



Pathogenic variation and genetic diversity of *Alternaria sesami*, causal agent of leaf spot of sesame (*Sesamum indicum*)

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ABSTRACT

Sesame (*Sesamum indicum* L.), is an economically important oilseed crop. In sesame, the most important fungal disease is Alternaria blight, caused by *Alternaria sesami*. The present study was carried out during 2020–21 and 2021–22 at Sri Karan Narendra Agriculture University, Jobner, Jaipur, Rajasthan to analyse morphological and genetic differences among the various isolates of *Alternaria sesami*, which will provide a path for improved management against the pathogen. The study was conducted under a pot condition using the Alternaria blight-susceptible sesame cultivar (RT-46). *A. sesami* isolates were collected from diverse geographical regions of Rajasthan. Cultural studies revealed significant variation in colony morphology, size of conidia, and number of septa. Pathogenicity tests identified varying degrees of virulence, with the isolate AsJP1 showing the highest virulence and disease intensity (63.29%). RAPD markers revealed substantial genetic variation among *A. sesami* isolates, suggesting multiple sources of the pathogen. Phylogenetic analysis delineated distinct genetic clusters, highlighting potential evolutionary relationships among isolates. RAPD primers, viz. OPD-03, OPD-04, OPD-08, and OPD-10, showed 100% polymorphism, and the least polymorphism was observed with primer OPD-09 (95.34%). The present study will provide a platform to develop effective management strategies by unveiling the genetic variation and pathogenic variability of *A. sesami*.

Keywords: Genetic diversity, Molecular Marker, Plant Disease, RAPD, Sesame

Sesame (*Sesamum indicum* L.) is known as the oldest native oilseed crop in the world with the longest history of cultivation. Because of sesame's abundant, high-quality oil and significant economic importance, it is known as the "queen of oilseeds" (Dossa *et al.* 2016). Sesame seeds contain 50% oils, 25% proteins, and 15% carbohydrates. It covered about 10,245,246 ha of area globally (Sharaby and Butovchenko 2019) and 18–20 lakh ha in India. The overall loss of sesame yield in all parts of India due to this disease is 20–40% (Kolte 2018). Among all the diseases in sesame, Alternaria leaf spot, which is caused by *Alternaria sesami*, is more destructive and is considered a major constraint on production and productivity. The *A. sesami* causes the dark irregular patches on the leaf to show a blighted appearance in humid conditions. Stem and seed rot at later stages of disease development were also observed (Singh *et al.* 2020). Nikam *et al.* (2015) found that *Alternaria* spp. exhibit a

great degree of variability in terms of traits like pathogenic, cultural, morphological, nutritional, physiological, and molecular traits. Genetic variability of *Alternaria* spp. has been done to evaluate the genetic differences among the isolates (Eswarappa *et al.* 2011) but specifically *A. sesami* was less explored and pint-size information on the genetic diversity of this particular pathogen has been available (Lupascu *et al.* 2019).

The overall aim of the study was to provide comprehensive morphological and genetic differences among the various isolates of *Alternaria sesami*, which will provide a path for improved management against the pathogen.

MATERIALS AND METHODS

Collection and isolation of Alternaria sesame: The present study was carried out during 2020–21 and 2021–22 at Sri Karan Narendra Agriculture University, Jobner, Jaipur, Rajasthan. The collection of samples was done from the different geographical locations of Rajasthan (latitude-25.8593–27.1556 and longitude-73.5554–75.7584) (Table 1). For the collection of samples, a stratified random sampling method was adopted during the crop's harvesting stage because of the highest visibility of symptoms. A

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typical symptom was collected in individual butter paper envelopes to ensure the purity of the infected samples and labeling was done. A standard method was used to isolate *Alternaria*, and later, a single-spore isolation technique was used. Later, purification was done in PDA media plates at $26 \pm 1^\circ\text{C}$. The purified culture was kept at 4°C in the test tubes for further use.

Cultural and morphological variability: Cultural characteristics observations, viz. colony diameter, colony colour, appearance, growth rate, shape, margin, zonation, and sporulation, were recorded. The morphological characteristics, viz. width of hypha, conidia size, and length of the beak of each test isolate, were done with the help of fluorescence microscopy at 40X and the eyepiece magnification was 10X. Similarly, the transverse and longitudinal septa/conidium numbers were counted. The filter used for fluorescence brighter 28 excitations of 330–385 nm, dichroic mirror DM 400 nm, and the barrier filter >420 nm.

Pathogenic variability of different isolates of *Alternaria* sesami: The susceptible sesame variety RT-46 was grown in pots at polyhouse conditions with the replication of 5 pots. The optimum temperature was $25 \pm 2^\circ\text{C}$ and the relative humidity was $>85\%$. The virulence of 28 isolates was checked on the sesamum plants. For this purpose, a spore suspension of 1×10^6 conidia/ml was obtained from the 15-day-old culture of the 28 isolates. The spore suspension was sprayed on 25 day old sesamum plant. Within a week after the inoculation, the sesamum plant showed the symptoms. Based on symptoms, disease intensity was recorded with the help of a severity scale (Zhao *et al.* 2014).

DNA isolation and purification: The purified culture of *Alternaria sesami* isolate was transferred into the conical flask of 250 ml containing potato dextrose broth and kept it in the rotatory shaker at 150 rpm for the uniform growth of the pathogen. After 20 days, the mycelium growth was harvested, and excess moisture was dried into the laminar airflow at 26°C . After that, the mycelium was crushed with liquid nitrogen. The powdered mycelium was transferred into the Eppendorf tube of the DNA isolation kit of Hi Media™, and the procedure for DNA isolation was then followed. After getting the DNA, the quality of the DNA was checked in the nanodrop as well as the gel electrophoresis unit. 1.8% of agarose gel was prepared. Pure DNA was diluted up to 20 ng/1 for further use in the PCR.

PCR analysis of different isolates of *A. sesami* using RAPD primers: From different pieces of literature, a total of 50 RAPD primers were assessed, and 10 selected RAPD markers were used in this study (Table 1). A thermocycler (BioRad™, USA) was used for the amplification process. A PCR master mix of 10 μl including 0.5 μl forward primer, 0.5 μl reverse primer, and 2 μl DNA template were used during the amplification process. The amplification was observed with the 35-step reaction cycle with conditions of 95°C for 5 min, annealing at an appropriate T_m for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 5 min with a holding temperature of 40°C for 10 min. The

PCR product was run into the gel electrophoresis unit in the 2.5% agarose gel. Differences in the base pairs among the isolates were scored in the Excel sheet by giving a 0 and 1 score. The similarity matrix was then subjected to the unweighted pair group method with an arithmetical mean (UPGMA) by DARwin software.

Population structure and gene flow: The Structure 2.3.4 software, which worked on the principle of inferred genome frequency, was used to identify the distinct population and admixture among the *Alternaria* isolates (Pritchard *et al.* 2010). The input data in text format were taken for the structure software 2.3.4, from the binary scoring of presence or absence of amplification of base pairs across the loci. The optimum population size was determined by evaluating $K = 1$ to $K = 10$. The length of the burning period was 1,50,000 iterations. The probability of log data $\{\text{LnP(D)}\}$ is used to calculate the value of K based on how quickly LnP(D) changes between iterations of K .

Gene diversity, principal coordinate analysis (PCoA), and analysis of molecular variance (AMOVA): The genetic diversity analysis was done based on major allele frequency, observed heterozygosity, gene diversity, and PIC (Polymorphic Information Content) of RAPD markers obtained by Power Marker™ software (Liu and Muse 2005). The AMOVA and PCoA were analyzed by using the Gene Alxv6.503 software. The other genetic parameters like F_{st} and Gene flow were also analyzed for more genetic diversity indication (Peakall and Smouse 2012).

RESULTS AND DISCUSSION

Cultural and morphological variability among various isolates of *Alternaria* sesame: Fluorescence Microscopy was used to examine the pathogen's cultural and morphological diversity. The result showed that isolates of *Alternaria sesami* varied in their colony nature and variations in colony diameter were displayed in Table 1 and Supplementary Fig. 1. Similar studies were also done by Al-Lami *et al.* (2020) and Manjunatha *et al.* (2022) where they identified *A. Sesame* based on conidial length, breadth beak length, and cultural characteristics.

Isolates AsPA-3, AsSM-2, AsSM-4, AsJD-3, AsKR-3, AsBK-4, AsAJ-2, AsAJ-3, AsJP-1, AsJP-3, and AsJP-4 had the highest radial expansion of colony diameter (90.00 mm) on the third-day after incubation, and these isolates were classified as fast-growing. Isolates AsPA-4, AsSM-1, AsJD-2, AsKR-2, AsKR-4, AsBK-2, AsBK-3, AsAJ-1, AsJP-2, AsPA-1, AsJD-4, AsAJ-4, AsPA-2, and AsJD-1, on the other hand, was classified as moderately growing isolates. While AsBK-1 (65.00 mm), AsKR-1 (78.00 mm), and AsSM-3 (81.00 mm) had the slowest colony diameter development, these isolates were all classified as slow-growing fungi. Most of the isolates showed a whitish colony color to grayish with a smooth and rough margin, a cottony to feathery surface, and a circular to irregular shape. Zonations were also observed in some isolates, viz. AsJD-1, AsJD-2, AsAJ-2, AsJP1, and AsJP-3.

Morphological observations of each isolate presented

in Table 1 and Supplementary Fig. 1 revealed that conidia were hyaline, straight to sub-straight, muriform, comprising 1–4, vertical and horizontal septa, and the size of conidia ranged from 12.39×5.90 (isolate AsAJ4) to 49.76×15.24 μm (isolate AsJP1) in size, respectively. Abo-Ghazala *et al.* (2019) studied the characterization of *Alternaria sesami* and differentiated them into fast-growing and slow-growing colonies, initially white but later stages grayish in colour, with conidia having hyaline beaks and the number of horizontal and vertical (both) septa ranges from four to twelve.

Pathogenic variability of different isolates of *Alternaria sesami*: Results on percent disease intensity showed that the isolates confirmed to be pathogenic to sesame and capable of producing all of the disease's symptoms, which included tiny isolated light brown to dark brown circular spots (1–2 mm dia.) on the lower leaves that eventually spread to the upper leaves. Semi-arid eastern plain isolate, i.e. AsJP1, had the highest percentage of disease intensity (63.29%), followed by AsJP2 (61.29%), AsJP3 (59.75%), and AsJP4 (58.28%). AsBK4 isolate from the region Hyper Arid Partially Irrigated Western Plain had the lowest disease (18.28%) (Table 1). In our study it was found that the semi-arid eastern plain region have highest disease severity than the other agro-climatic zones of Rajasthan. This variation could be possible due to favourable temperature (22°C to 29°C) and relative humidity (75–92%) at the semi-arid eastern plains i.e. Ajmer and Jaipur regions. Fagodiya *et al.* (2021) performed epidemiological studies in various parts of Rajasthan and they found the highest incidence of *Alternaria* leaf spot of soybean at the Udaipur region which shares the same weather conditions as in semi-arid regions of Rajasthan. Nayyar *et al.* (2017) studied pathogenic variability among *Alternaria brassicae*, *Alternaria brassicicola*, and *A. raphanin* causing leaf blight of mustard. They reported all three species as strongly pathogenic on mustard, which induced the symptoms within 72 h of incubation.

Molecular variability of *Alternaria sesami* isolates

Diversity analysis using RAPD markers and marker informativeness: The genomic DNA of 28 isolates of *A. sesami* isolated from leaf blight-diseased specimens of sesame were subjected to PCR amplification using RAPD primers. Ten random primers, viz. the OPD series, were tested. Such RAPD profiling of *Alternaria solani* was done by Lieminger *et al.* (2013) who found distinct genetic diversity among *Alternaria* spp., isolates originating from the same field. More studies on the exploration of genetic diversity were also done by SSR, inter primer binding site DNA profiling in other species of *Alternaria* (Adhikari *et al.* 2021). The average size of the amplicons generated by the test primers ranged from 0.1 kb to 10 kb. The RAPD-PCR amplification results showed that about 10 RAPD primers generated a total of 506 bands, which were found to be polymorphic with an average of 49.90 bands per primer. The primers OPD-03, OPD-01, OPD-05, and OPD-02 were found to be more informative, as they could generate

a total number of 69, 57, 55, and 53 bands, respectively, followed by the primers OPD-07 (48), OPD-10 (47), OPD-04 (46), OPD-08 (46), and OPD-09 (43) and the lowest number of bands generated by the primer OPD-06 (42). Primers OPD-03, OPD-04, OPD-08, and OPD-10 showed 100% polymorphism, whereas other primers, i.e. OPD-01 (98.24%), OPD-05 (98.18%), OPD-02 (98.11%), OPD-07 (97.91%), and OPD-06 (97.61%) showed polymorphism, and the least polymorphism was observed in primer OPD-09 (95.34%).

Various information from the RAPD markers was derived, and these values decipher the actual and virtual diversity in the isolate genome. The highest allele was observed at OPD1, with the highest band frequency of 0.38, while the lowest band frequencies were observed in OPD4 and OPD10 (0.38). Variations in the number of alleles (N_a) were observed and varied from 1.33–2.0. The effective number of alleles was highest in OPD1 (1.82), and the lowest was in OPD4 and OPD10. Polymorphic Information Content (PIC) was highest in the markers OPD1, OPD5, and OPD6 and lowest in OPD4 and OPD10. The small h denotes the diversity among the isolates, which was revealed by the OPD markers. The highest diversity was revealed by the marker OPD1 (0.44), while it was lowest in the markers OPD4 and OPD10 (Table 2). The RAPD markers have more coverage for amplifying the chromosomes of the targeted organism which is why the PIC content and the genetic diversity were found high in our study in the confined region isolates (Masiello *et al.* 2020). In this study, RAPD markers have been used, which do not reproduce the same bands across the genome of an organism, but have some qualities like excellent detection of polymorphism, being useful for studying unknown genomes, being a multilocus marker system, being effective with minimum DNA, being low-cost, rapid, and easy. Some other workers also used RAPD markers for genetic diversity analysis (Masurkar *et al.* 2022), and they observed these parameters in the population genetics of *Ustilagoidea virens*.

Cluster analysis of RAPD DNA fingerprint: The dendrogram was depicted using Jaccard's similarity coefficient and genetic similarity matrix. The dendrogram generated based on UPGMA analysis of RAPD data grouped all 28 isolates of *Alternaria sesami* into three major clusters (Fig. 1). These clusters were formed based on the genus as well as zones. In Cluster I, there were two isolates of *Alternaria sesami*, i.e. AsBK-2 and AsPA-1, whereas Cluster II comprised 12 isolates of *Alternaria sesami*, i.e. AsJD-1, AsAJ-2, AsJD-2, AsPA-2, AsSM-1, AsKR-2, AsAJ-1, AsKR-1, AsBK-, AsJP-1, and AsJP-2, while Cluster III comprised only one isolate, i.e., AsSM-2. These results were found similar to the study of Adhikari *et al.* (2021). They found the three clusters of *Alternaria* spp. in their studies.

Population structure: Population structure was determined based on maximum likelihood and maximum ΔK values.

The RAPD marker analysis showed a peak at $K = 2$, which means two sub-populations were present, i.e. sub-

Table 1 Cultural, morphological, and pathogenic variability of *Alternaria sesami*

Agro-climatic zones	Isolate code	Colony dia. (mm)	Colour	Surface	Shape	Margin	Zonation	Average size of conidia (μm)	No. of septa (Horizontal: Vertical)	Pooled PDI (%)
Transitional plain of luni basin	AsPA1	87	Whitish	Feathery	Circular	Smooth	Absent	27.56×11.23	3:2	54.2
	AsPA2	85	Greyish	Cottony	Irregular	Rough	Absent	29.21×13.78	4:3	51.84
	AsPA3	90	Light greyish	Feathery	Circular	Smooth	Absent	28.11×10.76	3:2	48.72
	AsPA4	88	Whitish	Feathery	Circular	Smooth	Absent	26.45×8.87	3:2	50.35
Humid S-E plain	AsSM1	88	White turned in to grey	Cottony	Circular	Smooth	Absent	23.43×7.54	3:2	45.92
	AsSM2	90	Dark grey	Cottony	Circular	Smooth	Absent	25.67×8.40	4:2	43.35
	AsSM3	81	White turned in to grey	Cottony	Circular	Smooth	Absent	24.90×7.87	3:2	42.21
	AsSM4	90	Whitish	Cottony	Circular	Smooth	Absent	25.75×8.23	3:1	46.47
Arid western plain	AsJD1	85	Greyish	Fluffy	Irregular	Rough	Present	48.15×16.29	5:3	39.34
	AsJD2	88	White turned to grey	Cottony	Circular	Smooth	Present	46.79×15.37	4:2	37.28
	AsJD3	90	Whitish	Cottony	Circular	Smooth	Absent	43.56×14.82	4:2	34.81
	AsJD4	87	Whitish	Feathery	Circular	Smooth	Absent	41.78×12.43	3:2	36.21
Flood prone eastern plain	AsKR1	78	Greyish	Cottony	Irregular	Rough	Absent	15.80×4.90	3:1	44.32
	AsKR2	88	Light grey	Cottony	Irregular	Rough	Absent	17.43×5.49	2:1	41.72
	AsKR3	90	Whitish	Cottony	Circular	Smooth	Absent	18.24×7.78	3:2	38.91
	AsKR4	88	Whitish	Cottony	Circular	Smooth	Absent	14.86×4.30	2:1	36.31
Hyper arid partially irrigated western plain	AsBK1	65	Whitish	Feathery	Irregular	Smooth	Absent	29.67×13.11	3:2	22.17
	AsBK2	88	Whitish	Feathery	Circular	Smooth	Absent	28.91×12.34	3:2	19.29
	AsBK3	88	Whitish	Feathery	Circular	Smooth	Absent	31.56×14.53	3:1	21.78
	AsBK4	90	Whitish	Feathery	Circular	Smooth	Absent	30.87×13.45	3:2	18.28
Semi-arid eastern plain	AsAJ1	88	Greyish	Cottony	Irregular	Smooth	Absent	13.78×4.67	2:1	36.39
	AsAJ2	90	Greyish	Cottony	Circular	Smooth	Present	14.20×5.34	3:2	34.9
	AsAJ3	90	Whitish	Fluffy	Circular	Smooth	Absent	13.60×4.35	2:1	33.2
	AsAJ4	87	Whitish	Cottony	Irregular	Rough	Absent	12.39×5.90	2:1	34.31
Semi-arid eastern plain	AsJP1	90	White turned to greyish brown	Cottony	Circular	Smooth	Present	49.76×15.24	4:3	63.29
	AsJP2	88	Whitish	Cottony	Circular	Smooth	Absent	47.34×14.20	3:2	61.29
	AsJP3	90	Whitish	Feathery	Circular	Smooth	Present	44.10×10.38	4:3	59.75
	AsJP4	90	Whitish	Feathery	Circular	Smooth	Absent	38.78×9.56	3:2	58.28

Table 2 Genetic variation among different isolates of *Alternaria sesami* through RAPD markers

Locus	BF	Sequence '5-3'	Tm	BP	N	Na	Ne	PIC	h	%P
OPD1	0.38	ACCGGAAGG	38	10	28.00	2.00	1.82	0.63	0.44	98.24
OPD2	0.34	GGACCAACC	38	10	28.00	2.00	1.78	0.62	0.43	98.18
OPD3	0.36	GTCGCCGTCA	38	10	28.00	2.00	1.78	0.62	0.43	98.11
OPD4	0.18	TCTGGTGAGG	32	10	28.00	1.33	1.44	0.37	0.25	97.91
OPD5	0.37	TGAGCGGACA	38	10	28.00	2.00	1.80	0.63	0.44	97.61
OPD6	0.36	ACCTGAACGG	32	10	28.00	2.00	1.81	0.63	0.44	96.23
OPD7	0.31	TTGGCACGGG	38	10	28.00	1.78	1.68	0.54	0.38	98.33
OPD8	0.17	GTGTGCCCCA	38	10	28.00	1.44	1.37	0.38	0.26	96.33
OPD9	0.21	CTCTGGAGAC	32	10	28.00	1.56	1.51	0.44	0.30	95.34
OPD10	0.18	GGTCTACACC	32	10	28.00	1.33	1.44	0.37	0.25	97.12
Ave.	0.29				28.00	1.74	1.64	0.52	0.36	0.37

*BF, Band frequency; Tm, Annealing temperature; BP, Base pair; Na, No. of different alleles; Ne, No. of effective alleles = $1 / (p^2 + q^2)$; PIC, Polymorphic information content; h, Diversity = $1 - (p^2 + q^2)$; %P, Percent polymorphism.

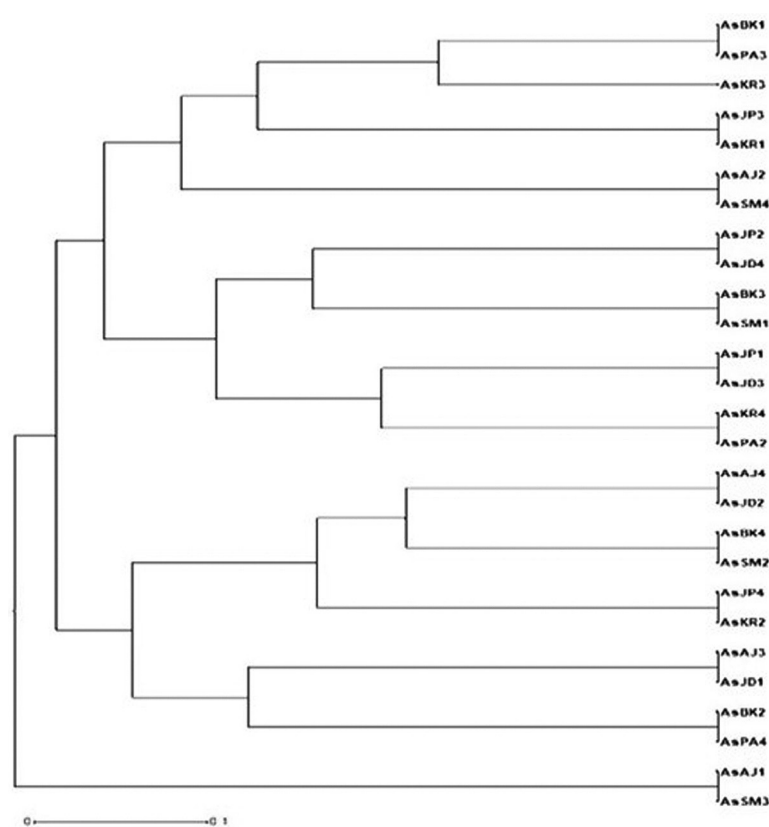


Fig. 1 Dendrogram based on RAPD analysis depicting relationship between 28 isolates of *Alternaria sesami*.

population 1 and sub-population 2. Each bar represented an individual isolate of the *Alternaria* population. The population structure was built on the genomic similarities between the genomes of the isolates. Samples with an inferred genome fraction value (membership proportion) of 0.66 or more subgroups are considered pure populations, and those less than 0.66 are considered admixtures. Generally, for differentiation between the sub-populations, the inferred genome frequency will be higher than 0.60.

In sub-population 1, isolates were viz. AsPA1, AsPA2, AsPA3, AsPA4, AsSM1, AsSM2, AsSM3, AsSM4, AsJD1, AsJD2, AsJD3, AsJD4, and in sub-population 2, isolates viz. AsKR1, AsKR2, AsKR3, AsKR4, AsBK1, AsBK2, AsBK3, AsBK4, AsAJ1, AsAJ2, AsAJ3, AsAJ4, AsJP1, AsJP2, AsJP3, and AsJP4. While the 13th isolate showed the admixture having an inferred genome frequency of 0.50, these all sub-populations were organized based on K= 2 populations and only one isolate as an admixture (Fig. 2). Zelman *et al.* (2021) evaluated the population structure of the *Alternaria* spp. and found more than two sub-populations in the collected Moroccan isolates.

PCoA (Principal co-ordinate analysis): Principal component analysis was performed to analyze the genetic relationship among the isolates of *Alternaria sesami*. The first three axes accounted for 52.80% of the total variation, with 21.28%, 18.00%, and 13.52%, for PC axes 1, 2, and 3, respectively. Each principal component was derived from the eigenvalue calculation of genomic data. PCoA was performed based on geographical location, viz. Pali (population 1), Sawai Madhopur (population 2), Ajmer (population 3), Karauli (population 4), Jaipur (population 5), Bikaner (population 6), and Jodhpur (population 7).

No distinct genetic clustering has been seen among these isolates (Fig. 3). The visualization of genetic clustering of *Alternaria solani*, *A. alternata*, and *A. linariae* was seen through principal components and suggested the genetic differentiation among them (Adhikari *et al.* 2021) which contradicts our research. PCoA as a method for genetic diversity exploration observed in *Cercospora beticola* with regional origin (Knight *et al.* 2019).

AMOVA: This analysis showed the portion of genetic

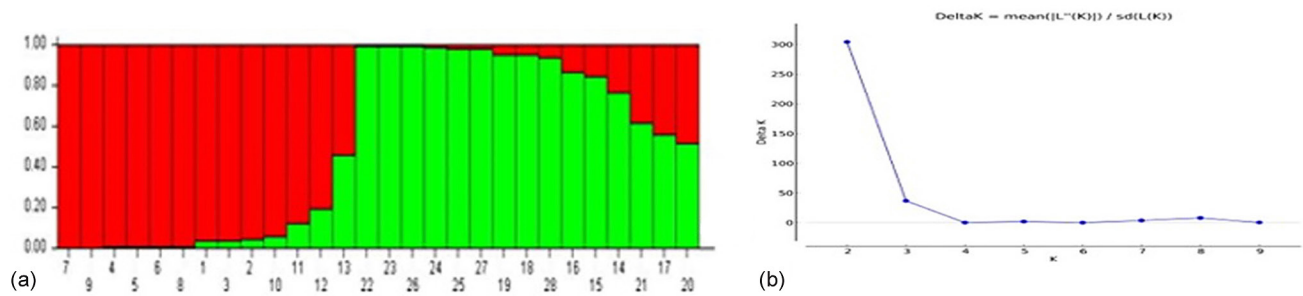


Fig. 2 Estimated population delta K obtained from 28 isolates of *Alternaria sesami* (a) The Bar diagram represents the isolates, those in red are one sub-population, and isolates in green one in another sub-population; (b) The maximum value of sub-populations present in 28 *Alternaria sesami* isolates.

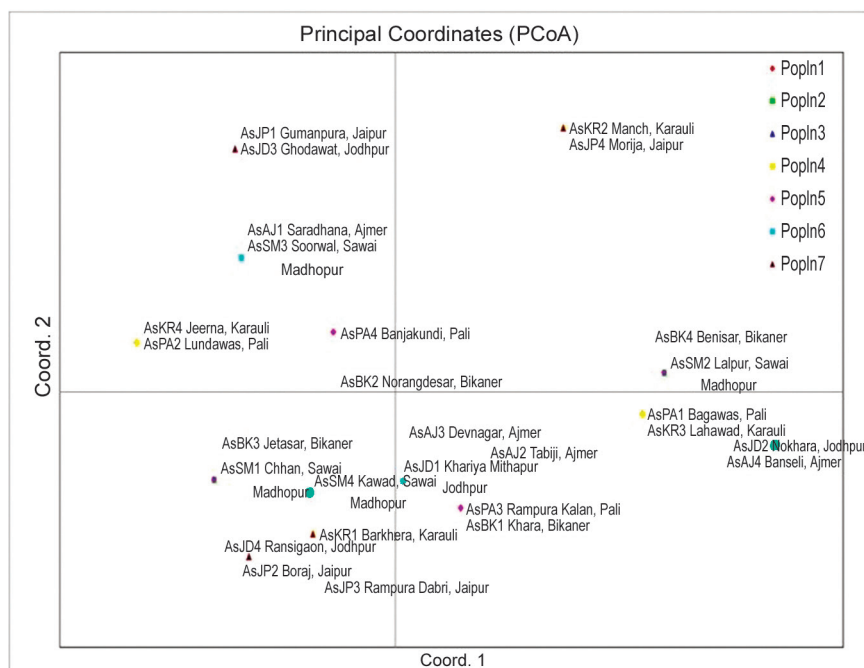


Fig. 3 Principal coordinate analysis based on the genetic clustering showing the tree-clustered sub-population.

relatedness among the population and within the population. In our experiment, the variation among the population was nil, but the variation within the population was high, i.e. 100% (Supplementary Fig. 2). Less than 5% genetic variance among the *Alternaria* isolates was observed (Adhikari *et al.* 2021), while Zelman *et al.* (2021) found 99% variation in the genetic structure of *Alternaria* spp. within the population in diverse geographical locations which was similar to our findings.

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