^{+}$ (GenBank accession number X64195), and all eight *MAT-2* alleles from the *G. fujikuroi* mating type tester have an intron at a conserved position within the HMG-domain (data not shown). Although there were some differences in the sequence (< 90% sequence similarity) of this intron among the *Fusarium* strains, there was no significant similarity (< 30% sequence similarity) to the intron from the other three ascomycetes. The ~800 bp *MAT-2* fragment was amplified from the "+" mating type tester strains from mating populations A, B, D, E and H, and from the "-" mating type tester strains from mating populations C, F and G.

Phylogenetic analysis based on the *MAT-2* sequence data, resulted in a single most parsimonious tree with three distinct clades (Fig. 1). The first clade included the isolates from mating populations A, F, and G; the second clade included the isolates from mating populations B, C, and D; the third clade included isolates from mating populations E and H.

Analysis of *MAT-1*. We designed a pair of primers, GFmat1a and GFmat1b, that are specific for the *MAT-1* alleles in *G. fujikuroi*. These alleles shared more than 94% nucleotide sequence similarity in their α-domains (GenBank accession numbers AF236757 to AF236764). The *G. fujikuroi MAT-1* α-domain contained an intron at a position similar to the intron in *N. crassa A* (GenBank accession number M33876), *C. heterostrophus MAT-1* (GenBank accession number X68399), and *P. anserina mat*$^{-}$ (GenBank accession number 64194). Although there was a significant amount of variation in the *G. fujikuroi* α-domain intron sequences (> 73% sequence similarity), these sequence shared little similarity (< 40% sequence similarity) with those from the other three ascomycetes. The ~200 bp *MAT-1* fragment was amplified from the "-" mating type tester strains from mating populations A, B, D, E and H, and from the "+" mating type tester strains from mating populations C, F and G.

Phylogenetic analysis of the *MAT-1* α-domain sequences resulted in a tree with three clades (Fig. 1). The composition of these clades were similar to those obtained from the *MAT-2* sequence

data in that mating populations A, F, and G, mating populations C and D, and mating populations E and H remained grouped. The sequence of the *MAT-1* α-domain from the B-mating population shared significant similarities with the α-domains in all the other mating populations, and could be basal to the other seven mating populations (Fig. 1.)

Diagnostic PCR for *MAT-1* and *MAT-2*. PCR reactions containing primers GFmat1a, GFmat1b, GFmat2c and GFmat2d, resulted in amplification of either the ~200 bp *MAT-1* or the ~800 bp *MAT-2* fragment (Fig. 2). We obtained better results when either primers GFmat1a and GFmat1b or GFmat2c and GFmat2d, were used as pairs rather than as multiplex reactions. The amount of DNA was an important variable. Results were more reproducible and there was less background with the 1:100 (~2 to 10 ng of DNA/μl) dilutions of initial DNA preparations, than with the 1:10 (~20 to 100 ng of DNA/μl) dilutions. Annealing temperatures also was an important variable. If only the *MAT-1* primers (GFmat1a and GFmat1b) were used, then a single ~200 bp product was detected at all four temperatures tested (61, 63, 65, and 67°C). If only the *MAT-2* primers (GFmat2C and GFmat2d) were used, then clear amplification of a single ~800 bp product was observed only at 65 and 67°C. Increasing the annealing time from 30 to 60 s resulted in more degenerate amplification products.

Blind tests. We tested the multiplex PCR amplification on 102 strains from mating populations A-F (24), and 60 strains from mating population H (4) and found that the amplification products detected were the same as those predicted based on the result of sexual crosses. The amplified *MAT* DNA fragments cosegregated with mating type in a genetic mapping cross (9, 24). Thus the amplified fragments map with 95% certainty to a 2.3-map unit region that includes *MAT* and are unlikely to map more than one map-unit from *MAT*, if they are not coincident with it.

***MAT* alleles in other *Fusarium* species and phylogenetic lineages.** We observed no amplification of either *MAT-1* or *MAT-2* fragments from the seven strains from species outside the *Liseola* or *Elegans* sections of the genus. These species were *F. acuminatum*, *F. avenaceum*, *F. crookwellense*, *F. culmorum*, *graminearum*, *F. pseudograminearum*, and *F. solani*. Of the five *F. oxysporum* strains, two were *MAT-1* (KSU O-02523 and O-2529) and three were *MAT-2* (KSU O-02332, O-02530 and O-02533). The *MAT-1* results were clear in the multiplex reaction. No amplification of the *MAT-2* allele was detected in the multiplex reaction, but clear bands were observed from all three strains following PCR amplification using the Kerényi et al. (9) primers. The 23 strains from the *G. fujikuroi* species complex represented 21 species or phylogenetic lineages other than the eight identified *G. fujikuroi* mating populations. We tested two isolates for each of *F. phyllophilum* and *F. pseudocircinatum*. Both *F. phyllophilum* isolates were *MAT-1*, while one *F. pseudocircinatum* isolate (KSU X-04379) was *MAT-1* and the other (KSU X-10761)

was *MAT-2*. Of the remaining 19 species, 9 were represented by a strain from which the *MAT-1* fragment could be amplified. They included *F. annulatum*, *F. anthophilum*, *F. begonieae*, *F. bulbicola*, *F. concentricum*, *F. lactis*, *F. napiforme*, *F. ramigenum*, and *F. succisae*. The other ten species were represented by a strain from which a *MAT-2* fragment could be amplified. These included *F. acutatum*, *F. beomiforme*, *F. brevicatenulatum*, *F. denticulatum*, *F. dlamini*, *F. guttiforme*, *F. nisikidoi*, *F. pseudoanthophilum*, *F. pseudonygamai*, *F. subglutinans* (mango). Representatives from all ten species also yielded a fragment when the Kerényi et al. (9) primers were used.

DISCUSSION

Kerényi et al. (9) described a primer pair that could be used to prime a PCR reaction that amplified the *MAT-2* idiomorph and they standardized the terminology for mating type in *G. fujikuroi* mating populations A to G. Covert et al. (6) identified a *MAT-2* allele in mating population H and adopted the Kerényi et al. (9) terminology. In this report we extend their results by developing primers for the α-box of the *MAT-1* idiomorph and by identifying limits on the diversity of species in which the primers will function. We developed a multiplex PCR reaction in which both *MAT-1* and *MAT-2* can be diagnosed as the positive outcome of a PCR amplification reaction without the worry that a lack of amplification, as with the Kerényi et al. (11) or Covert et al. (6) protocols, might have more than one meaning, i.e., no *MAT* sequence to amplify or *MAT-1* allele present.

We examined both of the strains from the B-mating population (MRC 6524 and MRC 6525) that Britz et al. (4) identified as, at least occasionally, homothallic. Both of these strains clearly yielded only a single product in the multiplex PCR amplification reaction. The strain MRC 6524 is *MAT-2* and strain MRC 6525 is *MAT-1*. Thus, the basis for homothallism in these strains cannot be due to mating type switching, as has been observed in some yeasts and a few filamentous fungi (7, 8, 21).

The fragment amplified by our *MAT-2* primers is larger than that of Kerényi et al. (9), ~800 bp and ~260 bp, respectively, and includes a ~560 bp region that is not a part of the conserved HMG-box. Because of the size of the *MAT-2* fragment generated with the Kerényi et al. (9) primers, it is not possible to use them in the multiplex reaction with our *MAT-1* primers. This is because the fragment sizes are similar enough to be difficult to distinguish easily on an agarose gel. Additionally, the annealing temperatures (61 and 67°C, respectively) for the Kerényi et al. (9) *MAT-2* primers and our *MAT-1* primers differ significantly. Nevertheless, the Kerényi et al. (9) primers and our primers do not differ significantly in their ability to amplify fragments from the eight identified mating populations of *G. fujikuroi*, but they do differ in their abilities to prime PCR reactions with DNA from strains of more distantly related species. For example, the Kerényi et al. (9) primers can be used to amplify a fragment from strains of *F. beomiforme* and *F. nisikadoi*. These two species are not closely related to the other species in the *G. fujikuroi* complex based on sequences from the 28S ribosomal DNA, the mitochondrial small subunit ribosomal DNA, and β-tubulin (17).

The conserved nature of the *MAT* alleles has led some to suggest their possible use in phylogenetic and taxonomic studies (21). The phylogenetic trees generated from the partial sequences of both *MAT-1* and *MAT-2* (Fig. 1) are similar to those of O'Donnell et al. (17) and

Steenkamp et al. (20, Chapter 2 of this thesis), with the exception of the placement of mating population B. The B-mating population groups with the isolates from the C- and D-mating populations based on *MAT-2* (Fig. 1), histone (21), and β-tubulin (17) DNA sequences. The partial *MAT-1* sequences, however, suggest that mating population B is approximately equally distant from the seven other mating populations (Fig. 1). Thus, the B-mating population α-domain could have resulted from a hybridization event between the α-domains of strains from the other mating populations, or it could be the basal progenitor of the α-domain in the other mating populations. To resolve this problem, a larger, and perhaps more variable, portion of the *MAT-1* idiomorph from more strains and species will have to be sequenced and analyzed.

Molecular scoring of mating type will reduce the amount of effort required to screen field populations for sexual fertility. Molecular screening should also increase the efficiency of the process through which new mating populations are identified. Diagnosis of the mating type of strains assigned to a known mating population using the PCR-based technique described here, can reduce the number of crosses needed in two ways. First, only crosses with the tester of the opposite mating type need to be made, thereby reducing the number of crosses by one half. Second, if the initial crosses are successful, then the crosses need not be repeated to confirm fertility, since the molecular diagnosis provides this confirmation.

For the identification of a new mating population, each putative member of the new mating population must be used as both a male and a female parent in crosses with all of the other putative members of that mating population to identify female-fertile strains. If a set of 60 strains is used, then 3600 crosses (60^2) are needed to test the 60 strains for the presence of female fertility at the 5% frequency level with 95% confidence. If mating type is scored molecularly, then the number of crosses that need to be made is significantly reduced. For example, if a 40:20 split at mating type is detected following PCR amplification, then only 1660 crosses would be needed.

The availability of molecular diagnostics for mating type also may enable the analysis of purportedly asexual fungi, such as *F. oxysporum*, and 12 of the 13 new *Fusarium* taxa (15, 16). There is circumstantial evidence in *F. oxysporum* for sexual reproduction in the form of high levels of diversity with respect to the multi-locus vegetative compatibility trait (see for example refs 8, 11, 12, 22), especially in populations of putatively nonpathogenic strains. Sexual reproduction need not be frequent to still play an important role in the maintenance and generation of genotypic diversity within field populations of these fungi (14), and the availability of mating type data should make it easier to identify potentially cross-fertile strains that can be used to test some of these hypotheses.

In conclusion, we developed a multiplex PCR reaction for scoring mating type within the

existing mating populations of *G. fujikuroi* that will speed the analysis of natural populations of these fungi. Additionally, *MAT-1* and *MAT-2* sequences may be useful in taxonomic and phylogenetic studies of this group of fungi, but sequences from more strains and species will need to be analyzed.

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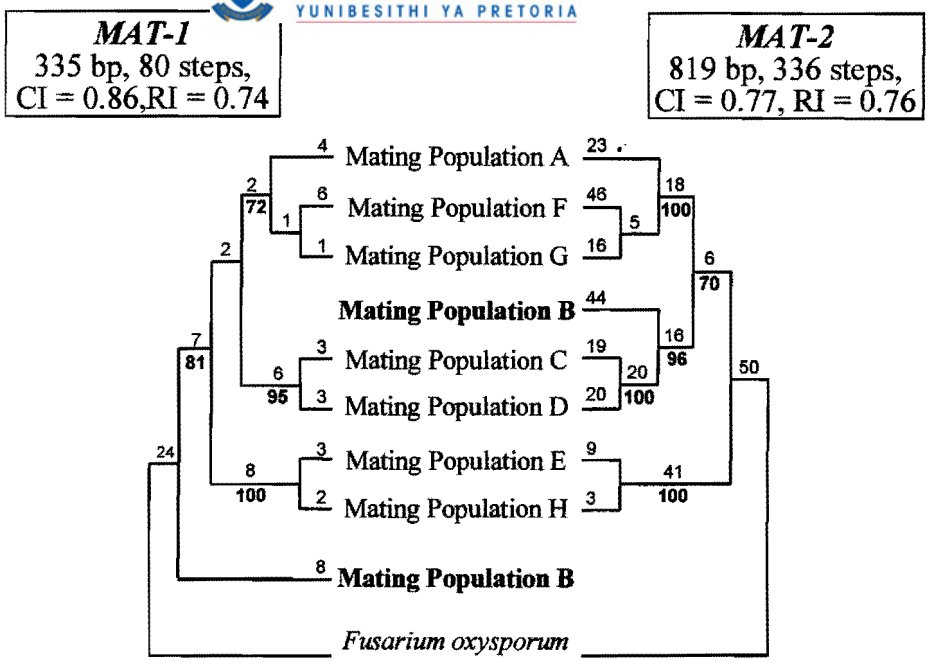

Figure 1. Phylograms generated from partial *MAT-1* (left) and *MAT-2* DNA sequence data for the eight identified mating populations (A to H) in the *G. fujikuroi* species complex. The trees were generated with PAUP and rooted to *F. oxysporum*. Bootstrap values are based on 1,000 replications and are indicated as percentages in bold below the branches. Branch lengths are indicated above the branches.

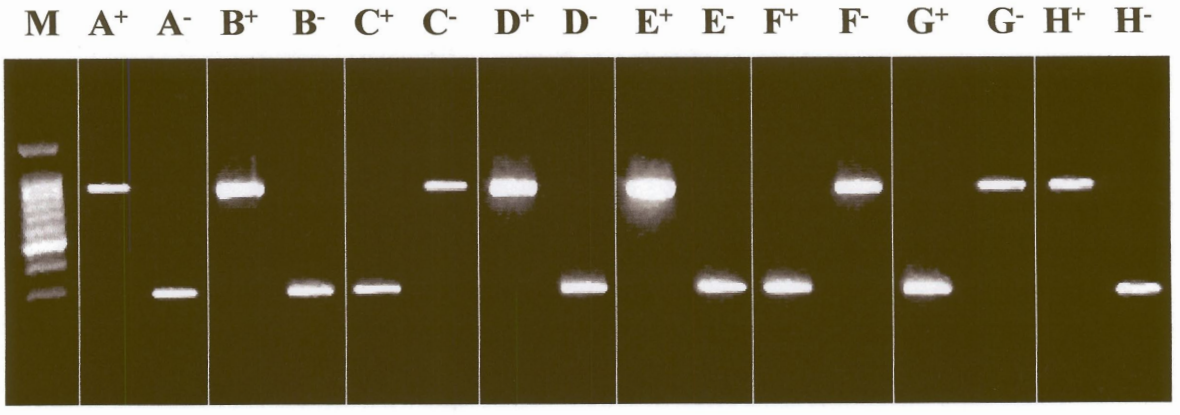

Figure 2. *PCR* amplification of the *MAT* region using the multiplex PCR described in this study. The ~800 bp fragment are amplified from the *MAT-2* regions of the *Gibberella fujikuroi* mating poputlations A to H, whereas the ~200 bp fragments are amplified from their *MAT-1* regions. Lane M, 100-bp ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp); lanes A^+, B^+, C^-, D^+, E^+, F^-, G^- and H^+, *MAT*-2 tester strains; lanes A^-, B^-, C^+, D^-, E^-, F^+, G^+ and H^-, *MAT-1* tester strains.