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# Genetic variability and relationship analysis of *Bipolaris sorokiniana* isolates causing spot blotch disease in wheat using random amplified polymorphic DNA (RAPD) markers

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**Twelve (12) isolates of *Bipolaris sorokiniana* (*Cochliobolus sativus*) of wheat were studied for their molecular characterization using random amplified polymorphic DNA (RAPD) techniques. Twenty (20) RAPD primers were tested for amplification of the genomic DNA of fungal isolates of wheat. A total of 77 bands were amplified, out of which 68 bands were polymorphic showing high range of variability (84.42% polymorphism) and nine bands were monomorphic. Average total number of bands generated per primer was 3.85, of which 3.4 and 0.45 were polymorphic and monomorphic, respectively. The genetic similarity coefficients for the 12 isolates of *B. sorokiniana* of wheat ranged from 0.526 to 0.842. The highest similarity occurred between DWR-Karnal versus Hisar-PBW-443 and Hisar-502 versus IARI with a coefficient value of 84.2% and the lowest similarity occurred between two fungal isolates that is, Hisar-WH-542 versus IARI and Meerut-Hallana versus Faizabad with a coefficient value of 52.6%. The study indicates that the RAPD is a good tool for determination of genetic variability and relationship of *B. sorokiniana*, which is already visible in the analysis of even such a small number of isolates.**

**Key words:** Wheat, *Bipolaris sorokiniana*, genetic variability, polymorphism, random amplified polymorphic DNA (RAPD) marker.

## INTRODUCTION

Wheat (*Triticum aestivum* L.) is the world's most important food crop; serve as the prime item in the diet of millions of people. Spot blotch is now considered to be the leading disease of wheat in India, which is caused by *Bipolaris sorokiniana*. Estimates of yield losses due to spot blotch were reported to vary from 15.5 to 19.6% (Dubin and van Ginkel, 1991), around 17% (Saari, 1998) and up to 100% under severe infection conditions (Srivastava et al., 1971).

The assessment of genetic diversity is required for the development of long-term disease management strategies. The role of polymerase chain reaction (PCR) based molecular markers like random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), intersimple sequence repeats (ISSR) and so forth in the study of variability of the isolates of pathogen has been established. The RAPD method has been successfully used to identify strains (Guzman et al., 1999; Pryor and Gilbertson, 2000), to characterize races (Malvic and Grau, 2001) and to analyze virulence

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**Table 1.** List of collection sites of *B. sorokiniana* isolates.

| S/N | Collection site |
|---|---|
| 01 | Karnal (Haryana) |
| 02 | Uttar Pradesh |
| 03 | New Delhi |
| 04 | SVPUAT, Uttar Pradesh |
| 05 | SVPUAT, Uttar Pradesh |
| 06 | Haryana |
| 07 | Haryana |
| 08 | Haryana |
| 09 | Haryana |
| 10 | Uttar Pradesh |
| 11 | Uttar Pradesh |
| 12 | Uttar Pradesh |

diversity related to genetic polymorphism (Chen et al., 1995; Chen et al., 1999; Chakraborty et al., 1999; Kolmer and Liu, 2000) in pathogenic fungi. *B. sorokiniana* has a high degree of phenotypic variability; however, the genetic diversity of this fungus has not been fully studied. Studies have been made of isozyme polymorphisms among isolates (Matsumura, 1991; Valim-labres, 1995).

The aim of the present study was to assess the genetic variability and relationships of *B. sorokiniana* isolates of major wheat growing areas using the RAPD technique.

## MATERIALS AND METHODS

### Collection of fungal isolates

Twelve (12) isolates of *B. sorokiniana* were used in this study collected from different geographic regions of North India (Table 1). These fungal isolates were maintained on potato dextrose agar (PDA) medium, purified by single spore isolation and stored at 5°C on PDA medium for long-term storage.

### Isolation of culture

For isolation of causal organism from diseased wheat leaf samples and small fragments of diseased portion sowing typical disease symptoms along with healthy leaf tissue, were cut into small pieces with the help of sterilized blade. These pieces were surface sterilized with 0.2 to 0.5% sodium hypochlorite for 1 to 2 min and then washed with sterilized distilled water for 3 to 4 times in order to remove the saprotrophs and other contaminants. Excess moisture was removed by drying these pieces in the folds of sterilized blotting paper. These treated pieces were placed on PDA medium under aseptic condition and incubated at 25°C for 8 days. Subcultures of fungi from the periphery of the mycelial growth were made on PDA medium and allowed to grow at 25°C.

### Single spore isolation

A method of single spore isolation was developed. A 0.1 µl spore suspension was placed on water agar above 50 to 100 circles marked on the bottom of a plate. After 12 to 24 h, the number of spores in each circle was counted. Single germinating spores in each circle were transferred separately. For all 12 isolates of fungi tested, the number of spores in each micro-drop ranged from 0 to 4. More than 50% of the micro-drops contained a single spore with an unbranched germ tube. This method made it easy to locate well separated spores for single-spore isolation, shortened the isolation time by half, and reduced the incubation period from 2 days to 1 day.

### Identification of *Bipolaris sorokiniana* isolates

Isolated and purified culture of test fungus were identified on the basis of morphological (Conidiophores, Conidia, Conidiophore-conidia attachment, hilum and germination) and cultural characters with help of available manual and books (Ellis, 1976; Sivanesan, 1987; Alcorn, 1988).

### Genomic DNA isolation of fungal isolates

Genomic DNA of all fungal isolates was isolated using cetyltrimethylammonium bromide (CTAB) method with some minor modifications in the protocol given by Murray and Thompson (1980). 0.5 g mycelium of *B. sorokiniana* isolates was ground in liquid nitrogen and powdered mycelium, transferred to DNA extraction buffer [50 mM Tris, 150 mM NaCl, 20 mM ethylene diamine tetraacetic acid (EDTA)] and 1 ml sodium dodecyl sulfate (SDS) (10%) was added and incubated at 37°C for 1 h. Again, 5 M NaCl was added to the above mixture and mixed well, after that 1.25 ml CTAB (10%) was added, mixed gently and incubated at 65°C for 20 min. Equal volume of chloroform:isoamyl alcohol (24:1) was added to each tube followed by centrifugation. Aqueous, viscous supernatant was removed to a fresh tube and precipitated with 0.6 ml volume of ice-cold isopropanol and 0.1 ml volume of sodium acetate and then it was kept inside the freeze for overnight at 4°C and again centrifuged. The DNA pellet was collected carefully and washed with 70% ethanol and dried at room temperature. Finally, the pellet was dissolved in 100 µl TE buffer and kept at -20°C. For purification of DNA, RNase solution 10 mg/ml was used. DNA was quantified using spectrophotometer and quality analysis done on 0.8% agarose gel.

### RAPD primers and PCR amplification

Total of 20 RAPD primers were tested for amplification of the genomic DNA of *B. sorokiniana* isolates of wheat. PCR was performed in LongGene Thermal cycler (Version 3.20). All PCR reactions of genomic DNA of *B. sorokiniana* isolates were carried out in a volume of 25 µl with 50 ng DNA, 10 mM deoxyribonucleoside triphosphates (dNTPs), 10 µM of primer, 2.5 µl of 10× assay buffer and 1 Unit of Taq DNA polymerase (Bangalore GeNei, India).

PCR reactions was carried out under the following touchdown programmes for 1 initial cycle with 1 min at 94°C, 5 min at 35°C, 2 min at 72°C and 45 cycles of 1 min at 94°C, 1 min at 35°C and 2 min at 72°C. Although the annealing temperatures for primer ranged from 22 to 32°C were used for all amplifications that is, all PCR reactions. The amplification products were visualized on 1.5% agarose gel, detected by staining with ethidium bromide and analyzed using the gel documentation system AlphaImager 2200 (Alpha Innotech Corporation, USA). Fragment sizes for each locus were measured using standard size markers. All PCR reactions were repeated at least twice and only clear and apparently unambiguous bands were scored.

**Table 2.** Number of bands (monomorphic and polymorphic) and percentage polymorphism of RAPD primers in isolates of *B. sorokiniana*.

| S/N | Primer | Sequence | Number of band | | | Percentage polymorphism |
|---|---|---|---|---|---|---|
| | | | Total | Monomorphic | Polymorphic | |
| 01 | OPW-02 | ACC CCG CCA A | 8 | 0 | 8 | 100.00 |
| 02 | OPW-03 | GTC CGG AGT G | 3 | 0 | 3 | 100.00 |
| 03 | OPW-04 | CAG AAG CGG A | 2 | 1 | 1 | 50.00 |
| 04 | OPW-05 | GGC GGA TAA G | 5 | 1 | 4 | 80.00 |
| 05 | OPW-06 | AGG CCC GAT G | 4 | 1 | 3 | 75.00 |
| 06 | OPW-07 | CTG GAC GTG A | 4 | 0 | 4 | 100.00 |
| 07 | OEP-03 | CCA GTA GCA C | 2 | 1 | 1 | 50.00 |
| 08 | OEP-07 | AGA TGC AGC C | 5 | 0 | 5 | 100.00 |
| 09 | OEP-11 | GAG TCT CAG G | 3 | 1 | 2 | 66.66 |
| 10 | OEP-12 | TTA TCG CCC C | 4 | 2 | 2 | 50.00 |
| 11 | OEP-14 | TGC GGC TGA G | 6 | 0 | 6 | 100.00 |
| 12 | OEP-16 | GGT GAC TGT G | 2 | 0 | 2 | 100.00 |
| 13 | OEP-17 | CTA CTG CCG T | 2 | 0 | 2 | 100.00 |
| 14 | OPP-01 | GAT GCA CTC C | 3 | 0 | 3 | 100.00 |
| 15 | OPP-02 | TCG GCA CGC A | 4 | 0 | 4 | 100.00 |
| 16 | OPP-03 | CTG ATA CGC C | 3 | 0 | 3 | 100.00 |
| 17 | OPP-04 | GTG TCT CAG G | 7 | 0 | 7 | 100.00 |
| 18 | OPP-05 | CCC CGG TAA C | 2 | 1 | 1 | 50.00 |
| 19 | OPP-06 | GTG GGC TGA C | 3 | 1 | 2 | 66.66 |
| 20 | OPP-07 | GTC CAT GCC A | 5 | 0 | 5 | 100.00 |
| Total | | | 77 | 9 | 68 | - |
| Mean | | | 3.85 | 0.45 | 3.4 | 84.42 |

**Data scoring and RAPD analysis**

The RAPD bands were scored as '0' (absence of the band) and '1' (presence of the band) for each DNA sample with 20 primers in a data matrix. Statistical analysis of the RAPD data was performed using the NTSYS-pc version 2.2 (Rohlf, 2000), through which similarity coefficient were calculated. Clustering of similarity matrices was done by Unweighted pair group with arithmetic average (UPGMA) and projection by the TREE programme of NTSYS-pc.

## RESULTS

### Polymorphism of RAPD markers

Twenty (20) RAPD primers were used for the DNA profiling of 12 isolates of *B. sorokiniana* causing spot blotch of wheat. A total of 77 bands were amplified among the isolates of *B. sorokiniana* of wheat through 20 RAPD primers, of which 68 bands were polymorphic showing high range of variability (84.42% polymorphism) and 9 bands were monomorphic (Table 2). On average, the total number of bands generated per primer was 3.85, of which 3.4 were polymorphic and the 0.45 were monomorphic.

In RAPD analysis, the average of polymorphism percentage was 84.42; ranged from 50.00 to 100.00. The lowest polymorphism percentage were recorded for primer OPW-04, OEP-03, OEP-12, OPP-05 and the highest polymorphism percentage were recorded for primers OPW-02, OPW-03, OPW-07, OEP-07, OEP-14, OEP-16, OEP-17, OPP-01, OPP-02, OPP-03, OPP-04 and OPP-07. Out of 20 RAPD primers, only 12 oligonucleotide primers generated polymorphic bands showing 100% polymorphism. The RAPD profiles showed a high level of genetic variability among the *B. sorokiniana* isolates. Primer OPW-02 generated maximum total number of bands (8) showing 100% polymorphism as shown in Figure 1.

### Cluster analysis

Both monomorphic and polymorphic DNA bands were used to generate a dendrogram by means of UPGMA cluster analysis. Cluster analysis is an important approach to order genetic variability and relationships by using computer algorithms developed in the fields of multivariate statistics. The cluster analysis generated showed a significant genetic variation among the *B. sorokiniana* isolates studied, with similarity coefficients which ranged from 0.526 to 0.842 (Table 3). The highest similarity percentage occurred between DWR-Karnal

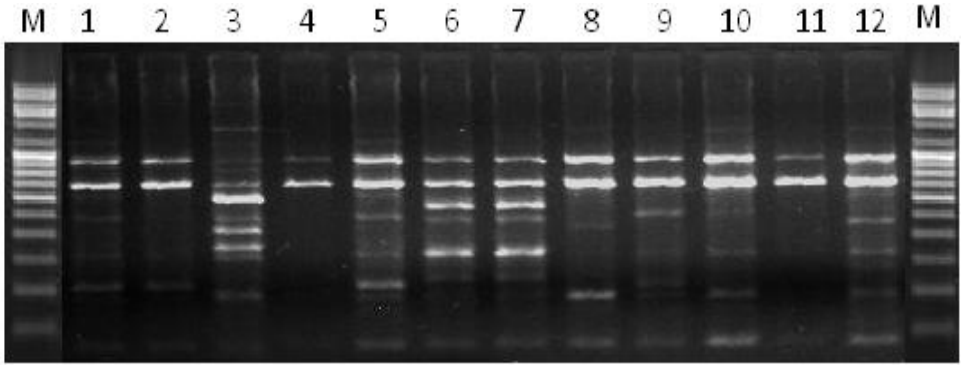


**Figure 1.** DNA profile of 12 isolates of *B. sorokiniana* with primer OPW-02.

**Table 3.** Genetic similarity coefficient obtained between 12 isolates of *B. sorokiniana* of wheat.

| S/N | Isolate | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 01 | DWR-Karnal | 1.000 | | | | | | | | | | | |
| 02 | Hisar-PBW-373 | 0.754 | 1.000 | | | | | | | | | | |
| 03 | Hisar-WH-542 | 0.544 | 0.614 | 1.000 | | | | | | | | | |
| 04 | Hisar-PBW-443 | 0.842 | 0.772 | 0.632 | 1.000 | | | | | | | | |
| 05 | Hisar-502 | 0.754 | 0.649 | 0.544 | 0.737 | 1.000 | | | | | | | |
| 06 | Meerut-PBW-343 | 0.702 | 0.737 | 0.596 | 0.789 | 0.702 | 1.000 | | | | | | |
| 07 | Meerut-Hallana | 0.632 | 0.596 | 0.632 | 0.684 | 0.561 | 0.614 | 1.000 | | | | | |
| 08 | IARI | 0.737 | 0.667 | 0.526 | 0.754 | 0.842 | 0.649 | 0.614 | 1.000 | | | | |
| 09 | Varanasi-HUW-234 | 0.719 | 0.719 | 0.649 | 0.737 | 0.789 | 0.667 | 0.632 | 0.807 | 1.000 | | | |
| 10 | Faizabad | 0.719 | 0.789 | 0.649 | 0.772 | 0.754 | 0.772 | 0.526 | 0.772 | 0.825 | 1.000 | | |
| 11 | Varanasi-2508 | 0.719 | 0.649 | 0.544 | 0.667 | 0.754 | 0.702 | 0.596 | 0.737 | 0.754 | 0.719 | 1.000 | |
| 12 | Varanasi-33 | 0.596 | 0.737 | 0.561 | 0.684 | 0.667 | 0.754 | 0.474 | 0.649 | 0.702 | 0.772 | 0.632 | 1.000 |

versus Hisar-PBW-443 and Hisar-502 versus IARI with a coefficient value of 84.2% and the lowest percentage of similarity occurred between two fungal isolates that is, Hisar-WH-542 versus IARI and Meerut-Hallana versus Faizabad with a coefficient value of 52.6%.

All 12 isolates of *B. sorokiniana* of wheat were distributed into two distinct clusters that is, Clusters I and II (Figure 2). Cluster I comprised of 10 fungal isolates which were further divided into three subclusters viz; IA, IB and IC. Subcluster IA consisted of three fungal isolates namely DWR-Karnal, Hisar-PBW-443 and Hisar-PBW-373, in which the minimum similarity coefficient occurred between DWR-Karnal versus Hisar-PBW-373 and the maximum similarity coefficient occurred between DWR-Karnal versus Hisar-PBW-443 with a value of 0.754 and 0.842, respectively. Subcluster IB consisted of five fungal isolates namely Hisar-502, IARI, Varanasi-HUW-234, Faizabad and Varanasi-2508, in which the minimum similarity coefficient occurred between Faizabad versus Varanasi-2508 and the maximum similarity coefficient occurred between Hisar-502 versus IARI with a value of 0.719 and 0.842, respectively. Subcluster IC consisted of only two fungal isolates of wheat namely Meerut-PBW-343 and Varanasi-33, which showed the similarity coefficient value of 0.754. Cluster II consisted of only two fungal isolates of wheat namely Hisar-WH-542 and Meerut-Hallana, which showed the similarity coefficient value of 0.632.

## DISCUSSION

In the present investigation, 20 RAPD primers were used to assess the genetic variability and relationship among 12 fungal isolates of wheat. Out of 20 primers, 12 RAPD primers generated polymorphic bands showing 100% polymorphism. 20 RAPD generated total 77 bands, of which 68 bands were polymorphic and nine were monomorphic. 68 polymorphic bands showed high range of variability (84.42% polymorphism). Average total number of bands generated per primer was 3.85, of which 3.4 were polymorphic and 0.45 were monomorphic. Similar results were also reported working on *B. sorokiniana* isolates by many other researchers

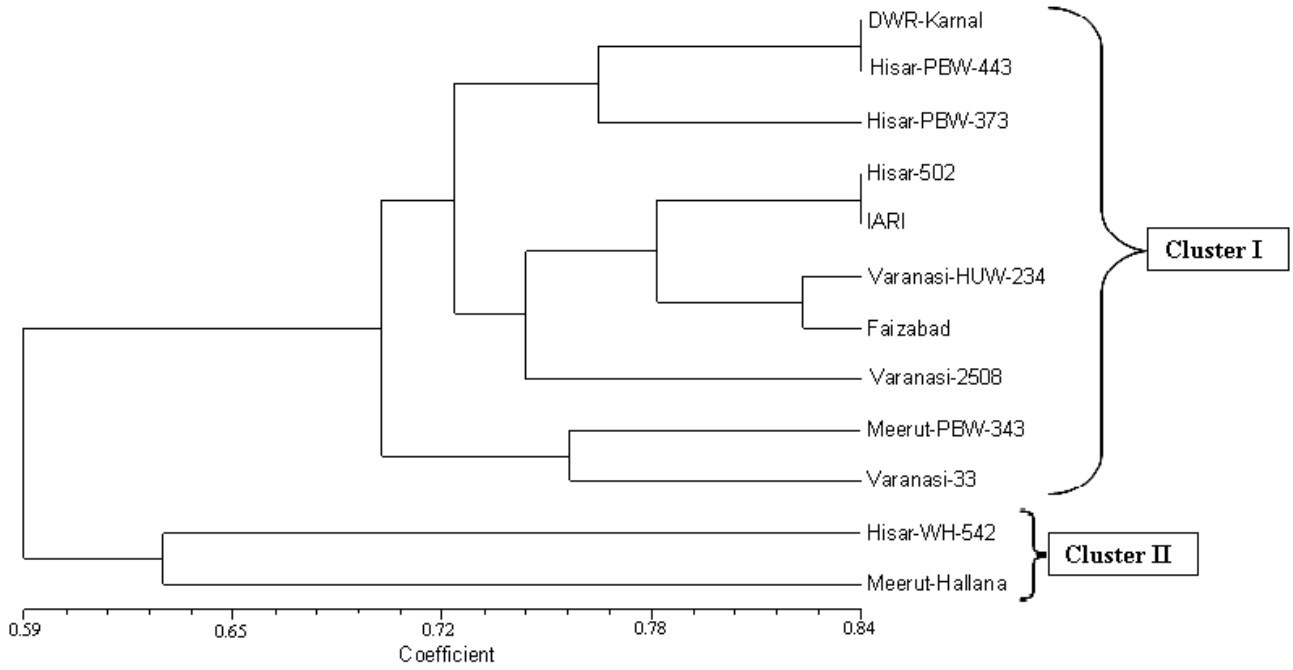


**Figure 2.** Dendrogram of 12 isolates of *B. sorokiniana* of wheat revealed by UPGMA cluster analysis of genetic similarities based on RAPD data of 77 fragments amplified with 20 arbitrary primers.

(Oliveira et al., 2002; Iram and Ahmad, 2004; Jaiswal et al., 2007; Aggarwal et al., 2010).

The polymorphism percentage ranged from 50.00 (OPW-04, OEP-03, OEP-12 and OPP-05) to 100.00 (OPW-02, OPW-03, OPW-07, OEP-07, OEP-14, OEP-16, OEP-17, OPP-01, OPP-02, OPP-03, OPP-04 and OPP-07) with an average of 84.42. Such similar results have been reported by Vicario et al. (1995) and Jaiswal et al. (2007) in case of *B. sorokiniana*. RAPD analysis showed that highest similarity percentage occurred between DWR-Karnal versus Hisar-PBW-443 and Hisar-502 versus IARI with a coefficient value of 84.2%. Therefore, DWR-Karnal versus Hisar-PBW-443 and Hisar-502 versus IARI pairs of fungal isolates show much variation at genetic level.

All 12 isolates of *B. sorokiniana* of wheat were grouped into two clusters that is, Clusters I and II comprising of 10 and two fungal isolates, respectively. The genetic diversity pattern revealed differences in all the isolates of *B. sorokiniana* of wheat. In this study, 12 fungal isolates showed genetic variability. In some of the isolates, the amplification profiles of the *B. sorokiniana* were highly polymorphic and some of the isolates showed quite monomorphic amplification profiles.

It can be concluded that application of RAPD technique is useful for genetic diversity of some *B. sorokiniana* isolates at the genetic level and provide good results for further studies including identification of susceptible verities.

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