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Genetic diversity and population structure among isolates of the brown spot fungus, *Bipolaris oryzae,* as revealed by inter-simple sequence repeats (ISSR)

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***Bipolaris oryzae*, the rice brown spot fungus is one of the pathological threats to rice crop worldwide. The genetic diversity among the Indian isolates of brown spot pathogen was studied using inter-simple sequence repeats (ISSR). Considerable intraspecific variability among the isolates of *B. oryzae* was revealed. Eighty-seven (87) highly reproducible fragments were amplified in all 69 isolates with an average of 13.8 markers per primer. Polymorphism range shown by ISSR primers was 83.33 to 95.45%, while the range of total loci scored was from 6 to 22. The molecular weight of scorable loci ranged from 150 to 2600 bp. The results obtained confirmed the genetic diversity of rice brown spot fungus among samples under study. The present study suggests that ISSR markers can be used as robust molecular markers for the population genetics, epidemiological and ecological studies of *B. oryzae* that will aid in designing improved methods for management of the rice brown spot disease.**

Key words: *Bipolaris oryzae*, rice brown spot, inter-simple sequence repeats (ISSR), dendrogram, polymorphism.

INTRODUCTION

Rice (*Oryza sativa* L.) is the most cultivated cereal crop worldwide and it has been estimated that half the world's population subsists wholly or partially on this crop (Nguyen and Ferrero, 2006). Rice cultivation takes place in all states of India, but West Bengal, Uttar Pradesh, Madhya Pradesh, Punjab, Orissa and Bihar are the major rice producing states. Rice crop is severely affected by brown spot disease. Brown spot is caused by heterothallic ascomycete *Cochliobolus miyabeanus* (Ito and Kurib.) Drechsler ex Dastur) (Anamorph: *Bipolaris oryzae* (Breda de Haan) Shoem. which is the most important constraint to rice production in all rice growing countries of the world (Shabana et al., 2008). Rice is the most common host of *B. oryzae*. All aerial parts of the plant can be affected in moistened environment. Early stage symptoms causes tiny water soaked lesions on the leaves, which at later stages coalesce to form typical elongated lesions half inch long. In case of severe infections, lesions cover the entire leaf thus reducing the nutrient absorption, photosynthetic area and results in the decrease of tillering nodes. Leaves may shrivel and die. Stems may become girdled and break causing the panicle to fall, and seed to be lost. If flowers and seed are infected, they become grey; often spore masses are present (Percich and Nyvall, 1995). The seeds produced by such infected seedlings show typical brown spot

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Abbreviations: RFLP, Restriction fragment length polymorphism; **RAPD,** random amplified polymorphic DNA; **AFLP,** amplified fragment length polymorphic DNA; **VNTR,** variable number tandem repeats; **ISSR,** inter-simple sequence repeats; **PDA,** potato dextrose agar; **PDB,** potato dextrose broth.

symptoms.

The disease was reported in West Bengal in 1943 and has also been reported in many countries around the world, especially under semi-dry conditions (Ou, 1985). An epidemic of this disease lead to the 'Great Bengal Famine' and killed nearly two million people due to starvation (Padmanabhan, 1973). In Asia, brown spot has been noted to reduce yield from 6-90% (Mew and Gonzales, 2002). Growers and experts estimate that typical losses are 5-30%, but without control losses may reach 75- 100% (Percich and Nyvall, 1995). The relative importance of brown spot on rice has increased, in part due to the growing population which has resulted in an increased demand for rice with high quality appearance and free of in-borne disease. Surveys confirmed that brown spot remains among the most serious constraints to yield in South Asia (Widawsky and O'Toole, 1990; Geddes and Iles, 1991). Host plant resistance is the most promising method to brown spot disease control (Bonman et al., 1992). The analysis of genetic variation in plant pathogen populations is an important prerequisite for understanding coevolution in the plant pathosystem (McDonald et al., 1989). Populations of rice brown spot pathogen throughout the world have been studied for the phenotypic and genetic variation (Ouedraogo et al., 2004; Motlagh and Kaviani, 2008; Kamal and Mia, 2009; Motlagh and Anvari, 2010).

The utility of DNA markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphic DNA (AFLP), variable number tandem repeats (VNTR) and inter-simple sequence repeats (ISSR) in detecting genetic variability is well established for many phytopathogenic fungi (Aminnejad et al., 2009; Barcelos et al., 2011; Sharma et al., 2009; Kamal and Mia, 2009; Bayraktar and Dolar, 2010). Molecular techniques have been used to develop faster and unambiguous methods for differentiation of populations of *B. oryzae* by employing RFLP (Ouedraogo et al., 2004; Siripornwisarn et al., 2009), RAPD (Kumar et al., 2011; Motlagh and Anvari, 2010), AFLP (Castell-Miller and Samac, 2012) and VNTR (Kamal and Mia, 2009; Paz et al., 2007) techniques. However, none of these methods have so far been able to differentiate isolates of *B. oryzae* from different geographical regions.

ISSR involves amplification of the DNA sequences between two adjacent and inversely oriented microsatellites usually by using 18-24 mer primer (Williams et al., 1990; Godwin et al., 1997). The potential supply of ISSR marker depends on the variety and frequencies of microsatellites between different individuals, population and species (Ye et al., 2005). ISSR is a popular DNA fingerprinting method because it is quick, inexpensive, highly polymorphic and do not require any sequence information about the genome under study. Although ISSR has been used to assess genetic variation in a number of phytopathogenic fungi, no study has so far determined genetic variation in fungus *B. oryzae*.

To the best of our knowledge, this is the first report of the use of ISSR marker to study the genetic diversity of brown spot pathogen *B. oryzae*. The objective of this study was to investigate the genetic variation with *B. oryzae* isolates using ISSR method.

MATERIALS AND METHODS

Collection of samples

The brown spot disease samples of rice were collected from 16 different rice growing states of India (Karnataka, Kerala, Assam, Andhra Pradesh, Manipur, Madhya Pradesh, Maharashtra, Chattisgarh, Tamil Nadu, Uttar Pradesh, West Bengal, Arunachal Pradesh, Orissa, Delhi, Bihar and Jharkhand). The rice seed samples were obtained from rice research institutes, agricultural research stations, plant breeding stations and farmer's holdings. Two hundred seeds per sample were surface disinfected in 1% freshly prepared sodium hypochlorite solution for 2 min. Seeds were rinsed with sterilized distilled water, air dried and then plated onto three layers of moistened filter paper (Whatman No. 2) in Petri dishes and incubated at 25°C for seven days under alternating near ultraviolet light (nuv) and dark conditions (nuv/dark, 12/12 h). The main objective of surface disinfection is to prevent the seed from saprophytic fungi and to detect internally seed-borne fungi. Rice seed samples were screened for the isolation of *B. oryzae* isolates as listed in Table 1. *Bipolaris sorghicola* was isolated from sorghum seed sample collected from Karnataka. *Alternaria padwickii* was isolated from rice cultivar and was used as an out group in the phylogenetic analysis. All isolates were propagated on potato dextrose agar (PDA) slants, covered with parafilm at 25±1°C and maintained in a collection at Asian Seed Health Centre, Department of Biotechnology, University of Mysore, Karnataka, India.

Genomic DNA isolation

Mycelium of each fungal isolate was obtained by growing each isolate in potato dextrose broth (PDB) (Himedia, Bangalore, India). For DNA extraction, 150 ml of PDB was inoculated with two colonized agar plugs of 5 mm diameter for each isolate, and cultures were incubated at 22 ± 2°C for 8 days under alternating 12 h durations of darkness and near ultra-violet (NUV) light. After incubation, the mycelial mat was harvested under aseptic conditions, and genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Bangalore, India) following instructions of the manufacturer. The purity of isolated DNA was checked by running 5 µl DNA sample on 0.8% agarose gel at 80 V for 45 min.

ISSR primers and PCR amplification

The sequences of seven ISSR primers of 16 mer (ISSR 08), 9 mer (ISSR 12, ISSR 14 and ISSR 15) and 12 mer (ISSR 20, ISSR 21 and ISSR 22) (Table 1) were synthesized from Sigma-Aldrich, Bangalore and screened in the present study. ISSR-PCR amplification was carried out in 20 µl reaction mixture in 200 µl PCR tubes. Each reaction mixture contained 20 ng genomic DNA, 200 µM of each dNTPs, 0.5 unit of Taq polymerase, 1 X Taq polymerase buffer solution and 0.2 µM of primer. The reaction mixture was overlaid by one drop of mineral oil. Amplifications were performed in a thermal cycler (Eppendorf® Mastercycler® Thermal Cyclers, Germany) programmed for an initial denaturation of 4 min at 94°C, 45 cycles of denaturation at 94°C for 1 min, annealing temperature specific for each primer for 1 min and extension at 72°C for 2 min followed by a final extension at 72°C for 5 min.

Table 1. Source of fungal isolates used in the present study.

Fungi	Host/Cultivar	Place of collection	Isolate ID	Year
Bipolaris oryzae	*Oryza sativa,* Phalgun	Andhra Pradesh	*BoAP1*	2009
Bipolaris oryzae	*Oryza sativa, Pankaja*	Andhra Pradesh	*BoAP2*	2009
Bipolaris oryzae	*Oryza sativa, Surekha*	Andhra Pradesh	*BoAP3*	2009
Bipolaris oryzae	*Oryza sativa,* Sabari	Kerala	*BoKer1*	2010
Bipolaris oryzae	*Oryza sativa, Aswathi*	Kerala	*BoKer2*	2010
Bipolaris oryzae	*Oryza sativa, Triveni*	Kerala	*BoKer3*	2010
Bipolaris oryzae	*Oryza sativa,* Mantriphukri	Manipur	*BoMani1*	2009
Bipolaris oryzae	*Oryza sativa, IR 36*	Manipur	*BoMani2*	2009
Bipolaris oryzae	*Oryza sativa, Phou-oibi*	Manipur	*BoMani3*	2009
Bipolaris oryzae	*Oryza sativa,* ADT 38	Tamil Nadu	*BoTN1*	2009
Bipolaris oryzae	*Oryza sativa,* ADT 36	Tamil Nadu	*BoTN2*	2009
Bipolaris oryzae	*Oryza sativa,* White Ponni	Tamil Nadu	*BoTN3*	2009
Bipolaris oryzae	*Oryza sativa,* Nati Masoori	Uttar Pradesh	*BoUP1*	2010
Bipolaris oryzae	*Oryza sativa,* Saryu	Uttar Pradesh	*BoUP2*	2010
Bipolaris oryzae	*Oryza sativa,* Nabin	Uttar Pradesh	*BoUP3*	2010
Bipolaris oryzae	*Oryza sativa,* Mahamaya	Chhattisgarh	*BoChattis1*	2010
Bipolaris oryzae	*Oryza sativa*	Chhattisgarh	*BoChattis2*	2010
Bipolaris oryzae	*Oryza sativa*	Chhattisgarh	*BoChattis3*	2010
Bipolaris oryzae	*Oryza sativa,* Piska Nagri	Ranchi	*BoRan4*	2011
Bipolaris oryzae	*Oryza sativa*	Ranchi	*BoRan5*	2011
Bipolaris oryzae	*Oryza sativa*	Ranchi	*BoRan6*	2011
Bipolaris oryzae	*Oryza sativa,* MR 219 Malaysia	Madhya Pradesh	*BoMP1*	2010
Bipolaris oryzae	*Oryza sativa,* P 14 60	Madhya Pradesh	*BoMP2*	2010
Bipolaris oryzae	*Oryza sativa,* PUSA Kranti	Madhya Pradesh	*BoMP3*	2010
Bipolaris oryzae	*Oryza sativa,* Swarna	West Bengal	*BoWB1*	2008
Bipolaris oryzae	*Oryza sativa,* Kankaj	West Bengal	*BoWB2*	2008
Bipolaris oryzae	*Oryza sativa, IET849*	West Bengal	*BoWB3*	2008
Bipolaris oryzae	*Oryza sativa,* Karishma	Madhya Pradesh	*BoMP4*	2011
Bipolaris oryzae	*Oryza sativa, Suraksha*	Madhya Pradesh	*BoMP5*	2011
Bipolaris oryzae	*Oryza sativa, Anrada*	Madhya Pradesh	*BoMP6*	2011
Bipolaris oryzae	*Oryza sativa,*	Jharkhand	*BoJhar1*	2010
Bipolaris oryzae	*Oryza sativa,*	Jharkhand	*BoJhar2*	2010
Bipolaris oryzae	*Oryza sativa,*	Jharkhand	*BoJhar3*	2010
Bipolaris oryzae	*Oryza sativa,* Telahamsa	Andhra Pradesh	*BoAP4*	2009
Bipolaris oryzae	*Oryza sativa, Kothamala*	Andhra Pradesh	*BoAP5*	2009
Bipolaris oryzae	*Oryza sativa*	Andhra Pradesh	*BoAP6*	2009
Bipolaris oryzae	*Oryza sativa,* Karuna	Tamil Nadu	*BoTN4*	2009
Bipolaris oryzae	*Oryza sativa,* Rasi	Tamil Nadu	*BoTN5*	2009
Bipolaris oryzae	*Oryza sativa, Bhavani*	Tamil Nadu	*BoTN6*	2009
Bipolaris oryzae	*Oryza sativa,*	Arunachal Pradesh	*BoArunPr1*	2007
Bipolaris oryzae	*Oryza sativa*	Arunachal Pradesh	*BoArunPr2*	2008
Bipolaris oryzae	*Oryza sativa*	Arunachal Pradesh	*BoArunPr3*	2010
Bipolaris oryzae	*Oryza sativa,* KRH 2	Arunachal Pradesh	*BoArunPr4*	2008
Bipolaris oryzae	*Oryza sativa,*	Arunachal Pradesh	*BoArunPr5*	2008
Bipolaris oryzae	*Oryza sativa*	Arunachal Pradesh	*BoArunPr6*	2008
Bipolaris oryzae	*Oryza sativa,* Padmini	Orissa	*BoOri1*	2008
Bipolaris oryzae	*Oryza sativa, Anjali*		*BoOri2*	2008
Bipolaris oryzae	*Oryza sativa, Lalat*		*BoOri3*	2008
Bipolaris oryzae	*Oryza sativa,*	Chattisgarh	*BoChattis4*	2008
Bipolaris oryzae	*Oryza sativa,*	Chattisgarh	*BoChattis5*	2008
Bipolaris oryzae	*Oryza sativa,*	Chattisgarh	*BoChattis6*	2008

Table 1. Contd.

Bipolaris oryzae	*Oryza sativa,Ratna*	Maharashtra	*BoMaha1*	2008
Bipolaris oryzae	*Oryza sativa,KRH2*	Delhi	*BoDel1*	2008
Bipolaris oryzae	*Oryza sativa,*	Arunachal Pradesh	*BoArunPr7*	2008
Bipolaris oryzae	*Oryza sativa, IR20*	Bihar	*BoBih1*	2008
Bipolaris oryzae	*Oryza sativa,Archana*	Bihar	*BoBih2*	2008
Bipolaris oryzae	*Oryza sativa,Rajeswari*	Orissa	*BoOri4*	2008
Bipolaris oryzae	*Oryza sativa,Sita*	Bihar	*BoBih3*	2008
Bipolaris oryzae	*Oryza sativa, Ketki Joha*	Assam	*BoAs1*	2008
Bipolaris oryzae	*Oryza sativa,BR 34*	Bihar	*BoBih4*	2010
Bipolaris oryzae	*Oryza sativa,* Unknown1	Karnataka	*BoU1Kar*	2010
Bipolaris oryzae	*Oryza sativa,* Unknown2	Karnataka	*BoU2Kar*	2010
Bipolaris oryzae	*Oryza sativa, Jaya*	Karnataka	*BoKar1*	2010
Bipolaris oryzae	*Oryza sativa,BR 8*	Bihar	*BoBih5*	2010
Bipolaris oryzae	*Oryza sativa, IR64*	Karnataka	*BoKar2*	2010
Bipolaris oryzae	*Oryza sativa, Bokul Borah*	Assam	*BoAs2*	2010
Bipolaris oryzae	*Oryza sativa, B 370*	Karnataka	*BoKar3*	2010
Bipolaris oryzae	*Oryza sativa, BR 2655*	Karnataka	*BoKar4*	2010
Alternaria padwickii	*Oryza sativa, Jaya*	Karnataka	*ApKar1*	2010

Table 2. List of ISSR primers used in the present study.

Primer name	Core sequence (5'-3')	Attached bases	Tm (°C)	Range of loci scored (bp)	Total loci	Polymorphism	
						No. of loci	%
ISSR08	(GACA)4	-	45.0	200-2400	14	12	85.71
ISSR12	(GTG)3	GC	43.5	400-2000	06	05	83.33
ISSR14	(GAG)3	GC	40.1	150-1900	22	21	95.45
ISSR15	(CAC)3	GC	45.5	210-2300	18	16	88.88
ISSR20	(CA)6	AG	40.6	300-2600	15	14	93.33
ISSR21	(CA)6	GT	39.2	400-1815	10	08	80.00
ISSR22	(CA)6	AC	40.1	220-2450	12	11	91.66
Total					97	87	88.33

Agarose gel electrophoresis

Horizontal submerged gel electrophoresis unit was used for fractionating ISSR primers on agarose gel. After amplification, 10 µl of each amplified product was electrophoresed in a 1.5% agarose gel prepared in 1 X TBE buffer, 6 X DNA loading dye was mixed in the ratio of 5 : 1 v/v to amplified product. Ethidium bromide was used in gel to stain DNA bands. Electrophoresis was performed at 80 V for 4 h in 1 X TBE buffer. Two hundred and fifty nanogram (250 ng) of 100 bp DNA ladder was also loaded in the same gel to estimate the molecular weight of the amplified product.

Data analysis

DNA banding pattern generated by ISSR primers were scored as "1" for presence of an amplified band and "0" for its absence. All ISSR assays were repeated twice and only the reproducible bands were scored. For genetic distance analysis, WINDIST software of the WINBOOT package was used with the NTSYS format. Cluster analysis was based on similarity matrices using the UPGMA program in the WINBOOT software package (Yap and Nelson, 1996). The Dice coefficient was used for dendrogram construction with a sample number of 100.

RESULTS AND DISCUSSION

The genetic diversity of brown spot fungi throughout India has not been reported, although it is a main problem of rice production in India. In the present study, *B. oryzae* isolates were collected from seed samples obtained from different geographical regions of India. All ISSR markers in this study depicted high polymorphism (83.33 to 95.45%). Different levels of polymorphism and number of bands were obtained with seven primers (Table 2). Bands ranging from 150-2600 bp were generated for each isolate (Figure 2). Only a few band differences were observed in *B. oryzae* isolates with ISSR 12. The results obtained with the primer ISSR 15 is shown in Figure 1. The primers produced 87 polymorphic markers at an

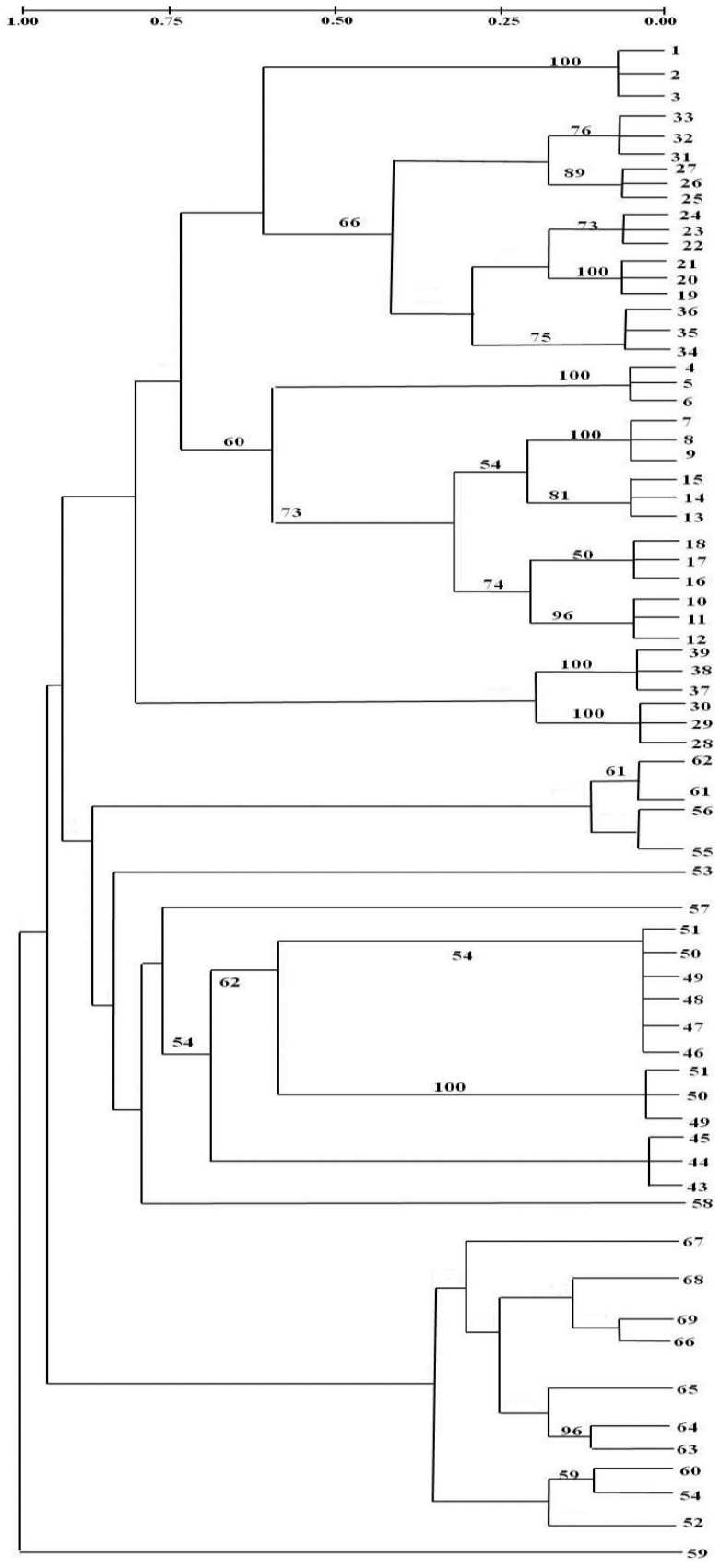

Figure 1. UPGMA dendrogram for ISSR markers based on dice genetic similarity coefficients. Bootstrap values above 50% are shown.

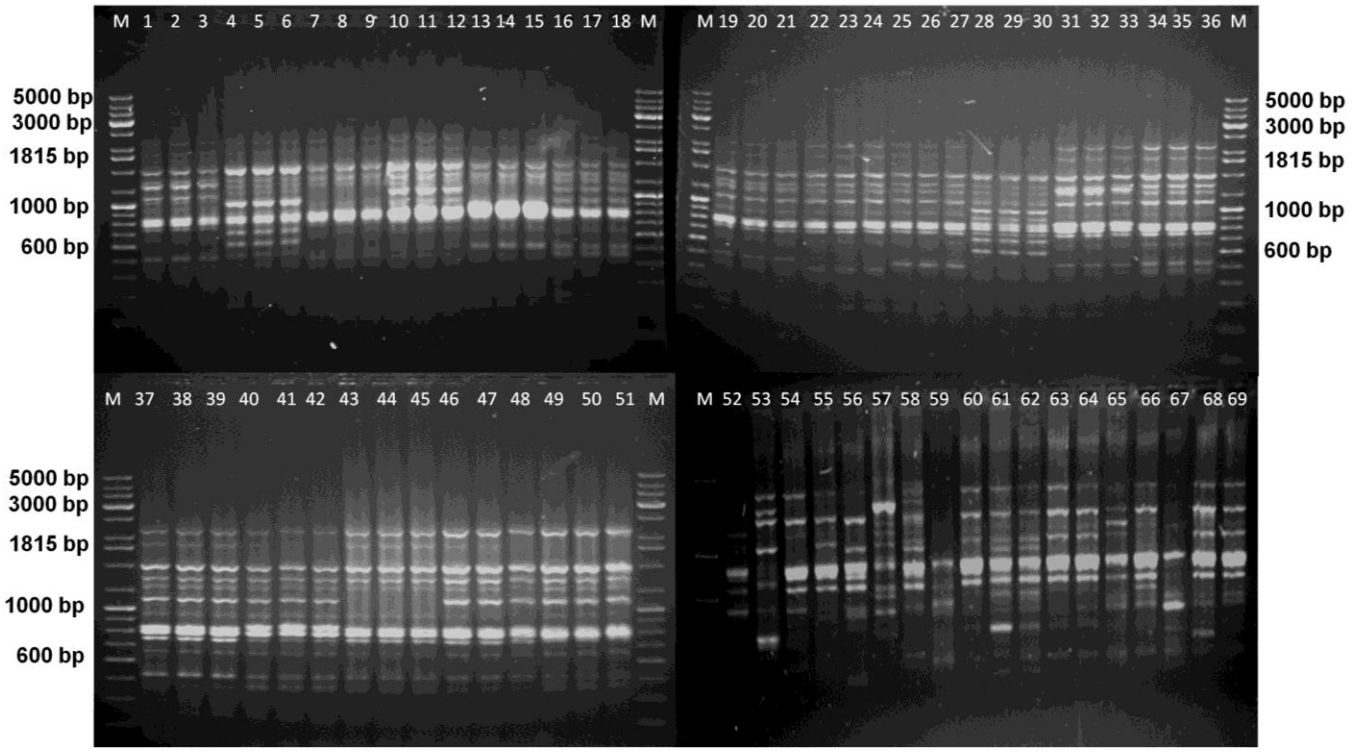

Figure 2. Electrophoretic banding pattern generated from *Bipolaris oryzae* isolates with ISSR 15. Lanes 1 to 58, *Bipolaris oryzae* isolates; Lane 59, *Alternaria padwickii*; Lanes 60 to 69, *Bipolaris oryzae* isolates; Lane M, medium range DNA marker.

average of 12.42 markers per primer. The highest numbers of markers were obtained with the primers ISSR 14 and ISSR 15, whereas the primer ISSR 12 resulted in the lowest number of markers. Among the large number of DNA bands specifically amplified in all *B. oryzae* isolates, DNA bands of approximately 800, 1500 and 2500 bp were more prominent. These bands were present in all *B. oryzae* isolates irrespective of their geographical origin and the rice variety from which they were isolated. The distance matrix based on ISSR data sets were used to construct a dendrogram which is shown in Figure 1.

No relationship was observed between the genetic profiles and geographic origin. This can be attributed to the fact that *B. oryzae* has frequently been reported to be seed-borne, so populations of *B. oryzae* in India may not remain geographically isolated for long due to possible exchange of infected seeds. Cultural practices, transportation of infected seeds, soil and plant materials also have contributed to the genetic diversity observed within geographic regions (O'Donnell et al., 1998). This could also account for the lack of correlation between genetic profiles and origin found among Indian isolates.

The genetic diversity of *B. oryzae* has been widely studied in Philippines (Paz et al., 2007; Burgos et al., 2009), Arkansas (Ouedraogo et al., 2004), Bangladesh (Kamal and Mia, 2009), Uttar Pradesh (Kumar et al., 2011) and Iran (Motlagh and Anvari, 2010). In the present study, the patterns generated from ISSR demonstrated variability among *B. oryzae* isolates. This variability may have arisen through point mutations, gene flow and/or recombination (Parry et al., 1995), but the exact mechanism responsible for this high degree of genetic variability in the present study could not be found. Further, the sexual cycle does not seem to be a source of variation for the rice brown spot pathogen in Philippines (Paz et al., 2007).

Nucleotide sequence analysis of all these isolates followed by multiple sequence alignment may provide an accurate pattern responsible for genetic variation. The dendrogram study in the present investigation revealed that geographic origin of isolates does not play crucial role in grouping of isolates, as in each group there were mixed population of 3 to 5 geographical regions. Similar results have been shown by Motlagh and Anvari (2010) in their study on isolates of *B. oryzae* from different regions of Guilan Province of North Iran. The topology of the dendrogram with bootstrap values indicates that both local and geographic polymorphism exists.

The analysis of ISSR polymorphism among populations of *B. oryzae* from different regions of India revealed the occurrence of high level of polymorphism, indicating a wide and diverse genetic base. ISSR markers used in the present study increases the marker density to find out genetic relationship. It also indicates that *B. oryzae* isolates from diverse geographical regions of India may

be genetically heterogeneous and the interrelationship amongst the different isolates can be easily, precisely and reliably explained by ISSR-polymerase chain reaction technology.

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