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Systematic Studies on Populations of *Medicago orbicularis* (L.) Bartal: Molecular, Morphological and Ecological Characterizations

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ABSTRACT

In this paper, pod morphological traits of eleven populations of *Medicago orbicularis* (L.) Bartal., collected from the West, Northwest, and Southwest of Iran, have been assessed. In addition, nuclear ribosomal Internal Transcribed Spacer (nrITS, ITS1+5.8s+ITS2) variation in four populations of *M. orbicularis* was analyzed. Molecular phylogenetic analysis showed that all accessions of *M. orbicularis* formed a well-supported monophyletic clade while no geographical patterning was observed. The result of Principle Components Analysis (PCA) and cluster analysis (Ward's method) (despite a negative Mantel test), suggested a relationship between the morphology and geographical distribution of the populations. Moreover, three distinctive geographical groups were determined using PCA and cluster analysis. Correlation analyses between ecological variables and morphological traits were often negative. However, altitude, average temperature, and average monthly soil temperature showed positive correlations. Wind's relative effect on all morphological traits has also been observed. Two morphological traits-Seeds Total Number (STN) per pod and Fruit Diameter (FDM)-indicated a significant variation. A direct relationship was observed between the two mentioned characters in all populations. Paveh (PVH) and Marivan (MRV) with the highest STN and FDM demonstrated useful potential for breeding and conservation programs in the future. Overall, it could be assumed that the high STN in *M. orbicularis* (comparison with other species of *Medicago* studied in Iran, such as *M. minima, M. sinskiae, and M. polymorpha*), as well as the effect of winds on seeds distribution, are two main influential factors in creating geographical pattern and morphological diversity.

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Introduction

The genus *Medicago* has been classified in various systems, and Small (2011) recognized 87 species indigenous in Eurasia and North Africa. This study focuses on analyzing the morphological and genetic diversity of *M. orbicularis* (L.) Bartal., the only representative of *Medicago* section *orbiculares*. *M. orbicularis* is a winter annual plant flowering in the spring and early summer. It has leafy stems, fimbriate stipules, oval non-hairy leaflets with toothed margins, orange-yellow flowers, and flat, coiled seed pods with no spines. This species is quite adventive and widespread outside of its indigenous area. The focus of this study is on the levels of diversity in pod morphological traits, especially the Seeds Total Number (STN) per pod and Fruit Diameter (FDM), assumed to be quite variable. Olivieri *et al.* (1991) suggested that the main reason for successful dispersal of *M. orbicularis* could be related to the high number of seeds per pods (about 20, instead of four or five for the most species). Our research examined the hypotheses that wind factor plays an important role in the distribution of *M. orbicularis* populations. High number of seeds per plant makes much more sense as a breeding criterion. Button clover produces high-quality forage for livestock (Derkaoui *et al*., 1993; Zhu *et al*., 1996), and also has high seed productivity (Conway *et al.,* 2001). In recent years, understanding the contemporary and historical ecological (climatic, geographical) factors shaping population genetic diversity, structure, and divergence is of great interest to molecular ecology, evolutionary biology and conservation biology (Loveless *et al*., 1984; Lester *et al.,* 2007; Holderegger *et al.,* 2010). Population genetic divergence can be influenced by both geographical and environmental factors (including climate and soil). However, few studies have examined how these factors contribute to genetic structure through time (Zhang *et al*., 2016). Koch *et al.* (2003), using ITS marker, demonstrate the variation of natural populations of *Arabis divaricarpa* (Brassicaceae) in North America. Lee *et al.* (2018) utilized the Internal Transcribed Spacer (ITS) DNA sequence to trace the geographical sources of Aquilaria Malaccensis populations.

The main purpose of the present study is to investigate the use of the ITS of the nuclear genome to clarify the different charactristics of *M. orbicularis* populations in Iran in relation to morphological and ecological variations. The project has also a secondary purpose, which is to clarify features that might be useful for breeding in future.

Materials and Methods

Collection sites

Our research area can be divided into three regions:1) northwest region, including populations from Ahar (AHR), Germi (GRM) and Meshkindasht (MSH) 2); west region, including Marivan (MRV), Sarvabad (SRV) and Paveh (PVH); and 3) southwest region, covering Darre-shahr (LPD), Khorram-abad (KHD), Pol-Dokhtar (PLS), Andimeshk (AND), Sepid-Dasht (SPD). Details of sampling sites are provided in Table 1. The samples were collected from five provinces of Iran: East Azerbaijan, Ardabil, Kurdistan, Kermanshah and Lorestan. Vouchers of each population were deposited at the herbarium of Islamic Azad University, Science and Research Branch Tehran, Iran (IAUH). The distance between the collected samples was at least 20 m. Morphological characters of at least 285 pods from 56 individuals of 11 populations were evaluated.

Data on ten ecological variables, including average wind speed, average, maximum, and minimum temperature, total monthly rainfall, average monthly humidity, average temperature, average north, west, and northwest wind speed, and also, average monthly temperature were acquired from http://data.irimo.ir to highlight the ecological variables affecting pods' morphological characters and population's diversity.

Morphological studies

Between August and September 2017, ripe pods of eleven populations of *M. orbicularis* were collected from West, Northwest, and Southwest of Iran (Table 1). For morphometric studies, nine morphological quantitative characters of above-mentioned populations were measured (Table 2). Four to five samples from each population were evaluated.

Our statistical analyses were carried out in SPSS Software, version 27.0, and Excel 2013. Analysis of Variance (ANOVA) was done based on Duncan's test to specify the differences between the mean values ($P< 0.05$). Principle Components Analysis (PCA) was performed for better understanding of delamination of each population based on morphological traits. The other software that supported our parsimony analysis was PAUP Software, version 4 OB 10 (Swofford, 2002). Overlapping of ecological traits with morphological characters was analyzed by correlation analysis in PAST Software, version 2.17 (Hammer *et al.,* 2012). To determinate the genetic relationships and categorized morphological traits, Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Ward procedure with minimum spherical variance (Podani, 2000) were used.

Table 1. Geographical features of the investigated populations.

Population	Location	Coordinates	Alt. (m)	IAUH*
AHR	East Azarbaijan: 25 km from Ahar to Meshkindasht	38° 30' N;47° 6.01' E	1070	IAUH-000014936
MSH	East Azarbaijan: 45 Km from meshkin shahr to Ahar	36° 23.792' N; 47° 17.038' E	1250	IAUH-000015036
GRM	Ardabil: Germi, 20 km from Germi to Ardabil	39° 10.859' N; 48° 5.222' E	380	IAUH-000015030
MRV	Kurdistan: Paveh, 20 km from Marivan to Paveh	35° 23.402' N; 48° 14.951' E	1200	IAUH-000015006
SRV	Kurdistan: Sarvabad	35° 19.023' N; 46° 21.264' E	360	IAUH-000015010
PVH	Kermanshah: 5 km from Paveh to Nusod	35° 3.777' N; 46° 20.252' E	1474	IAUH-000014957
KHD	Lorestan: Pol-Dokhtar, 85 km from Pol-Dokhtar to Khorram-Abad	33° 18.168' N; 47° 49.748' E	710	IAUH-000014948
LPD	Lorestan: Pol-Dokhtar, 5 Km from Pol-Dokhtar to Darre-Shahr	33° 7.508' N; 47° 30.663' E	780	IAUH-000015018
PLS	Lorestan: Pol-Dokhtar, 25 km from Pol-Dokhtar to Andimeshk	32° 58.429' N; 47° 48.872' E	800	IAUH-000014950
AND	Lorestan: 75 Km from Pol Dokhtar to Andimeshk	32° 45.450' N; 48° 14.745' E	450	IAUH-000014999
SPD	Lorestan: Sepid-Dasht, 5 km from Sepid-Dasht to Khorram-Abad	33° 13.175' N; 48° 51.778' E	1300	IAUH-000014963

* Islamic Azad University of Herbarium (IAUH) -herbarium code.

ITS sequences

For phylogenetic studies, four geographically well-separated populations (SRV, AND, SPD, PVH) were selected randomly. Total DNA was extracted from samples taken from fresh leaf fragments of mentioned populations. The samples were gradually dried in silica-gel pearls, following a modified version of CTAB procedure suggested by Şakiroğlu *et al.* (2010) and using Nucleospin© Plants kit (Machery-Nagel, Germany). The complete ITS region of DNA was amplified using the primer pair AB 101(5′- ACG AAT TCA TGG TCC GGT GAA GTGTTC G- 3) and AB102 (5′-TAG AAT TCC CCG GTT CGCTCG CCG TTA C– 3') (Douzery *et al.*, 1999), in a PCR reaction under the following conditions: a pretreatment of 5 minutes at 95°C, 35 cycles of 30 seconds at 95°C, 30 seconds at 50°C, and 1 minute and 30 seconds at 72°C, and a final extension of 7 minutes at 72°C. The complete ITS region was sequenced on an ABI 3730 sequencer machine (Applied Bio systems, Waltham, Massachusetts, USA). Sequences were visually checked and edited with Sequencer 4 (Gene Codes Corporation, Ann Arbor, MI USA), and then aligned using Mesquite, version 3.61. Moreover, additional sequences were taken from the GeneBank. *Medicago radiate* were chosen as outgroup taxa.

Molecular analyses

Species relationship by ITS sequences

In this research, different phylogenetic methods were used to study the species relationship: Maximum Parsimony (MP) and Bayesian Approach (BA). Using the PAUP Software, the MP was carried out with the following criteria: 100 heuristic searches, 100 replicates, and swapping method of Tree Bisection and Reconnection (TBR). Combining the shortest trees, the strict consensus tree was formed. Bootstrap Support (BS) for each branch was calculated conducting a complete heuristic search with 100 replicates (Felsenstein, 1985). The BA was conducted in MrBayes, version 3.12, (Ronquist and Huelsenbeck, 2003) after assessing the best DNA substitution model (Akaike, 1974) in PAUP* and MODELTEST, version 3.7 (Posada and 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 discarding 25% of the initial trees, the remaining trees sampled from each generation were combined into a 50% majority-rule consensus tree. Tree visualization was performed in Tree View, version 1.6.6 (Page, 2001).

Results

Species delimitation and inter-relationship

Obtained results by Duncan's test indicated the significant variations of pod morphological characters. FDM (by the highest total mean value of 12.5714 ± 0.08706) is one the most effective characters on *M. orbicularis* diversity. The maximum mean of FDM was observed in the populations of MRV (13.21mm), LPD (13.30 mm) and AND (13.020). MSH and GRM populations with the lowest FDM (10.23 mm and 10.77 mm, respectively), Fruit Length Trait (FLT), Number of Middle Coils (NMC), Seed Length Trait (SLT), Seeds Number on Middle Coil (SDN), and Seeds Total Number (STN) (as mentioned in Table 2) made a distinct group. It should be highly noted that other statistical analyses confirm this approach. Middle Coil Thickness (MCT) with a total mean of 0.321± 0.0104 and Fruit Adpression (FAP) with a total mean of 0.8002± 0.02212 showed the lowest effect on *M. orbicularis*. (All the above-mentioned statistical data are highlighted in Table 2.

Table 2. Descriptive statistics of the quantitative morphological traits.

Populations	**FLT**	**FDM**	**NMC**	**FAP**	**MCT**	**SLT**	**SWT**	**SDN**	**STN**
AHR	3.86± 0.12bcd	12.38± 0.16b	2.17± 0.08abc	0.85± 0.05bc	0.30± 0.01ab	1.92± 0.09ab	1.63± 0.07a	4.03± 0.21b	9.68± 0.67bcd
MSH	3.57± 0.269a	**10.23± 0.31a**	1.96± 0.03ab	0.73± 0.051ab	0.26± 0.03bc	**1.68± 0.27ab**	**1.47± 0.24a**	**2.07± 0.39ab**	**3.84± 1.03ab**
GRM	**2.39± 0.21b**	10.77± 0.44a	**1.94± 0.19abc**	0.53± 0.12abc	0.43± 0.08a	2.02± 0.081a	1.71± 0.06a	3.77± 0.43a	6.77± 0.86abcd
MRV	**5.45± 0.33f**	**13.21± 0.35b**	**2.57± 0.11cd**	0.81± 0.09bc	0.33± 0.04abc	**2.6± 0.08c**	2.21± 0.07a	**4.64± 0.19b**	**11.85± 1.03d**
SRV	4.86± 0.13	12.98± 0.19b	2.11± 0.051ab	0.89± 0.05c	0.32± 0.02abc	2.16± 0.06abc	**6.58± 4.78a**	4.29± 0.17b	11.21± 0.6cd
PVH	5.05± 0.46ef	12.49± 0.34b	2.16± 0.15abc	0.71± 0.07abc	0.27± 0.02a	2.18± 0.16abc	1.77± 0.14a	3.61± 0.36ab	7.66± 1.06abc
LPD	4.16± 0.30bcde	**13.30± 0.44b**	2.28± 0.16bcd	0.73± 0.08abc	0.15± 0.03a	2.4± 0.06bc	2.19± 0.06a	4.04± 0.29b	**12.75± 1.04bcd**
PLS	4.49± 0.17cdef	12.67± 0.188b	2± 0.07ab	**0.91± 0.06c**	0.35± 0.03abc	2.26± 0.1abc	1.95± 0.09a	4.4± 0.2b	11± 0.82cd
AND	4.19± 0.24bcdef	**13.020± 0.30b**	2.21± 0.07d	0.36± 0.09abc	0.31 ± 0.03abc	2.15± 0.1abc	1.84± 0.03a	4.13± 0.29b	9.83± 1.04abcd
KHD	4.57± 0.35def	12.43± 0.37b	2.08± 0.13ab	**0.92± 0.10c**	0.44± 0.07cd	2.39± 0.071bc	2.0633± 0.06a	4.33± 0.28b	8.83± 1.17abcd
SPD	4.96± 0.30ef	12.38± 0.32b	**2.71± 0.14d**	0.72± 0.09abc	**0.62± 0.08d**	2.28± 0.16abc	1.96± 0.12a	3.57± 0.61ab	9.71± 1.61bcd
Total mean	**4.3904 ±0.07472**	**12.5714± 0.08706**	**2.205± 0.032**	**0.8002± 0.02212**	**0.321± 0.0104**	**2.1693± 0.03681**	**2.5858± 0.73812**	**4.0263± 0.08456**	**9.6165± 0.28805**

Abbreviations: FLT= Fruit length; FDM= Fruit diameter; NMC= Number of coils; FAP= Fruit adpression; MCT= Middle coil thickness; SLT= Seed length; SWT= Seed width; SDN= Number of seeds on middle coil; STN= Total number of seeds; To provide visual guidance, in each column per trait, coloring has been used; the lowest means are 'red' and the highest means are 'green' in each trait.

WARD clustering, based on all the collected samples, separated plants of each population in a distinct cluster or group (Fig. 1). Therefore, the main cluster was divided into two main groups (in phenoline 25). Group A and Group B were divided into two clusters (in phenoline 15): cluster A1 was comprised of SRV, PLS, AHR and KHD associations and cluster A2 consisted of PVH, LPD, AND MRV and SPD populations. Group B was the well-separated group which remarkably distinguished in all of the analyses. Ecological correlation analyses was performed by PAST Software (Fig. 2). Correlation analyses between the morphological and ecological variables indicated that FDM, SDN and STN were influenced by ecological conditions such as *altitude, **average temperature (tm_m), and ***average monthly soil temperature (tsoilm_m) more than other morphological characters. These three ecological factors showed strong positive correlation with the following morphological traits, respectively: *STN, *SDN, **FDM, and ***FAP. Other effective ecological factors relatively common in all morphological traits were average speed of the west and northwest winds (ff_w_mm & ff_nw_mm). Noticeably, average wind speed (ffm_m) and average speed of the north winds (ff_n_m) with negative correlation showed the lowest effect or negative effect on all the morphological characters.

A comparative analysis of the ecological correlation and Ward clustering demonstrated remarkable results about the effect of ecological variables on clustering populations and at least their distributions.

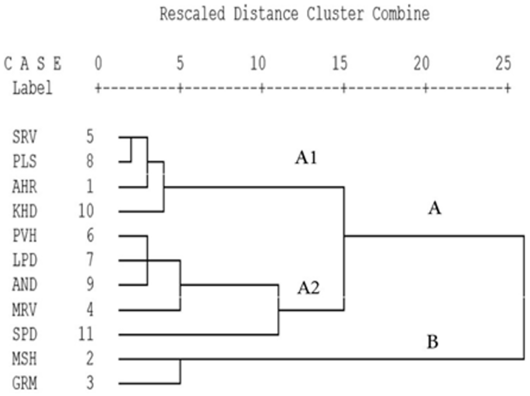

Fig. 1. WARD clustering of morphological characters revealing species delimitation

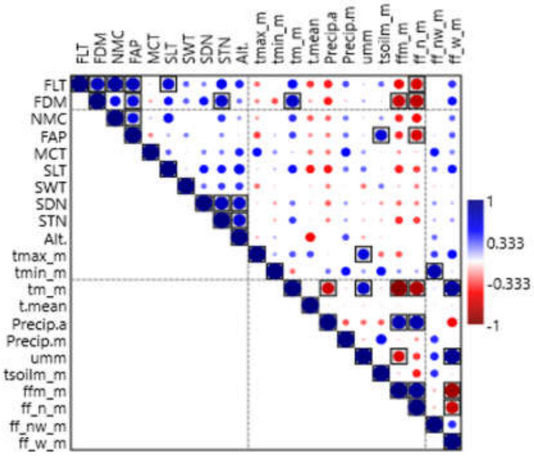

Fig. 2. Correlation analyses between ecological variables and morphological characters Abbreviations; Alt.: Altitude, tmax_m: Average of maximum tempuature, tmin_m: Average of minimum temperature, tm_m: Average temperature, Precip.a: Average precipitation, Precip.m: Average monthly precipitation, umm: Average monthly relatively humidity, tsoil_m: Average monthly soil temperature, ffm_m: Average wind speed, ff_n_m: Average speed of north winds, ff_nw_m: Average speed of northwest winds, ff_w_m: Average speed of west winds. To provide visual guidance, “red circles” presented negative correlations and “blue circles” determined positive correlations between the ecological variables and morphological traits.

As mentioned above, FDM, FAP, FLT and NMC showed positive correlation with ecological variables. Red cells at Table 2 shows populations with the highest FDM means (at the MRV, LPD, and AND populations). In the Ward analyses, at cluster A2 of Ward dendrogram, the MRV, LPD and AND populations were placed next to each other. The PVH and SPD are also in this cluster but placed far from each other. The research showed that besides FDM and FAP, another highly remarkable trait at the PVH population is FLT while NMC is noticeable at the SPD population. A correlation analysis showed that NMC and FLT are two main factors affected by the ecological traits. Cluster B included the GRM and MSH populations with the lowest FDM and the lowest ecological influence. These three analyses could support each other perfectly showing the positive ecological and morphological effects on clustering *M. orbiculairs* populations.

PCA analysis revealed the similarity between the individuals of each association (Fig.3). In the first PCA axis with 37.52% of total variance, morphological characters like FLT, FDM, SDN, STN, LST had the highest correlation (>0.6). In the second axis with 62.14% of cumulative variance, FLT, FAP, and NMC had the maximum similarity.

Morphometric observations showed the various ends of fruits shapes in each population. In the AHR, MRV and SRV populations, mode of fruit shapes was light convex. The MSH and GRM populations were truncate as the PVH, LPD and SND populations. These populations showed similarity in the analyses and were placed at the same group at Ward cluster and PCA analyses. The PLS and KHD populations had a quasi-convex shape, while the clearest convex shape was observed at the SPD population.

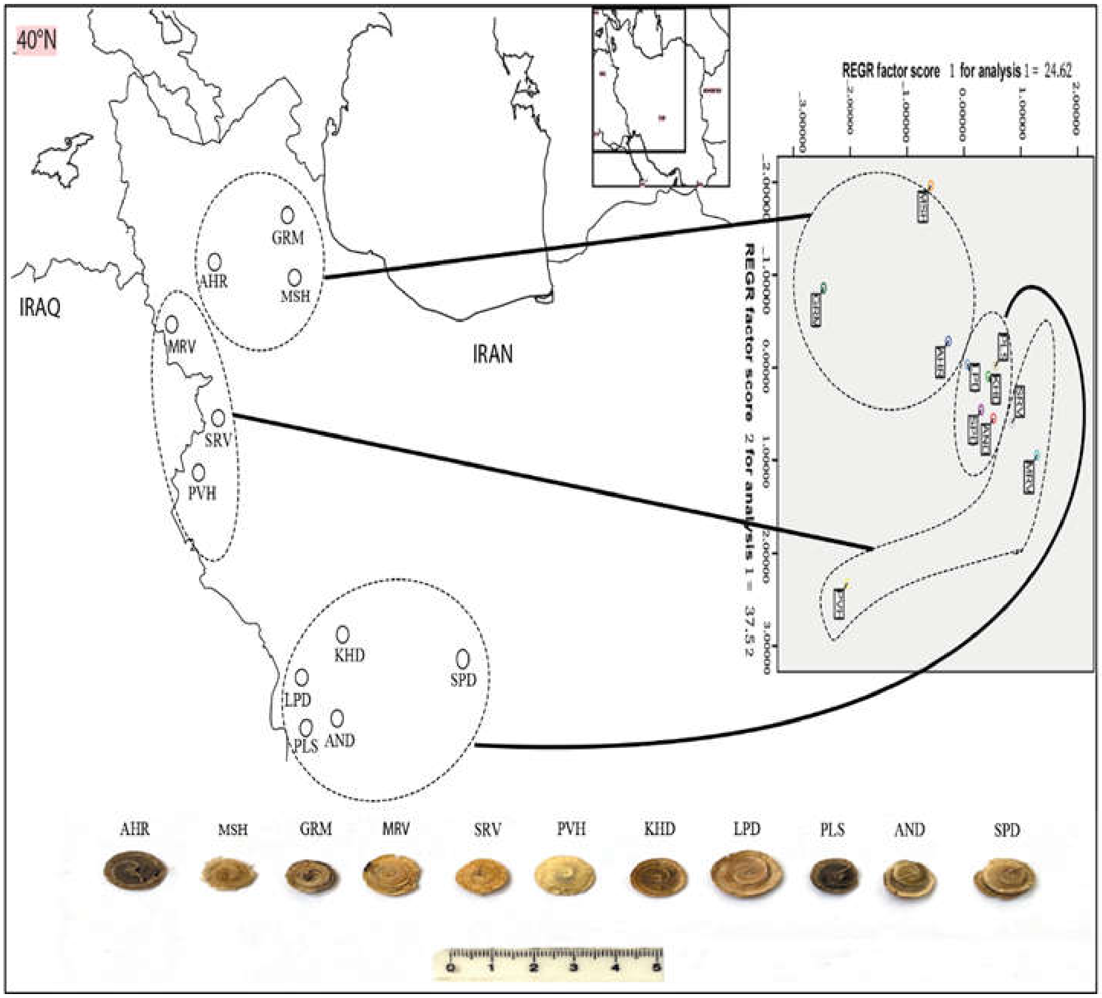

Fig. 3. PCA of morphological traits of *M. orbicularis* samples collected from North, West, and Southwest of Iran.

Molecular studies

ITS sequence-based phylogeny

The phylogenetic tree obtained showed that the species of the phylogram were predominantly categorized into two clades. Posterior Probabilities (PP) are indicated by numbers bellow each clade. Bootstrap Supports (BS) for those clades also retrieved in the Maximum Parsimony (MP) analysis are indicated by the numbers above each clade (100 replicates) (Fig. 4). Our ITS dataset included 368 characters. These 74 (20.1%) and 94 (25.5%) variables were parsimony informative and parsimony-uninformative, respectively. Furthermore, 200 characters were constants. Model of evolution was GRT+I+G with A-C constitution rate= 0.9863, A-G constitution rate= 1.9283, A-T constitution rate = 0.9917, C-G constitution rate= 0.5809, C-T constitution rate = 3.1511, G-T constitution rate= 1.0000, and Gamma distribution rate= 1.034.

Phylogenetic tree presented in Fig. 4 and species of the genus *Medicago* are mainly grouped into two clades. Clade A is a strongly supported clade in which all samples of *M. orbicularis* and *Medicago arabica are* located, and clade B includes 50 species of *Medicago*.

Clade A with BS= 51 and PP= 0.144, and clade B with BS= 53 and PP= 0.146 are clearly distinguished in Fig. 4. In this phylogenetic tree, species of *M. radiate* are indicated as an outgroup. Clade A includes samples from four populations of *M. orbicularis* and one sample from Genbank, creating a monophyletic clade. Clade B includes a polytomy of various species of *Medicago.*

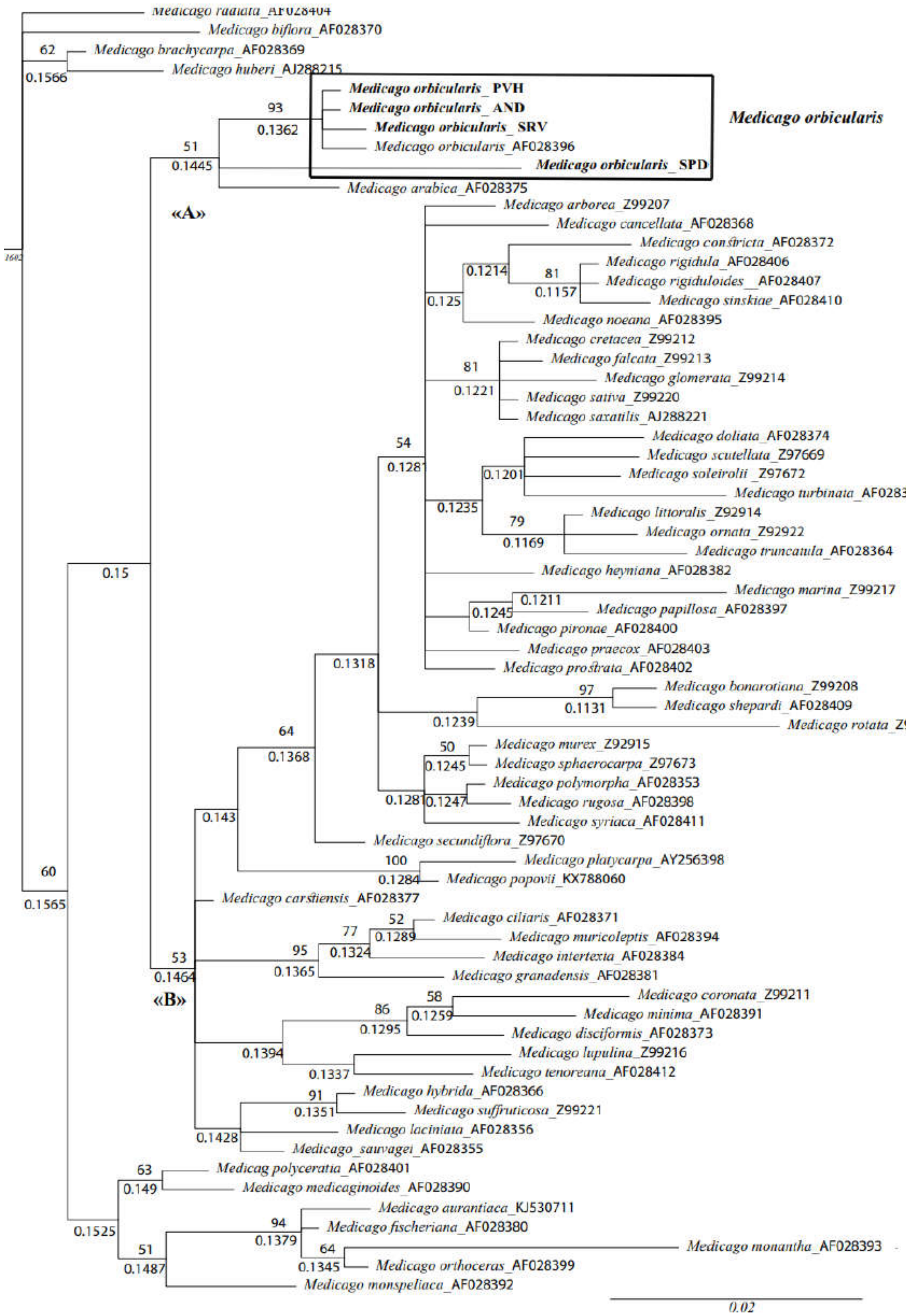

Fig. 4. Phylogenetic tree obtained from the maximum parsimony analysis of ITS region of different species of *M. orbicularis*: Total characters= 368, constants= 200, parsimony informatives= 74, parsimony-uninformative= 94. Numbers written above and below each branch indicated bootstrap support percentages and Posterior Probabilities (PP), respectively. *Medicago radiate* = outgroup.

Discussion and Conclusion

This study documents the occurrence of eleven populations belonging to the *M. orbicularis* found in our study area in the West, Northwest, and Southwest of Iran. Our evaluations indicated that the most valuable taxonomically characters in *M. orbicularis* are FLT (Fruit Length), FDM (Fruit Diameter), NMC (Number of Middle Coils), FAP (Fruit Adpression), MCT (Middle Coil Thickness), SLT (Seed Length Trait), SWT (Seed Width Trait), SDN (Seeds Number on Middle Coil), and STN (Seeds Total Number) (Table 2). High level of diversity observed in these characters. Our results also indicated that the STN with the mean of 9.6 per pods and FDM with the mean of 12.5 mm, are higher here compared to the reported values in *M. minima* (STN= 3.85, FDM= 4.06 mm) by Bagheri *et al.* (2020), *M. polymorpha* (STN= 3.03; FDM= 5.28 mm) by Emami-Tabatabee *et al.* (2021), and also *M. sinskiae* (STN= 4.67; FDM= 6.55 mm) by Zaraee *et al.* (2020). We could introduce STN and FDM as two main morphological characters affecting the *M. orbicularis* diversity. Based on the statistical results presented in Table 2, a direct relationship was observed between FDM and STN at all populations. It should be noted that, the PVH and MRV populations with the highest level of STN and FDM demonstrated remarkable potential for breeding and conservation programs in future. Positive correlations were observed between altitude & STN, and also, between average temperature & FDM. Besides, the relative correlations between west and northwest winds speed with all of the morphological traits could approve our hypothesis about the effective role of high number of seeds per pods and winds speed on the distribution of *M. orbicularis*.

Despite the negative results from the Mantel test, after 5000 permutations, (r= 0.02155, p= 0.3805) (r= -0.032524, p= 0.5751) clustering showed a pattern of relationship between morphology and geographical distribution of *M. orbicularis* populations. PCA results clearly indicated that the populations with the highest levels of mean value of morphological traits (MRV, SRV and PVH) belonged to the Western regions and also, populations with lowest level of mean value (MSH, GRM and AHR) belonged to the Southwestern regions. The role of climatic parameters in grouping populations is obvious. Although the molecular characterization has been the most efficient and reliable tool in studying the genetic diversity of populations and species, the impact of environmental factors on morphological traits is impressive. Badri *et al.* (2016) and Ferchichi *et al.* (2021) examined the morphological, ecological and molecular characterization of *M. tunetana and M. polymrpha,* respectively. Their results demonstrated variations of quantitative traits of populations influenced by the altitude, temperature and relative humidity. Therefore, in addition to molecular and morphological traits, climatic factors and their affecting role on species population diversities could be considered in future biodiversity studies.

In recent years, several phylogenetic analyses have been done based on nucleotide sequence data (e.g., Dowanie *et al.*, 1998; Bena, 2001; Steele and Wojciechowski, 2003; Maureira-Butler *et al.*, 2008; Steele *et al.*, 2010) to depict an appropriate infrageneric classification of *Medicago*. Depending on the nucleotide sequence examined, different results were obtained. All of the above-mentioned hypotheses approved the correct placement of *M. orbicularis* populations sequenced in this research and presented in Fig. 4. Colagar *et al*. (2016) noted that the development of DNA barcodes for situations where morphological evidence is deficient makes it possible to identify closely related species. Our ITS analysis result, coincides with Emami-Tabatabaei *et al.'s* (2021) report on *M. polymorpha,* indicating that "the measure of relative genetic distance among populations of *M. orbicularis* did not correlate well with geographical distance of their sampling locations."

Small (2011) referred to the association between geographical distance and similarity and geographical distance and intersterility in *Medicago* species, and showed the intimate association between the morphological divergence and geographical separation. Our current research expressed that the morphology-based clustering of genotypes has the best fit with the geographical origins of plant accessions. Therefore, morphological evaluation provides a rapid tool for discriminating different genotypes.

Conflicts of interest

The authors have declared no conflicts of interest.

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