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RAPD analysis and rDNA gene sequence based phylogeny of *Bipolaris sorokiniana*, a spot blotch pathogen of sorghum

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Abstract
Sorghum [*Sorghum bicolor* (L) Moench] is the one of the most important cereal crops in the world. It is the staple food grain for over 750 million people who live in the semi-arid tropics of Africa, Asia, and Latin America. Global pro-duction of sorghum is currently estimated to be 57.6 million tonnes, with Asian countries contributing 20% of the total production. Within Asia, India is the largest producer of sorghum grain. Recently there have been severe signs of sorghum decline caused by *Bipolaris sorokiniana* resulting in decreased production of sorghum in villages of Kalimpong and Darjeeling. In the present study, initially, several strains of the fungus were isolated from diseased leaves of *Sorghum bicolor* and *Triticum aestivum* which were morphologically identified as *Bipolaris sorokiniana*. Genomic DNA of *B.sorokiniana* isolated from infected leaves was purified and PCR amplification of 18s rDNA was done using specific primers. Amplified product (1190 bp) was sequenced and aligned against ex-type strain sequences of *B.sorokiniana* from NCBI GenBank using BLAST and phylogenetic analysis was done using MEGA4 software. RAPD PCR analysis and DGGE analysis of amplified genomic DNA were done.The evolutionary history was inferred using the UPGMA method. Amplification of ITS region of the rDNA can be considered as a rapid technique for identifying pathogens successfully in all cases.

Keyword: RAPD, MEGA 4.0, DGGE, UPGMA.

Sorghum [*Sorghum bicolor* (L) Moench] is the fifth most important cereal crop in the world; and its wide range of other applications are now being explored with worldwide interest in renewable resources (Dahlberg *et al*,2011).However, fungi as-sociated with sorghum are of serious concern due to their toxigenic potential. The risk of contamination by mycotox-ins in sorghum is related to the kind of fungi associated. *Bipolaris sorokiniana*, a spot blotch pathogen which is a major threat for cereals like wheat and barley has also now a days shown its harmful effects on other members of the family Poaceae and the mostly affected one is sorghum in case of our study area, hills of Darjeeling and Kalimpong where the crop is cultivated in replacement of rice. *Bipolaris sorokiniana* (Sacc.) Shoemaker (syn. *Helminthosporium sativum* teleomorph: *Cochliobolus sativus*), a hemibiotrophic phytopathogenic fungus is a well known cause of spot blotch disease in sorghum (Acharya *et al*., 2011). The symptom of spot blotch usually appears on the leaf, sheath and stem. Yield losses due to spot blotch vary from 16 to 35% in sorghum. *B. sorokiniana* is widely distributed in the areas where sorghum are grown and forms a continuous genetic pool of isolates varying in virulence and aggressiveness to various cereals and grasses. The infection process on the leaves usually occurs through natural wounding, stomata or with the use of an appresorium-like structure through the cell wall. The presence of other hosts plays an important role in disease epidemic.

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the detection of pathogens at the species level. These rDNA are highly stable at the species level and exhibit a mosaic of conserved and diverse regions with the genome. Internal transcribed spacer (ITS) regions have been used successfully to generate specific

primers capable of differentiating closely related fungal species. Phylogenetic species concept between five or more gene trees has been proposed by Taylor *et al.* (1999). Random amplified polymorphic DNA (RAPDs) analysis has attracted a lot of attention after its advent during the 90's .This marker system was developed by Welsh and McClelland (1990). Manulis *et al.*,(1994), applied RAPDs to the carnation wilt fungal pathogen *Fusarium oxysporum* f. sp. *Dianthi* and they were able to identify specific banding patterns that were subsequently used as probes to distinguish between races of the pathogen. In another study, genetic relationships could be inferred among the wheat bunt fungi using RAPD markers (Shi *et al.*, 1995). *Colletotrichum gloeosporioides* isolates from mango and cashew plants were separated in different groups based on their RAPD band profile (Serra *et al*, 2011). The PCR-DGGE method is mainly applied for the analysis of the genetic diversity of microbial communities without the need of any prior knowledge of the species (Portillo *et al.*, 2011). These molecular techniques which interrelations among spsecies combined with phenotypic characters, can lead to a reliable taxonomy that is reflective of phylogenetic relathionship. ITS sequences of rDNA analysis and universally primed polymerase chain reaction have been used to categorize the isolates of *Talaromyces flavus* and *Trichoderma* species (Chakraborty *et al.*, 2011).

The purpose of the present study was rapid identification of *Bipolaris sorokiniana*, spot blotch pathogen of *Sorghum bicolor* (L.) Moench in hill regions of North Bengal based on the sequence analysis of ITS regions of the rDNA gene, RAPD and DGGE analysis with universal primer and development of rDNA markers for analysis of genetic variability.

Materials and method

Fungal Culture

Fungal pathogen (*Bipolaris sorokiniana*) was isolated from samples of diseased leaves of *Sorghum bicolor* and *Triticum aestivum* grown in Kalimpong, Siliguri and adjacent areas by culturing pieces of internal tissues. Healthy sorghum seedlings (1 month old) were further inoculated with this isolated organism for completion of Koch's postulate. At the end of two weeks, the reisolated organism was examined, compared with the original stock culture and its identity was confirmed following microscopic observations as *Bipolaris sorokiniana*. Mycelia were septate, branched, hyaline when young becoming brown with age.

Assessment of spot blotch disease

Disease assessment was done on the basis of appearance of infection on leaves in glass house condition of two month old sorghum plants. The disease severity was measured in terms of lesion number per leaf and percentage disease index was calculated by following the method of Adlakha *et al.*, (1984).

The following formula was used for percent disease index (PDI) calculation -[(class rating x class frequency)/(total no. of leaves x maximum rating)] x 100. The mean PDI was transformed into disease reaction as: 0%= no infection/ immune; 0-10%= resistant response (R); 10.1-20.0%= moderately resistant (MR); 20.1-30.0%=moderately susceptible (MS); 30.1-50.0%= susceptible (S) and >50.0%= highly susceptible (HS).

Genomic DNA Extraction

Genomic DNA was isolated from 4 day old fungal culture of *B.sorokiniana* by a modified Raeder and Broda (1985) method. Fungal mycelia from 3-4 days old cultures grown on potato dextrose broth was crushed with liquid nitrogen and incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS, for 4 h at 65°C followed by centrifugation at 12,000 rpm for 15 minutes. The supernatant was extracted with equal volume of water saturated phenol, centrifuged at 12,000 rpm for 15 minutes and further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) by centrifugation at 12000 rpm for 15 minutes; the aqueous phase was transferred in a fresh tube and chloroform (in the ratio of 1:4 v/v) was added followed by 0.5 M Na-acetate (in the ratio of 1:10 v/v). Next, isopropanol was added to the above mixture (0.7 times the final volume) and centrifuged. DNA was precipitated from the

aqueous phase with chilled ethanol (100%) and pelleted by centrifugation at 12000 rpm for 15 minutes followed by washing in 70% ethanol and centrifugation. The pellets were air dried and suspended in TE buffer pH 8.

Qualitative and Quantitative Estimation of DNA

The extraction of total genomic DNA as per the above procedure was followed by RNAse treatment. Genomic DNA was re-suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 minutes with RNAse (60 µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

PCR amplification of ITS region and sequencing

Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq polymerase buffer, 1 µl of 1U Taq Polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers.

Oligonucleotide	Sequences (5'- 3')	GC %	Tm Value	Length
18s FP	GCC TGT CTC AAA GAT TAA GCC	48	52.4	21
18s RP	CAC CTA CGG AGA CTT TGT TAC	48	52.4	21

For amplification of the ITS regions of the ribosomal DNA Primer pairs, 18s FP and 18s RP were used. PCR was programmed with an initial denaturing at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler.

RAPD PCR analysis

For RAPD, random primers were selected. PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36oC for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min. in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8µl) containing 0.25 % bromophenol blue, 40 % w/vsucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

The following primers were used for RAPD analysis in the study:

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
OPA1	CAGGCCCTTC	10	38.2	70%
OPA-4	AATCGGGCTG	10	39.3	60%

Amplification conditions and Analysis of RAPD bands

Temperature profile, 94°C for 4 min followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. RAPD band patterns were initially assessed by eye and isolates were grouped according to their shared band patterns.

PCR amplification of genomic DNA of the isolates for DGGE analysis

Denaturing Gradient Gel Electrophoresis was performed according to the method of Zhao et al (2012). 18S DNA (200bp with GC clamp) was amplified with the forward primer containing GC clamp at 5' end) F352T : 5'- CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C-3' and 519r: 5'- ACC GCG GCT GCT GGC AC-3') in 25µl of reaction mixture containing 1X PCR buffer, 2.5mM MgCl2 (Bangalore Genei, India), 100 ng of the template DNA,25.0pmol each of the forward and reverse primers, 250µM each of the dNTPS, and 1U of Taq DNA polymerase (Bangalore Genei, India). The touchdown PCR program was performed which consisted of an initial denaturation at 95°C for 5min, followed by 6 cycles of 95°C for 1min,65°C for 1min, and 72°C for 1min, in which the annealing temperature was reduced by 0.5°C per cycle from the preceding cycle, and then 24 cycles of 95°C. Perpendicular DGGE was performed with "the decode universal mutation detection system" (Bio Rad laboratories, USA). A uniform gradient gel of 0% to 100% denaturant was prepared which was changed several times so as to optimise suitable concentration and finally 20% to 60% denaturant was found optimal for the best result.

Results and discussion

Ten isolates of fungus causing spot blotch were obtained from naturally infected sorghum leaves grown in field of Department of Botany North Bengal University, adjacent areas of Siliguri and Kalimpong (Fig. 1A). All the ten isolates after completion of Koch's postulate showed bipolar germination, simple conidiophores, either single or clustered and 6-10 with septations (Fig. 1B and 1C).

Fig. 1: Appearance of disease symptoms (A) in leaves of susceptible sorghum genotype. Microscopic characteristics of fungal isolate- (IP/BS/SB-01), isolated from the infected sorghum leaves of variety sudan grass; (B) Structure of spore under 10X showing germination; (C) Enlarged view of spore showing germination.

For assessment of growth rate and sporulation, different solid media, Potato dextrose Agar (PDA), Richard's Media (RMA) and Oat Meal Agar (OMA) in petridishes were inoculated with 4mm diameter plugs taken from the periphery of young fungal culture of specified media, incubated at 25°C, and growth rate was measured by taking readings at every 24hrs. Mycelial dry weight was measured for all ten isolates grown in liquid Richard's Solution. Spores of the isolates grown on PDA medium after 14 days of incubation were measured with the help of ocular micrometer. The spores of each isolate were germinated separately on glass slides at 25±2°C, and relative humidity at around 90% and their appressorial dimensions measured after 24hrs.

Growth rates were measured in the ten strains of *B.sorokiniana* in three different solid media – PDA, RMA and OMA. The results are presented in Table 1, it is evident that the isolates did not differ significantly with respect to their growth rates in solid media, however there was some difference observed with respect to the type of the solid media used. For all the three isolates growth rate was highest in PDA and lowest in OMA. The growth rates ranged from 15.01 mm to 10.20 mm. The patterns of growth differed depending upon the media used.

Table 1. Mycelial growth of *B.sorokiniana* isolates in different solid media.

Isolate	Diameter of mycelia (mm)		
	Media		
	Potato Dextrose Agar	Richard's Agar	Oat Meal Agar
IPL/BS/SB-01	15.01±0.23	12.92±0.07	10.57±0.05
IPL/BS/SB-02	12.77±0.16	10.68±0.06	09.59±0.05
IPL/BS/SB-03	13.09±0.21	11.19±0.09	09.90±0.03
IPL/BS/SB-04	11.67±0.19	10.34±0.08	09.80±0.23
IPL/BS/TA-01	12.45±0.23	11.65±0.07	09.80±0.14
IPL/BS/TA-02	13.67±0.22	11.73±0.15	10.89±0.03
IPL/BS/TA-03	11.35±0.17	10.01±0.12	10.70±0.20
IPL/BS/TA-04	10.91±0.36	09.50±0.28	09.80±0.14
IPL/BS/TA-05	11.48±0.26	10.16±0.16	09.60±0.29
IPL/BS/TA-06	13.82±0.16	11.79±0.15	10.20±0.08
10 days after incubation, mean±SD, Average of three replicates, Incubation at 25±2°C.			

Growth in liquid synthetic Richard's solution (RM) was measured for the ten isolates separately by taking dry weight. The resulting data is presented in Table 2. The dry weight accumulated was highest 15 days after inoculation in *B. sorokiniana*, irrespective of the isolate. However the dry weight values at that point differed significantly among the isolates. IPL/BS/SB-01 has the highest weight (293 mg) and IPL/BS/TA-04 has the lowest weight (197 mg).

Table 2. Mycelial growth of *B. sorokiniana* isolates in Richard's solution.

Isolates	Growth rate (mg)		
	Days after inoculation		
	5	10	15
IPL/BS/SB-01	120.83±0.60	232.33±1.45	293.33±7.68
IPL/BS/SB-02	106.26±0.81	199.00±2.08	238.00±7.57
IPL/BS/SB-03	117.53±0.78	212.33±8.68	257.00±1.52
IPL/BS/SB-04	105.83±0.60	185.66±2.96	199.00±2.08
IPL/BS/TA-01	99.50±0.76	203.66±3.17	255.00±2.88
IPL/BS/TA-02	102.00±1.15	205.33±3.71	291.66±6.00
IPL/BS/TA-03	109.66±0.88	146.66±7.26	267.33±5.92
IPL/BS/TA-04	93.50±0.76	159.66±5.78	197.33±3.71
IPL/BS/TA-05	107.16±0.92	117.66±1.76	213.33±8.81
IPL/BS/TA-06	112.33±1.45	152.33±2.02	280.00±5.00
Mean±SD, Average of three replicates, incubation at 25±2°C.			

Sporulation was assessed in *B.sorokiniana* isolates in three different media (PDA, OMA and RMA). Highest degree of sporulation was observed in PDA which was similar for all the isolates. Lowest sporulation was observed in OMA. Results are shown in Figure 2. IPL/BS/SB-01 exhibited highest amount of sporulation among the ten isolates.

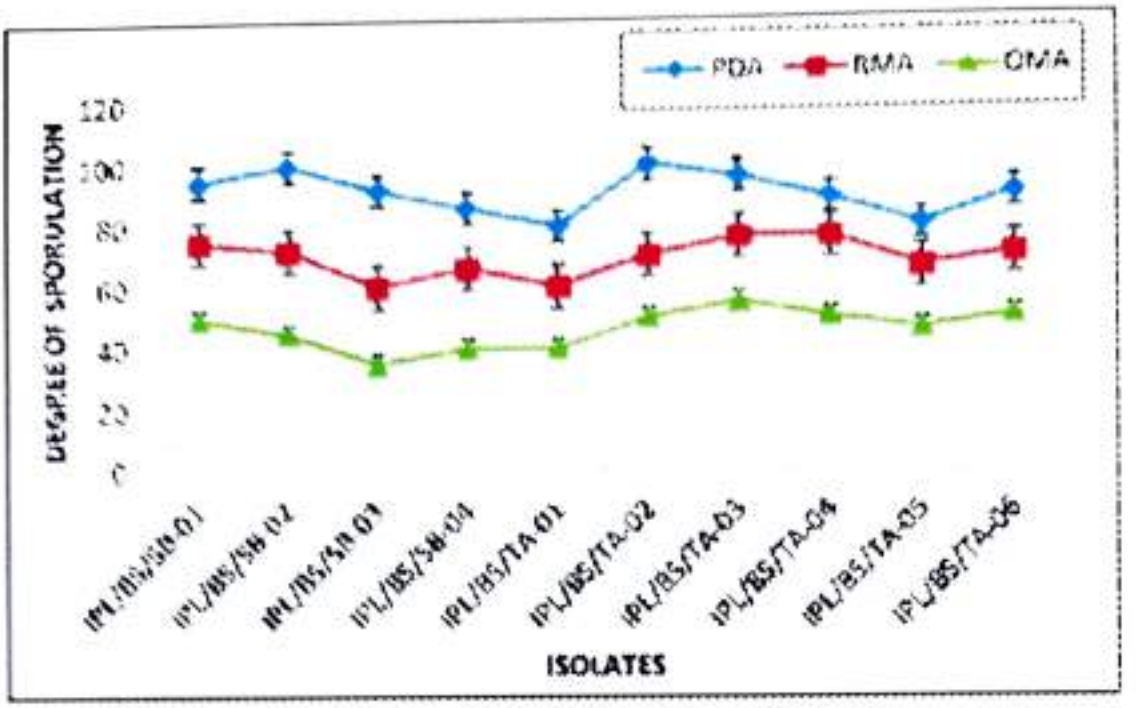

Fig. 2: Percentage sporulation of *B. sorokiniana* isolates in three different solid media (PDA – Potato Dextrose Agar, RM – Richard's Medium, OMA – Oat Meal Agar).

Under the natural condition the establishment of the spot blotch disease was observed after two month growth of the sorghum plants grown on glass house condition and Disease index (PDI%) was calculated. PDI of different sorghum variety was found to differ significantly from each other in comparison to check the susceptibility towards the infection. The maximum incidence (76.69%) was observed in CSV 30F followed by Sudan grass (74.03%) and CSV 15 (70.08%) (Fig. 3). The lowest incidence was observed in CSV 17 (42.04%) and CSV 29 R (49.62%) (Fig. 3) shows that among all the ten varieties one local (Sudan grass) and two hybrid sorghum variety (CSV 30 F and CSV 15) are highly susceptible to brown spot blotch pathogen as evident from the data on percent disease index. The variety Sudan grass was found to be most susceptible to spot blotch.

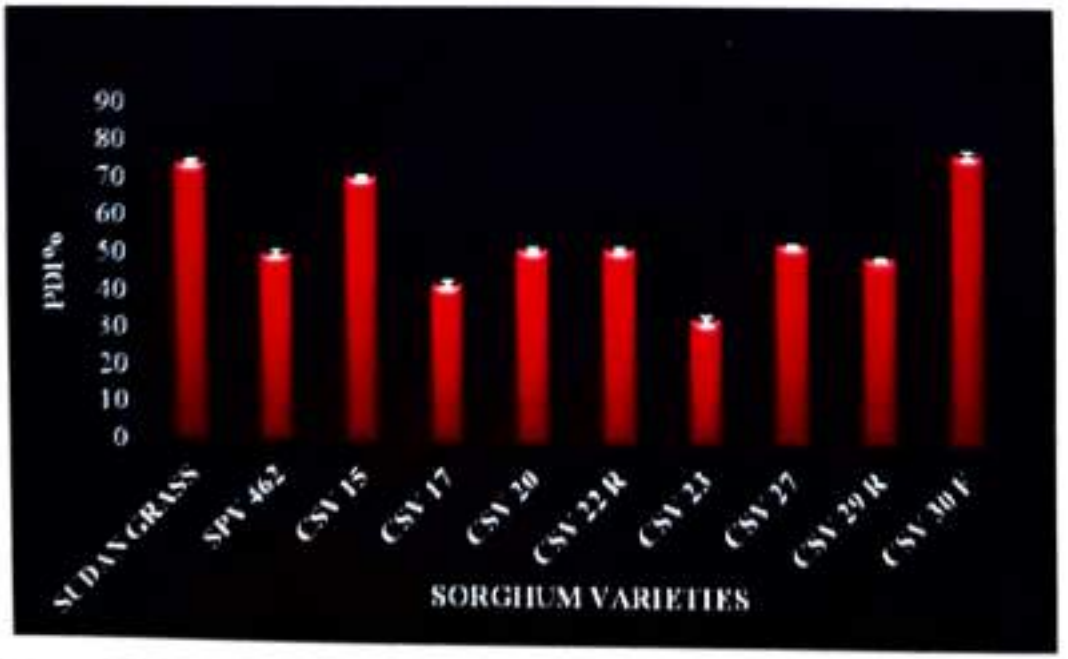

Fig. 3: Percent Disease Index (PDI) of spot blotch disease in nursery grown sorghum plants.

The isolate IPL/BS/SB-01 was identified as *Bipolaris sorokiniana* by 18S rDNA sequencing and deposited in NCBI Genbank and the accession number was obtained (MF927960). Earlier studies have also indicated a high level of morpho-pathological variability in the pathogen (Chand *et al.*, 2003). These authors also mentioned another possible cause of variability in spot blotch pathogen which was suggested to be the variable rearrangement of one to six nuclei per cell. Serological detection of the pathogen in terms of Dot blot and FITC of the strain was done previously by Bhattacharjee *et al.* (2016).

Genomic DNA of the organism *B. sorokiniana*, was amplified. Main focus was on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *B. sorokiniana*. ITS region of rDNA was amplified using specific 18sR and 18sF for sequencing of its 18s rDNA region (Fig. 4). Amplified products of size in the range was produced by the primer pairs.

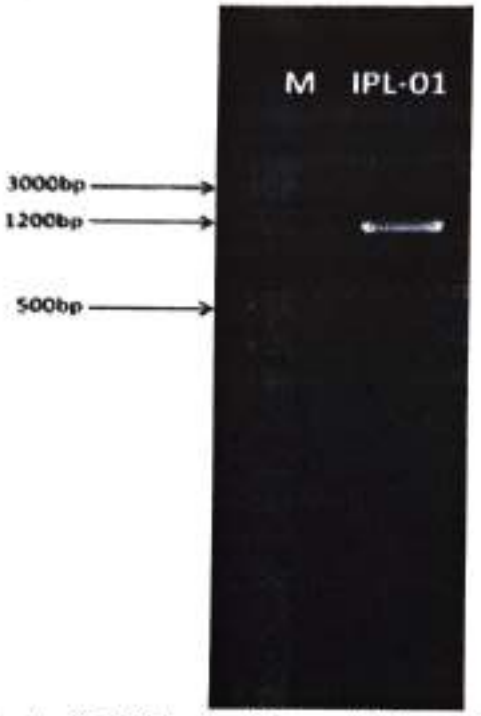

Fig. 4: ITS-PCR amplified products (1190 bp) of *B.sorokiniana* (IPL/BS/SB-01).Lane M-high range DNA ladder, lane 1-*B.sorokiniana.*

The sequenced PCR product was aligned with extype isolate sequences from NCBI GenBank for identification as well as for studying phylogenetic relationship with other ex-type sequences (Table 3). The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4. Phylogenetic analysis showed that *B. sorokiniana* (IPL/BS/SB/01) isolate is closely related to *B. sorokiniana* isolate-KJ396082.1 showing 78% similarity (Fig. 5). Multiple sequence alignment revealed that there were regions in the sequences which were not similar and, hence, gaps were introduced in these regions. Presence of regions with similar sequences indicated relationships among the isolates of *B. sorokiniana* (Fig. 5). Aggarwal *et al.*, (2010) studied molecular variability in *B.sorokiniana* using URP-PCR and grouped the isolates according to their geographic origin.

Table 3. GenBank accession numbers and geographic location of ex-type strains that showed homology with isolate IPL/BS/SB-01.

Sl No	Accession No	Strain or Isolate	rDNA Sequence	Country
1.	FJ864705	PPRI 6810	560 bp	South Africa
2.	KM066949	WH.PBW.IP.0	588 bp	India
3.	HM998314	MvNorthCarDukeForU13	566 bp	USA
4.	HM998310	MvNorthCarDukeForU8	578 bp	USA
5.	EU030351	FG42	566 bp	China
6.	GU073106	DH08015B	561 bp	China
7.	DQ367885	BS-54	590 bp	India
8.	KF765401	BS52M2P	569 bp	Brasil
9.	HM195258	Bs 72	570 bp	India
10.	HM195260	Bs 77	570 bp	India
11.	HM195250	Bs 03	571 bp	India
12.	KF765405	98026P	569 bp	Brasil
13.	KF765407	98023P	569 bp	Brasil
14.	KF765399	98012C	569 bp	Brasil
15.	GU480768	64	591 bp	India
16.	DQ229951	64	591 bp	India
17.	DQ229952	9	590 bp	India
18.	GU345084	7	589 bp	India
19.	KJ634700	ppl-1	554 bp	China
20.	GU080212	BsDR1	540 bp	Oman
21.	KJ767094	A1S6-2	598 bp	Malaysia
22.	DQ337383	99-36-2	2248 bp	China
23.	XM_007701880	ND90Pr	2510 bp	USA
24.	HM998314	MvNorthCarDukeForU13	566 bp	USA
25.	HQ611957	PSB3	554 bp	USA
26.	GU073106	DH08015B	561 bp	China
27.	GU073107	DH08122quan2	554 bp	China
28.	NR_147494	BRIP 15929	700 bp	Australia
29.	NR_147495	BRIP 12030	731 bp	USA
30.	NR_147496	BRIP 16226	723 bp	USA
31.	NR_147497	BRIP 12239	727 bp	USA

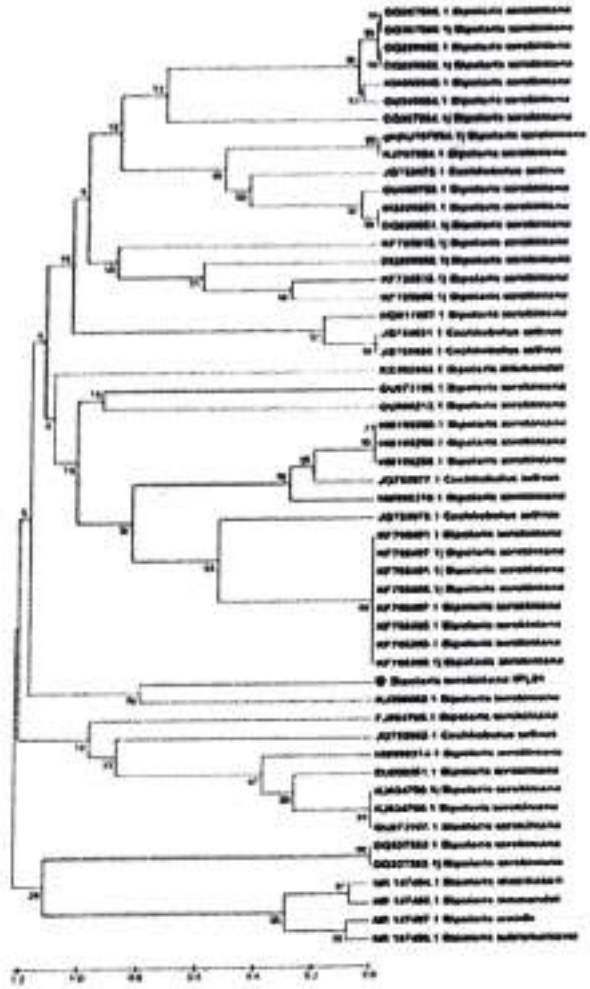

Fig. 5: Phylogenetic placement of *Bipolaris sorokiniana* with other ex-type strain sequences obtained from NCBI GenBank Database.

Multiple sequence alignment revealed that there were quite a number of gaps introduced in the alignment within the ITS region which were closely related. Similar sequence indicated that the isolates were closely related. From the sequence alignment, variations were observed between other *B. sorokiniana* isolates in species level (Fig. 6).

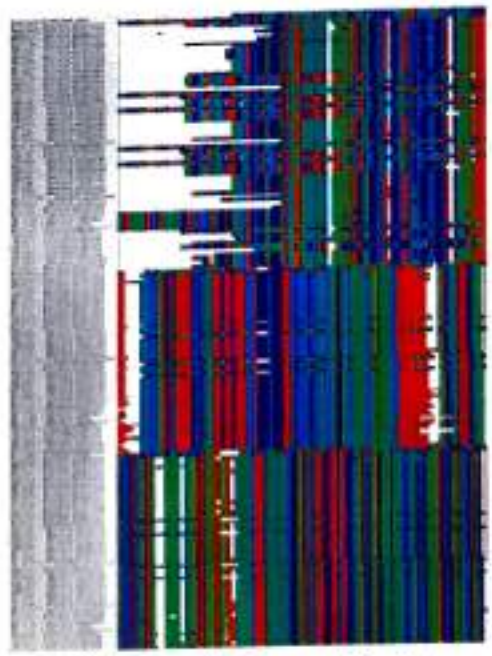

Fig. 6: 18S rDNA sequence alignments of *B. sorokiniana* with other ex-type strains obtained from NCBI GeneBank database. The conserved regions of the gene are demonstrated in different colour.

In the present study *B. sorokiniana* isolates were evaluated for their genetic variability using RAPD markers. The genetic relatedness among the isolates were analysed by random primers to generate reproducible polymorphism.

Among the two different primers (OPA-1, OPA-4), OPA-4 did not produce any polymorphic bands. However OPA-1 produced a total of 27 bands out of which 11 bands were polymorphic (Table 4). The RAPD profile showed that primer OPA-1 showed maximum polymorphism among the different isolates of *B. sorokiniana* (Fig. 7).Relationships among the isolates were evaluated by cluster analysis of the data based on the similarity matrix. Based on the results obtained all the ten isolates can be grouped into three main clusters. Similar result was reported by previous workers (Yadav *et al.*, 2013) in their study genetic variability and relationship analysis of *Bipolaris sorokiniana* isolates causing spot blotch disease in wheat using random amplified polymorphic DNA (RAPD) markers.

Table 4. Total number of polymorphic bands produced by different RAPD primers.

Primer	No. of RAPD products (bands)		
	Total bands	Polymorphic bands	% polymorphism
OPA-1	27	11	40%
OPD-4	20	0	-

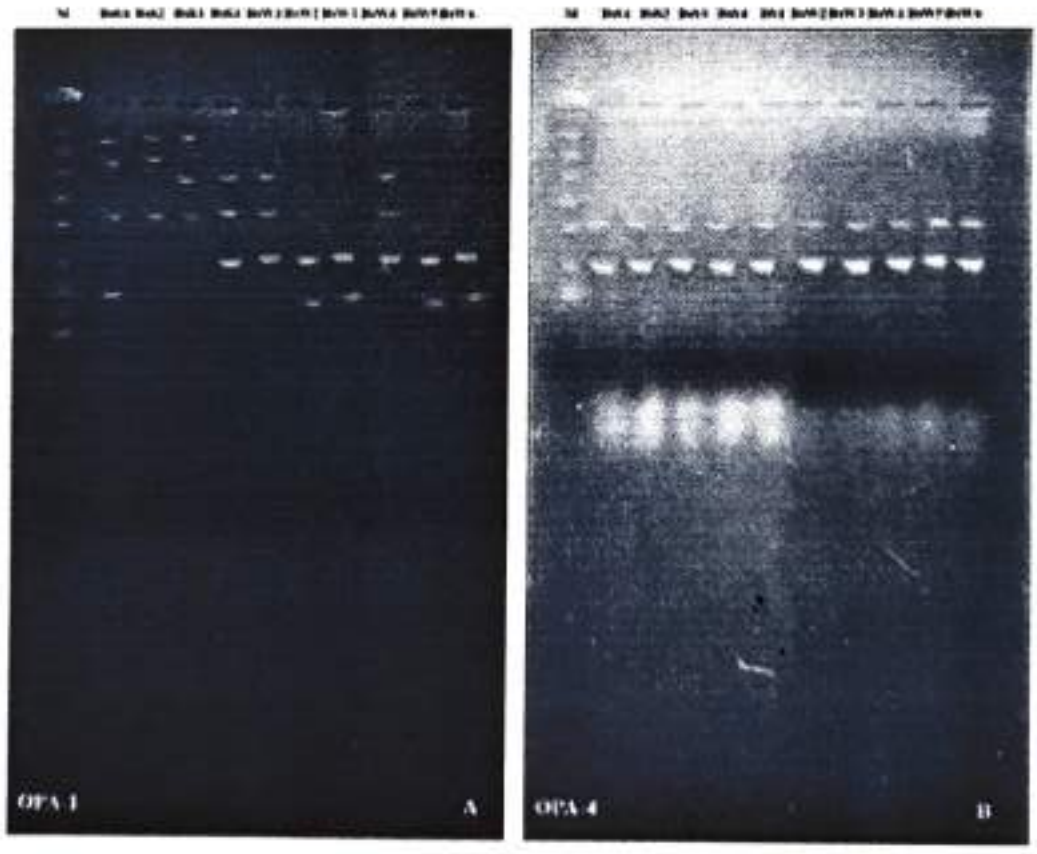

Fig. 7: RAPD-PCR analysis of *Bipolaris* isolates of sorghum and wheat plant using primers (A) OPA-1 and (B) OPA-4. Lane M – DNA ladder, Lane 1-4 *B. sorokiniana isolated from sorghum plant* BsS1, BsS2, BsS3, BsS1), Lane 5 – 10 *B. sorokinian* isolated from wheat plant (BsW1, BsW2, BsW3, BsW4, BsW5, BsW6).

Denaturing Gradient Gel electrophoresis analysis of ten different isolates of *Bipolaris sorokiniana* (IPL/BS/SB-01, IPL/BS/SB-02, IPL/BS/SB-03, IPL/BS/SB-04, IPL/BS/TA-01, IPL/BS/TA-02, IPL/BS/TA-03, IPL/BS/TA-04, IPL/BS/TA-05, IPL/BS/TA-06) was assessed using the GC flung primers as mentioned above. The uniform PCR product of 300bp was obtained. The DGGE electrophoresis yielded a uniform and unique banding pattern of each group of organism. In this uniform gradient gel of 20-60%, the banding pattern of four isolates of *B. sorokiniana* isolated from sorghum plants were identical to that of the reference isolates used for confirmation (Fig. 8). Out of ten isolates, only one has been sequenced and their GC content known. This isolate taken as reference isolate. The ten isolates have been separated into three distinct groups based on the migration rate of their amplified 18S fragment following DGGE. The amplified bands of three isolates (IPL/BS/TA-01, IPL/BS/TA-02, IPL/BS/TA-03) migrated faster than other isolates. This is due to the GC content of the isolates. DNA with higher GC content will always migrate faster in denaturing gel. The migration of amplified 18S rDNA samples within each group was different, suggesting that there was little intraspecific variation among the isolates. Fagbola and Abang (2004) distinguished *Colletotrichum circinans* and *C. coccodes* isolates based on DGGE analysis of their amplified fragments in spite of the failure of previous attempts at genetic differentiation of the two species based on RFLP analysis of the rDNA ITS region.

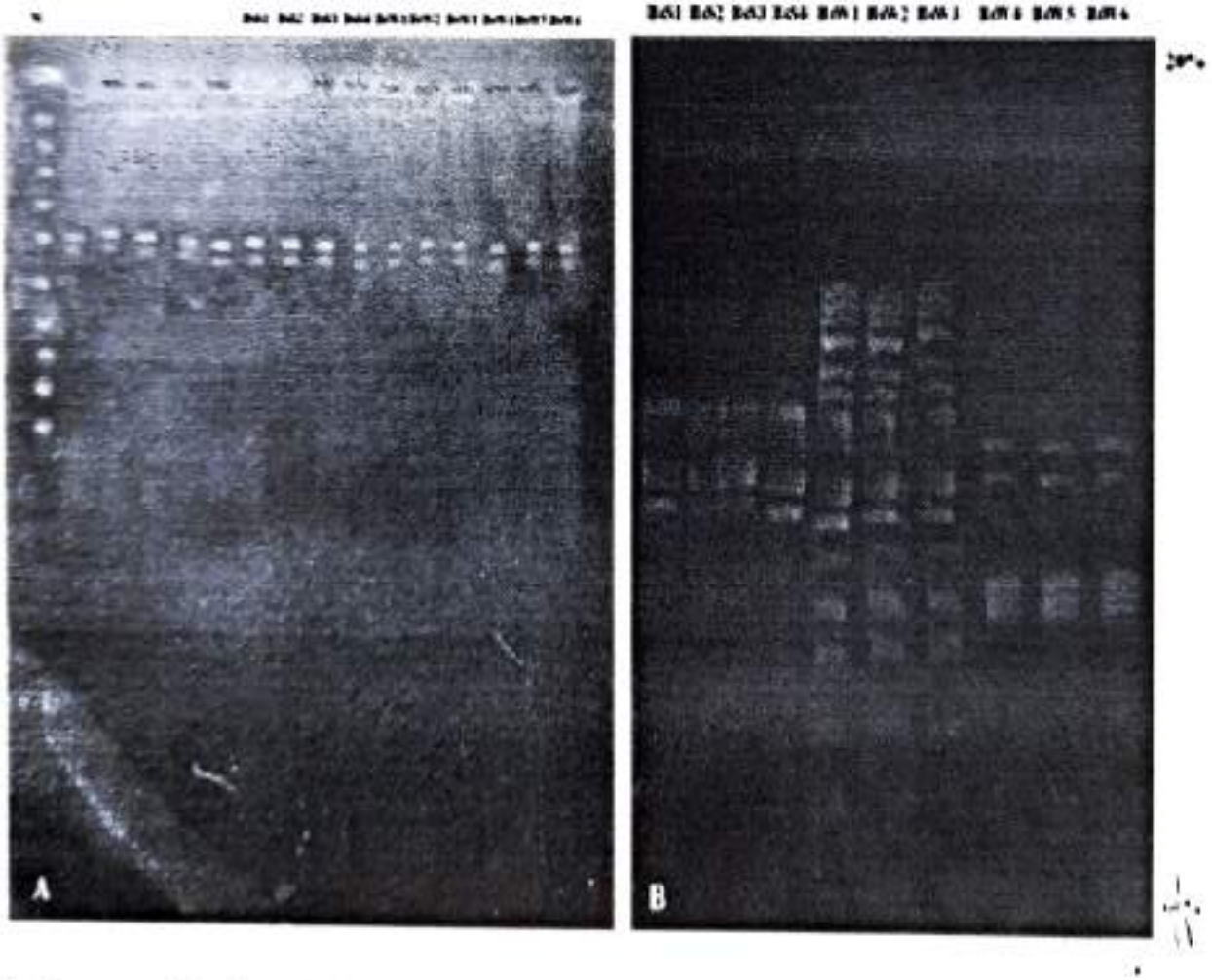

Fig. 8: Denature Gradient Gel Electrophoresis of the PCR amplified products of isolates of *Bipolaris sorokiniana* (BsS1, BsS2, BsS3, BsS4, BsW1, BsW2, BsW3, BsW4, BsW5, BsW6),(A) PCR products, 300bp; (B) Gradient 20-60%, 8hrs run, 100V.

Amplification of DNA fragments of *B. sorokiniana* with specific primers indicate the usefulness of molecular technique for their detection and identification. In RAPD and DGGE analysis also the isolates were clearly grouped according to the host origin. Additionally, little molecular variation was observed among the isolates tested by RAPD and DGGE. The overall study established the rDNA gene sequence of *Bipolaris sorokiniana* (Sacc.) Shoemaker a spot blotch pathogen of Sorghum *bicolor* (L.) Moench based on the bioinformatics tools with sequence analysis if ITS regions of the rDNA gene for rapid identification and development of rDNA markers for analysis of genetic variability within the out groups. We have demonstrated that the analysis of aligned rDNA sequences is a reliable clustering strategy for identification purpose in a variety of taxonomic groups and systemic levels. While this approach was previously applied in analyzing complete genome data, the present study shows that it is also applicable in analyzing much shorter DNA sequences from a single gene, which is going to be the fundamental block in the massive rDNA database.

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