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# Genetic Variation of Some Iranian Black Henbane Accessions (*Hyoscyamus niger* L.) using RAPD and SDS-PAGE of Seed Proteins

**Mohammad Javad Yousefi[1*] • Mohammad Esmaeil Hassani[1] • Gholamreza Salehi Jouzani[2] • Hassan Maddah Arefi[3] • Matin Mohammadipour[2]**

[1] Department of Horticultural Sciences, University College of Agriculture and Natural Resources, University of Tehran, P.O. Box 31587-77871, Karaj, Iran
[2] Department of Microbial Biotechnology and Biosafety, Agricultural Biotechnology Research Institute of Iran (ABRII), P.O. Box 21525-1897, Karaj, Iran
[3] Research Institute of Forests and Rangelands, P.O. Box 13185-116, Tehran, Iran

*Corresponding author*: * **javadyousefi_138@yahoo.com**, **mjyousefi1038@gmail.com**

## ABSTRACT

Genetic diversity of 20 accessions of Iranian black henbane (*Hyoscyamus niger* L.) collected from various agro-ecological regions was studied using RAPD DNA markers and SDS-PAGE of seed proteins. Application of 16 RAPD arbitrary primers resulted in a total of 208 bands, in which 196 bands were polymorphic (94.31%). Comparisons, based on Jaccard's coefficient and UPGMA clustering, revealed a considerable level of genetic diversity among accessions. Maximum similarity was observed between Roodbar-2 and Siahkal-1 accession with 80% similarity, while maximum differences were observed between the Isfahan accession and other accessions, placing it in a separate group. Electrophoresis of total seed proteins also revealed polymorphisms among accessions and resulted in three main clusters. Seed protein profiles of black henbane accessions showed a relatively high level of intraspecific variability which coincides with the results of RAPD analysis. An average genetic similarity of 0.52 (ranging from 0.16 to 1.0) was found among the accessions. Cluster 1 divided in two subgroups in which Khalkhal and Roodbar-2 accessions with 100% similarity were in one subgroup, even though they originated from different geographical regions. Finally, the results of this study indicate that RAPD DNA markers and seed protein profiles seem to be suitable for assessing genetic diversity among *H. niger* accessions for future breeding programs.

**Keywords:** diversity, RAPD markers, seed protein profiles
**Abbreviations: PCR**, polymerase chain reaction; **RAPD**, random amplification polymorphic DNA; **SDS-PAGE**, sodium dodecyl sulphate polyacrylamide gel electrophoresis; **TBE**, tris boric acid EDTA; **UPGMA**, unweighted pair group method with arithmetic mean

## INTRODUCTION

The genus *Hyoscyamus* belongs to the tribe *Hyoscyameae* Miers and family Solanaceae. More than 13 species of this genus are found in Iran, three of which are endemic. The most important species in this genus is *H. niger* (black henbane), widely distributed in different parts of Iran (Khatamsaz 1998). Black henbane is a well known, annual or biennial, herbaceous medicinal plant native to western Asia and southern Europe. The plant grows to 1 m, and has delicate and slightly lobed leaves. The flowers are bisexual, bell-shaped and pale yellow in color with fine purple veining (Chevallier 2001). The chromosome number of black henbane is 17 (2n=34) (Sheidai *et al*. 1999).

Black henbane has been regarded as a rich source of pharmaceutically important tropane alkaloids, such as hyoscyamine and scopolamine (Strauss 1989). The leaves, flowering tops and seeds of the plant are widely used in herbal medicine as a mydriatic, sedative, painkiller, analgesic and also to treat asthma and bronchitis (Strauss 1989; Bruneton 1999; Chevallier 2001; Duke 2002). In agreement with recent WHO and ESCOP regulations, the quality of medicinal plants and their products have to meet requirements of safety, efficacy and stability (Franz 2000). To meet these demands, homogenous genotypes with well known biological properties are needed.

In recent years several breeding and registration of medicinal plant cultivars were reported from different countries (Bernath 2002). Improvement of morphological, yield biological characteristics and modification of the accumulation level of biologically active agents are the most important goals of any medicinal plant breeding program. The study of genetic diversity of available plant germplasm is a prerequisite of each breeding program because it provides the basis for tailoring desirable genotypes (Bernath 1996, 2002). RAPD and SDS PAGE profiles were successfully used for analysis of genetic diversity in many plants including *Salvia fruticosa* Mill. (Melpomeni *et al*. 1999), *Capsicum annum* L. (Anu and Peter 2003), *Ocimum basilicum* L. (De Masi *et al*. 2006), *Digitalis obscura* L. (Nebauer *et al*. 1999), *Fagopyrum* species (Rout *et al*. 2007), *Arachis pintoi* and *A. repens* (Bertozo and Valls 2001) and *Satureja hortensis* L. (Hadian *et al*. 2008).

Unfortunately, in spite of its importance, black henbane has received very little scientific attention, particularly germplasm evaluation and diversification by DNA markers and seed protein SDS-PAGE and not much information is available on intra-specific variation in molecular fingerprints for this species. Interrelationships of some Iranian *Hyoscyamus* species were studied using seed storage proteins and numerical taxonomy (Sheidai *et al*. 2000). Furthermore, seven species of *Hyoscyamus* L. were studied using isozyme markers in order to clarify taxonomic relationships within the genus (Monatasser-Kouhsari *et al*. 2006; Sharifi *et al*. 2006).

The selection of appropriate molecular tools for screening accessions in gene bank collections and elucidation of intra-specific variations has always been an important consideration.

Molecular markers provide a powerful tool for assessment of genetic diversity of germplasm and their management. Among several markers available Random Amplified

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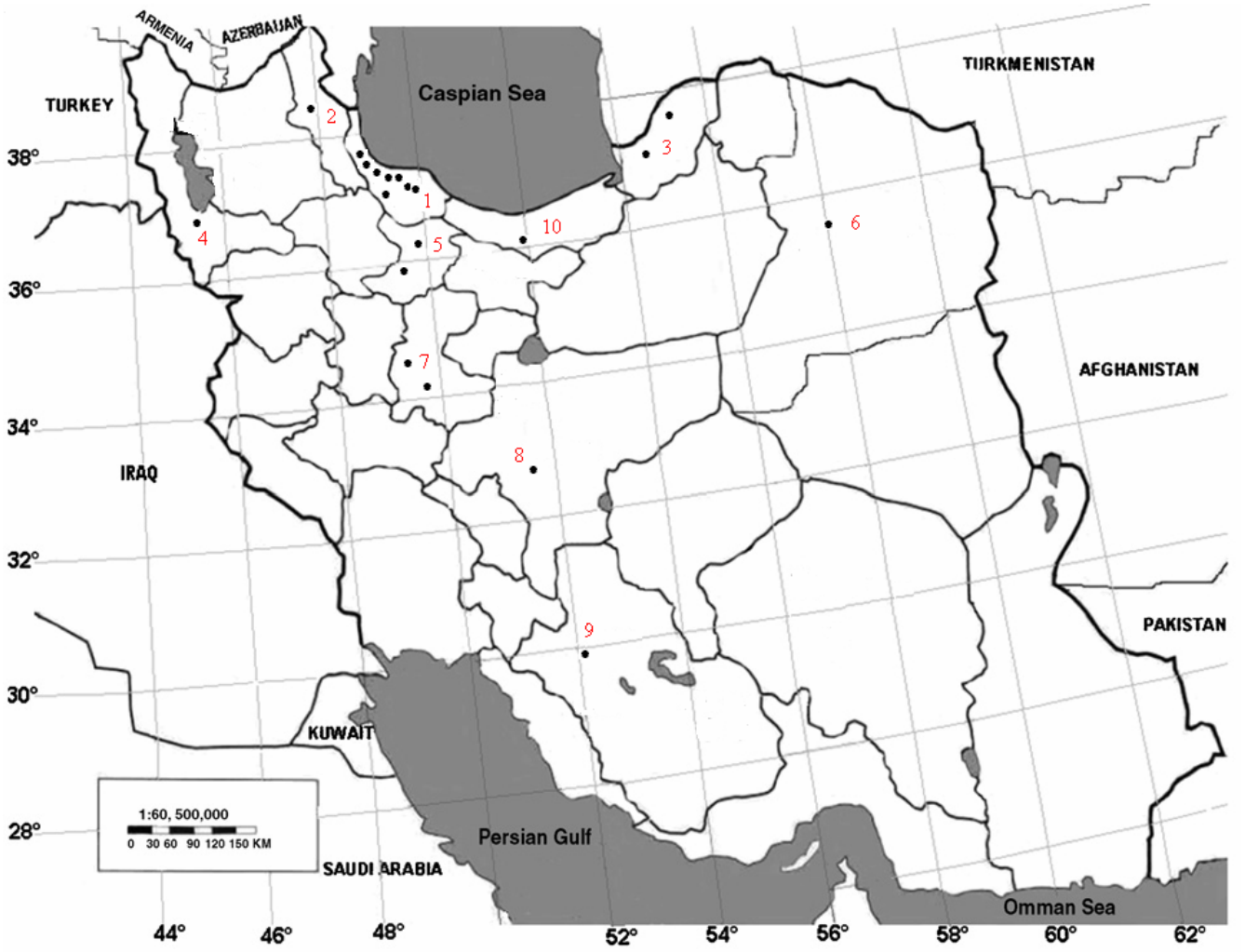


**Fig. 1 The geographical origins of the 20 *Hyoscyamus niger* accessions used in this study.** The numbers inserted in the map showing the provinces where accessions of black henbane were collected. 1: Guilan, 2: Ardebil, 3: Golestan, 4: West Azarbaijan, 5: Ghazvin, 6: Razavi Khorasan, 7: Markazi, 8: Isfahan, 9: Fars and 10: Mazandaran.

Polymorphic DNA (RAPD) is generally favored because of its sensitivity, simplicity, and cost-effectiveness, coupled with the fact that DNA sequence information is not required for primer design, no radioisotope labeling is needed for sample detection, and only a small amount of template DNA is required (Williams *et al*. 1990; Welsh and McClelland 1990). This technique is reliable when assaying a considerable number of primers and selecting a few repeatable and clearly interpretable RAPD fragments (Yang *et al*. 1999; Nayak *et al*. 2003; Lin *et al*. 2009). Seed proteins have the advantage of reflecting numerous variations as they belong to very polymorphic multigenic families (De Lumen 1990). Also SDS-PAGE of seed proteins provides polymorphisms on expressed genic regions which are very important (Doll and Brown 1979).

This research mainly focused on the analysis of genetic diversity of different Iranian accessions of *H. niger* L. using RAPD markers and SDS-PAGE of seed proteins in order to be applicable future breeding programs.

**Table 1** Accessions of black henbane used in this study.

| Origin (Province/City) | Source | Species | Accession code | No |
|---|---|---|---|---|
| Guilan (Roodbar-a) | RIFR[a] | *H. niger* | 21158 | 1 |
| Guilan (Roodbar-b) | RIFR | *H. niger* | 21155 | 2 |
| Guilan (Siahkal-a) | RIFR | *H. niger* | 21148 | 3 |
| Guilan (Siahkal-b) | RIFR | *H. niger* | 21149 | 4 |
| Guilan (Siahkal-c) | RIFR | *H. niger* | 21162 | 5 |
| Guilan (Talesh-a) | RIFR | *H. niger* | 21159 | 6 |
| Guilan (Talesh-b) | RIFR | *H. niger* | 21153 | 7 |
| Guilan (Fooman) | RIFR | *H. niger* | 15116 | 8 |
| Golestan (Golestan-a) | RIFR | *H. niger* | 18783 | 9 |
| Golestan (Golestan-b) | RIFR | *H. niger* | 19936 | 10 |
| Qazvin (Qazvin-a) | RIFR | *H. niger* | 23772 | 11 |
| Qazvin (Qazvin-b) | RIFR | *H. niger* | 22185 | 12 |
| Markazi (Markazi-a ) | RIFR | *H. niger* | 22288 | 13 |
| Markazi (Markazi-b) | RIFR | *H. niger* | 22290 | 14 |
| Isfahan (Isfahan) | RIFR | *H. niger* | 21178 | 15 |
| Fars (Shiraz) | RIFR | *H. niger* | 22254 | 16 |
| Ardebil (Khalkhal) | RIFR | *H. niger* | 13314 | 17 |
| West Azerbaijan (Uromieh) | RIFR | *H. niger* | 22851 | 18 |
| Mazandran (Baladeh) | — | *H. niger* | — | 19 |
| Razavi Khorasan (Kashmar) | — | *H. niger* | — | 20 |

[a]Research Institute of Forests and Rangelands of Iran.

## MATERIALS AND METHODS

### Plant material

Eighteen accessions of black henbane used for the present investigation were procured from gene bank of Research Institute of Forests and Rangelands of Iran (RIFR). Also, two accessions of wild growing *H. niger* were collected from northeast (Kashmar) and north (Baladeh) of Iran. The list of accessions and their original sites are shown in **Table 1** and **Fig. 1**.

### DNA extraction and PCR amplification

Genomic DNA was extracted from freshly collected leaves of individuals as described by Sharp *et al*. (1988). DNA was dissolved to an appropriate dilution in TE buffer and quantified in a spectrophotometer (Perkin Elmer, Lambda EZ201, USA). Fifty five arbitrary 10-mer primers (TIBMOLBIOL Co., Berlin, Germany) were

**Table 2** Selected primer combinations and level of polymorphism obtained by RAPD analysis.

| RAPD primer | Nucleotide sequence (5´-3´) | Total No. of bands | No. of Polymorphic bands | Polymorphism (%) |
|---|---|---|---|---|
| TIBMBA-19 | CCATCCGTTG | 9 | 9 | 100 |
| TIBMBA-13 | AGGGCGAATG | 14 | 14 | 100 |
| TIBMBA-14 | TCGGGAGTGG | 16 | 15 | 94 |
| TIBMBA-20 | GAGCGCTACC | 10 | 10 | 100 |
| TIBMBB-10 | ACTTGCCTGG | 12 | 12 | 100 |
| TIBMBB-14 | GTGGGACCTG | 12 | 11 | 91 |
| TIBMBC-03 | GGCTTGACCT | 16 | 16 | 100 |
| TIBMBC-14 | GGTCCGACGA | 13 | 11 | 85 |
| TIBMBC-20 | AGCACTGGGG | 15 | 14 | 93 |
| TIBMBD-16 | GAACTCCCAG | 6 | 6 | 100 |
| TIBMBD-19 | GGTTCCTCTC | 17 | 15 | 88 |
| TIBMBE-08 | GGGAAGCGTC | 14 | 14 | 100 |
| TIBMBE-12 | GGTTGTTCCC | 11 | 9 | 81 |
| TIBMBE-18 | CCAAGCCGTC | 16 | 15 | 94 |
| TIBMBE-19 | AGGCCAACAG | 12 | 10 | 83 |
| TIBMBE-20 | CAAAGGCGTG | 15 | 15 | 100 |
| Total | - | 208 | 196 | — |
| Average | - | 13 | 12.25 | 94.31 |

used for PCR amplification. PCR was performed based on the standard protocol of Williams *et al*. (1990). PCR was carried out in a thermocycler (iCycler, Bio Rad Co., Hercules, USA) at the following temperature profile: pre-denaturation for 4 min at 94°C; then 35 cycles consisting each of denaturation for 1 min; an annealing at 37°C for 1 min; an extension at 72°C for 2 min; and termination with a final extension at 72°C of 10 min. PCR products were resolved by electrophoresis using 1.6% (w/v) agarose gels (Biomol, Hamburg, Germany) in 1X TBE buffer (Tris-boric acid-EDTA), and photographed over UV light after ethidium bromide (100 mg $ml^{-1}$) staining. The reproducibility of the amplification products was checked thrice for each polymorphic primer and polymorphisms were scored from photographs.

## Seed protein extraction and electrophoresis

One hundred miligrams of each seed sample were selected to obtain a fine and homogenized whole mixture. Proteins were extracted in a pre-cooled mortar and pestle over ice with a 0.39 M Tris phosphate buffer (pH 8.3). The resulting mixture was centrifuged at 15,000 × *g* for 10 min. The crude extracts were boiled for 5 min in 77 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulphate (SDS), 10% 2-mercaptoethanol and 3% glycerol (Sanches-Yelamo *et al*. 1995). SDS-PAGE of the extracted proteins was carried out on a 12% polyacrylamide slab gel under reducing conditions as previously reported by Laemmli (1970). Samples containing 20 µg proteins were loaded on 1.5 mm-thick 12% acrylamide gels. Electrophoresis was carried out at a constant voltage of 100 V for 6 h. The gels were stained for 3 h in 0.25% (w/v) Coomassie brilliant blue R-250 followed by distaining in methanol: water: glacial acetic acid (4:5.3:0.7). Protein bands were visualized in a transilluminator under white light.

## Data analysis

Evaluation of genetic variations in RAPD profiles and the seed proteins was performed by calculating the individual band frequency for each accession. Polymorphism was manually scored as discrete variables, using '1' to indicate presence, and '0' to indicate absence of bands. Cluster analysis was performed on the similarity matrix based on Jaccard's coefficient and the UPGMA method using a NTSYS-PC version 2.1 software (Rohlf 1998). Principal Component Analysis (PCA) was performed to display the relationship among *H. niger* accessions in terms of their position relative to two coordinate axes. All PC analyses were carried out using SAS software (SAS Institute Inc., Cary, NC) procedures.

# RESULTS

## RAPD polymorphism

Results of PCR amplification using RAPD primers for primer screening showed that from 55 arbitrary primers, only 16 primers showed polymorphic bands (**Table 2**). Out of 208, 196 bands (94.31%) were detected as reproducible and polymorphic among the studied accessions, while the remaining 12 bands (5.69%) were monomorphic. The number of bands per primer varied from 6 (TIBMBD-16) to 17 (TIBMBD-19) with an average of 13. The average proportion of polymorphic markers across primers was 94.31%, ranging between 83% (TIBMBE-19) and 100% (TIBMBA-19, TIBMBA-13, TIBMBA-20, TIBMBB-10, TIBMBC-03 TIBMBD-16, TIBMBE-08, and TIBMBE-24). RAPD profiles obtained by TIBMBE-19 and TIBMBE-24 are shown in **Fig. 2**.

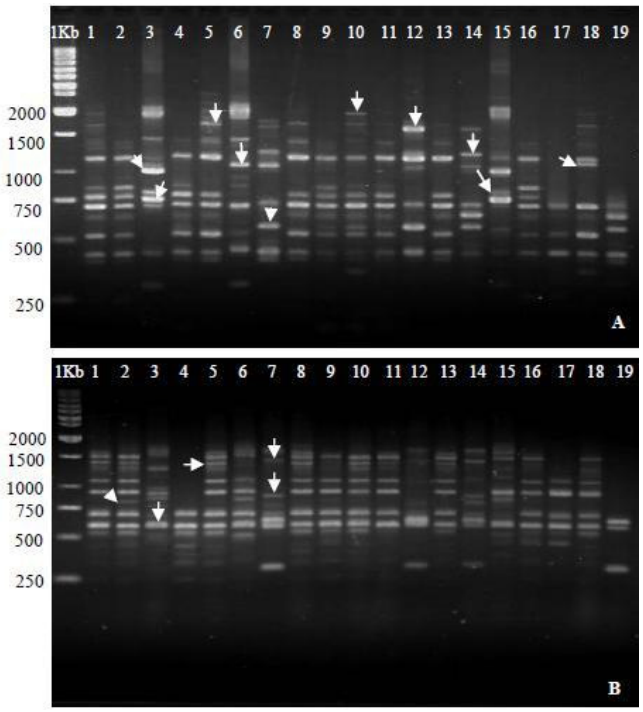


**Fig. 2** RAPD profiles of *H. niger* accessions amplified by 10-base primers TIBMBE-20 (**A**) and TIBMBE-19 (**B**). The numbers represent different *H. niger* accessions according to **Table 1**.

Estimates of genetic similarity of RAPDs based on the 196 polymorphic markers between 19 accessions of *H. niger* ranged from 0.23 for Fooman and Isfahan (minimum similarity) to 0.80 for Siahkal-a and Roodbar-b (maximum similarity). According to the dendrogram (**Fig. 3**), at a similarity level of 40% the accessions were divided in four clus-

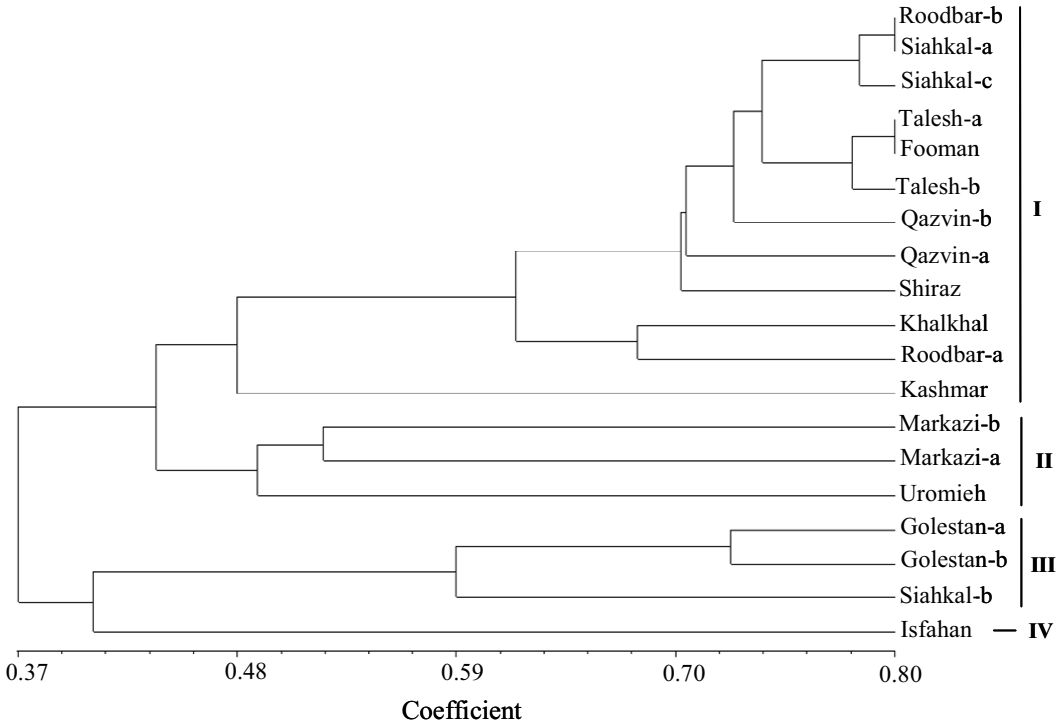


**Fig. 3 UPGMA dendrogram based on Jaccard's genetic similarities calculated from RAPD data, showing 4 groups and relationships among the 19 accessions of *H. niger*.**

ters, containing 12, 3, 3 and 1 accessions, respectively. As is clear in the dendrogram, the classified accessions showed a high level of variation. The most accessions collected from different regions of Guilan province, including Fooman, Siahkal-a, Siahkal-c, Roodbar-a, Roodbar-b, Talesh-a, Talesh-b (except Siahkal-2), were placed in relatively close branches. This was also observed for the accessions of Qazvin (a, b), Golestan (a, b) and Markazi (a, b) provinces. It is important to note that clustering showed that most of the accessions of Guilan and Qazvin provinces were placed in close branches which may indicate similarity in their genetic make-up and origin. The cophenetic correlation coefficient indicated a correlation of $r$ = 0.92 between the similarity matrix and the UPGMA dendrogram, indicating a good representation of the relationships among the accessions.

## Polymorphism of seed proteins

As a result of the SDS-PAGE of seed proteins, 10 or 17 different polymorphic bands were observed. Bands number 2 and 9 were just seen in Golestan-2 accession (**Fig. 4B**). These bands could be probably used as markers for identifying this accession. Band number 10 has been seen in Golestan-2 and Siahkal-3 (with less thickness in Siahkal-3). The size (molecular weight) of fractionated polypeptide bands was from 19 to 70 kDa. The most variation was observed in the range between 25 to 50 kDa. The profiles of seed proteins in some studied accessions are shown in **Fig. 4**.

The dendrogram on the basis of seed proteins profiles clustering (**Fig. 5**) included the accessions into three groups (similarity coefficient, 0.50). The first group contains two subgroups. Accessions Khalkhal and Roodbar-2 were clustered in the first subgroup and showed 100% similarity, though they are from different geographical origins. Three accessions from Guilan province were placed in this group. In the second subgroup, the accession of Markazi-2 make distinctive branch with the rate of 61% similarity in comparison with other accessions in this group. The second group is also divided into two subgroups and contains six accessions from different regions. Interestingly, this group

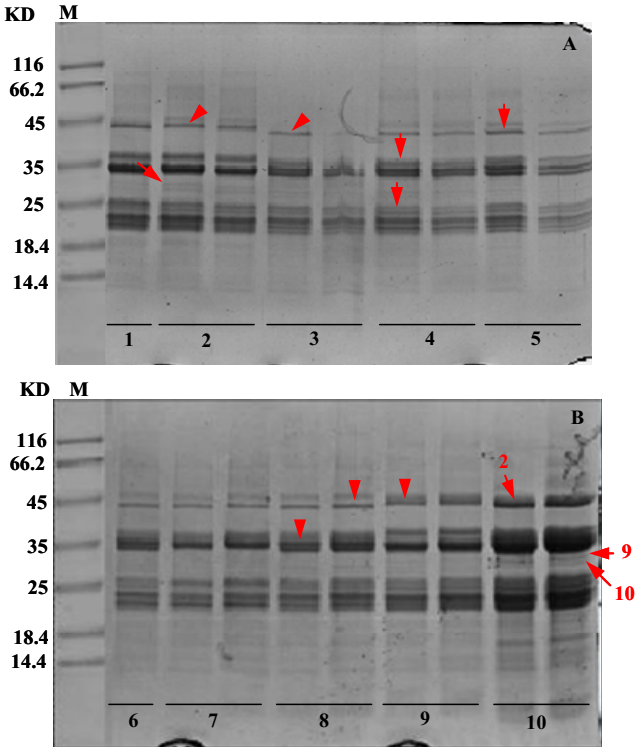


**Fig. 4 SDS-PAGE profile of seed proteins of some accessions of black henbane.** The numbers represent different *H. niger* accessions according to **Table 1**.

contains accessions form provinces in north and northwest of Iran (Guilan, Golestan, Qazvin and West Azerbaijan). Comparing with other accessions in this group, Qazvin-2 and Talesh-2 accessions form distinctive branches with the rate of 64 and 60% similarity, respectively. The most studied accessions (10 accessions) were placed in the third group. It is important to note that accessions included in this group were from different origins and therefore no geographical relations were found. This group contained four sub-

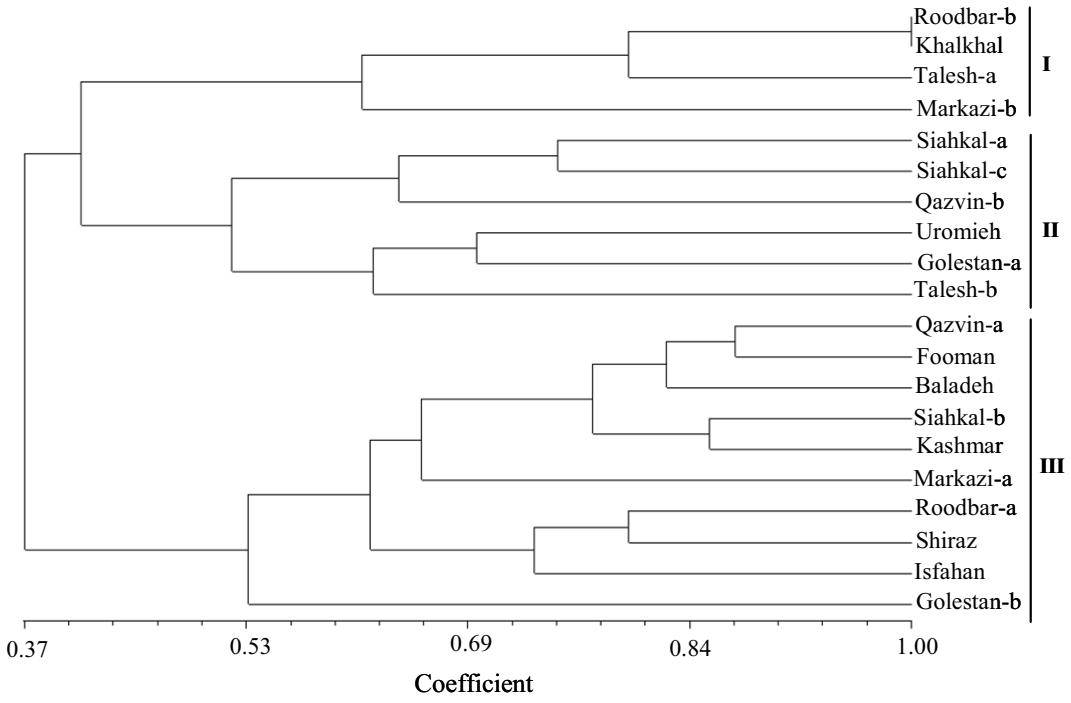


**Fig. 5 Cluster analysis of 20 *H. niger* accessions based on seed proteins profiles based on the UPGMA analysis and Jaccard's coefficient.**

groups in which accessions were classified in different subgroups with little difference (maximum similarity rate of 87%). For instance, its first subgroup contains five accessions, including Qazvin-1, Fooman, Baladeh, Siahkal-2 and Kashmar, though the recent accessions belong to different geographical regions. In this subgroup, Baladeh accession form separate branch with an 83% similarity rate in comparison with the other accessions. The accessions Golestan-2 and Markazi-1 are in distinctive subgroups with more differences and a similarity rate of 53 and 65% respectively, compared with other accessions in the third group. Totally, clustering analysis using seed proteins profiles showed approximately high genetic diversity among the studied black henbane accessions. Totally, eight accessions from Guilan province were placed in the close branches. However, this situation was not observed for accessions of Qazvin, Golestan and Markazi provinces, as those accessions were categorized in different groups and subgroups. In this experiment, correlation between similarity matrix and the dendrogram was high ($r = 0.90$).

PCA of the RAPD and seed protein SDS-PAGE profiles is illustrated in **Fig. 6**. Spreading accessions in the figure was in accordance with the distribution in dendrogram branches of the two experiments. In RAPD experiments, PCA1 and PCA2 showed the total variance of 21.11 and 12.49% (33.61), respectively. The RAPD markers almost had good distribution in genome and also in evaluating the genetic diversity of the accessions. Furthermore, in seed protein analysis, the total variance of PCA1 and PCA2 were 27.32 and 15.68% (43%), respectively.

## DISCUSSION

Research on genetic diversity in germplasm collections is important for germplasm collection, preservation and plant breeding. The efficiency and precision of genetic diversity estimate depends largely upon the tools available to the researchers and how they fit it into the breeding scheme. As the correlation between genetic diversity and the evolutionary changes done, is high, there is close relation among the efficiency of genetic improvement of a population and genetic variation for the considered character (Bernath 1996).

Genetic diversity of the native Iranian black henbane accessions was investigated using RAPD markers, and considerable polymorphism was observed among banding patterns of the studied accessions which showed high level of genetic diversity. A high relationship between molecular variation obtained from RAPD data and geographical origins of the accessions was observed. For example, clustering analysis showed that accessions from Guilan province were placed in close clusters, and also this subject was observed for the accessions from Golestan, Markazi and Qazvin provinces. These results may exhibit a possible genetic relationship among the accessions from one region. The results in RAPD analysis was in accordance with previous works by Vieira *et al*. (2001) and Hadian *et al*. (2008), which reported a relationship between genetic diversity and geographical diversity based on the dendrograms of RAPD data for *Ocimum gratissimum* and *Saturja hortensis* accessions, respectively. A small part of the dendrogram showed little relationship of geographical origins with molecular diversity gained from RAPD data. For instance, accession Siahkal-2 form distinctive branch and is apart from other accessions in Guilan province. Also, some accessions from different origins, such as Khalkhal and Roodbar-1, were classified in one subgroup. This may be because of the translocation of some accession during long time. Pezhmanmehr *et al*. (2009), Nebauer *et al*. (1999) and De masi *et al*. (2006) studied the *Bunim persicum*, *Digitalis obscura* and *Ocimum basilicum* genetic diversity by RAPD markers, respectively. Their results showed that there is a weak relation between geographical origin and clustering obtained from genetic studies among investigated accessions which are not similar with the results of our experiment.

According to the above explanations (the partial relation among black henbane accessions in geographical places) geographical distribution may be the reason for close or far genetic relationship and in breeding programs needs highly genetic diversity, the selection of accession can possibly be influential on the basis of geographical origins.

Genetic variation of the studied black henbane accessions using SDS-PAGE of seed proteins showed relatively high genetic variation. As the result of these experiments, accessions from the same origins mostly did not show genetic relationships (exception for some accessions from Guilan province). For instance, the accessions of Siahkal-2 and Roodbar-1 were clustered in separate group and were far from other accessions of Gulian province. In contrast, the accessions from different origins and provinces, such as

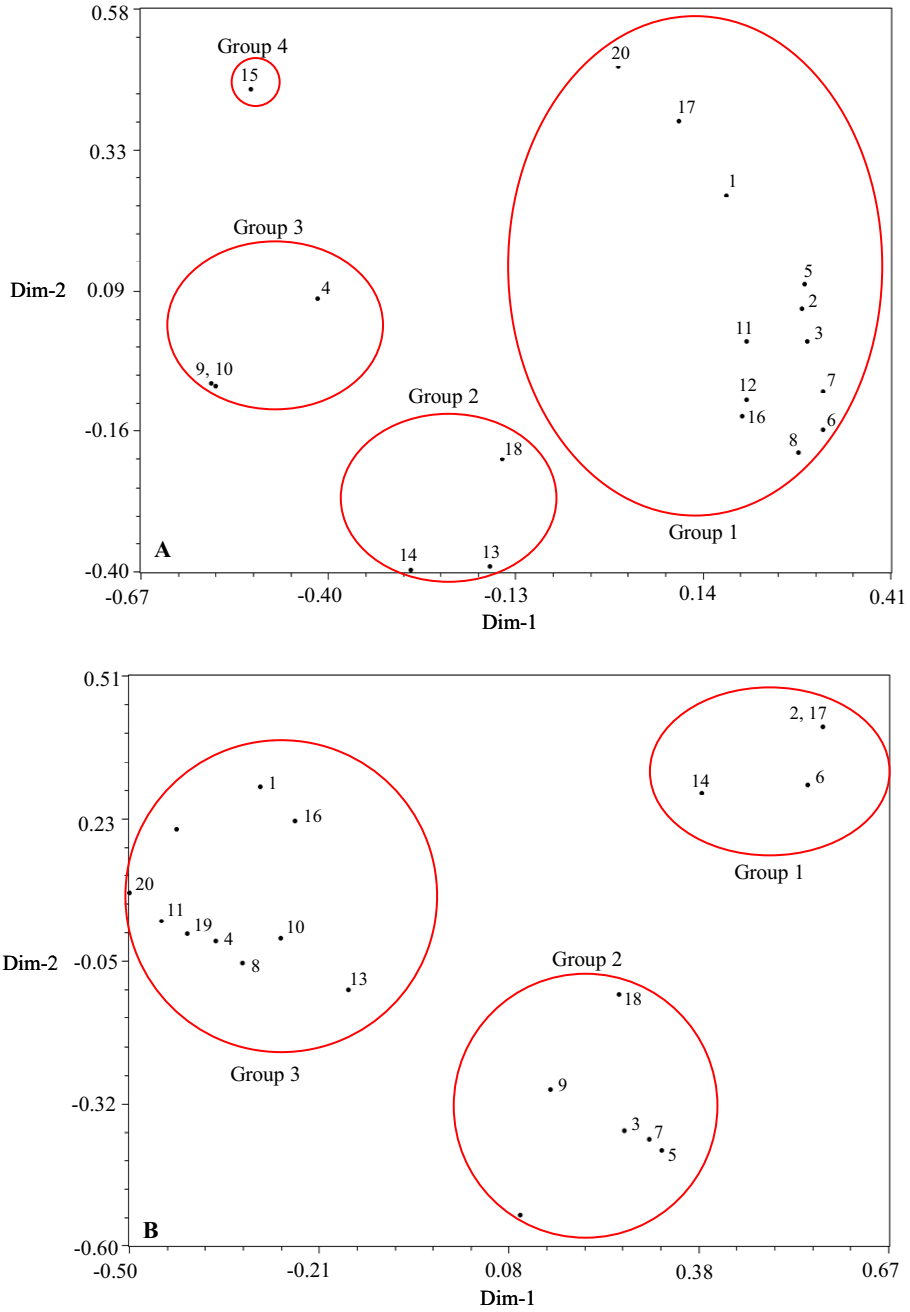


**Fig. 6** Plot of principal component analysis based on RAPD profile (**A**) and SDS PAGE profile of seed proteins (**B**) of different accessions of black henbane.

Uromieh and Golestan-1 were clustered in one subgroup. These findings showed that the seed proteins markers were not able to detect real genetic diversity of germplasm collections as seed protein markers targeted limit loci (gene expression is particularly in coding and non repetitive regions of genome) (Gepts 1990). In accordance with these results, investigating 28 accessions *Arachis pintoi* by seed proteins of electrophoresis, Bertozo *et al*. (2001) reported that there is relatively weak relation between geographical origins and the classification gained from genetic studies among accessions of *A. pintoi*. Small parts of the dendrogram in this experiment indicate that the molecular variation is corresponding with geographical distribution. For instance, the accessions of Roodbar-2 and Talesh-1 collected from different places of Guilan are adjacent and have been classified in one subgroup. This condition was observed for Siahkal-1, Talesh-2 and Siahkal-3 which classified in the second group. Karihaloo *et al*. (2002) studied the seed proteins of *Solanum melongena* and reported that the collected accessions from growing regions, geographically close, were placed in adjacent groups which are in contrast with our results in the experiment.

The combined investigation of RAPD markers and seed proteins data showed low correlation. This may be due to the extracted total seed proteins, as gene products, are not able to show all the changes in DNA; therefore, are not able to represent the whole genome.

Considering some disadvantages of RAPD and seed protein markers in clustering of some the collected accessions from growing regions of a province, it is necessary to use also morphological markers and other highly reliable and accurate markers such as AFLPs, SSRs or isozymes.

Finally, the result of the study showed that RAPD and seed protein markers are useful tools and are recommended with other markers to us to evaluate genetic variation and relationship among black henbane accessions and in breeding programs.

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