Molecular identification of *Macrophomina phaseolina* by microsatellite-based fingerprint

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The microsatellite primers (ATG)5, (TAGG)4 and a primer derived from the intergenic spacer regions (T3B) were employed to distinguish 16 fungal species. Distinctive and reproducible sets of amplification products were observed for the 16 species, with the numbers and sizes of the amplification products characteristic for each species. Microsatellite primer PCR yielded highly reproducible and complex genomic fingerprints, with several bands ranging in size from 200 to 3000 bp. Amplification products, regardless of the tested primers, were obtained from *Macrophomina phaseolina* DNA, each primer pair yielded a single DNA fragment of the expected size: 1100, 760, and 530 bp for (ATG)5, (TAGG)4, and (T3B), respectively. Cluster analysis separated the isolates into two major groups with intermix of isolates from two sampling locations. The three primers tested amplify species-specific microsatellites that unambiguously distinguish *M. phaseolina* from other fungal species tested. The unique banding patterns of *M. phaseolina* from 16 isolates make these primers valuable as diagnostic markers for the respective species. These distinct fingerprinting patterns can be used as diagnostic tools to local pathogen populations.

Keywords – Botryosphaeriaceae **–** cotton **–** Microsatellite markers **–** soil-borne fungi

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Introduction

Agricultural and wild ecosystems may interact through shared pathogens such as *Macrophomina phaseolina* (Tassi) Goid., a generalist clonal fungus with more than 284 plant hosts that it is likely to become more important under climate change scenarios of increased heat and drought stress (Ali & Dennis 1992, Saleh et al. 2010, Mihail & Taylor 1992). *M. phaseolina* is a seed and soil-borne pathogen with a wide distribution and host range (Dhingra & Sinclair 1978). The importance of *M. phaseolina* as the cause of ashy stem (charcoal rot) of cotton in Egypt is underestimated. This view has come from the observation that during the last 50 years, *M. phaseolina* on cotton was almost absent from the literature of cotton diseases in Egypt, and only a handful of studies, most of them not dealing with *M. phaseolina per se*, were found in this literature (Mostafa 1959, Sabet & Khan 1969, Aly et al. 1996, Abd-Elsalam 2011a). This lack of concern is not justifiable because this fungus is of widespread distribution in Egyptian soil and it is easily and

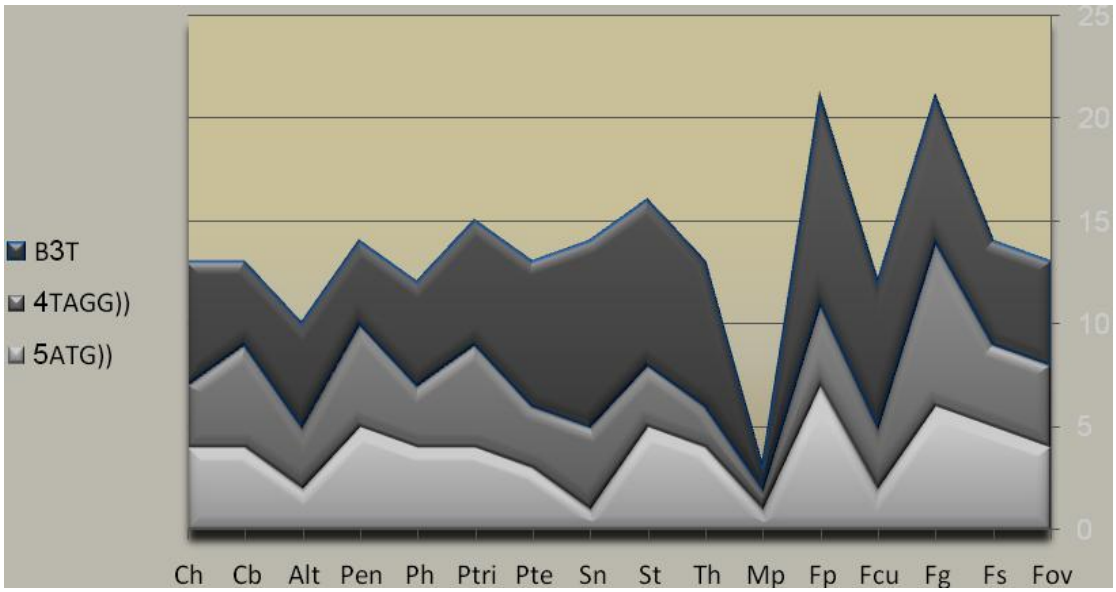

Fig. 1 – Polymorphism barcode obtained by three repeat-based primers among 16 fungal species isolates tested for MP-PCR specificty

frequently isolated from cotton roots particularly during the late period of the growing season. Molecular markers are useful tools for detecting genetic variation within populations of *M. phaseolina* (Jana et al. 2005). RAPD markers have been considered suitable for measuring genetic relatedness, detecting variation within and between *M. phaseolina* populations (Alvaro et al. 2003, Fuhlbohm 1997 and Jana et al. 2003, Omar et al. 2007, Su et al. 2001). AFLP genotype and geographical origin of *M. phaseolina* from Mexico and other countries gave a clear differentiation between Mexican and non-Mexican isolates (Reyes-Franco et al. 2006). Amplification of ITS1 region of *M. phaseolina*, root rot pathogen of *Citrus reticulata* the rDNA can be considered as a rapid technique for identifying pathogens successfully in all cases (Saleh et al. 2010, Chakraborty et al. 2011). Amplification of the ITS region using primers ITS1 and ITS4 produced only one DNA fragment of 620 bp. None of the isolates were differentiated through PCR-RFLP. The results demonstrated genetic variability among Brazilian isolates of *M. phaseolina* and showed that one single root can harbor more than one haplotype (Alvaro et al. 2003). The RFLP analysis was not suitable for detection of genetic diversity of *M. phaseolina* (Purkayastha et al. 2006).

Recently, microsatellite primer PCR (MP-PCR) has been extensively applied for various fungal molecular biological studies such as genetic diversity (Abd-Elsalam et al. 2010, Mwang'-Ombe et al. 2007) pathogen identification and differentiation (Alves et al. 2007) and fungal evolution (Wöstemeyer et al. 2002). Microsatellites have been used in several epidemiological studies of phytopathogenic fungi (Guerin et al. 2007, Raboin et al. 2007). Microsatellite dinucleotide specific-primers were designed based on microsatellite repeats of sequences present in the genome of *Mycosphaerella graminicola* (Abd-Elsalam et al. 2011a). Simple-sequence repeats (SSR) primers have been generated and then applied for the genetic analysis of *M. phaseolina* isolates (Vandemark et al. 2000, Su et al. 2001, Jana et al. 2003, 2005, Purkayastha et al. 2008, Baird et al. 2010, Ariasa et al. 2011, Mahdizadeh et al. 2012).

Table 1 Fungal isolates used in the current study.

Isolate No.	Fungal species abbrevation	Fungal species	Host	Geographic origin
1	Fov	*Fusarium oxysporum* f. sp. *vasinfectum*	Cotton	Egypt
2	Fs	*F. solani*	Cotton	Egypt
3	Fg	*F. graminearum*	Wheat	Germany
4	Fcu	*F. culmorum*	Wheat	Germany
5	Fp	*F. poae*	Wheat	Germany
6	Mp	*Macrophomina phaseolina*	Cotton	Egypt
7	Th	*Trichoderma harizinum*	Cotton	Egypt
8	St	*Septoria tritici*	Wheat	Germany
9	Sn	*Stagonospora nodorum*	Wheat	Germany
10	Pte	*Pyrenophora teres*	Barley	Germany
11	Ptr	*Pyrenophora tritici*-repentis.	Wheat	Germany
12	Ph	*Pseudocercosporella herpotrichoides*	Wheat	Germany
13	Pen	*Penicillium* sp.	Wheat	Germany
14	Alt	*Alternaria* sp.	Wheat	Germany
15	Cb	*Cercospora beticola*	Sugerbeet	Germany
16	Ch	*Chaetomium* sp.	Cotton	Egypt

Currently intensive work is ongoing for developing microsatellite primers as a diagnostic tool for detection of *M. phaseolina* in cotton. In the current study, three microsatellite primers designed based on microsatellite sequences (Bahkali et al. 2012) were used to identify *M. phaseolina* based on one single marker. To our knowledge, the use of microsatellite typing method for molecular diagnosis of *M. phaseolina* isolated from cotton has not been reported before.

Materials and Methods

Fungal isolates

Sixteen fungal species isolates used in this study are listed (Table 1) and 16 *M. phaseolina* isolates were obtained from different cotton-producing areas (Table 2). Pure cultures were grown on potato dextrose broth (PDB) for 10 days at 25–28°C in the dark. Mycelia were harvested by filtration through filter paper (Whatman No. 1). The harvested mycelia were either used immediately for DNA extraction or stored at –70°C until use.

DNA extraction

A modification of the traditional sodium dodecyl sulfate (SDS) extraction procedure was adopted. Fresh fungal mats (100 mg) were homogenized in 400 µL sterile salt homogenizing buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Next, 6 µL 20 mg/mL RNase A was added and mixed well. The samples were incubated at 65°C for 10 min, after which 130 µL 3 M sodium acetate, pH 5.2, was added to each sample. Samples were vortexed for 30 s at maximum speed, and incubated at -20°C for 10 min. The lysate was centrifuged at 13,000 rpm at 4°C for 15 min, and the supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample, and after mixing well, samples were incubated at -20°C for 10 min. Samples were then centrifuged for 20 min at 4°C, at 6000 rpm. The DNA pellets were washed twice using 700 µL washing solution (100 and 70% ethanol, respectively). The DNA pellets were subsequently air dried in an oven at 40°C for at least 10 min. The resultant DNA pellet was then resuspended in 100 µL 1X TE (10 mM Tris-HCl,

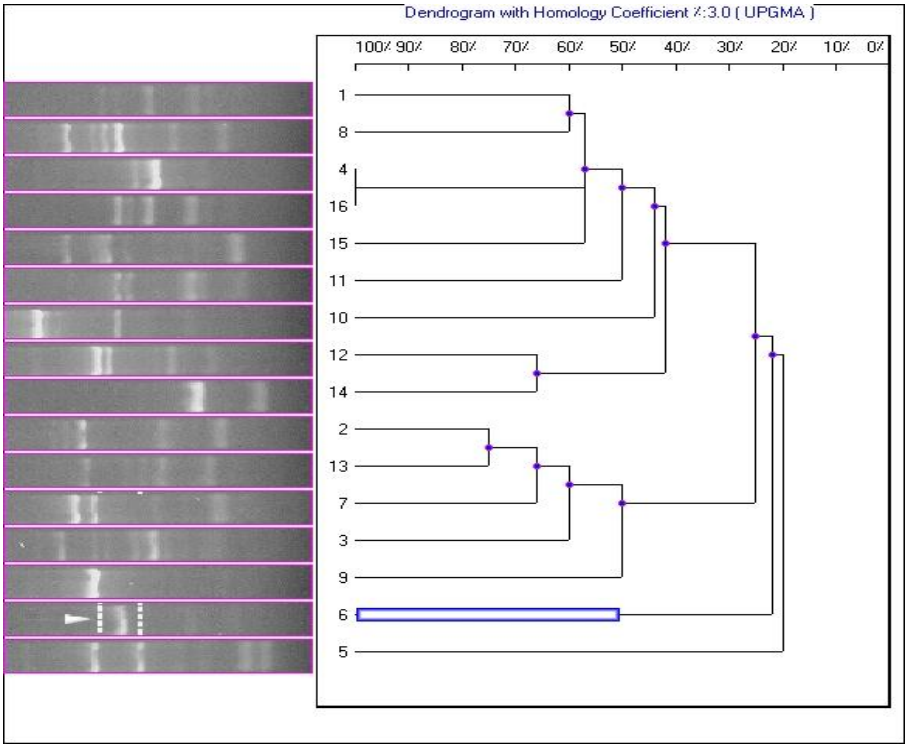

Fig. 2 – Digitized dendrogram from (ATG) 5 microsatellite-polymerase chain reaction profiles for 15 fungal species and *Macrophomina phaseolina* (6) created with Bionumerics using Pearson's correlation coefficient and unweighted pair group method with arithmetic mean (UPGMA).

1 mM EDTA) buffer, pH 8.0 (Abd-Elsalam et al. 2011b).

Microsatellite directed PCR

Microsatellite primed-PCR amplifications PCR was performed using a thermal cycler (Techne TC-312, Techne, Stone, UK) in 50 µl volumes containing 50 ng fungal DNA template, 10 pmol of the tested primers (Table 3), 0.2 mM of each dNTP, 2.0 mM MgCl2, 1 x Promega Taq Polymerase Buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, and 0.1% Triton X-100, Promega, WI, USA), and 1.5 U of Taq Polymerase (Promega). Amplification was performed using the following parameters: an initial pre-heat for 3 min at 95 °C, 37 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 10 min. PCR products were separated in 1.5% agarose gels in Tris–acetate (TAE) buffer. DNA was visualized by UV flourescence after staining with ethidium bromide. UVIsoft analysis packages (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK) were used to capture the image and calculate the molecular sizes.

Visual and computer-assisted analysis of MP-PCR patterns

Fragments profiles obtained were calculated for polymorphism by visual observation. Visible bands among isolates with the same migration distance were considered no different. Microsatellite fragments were scored as present (1) or absent (0) among the isolates. Only reproducible bands in repeated PCR amplification were considered for analyses. All MP-PCR patterns were analyzed with Fingerprinting Software (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK). Bands were automatically identified, verified and edited manually. Dendrograms were generated

Table 2 *Macrophomina phaseolina* (Mp) isolates collected from two major cotton-producing areas and total number of amplified fragments obtained by the tested primers used in this study.

Isolate No.	Mp isolates	Geographic origin	Region	Fragment number obtained		
				(ATG)5	(TAGG)4	T3B
1	*Macrophomina phaseolina*	Beheira	Lower Egypt	1	1	1
2	*Macrophomina phaseolina*	Beheira	Lower Egypt	1	1	1
3	*Macrophomina phaseolina*	Daqahliya	Lower Egypt	1	1	1
4	*Macrophomina phaseolina*	Daqahliya	Lower Egypt	1	1	1
5	*Macrophomina phaseolina*	Sharqiya	Lower Egypt	1	1	1
6	*Macrophomina phaseolina*	Sharqiya	Lower Egypt	1	1	1
7	*Macrophomina phaseolina*	Kafr El-Sheikh	Lower Egypt	1	1	1
8	*Macrophomina phaseolina*	Kafr El-Sheikh	Lower Egypt	1	1	1
9	*Macrophomina phaseolina*	Giza	Upper Egypt	1	1	1
10	*Macrophomina phaseolina*	Giza	Upper Egypt	1	1	1
11	*Macrophomina phaseolina*	Minya	Upper Egypt	1	1	1
12	*Macrophomina phaseolina*	Minya	Upper Egypt	1	1	1
13	*Macrophomina phaseolina*	Assiut	Upper Egypt	1	1	1
14	*Macrophomina phaseolina*	Assiut	Upper Egypt	1	1	1
15	*Macrophomina phaseolina*	Sohag	Upper Egypt	1	1	1
16	*Macrophomina phaseolina*	Sohag	Upper Egypt	1	1	1

by the hierarchic unweighted pair-group method with arithmetic averages (UPGMA) cluster algorithm.

Results

To determine whether the PCR fingerprint technique could be employed for species identification, each sample of genomic DNA from 16 fungal species was amplified separately with three primers, the microsatellite repeats (ATG)5, (TAGG)4, and a primer derived from the intergenic spacer regions (T3B). (ATG)5, (TAGG)4, and (T3B) primers gave single amplification products at 50° annealing temperatures, because it always led to high polymorphic banding patterns that were suitable for inter-species comparisons. The patterns resulting from the T3B and (TAGG) 4 test were more distinct.

The (ATG)5, (TAGG)4, and (T3B) primers amplified single unique DNA fragments of approximately 1100, 760, and 530 bp, respectively from *M. phaseolina* isolates (Table 2), whereas no amplification was achieved with DNA isolated from other fungal species. The numbers of scorable bands were between 1 to 10 depending on the primer combination and about 68% of the scored bands were polymorphic between different fungal isolates (Fig. 1).

To check the effectiveness of primers in the detection of intraspecific polymorphisms, experiments were performed by analysing isolates of *M. phaseolina*. Amplification of DNA from various *M. phaseolina* isolates using microsatellite repeat primers resulted in a single distinguishable marker. The discriminating powers of the three primers used in this study were nearly the same. Cluster analyses were performed on the genomic fingerprints generated by each of the primers tested. Three dendrograms were generated with the UPGMA method and are shown in Figs. 2, 3 and 4.

For all primers analyzed the clustering of the isolates was species-specific. Isolates belonging to the same species typically clustered together at similarity values greater than 80%. The observed intra-isolates homologies were mostly in the range of about 80 to 95%, similar to those obtained with the dendrogram produced by T3B-based fingerprinting. There was no clear-cut relationship between clustering in the MP-PCR dendrogram and geographic origin of

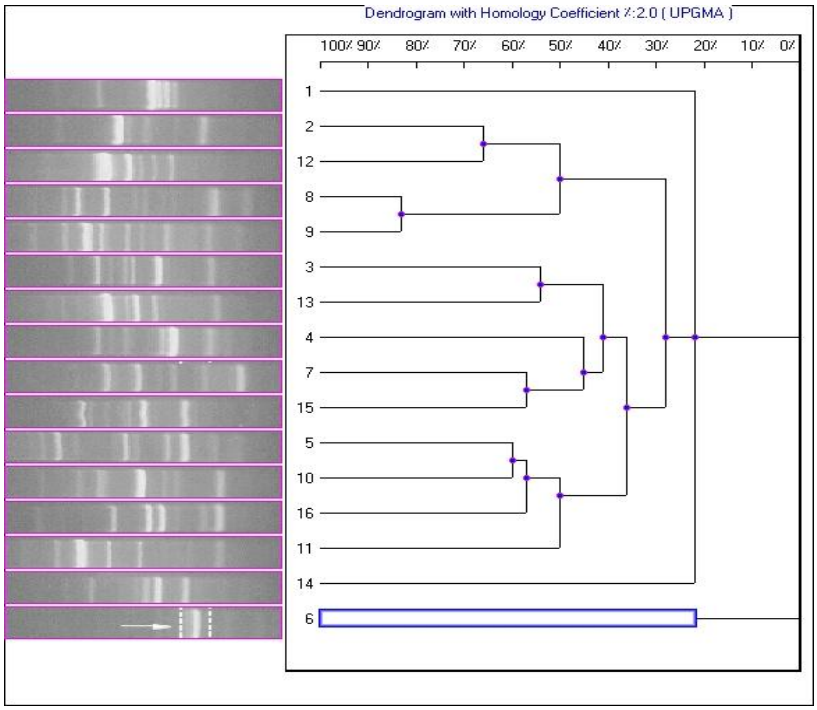

Fig. 3 – Digitized dendrogram from T3B microsatellite-polymerase chain reaction profiles for 15 fungal species and *Macrophomina phaseolina* (6) created with Bionumerics using Pearson's correlation coefficient and unweighted pair group method with arithmetic mean (UPGMA).

tested isolates.

Discussion

Ashy stem (charcoal rot) of cotton, caused by *M. phaseolina*, is an important and widely prevalent disease of cotton. Simple sequence repeats (SSRs) or microsatellites have been shown to be one of the most powerful genetic markers in mycology (Bahkali et al. 2012). Microsatellite markers have been generated for a number of plant pathogenic fungi (Saharan et al. 2007, Purkayastha et al. 2008, Baird et al. 2010, Ariasa et al. 2011, Mahdizadeh et al. 2012). However, there are only a few reports on the molecular characterization of *M. phaseolina* isolates in relation to host specificity (Vandemark et al. 2000, Su et al. 2001, Omar et al. 2007).

In this study, we have focused on microsatellite-based methods supplying patterns specific for particular *M. phaseolina* isolates. Based on the specific PCR fingerprints and the high interspecies variation of these banding patterns, a clear distinction between all species was possible. This study also revealed that a single marker can clearly distinguish *M. phaseolina* from other fungal species at the molecular level. The ability of the selected primers to produce species-specific fingerprints was apparent. Isolates that were deemed to represent different species according to conventional morphological as well as molecular phylogenetic criteria gave rise to distinct PCR fingerprints, whereas isolates of the same species had similar banding patterns.

T3B was the most successful primer because it always led to high polymorphic banding patterns that were suitable for interspecies comparisons. Complex fingerprints are generated, allowing detection of polymorphisms at inter- and intraspecific levels and subsequent identification (Godoy et al. 2004). The high discriminatory power (Burgess et al. 2001, Grünig et al. 2001) of this technique explains why it revealed a great heterogeneity

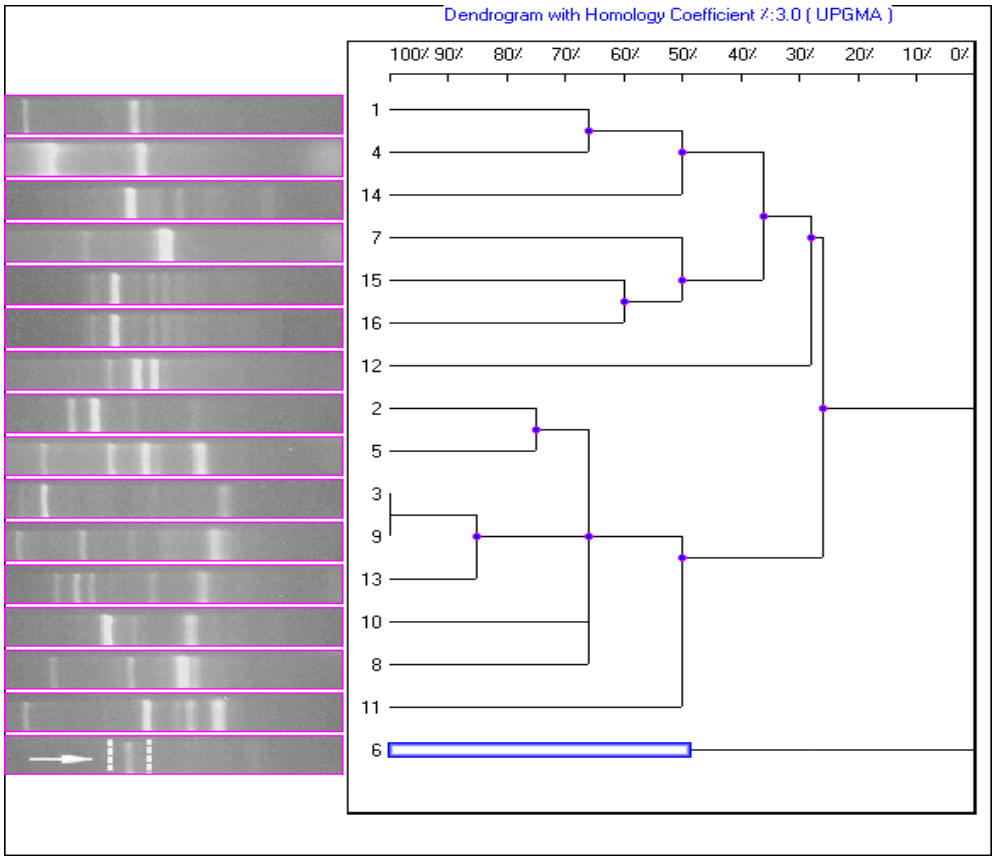

Fig. 4 – Digitized dendrogram from (TAGG)4 microsatellite-polymerase chain reaction profiles for 15 fungal species and *Macrophomina phaseolina* (6) created with Bionumerics using Pearson's correlation coefficient and unweighted pair group method with arithmetic mean (UPGMA).

among isolates. Single alleles per marker on each sample have been reported previously when 13 microsatellite markers were tested on *M. phaseolina* isolates from soybean (Baird et al. 2010) and from other plant hosts (Baird et al. 2010, Arias et al 2011, Mahdizadeh et al. 2012). Arias et al. (2011) provided 147 new microsatellite markers that will significantly enrich the molecular resources for *M. phaseolina*.

The low length polymorphism of tetranucleotide repeats obtained in the current study could be clarified by the fact that in fungi short stretches of trinucleotide repeat motifs exist as compared to higher organisms. Trinucleotide and dinucleotide repeat primers are the most frequent motifs in fungi (Karaoglu et al. 2005, Jany et al. 2006).

In conclusion, the results presented herein indicate that a microsatellite technique provides an efficient tool for the identification of poly and monomorphic loci that can be used to monitor the genetic differences between phytopathogenic fungi. In the next few years complete genome sequences might be available for *M. phaseolina* also, and a combination of microsatellites markers with other genomic methods will certainly accelerate the effort to characterize this fungus. Future research is warranted to develop more microsatellite primers with a wider array of *M. phaseolina*.

Table 3 Microsatellite primers used in the current study and their length, GC content, optimum annealing temperature (AT) , size range of fragments

Primers name	Sequence (5´-3´)	Length	GC Content (%)	Optimum AT (ºC)	Range of fragment size (bp)
(ATG)5	ATG ATG ATG ATG ATG	15	33.3	50	200-3000
(TAGG)4	TAGG TAGG TAGG TAGG	16	50.0	50	200-2000
T3B	AGG TCG CGG GTT CGA ATC C	19	63.1	50	100-3000

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