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Elucidation of genetic diversity and population structure of sixty genotypes of *Aloe vera* using AFLP markers

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ABSTRACT

Aloe vera has been used since ancient times for cosmetics and medicinal uses. In the present study, sixty genotypes were collected from different agro-climatic zones to evaluate genetic variation using amplified fragment length polymorphism markers. A total of ten primer combinations were used, which generated a total of 16,578 fragments from 419 loci with an average of 41.9 loci per primer. The resolving power of the primers ranged from 24.86 to 87.20 with an average of 55.23 and polymorphic information content ranged from 0.92 to 0.98 with an average of 0.95. Clustering pattern and principal coordinate analysis revealed a considerable genetic variability among the selected genotypes. The population genetic analysis was carried out in terms of some essential genetic diversity parameters *viz.*, Na, Ne, H, I, NPL, PPL, Hs, Ht, Nm, and Gst, which indicated a high degree of genetic diversity. Analysis of molecular variance revealed 7.46% genetic variation among the groups and 91.46% among genotypes. The degree of freedom and variance component observed were 2 and 3.496, respectively, among the groups and 57 and 42.858, among the genotypes. Population structure analysis assumes three populations that are consistent with an initial grouping of the genotypes based on their geographical regions. Furthermore, it is concluded that the AFLP markers were found an ideal tool for the genetic diversity in *Aloe vera* because it is a highly reproducible, highly polymorphic, high throughput, and cost-effective method. Observed results can be used for proper maintenance, validation, and effective utilization of *Aloe vera* genotypes.

1. Introduction

Medicinal plants have been a vital source of both curative and preventive medical therapy preparations for human beings. They are also a genetically diverse group due to more decadent gene combinations (Ercisli et al., 2011; Rasool, 2012; Cosge Senkal et al., 2019). *Aloe vera* has secured a special place for their use as food, medicines, and cosmetics among the available medicinal plants. The name *Aloe* derived from the Arabic word “Alloeh” meaning “shining bitter substance,” whereas “*vera*" in Latin means "true." The Greek researchers considered *A. vera* as the complete solution. The Egyptian people entitled aloe as "the plant of immortality" (Surjushe et al., 2008). It is indigenous to Africa and the Mediterranean. However, it is now cultivated worldwide except in tundra, deserts, and rainforests countries (Rajeswari et al., 2012). In India, *A. vera* is known as Komarika, Grithkumari, Vazha, Kattar, Korphad, and many other provincial names. Approximately 530 species of *Aloe* were reported and most of them are used worldwide for their therapeutic properties. Among these, *A. vera* Linn Syn., Common aloe (*A. vera* L.) is considered most potent compared to other species, like *Aloe ferox, Aloe arborescens, Aloe perryi*, and *Aloe greatheadii* (Babu and Noor, 2020). It belongs to the subfamily Xanthorrhoeaceae and family Asphodelaceae (Klopper et al., 2010). It is a shrub, xerophytic, succulent, perennial, pea-green color, a monocot, crassulacean acid metabolism (CAM) plant (Liu et al., 2011).

Today, apart from pharmacological activities, *A. vera* is used for cosmetic and toiletry industries. The beautifying preparations comprise various moisturizing creams, shampoos, cleansers, shaving creams, and bath soaps (Jadhav et al., 2020). Like vitamin A derivatives, it also has an anti-aging effect (Danhof, 2006) and used to treat even AIDS (Yamaguchi et al., 1993). The high concentration of *A. vera* in toothpaste reduces gingivitis and plaque (Sajjad and Sajjad, 2014). *A. vera* comprises countless medicinal properties due to their unique secondary metabolite content. For example, the *A. vera* gel comprises

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more than 75 active ingredients, which includes lignin, saponins, anthraquinones (aloe-emodin, aloeitic acid, anthranol, barbaloin, ester of chrysophanic acid, aloin, and ethereal oil), amino acids, vitamins (C, A, E, Choline, and B_{12}), minerals (iron, chromium, copper, zinc, potassium, calcium, sodium, manganese, magnesium), sugars (monosaccharides and polysaccharides), enzymes (lipase, peroxidase, aliases, catalase, cellulase, carboxypeptidase, alkaline phosphatase, and amylase), sterols, and salicylic acid (Hamman, 2008; Raksha et al., 2014).

Due to the commercial potential of *A. vera*, it is of utmost importance to access the genetic diversity in *Aloe* genotypes for its genetic improvement program (Arolu et al., 2015). Different physiological markers can measure genetic diversity in plants. However, the morphological markers are not reliable because they can be affected by climatic conditions and stages of development. In contrast, molecular markers are more prevailing tools for studying genetic diversity and relationships among genotypes (Bisrat et al., 2000). Molecular markers help in improving the effectiveness of molecular breeding to many folds because they are closely related to the trait of interest. Furthermore, transcriptome sequencing of *A. vera* carried out by Choudhri et al. (2018) will provide new insights for the development of advanced markers system as well as to study the genes involved in the production of essential metabolites.

Various types of molecular markers are available to analyze the genetic diversity in medicinal plants. Among them, the amplified fragment length polymorphism (AFLP) technique is considered a robust and highly polymorphic DNA fingerprinting method used to analyze genetic diversity, population genetic analysis, AMOVA, and population structure analysis of plants (Rozo et al., 2009). There is a tremendous advantage of AFLP; unlike the other DNA fingerprinting techniques for genetic variability study, it does not require the genome sequence's prior information (Blignaut et al., 2013). AFLP analysis consists of the complete digestion of DNA with restriction endonucleases, ligation of specific adapters designed from the restriction site of the enzymes to the digested fragments, followed by PCR amplification (Sorkheh et al., 2007). The amplified product from the ligated DNA can be separated by non-denaturing polyacrylamide gel electrophoresis (Hiransuchalert et al., 2020). AFLP markers contribute to preserving the genetic resources for the development of new varieties with improved characters.

Previously assessment of genetic diversity in *A. vera* has been carried out using RAPD markers (Darokar et al., 2003; Nejatzadeh Barandozi et al., 2012; Panwar et al., 2013; Chikkaswamy, 2014; Chandra and Choudhary, 2014; Das et al., 2017). However, until now few attempts were undertaken to study genetic diversity in *A. vera* using AFLP (Tripathi et al., 2011) and RAPD and ISSR markers (Rathore et al., 2011; Bhaludra et al., 2014; Kumar et al., 2016) with fewer genotypes. Studies on genetic diversity in *A. vera* are required not only for conservation but also for proper maintenance, validation, and effective utilization. Scanty information is available on genetic diversity analysis in *A. vera* using AFLP markers to the best of our knowledge.

The present study comprised of sixty *Aloe* genotypes collected from different agroclimatic regions of India and categorized into three groups based on their site of collection i.e. arid zone, temperate zone, and coastline zone of India to estimate the extent of genetic diversity and relatedness.

2. Materials and methods

2.1. Plant material and DNA isolation

The plant materials were procured from the National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India. *Aloe* genotypes were collected from different agro-climatic regions by NBPGR i.e., arid zone, temperate zone, and coastline zone of India. A total of sixty indigenous accessions were sampled and raised in natural conditions for study. Details of the genotypes with their accession number and site of the collection are shown in Table 1. Young leaf samples were used for the isolation of genomic DNA due to their lesser secondary

Table 1
Details of the 60 genotypes of *A.* vera with their accession number and their site of collection. All the 60 genotypes are collected from 3 agro-climatic zones: arid, temperate, and coastline.

Sr. No	Accession number	Collection site	State	Sr. No	Accession number	Collection site	State
1	IC 112514	Jodhpur	Rajasthan	31	IC 281340	Delhi	Delhi
2	IC 112521	Bikaner	Rajasthan	32	IC 326744	Khandwa	Madhya Pradesh
3	IC 112519	Nagaur	Rajasthan	33	IC 112530	Churu	Rajasthan
4	IC 112518	Nagaur	Rajasthan	34	IC 112515	Jodhpur	Rajasthan
5	IC 112511	Jodhpur	Rajasthan	35	IC 112539	Jhunjhunu	Rajasthan
6	IC 112523	Ganga Nagar	Rajasthan	36	IC 326744	Khandwa	Madhya Pradesh
7	IC 112513	Jodhpur	Rajasthan	37	IC 281340	Delhi	Delhi
8	IC 112512	Jodhpur	Rajasthan	38	IC 337889	Bolangir	Orissa
9	IC 112516	Jodhpur	Rajasthan	39	IC 436193	Pithoragarh	Uttarakhand
10	IC 112517	Jodhpur	Rajasthan	40	IC 436191	Pithoragarh	Uttarakhand
11	IC 112531	Churu	Rajasthan	41	IC 527335	Sawai Madhopur	Rajasthan
12	IC 112527	Ganga Nagar	Rajasthan	42	IC 520360	Puducherry	Tamil Nadu
13	IC 111280	Faridabad	Haryana	43	IC 520389	Puducherry	Tamil Nadu
14	IC 111267	Chittor	Rajasthan	44	IC 527344	Ajmer	Rajasthan
15	IC 111279	Anand	Gujarat	45	IC 527346	Ajmer	Rajasthan
16	IC 471886	Delhi	Delhi	46	IC 527160	Sawai Madhopur	Rajasthan
17	IC 471882	Delhi	Delhi	47	IC 520361	Vilupuram	Tamil Nadu
18	IC 111269	Chittor	Rajasthan	48	IC 520358	Puducherry	Tamil Nadu
19	IC 471883	Delhi	Delhi	49	IC 527342	Ajmer	Rajasthan
20	IC 112526	Hanumangarh	Rajasthan	50	IC 422483	Darjeeling	West Bengal
21	IC 111271	Banswara	Rajasthan	51	IC 527334	Sawai Madhopur	Rajasthan
22	IC 112533	Churu	Rajasthan	52	IC 520364	Vilupuram	Tamil Nadu
23	IC 112573	Bidar	Karnataka	53	IC 527336	Sawai Madhopur	Rajasthan
24	IC 112572	Bidar	Karnataka	54	IC 527328	Karoli	Rajasthan
25	IC 112522	Bikaner	Rajasthan	55	IC 527343	Ajmer	Rajasthan
26	IC 112534	Jhunjhunu	Rajasthan	56	IC 527340	Ajmer	Rajasthan
27	IC 471884	Delhi	Delhi	57	IC 527338	Sawai Madhopur	Rajasthan
28	IC 112520	Bikaner	Rajasthan	58	IC 524161	Allahabad	Uttar Pradesh
29	IC 471885	Delhi	Delhi	59	IC 524197	Rai Bareli	Uttar Pradesh
30	IC 112532	Churu	Rajasthan	60	IC 524163	Allahabad	Uttar Pradesh

metabolite content. Genomic DNA from the plant samples was isolated by the CTAB method (Murray and Thompson, 1980) with minor modifications. The recovered pallet was subjected to RNase *H* and proteinase *K* treatment to remove the possible contamination of RNA and proteins in the DNA. After treatment, the DNA samples were reprecipitated and dissolved in Tris EDTA (TE) buffer. The purity of DNA was tested on agarose gel and the quantity of DNA was done by a Nanodrop spectrophotometer. The observed values (A_{260}nm /A_{280} nm) range from 1.7 to 1.9, indicating the good quality of DNA (Sambrook et al., 1989). High purity of DNA was obtained and stored at -20 °C for AFLP analysis.

2.2. AFLP fingerprinting

AFLP was performed according to Vos et al. (1995) using Amplified Fragment Length Polymorphism AFLP® products of Invitrogen Life Science Technologies according to manufacturer protocol with minor modification (Blignaut et al., 2013). Extracted genomic DNA of 60 genotypes of *A. vera* were purified by Phenol: Chloroform:Isoamyl alcohol solution (25:24:1) to remove the contaminants. Dilution of good quality genomic DNA was carried out to a final concentration of 100 ng/μl. For the preparation of the template, isolated genomic DNA was digested with two restriction enzymes (*Eco*RI *and Mse*I, 1.25 U/μl each in, 50 mM NaCl, 10 mM Tris HCl (pH 7.5), 0.1 mM EDTA, 0.1 mg/ml BSA, 1 mM Dithiothreitol (DTT), 0.1% Triton® X-100, 50% (v/v) glycerol)) simultaneously in 0.6 ml centrifuge tube including the 5X reaction buffer (50 mM Mg acetate; pH 7.5, 250 mM *K*-acetate) and incubated at 37 °C for 3 h. After digestion, the samples were incubated at 70 °C for 15 min for the inactivation of the restriction enzymes. For the ligation reaction, the digested DNA was subjected to adapter/ligation solution (*Eco*RI/*Mse*I adapters, 10 mM Tris-HCl; pH 7.5, 0.4 mM ATP, 50 mM K-acetate and 10 mM Mg-acetate) and 1 μl of T_4 DNA ligase (1 U/μl in 10 mM Tris-HCl; pH 7.5), 1 mM DTT, 50 mM KCl, and 50% (v/v) glycerol) and incubated at 20 °C for 2 h. After ligation, the digested-ligated product was diluted (1:10) with TE buffer (0.1 mM EDTA and 10 mM Tris-HCl (pH 8.0) and used as a template for pre-amplification PCR. Pre-amplification was carried out in 15 μl pre-amplification reaction consisting of 2.5 μl of the diluted digestion-ligation reaction mixture, 1 mM *Eco*RI +0, 5 mM *Mse*I+0 primer, 1X Taq buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 2 U of Taq DNA polymerase. The amplified product was analyzed on agarose gel, and the observed smear confirmed the amplification. Ten combinations of selective AFLP primers were used in the present study and generated repeatable, unambiguous, and highly polymorphic fragments. Details of AFLP primers are included in Table 2.

The successfully amplified products were diluted (1:19) with nuclease-free water and used as the template for selective AFLP. The selective amplification reaction was performed with 1 mM *Eco*RI selective primer, 5 mM *Mse*I selective primers using touchdown PCR. Thermal profile for amplification includes 1 cycle at 95 °C for 5 min; 12 cycles at 95 °C for 60 s; annealing each cycle by 0.7 °C (65 °C to 56 °C); extension at 72 °C for 2 min; initial denaturation at 95 °C for 5 min, 25 cycles of final denaturation at 95 °C for 60 s, annealing at 56 °C for 60 s, initial extension at 72 °C for 60 s and one cycle of final extension at 60 °C for 30 min. The resulted PCR products from selective amplification were resolved by denaturing polyacrylamide gel on sequencing gel apparatus (Sequi Gen-GT, Bio-Rad). In order to get a better resolution (Vuylsteke et al., 2007; Kumar et al., 2018) 6% denaturing gel (29:1 acrylamide:bisacrylamide solution, 1X TBE buffer, 7 M urea, (0.15% (v/v) TEMED and 10% (w/v) APS) of 38 × 30 cm and 0.4 mm thickness was used. The amplified product was loaded onto the gel after adding 1X loading dye and electrophoresis for about 2 h at 50 W. After completion, the glass plates were separated, and the outer plate containing the gel was subjected to silver staining. The denaturing polyacrylamide gel was silver-stained by using the method of Kumar et al. (2015). Stained polyacrylamide gels were seen on a white light background and photographed.

Table 2
Details of the AFLP primers used in the study and their sequence.

Sr. No.	Primer Name	Sequence (5′-3′)
1	EcoR1 a	CTCGTAGACTGCGTACC
2	EcoR1 b	AATTGGTACGCAGTC
3	Mse I a	GACGATGAGTCCTGAG
4	Mse I b	TACTCAGGACTCAT
5	EA	GACTGCGTACCAATTCA
6	MC	GATGAGTCCTGAGTAAC
7	E1	GACTGCGTACCAATTCAAC
8	E3	GACTGCGTACCAATTCACA
9	E4	GACTGCGTACCAATTCACT
10	M2	GATGAGTCCTGAGTAACAC
11	M3	GATGAGTCCTGAGTAACAG
12	M6	GATGAGTCCTGAGTAACTC
13	M7	GATGAGTCCTGAGTAACTG

2.3. Genetic diversity analysis

2.3.1. Data recording and statistical analysis

The genotypes were grouped based on their geographical region, i.e., arid zone, temperate zone, and coastline zone of India (Table 3). The grouping of the genotypes was carried out only for population genetic analysis and analysis of molecular variance (AMOVA). The genetic diversity among the *Aloe* genotypes was assessed by comparing the size of the amplified fragments of AFLP markers by visual observation. The marker alleles were coded '0′ for the absence and '1′ for the presence. The alleles with negligible intensity, diffused or smeared, were considered missing data and excluded from the final data analysis (Pandey et al., 2015). Polymorphism and genetic diversity analysis was assessed by the total number of loci (TL), the number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), and total amplified fragment (TF) calculated for each primer. The resolving power of each primer was calculated according to Prevost and Wilkinson (1999). Polymorphic information content (PIC) for each marker was calculated by using the formula of Anderson et al. (1993). The number of polymorphic loci in the germplasm set of interest known as an effective multiplex ratio (EMR) is also calculated by the formula EMR (E) = np(np/n), Where np is the number of polymorphic loci and n is the total number of loci of the marker because many loci were monomorphic in germplasm (Varshney et al., 2007). The marker index for each marker was calculated to estimate the utility of the marker.

2.3.2. Phylogenetic, AMOVA, and population genetic analysis

A phylogenetic tree was constructed based on the unweighted neighbor-joining method, and the Dice coefficient was calculated based on the dissimilarity matrix. To get the better reliability of the construction, 1000 bootstrapping were used with DARwin (Version 6.0.14) the software program (http://darwin.cirad.fr/darwin/Home.php) (Moges et al., 2016). For evaluating the genetic variation among the population and within the population, non-parametric analysis of AMOVA was done via GenAlEx (version 6.5) (Peakall and Smouse, 2006). Two-dimensional PCA has also been constructed for accurately testing the relationships among 60 genotypes of *A. vera* using the EIGEN program analysis. Nei's unbiased genetic distance among genotypes was calculated using POPGENE software (version 1.32). Several other genetic diversity parameters *i.e.*, observed number of alleles (Na), the effective number of alleles (Ne), Nei's genetic diversity (H), Shannon's information index (I), number of polymorphic loci (NPL), and percentage polymorphic loci (PPL) were also analyzed (Zhao et al., 2006). Within species diversity (Hs), total genetic diversity (Ht) (Nei, 1978) and gene flow (Nm) were calculated within

Table 3
Grouping of the Aloe genotypes based on their geographical region.

Sr. no.	Groups	A. vera genotypes
1	Group 1 (36 genotypes) (Arid zone)	1,2,3,4,5,6,7,8,9,10,11,12,14,15,18,20,21,22,25,26,28,30,33, 34,35,41,44,45,46,49,51,53,54,55,56,57
2	Group 2 (15 genotypes) (Temperate zone)	13,16,17,19,27,29,31,32,36,37,39,40,58,59,60
3	Group 3 (9 genotypes) (Coastline zone)	23,24,38,42,43,47,48,50,52

Table 4
The sequence of primers used for AFLP amplification, the total number of loci (TL), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), total fragments amplified (TF), resolving power (Rp), polymorphic information content (PIC), and Marker index (MI).

Primer Name	Primer Sequence	TL	NPL	PPL(%)	TF	RP	PIC	MI
AFLP 1	E-AAC+M-CAG	16	2	12.5	746	24.86	0.92	0.11
AFLP 2	E-AAC+M-CTG	61	16	26.22	2616	87.2	0.98	0.25
AFLP 3	E-ACA+M-CAC	38	9	23.68	1726	57.33	0.96	0.22
AFLP 4	E-AAC+M-CAC	50	20	40	1814	60.46	0.97	0.38
AFLP 5	E-ACA+M-CAG	42	15	35.71	1592	53.06	0.96	0.34
AFLP 6	E-ACA+M-CTG	60	22	36.66	2316	77.2	0.97	0.35
AFLP 7	E-ACT+M-AAG	32	13	40.62	1198	39.93	0.95	0.38
AFLP 8	E-ACT+M-CAG	50	19	38	1921	64.03	0.97	0.37
AFLP 9	E-ACT+M-CTC	30	12	40	1115	37.16	0.95	0.38
AFLP 10	E-ACT+M-CTG	40	15	37.5	1534	51.13	0.96	0.36
	Total/Average	419	143	33.08	16,578	55.23	0.959	0.31

the species and within three major groups. Grouping of the genotypes was made based on different agro-climatic regions (arid zone, temperate zone, and coastline zone of India).

2.3.3. Population structure analysis

To better understand the genetic pattern of *Aloe* genotypes, STRUCTURE (version 2.3.4), a Bayesian clustering software was used. It identifies subpopulations; assigns individuals to different populations and hybrid zones. The populations always remain unknown for STRUCTURE analysis. STRUCTURE assumes *K* (unknown) populations for the given genotyping data (Pritchard et al., 2000). For this purpose, both the ancestry model (Admixture Model) and the frequency model were used with a simulation of 10 iterations, with each iteration, consisted of 10,000 'burnin' followed by 100,000 Markov Chain Monte Carlo (MCMC) replications. The data generated by the STRUCTURE were harvested by using Evanno's method (Evanno et al., 2005) based program STRUCTURE HARVESTER developed by Earl and Vonholdt (2012). In which, the value of estimated Ln probability of data-LnP (*K*) and to get the best fit value of K for the data was determined.

3. Results

3.1. Genetic diversity and AFLP polymorphism

A total of ten primer combinations were used for the amplification of DNA (Table 4). All the randomly selected primers produced reproducible and scorable fragments. The representative gel picture for sixty *A. vera* genotypes amplified with AFLP 2-E-AAC+M-CTG is shown in Fig. 1. Ten primer combinations generated a total of 16,578 fragments. The total number of loci detected was 419, out of which 143 loci were found polymorphic. The number of loci ranged from 16 to 61 with an average of 41.9 loci per primer. The minimum number of loci was detected by the primer E-AAC+M-CAG, whereas the maximum number of loci was detected by the primer E-AAC+M-CTG. The percentage of polymorphic loci (PPL) ranged from 12.50% to 40.62% with an average of 33.08%. The minimum percentage of polymorphic loci was detected by the primer E-AAC+M-CAG, whereas the maximum percentage of polymorphic loci was detected by the primer E-ACT+M-AAG. The resolving power of the primers ranged from 24.86 to 87.20 with an average of 55.23. The polymorphic information content (PIC) of the primers ranged from 0.92 to 0.98 with an average of 0.95. The marker index for the AFLP primers was also calculated, ranging from 0.11 to 0.38 with an average value of 0.31.

Details of the AFLP markers in terms of the total number of loci (TL), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), total fragments amplified (TF), resolving power (Rp), polymorphic information content (PIC), and markers index (MI) are collated in Table 4.

3.2. Phylogenetic analysis

The unweighted neighbor-joining method with 1000 bootstrapping was adopted for phylogenetic analysis. The Cluster analysis

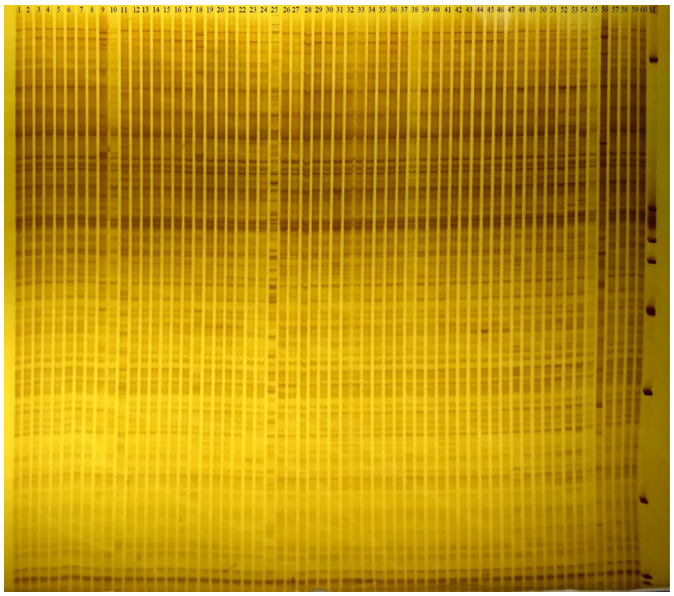

Fig. 1. Representative gel picture of sixty Aloe vera genotypes amplified with AFLP 2-E-AAC+M-CTG primer. *M* is the DNA size marker of 100 bp.

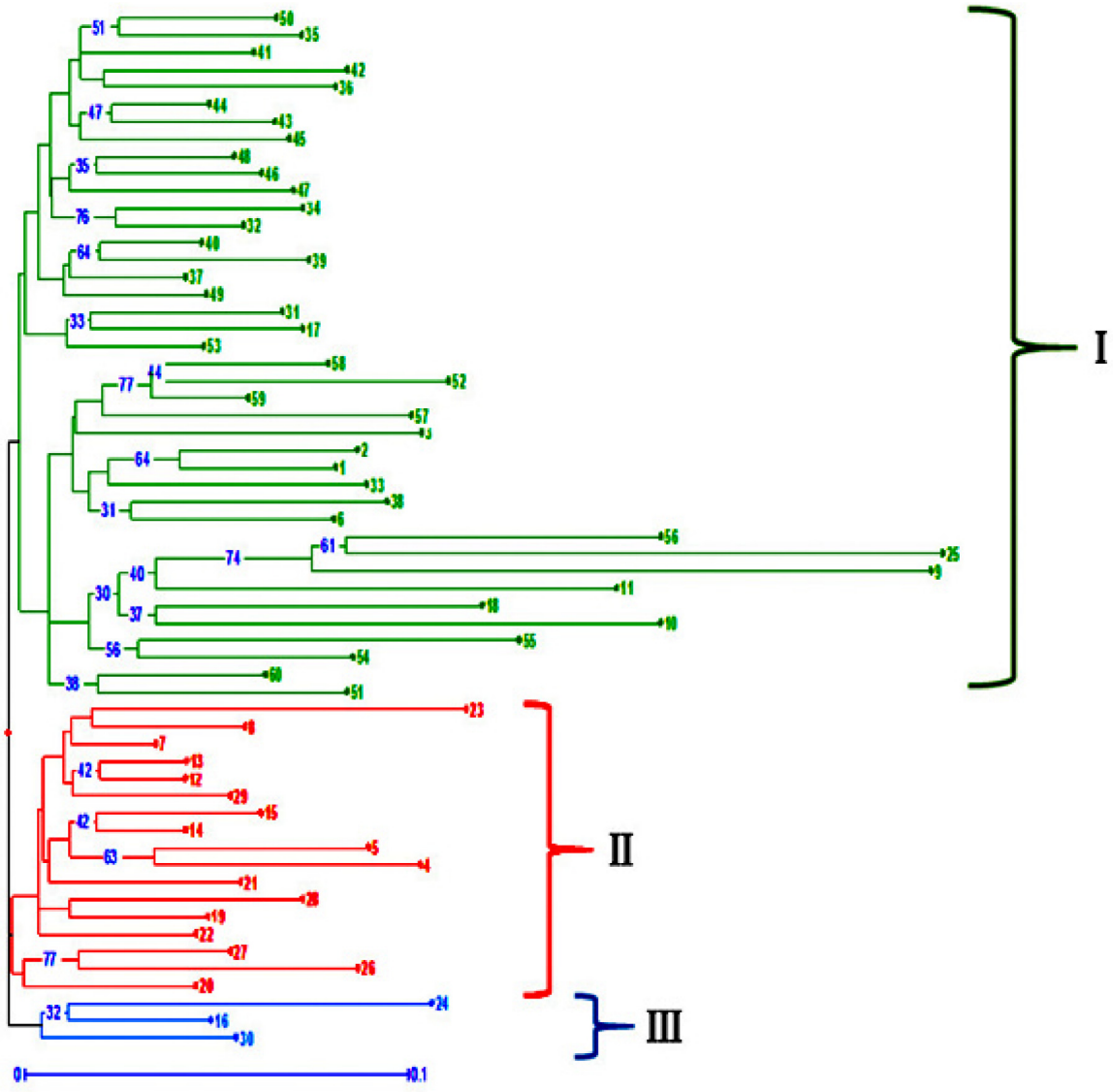

Fig. 2. Unweighted neighbor-joining dendrogram along with supported bootstrap showing the genetic relationship among sixty aloe genotypes using AFLP markers.

revealed the distribution of sixty *A. vera* genotypes into three major clusters (Fig. 2). The first major cluster consisted of a maximum number of genotypes (40) *viz.* 50, 35, 41, 42, 36, 44, 43, 45, 48, 46, 47, 34, 32, 40, 39, 37, 49, 31, 17, 53, 58, 52, 59, 57, 3, 2, 1, 33, 38, 6, 56, 25, 9, 11, 18, 10, 55, 54, 60, and 51. The first major cluster was further divided into two sub-clusters. The second major cluster consisted of seventeen genotypes *viz.* 23, 8, 7, 13, 12, 29, 15, 14, 5, 4, 21, 28, 19, 22, 27, 26 and 20. The third major cluster is the smallest cluster consisted of only three genotypes, *i.e.*, 24, 16, and 30. The clustering pattern suggested that there is a good fit genetic variability in the selected genotypes. Nevertheless, the distribution of the genotypes was not found exactly according to the initial grouping based on geographical locations.

3.3. Analysis of molecular variance

Analysis of molecular variance (AMOVA) was performed to estimate the genetic variability among genotypes and groups. Nine hundred ninety-nine permutations were used to establish the significance of the analysis. AMOVA analysis revealed 7.46% genetic variation among the groups and 91.46% among genotypes (Table 5). The degree of freedom and variance component observed among the

Table 5
Analysis of molecular variance (AMOVA) obtained in sixty *A.* vera genotypes using AFLP markers with respect to their geographical region.

Source of variation	Degree of freedom	Variance component	Percentage of variation	*P*-value
Among groups	2	3.496	7.46	< 0.001
Among genotypes	57	42.858	91.46	< 0.001

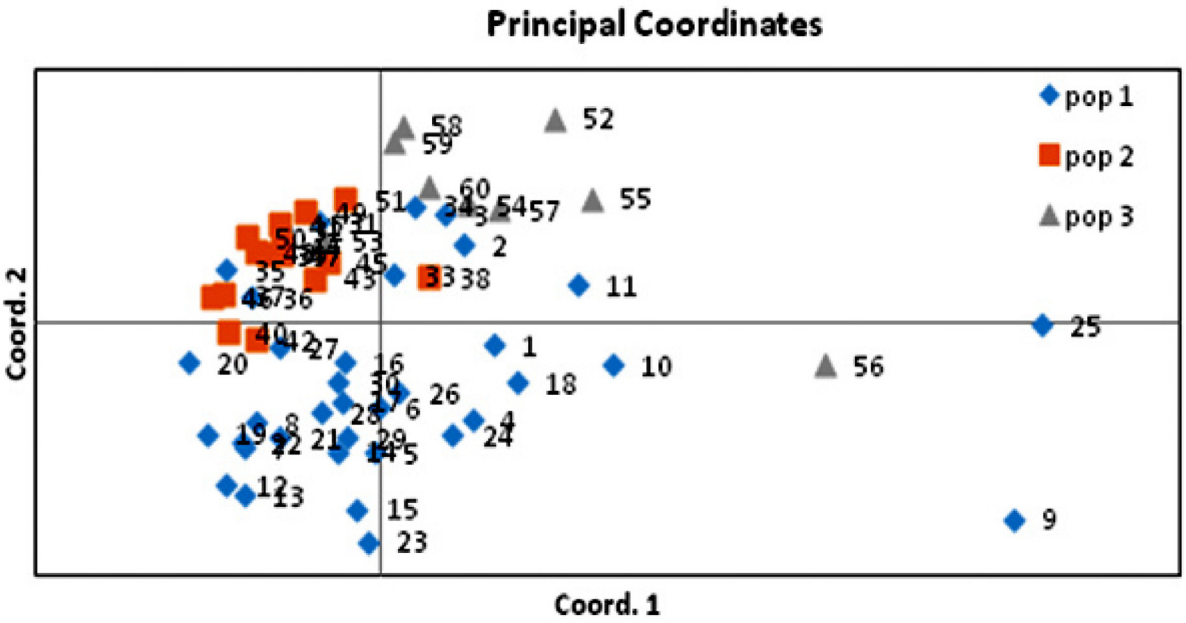

Fig. 3. Principal coordinates analysis of *A.* vera genotypes in two-dimensions space-based using AFLP data, where Pop 1, 2 and 3 represent arid, temperate and coastline zone groups, respectively.

groups were 2 and 3.496, respectively. The degree of freedom and variance component observed among genotypes are 57 and 42.858, respectively. The two-dimensional PCA was also constructed, which followed a similar distribution pattern observed from the dendrogram (Fig. 3). The percentage of variation for the two axes of PCA is 6.73% (coordinate 1) and 16.28% (coordinate 2).

3.4. Population genetic analysis

The population genetic diversity was carried out in terms of some crucial genetic diversity parameters and is collated in Table 6. The value of the observed number of alleles was 1.7780, 1.7613, and 1.8496 for arid, temperate, and coastline zone groups, respectively. The value of Ne was 1.2677 for arid, 1.3110 for temperate, and 1.3620 for the coastline zone group. The Nei's genetic diversity was found to be a maximum value of 0.2354 in the coastline, followed by 0.2011 in temperate and a minimum value of 0.1806 for the arid zone. Shannon's Information index was found to be 0.2957, 0.3208, and 0.3731 for arid, temperate, and coastline zones, respectively. The total genetic diversity was 0.1806, 0.2011, and 0.2354 for arid, temperate, and coastline zones, respectively. The PPL was found to be maximum in the coastline group (84.96%) followed by arid (77.80%) and temperate zones (76.13%) and indicated that a good fit genetic diversity in the *A. vera* genotypes. Values for all the parameters were found to be maximum in the coastline zone, indicated a high degree of genetic diversity among the genotypes.

The overall genetic variability across sixty genotypes in terms of genetic differentiation (Gst), gene flow (Nm), number of polymorphic loci (NPL), and percentage of polymorphic loci (PPL) are collated in Table 7. The value of Gst for overall genetic variability across sixty genotypes was found lower (0.0403), whereas the value of Nm was found higher (11.9105), which indicated significant genetic diversity. Out of 419, a total of 385 loci were detected as polymorphic with a polymorphic percentage of 91.89%, which also clearly indicates a high degree of genetic diversity among collected genotypes.

3.5. Population structure analysis

STRUCTURE analysis distributed the individuals to the possible population groups based on the allele frequencies of genotypes. Three clusters were observed without prior knowledge of the number of populations. With the use of the admixed model, the estimate of the likelihood of the data [LnP(D)] and value of ΔK was found maximum when K=3. For $K > 3$, LnP(D) increased slightly but more or less plateaued (Fig. 4 a,b). The observed values indicated that the software assumed three populations based on the allele frequency of the genotypes. The grouping of the bar plot was found similar to the neighbor-joining tree, but no clear demarcation was observed according to

Table 6
Summary of genetic variation statistics for all loci of AFLP among the Aloe genotypes with respect to their geographical region.

Geographical region	Sample size	Na	Ne	H	I	Ht	PPL
Arid	36	1.7780 (0.4161)	1.2677 (0.2662)	0.1806 (0.1489)	0.2957 (0.2142)	0.1806 (0.0222)	77.80
Temperate	15	1.7613 (0.4268)	1.3110 (0.2995)	0.2011 (0.1614)	0.3208 (0.2291)	0.2011 (0.0260)	76.13
Coastline	9	1.8496 (0.3578)	1.3620 (0.2820)	0.2354 (0.1500)	0.3731 (0.2092)	0.2354 (0.0225)	84.96

Na = Observed number of alleles; Ne = Effective number of alleles; H = Nei's gene diversity; I = Shannon's Information index; Ht = Total genetic diversity; PPL = percentage of Polymorphic Loci.

Table 7
Overall genetic variability across all the 60 genotypes of A. vera based on AFLP markers.

Sample size	Na	Ne	H	I	Ht	Hs	Gst	Nm	NPL	PPL (%)
60	1.9189 (0.273)	1.3139 (0.2699)	0.2104 (0.1421)	0.3439 (0.19)	0.2104 (0.0202)	0.2019 (0.0184)	0.0403	11.9105	385	91.89

Na = Observed number of alleles; Ne = Effective number of alleles; *H* = Nei's gene diversity; *I* = Shannon's Information index; Ht = Heterogeneity; Hs = Homogeneity; Gst = Gene differentiation; Nm = Gene flow (Nm = 0.5(1 - Gst)/Gst); NPL= Number of polymorphic loci; PPL = Percentage of Polymorphic loci.

arid, temperate, and coastline zone genotypes. A total of three clusters were observed in the bar plot with interference (Fig. 4c).

4. Discussion

In order to understand the degree of genetic variability among *A. vera* genotypes, a high-throughput AFLP maker was used at the accession level and the group level (based on the geographical regions). The results suggested that AFLP markers are effective tools to investigate genetic relationships among the genotypes of *A. vera*. Only a few reports are available to assess the genetic diversity in *A. vera* using AFLP markers. In contrast, several studies have been conducted for molecular characterization of genetic diversity in *A. vera* using RAPD and ISSR markers (Nejatzadeh-Barandozi et al., 2012, Panwar et al., 2013).

The present investigation establishes co-relation with the studies that have been conducted to evaluate genetic diversity in several medicinal plants by Sarwat et al. (2008), Shadia et al. (2014), Patil et al. (2015), Aichi-Yousfi et al. (2016), Jabri et al. (2016), Bhattacharyya et al. (2017), Szeliga et al. (2017) and Li et al. (2019) using AFLP markers in terms of the total number of loci (TL), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), total fragments amplified (TF), resolving power (Rp), polymorphic information content (PIC) and Markers index (MI) to assess the efficiency and effectiveness to evaluate genetic diversity. The polymorphic information content (PIC) of the primers in this investigation ranged from 0.92 to 0.98 with an average of 0.95, which is similar to the value reported by Shadia et al. (2014), El-Demerdash et al. (2019) and Nurmansyah-Alghamdi et al. (2020). PIC depends on the number of visible alleles, and the distribution of their frequency corresponds to the gene diversity. The present study demonstrated polymorphic loci ranging from 12.50% to 40.62% with an average of 33.08%, which is corroborated with the polymorphism reported by Domyati et al. (2011) for some medicinal plants. Our results also reveal similar

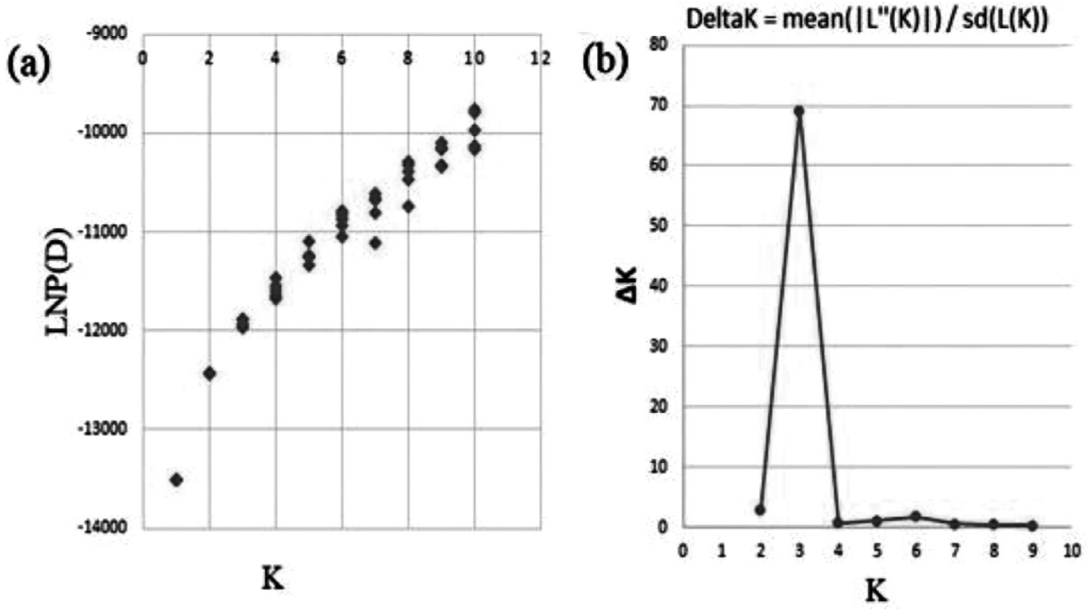

Fig. 4. a,b The relationship between the number of clusters (*K*) and the estimated likelihood of data [LnP(*D*)]; (b) Relationship between *K* and Δ*K* based on STRUCTURE analysis.

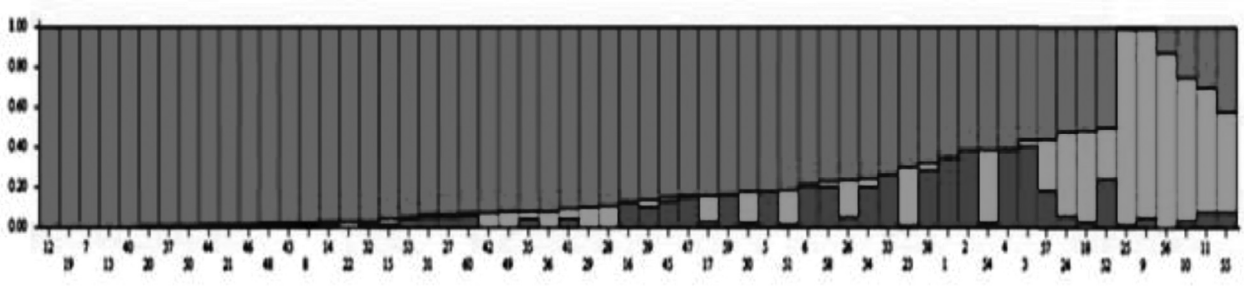

Fig. 4c. STRUCTURE analysis of *A.* vera genotypes based on AFLP data showing a grouping of genotypes when *K*=3.

polymorphism among *A. vera* accessions (48.5%) using AFLP markers, as reported by Tripathi et al. (2011). Clustering of 60 genotypes from different geographical regions of India by the unweighted neighbor-joining method with 1000 bootstrapping represented three major clusters I, II, and III consisted of 40, 17, and 3 genotypes, respectively. The genetic variation among the collected genotypes is in agreement with Nejatzadeh-Barandozi et al. (2012), Bhaludra et al. (2014), Kumar et al. (2016) and Rana and Kanwar (2017), who reported remarkable genetic variation among *Aloe* accessions based on RAPD and ISSR markers. Similarly, Kumar et al. (2015) and Das et al. (2017) grouped *A. vera* from geographically different regions of India in three major clusters with significant divergence by phylogenetic analysis yielded from SSR fragments and Internal Transcribed Spacers (ITS) based profiling, respectively.

Cluster analysis revealed that most genotypes of a specific geographical region were grouped, despite the mixing-in of some genotypes from different regions. For example, the genotypes collected from Rajasthan (Swai Madhopur and Ajmer), MP, Tamilnadu, UP, and UK showed a geographical significant relationship and grouped in one major cluster. However, the genotypes from Rajasthan (Churu, Jodhpur), Karnataka, and Delhi did not cluster together, suggesting that their geographical distances do not strictly relate to genetic distances revealed by AFLP markers. These findings can be evident from Tripathi et al. (2011), (2012) and Yu et al. (2014) clearly mentioned that a little or no genetic correlation was found with geographical distance when AFLP markers were applied to investigate genetic diversity. Results of the present study strongly support the findings of Panwar et al. (2013) in which grouping of *Aloe* genotypes from similar regions of India was not showing genetic relatedness at a gross level when analyzed by RAPD markers, but samples from Jodhpur and Ajmer showed 100% genetic similarity with each other as four genotypes from Jodhpur, and three from Ajmer shared one cluster. Nejatzadeh-Barandozi et al. (2012) also found little relationship between genetic divergence and geographical origins when RAPD based genetic diversity was assessed in *A. vera*. Recently, Kumar et al. (2019) determined the extent of morphological and biochemical diversity and relationships among 74 *Aloe* germplasm collected from the arid zone of India and revealed that the clustering pattern grouped the accessions partly as per geographical origin in respect of Jamnagar and Jalore. The resulted grouping pattern may be due to human interventions in the distribution of *Aloe*, such as selection and breeding.

Analysis of molecular variance (AMOVA) revealed the majority of genetic variation (94.59%) within genotypes compared to between the three clusters (5.79%), indicated that the level of intra-genotypic polymorphism is statistically significant. This pattern of genetic divergence was also found in previous studies on *A. vera* (Tripathi et al., 2011), *Disanthus cercidifolius* (Yu et al., 2014), *Dactylis glomerata* L. (Sun et al., 2017), and endangered *Glehnia littoralis* (Li et al., 2019). The degree of freedom and variance components are also consistent with the large scale of genetic variations among genotypes compared to among groups that could reflect gene flow among genotypes, especially at small distribution scales. The distribution of genotypes in two-dimensional PCA and clustering of genotypes in dendrogram confirm a similar variability pattern among 60 genotypes of *A. vera*. The exhibited pattern of genetic variation might be due to the vegetative propagation of *A. vera*, which is in agreements with the revealed variance in other vegetatively propagated species such as *Potamogeton maackianus* (Li et al., 2004), *Ligusticum chuanxiong* (Chen et al., 2010), *Chlorophytum borivilianum* (Tripathi et al., 2012), and *Tectona grandis* (Vaishnaw et al., 2015).

In population genetic analysis, different parameters observed indicated the degree of genetic diversity. The Shannon's diversity index, expected heterozygosity, and other genetic diversity parameters agree with the values reported in *S. purpurea* (Sulima et al., 2017) and *Indigofera pseudotinctoria* Mats (Fan et al., 2017). The observed value of Nei's genetic diversity, the observed number of alleles, expected heterozygosity, and homozygosity are higher in the current study than the values reported in *M. ciliaris* (Jabri et al., 2016), *Disanthus cercidifolius* (Yu et al., 2014) using AFLP markers. The observed results corroborate with the results of Kumar et al. (2016) using fifty-five *Aloe* genotypes using RAPD markers. However, all the genetic diversity parameters were inconsistent with the results of Hou and Lou (2011) except the value of gene flow, which indicated that more closely located populations tend to be more genetically similar to one another. The differences in the results might be due to differences in the types of plant species and the number of primers used in the investigation. The high value of Nm indicated the migration of more than one individual per population; therefore, the genotypes are expected to stay genetically stable for a long time. Lower gene differentiation causes higher gene flow. The observed values indicated the constant gene flow between the genotypes in *A. vera*. The reported high gene flow might be due to human interference, wind dispersal of propagules, or accidental transportation.

Three populations were observed in structure analysis which supports the initial grouping of the genotypes based on different agro-climatic conditions. The grouping of the bar plot was found similar to the neighbor-joining tree, but no clear demarcation was observed among the genotypes according to arid, temperate, and coastline zones. Similar results were observed in *Disanthus cercidifolius* (Yu et al., 2014) and *Rhodiola dumulosa* (Hou and Lou 2011) based on their geographical region. The observed results also supported the phylogenetic analysis. The distribution of genotypes in a bar plot is albeit different compared to the findings of Kumar et al. (2016) using RAPD and ISSR markers. The observed results follow the distribution pattern of dendrogram and PCA. The difference in the studied parameters may be due to different plant species for genetic diversity analysis. The interference might be due to the gene flow or human intervention through accidental transportation or excessive plantation.

In the present study, the AFLP technique was found to be informative and efficient to give good coverage of the genome of *A. vera*. Furthermore, it was helpful to elucidate the genetic variations and phylogenetic relationships among the genotypes collected from different locations in India. Observed resolving power and marker index reflected that AFLP is a great tool to evaluate the genetic diversity in *A. vera*. The ability to resolve genetic variation among different genotypes may be directly related to the number of polymorphisms detected with each marker technique. This is the first attempt to study genetic distance patterns using AFLP markers among a large population of *A. vera* from a natural location, which is very important for the plant breeding program.

Declaration of Competing Interest

The authors declare that they have no known competing financialinterestsor personal relationships that could have appeared to influence the work reported in this paper.

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