IRANIAN JOURNAL OF BOTANY 27 (2), 2021
DOI: 10.22092/ijb.2021.354838.1328

TERRITORIAL PARTITIONING OF THE CONTACT ZONE OF THE EUROPEAN MEDICAGO RIGIDULA AND THE CLOSELY RELATED ASIAN MEDICAGO RIGIDULOIDES IN IRAN

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Received 2021. 06. 08; accepted for publication 2021. 09. 21

Bayat, M., Assadi, M., Small, E & Mehregan, I. 2021. 12. 30: Territorial partitioning of the contact zone of the European Medicago rigidula and the closely related Asian Medicago rigiduloides in Iran. *-Iran. J. Bot. 27 (2): 115-129*. Tehran.

Medicago rigiduloides E. Small is a mostly Asian species that was recently separated from the extremely closely related, mostly European species, *M. rigidula* (L.) All. *Medicago* is a large genus of about 90 species, but there has never been a study of how any of these interact geographically where they meet. This, the first such study, documents the distribution ranges of the two species in Iran, based on 14 representative populations. The two species are extremely difficult to distinguish morphologically, so in addition to analysis of their morphology, molecular analysis of the internal transcribed spacer (ITS) region was conducted, and the degree of reliability of the diagnostic characters of the two taxa was assessed. *Medicago rigidula*, known to be mainly European, was found to have extended into Iran bordering the Caspian Sea. *Medicago rigiduloides* was found to be widely distributed in northwestern, western, southwestern, and central Iran, consistent with the hypothesis that the taxon is mainly Asian. Thus, the main distributions of the two taxa appear to be mainly parapatric, presumably related to different adaptations to the local ecology.

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Keywords: pod morphology traits; annual Medicago; genetic variation; geographical distribution

تقسیم‌بندی جغرافیایی نواحی تماس گونه اروپایی M. rigidula و نزدیک‌ترین گونه آسیای آن M. rigiduloides در ایران

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Medicago rigiduloides E. Small گونه‌ای عمدتاً آسیایی است که اخیرا از خویشاوند اروپایی بسیار نزدیک به آن یعنی M. rigidula (L.) All. تفکیک شده است. جنس Medicago جنسی بزرگ با حدود ۹۰ گونه است و لی تاکنون بر هم کنش آنها در نواحی تلاقی مطالعه نشده است. این اولین مطالعه از این نوع در ایران با بررسی ۱۴ جمعیت است. تشخیص دو گونه از هم با ویژگی‌های مورفولوژیک بسیار مشکل است و بنابراین علاوه بر ویژگی‌های مورفولوژیک، ناحیه فاصله انداز داخلی (ITS) آنها نیز بررسی شد. نتایج این بررسی نشان داد که پراکندگی گونه عمدتاً اروپایی Medicago rigidula به منطقه خزری امتداد یافته است. گونه Medicago rigiduloides به طور گسترده در شمال غرب، غرب، جنوب غرب و

مناطق مرکزی ایران پراکنده شده که این موضوع مؤید پراکندگی عمدتاً آسیایی این گونه است. بنابراین این به نظر می‌رسد که این دو گونه الگوی پراکندگی پاراپاتریک (parapatric) متأثر از سازگاری به شرایط اکولوژیک محلی دارند.

INTRODUCTION

The genus *Medicago* L. consists of about 90 species, with the center of distribution ranging from the Mediterranean area to Central and West Asia (Small & Jomphe 1989; Cannon & al. 2006; Small 2011). *Medicago*, *Trigonella* L., and *Melilotus* Miller all belong to the tribe Trigonellinae (Steele & al. 2010; Small 2011; Wu & al. 2016; Chen & al. 2021). *Medicago rigidula* (L.) All. and *M. rigiduloides* E. Small All. are annual, weedy, diploid (2n = 14), inbreeding species (Small 1990; Small 2011). *Medicago rigiduloides* is a mainly Asian taxon segregated from *M. rigidula* sensu lato. However, their morphological and geographical separateness requires study. An early study suggesting that what was subsequently recognized as *M. rigiduloides* should be segregated from *M. rigidula* was that of Lesins & Lesins (1963), who observed low pollen viability in hybrids produced by the crossing of European plants with 3-pored pollen × Asian plants with 4-pored pollen. Using these pollen characters as well as 30 other morphological characters, Small & al. (1990) separated *M. rigidula* sensu lato accessions from Europe and Africa (all with 3-pored pollen) as *M. rigidula* sensu stricto, from Asian accession (all with 4-pored pollen) as *M. rigiduloides* E. Small. Based on a large-scale study, Mehregan & al. (2002) treated *Medicago sinskiae* Uljanova and *Medicago canstricta* Durieu as subspecies of *M. rigidula*, while merging *M. rigidula* and *M. rigiduloides* (Small's classification) into subsp. *rigidula*. They argued that the Small & al. (1990) report indicates a clinal variation in *M. rigidula* from Europe toward Asia. While the previous studies (Small & al., 1990; Small, 1990; Heft & Groose, 1996) highly resolved the distinction between *M. rigidula* and *M. rigiduloides*, and pollen and pod morphology traits are relatively reliable key characters, the distribution range of the two species is unclear and the morphological distinctions require confirmation.

Particularly in the light of climate change, it is important to clarify the taxonomic status and geographical range of economically important species to conserve their valuable germplasm (Mace 2004). Particularly valuable in this respect are molecular (nucleic-acid-based) approaches (Hynniewta & al. 2014). For example, the internal transcribed spacer (ITS) is a useful genetic tool in resolving taxonomic relationships, such as in *Flemingia* L. and *Glycine* L. (Wu & al. 2013), Caraganeae tribe (Ranjbar & al. 2014) *Oxytropis* Sect. (Kholina & al. 2020), *Medicago* (Chen & al. 2021), *Trigonella foenum-graecum* L. (Kakani & al. 2011), *M. ruthenica* L. (Wu & al. 2016), *Oxytropis almaatensis* Bajt. (Almerekova & al. 2017), and *Trifolium resupinatum* L. (Ansari & al. 2018)). ITS markers provide valuable genetic information because of their fast evolution. Additionally, low genomic diversity of the nuclear ribosomal DNA arranged in tandem arrays results from concerted evolution in which they can be treated as a single locus (Baldwin & al. 1995). Morphological traits alone or in combination with molecular markers have successfully differentiated *Medicago* species in several cases (Chen & al. 2021). The primarily driven factors behind the morphological variation, among populations across the geographical distribution range of a specific species, could be broadly classified into the ongoing environmental conditions within habitats, and previous historical processes and phylogenesis (Peppe & al. 2011; Thorpe 1987). The populations of the same species under various environmental conditions may respond differently to the selection pressures in their genetic and morphology. Among the morphological traits, some may not be that responsive compared to others, especially if the traits are crucial for survival (Ramsey & al. 1994; Stenøien & al. 2002; Albarrán-Lara & al. 2019).

The geographical relationships between the very closely related *M. rigidula* and *M. rigiduloides* have never been examined in a region where they meet. This is the first such study and provides an opportunity to precisely locate the distribution ranges of the two species for the flora of Iran. Additionally, this study takes advantage of molecular examination of the ITS region in order to expand understanding of the morphological relationships of *M. rigidula* and *M. rigiduloides*.

MATERIALS AND METHODS

Site selection and plant material

Six populations of *M. rigidula* and eight of *M. rigiduloides* from different altitudes (500 to 2210 m) were identified in the north, northwestern, western, and southwestern Iran during May and July 2017. Individual plants were sampled at least 20 m apart. Fruit and leaf samples were collected from a minimum of 10 individuals per population with a total of 140 individuals sampled from 14 populations (fig. 1). The leaves were dried on silica gel for molecular studies.

Voucher samples were made and deposited in the Islamic Azad University Herbarium (IAUH) (table 1). Pod morphological traits were based on characters considered important by Lesins & Lesins (1979), Heyn (1984), Small & Jomphe (1989), Small & al. (1990), and Small (2011), and were searched to determine important morphological characters including those mentioned in tables 2 & 3. In addition to altitude, 10-year means of three ecological variables including annual precipitation and the average temperature of the growth seasons were obtained from www.en.climate-data.org.

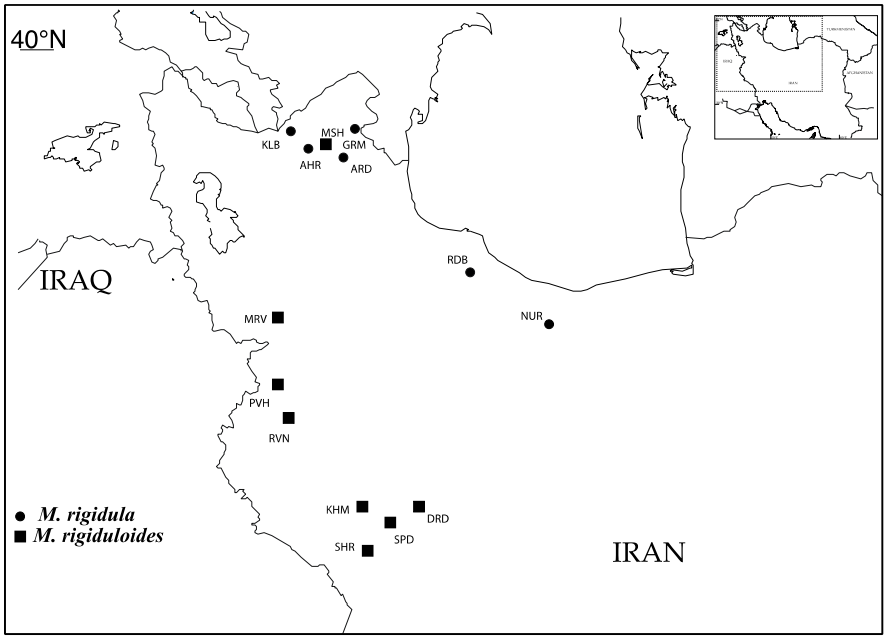

Fig. 1. The map showing the localities of populations of *M. rigidula* and *M. rigiduloides* collected for this study.

Table 1. Geographical and climatic details and voucher specimens in studied populations. Abbreviations: Pop.: Population; Av. Temp.: Average Temperature; Av. Prec.: Average Precipitation; Acc. No.: Genbank Accession Number.

Pop.	Locality	Code	Altitude (m)	Latitude	Longitude	Voucher no.	Av. Tem. (°C)	Av. Prec. (mm)	Genbank Accession Number
1	Khoramabad, Dorood	DRD	1555	33° 35.571´ N	48° 5.088´ E	IAUH 14960	14.90	375	OL455026
2	Kermanshah, Paveh	PVH	1450	35° 3.777´ N	46° 20.252´ E	IAUH 14956	12.50	674	OL455033
3	Azerbaiejan, Meshkinshahr	MSH	1250	38° 23.792´ N	47° 17.038´ E	IAUH 15035	9.70	356	-
4	Khoramabad, Poldokhtar	KHM	940	33° 57.121´ N	47° 57.328´ E	IAUH 14979	16.90	488	OL455029
5	East Azerbaiejan, Kaleybar	KLB	1200	38° 50.563´ N	47° 2.937´ E	IAUH 15042	11.20	422	OL455030
6	Ardabil, Germi	GRM	1300	38° 57.595´ N	47° 58.898´ E	IAUH 15040	11.70	485	OL455032
7	Kermanshah, Ravansar	RVN	1300	34° 33.827´ N	46° 45.767´ E	IAUH 14985	13.80	551	OL455031
8	Gilan, Rudbar	RDB	500	36° 50.03´ N	49° 25.163´ E	IAUH 14975	16.20	896	-
9	Kurdistan, Marivan	MRV	1260	35° 19.023´ N	46° 21.264´ E	IAUH 15009	14	878	OL455028
10	Lorestan, Sepiddasht.	SPD	1300	33° 13.175´ N	48° 51.778´ E	IAUH 14966	18.10	428	OL455027
11	Khoramabad, Shourab	SHR	1100	33° 27.124´ N	48° 10.9´ E	IAUH 15022	10.20	351	-
12	Azerbaijan, Ahar	AHR	1070	38° 23.654´ N	47° 20.6´ E	IAUH 14935	10.60	378	OL455025
13	Ardabil, Meshkinshahr	ARD	1110	38° 32.070´ N	47° 49.42´ E	IAUH 14938	9.50	325	-
14	Mazandaran, Nur	NUR	2210	26° 51.00´ N	47° 36´ E	IAUH 14968	16.10	927	-

Table 2. List of morphological traits investigated in this study and their abbreviations.

Measured traits of fruits	Abbreviation	States (quantitative characters only)
Fruit length	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 seed	SLT	-
Width of seed	SWT	-
Number of seeds in middle coil	SDN	-
Number of seeds	STN	-
Presence of simple hair (in mm^2)	PSH	1= 0, 2= 1-5, 3= 6-10, 4= 11<
Presence of glandular hair (in mm^2)	PGH	1= 0, 2= 1-5, 3= 6-10, 4= 11<
Shape of fruits' ends	FESH	1= truncate, 2= light convex1, 3= convex2, 4= more convex, 5= hemispherical
Shape of spines	SSH	1= straight, 2= slightly curved, 3= curved, 4= more curved, 5= hooked
Presence of lateral grooves	PGD	1=0 (mm), 2= 0.1-0.2, 3= 0.3-0.4, 4=0.5-0.6, 5= 0.7-1.5
Dorsal to middle vein protrusion	DSP	1=absent, 2= more absent2, 3= present3, 4= more present4, 5= more present5

DNA extraction, PCR and ITS sequencing

Nine out of 14 populations were chosen for phylogenetic study. Total genomic DNA was extracted from silica gel dried leaves according to the CTAB (cetyltrimethylammonium bromide) method of Doyle & Doyle (1987) using the Nucleospin© Plants kits (Machery-Nagel, Germany) following the manufacturer's instructions. Using 1% agarose gel, the concentration of the isolated DNA assessed. The ITS (ITS1-5.8S-ITS2) region of the nuclear ribosomal DNA (nrDNA) was amplified 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 & al. 1999). The PCR reactions were carried out in 25µL volumes consisted of 50Mm KLC, 10Mm Tris-HCl buffer at pH 8, 1.5Mm MgCl2, 0.2Mm of each Nucleoside triphosphate, 0.2 µL of a single primer, 20 ng genomic DNA, and 1 unite of Taq DNA polymerase. The program used composed of a 5 min at 95ºC, 35 cycles of 30 sec at 95ºC, 30 sec at 50ºC, and 90 sec at 72ºC, and a final extension of 7 min at 72ºC. The PCR products were evaluated qualitatively by electrophoresis on 1% agarose gel and quantitatively using the spectrophotometry method. Sequencing of amplicons was performed on an ABI 3730 DNA Analyzer (Hitachi-Applied Biosystems, Waltham, Massachusetts, USA).

Table 3. The Duncan's means of the traits, assessed from eight populations of *M. rigiduloides* and six populations of *M. rigidula*.

Pops	Alt.	FLT	FDM	NMC	FAP	MCT	LSL	SBT	SMC	ASP	SLT	SWT	SDN	STN
	M. rigiduloides													
DRD	1555	5.41±0.79^{e,f}	5.95±0.46^{e,f}	4.95±0.61^{d,e}	0.15±0.13^{c,d,e}	1.01±0.17^{a,b}	0.86±0.36^{d}	0.41±0.15^{c,d}	17.32±1.90^{e}	7.500±7.28^{d}	3.45±0.43^{f,g}	1.96±0.20^{f}	1.84±0.45^{a,d}	5.35±1.60^{d,e}
PVH	1474	4.85±0.85^{b,c,d}	5.64±0.44^{c,d,e}	5.25±0.62^{e,f}	0.11±0.07^{b,c,d}	0.97±0.21^{a}	0.43±0.21^{a}	0.31±0.10^{a}	16.07±2.00^{d,e}	2.11±1.78^{a}	3.30±0.48^{e,f}	1.82±0.24^{d,e}	1.63±0.49^{a}	4.08±1.55^{a,b}
MSH	1250	5.58±0.98^{f}	5.73±0.53^{c,d,e}	5.70±0.61^{g}	0.10±0.09^{b}	1.03±0.17^{a,b}	0.82±0.33^{c,d}	0.36±0.09^{a,b,c}	17.36±2.65^{e}	5.16±4.87^{b,c}	3.20±0.35^{d,e}	1.77±0.20^{b,e}	1.78±0.42^{a,c}	4.93±1.54^{b,d}
KHM	941	4.90±0.82^{b,c,d}	5.51±0.46^{b,c}	5.10±0.78^{e,f}	0.10±0.07^{b,c}	1.11±0.20^{b,c}	0.52±0.34^{a,b}	0.34±0.12^{a,b}	15.77±2.67^{d}	2.44±2.61^{a}	3.41±0.33^{f,g}	1.85±0.25^{e}	1.65±0.53^{a,b}	4.07±1.42^{a,b}
RVN	1300	5.00±0.60^{c,d,e}	5.72±0.36^{c,d,e}	5.21±0.67^{e,f}	0.13±0.08^{b,c,d}	1.02±0.03^{a,b}	0.43±0.19^{a}	0.32±0.08^{a}	16.49±2.68^{d,e}	2.34±1.94^{a}	3.58±0.37^{g}	1.99±0.20^{f}	1.60±0.50^{a}	4.34±1.63^{b,c}
MRV	1260	5.23±0.98^{d,e,f}	5.86±1.06^{d,e,f}	5.33±0.55^{f}	0.29±0.08^{g}	0.98±0.03^{a}	0.91±0.32^{d}	0.47±0.11^{d,e,f}	13.38±1.24^{b,c}	6.02±4.31^{c,d}	3.04±0.49$^{b,\ d}$	1.76±0.30^{b,e}	2.00±0.00^{c,e}	6.04±1.59^{e}
SPD	1300	4.47±0.84^{a,b}	5.00±0.47^{a}	5.28±0.55^{f}	0.12±0.05$^{b,c,\ d}$	0.95±0.04^{a}	0.47±0.23^{a}	0.34±0.12^{a,b}	13.97±1.54^{c}	1.84±2.18^{a}	2.99±0.26^{b,d}	1.66±0.15^{a,b}	1.77±0.43^{a,c}	4.37±1.40^{b,c}
SHR	1100	4.88±0.77^{b,c,d}	5.58±0.56^{b,c,d}	5.10±0.58^{e,f}	0.16±0.11^{d,e,f}	0.97±0.03^{a}	0.66±0.33^{b,c}	0.40±0.13^{c}	15.85±3.16^{d}	4.63±3.90^{b,c}	3.09±0.37^{d}	1.79±0.23^{c,e}	1.72±0.54^{a,b}	4.78±1.56^{b,d}
Mean		5.03	5.62	5.25	0.14	1.00	0.62	0.36	15.92	3.80	3.27	1.83	1.73	4.65
CV%		13.2	8.92	9.18	25.8	13.20	29.12	22.5	16.5	65.21	13.2	13	27.3	33.4
	M. rigidula													
KLB	1200	4.69±0.82^{a,b,c}	5.95±0.73^{e,f}	4.27±0.41^{b}	0.14±0.09^{b,c,d}	1.09±0.23^{b,c}	1.51±0.51^{f}	0.46±0.10^{d,e}	16.69± 3.44^{d,e}	3.17±1.93^{a,b}	3.07±0.41^{c,d}	1.81±0.25^{c,e}	2.00±0.34^{c,e}	5.57±1.42^{d,e}
GRM	1070	4.83±0.69^{b,c,d}	5.11±0.39^{a}	4.56±0.42^{c}	0.04±0.06^{a}	0.94±0.18^{a}	0.98±0.24^{b,c}	0.39±0.07^{b,c}	12.46±1.46^{b}	3.16±1.79^{a,b}	2.74±0.36^{a}	1.57±0.18^{a}	1.66±0.54^{a,b}	5.40±1.70^{d,e}
RDB	500	4.38±0.66^{a}	6.07±0.64^{f}	3.80±0.63^{a}	0.18±0.12^{e,f}	1.04±0.04^{a,b}	1.82±0.43^{f}	0.52±0.11^{f}	19.23±4.79^{f}	5.23±2.72^{b,c}	2.84±0.47^{a,b}	1.69±0.26^{b,c}	2.23±0.57^{e}	5.20±1.73^{c,e}
AHR	1070	4.52±0.79^{a,b}	5.93±0.60^{e,f}	4.57±0.55^{c}	0.20±0.09^{f}	0.98±0.04^{a}	0.84±0.50^{c,d}	0.46±0.10^{d,e}	12.35±2.20^{b}	14.93±8.74^{e}	2.85±0.34^{a,b}	1.74±0.17^{b,e}	2.06±0.60^{d,e}	5.97±1.66^{e}
ARD	1113	5.25±0.53^{d,e,f}	5.29±0.59^{a,b}	4.76±0.45^{c,d}	0.04±0.05^{a}	1.17±0.03^{c}	1.22±0.39^{e}	0.59±0.13^{g}	12.42±1.52^{b}	5.91±3.37^{c,d}	2.88±0.27^{a,c}	1.72±0.21^{b,d}	1.91±0.38^{b,d}	5.03±1.72^{c,d}
NUR	2210	4.77±1.10^{a,b,c}	5.47±0.67^{b,c}	4.74±0.47^{c,d}	0.05±0.06^{a}	0.96±0.05^{a}	1.31±0.33^{e}	0.48±0.14^{e,f}	9.59±2.09^{a}	2.50±2.54^{a}	2.71±0.48^{a}	1.55±0.29^{a}	1.60±0.51^{a}	3.40±1.88^{a}
Mean		4.73	5.65	4.43	0.11	1.03	1.26	0.48	14.10	6.10	2.87	1.69	1.94	5.27
CV%		16.6	12.9	13.1	56.23	22.6	42.5	27	33.2	55.23	14.3	14.9	26.3	31.2

Phylogenetic analysis

The ITS sequences of *M. rigidula* and *M. rigiduloides* were manually assessed, edited, and then aligned using Sequencher 4 software (Gene Codes Corporation, Ann Arbor, Michigan, USA). Additionally, ITS sequences of 63 accessions were obtained from the Genbank (see fig. 7). The accession numbers of those are added to fig. 6. To assemble and align the entire ITS sequences, MacClade 4 (Maddison & Maddison 2000) was used. *Medicago popovii* and *M. platicarpa* were chosen as outgroups following Bena & al. (1998a & 1998b). Maximum Parsimony analysis (MP) was performed using PAUP* software (Swofford 2002) with the following criteria: 100 heuristic search, 100 replicates, swapping method: TBR. The strict consensus tree was formed by combining the shortest trees recovered under MP analysis. Bootstrap support (BS) for each branch was calculated using a complete heuristic search with 100 replicates and a similar setting as mentioned above (Felsenstein 1985). The Bayesian analysis (BA) of the ITS dataset was performed using MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001). In order to find the appropriate model of DNA substitution, the Maximum Likelihood criteria for the dataset were determined by the Akaike information criterion (AIC; Akaike 1974) as implemented in ModelTest v3.7 (Posada & Crandall 1998). "TIMef "was selected as the best fit model for our dataset by ModelTest v3.7. The MCMC (Markov Chain Monte Carlo) process was set so that four chains ran simultaneously for 5,000,000 generations. 25 % of initial trees being discarded, the remaining trees sampled from each generation were combined into a 50 % majority rule tree.

Statistical analysis

Morphological results were presented as mean ± standard error observations (mean ± SE). Significance of differences among mean values were determined at $P < 0.05$ by Duncan's test. Principal components analysis (PCA) was carried out to see the distribution pattern of populations based on average values of morphological traits among the studied populations. SPSS 22 software (Green & Salkind 2016) was utilized for these analyses. For the cluster analysis based on Ward's method, PAST Software ver. 4.03 with 1000 bootstrap replicates was used (Hammer & al. 2001). To assess the correlation between different data sets, quantitative and qualitative, and ecological characteristics, Pearson's correlation coefficient was performed using "corrplot" package in R. To assess the relationship between morphological diversity and geographical distance, the Mantel test (Mantel 1967) was utilized. The Euclidian distance was first calculated for morphological traits using PAST ver. 3.24 (Hammer & al. 2012). The correlation between the two matrices was calculated using GenAlEx version 6.5 (Peakall and Smouse 2012).

RESULTS

Morphological analysis

The measurements of morphological characters examined in this study is summarized in tables 3 and 4. Comparison of means of pod morphology characteristics revealed significant variation among the populations. In general, *M. rigiduloides* had lower values of coefficient of variation (CV) compared to *M. rigidula* (table 4). For example, number of coils (NMC) and the length of longest spine (LSL) in *M. rigiduloides* were 9.18 % and 29.12 % respectively, compared to 13.1% and 42.5% in *M. rigidula.* Table 4 reports the variation of qualitative characters examined. Angle of spine insertion (ASP) in both species *M. rigiduloides* and *M. rigidula* showed relatively high amount of CV (65.12% and 55.23 %, respectively; table 3). Here the attempt was first to assess the more distinguishing traits such as LSL and NMC among quantitative and shape of spines (SSH) from qualitative characteristics, which Small (2011) mentioned as the primary identification diagnostic features between *M. rigidula* and *M. rigiduloides.* Overall, the traits mentioned above served to assign the populations to two main groups where the highest values for LSL were found to be in *M. rigidula* populations (e.g., Roudbar, Kaleybar, Nur, Ardebil, Germi, and Ahar) in the northwestern and northern Iran. Whereas the rest of the populations, mainly in the west and southwest, had higher values for NMC, a key for identifying *M. rigiduloides*. However, a population from the northwest (Meshkinshahr) with the highest NMC of 5.70 grouped with populations of west and southwest (*M. rigiduloides*). The average NMC of *M. rigiduloides* was 5.25 against 4.43 in *M. rigidula* populations. Another key character to discriminate two species is the shape of spines (SSH), which was found to be mainly straight in *M. rigiduloides* populations in the west and slightly curved in *M. rigidula* populations of northern and northwestern Iran. Notable variation was found in other traits among populations and between the two species. The highest length of fruit (FLT, 4.38 mm) and fruit diameter (FDM, 6.08 mm) for *M. rigidula* were found in Roudbar population. Whereas Meshkinshahr and Dorood showed the highest values for FLT (5.58mm) and FDM (5.95mm), respectively.

Table 4. Qualitative pod morphological characters of the studied populations of *M. rigidula* and *M. rigiduloides*.

Pop	PSH	PGH	FESH	SSH	DSP	PGD (mm)
M. rigiduloides						
DRD	1-5	11<	Light convex1	Straight	Peresent3	0
PVH	1-5	11<	Light convex1	Straight	Peresent3	0
MSH	6-10	11<	Light convex1	Straight	More present 4	0.1-0.2
KHM	1-5	11<	Light convex1	Straight	Present 3	0
RVN	1-5	11<	Light convex1	Straight	Present 3	0
MRV	1-5	11<	Truncate	Straight	More present 4	0.1-0.2
SPD	1-5	11<	Light convex1	Straight	Present 3	0
SHR	1-5	11<	Light convex1	Straight	More present 4	0.1-0.2
CV%	22.6	17.3	25.7	32.2	19.4	26.9
M. rigidula						
AHR	1-5	0	Truncate	Straight curved	More present 4	0.1-0.2
ARD	1-5	11<	Light convex1	Straight curved	Present 3	0
NUR	11<	11<	Truncate	Slightly curved	Present 3	0
KLB	1-5	11<	Truncate	Slightly curved	More present 4	0.1-0.2
GRM	11<	11<	Light convex1	Straight curved	More present 4	0.1-0.2
RDB	1-5	11<	Truncate	Slightly curved	More present 4	0.1-0.2
CV%	34.2	27.1	39.02	42.1	17.8	38.9

Adpression of fruits was relatively higher in *M. rigiduloides* populations (0.14) compared to 0.11 in *M. rigidula* populations. In the former, Marivan with 0.29 showed the highest value, while Ahar was found to have the highest value (0.20) in the latter. The number of spines on the middle coil (MCT) between both populations' values did not vary significantly, and Ardebil with 1.17 showed the highest value. In the case of the thickness of spine base (SBT) and the number of spines on the middle coil (SMC), values were higher in *M. rigidula* populations in a statistically significant manner for Ardebil (SBT=0.59) and Roudbar (SMC=19.23) while in *M. rigiduloides* populations of Marivan (SBT=0.47) and Meshkinshahr (SMC=17.36) indicated the highest values. The difference between the angle of spine insertion (ASP) of the two species was considerably high: *M. rigiduloides (*mean 3.80) vs. *M. rigidula* (mean 6.10). Seed length (3.58 mm) and width (1.99 mm) of Ravansar population were observed to be the highest among both species with *M. rigiduloides* having slightly higher average values. On average, *M. rigidula* displayed a higher number of seeds (SDN; 5.27). The variation of qualitative traits failed to follow a specific pattern between the two species except for SSH.

Correlation analysis revealed significant correlation between several cases of both species (fig. 2). The left triangle in figure 2 indicates the correlation status between quantitative and qualitative traits of pod morphology in *M. rigiduloides*. The correlations between pod morphology traits and ecological factors were mainly negative or non-significant at a 0.05% level; however, the correlation of precipitation with adpression of fruits (FAP) was strongly positive. Furthermore, the correlations between LSL and ASP, FLT, SDN, STN, FDM, and PGD were positive similar to the relationship of SSH with PSH and NMC. Despite the extreme similarity of *M. rigiduloides* and *M. rigidula*, their responses to ecological variables were different. The correlation matrix of *M. rigidula*, the right triangle in Figure 2, displays the relatively positive correlation between altitude and FLT, SBT, SLT, and MCT. The average temperature and precipitation indicated a significant positive relationship with LSL. Additionally, the seed morphological characters (such as SLT and SWT) showed positive correlations with STN, SDN, FDM, and FAP. Correlation between SSH and average temperature, precipitation, and presence of glandular hair (PGH) was also significant.

Cluster and principal component analyses

In the clustering analysis based on pod morphology traits, populations were grouped into two main clusters, following the species identity and geographical origin to a large extent (fig. 3). Cluster A mainly encompassed *M. rigiduloides* populations consisting of two sub-clusters where in A1 and A2, populations of western and southwestern Iran including Marivan, Paveh, Khormabad, Spiddasht, Dorood, Ravansar, and Shourab grouped. In cluster B, populations of *M. rigidula* are grouped, which is divided into two sub-clusters, subsequently. The first sub-cluster B1, includes populations of Nur, Germi, and Ardebil; second sub-cluster B2, includes populations of

Kaleybar, Rourbar, and Ahar. Meshkinshahr population, which was identified initially as *M. rigiduloides* however, clustered with *M. rigidula* populations. The clustering pattern illustrated in fig. 3 is chiefly in accordance with geographical proximity. The difference in pod morphology of the two species is further presented in fig. 4 for visual comparison.

Similarly, PCA analysis (fig. 5) revealed a grouping pattern in which western and southwestern populations presumably belonging to *M. rigiduloides* are grouped and occupy the upper part of the chart (see fig. 1 for localities). Assessing the relationship between geographical distance and morphological diversity matrices revealed the absence of a strong correlation (R^2: 0.052; fig.6). However, their relationship was relatively linear but not significant, indicating the pattern of variation among morphological traits mainly affected by species identity of the populations rather than the geographical location.

ITS region

The dataset developed based on ITS region consisted of 460 characters, of which 304 characters were constant (66%), 66 were parsimony-uninformative, and the remaining 90 characters were parsimony-informative. The strict consensus tree of 63 accessions had a length of 271 steps with a consistency index (CI) of 0.683 and retention index (RI) of 0.849. The accessions were divided into two main clades where all samples of *M. rigidula* complex formed a monophyletic clade with relatively low posterior probability (PP) of 0.64 and bootstrap support (BS) of 58% with *M. sinskiae* as sister (fig. 7, Clade B). This clade showed a robust PP support of 1.00 and BS of 90% sister to *M. constricta*. *Medicago rigidula*, *M. rigiduloides*, *M. sinskiae* and *M. constricta* collectively form the *M. rigidula* complex (Mehregan & al., 2002).

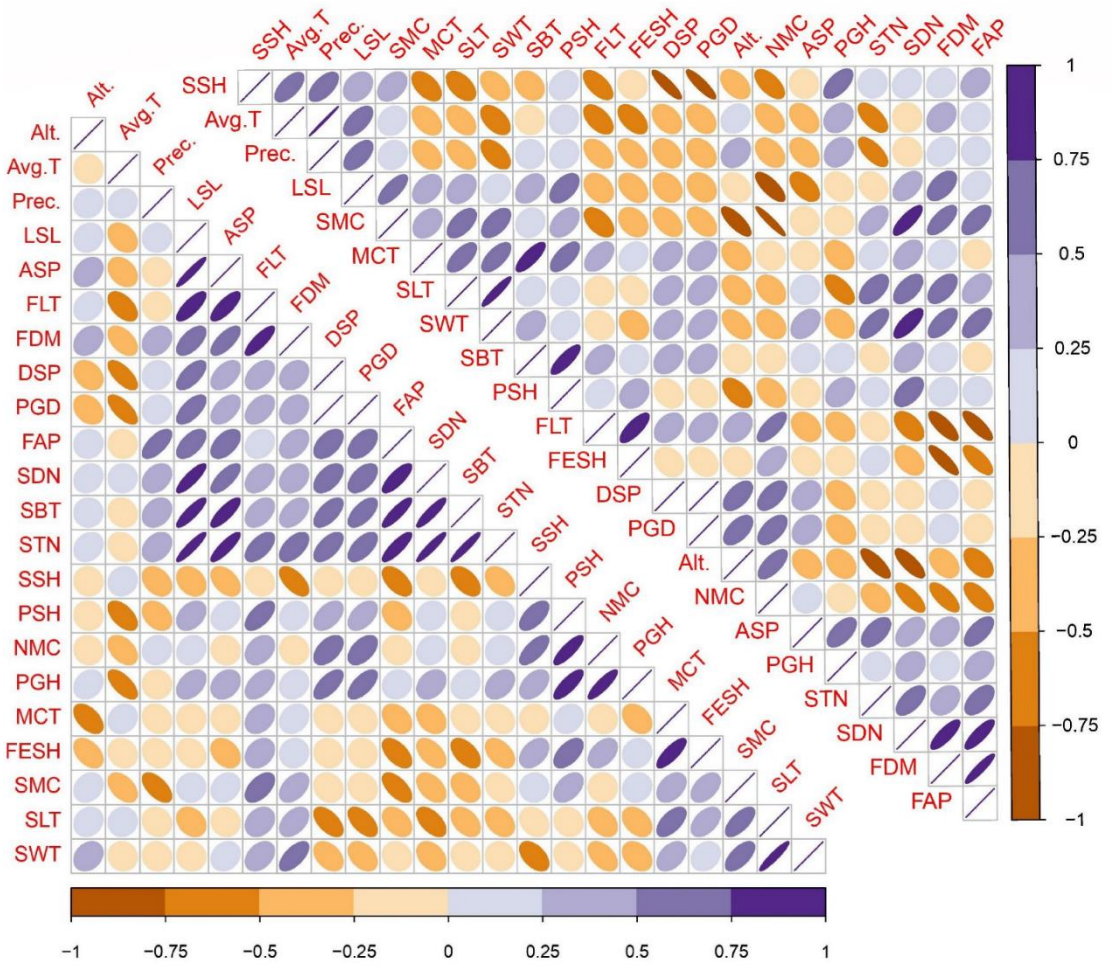

Fig. 2. Correlation analysis of quantitative and qualitative traits and ecological variables in *M. rigiduloides* (left triangle) and *M. rigidula* (right triangle). Different colors and shapes are used in accordance with the amount of correlation.

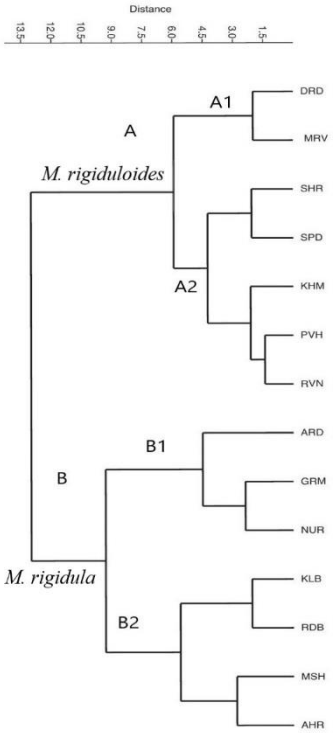

Fig. 3. Cluster analysis of *M. rigidula* and *M. rigiduloides* populations using Ward's method based on morphological data.

Fig 4. Morphological variation in pods of *M. rigiduloides (A)* and *M. rigidula* (B).

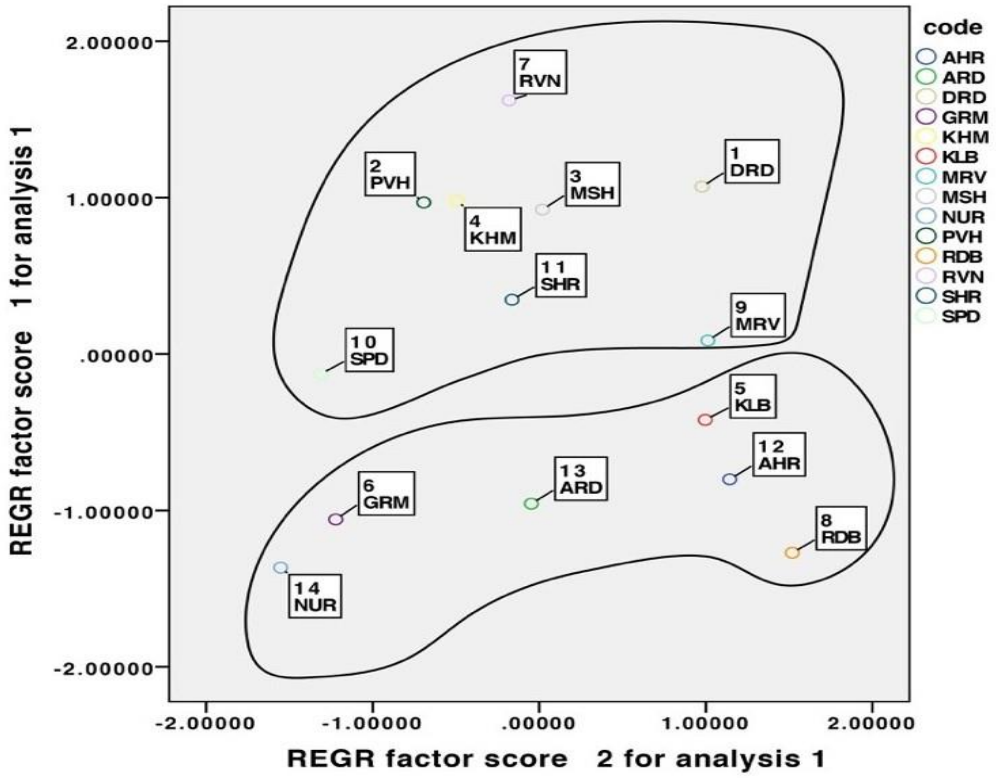

Fig. 5. Two-dimensional principal component analysis (PCA) of 14 populations of *M. rigidula* and *M. rigiduloides* based on 19 quantitative and qualitative morphological traits.

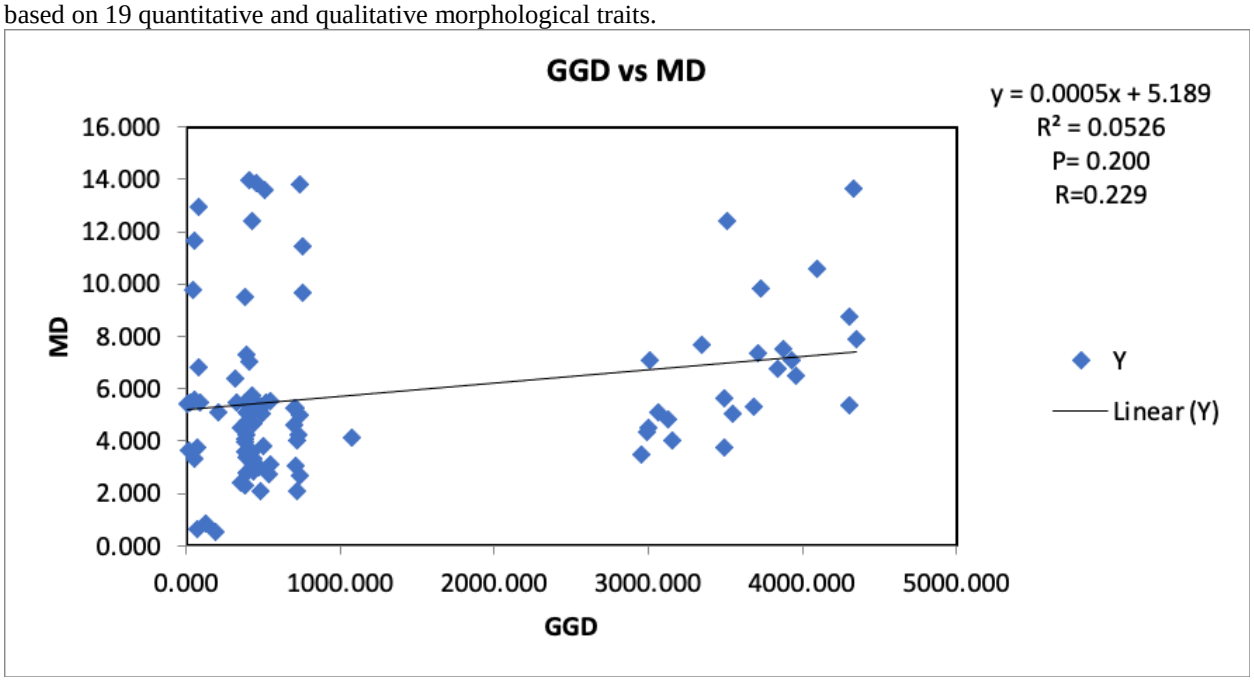

Fig. 6. Correlation between Euclidean distance of morphological traits (MD) and Geographic distance (GGD) using Mantel test.

Fig. 7. Phylogram based on the Bayesian analysis of ITS DNA sequences. The numbers above the branches are Posterior Probabilities (PP), and below the branches are bootstrap supports (%) of those branches reconstructed in the Maximum Parsimony (MP) analysis. MP tree based on the same ITS database is shown on below right Bootstrap values on the clades. Each accession taken from the Genbank is followed its Genbank accession number.

DISCUSSION

Small (2011) suggested that pod morphology traits, in particular shape of spines (SSH), length of longest spine (LSL) and number of coils (NMC) are critical characters to identify the two notably close species of *M. rigidula* and *M. rigiduloides*. This study confirmed that these were the most discriminating characters, useful for identification. In contrast, the other characteristics, such as the size and shape of seeds, were not notably discriminative (table 4). Despite reporting the importance of size of seed characters in differentiating *M. rigidula* populations, Medoukali & al. (2015) did not measure the important key features in this species' populations. The coefficient variation as an essential reflection of diversity of traits indicated that overall morphological diversity in *M. rigidula* was higher than *M. rigiduloides*. The results of this study can also on one hand indicate the reliability and stability of the three key characters LSL, NMC, and SSH under different environmental condition and on the other hand explain the considerable variability in pod morphology observed here. This is the most concerned attempt to date to determine the geographical distribution range of *M. rigidula* and *M. rigiduloides* in Iran. Indeed, there is no previous similar attempt to study the way any of the species of *Medicago* partition their areas of occupation at the interfaces of their natural distribution ranges. The results presented here suggest that the two species strongly tend to occupy different geographical ranges, probably because of different adaptation, although this needs to be confirmed. The results also are inconsistent with the Mehregan & al. (2002) treatment of *M. sinskiae, M.canstricta*, *M. rigidula*, and *M. rigiduloides* as one species, but to some extent confirm the clinal variation of *M. rigidula* from Europe to Asia as the populations in north and northwest identified as *M. rigidula*.

Monophyly of the *M. rigidula* complex is illustrated in some previous studies (Maureira-Butler & al. 2008; Yoder & al. 2013; Zareei & al. 2020). In contrast to the pod morphology, the ITS region was unable to clearly differentiate two species *M. rigidula* and *M. rigiduloides* (fig 7). Owing to the high mutation rate and lineage sorting, the ITS region often has been highly influential in delimitating species in angiosperms compared to other barcoding techniques such as cpDNAs (Hu & al. 2016; Lu & al. 2016; Yi & al. 2013). However, this marker is known to fail in differentiationg closely related species and species complexes. The inconsistency observed between ITS region and pod morphology structure in several studies can also be explained by two possible factors: hybridization and introgression and deep coalescence (Degnan & Rosenberg 2009; Lemmon & Lemmon 2013; Ren & al. 2015). Among closely related species with not completely isolated reproduction, hybridization or introgression can occur, causing accelerated introgression of the concerted evolution of the nuclear genes, capable of creating disconcert in phylogenetic relationships of closely related species (Abbott & al. 2010; Mallet 2007). The ongoing hybridization between lineages of *Medicago* has been found to exist (Maureira-Butler & al. 2008). Although hybridization has been blamed for incongruent placement of *Medicago* spp. (Chen & al. 2021) but Lesins & Lesins (1963) reported a significant reduction in pollen viability in crossing produced from *M. rigidula* × *M. rigiduloides*; therefore, the influence of hybridization in the absence of clear phylogenetic separation of these two species might be weak.

We here suggest that the use of other nuclear markers such as ETS (external transcribed spacer) and microsatellites beside improving sampling size in the future studies may be an essential factor in producing additional clarification of these two species.

CONCLUSIONS

This is the first study to document in *Medicago* how two species partition their distributions where they meet. Most of the annual species of *Medicago* are very weedy, and often congregate together, making it impossible to assess precisely their original distribution ranges. However, *M. rigidula* is known to be primarily European and *M. rigiduloides* is primarily Asian. Northwestern Iran seems to be a primary region where they meet. Our analysis suggests that the two species appear to remain mostly geograpahically separated, perhaps because of different ecological adapation, but this remains to be verified.

The present study also intended to examine the genetic relationships of the two taxa in northewestern Iran, given that this is a meeting area of the two species, suggesting the possiblity of genetic interchange and weakening of the morphological distinctiveness of the two species by introgression in Iran. However, the morphological characters known in the literature to distinguish the two species elsewhere were found to also reliably distinguish the two species in Iran.

ACKNOWLEDGMENTS

We would like to thank Prof. Ziba Jamzad and two anonymous reviewers for their valuable comments on the earlier versions of the manuscript. Thanks are due to the authorities of Islamic Azad university, Science and Research Branch for providing lab facilities.

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