Original Article

MOLECULAR ASSESSMENT OF GENETIC DIVERSITY IN INDIAN ACCESSIONS OF *ALOE VERA* USING SSR MARKER

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ABSTRACT

Objective: In this study *Aloe vera* (L.) Burm. f. collected from 12 states covering all the different agro-climatic zones of India were investigated for its genetic diversity analysis by using SSR marker assay.

Methods: Total genomic DNA was isolated from young leaf samples using CTAB method. Twenty primers were selected which were used for *Asparagus officinalis* L a related species of *A. vera* and others were developed from available *Aloe vera* plant sequences with the help of primer 3 software. Similarity matrices and dendrogram were constructed by using NTSys software to show a phenetic representation of the genetic relationship. Polymorphic Information Content (PIC), the effective multiplex ratio (EMR) and Marker Index (MI) were calculated for the assessment of genetic diversity.

Results: The neighbor-joining tree based on all SSR fragments of twelve *Aloe vera* germplasm accessions grouped into three major clusters. The similarity value ranged from 46 % to 100 %. The highest 100 % similarity was noted between Haryana and Uttar Pradesh accessions followed by 93% similarity between Haryana and Punjab accessions with Rajasthan. Minimum similarity was noted between Gujarat and Kerala accessions.

Conclusion: This study revealed the rich genetic diversity among *Aloe vera* accessions from different agro-climatic zones of India. It is also concluded that SSR marker analysis can be a useful tool for the assessment of genetic diversity of the medicinal plants.

Keywords: *Aloe vera*, Agro-climatic zones, SSR, Genetic diversity, Polymorphism.

INTRODUCTION

Genetic diversity is a fundamental component of biodiversity analysis. It represents all of the genetically determined differences that occur between individuals of a species in the expression of a particular trait or set of traits. The genetic variability is influenced by the geographical, seasonal and edaphic factors of the environment [1]. The quality of adaptation of a population to different environmental conditions is directly dependent on its amount of genetic diversity. In the wilderness of the tropics, plants grow in extreme situations along longitudinal, latitudinal and temperature gradients and therefore, variations within and in between the populations of a species are not uncommon [2]. Molecular markers are an important tool for biodiversity assessment as they are free from the influence of environment. Molecular markers help in pinpoint the location of desirable genetic traits. So, the study of polymorphism is best done at the level of the arrangement of nucleotide bases in DNA, the primary source of all biological information [3]. Microsatellite polymorphisms are based on different numbers of a short repeated motif at a given locus and have been shown to be one of the most powerful genetic markers in biology [4].

Simple sequence Repeats (SSRs) are stretches of DNA containing a varying number of tandem repeats of six or less bases, distributed throughout the eukaryotic genomes [5]. The variation/ polymorphism are determined using SSR markers by the number of time the base sequence repeat. SSR markers are considered highly polymorphic, so they are excellent tools for comparing the germplasm. They are desirable and markers of choice in many plant breeding programs because they are often co-dominant, highly reproducible, frequent in most eukaryotes, and reveal high allelic diversity [6]. Simple Sequence Repeat markers require only small amount of DNA sample, do not involve radioactive labels and are simpler and faster. SSR has proven to be quite efficient in detecting genetic fidelity even in closely related organisms such as near isogenic lines [7].

Aloe vera (L.) Burm. f. (Synonyms: *Aloe barbadensis* Miller), commonly known as 'Ghrith Kumari' has become naturalized almost in all parts of India [8]. It is a perennial, xerophytic, succulent plant belonging to the family 'Liliaceae' with turgid green leaves. *Aloe* species has been used medicinally for several thousand of years in folk medicine in many cultures from ancient Egypt, Greece, Rome, China to India [9].

The main chemical constituents of *Aloe* are anthraquinones, carbohydrates, amino acids, organic acids, active enzymes, vitamins, minerals, and microelements; among which anthraquinones are the most important active ingredients [10]. The four main anthraquinones showing quite high medicinal values are Aloin, Aloe emodin, Aloe bitter and Aloe lectin [12]. Now a day, *Aloe vera* is used as an important constituent in the pharmaceutical, cosmetic and food industries [12, 13].

The uncontrolled collection of *Aloe vera* by the local community leads to a serious threat to its population in the nature and biodiversity. An understanding of genetic relationship in germplasm is a valuable technique for biodiversity conservation and improvement strategies. Keeping in view of the medicinal values of *Aloe vera* and the potential use of molecular markers in breeding program, the present study was carried out to access the genetic diversity among *Aloe vera* genotypes using SSR markers. To the best of our knowledge, this is the first report on the characterization of *Aloe vera* genotypes based on commercially available SSR primers from India.

MATERIALS AND METHODS

Plant collection

A total of 12 sites from different climatic zones of India were selected for the collection of plant samples (fig. 1). The average temperature, average rainfall, latitude and longitude of the collection sites are given in table 1. Leaves were collected from healthy plants in Jan-Feb 2013. Healthy leaves of *Aloe vera* were collected from each location in sterile plastic bags. The plant material was identified and authenticated by comparing the herbarium specimen number MDU-6803 available in Department of Genetics, M. D. University, Rohtak (India). All samples were brought to the laboratory in an ice box and processed further.

Table 1: Details of geographical parameters of different collection sites

S. No.	Agro-climatic zones	Accessions	Place of collection	Latitude	Longitude	Average temp (°C)	Average rainfall (mm)
1	Highland	Jammu and Kashmir [J&K]	Jammu	32 ° 72' N	74 ° 85' E	7-20	1,011
2		Himachal Pradesh [H. P.]	Palampur	32 ° 11' N	76 ° 53' E	10-17	1,251
3	Semi-arid	Punjab	Sangrur	30 ° 24' N	75 ° 84' E	15-35	649
4		Haryana	Rohtak	28 ° 89' N	76 ° 60' E	20-34	617
5	Arid	Rajasthan	Jaisalmer	25 ° 55' N	70 ° 57' E	22-35	209.5
6		Gujarat	Gandhinagar	23 ° 21' N	72 ° 63' E	22-33	1,107
7	Humid Subtropical	Uttar Pradesh [U. P.]	Pratapgarh	25 ° 89' N	81 ° 94' E	19-32	904
8		Madhya Pradesh [M. P.]	Bhopal	23 ° 25' N	77 ° 41' E	19-32	1,146
9	Tropical wet & dry	West Bengal [W. B.]	Kolkata	22 ° 34' N	88 ° 24' E	22-32	1,582
10		Telangana	Hyderabad	17 ° 20' N	78 ° 30' E	23-30	812.5
11	Tropical wet	Kerala	Kochi	09 ° 93' N	76 ° 26' E	24-32	3,005
12		Goa	Vasco	15 ° 24' N	73 ° 50' E	23-32	3,055
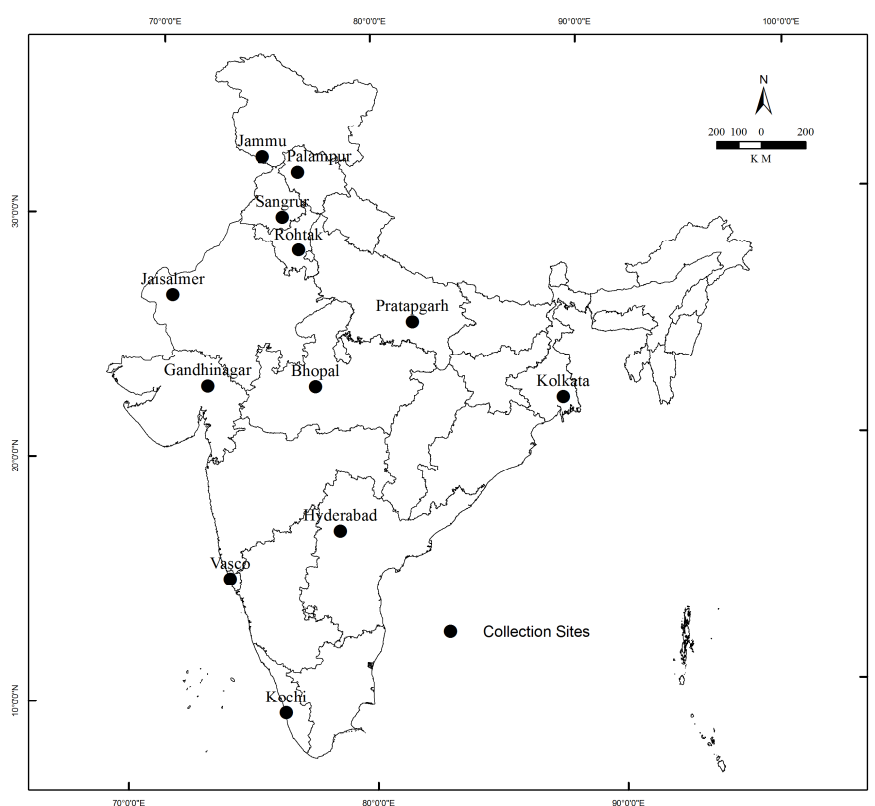
Fig. 1: Map showing *Aloe vera* accessions collection sites from different places of India

Genomic DNA isolation

The total genomic DNA was isolated from young leaf samples using the CTAB (Cetyltrimethylammonium bromide) method [14] with minor modifications. About 20 mg of macerated tissue was placed in a 1.5 ml microfuge tube and incubated in 300 µl of CTAB extraction buffer (2% CTAB, 100 mM Tris-HCL, pH 8.0, 1.4 M NaCl, 0.1% 2-mercaptoethanol) at 65 °C for 1h. Samples were then homogenized with a plastic pestle and incubated at 65°C for at least 10 min.

The extracted DNA was subjected to an additional step of purification with chloroform: isoamyl alcohol (24:1) treatment followed by precipitation with chilled solution of ethanol-NaOAc. The pellet was washed with 70% ethanol, dried to remove alcohol, and dissolved in 100 µl TE (10 Mm Tris, pH 8.0). The DNA concentration was about 40-50 ng/µl. The DNA was checked on 0.8 % agarose and visualized, photographed and further analyzed by a gel documentation system (Alpha Innotech; AlphaImager, USA).

SSR primers

There was no literature available on SSR genetic diversity analysis for the *Aloe vera* plant. So, out of 27 SSR primers, 20 were taken from another important related plant species (*Asparagus officinalis* L.) of the same family [15]. We have designed and developed the seven primers for the *Aloe vera* plant. Approximately 300 *Aloe vera* sequences were retrieved from NCBI database for SSR primer development. These sequences were analyzed by tandem repeat finder. After processing, SSR-containing sequences were identified by Gramene, SSRIT-Simple Sequence Repeat Identification Tool [16]. Primer pairs were successfully developed on the basis of standard parameters by using primer 3 software [17]. Primers were synthesized using the facility of Eurofins Pvt. Ltd (table 2).

Table 2: SSR primers used for detecting the genetic diversity among twelve *Aloe vera* accessions

S. No.	Name	Repeat	Forward primers			Reverse primers		
			Sequences	GC (%)	Tm (^{o}C)	Sequences	GC (%)	Tm (^{o}C)
1	AV1	(CCT)4	AAATAGACACCCCCTCCACA (20)	50	57.3	TCCCCAAAGAGAAACACTGG (20)	50	57.3
2	AV2	(GA)9	CGCGAGCGGAGTAATCAATA(20)	50	57.3	CTGGCAACTGAAACAGTCCA (20)	50	57.3
3	AV3	(CAG)4	AGCCAGCTGTGCCCGATA (18)	61.1	58.2	CAACAACAACCACCACCTTG (20)	50	57.3
4	AV4	(TA)6	TTACGTTGCGTTGGTAGCAG (20)	50	57.3	AGAGGGACTTGACCTCACGA (20)	55	59.4
5	AV5	(CA)6	AGCCAGCTGTGCCCGATA (18)	61.1	58.2	GAAGTTGAGCCTGGCGTAGT (20)	55	59.4
6	TC1	(TC)12	AGGTGGAGAACAAATGGCTG (20)	50	57.3	CGAGCTCAATTGAAATCCATAA (22)	36.4	54.4
7	AGA1	(AGA)11	CCGGTGCTTTGATTACTGCT (20)	50	57.3	GATCATCATCTTGCGCATTG (20)	45	55.3
8	TC2	(TC)10	GGCAGGATTAGGGTTTCG (18)	55.6	56.0	TCTCGCTCACCTTCTCATCC (20)	55	59.4
9	AAT1	(AAT)9	CTTTTGCTTCTGAACGCTCC (20)	50	57.3	TTGAAGGAGCCGTAAACTGG (20)	50	57.3
10	AG5	(TC)18	GATTAATAAAGCGCCGCTGA (20)	55.3	45	ACATAAGCCCATACTTGCGG (20)	50	57.3
11	AG7	(AG)10	TTTTGCTCCGATCATTTTCA (20)	35	51.2	CCTCTTCGTCTTCATCAGCC (20)	55	59.4
12	TC4	(CT)9	AGAGAGGAAGTTGTCGCTCG (20)	55	59.4	TGGGAAAATGGAAGAACCAA (20)	40	53.2
13	TC6	(TC)15	CATGCCCTAAAATCTCCAAGA (20)	42.9	55.9	GCCAGAGGCTGAAATAAACTG (20)	47.6	57.9
14	TC7	(CT)10	CGCCCCGAATCAACTAATAA (20)	45	55.3	TACTGCGGAGGTATGTGGGT (20)	55	59.4
15	AG10	(AG)9	CGCCCTTGTTCTTCTTCTTG (20)	50	57.3	CAGTTGTCTGCCGTCTTCAA (20)	50	59.4
16	AG11	(AG)9	AGGGGTCCGGATTAATTCAC (20)	50	57.3	GTCCTTGGCCATTAGAGCTG (20)	55	57.3
17	TC9	(TC)12	GTGATTCAAGGGGGAAAGGT (20)	50	57.3	TACACCAAAACCAGAAGGGC (20)	50	57.3
18	AG12	(AG)9	GACTAGCGCCATGAGAAAGG (20)	55	59.4	TTTTAGGGCATTTTAAACGCAT (22)	31.8	52.8

PCR amplification using SSR primers

The PCR (polymerase chain reactions) accomplished in a 25 µl reaction mixture containing 1X assay buffer, one unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), 200 µM of each dNTP (Genei Pvt. Ltd., India), 0.2 µM primers and 50 ng of template DNA. The PCR reaction was carried out in a thermal cycler (Bio-Rad; My Cycler, USA). The PCR amplification conditions for SSR consisted of the initial extended step of denaturation at 95°C for 5 min followed by 35 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and elongation at 72°C for 2 min and one cycle of final extension at 72 °C for 15 min. The PCR products were visualized on 2.5 % agarose gel and detected by ethidium bromide staining.

The 100 bp ladder was used for characterization of SSR amplified products. Primers that could amplify visible bands after preliminary screening were selected for further use. Different annealing temperatures were examined to optimize the amplification condition for the selected primers. Finally, primers that produced clear and reproducible bands were selected for the amplification of all samples. The PCR gels were photographed under UV light in a gel documentation system.

Statistical analysis

The SSR banding pattern was scored for presence (1) or absence (0) of a band. Each band was regarded as a locus. All calculations were done using computer program NTSys PC version 2.02 [18]. Pairwise similarity matrices were generated by Jaccard's similarity coefficient using SIMQUAL format of NTSys software. A dendrogram was constructed by using the UPGMA with SAHN module of NTSys software to show a phenetic representation of the genetic relationship. Polymorphism information content (PIC) was calculated according to the method given by Anderson *et al.* [19].

PIC: $1-\Sigma^{pi2}$ where P is the frequency of *i*-th allele [20].

The SSR markers were characterized by using Marker Index (MI), which is obtained by multiplying the Polymorphic Information Content (PIC) with the effective multiplex ratio (EMR). EMR is the average number of polymorphic loci in a single analysis for a particular set of objects [21, 22].

RESULTS

Genetic diversity assessment of *Aloe vera*

Total of 27 SSR primers were screened, out of which 18 primers showed amplification. Three primers amplified the similar loci in all the samples. 15 primers showed polymorphism and were selected for further study. These primers generated 134 amplicons, ranging from 40 bp (AG11) to 800 bp (AV4) in size, with an average of 8.9 bands per SSR primer.

Polymorphism information content (PIC)

Polymorphism Information Content (PIC) values of different primers were calculated for the assessment of genetic diversity between different accessions of *Aloe vera*. PIC (mean value) calculated for SSR were 0.442. PIC was calculated for each polymorphic locus and it ranged from 0.172 to 0.960. Maximum PIC value was obtained for primer AGA1 (0.960) which was followed by primer AV4 (0.95), AV2 (0.913) and AG12 (0.89).

Effective multiplex ratio (EMR)

Usually, SSR markers are locus specific. In the present study, 15 markers yielded 37 polymorphic loci. EMR is the average number of polymorphic loci in a single analysis for a particular set of object. The EMR value was 2.46 (table 3).

Marker Index (MI) calculated value was 1.08 (table 3).

Table 3: Characteristics of SSR markers used for genetic similarity analysis

Characteristics	Observed values
Number of primer or primer pairs	15
Number of polymorphic product	37
EMR	2.46
PIC range of values	0.172-0.960
PIC mean	0.442
MI (EMR×PIC)	1.08

Dendrogram

The neighbor-joining tree based on all SSR fragments grouped twelve *Aloe vera* germ plasm accessions into three major clusters

(fig. 2). The cluster I was consisting of two accessions, Jammu and Kashmir and Himachal Pradesh showing 77% similarity with each others. The Cluster II consisted of five accessions including Haryana, Uttar Pradesh, Rajasthan, Punjab and West Bengal joined to others

with much higher distance. In this cluster accessions from Haryana and Uttar Pradesh showed 100% similarity and were very close to each other. The cluster III comprised of four accessions viz. Madhya Pradesh, Kerala, Telangana and Goa. Among all the accessions present in the second cluster, accessions from Madhya Pradesh and Kerala had 75% similarity with each other and accessions from Telangana and Goa had also 75% similarity with each other.

The accession from Gujarat was represented by separate line in cluster tree analysis. Table 4 shows the Jaccard's similarity coefficient values of all accessions.

Principal component analysis (PCA)

Principal component analysis (PCA) based on genetic similarity (fig. 3) showed the relationship among accessions in two-dimensional space. The PCA of the SSR data produced a picture similar to the revealed from the cluster analysis. Fig. 2 showed the SSR markers data that contribute mostly to the observed clustering as shown by different clusters in cluster tree analysis. PCA analysis was compared with the cluster analysis because PCA is based on allele frequencies rather than distance measured and obviously it is more significant than UPGMA and neighbor joining.

Table 4: Showing Jaccard's similarity coefficient values among *Aloe vera* accessions using SSR primers

|	J&K	H. P.	Haryana	Punjab	Rajasthan	Gujarat	U. P.	M. P.	W. B.	Telangana	Kerala	Goa
J&K	1											
H. P.	0.77	1										
Haryana	0.72	0.73	1									
Punjab	0.63	0.73	0.88	1								
Rajasthan	0.66	0.77	0.93	0.93	1							
Gujarat	0.58	0.61	0.55	0.55	0.58	1						
U. P.	0.72	0.73	1	0.88	0.93	0.55	1					
M. P.	0.52	0.55	0.68	0.68	0.73	0.53	0.68	1				
W. B.	0.64	0.57	0.81	0.7	0.75	0.47	0.81	0.6	1			
Telangana	0.57	0.6	0.72	0.72	0.76	0.5	0.72	0.62	0.64	1		
Kerala	0.56	0.58	0.62	0.62	0.66	0.46	0.62	0.75	0.53	0.66	1	
Goa	0.47	0.5	0.61	0.61	0.64	0.47	0.61	0.71	0.52	0.75	0.64	1
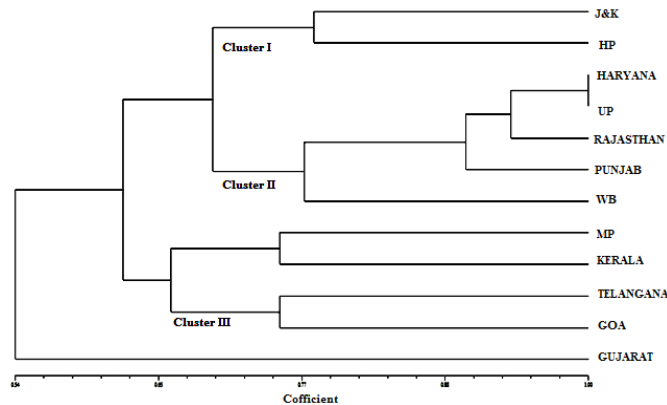
Fig. 2: Dendogram generated using UPGMA analysis showing relationship among *Aloe vera* accessions using SSR markers
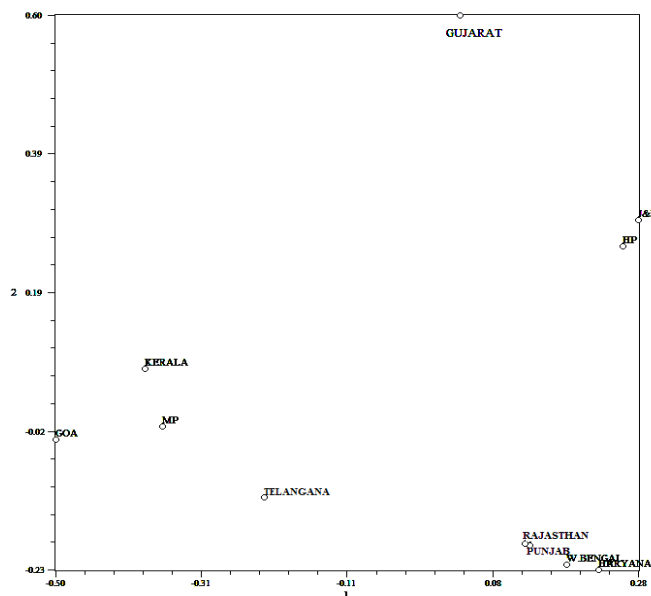

Fig. 3: Two-dimension scaling of *Aloe vera* genotypes by principal component analysis (PCA) using SSR marker

DISCUSSION

Aloe vera is a well-known medicinal plant and has a great importance in pharmaceutical as well as corporate sector. Mostly research has been done on *Aloe vera* is related to its phyto-constituents and medicinal properties. Geographical conditions are the main factors for genetic diversity that ultimately affect the phyto constituents and medicinal properties of a plant [23]. Diversity among organisms takes place as a result of variations in DNA sequences and environmental effects. Species with a wide geographic area generally have more genetic diversity [24]. Molecular techniques have been proven useful in the investigation of the origin and domestication of species and information on evolutionary relationships. Different molecular markers techniques i.e. RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphisms), RFLP (Restriction Fragment Length Polymorphism), ISSR (Inter Simple Sequence Repeat), SCAR (Sequenced Characterized Amplified Region) SSR, STS (Sequence-Tagged Sites), VNTR (Variable Number of Tandem Repeats), STR (Short Tandem Repeats), and SNP (Single Nucleotide Polymorphism) have been used to study genetic diversity and variations in different plant species [25-29]. Several medicinal and aromatic plant species, such as *Azadirachta indica* [30], *Artemisia annua* [31], *Cassia occidentalis* [32], *Tanacetum vulgare* [33], *Achillea millefolium* [34] and *Withania* sp. [35] have been observed for their genetic diversity and geographical distributions using various marker systems. Tripathi *et al.* employed AFLP to assess the genetic diversity in 12 elite accessions of *Aloe vera* collected from different locations of Madhya Pradesh, India and found significant variability among different accessions [36]. Darokar *et al.* used RAPD and AFLP markers to analyze the germplasm diversity in *Aloe* species [37]. Nayanakantha *et al.* also reported a good amount of genetic variability among *Aloe vera* accessions based on RAPD analysis [38]. Molecular characterization of multiple shoot culture of *Aloe* clones were tested for genetic integrity by using ISSR primers [39]. Recently, Nejatzadeh-Barandozi showed genetic diversity in *Aloe vera* accessions from Iran based on agro-morphological, phytochemical and random amplified polymorphic DNA (RAPD) markers but results did not reveal a clear relationship between diversity pattern and geographical origin [40].

In present study, SSR molecular marker is used for the analysis of genetic diversity among different *Aloe vera* accessions collected from different climatic regions of India. In order to improve the medicinal value of *Aloe vera* and to meet gap between the demand and supply of elite plant material, there is a need to conserve this species for sustainable use in nature. Microsatellite assays explain wide inter-individual length polymorphisms during PCR analysis of unique loci using discriminatory primers sets. High level of polymorphism and their co-dominant nature have made SSRs as ideal markers for studying genetic diversity in plants [41]. The SSR marker system has been successfully applied to detect the genetic variations in a number of species such as *Tinospora cordifolia, Mantisia spathulata*, and *Cymbopogon martini* etc [42, 43].

In our study, *Aloe vera* showed a high percentage of genetic polymorphism. High PIC values indicate rich heterozygosity which in turn is associated with a high degree of polymorphism [44]. This analysis showed high genetic diversity in *Aloe vera* accessions growing in different environments and low diversity in *Aloe vera* accessions collected from the same or adjacent regions with a few exceptions. The similarity value ranged from 46% to 100%. The highest 100% similarity was between Haryana and Uttar Pradesh followed by 93% similarity between Haryana and Punjab accessions with Rajasthan. The minimum similarity was showed between Gujarat and Kerala accessions. High diversity is the reflection of adaptation to the environment, which is beneficial to its propagation, resources conservation, domestication of wild species and the screening of specified locus [45]. However, Genetic diversity is best estimated if agro-morphological, biochemical and marker studies are used together [46]. This variability can be used for genetic improvement through breeding programs. The observations and interpretations of this investigation are interesting as a preliminary exploration analysis. The present findings make a strong point to enlarge the scope and size of collection throughout the distribution area of *Aloe vera* in order to detect and quantify the prevalent genetic diversity existing within this species at molecular level.

CONCLUSION

Studies indicate that SSR is sufficiently informative and powerful tool to access genetic variability of natural populations of *Aloe vera*. The results of our investigations on genetic diversity provide estimates on level of genetic variation among diverse materials that can be used in assessing the purity and stability of genotypes entering into a breeding or multiplication programs. This indicates the power of SSR marker system, presumably covering the entire genome. Thus, SSR marker analysis can be useful for designing collection strategies and germplasm conservation of medicinal plants.

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CONFLICT OF INTERESTS

There is no conflict of interest between authors.

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