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RESEARCH ARTICLE

Genetic diversity of *Fusarium oxysporum* f. sp. *lentis* populations causing wilt of lentil in India

Neelakanth S. Hiremani[1,2] · Sunil C. Dubey[1,3]

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Abstract

Fusarium oxysporum is associated with vascular wilt disease in most of the crops. Identification of *Fusarium* spp. by morphological characters are highly variable, thus DNA-based techniques such as RAPD and SSR markers are widely used in the detection and genetic diversity analysis. In this study, fifty *F. oxysporum* f. sp. *lentis* (*Fol*) isolates from 7 different lentil cultivating Indian states were analyzed for the genetic diversity by using 14 RAPD and 9 SSR primers. Results revealed the existence of high genetic variability among the isolates. A total of 125 reproducible bands were amplified by RAPD primers, out of which 121 were polymorphic (96.8%) and only four were monomorphic (3.2%). Primers OPA 12, OPC 14, OPF 8, OPM 12 and OPY 20 showed the highest polymorphism. UPGMA analysis differentiated the isolates into six clusters at 25% similarity coefficient. Nine SSR primers produced 59 bands with 96.6% polymorphism. The primers MB 2, MB 9 and MB 11 were highly informative and showed maximum polymorphism (100%). UPGMA analysis at 30% genetic similarity grouped the isolates into six clusters. It was evident that the isolates belonging to different locations were grouped into different clusters representing different races. However, the analysis partially corresponded to the races of *Fol* and the lentil growing regions of India. Nevertheless, this information is highly useful for the breeders to plan the breeding strategies to develop resistant cultivars against *Fol* looking into existence of races in a particular region and considering the diversity among them.

Keywords Genetic diversity · RAPD · SSR · *Fusarium oxysporum* f. sp. *Lentis* · Lentil wilt

Introduction

Fusarium oxysporum incites vascular wilt syndrome in many of the crop plants and '*formae specialis*' are specific strains infecting only a limited number of host plants as *Fusarium oxysporum* Schlecht. Emend Snyder and Hansen f. sp. *lentis* Vasudeva and Srinivasan (*Fol*) infecting lentil (*Lens culinaris* (L.) Medik). Lentil is one of the important legume crops in the farming and food systems of many countries worldwide. In India, lentil wilt is a major disease in almost all the lentil growing states including Uttar Pradesh, Madhya Pradesh, Bihar, West Bengal and 25 to 95% infection has been reported in some of the fields (Khare 1980; Agrawal et al. 1991). Morphological identification of *Fusarium* spp. by characters like conidial size, shape and pigmentation is variable as these characters may be influenced by nutritional composition of the medium and cultural conditions (Datta et al. 2011). However, DNA-based techniques have become the tool of choice in studying the genetic variability (O'Donnell 2000). For instance, RAPD and SSR markers are widely used in the detection and genetic characterization of many phytopathogenic fungi, particularly race differentiation in the *formae speciales* of *F. oxysporum* (Baayen et al. 1997; Sivaramakrishnan et al. 2002; Castano et al. 2014). According to Bayraktar (2010), RAPD-PCR proved suitable marker system to analyze the genetic variability among the *F. oxysporum* f. sp. *cepae* populations. A total 18 representatives of *F. oxysporum formae speciales* as well as sequences from *GenBank* representing the three phylogenetic clades were used for comparative analysis. The 18 *formae speciales* included did not separate according to host with any of the DNA-based techniques used (Bogale et al. 2006). *Fol* exhibits high genetic diversity (Mohammadi et al. 2011;

✉ Sunil C. Dubey
scdube2002@yahoo.co.in; sunil.dubey@icar.gov.in

[1] Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India

[2] Present Address: Division of Crop Protection, ICAR-CICR, Nagpur 440010, India

[3] Present Address: Division of Plant Quarantine, ICAR-NBPGR, New Delhi 110012, India

Al-Husien et al. 2017) and races/ pathotypes have been recognized (Pouralibaba et al. 2016; Hiremani and Dubey 2018). Therefore, the aim of this study was to know the relationship among the Indian population of *Fol* and to test whether the races identified in our earlier study (Hiremani and Dubey 2018) correspond to the genetic groups through RAPD and microsatellite marker analysis.

Materials and methods

Fungal cultures and production of mycelium

A total of 50 *Fusarium oxysporum* f. sp. *lentis* (*Fol*) isolates from 7 major states of India where lentil crop is being cultivated were used in the study (Table 1). The isolates those being maintained in Division of Plant Pathology (Pulse Pathology laboratory), IARI, New Delhi; RAK College, Sehore, Madhya Pradesh and IIPR, Kanpur, Uttar Pradesh, India were taken for the present study. The cultures were also isolated from the plants of lentil collected from fields showing wilt symptoms. The cultures (purified with single spore) of *F. oxysporum* f. sp. *lentis* isolates were grown in potato dextrose broth (100 ml) at 25 ± 1°C and 120 rpm in a shaking incubator for 7 days. After the incubation, the mycelium was harvested by filtering through a sterile Whatman® No. 1 filter paper, washed thoroughly with sterile distilled water thrice and blotted dry on a blotting paper. The mycelial mat was used for DNA extraction.

Genomic DNA extraction

The genomic DNA was extracted from the mycelium by CTAB method with slight modification of the protocol given by Murray and Thompson (1980). Briefly, about 1 g of mycelial mat was ground to a fine powder and transferred into Oakridge tubes containing 10 ml preheated CTAB extraction buffer (1M Tris–HCl, pH 8.0; 5M NaCl; 0.5M EDTA, pH 8.0 and 2% CTAB). The contents were gently mixed and incubated at 65°C for an hour followed by addition of equal volume phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 11,000 rpm for 10 min. This step was repeated after discarding the debris and centrifuged at 10,000 rpm for 5 min. Upper aqueous solution was transferred to another tube and precipitated with chilled 0.6 volume of isopropanol and 0.1 volume of sodium acetate. Post incubation, it was centrifuged at 12,000 rpm for 10 min and the pellet obtained was washed twice with 70% ethanol after discarding the supernatant. The extracted DNA was purified by RNase treatment (@ 1µl/100µl), then dissolved in Tris–EDTA (TE) buffer and kept at − 20°C for further use.

Table 1 Isolates of *Fusarium oxysporum* f. sp. *lentis* representing major lentil growing areas of India and used for genetic diversity analysis

State	District	Place	Accession no.
Uttar Pradesh	Karwi	Shivrampur	FLS 2
	Budaun	Dhanari	FLS 4
	Hamirpur	Muskara	FLS 5
	Lucknow	Rahmatnagar	FLS 8
	Gazipur	Shadi Badi	FLS 10
	Basti	Saoonghat	FLS 11
	Lalitpur	Lalitpur	FLS 12
	Lalitpur	Jakhaura	FLS 14
	Jhansi	Bamaur	FLS 15
	Varanasi	Chaubeypur	FLS 16
	Chandauli	Chandauli	FLS 17
	Bareilly	Bhagwanpur	FLS 19
	Jalaun	Orai	FLS 20
Bihar	Muzaffarpur	Muraul	FLS 22
	Khagaria	Bardaya	FLS 23
	Purnia	Bhatgaon	FLS 24
	Nawada	Nawada	FLS 25
	Samastipur	PUSA	FLS 26
	Patna	Mokama	FLS 27
Madhya Pradesh	Sehore	Sehore	FLS 29
	Hoshangabad	Rohana	FLS 30
	Damoh	Jabera	FLS 31
	Panna	Simariya	FLS 32
	Sagar	Sagar	FLS 34
	Rewa	Rewa	FLS 35
	Datia	Padokhar	FLS 36
	Bhopal	Jhikaria	FLS 37
	Dewas	Berakhedi	FLS 39
	Betul	Multai	FLS 40
	Damoh	Singrampur	FLS 43
	Burhanpur	Shahpur	FLS 47
Jharkhand	Hazaribagh	Hazaribagh	FLS 51
	Chatra	Chatra	FLS 52
	Giridih	Dumri	FLS 53
	Giridih	Bagodra	FLS 54
	Palamu	Daltonganj	FLS 56
	Ranchi	Ranchi	FLS 58
Chhattisgarh	Raigarh	Jamgaon	FLS 61
	Baloda Bazar	Bhatapara	FLS 62
	Raipur	Sankara	FLS 63
	Raigarh	Jamgaon	FLS 64
	Durg	Jeora	FLS 65
	Kabirdham	Kawardha	FLS 66

Table 1 (continued)

State	District	Place	Accession no.
Rajasthan	Nagaur	Mayapur	FLS 67
	Dholpur	Dholpur	FLS 69
	Bundi	Jarkhoda	FLS 70
	Nagaur	Dabra	FLS 71
	Jaipur	Durgapura	FLS 72
	Jaipur	Durgapura	FLS 74
Delhi	New Delhi	IARI	FLS 75

Table 2 Analysis of polymorphism obtained with random amplified polymorphic DNA primers in isolates of *Fusarium oxysporum* f. sp. *lentis*

Primer	Sequence (5′-3′)	Total number of bands	Polymorphism (%)	Size range of amplicons (kb)
OPA 3	AGTCAGCCAC	10	100.0	0.5–2.0
OPA 11	CAATCGCCGT	7	71.4	0.5–2.5
OPA 12	TCGGCGATAG	9	100.0	0.3–2.5
OPC 14	TGCGTGCTTG	12	100.0	0.3–2.5
OPD 4	TCTGGTGAGG	8	100.0	0.4–2.5
OPD 5	TGAGCGGACA	9	100.0	0.25–3.0
OPE 7	AGATGCAGCC	7	100.0	0.6–2.5
OPF 1	ACGGATCCTG	9	88.9	0.35–3.0
OPF 8	GGGATATCGG	11	100.0	0.25–2.5
OPF 12	ACGGTACCAG	6	100.0	0.5–2.0
OPF 16	GGAGTACTGG	10	90.0	0.4–3.0
OPM 12	GGGACGTTGG	9	100.0	0.45–2.0
OPY 10	CAAACGTGGG	7	100.0	0.3–3.0
OPY 20	AGCCGTGGAA	11	100.0	0.4–3.0
Total		129	–	–
Mean		–	96.8	–

Randomly amplified polymorphic DNA (RAPD) analysis

Various PCR protocols were evaluated for the amplification and the optimized reaction mixture and thermal cycler conditions were used for analyzing the genetic variability among the 50 isolates of *Fol* by using RAPD method (Williams et al. 1990) with 14 different 10-mer arbitrary oligonucleotide primers (Table 2). The RAPD-PCR reaction mixture (25 µl) consisted of template DNA (50 ng), *Taq* polymerase (1.5 U) from Bangalore Genei, India, $MgCl_2$ (3.5 mM), each dNTP (0.6 mM) and primer (10 pmol) in 1× *Taq* buffer. The PCR was conducted with reaction conditions as initial denaturation for 5 min at 94°C followed by 40 cycles each of denaturation for 1 min at 94°C, annealing for 1 min at 35°C and extension for 2 min at 72°C with a final elongation for 5 min at 72°C. The PCR products were electrophoresed on agarose gel (1.2%) in TAE buffer (1×) at 70 V for 40 min. A DNA ladder of 1 kb was used as a marker. Each primer was used two times for amplification before final scoring and the amplifications which were reproducible and scorable utilized in the analysis.

Simple sequence repeats (SSR) analysis

The SSR primers used for of *F. oxysporum* species complex (Bogale et al. 2005) were screened against the isolates (50) of *Fol*. The reaction mixture was optimized for all the primers as described earlier but the annealing temperature for each primer was standardized by gradient PCR analysis. Nine SSR primers (Table 3) were selected and were synthesized from Eurofins Genomics India Pvt. Ltd., Bangalore, India. Briefly, the reaction mixture (25µl) contained template DNA (50 ng), *Taq* polymerase (1.5 U), $MgCl_2$ (1.5 mM), each dNTP (0.4 mM) and each of forward and reverse primer (15 pmol) in *Taq* buffer (1x) from Bangalore Genei, India. The PCR was performed as 94°C for 5 min initial denaturation followed by 40 cycles each of denaturation at 94°C for 2 min, annealing at respective temperature for each primer (Table 3) for 2 min and extension at 72°C for 2 min with a final elongation for 5 min at 72°C. Post PCR protocol is same as for RAPD.

Scoring and data analysis

The reproducible and scorable amplifications obtained by the primers were used to analyze genetic variability existing in the selected pathogenic *Fol* isolates. The scoring was done on the basis of absence (0) or presence (1) of each band for all the isolates in each primer. The matrix for data created was used for analysis of Jaccard's similarity coefficient. UPGMA (Unweighted paired group method with arithmetic average) cluster analysis was performed using similarity coefficients and dendrogram was prepared by NTSYS pc using SAHN clustering program (Rohlf 1998).

Results and discussion

The diversity analysis of 50 *Fol* isolates using 14 RAPD primers (Table 2) revealed the existence of high genetic variability among the isolates. A total of 125 reproducible bands were amplified by the primers, out of which 121 were (96.8%) polymorphic and only four (3.2%) were monomorphic. The number of prominent DNA fragments varied from 6 to 12 with an average of 8.9 bands per primer and size between 0.25 and 3.0 kb. Primers OPA 12, OPC 14, OPF 8 (Fig. 1), OPM 12 and OPY 20 were highly informative and showed the highest polymorphism, whereas primers OPA

Table 3 Analysis of polymorphism obtained with simple sequence repeat primers in isolates of *Fusarium oxysporum* f. sp. *lentis*

Primer	Sequence (5′-3′)	Annealing temperature (°C)	Total bands (No.)	Polymorphism (%)	Size range of amplicons (kb)
MB 2	F-TGCTGTGTATGGATGGATGG R-CATGGTCGATAGCTTGTCTGAG	49.6	7	100.0	0.2–1.5
MB 5	F-ACTTGGAGGAAATGGGCTTC R-GGATGGCGTTTAATAAATCTGG	49.0	8	100.0	0.25–2.0
MB 9	F-TGGCTGGGATACTTGTGTAATTG R-TTAGCTTCAGAGCCCTTTGG	52.0	5	100.0	0.2–1.0
MB 10	F-TATCGAGTCCGGCTTCCAGAAC R-TTGCAATTACCTCCGATACCAC	54.0	5	100.0	0.25–2.0
MB 11	F-GTGGACGAACACCTGCATC R-AGATCCTCCACCTGCATC	48.0	8	100.0	0.2–2.0
MB 13	F-GGAGGATGAGCTCGATGAAG R-CTAAGCCTGCTACACCCTCG	49.6	4	100.0	0.25–1.0
MB 14	F-CGTCTCTGAACCACCTTCATC R-TTCCTCCGTCCATCCTGAC	52.0	8	87.5	0.2–2.0
MB 17	F-ACTGATTCACCGATCCTTGG R-GCTGGCCTGACTTGTTATCG	48.0	6	100.0	0.25–1.5
MB 18	F-GGTAGGAAATGACGAAGCTGAC R-TGAGCACTCTAGCACTCCAAAC	48.0	8	87.5	0.25–1.0
Total		59			
Mean	–	–	96.6	–	

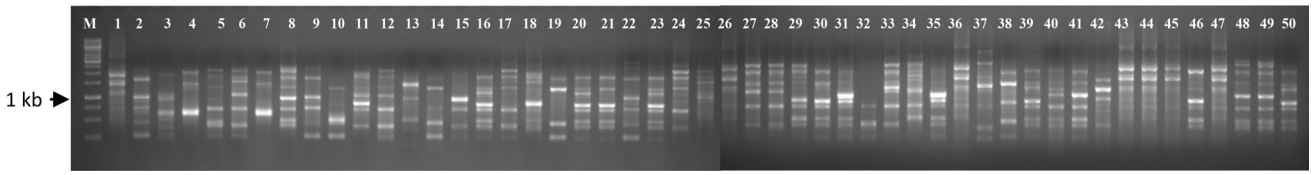

Fig. 1 DNA profile generated by random amplified polymorphic DNA (RAPD) primers OPF 8; M = 1 kb marker; lanes 1–13 (Uttar Pradesh), 14–19 (Bihar), 20–31 (Madhya Pradesh), 32–37 (Jharkhand), 38–43 (Chhattisgarh), 44–49 (Rajasthan) and 50 (Delhi) indicate the 50 isolates of *Fol*

11, OPF 1 and OPF 16 showed 71.4, 88.9, and 90% polymorphism, respectively.

Banding pattern obtained for 50 *Fol* isolates using primers (14) was analyzed using UPGMA analysis and generated a dendrogram (Fig. 2) differentiated the isolates into six clusters at 25% similarity. The first cluster had only two isolates namely, FLS 2 and FLS 20 both belonging to Uttar Pradesh but representing race 5 and race 1, respectively. The second cluster had 7 isolates, of which, 3 were from Uttar Pradesh namely, FLS 4, FLS 15 (representing race 2) and FLS 16 (race 3), FLS 31 from Madhya Pradesh (race 4), FLS 22 and FLS 27 from Bihar representing race 7 and race 4, respectively and FLS 65 from Chhattisgarh representing race 5. The third cluster had the highest number of isolates (28) belonging to all the seven states. While, the fourth cluster had eight isolates, of which three were from Rajasthan (FLS 67, 69 and 71), two each from Chhattisgarh (FLS 61 and 66) and Jharkhand (FLS 56 and 58), the fifth had only two isolates namely, FLS 35 (Madhya Pradesh) and FLS 53 (Jharkhand) belonging to races 1 and 6, respectively. Finally, the sixth cluster had three isolates namely, FLS 5, FLS 8 and FLS 12 from Uttar Pradesh representing three different races 1, 3 and 2, respectively.

It was evident that the isolates belonging to different locations were grouped into different clusters representing different races. Even, in one group for instance, sixth cluster had three isolates namely, FLS 5, FLS 8 and FLS 12 from Uttar Pradesh and each represented a different race namely, 1, 3 and 2, respectively. But, it was also observed that two isolates from a single place clustered together and represented a same race, for example isolates FLS 12 and FLS 14 are from Lalitpur in UP and they represented the race 2 and isolates FLS 72 and FLS 74 from Jaipur represented race 3. Thus, it was found that the random amplified polymorphic DNA analysis partially corresponded to the races as well as lentil growing regions of the country. Belabid et al. (2004)

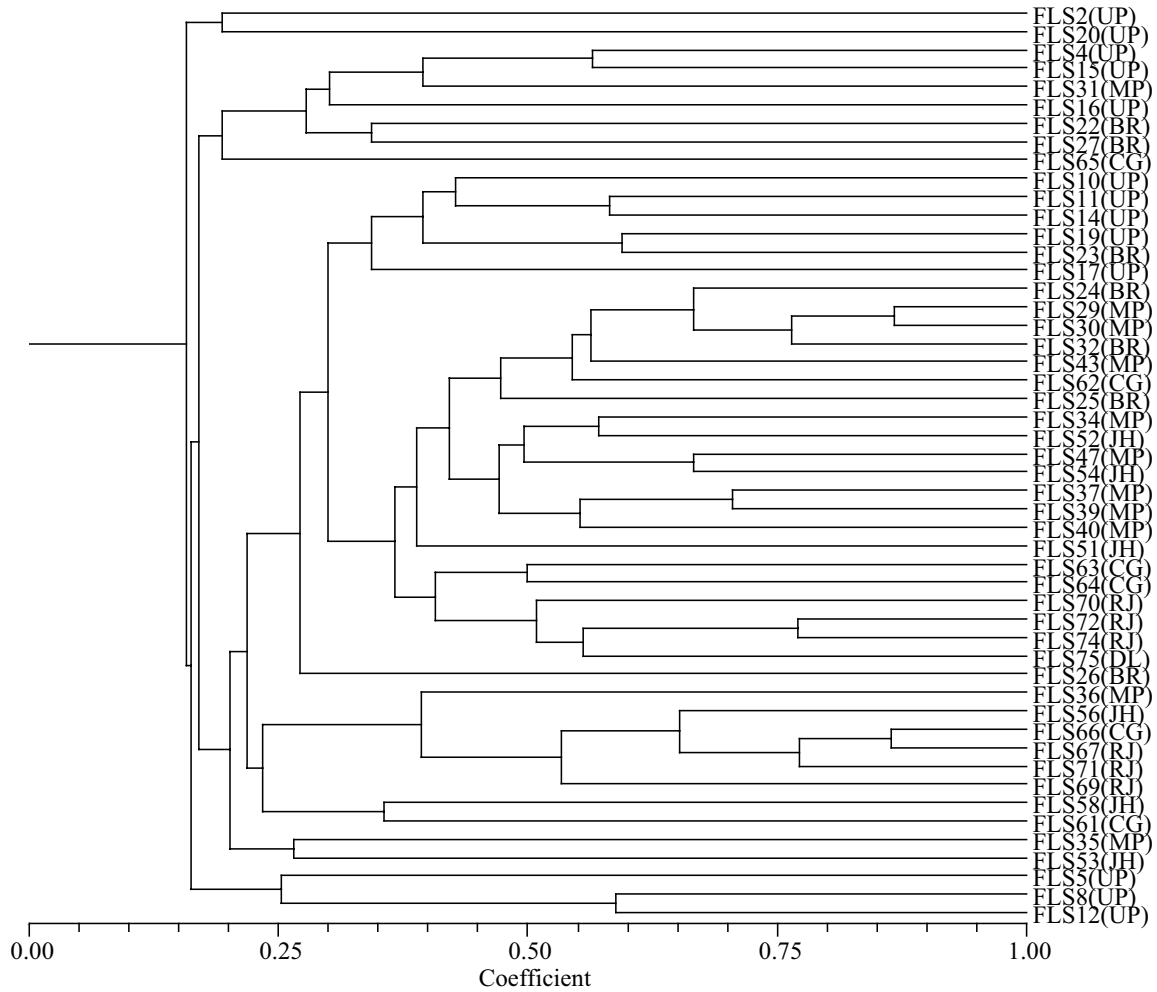

Fig. 2 Dendrogram derived from random amplified polymorphic DNA (RAPD) analysis of 50 isolates of *Fusarium oxysporum* f. sp. *lentis* with 14 primers by unweighted paired group method with arithmetic average (UPGMA). The bottom scale is the Jaccard's similarity coefficient. Abbreviations in bracket indicate states as *UP* Uttar Pradesh, *BR* Bihar, *MP* Madhya Pradesh, *JH* Jharkhand, *CG* Chhattisgarh, *RJ* Rajasthan and *DL* Delhi

reported that RAPD markers grouped the *Fol* isolates into two major groups on the basis of geographical location. Earlier, Dubey and Singh (2008) reported that the isolates of *F. oxysporum* f. sp. *ciceris* were clustered into two major groups at 30% genetic similarity using RAPD markers. In another study, RAPD analysis using 30 primers, the isolates of *F. oxysporum* f. sp. *psidii* separated into three groups wherein, *F. solani* isolates grouped in two different clusters, showing a higher degree of similarity, thus PCR-RAPD can be used for genetic variability studies (Gupta 2012).

Simple sequence repeats (SSR) analysis

Nine SSR markers specific to *Fusarium oxysporum* complex used against 50 *Fol* isolates belonging to different states of India showed good polymorphism (Table 3). A total of 59 bands with the size varied from 0.25 to 2.0 kb were obtained by the nine sets of primers out of which 57 were polymorphic (96.6%) and only two were monomorphic. The number of bands varied from four to eight with an average of 6.6 bands per primer. The primers MB 2, MB 9 (Fig. 3) and MB 11 were highly informative and showed maximum polymorphism (100%) while the primers MB 14 and MB 18 were also informative with 87.5% polymorphism and both produced a unique monomorphic band which was specific to all the *Fol* isolates used in the study.

Banding pattern of the 50 isolates was analyzed and the dendrogram derived from the nine primers based on UPGMA analysis revealed that the isolates were clustered into six groups at 30% genetic similarity (Fig. 4). Seven isolates representing six different races originated from five states were place in the first group. The second group had only three isolates belonging to two states namely, FLS 5

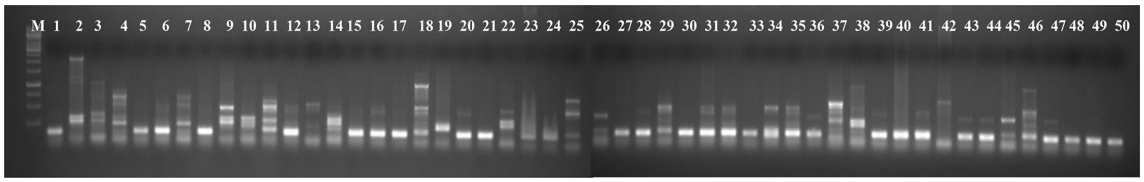

Fig. 3 DNA profile generated by simple sequence repeat (SSR) primers MB 9; M = 1 kb marker; lanes 1–13 (Uttar Pradesh), 14–19 (Bihar), 20–31 (Madhya Pradesh), 32–37 (Jharkhand), 38–43 (Chhattisgarh), 44–49 (Rajasthan) and 50 (Delhi) indicate the 50 isolates of *Fol*

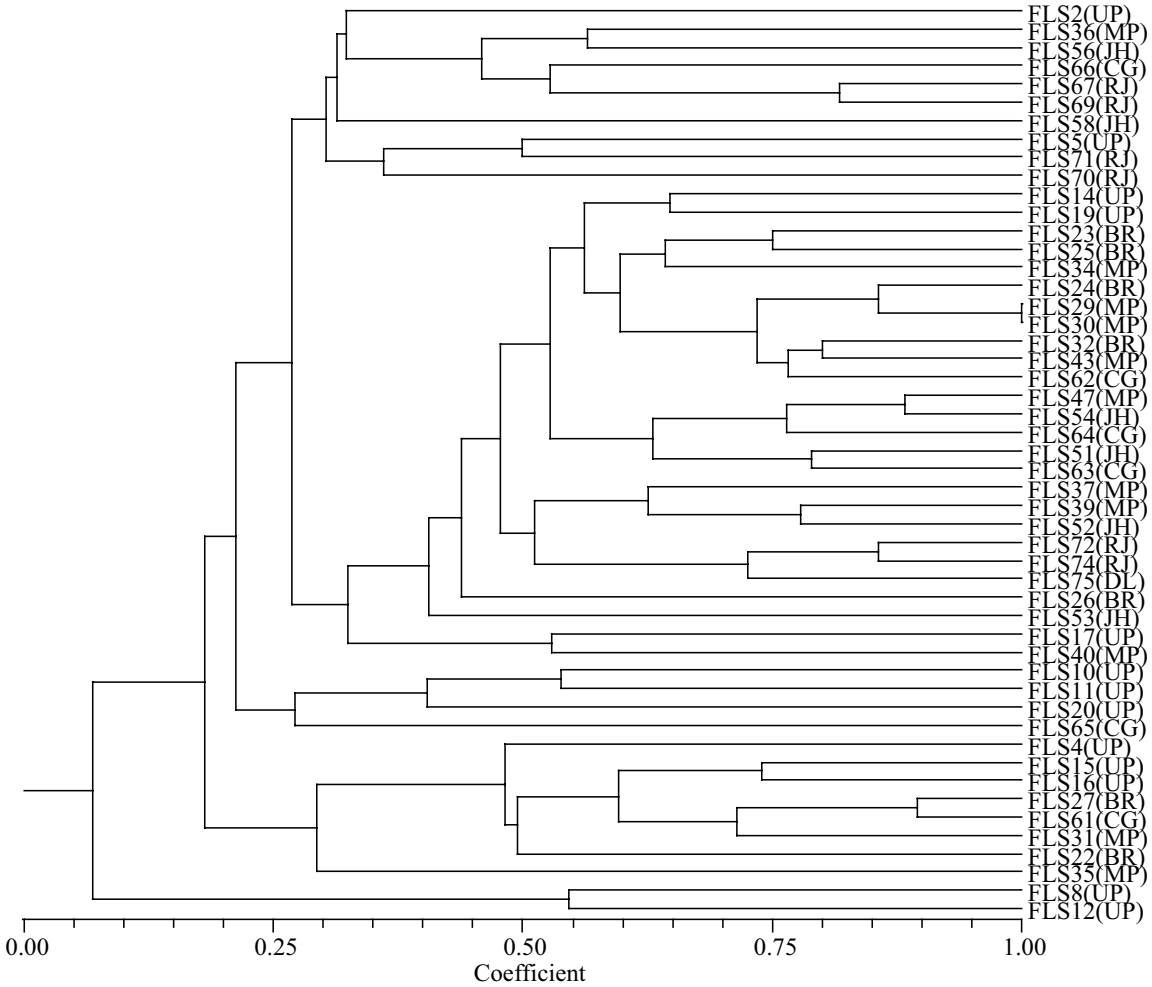

Fig. 4 Dendrogram derived from simple sequence repeats (SSR) analysis of 50 isolates of *Fusarium oxysporum* f. sp. *lentis* with 9 primers by unweighted paired group method with arithmetic average (UPGMA). The bottom scale is the Jaccard's similarity coefficient. Abbreviations in bracket indicate states as *UP* Uttar Pradesh, *BR* Bihar, *MP* Madhya Pradesh, *JH* Jharkhand, *CG* Chhattisgarh, *RJ* Rajasthan and *DL* Delhi

(Uttar Pradesh), FLS 70 and FLS 71 (Rajasthan) representing race 1 and race 2, respectively. The third group had the highest number of isolates (26) originating from seven different states including the only isolate FLS 75 from Delhi. Fourth cluster had four isolates namely, FLS 10, FLS 11, FLS 20 (from Uttar Pradesh) and FLS 65 (from Chhattisgarh). The fifth group had eight isolates from four Indian states and finally, the sixth group had two isolates namely FLS 8 and 12 both from Uttar Pradesh but representing races 3 and 2, respectively.

In the present study, nine SSR markers specific to *Fusarium oxysporum* complex (Bogale et al. 2006) used against 50 *Fol* isolates belonging to different states of India showed good polymorphism (96.6%). The primers MB 2, MB 9 and MB

11 showed 100% polymorphism which confirmed that these markers are highly suitable for genetic diversity studies in *Fusarium oxysporum* group. Earlier, Dubey and Singh (2008) reported MB 05, MB 14 and MB 17 as suitable in SSR markers for good allelic variation against *F. oxysporum* f. sp. *ciceris*. Although, SSR analysis also clustered the isolates into six groups but these were not exactly the same as RAPD groups. Earlier to this, several workers have used SSR markers to know the genetic diversity among the *Fusarium oxysporum* group (Bogale et al. 2006; Datta et al. 2011; Datta and Lal 2013). The results in the present study revealed that the SSR analysis partially corresponded to the races as well as the geographic locations from where the *Fol* isolates originated. Nevertheless, the SSR markers included in the study are proved highly suitable for genetic diversity studies in *Fusarium oxysporum* group as it has been reported earlier by many workers.

In the present study, *Fol* population representing major lentil growing regions of India was analyzed. Earlier to this study, the Indian populations of *Fol* were characterized into 8 races/pathotypes based on the differential reactions on a set of 10 lentil genotypes (Hiremani and Dubey 2018). Thus, the population proved to variable as different races/pathotypes prevalent in a state of India. The existence of genetic diversity among the *Fol* isolates was evident through the RAPD and SSR analyses which differentiated the *Fol* isolates into different clusters based on the location as well as races of *Fol*. However, the analyses partially corresponded to the races of *Fol* and the lentil growing regions of India. Nevertheless, this information is highly useful for the breeders to plan the breeding strategies to develop resistant cultivars against *Fol* looking into existence of races in a particular region and considering the diversity among them. Thus, the results of this study are also in agreement with the earlier reports that RAPD and microsatellite markers can be effectively utilized to know the relationship existing between the isolates belonging to different geographic locations and also are popular tool to identify the diversity present among the isolates.

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Compliance with ethical standards

Conflict of interest The authors do not have any conflict of interest.

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