UDC 575.630
https://doi.org/10.2298/GENSR2002787K
Original scientific paper

MICRO-MORPHOLOGY AND MOLECULAR INVESTIGATIONS IN SOME *CARDUUS* SPECIES IN IRAN

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Kazemian J., M. Sheidai, F. Koohdar, H. Azizi, S. Ghasemzadeh-Baraki (2020). *Micro-morphology and molecular investigations in some Carduus species in Iran*-.Genetika, Vol 52, No.2, 787-803.

The genus *Carduus* L. comprised about 90 species, some of which are medicinally important and have high content of flavonoids, and phenolic acids. There are about nine *Carduus* species growing in Iran, out of which one species is endemic. Biosystematic investigation of the genus has been confined to morphological and palynological studies only and we do not have information on molecular study, species relationships and genetic structure of *Carduus* taxa in the country. We therefore, carried out multiple data set analyses of these species for the first time by using micro-morphological and molecular characteristics and provide data on species diversification and their affinities for the first time. The results showed that micro-morphological data can differentiate *Carduus* species, while the species relationship obtained by different approaches may disagree in some points. The results suggest that complexity observed in the taxonomy of the genus *Carduus* may be due to historical introgression events occurred between nuclear and chloroplast genomes during species diversification.

Keywords: Carduus L., micro-morphology, ISSR, phylogeny, introgression.

INTRODUCTION

Tribe Cardueae (thistles) with 2400 species forms one of the largest tribes in the family Asteraceae, with representatives in almost every continent. The greatest species richness of Cardueae occurs in the Mediterranean region where it forms an important element of its flora (BARRES *et al.*, 2013).

The genus *Carduus* L. is native to the Old World but its present-day distribution extends to Europe, central Asia, East Africa, Australia and, South America (DESROCHERS *et al.*, 1987).

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The cardueae are traditionally subdivided into the four subtribes Echinopinae, Carlininae, Carduinae, and Centaureinae (HOFFMANN, 1890). This classification emphasizes the close relationship between Carduinae and Centaureinae. The smaller subtribes Echinopinae and Carlininae are regarded as basal within the Cardueae, whereas the Carduinae and Centaureinae together probably constitute a more advanced monophyletic group (BREMER and ANDERBERG, 1994; SUSANNA *et al.*, 1995).

The subdivision of this large group into Carduinae and Centaureinae is not entirely satisfactory. While there is hardly any doubt about the monophyly of the Centaureinae (SUSANNA *et al.*, 1995), the Carduinae are most likely paraphyletic assemblage. The phylogenetic relationships between the genera within Cardueae are unclear. DITTRICH (1970, 1977) has proposed a subdivision of the Carduinae into four groups based on morpological characters, which is not phylogenetic and some of the groups are certainly artificial. This classification contradicts with the conclusions of previous phylogenetic studies of the Cardueae (BREMER, 1994; PETIT, 1997) and therefore, a better understanding of the diversification processes within the Carduinae+Centaureinae is needed (HÄFFNER and HELLWIG, 1999).

The generic delimitations and the monophyly of some of the larger genera within the Carduinae are also uncertain and require more detailed investigation. Many complexes within this group consist of one large genus and several smaller satellite genera, which are closely related to the respective large genus but differ conspicuously in one or another character. A special case is the *Carduus-Cirsium* group, because this complex consists of two closely related large genera and some smaller ones (HÄFFNER and HELLWIG, 1999). It is not clear whether *Carduus* and *Cirsium* are sister groups or whether at least one of them is paraphyletic or even polyphyletic. Their separation is mainly based on a single character (scabrous or plumose pappus bristles respectively) and is often regarded as artificial (e.g. BREMER, 1994; HÄFFNER and HELLWIG, 1999). Close relationship between *Carduus* species from tropical East Africa and *Cirsium* was reported based on morphological and molecular data (HÄFFNER and HELLWIG, 1999; BARRES *et al.*, 2013).

According to the relationships obtained within the clade containing *Carduus* and *Cirsium* plus the monotypic genera *Notobasis* and *Tyrimnus* (BARRES *et al.*, 2013), thegenera *Carduus* and *Cirsium* are not monophyletic groups. The genus *Carduus* is monophyletic only when *Carduus* subg. Afrocarduus Kazmi is excluded.

The genus *Carduus* L. comprised about 90 species of which eight of them are present in Iran. From these only one species is endemic (AZIZI *et al.*, 2013). These species are of medicinal importance for example, *Carduus thoermeri*, *Carduus nutans* and *Carduus candicans* ssp. *globifer* have high content of flavonoids, while *Carduus armatus* has highe content of phenolic acids (ZHELEV *et al.*, 2013).

Biosystematic investigation of the genus has been confined to morphological and palynological studies only (AZIZI *et al.*, 2013), and we do not have information on molecular phylogeny, species relationships and genetic structure of *Carduus* taxa in the country. We therefore, carried out a multiple data set analyses of these species for the first time by using micromorphological and molecular characteristics and provide data on species diversification and their affinities for the first time.

MATERIALS AND METHODS

Plant materials

Plant specimens of 8 *Carduus* species were used for micro-morphological and ISSR studies. The species studied are presented in Table1.

Table1. Carduus species in micro-morphological and ISSR studies.

No	Species	No	Species
1	*C. arabicus*	5	*C. seminoudus*
2	*C. hamulosus*	6	*C. thoermeri*
3	*C. onopordioides*	7	*C. transcaspicus*
4	*C. pycnocephalus*	8	*C. getolos*

Micromorphology

For micro-morphological study we used seven *Carduus* species (Table1). We used AZIZI *et al.,* (2013) published data on pollen characteristics of the studied *Carduus* species to compare with the species relationships obtained based on seed micro-morphological data.

Seed samples were attached to aluminum stubs with double-sided cellophane tape and coated with a goal. The specimens were examined with a Phillips × L20 SEM. UTHSCSA Image Tool Version 3.0 was used to carry out required measurements.

Seed characters were randomly measured by using a minimum 10 seed and the means were used in phenetic analyses. Seed characters studied were: 1- qualitative characters: seed length (mm), seed width (mm), nectar length (mm), nectar width (mm), ring diameter (mm).

2- Quantitative characters: seed shape, seed symmetry, kurtosis, surface of Seed, ornamentation, surface of the Seed epidermis cell, anticlinal cell walls of the Seed epidermis, periclinal cell walls of the Seed epidermis.

Data were standardized (Mean = 0, variance = 1) and used for multivariate analyses. UPGMA (Unweighted paired groups using average method) clustering based on Euclidean distance was used for grouping of the accessions after 100 times bootstrapping (PODANI, 2000). Different ordination methods were applied on standardized data like PCA (Principal component analysis) to group the plant specimens. Data analyses were performed by PAST ver. 2.17 (HAMMER *et al.*, 2012).

ISSR Assay

For ISSR studies, the fresh leaves were randomly collected from 70 plants in the studied area and were dried in silica gel powder. The genomic DNA was extracted using CTAB-activated charcoal protocol (KRIŽMAN *et al.,* 2006). The extraction procedure was based on activated charcoal and polyvinylpyrrolidone (PVP) for binding of polyphenolics during extraction and under mild extraction and precipitation conditions. This promoted high-molecular-weight DNA isolation without interfering contaminants. Quality of extracted DNA was examined by running on 0.8% agarose gel.

Eight ISSR primers, UBC 807 (AG AG AG AG AG AG AGAGT), UBC 811(GAG AG AG AG AG AG AGAC), CAG(GA)7, (CA)7AC, (CA)7AT, (CA)7GT (GA)9A, and (GA)9T, commercialized by the University of British Columbia, were used (GODWIN *et al.,* 1997). Amplification reactions were performed in a Techne thermocycler (Germany) with the following program: 5 min for initial denaturation step at 94°C, 30 s at 94°C, 1 min at 57°C, and 1 min at 72°C. The reaction was completed by a final extension step of 7 min at 72°C. The amplification products were visualized by running on 2% agarose gel, followed by ethidium bromide staining. The fragments size was estimated by using a 100-bp molecular size ladder (Fermentas, Germany). The experiment was replicated 3 times and constant ISSR bands were used for further analyses.

The ISSR bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). The number of private bands versus common bands was determined. Genetic diversity parameters like: The percentage of allelic polymorphism, allele diversity (WEISING, 2005), Nei' gene diversity (He), and Shannon information index (I) (WEISING, 2005), were determined. We used GenAlex 6.4 for these analyses (PEAKALL and SMOUSE, 2006).

The Nei genetic distance (WEISING, 2005) was determined among the studied populations and was used for the grouping of the genotypes. Genetic differentiation of the studied populations was studied by AMOVA with 1000 permutations as performed in GenAlex 6.4 (PEAKALL and SMOUSE, 2006).

Networking method was used to study the species relationship and species delimitation by using Splits Tree4 program (HUSON and BRYANT, 2006).

Genetic structure of the populations was studied by model-based clustering as performed by STRUCTURE software ver. 2.3 (PRITCHARD *et al.,* 2000). We used the admixture ancestry model under the correlated allele frequency model. A Markov chain Monte Carlo simulation was run 20 times for each value of K (1-10) after a burn-in period of 105. Data were scored as dominant markers and analysis followed the method suggested by FALUSH *et al.* (2007).

ITS Sequences Analyses

For molecular phylogeny studies we also used DNA sequences (ITS1, 5.8.S, ITS2 region) published in NCBI (National Center for Bioinformatic Information). The accession numbers of taxa used have been provided (Table2).

The sequences were aligned by MUSCLE program as implemented in MEGA 7 (TAMURA *et al.,* 2012). Model test was performed as implemented in MEGA which revealed Kimura 2-parameters model as the best fit to our data.

Maximum parsimony tree was constructed followed by TBR branch swapping as implemented in PAUP software ver. 4 (SWOFFORD, 2002) with 1000 times bootstrapping. HGT tree obtained by T-Rex after 999 permutations.

BEAST v1.6.1 (DRUMMOND *et al.,* 2010a; DRUMMOND *et al.,* 2010b) was used for the Bayesian MCMC inferred analyses of the nucleotide sequence data (DRUMMOND and RAMBAUT, 2007).

BEAUti (Bayesian Evolutionary Analysis Utility version) v1.6.1 (DRUMMOND *et al.,* 2010a, 2010b) was utilized to generate initial xml files for BEAST. A Yule process of speciation (a 'pure birth' process) was used as a tree prior for all the tree model analyses.

Table2. Carduus and Cirsium species in phylogeny studies.

No	Species	Accession number	NO	Species	Accession number
1	*Cirsium canovirens*	AF443688	24	*C. nutans* subsp. *falcatoincurvus 3*	**KT013067.1**
2	*Cirsium congdonii*	AF443690.1	25	*C. acanthoides x Carduus nutans*	**KT013068**
3	*Cirsium tioganum*	AF443721.1	26	*C. candicans 1*	**KT363906.1**
4	*Carduus hamulosus* subsp. *hamulosus*	KT013062.1	27	*C. candicans 2*	**KT013061.1**
5	*Carduus hamulosus* subsp. *hystrix*	KT013063.1	28	*C. candicans* subsp. *globifer*	**KT013060.1**
6	*Carduus hamoulosus 1*	KT363909.1	29	*C. transcaspicus*subsp. *macrocephalus*	**KT013066.1**
7	*Carduus hamoulosus 2*	KT363911.1	30	*C. thracicus*	**KT363918.1**
8	*Carduus nervosus*	KT013070	31	*C. adpressus* subsp. *rhodopaeus*	**KT363917.1**
9	*Carduus pycnocephalus*	EF123105.1	32	*C. tmoleus*	**KT013085.1**
10	*Carduus pycnocephalus* subsp. *arabicus*	KT013082	33	*C. tmoleus*	**KT363905.1**
11	*C. pycnocephalus* subsp. *albidus 1*	KT013081.1	34	*C. kerneri*	**KT363913.1**
12	*C. pycnocephalus* subsp. *albidus 2*	KT013080.1	35	*C. carduelis*	**KT363907.1**
13	*C. pycnocephalus* subsp. *breviphyllarius*	KT013083.1	36	*Carduus sp.*	**KT013064.1**
14	*C. rechingerianus*	KT013084	37	*C. acanthoides* subsp. *sintenisii*	**KT013086**
15	*C. corymbosus*	AY780400	38	*C. crispus 1*	**EF010530.1**
16	*C. argentatus*	KT013058.1	39	*C. crispus 2*	**KT363908.1**
17	*C. onopordioides* subsp. *turcicus*	KT013079	40	*C. amanus*	**KT013057.1**
18	*C. onopordioides* subsp. *onopordioides*	KT013078.1	41	*C. personata*	**KT363915.1**
19	*C. nutans*	AY780401.1	42	*C. olympicus* subsp. *olympicus*	**KT013077.1**
20	*C. nutans* subsp. *leiophyllus 1*	EF543521.1	43	*C. olympicus* subsp. *hypoleucus*	**KT013076.1**
21	*C. nutans* subsp. *leiophyllus 2*	KT013072.1	44	*C. adpressus*	**KT013056.1**
22	*C. nutans* subsp. *leiophyllus 3*	KT363919.1	45	*C. nutans* subsp. *trojanus*	**KT013073**
23	*C. nutans* subsp. *falcatoincurvus 2*	KT013074.1	46	*C. nutans* subsp. *falcatoincurvus 1*	**KT013075.1**

The Yule tree prior is widely recognized as giving the best-fit model for trees describing the relationships between different species (DRUMMOND *et al.*, 2010a, 2010b) and can be regarded as explaining the net speciation rate (NEE, 2006). For the MCMC analyses, the chain length was

10000000. After discarding 100 trees representing the burn-in, 10000 trees were used for the analyses. The BEAUti xml file was run in BEAST v1.6.1 (DRUMMOND *et al.*, 2010a, 2010b). Because no fossils are available for the studied species, we assumed a rate of evolution of the plastid sequence ($u = 1.0 \times 10^{-9}$ s s^{-1} $year^{-1}$; ZURAWSKI *et al.*, 1984; MINAEIFAR *et al.*, 2016). This was included in the option of molecular clock model in BEAUti v1.6.1. The normal distribution (Mean = 0, St. dev = 1) was used for priors.

Tracer v1.5 (DRUMMOND and RAMBAUT, 2007) was used to examine sampling and convergence. Tree Annotator v1.6.1 (DRUMMOND and RAMBAUT, 2007) was used to annotate the phylogenetic results generated by BEAST to form a single 'target' tree (Maximum Clade Credibility tree, MCC) including summary statistics. FigTree v1.3.1 (RAMBAUT, 2009) was used to produce the annotated BEAST MCC tree.

RESULT

Seed data and selected SEM micrographs are presented in Table 3, 4 and Figure 1.

Table3. Qualitative characters studied in Carduus species.

characters / species	length (mm)	width (mm)	nectar\ length(mm)	nectar\ width(mm)	ring diameter(mm)
C.arabicus	4.56	1.8	0.66	0.54	1.13
C.hamulosus	4.07	1.84	0.38	0.64	1.3
C.onopordioides	4.55	1.9	0.4	0.6	1.39
C.pycnocephalus	5.66	2.04	0.43	0.49	1.52
C.seminodus	3.72	1.71	0.39	0.58	1.34
C.thoermeri	3.62	1.59	0.56	0.3	1.06
C.transcaspicus	4.7	2.01	0.3	0.74	1.36

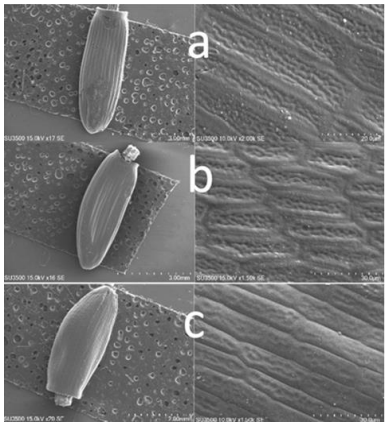


Figure 1. General appearances & exine ornamentations in SEM micrographs of seed: *C.pycnocephalus* (a), *C.arabicus* (b) *C.hamulosus* (c).

Table4. Quantitative characters studied in Carduus species.

Characters species	1	2	3	4	5	6	7	8
C.arabicus	narrowly oblong	±symetric	compressed	furrowed	scrobiculate-faveolate	pitted	angular	Concave or striate
C.hamulosus	narrowly oblong	asymetric	±compressed	striate	scrobiculate-foveolate	pitted	striate or angular	Concave
C.onopordioides	narrowly oblong	asymetric	±compressed	striate	scrobiculate-foveolate	pitted	angular	striate
C.pycnocephalus	oblong	±symetric	compressed	furrowed	scrobiculate-foveolate	pitted	striate or angular	striate
C.seminodus	oblong	asymetric	±compressed	striate	scrobiculate-foveolate	pitted and depressed	striate or angular	Concave
C.thoermeri	oblong	asymetric	compressed	furrowed	scrobiculate-foveolate	flat or depressed	angular	striate
C.transcaspicus	oblong	asymetric	compressed	striate	scrobiculate-foveolate	pitted	striate	Concave or striate

1-seed shape, 2-seed symmetry, 3- kurtosis, 4-surface of cypsela, 5-ornamentation, 6-surface of the cypselar epidermis cell, 7-anticlinal cell walls of the cypselar epidermis, 8-periclinal cell walls of the cypselar epidermis.

PCA analysis of seed morphological data (Figure2) revealed that the first two PCA components comprised about 61% of total variation. In the first component with about 36% of total variance, characters like seed width, umbo width and length, as well as ornamentation of epidermal cells are the most variable characters with the highest correlation to this axis (>0.70). The first PCA axis separates *Carduus thoeromeri* from the other studied species. Similarly, in the second PCA components with about 25% of total variance, characters like seed length, seed symmetry, and ornamentation of seed surface had the highest correlation (>0.70) and are the most variable characters among the studied species. The second PCA axis separates *C. arabicus* on one side, and three species of *C.hamulosu*s, *C. onopordioides* and *C. Seminodus* in the other side of PCA plot. These results are in agreement with the studies of AZIZI *et al.* (2013).

We used AZIZI *et al.* (2013) published data on pollen characteristics of the studied *Carduus* species to compare with the species relationships obtained based on seed micro-morphological data (Figure 3). These two kinds of data produced differed grouping of the studied taxa. For

example, close affinity between *C. arabicus*, *C. onopordiodes*, and *C. transcaspicus* shown by seed data, is not supported by pollen data.

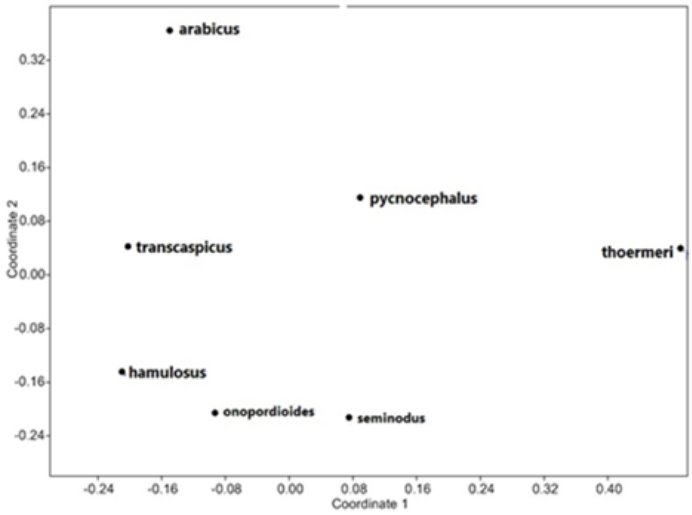


Figure 2. PCA plot of *Carduus* species studied based on seed micro-morphological features.

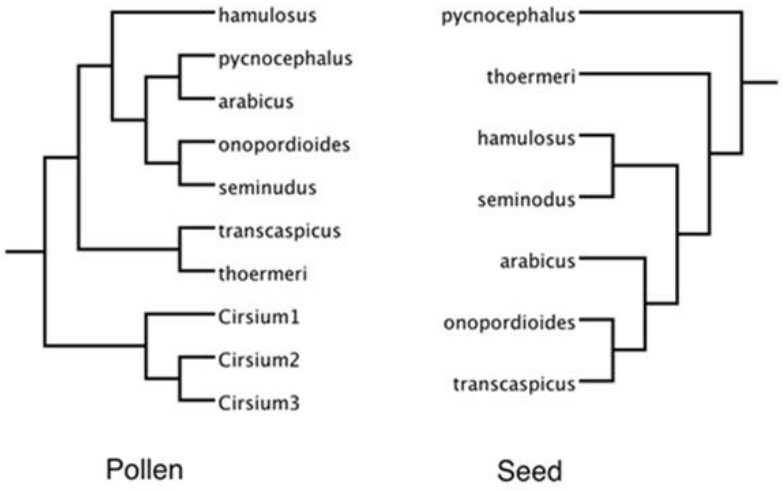


Figure 3. Comparison of seed and pollen data UPGMA trees in the studied *Carduus* species.

ISSR data

In total 51 ISSR bands were obtained in the studied *Carduus* species. Details of these bands are provided in Table5. Some bands were common in the studied taxa and are shared ancestral alleles, while some private bands occurred in *C. pycnocephalus* (13 bands) and *C. arabicus* (2 bands). These bands might have occurred during species diversification.

Table 5. ISSR bands in the studied Carduus species (the numbers are based on table1).

Species	1	2	3	4	5	6	7
No. Bands	32	29	31	13	24	24	30
No .Bands Freq%5 =< .	32	29	31	13	24	24	30
No .Private Bands	13	0	0	0	0	0	2
No .LComm Bands(%25=>)	2	1	1	0	0	0	1
No .LComm Bands(%50=>)	4	7	9	2	4	4	6
Mean He	0.16	0.19	0.23	0.06	0.11	0.15	0.15

The genetic diversity parameters determined in the studied *Carduus* species (Table 6) revealed that *C. seminodus* has the lowest percentage of genetic polymorphism (17.65), while *C. hamulosus* has the highest percentage (60.78). The other species have moderate degree of genetic polymorphism ranging from 45% to 56% (Due to single specimen studied in *C. getolos*, we could not determine genetic polymorphism). The same holds true for gene diversity (H). The mean Nm (No. of migrants) obtained was 0.65, which again indicates moderate gene flow among the studied species. Moderate to high genetic diversity of the studied taxa might be related to their open pollinatiion.

Table 6. Genetic diversity parameters determined in the studied Carduus species.

SP.	**N**	**Na**	**Ne**	**I**	**He**	**uHe**	**%P**
C. pycnocephalus	5	1.13	1.25	0.24	0.16	0.17	50.98
C. thoermeri	5	1.13	1.32	0.29	0.19	0.21	56.86
C. hamulosus	5	1.21	1.40	0.34	0.23	0.25	60.78
C. seminoudus	5	0.43	1.09	0.091	0.06	0.06	17.65
C. transcaspicus	5	0.92	1.16	0.19	0.11	0.13	45.10
C. onopordioides	5	0.92	1.25	0.23	0.15	0.17	45.10
C. arabicus	5	1.05	1.26	0.24	0.15	0.17	47.06
C. getolos	5	0.37	1.00	0.00	0.00	0.00	0.00

Na = No. of alleles, Ne = No. of effective alleles, He = Gene diversity, Uhe = Unbiased gene diversity, %P = Percentage of polymorphism, and Hs = Genetic diversity due to populations.

AMOVA revealed that the species differe significantly in their genetic content (PHIPT = 0.29. P = 0.01). It also reveald that 66% of total genetic variability occurred within species, while 34% was due to between species variation. These values therefore, indicate a high degree of both within species genetic variability as well as between species genetic divergence. Species dilimitatin by ISSR data (Figure 4) revealed that these molecular markers can partially delimit these tax. For example, samples of *C. pycnocephalus* were well differentiated by ISSR data and they were placed close to each other and formed a separate cluster. Similarly, most of the specimens in *C. arabicus* were placed close to each other in a single cluster. The clear cut separation of the other studied species was not observed as they showed some degree of intermixture. Therefore, ISSR molecular markers may be used for *Carduus* species differentiation along with addiotional morphological data.

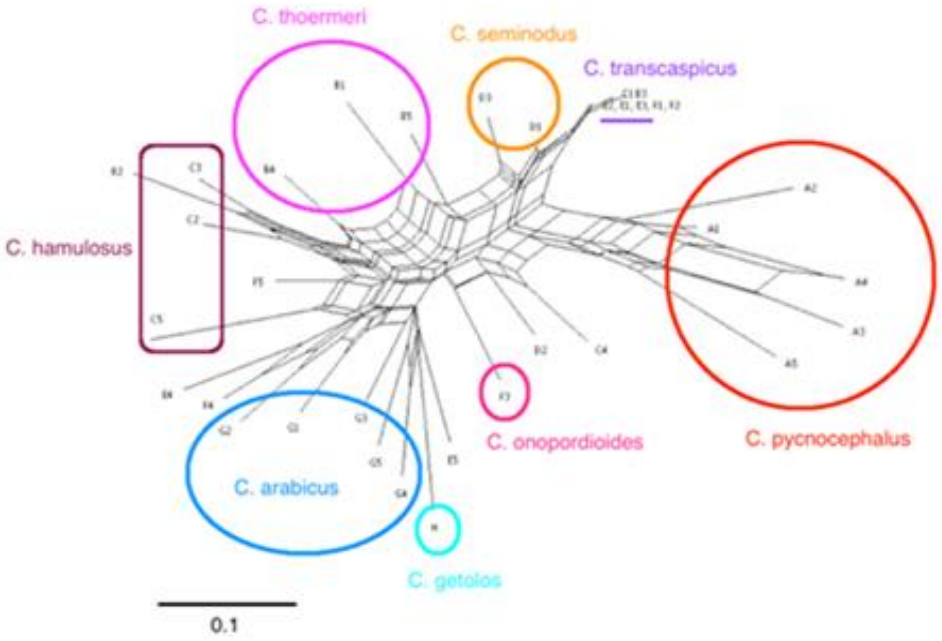


Figure 4. NeighberNet diagram of the studied *Carduus* species based on ISSR data revealing partial species differentiation.

The genetic affinity of the studied *Carduus* species based on ISSR data was determined by STRUCTURE analysis. The STRUCTURE plot obtained (Figure5), revealed that *C. pycnocephalus* and *C. arabicus* differ genetically from the others (having differently colored segments). This is further supported by high Fst values obtained by STRUCTURE analysis for *C. pycnocephalus* (0.93) and *C. arabicus* (0.50). *C. thoermeri* showed some degree of genetic similarity to *C. hamulosus* (have

similarly colored segments), while *C. transcaspicus* has closer genetic affinity to *C. onopordioides* .

STRUCTURE plot also revealed some degree of gene flow among the studied species (similarly colored segments). These are possibly shared ancestral common alleles in the *Carduus* taxa studied. These alleles differed in their frequency (extent of the colored segments) in *Carduus* species, which might happened during genetic restructuring of these taxa during species diversification. The genetic affinity of the studied *Carduus* species based on ISSR data was almost in agrrement with species relationship obtained by seed morphological data. Both analyses showed close affinity between *C. seminodus*, *C. transcaspicus*, *C. onopordiodes*, and *C. arabicus*.

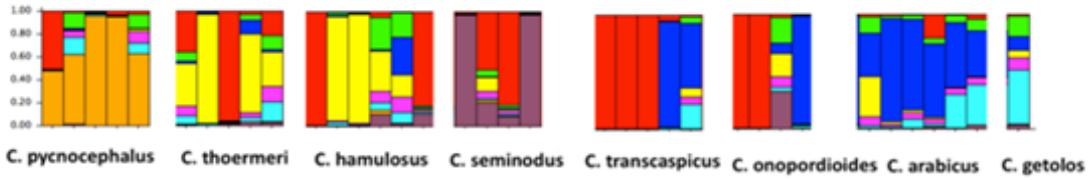
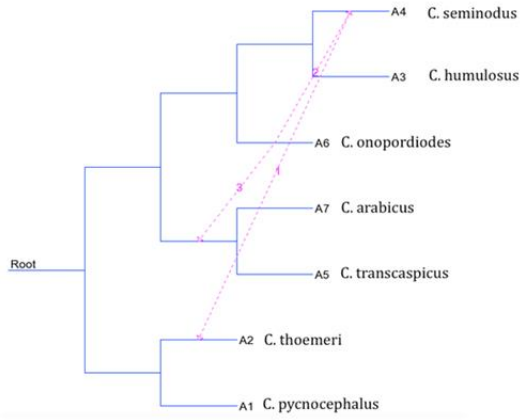


Figure 5. STRUCTURE plot of the studied *Carduus* species based on ISSR data showing their genetic affinities versus genetic difference.

Species molecular phylogeny based on ITS sequence data

In ITS phylogenetic tree obtained (Figure 6), The *Circium* species (outgroup taxa) were separated from *Carduus* species studied. However, AMOVA of ITS sequences between *Carduus* and *Circium* taxa did not produced significant difference (PhiPT =0.25, P = 0.11). This indicates close relationship between the two genera.



nity between *Carduus pycnocephalus*, and *C.* ılt. *C. transcaspicus* joined these two species as well as in seed and pollen-based trees. *C.* of taxa in trees obtained from ITS, ISSR and

ne species showed molecular variability. For *hamulosus*, *C. pycnocephalus*, *C.nutans*, and e phylogenetic tree.

ıay be due to introgression between different ween ISSR and ITS data for the same species between *C. thoemeri* and *C.sminodus*, and events happened between *C. transcaspicus*, C.

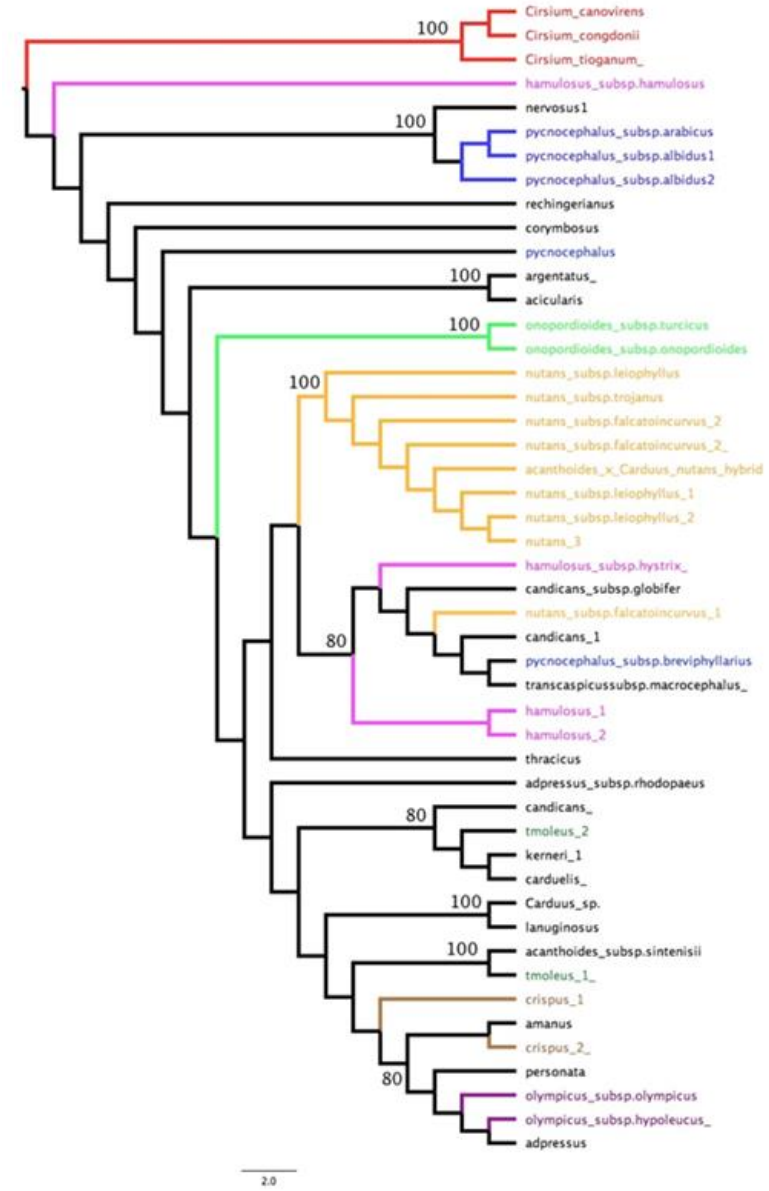


Figure 6. Maximum parsimont tree based on ITS sequences (Numbers above branches are bootstrap value).

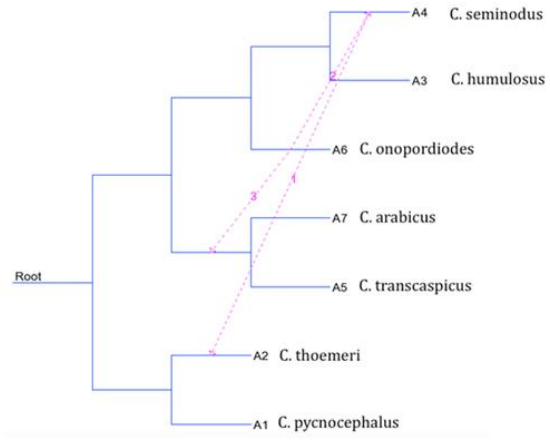


Figure 7. HGT peformed between ISSR and ITS data in *Carduus* species studied (Dashed lines indicate introgression vents).

Species date of divergence

The chronological tree obtained for the *Carduus* species based on ITS sequence data (Figure 8), suggested a probable date of divergence between *Cirsium* and *Carduus* somewhere around 10-11 Mya during Miocene. This tree also suggests that the earlier species divergence within the genus *Carduus* occurred sometime between 5-10 Mya during Pleiocene, while recent active speciation occurred between 1-4 Mya.

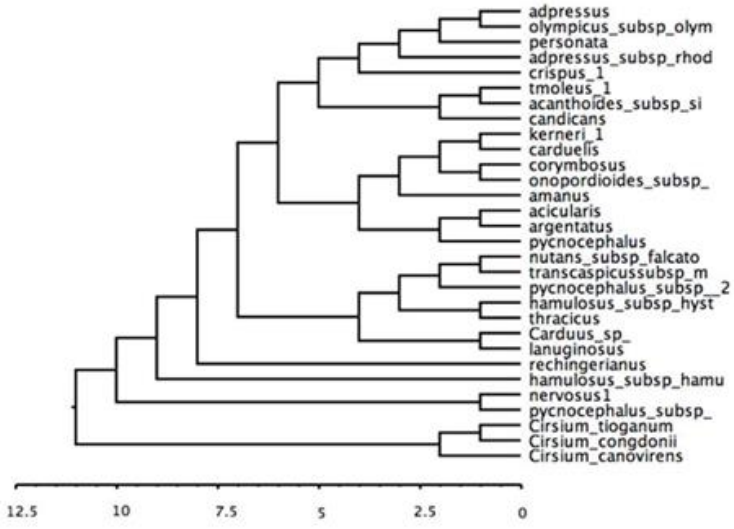


Figure 8. Chronological relax model-based BEAST tree in *Carduus* species based on ITS sequence data.

DISCUSSION

The present investigation revealed that a combination of micromorphological and molecular data could be used in species differentiation within the genus *Carduus*. Species identification and differentiation is an important task for systematic and evolutionary studies. This may be difficult in the genera with overlapping morphological characters among the species, and in those genera that encounter inter-specific hybridization as well as ancient-historic introgression events (SIAHKOLAEE *et al.* 2017).

The bulk of genera within the Cardueae is placed in the subtribes Carduinae and Centaureinae, which together probably form a monophyletic group. Especially within the Carduinae, the phylogenetic relationships are unclear (HÄFFNER and HELLWIG, 1999). HÄFFNER and HELLWIG (1999) used ITS sequence data and believe in the paraphyly ofthe Carduinae and that the genera *Carduus* and *Cirsium* are not monophyletic groups. This is supported by BREMER (1994), he does not agree with generic delimitation within the *Carduus-Cirsium* complex and emphasizes that the separation of *Carduus* and *Cirsium* is possibly not natural as it is based on a single character, scabrous and plumose pappus bristles respectively. Though in the present study, *Circium* species (out group taxa) were separated from *Carddus* species but AMOVA revealed that they did not differ significantly in molecular content and have close affinity.

The chronological tree obtained here suggested that the probable date of divergence between *Circium* and *Carduus* is somewhere around 10-11 Mya during Miocene, while the earlier species divergence within the genus *Carduus* occurred sometime between 5-10 Mya during Pleiocene. Our result is in agreement with the studies of BARRES *et al.* (2013). They used fossil evidence and phylogenetic analyses based on nuclear ribosomal DNA and chloroplast DNA markers and concluded that tribe Cardueae originated around the Mid Eocene in West Asia, which is also the ancestral area of most subtribes within Cardueae. Diversification within each sub tribe began during the Oligocene-Miocene period. Most diversification events within Cardueae are related to the continuous cycles of area connection and division between the Anatolian microplate and the western Mediterranean Basin during the Oligocene- Miocene and with the uplift of the Himalayan range from the Miocene onward. From these two regions, thistles dispersed and colonized the rest of the continents (e.g., the New World, Africa, and Australia), most likely during the colder Pliocene-Pleistocene Period.

In general, the present study produced some new information on the use of micro-morphological and molecular data for species differentiation within the genus *Carduus* and also supported divergence date for *Carduus-Circium* split some to be in Miocene.

Fossil records for Cardueae are rare, and the earliest of the known records is pollen of *Cirsium* from the early Pliocene (GRAHAM, 1996). It was hypothesized that the divergence of *Cirsium* and its sister *Carduus* occurred before the early Pliocene, and constrained 5.3 Ma as a minimum age to the stem node of*Cirsium*. At the same time, the stem age of Cardueae was estimated at 29–24 Ma in a recent dating of Asteraceae (Kim et al., 2005) and therefore this dating was used to calibrate the divergence of Cardueae from Tarchonantheae.

Combined analyses of nuclear ITS and plastid trn L-F and psbA-trnH fragments.

The ILD test showed that the two data sets, the nuclear rDNA ITS vs. the combination of the plastid trnL-F and psbA-trnH, were fully congruent, and it is therefore justifiable to combine them in a single data set for further analysis. The combined sequence matrix comprised 1848

characters after alignment, of which 189 sites were variable but uninformative, and 226 sites were variable and potentially parsimony-informative when gaps were treated as missing characters.

The heuristic search resulted in 78 trees (length. 1002 steps; CI. 0.555; RI. 0.553). Their strict consensus produced an identical topology to the majority consensus tree of the Bayesian inferences. The ML tree (TIM fl IflG model, lnL .8256.4629) also yielded a similar topology. The analyses of the combined data set confirmed all identified clades in both Figs 2 and 3, and most of them received elevated support.

Divergence times Because the ITS data matrix includes most representative genera of Cardueae, with a coverage of the wide morphological range of this tribe, only this data set was used to infer origin time scales of the Himalayan genera Fossil records for Cardueae are rare, and the earliest of the known records is pollen of *Cirsium* from the early Pliocene (GRAHAM, 1996). It was hypothesized that the divergence of *Cirsium* and its sister *Carduus* occurred before the early Pliocene, and constrained 5.3 Ma as a minimum age to the stem node of *Cirsium*. At the same time, the stem age of Cardueae was estimated at 29–24 Ma in a recent dating of Asteraceae and therefore this dating was used to calibrate the divergence of Cardueae from Tarchonantheae (Figure 2). The DIVTIME command of the r8s program with the cross-validation function enforced identified the smoothing parameter value of 1.6 as the optimal and it was then selected to execute the PL estimates. The results indicated that the divergences of *Dolomiaea*, *Diplazoptilon* and *Xanthopappus* from their sisters probably occurred between 13.6 and 12.2 Ma, 2.0 and 1.6 Ma or 5.7 and 4.7 Ma, respectively.

AZIZI *et al.* (2013) showed close morphological affinity between *C. Pycnocephalus* and *C. arabicus*, and also between *C. thoemeri* and *C. onopordiodes*, to which *C. transcaspicus* joined with some distance while, *C. humulus* was placed far from the other species. Close affinity between *C. transcaspicus* and *C. humulus* is in agreement with the ITS result presented here.

Received, August 13th, 2019
Accepted March 18th, 2020

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MIKRO-MORFOLOGIJA I MOLEKULARNA ISTRAŽIVANJA NA NEKIM VRSTAMA *CARDUUS* U IRANU

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Izvod

Rod *Carduus* L. sastoji se od oko 90 vrsta, od kojih su neke medicinski važne i imaju visok sadržaj flavonoida i fenolnih kiselina. U Iranu raste oko devet vrsta *Carduus* carduusa, od kojih je jedna endemska. Biosistematska ispitivanja roda ograničena su samo na morfološka i palinološka ispitivanja i nemamo podatke o molekularnim istraživanjima, odnosima vrsta i genetskoj strukturi *Carduus* taksona u zemlji. Stoga smo prvi put izvršili više analiza podataka tih vrsta koristeći mikro-morfološke i molekularne karakteristike i prvi put pružili podatke o diverzifikaciji vrsta i njihovim afinitetima. Rezultati su pokazali da se mikromorfološki podaci mogu razlikovati unutar vrste *Carduus*, dok se odnos vrsta koji se dobija različitim pristupima u nekim tačkama može ne slagati. Rezultati pokazuju i da složenost primećena u taksonomiji roda *Carduus* može biti posledica istorijskih događaja introgresije koji su se dogodili između genoma jedra i hloroplasta tokom diverzifikacije vrsta.

Primljeno 13.VIII.2019.
Odobreno 18. III. 2020