DOI: http://dx.doi.org/10.22092/ijb.2013.4157

RAPD ANALYSIS OF GENETIC VARIATION WITHIN AND AMONG NATURAL POPULATIONS OF TWO SPECIES OF DIANTHUS L. (CARYOPHYLLACEAE) IN NE IRAN

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Received 17.07.2013. Accepted for publication 06.11.213.

Behroozian, M., Jafari, A. & Farsi, M. 2013 12 31: RAPD Analysis of Genetic Variation within and among natural populations of two species of *Dianthus* L. (*Caryophyllaceae*) in NE Iran. *–Iran. J. Bot. 19 (2): 194-201.* Tehran.

In this study we investigated the genetic variation, using RAPD data in 69 individuals of nine natural populations representing two species of *Dianthus* L. growing in Northeast of Iran. Eleven RAPD primers generated 111 polymorphic DNA bands. The percentage of polymorphic bands, Nai[1]s genetic diversity (h), as well as Shannon index (I), were assessed. A dendrogram based on UPGMA segregated examined populations into two main clusters matching the two species and the two subspecies of *Dianthus polylepis* subsp. *polylepis* and *D. polylepis* subsp. *D. binaludensis* were to each other. AMOVA showed 82% and 18% of the variation within and among populations respectively. Furthermore, principal coordinate analysis (PCA) based on a Euclidean metric revealed that three populations of *D. crinitus* subsp. *turcomanicus* were genetically different from six populations of *D. polylepis*. According to our results, geographical distances, mating system and gene flow have important effects on genetic polymorphism of populations.

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Key words. *Dianthus*, *Caryophyllaceae*, Iran, RAPD markers, genetic variation.

بررسی تنوع ژنتیکی بین و درون جمعیت‌های Dianthus (Caryophyllaceae) در شمال‌شرق ایران با استفاده از آنالیزRAPD

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در این مطالعه تنوع ژنتیکی ٦٩ فرد از نه جمعیت طبیعی متعلق به دو گونه *Dianthus* موجود درشمال شرق ایران با استفاده از داده‌های PARD به منظور دستیابی به اطلاعات مهم ژنتیکی بررسی گردید. ١١١ باند چندشکلی با کمک ١١ آغازگر RAPD بدست آمد. درصد باندهای چند شکلی، تنوع ژنتیکی نی و اندیس شانون محاسبه گردید. دندروگرام بر اساس روش UPGMA جمعیتهای مورد بررسی را به دو گروه اصلی مطابق با دو گونه جدا نمود و نزدیکی دو زیرگونه *D. polylepis* subsp. *polylepis* و *D. polylepis* subsp. *binaludensis* را نسبت به هم نشان داد. بر اساس آنالیز AMOVA به ترتیب ٨٢ و ١٨ درصد تنوع ژنتیکی برای درون و بین جمعیتها بدست آمد. همچنین آنالیز مختصات اصلی (PCA) بر پایه فاصله اقلیدوسی اختلاف سه جمعیت زیرگونه *D. crinitus* subsp. *turcomanicus* را از نظر ژنتیکی با شش جمعیت از دو زیرگونه *D . polylepis* subsp. *polylepis* و *D. polylepis* subsp. *binaludensis* آشکار کرد. با توجه به نتایج به دست آمده، فاصله جغرافیایی، سیستم آمیزشی و جریان ژنی می تواند اثرات مهمی در تنوع ژنتیکی جمعیتهای جنس *Dianthus* داشته باشد.

INTRODUCTION

Dianthus L. (*Caryophyllaceae*), with over 300 species, is one of the largest genera of vascular plants in the world. The *Dianthus* species are adapted to the northern temperate regions of Europe, Asia, North America, North Africa and are also growing in Mediterranean coastal regions (Galbally and Galbally 1997; Jurgens et al. 2003). Thirty species of *Dianthus* grow in Iran, of which three species *D. crinitus* subsp. *turcomanicus* Schischk, *D. polylepis* subsp. *polylepis*, *D. polylepis* subsp. *binaludensis* (Rech. f.) Vaezi & Behroozian and *D. orientalis* subsp. *stenocalyx* (Boiss.) Rech. f. are distributed in Northeast Iran (Rechinger 1986, Farsi et al. 2013).

The genus *Dianthus* contains both annual and perennial caespitose plants which some of its species are cultivated for hundreds of years for ornamental purposes (Ingwerson 1949). This genus was traditionally prescribed to treat coronary and nervous disorders (McGeorge and Hammett 2002) and fevers (Bown 1995). *Dianthus* flowers are normally protandrous, typically outcross and insect-pollinated **(**Carine and Shykoff 2003). Absence of self-pollination and self-crossing causes highly heterozygote varieties in *Dianthus* species (Holley and Baker 1992), which in turn increases the morphological diversity in its populations.

Many studies indicated that some wild species in *Dianthus* show very similar patterns of morphological variation. Farsi et al. (2013) performed morphological and molecular analysis on *D. polylepis* complex. They found a high diversity of morphological characteristics among the populations.

Despite of the morphological (Farsi et al. 2013), ecological (Crespi et al. 2007) and cytological (Jafari and Behroozian 2010) studies performed on populations of *Dianthus*, limited information exists on the genetic variation in *Dianthus* wild populations. Most of the molecular studies available are related to hybridization and determination of modified genes (Lee et al. 2005; Nimura et al. 2006; Onozaki et al. 2006). Thus, in order to assess genetic diversity, determination of both interspecies and intraspecies genetic variation, there is an essential need to obtain genetical information on natural populations of *Dianthus.*

In recent decades many molecular markers have been used to detect the genetic diversity within and among plant populations (Lin et al. 1996; Bharmauria et al. 2009) Molecular markers such as SSR (Simple Sequence Repeats), ISSR (Inter Sequence Repeats) and RAPD (Random Amplified Polymorphic DNA) provide useful information related to evolution and population genetics (Kimura et al. 2009; Safari et al. 2013). Among molecular markers, RAPD markers have generally been used for the detection of genetic variation within and among populations in several plant species and populations without the need for detailed knowledge of DNA (Holsinger et al. 2002; Wei et al. 2008; Zarek, 2009).

Su Yeong (2002) analyzed genetic similarity in some wild species and cultivars of *Dianthus caryophyllus* (carnation) using isozyme and RAPD markers and showed that these markers can distinguish the cultivars and wild species of carnation.

Here, we evaluated a study on genetic diversity within and among natural populations of *Dianthus* using RAPD markers in Northeast of Iran. The aim of this work was to investigate genetic polymorphism and effect of geographical distance and reproduction process on it.

MATERIALS AND METHODS

Plant material

Nine natural populations (7-8 individuals per populations) of three subspecies belonging to *D. polylepis* and *D. crinitus* were collected from different regions of Northeast of Iran (Table1 and Fig. 1).

DNA extraction and PCR procedures

Total genomic DNA was isolated from young leaves of seven to eight individuals from nine populations according to the Dellaporta protocol (Dellaporta et al., 1983) with minor modifications. PCR reactions and selection of primers were carried out according to Lee et al. (2005). Eleven primers were selected according to the number and consistency of amplified fragments. PCR reactions were performed in a 25 µl volume each containing 1 U of Taq polymerase, 50 ng of genomic DNA as template, 1.5-2.5 Mm $MgCl_2$, 50 ng of primer, 0.2 µmol of dNTP mix and 2.5 µl of 10X PCR reaction buffer (Fermentas, USA). All reactions were run on an Ependrof Mastercycle Gradient. The PCR conditions used to amplify primer included a 5min denaturing step at 94ºC followed by 45 cycle of 94 ºC for 1 min, 30 sec at 37°C, 1 min at 72°C and 72°C for 5 min. Amplified products were separated by 1.2% agarose gel electrophoresis in TBE (Tris- Borate- EDTA) buffer. The PCR products were visualized by ethidium bromide staining and photographed under UV light by a Gel Doc system (UVP. BioDoc. USA).

Data analysis

RAPD bands were scored as present (1) or absent (0). Genetic diversity within populations was estimated in terms of polymorphic loci, Nei's genetic diversity coefficient and genetic distance and Shannon's indices (Lewontin,1972) using the program POPGENE 32 software. The program was used for the analysis of

Table 1. Location and number of samples collected for each of the nine populations of *Dianthus* L. species

No. pop.	species	population	Altitude (m)	Longitude	Latitiude	Location	Abv.	Number of samples
1	*D. polylepis* subsp. *binaludensis*	Moghan1	2600	59º22'51.6″	36º07'44.4″	Khorassan Razavi, S. Moghan Mts	P.B1	8
2		Bujan	1600	58º58'58.8″	36º14'31.2″	Khorassan Razavi, Boujan Mts. northern slopes	P.B2	8
3		Zoshk	2100	59º07'51.6″	36º18'43.2″	Khorassan Razavi, Mountains between Kang and Zoshk	P.B3	8
4	*D. polylepis* subsp. *polylepis*	Kardeh	1680	59º38'13.2″	36º40'33.6″	Khorassan Razavi, Kardeh Mts.	P.P1	8
5		Torbat	1800	59º11'38.4″	35º33'07.2″	Khorassan Razavi, N Torbat Heydariyeh, Khomari pass	P.P2	8
6		Fariman	1595	59º50'27.6″	35º38'38.4″	Khorassan Razavi, Fariman, Dam Mts.	P.P3	7
7	*D. crinitus* subsp. *turcamanicus*	Sarrud	1800	59º44'02.4″	36º52'55.2″	Khorassan Razavi, NW Kalat-e Naderi, Sarrud Mts.	C.T1	8
8		Moghan2	2300	59º22'51.6″	36º07'44.4″	Khorassan Razavi, S. Moghan Mts	C.T2	7
9		Ardak	1758	59º27'54.0″	36º40'44.4″	Khorassan Razavi, NW Mashhad, Ardak Mts.	C.T3	7

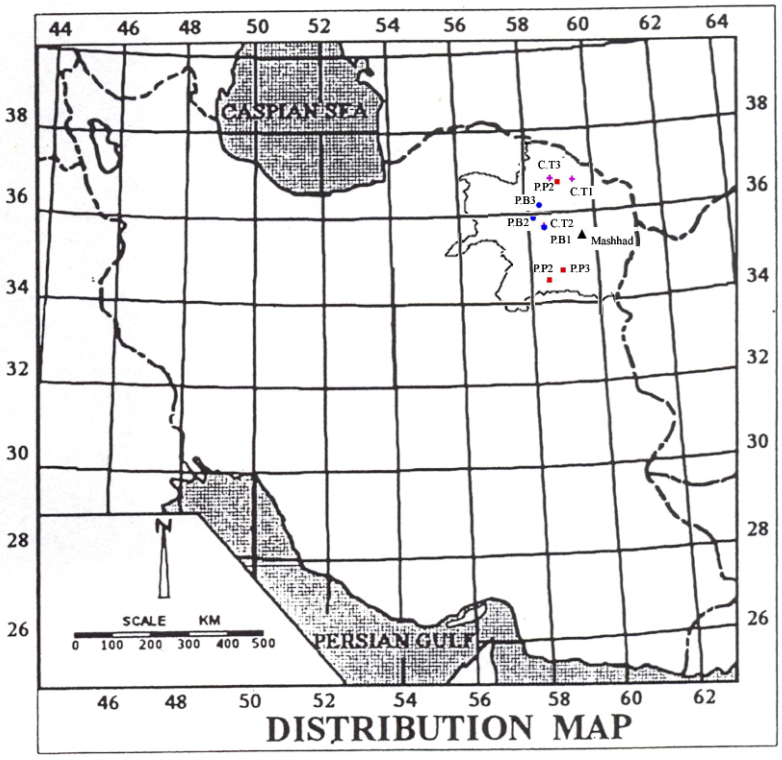

Fig. 1. Sampling site for the nine populations analyzed of *Dianthus* species in northeast of Iran. Populations per subspecies are indicated with different symbols.

Table 2. Number of polymorphic loci, percentage of polymorphic loci, Shannon's index I (Lewontin, 1972) and Nei's (1973) gene diversity within each of the nine populations.

No. pop.	species	h	I	Percentage of polymorphic loci	No. polymorphic loci
1	*D. polylepis* subsp. *binaludensis*	0.229	0.355	75.68	84
2		0.209	0.323	69.37	77
3		0.224	0.346	75.68	84
4	*D. polylepis* subsp. *polylepis*	0.242	0.371	78.38	87
5		0.218	0.336	72.97	81
6		0.196	0.301	63.06	70
7	*D. crinitus* subsp. *turcamanicus*	0.206	0.320	70.27	78
8		0.251	0.385	80.18	89
9		0.213	0.330	71.17	79
Total		0.220	0.304	72.97	72.9

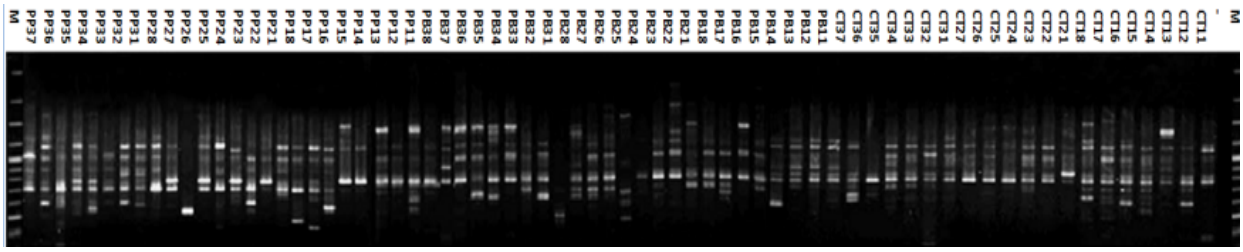

Fig. 2. DNA bands using OPF-09 primer within and among the populations, M: Master, -: negative control, D. *crinitus* subsp. *turcomanicus*, C11-C18: (Sarrud population), C21—C27: (Moghan population), C31-C37: (Ardak population). *D. polylepis* subsp. *binaludensis*, B11-B18: (Moghan population), B21-B28: (Bujan population), B31-B38: (Zoshk population). *D. polylepis* subsp. *polylepis*, P11-P18: (Kardeh population), P21-P28: (Torbat population, P31-P37: (Fariman population).

genetic variation among and within populations using dominant markers. The relationship between populations using UPGMA method was constructed with Poptree software. Also, cophenetic correlation coefficient (r) was computed. Genetic diversity within and among the populations were evaluated by analysis of molecular variance (AMOVA) (Excoffier et al. 1992). Principal Component Analysis (PCA) based on Eclidean metric (Gower, 1966) was also used to visualize relationship individuals using the program GenAlex (Peakall and Smouse, 2001). Moreover, mantel test was applied to estimate the correlation between genetic distance and geographic distance among populations**.**

RESULTS

Eleven primers were used for the 69 individuals representing three populations of each *D. polylepis* subsp. *polylepis, D. polylepis* subsp. *binaludensis* and *D. crinitus* subsp. *turcomanicus* produced 126 bands (Fig. 2). The percentage of polymorphic loci in the analyzed samples was 87.37%. The highest number of polymorphic loci (13) was obtained by primers OPR-20 and OPX-07 and the lowest (3) by primer OPR-09 (Table 2). Most of the primers generated a high percent of polymorphic loci, which means that genetic polymorphism in the population is generally high.

Table 3 describes the genetic parameters of nine populations generated based on RAPD markers. The total percentage of polymorphic loci among populations was 72.97%. For each population, the percentage of polymorphic loci varied from 63.06% (population Friman) to 80.18% (population Moghan2). Nei's gene diversity (h) ranged from 0.196 (Fariman) to 0.251 (Moghan2), with a mean of 0.220. The highest genetic diversity was in population C.T2 (Moghan2) and the lowest in population P.P3 (Fariman). A similar pattern was observed for the Shannon information index (I), with the highest value of 0.385 observed in population C.T2 (Moghan2) and the lowest value of 0.301 was observed in population P. P3 (Fariman).

Among populations of *D. crinitus* subsp. *turcomanicus*, the highest value of genetic diversity was observed in Moghan population based on Shannon's index (0.385), Nei's genetic diversity (0.2514), polymorphic loci percentage (80.18%) and 89 polymorphic bands. While Sarrud population had the lowest level of genetic diversity, as illustrated by Shannon's index (0.3208), Nei's genetic diversity (0.2064), polymorphic loci percentage (70.28%) and 78

Table 3. Analysis of molecular variance (AMOVA) for 69 individuals grouped in nine populations of *Dianthus* species.

Source of variation	d.f	SS	MS	Est. Var.	Total variance(%)	P - value
Among Pops.	8	362.785	45.348	3.757	18%	< 0.001
Within Pops.	60	993.679	16.561	16.561	82%	< 0.001

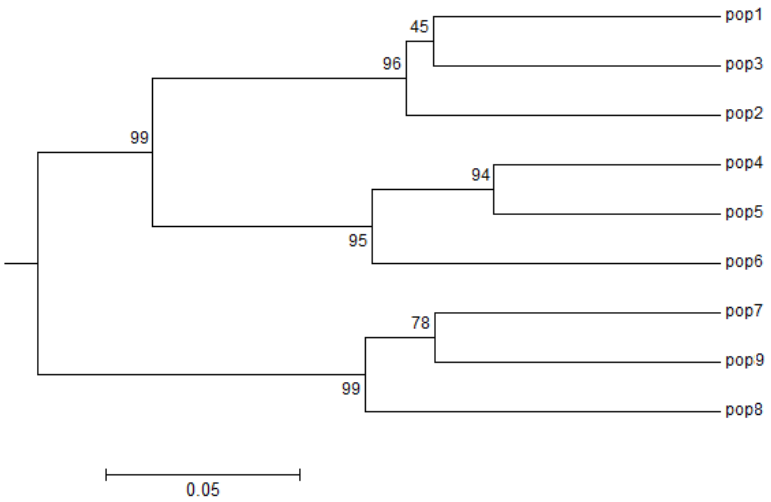

Fig. 3. UPGMA dendrogram of the populations studied based on Nei's genetic distance (population numbers as in Fig. 4).

polymorphic bands (bootstrap value 99%). The populations of Moghan and Bujan of *D. polylepis* subsp. *binaludensis* illustrated the highest and lowest value of genetic diversity based on Shannon's index (0.355, 0.323), Nei's genetic diversity (0.229, 0.2099), the percentage of polymorphic loci (75.68%, 69.37%) and 84,77 polymorphic bands, respectively (bootstrap value 96%). Finally, Kardeh population of *D. polylepis* subsp. *polylepis* had the highest value of genetic diversity, as determined by Shannon's index (0.0.371), Nei's genetic diversity (0.241), polymorphic loci percentage (78.38%) and 89 polymorphic bands, whereas Fariman population had the lowest level of genetic diversity, as illustrated by Shannon's index (0.3015), Nei's genetic diversity (0.1963), polymorphic loci percentage (63.06%) and 70 polymorphic bands (bootstrap value 95%).

AMOVA analysis indicated a significant partitioning of the genetic variation (P<0.0001) with the most variation (82%) among individuals within populations and lesser amounts (18%) among populations (Table 3).

The UPGMA dendrogram based on Nei's genetic distance is presented in Fig. 3. This dendrogram also indicated that the lowest genetic distance was between populations C.T1 and C.T2, but the highest distance was between populations C.T2 and P.B1 (Table 4). For the dendrogram, the cophenetic correlation coefficient was obtained (r = 0.9148). In the dendrogram the populations were divided into two main groups (Fig. 3). The first main group included the three populations of *D. crinitus* subsp. *turcomanicus* that were separated from the second group which contained six populations of *D. polylepis* subsp. *polylepis* and *D. polylepis* subsp. *binaludensis* (bootstrap value 99%). A Mantel test evaluated that genetic distance between populations were correlated to geographic distance (r = 0.871, P value = 0.001).

Principal Component Analysis (PCA) based on Euclidean metric evaluated 28.21, 24.13 and 13.43 of the total variance for studied individuals of the nine populations. According to PCA results, six populations of *D. polylepis* were closer to one another than populations of *D. crinitus*. These results showed a sufficient correspondence with obtained dendrogram based UPGMA. Both results indicated two subspecies of *D. polylepis* were genetically different from *D. crinitus* subsp. *turcomanicus* (Fig. 4).

DISCUSSION

In this study, RAPD analyses provided insight into the genetic diversity, an effect of geographical distances on nature populations of *Dianthus* species in Northeastern Iran. RAPD markers produced several polymorphic

Table 4 . Gene distance matrix among nine populations of *Dianthus* based Nei's gene diversity (1978).

pop ID	P.B1	P.B2	P.B3	P.P1	P.P2	P.P3	C.T1	C.T2	C.T3
P.B1	0.0000								
P.B2	0.0590	0.0000							
P.B3	0.0504	0.0585	0.0000						
P.P1	0.1445	0.1210	0.1066	0.0000					
P.P2	0.1151	0.1020	0.0808	0.0499	0.0000				
P.P3	0.1374	0.1167	0.0942	0.0467	0.0527	0.0000			
C.T1	0.1407	0.1575	0.1487	0.1161	0.1005	0.0941	0.0000		
C.T2	0.1730	0.1164	0.1147	0.0983	0.0715	0.0751	0.0420	0.0000	
C.T3	0.1508	0.1341	0.1317	0.1189	0.1038	0.1115	0.0566	0.0629	0.0000

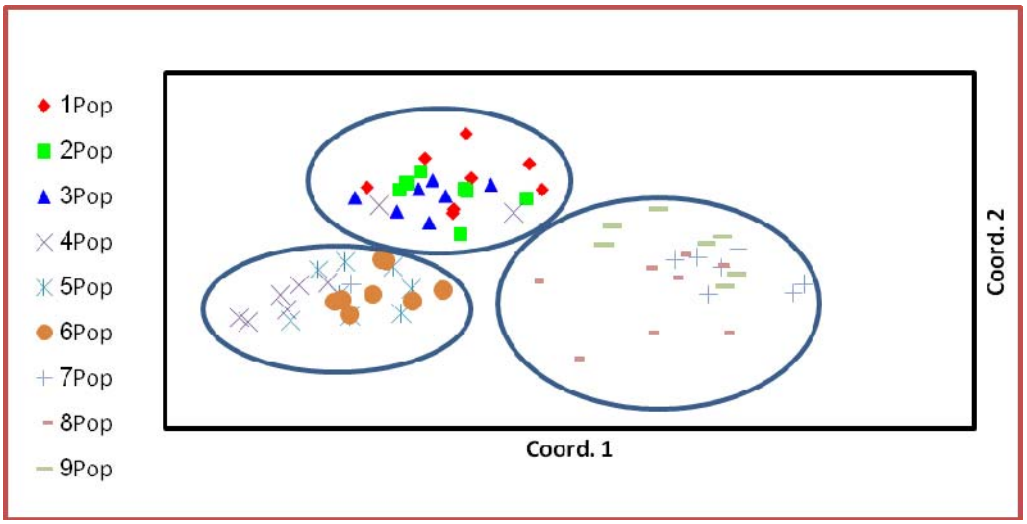

Fig. 4. PCoA plot of *Dianthus* species and populations studied. *D. polylepis* subsp. *binaludensis:* 1 pop: (Moghan population), 2 pop: (Bujan population), 3 pop: (Zoshk population). *D. polylepis* subsp. *polylepis:* 4 pop : (Kardeh population), 5 pop: (Torbat population, 6 pop: (Fariman population). *D. crinitus* subsp. *turcomanicus*: pop 7: (Sarrud population), pop 8: (Moghan population), pop 9: (Ardak population).

bands for estimating the populations' genetic diversity parameters. Similar results were reported in a study conducted by Lee et al. (2005) on the interspecific hybrid between *D. giganteus* d'Urv and *D. carthusianorum* L. They obtained 216 polymorphic bands and 18 monomorphic bands by 23 RAPD primers used. Su Yeong (2002) also obtained 80 polymorphic bands out of 82 observed bands (97.5% polymorphic loci) in wild species and cultivars of carnation. In the present study, RAPD markers revealed 72.97% of the total polymorphic loci among nine populations. Thus, high polymorphism obtained by RAPD markers could resolve the genetic variation among and within populations.

As shown in table 3, the range of Shannon's indices and Nei's gene diversity among populations appeared to be relatively limited. These results suggest that populations used in this study have very similar levels of within-population genetic variation. The populations with the highest Shannon's indices had the highest Nei's gene diversity and the percentage of polymorphic loci and vice versa. Among populations, Moghan population (C.T2 and P.B1) of *D. crinitus* subsp. *turcamanicus* and *D. polylepis* subsp. *binaludensis* and Kardeh population (P.P1) of *D. polylepis* subsp. *polylepis* had the highest value of parameters.

The Mantel test showed that there was significant correlation between genetic distance and geographic distance (r = 0.871, p value= 0.001). Among populations of *D. crinitus* subsp. *turcomanicus*, Moghan population (C.T2) was genetically distant from the two other populations. Bujan population of *D. polylepis* subsp. *binaludensis* and Kardeh population of *D. polylepis* subsp. *polylepis*, separated from other populations each subspecies. Geographically, populations of Moghan, Bujan and Kardeh are also distant from the other populations. Moghan population of *D. crinitus* subsp. *turcomanicus* is located in Binalud

Mountains and had the highest genetic variation, while two other populations grow in Koppeh-dagh Mountains. Moreover, all three populations of *D. polylepis* subsp. *binaludensis* are located in Bianalud Mountains. Nei's Both population of Zoshk and Moghan are found in South slopes while Bujan population is located in North slope. Finally, Torbat-Heidarie and Fariman populations are closely related to each other, while they are far from Kardeh population in term of geographical distance. According to obtained results, there is a significant positive correlation between geographic distance and genetic distance ($r = 0.871$).

In conclusion, there is high genetic variation among individuals within populations (82%) and low genetic variation among populations (18%) and all AMOVA variation show highly significant ($p < 0.001$).

There are many factors that have effect on genetic structure among populations, of which natural selection, mating system and gene flow are important (Ge, 1998). In general, predominantly outcrossed species have been found to exhibit most variation within populations (Loveless, 1992 and Chase et al. 1995). Moreover, high levels of variation found within populations suggest the unlimited gene flow (*Nm* = 1.66), which in turn increases the probability of outbreeding. *Dianthus* is a genus pollinated by insects and chemical materials as ethylene. So, it´s petals immediately senesce after pollination by pollination-induced ethylene which leads to an enormous evolutionary benefit to the species, by preventing of the pollinators to the same flower and increasing more-efficient pollination to the individual flowers in a population. Thus, phenotype and genotype variation occur within its populations (Woltering et al. 1994; Van Altvorst and Bovy, 1995). Likely, unlimited gene flow could be one factor contributing to *Dianthus* species's low level of intra-population differentiation.

ACKNOWLEDGMENTS

The authors thank Dr. Mahmood nia and Mr. Joharchi for their suggestions and technical helps and Mr. Feiz for his help in sampling the plant material. This study was financially supported by Department of Biology of Islamic Azad University of Mashhad and Research Center for Plant Sciences of Ferdowsi University of Mashhad.

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