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Genetic and morphological diversity in Iranian *Medicago polymorpha* populations by using morphological and ITS analysis

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Abstract

Concerning the increasing importance of feeding livestock with valuable fodder, the recent study was focused on characterizing the genetic and morphological diversity in bur clover (*Medicago polymorpha*) samples collected from different regions of Iran by using morphological traits and internal transcribed spacer (ITS) analysis. A total of 14 populations were collected and evaluated morphologically from three regions of Iran in June 2017. ITS-based phylogenetic analysis was carried out through maximum parsimony and Bayesian. The bur clover samples were morphologically categorized into some taxa related to the areas studied. From our results, a high level of diversity for most morphological characteristics was found among populations. Clustering analysis based on morphological information showed correlation with geographical distribution. Phylogenetic analysis based on the ITS data showed that, all accessions of *M. polymorpha* formed a monophyletic clade. The findings suggest the presence of high variation within bur clover samples accessible in different Iranian regions. The utilization of morphological traits coupled with ITS analysis found useful to track the evolution and origin of *M. polymorpha* in Iran.

Keywords: Annual medics, diversity, morphological traits, phylogenetic analysis, population

تنوع ژنتیکی و ریخت‌شناختی در جمعیت‌های ایرانی *Medicago polymorpha* با استفاده از آنالیز ریخت‌شناسی و نواحی ITS*

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خلاصه

با توجه به اهمیت روزافزون تغذیه دام با علوفه با ارزش، مطالعه اخیر در مناطق مختلف ایران، با هدف توصیف تنوع ژنتیکی و ریخت‌شناختی جمعیت‌های یونجه (*Medicago polymorpha* L.) با استفاده از صفات ریخت‌شناسی و تجزیه و تحلیل نواحی فاصله‌انداز داخلی (ITS) انجام گردید. بررسی ریخت‌شناسی ۱۴ جمعیت جمع‌آوری شده از سه منطقه کشور در خرداد ۱۳۹۶ و تجزیه و تحلیل فیلوژنتیک ماکسیمم پارسیمونی و بایسین ناحیه ITS آن‌ها انجام شد. نمونه‌های این گونه از جنبه ریخت‌شناسی به گروه‌های مختلفی دسته‌بندی شدند. طبق نتایج به دست آمده در مطالعه حاضر، میزان بالایی از تنوع در بسیاری از ویژگی‌های ریخت‌شناسی بین جمعیت‌ها مشاهده شد. تجزیه و تحلیل خوشه‌بندی براساس اطلاعات ریخت‌شناسی ارتباط نسبی بین توزیع جغرافیایی را نشان داد. تجزیه و تحلیل فیلوژنتیک براساس داده‌های آی.تی.اس. نشان داد که تمام نمونه‌های *M. polymorpha* در یک شاخه تک‌نیا تجمیع شدند. مجموع نتایج یافته‌های این مطالعه، حاکی از وجود تنوع زیاد در نمونه‌های *M. polymorpha* در مناطق مختلف ایران است. به علاوه، استفاده از صفات ریخت‌شناختی همراه با تجزیه و تحلیل آی.تی.اس. برای ردیابی منشا و تکامل گونه مذکور در ایران مفید است.

واژه‌های کلیدی: آنالیز فیلوژنتیک، تنوع، جمعیت، صفات ریخت‌شناسی، یونجه‌های یک‌ساله

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Introduction

The genus *Medicago* L. comprises 87 species originally native to the Mediterranean area (Small & Jomphe 1989, Small 2011). It belongs to *Fabaceae*, the largest Iranian family of flowering plants (Ghahremaninejad & Nejad Falatoury 2016). Bur clover (*M. polymorpha* L.) is an important forage plant (Clark 2014, Lopez *et al.* 2020), originated in the Mediterranean basin, expanded to other areas including Iran. *Medicago polymorpha* grows naturally in N, NW, W, and SW of Iran (Mehregan *et al.* 2002). This forage crop is very valuable because of its high nutritive value in animal feeding, efficiency in nitrogen fixation, and high adaptability to a variety of ecological situations (Guo *et al.* 2020). The advantages of the *Medicago* genus are not limited to animal husbandry and agriculture (Živković *et al.* 2012). These crops have considerable potential for application in molecular farming schedules for vaccine and monoclonal antibody production (Dus Santos *et al*. 2005). Moreover, their biomass containing a high level of fiber can be utilized for energy generation, like ethanol biofuel, and paper production (Adapa *et al.* 2007).

Genetic diversity is traditionally explored via analysis of morphological traits (Bolanos-Aguilar *et al*. 2000, Farshadfar & Farshadfar 2008, Touil *et al.* 2009, Rezaei *et al*. 2010a, Badri *et al.* 2016). This approach includes some restrictions like the effect of environmental factors, giving outputs that present just a fraction of total genetic variability (Falahati-Anbaran *et al.* 2007, Živković *et al.* 2012). As a complementary method, the exploring of genetic diversity and differences among and within populations by molecular analysis excludes the effect of environmental conditions (Talebi *et al.* 2011). As suggested by Veronesi *et al.* (2003) the application of DNA markers in *M. polymorpha* breeding programs could be divided into four classes: a) inbreeding and heterosis evaluation; b) marker-assisted selection and gene targeting; c) genetic linkage mapping; and d) germplasm management and characterization. To date, several molecular markers have been utilized for the above-mentioned purposes (Pupilli *et al*. 2000, Segovia-Lerma *et al.* 2003, Liu *et al.* 2007). As for other molecular markers (Zaccardelli *et al.* 2003, Bahar *et al.* 2006, Falahati-Anbaran *et al.* 2007, Rezaei *et al.* 2010b, Bayat *et al*. 2021), the applications of the internal transcribed spacer (ITS) in the breeding and genetic studies of *Medicago* genus have been recommended in the literature, especially for detecting the variability (Bena *et al*. 1998).

The internal transcribed spacer-based markers relatively swiftly evolve and can be beneficial in evaluating inter-species and sometimes intra-species relationships in plants (Alvarez & Wendel 2003). The pattern and rate of mutation in ITS sequences are generally suitable for exploring relationships among genera and species (Carvalho *et al.* 2009). Albeit many copies of ITS sequences are extent in a plant genome, they are usually homogenized through concerted evolution, an event where paralogous genes within one species are more closely associated with one another than to members of the same gene family in closely related species, therefore, ITS can be conceived as a single locus (Nalini *et al.* 2007). The rDNA gene complexes are tandem repeat units of one to several thousand copies and possess multiple domains, which evolved at different rates and thereby have varying phylogenetic efficiencies (Alvarez & Wendel 2003). This area of DNA was found to include remarkable phylogenetic data (Sharma *et al.* 2002). The ITS polymorphisms may happen at an individual, species, or genus level, making it helpful for bio-geographical diversity, evolution tracking, as well as phylogenetic research (Carvalho *et al*. 2009). To date, internal transcribed spacer-based markers have been broadly utilized in several species for phylogenetic inferences (De Bustos & Jouve 2002, Sharma *et al.* 2002).

With such a conception in mind, the current work was aimed to characterizing and classifying the genetic and morphological variability within bur clover (*M. polymorpha*) populations gathered from N, W, and SW regions of Iran, based on morphological characteristics and ITS sequences to assist bur clover breeders in the future breeding programs.

Materials and Methods

- Plant materials and morphological measurements

Fourteen natural populations of *M. polymorpha* were collected from three different regions in Iran (Table 1). These populations were sampled during July-August 2017 from ripening pods. The collected regions include three areas/ populations, viz., north, west, and southwest. The northern region including AMR, AGP, and GFM; the western region including KPA, QSR, and PVH; and the southwestern region including KHD, LPD, PLS, KHM, LPK, SHR, SPD, and SPC populations, respectively (Fig. 1). Ripe pods of each population were collected at a distance of at least 20 m from each other. Morphological characters of at least 30 pods from 10 individuals of each population were measured. Identification of *M. polymorpha* was confirmed employing different references (Boissier 1872, Parsa 1948, Small 1989, Mehregan *et al.* 2002). Morphological measurements were carried out on the mature pods (Table 2). The traits related to fruit were measured as described by Basafa & Taherian (2009). Vouchers were deposited at the herbarium of Islamic Azad University, Science and Research Branch, Tehran, Iran (IAUH; herbarium acronym follows Thiers 2021+).

Descriptive statistics like mean and deviation were calculated to compute variability for all morphological characteristics. Cluster analysis was conducted through the Ward procedure (Crochemore *et al.* 1998) and its dendrogram for evaluating the genetic relationships of samples and categorizing them according to phenotypic characteristics. Parsimony analysis was performed by using the PAUP program (Ver. 4.OB 10). Factor analysis was carried out on means of traits based on the varimax rotation procedure (Kakani *et al.* 2011) using a correlation matrix to decrease the data of samples. To decrease data dimensionality as well as achieve a better comprehending of the efficacy of morphological traits, PCA was carried out (Warburton & Smith 1993). Analysis of variance (ANOVA) and also mean comparison based on Duncan's multiple range test ($P<0.05$) were accomplished through the SPSS software (Ver. 27.0, IBM).

- Molecular evaluation

1. DNA extraction

Fresh leaves were randomly collected from five individuals from PVH, GFM, SPD, AGP, and LPD populations. The extraction of total DNA was carried out by using silica gel-dried leaf following a modified CTAB procedure of Şakiroğlu *et al.* (2010) by using Nucleospin© Plants kits (Machery-Nagel, Germany). The quality of DNA extracted, in turn, was evaluated on a 1% agarose gel. The concentration of DNA was also estimated through Nano-Drop™ 2020 (Thermo Fisher Scientific, USA) at 260 nm.

2. ITS assay

The ITS regions of the DNA were amplified via the primer pair AB102 (5' -TAG AAT TCC CCG GTT CGC TCG CCG TTA C- 3') and AB101 (5' -ACG AAT TCA TGG TCC GGT GAA GTG TTC G- 3') (Douzery *et al.* 1999, Mehregan & Assadi 2016), in a polymerase chain reaction under the following programs: an initial denaturation at 95 °C for 5 min, 30 cycles of 30 s at 95 °C, 30 s at 50 °C, 30 s at 72 °C, and eventually, the PCR was finished by final extension of 7 min at 72 °C. The complete region of the internal transcribed spacer was sequenced on a sequencer machine ABI 3730 (Applied Biosystems, United States). The resulted sequences were tracked visually and edited by suing Sequencer 4 (Gene Codes, United States), and then aligned via Mesquite Ver. 3.61, alongside additional sequences provided from the GeneBank.

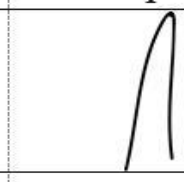
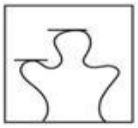
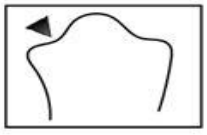
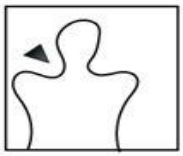
3. Molecular analysis

Medicago radiata L. was chosen as an outgroup taxon. The analysis of maximum parsimony (MP) of the ITS dataset was carried out. Then, each bootstrap value was estimated from one- hundred replicate analyses via a heuristic search approach, simple addition sequence of the taxa with the *Phylogenetic Analysis Using PAUP (PAUP*) (Swofford 2002). Bayesian analysis (BA) of the ITS dataset was performed using MrBayes Ver. 3.1.2 (Huelsenbeck & Ronquist 2001).

Table 1. Names and sampling places of the Iranian bur clover (*Medicago polymorpha*) samples

Sampling area	Location	Coordinates	Alt. (m)	Collecting code	Herbarium code	Genbank accession
North	Gilan prov.: Tonkabon, 10 km from Tonkabon to Ammarloo	49° 33.188' E; 36° 51.654' N	230	AMR	IAUH-14976	-
	Gilan prov.: Fooman, 15 km Fooman to Masooleh	49° 8.158' E; 37° 10.483' N	250	GFM	IAUH-15046	OK036668
	Ardebil prov.: Germi, 20 km from Germi to Pars-Abad	48° 5.222' E; 39° 10.859' N	380	AGP	IAUH-15029	OK036669
West	Kermanshah prov.: Paveh, Paveh Shahid Kazemi Forest Park	46° 20.396' E; 35° 1.812' N	1400	KPA	IAUH-14984	-
	Kermanshah prov.: Ghasre-Shirin	45° 34.376' E; 34° 29.661' N	360	QSR	IAUH-14953	-
	Kermanshah prov.: Ghasre-Shirin, 5 km from Paveh to Nusood	46° 20.252' E; 35° 3.777' N	1474	PVH	IAUH-14955	OK036670
Southwest	Lorestan prov.: Pol-e Dokhtar, 85 km from Pol-e Dokhtar to Khorram-Abad	47° 49.748' E; 33° 18.168' N	710	KHD	IAUH-14944	-
	Lorestan prov.: Pol-e Dokhtar, 5 Km from Darre-Shahr to Pol-e Dokhtar	47° 30.663' E; 33° 4.840' N	670	LDP	IAUH-15019	OK036671
	Lorestan prov.: Pol-e Dokhtar, 5 km from Pol-e Dokhtar to Andimeshk	47° 42.448' E; 33° 6.480' N	800	PLS	IAUH-15004	-
	Lorestan prov.: Khorram-Abad, 35 km from Khorram-Abad to Pol-e Dokhtar	47° 57.328' E; 33° 57.121' N	940	KHM	IAUH-14959	-
	Lorestan prov.: Khorram-Abad, 60 km from Pol-e Dokhtar to Khorram-Abad	48° 15.886' E; 33° 98.327' N	1370	LPK	IAUH-14983	-
	Lorestan prov.: Khorram-Abad, Shoorab	48° 10.286' E; 33° 27.407' N	1100	SHR	IAUH-14982	-
	Lorestan prov.: Sepid-Dasht, 15 km from Sepid-Dasht to Khorram-Abad	48° 50.649' E; 33° 13.292' N	1280	SPC	IAUH-14969	-
	Lorestan prov.: Sepid-Dasht, 5 km from Sepid-Dasht to Khorram-Abad	48° 51.778' E; 33° 13.175' N	1300	SPD	IAUH-14968	MZ356153

Table 2. Qualitative and morphological fruit traits studied in the Iranian bur clover (*Medicago polymorpha*) samples

Trait	1	2	3
SSH			
DSP			
PGD	 0 mm	 0.1–0.2 mm	

Abbreviations: SSH: Spine shape, DSP: Dorsal to middle vein protrusion, PGD: Presence of lateral grooves.

Results

- Morphological measurements

Mean values of the quantitative, phenotypic traits were represented in Table 3. From our results, a remarkable diversity was observed for these morphological traits. The PVH, KHD, and LPD populations had the highest fruit length (FLT), while the AMR, GFM, and AGP had the lowest. The highest fruit diameter (FDM) was recorded in the QSR and KHD populations, while the lowest in the GFM and AGP. Except for the AMR, GFM, KPA, and AGP populations, most populations had a similar number of middle coils (NMC), however, the LPD and PLS populations had the highest level. The KPA, KHD, and LPK populations had the highest adpression of fruit (FAP), while the GFM population had the lowest. Trait MCT or middle coil thickness was maximum in the KHD, LPD, SPC, and SPD populations and minimum in the GFM. Seed length (SLT) was the highest in the SPD population and lowest in the GFM. Seed width (SWT) trait appeared highest in the QSR, LPD, and SPD populations and lowest in the GFM. The number of seeds on the middle coil (SDN) was found the highest in the KPA, KHD, and LPD populations. The results from other morphological traits were presented in Table 3.

Our results indicated that the simple and glandular hairs are mostly absent on the fruit of most of the studied samples. The shape of the fruit spine was straight to slightly curved (Table 4). In the AMR, GFM, KPA, and PVH populations, the third type of fruit spine (curved) was observed; in the AGP, QSR, LPD, LPK, SHR, and SPC populations, the second type (slightly curved); and in the KHD, PLS, KHM, and SPD, the first type (straight) (Tables 2 & 4). In most populations (11 out of 14; ca. 86%), the groove between the dorsal and submarginal veins were obvious (type B), while only two populations KPA and LPK showed no visible groove (Tables 2 & 4). The PGD (presence of lateral grooves) trait showed the second type in most populations (ca. 93%); only the LPK population indicated mainly the first type (Tables 2 & 4).

Factor analysis was performed to examine the similarity between individuals in the populations. The main purpose of this analysis is to summarize the data and variables in a limited number of factors. Table 5 shows the two main factors resulting from this procedure. Based on this analysis, the traits such as fruit length and width, NMC, adpression of fruit, middle coil thickness (MCT), Spine base thickness, seed length and width, number of seeds in the middle coil (SDN), and the total number of seeds (STN) in the first component as well as the length of the longest spine (LSL) in the second component were known as distinguishing traits of different populations.

Table 3. Descriptive statistics of the quantitative and morphologic traits in bur clover (*Medicago polymorpha*) samples collected from N, W, and SW of Iran

Population	FLT	FDM	NMC	FAP	MCT	LSL	SBT	SMC	ASP	SLT	SWT	SDN	STN
AMR	1.98±0.08b	5.3±0.07bc	1.71±0.05b	0.58±0.03bc	0.91±0.05b	1.42±0.04ef	0.13±0.01a	**34.2±0.81c**	4.04±0.16bc	2.46±0.06b	1.43±0.04bc	1.78±0.09bc	3.02±0.14cde
GFM	1.34±0.062a	4.5±0.10a	1.37±0.05a	0.39±0.04a	**0.68±0.05a**	1.14±0.06bcd	0.14±0.01b	**33.74±1.12bc**	3.61±0.25b	2.09±0.072a	1.26±0.03a	1.82±0.11bc	2.04±0.07a
AGP	1.95±0.074b	4.56±0.10a	1.88±0.05c	0.47±0.03ab	1.11±0.05bcd	0.99±0.04bc	0.14±0.01b	31.12±1.08abc	2.55±0.35a	2.46±0.038b	1.41±0.03b	1.5±0.11a	2.15±0.17a
KPA	2.55±0.12cd	5.20±0.12bc	1.89±0.05bc	**0.9±0.04f**	0.98±0.04bc	1.46±0.05ef	0.16±0.01bcd	28.56±0.90a	4.38±0.16bc	2.61±0.11bcd	1.57±0.05defg	**2±0.09cd**	3±0.21cd
QSR	2.43±0.10c	**6.02±0.16d**	2.21±0.05d	0.68±0.04cd	1.11±0.07cde	**1.85±0.09g**	0.18±0.01bcde	32.03±0.97abc	3.91±0.32bc	2.91±0.05efg	**1.69±0.03g**	1.56±0.12a	2.6±0.23bc
PVH	**3.01±0.09ef**	5.35±0.07bc	2.12±0.07d	0.84±0.05e	1.17±0.05def	1.28±0.07de	0.15±0.01bc	29.66±0.85a	4.89±0.29c	2.87±0.05efg	1.53±0.02cdef	1.96±0.13bcd	3.27±0.22de
KHD	**3.09±0.12f**	**5.81±0.10d**	2.23±0.07d	**0.96±0.06f**	**1.32±0.08g**	1.45±0.06ef	0.15±0.01bcd	31.4±0.89abc	**10.16±0.69f**	2.82±0.05def	1.59±0.02efg	**2.23±0.12d**	3.56±0.18e
LPD	**3.09±0.12f**	5.51±0.13c	**2.51±0.05e**	0.78±0.05de	**1.38±0.05g**	1.50±0.10f	**0.29±0.01g**	31.97±0.76abc	3.53±0.21bc	2.94±0.05fg	**1.67±0.02g**	**2.63±0.15e**	**4.06±0.14f**
PLS	2.5±0.11cd	5.18±0.08b	**2.52±0.07e**	0.69±0.042cd	1.01±0.05bc	0.93±0.06a	0.21±0.02f	30.72±0.75ab	7.44±0.87de	2.52±0.063bc	1.45±0.03bc	1.83±0.13bc	3.16±0.18de
KHM	2.57±0.07cd	5.15±0.07b	2.16±0.04d	0.63±0.04cd	1.23±0.04efg	0.99±0.05bc	0.18±0.01cde	31.56±0.93abc	3.58±0.34bc	2.74±0.04de	1.46±0.03bcd	1.84±0.08bc	3.13±0.14cde
LPK	2.64±0.09cd	5.32±0.09bc	2.1±0.05d	**0.93±0.04f**	1.24±0.04efg	1.06±0.05bc	0.15±0.01bc	**33.2±1.05bc**	6.56±0.48d	2.67±0.07cd	1.5±0.04bcde	1.76±0.11bc	3.33±0.12de
SHR	2.77±0.07de	5.35±0.07bc	2.27±0.05d	0.68±0.05cd	1.1±0.03cde	1.16±0.04cd	0.16±0.01bcd	**33.35±0.81bc**	4.93±0.43c	2.88±0.05efg	1.55±0.02cdef	1.9±0.09bcd	3.43±0.17de
SPC	2.72±0.07cd	5.26±0.06bc	2.21±0.04d	0.72±0.05cde	**1.3±0.04g**	0.94±0.05a	0.19±0.01def	**33.9±0.84c**	8.52±0.68e	2.77±0.06def	1.59±0.04efg	1.78±0.08bc	2.88±0.13cd
SPD	2.62±0.06cd	5.44±0.05bc	2.18±0.05d	0.73±0.04de	**1.37±0.04g**	0.93±0.05a	0.21±0.01ef	32.34±0.89abc	3.71±0.33b	**3.02±0.04g**	**1.63±0.03fg**	1.90±0.11bcd	2.90±0.13cd

Abbreviations: FLT: Fruit length, FDM: Fruit diameter, NMC: Number of coils, FAP: Adpression of fruit, MCT: Middle coil thickness, LSL: Length of the longest spine, SBT: Spine base thickness, SMC: Number of spine on middle coil, ASP: Angle of spine insertion, SLT: Seed length, SWT: Seed width, SDN: Number of seeds on middle coil, STN: Total number of seeds.
Populations: AMR and GFM: Gilan, AGP: Ardebil, KPA, QSR, and PVH: Kermanshah, KHD, LPD, PLS, KHM, LPK, SHR, SPC, and SPD: Lorestan.

Table 4. The qualitative and morphologic traits in bur clover (*Medicago polymorpha*) samples collected from N, W, and SW of Iran

Pop	SSH	DSP	PGD (mm)
AMR	Curved	B (more present)	B (0.1–0.2)
GFM	Curved	B (more present)	B (0.1–0.2)
AGP	Slightly	B (more present)	B (0.1–0.2)
KPA	Curved	A (present)	B (0.1–0.2)
QSR	Slightly	B (more present)	B (0.1–0.2)
PVH	Curved	B (more present)	B (0.1–0.2)
KHD	Straight	B (more present)	B (0.1–0.2)
LPD	Slightly	B (more present)	B (0.1–0.2)
PLS	Straight	B (more present)	B (0.1–0.2)
KHM	Straight	B (more present)	B (0.1–0.2)
LPK	Slightly	A (present)	A (0)
SHR	Slightly	B (more present)	B (0.1–0.2)
SPC	Slightly	B (more present)	B (0.1–0.2)
SPD	Straight	B (more present)	B (0.1–0.2)

Abbreviations: SSH: Spine shape, DSP: Dorsal to middle vein protrusion, PGD: Presence of lateral grooves, Pop: Population, AMR and GFM: Gilan, AGP: Ardebil, KPA, QSR, and PVH: Kermanshah, KHD, LPD, PLS, KHM, LPK, SHR, SPC, and SPD: Lorestan.

Table 5. Factor analysis of the quantitative and morphologic traits in bur clover (*Medicago polymorpha*) samples collected from N, W, and SW of Iran

Trait	Component	
	1	2
Fruit length (mm)	**0.961	-0.066
Fruit diameter (mm)	**0.805	0.493
Number of coils	**0.843	-0.280
Adepression of fruit (mm)	**0.792	0.194
Middle coil thickness (mm)	**0.850	-0.240
Length of the longest spine (mm)	0.293	**0.801
Spine base thickness (mm)	**0.603	-0.458
Number of spines on middle coil	-0.296	-0.105
The angle of spine insertion (°)	0.415	-0.036
Seed length (mm)	**0.884	.086
Seed width (mm)	**0.879	0.273
Number of seeds in middle coil	**0.616	-0.270
Total number of seeds	**0.843	-0.203
Total	6.985	1.480
Variance	53.732	11.386
Cumulative	53.732	65.118

**Eigen values is significant at the ≥0.50 level.

Clustering analysis of characters of fruits was performed using the Ward method. PCA (Principal Components Analysis) biplot was used to identify the most variable morphological characters among the studied populations (Podani 2000). Measures carried out are summarized as two varimax-rotated principal components in figures 1 & 2. According to the dendrogram obtained from the cluster analysis (Fig. 1), in phenoline 25, the studied species were divided into two main groups A and B. The first major group (A), in phenoline 8, was further divided into two groups. Group a, in phenoline 7, in turn, was divided into two separate subgroups. The first subgroup consisted of four populations KPA, PVH, KHD, and QSR that are more similar to each other. The second subgroup included five populations KHM, SPD, LPK, SHR, SPC, and PLS. Group b consisted of only one population, i.e., LPD. Finally, the second major group (B), in phenomena 3, consists of three populations AMR, AGP, and GFM, which were more similar to each other. According to the results derived from principal component analysis, the bur clover samples can be considered into four groups: i) SPC, SPD, KHM, SHR, LPK, and PLS; ii) LPD; iii) KHD, PVH, KPA, and QSR; and iv) AGP, GFM, and AMR (Fig. 2). As a result, the same observations were achieved by using both the clustering method and principal component analysis, showing their potential for classifying the bur clover accessions.

- Molecular evaluation

Herein, the phylogenetic relationships of various populations of *M. polymorpha* were explored according to the Bayesian analysis (BA) of the internal transcribed spacer marker (Fig. 3). The species of *M. radiata* was in the tree as an out-group. Posterior probabilities (PP) are indicated by numbers above each clade. Bootstrap supports for this clade retrieved in the Maximum Parsimony (MP) assessment were exhibited by the numbers low each clade. The data matrix consisted of 58 taxa and 455 characters. Of the 338 constant positions, 54 were parsimony uninformative and the remaining 63 were parsimony informative. Based on the results of strict consensus phylogenetic tree obtained from the total number of the shortest trees, 10,000 trees with the following characteristics: Length: 228 steps, Stability Index (CI): 0.601, Index (RI): 0.779. The resulting conclusive consensus tree has the following characteristics: Length: 285 steps, Stability Index (CI): 0.663, Index (RI): 0.860. Bootstrap analysis was performed by fast stepwise method with 10,000 replications to obtain the estimated support of the branches in the phylogenetic tree. From the achieved phylogenetic tree, species of the cladogram were predominantly categorized into two clades: clade A. a firmly-supported clade encompassing all samples of *M. polymorpha* with posterior probability (PP) = 0.9 and bootstrap support (BS) = 59%, and clade B. another well-supported clade encompassing a polytomy of 24 species with different bootstraps.

Medicago polymorpha was found as a sister to the rest of the bur clover species. All of the five samples of *M. polymorpha* together with another sample of *M. polymorpha*-AF028353 (from the USA), and other three samples taken from the Genbank, formed a monophyletic clade. All five samples are taken from Kermanshah, Gilan, Lorestan, and Ardebil provinces (Iran). Our observations revealed that, bur clover accessions from the studied areas belong to one species. Phylogeny relationships are essentially resolved within this clade.

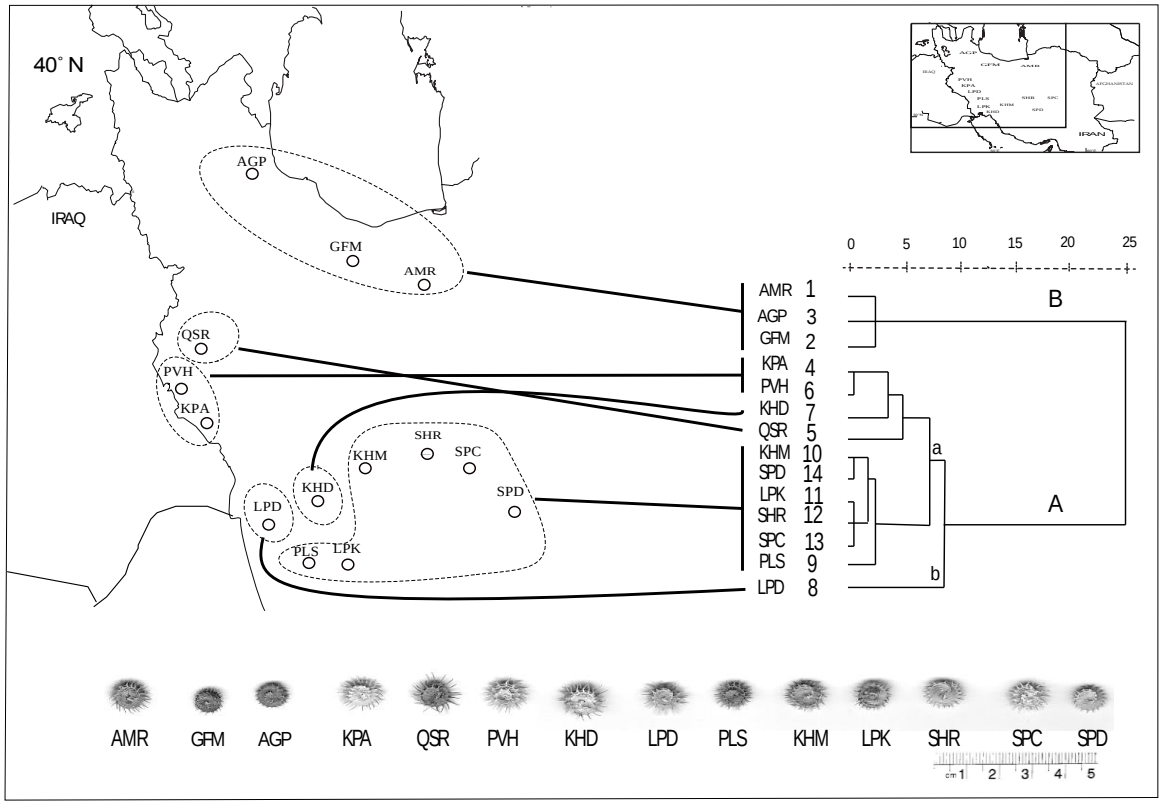


Fig. 1. Geographical distribution with Clustering analysis of morphological traits of *Medicago polymorpha* samples collected from N, W, and SW of Iran (Populations: AMR and GFM: Gilan, AGP: Ardebil, KPA, QSR, and PVH: Kermanshah, KHD, LPD, PLS, KHM, LPK, SHR, SPC, and SPD: Lorestan).

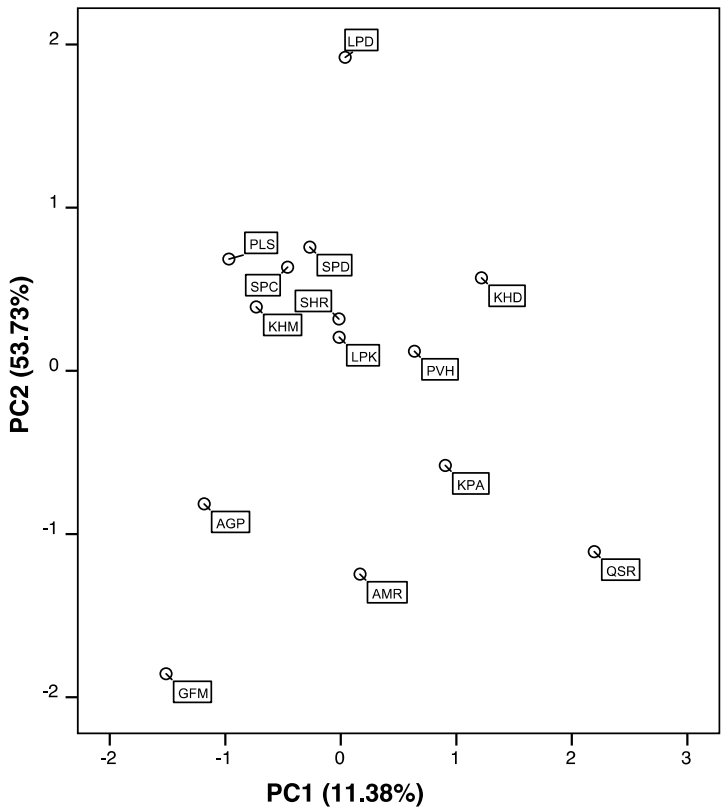


Fig. 2. Principal component analysis of morphological traits of *Medicago polymorpha* samples collected from N, W, and SW of Iran (Populations: AMR and GFM: Gilan, AGP: Ardebil, KPA, QSR, and PVH: Kermanshah, KHD, LPD, PLS, KHM, LPK, SHR, SPC, and SPD: Lorestan).

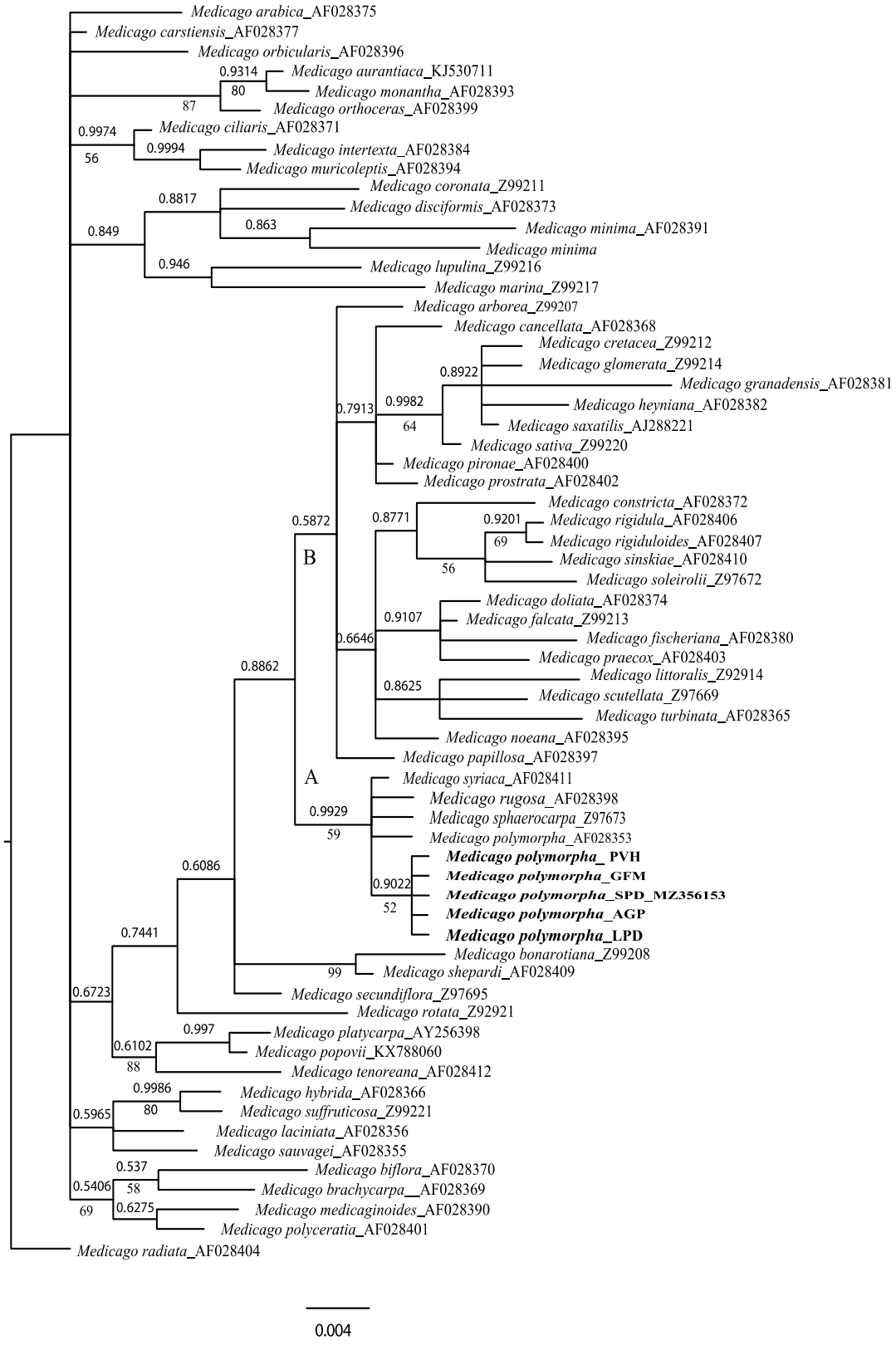


Fig. 3. Phylogenetic tree obtained from the maximum parsimony analysis of the internal transcribed spacer (ITS) region of different species of *Medicago polymorpha* (total characters = 455, constants = 338, parsimony informatives = 63, Model of evolution = GTR+I+G, A-C constitution rate = 0.7770, A-G constitution rate = 2.1783, A-T constitution rate = 1.0977, C-G constitution rate = 0.6219, C-T constitution rate = 5.0243, G-T constitution rate = 1.0000. Gamma distribution rate = 0.5820). Numbers above clades are posterior probabilities. Numbers below clades are the percentages of bootstrap supports (100 replicates) for those clades retrieved in the maximum parsimony analyses (bootstrap supports less than 50% are not shown).

Discussion

From our results, a high level of diversity for most morphological characteristics was present among populations. However, based on the findings of the morphological assessment alone, it is usually beyond the bounds of possibility to differentiate between each pair of accessions owing to an overlapping variation. The cause for this uncertainty is the presence of highly variable characteristics in *M. polymorpha*. High variability of morphological traits in *M. sativa* L. has been reported previously (Abbasi *et al.* 2006, Abbasi *et al*. 2007, Basafa & Taherian 2009) as an outcome of tetraploid nature and cross-pollinating. In line with our observations, Živkovića *et al.* (2012) demonstrated that morphology analysis-based clustering has a relative fit with the geographical origin of alfalfa accessions. As observed in our work, Smith *et al*. (1991), and Warburton & Smith (1993) assayed some accessions from the Middle East and India by PCA and clustering method and eventually demonstrated that, both analytical procedures were in line with each other.

Compared to the highly conserved rRNA sequences, their spacers like internal transcribed spacers (ITS) represent high variation and a more rapid rate of evolution (Carvalho *et al.* 2009). A high frequency of polymorphisms was observed in polymerase chain reaction-amplified product of ITS, facilitating exploration of close lineages of genetically distinguished accessions. The ITS polymorphisms and length variants have been identified for a couple of plant/crop species (Alvarez & Wendel 2003, Nalini *et al*. 2007, Saini *et al*. 2008). For instance, Carvalho *et al.* (2009) described that RFLP-ITS molecular marker provides a specific and feasible tool for genetic variation evaluation among wheat genotypes and higher taxa, like botanical lines, and validated its efficiency for the phylogeny approximation, as shown from our results. Ghanavati (2011) also revealed that, high phylogenetic relationships between different species of *Medicago* by using ITS analysis. These studies suggested a high potential of ITS polymorphisms for genetic variation evaluation among *Medicago* species.

From ITS analysis, the measure of relative genetic distance among bur clover populations did not correlate well with the geographical distance of their sampling locations. For example, research on *Medicago* plants has been demonstrated a low association between genetic distances via DNA sequences and geographic distance. For instance, Bena (2001) observed that, molecular phylogeny supports the morphological evaluation of *Medicago* plants; however they showed a relatively low association between geographical distribution and genetic distances calculated via ITS analysis. Using micro-satellite markers, Emami-Tabatabaei *et al*. (2021), however, showed a clear correlation between genetic distances and geographic distributions in *M. polymorpha*. As observed in the current study, morphological data and genetic markers revealed appreciable homology. The morphology-based clustering of genotypes possesses the best fit with the geographical origins of plant accessions. Therefore, morphological evaluation provides a rapid tool for discriminating genotypes.

Živković *et al.* (2012) demonstrated that, calculating the genetic distances via morphological markers exhibited a poor fit of distance over that of molecular markers. However, Disagreement on the relationship among *M. polymorpha* accessions (in the recent work), and other plants/crops recorded via molecular marker and morphological analysis has been elucidated previously (Greene *et al*. 2004, Tucak *et al.* 2008). Živkovića *et al*. (2012) supposed that, although this association between molecular markers and morphological data observes for *M. polymorpha*, it is not a usual formula for other plants. One caveat for this observation is that, marker-based analysis of the complicated genome in *M. polymorpha* may not encompass sufficient marker loci and, thus, merely incompletely reflect actual genetic associations among *M. polymorpha* genotypes of interest. *Medicago polymorpha* accessions possess a broad genetic

background (Julier *et al.* 2000), which is apparent from the phenotypic diversity in the recent work. As a result, a low association between morphological properties and DNA markers is expected.

We reported a broad genetic and morphological diversity in *M. polymorpha* accessions that can present potential in its use in a variety of geographical and climatic environments. Such a high level of variability can be beneficial in the choice of favorable phenotypic characteristics in bur clover domestication and breeding. All of the accounts, the knowledge of phylogeny and genetic relationship in this study can contribute to the modeling of specific crosses between *M. polymorpha* accessions with potential interest in breeding programs.

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