УДК 575.22

# A Study on the Genetic Variation in Licorice (*Glycyrrhiza glabra)* in Iran by Molecular DNA Markers (RAPD)

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Thirty ecotypes of licorice (a medicinal plant) from all of Iran were collected and genetically evaluated. To assess the genetic diversity of licorice, genomic DNA was extracted using Winnepenninckx method (CTAB method). 12 random primers were used to perform PCR. All 12 primers showed obvious and repeatable bands. Totally, 1343 bands were produced. Bands size varied from 250 to 5000 bp. Percentage of polymorphism and polymorphic loci was 88.83% and 95.5%, respectively. The highest number of band was related to primer OPN-08 (band 188). The highest similarity between Esfaraien and Bojnourd ecotypes is equal to 0.647. Kermanshah and Orumieh ecotypes had the lowest similarity that was 0.3. Dendrogram divided 30 ecotypes into 5 groups in terms of genetic distance. Generally, surveying the variation in genotypes of licorice using RAPD marker showed that this marker can be useful in identifying the polymorphism, estimating the genetic distance, and managing germplasm.

***Keywords***: licorice; genetic diversity; genetic relationships; UPGMA; molecular markers; RAPD

## Introduction

Medicinal plants have been used as the main factor in healing and pain treatment in great civilizations of the world (Craker and Gardner, 2006). one of the most popular medicinal plants of the Fabaceae family is Glycyrrhiza glabra. The extract of this plant is currently used in the pharmaceutical and functional food industries, as well as in food supplements (Pastorino, 2018).

Thirty species of glycyrrhiza are found worldwide. In Europe and China, the roots and rhizomes of Glycyrrhiza uralensis, Glycyrrhiza inflata and Glycyrrhiza glabra are used, while in Japan and the United States, two species, glabra and uralensis, are used (Li et al., 2019).

Glycyrrhizic acid is active substances of licorice root. It is 50 times sweeter than sugar (Lee, 2018). Licorice extract is used as an important ingredient in the tobacco, cosmetic industries and as a sweetener in beverage preparation and food .The root properties of this plant, in addition to protecting the bones, also show the effective role of anti-diabetic, anti-lipidemic and hypocholesterolemic actions (Galanis et al., 2019).

The origin of genetic diversity in plants is through three ways of genetic recombination, changes in chromosome number and mutations (Arzani & Mortazavi, 2002). In plant breeding, Genetic selection requires genetic diversity and increasing genetic diversity makes the range of our choice wider. On the other hand, characterization and grouping of germplasm allows reformers to avoid duplication in the sampling of the population. Heterosis increases function of biological quality in a hybrid offspring or hybrid superiority over average parents which depend on the genetic distance between parents. To investigate the genetic distance between parents, plant varieties should be classified (Nematzadeh & Kiani, 2004).

In recent years, the use of molecular markers is a very powerful method for analyzing genetic diversity because it shows the relationship between genetic diversity and phenotype (Garrido-Cardenas et al., 2018). Genetic, DNA-based molecular markers have been made since the 1990s, that (RAPD) random amplified polymorphic DNA being one of them. This marker is widely used alone or in combination with other markers for genetic evaluation of medicinal plants (Shangyi et al., 2017).





*How to Cite*

Ahangaran, M., Gharaviri, M., & Masoumian, M. (2020). A Study on the Genetic Variation in Licorice (Glycyrrhiza glabra) Iran by Molecular DNA Markers (RAPD). *Health, Food & Biotechnology, 2*(4). https://doi.org/10.36107/hfb.2020.i4.s76

RAPD a molecular marker is one of the most popular markers that have been used in different fields such as determining the components of the medicinal plants and in pharmaceutical formulations (Chawla, 2002; Adiguzel et al., 2006). RAPD technology with short, arbitrary, single-stranded synthetic oligonuclear primers (usually 10 bp in length) using polymerase chain reaction amplifier (PCR) can generate large numbers of anonymous DNA fragments (Heubl, 2010).

This study aimed to evaluate the genetic diversity of landraces of licorice and their grouping was performed using RAPD markers.

## Materials and Methods

### Plant material

30 different ecotypes of licorice were collected from research centers of, Gilan, Mazandaran, Golestan, Nºrth Khorasan, Semnan, Markazi, Hamedan, Yazd, Kerman, Hormozgan, Ardabil, East Azerbaijan, West Azerbaijan, Kermanshah, Chaharmahal and Bakhtiari, Khuzestan, Zanjan, Kurdistan, Lorestan, Ilam provinces.

The pots seeds and the soil were firstly disinfected and then, dormancy of the seeds was broken using the sand paper and each seed was planted separately in small pots. After two months, they grew up to their 4 leaf stage.

### DNA extraction

Plant tissues were powdered by liquid nitrogen, and DNA extracted from samples by Winnepenninckx Method (Winnepenninckx et al., 1993). DNA quality was determined by electrophoresis method. Gel was stained with ethidium bromide.

### Primers used in this study

12 RAPD random primers were used to amplify genomic DNA.

### RAPD analysis

PCR reaction buffer in a volume of 25 µl was contained 2.5 µl PCR buffer, 2mM MgCl2, 0.4 µM primer , 0.2 µM dNTP, a single tag enzyme of polymerase and 50 µg/ml DNA.

A thermocycler with the following conditions was used to amplify DNA: Initial denaturation at 94°C for 5 minutes and then 36 cycles consisted of the following denaturation (10 seconds, 94°C), primer annealing (45 seconds, while the temperature varied for each primer), primer extension (1 min 72°C) and final cycle of 5 minute at 72°C to develop the product. PCR products observed by electrophoresis with 1.5% agarose gel using an electric current with a voltage of 80 mA for 50 minutes.

1 Kb ladder was used in first well and staining was performed using ethidium bromide.

### Data analysis

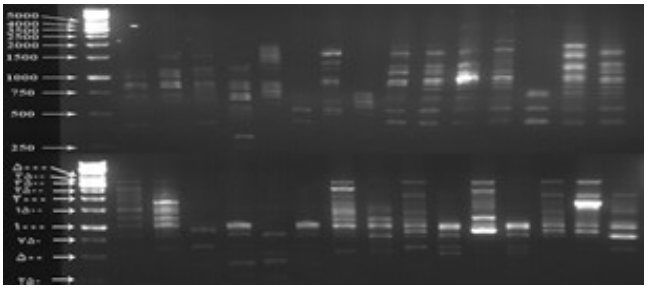
To analyze RAPD data NTSYS and PopGen software were used. Presence or absence of each observed bands were scored with the numbers 1 and 0, respectively. Similarity coefficient value was between zero and one, Zero value indicates the common bands (non-genetic similarity) and One- value shows the value of similar-band patterns (complete genetic similarity). Data analyzed based on Jaccard coefficient of similarity and unweighted pair-group method with arithmetic mean (UPGMA) method.

## Results

Genetic diversity of licorice was performed with PopGen32 and SPSS9. Three criteria were considered in this assessment: 1) A high level of polymorphism that was achieved. 2) Repeatable data which was produced. 3) A comparison between software that used different similarity coefficients (Nei and Jaccard).

Clustering resulted from both software divided 30 ecotypes into 5 separated groups. The percentage of polymorphism and the number of polymorphic loci was determined 88.83% and 95.5%, respectively. Cophenetic Correlation Coefficient for Jaccard and Nei coefficient of similarity was 0.79 that a good fitness between dendrogram (cluster analysis) and the original similarity matrix.

Figure1
*Amplification profiles of plants by primer OPA-16 For 30 licorice ecotypes*



A total of 1343 bands were produced by these indicators. The bands' size varies from 250 to 5000 bp. In this regard, the highest similarity for Bojnourd and Esfaraien ecotypes was 0.647 while the least similarity for the ecotypes of Kermanshah and Orumieh was 0.3. The highest rate of band was belonging to OPN-08 (band 188), so this initiator could determine better genetic distance of the ecotypes.

Table 1
*List of primers, their sequence, number of detected loci, number of polymorphic loci and size of amplified product generated by RAPD primers*

| S. NO. | Primer code | Primer Sequence (50–30) | Detected loci | Polymorphic loci | Total no. of bands | Range of amplification (kb) |
|---|---|---|---|---|---|---|
| **1** | **OPA-02** | **TGCCGAGCTG** | **10** | **10** | **96** | **250-3500** |
| **2** | **OPA-03** | **AGTCAGCCAC** | **8** | **7** | **119** | **200-2500** |
| **3** | **OPA-16** | **AGCCAGCGAA** | **10** | **10** | **109** | **500-3000** |
| **4** | **OPD-01** | **TTGGCACGGG** | **11** | **11** | **125** | **250-3000** |
| **5** | **OPD-02** | **GGACCCAACC** | **7** | **6** | **105** | **200-3000** |
| **6** | **OPF-16** | **GGAGTACTGG** | **9** | **9** | **46** | **500-3000** |
| **7** | **OPK-19** | **CACAGGCGGA** | **10** | **10** | **98** | **200-2500** |
| **8** | **OPN-05** | **ACTGAACGCC** | **9** | **8** | **108** | **200-2500** |
| **9** | **OPN-08** | **ACCTCAGCTC** | **10** | **9** | **188** | **200-2000** |
| **10** | **OPN-11** | **TCGCCGCAAA** | **9** | **8** | **110** | **250-2500** |
| **11** | **OPN-17** | **CATTGGGGAG** | **10** | **10** | **131** | **200-5000** |
| **12** | **OPU-12** | **TCACCAGCCA** | **9** | **9** | **108** | **300-2000** |

Figure 2
*Dendrogram depicting the genetic relationship among 30 G. glabra ecotypes were constructed using UPGMA method based on Jaccard's coefficient similarity.*

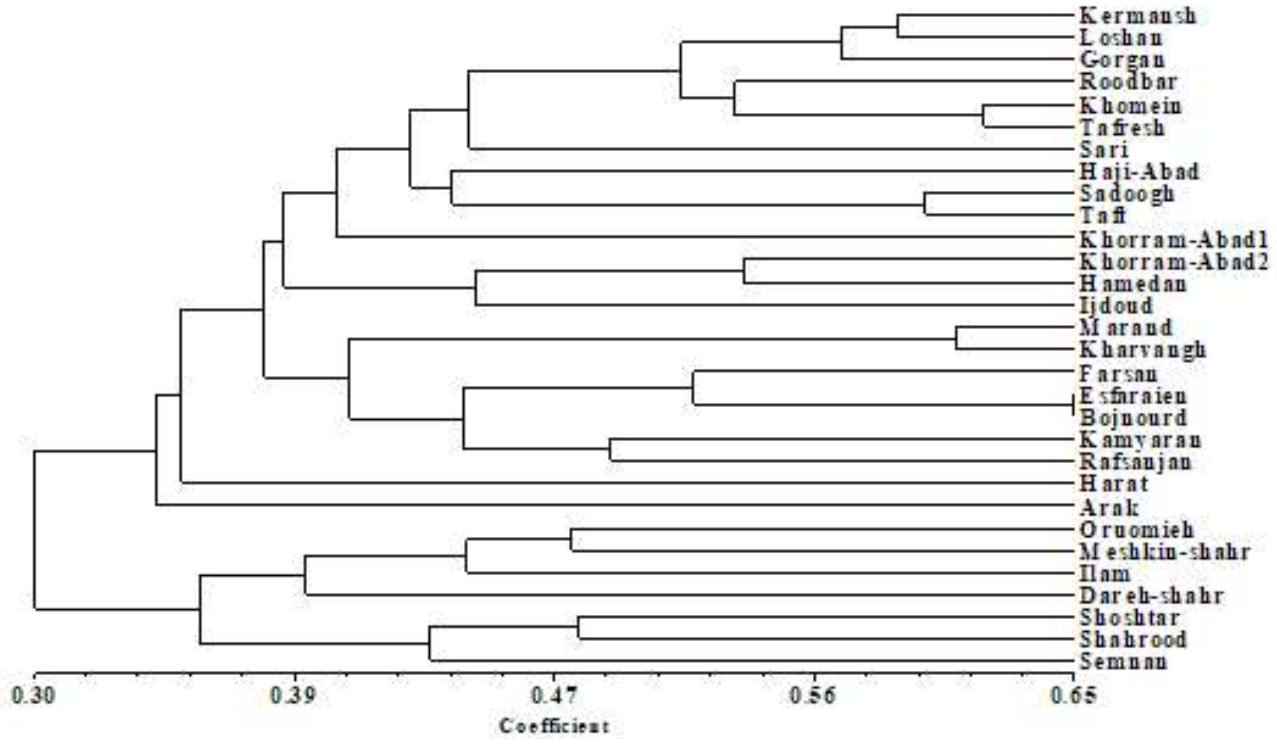


Table 2
*Jaccard similarity coefficients*

| Case | Kermanshah | Gorgan | Loshan | Roodbar | Khoram-Abad1 | Khoram-Abad2 | Sari | Arak | Khomein | Tafresh | Hamedan | Haji-Abad | Harat | Sadoogh | Taft | Marand | Kharvangh | Orumieh | Meshkin-Shahr | Dareh-Shahr | Ilam | Farsan | Esfaraien | Bojnourd | Shooshtar | Ijdoud | Semnan | Shahrood | Kamyaran | Rafsanjan |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kermanshah | 1.00 | .56 | .59 | .47 | .47 | .35 | .39 | .31 | .56 | .54 | .35 | .42 | .40 | .36 | .42 | .40 | .38 | .38 | .29 | .33 | .32 | .33 | .43 | .39 | .28 | .29 | .28 | .27 | .37 | .38 |
| Gorgan | .56 | 1.00 | .58 | .51 | .44 | .44 | .51 | .44 | .51 | .45 | .45 | .33 | .37 | .39 | .51 | .42 | .42 | .34 | .27 | .35 | .26 | .42 | .41 | .35 | .25 | .38 | .26 | .26 | .36 | .35 |
| Loshan | .59 | .58 | 1.00 | .55 | .45 | .36 | .46 | .31 | .48 | .56 | .32 | .38 | .34 | .41 | .49 | .47 | .45 | .33 | .30 | .30 | .26 | .29 | .37 | .32 | .23 | .32 | .24 | .22 | .35 | .38 |
| Roodbar | .47 | .51 | .55 | 1.00 | .43 | .34 | .42 | .32 | .51 | .56 | .40 | .43 | .37 | .39 | .37 | .45 | .36 | .34 | .26 | .26 | .33 | .34 | .39 | .29 | .32 | .34 | .21 | .24 | .38 | .43 |
| Khorram-Aba1 | .47 | .44 | .45 | .43 | 1.00 | .38 | .38 | .35 | .35 | .35 | .36 | .43 | .32 | .36 | .35 | .36 | .30 | .36 | .30 | .30 | .28 | .29 | .34 | .34 | .27 | .30 | .26 | .33 | .28 | .34 |
| Khorram-Aba2 | .35 | .44 | .36 | .34 | .38 | 1.00 | .38 | .38 | .41 | .43 | .54 | .42 | .31 | .45 | .44 | .32 | .31 | .32 | .28 | .41 | .29 | .36 | .43 | .35 | .30 | .44 | .30 | .32 | .38 | .37 |
| Sari | .39 | .51 | .46 | .42 | .38 | .38 | 1.00 | .39 | .43 | .45 | .39 | .44 | .29 | .38 | .40 | .40 | .36 | .30 | .34 | .27 | .29 | .41 | .39 | .31 | .26 | .36 | .20 | .27 | .38 | .36 |
| Arak | .31 | .44 | .31 | .32 | .35 | .38 | .39 | 1.00 | .35 | .26 | .37 | .34 | .24 | .40 | .40 | .28 | .32 | .25 | .24 | .24 | .24 | .35 | .27 | .34 | .29 | .34 | .23 | .31 | .35 | .36 |
| Khomein | .56 | .51 | .48 | .51 | .35 | .41 | .43 | .35 | 1.00 | .62 | .39 | .42 | .51 | .48 | .49 | .44 | .34 | .27 | .24 | .36 | .32 | .37 | .45 | .36 | .28 | .36 | .25 | .31 | .45 | .42 |
| Tafresh | .54 | .45 | .56 | .56 | .35 | .43 | .45 | .26 | .62 | 1.00 | .37 | .48 | .40 | .46 | .47 | .46 | .35 | .27 | .29 | .31 | .34 | .33 | .40 | .32 | .33 | .37 | .31 | .24 | .52 | .42 |
| Hamedan | .35 | .45 | .32 | .40 | .36 | .54 | .39 | .37 | .39 | .37 | 1.00 | .44 | .33 | .44 | .45 | .32 | .29 | .36 | .27 | .31 | .31 | .36 | .38 | .35 | .34 | .45 | .24 | .32 | .43 | .35 |
| Haji-Abad | .42 | .33 | .38 | .43 | .43 | .42 | .44 | .34 | .42 | .48 | .44 | 1.00 | .35 | .45 | .42 | .36 | .30 | .30 | .30 | .22 | .37 | .36 | .44 | .39 | .32 | .38 | .25 | .36 | .42 | .39 |
| Harat | .40 | .37 | .34 | .37 | .32 | .31 | .29 | .24 | .51 | .40 | .33 | .35 | 1.00 | .37 | .43 | .33 | .39 | .28 | .25 | .31 | .20 | .32 | .31 | .28 | .26 | .26 | .26 | .27 | .29 | .35 |
| Sadoogh | .36 | .39 | .41 | .39 | .36 | .45 | .38 | .40 | .48 | .46 | .44 | .45 | .37 | 1.00 | .60 | .33 | .31 | .26 | .23 | .25 | .31 | .38 | .33 | .38 | .28 | .31 | .20 | .29 | .45 | .35 |
| Taft | .42 | .51 | .49 | .37 | .35 | .44 | .40 | .40 | .49 | .47 | .45 | .42 | .43 | .60 | 1.00 | .37 | .33 | .21 | .23 | .20 | .22 | .42 | .36 | .36 | .24 | .44 | .20 | .26 | .44 | .37 |
| Marand | .40 | .42 | .47 | .45 | .36 | .32 | .40 | .28 | .44 | .46 | .32 | .36 | .33 | .33 | .37 | 1.00 | .61 | .30 | .32 | .32 | .31 | .40 | .41 | .44 | .26 | .40 | .29 | .36 | .47 | .48 |
| Kharvangh | .38 | .42 | .45 | .36 | .30 | .31 | .36 | .32 | .34 | .35 | .29 | .30 | .39 | .31 | .33 | .61 | 1.00 | .36 | .34 | .28 | .25 | .38 | .35 | .39 | .28 | .34 | .32 | .38 | .38 | .35 |
| Oruomieh | .38 | .34 | .33 | .34 | .36 | .32 | .30 | .25 | .27 | .27 | .36 | .30 | .28 | .26 | .21 | .30 | .36 | 1.00 | .48 | .45 | .43 | .29 | .39 | .32 | .38 | .28 | .33 | .38 | .23 | .28 |
| Meshkin-sha | .29 | .27 | .30 | .26 | .30 | .28 | .34 | .24 | .24 | .29 | .27 | .30 | .25 | .23 | .23 | .32 | .34 | .48 | 1.00 | .37 | .46 | .32 | .37 | .33 | .39 | .34 | .40 | .36 | .30 | .33 |
| Dareh-shahr | .33 | .35 | .30 | .26 | .30 | .41 | .27 | .24 | .36 | .31 | .31 | .22 | .31 | .25 | .20 | .32 | .28 | .45 | .37 | 1.00 | .35 | .19 | .37 | .31 | .29 | .25 | .26 | .32 | .30 | .29 |
| Ilam | .32 | .26 | .26 | .33 | .28 | .29 | .29 | .24 | .32 | .34 | .31 | .37 | .20 | .31 | .22 | .31 | .25 | .43 | .46 | .35 | 1.00 | .34 | .48 | .41 | .45 | .27 | .35 | .34 | .30 | .40 |
| Farsan | .33 | .42 | .29 | .34 | .29 | .36 | .41 | .35 | .37 | .33 | .36 | .36 | .32 | .38 | .42 | .40 | .38 | .29 | .32 | .19 | .34 | 1.00 | .48 | .56 | .38 | .44 | .36 | .40 | .44 | .40 |
| Esfaraien | .43 | .41 | .37 | .39 | .34 | .43 | .39 | .27 | .45 | .40 | .38 | .44 | .31 | .33 | .36 | .41 | .35 | .39 | .37 | .37 | .48 | .48 | 1.00 | .65 | .44 | .39 | .30 | .41 | .41 | .48 |
| Bojnourd | .39 | .35 | .32 | .29 | .34 | .35 | .31 | .34 | .36 | .32 | .35 | .39 | .28 | .38 | .36 | .44 | .39 | .32 | .33 | .31 | .41 | .56 | .65 | 1.00 | .37 | .44 | .27 | .44 | .46 | .47 |
| Shoshtar | .28 | .25 | .23 | .32 | .27 | .30 | .26 | .29 | .28 | .33 | .34 | .32 | .26 | .28 | .24 | .26 | .28 | .38 | .39 | .29 | .45 | .38 | .44 | .37 | 1.00 | .38 | .48 | .48 | .40 | .41 |
| Ijdoud | .29 | .38 | .32 | .34 | .30 | .44 | .36 | .34 | .36 | .37 | .45 | .38 | .26 | .31 | .44 | .40 | .34 | .28 | .34 | .25 | .27 | .44 | .39 | .44 | .38 | 1.00 | .36 | .38 | .44 | .41 |
| Semnan | .28 | .26 | .24 | .21 | .26 | .30 | .20 | .23 | .25 | .31 | .24 | .25 | .26 | .20 | .20 | .29 | .32 | .33 | .40 | .26 | .35 | .36 | .30 | .27 | .48 | .36 | 1.00 | .38 | .27 | .34 |
| Shahrood | .27 | .26 | .22 | .24 | .33 | .32 | .27 | .31 | .31 | .24 | .32 | .36 | .27 | .29 | .26 | .36 | .38 | .38 | .36 | .32 | .34 | .40 | .41 | .44 | .48 | .38 | .38 | 1.00 | .35 | .32 |
| Kamyaran | .37 | .36 | .35 | .38 | .28 | .38 | .38 | .35 | .45 | .52 | .43 | .42 | .29 | .45 | .44 | .47 | .38 | .23 | .30 | .30 | .30 | .44 | .41 | .46 | .40 | .44 | .27 | .35 | 1.00 | .49 |
| Rafsanjan | .38 | .35 | .38 | .43 | .34 | .37 | .36 | .36 | .42 | .42 | .35 | .39 | .35 | .35 | .37 | .48 | .35 | .28 | .33 | .29 | .40 | .40 | .48 | .47 | .41 | .41 | .34 | .32 | .49 | 1.00 |

## Discussion

According to the results, there is a good variation among Licorice in Iran. In this study, the ecotypes of Herat and Arak could be used to produce the parent ecotypes for heterozygous, because the two ecotypes are far apart in term of genetic similarities, so they have the greatest diversity. Few studies about genetic diversity of licorice with RAPD marker are available. One of them was the study in which researchers used RAPD marker with ten primers to study the genetic diversity of licorice. The results of the phylogenetic tree of 4 echotypes of licorice were divided into two groups. This study showed that RAPD markers were suitable to identify diversity of licorice (Saito et al., 1994). Another research which was used to differentiating *G. glabra* from *Abrus precatorius* that was alternatively used as a fake species instead of the true one, licorice. Among 52 RAPD primers, 16 primers showed visible bands of the specific species (Salim et al., 2009). In addition, identification of *Atractylodes* plants (Chen et al., 2001), screening an elite strain Aizu K-111 of *Panax ginseng* (Komatsu et al., 2001)2001, differentiating of *Senna surattensis* and *S.sulfuera* (Kumar et al., 2007) has been developed by RAPD. Therefore, RAPD was used as a complementary tool for quality control.

Base on the analysis 87.5% of the bands were polymorphic. However, the percentage of polymorphic bands in the other reported medicinal plants was 100% in *Poincianella pyramidalis* (Belarmino et al., 2017) and 51% chickpea (Yadav et al., 2015).

Mehrnia *et al*. (2005) studied relationship between related species of 20 Iranian *Astragalus microcephalus* and results showed that RAPD was the best molecular marker to classify systematic relationship between related species. Shakti et al., (2012) worked on *Glysyryza glabra* to study genetic variation using RAPD. Liquid medium was used to grow the explants taken from a two-year-old licorice.10 primers were used and 28.57% polymorphism was achieved. RAPD cluster analysis showed that all plants including the mother plant are placed in two main groups with a distribution of similarity coefficient between two extremes ranging from 0.91 to 0.96.

In a similar work, the RAPD analysis showed a high level of genetic diversity among 12 *Podophyllum hexandrum* collected from high-latitude regions. This study confirmed that RAPD markers were very useful tools for attributing to geographical and climatic conditions, genetic relationship and diversity. (Sultan et al., 2010).

Evaluating genetic similarity by RAPD marker was conducted among the four species of licorice and phylogenetic tree suggested that *G. glabra* and *G. uralensis* rich in glycyrrhizin have a closer relation compared to the G. *echinata* and *G. pallidiflora* (Yamazaki et al., 1994).

Moreover RAPD can be used to screen Inter and intra species diversity. Echinacea Interspecies diversity has been studied by RAPD markers (Kapteyn et al., 2002). it was the best tool for studying genetic diversity in the Tunisian bean population (*Vicia faba L*) (Backouchi et al., 2015), Chinese medicinal plant and three subspecies of *Meliss officinalis* (Ma et al., 2002), four *Glycyrrhiza* sp. and their relationship with commercial liquorice roots (Yamazaki et al., 1995), *Dendrobium officinale* (Ding et al., 2005), *Mimosae tenuiflorae* cortex (Rivera-Arce et al., 2007), *Rahmannia glutinosa* cultivars and varieties (Qi et al., 2008) and *Desmodium* species (Irshad et al., 2009).

## Conclusion

In conclusion RAPD molecular marker was the best and only useful tool that can be used in evaluating genetic distance among different samples of licorice. In this study, %95.5 of bands was polymorphic. Bojnourd and Esfaraien ecotypes have the highest similarity and the ecotypes of Kermanshah and Orumieh are placed at the farthest genetic distance from each other.

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# Исследование генетической изменчивости лакри́чника (*Glycyrrhiza glabra*) в Иране с помощью молекулярных ДНК-маркеров (RAPD)

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Тридцать экотипов лакри́чник (лекарственного растения) со всего Ирана Было собрано и генетически оценено. Для оценки генетического разнообразия Glycyrrhiza glabra геномную ДНК экстрагировали методом Winnepenninckx (метод CTAB). Для проведения ПЦР использовали 12 случайных праймеров. Все 12 праймеров показали очевидные и повторяющиеся полосы. В целом, Произведено 1343 полоса. Размеры полос варьируются от 250 до 5000 п.н. Процент полиморфизма и полиморфных локусов составил 83,88% и 95,5% соответственно. Наибольшее количество полос было связано с праймером OPN-08 (полоса 188). Наибольшее сходство между экотипами Эсфарайен и Боджнурд составляет 0,647. Экотипы Керманшаха и Орумие имели наименьшее сходство 0.3. Дендрограмма разделила 30 экотипов на 5 групп с точки зрения генетической дистанции. В общем-то, Изучение вариаций генотипов лакри́чник (Glycyrrhiza glabra) с использованием маркера RAPD показало, что этот маркер может быть полезен для идентификации полиморфизма, оценки генетического расстояния и управления зародышевой плазмой.

***Ключевые слова***: *Glycyrrhiza glabra*, генетическое разнообразие, генетические отношения, UPGMA , молекулярные маркеры , RAPD

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*Как цитировать*

Ахангаран, М., Гаравири, М., & Масумян, М. (2020). Исследование генетической изменчивости лакри́чник (*Glycyrrhiza glabra*) Иран с помощью молекулярных ДНК-маркеров (RAPD). *Health, Food & Biotechnology, 2*(4). https://doi.org/10.36107/hfb.2020.i4.s76

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