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Molecular characterization of *Bipolaris sorokiniana* populations from winter cereals

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ABSTRACT

Isolates of *B. sorokiniana* were obtained from infected leaf samples of bread wheat, durum wheat, barley, triticale and rye leaves collected randomly from Gurdaspur, Ludhiana and Ferozepur areas of Punjab. Based on qualitative colony parameters, 30 isolates were selected for further studies. Molecular characterization of the isolates was done using 30 RAPD primers and Polymorphic Information Content values for these ranged from 0.51 to 0.98 with an average of 0.72. A total of 197 alleles were amplified out of which 184 were polymorphic and 13 were monomorphic. The number of alleles amplified varied from 3-13 and size of amplified fragments varied from 3.5 Kb to 200bp.The similarity index values ranged from 0.48 to 0.78 indicating wide range of genetic diversity among isolates. On cluster analysis of the molecular data, the isolates were grouped into two major clusters at 50 per cent level of similarity, whereas at 60 per cent similarity coefficient, the isolates were grouped into 10 clusters. Five complete clusters were formed by 25 isolates whereas 5 isolates formed independent lineages. RAPD profiles, however, did not correlate polymorphism with the geographic source or host source of the isolates.

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INTRODUCTION

Bipolaris sorokiniana (Sacc.) Shoemaker [Teleomorph: *Cochliobolus sativum* (Ito and Kuribayashi) Drechs. ex Dastur, Syn. *Helminthosporium sativum* Pammel, C.M. King and Bakke causing spot blotch of cereals is a major production constraint in the intensive cropping systems affecting 1.2 crore ha in South Asia (Kumar *et al.,* 2002). In addition, it has expanded its area of colonization with the warming of the temperature towards temperate areas because high humidity with temperature between 22-25°C during growing season enhance damage caused by spot blotch (Kaur and Nanda, 2001). Conidial populations of *B. sorokiniana* have extensive variability in morphological and physiological traits (Christensen and Davies, 1937; Tinline, 1960; Wood, 1962; Nelson and Kline, 1962).

Characterization of inherent variability is fundamental for the study of host parasite interactions in order to develop appropriate strategies for plant breeding programmes and long-term management of disease. Analysis of the diversity of plant pathogens have been revolutionized by the molecular techniques particularly the Polymerase Chain Reaction (PCR). Among these PCR based molecular markers, Random Amplified Polymorphic DNA (RAPD) markers provide a mechanism for swiftly and easily characterizing isolates in terms of polymorphism of primer – defined DNA fragments and genetic polymorphism in phytopathogenic fungi (Malvickan and Grau, 2001) for inter and intra-specific variability among populations from different and from the same geographic regions (Vakalovnakis and Fragkiadakis, 1999; Oliveira *et al.,* 2002; Muller *et al.,* 2005).

There has been a limited effort on molecular study of Indian isolates of *Bipolaris sorokiniana* obtained from different cereal hosts and no information was available on these aspects from Western part of India. The present study was undertaken to assess genetic variability of *B. sorokiniana* isolates obtained from different cereal hosts (bread wheat, barley, durum, triticale and rye) from Punjab.

MATERIAL AND METHODS

Fungal isolates :

Isolations of the pathogen were made on potato dextrose agar (PDA) amended with 1 per cent finely ground wheat grains, from infected leaf samples of bread wheat, durum wheat, barley, triticale, and rye collected from different parts of Punjab (India) *viz.,* Gurdaspur, Ludhiana and Ferozepur (Fazilka/ Abohar) to represent foot-hills, Central and Southern parts of the region. The cultures were purified by single spore isolation method. From all the samples, thirty isolates of *Bipolaris sorokiniana* were selected for further studies based on

Table A : Sources (host and location) of *B. sorokiniana* isolates

Isolate No.	Colony colour	Colony margin	Host	Variety	Location
1.	White	Smooth	Barley	PL 426	Ludhiana
2.	Offwhite	Smooth	Barley	PL 481	Ludhiana
3.	Dull black	Irregular	Barley	PL 419	Ludhiana
4.	Mixture of black and white	Irregular	Bread wheat	PBW 343	Ludhiana
5.	Greenish white	Irregular	Barley	BL 286	Ludhiana
6.	Greenish white	Smooth	Barley	RD 2700	Ludhiana
7.	Offwhite	Irregular	Barley	PL 481	Ludhiana
8.	Black	Smooth	Triticale	TL 2708	Ludhiana
9.	Mixture of black and white	Irregular	Triticale	TL 2708	Ludhiana
10.	White	Smooth	Barley	RD 2503	Ludhiana
11.	White	Irregular	Barley	RD 2715	Ludhiana
12.	Grey	Smooth	Barley	PL 426	Ludhiana
13.	Black	Smooth	Barley	RD 2703	Ludhiana
14.	Black	Irregular	Barley	BS 169	Ludhiana
15.	Offwhite	Irregular	Bread wheat	WH 147	Gurdaspur
16.	Dull black	Smooth	Bread wheat	HP 1633	Gurdaspur
17.	Dull black	Smooth	Durum	PDW 291	Abohar
18.	Grey	Smooth	Triticale	TL 3358	Ludhiana
19.	Mixture of black and white	Smooth	Bread wheat	PBW 373	Abohar
20.	Black	Smooth	Bread wheat	WH 896	Fazilka
21.	Dull black	Smooth	Durum	PDW 291	Ludhiana
22.	Mixture of black and white	Irregular	Bread wheat	H P 1633	Fazilka
23.	Mixture of black and white	Smooth	Bread wheat	WL 1562	Fazilka
24.	Black	Smooth	Bread wheat	PBW343	Fazilka
25.	Dull black	Smooth	Rye	Rye	Ludhiana
26.	Dull black	Irregular	Rye	Rye	Ludhiana
27.	Black	Smooth	Bread wheat	HD 2687	Abohar
28.	Grey	Smooth	Bread wheat	HW 2021	Abohar
29.	Grey	Smooth	Triticale	TL 3358	Ludhiana
30.	Dull black	Irregular	Triticale	TL 3358	Ludhiana

their host source, colony colour, colony margin, variety and location (Table A). Single spore cultures of all the isolates were cultivated on liquid culture medium (potato dextrose broth, PDB) at 25±1^{0}C. These cultures were harvested after 7 days of growth for extraction of DNA.

Genomic DNA was extracted by using CTAB method (Hexa-decyl tri-methyl ammonium bromide) modified by Doyle and Doyle (1990) from each isolate grown on liquid culture medium (PDB).

RAPD analysis :

All the reactions were carried out in a volume of 20µl with 60 ng DNA, 1 unit of Taq DNA polymerase (Promega), 1.5 mM $MgCl_2$, 1.25 µM primer and 1X PCR buffer. A drop of low molecular weight mineral oil was then overlaid on the reaction mix. The reactions were performed on a thermocycler (Eppendorf mastercycler) programmed for the initial denaturation at 94^{0}C for 5 minutes, followed by 45 cycles of denaturation at 94^{0}C for 1 minute, annealing at 37^{0}C for 1 min and extension at 72^{0}C for 1 minute with final elongation at 72^{0}C for 5 minutes. Primer survey was carried out and 37 primers from OPE, OPW, OPP, OPC series (Operon technologies, USA) and A- 01 to A –08 (Biodynamics kits, S.R.L) were screened using DNA from all the 30 isolates. The primers that gave reproducible and scorable amplification products were used for characterization of all the isolates.

Agarose gel electrophoresis :

The amplified products were electrophoresed on a 1.5 per cent agarose gel in 0.5X TBE buffer. Gels were stained with ethidium bromide (1µg/ml). Electrophoresis was carried out at 60 Volts for 4 hours and the gels were visualized under UV light and photographed using UVP gel documentation system (FOTODYNE). One Kb ladder (MBI, Fermentas) was used as a marker.

Scoring of RAPD and data analysis :

The RAPD allele sizes were determined depending on the position of bands relative to the ladder (1KB). The banding pattern of each isolate was coded in binary form, 1 representing the presence and 0 the absence of each band. Dendrogram was generated with in the SHAN programme of NTSYS- pc (Extra software, Setauket, NY) using the unweighted pair group method based on arithmetic averages (UPGMA). Cluster analysis based on their similarities was carried out to group the isolates into different clusters.

RESULTS AND DISCUSSION

The findings of the present study as well as relevant discussion have been presented under the following heads:

RAPD analysis:

Thirty primers yielded multiple DNA amplification products ranging from 3 (OPC 13) to 13 (OPW 03) (Table 1). Out of the total 197 amplified fragments 184 were polymorphic and 13 were common in all the 30 isolates. The Polymorphic Information Content (PIC) values ranged from as low as 0.5085 in primer A 06 to as high as 0.9814 in primer OPW 03 with an average of 0.7224 for all 30 primers. A high degree of polymorphism, in general, was obtained with most of the primers. The variation in intensity of some fragments in this study was observed. Isolate 11 obtained from barley and 18 from triticale had identical banding pattern (Fig. 1). These isolates may have originated from the same parent during evolution although they have different host sources. In case of primer A06, a total of 6 bands, ranging from approximately 3500 bp to 1Kb size, were obtained all of which were polymorphic indicating a high level of variability among the isolates.

Cluster analysis :

Dendrogram obtained by cluster analysis of molecular data generated by 30 RAPD primers exhibited variable degree of relationship among the isolates (Fig. 2). PCR grouping based on RAPD analysis revealed two major clusters. At 60 per cent similarity coefficient, six clusters were formed by twenty five isolates and five isolates formed independent lineages (Table 2). So all the 30 isolates were grouped into ten clusters at 60 per cent similarity whereas all of them were associated at the 45 per cent similarity level. Isolates from bread wheat were most variable being present in five out of eleven clusters followed by triticale. All the isolates of triticale were present in different clusters indicating their greater variability. Barley and durum isolates were next to triticale in variability whereas isolates of rye were present in single cluster II indicating their greater similarity. With respect to location, isolates from Ludhiana were most variable followed by Abohar and Fazilka isolates whereas

isolates from Gurdaspur were least variable being present in single cluster I.

Plant pathogenic fungi most commonly rely on mutation and recombination as the main source of genetically based variation (Burdon and Silk, 1997). Variability was observed among isolates of *B. sorokiniana* originated from same cultivar as well as between different cultivars of wheat (Oliveira *et al.,* 2002) and RAPD profiles did not correlate polymorphism with geographic source of the isolates. Gene flow along with other evolutionary forces can result in the spread of single gene (DNA sequence) and even establishment of whole populations in different regions (Mc Dermott and Mc Donald, 1993). In the present study also, variability at molecular level was observed among isolates from same host as well as from different hosts. Muller *et al.* (2005) reported that genetic variability was low with a similarity coefficient of >78 per cent among *B. sorokiniana* isolates obtained from different regions of Brazil irrespective of their different host cultivars. Some workers have identified specific DNA bands for selected isolates, which appeared to be potential enough to be genetic fingerprints for future strain identification and classification (Jaiswal *et al.,* 2007; Pandey *et al.,* 2008). Thus RAPD primers provide an easy, rapid and simple technique for the preliminary assessment of genetic

Table 1 : PIC values of primers used for analysis of RAPD of *B. sorokiniana* and no. of fragments amplified

Sr. No.	Primer No.	Primer sequence (5'-3')	Polymorphic information content (PIC) values	No. of amplified fragments	No .of polymorphic bands	No. of monomorphic bands
1.	OPW03	GTCCGGAGTG	0.9814	13	13	0
2.	OPW04	CAGAAGCGGA	0.8185	8	7	1
3.	OPW05	GGCGGATAAG	0.7601	5	5	0
4.	OPW06	AGGCCCGATG	0.6927	7	7	0
5.	OPW07	CTGGACGTCA	0.7114	6	6	0
6.	OPW08	GACTGCCTCT	0.8119	8	8	0
7.	OPW09	GTGACCGAGT	0.7995	5	5	0
8.	OPW18	TTCAGGGCAC	0.7343	7	7	0
9.	OPW19	CAAAGCGCTC	0.8193	6	6	0
10.	OPW20	TGTGGCAGCA	0.6384	7	6	1
11.	OPE03	CCAGATGCAC	0.6317	4	4	0
12.	OPE07	AGATGCAGCC	0.7118	5	5	0
13.	OPE11	GAGTCTCAGG	0.7504	5	5	0
14.	OPE12	TTATCGCCCC	0.7243	6	5	1
15.	OPE14	TGCGGCTGAG	0.8114	7	6	1
16.	OPE16	GGTGACTGTG	0.6210	9	8	1
17.	OPE17	CTACTGCCGT	0.7632	8	7	1
18.	OPE 18	GGACTGCAGA	0.7690	7	6	1
19.	OPP01	GATGCACTCC	0.7517	8	7	1
20.	OPP02	TCGGCACGCA	0.7253	4	4	0
21.	OPP03	CTGATACGCC	0.5588	9	7	2
22.	OPP04	GTGTCTCAGG	0.7311	7	7	0
23.	OPP05	CCCCGGTAAC	0.7583	6	6	0
24.	OPP06	GTGGGCTGAC	0.7241	7	7	0
25.	OPP08	ACATCGCCCA	0.7418	4	4	0
26.	OPP14	CCAGCCGAAC	0.7157	6	6	0
27.	OPC13	AAGCCTCGTC	0.5667	3	3	0
28.	A01	CCCAAGGTCC	0.5535	5	4	1
29.	A02	GGTGCGGGAA	0.7856	9	7	2
30.	A06	GAGTCTCAGG	0.5085	6	6	0
			Total	197	184	13

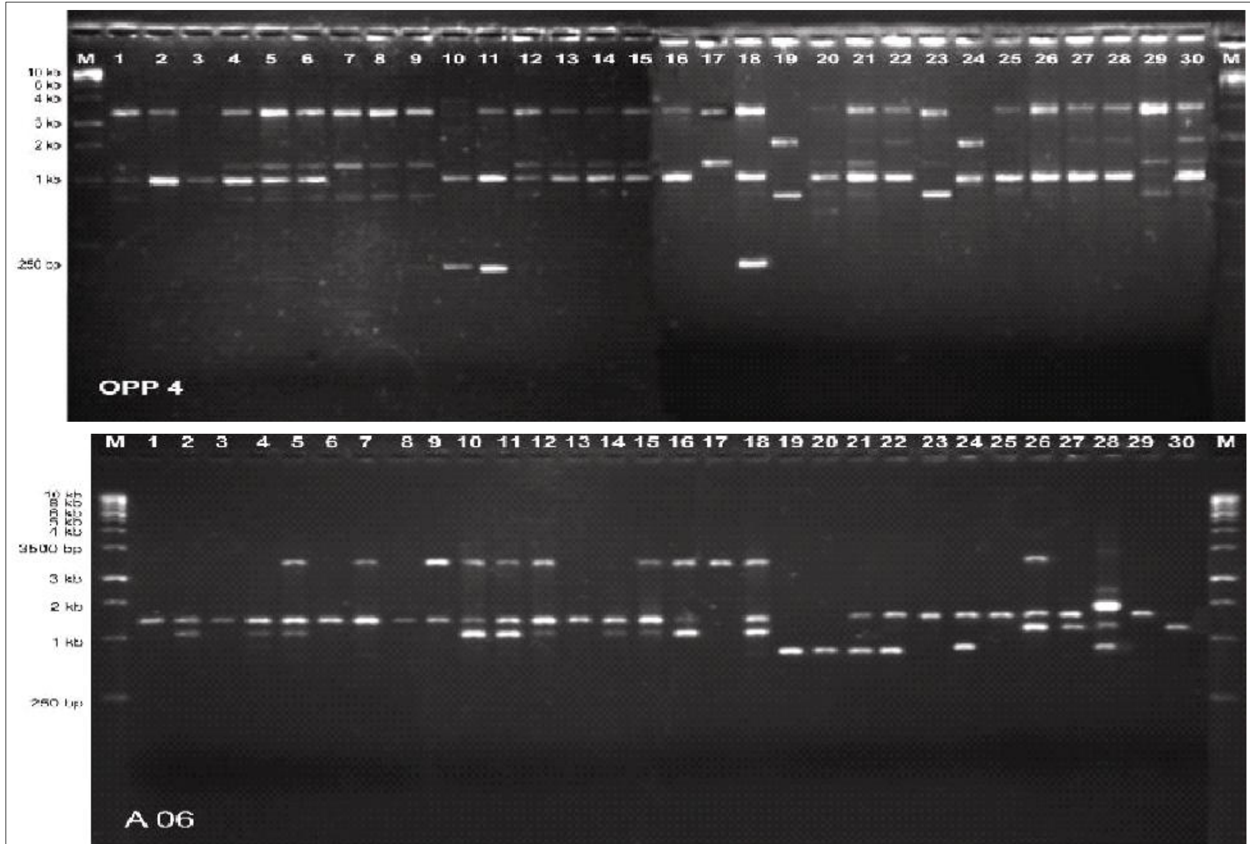

Fig. 1 : PCR amplification products of *B. sorokinana* isolates obtained by primer OPP 4 and A 06 (lane 1 and 31 is a 1 kb marker)

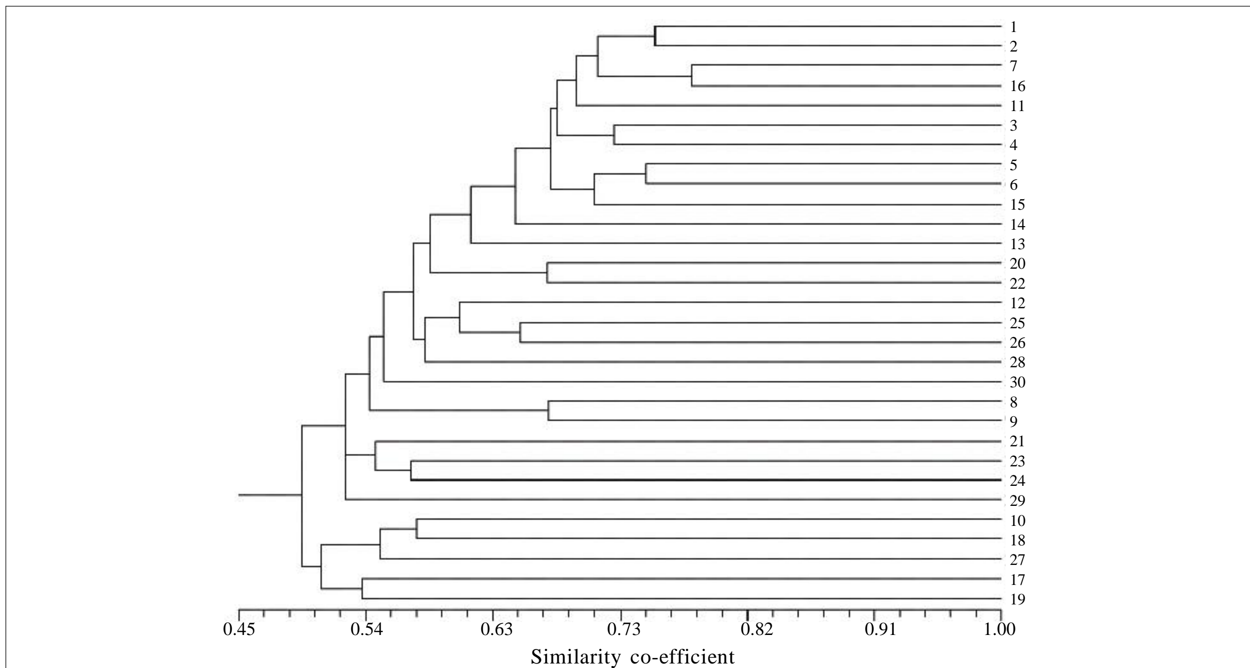

Fig. 2 : Dendrogram showing molecular diversity among *B. sorokiniana* isolates using RAPD markers

Table 2 : Clustering of *B. sorokiniana* isolates based on RAPD markers

Cluster	Isolates	Host*	Location**
I	1,2,3,4,5,6,7,11,13,14,15,16,20,22	B, BW	F, G, L
II	12,25,26,28	B, BW, R	A, L
III	30	T	L
IV	8, 9	T	L
V	21	D	L
VI	23, 24	BW	F
VII	29	T	L
VIII	10, 18	B, T	L
IX	27	BW	A
X	17,19	D, BW	A

* B- Barley, BW - Bread wheat, T- Triticale, D- Durum, R- Rye.
** A – Abohar, F- Fazilka, G - Gurdaspur, L- Ludhiana

diversity among fungal isolates.

Knowledge of genetic diversity in pathogen population is a prerequisite for effective management of diseases. DNA finger printing of 30 *B. sorokiniana* isolates of Punjab revealed genetic diversity at DNA level. The molecular profiles generated by RAPD markers showed presence of some common fragments in all the isolates. Although the molecular weight of this fragment is similar in all the isolates, the DNA sequence homology still needs to be confirmed. Nevertheless the intense and common amplification product might be species- specific and cloning and sequencing of such products might be useful in generating diagnostic marker. The identification of unique fragments for each isolate will help in rapid identification of the isolate and also can be further utilized to design a diagnostic marker which is isolate specific.

Relationship among isolates and lineages has been observed to be complex and similar observation was reported by Oliveira *et al.* (2002).The isolates from different locations *viz.*, Fazilka, Gurdaspur and Ludhiana are grouped together in cluster I indicating that these isolates were derived from same founder source population and disseminated from one area to another in association with their hosts. Similar results were obtained by Zhong and Steffenson (2001) where cluster analysis did not reveal a close correlation between pathotypes and AFLP groups.

Biological pathotyping can assess only a few genetic loci and which in tern may be subject to selection, thus making it difficult to infer the genetic diversity and evolution of the pathogen. The characterization of the isolates at molecular level may further help in delineating isolates belonging to same race or group, in studying the pathogenic diversity across the country and monitoring isolates at field level. Based on molecular typing, genetic diversity has been reported in the pathogen populations from different wheat growing countries (Zhong and Steffenson, 2001; Oliveira *et al.,* 2002; Muller *et al.,* 2005; Jahani *et al.,* 2008). Knowledge of spot blotch pathogen population can be useful in allowing a greater diversity of genes to be identified, mapped and characterized. The information generated could be applied in breeding programmes to develop resistant cultivars against spot blotch pathogen so that it could not become a problem in future when the ever-rising temperature will favour the establishment of this pathogen in non- traditional areas too.

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