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Genetic diversity in different accessions of *Aloe* sp. using Morphological and AFLP markers

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Aloe has medicinal and cosmetic properties. This plant has stiff, gray-green, lance-shaped leaves containing clear gel in a central mucilaginous pulp (Nayanakantha *et al.* 2010). The gel contains an emollient polysaccharide, Glucomannan, which is a good moisturizer utilized in many cosmetics. There are morphological variations in some economically important *Aloe* species and leaf phenolic constitution (Darokar *et al.* 2003). However, due to lack of expressions for reproductive characters in some of the species, it is impossible to distinguish them. Since morph-chemical characters are dependent on age and environment, it is essential to characterize this medicinally and economically important genus genetically.The success of any genetic conservation and breeding program depends largely on the identification of the amount and distribution of genetic diversity in the gene pool of the concerned plant. Knowledge on the genetic diversity and relationships among plant varieties is important to recognize gene pools, to identify gaps in germplasm collections and to develop effective conservation and management strategies. In this way, molecular evaluations can provide insights into the genetic structure and diversity within and among varieties from different geographical origins, producers and distributors (Nayanakantha *et al.* 2010). Without this information, breeders have no means of selecting appropriate plant material for the participation in screening and breeding programs, with a view to the introduction of novel varieties into a country. 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. To this day, only few studies on genetic diversity of *Aloe* species were reported in literature, Adams *et al.* (2000b) constructed a phylogenetic tree using 28 Aloe species by sequence analysis of the internal transcribed spacer (ITS) of 18S-5.8S-26S rDNA, and found the distribution of 18S-5.8S-26S rDNA was variable in number, location and size in different Aloe species. Afterwards, Shioda *et al.* (2003) investigated the phylogenetic relationship for different varieties of Aloe using random amplified polymorphic DNA technology.

Among various molecular markers, the AFLP ability was evaluated for *Aloe* genetic studies as well as for a discrimination of *Aloe* accessions, based on their geographical origin and producers. In recent years, AFLP technique was used to estimate the genetic diversity in *Aloe* sp. germplasm collections. The advantages of this technique include the large number of loci analyzed, high polymorphism levels, high reproducibility without prior sequence knowledge, and genome-wide marker distribution. Furthermore, The AFLP technique provides a useful approach for evaluating genetic differentiation, particularly in those species that are poorly known genetically and are propagated vegetative such as *Musa* and *Lilium*. Not only, has the extent of variation but also AFLP provided markers even for cultivar identification and germplasm evaluation (Shioda *et al.* 2003, Darokar *et al.* 2003).

Information about the genetic diversity of *Aloe* germplasm in Iran is particularly important for variety identification, to enhance the classification of germplasm collections and exploiting them in breeding programs and for the development and introduction of new accessions. Thus, the present study was taken to characterize the *Aloe* germplasm accessions collected from different geographical regions of Iran and maintained in Islamic Azad University for investigating genetic diversity in some *Aloe* sp. accessions using morphological and AFLP markers.

Thirty three *Aloe* accessions were collected from different geographical regions of Iran and were planted in the farmland of Iranshahr Islamic Azad University in September 2009.

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These accessions were including 31 wild genotypes and 2 commercial genotypes (Table 1).

Morphological characters such as sprig number, plant height, leaf length, leaf width, leaf thickness, leaf fresh weight, leaf dry weight, number of burr in leaf, number of leaf, mean of leaf burr length, mean of inter-burr length, stem length, stem diameter, mean of internode length, leaf cuticle thickness, leaf mesophyll thickness, leaf gel weight, the gel weight ratios to leaf through weight were recorded in all Aloe accessions for comparative studies. Morphological data were analyzed by SPSS version 19, MSTATC and SAS software version 9.1 Ref.

Total genomic DNA was extractedusing a modified 2 × CTAB method described by Doyle and Doyle (1990). First 3-4g of the leaf fresh tissue that gel like mesodermal region removed, and the upper surface cut into pieces (4 × 4 mm). The DNA was then purified by phenol: chloroform extraction and ethanol precipitation. Then, quality and purity checked by electrophoresis in an agarose gel in 1 × TAE buffer. Quantification of DNA was done in UV spectrophotometer by measuring optical densities at 260nm and the ratio of OD 260/280nm respectively. Finally, all DNA samples were diluted to get 50 ng μL^{-1} solutions and were stored at -20°C for use in AFLP assay.

Details of AFLP assay, adapter and primer sequences, PCR conditions for pre-selective and selective amplifications, and PCR product electrophoresis were performed according to Zabeau and Vos (1993). Genomic DNA was restricted with the *Eco*RI/*MseI* enzyme combination, double stranded adapters specific to each site were ligated, and pre-selective amplification was performed with primers complementary to the adaptors and with one selective base at the 3' end. Selective amplification was carried out with 16 primer combinations, which were synthesized by MWG (Germany). Fragments were resolved using acrylamide sequencing gels containing 7 M urea in 1 × TBE buffer. Gels were run for 1.5–2 h at 100 W until the forward running to Dye (Bromophenol blue) reached the end of the gel. The DNA bands were visualized by silver staining. Fragments in the range 59-388 base pairs were scored. The fragments produced by each primer combination were treated as characters and numbered sequentially. Genotypes/individuals were scored for the presence (1) or absence (0) of good-quality peaks. Binary data matrix was constructed based on band scores and were analyzed in NTSYS-pc version 2.02 software package. Different polymorphic fragments produced by each primer were treated as the unit and numbered sequentially. Monomorphic fragments and those with low intensity were not taken into account. Similarity indices were calculated using the coefficients of Jaccard to estimate relationships between accessions. The similarities' matrix was the basis for cluster analysis. A dendrogram of genetic relationship was produced by clustering around the data using the unweighted pair group method with an arithmetic average UPGMA (Rohlf 2000). The cophenetic correlation coefficient was calculated, and the Mantel test was performed to check the goodness of fit of cluster analysis to the similarity matrix on which it was used. By the Mantel test, the correlation between the similarity matrix and the cophenetic matrix obtained by the Jaccard's coefficient, respectively, is calculated separately. Then the method by which the correlation coefficient is higher is selected for analysis(Rohlf, 2000). A principal coordinated analysis (PCO) was performed on the similarity matrix using the DCENTER and EIGEN procedures; and two and three-dimensional scatter plots were constructed by the Matrix Plot option (Rohlf 2000).

Morphological analysis

In the present study, thirty three *Aloe* accessions were studied with some morphological traits that some of those were easily separated from other accessions by their distinct phenotypic characteristics stem from development. Morphological analysis indicated that all the studied various characters have a significant difference at $P < 0.01$ among *Aloe* accessions. Sarbaz-6 accession was found to be the tallest (77 cm) as it possesses a distinct stem (calescent) with long internodes. Minimum leaf thickness (1.23 cm) and wideness (1.680 cm) was recorded for zehkalot accessions and chanf-2 accessions then. Therefore, these accessions contained a lesser amount of gel. Maximum amounts for leaf fresh weight, leaf dry weight, gel weight, number of leaf, stem length. Mean of internode length, leaf mesophyll thickness, leaf length and leaf width characteristics were recorded for Sarbaz-6 that it was a wild accession. Maximum sprig number and gel weight ratio to leaf through weight for Chanf-1 accession and maximum mean of inter-burr length in the Zaboli-2 accessions were measured. Furthermore, maximum leaf cuticle thickness and number of burr in leaf were found in the Iranshahr-1 accession and maximum mean of leaf burr length and minimum stem diameter were recorded in the Sarbaz-2 accession. In this paper, we used accession label instead of accessions name for comfortable explanation (Table 1).

Genetic diversity studied based on morphological various characters using analysis of the cluster among *Aloe* accessions. Dendrogram using average linkage (between groups) indicated that all accessions were divided into three major groups. There were commercial accessions (31 & 32 numbers) in the third group, and the best wild accession (number of 29) was in this group. This clustering indicated that there were differences among all accessions. Commercial accessions performed better than wild accessions and were ranked higher; whereas the most wild-accessions performed worse than commercial accessions and were ranked lower (Fig 1). According to them, the similarity mean between commercial accessions was 97% while the similarity mean among wild accessions was 79%. Thus, the observed morphological differences and similarity values of *A. vera* strains maintained

Table 1 *Aloe* accessions name and their given label

Accession's name	Label	Accession's name	Label
chanf-1	1	segar	18
gotij-1	2	zaboli-3	19
phanouch-1	3	zaboli-4	20
sarbaz-1	4	chanf-2	21
sarbaz-2	5	spakeh-2	22
sarbaz-3	6	sarbaz-5	23
sarbaz-4	7	chabahar-1	24
spakeh-1	8	chabahar-2	25
iranshahr-1	9	iranshahr-2	26
bent	10	gotij-3	27
zaboli-1	11	spakeh-3	28
zaboli-2	12	sarbaz-6	29
lashar	13	khash	30
saravan	14	zehkalot	31
nikshahr	15	control-1	32
phanouch-2	16	control-2	33
gotij-2	17		

at Iran suggest that they all possess a useable genetic diversity.

Phenotypic correlations among morphological various characters indicated that there were highly significant correlations at P< 0.05 or P< 0.01 among the most studied characters. For example, a highly significant positive correlation at P < 0.01 among the gel weight and all the studied characters except the numbers of burr in leaf were observed. These resultsindicated that some associated factors correlating with each other contribute in the occurring of these characters (saljooghianpour *et al.* 2010). *Aloe* accessions were more separated with the help of their leaf morphology. Although, common *Aloe* accessions exhibited some variations in height, leaf size and gel weight, these could be attributed to the adaptations of their original geographical and environmental conditions. Complete phenotypic expression of vegetative characters that show variations, 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. Hence, they lack the resolving power needed to identify individual genotypes (Nayanakantha *et al.* 2010). There are other Aloe species, which resemble *A. vera* accessions in growth habits and morphological traits. More, identifying *Aloe* accessions at its early stage is also sometimes ambiguous. These

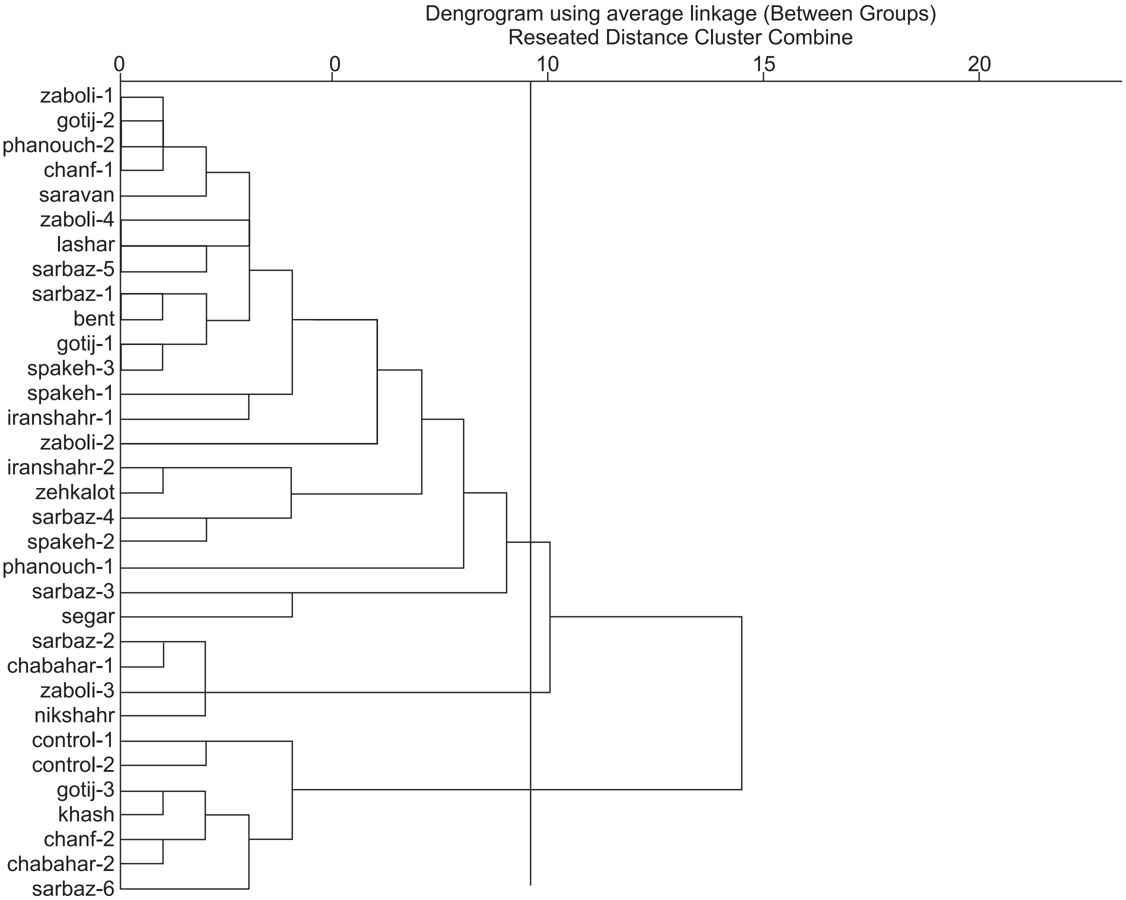

Fig 1 Dendrogram cluster analysis of 33 *Aloe* accessions based on morphological characters

accessions were further subjected to AFLP analysis for the assessment of genetic variation at the molecular level. Thus, in order to determine genetic variations of these accessions, AFLP markers were used in the present study.

Levels of AFLP polymorphism

In this study, we applied AFLP fingerprinting to evaluate diversity in *Aloe* accessions. The 16 primer combinations generated 411 polymorphic and clearly scorable fragments in across the 33 accessions. The number of polymorphic fragments ranged from 19 for the primer combination E+ACT/M+GAG and E+AGG/M+CAA to 31 for E+AAC/M+CAT and E+AAC/M+ACG, with a mean of 62.12 fragments and 25.687 polymorphic fragments per primer combination (Table 3).

Results show that most of the markers have a suitable discriminating power. The high number of polymorphic bands and the suitable level of polymorphism within *Aloe* accessions suggest that AFLPs are highly discriminatory and powerful markers for classification, fingerprinting and diversity analysis. Furthermore, the high polymorphism production makes AFLP markers a powerful tool for genotyping a large number of accessions and suitable for the evaluation of genetic diversity in *Aloe* gene banks. There were no region-specific (diagnostic) markers (present in accessions from the same geographical origin and absent in others), and no AFLP marker could be clearly discriminate commercial accessions and wild accessions.

Cluster analysis using the Jaccard's matrix showed a clear separation of the 33 *Aloe* accessions. *Aloe* accessions classified into three major clusters (Fig 2). Within each cluster, almost accessions from the same geographical origins however tended to group together, and the overall geographic

Table 3 AFLP primer combinations and percentage of polymorphism

Primer combinations	Total number of band	Number of polymorphic band	Polymorphism (%)
E+GTC/M+GAG	58	23	39.6
E+AGG/M+GAG	62	25	40.3
E+ACT/M+GAG	45	19	42.2
E+GTC/M+ATA	51	20	39.2
E+AGG/M+ATA	60	26	43.3
E+GTC/M+CCC	68	27	39.7
E+ACT/M+CCC	59	30	50.8
E+GTC/M+CAT	70	29	41.4
E+AGG/M+CAT	55	27	49
E+ACT/M+CAT	63	28	44.4
E+AAC/M+CAT	78	31	39.7
E+GTC/M+CAA	57	26	45.6
E+AGG/M+CAA	49	19	38.7
E+AAC/M+CAA	66	22	33.3
E+ACT/M+ACG	74	29	39.1
E+AAC/M+ACG	79	31	39.2
Total	994	411	
Mean	62.1	25.68	41.3

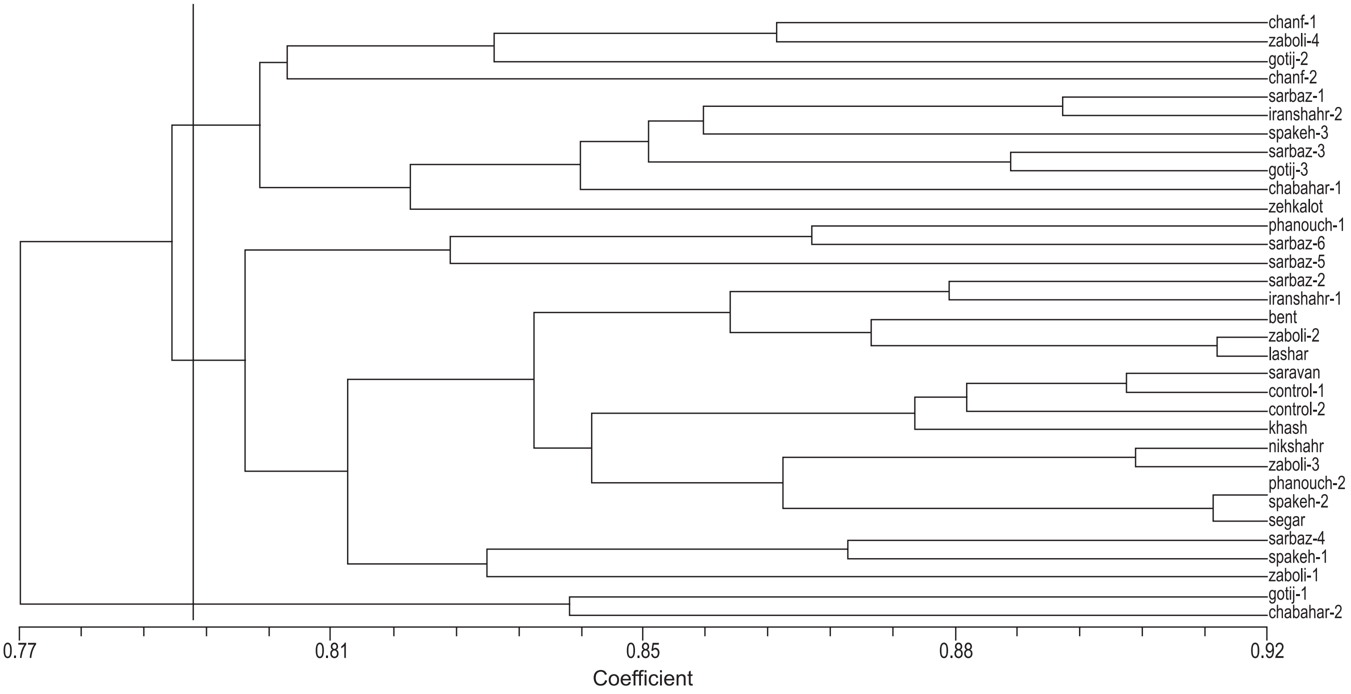

Fig 2 Cluster analysis of 33 *Aloe* accessions revealed by AFLP data based on Jaccard's coefficient.

proximity was rather high, as shown by the AFLP dendrogram. Commercial accessions (31 and 32 numbers) tended to group together, and this cluster was more clearly distinguished. However, the similarity mean and the UPGMA dendrogram both suggested that the genetic diversity is not very remarkable. According to them, the similarity mean between commercial accessions was 89% while the similarity mean among wild accessions was 79%. Thus, the observed molecular differences and similarity values of *A. vera* strains maintained at Iran suggest that they all possess a useable genetic diversity.

The result of PCO analysis was in agreement with what was found in the molecular data analysis. Three PCO axes explained 21.8714%, 5.9798% and 5.0917% of the total variance in the data set, respectively, resulting in a cumulative value of 32.9429%. PCO axes separated *Aloe* accessions into three groups and occupy the plot intermediate position less. On the other hand, separated commercial accessions into one group indicated that PCO analysis is suitable and efficiency analysis for molecular data.

Although the commercial accessions are known to have arisen from a limited number of introductions resulting in a lower level of genetic diversity compared to wild accession's gene pool, the slight difference among all studied accessions in genetic diversity indicates probably their main origin are similar. Just, the same, we suggest that wild accessions are still preferable for introduction into breeding programs and further improvement of *Aloe* accessions. These results may indicate the role of factors other than the origin in the determination of genetic similarities, which may contribute to a further separation of genetically homogeneous *Aloe* germplasm (Nayanakantha *et al.* 2010). However, that wild accessions and its selection were not genetically divergent enough to be considered as different accessions and their similarity is firmly supported (Fig 2). Besides, in other field experiments, no obvious phenotypic dissimilarity in apparent characteristics could be observed. These results may be considered as a confirmation of the suitability of AFLP in discriminating *Aloe* accessions based on their effective genetic differences (Esfahani *et al.* 2009). The most surprising result of this analysis was the high genetic diversity observed in some of the wild accessions (Fig 2). In field experiments (morphological studies), no extreme dissimilarities with other accessions were observed in the phenotypic traits, and the appearance was completely normal. Due to this measured genetic distance, the accession may serve as a valuable genetic source and promising parental material in *Aloe* breeding programs, based on further research in future genetic, physiological and morphological studies.

The high similarity values among accessions indicate that there are not remarkable genetic variations in the present study. The higher mean genetic similarities and the lower genetic diversity among wild accessions reflect their genetic potential slightly. Thus, all the 16 primer combinations employed in the present study could not be very effectively used to distinguish *Aloe* accessions.

Therefore, the *Aloe* genetic background in Iran is much more limited than the world other regions. However, this should be further evaluated and confirmed based on a larger number of accessions from geographically more diversified regions.

Hence, phenotypic characters combined with AFLP analysis provided a better relationship to identify these accessions. However, AFLP and detailed morphological study are also desirable in order to understand all aspects of these variations.

The more researches on these accessions using other molecular markers could be useful for genetic resource identification and introduction.

SUMMARY

Thirty three *Aloe* accessions were collected from different geographical regions of Iran. Morphological evaluation and AFLP finger printing were applied for showing genetic variations among the studied accessions. Morphological analysis indicated that all the studied various characters have a significant difference P<0.01 among *Aloe* accessions and cluster analysis indicated that there are differences among all accessions, commercial accessions performed better than wild accessions and were ranked higher; whereas, the most wild-accessions performed worse than commercial accessions and were ranked lower. AFLP finger printing indicated that, there were no region-specific markers, and no AFLP marker can distinguish commercial accessions and wild accessions clearly. The higher mean genetic similarities and the lower genetic diversity among wild accessions reflect their genetic potential slightly. Thus, all the 16 primer combinations employed in this study could not be effectively used to distinguish *Aloe* accessions. The high similarity values among accessions indicate that there are not remarkable genetic variations in the present study. Furthermore, the result of PCO analysis was in agreement with what was found in the molecular data analysis. Phenotypic characters combined with AFLP analysis provided a better relationship to identify these accessions. However, AFLP and detailed morphological study are also desirable in order to understand all aspects of these variations.

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