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27/3/2001

Dear Mr. DuPlessis,

Thank you for your letter dated 23/2/2001
I have enclosed copy of the publication from the maize project partially funded by Maize Trust. This is a widely circulated journal locally and internationally. However, if you send me a list of names to whom I should send in the maize industry, I will do so.

Please do note that Molecular detection of S. maydis in maize is the "top of the range method" of detection It is quick, sensitive, reliable and economical and thus should be recommended in terms of seed Health Testing and economical.

Thank you for the carry over of funds for 2001. I will keep in touch with you.

Prof Acha

* Note the Change of address since I am on leave

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Random Amplified Polymorphic DNA and Polymerase Chain Reaction Markers for the Differentiation and Detection of *Stenocarpella maydis* in Maize Seeds

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With 9 figures

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Abstract

The genetic relationship of 34 isolates of *Stenocarpella maydis* from different geographic regions in South Africa was analysed by random amplified polymorphic DNA (RAPD) and ribosomal DNA markers. Two genetic groups were differentiated by using three RAPD primers and correlated to the cultural morphology of the isolates. Of all the isolates tested, 79.4% were clustered into RAPD group I (RG I), which did not sporulate when cultured on potato dextrose agar (PDA) at 25°C for 10 days. The rest of the isolates designated as RG II sporulated on PDA medium and showed a higher genetic variation. Ribosomal DNA (rDNA) was amplified using polymerase chain reaction (PCR) with the universal primers, internal transcribed spacer (ITS) 1 and ITS 4. Restriction digestion of PCR products displayed three types (RF A, RF B and RF C) of profiles. RF A was in accordance with RG I. RF B was consistent with RG II except for one isolate, U5. However, U5 displayed a unique profile and had no restriction sites for *Hpa* II and *Hae* III. The results indicate that two distinct genetic groups exist among *S. maydis* isolates from maize in S. Africa. The ITS1 and ITS2 regions of rDNA were sequenced and primers were designed. The designed primer pair P1/P2 permitted a sensitive and specific detection of *S. maydis*.

Zusammenfassung

RAPD-und PCR-Marker zur Differenzierung und Detektion von *Stenocarpella maydis* in Maiskörnern

Mittels RAPD-und ribosomalen DNA-Markern wurden die genetischen Verwandtschaftsverhältnisse zwischen 34 *Stenocarpella-maydis*-Isolaten untersucht, die aus geographisch unterschiedlichen Gebieten Südafrikas stammten. Bei Verwendung von drei RAPD-Primern wurden zwei genetische Gruppen differenziert und mit den morphologischen Merkmalen der Isolate in Kultur in Verbindung gesetzt. 79, 4% aller untersuchten Isolate wurden in der RAPD-Gruppe I (RG I) zusammengefaßt. Sie sporulierten nicht, nachdem sie für 10 Tage bei 25°C auf PDA kultiviert worden waren. Die übrigen Isolate fielen in RG II, sie sporulierten auf PDA und zeigten eine stärkere genetische Variation. Ribosomale DNA (rDNA) wurde amplifiziert, wobei eine PCR mit den Universalprimern ITS 1 und ITS 4 ('internal transcribed spacer') durchgeführt wurde. Ein Restriktionsverdau der PCR-Produkte ergab drei Profiltypen (RF A, RF B und RF C). RF A stimmte mit RG I überein, RF B mit RG II (ausgenommen das Isolat U5). U5 zeigte jedoch ein einzigartiges Profil und besaß keine Restriktionsstellen für *Hpa* II und *Hae* III. Die Ergebnisse zeigen, daß bei südafrikanischen *S.-maydis*-Isolaten von Mais zwei verschiedene genetische Gruppen existieren. Die ITS1-und ITS2-Regionen der rDNA wurden sequenziert, und Primer wurden hergestellt. Das hergestellte Primerpaar P1/P2 bildete die Grundlage für ein empfindliches und spezifisches Verfahren zur Detektion von *S. maydis*.

Introduction

Molecular techniques allow more specific and sensitive identification and detection of pathogens, although serological and conventional methods are often used in routine detection of micro-organisms from plants or the environment. In order to establish detection and identification systems, information regarding the variability within fungal populations of interest is a prerequisite (Brunting et al., 1996). Random amplified polymorphic DNA (RAPD) markers have been widely used for assessing genetic diversity, genome mapping and molecular diagnostics of many fungal species (Assigbetse et al., 1994; Beck and Ligon, 1995; Brisbane et al., 1995; Achenbach et al., 1996; Shi et al., 1996; Chew et al., 1998). The technique, which does not

require previous molecular genetic information, often leads to a large number of discriminating markers and is technically simple (Williams et al., 1990). The ribosomal DNA (rDNA) of fungal genomes is also useful for detection purposes. This region is highly conserved, and is easily accessible for investigation by polymerase chain reaction (PCR) amplification (Toth et al., 1998). The 18 S, 5.8 S and 28 S genes evolve at a slow rate and are used for separating organisms at the level of class and/or family (Moukhamdov et al., 1994; Brunting et al., 1996). In contrast, the internal transcribed spacers (ITS) and intergenic spacer evolve at a more rapid rate (Trout et al., 1997; Hyun and Clark, 1998). Variations in ITS sequences can be most informative for closely related organisms. In many fungi, analysis of the ITS region has been used for differentiation of species (Brisbane et al., 1995; Lovic et al., 1995; Shi et al., 1996; Hyun and Clark, 1998; Guzmán et al., 1999). Up to now, molecular detection systems have been successfully established for a number of organisms (Moukhamdov et al., 1994; Smith et al., 1995; Lovic et al., 1995; Brunting et al., 1996).

Previous reports indicated that *Stenocarpella maydis* is an important pathogen on maize in USA, South Africa and other countries (Sutton and Waterston, 1966; McGee, 1988; Flett et al., 1992; Rheeder et al., 1993; Bensch, 1995). Biological characterization, colonization of the fungus and control measures for the disease have been studied (Latterell and Rossi, 1983; Flett et al., 1992; Rheeder et al., 1993; Dorrance et al., 1999). However, a sensitive and specific molecular detection system has not been established (Blakemore et al., 1994). The main aim of this study was to design specific PCR primers for the detection of *S. maydis* on the basis of the sequence information of the ITS regions of rDNA.

Materials and Methods

Maintenance and colony morphology of isolates

Thirty-four isolates of *S. maydis* used in this study, together with their geographic origins, source and colony characteristics are listed in Table 1. Isolates were maintained at 4°C on potato dextrose agar (PDA) slants and subcultured regularly. The isolates were

Table 1
The geographic origins, sources, colony characteristics and RAPD groups of *Stenocarpella maydis* isolates from maize cultivars

Isolates	Geographic origin	Source [a]	Colony coloration [b]	Sporulation on PDA [c]	PCR-RFLP	RAPD group
D72	Potchefstroom, North-west	SGC	Grey-white	No	A	I
D74	Potchefstroom, North-west	SGC	Grey-white	No	A	I
D75	Potchefstroom, North-west	SGC	Grey-white	No	A	I
D76	Potchefstroom, North-west	SGC	Grey-white	No	A	I
D78	Potchefstroom, North-west	SGC	Grey-white	No	A	I
D79	Potchefstroom, North-west	SGC	Grey-white	No	A	I
D80	Potchefstroom, North-west	SGC	Pink	Yes	B	II
D81	Potchefstroom, North-west	SGC	Grey-white	No	A	I
MC34	Potchefstroom, North-west	SGC	Grey-white	No	A	I
MC35	Potchefstroom, North-west	SGC	Grey-white	No	A	I
MC43	Potchefstroom, North-west	SGC	Grey-white	No	A	I
MC50	Potchefstroom, North-west	SGC	Grey-white	No	A	I
C1A	Cedara, KwaZulu Natal	Cedara	Grey-white	No	A	I
C3B	Cedara, KwaZulu Natal	Cedara	Grey-white	No	A	I
C3C	Cedara, KwaZulu Natal	Cedara	Grey-white	No	A	I
U5	Shongweni, KwaZulu Natal	This study	Pink-white	Yes, sparse [d]	C	II
U2	Shongweni, KwaZulu Natal	This study	Pink	Yes	B	II
U3	Shongweni, KwaZulu Natal	This study	Grey-white	No	A	I
U3H	Shongweni, KwaZulu Natal	This study	Grey-white	No	A	I
U7B	Shongweni, KwaZulu Natal	This study	Pink	Yes	B	II
U6H2	Shongweni, KwaZulu Natal	This study	Pink	Yes	B	II
UNK	Shongweni, KwaZulu Natal	This study	Pink	Yes	B	II
S6	Shongweni, KwaZulu Natal	This study	Grey-white	No	A	I
S8H2	Shongweni, KwaZulu Natal	This study	Pink	Yes	B	II
Y1	Transkei, Eastern Cape	MRC	Grey-white	No	A	I
Y2	Transkei, Eastern Cape	MRC	Grey-white	No	A	I
Y3	Transkei, Eastern Cape	MRC	Grey-white	No	A	I
Y4	Transkei, Eastern Cape	MRC	Grey-white	No	A	I
Y5	Transkei, Eastern Cape	MRC	Grey-white	No	A	I
Y6	Transkei, Eastern Cape	MRC	Grey-white	No	A	I
Y7	Transkei, Eastern Cape	MRC	Grey-white	No	A	I
Y8	Transkei, Eastern Cape	MRC	Grey-white	No	A	I
Y9	Transkei, Eastern Cape	MRC	Grey-white	No	A	I
Y10	Transkei, Eastern Cape	MRC	Grey-white	No	A	I

[a] Isolates were provided by Summer Grain Center, Grain Crops Institute, Agricultural Research Council, Private Bag X1251, Potchefstroom 2520; Cedara, Cedara Agricultural Development Institution, Private Bag X 9059, KwaZulu Natal, S. Africa. MRC: Dr Rheeder J.P., Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical research Council, Tygerburg, S. Africa; [b] Colony coloration of isolates was observed from top of Petri dish (aerial mycelium) on 10th day on PDA; [c] Sporulation were observed with light microscopy; [d] Scolecospores.

transferred onto PDA plates and then incubated at 25°C for 1 week. The colony characteristics were observed visually. The sporulation of each isolate and the spore diameter were investigated under light microscopy.

DNA extraction

Fungal DNA was extracted using the method described by Lee and Taylor (1990). Following phenol-chloroform extraction, DNA concentration was determined in GeneQuant (Pharmacia LKB Biochrom Ltd, Cambridge, England) or estimated by running an agarose gel. The DNA was diluted to 50 ng/μl concentration and stored at −20°C.

RAPD and PCR-restriction fragment length polymorphism analysis

PCR amplification of genomic DNA was performed using arbitrary primers and universal primers listed in Table 2. The UBC RAPD primer set was originally obtained from the University of British Columbia RAPD Synthesis Project.

Amplification reactions were performed in 50 μl reaction volumes containing 1 × *Taq* incubation buffer, 100 μM each of dATP, dCTP, dGTP and dTTP, 0.5 μM primer, 100 ng of genomic DNA, 1 U *Taq* DNA polymerase (Gibco, BRL, Paisley, UK) and sterile deionized water.

A hot-start RAPD procedure was used for amplification with Omnigene thermal cycler (Hybaid Ltd, Ashford, Middlesex, UK). The reaction conditions were as follows: denaturation at 96°C for 5 min followed by a 'hold' step at 90°C; to allow addition of *Taq* DNA polymerase, 35 cycles of 1 min at the annealing temperature, 34°C; extension for 2 min at 72°C; and denaturation for 15 s at 92°C. The final cycle consisted of 1 min of annealing followed by 5 min at 72°C to produce fully double-stranded fragments. Ten microlitres of each reaction was analysed on a 1.5% agarose gel run at 10 V/cm for 3 h in 0.5 × Tris boric EDTA buffer. All reactions were repeated at least three times including negative controls (no template DNA). DNA markers used for electrophoresis were Marker VI (Boehringer Mannheim, Germany) and lambda DNA restricted with *BstE* II (Boehringer Mannheim). Following electrophoresis, gels were stained with 2 μg/ml ethidium bromide for 20 min, destained in water, and bands were visualized with a UV transilluminator (UVP, Inc., California, USA).

Table 2
Primers used in this study

Primer	(G+C)%	Sequence 5′-3′
UBC 228	80	GCTGGGCCGA
UBC 245	80	CGCGTGCGAG
UBC 300	80	GGCTAGGGCG
ITS1	63	TCCGTAGGTGAACCTGCGG
ITS4	45	TCCTCCGCTTATTGATATGC
P1	60	GTTGGGGGTTTAACGGCACG
P2	75	GTTGCCTCGGCACAGGCCGG

Dots and Southern hybridization

Probe preparation and hybridization: The RAPD band of interest was extracted with QIAEX II Gel Extraction Kit (Qiagen, Crawley, UK) according to the manufacturer's instruction. Probes were labelled with digoxigenin-11-dUTP using DIG High Prime Labeling and Detection Starter Kit I (Boehringer Mannheim). Overnight hybridization was performed at 65°C.

Restriction digestion of PCR products.

The PCR products amplified with primers ITS1 and ITS 4 were prepared for restriction analysis by extracting with 100 μl of chloroform. The aqueous phase was transferred to a fresh tube and precipitated with sodium acetate and isopropanol as described previously. The DNA was resuspended in 50 μl distilled water and was subjected to restriction digestion with the four- and five-base recognition endonucleases, *Hae* III and *Hpa* II (Boehringer Mannheim), according to the supplier's instruction. Digestion products were electrophoresed in a 2.5% agarose gel.

Cloning of PCR products

Purified PCR products were directly cloned using the pCR-Script TM SK(+) cloning Kit (Stratagene, California, USA) according to manufacturer's instruction. The X-gal and IPTG system was used to select positive clones. Target fragments were further confirmed by PCR with primer pair, ITS1 and ITS4.

Sequencing

Either purified PCR products or cloned plasmids were subjected to sequencing. A total volume of 12 μl for each sample contained about 100 ng of DNA and 50 pmol primer. Universal primers, ITS1 and ITS4 were used for sequencing both purified or cloned PCR products. In addition, sequencing primers KS, and T3 for pBlue script II SK(+) vector were purchased and were used to sequence cloned PCR products. Fluorescence-based DNA sequencing was conducted at the Medical School, University of Cape Town.

Primers design

Database searches were carried out with the complete sequence using two different methods on remote computers via the internet at Blast@ncbi.nlm.nih.gov. PCR primers were designed visually and double-checked for potential nonspecific priming sites and primer dimer formations at http://www.williamstone.com/primers. The primers were designed so they had sufficient GC contents and enough length to withstand highly stringent PCR conditions. Primer pair, P1 and P2 were designed on the basis of sequence of ITS region and were custom synthesized at Gibco BRL, England.

Table 3
Fungi other than *S. maydis* isolated from maize tissues which were used in this study

Isolates	Species	Geographic origin	Year	Source
SC1-SC10	*Stenocarpella macrospora*	Eastern Cape	1998	MRC
FB	*Fusarium moniliforme*	KwaZulu Natal	1998	This study
FG	*Fusarium graminearum*	KwaZulu Natal	1998	This study
F	*Fusarium* spp.	KwaZulu Natal	1998	This study
AL	*Alternaria* spp.	KwaZulu Natal	1998	This study
RS	*Rhizoctonia solani*	KwaZulu Natal	1998	This study
CG	*Colletotrichum graminicola*	KwaZulu Natal	1998	This study
AS	*Aspergillus* spp.	KwaZulu Natal	1998	This study
TL	*Trichoderma lignorum*	KwaZulu Natal	1998	This study
PH	*Phoma* sp.	KwaZulu Natal	1998	This study
PE	*Penicillium* sp.	KwaZulu Natal	1998	This study
NI	*Nigrospora* sp.	KwaZulu Natal	1998	This study
AU	*Aureobasidium zeae*	KwaZulu Natal	1998	This study
CZ	*Cercospora zeae-maydis*	KwaZulu Natal	1998	This study

Specificity and sensitivity of primers to *S. maydis*

The applicability of PCR primer pair P1/P2 in amplifying the desired DNA fragments from genomic DNA of *S. maydis* isolates was examined by PCR under the condition described above, except that various annealing temperatures (55–60°C) were tested. PCR was carried out with DNA preparations from other fungal isolates (Table 3) that are commonly found in maize, both pathogenic and nonpathogenic.

Detection of *S. maydis* in maize plants and seeds

Both artificially inoculated maize plants in the greenhouse and naturally field-grown maize samples were used in this experiment. To prevent cross-contamination, plant and seed tissues were sampled and placed in 1.5 ml Eppendorf tubes at −20°C. Tissues were crushed with plastic rods, which served as pestles, under liquid nitrogen. DNA extraction and subsequent PCR amplifications were conducted using the procedures described above.

Results

RAPD profile

Of 10 UBC RAPD primers assessed for their suitability in generating RAPDs with three isolates of *S. maydis* (D79, D76 and U2), primers UBC 228, UBC 245 and UBC 300 yielded satisfactory polymorphisms and were used further to differentiate the isolates of *S. maydis* genetically. The other seven primers amplified a few nonpolymorphic products. No amplified bands were observed in any of the control reactions.

Nineteen, 20 and 21 bands were amplified from *S. maydis* using primers UBC 228, UBC 300 and UBC 245, respectively. All isolates of *S. maydis* were classified into two RAPD groups (RG I and RG II) based on the profiles generated with the above three primers (Table 1 and Fig. 1). RG I was the basic profile and comprised 79.4% of all isolates tested. Consistently, RG I isolates were characterized as a grey-white colony on PDA (Table 1). The remaining isolates with a relatively higher genetic variation were designated as RG II (Fig. 1). Consistently, isolates belonging to sporulation type (pink colonies) were grouped into RG II.

PCR-restriction fragment length polymorphism profile

After PCR amplification with ITS1 and ITS4, all isolates revealed the same banding patterns of a single 575 bp band (Fig. 2). This restriction digestion of PCR products showed three types of banding patterns which were termed as RF A, RF B and RF C (Fig. 3).

The PCR-restriction fragment length polymorphism (RFLP) result was correlated to the RAPD classification. RF A exactly corresponded to RG I. RF B was almost consistent with RG II except for one isolate, U5, which displayed a unique profile and was designated as RF C. The isolate, U5, previously identified as *S. maydis*, was re-examined. Indeed, the isolate displayed a unique cultural morphology which differed from the two distant cultural phenotypes (Table 1). They only formed scolecospore-like spores and not the *S. maydis*-specific two-cell conidia or pycnida were found when cultured on PDA.

Hybridization

Amplification with primer UBC 228 gave rise to a band of approximately 0.38 kb in all *S. maydis* isolates. In addition, a band of about 1.01 kb was found to be present in RG I isolates.

Hybridization occurred only with the 375 bp band generated from UBC 228 for all 34 *S maydis* isolates and not with other amplified products from fungi such as *Fusarium* spp. and *Aureobasidium zeae* (Table 3), suggesting that this product was uniquely amplified from the genomic DNA of the *S. maydis*. Dot and Southern blot hybridization results are shown in Fig. 4 and Fig. 5. Similarly, the presence of a 1.01 kb unique band in all RG I isolates was also confirmed by the same technique (Fig. 5).

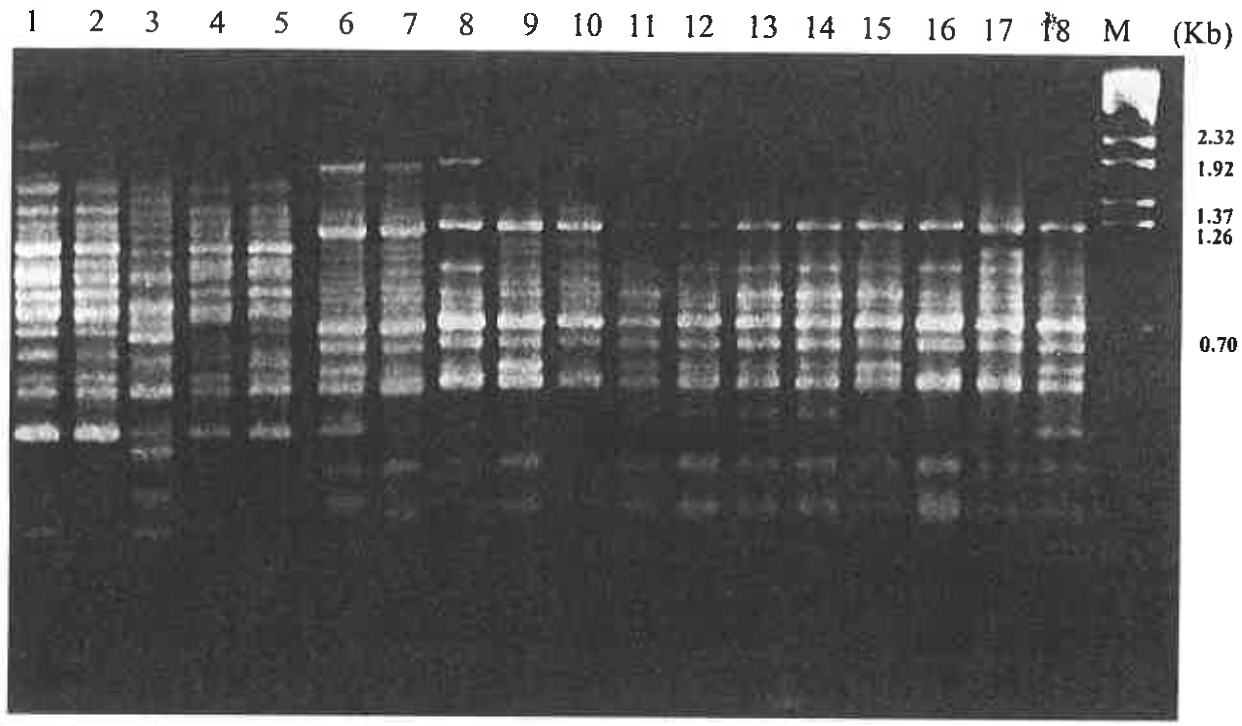

Fig. 1 Electrophoresis of random amplified polymorphic DNA fragments with primer UBC 245 on a 1.5% agarose gel. Lanes 1 to18: isolates U2, D80, UNK, U6H2, U7B, C3r°C MC50, C3B, MC35, MC43, Y5, C1A, D81, D76, D75, D74, D72, U3H of *Stenocarpella maydis*, respectively. Lane M: lambda Phage DNA digested with *Bst* E II (Boehringer Mannheim) as mol. wt. marker (kilobases)

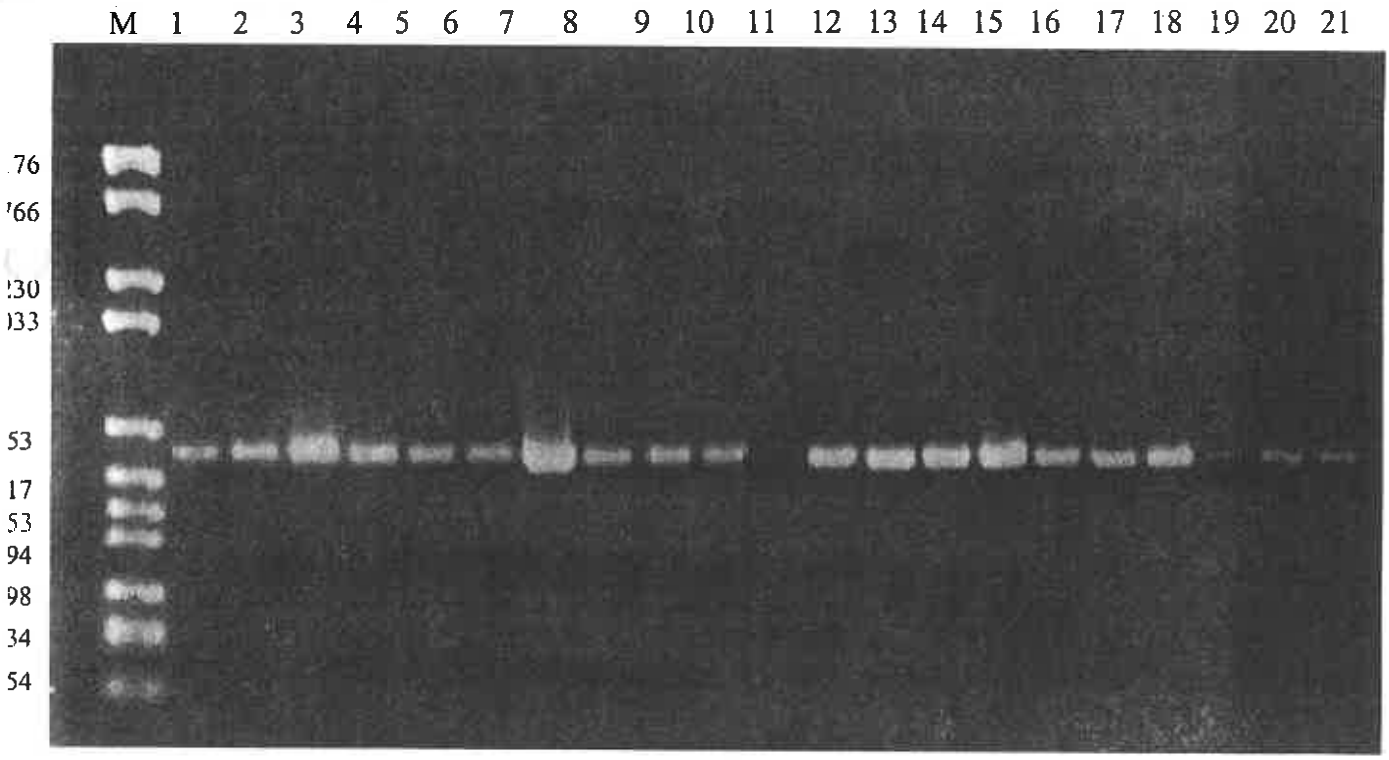

Fig. 2 Agarose gel illustrating the sizes of polymerase chain reaction (PCR) products. The amplified products were DNA regions of the internal transcribed spacer (ITS1/ITS4) and were electrophoresed on 1.5% agarose gels. Lanes 1 to 10: *Stenocarpella maydis isolates* U2, D80, UNK, U6H2, U7B, C3r°C MC50, C3B, MC35, MC43, respectively. Lanes 12 to 21: strains, SC1-SC10 of *Stenocarpella macrospora*, respectively; Lane 11 native control; Lane M: Marker VI (Boehringer Mannheim)

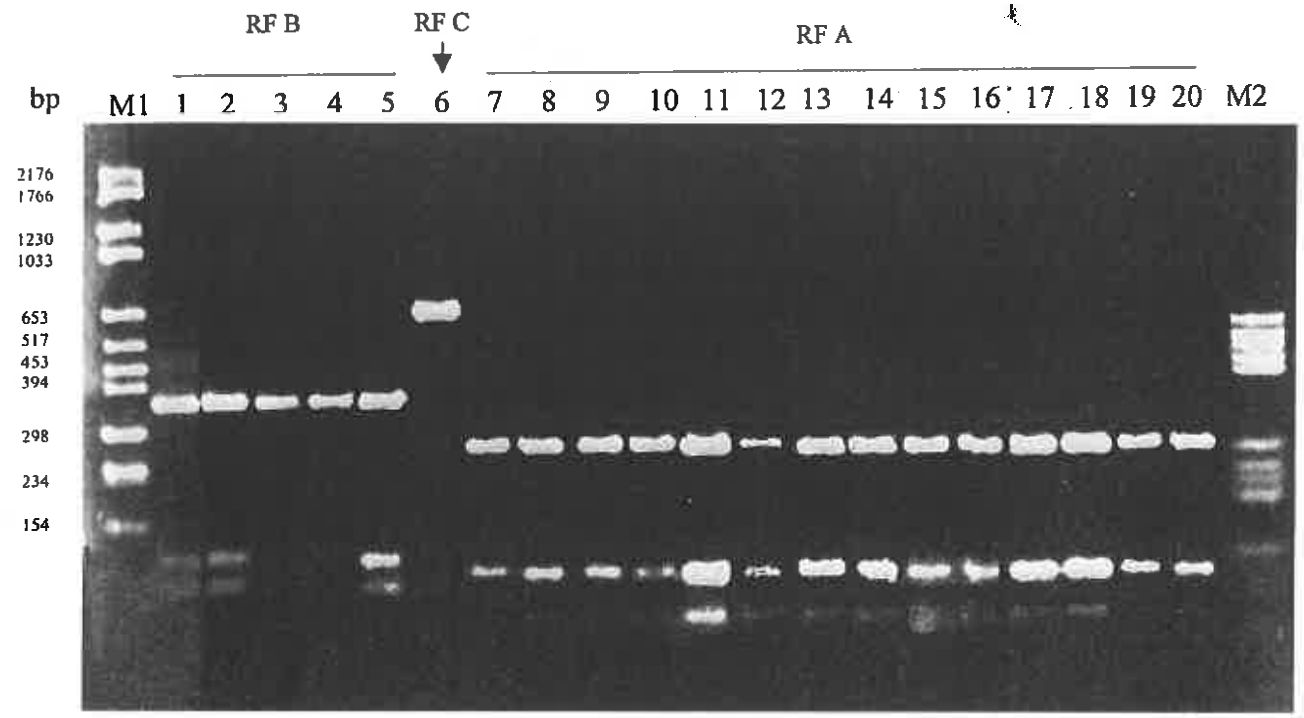

Fig. 3 Electrophoretic patterns on 2.5% Agrose gel of RFLPs of the amplified internal spacer (ITS) region from isolates of *Stenocarpella maydis* from maize digested with *Hae* III. Lane 1–5: RG Lane 1 to 5: D80, U7B, UNK, U8H2, U2. Lane 7 to 20: D72, D74, D75, D79, MC34, MC35, MC43, MC50, C1A, C3B, U3H, S6, Y4, Y10, respectively. Lane 6: PCR product without restriction. Lane M1 and M2: Mark VI and V(Boehringer Mannheim)

Cloning and sequencing of RAPD and PCR markers

The partial sequence of PCR products (ITS1 region, 5.8 S rDNA and ITS2 region) are shown in Fig. 6. These sequences and published ITS sequences of other fungi from GenBank were used to design primers that specifically amplify rDNA of *S. maydis* from maize seed samples. The ITS sequence of isolates, C1A, D72 were compared with the published ITS sequences through internet access. There was no sequence information on *S. maydis* or *S. macrospora* stored in the databases. Surprisingly, the sequence of the ITS region of *S. maydis* isolates demonstrated a high homogeneity

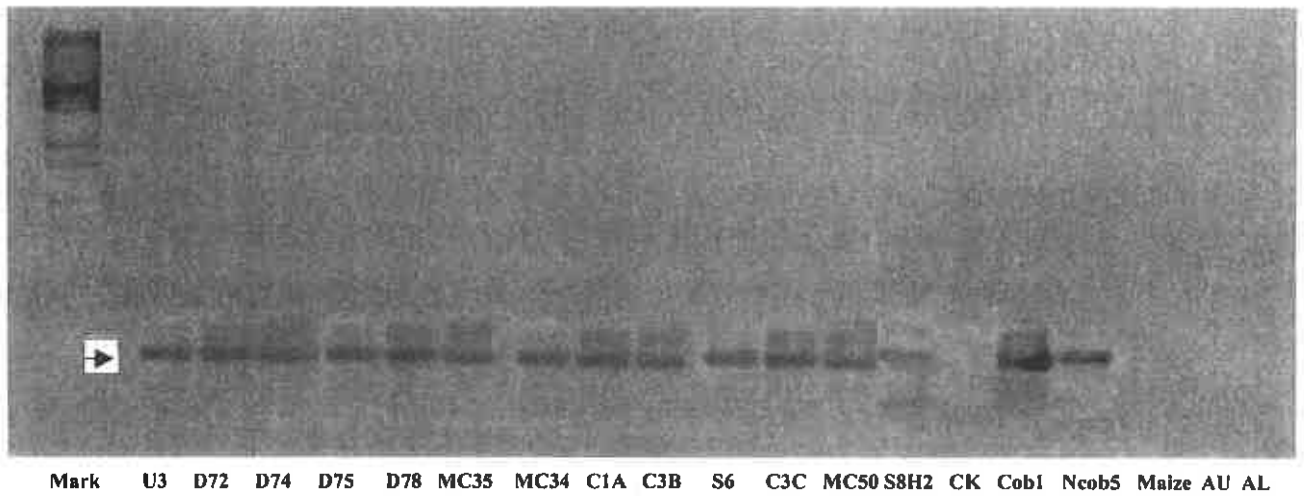

Fig. 4 Hybridization of *S. maydis* specific RAPD fragment (arrow) to a Southern blot of RAPD products of *Stenocarpella maydis* isolates and other fungus generated with primer 228. Mark: Lamda DNA DIG labelled; U3, D72, D74, D75, D78, MC35, MC34, C1A, C3B, S6, C3r°C, MC50, S8H2, Cob1, Ncob5: *Stenocarpella maydis*; AU: *Aureobasidium zeae*; AS: *Aspergillus* sp.; AL:*Alternaria* sp.; Maize: *Zea mays*; CK: no template DNA control

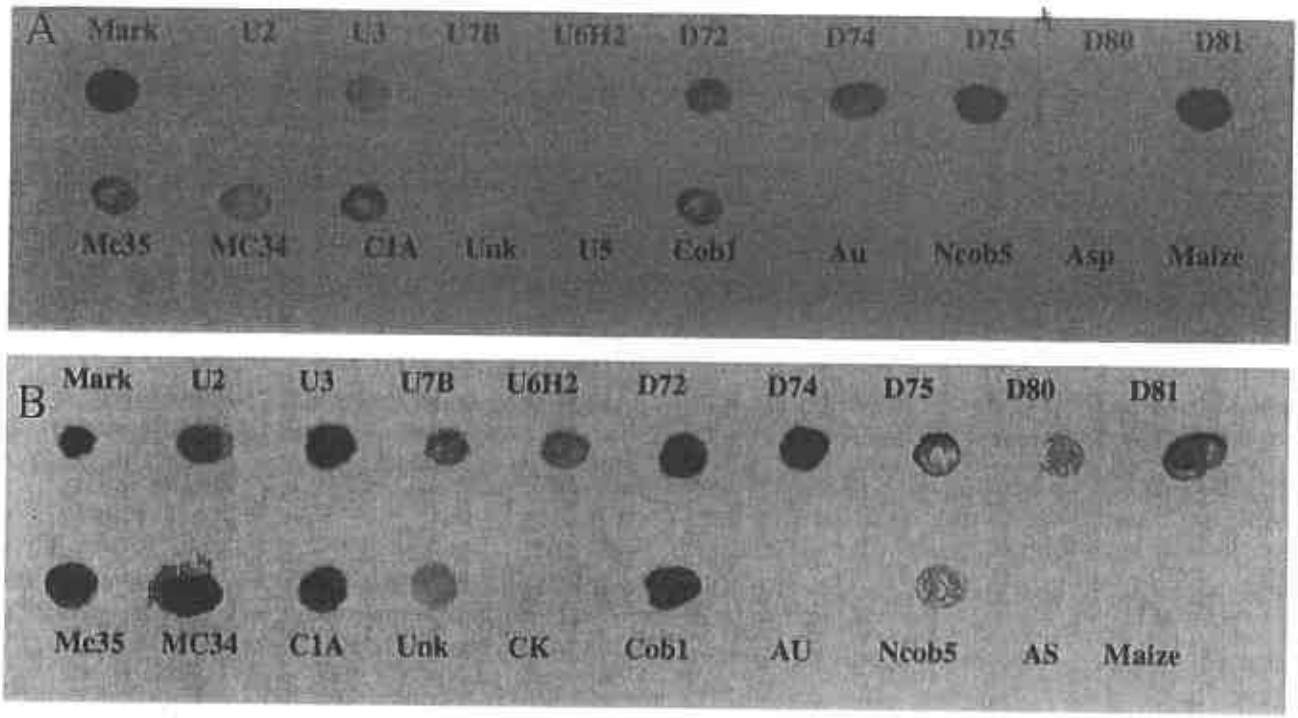

Fig. 5 Dot hybridization of two RAPD fragments labelled with DIG to RAPD product from *Stenocarpella maydis* and other fungus using primer UBC 228. Profile A: showing group I specific and B: showing *S. maydis* species-specific hybridization. Mark: Lamda DNA DIG labelled; U2, U3, U7B, U6H2, D72, D74, D75, D80, D81, MC35, MC34,C1A, UNK, U5, Cob1, Ncob5: *Stenocarpella* maydis; AU: *Aureobasidium zeae*; AS: *Aspergillus* sp.; Maize: *Zea mays*; CK: no template DNA control

to that of several *Phomopsis* (*Diaporthe*) species published in GenBank. For example, the most similar sequences in the GenBank were of the *Diaporthe* (*Phomopsis*) *phaseolorum* isolate sw93–13 (AF001018), which showed 91% similarity in a 242-base stretch.

Detection of *S. maydis* using designed primers

Specificity of PCR

For primer pair P1/P2, the expected 423 bp size fragments were amplified from genomic DNAs of *S. maydis* (Fig. 7). However, at the annealing temperature of 55°C some spurious bands were amplified from the genomic DNA mixture of maize plant and *S. maydis*. Although of different sizes, these spurious bands could still give rise to ambiguities and uncertainties in the results. When the annealing temperature was raised to 60°C, these bands disappeared and only one band was amplified from *S. maydis* in the presence of maize genomic DNA. No amplification was found from DNA of other fungi including *S. macrospora* that may

ITS1 P1

5' TCCGTAGGTGAACCTGCGG•••AAACCCTTTGTGAATTATACCTGACTGTTGCCTCGG
CACAGGCCGGCCCCCCATGGGGGGCCCCTCGGAAACAAAGAACAGGCCCCCC•••AATAC
GGAAATTCTGGAGCAGGAAAAACACCAAATGAATCAAAAACTTTCYAACAACGGATCTC
TTGGTTCTGGCATCGGATGCCGAACGCAGCGAAAAAAAAAAAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTTCCGGAGGGCAT
GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGTTGGGGCACTGCCTGTACA
GAAGGCAGGCCCTGAAATTCAGTGGCGAGCTCGCCAGGACCCCTAGCGCAGTAGTTAAAC

P2

CCTCGGTCGGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTTTTGTAAGGGG

ITS4

GASCTCGGATCAGG•••GCATATCAATAAGCGGAGGA 3'

Fig. 6 Alignment of DNA partial sequences of the internal transcribed spacer region of isolate MC43 of *Stenocarpella maydis*. The location of primers ITS1, ITS4, P1 and P2 are indicated. The sequences of P2 and ITS4 are complementary to those indicated

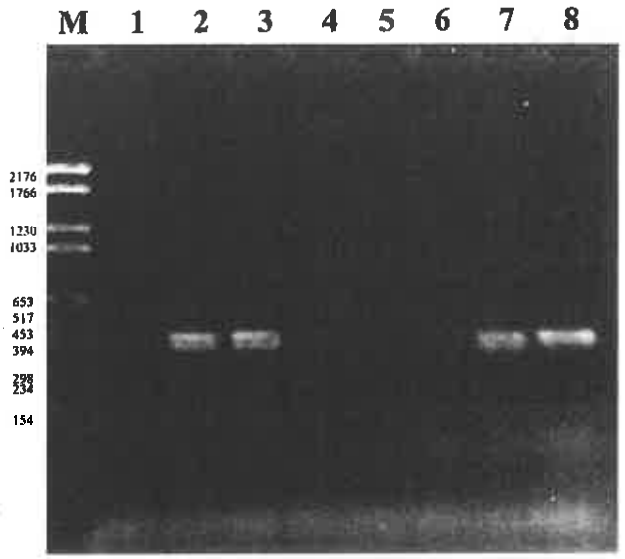

Fig. 7 Ethidium bromide-stained PCR products following amplification with primers P1/P2 from genomic DNA of *Stenocarpella maydis and* other fungal isolates (annealing temperature at 60°C). Lanes 1 to 8: SC4 (*Stenocarpella macrospora*), MC35, U7B (*S. maydis*), AU (*Aureobasidium zeae*), F (*Fusarium* sp.), Maize, MC34 and UNK (*S. maydis*), respectively. Lane M: Molecular DNA Marker VI (Boehringer Mannheim)

be found in a maize field (Table 3). Detection limits for isolates D80 and Y4 were approximately 10 pg genomic DNA for a 50 µl reaction volume (Fig. 8). For isolates MC35, the detection limit reached 0.1 pg DNA.

Field detection

DNA extracts from maize seeds from plants infected by *S. maydis and* field seed samples were subjected to PCR amplification using primer P1 and P2. The expected PCR products were obtained from the DNA extracts of infected seed samples. No amplification was found from DNA extracts of healthy plants from greenhouse. In three out of 10 field seed samples (asymptomatic) the presence of *S. maydis* (Fig. 9) was detected.

Discussion

The size of the ITS region of rDNA, amplified from all isolates tested, was very similar at approximately 575 bp. However, differences were detected by restriction digestion of the amplicon. The RF A was consonant with RAPD profile of RG I. RF B was concordant with RG II except for one isolate, U5. RG I isolates, with similar genetic background and found distributed in all four geographic areas, may be considered as a basic phenotype and probably developed from a common ancestor. It was also noted in the present study that RG II is strongly associated with a greater affinity to sporulate. Most of the RG II isolates appeared pink and produced a large number of spores without forming any pycnidia. RG I and RG II may thus be considered as being two genetically distinct groups.

The PCR-RFLPs from ITS regions does not take into account variation of the entire genome, but instead relies on only one specific area for differentiation (Lovic et al., 1995; Achenbach et al., 1996; Hyun and Clark, 1998). Sequence changes can occur relatively

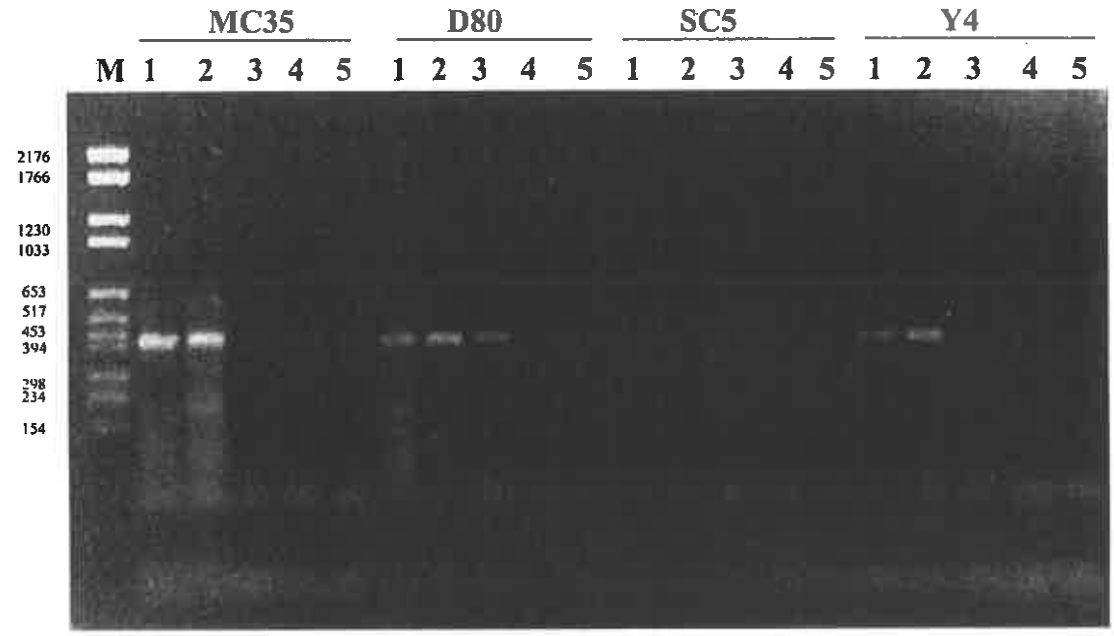

Fig. 8 Ethidium bromide-stained PCR products following amplification with primers P1/P2 from genomic DNA of *Stenocarpella maydis and S. macrospora* at annealing temperature of 60°C. Lanes 1 through 5 for all isolates: genomic DNA 1 ng, 100 pg, 10 pg, 1 pg and 0.1 pg. Lane M: Molecular DNA Marker VI (Boehringer Mannheim). MC35, S80 and Y4: *S. maydis*; SC5: *S. macrospora*

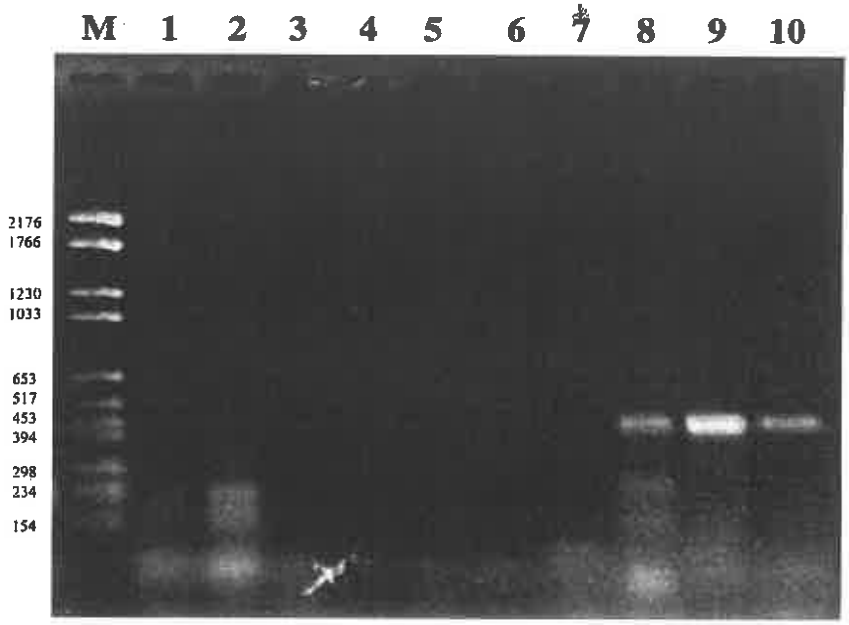

Fig., 9 Detection of seed samples (asymptomatic) with primer pair P1/P2. Three out of 10 samples showed positive amplification. Lane M: Molecular DNA Marker VI (Boehringer Mannheim)

quicker in spacer regions than in the coding region, and the way in which they change, within a species and between species, has been studied in large numbers of organisms (Lovic et al., 1995; Brunting et al., 1996). RAPD analysis, however, takes the entire genome into account and provides a more accurate reflection of the extent of variation, thus presenting a more useful method of differentiating isolates. The RAPD-PCR analysis is specific since a single-base mismatch in the primer annealing site can inhibit the amplification of DNA fragments (Lovic et al., 1995; Brunting et al., 1996; Hyun and Clark, 1998). Previous reports (Moukhamdov et al., 1994; Shi et al., 1996) support the findings of this study that RAPD-PCR is very useful for genetic analysis among closely related isolates and species. Thus, RAPD analysis is suitable to fill the analytical gap at the subspecies level where conventional methods fail (Tham et al., 1994).

Stenocarpella macrospora, is very similar to *S. maydis* in many aspects except for the size of conidia and additional requirement for biotin, *in vitro* (Latterell and Rossi, 1983; Dorrance et al., 1999). Further comparison of RAPD profiles using three UBC RAPD primers and PCR-RFLP clearly revealed that *S. macrospora* isolates were different from RG I and RG II of *S. maydis* (Xia and Achar, unpublished data).

The reliability and sensitivity of detecting *S. maydis* by light microscopy is limited by the amount of material which can be examined, and with difficulties in observing mycelium without pycnidia or spores. In the present study, primer pairs, P1/P2 permitted specific detection of *S. maydis* at annealing temperature of 60°. Plant tissues always contain inhibitors that could hamper the PCR detection of pathogens in those tissues. The inhibition might be due to tannins, humic acids, polysaccharides or phenolic compounds, which affect enzymatic activities and bind to RNA and DNA (Huff et al., 1994; Lovic et al., 1995). Several procedures have been proposed to overcome these problems. More recently, a combined biological and enzymatic amplification, magnetic capture–hybridization–PCR, and immunocapture–PCR were developed to circumvent inhibiting compounds (Smith et al., 1995). In the present study, such problems were not encountered while detecting the pathogen in the seeds. However, sensitivity of detecting of *S. maydis* in leaves or husks was much lower than in the seeds.

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