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جمعیت‌های *Medicago minima* (L.) Bartal در ایران: تنوع مورفولوژیکی بسیار و غیر مرتبط با توالی ناحیهٔ ITS و پراکندگی جغرافیایی

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چکیده

تاکنون به گونهٔ *Medicago minima* (L.) Bartal. ازلحاظ کشاورزی توجه کمی شده است؛ با این حال این گونه صفات ارزشمندی در مقایسه با سایر گونه‌های یک‌سالهٔ یونجه دارد و بررسی میزان تنوع صفات در این گونه نیازمند انجام این پژوهش بود. در این مطالعه، تنوع صفات ریخت‌شناسی نیام ۱۳ جمعیت از جنوب غرب، غرب، شمال غرب و شمال ایران همراه با تنوع توالی ناحیهٔ ITS ارزیابی شد. الگوی جغرافیایی برای تنوع صفات نیام مشاهده نشد. مطالعهٔ فیلوژنی مولکولی ناحیهٔ ITS، تنوع زیادی در این ناحیه نشان نداد و همهٔ آنها در گروهی تک‌نیا با درجهٔ حمایت زیاد قرار گرفتند؛ با این وجود ریخت‌شناسی نیام تنوع بسیاری به‌ویژه در صفات کرک‌ها با ضریب تنوع ۵۸ درصد نشان داد. این جمعیت‌ها به‌منظور استفاده در برنامه‌های زادآوری و حفاظتی، صفات مناسبی را نشان دادند: ۱. جمعیت باغچه با بیشترین تعداد دانه در نیام و زاویهٔ اتصال خار به نیام؛ ۲. جمعیت خرم‌آباد با بیشترین تعداد خار روی نیام؛ ۳. جمعیت سروآباد با بزرگ‌ترین ابعاد نیام و دانه. جمعیت‌های خرم‌آباد، باغچه، مریوان و پاوه بیشترین میزان کرک روی نیام را نشان دادند؛ اگرچه نتایج آنالیزهای همبستگی اغلب صفر یا منفی بود، میزان کرک‌های ساده و غده‌ای روی نیام ارتباط مثبتی با ارتفاع محل جمع‌آوری داشت. همبستگی واضحی بین تنوع ریخت‌شناسی و توالی ناحیهٔ ITS با الگوی پراکندگی جغرافیایی جمعیت‌های این گونه مشاهده نشد.

واژه‌های کلیدی: مورفولوژی نیام، تنوع مورفولوژیک، اثر ارتفاع.

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Populations of *Medicago minima* (L.) Bart. in Iran: High Morphological Variability Irrelevant to ITS Sequences and Geographical Proximity

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Abstract
Medicago minima (L.) Bartal. has received little agricultural development to date, while having potentially useful traits compared to other "medics", e.g., annual species of section *Spirocarpos*. However, the range of variation in *M. minima* requires further studies. This research assessed the diversity of the pod morphological traits of 13 populations in the southwest, west, northwest, and north of Iran. In addition, variation in nuclear ribosomal Internal Transcribed Spacer (nrITS, ITS 1+5.8s+ITS 2) was evaluated. No geographical patterns of variation in the pod traits were found. The molecular phylogenetic assessments of 7 populations indicated low variability, thus being grouped into a well-supported monophyletic clade (99% Bootstrap support/1.00 Posterior Probability (PP)). Nevertheless, the pod morphological traits exhibited significant variations, most notably in Glandular Hairs (GH), with a coefficient variation of 58%. For breeding and possibly conservation purposes, the following populations demonstrated potentially useful characteristics: (1) Baghcheh population with the highest number of seeds per pod (4.6) and angle of spine insertion (2.87◦); (2) Khoramabad population with a higher number of spines on the middle coil (65.27); and (3) Sarvabad population with the greatest fruit length (3.71 mm), fruit diameter (4.17 mm), Seed Length Trait (SLT) (2.37 mm), and Seed Width Trait (SWT) (1.35 mm). In qualitative traits, Khoramabad, Baghcheh, Marivan, and Paveh populations had higher scores, particularly for glandular pubescence and Simple Hairs (SH) on their pods. Results of the correlation analysis were often null or negative; however, altitude indicated a positive relationship with glandular pubescence and SH. No obvious correlations were found between pod morphology and ITS variation and geographical proximity.
Keywords: pod morphology, morphological variation, altitude effect.

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Introduction

Medicago is the Old World genus of the Fabaceae, consistings of 87 species and is best known for its perennial species complex of alfalfa (*M. sativa*) (Small and Jomphe, 1989; Mehregan *et al.*, 2002; Small, 2011). Section *Spirocarpos* makes up a group of about 30 species, which are annuals with distinctively coiled fruits. These are mostly native to the Mediterranean area and are called "Medics", but are sometimes known as world-wide weeds. About a dozen of them, including *M. minima*, (L.) Bartal., have been grown for forage. *Medicago minima* is a herb with yellow flowers and a recumbent growth habit. It is distinguishable from its relatives in section *Spirocarpos* by technical fruit characters. Like other legumes, it is valued for its nitrogen-fixing ability, which compensates for low-nitrogen soils. Agricultural studies have suggested that this species is useful as forage for sheep and as rotational green manure for grain crops (Crawford *et al.*, 1989; Ocumpaugh *et al.*, 2007). Several previous studies have addressed the morphological diversity of *M. minima*. For instance, Chebouti *et al.* (2015) surveyed the morphological characters of 5 populations from various sites in Algeria and observed a high level of diversity. In a most recent report, Kabtni *et al.* (2020) similarly expressed a significant variation among 12 Tunisian populations of *M. minima* sampled from environmentally diverse areas and identified 3 morphotypes. Nonetheless, most studies on *M. minima* have been ecological research (Busso *et al.*, 1998; Fresnillo-Fedorenko *et al.*, 2011; Martínez & Manzano-García, 2019). These reports have mostly attempted to assess phenological traits so as to identify ecological systems in the southern hemisphere, especially in Argentina and Australia (Giorgetti *et al.*, 2000; Fresnillo-Fedorenko *et al.*, 2011; Busso *et al.*, 2013). They have revealed that *M. minima* mainly occupies the brush and pasture lands of arid and semi-arid bioclimatic zones with an average annual precipitation of 50-225 mm/year. This species prefers clay or loamy-clay soils with poor phosphorus contents (Fresnillo-Fedorenko *et al*., 2011). *Medicago minima* is known for its significant tolerance against drought and cold temperature (Dölarslan et al., 2018; Woods & Orcutt, 2017), making it a notable genetic resource for developing superior pasture plants. Annual medics often exhibit considerable morphological variations, particularly in pod characteristics, such as size and shape of spines (SSH) (Bena *et al.*, 1998a,b; Mehregan *et al.*, 2002, 2003; Small, 2011). Kabtni *et al*. (2020) suggested that traits like plant shape, internode length, and blooming date could be regarded as great indicators for differentiating and classifying the varieties of *M. minima* plants.

Despite the significant contribution of morphological characters to understanding taxonomic relationships and genetic diversity at both inter- and intra-species levels, they come with limitations (Hynniewta *et al.*, 2014; Zareei *et al.*, 2020). Thus, alternate methodologies, such as DNA sequencing, RFLP, and SSR, have now been increasingly utilized to identify inter- or intra-species relationships in a more reliable manner (Valizadeh *et al.*, 1996; Falahati-Anbaran *et al.*, 2006; de Sousa *et al.*, 2016; Emami-Tabatabaei *et al.*, 2021). Still, there are numerous unresolved phylogenetic issues at either inter- or intra-species level.

Considering its fast evolution, Internal Transcribed Spacer (ITS) can be beneficially applied for resolving differences between inter-species, including Musaceae (Hřibová *et al.*, 2011), *Flemingia* L. and *Glycine* L. (Wu *et al.*, 2013), and *Citrus* L. (Hynniewta et al., 2014) and relationships between intra-species, such as *Oxalis tuberosa* Molina (Tosto & Hopp, 1996), *Commiphora wightii* (Arn.) Bahandari. (Haque *et al.*, 2009), and *Trigonella foenum-graecum* Sm. (Kakani *et al.*, 2011). Low genomic diversity of nuclear ribosomal DNA (nrDNA) arranged in tandem arrays resulting from concerted evolution has resulted in treating them as a single locus (Baldwin *et al.*, 1995). On the one hand, the stable nature of rDNA locus in the inter- and intra-species and proportionally accelerated evolutions of ITS1 and ITS2, which are exposed to less selective pressure, have turned the ITS1-5.8S-ITS2 region into one of the most used molecular markers in phylogenetic studies (Francisco-Ortega *et al.*, 2001; Alvarez & Wendel, 2003; Pettengill & Neel, 2008).

Therefore, to generate comprehensive and reliable information about the quantity of genetic variation and the relation of *M. minima* with other annual medics, a combined methodology composed of morphological and molecular phylogenetic procedures was utilized in this research.

Material and Methods

Plant material

In this research, 13 populations were collected from different areas in Iran (Fig. 1 and Table 1). Leaf samples were taken from 5 or 6 *M. minima* individuals separated by at least 20 m in each population and 67 samples were totally obtained. They were dried on silica-gel for molecular studies. Voucher samples were deposited in the Islamic Azad University Herbarium (IAUH) (Table 1). A list of 17 important morphological characters considered in the literature are presented in Tables 2 and 3 (Mehregan *et al*., 2002; Small, 2011). Additionally, the 10-year (2009-2019) means of 3 ecological variables, including altitude, annual precipitation, and average temperature in the growth seasons, were obtained from www.en.climate-data.org.

Table 1. Locality details and voucher numbers of the studied *M.minima* populations

	City code	Locality	Coordinates	Altitude	Herbarium code	Genbank accession
1	RDB	Gilan: Rudbar, Darestan	49° 25.163′ E 36° 50.03′ N	500 m	14974	MZ513909
2	MSH	Ardabil: 75 km from Aslanduz to Meshkinshahr triod to Meshkinshahr	47° 39.915′ E 39° 1.362′ N	780 m	14929	-
3	ASL	Ardabil: 20 km from Aslanduz to Khoda Afarin	47° 17.223′ E 39° 21.074′ N	220 m	15034	MZ513910
4	PVH	Kurdistan: 20 km from Marivan to Paveh	48° 14.951′ E 35° 23.402′ N	1200 m	15005	MZ513911
5	PLN	Lorestan: 10 km from Poldokhtar to Khorramabad	47° 43.433′ E 33° 48.01′ N	670 m	14931	MZ513914
6	AND	Khuzistan: 55 km from Andimeshk to Poldokhtar	48° 14.951′ E 35° 23.402′ N	310 m	14941	-
7	QRH	Ardabil: 55 km from Aslanduz to Meshkinshahr, Ardebil, Meshkinshahr	47° 39.062′ E 39° 7.803′ N	500 m	14940	-
8	SHR	Lurestan: Khorramabad, Shorabad	48° 10.286′ E 33° 27.407′ N	1100 m	14981	-
9	SRV	Kurdistan: Sarvabad, Trooper of Sarvabad to Marivan	46° 21.264′ E 35° 19.023′ N	1260 m	15011	MZ513912
10	ABD	Ilam: Abdanan towards Kabir-Kouh	49° 25.531′ E 33° 0.263′ N	1000 m	14973	MZ513913
11	BAG	Lurestan: 5 km from Poldokhtar to Andimeshk, Baghcheh	48° 14.951′ E 35° 23.402′ N	800 m	15001	-
12	KHD	Lurestan: 5 km from Khorramabad to Kohdasht, Kouhdasht	48° 15.164′ E 33° 28.917′ N	1220 m	15015	MZ513915
13	MRV	Kermanshah: 30 km from Paveh to Marivan	49° 12.799′ E 36° 08.115′ N	1180 m	15007	-

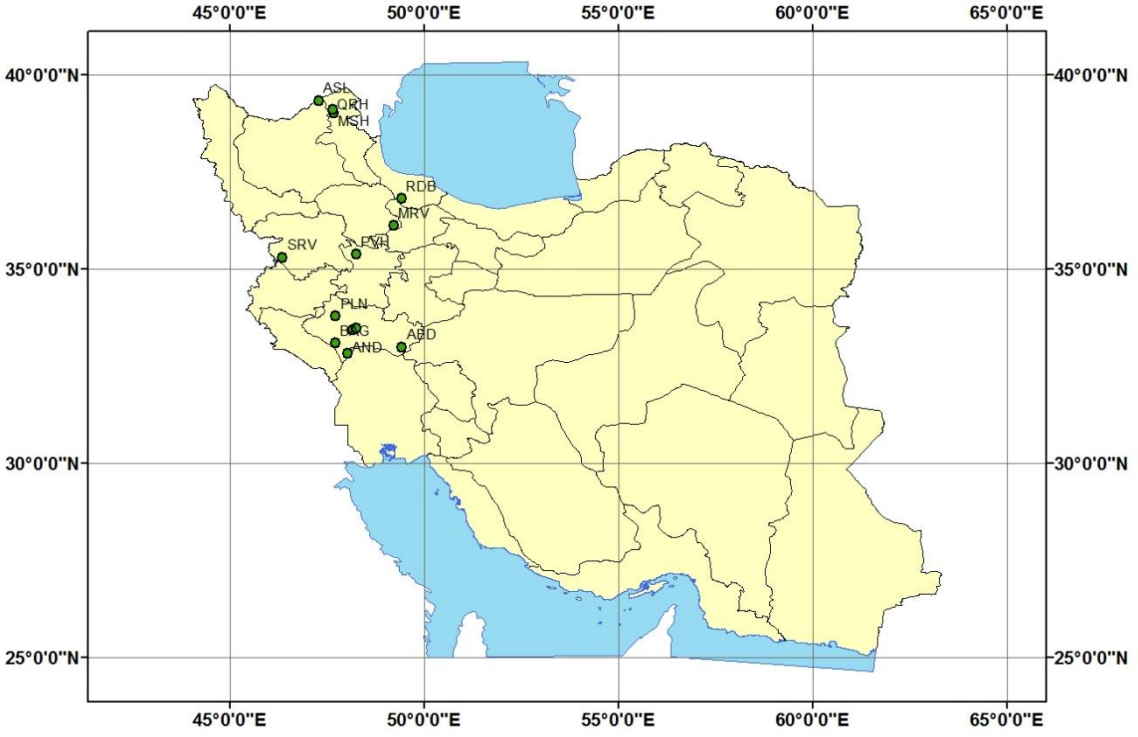

Figure 1. Map of Iran showing localities of the 13 experimental populations of M. minima collected from the study areas

Statistical analysis

The statistical analyses, including variance analysis and mean comparisons, were performed using SPSS 22 software (Green & Salkind, 2016) and Excel 2013. The results were shown as mean±Standard Error (mean±SE). The differences among the mean values obtained were determined at $P<0.05$ by using Duncan's test. Principal Components Analysis (PCA) was carried out on the morphological traits based on populational means. Custer analysis was conducted based on Ward's method by applying PAST Software ver. 4.03 with 1000 bootstrap replicates (Hammer et al., 2001). To assess the correlation between the different datasets, including quantitative and qualitative data and ecological characteristics, Pearson's correlation coefficient was determined by using the "corrplot" package in R software.

DNA extraction for PCR amplification and ITS sequencing

7 geographically well-separated populations were chosen for phylogenetic studies. Total genomic DNA was extracted from silica gel-dried leaves according to the cetyltrimethylammonium bromide (CTAB) method of Doyle & Doyle (1987) by using Nucleospin© Plant kits (Machery-Nagel, Germany) following the manufacturers' instructions. Using 1% agarose gel, the isolated DNA concentration was assessed. The ITS region of ITS1-5.8S-ITS2 of the nrDNA was amplified by utilizing the forward primer AB101 (5'- ACG AAT TCA TGG TCC GGT GAA GTG TTC G -3') and the reverse primer AB102 (5'-TAG AAT TCC CCG GTT CGC TCG CCG TTA C -3') (Douzery *et al.*, 1999; Mehregan & Kadereit, 2009). PCR was carried out in a 25-µL volume of 50 Mm KLC, 10 Mm Tris-HCl buffer at pH 8, 1.5 Mm $MgCl_2$, 0.2 Mm of each Nucleoside triphosphate, 0.2 µL of a single primer, 20 ng genomic DNA, and one unit of Taq DNA polymerase. The procedure was taken as follows: 5 min at 95ºC, 35 cycles of 30 sec at 95ºC, 30 sec at 50ºC, 90 sec at 72ºC, and final extension of 7 min at 72ºC. The PCR products were evaluated qualitatively by electrophoresis in 1% agarose gel and quantitatively through the spectrophotometry method. Sequencing of amplicons was performed on an ABI 3730 DNA Analyzer (Hitachi-Applied Biosystems, Waltham, Massachusetts, USA).

Phylogenetic analysis

The forward and reverse sequences were visually evaluated and edited and then initially assembled using Sequencer 4 software (Gene Codes Corporation, Ann Arbor, Michigan, USA). In addition to our sequences, ITS sequences of 63 accessions were obtained from the Genbank (see Fig. 3). The accession numbers are added in Fig. 3. The chosen outgroup accessions were *Medicago popovii* and *Medicago platycarpa* (Bena *et al.*, 1998). Sequences were assembled and aligned using MacClade 4 (Maddison & Maddison, 2000). Maximum Parsimony (MP) analysis was performed using PAUP* software (Swofford, 2002) with the following criteria: 100 heuristic searches, 100 replicates, and swapping method of Tree Bisection and Reconnection (TBR). The strict consensus tree was formed by combining the shortest trees recovered under MP. Bootstrap Support (BS) for each branch was calculated by using a complete heuristic search with 100 replicates and a similar setting as above

(Felsenstein, 1985). The Bayesian Analysis (BA) of the ITS dataset was performed using MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001) after assessing the best DNA substitution model (AIC; Akaike, 1974) using PAUP* and ModelTest v3.7 (Posada & Crandall, 1998). The analyses resulted in "TIMef" as the best fit model for our dataset. The Markov Chain Monte Carlo (MCMC) process was set in a way that 4 chains ran simultaneously for 5,000,000 generations. With 25% of the initial trees being discarded, the remaining trees sampled from each generation were combined into a 50% majority-rule consensus tree.

Results

Morphological traits

The descriptive statistics for the studied traits are presented in Table 2. The higher values of Coefficient of Variation (CV) were observed for Glandular Hairs (GH) (55.8%), angle of spine insertion (ASP°) (38.3), quantitative traits, and SSH (34.3%). The lowest CV was displayed by the number of coils (NMC, 8.4%).

Comparison of the means was performed by using Duncan's test to elucidate the change patterns of the quantitative and qualitative traits among the studied populations (Table 3). The pod morphology traits showed a significant variation; for instance, Fruit Length Trait (FLT) indicated a range of 2.90 mm in Meshkinshahr (MSH) to 3.71 mm in Sarvabad (SRV) or 3.70 mm in Marivan (MRV). Similarly, the highest Fruit Diameter Means (FDMs) of 4.47 and 4.32 mm were obtained for SRV and MRV, respectively. The lowest FDM (3.57 mm) was achieved for MSH population. Interestingly, the number of coils (NMC) among almost all the populations was the same (3.5), except for Andimeshk (AND), which was 4.1. The means of Fruit Adpression (FAP) were varied ranging from the highest FAPs of 0.26 and 0.25 mm in MSH and Abdanan (ABD) populations, respectively, to the lowest FAP of 0.14 mm in Aslanduz (ASL) population. On the contrary, the lowest (0.82 mm) and highest (1.16mm) values of Middle Coil Thickness (MCT) were observed for MSH and ASL, respectively. ABD and Baghcheh (BAG) populations exhibited the lowest and highest Longest Spine Lengths (LSLs) of 1.67 and 2.87 mm, respectively. The average Spine Base Thickness (SBT) did not vary considerably (0.11 mm in MSH, BAG, and ABD to 0.16 mm in ASL). The lowest and highest numbers of spines on the middle coil (SMC) were related to ASL (44.62) population and Khoramabad (KHD) (65.71) and ABD (65.27) populations, respectively. The means of angle of spine insertion (°, ASP) ranged from 11.17 in MRV to 17.74 in the north of Poldokhtar (PLN). SRV and MRV populations displayed the highest means of 2.37 and 2.34 mm for Seed Length Trait (SLT) and 1.35 and 1.34 mm for Seed Width Trait (SWT), whereas MSH exhibited their lowest means of 1.90 and 1.09 mm, respectively. The means of seed number in the middle coil (SDN) ranged from 1.79 and 1.80 in Roudbar (RDB) and AND populations to 2.06 and 2.03 in PLN and BAG populations, respectively.

Table 2. Descriptive statistics of the studied traits among the 13 populations of *M. minima*

Traits	**FLT**	**FDM**	**NMC**	**FAP**	**MCT**	**LSL**	**SBT**	**SMC**	**ASP**	**SLT**	**SWT**	**SDN**	**STN**	**SH**	**GH**	**FESH**	**SSH**
Min	2.02	2.60	2.5	0.05	0.6	0.70	0.06	28	4.00	1.18	0.76	1.00	1.00	1	0	1.00	1.00
Max	5.08	5.34	5.5	0.44	1.7	3.99	0.28	80	38.00	2.82	1.73	4.00	8.00	4	3	5.00	4.00
Mean Statistic	3.4198	4.0558	3.543	0.2132	0.973	2.1299	0.1255	59.65	14.6888	2.1715	1.2316	1.9237	3.8508	3.32	0.94	2.7117	2.6020
Mean Std.Error	0.02636	0.02335	0.0150	0.00363	0.0078	0.02915	0.00151	0.474	0.28425	0.01271	0.00837	0.2054	0.6359	0.043	0.046	0.03758	0.04511
Variance Statistic	0.272	0.214	0.088	0.005	0.024	0.333	0.001	88.183	31.673	0.062	0.027	0.160	1.545	0.727	0.845	0.554	0.798
Stand. dev	0.52196	0.46222	0.2960	0.07191	0.1549	0.57720	0.02989	9.391	5.62785	0.24846	0.16366	0.40048	1.24282	0.852	0.919	0.74399	0.89317
Skewnessn statistic	0.330	0.006	5.402	0.395	0.751	0.262	1.630	-0.992	0.856	-0.208	0.100	0.127	-0.226	-0.704	0.285	0.447	0.112
Skewness Std.Error	0.123	0.123	0.123	0.123	0.123	0.123	0.123	0.123	0.123	0.125	0.125	0.125	0.125	0.123	0.123	0.123	0.123
Kurtosis Statistic	0.282	0.157	33.895	-0.139	1.339	0.242	4.262	1.484	1.272	0.407	-0.232	7.168	0.208	-1.118	-1.418	-0.337	-0.831
Kurtosis Std.	0.246	0.246	0.246	0.246	0.246	0.246	0.246	0.246	0.246	0.249	0.249	0.250	0.249	0.246	0.246	0.246	0.246
CV%	15.3	11.4	8.4	33.7	15.9	27.1	23.8	15.7	38.3	11.4	13.3	20.8	32.3	25.7	55.8	27.4	34.3

Length of fruits: FLT, Fruit diameter: FDM, Number of coils: NMC, Adpression of fruits: FAP, Thickness of middle coil : MCT, Length of longest spine: LSL, Thickness of Spine base: SBT, Number of spines on middle coil: SMC, Angle of spine insertion: ASP, Length of seeds: SLT, Width of seeds: SWT, Number of seeds in middle coil: SDN, Number of seeds: STN, simple hairs: SH, glandular hairs: GH, Shape of ends of fruits (larger number means more convex): FESH, Shape of spines (larger number means more curved): SSH. To provide visual guidance, in each column per trait, conditional coloring was used indicating the lowest means as 'red' and highest means as 'green' in each trait among the populations.

Table 3. Means of the quantitative and qualitative traits measured from 67 individuals of the 13 populations of *M. minima*

Code	FLT	FDM	NMC	FAP	MCT	LSL	SBT	SMC	ASP	SLT	SWT	SDN	STN	PSH	PGH	FESH	SSH
ASL	3.44 ±0.37^{b,c}	3.74 ±0.36^{a,b}	3.5 ±0^{a}	0.14 ±0.05^{a}	1.16 ±0.20^{f}	1.84 ±0.40^{a-c}	0.16± 0.05^{d}	44.62 ±12.85^{a}	16.69 ±4.78^{d,e}	2.11 ±0.30^{a}	1.20 ±0.16^{a,b}	1.97± 0.48^{b-e}	3.90 ±1.19^{b-e}	2	1	3	3
SRV	3.71 ±0.52^{c}	4.47 ±0.47^{f}	3.5 ±0^{a}	0.19 ±0.08^{b}	1.08 ±0.16^{e}	2.45 ±0.45^{d}	0.14± 0.02^{b,c}	57.18 ±10.88^{c,d}	15.79 ±4.52^{c-e}	2.37 ±0.22^{d}	1.35 ±0.13^{a,b}	1.97± 0.30^{b-e}	4.03 ±0.90^{b-e}	3	1	3	4
RDB	3.36 ±0.56^{b}	4.05 ±0.50^{c,d}	3.5 ±0^{a}	0.24 ±0.08^{d}	0.96 ±0.13^{c,d}	2.00 ±0.44^{c}	0.12± 0.02^{a}	60.29 ±7.53^{b-d}	16.24 ±6.01^{d,e}	2.05 ±0.26^{b}	1.19 ±0.22^{a}	1.79± 0.41^{a-c}	3.5± 1.46^{a-c}	3	2	3	4
SHR	3.54 ±0.48^{b,c}	4.20 ±0.29^{d,e}	3.5 ±0^{a}	0.22 ±0.08^{b-d}	0.98 ±0.13^{c,d}	1.94 ±0.57^{b,c}	0.12± 0.03^{a,b}	64.15 ±7.23^{d-f}	16.63 ±7.51^{d,e}	2.21 ±0.17^{c}	1.27 ±0.13^{a}	1.81± 0.483^{a-c}	3.56 ±1.19^{a-c}	4	1	2	2
QRH	3.24 ±0.46^{b}	3.78 ±0.43^{a,b}	3.5 ±0^{a}	0.20 ±0.06^{b,c}	1.01 ±0.14^{d}	1.71 ±0.45^{a,b}	0.123 ±0.020^{a,b}	60.07 ±5.57^{b-d}	17.53 ±7.25^{-e}	1.90 ±0.21^{a}	1.12 ±0.19^{a,b}	1.92± 0.58^{a,b}	3.32 ±1.28^{a,b}	2	1	2	2
PLN	3.44 ±0.48^{b,c}	4.05 ±0.49^{c,d}	3.5 ±0^{a}	0.19 ±0.05^{b}	1.02 ±0.15^{d,e}	2.08 ±0.68^{c}	0.12± 0.02^{a}	59.03 ±7.83^{c,b}	17.74 ±6.92^{e}	2.18 ±0.19^{c}	1.23 ±0.12^{b}	2.06± 0.44^{b-e}	3.90 ±0.98^{b-e}	4	5	2	2
ABD	3.27 ±0.25^{b}	4.03 ±0.34^{c,d}	3.5 ±0^{a}	0.25 ±0.07^{d}	0.95 ±0.10^{b-d}	1.67 ±0.51^{a}	0.11± 0.02^{a}	65.27 ±6.27^{e,f}	13.03 ±3.77^{a-c}	2.33 ±0.17^{d}	1.33 ±0.10^{a,b}	1.89± 0.42^{a}	3.17 ±1.10^{a}	4	5	2	2
AND	3.53 ±0.82^{b,c}	3.88 ±0.40^{b,c}	4.1 ±0.94^{b}	0.22 ±0.08^{b-d}	0.92 ±0.13^{b,c}	1.96 ±0.39^{b,c}	0.15± 0.05^{c,d}	56±8.204^{b}	14.67 ±5.05^{b-e}	2.20 ±0.17^{c}	1.19 ±0.12^{a}	1.8±0.41^{c-e}	4.17 ±1.34^{c-e}	2	5	2	2
BAG	3.36 ±0.40^{b}	3.92 ±0.26^{b,c}	3.5 ±0^{a}	0.19 ±0.07^{b}	0.87 ±0.11^{a,b}	2.87 ±0.56^{e}	0.11± 0.02^{a}	61.2± 5.47^{c-e}	14.57 ±3.88^{b-d}	2.10 ±0.17^{b,c}	1.14 ±0.15^{a,b}	2.03± 0.32^{e}	4.6± 1.24^{e}	4	5	2	2
PVH	3.51 ±0.47^{b,c}	4.29 ±0.37^{e,f}	3.5 ±0^{a}	0.24 ±0.06^{d}	0.94 ±0.10^{b-d}	2.44 ±0.37^{d}	0.12± 0.02^{a,b}	58.97 ±6.13^{c,d}	12.1± 3.517^{a,b}	2.17 ±0.14^{c}	1.23 ±0.11^{a,b}	1.93± 0.37^{d,e}	4.33 ±1.12^{d,e}	4	1	3	4
MRV	3.70 ±0.41^{c}	4.32 ±0.36^{e,f}	3.5 ±0^{a}	0.23 ±0.06^{c,d}	0.95 ±0.08^{b-d}	2.36 7±0.41^{d}	0.12± 0.02^{a}	64.27 ±6.30^{d-f}	11.17 ±5.11^{a}	2.34 ±0.16^{d}	1.34 ±0.18^{a,b}	1.97± 0.18^{c-e}	4.13 ±1.19^{c-e}	4	5	2	3
KHD	3.26 ±0.51^{b}	4.19 ±0.46^{d,e}	3.5 ±0^{a}	0.20 ±0.04^{b,c}	0.92 ±0.13^{b,c}	2.44 ±0.29^{d}	0.12± 0.01^{a}	65.71 ±5.43^{f}	12.06 ±3.73^{a,b}	2.18 ±0.28 9^{c}	1.24 ±0.13^{a,b}	1.94± 0.24^{a-d}	3.69 ±1.16^{a-d}	4	4	2	3
MSH	2.90 ±0.46^{a}	3.57 ±0.36^{a}	3.5 ±0^{a}	0.26 ±0.08^{d}	0.82 ±0.08^{a}	1.70 ±0.44^{a,b}	0.11± 0.02^{a}	59.16 ±7.13^{c,d}	11.68 ±3.76^{a,b}	1.90 ±0.16^{a}	1.09 ±0.08^{a,b}	1.88± 0.49^{a-c}	3.53 ±1.37^{a-c}	4	3	2, 3	2
Mean	3.42 ±0.52	4.06 ±0.46	3.54 ±0.30	0.21 ±0.07	0.97 ±0.15	2.13 ±0.58	0.13± 0.03	59.65 ±9.39	14.69 ±5.63	2.17 ±0.25	1.23 ±0.16	1.92± 0.40	3.85 ±1.24				

Mean values with the same letters indicate homogeneous subsets for P≤0.05 according to the Duncan's test. Means in the same columns with different superscripts differ at P≤0.05. See Tables 1 and 2 for trait populations and abbreviations, respectively. To provide visual guidance, conditional coloring in 'red' and 'green' was used to indicate the lowest and highest means for the traits among the populations in each column, respectively.

BAG and ABD populations exhibited the highest (4.6) and lowest (3.17) numbers of seeds (STNs), respectively. Variations were also recorded among the qualitative traits. Only 3 populations (Qareh Aghaj: QRH, ASL, and AND) showed a score of 2 (1-5) for SH, while the rest displayed a score of 4 (11<), except for SRV and RDB, which got a score of 3 (6-10). In terms of GH, the 6 populations of PLN, ABD, AND, BAG, PVH, and KHD received a score of 5 (1-5) and KHD, RDB, and the rest obtained the scores of 3, 2, and 1, respectively. The fruit end shapes (FESH) of ASL, SRV, RDB, and PVH populations were found to have more convex ends. For SSH, the 3 populations of SRV, RDB, and PVH and 2 populations of ASL and MRV were scored as 4 and 3, respectively (Table 3).

Cluster analysis and PCA based on morphological traits

Cluster analysis based on Ward's method and PCA revealed almost a similar grouping pattern based on morphological information among the 13 populations of M. minima (Figs. 2 and 3) with the cophenetic index of 86%, indicating a strong correlation between similarity matrices and the dendrogram-driven cluster. In both methods, the populations were divided into 2 main groups. In the cluster analysis, the west and southwest populations were placed in a larger cluster, while the west populations (SRV, MRV, and PVH) were separately grouped. The southwest populations (ABD, AND, KHD, and PLN) were placed in another major branch of this cluster. Additionally, those from north and northwest (MSH, QRH, and RDB) were grouped in another group, except for ASL that was outgrouped. In PCA, the populations were grouped in 2 groups mainly with a strong geographical affinity (Fig. 3). The northwest and west populations were entered majorly on the lower side of the plot, while the rest of the west and southwest populations were entered on the upper side of the plot. The studied populations were successfully grouped based on their vegetative traits and according to their geographical distances to a large extent through Cluster analysis and PCA.

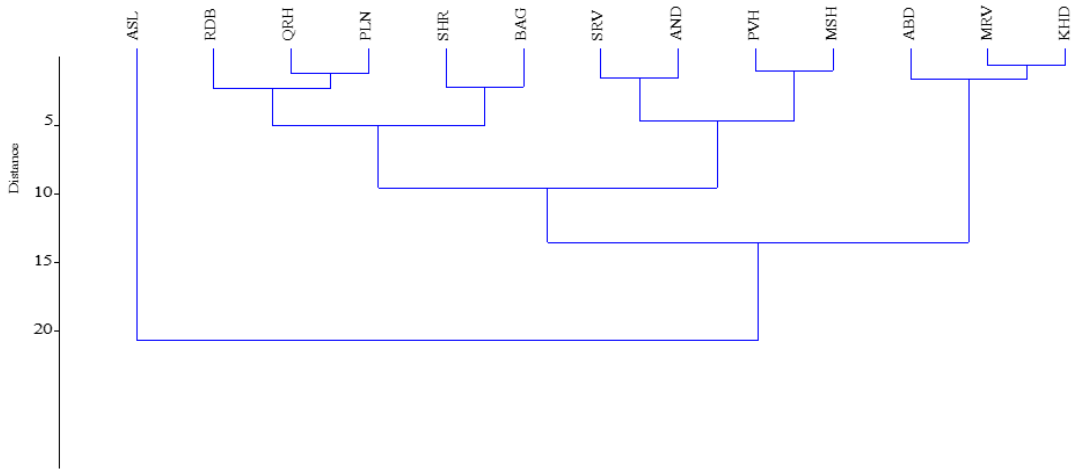

Figure 2. Cluster analysis based on morphological data using ward's method

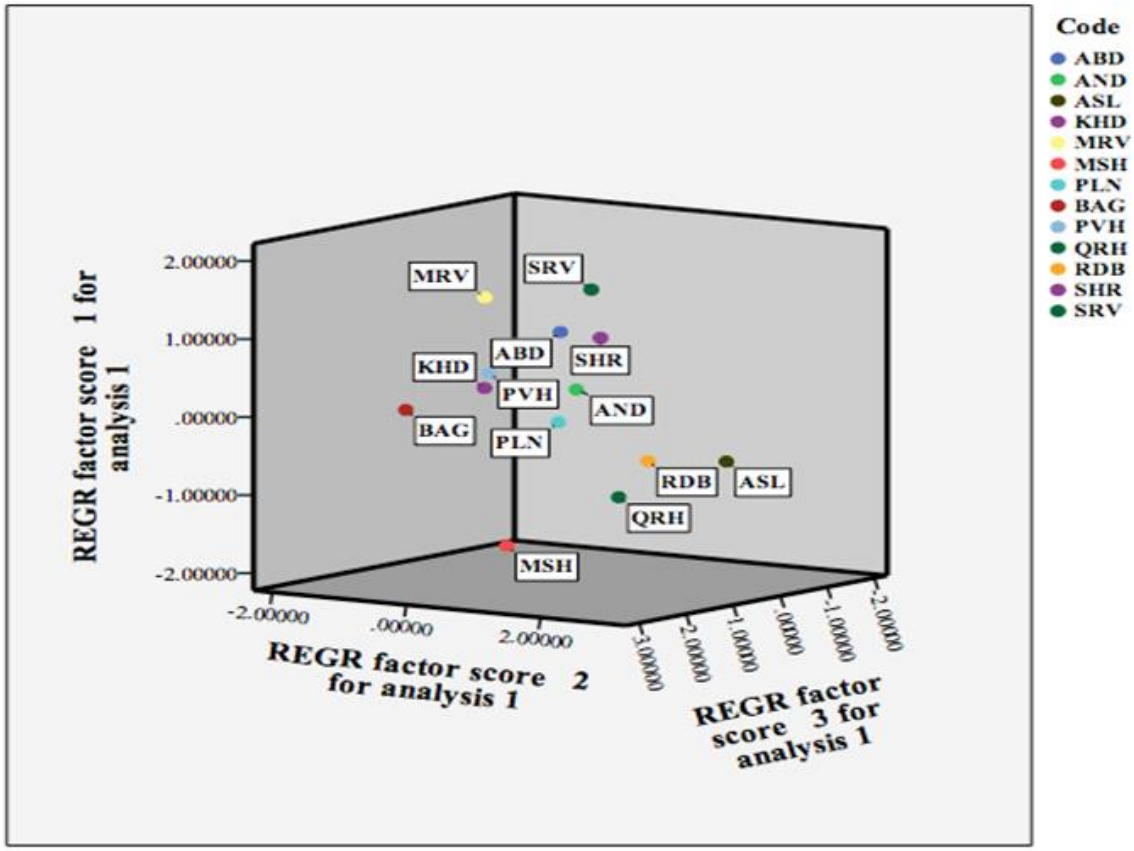

Figure 3. The 3-dimensional PCA of the morphological data obtained from the 13 Iranian populations of *M. minima*

Correlation analysis

Evaluation of the relationships between the morphological traits and ecological variables revealed null or negative correlations for many of the relationships (Fig. 4). However, in some cases, reasonable correlations were found, among which significant positive correlations between altitude and FDM, SMC, and SLT and SWT were witnessed (Fig. 4). The significant correlations between altitude and SH and GH were notable. Furthermore, the morphological responses to precipitation seemed substantial in a few cases, such as SSH, FLT, and FDM. Also, a weak correlation between temperature and number of seeds (STN) was observed (Fig. 4). Noticeably, a strong positive correlation between quantitative vegetative characters was witnessed. FLM, FDM, or SLT, and SWT with FLT and FDM were the cases in point.

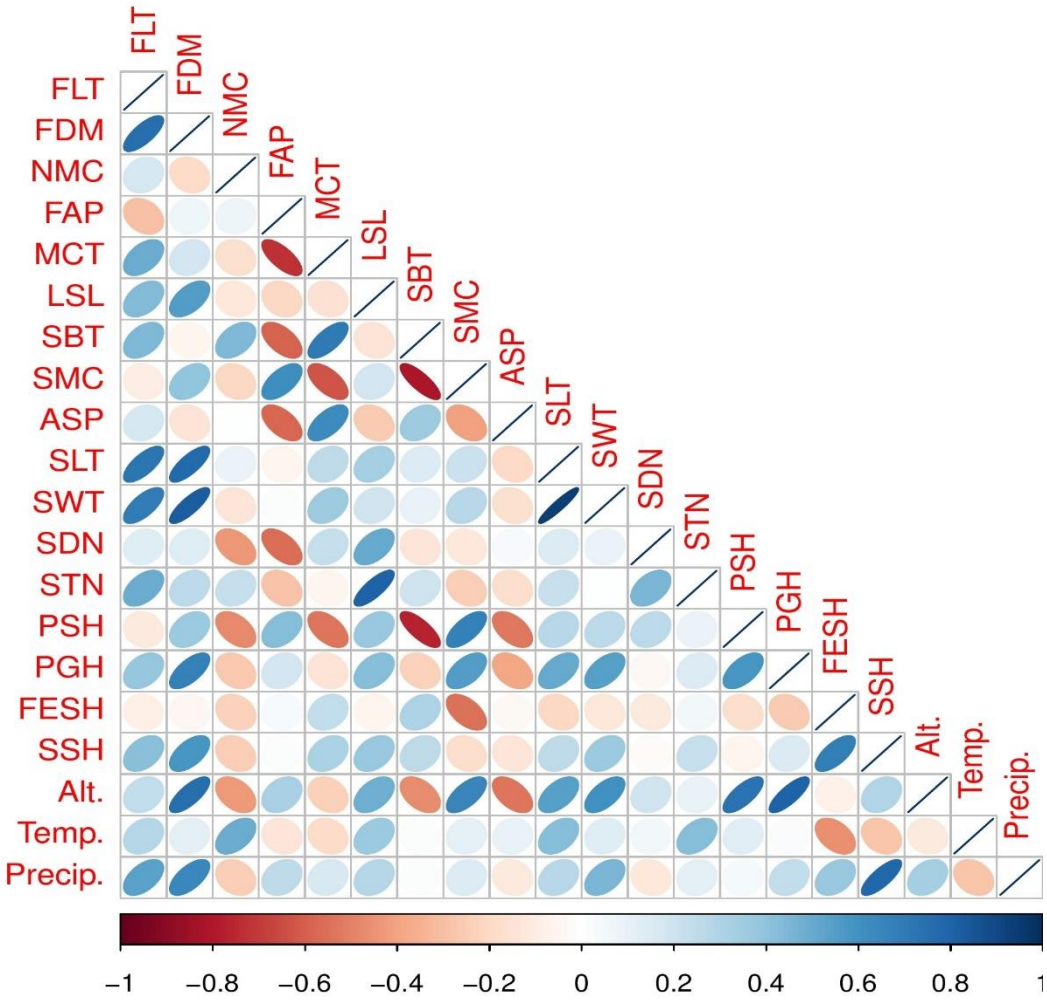

Figure 4. Pearson's correlation coefficients between ecological variables and morphological traits obtained from the 13 populations of *M. minima.*

Phylogenetic study

The aligned ITS dataset (ITS1+ITS2) included 476 characters, of which 65 (13.66%) and 74 (15.54%) variables were parsimony-uninformative and parsimony-informative characters, respectively. A strict consensus tree of the shortest trees (length=285 steps, Consistency Index (CI)=0.663, and Retention Index (RI)=0.859) was constructed (length=285 steps, CI=0.664, and RI=0.821) (Fig. 5). The generated sequences of 7 accessions of *M. minima* were grouped in a strongly supported monophyletic clade (PP=1.00, BS=99%) (Fig. 5) with 2 subsequent subclades of KHD and SRV (PS=62%) and ASD, PVH, PLN, RBD, and ABD (BS=63%).

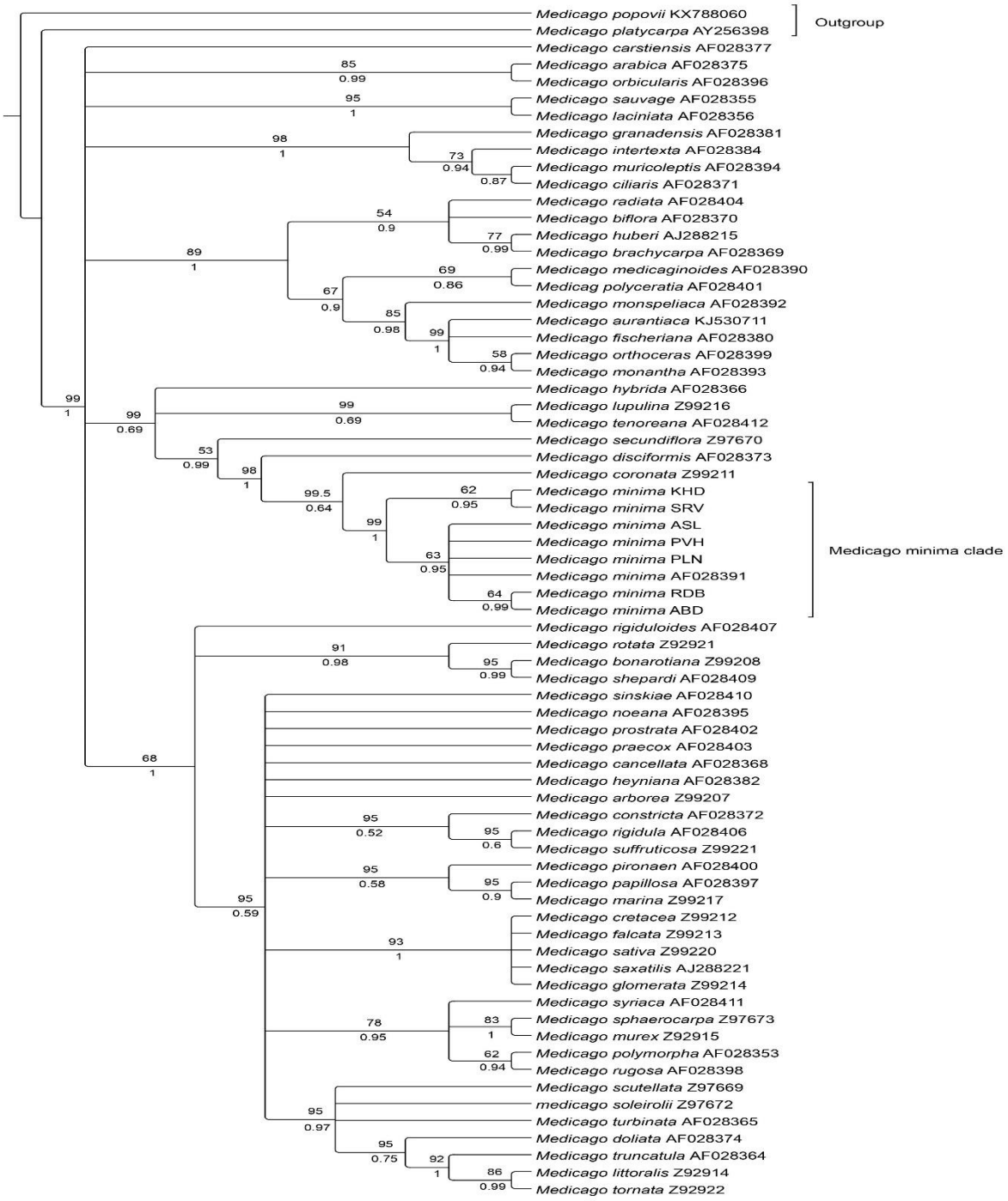

Figure 5. Taxonomic position of *M. minima* in a phylogenetic tree based on Bayesian analysis of ITS DNA sequences. The subclade bracket shows *M. minima* clade. The numbers below and above the branches are Posterior Probabilities (PPs) and bootstrap support percentages, respectively. The accession numbers of those samples taken from the Genbank are shown after each taxon name.

Discussion

Morphological diversity

A notable level of variation in pod morphology among *M. minima* populations was observed in this study. Although a firm pattern was lacking among the means of the traits, it seemed that the west populations (MRV, SRV, and PVH) had a higher average of the traits (Table 2). Significant morphological variations have been also reported in several studies on Moroccan *M. truncatula* populations (Hansali *et al.*, 2007; Haddioui *et al.*, 2012). Chebouti *et al*. (2015) observed a high variability in pod and seed traits among 5 *M. minima* populations. They reported an average of 5.16 seeds per pod, which was higher than our result (3.85) from the 13 populations. Additionally, seed size was slightly smaller in their research. SLT and SWT in this study were 2.17 and 1.23 mm vs. 2.23 and 1.24 mm in the study of Chebouti *et al*. (2015), respectively. Bullitta *et al.* (1994) and Graziano *et al.* (2010) recoded their analogous results from Sardinian populations of *Medicago polymorpha* L. The massive and usually inter-population variations among medic species, particularly in pod morphology (e.g., size and shape of spines on the pods), have attracted many scholars' attention (Bena *et al*., 1998; Small, 2011). This unique feature confers a significant option to determine the self-reseeding potential (Graziano *et al*., 2010). Additionally, variation in pod morphology at the population level is critical for making breeding attempts and developing cultivars with high self-reseeding capability (Bullitta *et al*., 1994).

Identifying morphological markers for natural populations is a cost-effective approach (Nadeem *et al.*, 2018) for studying genetic diversity and conservation and following plant breeding purposes (Ao *et al.*, 2013; Karaköy *et al.*, 2014). Recently, Kabtni *et al*. (2020) has utilized morphological traits to study Tunisian populations of *M. minima*. In consistent with our results, they revealed a significant variation among their studied populations in addition to confirming the effectiveness of pod morphology in morphological investigations of annual medics. However, similar to Chebouti *et al*. (2015), the average of important traits like number of seeds per pod was higher (5.5) in Tunisian populations of *M. minima* (Kabtni *et al*., 2020) compared to our result (3.8). These findings were also observed in some studies on *Medicago* gender, such as Tunisian *Medicago ciliaris* Luce. populations (Jabri *et al.*, 2016), Algerian *M. minima* populations (Chebouti *et al*., 2015), and Italian *M. polymorpha* populations (Graziano *et al*., 2010).

Interestingly enough, here, we saw a positive correlation between altitude and SH and GH. The presence of hairs in medics is often associated with a defense strategy that protects plants and pods, particularly against pests (Shade *et al.*, 1975). Following this idea, Small (1985) showed that these hairs might be few, but are still capable of playing the role of a mechanical barrier against small insects as he found the mummified remnants of aphids caught up in the sticky hairs. Additionally, Shade and Kitch (1983) evaluated the importance of glandular trichomes providing a protection mechanism for the pea aphid. The presence and density of GH have been reported to be affected by growth conditions, i.e., greenhouse vs. field condition, in annual medics (Othman *et al.*, 1981). Furthermore, these authors recorded the highest density of GH on pods in *Medicago scutellata* Mill. collected from Turkey. Glandular trichomes hold an important breeding trait as they have been considered key for developing resistant crop cultivars to herbivores or sub-sucking pests (Glas *et*

al., 2012). *M. scutellata* is probably the most studied annual medics regarding GH. There has been some strong evidence of erect GH in this species serving as a defense system against alfalfa weevil *Hyperapostica* Gyll. (Shade *et al*., 1975; Johnson *et al.*, 1980). Although the presence of SH and GH is linked to pest resistance in *M. minima*, no research on this species as a host for alfalfa weevil has been reported. Yet, strong correlations of trichomes with elevations of *Acanthophyllum squarrosum* Boiss.(Chen *et al.*, 2014), *Lippia origanoides* Kunth (Tozin *et al.*, 2015), and *Herpetospermum pedunculosum* (Ser.) Baill. populations (Zhao *et al.*, 2019) are well proven; however, contradictory instances exist as well. As a case in point, Kabtni *et al*. (2020) indicated the lack of fluctuation in the hardiness of Tunisian populations of *M. minima*, possibly due to the locations of the majority of the populations at low elevations. Such flexibility in SH and GH can be also taken as a response to winds. Although various factors, such as light, temperature, altitude, and availability of nutrients can be effective in plant hardiness (Cui *et al.*, 2018; Pérez-Estrada *et al.*, 2000), the correlations related to temperature in this research were insignificant, which indicated the need for more comprehensive studies on these populations by considering all of the climatic variables. Expectedly, FLT and FDM positively correlated with annual precipitation as higher soil moisture means availability of a vital source of water for producing larger fruits. Consistent with our study, Kabtni *et al*. (2020) recorded that greater pod diameters and seed lengths in sites correlated with relatively higher annual precipitation.

They were distinguished by dendrogram pattern of populations according to the geographical location via PCA and ward's method. Considering that morphological traits in natural populations may not be as stable as evidenced by molecular data, having such a relatively clear-cut grouping through both methods could suggest the existence of a sizeable genetic background supporting morphological diversity. In an investigation conducted by Kabtni *et al*. (2020), wild populations of *M. minima* grown under greenhouse conditions still exhibited high morphological polymorphism in addition to relatively discriminating the populations into three phenotypes close to the origins. Similar results were documented by studying Moroccan populations of *M. truncatula* (Haddioui *et al*., 2012). The ability of pod morphology in discriminating *M. ciliaris* populations was reported by Cheima *et al.* (2012). The classification approaches used in this research provided distinct grouping patterns, possibly because the clustering procedure taken through Ward's method inclines to outline clusters, which were visually consistent to areas of highly dense points in the PCA ordination diagram (Murtagh & Legendre, 2014)**.**

Phylogenetic study

Unlike their highly variable pod morphology, *M. minima* populations exhibited low variability in nuclear ribosomal DNA ITS (nrITS). The 7 populations analyzed in this work were placed in a well-supported cluster (99% Bootstrap), confirming the species identity of these populations (Fig. 3). However, subsequent grouping within this clade seemed not to be related to the geographical distributions of the populations. Although pod morphology traits have been widely utilized in taxonomic studies of annual medics, the similarities or vitalities of these traits sometimes bring studies to a halt. Thus, nrITS are capable of giving an insight into the taxonomic situation of

species. Application of nrITS has successfully provided taxonomic resolution, which is congruent with the results of this study. By employing nrITS, Bena *et al*. (1998) generated phylogenies that indicated lack of monophyly in most of the already known groups ranking below the genus. Similar results were observed by Downie *et al.* (1998). Separation of different closely related species of *Medicago* is nevertheless possible by using nrITS data (Zareei *et al.*, 2020).

Conclusion

M. minima populations collected from the southwest, west, north, and northwest of Iran exhibited significant morphological diversities in terms of pod traits. Among them, GH had the highest variation (CV, 58%). No clear geographical patterns were evidenced through the comparison of means. However, it seemed that the populations from the west (BAG, KHD, SRV, MAR, and PAV) were relatively more variable in general. Correlation analysis showed strong relations between altitude and GH and SH. This information might be exploited for conservation programs in addition to selection and breeding attempts. The phylogenetic results of nrITS revealed that the 7 populations analyzed in the well-supported clade (99%) belonged to *M. minima*, while they were divided into 2 separate groups, one of which encompassing 5 populations was further divided into 2 subgroups. No obvious correlations were found between pod morphology and ITS variation and geographical proximity. Application of other molecular markers, such as External Transcribed Spacers (ETSs) and chloroplast DNA spacers, is highly recommended for future studies so as to corroborate the consistency of the results of this research. Investigations of edaphic factors and more traits of pods and leaves are additionally encouraged for future research.

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