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Full Length Research Paper

Direct colony polymerase chain reaction (PCR): An efficient technique to rapidly identify and distinguish *Mycosphaerella fijiensis* and *Mycosphaerella musicola*

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Sigatoka disease is the most important threat for banana production worldwide. Many species of Mycosphaerella have been described from banana but, to date, the three species *Mycosphaerella fijiensis*, *M. musicola* and *M. eumusae* are the only species found to be pathogenic to banana. Reliable identification by classical methods requires expertise because these fungi produce similar symptoms and they are morphologically similar. For studies of ecology, genetic diversity and epidemiology their differentiation is crucial. Several laboratories have developed molecular protocols to differentiate these fungi. Currently, a number of primers targeting ribosomal sequences, actin, tubulin and histone 3 genes are available for diagnosis of the Sigatoka complex. In the present work, we report a direct colony-polymerase chain reaction (DC-PCR) approach to rapidly distinguish *M. fijiensis* and *M. musicola* strains in multiplex PCR reactions. This is the most economical and the fastest procedure reported so far for diagnosis of these two *Mycosphaerella* species, which are distributed in banana-growing regions in the world; the DC-PCR technique was also found to be amenable for the identification of mating type of *M. fijiensis* isolates. This DC-PCR may also be applicable to prepare DNA templates for basic PCR-based analyses in other fungi.

Key words: Sigatoka diseases, Banana's *Mycosphaerella*, molecular diagnosis, direct colony PCR.

INTRODUCTION

The causal agents of the most important fungal leaf diseases of banana worldwide, comprise *Mycosphaerella fijiensis* which causes the black Sigatoka disease, *Mycosphaerella musicola* (yellow Sigatoka disease) and *Mycosphaerella eumusae* (leaf spot disease) (Carlier et al., 2000; Arzanlou et al., 2007, 2008, Churchill, 2010). *M. fijiensis* and *M. musicola* are distributed worldwide in banana-growing regions (Jones, 2000), while *M. eumusae* has been so far described in South-East Asia, some Indian Ocean Islands and Nigeria (Arzanlou et al., 2010; Churchill, 2010). In addition to these three fungi, recently Arzanlou et al. (2008) reported three additional *Mycosphaerella* species occurring in banana, although there are no reports that these are pathogenic.

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Abbreviations: ITS, Internal transcribed spacer; **PCR,** polymerase chain reaction; **DC-PCR,** direct colony PCR; **dNTPs,** deoxynucleotide triphosphates; **bp,** base pairs.

Table 1. List of *Mycosphaerella* isolates used in this study.

Isolate	Fungi	Geographical origin
Sa-1	*M. fijiensis*	Uxmal, Yucatan, México
Ma-2	*M. fijiensis*	Uxmal, Yucatan, México
Pb-7	*M. fijiensis*	Uxmal, Yucatan, México
Ya-8	*M. fijiensis*	Uxmal, Yucatan, México
C1233	*M. fijiensis*	Uxmal, Yucatan, México
Veracruz	*M. fijiensis*	Martínez de la Torre, Veracruz, México
19H2	*M. musicola*	Tapachula, Chiapas, México
19H6	*M. musicola*	Tapachula, Chiapas, México

Specialists are able to distinguish different *Mycosphaerella* species based on disease symptoms and small differences in ascospore sizes and shapes; however, these structures are difficult to produce in culture. In addition, these fungi produce similar symptoms and are morphologically highly similar, which makes identification extremely difficult (Jones, 2000). Furthermore, these fungi can co-exist in the same lesion (Crous, 1998; Arzanlou et al., 2008) and because of their morphological and epidemiological similarities, *Mycosphaerella* species can be misidentified (Maxwell et al., 2005; Arzanlou et al., 2008). However, molecular identification of fungi overcomes these problems aforementioned. Numerous DNA-based methods have shown to be useful in diagnosis, identification and for taxonomy studies of pathogenic fungi given their simplicity, specificity and sensitivity (Luo and Mitchell, 2002; Schaad and Frederick, 2002; Atkins and Clarck, 2004). The molecular techniques include the sequencing of the internal transcribed sequence (ITS) region (Turenne et al., 1999; Landeweert et al., 2003; Martin and Rygiewicz, 2005), molecular fingerprinting (Plaza et al., 2004; Gente et al., 2006) and the use of polymerase chain reaction (PCR) amplification technique with specie-specific primers (Bindslev et al., 2002; Luo and Mitchell, 2002; Maxwell et al., 2005).

Isolation and purification of DNA is a key step in molecular protocols (Mirhendi et al., 2007; Deepak et al., 2007; Alshahni et al., 2009), and usually requires a number of laborious steps. There are some reports of protocols for extraction of DNA from *Mycosphaerella* fungi (Dellaporta et al., 1983; Johanson et al., 1994; Carlier et al., 2000; Rivas et al., 2004; Conde-Ferráez et al., 2008), but the isolation and purification of DNA is laborious and time consuming. Direct colony PCR (DC-PCR) of fungal strains is an ideal alternative, being simpler, quicker and more efficient when compared to standard methodologies. Fungal pathogens can be directly identified from primary isolation plates (Luo and Michell, 2002; Calmin et al., 2007; Mirhendi et al., 2007). Its main characteristic is the omission of the DNA extraction procedure which significantly decreases time and cost; moreover, it avoids the risk of contamination during the DNA extraction process (Calmin et al., 2007; Mirhendi et al., 2007; Lau et al., 2008; Alshahni et al., 2009). For population studies (population genetics, fungicide resistance, and epidemiological studies), rapid isolation and identification of a large number of strains are necessary. For these purposes the speeding up of conventional DNA extraction methods is a requisite.

Recently, Arzanlou et al. (2007) developed a molecular diagnostic approach for reliably distinguishing between *M. fijiensis*, *M. musicola* and *M. eumusae*. These authors extracted fungal genomic DNA by using the PureGene DNA isolation kit. To achieve this identification faster and cheaper, we used the specie-specific actin primers developed by these authors, but combined them with a simple in-house method for DC-PCR. This direct protocol is suitable for multiplex analysis, enabling rapid classification of *Mycosphaerella* strains isolated from banana with Sigatoka disease symptoms. In addition, the protocol is also applicable to other fungi.

MATERIALS AND METHODS

Biological material

For the development of this PCR assay, previously identified Mexican *Mycosphaerella* strains were selected. The “Veracruz” strain was identified by sequencing the ITS region; the other strains were identified by molecular diagnosis using primers from Johanson and Jaeger (1993) and Arzanlou et al. (2007). The strains used here are listed in Table 1. All *Mycosphaerella* strains were grown on sterile solid medium containing 200 ml/L V8 juice added with 2 g/L $CaCO_3$ and 2% agar–agar. Individual plates were inoculated with 16 mm^2 mycelium and growth conditions were according to Islas-Flores et al. (2006). For testing the technique with other fungi, the following species and lines were used: *Trichoderma reesei* CDBB-H-353, *Phanerochaete chrysosporium* ATCC34540, *Pleurotus ostreatus* CDBB-H-686 and *Trametes versicolor* PUJ3, obtained from the microbial collection of CINVESTAV, Mexico, and *Fusarium* sp. isolated in our laboratory from banana lignocellulosic waste collected in the field. These fungal strains were cultured on potato-dextrose-agar medium (PDA) for 7 days.

Template preparation

A small amount of mycelium (approximately 2 mm^2) was picked

Table 2. List of primers used in this study.

Primer	Sequence (5′→3′)	Target and location	Expected size product (bp)	References
ITS1 ITS4	TCC GTA GGT GAA CCT GCG G TCC TCC GCT TAT TGA TAT GC	General in fungi. ITS1-5.8S- ITS2 region of the ribosomal operon.	Highly variable (380-900)	White et al. (1990); Korabecna (2007)
MFactF ACTR	CTCATGAAGATCTTGGCTGAG GCAATGATCTTGACCTTCAT	*M. fijiensis*. Actin (fragment)	500	Arzanlou et al. (2007)
MMactF2 MMactRb	ACGGCCAGGTCATCACT GCGCATGGAAACATGA	*M. musicola*. Actin (fragment)	200	Arzanlou et al. (2007)
ACTF MEactR	TCCAACCGTGAGAAGATGAC GAGTGCGCATGCGAG	*M. eumusae*. Actin (fragment)	630	Arzanlou et al. (2007)
ACTF ACTR	TCCAACCGTGAGAAGATGAC GCAATGATCTTGACCTTCAT	Mycosphaerella genus (general). Actin (fragment)	820	Arzanlou et al. (2007)
2723Mt1-F 3137Mt1-R	AGCACCTGGCATGGCTGTGTCACC GCGCAGATGGCGAAGAACTCG	*M. fijiensis*. Idiomorph mat1-1 (fragment)	414	Conde-Ferráez et al. (2010)
4327Mt2-F 5027Mt2-R	ATGTCGTCAACCAGCAGAAGAACG GGTCATGCGCTTCTTCTTCTCG	*M. fijiensis*. Idiomorph mat1-2 (fragment)	700	Conde-Ferráez et al. (2010)
flan4739-F inver5656-R	GCGGTTTTGGAGCGGTCAGG AAGCTCTGGGTATCTCAGCACAGG	*M. fijiensis*. Idiomorph mat1-2 (fragment)	917	Conde-Ferráez et al. (2007)

with a micropipette tip and suspended in 25 µL of ultrapure water in a 1.5 ml Eppendorf tube. The mycelium was manually ground with a plastic pestle tip, vortexed thoroughly and then spun in a minicentrifuge. Mycelium suspension was used as template in PCR reactions. As a positive control template, genomic DNA from *M. fijiensis* Veracruz strain was prepared according to Johanson (1995).

PCR reactions

For PCR reactions, the mixture contained 3 µL of fungal suspension, 3 µL of 5X PCR buffer, 0.6 U of *Taq* DNA polymerase (BioLine), 2 mM $MgCl_2$, 0.2 mM dNTPs and ultra purified water for a final reaction volume of 15 µL. For amplification of intergenic spacer region 0.8 µM each of the primers ITS1 and ITS2 were used. PCR amplification conditions were 5 min of denaturation at 95°C, followed by 30 cycles of 94°C for 20 s, 55°C for 25 s, 72°C for 50 s and a final extension step of 72°C for 10 min. Independent identification of *M. fijienis* and *M. musicola* was performed by amplifying the actin gene (0.4 µM each primer). For *M. fijiensis*, the primer pair was MFactF and ACTR; for *M. musicola*, MMactF2 and MMactRb. For simultaneous screening of *M. fijiensis* and *M. musicola* by multiplex PCR, 0.4 µM of each MFactF, ACTR, MMactF2 and MMactRb primers were mixed in a single reaction. PCR amplification conditions was 5 min of denaturation at 95°C, followed by 36 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s and an extension step of 72°C for 10 min.

Assignment of mating type for *M. fijiensis* (idiomorphs *mat1-1* or *mat1-2*) was done by multiplex, using 0.8 µM each of the primers 2723Mt1-F and 3137Mt1-R (*mat1-1*) and 1 µM each of flan4739-F and inver5656-R (*mat1-2*). The cycling conditions were: 94°C denaturation 4 min, 94°C 40 s, 55°C annealing temperature 40 s, 72°C extension 50 s, 40 cycles; final extension 72°C, 7 min. The primer sequences are shown in Table 2. In all cases, water

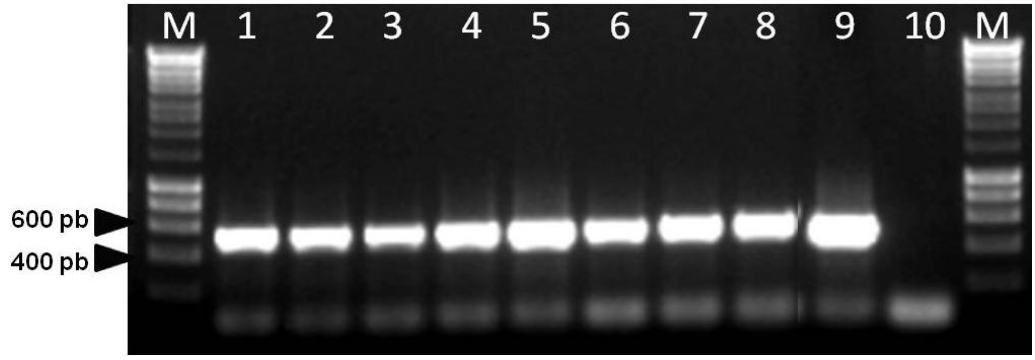

Figure 1. DC-PCR amplification of ITS region from different strains of *M. fijiensis* and *M. musicola*. Lanes 1 to 6, *M. fijiensis*: Sa-1; Ma-2; Pb-7;Ya-8; C1233 and Veracruz respectively. Lanes 7 and 8; *M. musicola*: 19H2 and 19H6 respectively. Lane 9, Positive control, genomic DNA from *M. fijiensis* Veracruz strain prepared according to Johanson (1995). Lane 10, Negative control (water). Lane M, Marker HyperLadder I (BioLine)

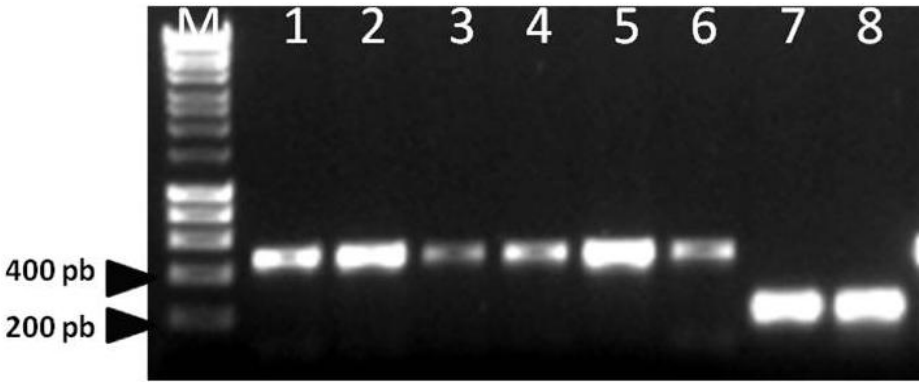

Figure 2. DC-PCR for diagnostic molecular identification of *M. fijiensis* and *M. musicola* in multiplex reactions. Lanes 1 to 6, *M. fijiensis* strains Sa-1, Ma-2, Pb-7, Ya-8, C1233 and Veracruz, respectively. Lanes 7 and 8, *M. musicola* strains 19H2 and 19H6 respectively. Lane M, Marker HyperLadder I (BioLine). Amplification was conducted using Arzanlou et al. (2007) primers targeting the actin gene in these fungi

was used as negative control (no template DNA).

Agarose gel electrophoresis

Detection of PCR-amplified products were performed by electrophoresis on a 1% (wt/vol) agarose gel stained with ethidium bromide. A volume of 5 µL of PCR product was loaded per lane.

RESULTS

Quick test for application of DC-PCR on *M. fijiensis* and *M. musicola* fungi - Amplification of ITS

The first PCR reactions were conducted using the ITS1 and ITS4 primers which amplify the ITS1-5.8S-ITS region of the ribosomal operon. Six strains of *M. fijiensis* and two of *M. musicola* were included. All of them produced an amplicon of approximately 500 bp (Figure 1), showing that no inhibitor of PCR amplification was evident. Although DNA was not purified, the direct PCR resulted in a single band with the expected size.

Diagnosis of *M. fijiensis* and *M. musicola* by DC-PCR using specific primers

To distinguish *M. fijiensis* and *M. musicola* using PCR, we used the primers pairs ACTR/MFactF and MMactF2/ MMactRb reported by Arzanlou et al. (2007) which amplify fragments of the actin gene specifically in *M. fijiensis* and *M. musicola* templates. Amplifications were in both cases successful, observing in the gel the expected size-diagnostic bands,500 bp for *M. fijiensis* and 200 bp for *M. musicola*. As above, no unspecific bands were observed. To investigate whether this methodology is suitable for high throughput, a multiplex assay was tried. Figure 2 shows the result of multiplex.

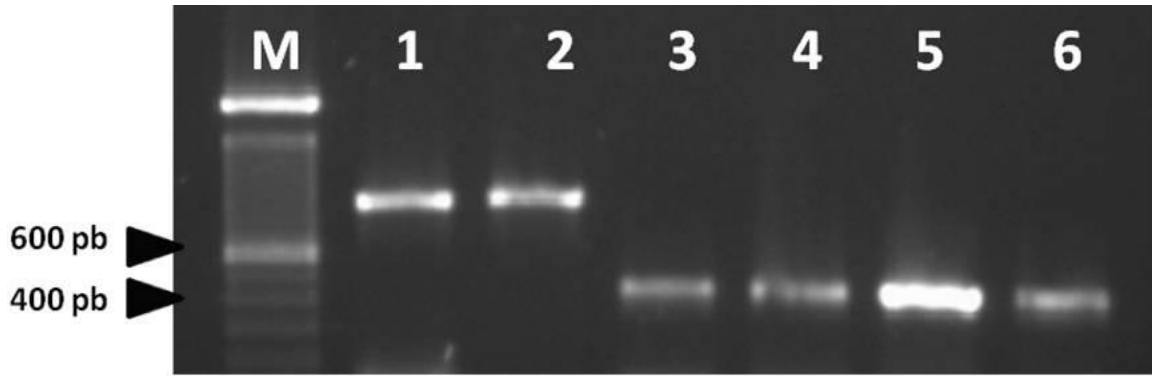

Figure 3. DC-PCR amplification for mating type determination in *Mycosphaerella fijiensis*. Lanes 1 and 2, idiomorph *mat1-2* (strains Sa-1 and Ma-2); lanes 3 to 6 idiomorph *mat1-1* (strains Pb-7, Ya-8, C1233 and Veracruz, respectively). Lane M, 100 bp DNA ladder (Gibco). Reactions were by multiplex as based on Conde-Ferráez et al. (2007, 2010)

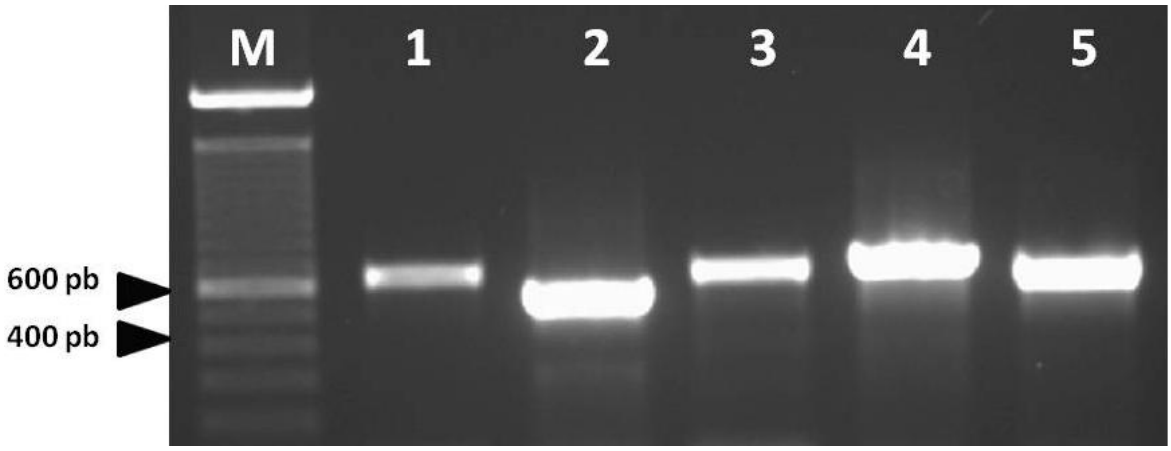

Figure 4. DC-PCR amplification of ITS region in different fungi. Lanes 1 and 2, Ascomycetes (*Trichoderma reesei* and *Fusarium* sp., respectively); lanes 3 to 5, Basidiomycetes: *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor*. Lane M, 100 bp DNA ladder (Gibco).s

The results were identical when both pairs of primers were pooled in a single PCR reaction, in comparison with reactions with separate pairs of primers (data not shown).

DC-PCR based assay for mating type of *M. fijiensis*

Suitability of this DC-PCR for rapid assignment of mating type in *M. fjiensis* strains was explored with the multiplex PCR assay developed by Conde et al. (2010). Attempts with this assay were unsuccessful; only *mat1-1* strains were positively amplified (data not shown). Modifications in *mat1-2* primer concentration in the mixture, and/or changes in the annealing temperature in the cycling were also negative for *mat1-2* strains. Primers 4327Mt2-F and 5027Mt2-R (Conde et al., 2010) were changed for flan4739-F and inver5656-R (Conde et al., 2007). These primers were selected because they are specific for the *M. fijiensis mat1-2* idiomorph and could also be suitable for multiplex with the *mat1-1* primers 2723Mt1-F and 3137Mt1-R. This new combination of primers works with the DC-PCR screening (Figure 3), producing the expected products (414 bp for *mat1-1* and 917 bp for *mat1-2*). Mating type of Sa-1 and Ma-2 is *mat1-2* and the other strains evaluated here are *mat1-1.*

Application of DC-PCR on other fungi

To explore if this quick and simple protocol can be extrapolated to other fungi, we tested the direct colony PCR-based ITS amplification on *T. reesei* and *Fusarium* sp. (Ascomycetes) and *P. chrysosporium*, *P. ostreatus* and *T. versicolor* (Basidiomycetes). Amplification of the ITS1-5.8S-ITS2 genomic region was successful with these fungi (Figure 4). The amplicons vary in size from approximately 400 to 700 bp, but were congruent with the expected range (Table 1). Mycelia suspensions were

stored at -20°C and used six months later for PCR reactions with ITS primers. Amplifications were positive in all cases (data not shown).

DISCUSSION

PCR-based screening of many samples requires rapid and simple protocols to obtain the DNA templates. In the literature, there are a number of protocols available to extract DNA from *Mycosphaerella* species (Johanson et al., 1994; Carlier et al., 1994; Guo et al., 2005; Maxwell et al., 2005; Conde-Ferráez et al., 2008; Motteram et al., 2009). Supplementary Table 1 summarizes the shorter protocols available to obtain DNA in these fungi. These are rapid protocols amenable for PCR approaches. However the procedure presented here is faster. The technical modification is extremely simple (substitution of extracting buffers and multi-step extraction procedures by mechanical mycelium maceration in water), but the value is precisely in the simplicity and avoidance of special equipment, kits or reagents (for example special columns for DNA purification), and it requires only a few seconds to prepare the template. Therefore, the protocol is not only fast and simple, but also cheap. The procedure starts from a tiny piece of fungal tissue harvested from solid cultures. This overcomes the necessity of sub-culturing in liquid medium to obtain fungal biomass, and it is suitable for applying directly on primary cultures. Its simplicity makes it convenient to apply to a large number of samples. Lack of expertise in *Mycosphaerella* taxonomy is not a limitation to conduct this molecular identification since production and analysis of conidia is not necessary.

Diverse protocols for distinguishing *M. fijiensis* and *M. musicola* have been published, based on molecular techniques or microscopic observations, as shown in the supplementary Table 2. Aguirre-Gaviria et al. (1999) were able to improve microscopic diagnosis with respect to other similar approaches [example that published by Jacome and Schuch (1993)], but diagnosis still requires experience to prevent misidentification of these fungi. Molecular approaches are more reliable since they are based on specific sequences in each species. Johanson et al. (1994) and Carlier et al. (1994) developed molecular diagnostic methods based on Southern blot and hybridization, but these techniques are laborious and require large amounts of DNA. In addition, purification of the DNA was necessary using CsCl to prevent inhibition of restriction enzyme digestions. The easiest protocols are based on the use of specific primers and analysis in gels of the PCR products or real time PCR diagnosis (Henderson et al., 2006). We chose the primers recently published by Arzanlou et al. (2007) because they developed species-specific primers after alignment of the actin gene for 17 *Mycosphaerella* spp. found in banana leaves and because they produced different amplicon sizes in PCR products which make them of potential use in multiplex analysis. The first attempt was a triple multiplex, combining primers targeting actin in *M. fijiensis*, *M. musicola* and *M. eumusae*, but the results were unsatisfactory. Arzanlou et al; 2007 diagnosis used the primer ACTF in combination with one specific primer of *M. eumusae* to amplify the actin in this fungus, and ACTR with one specific primer to amplify actin in *M. fijiensis*. However, both ACTF and ACTR together amplify actin in the *Mycosphaerella* genus, producing an expected amplicon of 820 bp. Therefore, the expected result for the multiplex is the 820 bp (general) band plus the specific band expected in each case. However, the *M. fijiensis* DNA template resulted in five bands instead of two, whilst in *M. musicola* there were three bands; the specific 200 bp band expected in *M. musicola* was the poorest amplified with *M. musicola* isolates. We obtained similar results when using pure DNA isolated from *M. fijiensis* and *M. musicola* and modifications in the preparation of the PCR mixture or cycling conditions were unsuccessful (data not shown). On these grounds the triple multiplex assay was discarded.

So far, there is no report about *M. eumusae* in Mexico. Therefore, the majority of *Mycosphaerella* species in Mexican banana samples with Sigatoka symptoms are assumed to be *M. fijiensis* and *M. musicola*. Double multiplex reactions combining primers for *M. fijiensis* and *M. musicola* were conducted. In this case, the assay worked well between these two fungi for distinguishing the two species in a single reaction; results for separate or multiplex reactions were identical. This multiplex has enabled us to rapidly screen a fungal collection obtained from diseased banana leaves. If *M. eumusae* is suspected to be present, it can be identified in separate PCR reactions after negative results for the *M. fijiesis*/*M. musicola* complex. Other primers should be further developed for the triple multiplex. Recently, Jaufeerally-Fakim et al. (2009) developed two primer pairs based on the ITS of *Mycosphaerella* species from *Musa*. One pair amplifies one band (800 bp) in *M. eumusae* and two bands (1200 and 1600 bp) in *M. musicola*. The other pair produces one band (900 bp) in *M. eumusae* and one (1500 bp) in *M. musicola* but neither pairs of primers amplify the ITS from *M. fijiensis*.

Evaluation of the protocol for rapid assignment of mating type in *M. fijiensis* strains was conducted. The first multiplex assay published by Conde-Ferrárez et al. (2010) was evaluated but only *mat1-1* strains were identified, although modifications in the PCR mixture and cycling conditions were tried. Probably, the *mat1-2* primers require purer templates to achieve annealing since the authors reported lower efficiency of the *mat1-2* primers in comparison with the *mat1-1* primers. Other primers for specific amplification of *M. fijiensis mat1-1* or *mat1-2* idiomorphs are available in the literature. We chose two *mat1-2*-specific primer pairs from Conde-Ferráez et al. (2007), f lan4739F and inver5656R, and

inver8486F and flan9352R, and tested in single reactions with the DC-PCR. Best results were observed with the former pair and these primers were then mixed with the *mat1-1* primers. In this case the mating type multiplex assay worked with the DC-PCR. Recently, Arzanlou et al. (2010) reported the sequences of *M. eumusae* and *M. musicola mat1-1* and *mat1-2* idiomorphs; therefore, the development of primers for quick PCR mating type assignment in these fungi is possible. With regard to the efficiency of the resulting primers, this DC-PCR approach could be probably applied for rapid idiomorphic classifications.

The success of DC-PCR with *M. fijiensis*, a difficult fungus for molecular manipulations, may indicate that the technique will probably work with other fungi. The protocol was tested on two other Ascomycete and three Basidiomycete fungi. The amplification of the ITS region with the universal primers ITS1-ITS4 demonstrated this to is a simple and fast procedure, which could be applicable to amplify the ITS1-5.8S-ITS2 in other Ascomycete and Basidiomycete fungi. Since the DC-PCR established here works with different primer-based assays, this procedure may also be suitable for other PCR-based applications, example quick confirmation of fungal transformation. For example, Suzuki et al. (2006) used one approach of "colony direct PCR" for the screening of *Aspergillus oryzae* mutants. In that report, the mutants were picked from the Petri dish, extracted by microwave oven and applied and dried on FTA cards. Their procedure was fast and simple, but still more expensive than that presented here. The DC-PCR established here may also be advisable for further screening of *M. fijiensis* mutants and other fungal mutants.

The DC-PCR reduces sampling handling to the minimum, eliminating the use of mortar and pestle. This prevents contamination and enables the protocol to be amenable for environmental and population studies. Currently, this procedure is being employed to identify *M. fijiensis* and *M. musicola* and other fungal isolates in our laboratory.

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Supplementary Table 1. Selected protocols from the literature for quick preparation of Mycosphaerella DNA suitable for PCR approaches.

Brief description	Time for preparation of template (before PCR)*	Reference
Used liquid-cultured mycelium (50 mg), ground in liquid nitrogen and lysis in 1 ml DNAzol reagent. After washing with chloroform, the DNA is precipitated with ethanol.	3 h	Guo et al. (2005)
DNA extraction using TENS buffer** followed by DNA purification using the silica binding kit from Mo Bio.	Not specified. Estimation is less than 30 min,	Maxwell et al. (2005)
Extraction of liquid-cultured mycelium (100 mg fresh weight) grinding in the presence of TENS buffer** and using a manual homogenizer with plastic pestle tips and quartz sand. After purification by phenol: chloroform: isoamyl alcohol, the DNA is precipitated with one volume of cold isopropanol.	3 h	Conde-Ferráez et al. (2008)
Ground by Fastprep FP120, 5 to10 mg of freeze-dried mycelium in presence of TENS buffer**, followed by 30 min incubation at 65°C. Subsequent step is 20 min incubation after adding ice-cold ammonium acetate. DNA is further precipitated from supernatant by adding one volume of cold isopropanol.	Not specified. Estimation is 1.5 to 2 h	Motteram et al. (2009)
Uses primary solid-cultured mycelium (tiny sampling by pipette tip). Ground manually (in water) inside an Eppendorf tube by using a plastic pestle tip.	Seconds	This study

*Time for growing the fungal culture was not considered. ** TENS buffer: **T**ris-HCl, **E**DTA, pH 8.0, **N**aCl, **S**DS. Concentration of the components could vary**.**

Supplementary Table 2. Methods for rapid diagnosis of *M. fijiensis* and *M. musicola.*

Brief description	Observation	Reference
Development of specific *M. fijiensis* and *M. musicola*. Forward primers designed on a variable region on ITS1. Use of same reverse primer on 25S rDNA.	Both pairs of primers amplify 1000 bp. Therefore not suitable for multiplex.	Johanson and Jaeger (1993)
Developing species-specific probes based on RAPD-PCR	Identification requires Southern blot and hybridization (laborious). After extraction, the DNA is purified on CsCl gradients to be suitable for restriction enzyme digestions.	Johanson et al. (1994)
Development of cosmid libraries and selection of clones corresponding to single or low-copy nuclear DNA to use as probes,	Identification requires Southern blot and hybridization. After extraction, the DNA is purified on CsCl gradients.	Carlier et al. (1994)
Use of needle-free disposable plastic syringe full with agar-crystal violet, streptomycin and benomyl. Two millimeters is exposed and pressed onto necrotic areas to collect conidia. The disc is removed with a scalpel, mounted on slide, placed in a humid chamber and observed with X40 lens.	These authors improved other microscopy-based identification of these fungi, which was fast in sampling but time-consuming at microscopy level. It also requires experience to distinguish the relative intensities of staining and morphological differences in the conidia (presence/absence of hilum).	Aguirre-Gaviria et al. (1999)
Development of specific primers targeting respectively *M. fijiensis*, *M. musicola* and *M. eumusae*. These authors conducted diagnosis and quantification of fungal biomass in field samples by qPCR*.	Fungal DNA extracted by using the PureGene kit (Gentra Systems Inc., Minneapolis, MN).	Arzanlou et al. (2007)
Use of species-specific primers developed by Arzanlou et al. (2007) on direct PCR and multiplex-direct PCR reactions, but preparation of template is simpler.	The cheapest and fastest procedure reported so far for molecular identification of *M. fijiensis* and *M. musicola* isolates.	This study

*qPCR, Quantitative PCR or real time PCR.