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ORIGINAL PAPER

Molecular identification of isolates of *Peronosclerospora sorghi* from maize using PCR-based SCAR marker

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Abstract The fungus *Peronosclerospora sorghi* [Weston and Uppal (Shaw)] infects both sorghum and maize and incites downy mildew disease. Pathogenic and molecular variability among isolates of *P. sorghi* from sorghum and maize has been reported. In the present study we developed a DNA sequence characterized amplified region (SCAR) marker for identification of isolates of *P. sorghi* from maize by using polymerase chain reaction (PCR). The random amplified polymorphic DNA (RAPD) primer OPB15 consistently amplified a 1,000 base pairs (bp) product in PCR only from DNA of *P. sorghi* isolates from maize and not from isolates of sorghum. The PCR-amplified 1,000-bp product was cloned and sequenced. The sequence of the SCAR marker was used for designing specific primers for identification of maize isolates of *P. sorghi*. The SCAR primers amplified a 800 bp fragment only from genomic DNA of maize isolates of *P. sorghi*. The SCAR primers developed in this study are highly specific and reproducible, and proved to be powerful tool for identification of *P. sorghi* isolates from maize.

Keywords Downy mildew · Maize · Molecular detection · *Peronosclerospora sorghi* · SCAR marker · Sorghum

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Introduction

Downy mildew a plant disease, caused by *Peronosclerospora sorghi* [Weston and Uppal (Shaw)], is a serious problem on sorghum and maize worldwide (Williams 1984). Morphological variation between strains of *P. sorghi* is limited (Bock 1995). However, pathogenic and molecular variability among the isolates of maize and sorghum has been well documented (Payak 1975; Dange 1976; Frederiksen and Renfro 1977; Bock et al. 2000; Mathiyazhagan et al. 2006). Dange (1976) demonstrated that the form of *P. sorghi* present in Rajasthan, India, was pathogenic to maize and tanglehead [*Hereropogon contortus* (L.) Beauv.] but not to sorghum. In contrast, Frederiksen and Renfro (1977) reported that *P. sorghi* found in Karnataka, India attacks maize and sorghum but not *H. contortus*. Payak (1975) postulated that two races of *P. sorghi*, differentiated by pathogenicity to maize and sorghum occurred in India.

Schmitt and Freytag (1977) compared the isolates of *P. sorghi* from Thailand and Texas, USA and demonstrated that the Thai strain of *P. sorghi* differed from the Texas strain in its ability to infect sorghum and its greater virulence to maize differentials. Craig and Frederiksen (1980) identified two pathotypes (Pathotype 1 and 2) of *P. sorghi* in Texas by differential pathogenicity on sorghum. Among them, Pathotype 2 caused higher percentage of mildew in differential sorghum cultivars than did Pathotype 1. Subsequently, Craig and Frederiksen (1983) demonstrated the existence of three pathotypes of *P. sorghi* (P1, P2 and P3) in Texas. Pawar (1986) reported that the pathotypes form Africa and India had a much wider range of virulence than did pathotypes from the Americas. Isakeit and Jaster (2005) reported the existence of a new pathotype of *P. sorghi* in Texas which showed metalaxyl resistance. Perumal et al. (2006) analyzed the genetic variability among the 14

isolates of *P. sorghi* including metalaxyl resistant and susceptible as well as common pathotypes 1 and 3 and reported that approximately 25% of the bands were polymorphic across the isolates tested with the majority of differences specific to the pathotype P1. Mathiyazhagan et al. (2006) reported the genetic variability between the isolates from sorghum and corn using restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR)-amplified internal transcribed spacer (ITS) region of ribosomal DNA. In the present study we developed a sequence characterized amplified region (SCAR) marker for identification of isolates of *P. sorghi* from maize by using PCR. To our knowledge, SCAR markers have not previously been used to compare *P. sorghi* isolates from sorghum and maize.

Materials and methods

Sources of fungal strains and extraction of DNA

Peronosclerospora sorghi isolates S1, S2, S3, S4, S5, S6 and S7 were collected from different sorghum genotypes and M1, M2, M3, M4, M5, M6 and M7 were collected from different maize genotypes in fields near Coimbatore, India and DNA was extracted from conidia of each isolate according to the method described by McDermott et al. (1994). Briefly, conidia were collected from the infected leaves using camel hair brushes and transferred the conidia into Eppendorf tubes containing 500 µl of extraction buffer (50 mM Tris–HCl, pH 8.0; 10 mM EDTA; 0.7 M NaCl; 1% Cetyltrimethylammonium bromide (w/v) and 1% 2-mercaptoethanol) (1×10^8 spores/ml), and vortexed for 30 s and incubated at 60°C for 1 h. The mixture was centrifuged at 13,000*g* for 10 min, and the aqueous phase was added with an equal volume of chloroform:isoamylalcohol (24:1 v/v) and incubated on a shaker (100 rpm) at room temperature for 1 h. The mixture was centrifuged at 13,000*g* for 10 min and the aqueous phase was transferred to a new Eppendorf tube and re-extracted with chloroform:isoamylalcohol. The aqueous phase was transferred to a new tube and the DNA was precipitated with equal volume of isopropanol and centrifuged at 13,000*g* for 10 min. The pellet was washed with 70% ethanol, dried and dissolved in 50 µl of Tris–EDTA buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0).

RAPD PCR

The 10-mer primers used in RAPD PCR are shown in Table 1. All the RAPD primers were purchased from Operon (Operon Biotechnologies, Cologne, Germany) and used as single primers. PCR was under taken in 20 µl volume consisting of 5 mM each dNTPs, 10 pmol of primer, 0.5 U of Taq DNA polymerase and 50 ng of template. The PCR was performed in a Palm-Cycler Model CGI-960 (Corbett Research, Mortlake, NSW, Australia) under the following conditions; 1 cycle of 94°C for 3 min; 40 cycles at 94°C for 1 min, 37°C for 2 min and 72°C for 2 min; and a final extension cycle at 72°C for 10 min. Following amplification, 10 µl of each PCR product was electrophoresed on 2% agarose gel in Tris–acetic acid–EDTA (TAE) buffer. The DNA fragments in the gels were stained with ethidium bromide, visualized under ultraviolet light (UVL), and recorded with an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA). Selected RAPD amplifications were repeated to ensure reproducibility.

Table 1 Sequences of RAPD primers used to study the genetic variability among isolates of *P. sorghi* from sorghum and maize

Primer	Nucleotide sequence
OPL-08	5′AGCAGGTGGA3′
OPE-01	5′CCCAAGGTCC3′
OPE-18	5′GGACTGCAGA3′
OPL-05	5′ACGCAGGCAC3′
OPL-07	5′AGGCGGGAAC3′
OPL-12	5′GGGCGGTACT3′
OPB-15	5′GGAGGGTGTT3′

Data analysis

The amplified fragments of each isolate were scored as 1 (present) or 0 (absent). Comigrating bands were considered homologous characters. Faint bands and bands showing variable levels of intensity were not considered for scoring. Pairwise comparisons were made by using the Jaccard similarity coefficient and the NTSYS-PC programme (Version 2.02) developed by Rohlf (1990). Similarity coefficients were used to construct the UPGMA (unweighted pair-group method with arithmetic means) dendrogram (Jaccard 1901).

Cloning and sequencing

The 1,000 bp PCR product amplified by the RAPD primer OPB15 from the DNA of maize isolate (M1) of *P. sorghi* was purified from the gel using QIAquick Gel extraction kit (QIAGEN, Hilden, Germany) and ligated into the pGEM-T easy vector (Promega Corporation, Madison, WI, USA) at 4°C overnight. Ligated DNA was used for transformation of competent *Escherichia coli* DH5α cells.

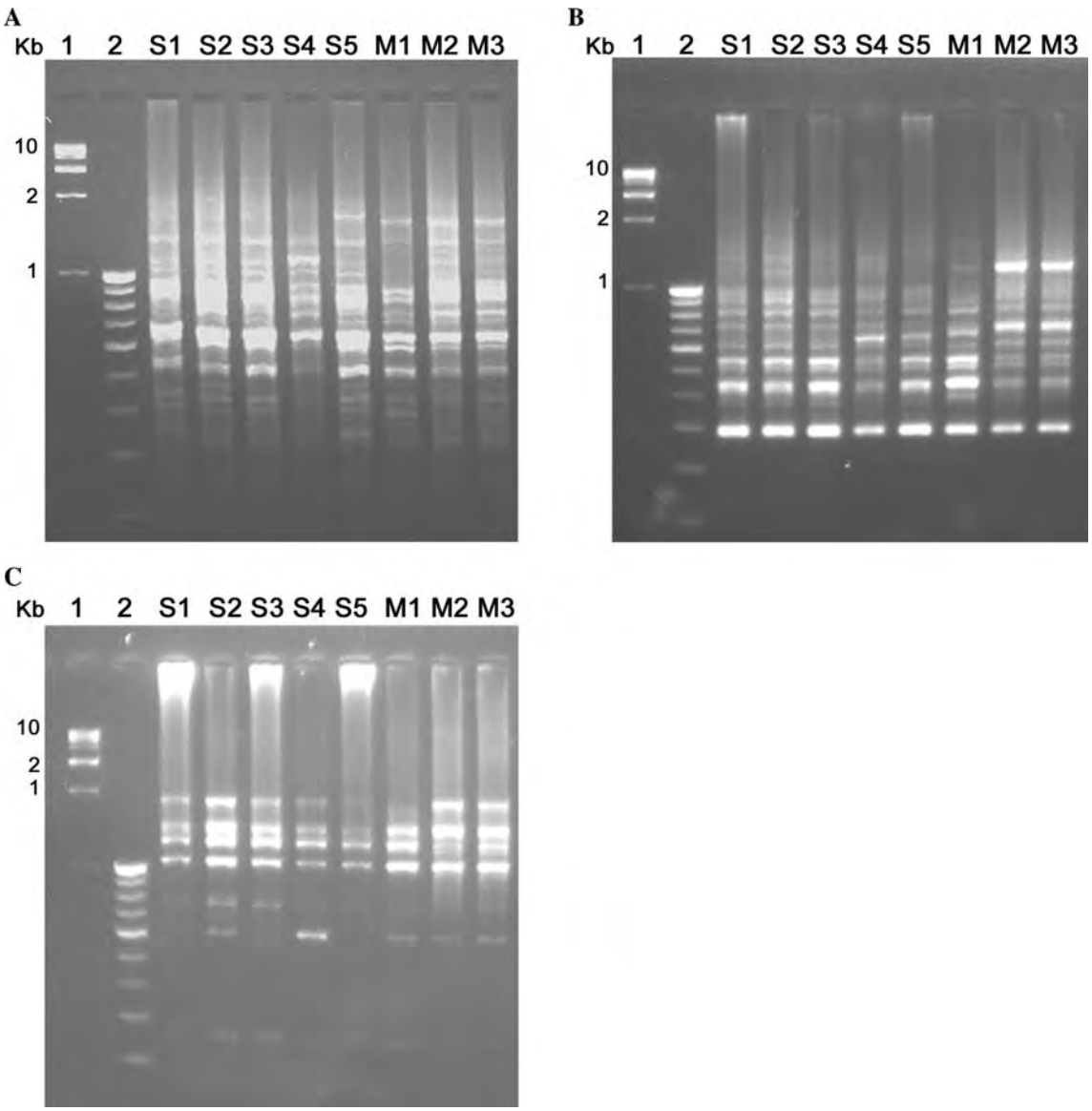

Fig. 1 Agarose gel electrophoresis of PCR-amplified products from genomic DNA of *P. sorghi* using the RAPD primers OPL-07 (**a**), OPL-08 (**b**) and OPE-01 (**c**). *Lane 1*, 1.0 kb DNA ladder; *Lane 2*, 100 bp DNA ladder; *Lanes S1* to *S5*, *P. sorghi* isolates from sorghum; *Lanes M1* to *M3*, *P. sorghi* isolates from maize

Plasmid DNA was isolated from the clones by using Wizard Plus plasmid DNA purification kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's recommendations. The clones were sequenced in both the forward and reverse directions, by the use of M13 forward and reverse primers (SP6 and T7). Sequencing was done at 1st Base Pte Ltd, Singapore. Database search was performed with the BLAST 2.0 program from the National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA, World Wide Web server.

SCAR reactions

SCAR primers (SCAR-F 5′TTG CAC AGC CAC TCT ATT G3′ and SCAR-R 5′AGT ATT TGG CAT CAA CTC C3′) for PCR assays were designed based on the nucleotide sequence and synthesized by Operon Company (Operon Biotechnologies, Cologne, Germany) in salt free status. The reaction was carried out in a Palm-Cycler Model CGI-960 (Corbett Research, Mortlake, NSW, Australia) programmed with initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for

1 min, 60°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min. Following amplification, 10 µl of each PCR product was electrophoresed on 2% agarose gel in TAE buffer. The DNA fragments in the gels were visualized by staining with ethidium bromide.

Results and discussion

A total of 7 RAPD 10-mer primers were tested for their ability to produce informative profiles from genomic DNA of *P. sorghi* isolates from sorghum and maize. A total of 126 clear and reproducible bands ranging between 150 and 2,500 bp were scored from the RAPD profiles and used in the analysis. The results indicated that all RAPD primers tested produced profiles that clearly showed variability between isolates of *P. sorghi*. Within the isolates of *P. sorghi* from maize, RAPD profiles seemed to be considerably less variable. The RAPD profiles produced with the primers OPL-07 (a), OPL-08 (b) and OPE-01 (c) are shown in Fig. 1. The RAPD primer OPB15 consistently amplified a 1,000 bp product in PCR only from DNA of *P. sorghi* isolates from maize and not from isolates of sorghum (Fig. 2a, b). Analysis of the genetic coefficient matrix derived from the scores of RAPD profiles showed that minimum and maximum percent similarities among the *P. sorghi* isolates tested were in the range of 37 and 87%, respectively (Fig. 3). Cluster analysis using UPGMA method clearly separated the isolates into three groups (Group I–III) (Fig. 4). The sorghum isolate S4, clustered into a separate group (Group I). All maize isolates were clustered together in Group II and the remaining sorghum isolates belonged to Group III. These results provide evidence of host specialization within the isolates of *P. sorghi*. Isolates from maize formed a single group and these isolates were placed separately from the isolates of *P. sorghi* from sorghum. This study has shown that RAPD analysis of the isolates of *P. sorghi* can be used effectively to distinguish between isolates of *P. sorghi* from sorghum and maize.

In order to develop a SCAR marker, the unique band (1,000 bp) specific to maize isolate (M1) of *P. sorghi* amplified by RAPD primer OPB15 was cloned and sequenced. The nucleotide sequence (Fig. 5) of the insert fragment was compared with all sequence data available in the GenBank (www.ncbi.nlm.nih.gov) sequence database. However, no identical or nearly identical sequences were found. The sequence of the SCAR marker was used for designing specific PCR primers (SCAR-F and SCAR-R). These primer pairs specifically amplified a 800 bp fragment from the genomic DNA of all *P. sorghi* isolates from maize (Fig. 6). However, these primers did not amplify the expected size (800 bp) fragment from genomic DNA of sorghum isolates. The specificity of the primers was confirmed by repeated testing to ensure reproducibility. This study demonstrated that SCAR primers distinguished the maize isolates from sorghum isolates. These results illustrated new opportunities for tracking the population dynamics of this important pathogen.

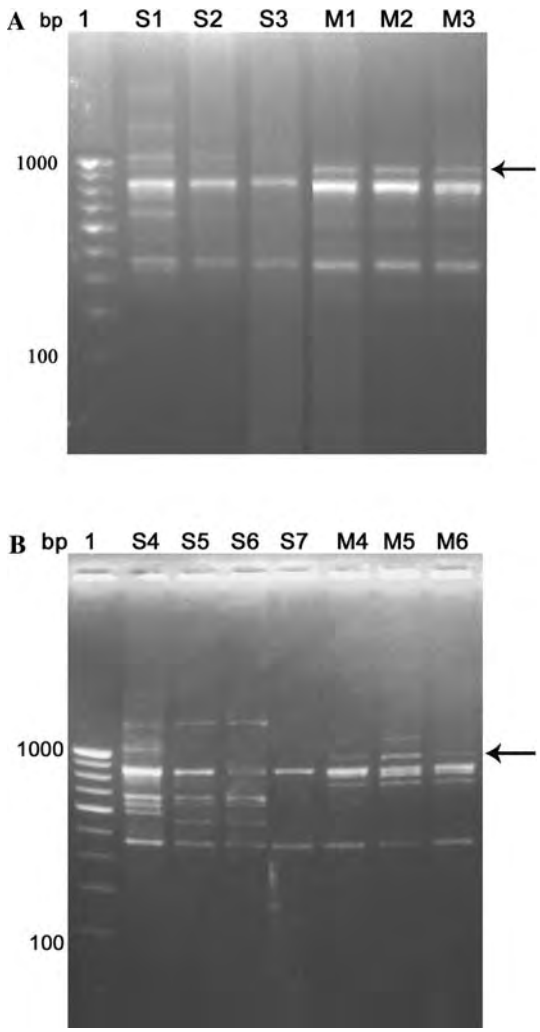

Fig. 2 Ethidium bromide-stained agarose gel showing amplification products from PCR of genomic DNA of *P. sorghi* using the RAPD primer OPB15. **a** *Lane 1*, DNA size marker; *Lanes 2–4*, *P. sorghi* isolates from sorghum (S1–S3); *Lanes 5–7*, *P. sorghi* isolates from maize (M1–M3). **b** *Lane 1*, DNA size marker; *Lanes 2–5*, *P. sorghi* isolates from sorghum (S4–S7); *Lanes 6–8*, *P. sorghi* isolates from maize (M4–M6). *Arrow* indicates the 1.0 kb fragment of *P. sorghi* to design SCAR markers

The *P. sorghi* infecting sorghum and maize are difficult to distinguish from each other morphologically. However, the existence of pathogenic and molecular variability among the isolates of *P. sorghi* from maize, sorghum and wild sorghum has been reported (Bock et al. 2000;

Fig. 3 Genetic similarity coefficient matrix for *Peronosclerospora sorghi* isolates from sorghum and maize based on RAPD profile

Isolates	S1	S2	S3	S4	S5	M1	M2	M3
S1	1.000							
S2	0.608	1.000						
S3	0.639	0.791	1.000					
S4	0.539	0.418	0.458	1.000				
S5	0.604	0.684	0.790	0.457	1.000			
M1	0.462	0.564	0.554	0.367	0.590	1.000		
M2	0.481	0.612	0.576	0.372	0.563	0.639	1.000	
M3	0.531	0.625	0.604	0.413	0.589	0.712	0.868	1.000

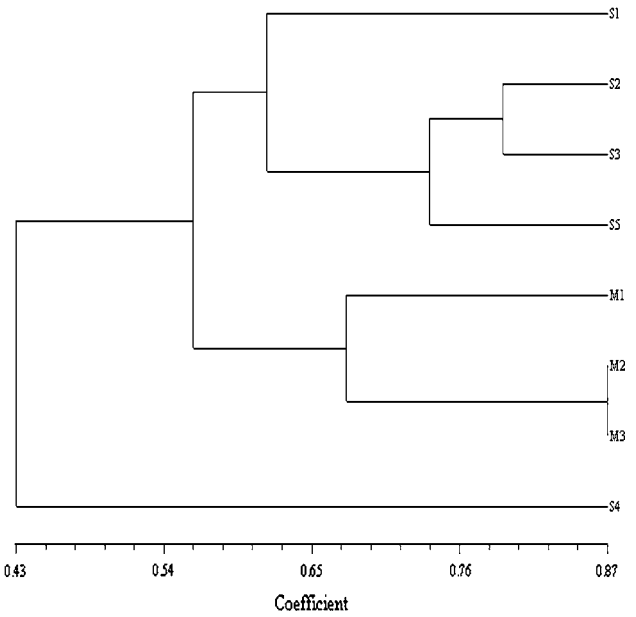

Fig. 4 Unweighted pair group method arithmetic average dendrogram constructed from RAPD data indicating the relationship among the isolates of *Peronosclerospora sorghi* from sorghum (S1–S5) and maize (M1–M3)

Mathiyazhagan et al. 2006; Perumal et al. 2006). A rapid, accurate, and sensitive method for differentiation of maize and sorghum isolates is essential to disease management and is a prerequisite for studies on environmental and cultural factors related to disease occurrence. Yao et al. (1991) identified an A–T rich DNA clone for identification and detection of *P. sorghi*. This clone, hybridizes only to DNA of *P. sorghi* or to DNA from leaves infected with *P. sorghi* and not to DNA of *P. sorghi* Thailand isolate, *P. philippinensis, P. sacchari* and *P. maydis*. The analysis of genomic DNA using PCR-based methods has proven to be a fast, sensitive and reliable method for determining genetic relationships among strains of the same phytopathogenic organisms (Ma and Michailides 2007). RAPD has the potential to detect polymorphism throughout the entire genome as compared to other PCR-based techniques. SCAR markers have been used to detect, characterize and identify a variety of plant pathogens (Nicholson et al. 1998; Garcia-Pedrajas et al. 1999; Moller et al. 1999; Forster and Adaskaveg 2000; Vandemark et al. 2000; Chioccetti et al. 2001; Pryor and Gilbertson 2001; Rigotti et al. 2002). The development of PCR primers specific to target organisms is one of the most important steps in PCR diagnosis. In the present study, RAPD fingerprinting confirmed the heterogeneity among the isolates of *P. sorghi* from sorghum and maize. The SCAR marker developed in the study could be used effectively to distinguish the strains of *P. sorghi* from sorghum and maize and to develop diagnostic methods for sensitive detection of the pathogen in infected seeds and soil.

Fig. 5 Nucleotide sequence of the PCR-amplified product obtained from genomic DNA of *P. sorghi* maize isolate (M1) using the RAPD primer OPB15

TGGAGGGTGTTGAGGGGTACTTCTGATGCTTTGACTTGTGCAGTAGCCGGCA
SCAR F
AATTGAGAAGTGCGCAGC**TTGCACAGCCACTCTATTG**GCTCCTGGTATAAT
TACTACAGGATAATGCTCCAACGGCCCAATCGAAGCCCGGTCGGGTAGGACA
GGTGTACAATGCCACCGTGGAACAAGCGGGCGTCCACACCAGTAGGTGTGCG
ATCCCCGGCTGGGCGATTGGTGTTCTACTTGTCTACCACAAAGACTAAAAAG
GGAGGACAAGCAGTGGCAAGATTTCCAGTTGGGTATCCATTTGAATACATGC
AAACCTTCATCAAGGAGGTGAAGCAAGTTGTTAAGTTCTTTAACAACCATCA
TTGCAATCAAGGCCAGATTGTCCCAACTACAAGATCTTGAAAAATTGCGTCG
ACTGGCTCTACCAGCGCCAACTCGTTGGGGTTCGCTCCAGAATTTCTTGAAGA
CTGTCTTGAAATCGGAACACTTGCTGCACAGCTTGGTGTGCGAGTGCAATTTT
ATTACCGGTAATACAGACCAAAGAAAACAACTTGAAGAAGTCCAAGCTACTA
CATTTTCCAAAAAACTCAACACCTTATTTCAGAAGGCACTGCCTTTGCTGATA
CCACTGGTCATGTTAATTATCTAATATCAAAATTACAGCTGTGCCGCTAGTGA
GGTTTACCCTGATTTCAATAGCTTGCCGCTAAGTACCTACAACTGAAGAATCA
AGGAACTATCACCCAGCAGTAATATGACTACATTTACCATTTAGTGATGGAA
CGCTTTCCGTTTATTTAAGGACCTGCTCATGGTTTCTCCTACATGCTGGACCCA
CGATTCATAGC**GGAGTTGATGCCAAATACT**GTCAAAAGAACTCTGGAGGAA
SCAR R
AAATTGTGCAAAATTCCCTTAAATGACACCCCACCCCTTTATCGCTGCTCGA

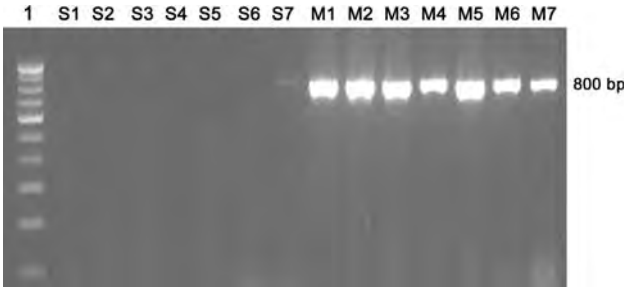

Fig. 6 Agarose gel electrophoresis of PCR products using SCAR primers with genomic DNA from *P. sorghi* isolates. *Lane 1*, DNA size marker; *Lanes S1–S7*, P. *sorghi* isolates from sorghum; *Lanes M1–M7 P. sorghi* isolates from maize

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