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The study of genetic diversity in different plant populations of *Artemisia Annua* L. native to northern Iran by molecular marker AFLP

Bovard Reza[1], Naghavi Mohammad Reza[2], Omidi Mansoor[3], Torabi Sepide[4], Rezazadeh Shamsali[5], Hariri Akbari Farhad[*1], Shafie Alavijeh Mehdi[6], Behjat Sasan Baharak[7], Parvaneh Saeed[8], Safakish Kashani Mohammad Bagher[9]

[1]*Department of Agricultural Biotechnology, Islamic Azad University, Research and Science Branch, Tehran, Iran*

[2]*Department of Biotechnology, University of Tehran, Karaj, Iran*

[3]*Department of Agronomy and Plant Breeding, University of Tehran, Karaj, Iran*

[4]*Department of Agriculture & Plant Breeding, Islamic Azad University, Research & Science Branch, Tehran, Iran*

[5]*Department of Pharmacognosy, Institute of Medicinal Plants, Jahad Daneshgahi, Karaj, Iran*

[6]*Department of Biotechnology, University of Zabol, Zabol, Iran*

[7]*Department of Horticultural, Islamic Azad University, Research and Science Branch, Tehran, Iran*

[8]*Department of Gardening, University of Zabol, Zabol, Iran*

[9]*Department of Agronomy, University of Tehran, Karaj, Iran*

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Abstract

Artemisia annua L. is an annual, medicinal and aromatics plant that is widespread in Asia, Europe and North America and has been wide distribution in northern areas of Iran. In this study, AFLP markers were used to investigate the genetic diversity of 15 populations of *A. annua*. 86 scorable bands were produced by using four primer combinations (E/M), that 58 of them) were polymorphic. The highest number of polymorphic bands (24 bands) using primer combination EcoR11-Mse20 and the lowest number of polymorphic (6 bands) using primer combinations EcoR2-Mse35 were produced. Data matrix using similarity coefficient was converted to similarity matrix (distance matrix), and then using the algorithm (UPGMA) was drawn relevant dendrogram. Dendrogram obtained from UPGMA put 15 populations in four groups. The obtained cluster of cases was consistent with the geographic diversity in some cases. Then correlation coefficient based on the Mantel test was obtained 7.0, indicating good agreement drawn dendrogram with the similarity matrix. The results of principal component analysis and almost coincided with the results of cluster analysis. The results showed that the use of AFLP method to assess molecular diversity in these masses is appropriate.

***Corresponding Author:** Akbari Farhad ✉ Hariri.a.farhad@gmail.com

Introduction

Among the 45000 plant species, approximately 15000 are flowering plants and nearly 3000 of these are known to be of medicinal value (Sarwat *et al.,* 2012), All over the world, plant based medicines are gaining popularity due to their almost negligible or no side effects (Hoareau and DaSilva, 1999). These plants are traditional source of quite a big number of biologically active substances used in the pharmaceutical industry. The most valuable phytochemicals are products of the secondary metabolism. Most of them have complicated structure which makes their chemical synthesis very difficult and even impossible (Tasheva and Kosturkova, 2012). Biotechnological tools have potential to overcome the problems of traditional methods of propagation for large scale production of high quality planting material, (Kant and Parmar, 2014). These tools are important for the multiplication and genetic enhancement, for the medicinal plants by adopting techniques such as *in vitro* regeneration and molecular methods. It could also be harnessed for the production of secondary metabolites using plants as bioreactors (Hoareau and DaSilva, 1999; Siahsar *et al.,* 2011). One of the most applicable methods is, assessment and characterization of the germplasm, can identify novel genotypes and aid future plant improvement programs. There is a need for the assessment and characterization of genetic diversity that is an essential prerequisite for systematic documentation of plant species (Sarwat *et al.,* 2012; Schippmann *et al.,* 2002).

Genus *Artemisia* belongs to the family Asteraceae has more than 444 species in the world (Wright, 2002) that 34 species has wide distribution wildly in Iran in all areas (Asghari and Razban Haghighi, 1999; Verdianrizi, 2008). The great importance of *Artemisia* species for its application in various fields has caused to have been planted as economic and commercial product widely. Among the most famous commercial species of the genus *Artemisia* can be pointed to anti-malarial species (*A. annua*), species used in food and cooking (*A. dracunculus*), insect repellent species (*A. vulgris*), and ornamental species (*A. abrotanum*) (Mueller *et al.,* 2004; Tzenkova *et al.,* 2010;). A. annua species is an annual, aromatic and medicinal plant. This plant is native to Asia, particularly China and widely distributed in Asia, Europe and North America (Bertea *et al.,* 2006; Majruhi, 2008). The mentioned species in Iran is widely grown in northern strip of country (Sharafi *et al.,* 2006). The major importance of this species in the pharmaceutical industry is due to the presence of a sesquiterpene lactone endoperoxide compound called *Artemisinin* that has antimalarial activity (Cafferata *et al.,* 2010). Essence of the *A. annua* species has been used in the cosmetics, health and drug industry (Lari Yazdi *et al.,* 2001; Gupta *et al.,* 2002) and has anti-bacterial, anti-viral and anti-tumor properties (Bhakuni *et al.,* 2001; Santos and Simon, 2002). Essence of this species contains terpenoids (including monoterpenes, sesquiterpene and triterpene), flavonoids and coumarin (Bagchi *et al.,* 2003; Bovard *et al.,* 2014). Compounds which are reported from various geographic regions as a major component in essence of the *A. annua* are very different.

This shows the great influence of environmental conditions on the type and percentage of composition of plant essence. The studies indicate that the essence is distributed in all of plant organs but the major part of it is concentrated in the leaves and flowers, and tiny amounts are in the main stem, branches and roots (Charles *et al.,* 1991; Bovard *et al.,* 2014). The objective of this study was to estimate the genetic diversity among the 15 native populations of the medicinal plant *Artemisia annua* from North of Iran with using the Amplified Fragment Length Polymorphism (AFLP).

Materials and methods

Plant material and collecting population of Artemisia annua

15 native populations were collected from different cities from North of Iran. And cultivated in order to perform tests and take samples in the Research farm of Institute of Medicinal Plant, Karaj, Iran.

In order to achieve accurate and comprehensive information regarding to north Iranian Populations of *A. annua*, we have attempted that collected native populations to be appropriate for the geographic distribution throughout the region (Table 1).

Cultivation and production of plant material

First the seeds were collected from plants of natural habitats. The seeds were put in Petri dishes on wet filter paper and transferred to plastic pots after germination in order to grow these plants to a height of 10 to 15 cm and also leaves grow enough. After three weeks, fresh leaves from each pot were picked for genomic DNA extraction.

Genome extraction and purification

Total genomic DNA was extracted and the quality of the extracted DNA was tested on 1% agarose gel electrophoresis and quantity was measured with a spectrophotometer (Hariri Akbari *et al*., 2012). The fresh and young leaves of each sample produced in a mortar were ground to a fine powder in liquid nitrogen, and transferred to the tubes 1/5 ml. 900 µl of SDS 4% extraction buffer was added per tube and placed for 45 Min in water bath at 65 ° C. and then added 300 µl of potassium acetate per tube for 25 Min under ice container. The tubes were centrifuged at a temperature of 3 ° C for 15 Min and at 12000rpm. 750 µl of the upper liquid transferred to a new tube and 750 µl of cold isopropanol added to it and placed 5 Min at laboratory temperature (25 ° C).

Then the tubes were centrifuged at a temperature of 3 ° C for 15 Min and at 12000 rpm and after removing supernatant and tubes containing plates were incubated at 37 ° C for 20 to 30 Min. In order to purify, 700 µl of autoclaved deionized -distilled water (DDW) was added to each tube that has been kept for 1 h at 4 ° C. and then added the chloroform isoAmyl alcohol solution (Powell *et al.,* 1996) volume of 700 µl and shaken well until a uniform emulsion was obtained. Centrifugation was performed for 10 Min at 13000 rpm, the lower phase is chloroform Isoamyl alcohol and upper phase is solution containing DNA.

The upper phase are transferred to a new 2 ml tube and was added 0/1 volume of sodium acetate supernatant liquid (3 M, pH =8). Absolute alcohol (100%) that is 2/5 times volume of supernatant liquid was added and then dehydrated after 5 Min. centrifuge was performed for 10 Min at 13000 rpm and discarded the supernatant liquid and added 100 ml 70% alcohol to the sediment. The alcohol was removed and the samples incubated at 37 ° C for 20 to 30 Min to dry completely and then added TE buffer (100 µl) or deionized -distilled water (DDW). The quality of the extracted DNA was tested on 1% agarose gel (Fig. 1) electrophoresis and quantity was measured with a spectrophotometer (Hariri Akbari *et al*., 2012).

Statistical analysis

The similarity matrix and cluster analysis was performed using NTSYS-pc analytical software v.2. and dendrogram was constructed based on DICE similarity coefficient and the accessions were grouped by cluster analysis using the UPGMA algorithm method. To determine the correlation between similarity matrices and cophenetic, Mantel test was used that MAXCOMP coefficient obtained via COPH and SM coefficients was applied for the Mantel test (Powell *et al*., 1996).

Results

DNA extraction

Extracted samples by Hariri *et al*.,(2012) method on 1% agarose gel showed sharp bands and no fracture (Fig. 1). The DNA fracture on agarose gel can be investigated by this method. Broken DNA in the form of spots or smears can be seen as pieces with low molecular weight (Charles *et al.,* 1991; Hariri Akbari *et al.,* 2012).

AFLP reaction

Molecular markers, in particular DNA based markers provide reliable genetic information because of the in dependence of the confounding effects of environmental factors (Powell *et al.,* 1996).

Table 1. Province, city and coordinate for 15 populations of North of Iran.

Row	Province	City	Coordinate
1	Golestan	Aliabad	36°54′30″N 54°52′08″E
2	Golestan	Azadshahr	37°05′13″N 55°10′26″E
3	Golestan	Gonbad-e Kavus	37°15′00″N 55°10′02″E
4	Golestan	Gomishan	37°04′18″N 54°04′36″E
5	Golestan	Maraveh Tappeh	37°54′15″N 55°57′21″E
6	North Khorasan	Ashkhaneh	37°33′41″N 56°55′16″E
7	Mazandaran	Sari	36°33′48″N 53°03′36″E
8	Gilan	Asalem	37°43′50″N 48°57′13″E
9	Gilan	Lowshan	36°37′14″N 49°30′38″E
10	Mazandaran	Kiasar	36°14′17″N 53°32′28″E
11	Ardabil	Shabil	
12	Gilan	Sangar	37°10′52″N 49°41′38″E
13	Alborz	Gach Sar	36°06′13″N 51°18′17″E
14	Mazandaran	Amol	36°28′11″N 52°21′03″E
15	Mazandaran	Babol	36°33′05″N 52°40′44″E

Analysis of RFLP 15 *A. annua* plant population was performed using 4 primer pairs. The Primer name, total number of bands, the number of polymorphic bands and the percentage of polymorphic for each primer is shown in Table 2. A total of 86 scorable bands were observed of these markers that 58 bands were polymorphic, therefore the percentage of polymorphic bands was 5/64%, so that the number of bands for each marker varied from 12 to 31 bands. The average number of bands for each marker was 21/5. Most polymorphisms related to EcoR11-Mse20 with 24 polymorphic bands and lowest polymorphism related to EcoR2-Mse35 with 6 polymorphic bands. In Figure 2, banding pattern of the number of samples examined in primer marker pairs EcoR2-Mse22 is shown (Powell *et al.*, 1996) and (Etminan *et al.*, 2012).

Table 2. Specifications of primer, the total number of bands, the number of polymorphic bands and the percentage of polymorphic.

No.	Name of primer	Total number of band	Number of polymorphic bands	percentage of polymorphic
1	EcoR2-Mse22	20	14	70
2	EcoR2-Mse22	31	24	77/24
3	EcoR8-Mse35	14	23	60/86
4	EcoR4-Mse17	12	6	50

Data matrix using similarity coefficient SM was converted to similarity matrix (distance matrix), and then using the algorithm (UPGMA) was drawn relevant dendrogram (Fig. 3).

Then correlation coefficient based on the Mantel test was obtained 7.0, indicating good agreement drawn dendrogram with the similarity matrix. The results of principal component analysis and almost coincided with the results of cluster analysis (Fig. 4) (Powell *et al.*, 1996; Etminan *et al.*, 2012).

Discussion

The results showed that AFLP molecular markers are useful tools for studying the genetic diversity of medicinal plants.

The results of other studies have revealed that several factors have affected estimation of genetic relationships among individuals that some of them include the number of markers have been used, distribution of the markers in the genome and the nature of evolution mechanisms that basis for causing diversity (Lari Yazdi *et al.,* 2001; Kermani *et al.,* 2006; Rezaei *et al.,* 2012).

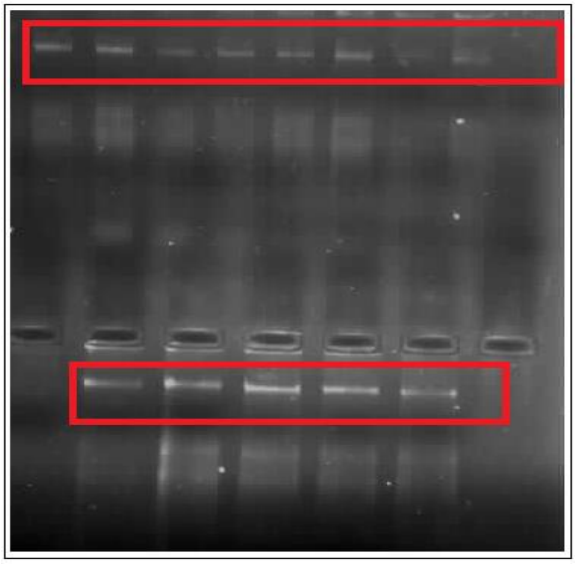

Fig. 1. The extracted genome samples in 1% agarose gel electrophoresis.

Based on molecular data, similarity range between the samples was variable from 65/0 to 90/0. samples of Aliabad and Azadshahr (both of Golestan province) had the most genetic similarity with the zero genetic difference and the lowest genetic similarity between samples of Aliabad and Azadshahr (Golestan province) with Babol (Mazandaran province) were observed about 25 percentage genetic difference.

The obtained dendrogram divided 15 *A. annua* plant populations in 4 classes. The masses of Aliabad, Azadshahr, Gonbad-e Kavus, Gomishan, Maraveh Tappeh of Golestan province and Ashkhaneh in northern Khorasan in class 1; masses of Sari (Mazandaran) and Asalem (Gilan province) in Class 2; masses of Lowshan (Gilan) and Kiasar (Mazandaran) in class 3; masses of Kiasar, Amol and Babol (Mazandaran Province), Gachsar (Alborz), and Shabil (Ardabil province) were placed in class 4.

It is expected that exists direct relationships between the geographic distances and genetic distances, i.e., samples are in a geographical region are grouped together in terms of genetic distance (Hashemi *et al.,* 2009; Khunani, 2008).

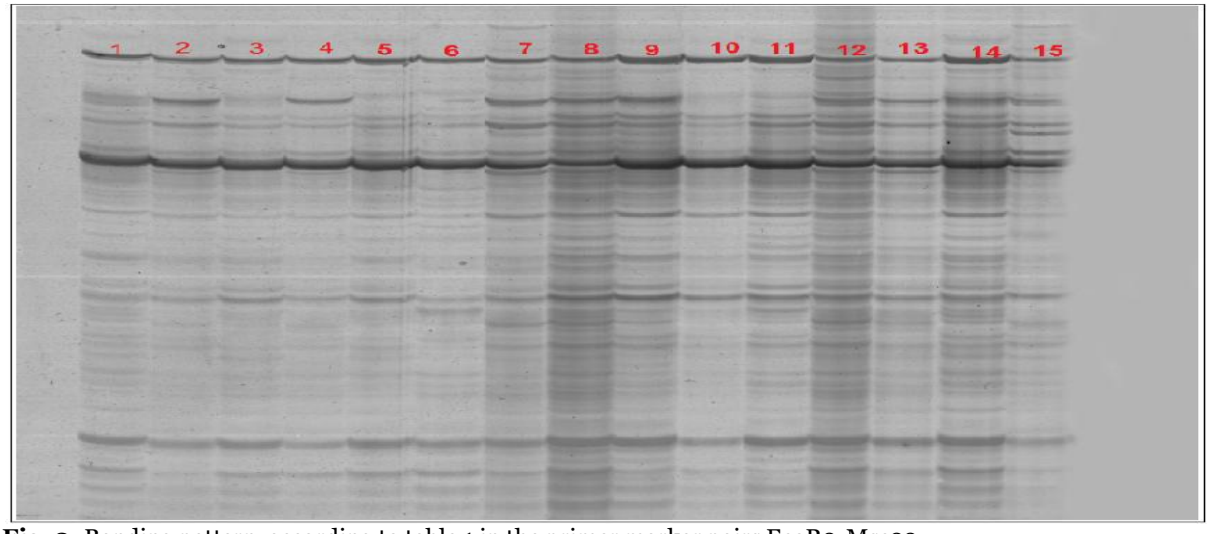

Fig. 2. Banding pattern according to table 1 in the primer marker pairs EcoR2-Mse22.

In the current study, this relationship was observed in some cases, as class 1 and in some cases this relationship was viewed contrariwise as classes 2 and 3.

For example, in Class 1, all masses were related to Golestan province, also Ashkhaneh sample (of North Khorasan province) was placed in this class, which indeed is near the border of Golestan province.

Furthermore the masses of the Mazandaran province (including masses of Kiasari, Amol and Babol) have been located in Class 4, Gachsar which is also located on the border of Mazandaran province but Shabil belongs to Ardebil province. Therefore direct relationship was observed between geographical and genetic distances in these cases (other than Shabil).

In Class 2 and 3, two examples of Gilan and Mazandaran provinces were located in a group adjacent to each other, but in class 2, 3 and 4 samples related to different provinces were located together, as in class 2 of Sari in Mazandaran province was placed next to the sample of Aslem in Gilan province that most likely these masses had the same origin.

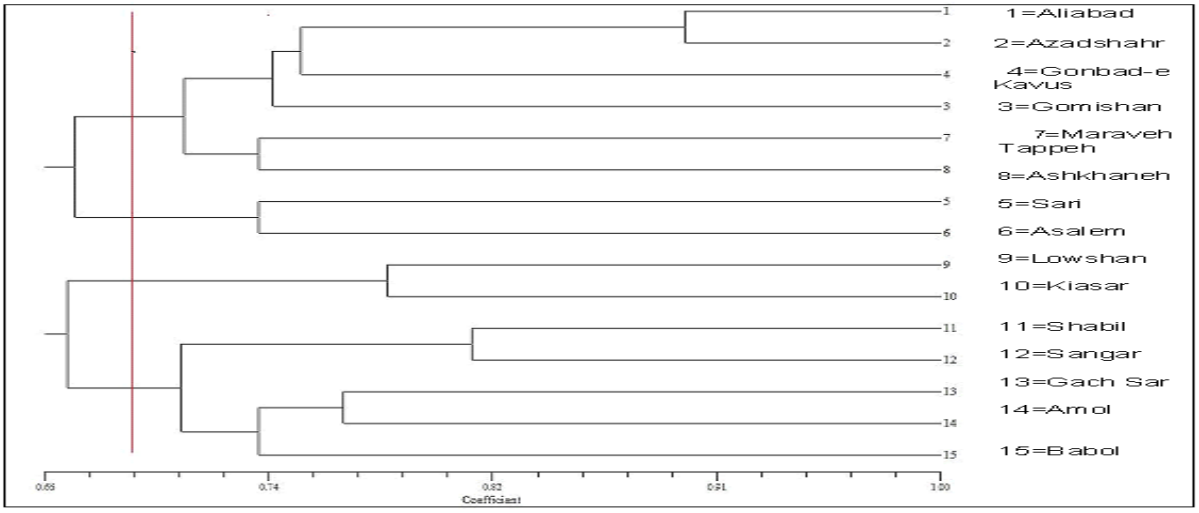

Fig. 3. Grouping of different samples examined using SM and UPGMA algorithm obtained from 4 AFLP primers.

Also in the class 4, masses of Shabil and Sangar of Ardebil and Gilan provinces were located next to masses of Gachsar belongs to Alborz province, Amol and Babol of Mazandaran province. These cases illustrate the lack of a direct relationship between geographic distance and genetic distance.

In research conducted by Hashemi *et al.,* (2008) and Hariri *et al.,* (2014), the results of molecular analysis were adapted with geographic distances with Persian cumin and study of genetic variation of *Anethum graveolens* plant populations of Iran. The lack of direct relationship between geographical distance and genetic distance in the studies of genetic diversity of *Ferula gummosa* populations of Iran by AFLP molecular markers have been reported (Khunani, 2008). Totally several factors influence estimation of genetic relationships between individuals that include: 1. The number of used markers 2. distribution of the markers in the genome 3. The nature of evolutionary mechanisms (Powell *et al.,* 1995).

About the number of used markers, it should be noted that the amount of information obtained from AFLP method depends largely on the number of used primers.

Khunani cited by Ellis *et al.,* (1997) showed that selection of six combinations of the best primers could justify 80% of considered relationships (Khunani, 2008; Yu *et al.,* 2011). In this study, while a little primer was used but AFLP molecular markers could divide the studied populations in different groups and in most cases the direct relationship between geographical distance and genetic distance were observed.

The correlation coefficient (r) was calculated to be 7.0, which indicates a good correlation of obtained dendrogram and similarity matrix. In the cluster analysis using molecular data, high correlation coefficient is the reason of efficiency of obtained dendrogram. If r>9/0 is very good correlation, 9/0 <r < 7.0 is good correlation, r < 7/0 is correlation weak (Hariri Akbari *et al.,* 2014; Khunani, 2008).

It is essential to investigate phytochemical variation and the routes of metabolite synthesis among the samples in order to introduce appropriate varieties and perform breeding programs (Hariri Akbari *et al.,* 2014). In conclusion, AFLP data sets showed a high level of polymorphism among north native Iranian population of *Artemisia annua* reflecting their efficiency in the assessment of the genetic diversity. These results can be used for germplasma management, biotechnology plant breeding programs.

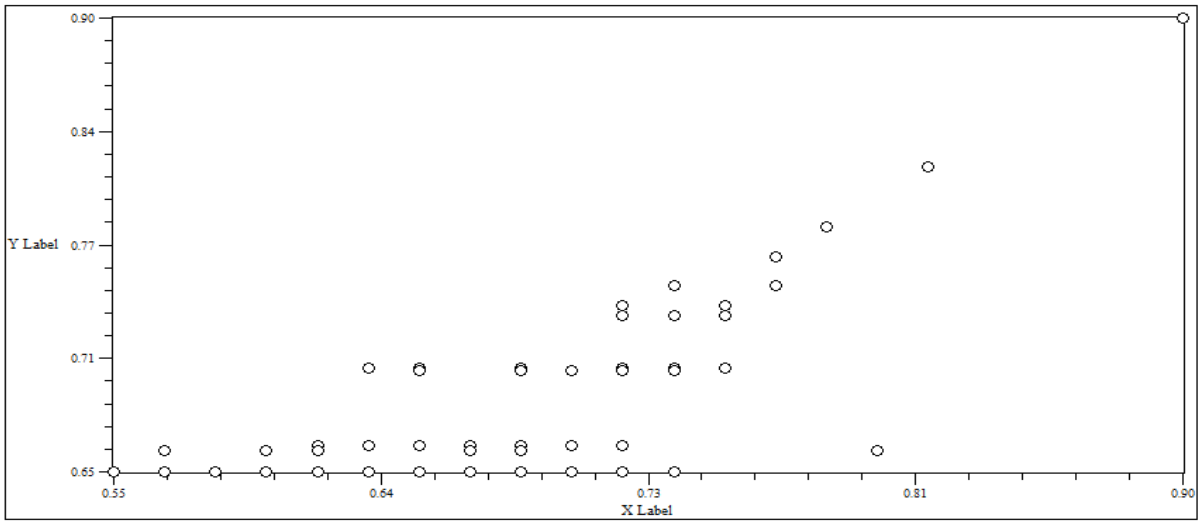

Fig. 4. Two-dimensional plot of principal component analysis.

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