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A SPECIES-SPECIFIC PCR ASSAY BASED ON THE INTERNAL TRANSCRIBED SPACER (ITS) REGIONS FOR IDENTIFICATION OF *Mycosphaerella eumusae*, *M. fijiensis* AND *M. musicola* ON BANANA

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ABSTRACT

A study on development of a rapid PCR-based detection method based on ITS region of *M. eumusae*, *M. fijiensis*, and *M. musicola* on banana was carried out. The main objecive of this study was to develop a fast and species-specific PCR-based detection method for the presence of *Mycosphaerella* species on banana. The methods include collection of specimens, morphological identification supported by molecular phylogenetic analysis, RFLP analysis, species-specific primers development, and validation. Two species of *Mycosphaerella*, namely, *M. fijiensis* and *M. musicola*, and one unidentified *Pseudocercospora* species were found in Java Island. Three restriction enzymes used in the RFLP analysis, viz, AluI, HaeIII, and TaqI were capable to discriminate *M. eumusae*, *M. fijiensis*, and *M. musicola*. Two species-specific primer pairs, viz, MfijF/MfijR and MmusF/MmusR have been successfully developed to detect the presence of *M. fijiensis* and *M. musicola*, respectively.

Key words: banana, detection, fungi, *Mycosphaerella* leaf spot, phytopathology

INTRODUCTION

Indonesia is one of banana production zones in Southeast Asia. However, crop losses from global climate change and fungal pathogens pose a serious threat not only to Indonesia, but also to global food security. Therefore, these threats should not be underestimated. Among the banana pathogens, three morphologically similar species, viz, *Mycosphaerella fijiensis* (black leaf streak disease/black Sigatoka), *M. musicola* (yellow Sigatoka disease), and *M. eumusae* (Eumusae leaf spot) are well known as important plant pathogens (Crous & Mourichon 2002). In Indonesia, these pathogens are considered as quarantine organisms (http://www.karantina.deptan.go.id/optk/

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detail.php?id=731). Therefore, it is important to prevent introduction (entry and establishment) and to limit dissemination of these pathogens in many Indonesian banana-producing regions.

Correct and rapid identification is a fundamental step for limiting the dissemination of the plant pathogens (Arzanlou *et al.* 2007). Failure to manage the pathogens would have far reaching effects on the industry. The 10-14 days incubation and classical isolation of the pathogens by culturing on appropriate media followed by morphological characters examination is a standard method currently used in Indonesia for the imported crops inspection. However, an accurate detection and diagnosis of the *M. fijiensis, M. musicola,* and *M. eumusae* based on the conventional method are complicated due to the similarity in morphological characters (Arzanlou *et al.* 2007). Consequently, this problem yield difficulties for Indonesian quarantine in inspecting imported banana seeds or crops.

Many PCR-detection methods for fungi have shown to be accurate and sensitive in detection various plant pathogens (Bonants *et al.* 1997; Mumford *et al.* 2006). ITS sequence analysis has shown that *M. fijiensis, M. musicola*, and *M. eumusae* are only distantly related in terms of phylogeny (Crous *et al.* 2002). However, the phylogeny method was still time consuming and lacked specificity to differentiate among the *M. fijiensis, M. musicola*, and *M. eumusae* (Arzanlou *et al.* 2007). The lack of specificity was possibly due to the high variability among those three pathogens. Therefore, it is necessary to develop a fast and specific PCR-based detection method with the aim of improving the specificity of the diagnostic procedure and increasing throughout readiness for outbreaks of the disease.

MATERIALS AND METHOD

Fungal materials

Fungal materials were collected from several locations in Bogor and Cibinong (West Java), and one specimen was collected from Wonosobo (Central Java). Specimens with black leaf streak diseases symptoms of *M. fijiensis* were collected during the course of field trips by using a 10×/20× magnifying lens. Specimens were kept in resealable plastic bag. The bags were labelled by adding all necessary information such as location, collector/s, collection date, host name, etc. Microscopic examination of materials was referred to Hidayat *et al.* (2007). Ascomata appearances of *Mycosphaerella* spp. and caespituli of anamorphic states (*Pseudocercospora* spp.) on the host surface were observed by using stereo microscope (OLYMPUS SZX7). Detailed observations of morphological characters was carried out by means of an OLYMPUS CX31 light microscope using oil immersion (1000×). Water and lactophenol were used as mounting media. Measurements of all important characters and photographing/line drawings were conducted at a magnification of 1000×. Single spore isolation was referred to Choi *et al.* (1999). Voucher specimens were deposited at the Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences-LIPI, Cibinong, West Java, Indonesia. Living cultures were deposited at the

LIPIMC microbial culture collection, Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences-LIPI, Cibinong, West Java, Indonesia. Fungal species found in this study were compared to isolate of the *M. eumusae*, *M. fijiensis*, and *M. musicola* obtained from CBS culture collection (Table 1).

DNA extraction and sequencing

DNA from fungal cultures was extracted using cetyltrimethylammonium bromide (CTAB) protocols (Rogers & Bendich 1994). The primers ITS1 (5'-GAAGTAAAAG TCGTAACAAG-3') and ITS4 (5'- CCTCCGCTTATTGATATGC-3') (White *et al.* 1990) were used to amplify the ITS area. The PCR reaction mixture contained 5µL DNA suspension; 2.5µL of 10×concentrated reaction buffer containing 1.5mM $MgCl_2$; 2.5µl 600µMdNTPs; 0.25µL of each primer at 60µM; 0.2µL Taq DNA polymerase (5 U/µL); 0.25µl internal control, and was filled up with MilliQ water to a final volume of 25µL. The PCR reaction was performed as follows: 1 cycle of 5 min at 94ºC followed by 40 cycles of 30s at 94ºC, 30s at 52ºC, and 30s at 72ºC. One cycle of 7 min at 72ºC was conducted. After amplification, 5µL of the reaction mixture was loaded onto a 1.0% agarose gel in 0.5×TBE buffer, separated by electrophoresis, stained with ethidium bromide, and viewed and photographed under UV light. A negative control (no DNA target) was included in every experiment to test for contamination, as well as a positive control (DNA from a reference strain of the pathogen). The amplicons was sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham, Biosciences) according to the manufacturer's recommendations. The products were analyzed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Foster City, CA). A consensus sequences were computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNAstar, Madison, WI).

Sequence alignment and phylogenetic analysis

The sequences obtained from the respective primers (ITS5 and ITS4) were aligned in Clustal X (Thomson *et al.* 1997) and Bioedit (Hall 1999). Phylogenetic analysis was performed in PAUP* (Swofford 2002). Ambiguously aligned sites were excluded from all analyses. Unweighted parsimony (UP) analysis were performed. Gaps were treated as missing data. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 1000 random taxa additions and tree bisection and reconstruction as the branch-swapping algorithm. Branches of zero length was collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1,000 bootstrap replications. Other measures calculated include tree length, consistency index, retention index, and rescaled consistency index (TL, CI, RI, and RC, respectively). The resulting phylogenetic tree was printed with TreeView version 1.6.6 (Page 1996).

Restriction Fragment Length Polymorphism (RFLP) analysis

Restriction digestion of PCR products was conducted directly without further purification with restriction endonucleases to obtain RFLPs; each sample was digested

with *Alu*I, *Hae*III, *Taq*I, or *Rsa*I in single enzyme digests. Per each 20mL restriction digest, 10 mL of unpurified, amplified PCR reaction was mixed with the appropriate restriction reaction buffer and 10 U of the appropriate enzyme and then incubated for 6h at 37°C for the *Alu*I, *Hae*III, or *Rsa*I digests or at 65°C for the *Taq*I digests. Restriction fragments were separated by electrophoresis in 2% (wt/vol) and 2.5% (wt/vol) Sepharide Gel Matrix in 1× TAE (40mM Tris acetate, 1mM sodium EDTA) with EtBr at 100 ng/mL in the gel and running buffer. DNA bands were visualized by fluorescence under UV light and photographed.

Development of specific PCR primers

Sequences obtained from ITS region were aligned with Clustal X (Thomson *et al.* 1997) dan Bioedit (Hall 1999). A series of species specific primers were designed using Vector NTI software (Invitrogen, Sigma-Aldrich), based on sequence differences among the *M. fijiensis*, *M. musicola*, and *M. eumusae*. The robustness and specificity of various primer combinations were evaluated using DNA from isolates of the *M. fijiensis*, *M. musicola*, and *M. eumusae*. DNA extraction and PCR amplification of these isolates were performed as described above.

Table 1. List of *Mycosphaerella* and *Pseudocercospora* obtained in this study.

No.	Name	Origin	Culture Collection Number
1	*Mycosphaerella musicola* (*Mycosphaerella* sp.1)	Cibinong, West Java, Indonesia	LIPIMC 0598
2	*Mycosphaerella fijiensis* (*Mycosphaerella* sp.2)	Cibalagung, West Java, Indonesia	LIPIMC 0599
3	*Mycosphaerella musicola* (*Mycosphaerella* sp.3)	Wonosobo, Central Java, Indonesia	LIPIMC 0600
4	*Mycosphaerella eumusae*	Unknown	CBS 114825
5	*Mycosphaerella fijiensis*	Cameroon	CBS 120258
6	*Mycosphaerella musicola*	Cuba	CBS 116634
7	*Pseudocercospora* sp.	Cibinong, West Java, Indonesia	LIPIMC 0601

RESULTS AND DISCUSSIONS

Fungal materials and phylogenetic analysis

Three isolates of *Mycosphaerella* and one isolate of *Pseudocercospora* were isolated from specimens collected. The cultures of *Mycosphaerella* species collected in this study were compared morphologically to the three *Mycosphaerella* species from banana obtained from CBS culture collection (Netherlands). All isolates are listed in Table 1. BLAST result from NCBI GenBank database showed that sequences of *Mycosphaerella* sp.1 has 100% similarity to the *M. musicola* (AY646445) (Fig. 1), and *Mycosphaerella* sp.2 has 99% similarity to the *M. fijiensis* (Gq169763) (Fig. 2).

The alignment data matrix of newly ITS sequences of three *Mycosphaerella* species and one *Pseudocercospora* Speg. species from banana were aligned with sequences of *Mycosphaerella*, *Cercospora* Fresen., *Pseudocercospora* and *Passalora* Fr. retrieved from NCBI GenBank DNA database. The alignment consists of 47 taxa including sequences of *Cladosporium cladosporioides* (Fresen.) G.A. de Vries and *Davidiella tassiana* (De Not.) Crous & U. Braun as outgroup. The data matrix yielded 510 total characters included in the analysis of which 320 characters were constant, 27 characters were variable and parsimony-uninformative and 137 characters were parsimony-informative. Twenty-six of the informative characters which were positioned within small insertion/deletions or ambiguous regions were excluded from the analysis. Two maximum parsimonious trees were generated from the analysis. Sum of minimum possible lengths is 232, and sum of maximum possible length was 1091. The best parsimonious tree selected by using KH test was generated in 360 steps (CI = 0. 644, RI = 0. 851, RC = 0.548, HI = 0. 356). The best phylogenetic trees obtained from unweighted maximum parsimony analysis is shown in Figure 3.

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                    ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I
                            5         15         25         35         45         55         65
Mycosphaerella_sp1  CCCTTTGTGA ACCACACCTG TTGCTTCGGG GGCGGCCCTG CCGGCGAACT CGTCGCCGGG CGCCCCCGGA
AY646445_M_musicola CCCTTTGTGA ACCACACCTG TTGCTTCGGG GGCGGCCCTG CCGGCGAACT CGTCGCCGGG CGCCCCCGGA
Clustal Consensus   ********** ********** ********** ********** ********** ********** **********

                    ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I
                           75         85         95        105        115        125        135
Mycosphaerella_sp1  GGTCTCCTTA ACACTGCATC TCTACGTCGG AGTTCCAAAC AAATCGGACA AAACTTTCAA CAACGGATCT
AY646445_M_musicola GGTCTCCTTA ACACTGCATC TCTACGTCGG AGTTCCAAAC AAATCGGACA AAACTTTCAA CAACGGATCT
Clustal Consensus   ********** ********** ********** ********** ********** ********** **********

                    ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I
                          145        155        165        175        185        195        205
Mycosphaerella_sp1  CTTGGTTCTG GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT
AY646445_M_musicola CTTGGTTCTG GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT
Clustal Consensus   ********** ********** ********** ********** ********** ********** **********

                    ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I
                          215        225        235        245        255        265        275
Mycosphaerella_sp1  CATCGAATCT TTGAACGCAC ATTGCGCCCT TTGGTATTCC GAAGGGCATG CCTGTTCGAG CGTCATTTCA
AY646445_M_musicola CATCGAATCT TTGAACGCAC ATTGCGCCCT TTGGTATTCC GAAGGGCATG CCTGTTCGAG CGTCATTTCA
Clustal Consensus   ********** ********** ********** ********** ********** ********** **********

                    ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I
                          285        295        305        315        325        335        345
Mycosphaerella_sp1  CCACTCAAGC CTAGCTTGGT ATTGGGCGCC GCGGTGCTCC GCGCGCCCCA AAGTCTCCCG GCTGAGCCGT
AY646445_M_musicola CCACTCAAGC CTAGCTTGGT ATTGGGCGCC GCGGTGCTCC GCGCGCCCCA AAGTCTCCCG GCTGAGCCGT
Clustal Consensus   ********** ********** ********** ********** ********** ********** **********

                    ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I
                          355        365        375        385        395        405        415
Mycosphaerella_sp1  CCGTCTCTAA GCGTTGTGGA TTTTTCAGTT CGCTCCGGAG TGCGGGTGGC CGCGGCCGTT AAATCTTCAA
AY646445_M_musicola CCGTCTCTAA GCGTTGTGGA TTTTTCAGTT CGCTCCGGAG TGCGGGTGGC CGCGGCCGTT AAATCTTCAA
Clustal Consensus   ********** ********** ********** ********** ********** ********** **********

                    ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I ....|....I .
                          425        435        445        455        465        475
Mycosphaerella_sp1  AGGTTGACCT CGGATCAGGT AGGGATACCC GCTGAACTTA AGCATATCAA TAAGCGGAGG A
AY646445_M_musicola AGGTTGACCT CGGATCAGGT AGGGATACCC GCTGAACTTA AGCATATCAA TAAGCGGAGG A
Clustal Consensus   ********** ********** ********** ********** ********** ********** *
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Figure 1. Pairwise alignment showed 100% similarity between *Mycosphaerella* sp.1 and *M. musicola* (Ay646445).

Based on this analysis, three major clades were performed. These included *Mycosphaerella* clade with *Pseudocercospora* anamorph (Clade I) with 61% bootstrap support. Another clade was *Mycosphaerella* and *Cercospora* anamorph (Clade II, 54% bootstrap support) which is also sister clade to *Mycosphaerella*-*Pseudocercospora* clade with 61% bootstrap support. The last clade is *Mycosphaerella* and *Passalora* anamorph (Clade III) with 90% bootstrap support. The *Mycosphaerella* sp.1 from this study nested together with species in the *M. musicola* clade with 100% bootstrap support. Another *Mycosphaerella* species from this study, *Mycosphaerella* sp.3 nested together with species within *M. fijiensis* clade with 92% bootstrap support. This information confirms the

species name of *Mycosphaerella* sp.1 as *M. musicola*, and *Mycosphaerella* sp.3 as *M. fijienssis*. Furthermore, this finding confirms that *M. fijiensis* and *M. musicola* exist in Indonesian banana plantation (Java). Another isolate, *Pseudocercospora* sp.1 needs more detailed examination as this species does not form monophyletic group with any clades in the phylogenetic tree. All *Mycosphaerella* species from banana, viz, *M. eumusae*, *M. fijiensis*, and *M musicola* form a monophyletic clade with 52% bootstrap support. This finding has shown that *Mycosphaerella* species from banana is a distinct group of species among the *Mycosphaerella* species from various hosts. It has also indicated that the three species of *Mycosphaerella* from banana are host specific to the banana trees. Further analysis such as pathogenicity test is necessary to carry out in order to justify the specificity of the three *Mycosphaerella* species from banana.

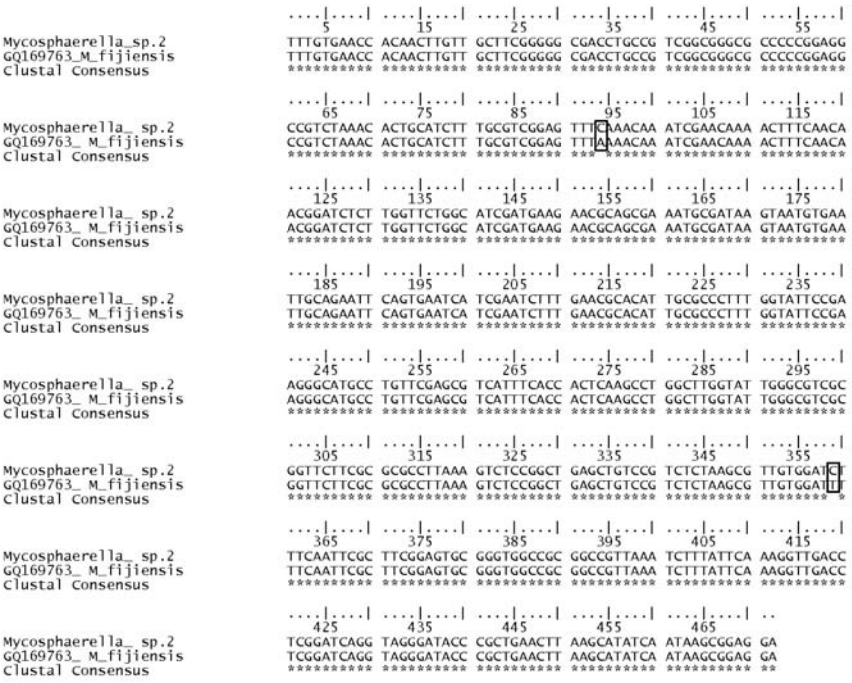

Figure 2. Pairwise alignment showed two nucleotides differences between *Mycosphaerella* sp.2 and *M. fijiensis* (GQ169763) (boxes).

Restriction Fragment Length Polymorphism (RFLP) analysis

Polymorphism of fragment size of ITS regions was recognized as reported previously in other fungal group (Gardes & Bruns 1993; Sreenivasadprasad *et al.* 1996), and it was thought to be variable in the sequences of ITS region because of nucleotide deletions and insertions. In order to identify the *Mycosphaerella* species detected by PCR using primers ITS5 and ITS4, the RFLPs of the ITS region were generated using four restriction enzymes, namely, *Alu*I, *Hae*III, *Taq*I, or *Rsa*I. From the analysis, only *Rsa*I was not very useful because it did not cut the amplicon of all *Mycosphaerella* species

from banana (Fig. 4a). Other restriction enzymes, namely, *Alu*I, *Hae*III, and *Taq*I, generated more fragment per digest, so that the ITS sequences of the *Mycosphaerella fijiensis*, *M. musicola*, and *M. eumusae* could be separated using each RFLPs profile. The *Hae*III had two recognition sites in the fragments of *M. eumusae*, and had three recognition sites in the fragments of *M. musicola* and *M. fijiensis* (Fig. 4b). One isolate of *M. musicola* can also be separated from three other isolates of *M. musicola* using the *Hae*III restriction enzyme because it only had two recognition sites in the fragments. A similar result was also found in the restriction fragments of *M. musicola* generated by *Alu*I and *TaqI* (Fig. 4c-d). The majority of RFLPs profiles generated from *Hae*III were unique for each *Mycosphaerella* species. For *M. musicola*, the given enzymes (*Alu*I and *Taq*I) probably generated RFLPs profiles which separated isolates at the subspecies level (Fig. 4c-d), but further analysis will be required to justify this result.

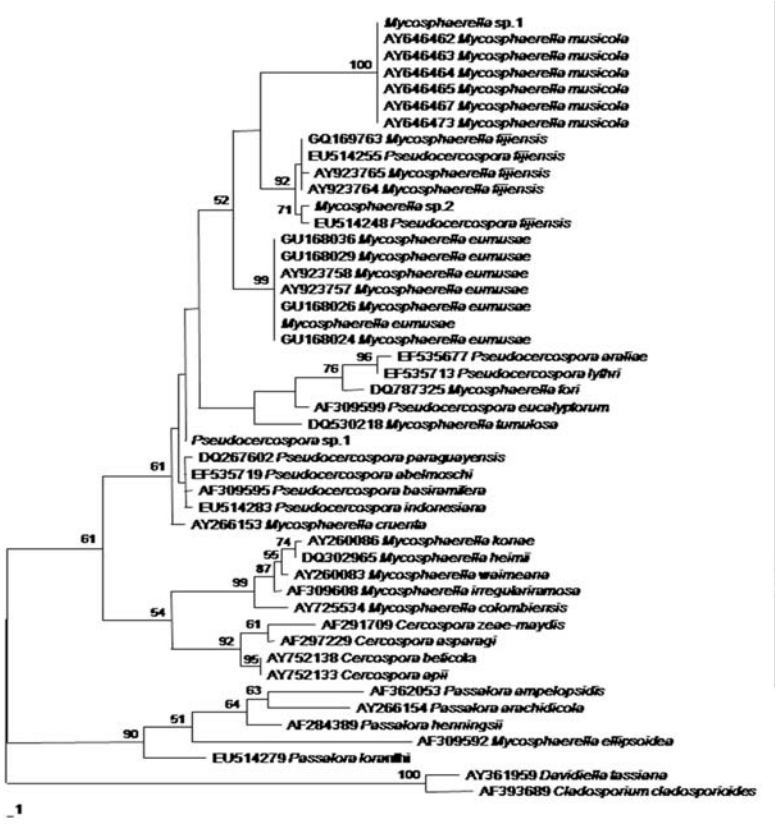

Figure 3. Single parsimonious tree based on ITS nrDNA sequence data representing placement of *Mycosphaerella* spp. and *Pseudocercospora* sp.1 found in this study within representatives of the family *Mycosphaerellaceae*. The tree is obtained from heuristic search with 1000 random taxon addition of the sequences alignment. Bootstrap values (>50%) from 1000 replicates of Unweighted Maximum Parsimony (UMP) analyses are shown above internodes.

Development of Specific PCR Primers

Understanding the banana Sigatoka disease complex is a challenge for plant pathologists (Arzanlou *et al.* 2007). Therefore, in this study we developed rapid and specific detection method with the feasibility of wide application. Even though a PCR-based detection tool has been developed previously (Johanson & Jeger 1993), those primers could only differentiate *M. fijiensis* from *M. musicola*. Three species-specific primers were designed, namely, MeuF/MeuR, MfijF/MfijR, and MmusF/MmusR, respectively (Table 2). All primers were designed to operate at relatively high annealing temperatures (54°C-55°C), thereby preventing the co-amplification of non-specific DNA targets. Primer sequences were compared against existing sequences in NCBI GenBank Data Base (http://www.ncbi.nlm.nih.gov/) and DDBJ DNA Data Base of Japan (http://www.ddbj.nig.ac.jp/), and a result of BLAST (Basic Local Alignment Search Tool) showed 100% homology of the primers with sequences of strains belonging to the species of which primers were designed. Single bands of correct size were obtained with species-specific primers from all strains belonging to the three *Mycosphaerella* species from banana.

Table 2. Primer pairs designed in this study.

No.	Name	Species target	Notes
1	MeuF (Forward) (5'-CATCTTTGCGTCGGAGTTCA-3') MeuR (Reverse) (5'-CCGAAGCGAATTGAAGAATCC-3')	*Mycosphaerella eumusae*	Not species-specific (cross reactions with *M. fijiensis*)
2	MfijF (Forward) (5'-TCTTTGCGTCGGAGTTTCA-3') MfijR (Reverse) (5'-TCCGAAGCGAATTGAAAGATC-3')	*Mycosphaerella fijiensis*	Species-specific
3	MmusF (Forward) (5'-TCCTTAACACTGCATCTCTACG-3') MmusR (Reverse) (5'-TCAGCCGGGAGACTTTGG-3')	*Mycosphaerella musicola*	Species specific

Validation assay on pure cultures of *M. eumusae*, *M. fijiensis*, and *M. musicola* showed that primer pairs of *M. fijiensis* (MfijF/MfijR) (Fig. 5a) and *M. musicola* (MmusF/MmusR) (Fig. 5b) are specific to the fungal pathogens as no cross-reactions with others *Mycosphaerella* species were observed in the amplification bands. Unfortunately, primer pairs of *M. eumusae* (MeuF/MeuR) failed to show specificity as cross-reactions were found with sequences of *M. fijiensis* (Fig. 5c). It is probably due to small nucleotide differences between DNA sequences *M. eumusae* and *M. fijiensis* from ITS region. The specificity of primer pairs of MfijF/MfijR to *M. fijiensis* and MmusF/MmusR to *M. musicola* is good indication for the development of molecular diagnosis technique and understanding of the Sigatoka diseases complex of banana in Indonesia. The molecular technique developed in this study may also significantly contribute to plant quarantine because of its reliability, specificity and simplicity. This assay could be done within 1-2 days laboratory works and analysis.

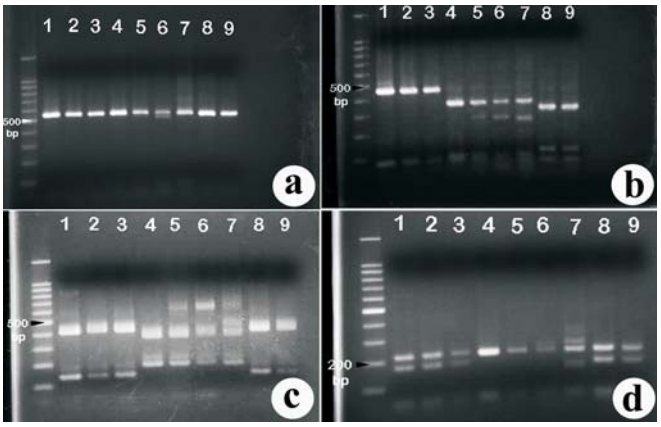

Figure 4. Restriction patterns of internal transcribed spacer (ITS) regions of ribosomal DNA amplified from three *Mycosphaerella* species from banana. a. *Rsa*I b. *Hae*III c. *Alu*I d. *Taq*I (1-3: *M. eumusae*; 4-7: *M. musicola*; 8-9: *M. fijiensis*).

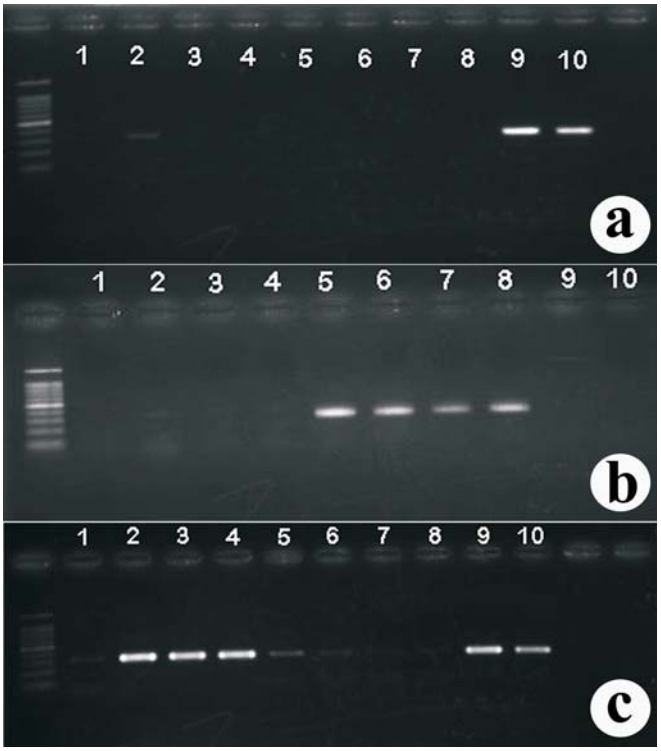

Figure 5. Species-specific amplification of *Mycosphaerella* species from banana using specific primers. a. Primer pairs MfijF/MfijR to the *M. fijiensis* b. primer pairs MmusF/MmusR to the *M. musicola* c. primer pairs MeuF/MeuR to the *M. eumusae* and *M. fijiensis* (2-4: *M. eumusae*; 5-8: *M. musicola*; 9-10: *M. fijiensis*).

CONCLUSIONS

Diagnosis of the banana Sigatoka disease complex is a challenge for plant pathologists. RFLP analysis using *Hae*III *restriction enzyme* is capable in discriminating the *M. eumusae, M. fijiensis,* and *M. musicola*. The rapid and specific PCR-based detection method using species-specific primers of MfijF/MfijR and MmusF/MmusR has been successfully developed to detect *M. fijiensis* and *M. musicola*, respectively, from pure cultures. Further examination/validation directly on samples from infected banana leaves with *Mycosphaerella* diseases symptom are necessary to test the sensitiveness of this method.

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