Morpho-cultural, pathological and molecular variability in Phloeospora maculans causing leaf spot of mulberry (Morus species) in India

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Research Article

Keywords: Morus species, leaf spot, Phloeospora maculans, variability, ISSR, Polymerase chain reaction

Posted Date: May 23rd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2277225/v1

Version of Record: A version of this preprint was published at Molecular Biology Reports on August 18th, 2023. See the published version at https://doi.org/10.1007/s11033-023-08677-x.

Abstract

Background:

Leaf spot disease (LSD) of mulberry caused by *Phloeospora maculans* is a major threat to the silk industry of Jammu and Kashmir, India, therefore, it was necessary to study the population structure of the pathogen for successful management of the disease.

Methods and Results

To understand the diversity in the *Phloeospora maculans*, a combination of conventional (morphological, cultural and pathological) and molecular (ISSR markers) approaches were employed to discern the variability in 27 isolates collected from Srinagar, Bandipora, and Baramulla districts of Jammu and Kashmir, India. The studies revealed a high level of variability in the pathogen. Based on the morpho-cultural and pathological studies, the pathogen isolates were grouped into different categories based on colony growth, texture, margin and colour besides changes in colour of medium, incubation period, leaf area infected, etc.A high level of polymorphism was observed in different isolates of *P. maculans* using ISSR markers, which indicated that these markers are suitable for studying the genetic diversity in this pathogen. All the isolates (27) of *P. maculans* were clustered into two groups or populations as indicated by mean delta K value. Analysis of molecular variance revealed the low genetic variation among the populations (1.08%) and a high level of genetic variation within the populations (98.91%). F_{st} value was found to be 0.01 indicating smaller amount of genetic differentiation between the populations against calculated P-value of 0.29

Conclusion

A high level of diversity based on morphological, cultural, pathological and molecular levels was observed in *Phloeospora maculans* collected from various districts of Kashmir valley, which indicates that the study of population structure is necessary for successful management of the disease.

Introduction

Mulberry (*Morus species*) is the only food for silkworm (*Bombyx mori* L.) which belongs to family *Moraceae* and genus *Morus*. It is a woody perennial plant exclusively grown for its foliage, which is the economic product of this plant [1]. The sericulture is being practiced in more than 58 countries in the world [2].. In India, 2.82 lac hectares are under mulberry cultivation where the traditional sericulture is practiced in Karnataka, Tamil Nadu, Andhra Pradesh etc. under tropical climate and under temperate to sub-tropical conditions, the commercial sericulture is being practiced in Himachal Pradesh, West Bengal and Jammu & Kashmir [3]. In Jammu and Kashmir, an area of 4,717 hectares is under mulberry cultivation. In addition, thousands of scattered mulberry trees are growing on hill and roadsides and river-banks etc. The foliage of these trees is mainly utilized for silkworm rearing and silk production.

Number of plant pathogens are responsible for causing various diseases in mulberry resulting in 15–20 per cent loss in leaf yield [4]. Among these, leaf spot disease (LSD) caused by *P. maculans* is a serious threat to the sericulture industry in Kashmir valley, where the environmental conditions are predominantly favourable for the disease development [5]. The disease initially appears in the month of May with maximum intensity during July-September [6]. The disease spreads primarily with the help of rain droplets and wind. The disease appears as circular or irregular brownish spots of varying size with ash grey centre. As the disease advances, the spots enlarge, coalesce and result in the formation of shot holes. Severely affected leaves become yellowish and fall off prematurely [7]. The disease directly affects leaf yield of about 5–10 per cent due to defoliation, which may however, reach upto 35 per cent in the most severe conditions resulting in severe economic loss to the farmers [8].

Crop failures due to LSD necessitates to study the diversity in the pathogen for better understanding of the pathogen population. Studies on the genetic diversity and phylogeny of the pathogen based on internal transcribed spacer (ITS) sequencing and inter-simple sequence repeat (ISSR) markers has not been studied in this pathogen till date. Traditionally, studies on phenotypic differences in morphology, physiology and pathogenicity of the fungal populations are influenced by environment and therefore, difficult to standardize and analyse. In recent years, there has been substantial development in innovative methods for population genetic analysis and evolutionary biology of fungi at molecular levels [9]. ISSR marker system is extensively used for investigating phylogeny, evolutionary relationships and genetic variability among plants [10, 11, 12]. Dearth of information about the biological, pathological and population genetic studies in *P. maculans* warrants detailed investigation. Since, the quality and quantity of mulberry leaf decides the quality of cocoon crop, therefore, becomes necessary to generate information on degree of pathogenicity inflicted by *P. maculans* isolates/ population on mulberry leaves. The information in this direction can open new avenues for silk cocoon production through the development of strategic control measures. Thus, the present study was carried out to ascertain the diversity in *P. maculans* isolates using morphological and cultural, pathological and molecular approaches.

Materials and methods

Collection of diseased samples and isolation of the pathogen

The infected mulberry leaves showing typical leaf spot symptoms were collected from three commercially mulberry growing districts of Kashmir valley *viz*., Srinagar, Bandipora and Baramulla. The leaf bits containing young leaf spot lesions were aseptically plated on potato dextrose agar (PDA) medium in Petri dishes/ test tubes and incubated at 24 ± 1 ˚C to isolate the causal fungal pathogen following appropriate plant pathological techniques. Pure culture of different isolates of the pathogen were obtained by hyphal tip method [13] and incubated at 24 ± 1˚C for seven days. The details of the pathogen isolates along with their latitude and longitude, coding etc. is given in Table 1.

Table 1

Phloeospora maculans isolate details collected from different mulberry growing areas of Kashmir and their pathological variability

District	Latitude and longitude of locations	Name of cultivar	Isolate code	Lesion diameter (mm)**	Incubation period (days)
Srinagar	Lat. 34.059240Long. 74.802980	Goshoerami	StG	13.0^{f}	7
		Ichinose	StI	14.5^{f}	4
		PPR-1	StP	14.5^{f}	4
	Lat. 34.149220 Long. 74.839140	Goshoerami	SbG	12.2^{g}	7
		Local-1	SbL_1	21.0^{c}	2
		Local-2	SbL_2	16.5^{e}	5
	Lat. 34.164940 Long. 74.905790	Goshoerami	ShG	11.3^{g}	7
		Local-1	ShL_1	13.0^{f}	4
		Local-2	ShL_2	13.0^{f}	4
Bandipora	Lat. 34.251491 Long. 74.653595	Goshoerami	B_am_aG	15.8^{e}	6
		Local	B_am_aL	21.8^{c}	2
		PPR-1	B_am_aP	14.8^{e}	4
	Lat. 34.423200 Long. 74.636000	Goshoerami	B_adG	24.7^{b}	2
		Local	B_adL	14.3^{f}	6
		TR-10	B_adT	23.3^{b}	3
	Lat. 34.267814 Long. 74.669215	Goshoerami	B_apG	31.0^{a}	2
		Local	B_apL	18.7^{d}	4
		PPR-1	B_apP	15.2^{e}	6
Baramulla	Lat. 34.141357 Long.74.648539	Goshoerami	BmG	18.0^{d}	4
		Local	BmL	14.3^{f}	4
		TR-10	BmT	16.0^{e}	4

** Recorded after 10 days of inoculation

District	Latitude and longitude of locations	Name of cultivar	Isolate code	Lesion diameter (mm)**	Incubation period (days)
	Lat. 34.203900 Long. 74.354400	Goshoerami	Bt_aG	16.5^{e}	2
		Local	Bt_aL	18.7^{d}	4
		Ichinose	Bt_aI	14.8^{e}	4
	Lat. 34.148458 Long. 74.526447	Goshoerami	BnG	11.7^{g}	4
		Local	BnL	11.0^{g}	6
		TR-10	BnT	15.7^{e}	7
	C.D. ($p \leq 0.05$)			1.98	-
** Recorded after 10 days of inoculation					

Pathogenicity tests and pathological variability

Pathogenicity and pathogenic variability of the pathogen isolates was determined by detached leaf technique on healthy young leaves of susceptible variety (Serpentina) of mulberry [14]. Inoculations were carried out with mycelial bit of 5-millimetre (mm) diameter on upper and lower surfaces of leaves with and without injury. A control (check) was also maintained by placing sterilized water drops on leaves instead of mycelial bits. Inoculated trays were incubated at 29 ± 1°C for the development of symptoms. Observations on incubation period were recorded on daily basis, whereas the lesion diameter was recorded 7 days post inoculation. The data was analysed in completely randomized block design (CRD) [15].

Morpho-cultural variability

Morphological and cultural characteristics of each isolate were observed on PDA medium. Two millimetre (mm) mycelial discs were cut aseptically from the actively growing region of seven-days-old culture, transferred to Petri dishes containing PDA medium and incubated at 24 ± 1 °C. Observations on colony size, margins, texture and colour were recorded after 3 days of incubation. Average colony diameter was also recorded daily basis up to 72 hours and growth rate calculated. There were five replications for each isolate. Conidial morphology was examined by preparing spore suspension from seven-days-old culture of each isolate and observed under microscope (Olympus, Tokyo, Japan using Magnus Pro software after calibration) to record shape, colour, septation and size (length and width) for at least 15 conidia per isolate.

Molecular variability

Genomic DNA extraction

All the pathogen isolates were separately cultured on potato dextrose broth (PDB) in 100 ml Erlenmayer flasks. After sterilization of PDB at 15 lbs psi, flasks were inoculated with 5 mm mycelial discs of each pathogen isolate aseptically and incubated at 24 ± 1 ˚C for a period of two weeks. Mycelium was filtered through double-layered sterilized filter paper, dried between two layers of filter paper in a laminar airflow cabinet and stored separately at − 80 ˚C for further use. The total genomic DNA of each isolate was extracted using CTAB (Cetyl trimethyl ammonium bromide) method [16] and diluted to a final concentration of 10µg/ml (Thermo Scientific). It was mixed and then incubated at 37 ˚C for 2 hours @ 500rpm in an Eppendorf thermomixer. DNA of all the 27 isolates was obtained in similar way and stored at -80 ˚C for further use.

Assessment of quality and quantity of DNA

The quality of genomic DNA was observed on 1.0 per cent agarose by gel electrophoresis in 1X TAE (Tris-acetate-EDTA) buffer [17, 18]. The quantity of the extracted genomic DNA was determined by measuring the absorbance using Nano Drop spectrophotometer (Eppendorf Biospectrophotometer, Germany). The DNA of each sample was diluted to 25ng/ µl by nuclease free water for polymerase chain reaction (PCR) amplification.

PCR amplification and visualization of the amplified products

The PCR amplification was performed in 0.2 ml PCR tubes in a thermal cycler (Eppendorf Germany) using 40–50 ng of genomic DNA for inter simple sequence repeat (ISSR) analysis of different pathogen isolates in a 15 µl per reaction containing DNA template (20-25ng/ µl) − 2 µl; 10X PCR buffer (10mM Tris HCl, pH 8.3, 50 mM KCl) − 1.5 µl; MgCl (25 mM) − 0.9 µl; dNTP's (2.5 mM) − 1.0 µl; primer (10 pmol) − 1.0 µl; *Taq* polymerase (Thermo Scientific) (5U/µl) − 0.12 µl and nuclease free water (Qiagen) − 8.48 µl. The reaction mixture of 15 µl in PCR tubes were given a short spin in vortex multispin (Thermo Scientific, Thermo Electron Corporation) and placed in 96 well thermal cycler. The PCR cycling conditions consisted of an initial denaturation step at 94 ˚C for 5 min followed by 35 cycles with denaturation at 94˚C for 1 min, annealing at 37 ˚C for 1 min, extension at 72 ˚C for 2 min and final extension at 72 ˚C for 10 min and hold at 4 ˚C.

Out of 15 µl of the PCR amplified product, 2 µl added with 6X loading dye was resolved on 1.0 per cent agarose gel. The gel was prepared in 1X TAE buffer. After loading PCR product, the gel was run at 80V, visualized under UV light and photographed using Alfa Imager gel documentation system (Protein Simple, USA).

Statistical analysis

The data from all the primers was recorded as 1 (band present) and 0 (band absent) in a binary matrix. A similarity matrix was generated using Jaccard's coefficient, and the dendrogram constructed using UPGMA (Unweighted Pair Group Method using Arithmetic Averages) available in NTSYS 2.0 software [19]. The dendrogram with best fit to similarity matrix was chosen. The population structure of different pathogen isolates was evaluated using Analysis of Molecular Variance (AMOVA) in STRUCTURE v.2.3 software and fixation indices (F_{st}) was estimated. The statistical significance of the total and pair-wise fixation indices was estimated by comparing the observed distribution with the null distribution.

Results

Twenty-seven isolates of the pathogen collected and isolated from different commercial mulberry growing areas of Kashmir valley (India) were identified as *Phloeospora maculans* (Bereng) Allesch on the basis of pathological and morpho-cultural characteristics after comparing with the literature [20, 21, 22, 23, 24].

Pathogenicity tests and pathological variability

All the 27 isolates of *Phloeospora maculans* produced round to irregular lesions, brown to dark brown in colour surrounded by a yellow zone symptoms, both on injured as well as uninjured leaves and on both (upper and lower) the surfaces of leaf (Fig. 1). The isolates varied significantly in respect of their incubation period and subsequent size (diameter) of the lesions produced on inoculated leaves of mulberry. The lesion diameter on mulberry leaves varied from 11.0–31.0 mm by different pathogen isolates (Table 1). The highest lesion diameter of 31.0 mm was observed in isolate B_apG followed by isolate B_adG (24.7 mm) which was at par with B_adT (23.3 mm) and the lowest lesion diameter of 11.0 mm was observed in isolate BnL followed by isolate ShG (11.3 mm) and were at par with BnG (11.7 mm) and SbG (12.2 mm). Incubation period varied from 2–7 days in different isolates and was 7 days in four isolates (StG, SbG, ShG and BnT), 6 days in four isolates (B_am_aG, B_adL, B_apP and BnL), 5 days in only one isolate (SbL_2), 4 days in twelve isolates (StI, StP, ShL_1, ShL_2, B_am_aP, B_apL, BmG, BmL, BmT, Bt_aL, Bt_aI and BnG), 3 days in only one isolate (B_adT) and a minimum incubation period of 2 days was observed in five isolates (SbL_1, B_am_aL, B_adG, B_apG and Bt_aG). These results indicated that the isolates with maximum lesion diameter and minimum incubation period were the virulent isolates compared to others. The shape of the lesions was circular to irregular in almost all the isolates of the pathogen.

Morpho-cultural variability

The morpho-cultural variability was recorded to exist among different isolates of the pathogen. Based on morphological characters such as colony shape, texture and colour and colour change in culture medium

after 3 days of incubation at 24 ± 1 ˚C, the pathogen isolates were divided into different groups (Table 2, Fig. 2). White mycelial colour was observed in 7 isolates (StP, $ShL_{1,}$ B_adL, BmL, BtaI, BnL and BnT), off-white in 10 isolates (SbG, $SbL_{1,}$ B_am_aL, B_am_aP, B_adT, B_apG, B_apL, BmT, Bt_aG and Bt_aL), white with grey center in 8 isolates (StG, StI, ShG, ShL_2, B_am_aG, B_adG, B_apP and BnG), off-white with grey center and off-white with white center in one isolate each *viz.*, SbL_2 and BmG, respectively. Colony texture such as raised, flat and granular were observed in different isolates of *Phloeospora maculans*. Raised texture was observed in most of the isolate (22) such as StG, StI, StP, $SbL_{2,}$ ShG, $ShL_{1,}$ $ShL_{2,}$ B_am_aG, B_adL, B_adT, B_apG, B_apL, B_apP, BmG, BmL, BmT, Bt_aG, Bt_aL, Bt_aI, BnG, BnL, BnT whereas, only four isolates namely SbG, $SbL_{1,}$ B_am_aP and B_am_aL showed flat texture. However, only one isolate B_adG showed granular texture. Most of the pathogen isolates (25) showed regular colony margins except two isolates (BmG and Bt_aG) which showed irregular margins. The underside colour of different isolates grown in Petri plates showed different colour shades (Table 2). Off-white colour of underside of Petri plates was observed in nine isolates (SbL_2, BamaL, BamaP, BapG, BapL, BmT, BtaG, BtaL and BnG), off-white with olive green center in seven isolates (StG, ShG, ShL_1, BamaG, BadG, BmL and BtaI). Off-white with olive brown center was observed in ten isolates (StI, StP, SbG, SbL_1, ShL_2, BadL, BapP, BmG, BnL and BmT), and only one isolate (BadT) showed golden brown and off-white with concentric circles after three days of incubation at 24 ± 1˚C.

Table 2
Growth characteristics of different isolates of *Phloeospora maculans* on potato dextrose agar medium at 24 ± 1°C

Isolate*	Mean colony diameter (mm)	Growth rate per hour (mm)	Mycelial dry wt. (mg)**
StG	12.61^{b}	0.910^{f}	184.82^{d}
StI	8.44^{c}	0.634^{g}	241.40^{b}
StP	16.17^{b}	1.266^{d}	229.35^{b}
SbG	15.83^{b}	1.190^{e}	204.33^{c}
SbL_1	8.50^{c}	0.718^{g}	201.51^{c}
SbL_2	6.42^{c}	0.520^{h}	187.32^{d}
ShG	13.89^{b}	0.993^{f}	119.10^{g}
ShL_1	14.61^{b}	1.146^{e}	235.54^{b}
ShL_2	7.17^{c}	0.576^{h}	136.68^{f}
B_am_aG	9.92^{c}	0.925^{f}	250.70^{b}
B_am_aL	21.67^{b}	1.843^{b}	194.19^{d}
B_am_aP	7.83^{c}	0.630^{g}	154.79^{e}
B_adG	22.00^{a}	1.662^{c}	162.43^{e}
B_adL	7.83^{c}	0.525^{h}	242.52^{b}
B_adT	11.92^{c}	0.948^{f}	134.26^{e}
B_apG	31.24^{a}	2.494^{a}	216.30^{c}
B_apL	8.94^{c}	0.764^{g}	235.94^{b}
B_apP	19.17^{b}	1.398^{d}	210.88^{c}
BmG	10.06^{c}	0.813^{f}	203.81^{c}
BmL	12.44^{b}	0.870^{f}	64.56^{h}
BmT	9.08^{c}	0.678^{g}	143.29^{f}

**after 15 days

Isolate*	Mean colony diameter (mm)	Growth rate per hour (mm)	Mycelial dry wt. (mg)**
Bt_aG	6.17^{c}	0.574^{h}	176.72^{d}
Bt_aL	10.72^{c}	0.856^{f}	293.46^{a}
Bt_aI	15.61^{b}	1.211^{e}	137.16^{f}
BnG	5.06^{c}	0.412^{h}	149.27^{e}
BnL	14.06^{b}	1.072^{e}	179.85^{d}
BnT	16.89^{b}	1.218^{d}	127.50^{f}
C.D. ($p \leq 0.05$)	9.30	0.994	28.20
**after 15 days			

Colony growth

Significant variations were recorded in mycelial growth (colony diameter, mycelial dry weight and growth rate) of different isolates of *P. maculans* (Table 2). The mean colony diameter per day ranged from 5.06 mm to 31.24 mm in different isolates. The results revealed that the isolates B_apG and B_adG showed maximum mean colony diameter of 31.24 mm and 22.00 mm, respectively, whereas the lowest colony diameter was observed in BnG isolate (5.06 mm) (Fig. 2). The mean growth rate per hour of *P. maculans* on PDA medium varied significantly from 0.412 to 2.494 mm (Table 2). The maximum mean growth rate of 2.494 mm was observed in isolate B_apG followed by isolate B_am_aL (1.843 mm) and the lowest mean growth rate of 0.412 mm was observed in isolate BnG. The mycelial dry weight of different *P. maculans* isolates varied significantly (64.56 mg to 293.46 mg) (Table 2). The highest mycelial dry weight of 293.46 mg was observed in isolate Bt_aL and the lowest mycelial dry weight of 64.56 mg was observed in isolate BmL.

Conidial characteristics

Conidial characteristics such as shape, size, colour and septation were observed in different isolates of *P. maculans* (Table 3, Fig. 3). Conidial shape of most of the isolates was falcate, gradually tapered at the apex except in isolate StI in which conidia were slightly curved, fusiform to clavate. Conidia were curved, falcate and apex obtuse in ShL_1 and in Bt_aL, conidia were straight, tapered towards the apex or cylindrical and obtuse. Conidal were hyaline and guttulate in all the isolates of the pathogen. Conidial septation varied from 1–7 except in four isolates namely ShL_1, B_am_aL, BmG and BnT in which 3–5 septa were observed. The highest number of septa (1–7) were observed in only one isolate SbL_1 and the number of

septa (1–3) were observed in four isolates *viz*., StI, B_apL, Bt_aL and BtaI. Conidial size in different isolates varied from 5–40 × 1–4 µm with an average size of 20.3 × 2.5 µm in different isolates (Table 3). The maximum mean length of 27.1 µm was observed in SbG and minimum mean length 14.6 µm of conidia was observed in Bt_aL. Similarly, maximum mean conidial width of 3.1 µm was observed in $SbL_{1,}$ whereas the lowest mean conidial width of 2.0 µm was observed in two isolates B_am_aG and BtaG.

Table 3
Conidial characteristics of different *Phloeospora maculans* isolates

Isolate*	Conidial Septation**	Conidia size (µm) Range [L (av) × W (av)]**
StG	1–4	5–33 (22.1) × 2–3 (2.2)
StI	1–3	10–27 (19.2) × 2–4 (3.0)
StP	1–5	8–29 (19.7) × 2–4 (2.9)
SbG	1–5	15–35 (27.1) × 2–3 (2.2)
SbL_1	1–7	10–31 (22.4) × 2–4 (3.1)
SbL_2	1–4	7–37 (21.4) × 1–3 (2.1)
ShG	1–5	9–28 (16.6) × 2–3 (2.4)
ShL_1	3–5	9–38 (17.5) × 2–3 (2.1)
ShL_2	1–5	7–39 (19.8) × 1–4 (2.6)
B_am_aG	1–5	10–40 (25.2) × 2–3 (2.0)
B_am_aL	3–5	15–31 (24.6) × 2–3 (2.3)
B_am_aP	1–6	8–32 (19.9) × 2–4 (3.0)
B_adG	1–5	11–29 (21.6) × 2–3 (2.4)
B_adL	1–5	11–36 (19.9) × 2–3 (2.8)
B_adT	1–4	9–31 (18.1) × 2–3 (2.2)
B_apG	1–4	10–30 (19.9) × 2–3 (2.6)
B_apL	1–3	7–32 (14.8) × 1–3 (2.4)
B_apP	1–5	10–29 (20.3) × 2–4 (2.4)
BmG	3–5	9–37 (22.0) × 2–3 (2.1)
BmL	1–4	8–35 (24.1) × 2–3 (2.3)
BmT	3–6	7–31 (19.7) × 2–3 (2.8)
Bt_aG	1–4	9–34 (20.4) × 2–3 (2.0)
Bt_aL	1–3	7–25 (14.6) × 2–3 (2.2)

**Average of 50 spores

Isolate*	Conidial Septation**	Conidia size (µm) Range [L (av) × W (av)]**
Bt_aI	1–3	6–30 (17.5) × 2–3 (2.4)
BnG	1–5	11–33 (19.9) × 2–3 (2.2)
BnL	1–5	10–31 (19.1) × 2–3 (2.8)
BnT	3–5	8–26 (20.6) × 2–4 (2.9)
**Average of 50 spores		

Molecular characterization

ISSR analysis

Molecular variability in the *P. maculans* isolates was assessed by genotyping with five polymorphic ISSR primers (Table 4). Initially, 15 ISSR markers were screened for PCR amplification of the *P. maculans* isolates. Out of 15 ISSR markers, only five markers were found polymorphic. The PCR amplification of different isolates showed a significant variation in their banding pattern (Fig. 4). The polymorphic information content (*PIC*) values varied from 0.219 to 0.395 in different ISSR primers. The highest *PIC* value of 0.395 was observed in primer UBC-889 (Table 4) therefore, indicated that this primer was the best one for studying the polymorphism in different isolates of *P. maculans* followed by primer UBC-840 with a *PIC* value of 0.350, UBC-834 and UBC-835 with *PIC* values of 0.346 and 0.317, respectively. The minimum *PIC* values of 0.0.219 was observed in primer UBC-836. Phylogenetic analysis was conducted on the taxonomic distance matrix with the Unweighted Pair Group Method based Arithmetic Average (UPGMA) using binary matrix (1, 0) obtained from PCR amplified products (gels) and dendrogram generated (Fig. 5). The constructed dendrogram revealed that the similarity among the *P. maculans* isolates ranged from 18 to 100 per cent. The molecular characterization based on ISSR markers clustered the pathogen isolates into one major cluster (Cluster I) accommodating all the isolates except one isolate StG which represented as an independent lineage in the dendrogram (Fig. 5) at a genetic similarity of approximately 40 per cent. Cluster I was further sub-divided into two sub-clusters sub-cluster 1 (Sc-1) and sub- cluster 2 (Sc-2) at approximately 45 per cent similarity. Sub-cluster- 1 accommodated most of the isolates, whereas Sc-2 accommodated only two isolates. Similarly, sub-cluster I was further sub-divided into five sub-clusters *viz*., Sc-1a, Sc-1b, Sc-1c, Sc-1d, Sc-1e and two independent lineages as L-a (B_apG) and L-b (B_adT) while as sub-cluster-2 accommodated only two isolates *viz*., Bt_aG and Bt_aL. Sub-cluster-1a accommodated ten isolates (StI, BmT, StP, BmL, BnL, Bt_aI, B_adL, ShL_2, B_am_aG and BnG), sub-cluster-1b accommodated two isolates (SbL_2 and ShL_1), sub-cluster-1c accommodated five isolates (SbG, ShG, B_apP, B_adG and B_am_aL), sub-cluster-1d accommodated only two isolates (SbL_1 and B_apL), sub-cluster-1e accommodated three isolates (B_am_aP, BmG and BnT). Two isolates SbL_2 and ShL_1 showed 100 per cent genetic similarity with each other.

Table 4
Polymorphism survey in *Phloeospora maculans* isolates based in ISSR genotyping

ISSR Primer	Sequence	*PIC* value
UBC-834	5 -AGAGAGAGAGAGAGAGYT-3	0.346
UBC-835	5 -AGAGAGAGAGAGAGAGYC-3	0.317
UBC-836	5 -AGAGAGAGAGAGAGAGYA-3	0.219
UBC-840	5 -GAGAGAGAGAGAGAGAYT-3	0.350
UBC-889	5 -DBDACACACACACACAC-3	0.395

Clustering of the pathogen isolates based on their geographical distribution

All the 27 isolates of *P. maculans* were clustered into two groups or populations as indicated by the mean delta K value (Fig. 6). Analysis of molecular variance (AMOVA) revealed the low genetic variation among the populations (1.085%) and a high level of genetic variation within the populations (98.91%) (Table 5). F_{st} value was found to be 0.01 that indicated the smaller amount of differentiation between the populations against calculated P value of 0.29. The various isolates of *P. maculans* showed a mixed differentiation among the populations indicated by different colours in structure plot (Fig. 7). The variation within the population may be due to the flow of infected planting material from one location to another location or across the districts in Kashmir. It can be due to flow of infection or spores from one place to another with the help of wind, water or equipment.

Table 5
Analysis of molecular variance (AMOVA) of *Phloeospora maculans* isolates

Source of variation	Degree of freedom	Sum of squares	Variance of component	Percentage of variation
Among populations	2	12.872	0.064	1.085
Within populations	24	130.252	5.903	**98.91**
Total	**26**	**143.124**	**5.967**	-
P value: 0.29; Fixation indices (F_{st}): 0.01				

Discussion

Twenty-seven isolates of *P. maculans* causing LSD of mulberry in Jammu and Kashmir, India were identified based on morpho-cultural characters [20, 21, 22, 23, 24]. The inoculation of *P. maculans* isolates on the young leaves of susceptible variety (Serpentina), revealed the consequential variation in incubation period and lesion development, indicating varied pathogenicity and virulence. Disease development on injured- and uninjured- leaves substantiated the pathogen entry through the natural openings (stomata) [5, 14, 24, 25, 26]. The infection was more on injured leaves inoculated on the under-surface containing more number of stomata that facilitated the entry of the pathogen [27, 28]. Significant variation in lesion size (diameter) produced by different *P. maculans* isolates was also observed. The lesion diameter varied from 11.0 to 31.0 mm, and the highest lesion diameter of 31.0 mm was observed in isolate B_apG, while as the lowest (11.0 mm) was in isolate BnL indicating that the B_apG as the most virulent isolate with maximum lesion diameter. *Cercospora zaea-maydis* isolates were similarly categorised as low, moderate and highly virulent based on lesion diameter [22] and used symptom severity to categorise isolates of *Cercospora zaea-maydis* infecting maize. The symptoms produced on the detached leaves were round to irregular lesions, brown to dark brown in colour surrounded by yellow zone. Similar observations were also reported [27, 28] while conducting the pathogenicity test in *Morus nigra* seedlings under greenhouse conditions. The variability in virulence of isolates observed in the present study indicates that there must be the presence of different pathotypes and races in *P. maculans* populations, thereby requiring carrying out the pathogen population studies on large scale including pathogenicity assays on live plants/seedlings of different cultivars.

Significant variation was recorded in morphological and cultural characteristics *viz*., colony texture, margins and colour, colour change in media, colony diameter, growth rate and spore characteristics in different isolates of *P. maculans*. Variations in cultural characteristics have also been observed in isolates of *Cercospora kikuchii* [27] *Cercospora canesens* [29], *Cercospora beticola* and *P. paramaculans* [30] and *P. salmali* [31, 32]

In order to discern the variability at molecular level, studies on pathogen were conducted for the first time using ISSR markers. Out of 15 ISSR primers, five polymorphic ISSR primers used in present study revealed a high level of polymorphism in different isolates of *P. maculans* in Kashmir, which indicated that these markers are suitable for studying the genetic diversity in mulberry leaf spot pathogen. Various researchers have also reported ISSR markers suitable for studying the genetic diversity in different phytopathogenic fungi [29, 33, 34]. The Polymorphic Information Content (*PIC*) values varied from 0.219 to 0.395 in different ISSR primers with the highest value of 0.395 in primer number UBC-889 followed bu UBC-840 with *PIC* value of 0.350 indicating their suitability for studying the genetic variation in this pathogen. The constructed dendrogram revealed that the similarity among the *P. maculans* isolates ranged from 18 to 100 per cent. The 27 isolates of the pathogen were clustered into one major cluster (Cluster-I) accommodating all the isolates except one isolate StG which represented as an independent lineage at genetic similarity coefficient of 40 per cent. Two isolates SbL_2 and ShL_1 showed 100 per cent genetic similarity with each other. All the 27 isolates of *P. maculans* were clustered into two groups or populations as indicated by the mean delta K value. Analysis of molecular variance (AMOVA) revealed

the low genetic variation among the populations (1.085%) and a high level of genetic variation within the populations (98.91%). F_{st} value was found to be 0.01 indicating smaller amount of genetic differentiation between the populations against calculated P-value of 0.29. The various isolates of *P. maculans* showed a mixed differentiation among the populations as indicated by colour admixtures in structure plot. The variation and mixture within the population may be due to the flow of infected planting material from one location to another location or across the districts in Kashmir. The aerial pathogens including *P. maculans* can also disseminate from one place to another along with the infected mulberry leaves collected for feeding/rearing of silkworms. The pathogen inoculum can also spread through wind, water and other means. Similarly, a high level of genetic diversity was reported in 32 isolates of *Alternaria brassicae* infecting rapeseed-mustard using ISSR markers [35], Sharma *et al.* while studying the *Alternaria alternata* using RAPD and AFLP markers reported a high level of genetic diversity among the isolates of this pathogen [33]. Analysis of molecular variance (AMOVA) also demonstrated the highest level of genetic diversity within the populations of *Mycosphaerella fijiensis* [34]. High genetic diversity in *P. maculans* may be attributed to mutation, recombination (sexual or parasexual), and migration (gene flow). To our knowledge, this is the first study conducted to study phylogenectics and genetic diversity in mulberry leaf spot pathogen using ISSR markers.

An appreciable morphological, pathological and molecular variability was observed in different isolates of the *P. maculans.* The studies further indicated that *P. maculans* is a quickly evolving fungus and has potential to overcome management strategies using fungicides and resistant varieties; thus management strategies need to be designed wisely to ensure effective and economical disease control with assured crop harvest. Besides, quarantine measures are necessary while importing planting material from other state [36, 37]. Further studies are needed to study genetic diversity and population structure with large sample size and using different markers.

Conclusion

A high level of diversity based on morphological, cultural, pathological and molecular levels was observed in *Phloeospora maculans* causing LSD on mulberry in Kashmir valley, which indicates that the study of population structure of the pathogen is necessary for the successful management of the disease. All the 27 isolates of *P. maculans* were clustered into two groups or populations as indicated by mean delta K value, but the colour mixture indicated admixture population distributed in different districts of Kashmir. Analysis of molecular variance revealed a low level of genetic variation among the populations (1.08%) and a high level of genetic variation within the populations (98.91%). F_{st} value was found to be 0.01 indicating smaller amount of genetic differentiation between the populations against calculated P-value of 0.29.

Declarations

Acknowledgements

Funding: Division of Plant Pathology, SKUAST-Kashmir, supported the research. The help is dully acknowledged.

Compliance with ethical standards

Ethical statement: This research did not involve any human and/or animal participants.

Conflict of interest: All authors declare that they don't have any conflict of interests.

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Figures

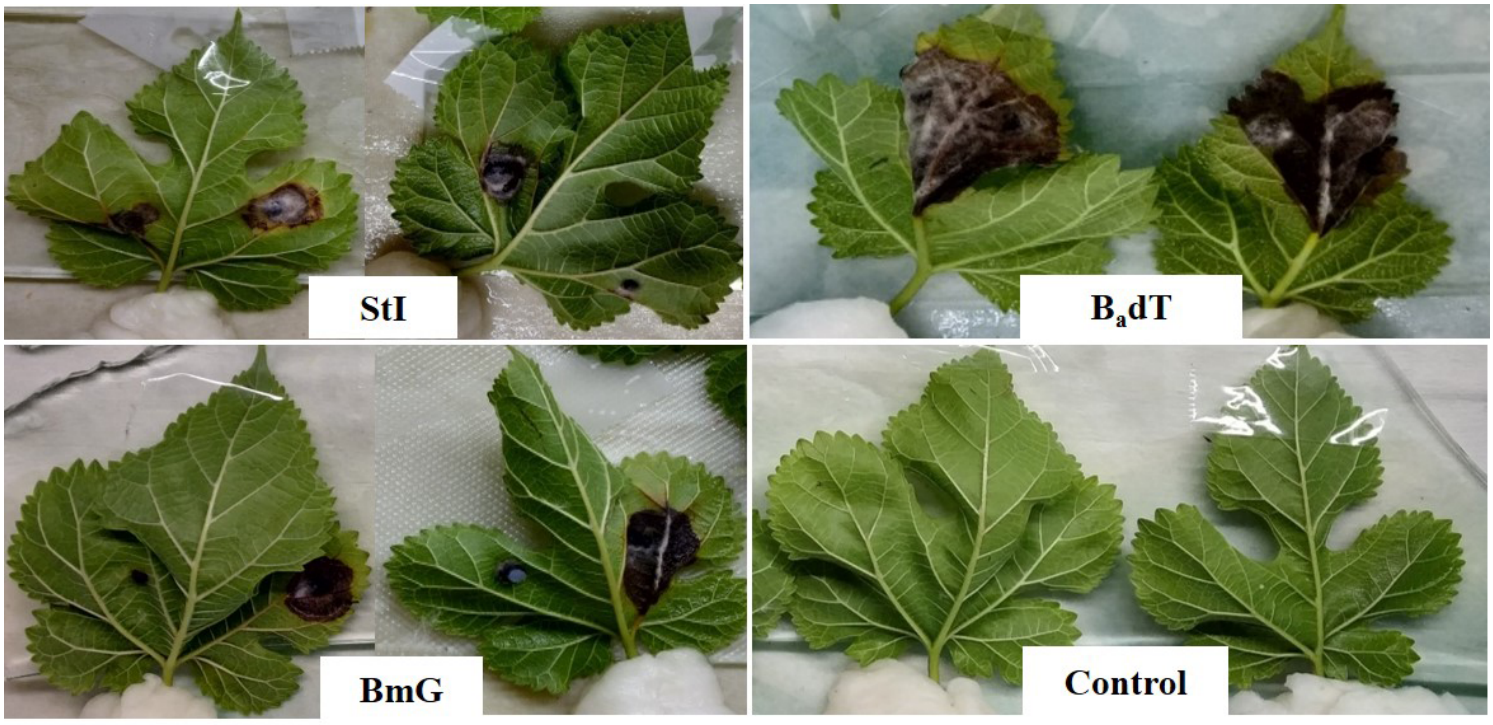

Figure 1

Pathogenicity and pathological variability of *Phloeospora maculans* using detached leaf technique

Figure 2

Growth characteristics of *Phloeospora maculans* isolates from Srinagar (1, 4, 7); Bandipora (2, 5, 8) and Baramulla (3, 6, 9) after 7 days (1-3), 10 days (4-6) and 20 days (7-9) of incubation at 24±1˚C on PDA medium

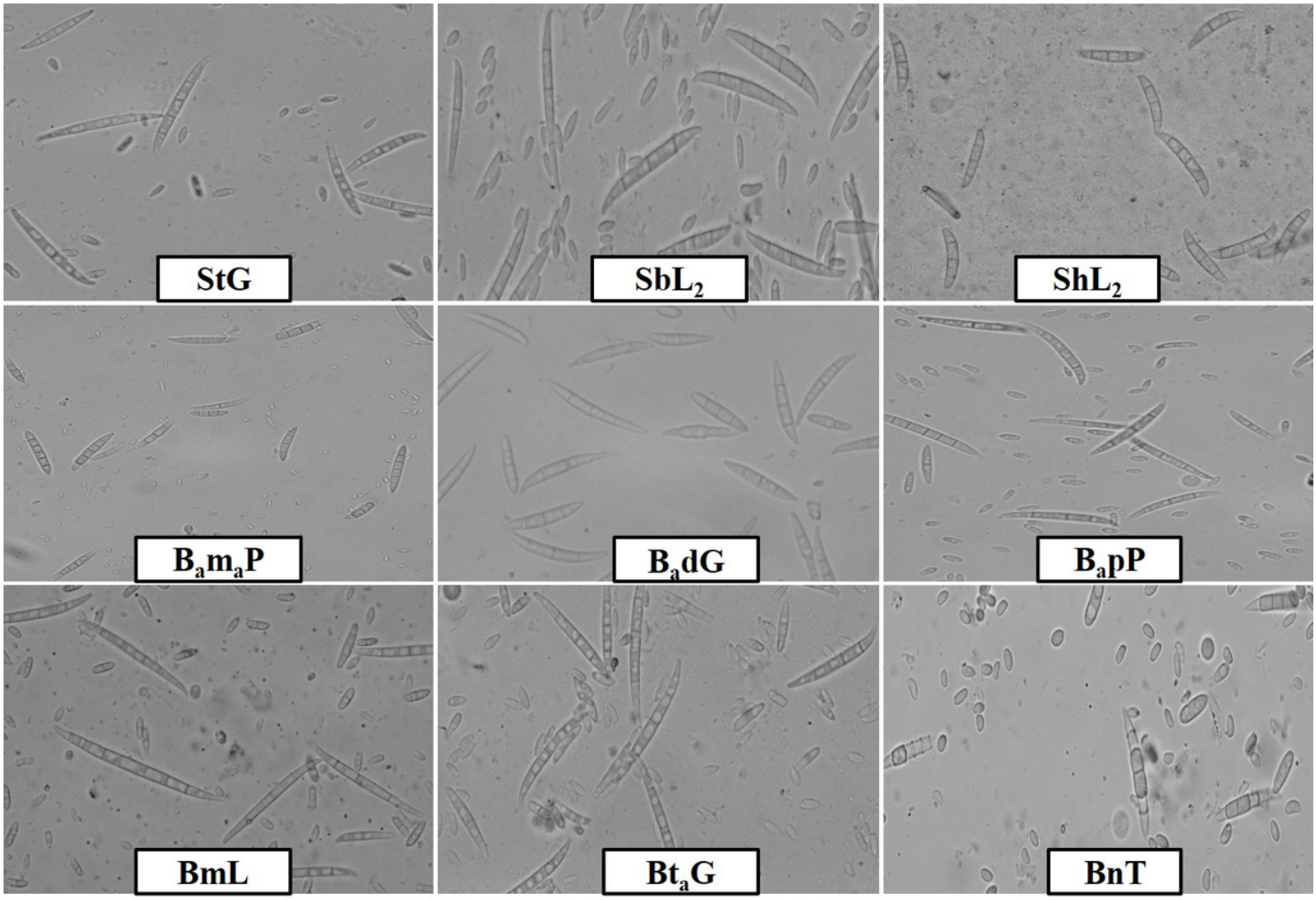

Figure 3

Conidia of of *Phloeospora maculans* from mulberry host collected from three districts of Kashmir

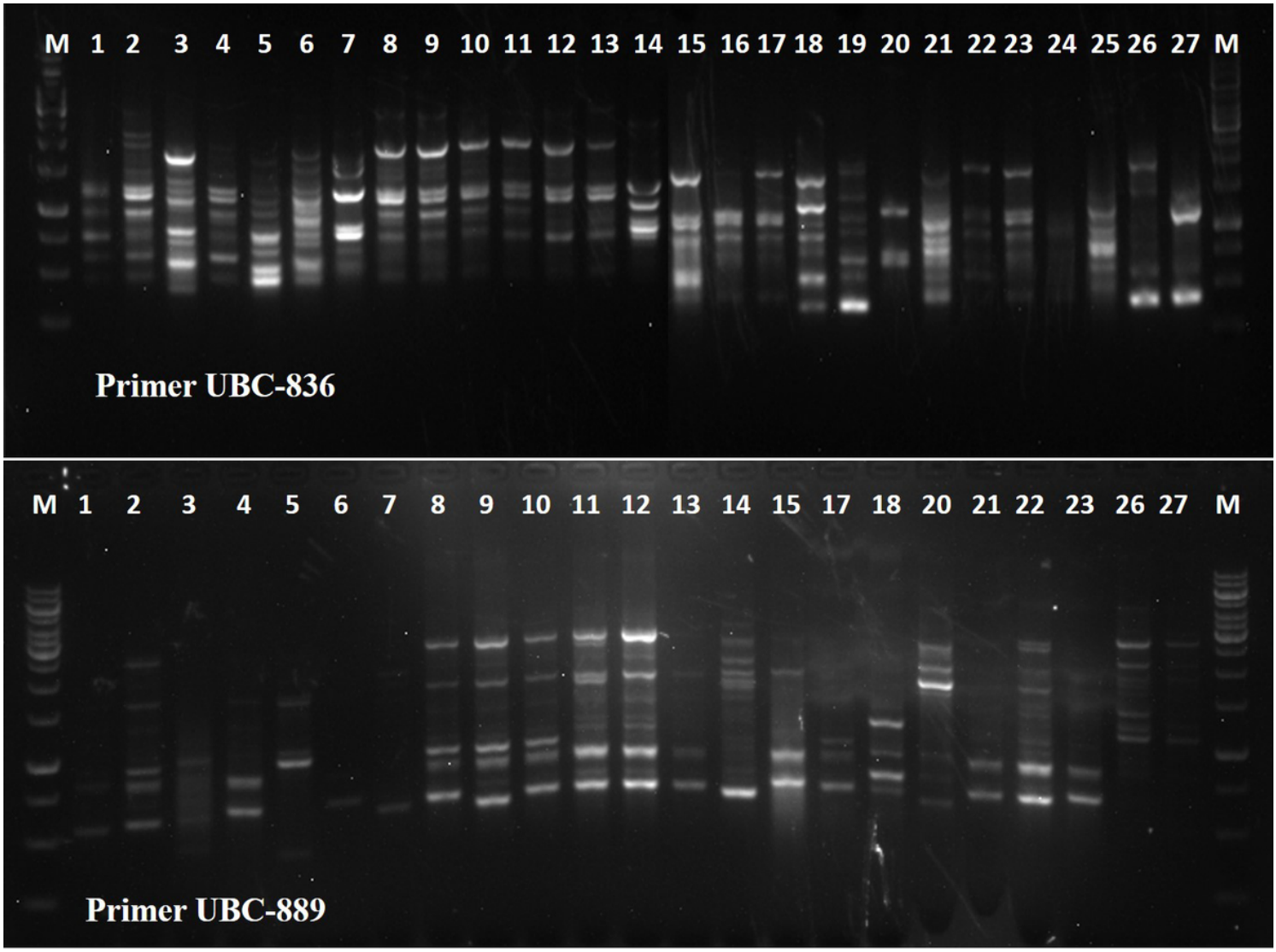

Figure 4

PCR amplification of *Phloeospora maculans* isolates using ISSR markers

Lane M: 1Kb DNA Ladder; Lane 1-27: Phloeospora maculans isolates

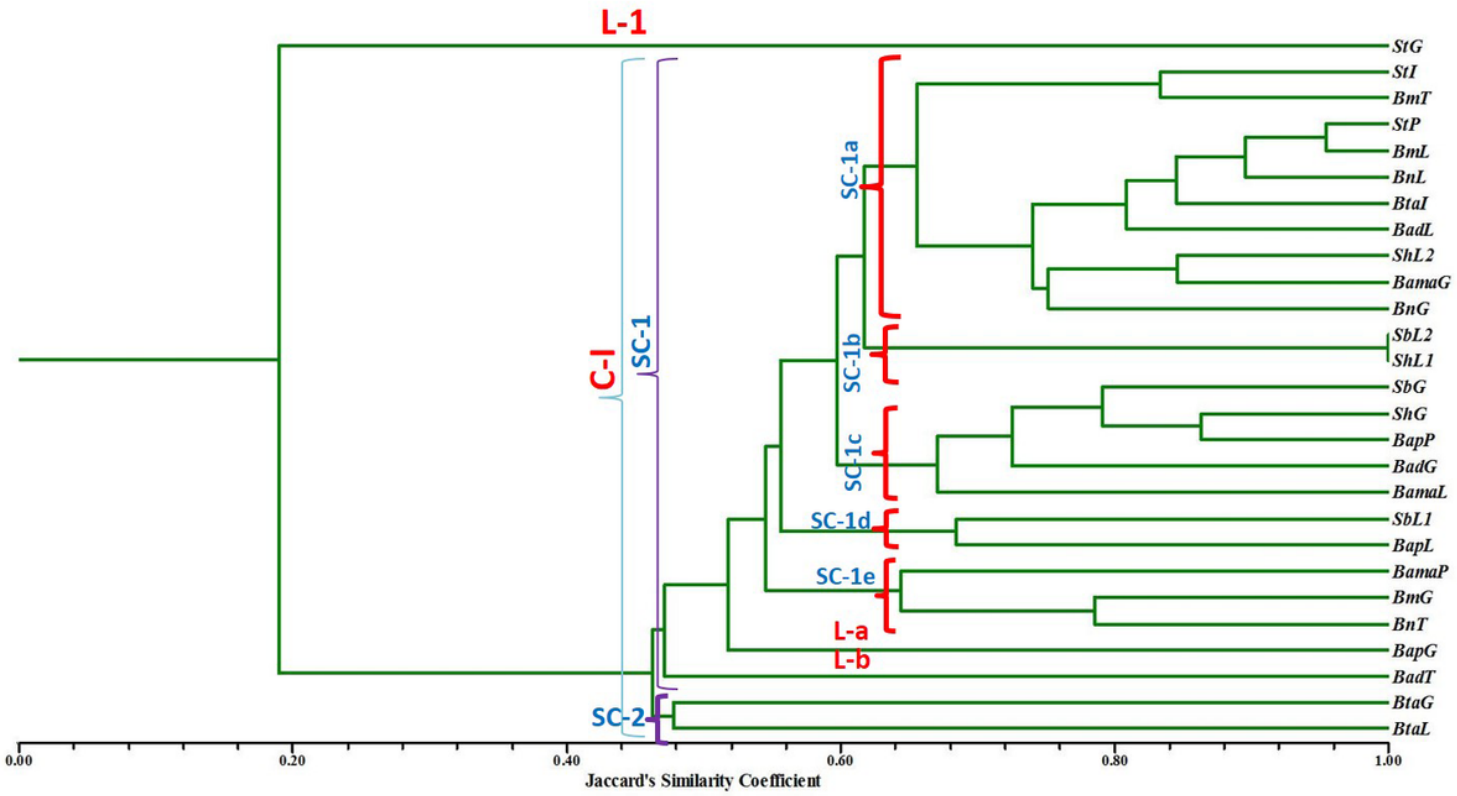

Figure 5

Phylogenetic relationship of *Phloeospora maculans* isolates using ISSR markers collected from different locations of mulberry growing areas of Kashmir

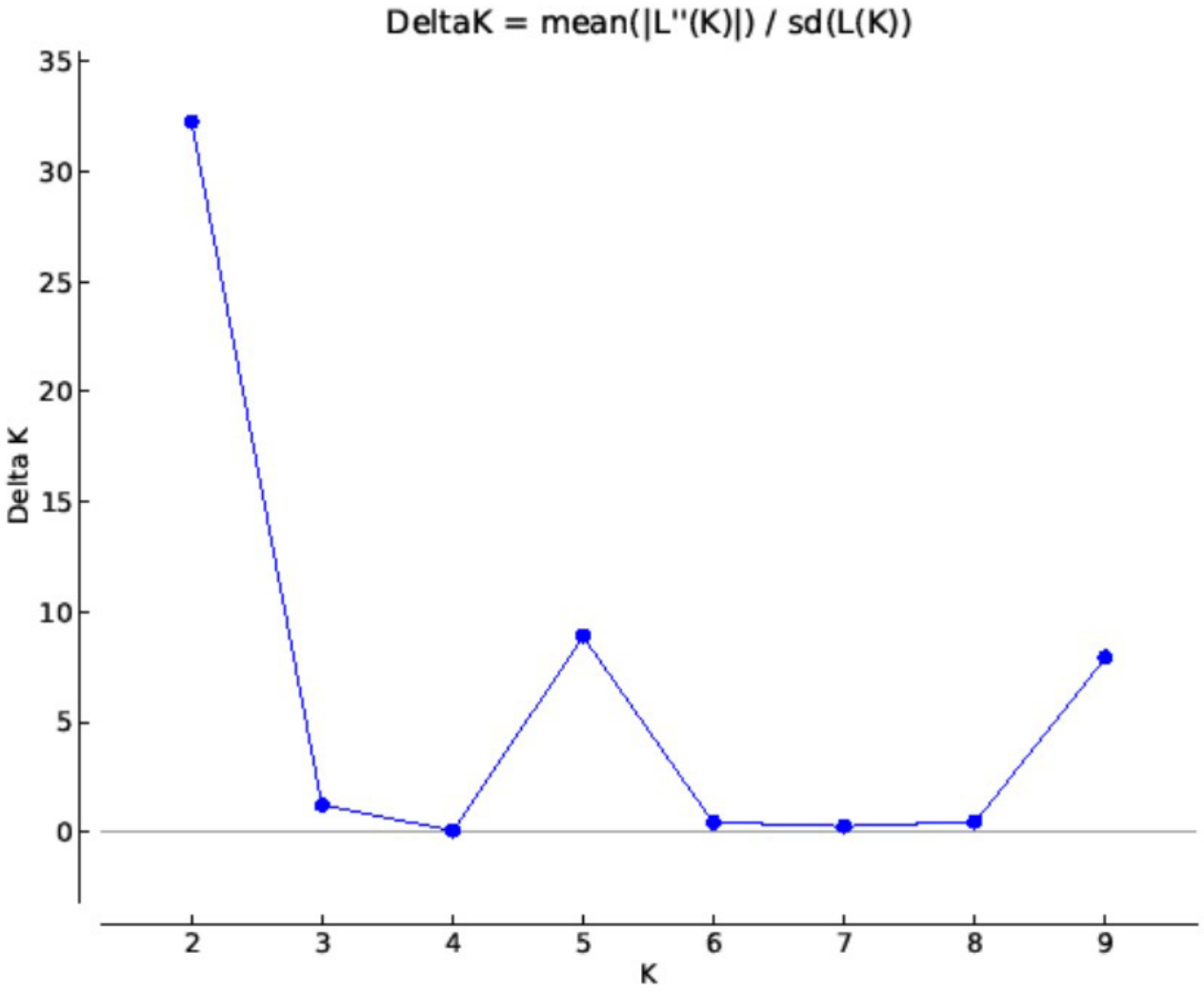

Figure 6

Delta K plot for *Phloeospora maculans* isolates of Kashmir valley

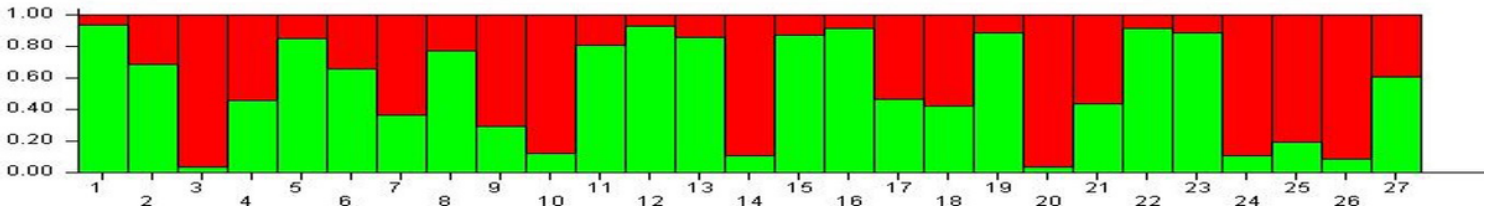

Figure 7

Structure plot showing *Phloeospora maculans* populations (red and green) prevalent in Kashmir valley