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ASSESSMENT OF GENETIC DIVERSITY IN *ALOE* GERMPLASM ACCESSIONS FROM INDIA USING RAPD AND MORPHOLOGICAL MARKERS

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ABSTRACT

Eleven *Aloe* germplasm accessions; *A. vera*, *A. perryi*, *A. lotus*, *A. zeylanicum* and seven strains of *A. vera* available at the Defense Agricultural Research Laboratory (DARL), Pithoragarh, and Medicinal and Aromatic Plant Research Development Centre (MRDC), Pantnagar, Uttaranchal, India were subjected to Random Amplified Polymorphic DNA (RAPD) analysis in relation to morphometric parameters for estimating the extent of diversity within and between species. Morphological evaluation of the 11 accessions for selected characters showed qualitative variation among the accessions studied. The RAPD analysis revealed comparable inter and intra species variation. A total of 192 bands were amplified with 7 primers. Out of 192 bands amplified, 89% was polymorphic and 10.9% was unique to a particular accession which made it distinct from all other accessions. Maximum similarity of 61% was observed between DARL 1 and DARL 3 (*A. vera*) and minimum similarity of 6.8 % was observed between *A. lotus* and *A. perryi*. Thus, *Aloe* accessions maintained at DARL showed high genetic diversity. The RAPD profiles would be useful in genetic improvement and authentication of species and genotype of this medicinally and economically important genus.

Key words: *Aloe vera*, cluster analysis, genetic similarity

INTRODUCTION

Aloe, belonging to the family Liliaceae, is a genus of herbaceous and succulents found in tropical and subtropical areas, particularly South Africa and Arabia. Although about 360 species of *Aloe* have been reported, only *Aloe vera* (L.) Burm f. (Synonyms : *Aloe barbadensis* Miller), commonly known as "*Komarika*" (in Sinhala) and "*Ghrith Kumari*" (in Hindi), has become naturalized almost in all parts of India (Klein and Penneys, 1988). *Aloe vera* is the most important among the *Aloe* species as it has been used medicinally for several thousands of years in folk medicine in many cultures from ancient Egypt, Greece, and Rome to China and India (Kemper and Chiou, 1999).

The plant has stiff, gray-green, lance-shaped leaves containing clear gel in a central mucilaginous pulp. The gel contains an emollient polysaccharide, glucomannan, which is a good moisturizer utilized in many cosmetics (Henry, 1979). Acemannan, the major carbohydrate fraction in the gel demonstrates antineoplastic and antiviral effects (Mc Daniel *et al.*, 1990). The gel also contains bradykininase, an anti-inflammatory**,** which prevents itching, and salicylic acid and other antiprostaglandin compounds that relieves inflammation (Yagi *et al.*, 1982). Other important pharmacological activities of *Aloe vera* are anti diabetic (Rajasekaran *et al.*, 2006)**,** antiseptic (Capasso *et al.*, 1998), anti- tumor (Winter *et al.*, 1981) and wound and burn healing effect (Heggers *et al.*, 1993).

Apart from *Aloe vera*, other economically important species of *Aloe* include *A. ferox* Mill., *A. Africana* Mill., *A. perryi* Back., *A. arborescence* Mill., *A. zeylanicum*, etc. The different species of *Aloe* have somewhat different concentrations of active ingredients (Yagi *et al.*, 1998). There are morphological variations in some economically important *Aloe* species (Darokar *et al.*, 2003) and leaf phenolic constitution (Van Der Bank *et al.*, 1995; Viljoen, 1999). However, due to lack of expressions for reproductive characters in some of the species, it is not possible to distinguish them (Reynolds,

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1990). Since morpho-chemical characters are dependent on age and environment, it is essential to characterize this medicinally and economically important genus genetically.

Various molecular markers have been developed as powerful tools for diversity analysis and establishing relationships between species and cultivars. The assessment of genotypic identity among individuals of a species is central to making valid biological interpretations about population structure, breeding systems, reproductive biology and micro evolutionary processes within and among the species. Among various molecular markers, the Random Amplified Polymorphic DNA (RAPD) is most widely used because it allows a rapid and inexpensive assay with different primers (Williams *et al.,* 1990). Due to the technical simplicity and speed of RAPD methodology, it has been successfully used for the assessment of genetic structure and phylogenetic analysis (Gepts, 1993). It has been successfully applied to studies of genetic differentiation in some genera- like *Mangifera* (Karihaloo *et al.,* 2003); *Asparagus* (Shasany *et al.,* 2003); *Eucalyptus* (Keil and Griffin, 1994) and *Gossypium* (Multani and Lyon, 1995), etc.

In recent years, RAPD technique was used to estimate the genetic diversity in the germplasm collections in *Aloe* spp. at different institutions (Shioda *et al.,* 2003; Darokar *et al.,* 2003). Thus, the present study was taken to characterize the *Aloe* germplasm accessions collected from different geographical regions of India and maintained at the Defense Agricultural Research Laboratory (DARL), Pithoragarh, and Medicinal and Aromatic Plant Research Development Centre (MRDC), Pantnagar, Uttaranchal, at molecular level in relation to morphological variation.

MATERIALS AND METHODS

Plant Material

Of the available *Aloe* germplasm accessions at DARL, Pithoragarh, India, nine accessions representing different geographical regions of India were used for the study. These accessions included two species namely *A. lotus* and *A. perryi* and seven strains of *A. vera* viz. DARL 1, DARL 2, DARL 3, DARL 4, DARL 5, DARL 6 and DARL 8. *Aloe zeylanicum* and commonly grown *A. vera* obtained from MRDC, Pantnagar, India were also used. All the plants used in the present study were about one year of age.

Morphological characteristics

Characters such as plant height, leaf length, leaf width and leaf thickness were recorded in all *Aloe* accessions for comparative studies. Leaf colour was recorded with the help of New Ornamentals Society (NOS) colour chart.

Isolation of DNA

DNA was isolated from 3 g of the leaf tissue (excluding the gel like mesodermal region) according to the protocol described by Khanuja *et al.* (1999) and dissolved in 200 µL of high salt TE buffer (pH 8.0)**.** For the removal of RNA, 10 µg of RNAse A was added to the DNA solution and incubated for 10 minutes at 37^0C. The DNA was then purified by phenol: chloroform extraction and ethanol precipitation. The quantity and purity of the DNA were assessed, checked for quality and purity by electrophoresis in a agarose gel in 1 x TAE buffer (Sambrook *et al.,* 1989). Quantification of DNA was done in UV spectrophotometer (Elico SL 159, India) by measuring optical densities at 260nm and the ratio of $OD_{260/280nm}$ respectively. Agarose gel electrophoresis 1% (w/v) with λ DNA standards of known concentrations was also carried out to check quantity and quality of extracted DNA. Finally all DNA samples were diluted to get 50 ng μL^{-1} solutions and were stored at -20 ^{0}C for use in RAPD assay.

RAPD assay

A set of ten decanucleotide random RAPD primers (Life Technology, India) were employed for PCR amplification. The sequence and the GC content of primers are given in Table 1.

Table 1. Sequences and GC content of random 10-mer primers (Life Technology, India) used in the analysis.

Primer	*Sequence 5' to 3'*	*GC content **(%)***
LC-76	5' GTGACGTAGG3'	60
LC-77	5' GGGTAACGCC3'	70
LC-80	5' CAGCACCCAC3'	70
LC-81	5' TCTGTGCTGG3'	60
LC-82	5' TTCCGAACCC3'	60
LC-83	5' AGCCAGCGAA3'	60
LC-87	5' AGGTGACCGT3'	60
LC-89	5' AGTCAGCCAC3'	60
LC-90	5' GTGAGGCGTC3'	70
LC-91	5' TGGACCGGTG3'	70

DNA amplification was performed in 25 µL reaction volume containing 50 ng of genomic DNA, 100 µM of each dNTPs, 1.5 mM $MgCl_2$, 30 ng of each primer, 1 unit of Taq DNA polymerase and 10X incubation buffer. The content (25 µL in each tube) was gently mixed

and centrifuged for few seconds. The PCR amplification was achieved in a Biometra T-gradient DNA thermocycler and the cycling conditions were: Initial denaturation at 94^0C for 5 min followed by 36 cycles of 94^0C for 30 sec, 41^0C for 1 min, 72^0C for 2 min and finally 1 cycle of 72^0C for 5 min. 12 µL aliquots of amplification products were separated on 1.6 (w/v) agarose gels containing ethidium bromide (0.5 µg/mL) in 1X TAE buffer and gels were photographed using an Alpha Image Gel Documentation System. Amplified fragments were scored as presence (1) or absence (0) of individuals. Initially a genetic similarity matrix was constructed using Jaccard's Similarity Coefficient (Sokal and Sneath, 1963). The similarity matrix was subsequently used to construct a tree for cluster analysis by UPGMA (Unweighted Pair Group Method with Arithmetic average) method using the computer package NTSYS 2.1 (Rohlf, 2000).

RESULTS

Morphometric analysis

Significant variations were observed for various characteristics studied among *Aloe* accessions. *Aloe zeylanicum* was found to be the tallest (53 cm) as it possesses a distinct stem (caulescent) with long internodes. The leaf shape was linear in this species compared to that of linear-lanceolate shaped leaves in others except *A. lotus* (Table 2). Minimum leaf thickness (0.4 cm) and wideness (1.0 cm) were recorded in this species among all *Aloe* accessions and therefore, it contained a lesser amount of gel. *Aloe lotus* was the shortest plant type with a height of 17 cm. It more or less resembles the common cactus plant in appearance than other *Aloe* accessions in possessing the ovate-lanceolate shaped and broadest (4.5 cm) leaves (Table 2 and Fig. 1a, b, c and d).

Among all *Aloe* accessions, *A. perryi* was considered as a blue green variety because of its blue-green appearance in leaf (Table 2). It is also a caulescent type plant but the internodal length was low compared to that in *A. zeylanicum*. Leaf width and size were slightly deviated from other *A. vera* strains (Fig. 1d).

Among *A. vera* strains, conspicuous variations were observed in plant height, leaf size, thickness and width while very little variations were observed in leaf colour (Table 2). In terms of leaf thickness, DARL-3 showed the highest thickness (1.1 cm) than other accessions. These accessions were further subjected to RAPD analysis for the assessment of genetic variation at molecular level.

RAPD analysis

The RAPDs generated were used to determine the genetic diversity among 11 accessions of *Aloe* as depicted in Table 3. Out of 10 primers tested, only seven primers (70%) generated strong amplification and resulted in informative scorable bands. A total of 192 bands were amplified with an average of 27.4 bands per primer and these were in the size range from 0.15 kb-1.5 kb. The individual primers generated bands ranged from 8 (with the primer LC- 91) to a maximum of 44 (with primers LC- 77 and LC-83) (Table 3).

Out of 192 bands, 171 (89%) were found to be polymorphic for one or more accessions. The primer LC-77 showed the highest polymorphism of 97% followed by LC-81 (95%), LC-76 (93%) and LC-83 (89%) respectively (Table 3). Results of the present study revealed an average of 24.4% polymorphic bands per primer. LC-91 amplified the least polymorphism of 75%. Amplification profiles observed with two primers (LC-77 and LC-83) are shown in Fig. 3a and b, respectively.

Twenty one unique (specific to an accession) bands (10.9%) were also identified. LC-90 amplified maximum number of accessions specific bands of 6 for 3 different accessions while LC-83 and LC-87 amplified maximum number of unique accessions specified bands of 5 and 4, respectively. Moreover, all the seven primers tested produced at least one unique band (Table 3).

Significant inter and intra specific variations could be visualized as evident from the similarity coefficients (Table 4) developed on the basis of relative indices among all possible pairs. Similarity values varied from 0.068 to 0.61. Maximum similarity of 61% was observed between DARL 1 and DARL 3 while lowest similarity of 6.8% was observed between *A. lotus* and *A. perryi*.

Cluster analysis using UPGMA (Fig. 2) generated from this matrix classified these accessions in to two major clusters. *Aloe lotus* and *A. perryi* branched out from the main clusters containing other accessions with distant similarity values of 14% and 21%, respectively. *Aloe vera* obtained from MRDC distinctly

formed a major cluster with other *A. vera* strains obtained from DARL with intra-specific diversity ranging from 37% (for the sub cluster containing DARL 1, 2 and 3) to 45% (for the sub cluster containing DARL 4, 5, 6). The other major cluster contained DARL-8 and *A. zeylanicum*. However, a distant relationship with a low similarity value of 30% was recorded between these two accessions.

Table 2. Morphological description of *Aloe* accessions used in the present study (Number given for leaf colour is according to New Ornamentals Society (NOS) colour chart).

Accessions	*Species*	*Plant type*	*Plant height (cm)*	*Leaf thickness (cm)*	*Leaf width (cm)*	*Leaf shape*	*Leaf length (cm)*	*Leaf colour*
DARL-1	*A. vera*	Acaulescent	22	0.9	1.5	Linear-lanceolate	22	007000
DARL-2	*A. vera*	Acaulescent	21	0.6	1.5	Linear-lanceolate	15	007000
DARL-3	*A. vera*	Acaulescent	23	1.1	2.0	Linear-lanceolate	33	008000
DARL-4	*A. vera*	Acaulescent	32	0.8	2.2	Linear-lanceolate	38	006000
DARL-5	*A. vera*	Acaulescent	31	1	1.8	Linear-lanceolate	34	004000
DARL-6	*A. vera*	Acaulescent	32	0.8	1.7	Linear-lanceolate	28	007000
DARL-7	*A. perryi*	Caulescent	24	0.7	1.6	Linear-lanceolate	21	004020
DARL-8	*A. vera*	Acaulescent	24	0.9	1.5	Linear-lanceolate	22	004000
AL	*A. lotus*	Acaulescent	17	0.9	4.5	Ovate-lanceolate	14	009000
AV	*A. vera*	Acaulescent	30	0.9	2.5	Linear-lanceolate	46	007000
AZ	*A. zeylanicum*	Caulescent	53	0.4	1.0	Linear	24	002000

Table 3. List of RAPD primers along with percentage of polymorphism and unique bands detected.

Primer	Sequence (5’ to 3’)	Number of scored bands	Number of Polymorphic bands	Polymorphism (%)	Number of Unique bands	Unique rate (%)
LC-76	GTGACGTAGG	32	30	93.7	2	6
LC-77	GGGTAACGCC	44	43	97.7	1	2
LC-81	TCTGTGCTGG	20	19	95	1	5
LC-83	AGCCAGCGAA	44	39	89	5	11
LC-87	AGGTGACCGT	17	13	76	4	23
LC-90	GTGAGGCGTC	27	21	77.7	6	22
LC-91	TGGACCGGTG	8	6	75	2	25
Total		192	171	89	21	10.9
Average		27.4	24.4	12.7	3	1.5
Range		8-44	6-43	75-97.7	1-6	1.5-25

Table 4. Genetic similarity matrix for Jaccard's Coefficient. Accession numbers 1-DARL-1, 2-DARL-2, 3-DARL-3, 4-DARL-4, 5-DARL-5, 6-DARL-6, 7- *A. perryi*, 8 -DARL-8, 9- *A. lotus*, 10 - *A. vera* and 11- *A. zeylanicum*.

	1	2	3	4	5	6	7	8	9	10	11
1	1.000										
2	0.529	1.000									
3	0.610	0.434	1.000								
4	0.545	0.428	0.518	1.000							
5	0.409	0.285	0.357	0.590	1.000						
6	0.375	0.160	0.290	0.480	0.478	1.000					
7	0.173	0.150	0.133	0.192	0.217	0.200	1.000				
8	0.214	0.153	0.242	0.310	0.346	0.423	0.240	1.000			
9	0.133	0.153	0.108	0.151	0.250	0.156	0.068	0.085	1.000		
10	0.454	0.333	0.392	0.500	0.434	0.458	0.380	0.384	0.125	1.000	
11	0.083	0.093	0.121	0.162	0.176	0.105	0.161	0.303	0.162	0.205	1.000

Figure 1. Typical morphotypes shown by four species of *Aloe* in the germplasm collection. (A): *A. lotus*, (B): *A. zeylanicum*, (C): *A. vera* and (D): *A. perryi*.

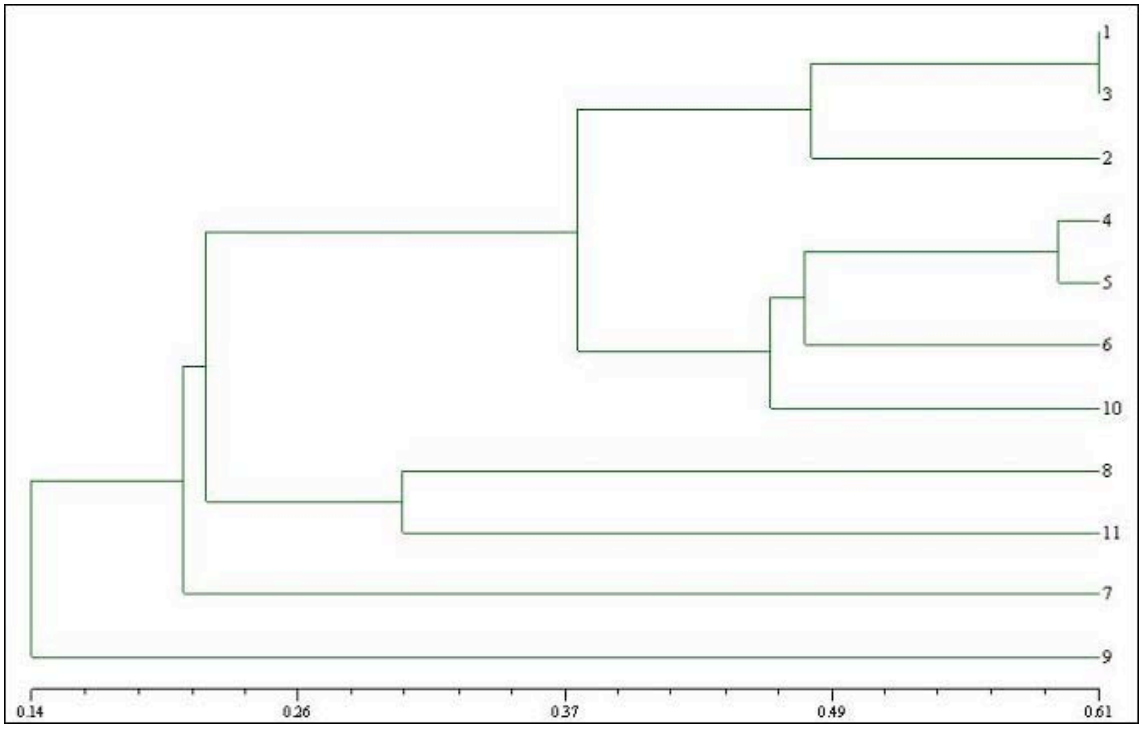

Figure 2. UPGMA cluster analysis-based dendrogram depicting genetic relationships among 11 accessions of *Aloe*. Accession numbers 1-DARL-1, 2- DARL-2, 3-DARL-3, 4-DARL-4, 5-DARL-5, 6-DARL-6, 7- *A. perryi*, 8 -DARL-8, 9- *A. lotus*, 10 - *A. vera* and 11- *A. zeylanicum*.

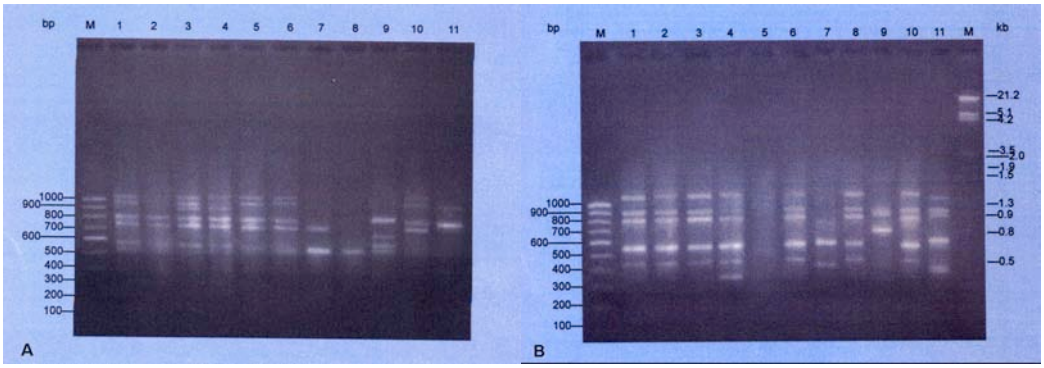

Figure 3. RAPD profiles generated from 11 *Aloe* accessions with primer LC- 77 (A) and primer LC-83 (B). Lanes 1-11 are DARL-1, DARL-2, DARL-3, DARL-4, DARL-5, DARL-6, *A. perryi*, DARL-8, *A. lotus*, *A. vera* and *A. zeylanicum* respectively. Lane M (left) is 100bp DNA ladder and Lane M (right) is *Eco* RI + *Hind*III double digested λ DNA.

DISCUSSION

In the present study, four *Aloe* species viz. *A. vera, A. perryi, A. lotus and A. zeylanicum* and seven strains of *A. vera* were studied with some characteristic morphotypic traits but still showing some overlaps across species and strains of *A. vera*. *Aloe zeylanicum* and *A. perryi* were easily separated from other accessions by their distinct stem development. *Aloe lotus* was also separated with the help of leaf morphology. Although, common *A. vera* and other strains of *A. vera* exhibited some variations in height, leaf size, and colour, these could be attributed to the adaptations of their original geographical and environmental conditions.

Vegetative characters being true phenotypic expression show variation, which makes the identification more difficult. Moreover, traditional morphological observations and chemical characters alone cannot determine the roles of phenotypic plasticity and genetic differentiation on population variation and adaptation (Gepts, 1993). Hence, they lack the resolving power needed to identify individual genotypes.

There are other *Aloe* species which resemble *A. vera* in growth habits and morphological traits (Reynolds, 1966). Moreover, identifying *Aloe* species at its early stage from other members of the Cactus family is also sometimes ambiguous. Thus, in order to determine genetic variations of these accessions, RAPD markers were used in the present study.

DNA-based markers such as RAPD, are practically unlimited in number, remain unaffected by environment and growth conditions and are simply inherited (Karihaloo *et al.*, 2003). Thus, this RAPD analysis provided phylogenetic relationships as well as some unique DNA bands to quantify the genetic variation.

Out of 10 primers tested, seven primers produced polymorphic bands giving a range of polymorphism from 75% to 97.7%. Moreover, all the seven primers tested produced at least one unique band. Therefore, higher polymorphism percentage reported in the present study demonstrates the high level of genetic diversity that exists among *Aloe* accessions maintained at DARL, India. The polymorphism generated by the primers LC-77, LC-81, LC-76 and LC-83 were 97.7%, 95%, 93% and 89%, respectively. The number of unique bands obtained from them were 1, 1, 2 and 5, respectively. On the other hand, the primers LC-90, LC-87 and LC-91 produced unique bands of 6, 4 and 2 respectively. Thus, all the seven primers employed in the present study could be effectively used to distinguish *Aloe* accessions.

The similarity values ranging from 6.8% to 61% indicate that there is a remarkable genetic variation among *Aloe* accessions used in the present study. The highest similarity recorded in the present study was 61% (between DARL-1 and DARL-3) followed by 59% (between DARL-4 and DARL-5). Other *A. vera* strains showed even lesser similarity values with each other and also with common *A. vera*. Furthermore, these similarity values are par less compared to that with higher similarity (78.8% - 99%) recorded among *A. vera* accessions maintained at the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India (Darokar *et al.*, 2003). According to them the similarity between *A. saponaria* and *A. perryi* was 45% while the similarity between *A. saponaria* and *A. vera* was 47%. Thus, the observed morphological differences and similarity values of *A. vera* strains maintained at DARL suggest that they all possess a genetic diversity to a great extent.

Cluster analysis clearly branched out *A. lotus* and *A. perryi* from rest of *Aloe* accessions suggesting that these two are more divergent from other accessions. Hence, phenotypic characters combined with RAPD analysis provided a better relationship to identify these species. *Aloe zeylanicum* formed a separate cluster with DARL-8 but with a distanced similarity of 30%. This cluster combined with the other major clusters containing *A. vera* and strains of *A. vera* (from DARL 1 to 6) with a distanced similarity of 21%. Thus, it is evident that *A. zeylanicum* is also more divergent from *A. vera* in the cluster analysis. However, the strains of *A. vera* (from DARL 1 to 6) showed a closest relationship to the common *A. vera* than to the other *Aloe* species suggesting that they all might have originated from *A. vera* but grown in different geographical localities in India. However, strain DARL-8 should be paid special attention as it deviated from rest of *A. vera* strains.

RAPDs amplify randomly across the genome. Since the majority of the genome is composed of non-coding regions, one would expect the majority of RAPD markers to be

amplified from them. These regions have higher random mutation rates and may have only a few phenotypic consequences (Wen and Hsiao, 2001). The RAPD technique provides a useful approach for evaluating genetic differentiation, particularly in those species that are poorly known genetically and are propagated vegetatively such as *Musa* (Bhat and Jarret, 1995) and *Lilium* (Haruki *et al.*, 1998). Not only the extent of variation but also RAPD provides markers even for cultivar identification (Torres *et al.*, 1993) and germplasm evaluation (Shioda *et al.*, 2003; Darokar *et al.*, 2003)**.**

Apparently, the present work constitutes the first application of RAPD markers for diversity analysis in *A. zeylanicum* and *A. lotus* in relation to *A. perryi* and *A. vera*. In the present study, highly reproducible amplification profiles produced under constant conditions suggested that RAPD markers reveal sufficient genetic diversity and a high level of genetic polymorphism among *Aloe* accessions evaluated. The RAPD technique is therefore expected to be useful in detecting small genetic variations within and among populations. However, detailed morphological study is also desirable in order to understand all aspects of this variation.

The species specific bands that could be identified in this study will provide tags for future genetic improvement as well as in authenticating the species and genotypes and hence an important tool for molecular systematic in this important genus.

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