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MOLECULAR ASSESSMENT OF *ALOE* SPECIES REVEALED BY RAPAD MARKERS

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ABSTRACT

The purpose of this study was to analyse genetic variation between fifteen *Aloe* species. The molecular marker technique: random amplified polymorphic DNA (RAPD) was performed. *Aloe* samples were obtained from DHA Karachi and the Centre for plant conservation, University of Karachi. DNA was isolated using a modified CTAB method. RAPD was performed to analyse diversity/similarity among fifteen *Aloe* species. Genetic diversity and phylogenetic analysis using nineteen RAPD primers showed 100% polymorphism. The pairwise similarity between analysed *Aloe* species ranged from 0.111 to 0.621. Dendrogram analysis using NTSYSpc software revealed that fifteen *Aloe* species can be divided into two main clusters and sub-clusters.

Keywords: *Aloe* species, Molecular marker, RAPD, Genetic diversity, Polymorphism

INTRODUCTION

Aloe belongs to the class Magnoliopsida and the family Liliaceae. The leaf-succulent genus *Aloe* L. (Grace, 2011) comprises a total of 548 accepted species (Grace *et al*., 2009; Klopper, 2009). Aloe varies in size ranging from very small shrubs of a few centimeters to large trees (Eshun and He, 2004; Grace, 2011). Aloe species continue to live for three or more seasons (Smith and Van Wyk, 2008; Cousins and Witkowski, 2012). The genus Aloe covers large variability in morphologies and different forms of growth including single and multi-stemmed tree, creeping, stemless rosette and rambling types (Smith and Van Wyk, 2009; Holland, 1978).

Aloe is a tropical and subtropical plant, dwelling in an extensive range of habitats. It is distributed throughout Africa mainly in Southern, Eastern and Western Africa, Madagascar, Arabian Peninsula, Indian Ocean Islands, Saudi Arabia, and Yemen (Reynolds, 2004; Klopper, 2009; Grace, 2011;). It is basically a xerophyte that prefers a dried arid climate (Urch 1999; Eshun and He, 2004). There are three main factors that influence the distribution of *Aloe* species, temperature (cold tolerance), fire, and moisture. Its distribution is also affected by seed pollinators; some seeds have an efficient wing that accounts for widespread distribution. On the other hand, some seeds are wingless which limits their dispersal and are confined to a certain area (Cousins and Witkowski, 2012).

In Pakistan, *Aloe* species are used as a folk medicine for the treatment of constipation, rheumatism, mucus, indigestion, back problem, skin cancer problems, inflammation, burns, eczema, blood pressure and diabetes (Loots *et al.*, 2007; Abbasi *et al.*, 2011). Furthermore, *Aloe arborescens* leaves are also good for minor burns, sunburns, cuts and scrapes while the leaf juice is used to cure conjunctivitis, rashes, skin burns and lesions.

Morphological or phenotypic markers are classical markers routinely used for the identification of various species. Phenotypic characteristics like leaf arrangements, leaf thickness and spines, flower shape, stalk, flower colour, seed shape, growth habits and pigmentations can be usually monitored without specialized biochemical or molecular techniques (Collard *et al*, 2005; (Ullah, 2009). (http://greenhouse.ucdavis.edu/files/botnot_01-01.00.pdf). Plant breeders are interested in investigating the morphological traits as they are low in price, speedy and simple to score. Moreover, such studies neither require sophisticated methods nor complex equipment (Sohrabi, 2012).

A molecular marker is determined as a segment of DNA that provides information on the variations at the genome level (White *et al.,* 2007). DNA markers originate from distinct classes of DNA mutations for example substitution, alterations (insertion or deletions), or mistakes in tandem repeats during DNA replication. They are basically infinite in number and are unaffected by the environment and plant growth stages (Agarwal *et al*., 2008). Polymorphic markers reveal differences among or different species, while, monomorphic markers do not differentiate between genotypes (Collard *et al*, 2005; Winter and Kahl, 1995). Molecular marker assays are applied for the determination of varieties, genetic constitution, pathological resistant genes, sex determination, evolutionary assessment, *etc* (Sharma *et al*., 2008). Various DNA-based markers have been developed to assess genetic diversity and to establish relationships between species and cultivars. These markers are advantageous over environmental markers as they are not influenced by environmental factors.

From these molecular markers, Random Amplified Polymorphic DNA (RAPD) is routinely used because it provides quick, simple and low-cost analysis of genetic structure and phylogenetic relationship (Nayanakantha *et al*., 2010).

RAPD has been extensively used for the genetic screening of unknown medicinal plant species. Moreover, RAPD relies on arbitrary primers and can be applied to any organism for genetic screening for example *Curcuma longa* L. (Khan *et al*., 2013) and *Asparagus officinalis* (Shasany *et al*., 2003) and genetic diversity of *Aloe vera* accessions (Nayanakantha *et al*., 2010; Tripathi *et al*., 2011; Panwar *et al*., 2013; Chandra and Choudhary, 2014) and Aloe species (Shioda *et al*., 2003; Van Der Bank *et al*., 1995). RAPD genetic markers have an extensive range of applications in agriculture and horticultural food technology.

The present study was intended to investigate the genetic variation between 15 *Aloe* species to evaluate polymorphism as well as similarity and determined the phylogenetic relationship between medicinally important Aloe species employing RAPD-PCR based DNA markers.

MATERIALS AND METHODS

Leaf samples of 15 *Aloe* species were collected from DHA Karachi and from the Centre for Plant Conservation, University of Karachi. The plants were identified, and voucher specimens were kept in the herbarium of the Institute of Plant Sciences, University of Sindh, Jamshoro. Young leaves were obtained from mature succulent aloes and stored at -20°C. All fifteen *Aloe* species employed in this assay are named in (Table 1).

Table 1. *Aloe* species used in the study.

S.NO.	Plant Sample	Voucher No.
1.	*Aloe vareigata*	SINDH020032
2.	*Aloe rauhii*	SINDH020036
3.	*Aloe brevifolia*	SINDH020040
4.	*Aloe descoingsii*	SINDH020035
5.	*Aloe juvenna*	SINDH020044
6.	*Aloe deltoideodonta*	SINDH020037
7.	*Aloe humilis*	SINDH020038
8.	*Aloe arborescens*	SINDH020042
9.	*Aloe camperi*	SINDH020045
10.	*Aloe barbadensis*	SINDH020039
11.	*Aloe jucunda*	SINDH020034
12.	*Aloe wickensii*	SINDH020041
13.	*Aloe spinosissima*	SINDH020033
14.	*Aloe branddraaiensis*	SINDH020043
15.	*Aloe saponaria*	SINDH020046

DNA extraction and estimation:

Cetyl trimethyl ammonium bromide (CTAB) method (Sambrok *et al*., 1989) was used for the extraction of DNA from Aloe leaves. Isolated DNA was checked at 260nm and 280nm using UV Spectrophotometer (JENWAY, Model Genova, Serial No. 1489). The quality of isolated DNA was observed by loading it onto a 0.8% agarose gel and observed under a gel documentation system (UV Tech™, UK). For RAPD analysis working DNA concentration was maintained at 10 ng/µL in PCR PCR-grade. All DNA samples were stored at -20°C.

RAPD PCR amplification:

A total of nineteen random primers were applied to amplify fifteen DNA samples from Aloe species. The master mix was prepared containing 2 µL (10 µM) of primers, 7.5 µL (2 x) PCR buffer (GoTaq Green Master Mix), 1 µL containing 10 ng DNA, and 4.5 µL PCR grade water to make a final volume of 15 µL. Amplification was performed in a Thermal cycler (Master Cycler, Eppendorf, Germany) with the following PCR program. Initial Denaturation was done at 94°C for 05 minutes, followed by 45 cycles of denaturation at 94°C for 1 min, primers annealing is shown in (Table 2), and the extension was done at 72°C for 2 minutes followed by a final extension at 72°C for 4 minutes, then held at 4°C till recovery. The PCR lid temperature was set as 105°C.

Electrophoresis:

PCR products were analysed using agarose gel electrophoresis (Gene-Link). PCR products were visualised with 5 µL ethidium bromide (0.05 µg/mL) in a 1% agarose gel, run on 1x TBE (Tris Borate EDTA) buffer (pH 8.0). To identify the molecular weight of PCR amplicons 100 bp ladder was used.

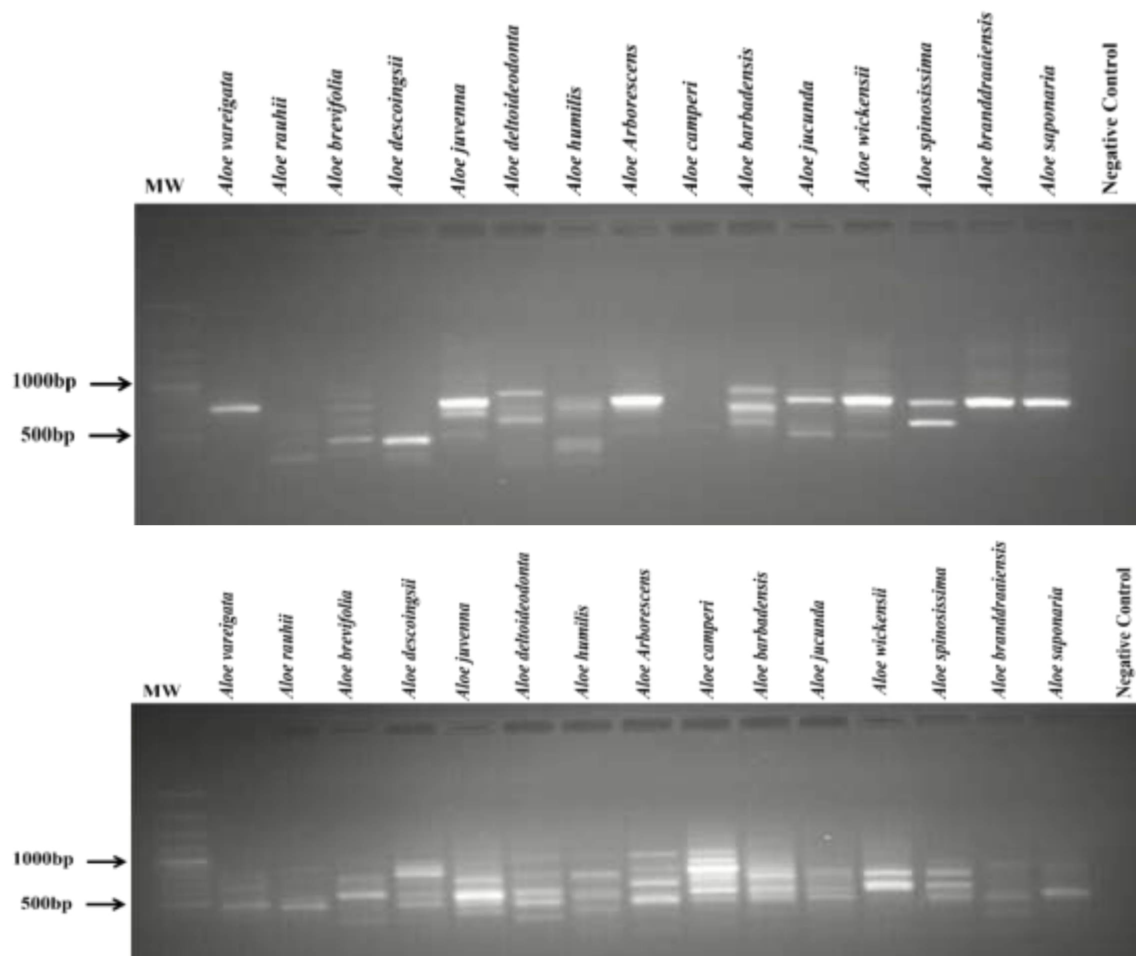

Fig. 1. RAPD-PCR amplified DNA gel image of A3 and OPA-1 primer.

Table 2. Polymorphism percentage in 15 *Aloe* species.

S.No.	Primer Name	Primer Sequence 5'to 3'	Annealing temperature	Scored Bands	Monomorphic bands	Polymorphic bands	Polymorphism %
1.	LC-76	GTGACGTAGG	41°C	6	0	6	100
2.	LC-77	GGGTAACGCC	41°C	5	0	5	100
3.	LC-83	AGCCAGCGAA	41°C	9	0	9	100
4.	LC-87	AGGTGACCGT	41°C	8	0	8	100
5.	LC-90	GTGAGGCGTC	41°C	7	0	7	100
6.	OPL-1	GGCATGACCT	32°C	5	0	5	100
7.	OPQ-2	AGTAGGGCAC	32°C	4	0	4	100
8.	OPA1	CAGGCCCTTC	36°C	8	0	8	100
9.	OPA4	AATCGGGCTG	36°C	6	0	6	100
10.	OPA10	GTGATCGCAG	36°C	7	0	7	100
11.	OPB4	GGACTGGAGT	36°C	5	0	5	100
12.	OPB7	GGTGACGCAG	36°C	6	0	6	100
13.	A3	GGGTAACGCC	36°C	8	0	8	100
14.	B1	GTTGCGATCC	36°C	8	0	8	100
15.	OPA-15	TTCCGAACCC	36°C	9	0	9	100
16.	OPA-20	GTTGCGATCC	36°C	7	0	7	100
17.	UBC-155	CTGGCGGCTG	36°C	7	0	7	100
18.	D-15	CATCCGTGCT	41°C	7	0	7	100
19.	D-20	ACCCGGTCTT	41°C	8	0	8	100
Total				**130**	**---**	**130**	**100**
Average				**6.84**	**---**	**6.84**	**---**
Range				**4-9**	**---**	**4-9**	**100-100**

RAPD Data Analysis:

After successful amplification reaction, amplicon fragments were counted from gel images. Amplified DNA bands were scored for presence (1) and absence (0) for each marker allele-genotype combination. Dice's coefficient was calculated individually for genetic similarity for all the fifteen Aloe samples, followed by the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) option of Sequential Agglomerative, Hierarchical and Nested Cluster (SAHN) (Sneath and Sokal, 1973). Dendrogram was constructed using the Numerical Taxonomy and Multivariate Analysis (NTSYS-pc version 2.0) (Rohlf, 1998). The percentage of polymorphism was calculated as the proportion of the total number of polymorphic bands amplified by the primer to that of the total number of bands produced by the same primer.

Table 3. Similarity matrix based on the number of shared fragments of RAPD-PCR among 15 *Aloe* species.

		Aloe vareigata	*Aloe rauhii*	*Aloe brevifolia*	*Aloe descoingsii*	*Aloe juvenna*	*Aloe deltoideodonta*	*Aloe humilis*	*Aloe arborescens*	*Aloe comperi*	*Aloe barbadensis*	*Aloe jucunda*	*Aloe wickensii*	*Aloe spinosissima*	*Aloe branddraaiensi*	*Aloe saponaria*
1	*Aloe vareigata*	1.000														
2	*Aloe rauhii*	0.304	1.000													
3	*Aloe brevifolia*	0.280	0.350	1.000												
4	*Aloe descoingsii*	0.253	0.476	0.378	1.000											
5	*Aloe juvenna*	0.205	0.352	0.405	0.494	1.000										
6	*Aloe deltoideodonta*	0.237	0.338	0.400	0.500	0.469	1.000									
7	*Aloe humilis*	0.250	0.250	0.388	0.383	0.461	0.463	1.000								
8	*Aloe arborescens*	0.301	0.188	0.250	0.257	0.293	0.181	0.285	1.000							
9	*Aloe comperi*	0.260	0.231	0.375	0.395	0.527	0.341	0.354	0.210	1.000						
10	*Aloe barbadensis*	0.233	0.233	0.253	0.363	0.365	0.383	0.428	0.298	0.289	1.000					
11	*Aloe jucunda*	0.169	0.135	0.257	0.263	0.395	0.222	0.289	0.242	0.390	0.356	1.000				
12	*Aloe wickensii*	0.163	0.163	0.333	0.358	0.481	0.378	0.366	0.294	0.380	0.426	0.621	1.000			
13	*Aloe spinosissima*	0.296	0.111	0.246	0.309	0.315	0.298	0.281	0.426	0.337	0.411	0.417	0.434	1.000		
14	*Aloe branddraaiensis*	0.150	0.188	0.250	0.257	0.320	0.242	0.349	0.300	0.342	0.328	0.333	0.323	0.491	1.000	
15	*Aloe saponaria*	0.222	0.111	0.297	0.188	0.310	0.244	0.304	0.232	0.271	0.280	0.326	0.235	0.318	0.372	1.000

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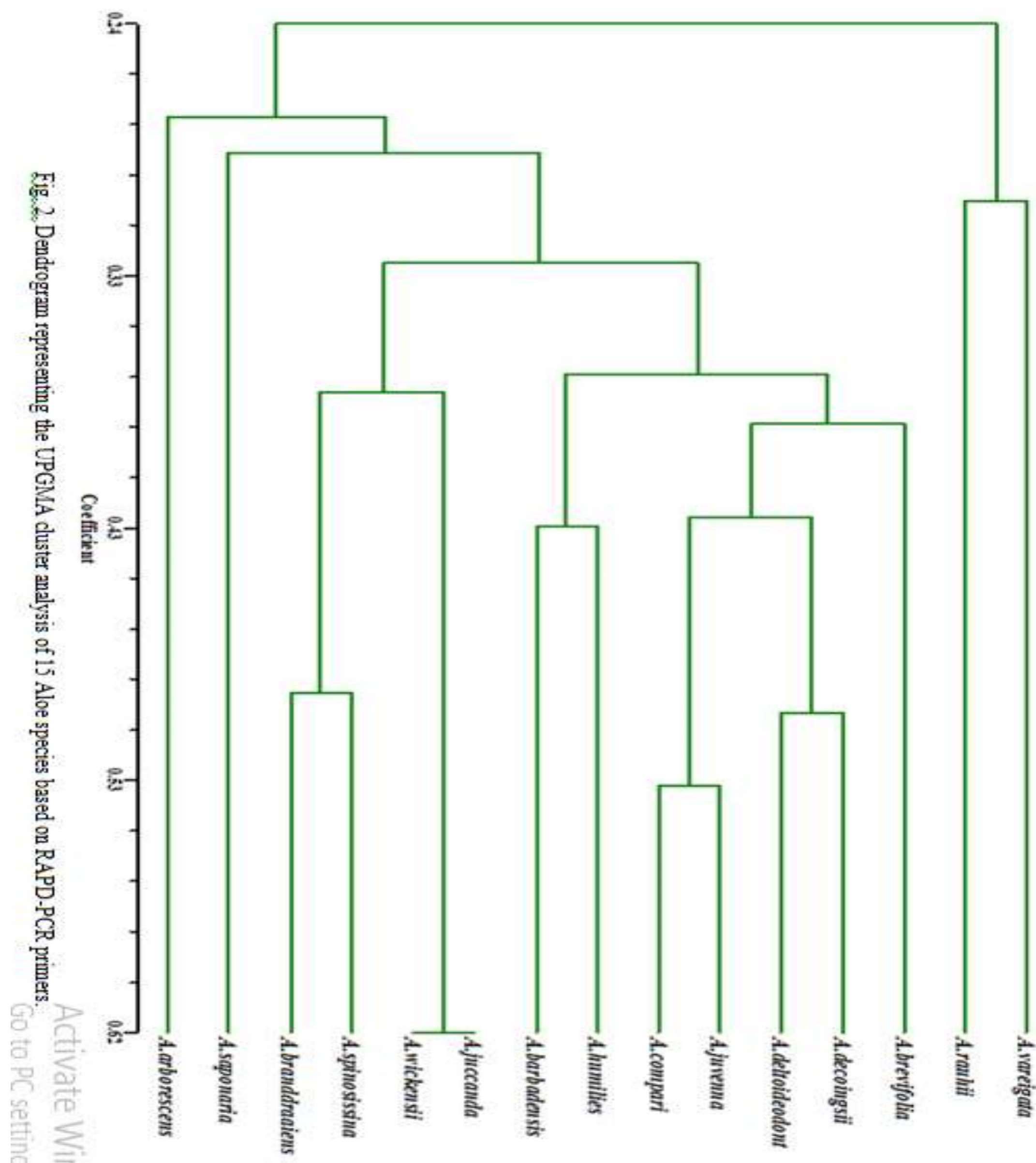

Fig. 2. Dendrogram representing the UPGMA cluster analysis of 15 Aloe species based on RAPD-PCR primers.

Results of RAPD

The aim of this study was to develop an accurate, cost-efficient and robust molecular marker-based method to test genetic diversity among fifteen *Aloe* species. RAPD is routinely used for the detection of genetic diversity (Asif *et al*., 2005), to establish phylogenetic relationships and for cultivar identification. Nineteen RAPD primers were applied to analyse genetic similarity/diversity among fifteen Aloe species. These nineteen decamer primers gave reproducible polymorphic amplicons (Table 2). Polymorphic bands were scored in the range of 4-9. The maximum 9 amplicons were generated from LC-83 and OPA-15 while the lowest number of polymorphic bands were observed in primer OPQ-2 with 4 bands followed by LC-77, OPL-1, and OPB-4 with 5 bands. An average number of 6.84 bands was observed by each primer. RAPD profile is shown in (Fig. 1). A total of 492 amplicons (number of species analysed x number of amplified products by all the primers) were produced using nineteen primers. The polymorphism percentage was observed at 100% with all nineteen RAPD markers. No monomorphic band was generated.

Genetic similarity indices were evaluated using RAPD markers profile. The recorded data were examined by means of Dice's coefficient to generalize the similarity matrices of the fifteen Aloe species. The genetic resemblance values varied from 0.111 *Aloe spinosissima* and *Aloe rauhii, Aloe saponaria* and *Aloe rauhii* to 0.621. The high genetic similarity of 0.621 was analysed between *Aloe wickensii* and *Aloe jucunda.* 02. Aloe species showed genetic similarity in the range of 0.50-0.527, out of the 15 Aloe species, 12 species showed greater than 0.40 genetic similarity, while 02 species showed 0.30 genetic similarity. The 0.205 genetic similarity was determined between *Aloe juvenna* and *Aloe variegata* (Table 3).

The cluster analysis by Un-weighted Pair Group Method of Arithmetic Averages (UPGMA) consisted of two clusters (Fig. 2). Cluster one consisted of two species *Aloe variegata* and *Aloe rauhii* showed 30% genetic similarity, while cluster two consisted of 13 Aloe species which were further divided into sub-clusters. In cluster two, species *Aloe juvenna* and *Aloe camperi* have 53% similarity. However, in the same cluster maximum genetic similarity of 62% was observed between *Aloe jucunda and Aloe wickensii.* Our results revealed a high level of polymorphism with a high level of genetic diversity among all fifteen *Aloe* species.

DISCUSSION

In the present study, DNA fingerprints were analysed and phylogeny was constructed using RAPD technique. For that purpose, fifteen Aloe species (Table 1) were collected from DHA Karachi and from the Centre for Plant Conservation, University of Karachi. Sindh, Pakistan. All selected nineteen primers yielded clear and reproducible banding patterns. Recently, DNA fingerprinting has been used as a competent technique in many areas to investigate genetic variations, genome profiling, gene determination, population genetics, classification, and diagnosis (Ahmad *et al*., 2017).

Molecular markers have been used extensively for the analyses of specific genetic structures because these markers can be detected at any stage of plant growth as these are neither tissue specific nor influenced by physiological or environmental circumstances. Moreover, these markers are more effective for confirmation of medicinal plants as a new pharmacognostic measure for quality control and certainty in medicinal plant research (Ezzat *et al*., 2016; Kumar *et al*., 2014).

Many studies have determined the genetic diversity of *Aloe* species with the influence of environmental conditions. RAPD-based; previous studies have also been reported such as Panwar *et al*. (2013) analyzed genetic diversity in 46 accessions of *Aloe. vera* collected from different locations in India and found 96% polymorphism with 10 primers. In addition, Pushpa and Samantaray (2015) used 14 primers to assess genetic diversity in 38 accessions of *Aloe vera* and observed 83% polymorphism. Recently, genetic variability in 8 *Aloe* species from Egypt was also studied and detected 100% polymorphism through RAPD technique (Ezzat *et al*., 2016) while from Iran 10 accessions of *A. vera* were characterized using RAPD markers. (Nejatzadeh-Barandozi, 2013).

However, the present study revealed that nineteen RAPD primers produced 492 amplicons across fifteen Aloe species with an average of 6.84 bands per primer. The nineteen RAPD primers showed 100% polymorphism level (Table 2). Das *et al*. (2017) also conducted studies on eleven *Aloe vera* accessions from India and obtained 87% polymorphism using eleven primers, these outcomes were in concordance with the current study. Variations in the DNA sequences directed to polymorphism revealed higher genetic diversity. As the markers are dispersed widely throughout the genome, they might be linked with functionally important loci (Pushpa and Samantaray, 2015 and Penner 1996).

The RAPD markers recognize throughout the genome in coding and noncoding sites as well as repetitive or single-copy sequences, while ISSR markers identify polymorphism between the microsatellite primer regions (Pushpa and Samantaray, 2015; Zietkiewicz *et al*., 1994; Williams *et al*., 1990). Since the majority of the genome consists of non-coding sites, mostly RAPD markers are also assumed to amplify these sites. These regions have larger random mutation rates and may have few phenotypic outcomes (Wen and Hsiao, 2001; Nayanakantha *et al*., 2010). The RAPD approach is considered very useful for assessing genetic variations, especially in poorly identified species (Bhat and Jarret, 1995) not just the variations level, RAPD markers also offer cultivar identification (Nayanakantha *et al*., 2010; Torres *et al.,* 1993).

In the current study, nineteen RAPD primers produced an average of 6.84 bands per primer contrary to 5.3 bands via ten primers with 10 Aloe accessions (Chandra and Choudhary, 2014).

In addition, the fifteen Aloe species showed 100% polymorphism similar to Nayanakantha *et al*. (2010) as 97 % polymorphism was obtained here through RAPD markers. The RAPD primers produced DNA bands ranging from 200 to 1200bp, consistent with Rathore *et al*. (2011) and Nayanakantha *et al.,* (2010). The

genetic similarity varied from 0.111 (*Aloe spinosissima* and *Aloe rauhii; Aloe saponaria* and *Aloe rauhii*) to 0.621 (*Aloe wickensii* and *Aloe jucunda*) (Table 3). These results are similar to the study reporting genetic similarity ranging from 0.54-0.72 (Ezzat *et al*., 2016). The present study showed the highest genetic diversity between Aloe species in concordance with Panwar *et al.* (2013) and Nayanakantha *et al.* (2010).

The genetic similarity was calculated and used to build a dendrogram to unveil the genetic relationships among these Aloe species. Fifteen species were clustered into two groups (Fig. 2). Cluster one consisted of two species *Aloe variegata* and *Aloe rauhii* showed 30% genetic similarity, while cluster two consisted of 13 *Aloe* species which were further divided into sub-clusters. However, the evaluated Aloe species have high morphological similarities in dried conditions, especially their leaves. Whereas in RAPD analyses great genetic diversity was observed among *Aloe* species.

Conclusions

Significant genetic diversity was observed among fifteen *Aloe* species through RAPD markers. In addition, polymorphic loci were identified and used to construct the phylogenetic tree. In this study, two clusters were detected. Cluster one contained two *Aloe* species while cluster two determined genetic similarity between 13 *Aloe* species which were further divided into sub-clusters. However, different *Aloe* species showed genetic diversity according to their respective origins. Moreover, RAPD marker systems may detect the genetic variations among the species indicating an efficient, accurate, and cost-effective testing method.

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