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Molecular characterization of *Sarocladium oryzae* isolates causing sheath rot in paddy (*Oryza sativa)*

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ABSTRACT

Sheath rot, caused by *Sarocladium oryzae* (Sawada) W. Gams & D. Hawksw, is an important seed borne fungal disease of paddy (*Oryza sativa* L.). In the present investigation, diseased specimens were collected in *kharif* 2016 and 2017 from more than 55 different geographical locations spread across 15 states of the country and variability in 30 isolates of *Sarocladium oryzae* was studied by molecular methods. The molecular characterization using two marker systems, i.e. Inter-Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) was carried out which revealed differences in isolates collected from different geographical locations. After preliminary screening, out of 22 ISSR and 24 RAPD primers, 8 ISSR and 7 RAPD primers were used against all the isolates. Percent polymorphic ranging between 53.1–100% in ISSR and 61.4–100% in RAPD was recorded with all the isolates. The average number of bands in ISSR markers was 63.3, and 71.8 in RAPD markers. All RAPD primers showed 100% polymorphism except OPS13 (61.4%) and OPD5 (74%). Among ISSR primers ISSR5 and ISSR18 showed 100% polymorphism. Cluster analysis of individual and combined primers was done. In the combined cluster analysis of RAPD and ISSR markers, the isolates from same geographical region were in same cluster as evident from the cluster I that includes Kadapa, Ragolu, Maruteru, West Godavari and Adutharai, which are from southern plain region of the country. Similar observations were recorded when data analysis of individual marker system was carried out which revealed that the two different marker systems applied complement each other in characterizing various isolates.

Key words: Genetic diversity, Molecular markers, *Sarocladium oryzae*, Sheath rot of paddy

Rice (*Oryza sativa* L.) is attacked by many diseases, and fungal diseases are known to cause maximum damage. Many of these fungal diseases are seed borne in nature. Sheath rot caused by *Sarocladium oryzae* (Sawada) W. Gams & D. Hawksw is an important seed borne fungal disease of paddy which is becoming a major concern to the rice growing farmers in our country. Due to the introduction of high-yielding and semi-dwarf rice cultivars, sheath rot pathogen of rice has become a major production constraint in all rice-growing countries of the world (Ou 1985). Dwarf cultivars are susceptible in comparison to tall ones (Amin 1974).

Though the disease is considered to be minor one, it is posing a serious emerging threat. The fungus is detected frequently during routine seed health testing and causes empty grain production (Kulwanth and Mathur 1992). The fungus produces characteristic greyish brown lesions on the uppermost flag leaf enclosing the panicle. Severe infection has been reported to produce partially emerged or totally compressed panicles with chaffy grains. The rotting occurs on the uppermost leaf sheath enclosing the young panicle. Sometimes oblong or irregular brown spots appear which enlarge and cover most of the leaf sheath. The young panicles are forced to remain enclosed within the leaf sheath or emerge partially. In severe cases, the panicles rot completely and the grains turn deep brown in colour and become chaffy (Singh and Dodan 1995). Although *S. oryzae* is seed-borne and seed transmitted, the fungus also survives as mycelium in infected plant residues, weed hosts, and soil (Chien and Huang 1979). The fungus causes empty grain production (Kulwanth and Mathur 1992), glume discolouration (Sachan and Agarwal 1995) and seed discolouration (Reddy *et al.* 2000). Sheath rot infection reduces the seed viability and nutritional value of rice. It also causes poor grain filling and reduction in seed germination (Vidhyasekaran *et al.* 1984). Seeds from infected panicles became discoloured and sterile (Mewand Gonzales 2002). Keeping this in view, molecular characterization of the fungus causing sheath rot

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in paddy, i.e. *Sarocladium oryzae*, was carried out in the present study.

MATERIALS AND METHODS

The diseased specimens (both sheath and seeds) showing typical sheath rot symptoms were collected from 55 different places of the country during *kharif* 2016 and 2017. The pathogen was isolated using blotter method. Identity of the culture was confirmed on the basis of morphological characters. Spore size was measured at 400X magnification under compound microscope. Thirty isolates which were geographically distant and had maximum disease incidence were selected for carrying out variability using molecular markers (Table 1).

Quantification of DNA was done by nano drop spectrophotometer (NanoDrop, USA) using 1 µl of sample for each isolate. Readings were taken at two wavelengths (260 nm and 280 nm). Working solutions (25 ng/µl) were prepared for optimization of polymerase chain reaction (PCR) reaction by adding double sterilized nuclease free water.

Preliminary primer screening was carried out with 24 RAPD and 22 ISSR primers of which 7 RAPD and 8 ISSR primers gave best amplification. The primers that gave scorable amplifications were further used in the analysis of genetic variability of *Sarocladium oryzae* isolates. Protocol for PCR was optimized by varying the parameters. Composition of PCR reaction was optimized by varying the concentration of template DNA (25 ng, 50 ng, 75 ng, 100 ng), Taq DNA polymerase (0.2 µl, 0.5 µl and 1.0 µl), and $MgCl_2$ concentration (1.5 µl, 2.5 µl, 3.0µl and 3.5 µl). Different PCR profiles were tested for obtaining best amplification of the isolates under investigation. The amplification assay with the following conditions were formulated: Template DNA 50 ng, Taq DNA polymerase 0.2 µl, $MgCl_2$ 3.0 µl, dNTP 0.2 µl, primer (0.4 µM) 2.5 µl, buffer 2.5 µl. Different PCR profiles were tested for best amplification of markers. The standardized temperature profiles of 94ºC for 5 min followed by 40 cycles of 94ºC for 1 min, 45ºC for 1 min, 72ºC for 2 min, with an elongation of 72ºC for 5 min gave best results and was used for further experiments.

Binary matrices were analysed by NTSYS-PC (Version 2.02; Exeter Biological Software, Setauket, NY). The Jaccard similarity coefficients were clustered to generate a dendrogram using SHAN clustering programme and

Table 1 Isolates of *Sarocladium oryzae* used in the study

Isolate	Place, State
SO1	Shimoga, Karnataka
SO2	Raipur, Chattisgarh
SO3	Pattambi, Kerala
SO4	Genetics field, IARI, NCR
SO5	Mandya, Karnataka
SO6	Kolar, Karnataka
SO7	Physiology field, IARI, NCR
SO8	Pathology field, IARI, NCR
SO9	Chiplima, Odisha
SO10	Lonavala, Maharashtra
SO11	Aduthar ai, Tamil nadu
SO12	West Godavari, Andhra Pradesh
SO13	Maruteru, Andhra Pradesh
SO14	Ragolu, Andhra Pradesh
SO15	Ranchi, Jharkhand
SO16	Bankura, West Bengal
SO17	Chinsurah, West Bengal
SO18	Puducherry, Puducherry
SO19	Karnal, Haryana
SO20	Kaul, Haryana
SO21	Noida, NCR
SO22	Agra, Uttar Pradesh
SO23	Jhansi, Uttar Pradesh
SO24	Khudwani, Jammu & Kashmir
SO25	Navsari, Gujarat
SO26	Nawagam, Gujarat
SO27	Raichur, Karnataka
SO28	Pusa, Bihar
SO29	Kadapa, Andhra Pradesh
SO30	Coimbatore, Tamil nadu

Table 2 RAPD primers and ISSR primers used in study and their analysis

Primer	Sequence	Annealing temp (°c)	Band size range (kb)	No. of bands	No. of polymorphic bands
RAPD Primers					
OPD3	TCGGCGATA G	45	0.4 -1.5	67	67
OPD5	CAGCAGCCA C	45	0.375-1.7	117	87
OPE14	TTCCGAACC C	45	0.74-1.54	51	51
OPE18	AGGTGACCG T	44	0.38-2.0	91	91
OPS28	GTGACGTAG G	42	0.3-0.9	33	33
OPS30	GTGATCGCA G	43	0.38-2.2	66	66
OPS13	GTTGCGATC C	45	0.45-1.30	78	48
Average number of Bands				71.8	63.3
ISSR Primers					
ISSR4	(AG)8YT	45	0.26-1.3	118	88
ISSR5	(GA)8YT	42	0.25-0.9	57	57
ISSR6	(GT)8YC	44	0.28-2.2	167	77
ISSR7	(ACC)6	45	0.75-2.1	82	52
ISSR18	(GA)8 T	45	0.74-1.2	47	47
ISSR22	(AC)9T	45	0.24-1.52	98	68
ISSR23	(AC)9G	42	0.4-1.4	115	85
ISSR13	(TC)8 A	43	0.35-1.2	64	34
Average number of bands				93.5	74.7

selecting UPGMA (Unweighted Paired Group Method with Arithmetic Averages) (Rohlf 1998). DNA bands that could be scored unequivocally for their presence or absence were included for analysis. Data analysis of individual marker system as well as combined data were analysed with Jaccard genomic similarity coefficient and three dendrograms were constructed.

RESULTS AND DISCUSSION

Genetic diversity was analysed among 30 isolates of *Sarocladium oryzae* by using RAPD and ISSR markers. The 7 RAPD and 8 ISSR primers were used against all the thirty isolates. Total number of bands was 503 with average of 71.85 in RAPD and 748 with average of 93.5 in ISSR markers. The band size range of various isolates among RAPD primers and ISSR primers is given in Table 2.

Cluster analysis using RAPD markers revealed seven different groups. As expected most of the isolates from similar geographical regions were in the same cluster like SO13 and SO14 in the cluster I from Andhra Pradesh. Cluster II had SO3 and SO18 from southern plains, similarly Cluster III had all isolates from Andhra Pradesh (West Godavari and Kadapa). Cluster V had many isolates from northern plains like Kaul, Haryana (SO20), Noida, NCR (SO21), Physiology IARI, NCR (SO7), Genetics field, IARI, NCR (SO4), Jhansi, UP (SO23). Cluster VI includes isolates Nawagam, Gujarat (SO26); Lonavala, Maharashtra (SO10); Raipur, Chattisgarh (SO2). Some isolates belonging to same geographical region fall under many different clusters like isolates from Karnataka, Shimoga (SO1) in Cluster VII, Raichur (SO27), Kolar (SO6) and Mandya (SO5) isolates in Cluster V. This indicates the variability of pathogen within same geographical region.

Cluster analysis using ISSR markers also revealed seven different clusters. As expected most of isolates belonging to same geographical region were under same cluster. Like Cluster I had West Godavari (SO12), Kadapa, Andhra Pradesh (SO29); Adutharai, TN (SO11); Ragolu, Andhra Pradesh (SO14); Maruteru, Andhra Pradesh (SO13) all from southern plains and except SO11, all isolates belonged to Andhra Pradesh. Similarly Cluster II includes Bankura, West Bengal (SO16); Chinsurah, West Bengal (SO17) and belong to same state of West Bengal. In cluster V most of isolates belong to northern plains like Khudwani, J&K (SO24); Jhansi, UP(SO23); Hapur, UP (SO22); Except one isolate, i.e. Navsari, Gujarat (SO25) which comes under central India. All the isolates from Karnataka like Raichur (SO27); Kolar (SO6); Mandya (SO5) are fit into

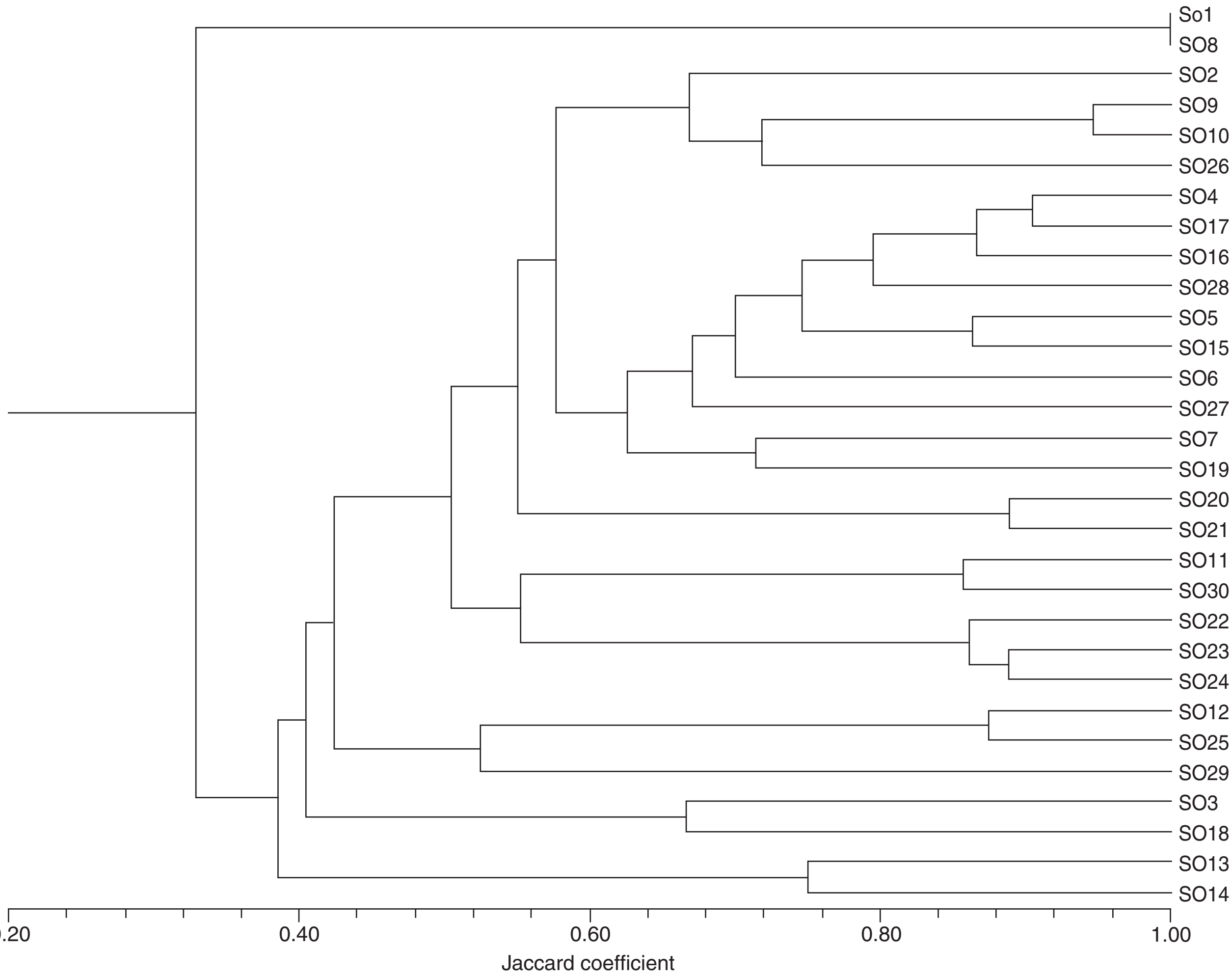

Fig 1 Dendrogram based on individual data analysis of RAPD Primers.

same cluster, i.e. Cluster VI, except shimoga isolate comes under cluster VII. Isolates from north eastern India fit into different clusters like Ranchi, Jharkhand (SO15) in Cluster VI, Pusa, Bihar (SO28) in Cluster IV, Bankura, West Bengal (SO6); Chinsurah, West Bengal (SO17) in Cluster II show the variability of pathogen in same geographical region.

In the cluster analysis using RAPD and ISSR markers, as expected most of the isolates from the same geographical regions fit into same cluster. Like, cluster I includes Kadapa, Andhra Pradesh (SO29); Ragolu, Andhra Pradesh (SO14); Maruteru, Andhra Pradesh (SO13); West Godavari, Andhra Pradesh (SO12); Adutharai, Tamil Nadu (SO11). All these isolates are from southern plain region. In cluster II includes isolates from Coimbatore, Tamil Nadu (SO30); Puducherry (SO18); Pattambi, Kerala (SO3) and these all isolates are from southern plains. In cluster III, Kaul, Haryana (SO20); Noida, NCR (SO21) both the isolates belonging to Northern plains. In cluster IV, most of isolates from Northern plains like Pathology field, IARI, NCR (SO8); Karnal, Haryana (SO19); Physiology field IARI (SO7) and cluster IV also includes two isolates from Karnataka Raichur (SO27), Kolar (SO6). The cluster V consists of 3 isolates Nawagam, Gujarat (SO26); Lonavala, Maharashtra (SO10); Chiplima, Odisha (SO9). Cluster VI also consists of 3 isolates from North eastern India like Pusa, Bihar (SO28); Chinsurah, West Bengal (SO17); Bankura, West Bengal(SO16).

Individual data analysis of RAPD marker (Fig 1) showed Jaccard's genetic similarity coefficient ranging from 8–100%. Highest similarity (100%) observed between SO1 and SO8 strains, collected from Shimoga (Karnataka) and Pathology Field, IARI (NCR), and SO10 and SO9 isolates shown 95% similarity collected from Lonavala (Maharashtra) and Chiplima (Odisha) respectively. Followed 90% similarity between SO4 (Genetics field, IARI, NCR) and SO17 (Chinsurah, West Bengal), followed by 89% between SO23(Jhansi, Uttar Pradesh) and SO24 (Khudwani, Jammu & Kashmir).

Individual data analysis of ISSR marker (Fig 2) showed Jaccard's genetic similarity coefficient ranging from 30% to 96%. The Highest similarity, i.e. 96% observed between SO23 and SO24 which are collected from Jhansi, Uttar Pradesh and Khudwani, Jammu & Kashmir. Followed by 92% between SO6 (Kolar, Karnataka) and SO7 (Physiology field, IARI, NCR), Followed by 88% between SO9 (Chiplima, Odisha) and SO10 (Lonavala, Maharashtra).

Combined data analysis of RAPD and ISSR markers

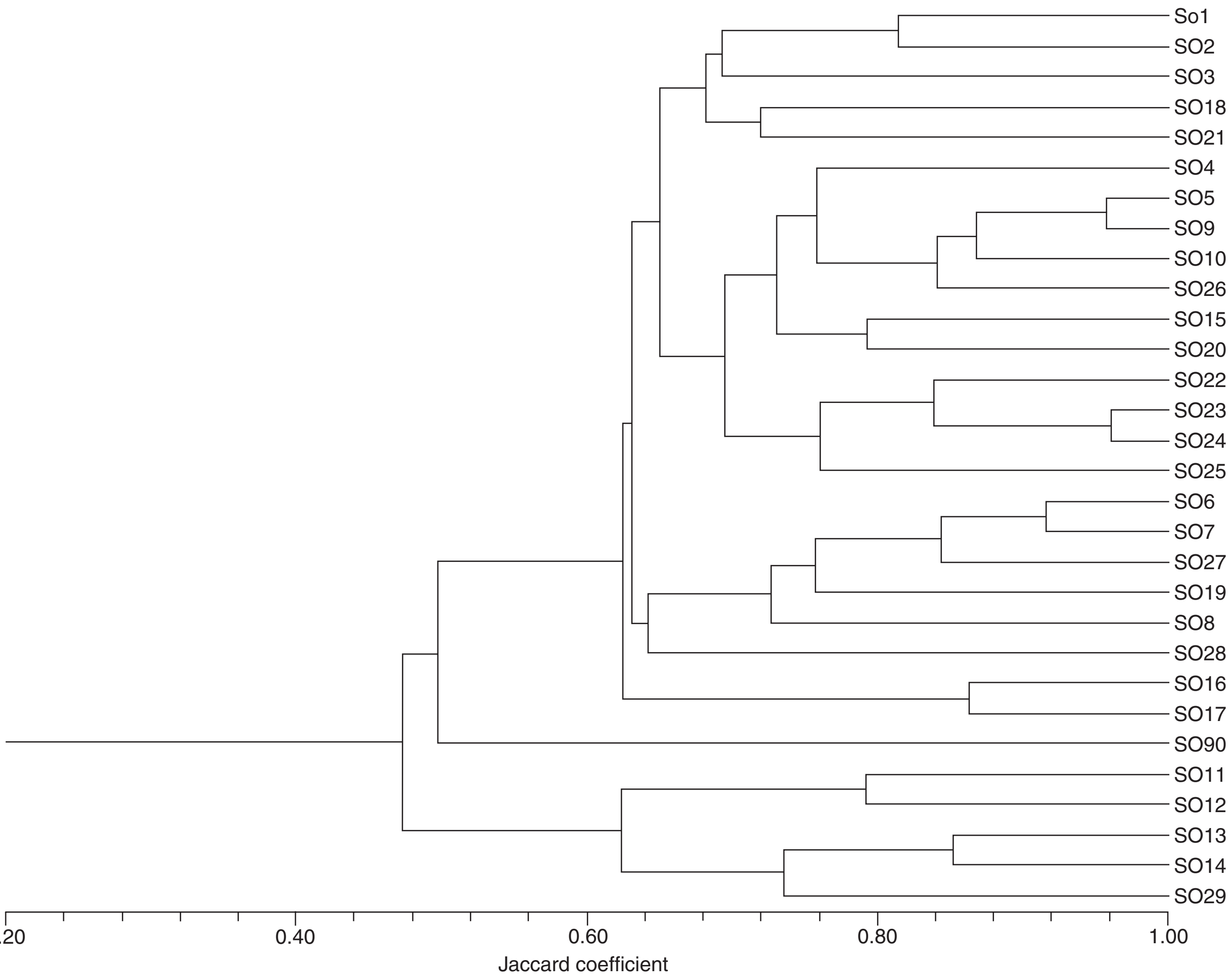

Fig 2 Dendrogram based on individual data analysis of ISSR Primers.

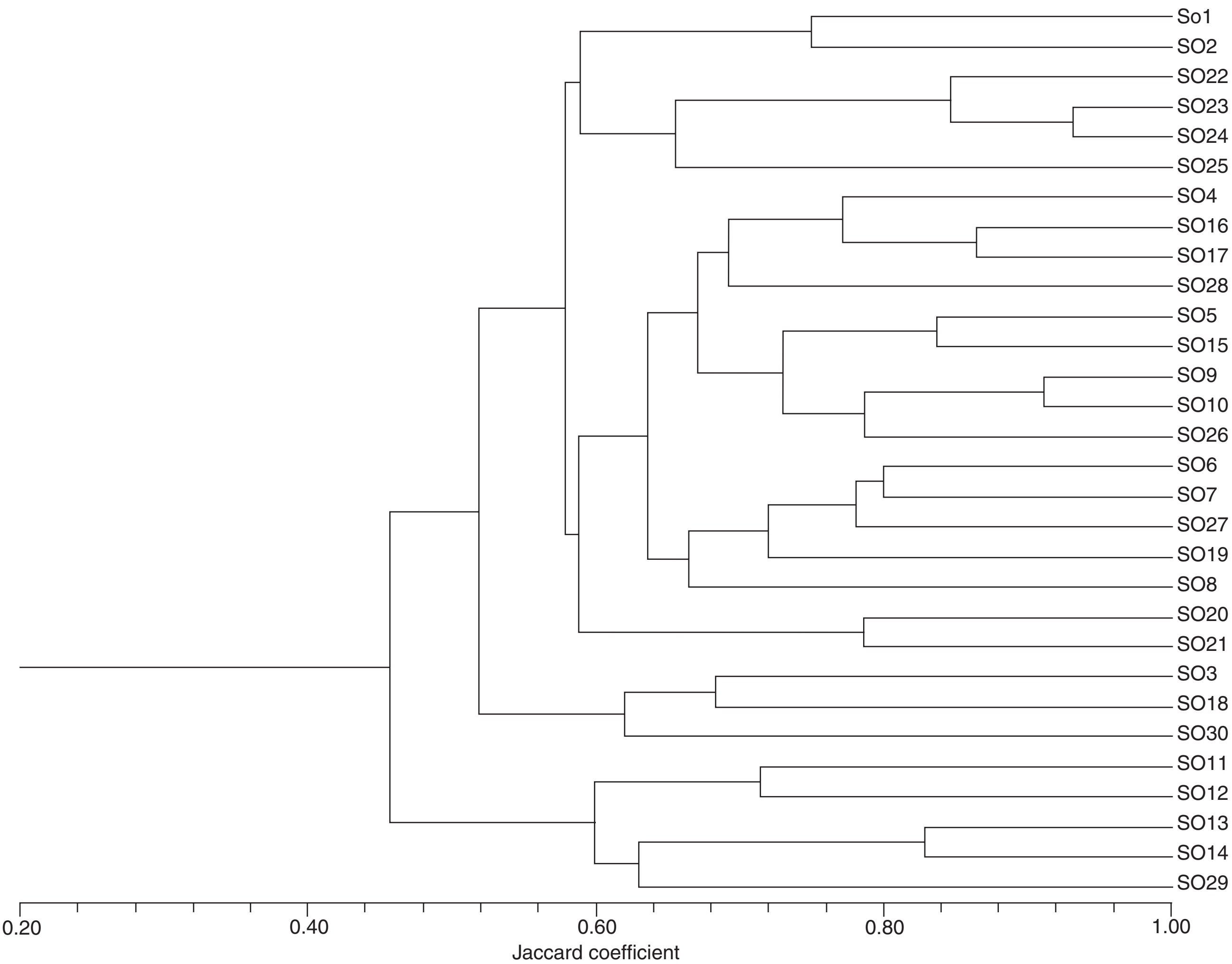

Fig 3 Dendrogram based on the combined data analysis of RAPD+ISSR primers.

(Fig 3) showed Jaccard's genetic similarity coefficient ranging from 29% to 93%. The Highest similarity (93%) seen between SO23 and SO24 which are collected from Jhansi, Uttar Pradesh and Khudwani, Jammu & Kashmir. Followed by 91% between SO9 (Chiplima, Odisha) and SO10 (Lonavala, Maharashtra).

In the present scenario, when we have the advantage of availability of techniques to study variability amongst fungi based on DNA polymorphism, it is imperative to employ such techniques for an important pathogen like *Sarocldium oryzae* which is becoming a threat to rice production. The RAPD technique has been used to detect genetic variation amongst isolates within a species (Cooke *et al.* 1996, Boyd and Carris 1997). To our knowledge no reports are available for variability studies of this pathogen by employing molecular techniques except one by Ayyadurai *et al.* (2005). They studied pathogenicity, phytotoxic metabolites and used Random Amplified Polymorphic DNA (RAPD) markers to assess the level of genetic variability of *S. oryzae* derived from the rice cultivars CR 1018, IR 36 and IR 50 of different locations in North East and South India. They reported that variability exists in pathogenicity, phytotoxic metabolite production and DNA polymorphisms amongst isolates of *S. oryzae.* Though there are no previous reports of similar nature against *Sarocladium oryzae,* similar findings have been reported in other fungi, *Ascochyta rabiei* by Kumar *et al.* (2005), *Bipolaris oryzae* causing brown spot in paddy by Kandan *et al.* (2014). They used three different set of primers, viz. RAPD, ISSR and URP and all gave polymorphism against *Bipolaris oryzae* establishing variability amongst various isolates. Similar findings have been observed in the present study also. As far as studies on variability of this pathogen based on molecular markers against isolates collected from different geographical locations covering major rice growing areas are concerned this is the first attempt made in India as the work carried out by Ayyudurai *et al.* (2005) was restricted on isolates of two specific regions and use of only RAPD primer was done and that too only two primers. This studies generated information regarding variability in *Sarocladium oryzae* with 30 isolates collected from different geographical locations covering major rice growing areas of the country using RAPD and ISSR primers. Thus, it is the first report of such nature in India as far as this pathogen is concerned. This is for the first time that clusters have been made for this pathogen and majority of isolates belonging to a particular region fall into the same clusters.

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