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Molecular Variability Study among *Bipolaris sorokiniana* (Sacc) Shoem Isolates-Incitant of Spot Blotch of Wheat

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ABSTRACT

Spot blotch caused by Bipolaris sorokiniana is an important disease of wheat. Assesment of molecular variability among 14 isolates of Bipolaris sorokiniana using ten SSR markers revealed high degree of genetic variability. A total of 40 polymorphic bands were produced out of 50 amplicons resulted from 10 different markers. The primer BS035 produced the highest number of amplicons (Twelve) followed by BS074 with nine amplicons. The lowest of one marker level amplicon was produced by BS065.Based on simple matching coefficient a genetic similarity matrix was constructed to assess the genetic relatedness among isolates of B. sorokiniana. The similarity coefficients were ranged from 0.53 to 0.98 representing less than 53 per cent genetic variability among 14 isolates. Further, the dendrogram constructed by UPGMA from the pooled data clearly showed 2 major clusters viz., A and B at a similarity co efficient of 0.53. Cluster A comprised of eight isolates viz., Bsm1, Bsg, Bsm2, Bsa, Bsj, Bsu, Bsd1and Bsn at a similarity co efficient of 0.64 while, cluster B comprised of six isolates viz., Bsd2, Bsp1 Bsdbl1, Bsdbl2, Bsb and Bsp2 at a similarity co efficient of 0.66.

Key words: *Bipolaris sorokiniana, Amplicons, Cluster, Similarity coefficient.*

INTRODUCTION

Wheat (*Triticum* spp.; family: Poaceae; centre of origin: Abyssinia) the versatile cereal crop is also described as "the shuffle of life" or "king of cereals. In India, wheat is the second most important food crop being next to rice and it contributes nearly 25 per cent to the total food grain production. Due to continuous rise in temperature during the wheat growing season and high humidity coupled with winter rains, spot blotch caused by *Bipolaris sorokiniana* is getting favourable conditions to develop aggressively and cause damage to wheat crop at larger scale by affecting significant yield loss up to 18-50 per cent under favourable conditions[1].

Traditional methods used to study variability in pathogens were based on morphological, pathogenicity or biochemical tests. These methods distinguish pathogen isolates on the basis of their physiological characters *viz.*, pathogenicity and growth behaviors.

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However, these are highly influenced by the host age, inoculum quality and environmental conditions. DNA markers are not influenced by any such conditions and therefore provide more reliable results. Part of variability in *B. sorokiniana* has been attributed to heterokaryosis and parasexual mechanism[8]. Sexual reproduction in *B. sorokiniana* is rare and reported only from Zambia[6]. Simple sequence repeats (SSRs), also known as microsatellites, are repetitive 2-6 base pair sequences of DNA that are abundant and highly polymorphic in many eukaryotic genomes[2]. These are co-dominant markers that provide more information on allelic variation per locus and distinguish among homozygote and heterozygote genotypes[4]. SSRs have proven to be used markers for genetic characterization of fungus species[3].

The information on molecular variability existing in *B. sorokiniana* can be utilized by breeders and pathologists for resistance breeding so the present study highlights the molecular variability within the *B. sorokiniana* isolates.

MATERIAL AND METHODS

The 14 isolates of *B. sorokiniana* were assessed for genetic diversity study by using Simple Sequence Repeats (SSR) markers. Isolates were multiplied in potato dextrose broth (PDB) medium at 26 ± 1 ^{0}C and DNA was extracted using the CTAB (N, N, N, N, -Cetyl Trimethyl Ammonium Bromide) method. DNA was quantified using Nano drop instrument and quality analysis done on 0.8 per cent agarose gel.

Growth of isolates on PDB

Pure cultures of *B. sorokiniana* isolates which were grown on PDA for eight days were transferred onto liquid potato dextrose medium aseptically and allowed to grown for ten days. The mycelium was filtered through Whatman No. 1 filter paper, the harvested fungal mat stored for a week at -20 ºC after wrapped with aluminum foil to make it brittle.

DNA extraction by CTAB method

Isolation of total DNA from the stored mycelial mat of *B. sorokiniana* isolates was done as per the procedure given below with slight modifications[5].

1. Fungal mat (2-3 g) grown on potato dextrose broth was taken and homogenized using pestle and mortar with liquid nitrogen.
2. To the above solution 1 ml of DNA extraction buffer (0.1M Tris, 1.5M NaCl, 0.01M EDTA) was added and incubated for 60 min in hot water broth.
3. The suspension in pestle and mortar was extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 V/V) in centrifugation tube, centrifuged at 10,000 rpm for 20 min at 4 °C.
4. Supernatant was taken in fresh centrifuge tube and 2.5 µl RNase and 2.5 µl protienase-K were added and incubated at room temperature for 30 min.
5. Cool isopropanol of about 1/3rd volume (300-400 µl) was added and centrifuged at 10, 000 rpm for 15 min. at 4 °C.
6. Wash buffer (500 µl) was added and centrifuged at 10, 000 rpm for 5 min at 4 °C.
7. Pellet was washed with 70 per cent ethanol, air dried and resuspended in 100 µl of $T_{10}E_1$ buffer.
8. DNA obtained was further quantified by agarose gel electrophoresis.

Purification of DNA

RNA in the total nucleic acid extracted was removed by *RNase* treatment. A stock solution of *RNase-A* (Genei) was prepared at 10 mg/ml in 10 mM Tris HCI, pH 8.0 and 15 mM NaCl. The solution was boiled by keeping in hot water bath for 10 min deactivating *DNase* activity and allowed to cool slowly at room temperature. From the stock, 2µl of *RNase* was added to the crude DNA samples and incubated at 37 °C for 1 hour resulting in pure DNA suitable for PCR.

Qualitative and quantitative verification of DNA

The quality and quantity of DNA was analysed by running 2 µl of each sample mixed with 2 µl of 10X loading dye in one per cent agarose gel. The DNA from all isolates produced clear sharp bands in one per cent agarose gel

indicating the good quality of DNA. The DNA was quantified by comparing with the 100 bp size marker (Genei, Bangalore) and by spectrophotometer (Nanodrop ND1,000).

Standardization of template DNA, primer and dNTPs concentration

Two concentrations of the template DNA (40 ng and 80 ng) and primer (2 pmol, 5 pmol and 10 pmol) were checked for good amplification and 40 ng of template and 5 pmol of primer concentration were selected for further standardization of dNTPs concentration. Three concentrations of dNTPs (100, 150 and 200 µl) were used. Based on the preliminary results, 150 µl of dNTPs concentration was selected.

Polymerase chain reaction

Ten SSR markers developed from a small insert genomic library for *B. sorokiniana* were used for the analysis of genetic variability of the isolates of *B. sorokiniana* and obtained from Imperial Life Sciences (P) Limited, Gurgaon, Haryana, India. Primer sequences used are given below.

Details of SSR markers used for genetic diversity study among *Bipolaris sorokiniana* isolates

Name of marker (Locus)	Primer Sequence (5' to 3')		Motif	Allele size
	Forward	Reverse		
*S*001	ATGAGTGATCAGAGCAGGACTTTT	CATACAGGGTAATGTGTAGGTAGTG	$(CA)_6$	243-268
*BS*027	ACTGAAGAGACCCAGATGTCGTAG	GGATATCCTTTGAGTTGATCTCTCC	$(TG)_6$	178-214
*BS*035	GAGCAAGCCGAGTAGACCAC	GCATGGATACACATACACACACAC	$(CAGTCCAGCG)_4$	228-251
*BS*036	ATTACCCTATGGCAGCAATCTG	ACCCACTCTGTTCCTTTCTCATC	$(GT)_6$	160-189
*BS*051	ACCCACTCTGTTCCTTTCTCATC	CCGAGTCAGTTCTTAATGCTATCAG	$(GT)_6$	175-223
*BS*065	TCAATGCTAGTATTTTTCTCATTCG	ACAATAATGACGTCACCATCTCAC	$(CA)_9$	182-215
*BS*070	AAACAAGAATGCTCCGAAGTTG	CCCGTCCTCATTACCCAGTAT	$(AC)_7$	219-265
*BS*074	ACGTAAGGAAAAACACCTCGAGTC	ACTTTATCCGTGTGCATCTTCAAC	$(CA)_8$	161-202
*BS*098	GGTAAGCTTTCACGCTAACAACTC	ACACTTGGATAGGCGTTGAGATACT	$(AC)_{23}$	132-180
*BS*103	ACATATCCTTGCCCTAAACACAAT	TAGGCAGAGCTTGGATATGACTACT	$(ACA)_{10}$	169-210
*BS*104	TATGACTATAGTGTCTTGGGCACAC	CCCACAGGCAAAGGTATATAATAAG	$(AC)_7$	150-175

dNTps (dATP, dGTP and dTTP) and Taq DNA polymerase 12.5U units per µl and 10X Taq buffer were obtained.

Thermo cycler

The optimum condition for DNA amplification used was as follows.

Step	Temperature (^oC)	Duration	Number of cycles
Initial denaturation	94	3 min	1
Denaturation	94	40 sec	35
Annealing	58	40 sec	
Extension	72	50 sec	
Final extension	72	5 min	1
Total temperature hold	4	-	-

After the completion of the PCR, the products were stored at 4 °C until the gel electrophoresis was done.

PCR reaction mixture

Reaction mixture	Quantity (µl)
Template DNA (40 ng)	1.0
Primer (5 pm)	1.0
Taq buffer A (10 X)	2.0
dNTP's (2 mM)	2.0
Taq DNA polymerase (3 U/µl)	0.3
Sterile water	13.7
Total	**20.0**

Agarose gel electrophoresis

Separation of amplified products by agarose gel electrophoresis

Requirements

- Electrophoretic unit, gel casting, gel comb, power pack and UV-trans illuminator
- Agarose 1 per cent
- Bromophenol blue
- Ethidium bromide (0.5 µg /ml)
- 50X TAE (stock): Tris-free base-60.5 g
- Glacial acetic acid - 14.25 ml
- 0.5 M EDTA - 25 ml
- Made up the volume to 250 ml, PH - 8.0
- Working solution (1X TAE): 20 ml of 50X TAE was made up to 1,000 ml by using distilled water (Appendix II)

Procedure

- One gram of agarose was weighed and added to a 250 ml conical flask containing 100 ml of 1X TAE buffer.
- The agarose was melted by heating the solution in an oven and the solution was stirred to ensure even mixing and complete dissolution of agarose.
- The solution was cooled to about 50 ^{0}C.
- Two to three drops of ethidium bromide (0.5 µg/ml) was added.
- The solution was mixed and poured into the gel casting platform after inserting the comb in the trough.
- While pouring, sufficient care was taken for not allowing the air bubbles to trap in the gel.
- The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus, containing sufficient buffer (1X TAE) so as to cover the wells completely.
- The amplified products (20 µl) to be analyzed were carefully loaded into the sample wells, after adding bromo phenol blue with the help of micropipette.
- Electrophoresis was carried out at 60 volts, until the tracking dye migrated to the end of the gel.
- Ethidium bromide stained DNA bands were viewed under UV-trans illuminator and photographed for documentation.

Scoring the amplified fragments

The amplified fragments were scored as '1' for the presence and '0' for the absence of a band generating the 0 and 1 matrix and per cent polymorphism was calculated using formula.

$$\text{Per cent polymorphism} = \frac{\text{Number of polymorphic band}}{\text{Total number of bands}} \times 100$$

Data analysis

All gels showing DNA bands scored twice independently. Presence and absence of unique and shared polymorphic as well as monomorphic products were used to generate similarity coefficient. The similarity coefficients were then used to construct a dendrogram UPGMA (Unweighted Pair Group method with arithmetical averages) using a Computer Programme NTSYSPC Version 2.1[7].

RESULTS AND DISCUSSION

Ten SSR markers were screened for polymorphism in fourteen isolates of *B. sorokiniana*, all the 10 gave scorable and reproducible amplification products which were polymorphic in nature (Table 1). A total of 40 polymorphic bands were produced out of 50 amplicons resulted from 10 different markers. The primer BS035 produced the highest number of amplicons (Twelve) followed by BS074 with nine amplicons. The lowest of one marker level amplicon was produced by BS065 (Table 2 and Plate 1).

Based on simple matching coefficient, a genetic similarity matrix was constructed to assess the genetic relatedness among isolates of *B. sorokiniana.* The similarity coefficients were ranged from 0.53 to 0.98 representing less than 53 per cent genetic variability among 14 isolates. Further, the dendrogram constructed by UPGMA from the pooled data clearly showed 2 major clusters *viz.*, A and B at a similarity co efficient of 0.53. Cluster A comprised of eight isolates *viz.*, *Bs*m1, *Bs*g,

*Bs*m2, *Bs*a, *Bs*j, *Bs*u, *Bs*d1and *Bs*n at a similarity co efficient of 0.64 while, cluster B comprised of six isolates *viz.*, *Bs*d2, *Bs*p1 *Bs*dbl1, *Bs*dbl2, *Bs*b and *Bs*p2 at a similarity co efficient of 0.66. Further, within cluster A two sub clusters A1, A2 were noticed at coefficient of 0.77, within cluster B two sub clusters B1 and B2 were noticed at coefficient of 0.72 and 0.77, respectively indicating more variations at genetic level (Fig. 1). The results are in agreement with Yadav *et al.*[9] who reported that 12 isolates of *B. sorokiniana* of wheat were grouped into two clusters as Clusters I and II.

The genetic similarity coefficient varied between 0.53 and 0.98 indicating much variation among the isolates. All 14 isolates of *B. sorokiniana* fall separately in two major clusters. Eight isolates come in cluster A representing *Bs*m1, *Bs*g, *Bs*m2, *Bs*a, *Bs*j, *Bs*u, *Bs*d1and *Bs*n where in maximum of 88 per cent similarity coefficient was observed among the isolates *Bs*m1 (isolated from spot blotch infected leaf samples collected from Mudhol taluk of Bagalkote district) and *Bs*g (Gokak taluk of Belagavi district) ; *Bs*m2 (Mudhol taluk of Bagalkote district) and *Bs*a (Agricultural Research Station, Arbhavi) ; *Bs*j (Bailhongal taluk of Belagavi district) and *Bs*u (Athani taluk of Belagavi district) followed by 77 per cent similarity coefficient among the isolates *Bs*d1 (Main Agricultural Research Station, University of Agricultural Sciences, Dharwad) and *Bs*n (Agricultural Research Station, Niphad, Maharashtra).

Remaining six isolates come in cluster B representing isolates *Bs*d2, *Bs*p1 *Bs*dbl1, *Bs*dbl2, *Bs*b and *Bs*p2 where in maximum of 88 per cent similarity coefficient was observed among the isolates *Bs*d2 (Main Agricultural Research Station, University of Agricultural Sciences, Dharwad) and *Bs*p1 (Hole farm, Baramati, Pune) followed by 77 per cent per cent similarity coefficient between isolates *Bs*b (Agricultural Research Station, Bagalkote) and *Bs*p2 (Hole farm, Baramati, Pune). The isolate *Bs*dbl2 isolated from the barley spot blotch resistant variety IBDSN 140 grown at Main Agricultural Research Station, University of Agricultural Sciences, Dharwad separates out from rest of the clusters showing only 66 per cent similarity with all other isolates and was the most diverse. The reason for this is *Bs*dbl2 isolate was obtained from barley while rest of 12 isolates were from wheat, further *Bs*dbl2 didn't clustered with *Bs*dbl1 because *Bs*dbl1 was isolated from variety, jyothi which is susceptible genotype to spot blotch while *Bs*dbl2 was isolated from the barley spot blotch resistant variety IBDSN 140. The results are in agreement with Aggarwal *et al.* who reported less than 60 per cent genetic variability among isolates representing different agro-ecological zones of India.

It was not possible to group the isolates according to geographical areas from which they were collected because of the genotypic diversity that existed among isolates, further geographical areas may not have any influence on genomic characers as the genetic variability could be attributed to interactions between genes. These results are in agreement with Zhong and Steffenson[10] who assessed the genetic diversity of *Cochhliobolus sativus* based on virulence and AFLP markers and found no correlation between genetic similarity and geographic origin.

SSR results obtained in the present study enabled a fast and efficient variability analysis for *B. sorokiniana*. These results also led to the generation of electrophoretic profiles, which discriminated intra-specific polymorphism in the isolates studied. Also, in order to find more variability among the isolates of *B. sorokiniana* and may be to separate them in races.

In this study, the diversity observed among single spore *B. sorokiniana* isolates using the SSR markers might be a result of many factors such as population dynamics, gene flow, mutations and the multinuclear nature of conidia, which can lead to high polymorphism. Gene flow is especially important in relation to plant pathogens in agro-ecosystems because it is the process that introduces new genes into agricultural fields

distant from the site of the original mutation what could be one of the reasons to explain why some isolates from two different geographical areas cluster together. Further work must be done on the genetic characterization of *B. sorokiniana* isolates and then the identification of the pathogen will be more fast and accurate.

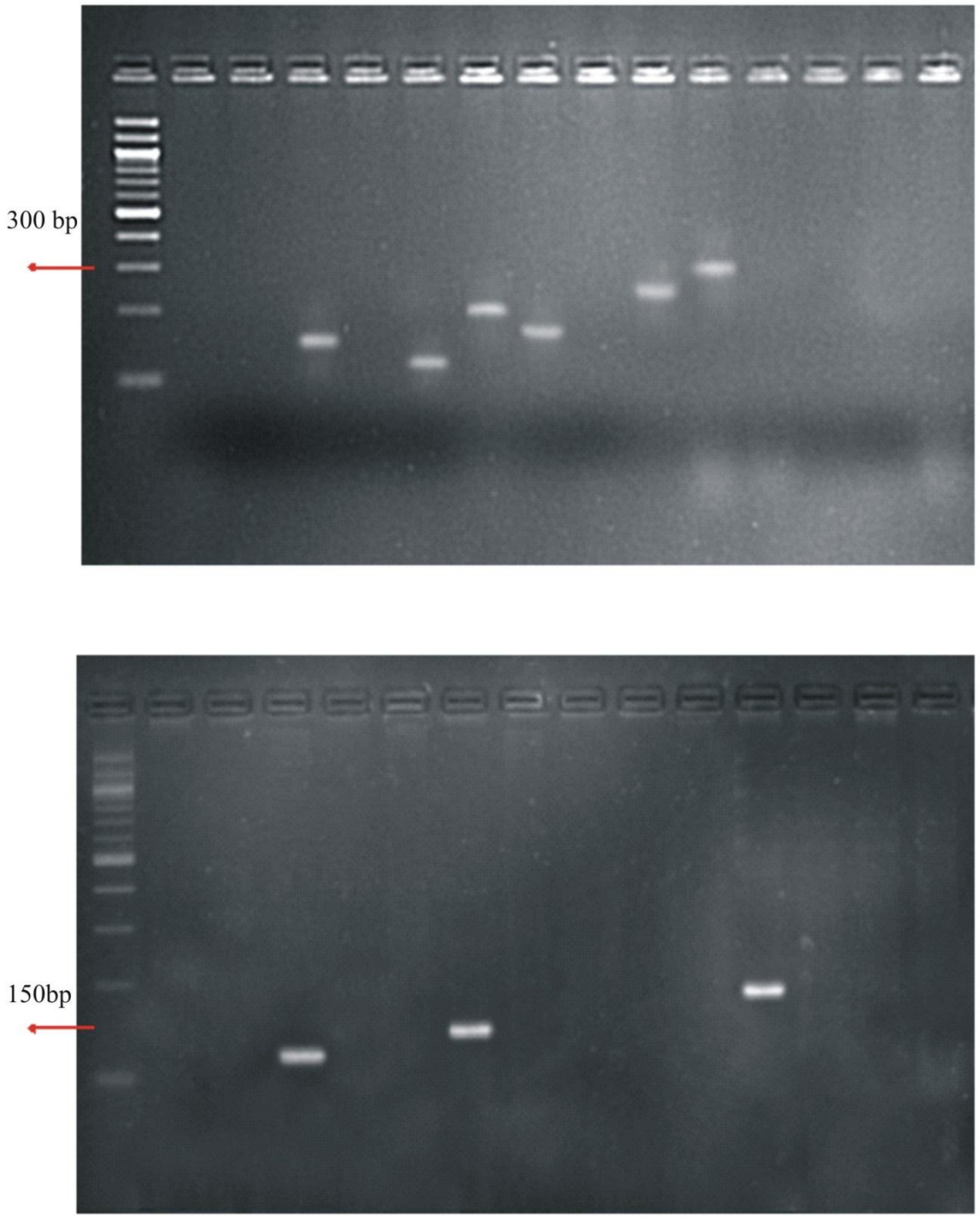
It was found that there was linkage of cultural characters of the various isolates *viz.*, *Bs*m1 and *Bs*g within subcluster A1; *Bs*d1 and *Bs*n within subcluster A2; *Bs*d2 and *Bs*p1 within subcluster B1; *Bs*b and *Bs*p2 within subcluster B2 with genetic diversity/molecular variability except for few isolates like *Bs*m2 and *Bs*a; *Bs*j and *Bs*u which were culturally different but genotypically they exhibited similarity of 0.88 per cent. Thus suggesting that although isolates may be morphological/culturally similar but they exhibit diversity at molecular level and *vice-versa*.

Table1. Summary statistics on general information of marker analysis for genetic diversity of *Bipolaris sorokiniana* isolates

Sl. No.	Particulars	
1	Total number of markers used	10
2	Number of markers produced scorable bands	10
3	Number of markers found polymorphic	07
4	Total number of amplicons from 10 markers	50
5	Total number of polymorphic bands	40
6	Maximum number of amplicons observed in individual markers	12
7	Minimum number of amplicons observed in individual markers	01
8	Maximum per cent polymorphism observed	100
9	Minimum per cent polymorphism observed	50

Table2. Genotypic information generated for SSR markers in *Bipolaris sorokiniana*

Marker	Number of amplicons	Number of polymorphic amplicons	Per cent polymorphism
*S*001	4	4	100.0
*BS*027	4	4	100.0
*BS*035	12	8	66.6
*BS*036	6	6	100.0
*BS*051	2	2	100.0
*BS*065	1	1	100.0
*BS*070	3	3	100.0
*BS*074	9	7	77.7
*BS*098	6	3	50.0
*BS*104	3	3	100.0



1 - *Bs*m1 2 - *Bs*m2 3 - *Bs*d1 4 - *Bs*j 5 - *Bs*d2 6 - *Bs*b
7 - *Bs*n 8 - *Bs*g 9 - *Bs*p1 10 - *Bs*p2 11 - *Bs*u 12 - *Bs*a
13 - *Bs*dbl1 14 - *Bs*dbl2

Plate 1: SSR profile of *Bipolaris sorokiniana* isolates on agarose gel

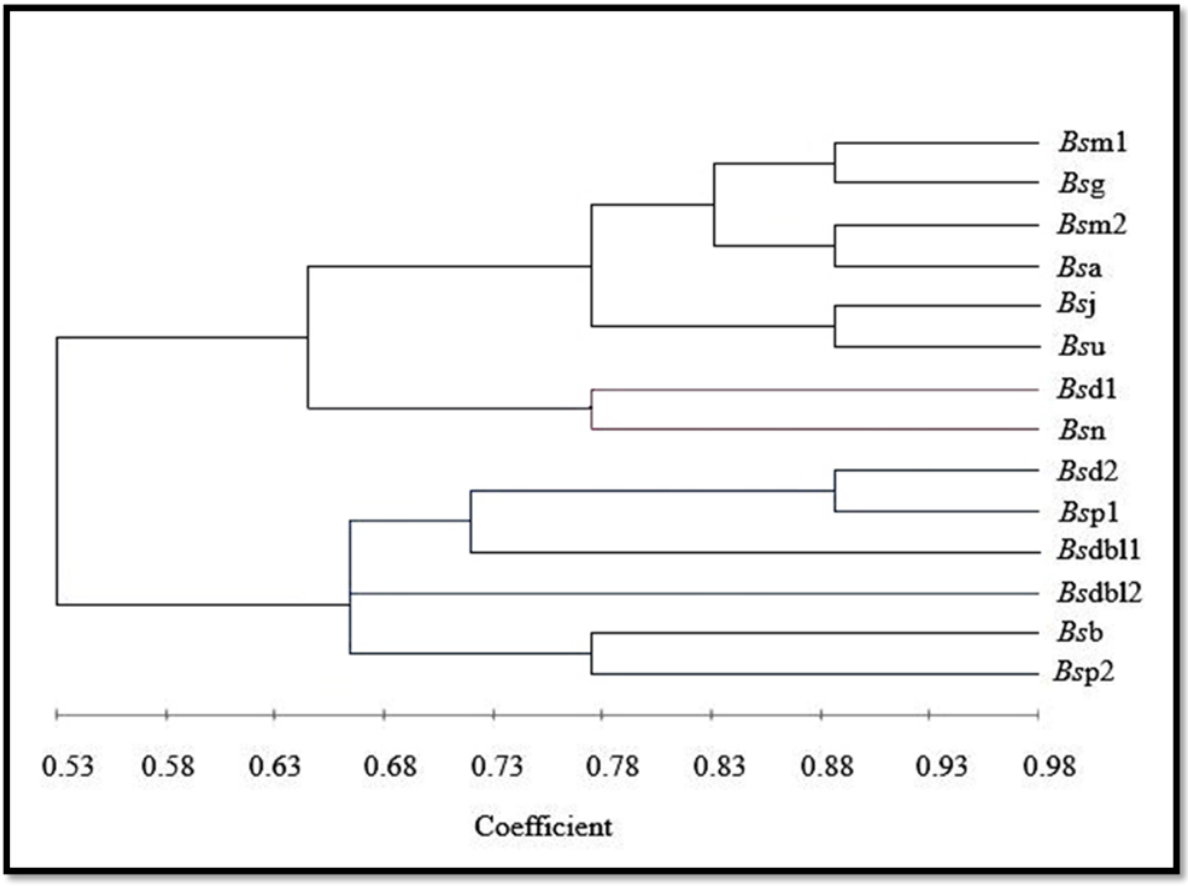


Fig. 1: Dendrogram for diversity of *Bipolaris sorokiniana* isolates as revealed by SSR markers

CONCLUSIONS

Assessment of genetic variability through ten SSR markers in 14 isolates showed polymorphism. The genetic similarity coefficient varied from 0.53 to 0.98 representing less than 53 per cent genetic variability among them. The monosporic isolates examined in this study showed very high genomic diversity. The profiles generated resulted in the difference among the monosporic isolates from each other. The dendrogram analysis showed diversity for isolates collected from different locations.

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