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ASSESSMENT OF GENETIC DIVERSITY OF SOME WILD POPULATIONS OF *THYMUS KOTSCHYANUS* USINGRAPD MOLECULAR MARKERS

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ABSTRACT. RAPD molecular markers were used to assess the genetic diversity of some population of thyme (*Thymus kotschyanus*) from Iran. The 17 ten-nucleotide primers used that produced 185 high-resolution bands, which 21 of these were monomorphic and 162 were polymorphic. An average, 10.88 bands were obtained per primer and 9.52 of these were polymorphic. POPGENE software was used to calculate Nei's genetic distance among populations and based on the cluster analysis of this matrix, a UPGMA dendrogram was drawn using the software NTSYS-pc. On the basis of dendrogram among populations of wild mountain thyme, Avan (Qazvin) and Siahkal (Gilan province) populations was the most similar between studied populations with the distance of 0.094 high disimilarity was observed between Qazvin and Mazandaran 2 populations with the distance of 0.185 within populations variation based on Shannon's information index and Nei genetic diversity index showed that Takestan (I= 0.26; H= 0.3) and Alamut (I= 0.24; H= 0.15) had a highest and lowest within populations variation compared to other populations, respectively. Average of Fst and Nm indices, which represent the amount of gene flow between populations, were recorded as 0.26 and 1.361, respectively, which reflects the high level of gene exchange between ten populations of *Thymus kotschyanus*.

Key words: Genetic diversity; *Thymus kotschyanus*; RAPD; Population; Medicinal plant.

PCR - The polymerase chain reaction
RAPD - Randomly Amplified Polymorphic DNA

INTRODUCTION

Given the economic importance of plants of *Thymus* genus, including mountain thyme (*Thymus kotschyanus*), in domestication program and improvement of homogeneous varieties from wild populations and ecotypes, correct knowledge and identification of botanical, genetical and

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phytochemical characteristics of populations and ecotypes, in terms of medicinal, horticultural and industry use, as a first step of improvement, are of high importance. Genetic diversity is the basis of plant breeding, so the creation of superior variety relied on the abundant and desirable germplasm. Finding of these desirable germplasm requires identifying the native and wild plants of each region's that store some traits over the years and remain stable. If the genetic diversity of a species is higher, then it is likely to plant breeders in selection and breeding programs will be more successful (Bernath, 1986; Nemeth *et al.,* 2000; Bernath, 2002).

Genetic diversity in plant germplasm was affected by evolution in both inter/intra species. This diversity is a right tool for improving the specific plants using breeding methods (Stebbins, 1971). Different marker systems have been used for the analysis and identification of genetic variations. Efficiency and reliability of any genetic marker depends on its heritability and polymorphism (Porter *et al.,* 1982). Among the molecular markers, RAPD markers, that based on multiplying DNA parts with random primers using the PCR, has considerable potential for the study of genetic variation and at least they have less need to advanced technology, labor and costs compared with other molecular markers (Kumar *et al.,* 2008; Ipek and Simon, 2003). Information about genetic relationships and diversity within species of *Thymus kotschyanus* are now limited. Therefore, the aim of the present study was to evaluate the efficiency of molecular markers in within species separation of *Thymus kotschyanus* and genetic diversity of this species.

MATERIALS AND METHODS

Plant material

Among the 100 genotypes that the morphological traits of them were studied, 50 genotypes belonging to 10 populations were selected. Five genotypes were selected from each population.

DNA extraction

The DNA was extracted from the dried leaves of genotypes collected from 10 habitats of five provinces using (Sharp *et al.,* 1998) method, except that some correction was applied on the amounts and types of materials used in the extraction buffer and the protocol. At first, 0.3 grams of herbarium-dried leaf samples were weighed and powdered under liquid nitrogen in a mortar. Then 1 ml of extraction buffer and 150 µl of SDS 10% added to solution and the resulting paste solution. The resulting solution was transferred to 2 ml tubes. Then 15 µl mercaptoethanol was added to each tube. Tubes containing a mixture of leaves and extraction buffer were placed for 15 minutes in water bath at 65°C and during this period, several times were shaken upside down. 200 µl of potassium acetate 5M were then added and were shaken upside down for 15 minutes. After this step, 1 mL of chloroform isoamyl alcohol (1:24 v/v) were added to each tube and after shaken upside down for 15 minutes, the tubes were centrifuged at 12,000 rpm for 10 minutes. Then supernatant solution was transferred to new 2 ml tubes with

sampler. With regard to high phenolic compounds, supernatant at the end of extraction had a dark color, which indicates the low quality and the presence of impurities in extracted DNA, thus CTAB 5% + NaCl 7 M was used to remove impurities from the solution. For this purpose, by adding one ml of the above solution to supernatant and centrifuged at 10,000 rpm for 15 minutes in three times used for remove impurities from DNA. After three washing steps and remove impurities, to precipitate of DNA equal to 0.6 volume of each tubes cold isopropanol added and the tubes shaken upside down for several times. In this step jelly-colored bulk of DNA is somewhat visible. Finally, for precipitate of DNA, the tubes were centrifuged for 15 minutes at 10,000 rpm. Then the supernatant solution is discarded and DNA pellet were washed 2 to 3 times with ethanol 75% and dried with sterile filter paper. In the final step to dissolve DNA, 200 ml of distilled water are added to each of the tube and refrigerated at -4°C. Gel electrophoresis of DNA and spectrometry methods were used to determine the DNA quality and quantity. Agarose gel 0.8% was transferred to electrophoresis gel tray that placed on the electrophoresis combs and after gel fixation, combs slowly pulled out. Mixed of distilled water, 2 µl of dye loading buffer, and 1 to 2 µl DNA genomic from each sample were placed into the wells of the agarose gel.

After electrophoresis, the gels were stained with ethidium bromide (0.5 µg/ml) for 20 minutes, then washed with distilled water for 10 minutes. Gels were then photographed using UVP GelDoc-It Imaging System (UVP Inc. USA) under an ultraviolet lamp. DNA concentration and quality were assessed from gel image. Given that the agarose gel electrophoresis method is not very accurate for quantifying DNA, we used spectrophotometer (nanodrop device), as a complementary method for determining the concentration of DNA. Nanodrop device shows concentration as ng/µl. According to the initial concentration of DNA, diluted solution with same concentration (15 ng/µl) was prepared by adding distilled water.

PCR amplification

The reaction mixture in final volume of 15 µl contained 3 µl DNA tamplet (15 ng), 1.5 µl of RAPD random primers (0.2 µM), 3 µl of double-distilled water and 7.5 µl PCR Kit (CinnaGen Co., Iran). To investigate the DNA polymorphism among studied populations, 40 ten-nucleotide primers, including 15 operon primers and 25 Tib Molbiol primers was used for initial screening. In the end, 17 primers that showed high polymorphism were selected for RAPD analysis. The polymerase chain reactions were carried out in a thermocycler (i-Cycler). The reaction is driven by temperature cycling as shown in *Table 1*.

After PCR, the reaction products were loaded on agarose gel 1.2% wells (3.6 g in 290 ml of TBE buffer) prepared with TBE buffer (Tris- Boric acid-EDTA) and electrophoresed at 120 V for 150 min. 1 Kb size marker (Fermentas, SMO311) was loaded on the first well and PCR products from each population were loaded on another wells. The gels were stained with ethidium bromide (0.5 µg/ml) for 10 minutes, and then washed with distilled water for 10 minutes. After electrophoresis, DNA was visualized under ultraviolet light and photographed.

Table 1 - The time and required temperature for the three-step (denaturation, annealing and elongation) in each PCR thermal cycles

Cycle	Repeat	Stage	Temperature (^{0}C)	Time
1	1	Denaturation	94	3 min
2	5	Denaturation	92	1 min
		Annealing	5.39	1 min
		Elongation	72	2 min
3	37	Denaturation	92	30 s
		Annealing	5.39	45 s
		Elongation	72	2 min
4	1	Final elongation	72	7 min

Statistical analysis

After the RAPD analysis to evaluate polymorphic among genotypes, the presence or absence of a particular band was recorded as 1 and 0, respectively. After preparation of 0-1 matrix, statistical analysis was performed using the Popgene software version 1.31 (Yeh *et al.,* 1999). Population distance matrix was calculated by Nei's method (Nei, 1972) and based on the cluster analysis of this matrix, A dendrogram was generated by UPGMA using NTSYS-pc software (Rohlf, 2000). Genetic diversity calculated for all allele locations using Nei's analysis (Nei, 1987), in evaluation of the observed number of alleles, number of effective alleles (Kimura and Crow, 1963), Nei's genetic diversity index (Nei, 1973) and Shannon information index (Lewontin, 1972) were calculated for each population. Also, total heterozygosity (Ht), heterozygosity within populations (Hs), diversity coefficient among populations (Gst), estimation of gene flow from Gst or Gcs (Nm) For each allele were calculated using Popgene software (Nei, 1987) as follows:

Ht = 0.31, Hs = 0.23,

Gst = 0.26, Nm= 1.36

Fst index (Wright, 1951) was measured via this formula (Lynch and Milligan, 1994):

Fst = 1 – Hs / Ht

This index shows the structure and distance between populations by calculating the allelic heterozygosity, allele frequency and diversity in the populations (Wright, 1951).

RESULTS AND DISCUSSION

RAPD molecular markers were used to assess the genetic diversity of some population of wild mountain thyme. A total of 185 bands with high resolution from 17 primers were used for RAPD analysis, which 21of these were monomorphic and 162 (86.17%) were polymorphic. An average 10.88 bands were obtained per primer and 9.52 of these were polymorphic. OPE-17 primer and OPN-18 primer yielded the lowest (5) and highest (17) number of bands, respectively as shown in *Table 2*. Primers BC11 and OPN-7 and OPK-10 gave the highest percentage of polymorphism (100) while the lowest percentage was obtained by using the primers TIBMBA-8 (71%) and TIBMBA-9 (75%) as shown in *Table 2*.

Table 2 - The percentage of polymorphic sequences of used primers in the RAPD analysis of *Thymus kotschyanus* populations

No.	Primer	Sequences	Total number of bands amplified (a)	Polymorphic bands (b)	Percent polymorphism (b/a x 100)
1	TIBMBA- 5	TGCGTTCCAC	14	13	92
2	OPN- 19	GTGCAACGTG	11	9	81
3	TIBMBA- 8	CCACAGCCGA	7	5	71
4	TIBMBA- 9	GGAACTCCAC	8	6	75
5	OPAB- 18	CTGGCGAATG	10	9	90
6	OPN- 7	CAGCCCAGAG	10	10	100
7	OPB- 17	AGGGAACGAG	9	7	77
8	OPAB-4	GGCACGCGTT	14	12	85
9	OPG- 11	TGCCCGTCGT	10	9	90
10	OPN-18	GGTGAGGTCA	17	15	88
11	TIBMBC-11	TTTTGCCCCC	12	12	100
12	TIBMBA-13	GTGCGAGAAC	11	10	90
13	OPB- 16	TTTGCCCGGA	11	10	90
14	TIBMBC- 15	CCAGACTCCA	11	10	90
15	TIBMBC- 20	AGCACTGGGG	14	1	85
16	TIBMBA-14	TCGGGAGTGG	11	9	81
17	OPE- 17	CTACTGCCGT	5	4	80
Average			88/10	9/52	86/17
Sum			185	162	-

Then, the similarities or differences among populations were determined by distance matrix and also obtained dendrogram as shown in *Table 3* and *Fig. 1*.

The evaluation of populations distance matrix of shows that a high similarity was between Avan (Qazvin) and Siahkal (Gilan province) populations with the distance of 0. 094 and between Rudbar and Evan populations with the distance of 0.095 given that Siahkal and Rudbar populations belong to Gilan province, probably similarities among these populations may be related to gene exchange between plants of Avan region and Gilan province (Rudbar and Siahkal populations). Distance matrix results show that high dissimilarity was observed between Qazvin and Mazandaran 2 populations with the distance of 0.185 that could be related to large geographical distance between the two provinces. Then, Mazandaran1 and Qazvin also showed the high difference 0.167 that there is proof to much difference between the populations of the two provinces.

The calculated distance based on polymorphic bands was ranged from of 0.094 to 0.185 in dendrogram obtained from the populations (*Fig. 1*), Qazvin and Piranshahr populations showed the highest differences with other populations and were placed in a separate group. Among *Thymus*

kotschyanus populations, Evan and Siahkal populations were grouped together and Takestan, Rudbar and Mazandaran1 populations were placed in a separate group. Alamut, Semnan and Mazandaran 2 populations formed another group. The results of this study showed incoherence between genetic diversity and geographic diversity. In other words, the lack of coincide between molecular diversity and geographic diversity (climatically) in mountain thyme, that had a high cross-pollination, is could be due to flow of germplasm.

Table 3 - Genetic distances among populations of *Thymus kotschyanus*

Population	Qazvin	Piranshahr	Rudbar	Takestan	Avan	Siahkal	Mazandaran 1	Mazandaran 2	Semnan	Alamut
Alamut										1
Semnan									1	0.102
Mazandaran 2								1	0.112	0.108
Mazandaran 1							1	0.128	0.118	0.128
Siahkal						1	0.127	0.121	0.107	0.119
Avan					1	0.094	0.128	0.132	0.116	0.148
Takestan				1	0.117	0.129	0.123	0.121	0.139	0.131
Rudbar			1	0.109	0.095	0.131	0.105	0.108	0.114	0.147
Piranshahr		1	0.13	0.132	0.164	0.159	0.111	0.157	0.133	0.167
Qazvin	1	0.123	0.165	0.154	0.159	0.159	0.167	0.185	0.164	0.137

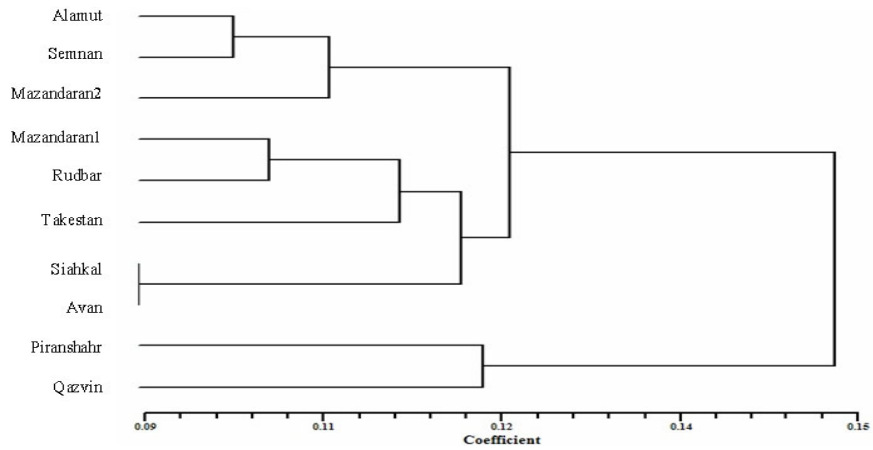

Figure 1 - Cluster analysis of based on distance matrix of RAPD data

Note that *Thymus kotschyanus* populations were cultivated for two year (since 2004) in the area research station of Homand Absard in Damavand and after seeding were planted in Engineer Badiei Research Station. Gene transfer by honey bees as the major pollen transfer in thyme, and subsequently reduction of genetic distance between populations may be another reason of incoherence between genetic diversity and geographic diversity. The observed number of alleles (na) in *Thymus kotschyanus* populations was the highest in Takestan population (1.72) and lowest in Alamut population (1.47) in terms of the number of effective alleles (ne), namely alleles that had equal frequencies and favorable distribution, Takestan and Avan populations showed the highest (1.45) and Alamut population showed the lowest (1.26) of non- uniformity of each population were calculated by dividing the ratio of the number of observed alleles to number of effective alleles as shown in *Table 4*.

Table 4 - Parameters of genetic diversity in populations of *Thymus kotschyanus*

Population	Number of genotypes	na	ne	h	I
Alamut	5	47.1	26.1	150.	240.
Semnan	5	66.1	39.1	230.	340.
Mazandaran 2	5	62.1	36.1	210.	330.
Mazandaran 1	5	59.1	33.1	20.	30.
Siahkal	5	69.1	44.1	250.	380.
Avan	5	68.1	45.1	250.	380.
Takestan	5	72.1	45.1	260.	390.
Rudbar	5	71.1	43.1	250.	380.
Piranshahr	5	68.1	42.1	240.	370.
Qazvin	5	67.1	41.1	240.	360.

Among *Thymus kotschyanus* populations, in Alamut population, 0.85 from observed alleles had a equal distribution, indicating that this population has a more uniform distribution than other populations because in Alamut population proportion of allele that had equal frequencies to total alleles are greater than other populations. While in Rudbar, Semnan and Mazandaran 2, 0.83 from observed alleles had a equal distribution, indicating that the homogeneity of these population were less compared to other studied populations. In general, the number of observed alleles in populations of *Thymus kotschyanus* was ranged between 1.47-1.72, number of effective alleles ranged between 1.26-1.45 and ratio of these ranged between 0.83-0.85. This closeness of numbers and small deviation represent a relatively equal distribution of alleles among *Thymus kotschyanus* populations and these ten people are roughly placed in same level in terms of allele distribution.

According to the *Table 4*, in all ten populations of *Thymus kotschyanus*, more than 0.82 of observed allele are effective alleles, that suggest that the allelic distribution within ten populations is relatively equal and these populations had a balanced distribution. Given the cross-pollination of various species of *Labiatae* family, including various species of the genus of the thymus genus, in balanced cross-pollinate populations, like ten studied populations, gene and genotype frequencies do not change from generation to generation (Ghahreman, 1994; Farsi and Zollali, 2003) and with regard to seed dispersal of *Thymus kotschyanus* this results is quite logical and expected. It can be used to study and better understanding of relationships between populations of wild thyme.

Assessment of genetic diversity in the population has some complexity due to the involvement of several factors, including the continuity, convergence of kinship, migration and differences in individual (Mohamadi and Prasana, 2003). In general, these studies depends on number of studied individual in each population, the number of studied allelic location, genotypic and allelic status of the population, type of crosses and population size (Weir, 1990). In a cross pollinated populations, there is a high heterozygosity, which provides an opportunity to adapt and evolve in the population, so there is an obvious variation within these populations. Within populations variation based on Shannon's information index and Nei genetic diversity index showed that Takestan (I = 0.26; H = 0.3) and Alamut (I = 0.24; H = 0.15) had a highest and lowest within populations variation compared to other populations, respectively. In general, in wild populations, geographic distance and gene flow between populations is determined genetic distance. In cross-pollinated species due to high gene flow, the genetic distance between populations are low and genetic diversity within populations rather scattered. In these species, if gene flow between habitats discontinued by unnatural factors such as habitat destruction caused by indiscriminate harvesting, excessive grazing and etc, genetic distance increased between discontinuous populations and due to the increasing homogenization and differentiation between populations genetic eroding will begin (Hamrick and Godt, 1989). To estimate gene flow among studied populations, Ht, Hs, Fst, Nm indexes were calculated. If Nm is close to zero, ie Fst is at its maximum and this mean that population heterozygosity has been very little. When Nm is at its maximum and Fst is at its minimum, this means that the heterozygosity of each population and the total population is close. Genetical differentiation of populations may be related to differences in genetic traits, that have been created by changing environmental conditions and natural selection. Also, related to random processes such as mutation, migration

and degree of differentiation of populations, which it can be estimated by different parameters (Bossdorph*et al.,* 2005).

In the present study, the average of Fst and Nm indexes, that showed the amount of gene flow between populations, are recorded 0.26 and 1.36, respectively, that indicating high genetic exchange among ten habitats of mountain thyme. Moreover, given that the rate of total heterozygosity was 0.23 (Ht = 0.23), therefore the amount of heterozygosity within populations is 0.8 (Hs=0.8), that express high levels of diversity within populations than among populations in ten studied mountain thyme population. This high genetic diversity may allow them to easier adapt to environmental changes and comprehend such a large variation may be helpful in the management and conservation of germplasm of mountain thyme.

Assess the diversity of plant genetic resources (germplasm), that have a great chance to occurrence of useful gens, is the first essential prerequisite to plan for the conservation, sustainable use and domestication of them. Morphological traits are influenced by several factors, that this factors are ineffective in DNA level. The combination of these factors cause that genotypes are close together in basis of DNA data may be different in terms of morphological traits. It should be noted that the amplified fragments in RAPD are not necessarily identical in terms of nucleotide sequence and fragments with the same size may obtained from different parts of the genome and had a different sequenced (Hayward *et al.,* 1993). Thus, the RAPD data may be had not expected align with the morphological characteristics. This is not unexpected considering the environmental effects on morphological traits, then in breeding programs if the choice made only based on morphological traits, it may not get the desired results. RAPD molecular markers, in spite of criticism, can be used as simple and efficient tool to assess the genetic diversity of plant resources that any initial data for DNA genomic and diversity among them are not present.

In this study, RAPD molecular markers were used to genetic variation of germplasm of some populations of wild mountain thyme. This marker system in addition of effective differentiation of studied populations, they also revealed the genetic diversity among and within populations. Knowledge of the genetic relationships of wild species for successful and sustainable utilization of them genetic variation is essential.

CONCLUSION

Generally, in all ten populations of *Thymus kotschyanus*, more than 0.82 of observed allele are effective alleles that suggest, that the allelic distribution within ten populations is relatively equal and these populations had a balanced distribution. Distance matrix results show that high

dissimilarity was observed between Qazvin and Mazandaran2 populations with the distance of 0.185, that could be related to large geographical distance between the two provinces. The amount of heterozygosity within populations is 0.8 (Hs = 0.8), that express high levels of diversity within populations than among populations in ten studied mountain thyme population.

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