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Study on genetic diversity of *Gibberella moniliformis* and *G. intermedia* from corn and rice, and determination of fertility status and of mating type alleles

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Abstract

Genetic diversity, fertility status and distribution of the mating type alleles of *Gibberella moniliformis* and *G. intermedia* from corn and rice were studied. One hundred and twenty one isolates of these fungi were collected from rice growing areas of Guilan and Mazandaran provinces, and from various corn growing areas of Iran. 46 isolates of *G. moniliformis* and *G. intermedia* from rice and corn were studied by RAPD-PCR using four random primers. The isolates of *G. moniliformis* were separated from the isolates of *G. intermedia* on the basis of their hosts. Furthermore, fertility status of *G. intermedia* from corn and rice, and of *G. moniliformis* from rice were surveyed using standard tester isolates. A multiplex PCR technique was used to distinguish and amplify the fragments of the *MAT-1* and *MAT-2*. The results of the field isolates with opposite mating types that were crossed each other showed that all of the isolates were male fertile. In spite of the existence of the *MAT-1* and *MAT-2* in populations of both fungi, the sexual reproduction did not occur among field isolates. Considering male fertile of the above mentioned isolates, genetic diversity resulted from the RAPD-PCR cannot necessarily show the occurrence of the sexual reproduction.

Keywords: bakanae, *Fusarium verticilioides*, *F. proliferatum*, PCR, stalk and ear rot.
Abbreviations: AFLP- Amplified Fragment Length Polymorphism; CTAB- Cetyl Trimethyl Ammonium Bromide; EDTA-EthyleneDiamineTetraacetic Acid; HMG- High-mobility-group; IGS- Intergenic Spacer region; MAT- Mating Type allele; NTSYSpc-2.02e- Numerical taxonomy system; PDA - Potato Dextrose Agar medium; PDB- Potato Dextrose Broth medium; RAPD-Rapid Amplified Polymorphic DNA; RFLP-Restriction Fragment Length Polymorphism; UPGMA- Unweighted Pair Group Method with Arithmetic Mean.

Introduction

Gibberella fujikuroi (Sawada) Ito in Ito & Kimura is a heterothalic complex species containing at least nine genetically distinct mating populations (biological species) (Kedera et al., 1994). Species of *Fusarium* in the *Liseola* section generally belong to the different mating populations of *G. fujikuroi* (Britz et al., 1998). Strains of *G. moniliformis* Wineland are assigned to the mating population A and strains of *G. intermedia* (Kuhlman) Samuels, Nirenberg & Seifert are assigned to the mating population D (Gerlach and Nirenberg, 1982). Members of *Fusarium verticillioides* (Saccardo) Nirenberg (teleomorph: *G. moniliformis*) and *F. proliferatum* (Matsushima) Nirenberg (teleomorph: *G. intermedia)* belonging to the *Liseola* section of *Fusarium* (Nelson et al., 1983), are well-known pathogens of maize, causing stalk and ear rot (Nelson et al., 1981; Leslie et al., 1990) and foot rot or stunting of rice (bakanae) (Hsieh et al., 1977). Variability, taxonomic and evolutionary studies in microorganisms have increased with the development of molecular techniques. The Random Amplified Polymorphic DNA (RAPD) is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of the arbitrary nucleotide sequence. In *Fusarium* sp., the RAPD has been used to characterize races (Migheli et al., 1998), species (Moller et al., 1999), populations (Hering and Nirenberg, 1995) and pathogenicity (Vakalounakis and Fragkiadakis, 1999). The RAPD-PCR has also been used to assess the relationship between *Fusarium* and its host (Viljoen et al., 1997). Amoah et al. (1995) reported the genetic variability among *Fusarium verticillioides* isolates from different hosts in Ghana by DNA fingerprints detected as the RFLPs of the ribosomal DNA and the RAPDs analysis. They used the RAPD analysis to determine the mating populations of several members of the *Fusarium* in *Liseola* section (Amoah et al., 1996) and they determined differences between A, D, and F mating population members. Voigt et al. (1995) reported the genetic variability in the *G. fujikuroi* using the RAPD. On the basis of such studies, it could be said that the knowledge of the genetic structure of pathogen populations has direct agricultural applications. For instance, the genetic variation maintained within a population indicates the speed at which a pathogen evolves (McDonald and McDermott, 1993). This information might eventually be used to predict the efficacy of control measures, e.g. the use of resistant cultivars or fungicides application. The mating type in filamentous ascomycetes is usually dimictic with one locus and two functional alleles often designated as *MAT-1* and *MAT-2* following the convention proposed by Yoder et al (Kerenyi et al., 1999). The alleles are idiomorphs, which means that they share no significant sequence similarity but do map to the same position on homologous chromosomes

(Kerenyi et al., 1999; Steenkamp et al., 2000). The *MAT-2* idiomorph has a conserved HMG (high-mobility-group) domain; the *MAT-1* idiomorph has a conserved α-domain (Steenkamp et al., 2000). Fertility is at the basis of biological species concepts and sexual studies have been most important for differentiation species in the *G. fujikuroi* species complex (Leslie and Summerell, 2006). The most reliable method for distinguishing the *G. fujikuroi* biological species is in cross with standard fertile tester isolates (Leslie, 1991). Assessing the mating groups by testing the fertility of strains belonging to the *Liseola* section (Leslie, 1995), together with morphological identification are the current methods for detecting the occurrence of the *G. fujikuroi* mating groups. Hsieh et al. (1977) identified three different mating populations termed A, B and C from corn, sugarcane, and rice, respectively by using crossing method. Kuhlman (1982) extended the work of Hsieh et al. (1977) to additional strains and identified mating population D by sexual crosses. Several studies have recorded the genetic distinction of the mating populations of the *G. fujikuroi* complex species, including vegetative compatibility (Leslie, 1993), isozyme analysis (Huss, 1996) and electrophoretic karyotyping (Xu and Leslie, 1996). These methods are time consuming and labor intensive, because these species are morphological nearly similar together and it is difficult to distinguish them from each other. Therefore, there is a need for reliable and simple methods for the detection of such species using DNA techniques. Arie et al. (1997), designed degenerate PCR primers that could be used to amplify the HMG box of the *MAT-2* region of various ascomycetes including *Cryphonectria*, *Gaeumannomyces*, *Mycosphaerella*, *Nectria*, *Neorospora*, *Pyrenophora* and *Steosphaeria* and 18 species of the genus *Cochliobolus*. Kerenyi et al. (1999) described a pair of PCR primers that could be used to amplify the *MAT-2* idiomorph. Steenkamp et al. (2000) sequenced *MAT-1* and *MAT-2* alleles from eight mating populations and developed a multiplex PCR technique to distinguish these idiomorphs.
The objectives in this study were (i) to study the genetic variability within *G. moniliformis* and *G. intermedia* and between these species isolated from corn and rice as well, (ii) to determine mating type frequencies of *G. moniliformis* and *G. intermedia* isolated from corn and rice by using the cross fertility and the multiplex PCR.

Results

DNA polymorphisms detected by RAPD-PCR

A total of 67 polymorphic DNA bands between 480-1800 bp sizes and six monomorphic bands were produced by four RAPD primers (Fig.1). At least four groups were identified within 46 isolates of *G. moniliformis* and *G. intermedia* from rice and corn based on analyzing the results of the RAPD polymorphism. In fact, a dendrogram generated from UPGMA cluster analysis recognized four distinct clusters of *G. moniliformis* and *G. intermedia* isolates according to the host (rice and corn) and species (Fig.2). In other words, *G. moniliformis* and *G. intermedia* isolates from corn were separated into two distinct clusters (Fig.3A); moreover, these species from rice were divided into two distinct clusters (Fig.3B). The Similarity index was 69% between rice and corn isolates. A dendrogram obtained by *G. moniliformis* isolates from rice and corn showed that these isolates were divided into two groups according to the host (rice and corn) with five fingerprinting groups (A-E) at 80% similarity level. Fingerprinting groups A-D encompassed all isolates from corn, and fingerprinting group E included all isolates from rice (Fig.4A). Also, a dendrogram obtained by *G. intermedia* isolates from rice and corn showed that these isolates were separated into two groups according to the host (rice and corn), and three fingerprinting groups (A-C) at 80% similarity level were observed. All isolates from corn distributed in two fingerprinting groups A and B; also, fingerprinting group C included all isolates from rice (Fig.4B).

Fertility studies

The isolates of this study were verified by sexual crosses with standard tester isolates. Four to six weeks after crossing perithecia containing asci were observed on Carrot Agar culture, and asci contained eight mature two-celled ascospores (Fig.5). Out of 12 isolates of *G. intermedia* from rice seven isolates (58%) were identified as the *MAT-1* and five isolates (42%) were belonged to the *MAT-2*. Among 30 isolates of *G. intermedia* from corn, 19 isolates (63%) were identified as the *MAT-1* and 11 isolates (37%) were belonged to the *MAT-2*. Among *G. moniliformis* isolates, fertility was not identified for five isolates from rice because they did not form any perithecia when they were crossed with standard tester isolates. However, the field isolates identified as the *MAT-1* and the *MAT-2* by crossing with standard tester isolates were crossed with each other, but any perithecia did not form.

Molecular assay to determination of mating type alleles

The mating types of the isolates tested by sexual crosses identified; furthermore, they were tested by the multiplex PCR. In all cases the molecular mating type assay agreed with the biological assay. The multiplex PCR were performed by using GFmat1a, GFmat1b, Gfmat2c and Gfmat2d primers to amplify the *MAT-1* and *MAT-2* idiomorphes from the mating populations A and D. All of the isolates produced a bright band including ~ 200-bp fragment from the *MAT-1* and ~800-bp fragment from the *MAT-2* (Fig.6). Among 68 isolates of *G. moniliformis* from rice and corn, 47 isolates (69%) were identified as the *MAT-1* and 21 isolates (31%) were belonged to the *MAT-2*. Among 53 isolates of *G. intermedia* from rice and corn, 33 isolates (62%) were identified as the *MAT-1* and 20 isolates (38%) were belonged to the *MAT-2*.

Discussion

The two goals of this study were to study on the genetic diversity of *G. moniliformis* and *G. intermedia* from rice and corn in the agricultural fields of Iran using the RAPD-PCR, also assess fertility status and mating type distribution of these species by using crossing field isolates with standard tester isolates and the multiplex PCR. 46 isolates of *G. moniliformis* and *G. intermedia* from rice and corn were tested using the RAPD-PCR. The results showed that the RAPD-PCR technique was able to separate *G. moniliformis* and *G. intermedia* from rice and corn according to the host and species. In the present study, in spite of *G. moniliformis* and *G. intermedia* from rice divided into two clusters but their similarity at 85 % level was high; therefore, it has been suggested that maybe the RAPD-PCR did not be able to separate these species because of using not many numbers of isolates or primers. Maybe the other techniques, e.g. AFLP, will be able to separate these species. On the other hand, *G. moniliformis* and *G. intermedia* from corn recognized two distinct groups at 67 % similarity level, and RAPD-PCR was

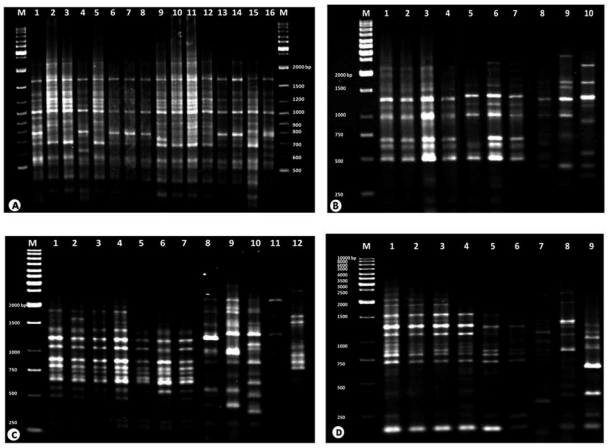

Fig1. The RAPD profiles of *G. moniliformis* and *G. intermedia* isolates from corn and rice using CS24 (A), CS29 (B), CS34 (C) and Fus01 (D) primers. M: DNA size marker (Gene Ruler^{TM} 1 kb DNA Ladder Mix).

able to distinguish these species. Assessment the isolates of *G. moniliformis* from rice and corn by using RAPD-PCR showed that this technique was able to separate this species into two distinct clusters at 64 % similarity level according to the host. These data proved that there were high genetic variation among the isolates of *G. moniliformis* from rice and corn. Also, four fingerprinting groups and 11 RAPD haplotypes that were detected from corn isolates suggested high genetic variation in *G. moniliformis*. However, One fingerprinting group and 12 RAPD haplotypes in the isolates from rice showed that genetic variation among the isolates of rice was fewer than corn. Also, consideration the isolates of *G. intermedia* from rice and corn proved that they were separated into two clusters according to the host at 69 % similarity level. Two fingerprinting groups, 11 RAPD haplotypes in the isolates from corn and one fingerprinting group, 11 RAPD haplotypes in rice demonstrated that the genetic variation in the isolates from corn was higher than rice and there were less relationship and similarity among the isolates of corn. Moretti et al. (2004), working with *F. verticillioides* from banana and corn using AFLP indicated that the isolates from banana and corn produced two distinctly different clusters. The isolates from banana showed the specific traits (toxin production, *in vitro* fertility, pathogenicity and molecular profiles), that were different to those of the isolates from corn. PCR-RFLP analysis of the IGS region of *F. verticillioides* isolates from cereals and banana allowed the identification of two distinct clusters regarding fumonisin production and host (Patino et al., 2006). Consequently, in this study RAPD-PCR revealed that there was relatively high genetic variation among two populations of *G. moniliformis* (mating population A) and *G. intermedia* (mating population D) from rice and corn. Based on these data, it seems that the isolates of *G. moniliformis* and *G. intermedia* from rice and corn are distinct each other and the RAPD-PCR was able to distinguish it. Crossing experiments showed that the isolates of mating population D (*G. intermedia*) from rice and corn were both *MAT-1* and *MAT-2* idiomorphs. The mating types segregated 7:5 *MAT-1-MAT-2* for isolates from rice and 19:11 *MAT-1-MAT-2* for isolates from corn. The mating type ratio in this study was the inverse of the ratio reported by Leslie and Klein (1996) from mating population A and by Danielsen et al. (1998). Crossing the field isolates with opposite mating types with each other showed that any perithecia did not form, so all of the isolates of this study were female-sterile or male-fertile. Leslie (1995), Leslie and Klein (1996) provided experimental data that support the hypothesis that the proportion of female-fertile isolates (hermaphrodites) in a population is an indicator of the frequency at which sexual reproduction occurs. They stated that the proportion of female-fertiles reaches its maximum following sexual reproduction and that the number of female-fertiles decreases during vegetative propagation because of either mutations or selective advantage of the female-sterile (FS) isolates. We tested on 121 isolates of mating populations A and D from rice and corn by the multiplex PCR, and found that the amplification products detected were the same as those predicted based on the results of sexual crosses. Also, the proportion of *MAT-1* was higher than *MAT-2*, that similar to the ratio reported by Chulze et al. (2000). Mating type of five isolates of *G. moniliformis* from rice was not identified by crossing with standard tester isolates and we suggested that these isolates are infertile or sterile. When we tested them by multiplex PCR, three isolates of them produced a bright band. Thus, there were DNA sequences of the *MAT* alleles in these isolates. In spite of the existence of *MAT* alleles in fungal isolate, fungal mating systems are controlled by complex molecules such as carotenes or steroids and by molecular signaling pathways that are still being unraveled (Leslie and Summerell, 2006). Maybe lack of these cases in those three isolates of *G. moniliformis* led to form sterile isolates. The results from fertility status using morphological and molecular assays showed that there are *MAT-1* and *MAT-2* among these two mating populations. These data showed that there were sexual reproduction and genetic variation among these species. Nevertheless, for sexual reproduction to occur both isolates must be in the same mating group, one isolate must carry the *MAT-1* allele and the other the *MAT-2* allele and one of the isolates is competent to act as a female parent and the other as a male parent (Leslie et al., 2006). But all of the isolates were used in current study were female-sterile or male-fertile. The results suggested that sexual reproduction did not occur frequently in the populations; therefore, genetic variation among these species may be as a result of other mechanisms; for example, mutation or parasexual cycle. If

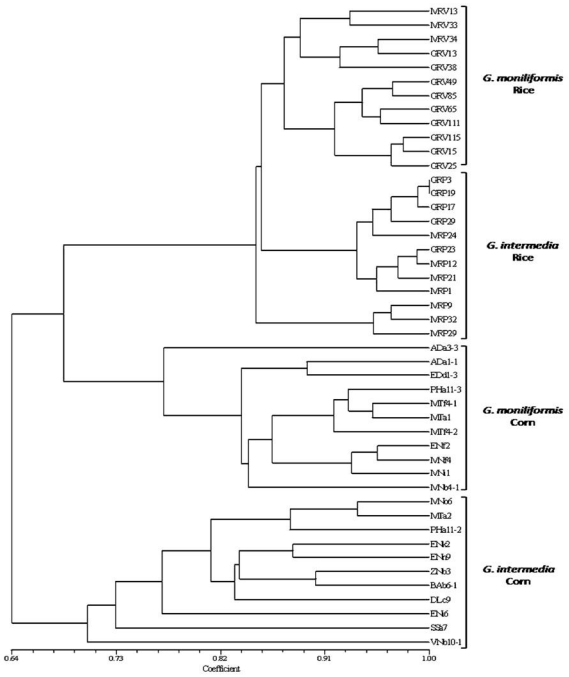

Fig 2. Dendrogram generated from the UPGMA cluster analysis for 46 isolates of *G. moniliformis* and *G. intermedia* from corn and rice using DICE similarity coefficient, showing four groups according to the host and species.

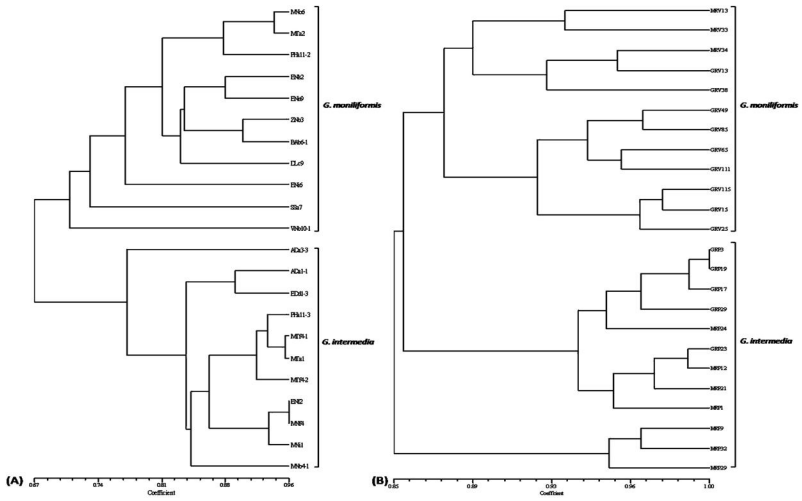

Fig 3.Dendrograms generated from UPGMA cluster analysis for *G. moniliformis* and *G. intermedia* isolates from corn (A) and rice (B) based on DICE similarity coefficient, showing two groups according to the species.

migration into the populations, for example, on the seeds planted each year, is an important source of the variation so the variation could be high even though sexual reproduction is relatively rare. Distinguishing these hypotheses would require further studies and monitory of the populations in the field.

Materials and methods

Fungal isolates

One hundred and twenty one single-spore isolates collected from the rice growing areas of Guilan and Mazandaran provinces and from the various corn growing areas of Iran during 2004- 2005 were used. 68 isolates were belonged to *G. moniliformis* (mating population A), including 14 isolates from rice and 54 isolates from corn. 53 isolates were belonged to *G. intermedia* (mating population D) including, 23 isolates from rice and 30 isolates from corn. Among 121 isolates, 46 isolates belonging to two species were analyzed for genetic relatedness by using the random amplified polymorphic DNA (RAPD). 47 isolates were examined for sexual fertility by crossing with standard tester isolates (obtained from Gordon Shephard of PROMEC, Cape town, South Africa, including 8549 (*MAT-1*) and 8550 (*MAT-2*) as standard *G. intermedia* mating-type tester strains, 8559 (*MAT-1*) and 8560 (*MAT-2*) as standard *G. moniliformis* mating-type standard strains) and determination of the mating

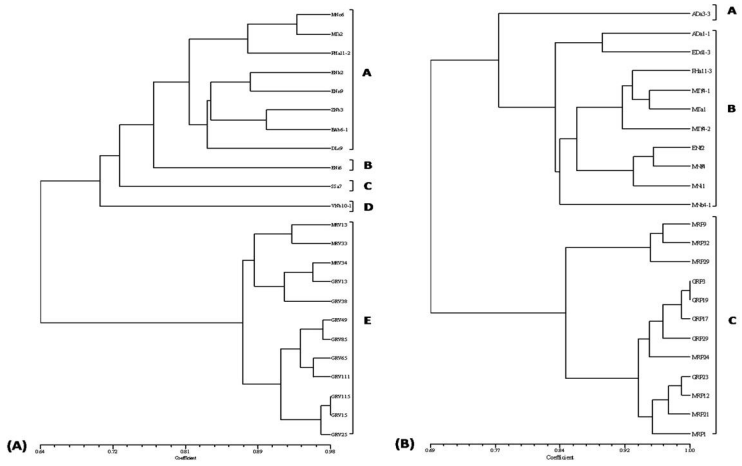

Fig 4. Dendrograms generated from UPGMA cluster analysis for *G. moniliformis* isolates from rice and corn (A) with five fingerprinting groups (A-D fingerprinting groups including corn isolates, and E including rice isolates), and *G. intermedia* isolates from rice and corn (B) with three fingerprinting groups (A and B fingerprinting groups including corn isolates, and C including rice isolates).

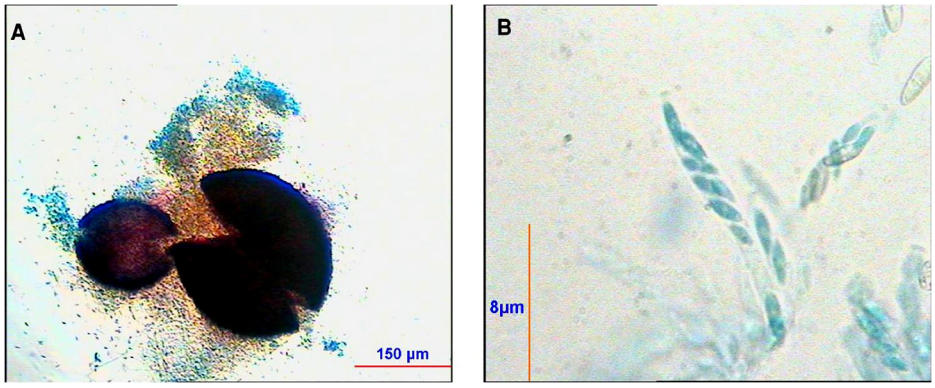

Fig 5. A) perithecia of *G. intermedia* formed from crossing between field isolates as the male parents and standard tester isolates as the female parents on the Carrot Agar culture six weeks after crossing, and oozing ascospores in the cirhhus B) asci of *G. intermedia* containing mature two-celled ascospores.

type alleles of 121 isolates were surveyed by using the multiplex-PCR.

DNA extraction

Fungal isolates were grown in PDB liquid medium (Potato Dextrose Broth) at 28-30˚C for five days. Mycelia were harvested by the vacuum filtration, washed with the sterile distilled water and pressed between the layers of paper towels to remove excess liquid. The genomic DNA was extracted by using a cetyltrimethylammonium bromide (CTAB) procedure as described by Leslie and Summerell (2006) with modification. The frozen mycelium sample (approximately 200 mg wet weight) was removed from -20˚C and ground to a fine powder with a mortar and pestle. Then, 1000 µl CTAB buffer (2% CTAB, 100 mM Tris-HCl [PH 8.0], 20 mM EDTA [PH 8.0], 1.4 M NaCl) with 1% 2-mercaptoethanol was added to sample and incubated in a water bath at 65˚C for 30 min. A half volume of phenolchloroform was added, mixed and centrifuged (10,000 × g) for 5 min. The supernatant was transferred to another tube and a half volume of absolute chloroform was added after that, the suspension was mixed and centrifuged (10,000 × g) for 5 min. The supernatant was collected and an equal volume of propanol was added and kept for 15 min at 4˚C. The aqueous layer was removed and the remaining pellet were precipitated and washed with 1 ml 70% ethanol, dried briefly and resuspended in 30 to 50 µl of deionized water and maintained in -20˚C.

RAPD analysis

Out of six primers tested, four primers CS24 (5´-GCG GCA TTG T-3´), CS29 (5´-CCA GAC AAG C-3´), CS34 (5´-GAT AGC CGA C-3´) and Fus01 (5´-CCG CAT CTA C-3´) (MWG-Biotech) (Cramer et al., 2003), which generated robust and reproducible bands were selected to characterize the 46 isolates. Reactions were performed in 20 µl volumes containing approximately 10 ng genomic DNA, 0.6 mM dNTP (made up of equal concentrations of dATP, dGTP, dTTP and dCTP), 2 µl (10 × PCR) buffers, 2.75 mM $MgCl_2$,

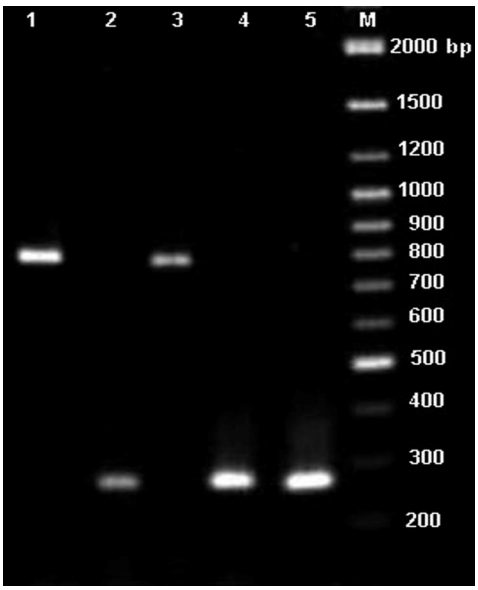

Fig 6. PCR amplification of the *MAT* locus by using multiplex PCR. The larger differential band (~800-bp) is from amplification of the *MAT-2* region [lanes 1 (*G. intermedia*) and 3 (*G. moniliformis*)], and the smaller differential band (~ 200-bp) is from amplification of the *MAT-1* region [lanes 2, 4 and 5 (*G. moniliformis*)]; M: DNA size marker (Gene Ruler^{TM} 1 kb DNA Ladder Mix).

2.5 unit of Taq DNA Polymerase and 1.5 µl primer (7.5 picomoles). The mixture was made up to final volume with sterile double distilled water. The amplification was performed in a thermal cycler (Palm cycler CG1-96) according to the following program: the initial denaturation at 94˚C for 3 min, followed by 45 cycles of 94˚C for 1 min, 34˚C for 1 min, and 72˚C for 2 min. A final extension was performed at 72˚C for 10 min. The amplification products were resolved by electrophoresis in 1.2% agarose gel and viewed over a UV light source following ethidium bromide staining. A Gen Ruler™ 1 kb DNA Ladder Mix was used as weight marker to determine the size of PCR products. RAPD bands data were analyzed by comparing RAPD profiles in terms of presence ('1') or absence ('0') of each reproducible DNA fragment. The similarity among isolates was calculated by Dice's coefficient, while UPGMA cluster analysis was performed with NTSYSpc-2.02e software.

Sexual crosses

Crosses were made on Carrot Agar as described by Leslie and Summerell (2006). The standard tester isolates were as the female parents are inoculated into Petri dishes containing Carrot Agar, and field isolates as the male parents are inoculated on a slant (6-8 ml of medium in a 16×150 mm test tube) of the PDA medium (Potato Dextrose Agar) on the same day. The isolates were allowed to grow for one week at an appropriate temperature. After seven days, the female and male parents were crossed each other and incubated at 22-23˚C with a mix of cool-white and near-UV fluorescent light bulbs. Crosses were retained for up to 6 weeks after crossing. Developing of perithecia was taken 2-4 weeks after crossing and the ascospore cirrhus usually could be observed 1-3 weeks after perithecium formation.

Mating type assay using PCR

The PCR based mating type assay for *G. moniliformis* and *G. intermedia* isolates was carried out by using four specific primers (Steenkamp et al., 2000) in the single reaction tube. GFmat1a (5′-GTTCATCAAAGGGCAAGCG-3′) and GFmat1b (5′-TAAGCGCCCTCTTAACGCCTTC-3′) were used to amplify an ~200 bp fragment of the *MAT-1* allele, and Gfmat2c (5′-AGCGTCATTATTCGATCAAG-3′) and Gfmat2d (5′-CTACGTTGAGAGCTGTACAG-3′) were used to amplify an ~800 bp fragment of the *MAT-2* allele. The PCR reaction mixture (20 µl) contained 10 ng of the fungal DNA, 2.5 mM $MgCl_2$, 0.75 mM dNTP (made up of equal concentrations of dATP, dGTP, dTTP, dCTP), 1 µl of each concentrations primer (5 picomoles), 2.5 unit of smar *Taq* DNA polymerase. PCR products were amplified according to the following program: an initial denaturation at 94˚C for 1 min, followed by 35 cycles of 1 min at 94˚C, 1 min at 58˚C and 1 min at 72˚C. After the last cycle, there was a final extension step for 10 min at 72˚C. The PCR products were resolved in 1.2 % Agarose Gel and were visualized with ethidium bromide solution and ultraviolet illumination. A Gene Ruler^{TM} 1 kb DNA Ladder Mix was used as molecular weight marker to determine the size of the PCR products.

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