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Assessment of diversity in *aloe barbadensis* miller using morphological and molecular markers

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Abstract

In this study, genetic diversity of 38 accessions of *Aloe barbadensis*, collected from different parts of India were evaluated using morphological and random amplified polymorphic DNA (RAPD) data. Statistical analysis showed significant differences for all morphological characteristics among the accessions, suggesting that selection for relevant characteristics could be possible. The analysis of molecular diversity was used the RAPD technique, with 14 random primers of 10-mer oligonucleotides. RAPD analysis was performed with 14 primers chosen a t er a previous screening. Significant genetic variability among those 38 cultivars was obtained both at the morphological and molecular level. This study demonstrated that for determining the genetic variability among some cultivars, the molecular markers technique is more precise than the morphological traits.

Highlights

- Morphological markers showed the maximum inter cluster distance D = 1.62
- 14 RAPD produced 89 DNA fragments, with average percentage polymorphism of 82.76.
- RAPD and morphological markers were sufficient to assess variability among 38 *Aloe* genotypes

Keywords: Aloe, RAPD analysis, dendrogram, genetic diversity

Aloe barbadensis Miller belongs to the family 'Liliaceae' and order 'Asparagales' (Anonymous 1985).Genus *Aloe* comprises of 275 species distributed in Africa, Asia and America. In India, only four species occur and of these *Aloe barbadensis* is the most widely naturalized species. *Aloe barbadensis* var. chinensis is commonly found in Maharashtra, *A.barbadensis* var. lit oralis is found in Tamil Nadu. One variety of Jafarabad aloe thrives in Saurashtra coast. Three varieties of aloe are official in the Indian Pharmacopoeia (Kurian and Sankar 2007). The *aloe* leaf can be divided into two major parts, namely the outer green rind, including the vascular bundles, and the inner colorless parenchyma containing the aloe gel. These three components of the inner leaf pulp have been shown to be distinctive from each other both in terms of morphology and sugar composition. (Ni *et al.* 2004). Moreover, the polysaccharides in *A. vera* gel have therapeutic properties such as wound healing, anti-inflammatory effects (Vazquez *et al.* 1996), immunostimulatory, anti-diabetic (Reynolds and Dweck 1999), promotion of radiation damage repair, anti-bacterial, anti-viral, anti-fungal, and anti-neoplastic activities, stimulation of hematopoiesis and anti-oxidant effects (Talmadge *et al.* 2004), anti-cancer activity (Steenkamp and Stewart 2007). It has been found useful for X-ray burns, dermatitis, cutaneous leishmaniasis and other disorders of the

skin (Kirtikar and Basu 1935; Chopra *et al.* 1956). Based on finished products, the world market for value-added products of *A. vera* is very large (Adams *et al.* 2000). Thus commercial cultivation and the application of biotechnology to utilization of this plant would generate great value.

In recent years, the breeding and registration of medicinal plant cultivars have been reported from several different countries (Bernath 1996). The most important goals of any medicinal plant breeding program are to improve the morphological characteristics and increase the accumulation of biologically active agents. Studying the genetic diversity of available plant germplasm is a prerequisite for each breeding program (Bernath 2002). *A. vera* is cultivated on a large scale in South Africa, Madagascar, Arabia and India (Adams *et al.* 2000). So far, several researchers have attempted to study the morphological and pharmaceutical characteristics of *Aloe* germplasm in different countries; as a result of their work, cultivars such as *A. ferox* and *Aloe claviflora* have been registered (Viljoen *et al.* 1999; Rajasekaran *et al.* 2006; Nayanakantha *et al.* 2010). Kuzuya *et al.* (2001) investigated the suitability of five different *Aloe* populations to be the production cultivar, looking for good horticultural traits and a high leaf-phenolic component. Van Der Bank *et al.* (1995) studied *Aloe* accessions and introduced cultivars with a high leaf phenolic constitution suitable for medicinal application. Darokar *et al.* (2003) compared the yields of leaf and morphological variations in some economically important *Aloe* species. Nayanakantha *et al.* (2010) examined 18 Indian populations of *Aloe* spp.; among these they found four populations with upright growth and high leaf-phenolic component to use as a good base for a further breeding program. The variation of morphological and pharmaceutical characteristics has been attributed to environmental and/or genetic factors (Hay and Waterman 1993). In recent years, DNA-based molecular markers have been used to assess the genetic diversity among the germplasm in many plant species. DNA-based molecular markers have the advantage of being free from environmental modulations. Random amplified polymorphic DNA (RAPD) markers have proved to be a very useful tool to provide a convenient and rapid assessment of the genetic differences between genotypes (Williams *et al.* 1990). Moreover, RAPDs use arbitrary primers that provide a large number of multilocus markers and can be applied to analyze almost any organism, even those for which no previous genetic or molecular information is available. RAPD markers have been used efficiently to study the genetic diversity among different plant species, such as *Asparagus officinalis* (Shasany *et al.* 2003) *Eucalyptus globulus* (Keil and Griffin, 1994), *Gossypium hirsutum* (Multani and Lyon 1995) and *Cajanus cajan*(Prajapti *et al.* 2015).

The objective of our study was, to characterize the genetic variation of thirty eight accessions of *Aloe barbadensis* Mill. collected from different places of Gujarat, Rajasthan and NBPGR, New Delhi grown at Directorate of Medicinal and Aromatic Plants Research, Anand, Gujarat in order to support a breeding programme.

Materials and Methods

Plant material

For the present investigation, 38 *Aloe* accessions collected from different places of Gujarat, Rajasthan, Madhya Pradesh and NBPGR, New Delhi and maintained under uniform growth conditions at DMAPR, Anand Gujarat. Measurements and counts were made at each genotype and comprised 20 plants per genotype.

Morphological trait analysis

The suckers of 38 accessions were planted in propagation pots and then transplanted to field with 50 cm × 30 cm spacing in a randomized complete block design (RCBD). The investigated morphological traits, plant height (cm), leaf width (cm) and leaf thickness and number of leaf per plant, were recorded for all *Aloe* accessions to allow comparative studies. These traits were evaluated for 38 accessions and the data collected were analyzed (Table 1).

Table 1. The average of the values for morphological traits of 38 accessions of *Aloe barabadensis*

Sr. No.	Accession	Place of collection	Plant height (cm)	Leaf width (cm)	Leaf Thickness (mm)
01	IC310611	Gujarat	49.00	6.24	11.52
02	IC310617	Gujarat	42.30	5.00	14.60
03	IC310618	Gujarat	45.80	5.80	13.50
04	IC310609	Gujarat	51.62	5.56	12.20
05	NR127	Gujarat	51.22	7.34	13.40
06	IC1112521	NBPGR, New Delhi	59.56	5.32	13.00
07	IC1112532	NBPGR, New Delhi	48.78	4.24	11.40
08	IC1112527	NBPGR, New Delhi	53.20	6.70	16.10
09	IC283671	Gujarat	46.30	5.60	14.20
10	NMRM2	Gujarat	61.40	6.26	14.60
11	IC285630	Gujarat	45.80	5.70	15.00
12	NR72	Gujarat	52.00	7.22	12.00
13	IC285626	Gujarat	47.40	6.62	13.40
14	IC310623	Gujarat	54.60	6.40	13.80
15	IC310619	Gujarat	54.40	5.10	15.80
16	MP1	Madhya Pradesh	48.00	5.52	14.20
17	GUJ2	Gujarat	55.20	5.14	16.60
18	NR63	Gujarat	45.40	5.62	15.20
19	IC283670	Gujarat	49.00	4.98	12.00
20	NR57	Gujarat	54.50	6.14	15.00
21	IC283655	Gujarat	42.70	5.18	10.00
22	NR74	Gujarat	53.20	5.54	16.80
23	GUJ1	Gujarat	56.64	5.80	12.40
24	NR61	Gujarat	61.44	5.34	18.60
25	IC310906	Gujarat	48.00	5.54	17.00
26	IC310903	Gujarat	54.40	6.26	13.20
27	IC310904	Gujarat	46.40	4.32	13.20
28	IC310908	Gujarat	60.12	5.00	17.00
29	IC310614	Gujarat	50.80	5.32	14.90
30	IC112531	NBPGR, New Delhi	52.28	4.34	14.40
31	IC112517	NBPGR, New Delhi	53.80	5.10	16.60
32	K98	Gujarat	57.20	4.70	14.80
33	GUJ6	Gujarat	51.28	4.80	12.40
34	IC285629	Gujarat	55.24	5.06	15.80
35	N129	Gujarat	41.86	6.96	14.00
36	GUJ3	Gujarat	51.20	5.14	17.00
37	RAJ1	Rajasthan	58.00	4.26	14.20
38	RAJ2	Rajasthan	52.40	6.30	16.60

DNA extraction and RAPD analysis

Total genomic DNA was isolated from young leaves of plants according to the cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1990), with minor modifications. To reveal the level of genetic variation for each population, DNA of 05 plants were bulked and analyzed. PCR amplification (Williams *et al.* 1990) was performed with 14 arbitrary decamer primers obtained from Operon Technologies, Alameda, USA (Table 2). Amplifications were performed in 25 µl reaction volumes containing 2.5 µl of 10X assay buffer (100 mMTris-Cl; pH 8.3, 500 Mm KCl, 15mM $MgCl_2$), 0.2mM of each dNTPs (dATP, dCTP, dGTP and dTTP), 5 pg of primer, 1.0 unit of Taq DNA polymerase and 25ng template DNA. The amplification reaction was carried out in a DNA Thermal Cycler (Eppendorf AG, Hamburg, Germany) programmed for 43 cycles using the following amplification profile: initial denaturation of template DNA at 94°C for 5 minutes followed by 43 amplification cycles of denaturation at 92°C for 1 minute primer annealing at 37°C for 1 minute and elongation at 72°C for 2 minutes followed by a final extension step at 72°C for 7 minutes.

After completion of amplifications, 2µl of 6x loading dye was added to each sample and 27µL volume was resolved on 1.5% agarose gel in 1×Tris–acetate–ethylenediaminetetraacetic acid (TAE) buffer. The size of amplified DNA fragments was estimated with 100-bp ladders (Fermentas, USA). The gels were visualized under UV using gel documentation system (Syngene, UK).

Table 2. Primers used for generating RAPDs in *Aloe barbadensis* genotypes

Sr. No.	Primer	5' -3' Sequence	Approx. Fragment Size (bp)	Total number of loci	Number of Polymorphic loci	Percent of polymorphic loci	Total number of fragments amplified	Primer index
01	OPA09	GGGTAACGCC	250-2000	07	06	85.71	235	0.185
02	OPA20	GTTGCGATCC	250-1550	09	09	100	198	0.499
03	OPJ07	CCTCTCGACA	690-2980	07	05	71.42	77	0.306
04	OPJ09	TGAGCCTCAC	600-1350	05	04	80	126	0.447
05	OPJ10	AAGCCCGAGG	430-3000	05	04	80	143	0.468
06	OPJ11	ACTCCTGCGA	390-2550	11	11	100	284	0.436
07	OPJ12	GTCCCGTGGT	490-3000	04	03	75	141	0.383
08	OPJ13	CCACACTACC	600-2000	07	07	100	168	0.465
09	OPJ17	ACGCCAGTTC	500-3000	08	07	87.5	189	0.494
10	OPJ18	TGGTCGCAGA	190-1250	06	4	66.67	159	0.424
11	OPJ19	GGACACCACT	590-2000	05	4	80	73	0.473
12	OPJ20	AAGCGGCCTC	500-1200	03	2	66.67	78	0.500
13	OPN02	ACCAGGGGCA	490-1600	07	6	85.71	173	0.455
14	OPN16	AAGCGACCTG	190-1200	05	4	80	98	0.500
Total				89	76	983.68	2142	6.035

Data analysis

Quantitative analyses of morphological traits were carried using the Mahalanobis D^2 statistics (Mahalanobis, 1936). Tracing D^2 as a generalized distance, the criterion used by Tocher as described by Rao (1952) was applied for determining the clustering group.

Polymorphic RAPD fragments were scored as either present (1) or absent (0) across all accessions. Only distinct, well resolved fragments were scored. A binary matrix employing the Jaccard index (Jaccard, 1908) was used to estimate the genetic similarities Polymorphic RAPD fragments were scored as either present (1) or absent (0) across all accessions. Only distinct, well resolved fragments were scored. A binary matrix employing the Jaccard index (Jaccard, 1908) was used to estimate the genetic similarities between pairs. These similarity coefficients were used to construct dendrogram using the unweighted pair group method with arithmetic averages (UPGMA) and employing the SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering) protocol from the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.02 (Applied Biostatistics) program (Rohlf, 1998).

Results and Discussion

Variability of morphological traits

The results showed that the genotypes of *A. barbadensis* were significantly different in all evaluated morphological traits (Table 1), suggesting that selection for relevant characteristics could be possible. The height and mature leaf width were ranged from 41.86 cm (RAJ-1) to 61.44 cm (NR-57) and 4.24 cm (IC1112521) to 7.34cm (IC310609), respectively.

Mature leaf thickness was ranged from 0.1cm (GUJ-2) to 1.86cm (NR-57). A high mature leaf length and mature leaf width is important in particular for

cultivars for medicinal components.

Nayanakantha *et al.* (2010) identified yields of the leaf length and leaf thickness among eighteen cultivars between 14–46 cm and 0.4–1.1 cm respectively and Rajasekaran *et al.* (2006) among 5 cultivars and accessions between 10–39 cm and 0.7–2.1 cm. These differences might be related to the utilization of *A. vera* accessions from different geographical origins with different environmental conditions.

Based upon mean observations of three characters from thirty eight genotypes the Mahalanobis D^2 statistic was computed in order to assess the genetic diversity present among the genotypes under the study. In all, seven clusters were formed in which, cluster I was the largest cluster containing twenty six accessions. Cluster II comprised of seven accessions consisting of. Cluster III comprised of only single, similarly cluster IV, cluster V, cluster VI, cluster VII having only single accessions (Figure 1). The maximum inter cluster distance (D = 1.62) was observed between cluster V (NR61) and cluster VII (N129) which indicated the presence of wider genetic diversity among the accessions included in these clusters. Inter cluster distance was minimum (D = 0.43) between cluster IV (IC310908) and cluster VI (IC283655), which indicated close relationship between the accessions of these clusters. Considerably higher inter cluster D values revealed a very interesting trend of genetic diversity. Intracluster D values of cluster I showed genetic diversity even within a cluster (Table 3). The results obtained are in agreement with Bhutia *et al.* (2005), Das *et al.* (2005), and Dias and Kageyama (1997), in rice.

RAPD analysis

PCR amplification of DNA, using 14 primers for RAPD analysis, produced 89 DNA fragments that could be scored in all genotypes. All the selected primers amplified DNA fragments across the 38 genotypes studied, with the number of amplified fragment varying from three (OPJ20) to eleven (OPJ11), with size ranging from 190 to>3000bp. Of the 89 amplified bands, 76 were polymorphic, with an average of 5.429 polymorphic bands per primer. Percent polymorphism ranged from 66.67 (OPJ18) to 100 (OPJ13), with an average percentage polymorphism of 82.76.

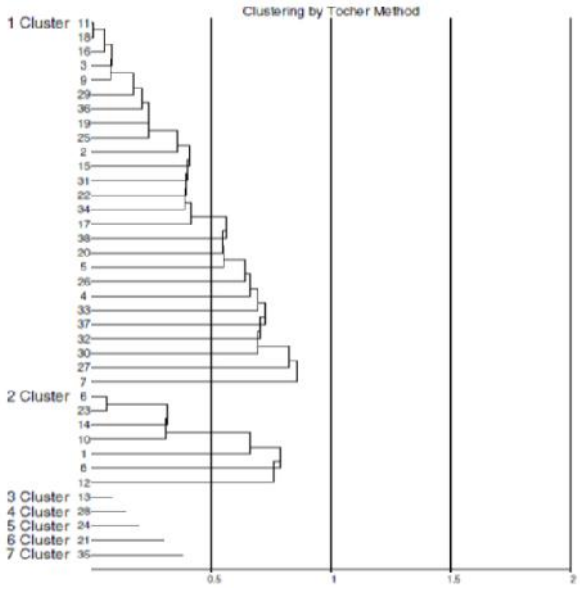

Fig. 1. Dendrogram constructed for 38 *Aloe* cultivars based on morphological traits

Table 3. Average intra and inter cluster distance (D) = D^2 value (Tocher's method)

Cluster	I	II	III	IV	V	VI	VII
I	0.45	0.82	0.89	0.79	0.97	0.84	0.81
II		0.55	1.00	0.85	1.31	1.12	0.93
III			0.64	0.86	1.04	0.77	1.38
IV				0.00	1.46	0.43	0.78
V					0.68	1.35	1.62
VI						0.00	1.07
VII							0.00

A dendrogram based on UPGMA analysis grouped the 38 genotypes into five main clusters, with Jaccard's similarity coefficient ranging from 0.42 to 0.93 (Figure 2). Germplasms no.1 and 11 were grouped in one cluster. Clusters II comprised of (2, 3, 4, 5, 6, 7 and 8). Cluster III consisted of (10, 18, 23 and 33). Cluster IV consisted of five subclusters. Subcluster I, II, III, and IV consisted of germplasms (15, 16, 17, 19), (25, 26, 27, 31), (32, 35, 34, 36, 37, 38) and (20, 21) respectively. Cluster V contained subcluster in which germplasms no. (24, 30, 29) and

(13, 14) clustered together while no.28, 22, 9, and 12 remained in separate cluster with 39% diversity.

Looking at the two dendrograms based on the morphological traits and on the molecular markers we can observe that both dendrograms shows a genetic variation among the cultivars. Both RAPD and morphological characters were sufficient to assess variability among 38 *Aloe* cultivars. No correlation was found between the matrices obtained by molecular and morphological traits. Despite the low correlation between those two dendrogram it can be observed that some cultivars were grouped in the same cluster in both dendrogram. For example the cultivars no. 7, 28, 35 appear to be grouped in the separate cluster in both dendrograms. The same results were obtained by other authors in different studies (Maric *et al.* 2004). The low correlation between RAPD dendrogram and morphological dendrogram had been also reported in other studies in European barley varieties (Schut et al, 1997), synthetic hexaploid wheat and their parents (Lage *et al.* 2003) and Squash (Ferriol *et al.* 2004). Normally until now, germplasm has been classified on the basis of morphological and agronomical traits, but recently the use of molecular markers to study diversity and characterization of the plants has become more common. This difference between the two dendrogram can be due to the fact that the morphological traits can be influenced by many factors such as: environmental conditions, the sample size, the time of making the measures etc.

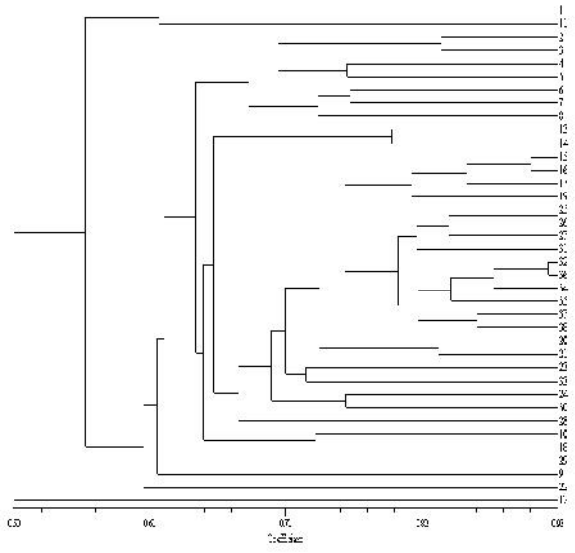

Fig. 2. Dendrogram showing the genetic relationship among *Aloe* genotypes using UPGMA analysis of RAPD data

Conclusion

Diversity analysis in Aloe barbadensis Miller using morphological and molecular markers revealed that, the Aloe genotypes showed both morphological and molecular diversity, but the molecular analysis is more precise, due to the fact that the genotype is not influenced by the environmental conditions. Also, this study shows that PCR-based techniques such RAPD can be successfully used for detecting genetic variability in Aloe. This study demonstrated that for determining the genetic variability among some cultivars, the molecular markers technique is more precise than the morphological traits.

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