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MOLECULAR AND PATHOLOGICAL CHARACTERIZATION OF ISOLATES OF C*olletotrichum falcatum* WENT. CAUSING RED ROT IN SUGARCANE

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ABSTRACT

Red rot also known as cancer of sugarcane is caused by a pathogen *Colletotrichum falcatum* (Went.). The disease has a devastating effect as it hinders sugarcane cultivation worldwide. The major way to manage the disease is to develop the disease resistant varieties which require the study of genetic diversity of the pathogen. The present research aim was to study the pathogenic and molecular diversity among the isolates of red rot pathogen collected during surveys of major sugarcane growing areas of Punjab, Haryana and Uttar Pradesh states. Pathogenic assay of isolates on fourteen host differentials grouped them into eight groups on the basis of reaction expressed and virulence frequency. The molecular variation among the isolates using ISSR marker analysis showed high level of polymorphism, which confirmed the variation in virulence pattern of isolates. Polymorphism information content (PIC) and similarity coefficient values varied in the range of 0.227 to 0.321 and 0.29 to 0.93, respectively. The dendrogram grouped isolates in different clusters which reveal considerable level of molecular diversity among the isolates. However, the genetic diversity estimates based on ISSR markers did not show any correspondence with pathogenic variation of the isolates which could be due to same geographical conditions. Nucleotide sequences analysis of the ribosomal DNA (rDNA) genes using universal ITS primers showed the nucleotide variation in the isolates which could be due to base substitution at one to four sites which suggested its role in the origin of virulence and diversity in *Colletotrichum falcatum*. The presence of ITS variation in the virulent isolates suggested its role in the origin of virulence and diversity in *Colletotrichum falcatum.* But this needs further verification using proteomic approach for the identification of proteins expressed in relation to pathogenicity.

Keywords: *Colletotrichum falcatum*, ISSR, 5.8s-ITS, Polymorphism, Sugarcane

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Sugarcane (*Saccharum* sp. hybrid complex) is one of the essential commercial crops of India serving as a source of sugar and bioenergy. It is challenged by number of stresses, both biotic and abiotic. Sugarcane crop is infested by many diseases caused by number of bacteria, fungi, virus and phytoplasma that directly lowers the cane yield. Amongst the major fungal diseases of sugarcane, red rot disease (*Colletotrichum falcatum* (Went.)) poses serious threat to its cultivation (Alexander and Viswanathan, 1996; Beniwal *et al*., 1989; Duttamajumdar, 2008; Freeman, 1998; Patel *et al*., 2019) which causes up to 29.07% losses in weight of cane and 30.08% in sugar yield (Hussnain and Afghan, 2006).

Red rot accounts for major economic losses and several epidemics in both sub-tropical and tropical parts of India that have resulted in complete elimination of many commercial varieties (Lewin *et al*., 1976; Nair *et al*., 1984; Satyavir *et al*., 1984; 2001). The major strategy to manage the disease under endemic condition, is the development and use of resistant cultivars (Sengar *et al*., 2009). However, the major bottleneck is the adaptive changes of the pathogen in relation to the host varieties that alters the virulence pattern of the fungus (Abbas *et al*., 2010; Duttamajumdar, 2008; Singh *et al*., 2012; Singh and Lal, 1999).

There are many reports related to variability study of the pathogen on the basis of source of collection and reaction on host differentials. Many workers earlier classified its isolates/races on the basis of their cultural/morphological studies which are time consuming and expertise specific. Thereafter, the study on differential hosts was found as a possible option for variability studies (Beniwal *et al*., 1989). However, pathogenic variability studies are also time consuming, expertise specific and virulence assay sometime leads to disease escape.

Earlier various DNA-based methods have been tested for characterization of number of *Colletotrichum* species (Crouch *et al*., 2008; Guerber *et al*., 2003; Latha *et al*., 2003; Padder *et al*., 2007). There are reports of genetic studies on many species of *Colletotrichum*, but very little on *Colletotrichum falcatum*. There are some reports on variability studies of *Colletotrichum falcatum* isolates using cultural/morphological characters, response on host differentials, by using arbitrary markers like Random Amplified Polymorphic DNA (RAPD; Madan *et al*., 2000; Suman *et al*., 2005), Inter

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Simple Sequence Repeat (ISSR) and Universal Rice Primers (URPs; Kumar *et al*., 2010) but the complete genetic basis has not defined so far. The proposed research aims at to investigate the pathological and molecular variability of *Colletotrichum falcatum* isolates in sub-tropical region of India.

MATERIALS AND METHODS

Collection, isolation and maintenance of isolates

Fifty seven isolates were isolated from infected samples of red rot collected from different varieties/ clones grown in sub-tropical regions of India. Single spore isolations were made from the cultures and stored at 4±1ºC for subsequent use (Kaur *et al*., 2014).

Pathogenicity sssay

Fourteen sugarcane differentials namely Baragua (*S. officinarum*), Khakai (*S. sinense*), SES 594 (*S. spontaneum*), CoS 767, Co 975, BO 91, CoC 671, Co 7717, Co 997, CoJ 64, Co 1148, Co 419, Co 62399 and CoS 8436 were planted in the field area as per recommended practices to study pathogenic variability in isolates under field conditions. Twenty canes of each differential were inoculated by plug method by all the isolates. Disease data were recorded after 60 days of inoculation (Anonymous, 2018; Srinivasan and Bhatt, 1961)

Molecular characterization

The fungal cultures of all the isolates were allowed to grow on oat meal broth for seven days at a temperature of 25±1 °C. Total genomic DNA of individual isolate was extracted using modified method of CTAB (Cetyl trimethyl bromide; Saghai-Maroof *et al*., 1984) and purified by 1 µl RNase (50 µg/ml). Electrophoresis was carried out in agarose gel (0.8%) to assess quantity and quality of DNA.

PCR amplification for ISSR

The polymorphism among the collected isolates of red rot was studied using eight ISSR primers (Arade *et al*., 2014; Gupta *et al*., 1994; Kumar *et al*., 2010). For amplification, 20 µl of reaction volume was prepared, consisting of 4 µl 10x Taq buffer, 1.2 µl 25 mM $MgCl_2$, 3 µl 1 mM dNTPs mix, 3 µl 10 µM primer, 2.0 µl DNA (20 ng/ µl), 0.2 µl 5U/µl Taq polymerase (Merck Genei) and final volume was adjusted with nuclease free water. Amplification was initiated by denaturation for 5 min at 94°C, then 35 cycles of 1 min denaturation at 94°C, primer specific annealing temperature i.e. at 43.4°C for primers $(GACA)_4$, $(ACTG)_4$ and $(TGTC)_4$, 44.7°C for primers $(GACAC)_3$, $(GT)_8T$ and $(AC)_8T$ and 47.4°C for $(CAG)_5$ and $(CAC)_5$ for 1 min and extension for 1 min at 72°C followed, lastly final extension was carried out at 72°C for 10 min.

Electrophoresis was carried out on agarose gel (1.5 %) prepared in 0.5X TBE buffer for 2-3 h and separated bands were visualized and photographed under gel documentation system (UVP Transillumination).

PCR amplification for Internal Transcribed Spacer Analysis (ITS)

Universal ITS primers were used for the amplification of regions ITS-1 and ITS-2 and the 5.8S ribosomal DNA i.e. a complete rDNA region. PCR reaction mixture of 4 µl 10x Taq buffer, 1.2 µl 25 mM $MgCl_2$, 3 µl 1 mM dNTPs mix, 1.5 µl 10 µM each of the conserved ITS-4 (5'-TCCTCCGCTTATTGATATGC- 3') and ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG -3') primers, 2.0 µl DNA (20 ng/ µl), 0.2 µl 5U/µl Taq polymerase was prepared and nuclease free water was used to make final volume of 20 µl. Amplification was initiated with profile of denaturation of 94°C for 5 min, then 35 cycles of 1 min at 94°C, 1 min annealing at 48°C for 40 sec and 1 min extension at 72°C followed, final extension was carried out for 10 min at 72°C. Electrophoretic separation was carried out on 1.0 per cent agarose gel and photographed under gel documentation system (UVP Transillumination). Desired products of expected size of 590 base pair (bp) were eluted and sequenced.

Data analysis

Banding profile obtained during amplification using ISSR primers were scored according to presence (1) or absence (0) of bands. Relative mobility of a resulted band was taken as the identification criteria for comparison. Polymorphic Information Content (PIC) value was computed using equation; PIC = 1− [f 2 + (1− f)2], where 'f' is the marker frequency in the data set. The statistical data analysis was performed using NTSYSpc software (version 2.02e; Rohlf 1998) by using Jaccard's coefficient. Similarity analysis was done taking Dice coefficient as a base using SIMQUAL (Similarity for Qualitative Data Programme) module (Sneath and Sokal, 1973). The dendrogram, was constructed using SAHN (Sequential Agglomerative Heirarchial and Nested) module based on UPGMA (Unweighed pair group method with arithmetic mean).

The multiple sequence analysis was initially carried out by ClustalW and phylogenetic tree was generated using bootstrapped (1000 replicates) by Molecular Evolutionary Genetic Analysis (MEGA, Kumar et al. 2018) software employing Neighbor-joining method.

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RESULTS AND DISCUSSION

Pathogenicity assay

Pathogenic variability of 57 collected isolates on the 14 host differentials clearly demonstrated, that no isolate exhibited resistant (R) behavior on all the 14 differentials (Table 1). Based on the disease severity, isolates RI-17, RI-19, RI-22 and RI-23 were found to be the most virulent (71.43% virulence frequency). On the basis of reaction expressed and virulence frequency of 57 isolates, isolates can be grouped into eight groups (Table 2). Isolates of Group 1 were highly virulent whereas the isolates of Group 8 were least virulent which produced susceptible reaction on three differentials only (Table 2).

Variation in red rot pathogen is known since 1940s and study of the pathogenic variability based on virulent pattern has also been reported by earlier workers who used different sets of differentials using plug and nodal methods of inoculation (Kaur *et al*., 2014; Padmanaban *et al*., 1996). Our results relate to the findings of Prakasam *et al*., (2000) who reported identification of five races of the pathogen based on host differential studies.

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Molecular characterization of the Red Rot pathogen

Inter-Simple Sequence Repeats (ISSR) analysis

Eight primers were tested for screening of *Colletotrichum falcatum* isolates to find out molecular variation among *Colletotrichum falcatum* isolates collected from different geographical locations. Out of these, only two (UBC-825: 5'-ACACACACACACACACT-3' and UBC-873: 5'-GACAGACAGACAGACA-3') gave reproducible results and were subsequently used (Fig. 1). These two primers amplified 14 scorable bands of which only 10 bands were polymorphic with 50.0 to 87.5 per cent of polymorphism. The two primers under study showed polymorphism information content (PIC) value of 0.227 to 0.321. PIC value determination for dominant markers (such as ISSR) should be between 0 and 0.5 (Bolaric *et al*., 2005; De Riek *et al*., 2001) while for codominant markers it should give a PIC between 0 and 1. The combined data of 14 fragments generated by the ISSR primers were used for the analysis of genetic diversity using the NTSYSpc software version 1.8. The similarity coefficient estimates by Jaccard's coefficient of similarity were found to be 0.29 to 0.93. Based on molecular data, it was observed that isolates RI-1, RI-8, RI-9, RI-16, RI-19 and RI-20 from Punjab, RI-24, RI-25, RI-26, RI-34, RI-41 and RI-42 from UP and RI-51 from Haryana were found to be most divergent while isolates RI-2, RI-3, RI-14, RI-15 from Punjab and RI-47, RI-48 and RI-49 from Haryana were found to be closely related to each other and other isolates with high similarity coefficient.

Cluster analysis using UPGMA was carried out to construct a dendrogram on the basis of the genetic distance matrix (Fig. 2). The dendogram clearly placed the 12 isolates in one cluster and 45 isolates in another big cluster. The cluster I comprised of isolates RI-1, RI-8, RI-9, RI-16, RI-19, RI-20, RI-24, RI-25, RI-26, RI-34, RI-41 and RI-42. The isolates of cluster I were further categorized into sub cluster A and sub cluster B. The sub cluster A contains seven isolates while sub cluster B contains five isolates. The cluster II comprised of 45 isolates which were similarly sub-divided into two sub clusters (Fig. 2).

The grouping of the Punjab, Haryana and Uttar Pradesh isolates together in the same clusters, suggests the underlying genetic similarity among the isolates across geographic limits. The genetic diversity estimates based on ISSR markers lacks any correlation with pathogenic variation of the isolates. The findings of this study were in agreement with the finding of Ratanacherdchai *et al*. (2010) and Sharma *et al*. (2005) on *C. capsici* isolates and Kaur *et al*. (2014) on *Colletotrichum falcatum* isolates. In our present study, isolates showing similar virulence pattern on host differential showed dissimilarity during genetic study. Furthermore, genetic diversity based on ISSR was somewhat correlated with geographic distribution in accordance with results of study conducted by Ratanacherdchai *et al*. (2010). During cluster analysis, dendrogram clearly placed some isolates of only Punjab and Uttar Pradesh in Cluster 1 whereas all the isolates collected from Haryana were placed in Cluster 2. Though isolates collected from Punjab were also placed in cluster 2 along with Haryana isolates which were further sub-grouped. This may be due to similar climatic conditions of the isolates collection areas. The study clearly authenticates the application of ISSR markers as a tool for genetic diversity studies in *Colletotrichum* spp.

Internal Transcribed Spacer (ITS) analysis

Universal ITS primers (ITS-4 and ITS-5) in all the isolates generated the ITS region of 590 nucleotides as expected, which include the regions ITS-1 and ITS-2 and the intervening 5.8S ribosomal DNA gene. The amplification products were sequenced. The sequences were aligned for comparison among themselves and with the available sequences of *Colletotrichum falcatum* in the NCBI GenBank database using multiple sequence alignment software ClustalW.

Multiple sequence alignment showed 96-100 per cent similarity among the red rot isolates. Mostly isolates showed complete homology of the ITS region

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Table 1. Expression and virulence frequency of red rot isolates on sugarcane differentials by plug method of inoculation (after 60 days)

Sr. No.	Isolate	Source	Reaction on host differentials														Virulence frequency
			Co 419	Co 975	Co 997	Co 1148	Co 7717	Co 62399	CoC 671	CoJ 64	CoS 767	CoS 8436	BO 91	Bara-gua	Khakai	SES 594	
Punjab																	
1	RI- 1	CoPb 91	S	S	S	X	S	S	S	S	R	R	R	R	S	R	57.14
2	RI-2	CoJ 88	X	S	S	S	S	S	S	S	R	R	R	R	S	R	57.14
3	RI-3	CoPb 13183	S	S	S	S	S	S	S	S	R	R	R	R	S	R	64.29
4	RI-4	CoPb 91	S	S	S	X	S	S	S	S	R	R	R	R	S	R	57.14
5	RI-5	CoJ 85	S	S	S	S	S	S	S	S	R	R	R	R	S	R	64.29
6	RI-6	CoJ 85	S	S	S	S	S	S	S	S	R	R	R	R	S	R	64.29
7	RI-7	CoJ 85	S	S	X	S	S	S	S	S	R	R	R	R	S	R	57.14
8	RI-8	CoS 8436	S	S	S	R	S	S	S	S	R	S	R	R	S	R	64.28
9	RI-9	CoJ 64	S	X	S	S	S	S	S	S	R	R	R	R	S	R	57.14
10	RI-10	Co 89003	R	X	S	X	R	S	S	S	R	S	R	R	S	R	42.86
11	RI-11	CoJ 88	X	S	S	S	S	S	S	S	R	R	R	R	S	R	57.14
12	RI-12	Co 89003	X	X	S	S	S	R	S	S	R	R	R	R	S	R	42.86
13	RI-13	CoJ 64	S	S	S	S	S	S	S	S	R	R	R	R	S	R	64.29
14	RI-14	CoJ 85	S	S	S	S	S	S	S	S	R	R	R	R	S	R	64.29
15	RI-15	CoJ 83	S	S	S	S	S	S	S	S	R	R	R	R	S	R	64.29
16	RI-16	CoPb 91	S	S	S	X	S	S	S	S	R	R	R	R	S	R	57.14
17	RI-17	CoJ 64	S	S	S	S	S	S	S	S	S	R	R	R	S	R	71.43
18	RI-18	CoJ 88	X	S	S	S	S	S	S	S	S	R	R	R	S	R	64.29
19	RI-19	CoJ 85	S	S	S	S	S	S	S	S	S	R	R	R	S	R	71.43
20	RI-20	CoPb 91	S	S	S	X	S	S	S	S	S	R	R	R	S	R	64.29
21	RI-21	Co 89003	X	X	S	X	R	S	S	S	S	R	R	R	S	R	42.86
22	RI-22	Co 89003	S	S	S	S	S	S	S	S	S	R	R	R	S	R	71.43
23	RI-23	Sel. K 2/3	S	S	S	S	S	S	S	S	S	R	R	R	S	R	71.43
Uttar Pradesh																	
24	RI-24	CoS 8436	S	R	R	R	S	X	S	S	R	R	S	R	S	R	42.86
25	RI-25	CoS 8436	S	R	R	R	S	R	S	S	R	R	X	S	S	R	42.86
26	RI-26	CoS 8436	S	R	R	R	R	S	S	S	R	R	S	R	S	R	42.86
27	RI-27	CoS 92423	S	R	R	R	R	R	S	S	R	R	X	R	S	R	28.57
28	RI-28	CoS 92423	S	R	R	R	R	S	S	S	R	R	R	R	S	R	35.71
29	RI-29	CoS 92423	S	R	R	R	X	S	S	S	R	R	R	R	S	R	35.71
30	RI-30	CoS 91269	S	R	R	R	S	X	S	R	R	R	R	R	S	R	28.57
31	RI-31	CoJ 85	S	X	R	S	S	S	S	S	R	R	R	R	S	R	50.00
32	RI-32	CoJ 85	X	R	R	X	R	S	X	S	R	R	R	R	S	R	21.43
33	RI-33	CoLk 8102	S	R	X	R	S	S	S	X	R	R	R	R	X	R	28.57
34	RI-34	CoLk 8102	S	R	X	R	S	S	S	X	R	R	R	R	X	R	28.57
35	RI-35	CoLk 8102	S	R	X	R	S	S	S	S	R	R	R	R	X	R	35.71
36	RI-36	CoLk 8102	S	R	X	R	S	S	S	X	R	R	R	R	X	R	28.57
37	RI-37	CoLk 8102	X	R	X	R	S	S	S	S	R	R	R	R	X	R	28.57
38	RI-38	CoLk 8102	S	X	X	R	S	S	S	S	R	R	R	R	S	R	42.86
39	RI-39	CoLk 94184	X	R	R	R	R	S	X	S	R	R	R	R	S	R	21.43
40	RI-40	CoLk 94184	S	R	R	R	R	R	R	S	R	R	R	R	S	R	21.43
41	RI-41	CoLk 94184	S	R	R	R	R	R	R	S	R	R	R	R	S	R	21.43
42	RI-42	CoLk 94184	X	R	R	R	R	R	R	S	R	R	R	R	S	R	21.43
Haryana																	
43	RI-43	CoSe 95422	S	R	S	R	R	S	S	X	R	R	R	R	R	R	28.57
44	RI-44	CoS 8436	S	S	S	R	S	S	S	S	R	S	R	R	S	R	64.29
45	RI-45	CoS 8436	S	R	S	S	R	S	S	S	R	R	R	R	S	R	50.00
46	RI-46	CoS 8436	X	S	R	R	R	S	S	R	R	R	R	R	X	R	21.43
47	RI-47	CoS 8436	S	X	S	X	X	S	S	R	R	R	R	R	X	R	28.57
48	RI-48	CoS 8436	S	R	S	R	R	S	X	R	R	R	R	R	R	R	21.43
49	RI-49	CoS 8436	S	S	S	S	S	S	S	X	R	R	R	R	S	R	57.14
50	RI-50	CoJ 64	S	R	S	R	X	S	S	S	R	S	R	R	S	R	50.00
51	RI-51	CoJ 64	X	R	S	X	R	X	S	S	R	R	X	R	S	R	28.57
52	RI-52	CoJ 64	R	R	X	R	R	R	S	S	R	R	R	R	S	R	21.43
53	RI-53	CoJ 64	S	R	R	R	R	R	S	S	R	R	R	R	R	R	21.43
54	RI-54	CoJ 64	S	R	R	X	R	X	S	S	R	R	R	R	R	R	21.43
55	RI-55	CoJ 64	S	R	X	R	R	R	S	S	R	R	R	R	S	R	28.57
56	RI-56	CoJ 64	S	R	R	R	R	R	S	S	R	R	R	R	R	R	21.43
57	RI-57	Co 89003	S	R	S	S	S	S	S	S	R	R	X	R	S	R	57.14

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Table 2. Pathogenic groups of various isolates of *Colletotrichum falcatum* formed on the basis of virulence

Group No.	Isolates
Group I	RI-17, RI-19, RI-22 and RI-23
Group II	RI-3, RI-5, RI-6, RI-8, RI-13, RI-14, RI-15, RI-18, RI-20 and RI-44
Group III	RI-1, RI-2, RI-4, RI-7, RI-9, RI-11, RI-16, RI-49 and RI-57
Group IV	RI-31, RI-45 and RI-50
Group V	RI-21, RI-24, RI-25, RI-26 and RI-38
Group VI	RI-28, RI-29 and RI-35
Group VII	RI-27, RI-30, RI-33, RI-34, RI-36, RI-37, RI-43, RI-47, RI-51 and RI-55
Group VIII	RI-32, RI-39, RI-40, RI-41, 42, RI-46, RI-48, RI-52, RI-53, RI-54 and RI-56

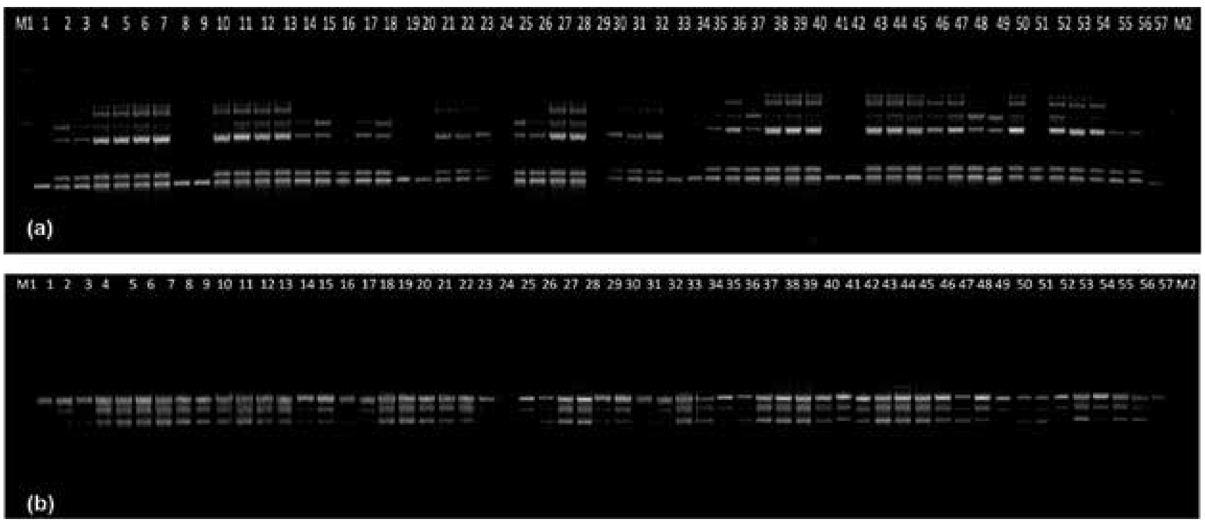

Fig. 1. Isolates of *Colletotrichum falcatum* showing amplification with primers UBC-825 (a) and UBC-873 (b)

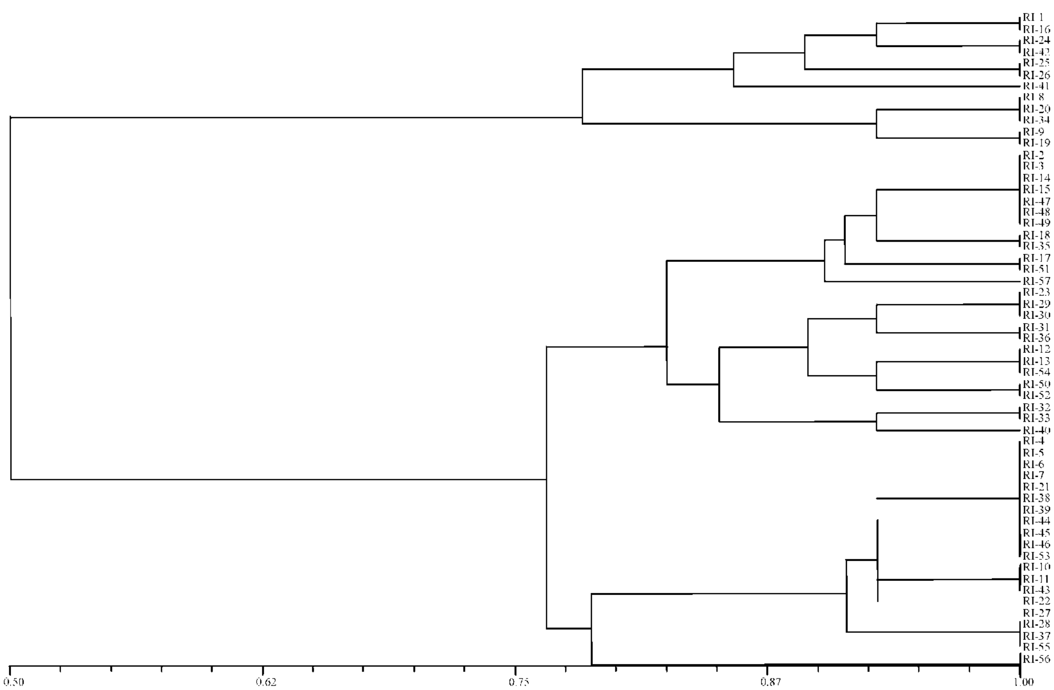

Fig. 2. UPGMA based dendrogram showing molecular diversity among different isolates of *Colletotrichum falcatum*

Fig. 3. Phylogenetic tree showing intra-species diversity among different isolates of *Colletotrichum falcatum* based on ITS sequence analysis using MEGA software

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and the variations observed in the isolates were within the ITS-1 region. The ITS-1 region shows a greater degree of nucleotide variation in *Colletotrichum* species (Martinez-Culebrass *et al.*, 2003).

The phylogenetic analysis characterized the isolates into three groups (Fig. 3). Group I constituted 45 isolates, which were further clustered into sub-groups. Group II comprised of twelve isolates (RI-2, RI-4, RI-7, RI-14, RI-18, RI-19, RI-23, RI-28, RI-44, RI-51, RI-55 and RI-56). The GenBank isolate (AJ536231) was grouped separately in 3rd cluster in the phylogenetic tree (Fig. 3).

Although ITS sequence analysis grouped isolates into separate clusters, the nucleotide variation was least. The variation between the isolates and their grouping into separate groups could be due to base substitution at one to four sites. This is supported by the study conducted by Malathi *et al*. (2011), who characterized the isolates of *Colletotrichum falcatum* into separate groups based on one bp difference between the isolates.

It can be concluded from the present study that molecular characterizations using ISSR and ITS markers are useful methods for rapid recognition of genetic diversity in *Colletotrichum* spp. along with pathogenic variability studies. However, ISSR markers study didn't show any correlation with pathogenic grouping of isolates using host differentials and similar geographical conditions could be the possible cause. The nucleotide variation in the ITS region of the isolates suggested its role in genetic variation in the isolates. But this needs further verification using further proteomic approaches.

Authors' contribution

Conceptualization of research work and designing of experiments (A, RK); Execution of field/lab experiments and data collection (A, LK, RK); Analysis of data and interpretation (A, LK, PS); Preparation of manuscript (A, LK, RK)

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