Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Huang Jing, Somayeh Esfandani-Bozchaloyi (2022) Genetic diversity and gene-pool of *Medicago polymorpha* L. based on retrotransposon-based markers. *Caryologia* 75(1): 131-140. doi: 10.36253/caryologia-1428

Received: October 19, 2021

Accepted: March 31, 2022

Published: July 6, 2022

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Genetic diversity and gene-pool of *Medicago polymorpha* L. based on retrotransposon-based markers

Huang Jing[1,*], Somayeh Esfandani-Bozchaloyi[2]

[1] *Department of information and Electronic Engineering, Hunan City University, Hunan 413000, China*
[2] *Faculty Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran*
*Corresponding author. E-mail: hyrwss@163.com

Abstract. The genus *Medicago* L. (Fabaceae) comprises approximately 87 different species of herbs and shrubs widespread from the Mediterranean to central Asia. *Medicago polymorpha* is a herbaceous legume that can be a useful pasture plant, in particular, in regions with a Mediterranean climate. It had aroused great interest due to high nutritious quality, highly palatability and N-fixing plan in neutral soil. There is no information on its population genetic structure, genetic diversity, and morphological variability in Iran. Due to the medicinal importance of this species, a genetic variability and populations' structure study is performed studying 15 geographical populations of *Medicago polymorpha*. Therefore, we used six inter-retrotransposon amplified polymorphism (IRAP) markers and 15 combined IRAP markers to reveal within and among population genetic diversity in this plant. AMOVA test produced significant genetic difference (PhiPT = 0.46, P = 0.010) among the studied populations and also revealed that, 66% of total genetic variability was due to within population diversity while, 34% was due to among population genetic differentiation. Mantel test showed positive significant correlation between genetic distance and geographical distance of the studied populations. STRUCTURE analyses and population assignment test revealed some degree of gene flow among these populations. PCoA plot of populations was in agreement with UPGMA clustering of molecular data. These results indicated that geographical populations of *Medicago polymorpha* are well differentiated based on (IRAP) markers.

Keywords: gene flow, IRAP, Medicago polymorpha, population differentiation.

INTRODUCTION

Knowledge of spatial genetic structures provides a valuable tool for inferring the evolutionary forces such as selective pressures and drift (bi *et al*, 2021; cheng *et al*, 2021; khayatnezhad and gholamin, 2020, 2021a, 2021b). Low gene flow due to spatial isolation of populations may even increase the degree of local differentiation (karasakal *et al*, 2020a, 2020b; huang *et al*, 2021; hou *et al*, 2021, guo *et al*, 2021). Nevertheless, phenotypic plasticity rather than genetic differentiation may be an alternative way of matching

genotypes to environment; indeed increasing environmental variation favors higher levels of plasticity (MA et al, 2021; peng *et al*, 2021; Si *et al*., 2021; sun *et al*., 2021; miao *et al* 2018; zou *et al*, 2019; wang *et al* 2020; xiaolong *et al*, 2021; hou *et al,* 2021).

The genus *Medicago* L. (Fabaceae) comprises approximately 87 different species of herbs and shrubs widespread from the Mediterranean to central Asia (Small, 2010), including the widely cultivated forage crop and weedy species *M. sativa* L. (commonly named alfalfa or Lucerne) and the legume model species *M. truncatula* Gaertn. (Steele et al., 2010). The annuals species collectively known as "medics" are naturally distributed over a very wide range of environmental conditions in the Mediterranean basin. Some medics have been introduced to regions of Australia, Chile, South Africa and United States with Mediterranean-type climate. Medics, as well as other annual pasture legumes, have a high feeding quality, determined by higher protein, mineral and vitamin contents (Keivani et al., 2010). Due to their capacity to fix atmospheric nitrogen and improve soil fertility in symbiosis with soil bacteria collectively known as 'rhizobia', *Medicago* species do not need costly and polluting chemical nitrogen fertilizer (Small and Jomphe, 1989).

The genus *Medicago* in Iran has been revised by different authors. Boissier (1872), in his Flora Orientalis, published 11 *Medicago* species for Iran. Parsa (1948), Moussavi (1977) and Heyn (1984) recognized 14, 16 and 11 species in Iran, respectively. Mehregan & al. (2001) reported 18 species of the genus *Medicago* from Iran. Two main reasons can be accounted for the disagreements over the taxonomic status of this genus in Iran: (1) incomplete collecting; and (2) taxonomic confusions encountered in *Medicago*.

Medicago polymorpha L. is an annual herbaceous and can be a useful pasture plant, in particular, in regions with a Mediterranean climate, self-compatible and diploid (2n = 14) (Salhi Hannachi et al., 1998). It had aroused great interest due to high nutritious quality, high palatability and N-fixing capability in neutral soil (Abdelkefi et al., 1996). *M. polymorpha* is a species of Mediterranean origin, but its species range is wide spread throughout the world. The wide diffusion and adaptability can be explained by its low sensitivity to photoperiod and vernalization (Aitken, 1981). Three botanical varieties of this species were identified by Heyn (1963): *brevispina*; *polymorpha* and *vulgaris*. In Iran, *M. polymorpha* grows in a range of environments from humid to arid.

In recent years, molecular marker systems such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeat (SSR) and inter-retrotransposon amplified polymorphism (IRAP) have been used to measure genetic variation and relationships in cultivars and landraces of *Medicago* species. For instance, Genetic diversity among and within 10 populations of Iranian alfalfa, from different areas of Azarbaijan was analyzed by screening DNA from seeds of individual plants and bulk samples (Mohammadzadeh et al, 2011). Morpho-phenological diversity among natural populations of *Medicago polymorpha* of different Tunisian ecological areas (Badri et al, 2016). Their results from analysis of variance (ANOVA) showed that differences among populations and lines existed for all traits, with population explaining the greatest variation for measured traits. Genetic relationships of 98 alfalfa (*Medicago sativa* L.) germplasm accessions examined using morphological traits and SSR markers from Europe, USA, Australia, New Zealand and Canada (Cholastova, Knotova, 2012). Moreover, due to extensive morphological variability of this species in the country, there is possibility of having infra-specific taxonomic forms. Therefore, we carried out population genetic analysis and morphometric study of 15 geographical populations for the first time in the country.

For genetic study, we used the inter-retrotransposon amplified polymorphism (IRAP) method that displays insertional polymorphisms by amplifying the segments of DNA between two retrotransposons. It has been used in numerous studies of genetic diversity (Smykal *et al*., 2011).

The objectives of this research were to study genetic diversity among *Medicago polymorpha* cultivars/population with a different geographical origin by inter-retrotransposon amplified polymorphism (IRAP) method, to determine genetic variation among and within materials using IRAP markers.

MATERIALS AND METHODS

Plant materials

A total of 89 individuals were sampled representing 15 natural populations of *Medicago polymorpha* in East Azerbaijan, Lorestan, Kermanshah, Gilan, Mazandaran, Golestan and Ardabil Provinces of Iran during July-Agust 2019-2020. Fresh leaves of 5-8 individuals from each population, were collected, and immediately dried in Silica Gel. Different references were used for the correct identification of species (*Medicago polymorpha*) (Boissier ,1872; Parsa 1948).

DNA extraction and IRAP assay

Fresh leaves were used randomly from 5-10 plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA. The quality of extracted DNA was examined by running on 0.8% agarose gel. A set of six outward-facing LTR primers (Smykal *et al*., 2011; Table 1) were used for IRAP analysis. We also used 15 different combinations of outward-facing LTR pair primers. PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The thermal program was carried out with an initial denaturation for 1 min at 94°C, followed by 40 cycles in three segments: 35 s at 95°C, 40s at 47°C and 55s at 72°C. Final extension was performed at 72°C for 5 min. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Molecular analyses

The IRAP profiles obtained for each samples were scored as binary characters. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism were determined (Weising *et al*., 2005).

Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Huson and Bryant, 2006). Mantel test checked the correlation between geographical and genetic distance of the studied populations (Podani, 2000). These analyses were done by PAST ver. 2.17 , DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse, 2006), and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) (Meirmans and Van Tienderen, 2004) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation (Hedrick, 2005), and D_est = Jost measure of differentiation (Jost, 2008).

The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (Pritchard *et al*. 2000), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2. (2013). For STRUCTURE analysis, data were scored as dominant markers. The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value (Evanno *et al.,* 2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k.

Table 1. *M. polymorpha* IRAP primers based on Smykal et al. (2011) study.

IRAP	Sequence (5′-3′)
GU735096	ACCCCTTGAGCTAACTTTTGGGGTAAG
GU980589	AGCCTGAAAGTGTTGGGTTGTCG
GU929878	GCATCAGCCTGGACCAGTCCTCGTCC
GU735096	CACTTCAAATTTTGGCAGCAGCGGATC
GU929877	TCGAGGTACACCTCGACTCAGG
GU980590	ATTCTCGTCCGCTGCGCCCCTACA

Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers equal amount of gene flow among all populations. (ii) Population assignment test based on maximum likelihood as performed in Genodive ver. in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5. (2012).

RESULTS

Populations' genetic diversity

Genetic diversity parameters determined in 15 geographical populations of *Medicago polymorpha* are presented in Table 2. The highest value of percentage polymorphism (53.75%) was observed in Ardabil, Khalkhal-Asalem Road (population No.1) which shows high value for gene diversity (0.32). and Shanon' information index (0.39). Population Kermanshah: Ghasre-Shirin, 5 km from Paveh to Nusod (No.9) has the lowest value for percentage of polymorphism (31.43%) and the lowest value for Shanon, information index (0.030), and He (0.011).

Population genetic differentiation

AMOVA (PhiPT = 0.74, P = 0.010), and Gst analysis (0.367, p = 0.001) revealed significant difference among the studied populations (Table 3). It also revealed that, 66% of total genetic variability was due to within population diversity and 34% was due to among population genetic differentiation. Pairwise AMOVA produced significant difference among the studied populations.

Table 2. Genetic diversity parameters in the studied populations *Medicago polymorpha* (N = number of samples, Na= number of different alleles; Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

Pop	Na	Ne	I	He	UHe	%P
Pop1	0.341	1.058	0.39	0.32	0.31	53.75%
Pop2	0.455	1.077	0.277	0.24	0.22	55.05%
Pop3	0.499	1.067	0.24	0.23	0.24	49.26%
Pop4	0.555	1.020	0.22	0.25	0.28	43.53%
Pop5	0.431	1.088	0.20	0.22	0.25	41.53%
Pop6	0.255	1.021	0.25	0.28	0.22	47.15%
Pop7	0.261	1.024	0.292	0.23	0.23	43.15%
Pop8	0.886	1.183	0.184	0.116	0.122	44.29%
Pop9	0.686	1.157	0.030	0.011	0.022	31.43%
Pop10	0.643	1.173	0.154	0.102	0.109	30.00%
Pop11	0.243	1.033	0.026	0.018	0.029	34.29%
Pop12	0.400	1.087	0.076	0.051	0.057	40.29%
Pop13	0.286	1.046	0.040	0.027	0.032	37.14%
Pop14	0.400	1.112	0.090	0.062	0.069	35.71%
Pop15	0.576	1.144	0.122	0.083	0.095	39.18

Moreover, we got high values for Hedrick standardized fixation index after 999 permutation (G'st = 0.367, P = 0.001) and Jost, differentiation index (D-est = 0.176, P = 0.001). These results indicate that the geographical populations of *Medicago polymorpha* are genetically differentiated from each other.

Table 3. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	20	216.576	21.327	9.082	66%	66%
Within Pops	59	114.767	9.530	1.530	34%	
Total	79	321.342		10.613	100%	

df: degree of freedom; SS: sum of squared observations; MS: mean of squared observations; EV: estimated variance; **Φ**PT: proportion of the total genetic variance among individuals within an accession, ($P < 0.001$).

Populations genetic affinity

In UPGMA tree, plant samples of each populations, were grouped together and formed separate cluster. In the studied specimens we did not encounter intermediate forms. These results showed that IRAP data can differentiate the populations of *Medicago polymorpha* in two different major clusters or groups (Figure 1). The first major cluster that was supported with significant bootstrapping values of higher than 50%, was divided into two main sub-clusters so that plants of Ardabil,Meshkin shahr, hatam Forest, Ardabil, Meshkin shahr, Sabalan MT, Shahbil, Qotursooi Villageand and Ardabil: Germi, 20 km from Germi to Pars-Abad (P8- P 9, 12; Province Ardabil) and West Azerbaijan, Kaleybar and Azarbaijan (E): Ahar, 45 Km from Meshkin-Shahr

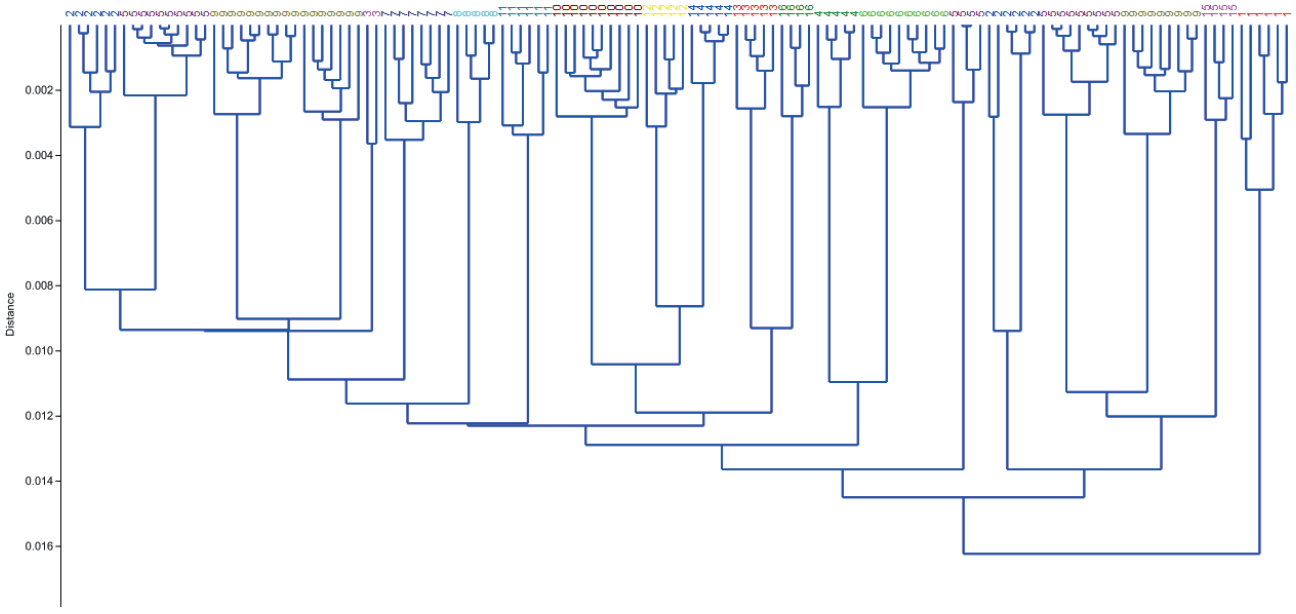

Figure 1. UPGMA clustering of populations in *Medicago polymorpha* based on IRAP data. Bootstrap value from 1000 replicates are indicated below branches (Population numbers are according to Table 1).

to Ahar (P10,15; Province West Azerbaijan) comprised the first sub-cluster due to morphological similarity, while the plants of Ardabil, Khalkhal-Asalem Road (P1) formed the second sub-cluster. Similarly, the second major cluster included two sub-clusters too: the first sub-cluster contained Lorestan: Khorram-Abad, 60 km from Pol-Dokhtar to Khorram-Abad (P11) and Kermanshah: Paveh, Paveh Shahid Kazemi Forest Park (P3) , while plants of Gilan, Mazandaran and Golestan Provine (Northen Iran) (P2- 4,5,6,7,13,14) were grouped into the second sub-cluster.

Genetic divergence and separation of populations Lorestan (P11) and Kermanshah (P3) as well as P8- P 9, 12 (Province Ardabil) from the other populations is evident in PCoA plot of IRAP data after 900 permutations (Figure.3). The other populations showed close genetic affinity. Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.48, P = 0.001). Therefore, the populations that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in *Medicago polymorpha*.

Populations genetic structure

K = 3 reveal the presence of 3 genetic group. Similar result was obtained by Evanno test performed on STRUCTURE analysis which produced a major peak at k = 3 (Figure.3). Both these analyses revealed that *Medicago polymorpha* populations show genetic stratification.

STRUCTURE plot based on k = 3, revealed genetic difference of populations 11 and 12 (differently colored), as well as 13 and 14 (Figure.4). But it showed genetic affinity between populations 1-10 and 15 (similarly colored). The mean Nm = 0.654 was obtained for all IRAP loci, which indicates low amount of gene flow among the populations and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. Population assignment test also agreed with Nm result and could not identify significant gene flow among these populations. However, reticulogram obtained based on the least square method (Figure not included), revealed some amount of shared alleles among populations 1 and 5, and between 13 and 6 and 7, also between 8, and 9. This result is in agreement with grouping we obtained with PCoA plot, as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in these populations and all these results are in agreement in showing high degree of genetic stratification within *Medicago polymorpha* populations.

In total 120 IRAP bands (loci) were obtained, out of which 34 bands were private. Populations 1-7, 8, 14 and 15 contained 1-4 private bands.

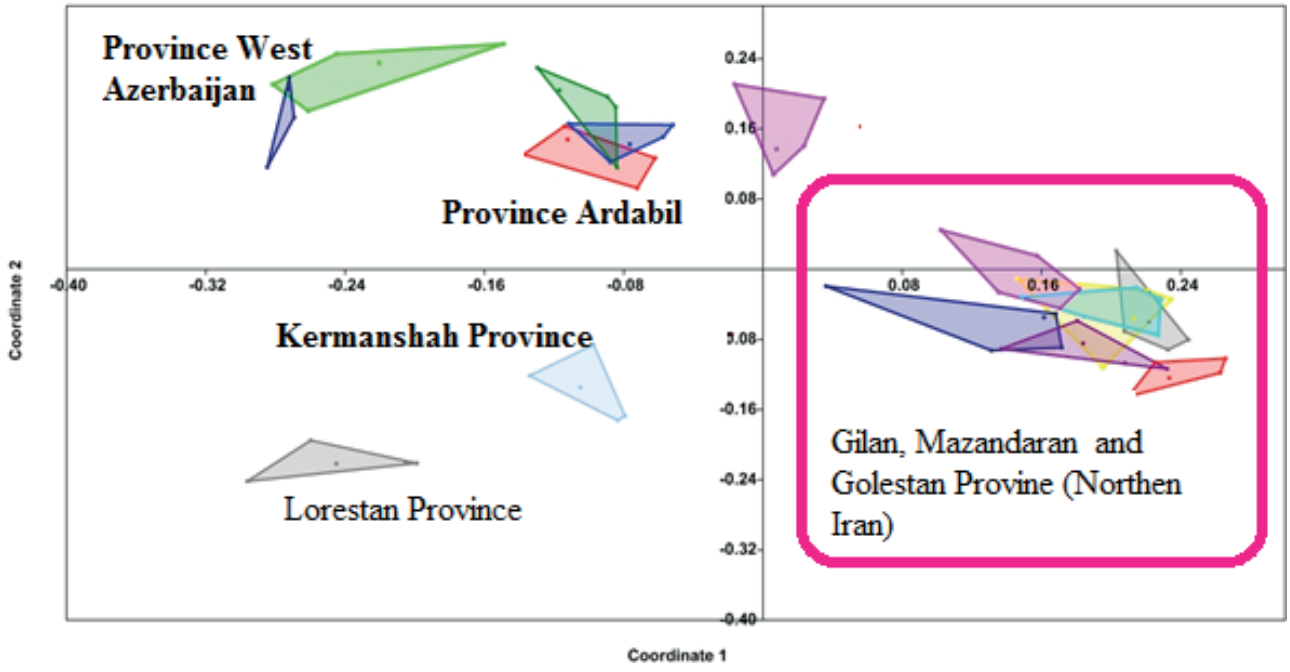

Figure 2. PCoA plot of populations in *Medicago polymorpha* based on IRAP data.

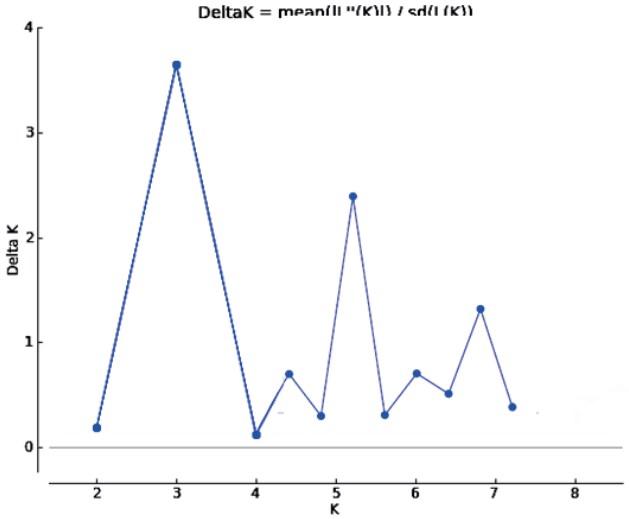

Figure 3. Evanno test of *Medicago polymorpha* populations based on k = 3 of IRAP data.

DISCUSSION

Population genetics analyses are important in genetic and breeding studies (Kizzie-Hayford et al 2021; Wasana et al 2021; Sawadogo et al., 2021; Paul et al 2021; Mieso & Befa et al 2020). They provide information on the levels of genetic variation, partitioning of genetic variability within/between populations, inbreeding or outcrossing, effective population size and population bottleneck (GHOLAMIN and KHAYATNEZHAD, 2020a; 2020b, 2020c). The advent of molecular markers has greatly improved population genetic studies. These markers have been used to identify potentially novel genotypes among the many *Medicago polymorpha* accessions. In recent years, molecular marker systems such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeat (SSR) and inter-retrotransposon amplified polymorphism (IRAP) have been used to measure genetic variation and relationships in cultivars and landraces (ren and khayatnezhad 2021; khayatnezhad and Nasehi 2021, i *et al*., 2021; jia *et al*, 2021). Transposable elements, particularly retrotransposons, comprise most of plant genomes. Their replication generates genomic diversity and makes them an excellent source of molecular markers (Smykal *et al*., 2011). The inter-retrotransposon amplified polymorphism (IRAP) method displays insertional polymorphisms by amplifying the segments of DNA between two retrotransposons. It has been used in numerous studies of genetic diversity (Smykal *et al*., 2011).

In China a population genetic study of two species, *M. lupulina* and *M. ruthenica*, reported that these types germplasm were valuable resources for improving *medicago* forage crops (Badri et al., 2011). This information has different applications, and from pure understanding of biology of the species to conservation of endangered species, choosing of proper parents for hybridization and breeding and phylogeography and mechanism of invasion. In this study, we investigated the genetic diversity of *M. polymorpha* populations. The main aim of our study was to evaluate the genetic diversity of *M. polymorpha* genotypes. To reach this objective, and to be able to detect segregating populations, we used the available inter-retrotransposon amplified polymorphism (IRAP) marker. The results of Diwan et al. (2000) study showed that SSR markers produced by *M. truncatula* are valuable genetic markers for the genus *Medicago*. These markers will be useful in establishing the genomic relationships important forage such as alfalfa and other annual medics. Among the 120 studied lines of *M. polymorpha*, there was no spineless line.

Our studies showed that the average number of 6.7 alleles per locus may be due to the high level of homozygous nature of *M. polymorpha*. Acording to Flajoulot et al. (2005) the number of allels per locus ranging were 3_24 in *Medicago sativa*. In contrast to work by Baquerizo et al. (2001) used six simple sequence repeat to analyse the genetic diversity and relationships between individuals of *Medicago truncatula,* showed to be highly diverse with an average of 25 alleles per locus. As a result, our studies emphasize that genetic variation has

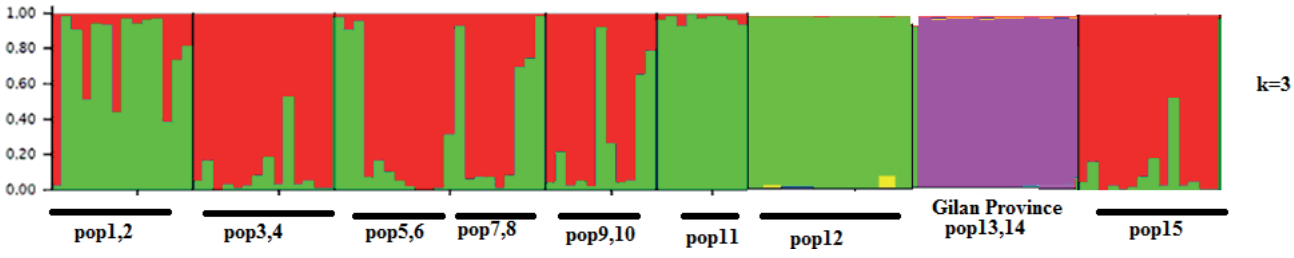

Figure 4. STRUCTURE plot of *Medicago polymorpha* populations based on k = 3 of IRAP data. (Population numbers are according to Table 1).

been effective in determining population relationships and the AMOVA results was level of among populations diversity (66%) is higher than within populations diversity (34%). The markers used in this study were highly effective in detecting the level of genetic diversity in the polymorphic and studied populations. Also the Ardabil, Khalkhal-Asalem Road population was high gene diversity and high polymorphism percentage. Min et al. (2017) investigated the extensive development of genes with micro-RNA-based SSR markers in *M. trunculata*. The mean value of information content of their polymorphisms was 0.71, indicating a high level of information. In other study the average of polymorphism information was 78.75% in *M. trunculata* and a total of 24 alleles were amplified with an average of 3 alleles per locus (Jafari et al. 2013). Also in other study reported informations polymorphism by SSR marckers indicating a high level of polymorphism (> 70%) for *M. trunculata* for *M. trunculata* and other annual medics (Diwan et al. 2000).

Genetic diversity is of fundamental importance to the survival of a species (sun and khayatnezhad 2021; tao *et al*, 2021; wang *et al*, 2021; xu *et al*., 2021; yin *et al*., 2021; zhang *et al,* 2021). Degree of genetic variability within a species is highly correlated with its reproductive mode, the higher degree of open pollination/cross breeding generally producing higher levels of genetic variability. According to Hamrick and Godt (2012) species that have selfing or mixed mating systems have lower levels of genetic variability then predominantly outcrossed species and 51% of their total genetic diversity is apportioned between populations in comparison to 10% for outcrossed species. Our study indicated a low level of heterozygosity (He = 0.01-0.32) in *M. polymorpha*. The substantially higher selfing rate in *M. polymorpha* likely contributed to a lower overall level of estimated heterozygosity. Like this our study a low level of He reported 0.246 in *M. lupulina* (Badri et al., 2011). The our study degree average of selfing rate (18.78) levels outcrossing (-10.78). The mean Nm = 0.654 was obtained for investigated IRAP loci, which indicates low amount of gene flow among the populations and supports genetic stratification as indicated by STRUCTURE analyses. By examining the biological results, it can be observed that the smaller the genetic distance between populations, they are more similar to each other, because of the shape of the seed of the species studied, it is light and easy to move and propagated by wind and other factors. This confers diversity, which results in AMOVA analysis showing that percentages within and among populations are relative, and since *M. polymorpha* is a selfing plant and regeneration occurs within the species population, which causes. Among-population differentiation in phenotypic traits and allelic variation can be the result of drift, founder effects and local selection.

According to Badri et al. (2016), among the 120 studied lines of *M. polymorpha* that they studied, environmental variance was higher than genetic variance for most traits and consequently had a relatively low average of heritability. Also they showed that there was no significant association between population differentiation and geographical distances.

These results are consistent with previous findings showing an absence of significant correlation between geographical distance and population differentiation in annual *Medicago* species (Badri et al. 2008, 2010; Zheng, et al., 2021; Zhu *et al*, 2021) and *Brachypodium hybridum* Catalán, Joch. Müll., Hasterok & Jenkins (Neji et al. 2014).

ACKNOWLEDGMENT

The authors thank anonymous reviewers for valuable comments on an earlier draft.

REFERENCES

Abdelkefi, A., M. Boussaid, A. Biborchi, A. Haddioui, A. Salhi-Hanachi and M. Marrakchi, 1996. Genetic diversity inventory and valuation of spontaneous species belonging to *Medicago* genus in Tunisia. Cahiers Options méditerranéennes, 18: 143-150.

Aitken Y (1981). Temperate herbage grasses and legumes. In Handbook of Flowering. Halevy, CRC, Boca Raton, Florida.

Baker, H.G., *Geranium purpureum* Vill. and *G. robertianum* L. in the British flora. II: *Geranium purpureum, Watsonia*, 1955, vol. 3, pp. 160–167.

Boissier, E. 1872: Medicago in Flora Oriental is 2: 90-105.-Rep. 1975, by A. Asher & Co. B. V., Amsterdam.

Badri M, Zitoun A, Soula S, Ilahi H, Huguet T, Aouani ME (2008). Low levels of quantitative and molecular genetic differentiation among natural populations of *Medicago ciliaris* Kroch. (Fabaceae) of different Tunisian eco-geographical origin. Conserv. Genet. 9:1509-1520.

Badri M, Arraouadi S, Huguet T, Aouani ME (2010). Comparative effects of water deficit on *Medicago laciniata* and *M. truncatula* lines sampled from sympatric populations. J. Plant Breed. Crop Sci. 2:192-204

Badri M, Chardon F, Huguet T, Aouani ME (2011). Quantitative Trait Loci associated with drought tol-

erance in the model legume *Medicago truncatula*. Euphytica 181:415-428.

Badri, Najah Ben Cheikh, Asma Mahjoub and Chedly Abdelly (2016). Morpho-phenological diversity among natural populations of *Medicago polymorpha* of different Tunisian ecological areas., *Watsonia*, Vol. 15(25), pp. 1330-1338.

Bi, D., C. Dan, M. Khayatnezhad, Z. Sayyah Hashjin, Z. Y. Ma (2021): Molecular Identification And Genetic Diversity In Hypericum L.: A High Value Medicinal Plant Using Rapd Markers Markers. Genetika *53*(1): 393-405.

Cheng, X., X. Hong, M. Khayatnezhad, F. Ullah (2021): Genetic diversity and comparative study of genomic DNA extraction protocols in Tamarix L. species." Caryologia *74*(2): 131-139.

Evanno, G, S., Regnaut, J., Goudet (2005): Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol., *14*:2611-2620.

Gholamin, R. M. Khayatnezhad (2020a): Assessment of the Correlation between Chlorophyll Content and Drought Resistance in Corn Cultivars (Zea Mays). Helix 10(05): 93-97.

Gholamin, R. M. Khayatnezhad (2020b): The effect of dry season stretch on Chlorophyll Content and RWC of Wheat Genotypes (Triticum Durum L.). Bioscience Biotechnology Research Communications *13*(4): 1833-1829.

Gholamin, R. M. Khayatnezhad (2020c): Study of Bread Wheat Genotype Physiological and Biochemical Responses to Drought Stress. Helix 10(05): 87-92.

Guo, L.-N., C. She, D.-B. Kong, S.-L. Yan, Y.-P. Xu, M. Khayatnezhad F. Gholinia (2020). Prediction of the effects of climate change on hydroelectric generation, electricity demand, and emissions of greenhouse gases under climatic scenarios and optimized ANN model. Energy Reports 7: 5431-5445.

Hou, R., S. Li, M. Wu, G. Ren, W. Gao, M. Khayatnezhad. F. Gholinia (2019: Assessing of impact climate parameters on the gap between hydropower supply and electricity demand by RCPs scenarios and optimized ANN by the improved Pathfinder (IPF) algorithm. Energy 237: 121621

Huson, D.H., D., Bryant (2006): Application of Phylogenetic Networks in Evolutionary Studies. Mol. Biol. Evol., *23*: 254–267.

Heyn CC (1963). The Annual Species of *Medicago*. Scripta hierosolymitana Hebrew University Press Jerusalem.

Heyn, C. C. 1984: Medicago in, K. H. Rechinger (Ed.), Flora Iranica 157: 253-271. -Akadernische Druch- u. Verlagsanstalt, Graz.

Huang, D., J. Wang, M. Khayatnezhad (2020): Estimation of Actual Evapotranspiration Using Soil Moisture Balance and Remote Sensing" Iranian Journal of Science and Technology, Transactions of Civil Engineering: 1-8.

I, A., X. Mu, X. Zhao, J. Xu, M. Khayatnezhad and R. Lalehzari (2020). Developing the non-dimensional framework for water distribution formulation to evaluate sprinkler irrigation. Irrigation and Drainage.

Jia, Y., M. Khayatnezhad, S. Mehri (2020). Population differentiation and gene flow in Rrodium cicutarium: A potential medicinal plant. Genetika 52(3): 1127-1144.

Jost, L. (2008): GST and its relatives do not measure differentiation. Mol. Ecol., *17*: 4015–4026.

Keivani, M. S. Sanaz Ramezanpour, H. Soltanloo, R. Choukan, M. Naghavi and M. Ranjbar, "Genetic diversity assessment of alfalfa (Medicago sativa L.) populations using AFLP markers," Australian Journal of Crop Science," vol. 4, no. 7, pp. 491-497, 2010.

Karasakal, A., M. Khayatnezhad, R. Gholamin (2020a). The Durum Wheat Gene Sequence Response Assessment of Triticum durum for Dehydration Situations Utilizing Different Indicators of Water Deficiency. Bioscience Biotechnology Research Communications 13(4): 2050-2057.

Karasakal, A., M. Khayatnezhad, R. Gholamin (2020b): The Effect of Saline, Drought, and Presowing Salt Stress on Nitrate Reductase Activity in Varieties of Eleusine coracana (Gaertn). Bioscience Biotechnology Research Communications 13(4): 2087-2091.

Khatamsaz, M. (1995): *Caprifoliaceae*. In: Assadi, M. & al. (eds), Flora of Iran, no. 13. Tehran.

Khayatnezhad, M. R. Gholamin (2020): Study of Durum Wheat Genotypes' Response to Drought Stress Conditions. Helix, *10*(05): 98-103.

Khayatnezhad, M. And R. Gholamin (2021a): The Effect of Drought Stress on the Superoxide Dismutase and Chlorophyll Content in Durum Wheat Genotypes. Advancements in Life Sciences, *8*(2): 119-123.

Khayatnezhad, M. And R. Gholamin (2021b): Impacts of Drought Stress on Corn Cultivars (Zea mays L.) At the Germination Stage. Bioscience Research 18(1): 409-414.

Khayatnezhad, M. F. Nasehi (2021): Industrial Pesticides and a Methods Assessment for the Reduction of Associated Risks: A Review." Advancements in Life Sciences 8(2).

Kizzie-Hayford, N., Ampofo-Asiama, J., Zahn, S., Jaros, D., Rohm, H. (2021). Enriching Tiger Nut Milk with Sodium Caseinate and Xanthan Gum Improves the Physical Stability and Consumer Acceptability. Journal of Food Technology Research, 8(2), 40–49.

Ma, S., M. Khayatnezhad, A. A. Minaeifar (2021): Genetic diversity and relationships among Hypericum L. species by ISSR Markers: A high value medicinal plant from Northern of Iran. Caryologia, *74*(1): 97-107.

Mieso, B., Befa, A. 2020. Physical Characteristics of the Essential Oil Extracted from Released and Improved Lemongrass Varieties, Palmarosa and Citronella Grass. Agriculture and Food Sciences Research, 7(1), 65–68.

Moussavi M. 1977: A help to Identification of *Medicago* species in Iran. -Ministry of Agriculture, Tehran. [In Persian].

Neji M, Geuna F, Taamalli W, Ibrahim Y, Smida M, Badri M, Abdelly C, Gandour M (2014). Morpho-phenological diversity among Tunisian natural populations of *Brachypodium hybridum*. J Agric. Sci. 153(6):1006-1016.

Parsa, A. 1948: Medicago in Flora de l'Iran 2: 171- 181. - Publication du Ministere de r Education, Museum l'Histoire Naturelle de Tehran, Tehran.

Paul, S., Ara, R., Ahmad, M.R., Hajong, P., Paul, G., Kobir, M.S., Rahman, M.H. (2021). Effect of Blanching Time and Drying Method on Quality of Black Pepper (Piper nigrum). Journal of Food Technology Research, 8(1), 18–25.

Peng, X., M. Khayatnezhad, L. Ghezeljehmeidan (2021): Rapd profiling in detecting genetic variation in stellaria l. (caryophyllaceae). Genetika-Belgrade, *53*(1): 349-362.

Peakall, R., P.E., Smouse (2006): GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes, *6*: 288–295.

Podani, J. (2000): Introduction to the Exploration of Multivariate Data. Backhuyes, Leiden, pp.407.

Pritchard, J.K., M., Stephens, P., Donnelly (2000): Inference of population structure using multilocus genotype Data. Genetics, *155*: 945–959.

Ren, J. M. Khayatnezhad (2021): Evaluating the stormwater management model to improve urban water allocation system in drought conditions. Water Supply.

Sawadogo, Y.A., Cisse, H., Oumarou, Z., Nikiema, F., Traore, Y., Savadogo, A. (2021). Reduction of Aflatoxins and Microorganisms in the Koura-Koura Produced in Burkina Faso with Spices and Aromatic Leaves. Journal of Food Technology Research, 8(1), 9–17.

Si, X., L., Gao, Y. Song, M, Khayatnezhad, A.a. Minaeifar (2020): Understanding population differentiation using geographical, morphological and genetic characterization in *Erodium cicunium*. Indian J. Genet., *80*(4): 459-467.

Sun, Q., D. Lin, M., Khayatnezhad, M. Taghavi (2020): Investigation of phosphoric acid fuel cell, linear Fresnel solar reflector and Organic Rankine Cycle polygeneration energy system in different climatic conditions. Process Safety and Environmental Protection, *147*: 993-1008.

Sun, X. And M. Khayatnezhad (2019): Fuzzy-probabilistic modeling the flood characteristics using bivariate frequency analysis and α-cut decomposition. Water Supply.

Small E (2010). Alfalfa and relatives: Evolution and classification of *Medicago*. NRC Research Press Ottawa Ontario Canada.

Salhi Hannachi, A., M. Boussaid and M. Marrakchi, 1998. Genetic variability organisation and gene flow in natural populations of *Medicago polymorpha* L. prospected in Tunisia. Genetics Selection Evolution, 30(Suppl. 1): S121-S135.

Steele K.P., Ickert-Bond S.M., Zarre S. and Wojciechowski M.F. (2010) Phylogeny and character evolution in Medicago (Leguminosae): evidence from analyses of plastic TRNK/MATK and nuclear GA3OX1 sequences. American Journal of Botany 97(7): 1142–1155.

Smykal, P., N., Bacova-Kerteszova, R., Kalendar, J., Corander, A.H., Schulman, M., Pavelek (2011): Genetic diversity of cultivated flax (*Linum usitatissimum* L.) germplasm assessed by retrotransposon-based markers. TAG, *122*: 1385–1397.

Tao, Z., Z., Cui, J., Yu, M., Khayatnezhad (2021): Finite Difference Modelings of Groundwater Flow for Constructing Artificial Recharge Structures. Iranian J. Sci. Techn., Transactions of Civil Engineering.

Wang, C., Y. Shang, M. Khayatnezhad (2021): Fuzzy Stress-based Modeling for Probabilistic Irrigation Planning Using Copula-NSPSO. Water Resources Management.

Wasana, W.L.N., Ariyawansha, R., Basnayake, B. 2021. Development of an Effective Biocatalyzed Organic Fertilizer Derived from Gliricidia Sepium Stem Biochar. Current Research in Agricultural Sciences, 8(1), 11–30.

Xu, Y.-P., P. Ouyang, S.-M., Xing, L.-Y., Qi, M., Khayatnezhad, H., Jafari (2020): Optimal structure design of a PV/FC HRES using amended Water Strider Algorithm. Energy Reports, *7*: 2057-2067.

Yin, J., M. Khayatnezhad, A. Shakoor (2020): Evaluation of genetic diversity in Geranium (*Geraniaceae*) using rapd marker. Genetika, *53*(1): 363-378.

Zhang, H., M. Khayatnezhad, A. Davarpanah (2019): Experimental investigation on the application of carbon dioxide adsorption for a shale reservoir. Energy Science & Engineering n/a(n/a).

Zheng, R., S. Zhao, M. Khayatnezhad, S, Afzal Shah (2020): Comparative study and genetic diversity in

Salvia (Lamiaceae) using RAPD Molecular Markers. Caryologia, *74*(2): 45-56.

Zhu, K., L. Liu, S. Li, B., Li, M. Khayatnezhad, A. Shakoor (2019): Morphological method and molecular marker determine genetic diversity and population structure in Allochrusa. Caryologia, *74*(2): 121-130.

Zhu, P., H. Saadati, M. Khayatnezhad (2020): Application of probability decision system and particle swarm optimization for improving soil moisture content. Water Supply.