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Molecular phylogeny and morphometric analyses in the genus *Cousinia* Cass. (Family Asteraceae), sections *Cynaroideae* Bunge and *Platyacanthae* Rech. f.

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Abstract. Taxonomy and molecular phylogeny of the genus *Cousinia* are complicated and unresolved mainly because of disagreement between morphological and molecular phylogenetic studies. The genus *Cousinia* has approximately 700 species, which makes it one of the most varied genera found in central and southwest Asia. Section *Cynaroideae*, containing 89 species, is considered the largest section of the genus. Identification and delineation as well as classifying the section and the species' relationships within the genus *Cousinia* generally remain debatable. Therefore, the present study aims to: 1) identify and delineate the species within the two sections *Cynaroideae* and *Platyacanthae*; 2) study the species relationships based on both morphological and molecular features (Internal Transcribed Spacer (ITS) marker); 3) study the sectional delimitation and its monophyly; and 4) estimate the divergence time of the studied sections. To this end, 50 *Cousinia* species occurring in Iran were investigated for the first time. A maximum parsimony tree of the morphological features separated the species of the two sections from each other. However, the ITS-based phylogenetic tree did not delimit the two studied sections. The relationships among the studied *Cousinia* species in the genetic trees were generally not congruent with the obtained morphological tree. The divergence time of the studied species within the *Cynaroideae* and *Platyacanthae* sections determined using Bayesian Evolutionary Analysis Sampling Trees (BEAST) was estimated to be around 3.5 million years ago (Mya).

Keywords: *Cousinia*, *Cynaroideae*, ITS, Phylogeny, *Platyacanthae*.

INTRODUCTION

The genus *Cousinia* Cass. of the tribe Cardueae (family Asteraceae) has approximately 700 species, which makes it one of the most diverse genera following *Senecio* L. (c. 1500 species) and *Vernonia* Schreb. (c. 1000 species) in central and southwest (SW) Asia (Tscherneva 1962; Rechinger 1972, 1979; Frodin 2004; Attar and Ghahreman 2006; Susanna and Garcia-Jacas 2007; Attar

and Djavadi 2010; Mehregan and Assadi 2016; Minaeifar *et al.* 2016; Rastegar *et al.* 2017, 2018). This genus has the greatest prevalence in the Flora Iranica area, with more than 400 different species distributed in SW Asia, of which, 379 are considered endemic. These species are distributed in mountainous areas of Iran, Afghanistan, and Turkmenistan (Rechinger 1986; Knapp 1987).

The genus *Cousinia* is not considered to be monophyletic, and comprises the *Arctium-Cousinia* complex as well as the genus *Arctium* L. (Susanna *et al.* 2003; Lopez-Vinyallonga *et al.* 2009).

Although the exact number of species in this genus in Iran is in dispute (Attar 2000; Mehregan 2008; Mehregan and Kadereit 2008; Assadi 2009; Attar and Mirtadzadini 2009; Mehregan and Assadi 2009; Attar and Djavadi 2010), approximately 270 *Cousinia* species have been reported of which, almost 200 species are considered endemic (Djavadi *et al.* 2007; Zare *et al.* 2013).

Cousinia species could be taxonomically categorized into 70 sections (Rechinger 1986), with section *Cynaroideae* being the largest section of the genus containing 89 species (Tscherneva 1962; Rechinger 1972, 1979; Huber- Morath 1975; Attar and Djavadi 2010; Rastegar *et al.* 2017, 2018). This section includes species consisting of decurrent leaves and appendiculate bracts (Tscherneva 1962; Rechinger 1972, 1979; Huber- Morath 1975), which are recognized as the Irano-Turkestanian elements (Djamali *et al.* 2012; Dehghani *et al.* 2017). Iran has 77 taxa, 66 of which are endemic, thus appearing to be the variety center of the section (Attar and Ghahreman 2006; Attar and Djavadi 2010).

The extensive morphological variability in the genus renders the taxonomy of *Cousinia* complicated and controversial (De Candolle 1837; Boissier 1875; Winkler 1892, 1897; Tscherneva 1962; Rechinger 1972, 1979; Huber-Morath 1975; Haffner 2000; Susanna *et al.* 2003; Mehregan 2011; Mehregan and Assadi 2016; Mabberley 2018; Atazadeh *et al.* 2020).

There is a controversy over the number of species within a single section, too; for instance, Mehregan and Kadereit (2008), in a taxonomic revision of the section *Cynaroideae*, reduced the number of species occurring in Iran to 31, while Attar and Djavadi (2010) reported 77 *Cousinia* species in this section as present within the country.

The sect. *Platyacanthae* Rech. f. has six species in Flora Iranica and is considered the sister group of the sect. *Cynaroideae* (Lopez-Vinyallonga *et al.* 2009), five of the six species of which are endemic in Iran (Rechinger 1972).

Species identification and delineation as well as classification of the sections and the species relationships within the genus *Cousinia* generally remain debat-

able, even after molecular investigations (Susanna *et al.* 2003; Ghaffari *et al.* 2006; Lopez-Vinyallonga *et al.* 2009; Mehregan and Assadi 2016; Galtier 2019). Therefore, the present study aims to: 1) identify and delineate the species based on differentiating taxonomical features within the two sections *Cynaroideae* and *Platyacanthae*; 2) study the species relationships based on morphological and molecular features (Internal Transcribed Spacer (ITS) marker); 3) study sectional delimitation and its monophyly, and 4) estimate the divergence time of the studied species within the *Cynaroideae* and *Platyacanthae* sections for the first time.

Molecular information has been commonly utilized to create a system for phylogenetic classification. Specifically, the ITS regions are considered as nuclear DNA regions described by parental inheritance patterns and can be changed faster compared with the coding regions, which results in higher levels of disparity among those narrowly-related individuals. Therefore, the ITS regions were used herein to study the interspecific and intergeneric relationships along with developmental styles and patterns in genetic variation (Baldwin 1992; Alvarez and Wandel 2003; Felner and Rossello 2007).

Molecular studies in the two sections *Cynaroideae* and *Platyacanthae* have not been fully performed until now. Therefore, present study has attempted to investigate 50 species of both studied sections based on molecular features (ITS) for the first time and specifically collect new information on molecular phylogeny, evolution, divergence time, and species relationships. These findings can enhance our knowledge of the true evolutionary pathway of the genus *Cousinia*.

MATERIALS AND METHODS

Plant material

Morphological studies were conducted on 150 plant specimens, of which 138 belonged to 46 species of *Cynaroideae* and 12 belonged to 4 species of *Platyacanthae* sections (Table 1). One specimen of each species was used for the ITS marker. The voucher specimens were deposited in the Herbarium of Tehran University (TUH). *Arctium umbrosum* Bung (accession number: AY373745; AY373712) and *Arctium lappa* L. (accession number: EU923773; EU923887) were obtained as out groups based on studies by Susanna and Garcia-Jacas (2007) and Lopez-Vinyallonga *et al.* (2009). ITS sequences for all of the species except for the out groups were newly generated.

Table 1. Investigated *Cousinia* species and their voucher information as well as the accession numbers of taxa in phylogeny studies.

R. Taxa	Section	Locality	Voucher number	Accession number	Abbreviation
1 <i>Cousinia carolihenrici</i> Attar & Ghahreman	<i>Cynaroideae</i> Bunge	Kurdistan	22455 (TUH)	MH992748	carolihenrici
2 <i>Cousinia fursei</i> Rech. f.	<i>Cynaroideae</i> Bunge	Kurdistan-Marivan	18314(TUH)	MH992734	fursei
3 <i>Cousinia millefontana</i> Rech. f.	<i>Cynaroideae</i> Bunge	Kurdistan-Marivan	20227(TUH)	MH971223	millefontana
4 <i>Cousinia concinna</i> Boiss. & Hausskn.	<i>Cynaroideae</i> Bunge	Kurdistan	20562(TUH)	MH992735	concinna
5 <i>Cousinia subinflata</i> Bornm.	<i>Cynaroideae</i> Bunge	Kermanshah	(TUH)	MK005181	subinflata
6 <i>Cousinia hamadanensis</i> Rech. f.	<i>Cynaroideae</i> Bunge	Hamadan - Malayer	46290(TUH)	MK005182	hamadanensis
7 <i>Cousinia barbeyi</i>	<i>Cynaroideae</i> Bunge	Boyer-Ahmad	22494(TUH)	MK005164	barbeyi
8 <i>Cousinia denaensis</i> Attar & Djavadi	<i>Cynaroideae</i> Bunge	Boyer-Ahmad	22495(TUH)	MH992739	denaensis
9 <i>Cousinia sardashtensis</i> Rech. f.	<i>Cynaroideae</i> Bunge	Chahar Mahal& Bakhtiari	20073(TUH)	MK005184	sardashtensis
10 <i>Cousinia dalahuensis</i> Attar & Ghahreman	<i>Cynaroideae</i> Bunge	Kermanshah- Mahidasht	19929(TUH)	MH992747	dalahuensis
11 <i>Cousinia grandis</i> C. A. Mey.	<i>Cynaroideae</i> Bunge	Azarbaijan	21343(TUH)	MH992738	grandis
12 <i>Cousinia grantii</i> Rech. f.	<i>Cynaroideae</i> Bunge	Azarbaijan	22490(TUH)	MK005183	grantii
13 <i>Cousinia gaharensis</i> Attar & Djavadi	<i>Cynaroideae</i> Bunge	Lorestan- Shulabad	38259(TUH)	MK005166	gaharensis
14 <i>Cousinia keredjensis</i> Bornm. & Gauba	<i>Cynaroideae</i> Bunge	Tehran	21807(TUH)	MH992732	keredjensis
15 <i>Cousinia zardkuhensis</i> Attar & Ghahreman	<i>Cynaroideae</i> Bunge	Chahar Mahal& Bakhtiari	21887(TUH)	MH990788	zardkuhensis
16 <i>Cousinia lordeganensis</i> Mehregan	<i>Cynaroideae</i> Bunge	Chahar Mahal& Bakhtiari	46301(TUH)	MK005173	lordeganensis
17 <i>Cousinia elwendensis</i> Bornm.	<i>Cynaroideae</i> Bunge	Hamadan-Alvand Mountains	20566(TUH)	MH992741	elwendensis
18 <i>Cousinia khorramabadensis</i> Bornm.	<i>Cynaroideae</i> Bunge	Lorestan	21851(TUH)	MH992737	khorramabadensis
19 <i>Cousinia phyllocephala</i> Bornm. & Gauba	<i>Cynaroideae</i> Bunge	Lorestan - Khorram Abad	46292(TUH)	MK005168	phyllocephala
20 <i>Cousinia macrocephala</i> C. A. Mey.	<i>Cynaroideae</i> Bunge	Ardebil- Meshkin shahr	42925(TUH)	MH990319	macrocephala
21 <i>Cousinia lurestanica</i> Attar & Djavadi	<i>Cynaroideae</i> Bunge	Lorestan	21824(TUH)	MH992746	lurestanica
22 <i>Cousinia iranica</i> C. Winkl. & Strauss.	<i>Cynaroideae</i> Bunge	Arak	21881(TUH)	MK005174	iranica
23 <i>Cousinia parsana</i> Ghahreman	<i>Cynaroideae</i> Bunge	Hamadan	20553(TUH)	MK005169	parsana
24 <i>Cousinia kornhuberi</i> Heimerl	<i>Cynaroideae</i> Bunge	Hamadan	22372(TUH)	MK005185	kornhuberi
25 <i>Cousinia ecbatanensis</i> Bornm.	<i>Cynaroideae</i> Bunge	Hamadan	22371(TUH)	MH988770	ecbatanensis
26 <i>Cousinia verbascifolia</i> Bunge	<i>Cynaroideae</i> Bunge	Khorasan-Mashhad	43013(TUH)	MK005179	verbascifolia
27 <i>Cousinia disfulensis</i> Bornm.	<i>Cynaroideae</i> Bunge	Lorestan- Khorram Abad	27589(TUH)	MH992742	disfulensis
28 <i>Cousinia shulabadensis</i> Attar & Ghahreman	<i>Cynaroideae</i> Bunge	Lorestan- Shul Abad	21874(TUH)	MH992744	shulabadensis
29 <i>Cousinia sahandica</i> Attar & Djavadi	<i>Cynaroideae</i> Bunge	Azarbaijan	46272(TUH)	MK005175	sahandica
30 <i>Cousinia gilliati</i> Rech. f.	<i>Cynaroideae</i> Bunge	Azarbaijan	21967(TUH)	MK005170	gilliati
31 <i>Cousinia algurdina</i> Rech. f.	<i>Cynaroideae</i> Bunge	Azarbaijan- Tabriz	30533(TUH)	MK005165	algurdina
32 <i>Cousinia cynaroides</i> C. A. Mey	<i>Cynaroideae</i> Bunge	Ardebil	22581(TUH)	MK005167	cynaroides
33 <i>Cousinia kotschy</i> Boiss.	<i>Cynaroideae</i> Bunge	Azarbaijan	46244(TUH)	MK005171	kotschy
34 <i>Cousinia nana</i> Attar	<i>Cynaroideae</i> Bunge	Arak	14347(TUH)	MK005172	nana
35 <i>Cousinia shebliensis</i> Ghahreman	<i>Cynaroideae</i> Bunge	Azarbaijan- Tabriz	20580(TUH)	MK005177	shebliensis
36 <i>Cousinia calocephala</i> Jaub. & Spach	<i>Cynaroideae</i> Bunge	Azarbaijan-Mianeh	46276(TUH)	MH992749	calocephala

R. Taxa	Section	Locality	Voucher number	Accession number	Abbreviation
37 <i>Cousinia behboudiana</i> Rech. f. & Esfand.	<i>Cynaroideae</i> Bunge	Ghazvin	27629(TUH)	MH992736	behboudiana
38 <i>Cousinia kirrindica</i> Bornm. & Rech. f.	<i>Cynaroideae</i> Bunge	Ilam	19711(TUH)	MK005163	kirrindica
39 <i>Cousinia mobayenii</i> Ghahreman & Attar	<i>Cynaroideae</i> Bunge	Kermanshah- Eslamabad	20569(TUH)	MK005180	mobayenii
40 <i>Cousinia sanandajensis</i> Rech. f.	<i>Cynaroideae</i> Bunge	Hamadan	46287(TUH)	MH992743	sanandajensis
41 <i>Cousinia lurorum</i> Bornm.	<i>Cynaroideae</i> Bunge	Kermanshah- Mahidasht	20568(TUH)	MK005176	lurorum
42 <i>Cousinia kurdistanica</i> Attar	<i>Cynaroideae</i> Bunge	Kurdistan- Maryvan	3232(TUH)	MK005178	kurdistanica
43 <i>Cousinia bornmulleri</i> C. Winkl.	<i>Cynaroideae</i> Bunge	Esfahan	22532(TUH)	MH992750	bornmulleri
44 <i>Cousinia farsistanica</i> Bornm.	<i>Cynaroideae</i> Bunge	Kerman	28636(TUH)	MH992733	farsistanica
45 <i>Cousinia lactiflora</i> Rech. f.	<i>Cynaroideae</i> Bunge	Lorestan	46299(TUH)	MK005156	lactiflora
46 <i>Cousinia aligudarzensis</i> Attar & Ghahreman	<i>Cynaroideae</i> Bunge	Lorestan-Aligudarz	27613(TUH)	MH992745	aligudarzensis
47 <i>Cousinia platyacantha</i> Bunge	<i>Platyacanthae</i> Rech. f.	Khorasan	43212(TUH)	MK005187	platyacantha
48 <i>Cousinia freynii</i> Bornm.	<i>Platyacanthae</i> Rech. f.	Semnan- Shahrud	27675(TUH)	MK005186	freynii
49 <i>Cousinia bienerti</i> Bunge	<i>Platyacanthae</i> Rech. f.	Khorasan-Neyshabur	28682(TUH)	MH992740	bienerti
50 <i>Cousinia reshingerorum</i> Bornm.	<i>Platyacanthae</i> Rech. f.	Khorasan-Torbate Jam	39729(TUH)	MH992700	reshingerorum

DNA extraction, amplification and sequencing

Garden-fresh leaves were dried in powder of silica gel. Cetyltrimethyl-ammonium bromide (CTAB) with activated charcoal protocol was used to extract genomic DNA (Murray and Thompson 1980). The quality of the extracted DNA was examined by running on 0.8% agarose (Sheidai *et al.* 2013).

ITS region (ITS1, 5.8S, ITS2) was amplified using 0.2 μ M primer ITS1 (5'- TCCGTAGGTGAACCTGCGG-3'; Bioron, Germany), and primer ITS4 (5'- TCC GCT TATTGA TAT GC -3') (Chen *et al.* 2010). PCR reactions were accomplished in a 25 μ L volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl₂; 0.2 mM of each dNTP (Bioron, Germany); 20 ng genomic DNA; and 3 U of Taq DNA polymerase (Bioron, Germany). The mentioned reactions were amplified in a Techne thermocycler (Germany) by applying the process as follows: 5 min at 94 °C, followed by 35 cycles for 30 s at 94 °C, 1 min at 52 °C, and 1 min at 72 °C, followed by one ultimate extension for 10 min at 72 °C. The process was run on 2% agarose gel to picture the amplification result, followed by the staining with ethidium bromide. A 100-base pair (bp) molecular-sized ladder (Fermentas, Germany) was used to determine fragment size.

Data analysis

Morphological analysis

In all, 19 morphological characteristics were investigated (quantitative and qualitative) and coded (Table 2). After, the obtained data was standardized (mean = 0, variance = 1) and applied to perform multivariate analyses. The species categorization was performed using the WARD (minimum spherical cluster method), UPGMA (unweighted paired group using average), PCoA (principal coordinate analysis), and MDS (multidimensional scaling) methods (Podani 2000). Paleontological Statistics software (PAST), ver. 2.17 (Hammer *et al.* 2012) was used for analysis. Moreover, morphological characteristics were coded for the maximum parsimony (MP) tree, and then created after tree bisection reconnection (TBR) branch swapping by applying them along with bootstrapping 1000 times in PAUP (phylogenetic analysis using parsimony) software, ver. 4 (Swofford 2002).

Molecular analysis

To assess homology, ITS sequences were aligned with MUSCLE implemented in MEGA 7 (Tamura *et al.*

Table 2. Morphological characters and their codes.

Characters	Codes				
	1	2	3	4	5
Head diameter	x<3	3≤x≤6	x>6	-	-
Flowers number	x<80	80≤x≤150	x>150	-	-
Bracts number	x<80	80≤x≤120	x>120	-	-
Appendages length of median bracts	x<9	9≤x≤15	x>15	-	-
Appendages width of median bracts	x< 5	5≤x≤15	x>15	-	-
Corolla length	x< 20	20≤x≤25	x>25	-	-
Habitat	Woodland	Alpine	Steppe	-	-
Leaves indumentum	Present	Absent	-	-	-
Stem leaves	Interruptedly decurrent	Continuously decurrent	Nondecurrent	-	-
Uppermost leaves	Distant from the head	Close to the head	Surrounding the head	-	-
Appendages	Present	Absent	-	-	-
Inner bracts indumentum	Smooth	Scabrous	-	-	-
Position of median bracts	Imbricated	Spreading	Recurved	Spreading-recurved	Imbricated-spreading
Shape of appendages of median bracts	Sagitate	Triangular	Rhombic	Ovate	Lanceolate
Margin of appendages of median bracts	Smooth	1-2 spines	Spinose	-	-
Receptacle bristles	Smooth	Scabrous	-	-	-
Corolla color	Yellow	Pink	Purple	White	-
Ratio limb to Anther tube	Longer	Shorter	As long as	-	-
Anther tube color	Yellow	Pink	Purple	White	-

2012). The test was conducted by comparing the maximum likelihood (ML) values for the known topology in the presence and absence of the molecular clock constraints under Tamura and Nei (1993). The similar rate of evolution of the investigated sequences was rejected by setting the significance level at 5%, and consequently, the relaxed molecular clock model was utilized in further analyses (Minaeifar *et al.* 2016). The HKY model was identified as the best substitution model as implemented in MEGA 7 (Tamura *et al.* 2011). Bootstrap analysis (BS) (Felsenstein 1985) was completed to attain support estimates for the nodes in the ML tree.

ITS sequences were also analyzed by TCS Networking as implemented in the PopART (Population Analysis with Reticulate Trees) program (<http://popart.otago.ac.nz>).

Estimation of species time of divergence

BEAST v1.6.1 (Drummond *et al.* 2012a, b) was applied for the Bayesian MCMC inferred analyses of the nucleotide sequence data. BEAUti (Bayesian Evolutionary Analysis Utility version) v1.6.1 (Drummond *et al.* 2012a, b) was used to generate initial xml files

for BEAST. A Yule speciation process ('a pure birth' process) was utilized as a tree prior for all tree model analyses. For the MCMC posterior analyses, the length of chain was 10,000,000. After 100 trees burn-in processing, 10,000 trees were utilized for the analyses. The BEAUti xml file was run in the BEAST v1.6.1 program, and the maximum clade credibility (MCC) chain generations were repeated five times for each molecular clock model with separate runs to confirm suitable convergence and sufficient mixing. The MCC tree was generated under the relaxed clock model (HKY substitution). ITS substitution rates were applied between 1.72×10^{-9} to 8.34×10^{-9} (mean = 4.13×10^{-9}), according to Lopez-Vinyallonga *et al.* (2009).

Tracer v1.5 software (Rambaut and Drummond 2007) was utilized for the production of the model parameters to assay the sampling and convergence results obtained from BEAST. TreeAnnotator v1.6.1 software (Drummond *et al.* 2012a, b) was utilized to annotate the phylogenetic results generated by BEAST as a form of single 'target' tree. On the target trees are shown summary statistics of posterior probabilities of the nodes: the 95% highest posterior density (HPD) limits of the node heights, rates, and the posterior estimates.

FigTree v1.3.1 (Rambaut 2009) program was also applied for the annotated BEAST MCC tree production analyses. The posterior probability was fix to 0.5, which is equal to the bootstrapping value in PAUP (Phylogenetic Analysis Using Parsimony analysis) analyses (Hong and Jury 2011).

RESULTS

Morphometry

PCA analysis of morphological features showed that the first two PCA components included about 79% general alteration. Morphological features like the shape and length of the appendages of the median bracts, diameter of the heads, number of the flowers, and length of the corolla were the most variable morphological features among the investigated plants. In fact, these morphological features are of taxonomic value in the two sections *Cynaroideae* and *Platyacanthae*.

An MP tree (Figure 1) of morphological characteristics can delimit the two studied sections *Cynaroideae* and *Platyacanthae* because of difference in traits, like stem leaves (sect. *Cynaroideae*: decurrent; sect. *Platyacanthae*: nondecurrent) and the appendages of the median bracts (sect. *Cynaroideae*: present; sect. *Platyacanthae*: absent).

In the MP tree, within the sect. *Platyacanthae*, *C. platyacantha* Bunge and *C. freynii* Bornm. were located close to each other due to similarity in all characters except for the color of the corolla (*C. platyacantha*: white; *C. freynii*: purple). Likewise, *C. reshingerorum* Bornm. and *C. bienerti* Bunge exhibited morphological similarity in traits like number of flowers, length of the corolla, color of the anther tube, median and the inner bracts, receptacle bristles, and the diameter of the head and ratio of the limb/tube.

In the sect. *Cynaroideae*, *C. grandis* C. A. Mey. and *C. grantii* Rech. f. were located close to each other because of similar morphological traits such as the shape of the appendages of the median bracts (ovate) and leaves indumentum (glabrous). The same applies for *C. ecbatanensis* Bornm., *C. kornhuberi* Heimerl, *C. elwendensis* Bornm., *C. parsana* Ghahreman, *C. denaensis* Attar & Djavadi and *C. khorrabadensis* Bornm.; these species have similar morphological characters like the color of the corolla (white) and the position of the median bracts (spreading). *C. millefontana* Rech. f., *C. fursei* Rech. f., *C. sardashtensis* Rech., *C. carolihenrici* Attar & Ghahreman, *C. dalahuensis* Attar & Ghahreman and *C. concinna* Boiss. & Hausskn. were also close to each other because of the similarity in morphological features

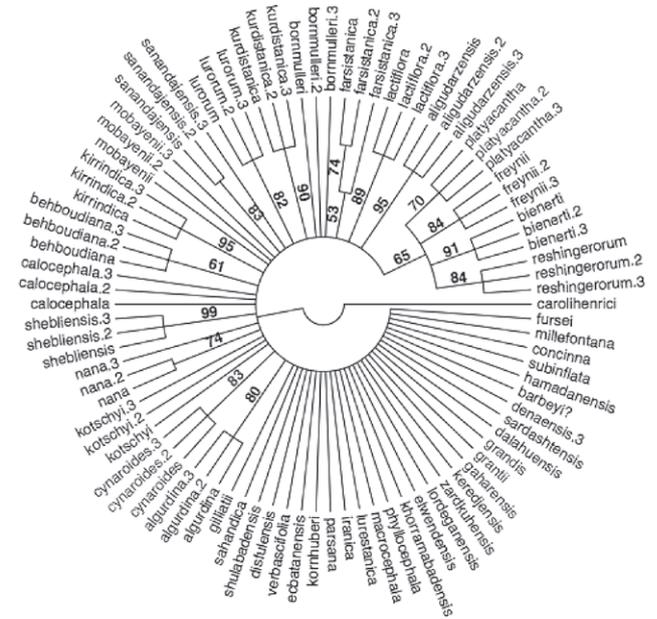


Figure 1. Maximum Parsimony tree of the studied *Cousinia* species based on morphological data. (species are according to Table 1). Values above branches are bootstrap value.

such as the position of the median bracts (imbricated). The same applies for *C. calocephala* Jaub. & Spach and *C. behboudiana* Rech. f. & Esfand. because of their similar morphological traits, like the position of the median bracts (recurved) and the color of the corolla (yellow).

ITS sequence analysis

The pair-wise genetic distances determined for the studied *Cousinia* species arranged from 0.01 (the lowest value between *C. ecbatanensis* and *C. kotschyi* Boiss.) to 0.50 (the highest value between *C. shebliensis* Ghahreman and *C. behboudiana*). These values showed the degree of sequence variability within species. The ML tree (Figure 2) and TCS network (Figure 3) of the studied species based on ITS sequences produced similar results. In these trees, the outgroups (*A. umbrosum* and *A. lappa*) were basically separated from the other species.

Based on these results, the ITS marker did not delimit the two studied sections. In the obtained genetic trees, the species of the sect. *Platyacanthae*, such as *C. platyacantha*, *C. reshingerorum*, *C. bienerti* and *C. freynii*, were placed among the species of the sect. *Cynaroideae*. These trees exhibited a close genetic affinity between *C. platyacantha*, *C. reshingerorum* and *C. bienerti*, which is in agreement with their morphological similarities. However, *C. freynii* is located far from the others in the genetic tree but close to them in the morphological tree.

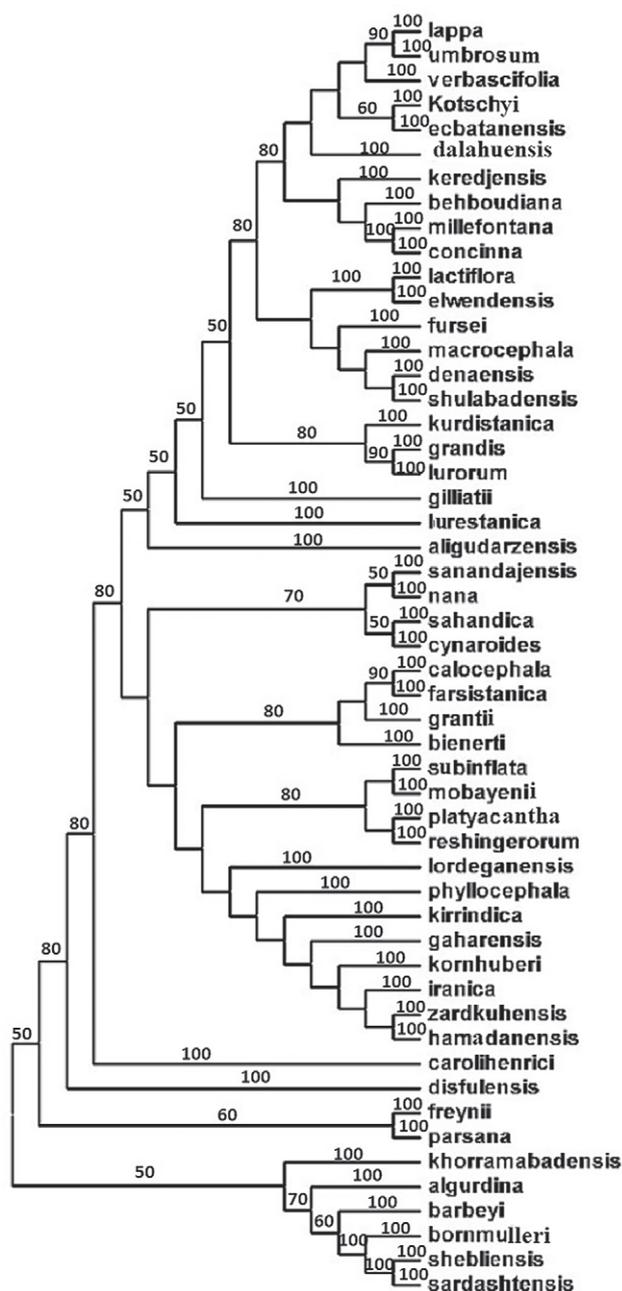


Figure 2. Maximum likelihood tree of the studied *Cousinia* species based on ITS data. (species are according to Table 1).

In the ML tree based on ITS sequences, *C. verbascifolia* was placed far from the other species. It differs morphologically from the other studied species in the color of corolla (light pink), bracts (numerous), the appendage of the median bracts (triangular), presence of long spine found at the apex, and the spinulose at the margin.

Both MP and ML trees exhibited an affinity between *C. kotschy* and *C. ecbatanensis*. These species are simi-

lar in morphological traits such as shape of the appendages in the median bracts (Lanceolate) and position of the median bracts (Spreading). Similarly, *C. elwendensis* and *C. denaensis* are related to *C. ecbatanensis*. They have morphological similarities in the color of the corolla (white) and the position of the median bracts (spreading).

The ITS-based phylogenetic tree showed a close affinity between *C. millefontana* and *C. concinna*, which have similar morphological characters such as the position of the median bracts (Imbricated). *C. keredjensis* Bornm. & Gauba is well separated from the two species mentioned above (*C. millefontana* and *C. concinna*) and also differs in its morphological traits, like the color of the corolla (white), the number of flowers (ca. 125), and the number of bracts (ca. 130). The same applies between *C. sahandica* Attar & Djavadi and *C. sanandajensis* Rech. f., as well as for *C. nana* Attar and *C. cynaroides* C. A. Mey.

Disagreement was observed between the other studied species of *Cousinia* based on morphological and genetic characters.

The TCS network exhibited the process of speciation and the number of nucleotide substitution in ITS sequences among the studied species. The highest number of nucleotide substitutions in ITS occurred in *C. khorrabadensis* (9).

The Bayesian tree (Figure 4) obtained with BEAST based on ITS sequences estimated the divergence times of the studied species within the *Cynaroideae* and *Platyacanthae* sections to be approximately 3.5 mya.

DISCUSSION

Taxonomy, molecular phylogeny, and the species relationships of the genus *Cousinia* are complicated and unresolved, mainly because of disagreement between morphological and molecular phylogenetic studies (Sausana *et al.* 2003; Lopez-Vinyallonga *et al.* 2009; Mehregan and Assadi 2016). Moreover, several overlapping morphological characteristics at the species level hinder species identification and delineation (Attar and Djavadi 2010; Minaeifar *et al.* 2016; Atazadeh *et al.* 2020).

Susanna *et al.* (2003), performed an extensive investigation on the evolution and generic delineation in the *Arctium-Cousinia* complex, based on two very important characters: pollen type and chromosome number. They divided all studied species into two major lineages: The Arctioid clade (including: *Arctium*, *Sehmalhallsenia*) with *Arctiastrum* pollen type and $x=18$ and the Cousinioid clade (including: *Cousinia* subg. *Cousinia*) with *Cousinia* pollen type and $x=11, 12, 13$. They also showed that the palynological and chromosome number results

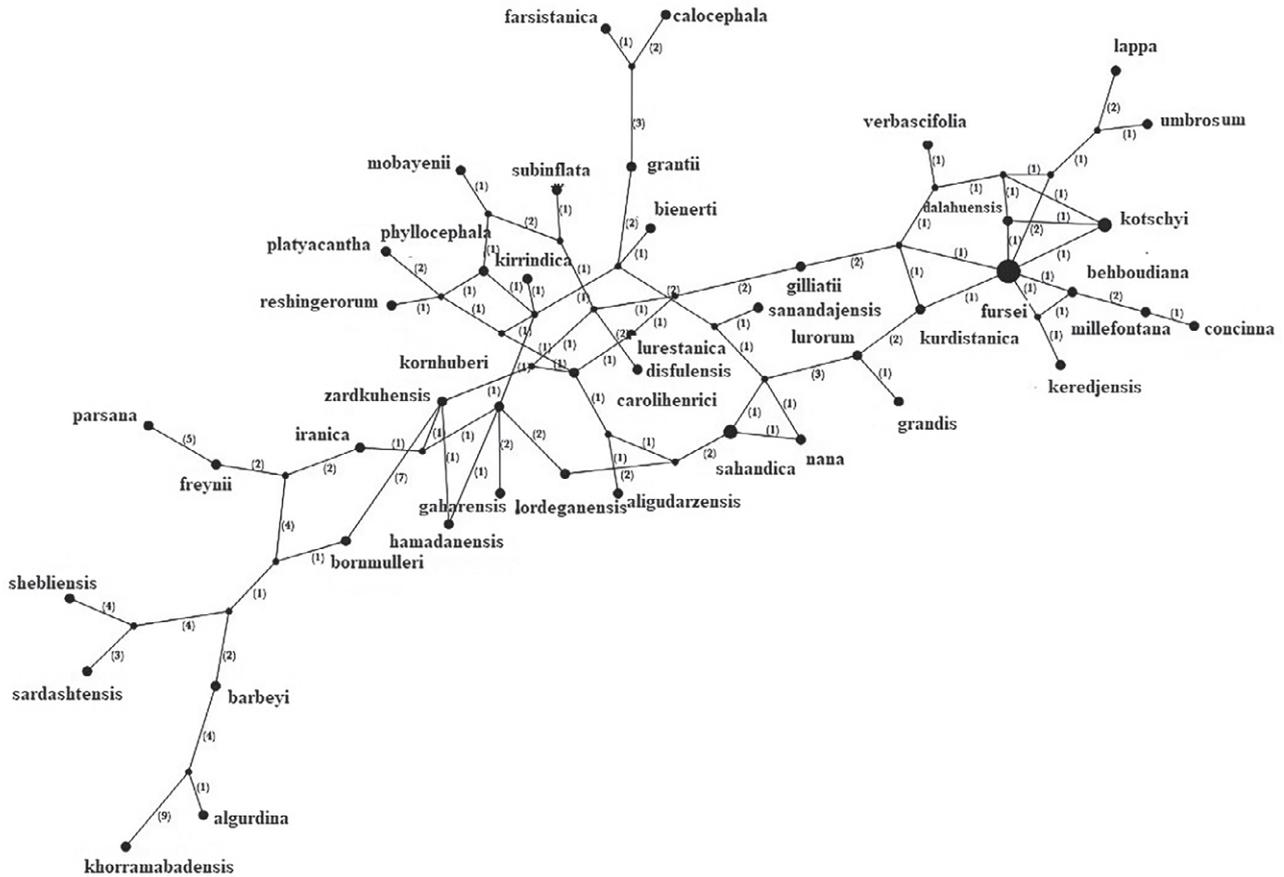


Figure 3. TCS Network of studied *Cousinia* species based on ITS sequences (species are according to Table 1).

are incongruent with morphological and molecular data in the *Arctium-Cousinia* complex and considered to be homoplasious morphological characters. These results were later confirmed by Lopez-Vinyallonga *et al.* (2009).

In the present study, the studied *Cousinia* species were delimited and useful taxonomic features, including the shape and length of the appendages of the median bracts, position of the median bracts, diameter of the heads, number of flowers and bracts, and the color and length of the corolla were identified.

The species relationships obtained based on the morphological features within sect. *Platyacanthae* are in agreement with those reported in previous studies (Asaadi and Mehregan 2017). For instance, within sect. *Platyacanthae*, *C. Platyacantha* and *C. freynii* were placed close to each other because of the similarity found in all features except color of the corolla (*C. Platyacantha*: white; *C. freynii*: purple). Asaadi and Mehregan (2017) also noticed their close affinity to each other. Likewise, *C. reshingerorum* and *C. bienerti* exhibited morphological likenesses in traits such as number

of flowers, length of the corolla, color of the anther tube, the median and inner bracts, receptacle bristles, diameter of the head, and the ratio of the limb/tube. Asaadi and Mehregan (2017) also showed the close morphological affinity among these species.

The morphological results in sect. *Cynaroideae*, were in complete agreement with the obtained results by Attar and Djavadi (2010).

The relationships among the studied *Cousinia* species in the genetic trees were generally incongruent with those in the obtained morphological tree.

The present study revealed that the studied sections within the genus *Cousinia*, including *Cynaroideae* and *Platyacanthae*, are not monophyletic. This is fully consistent with the results reported by Susanna *et al.* (2003) and Lopez-Vinyallonga *et al.* (2009), who also showed that morphological traits are highly incongruent with molecular data in *Arctium-Cousinia* complex and considered homoplasious morphological characters.

Various reasons are suggested for the disagreement between “gene tree” and “species trees”, including the

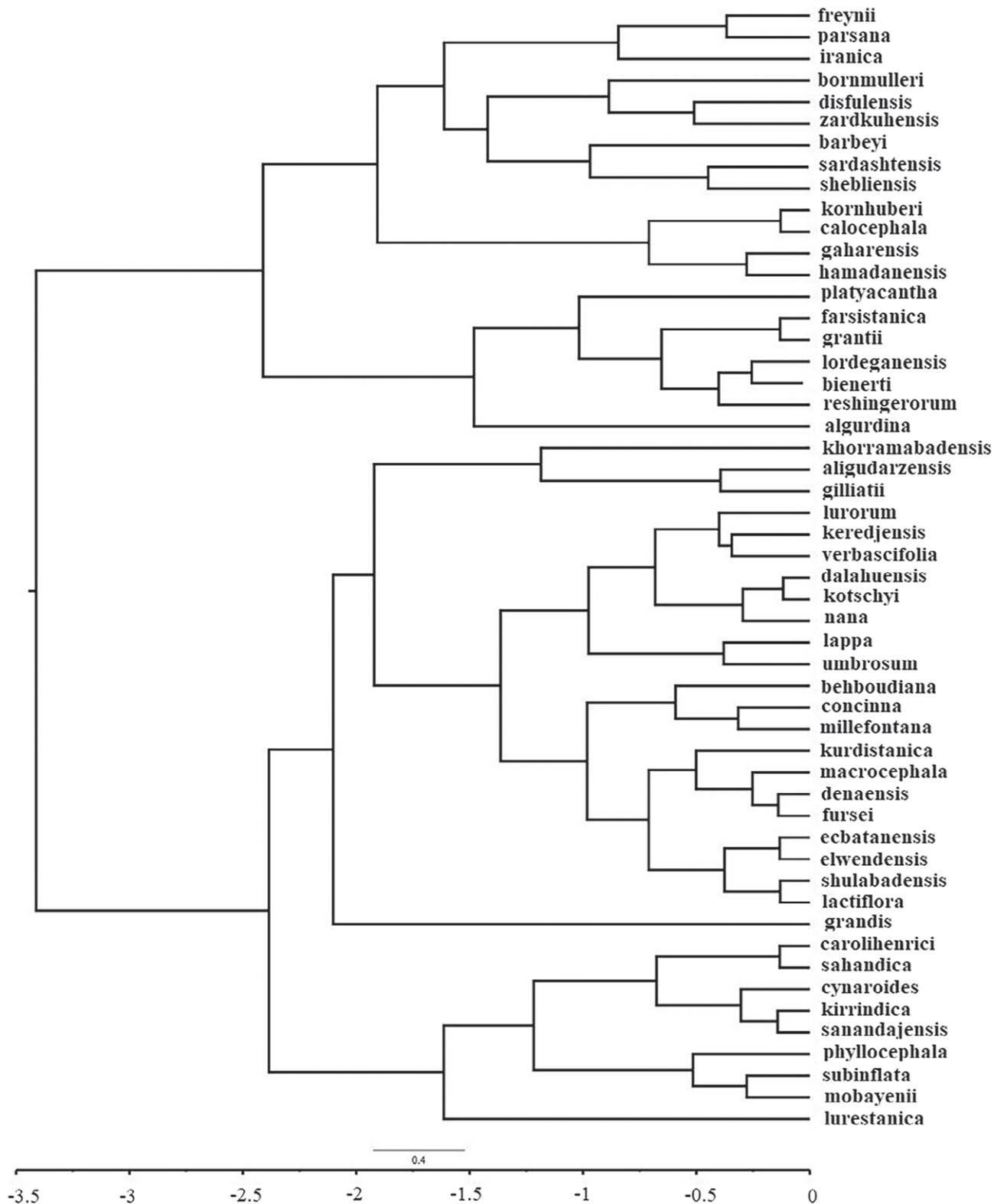


Figure 4. BEAST chronogram of studied *Cousinia* species based on ITS sequences (species are according to Table 1, numbers at the base of tree represents Mya).

high number of taxa in *Cousinia* (Susanna *et al.* 2003; Lopez-Vinyallong *et al.* 2009; Mehregan and Assadi 2016), interspecific hybridization, the occurrence of the intermediate forms (Mehregan and Kadereit 2009) and homoploid hybrid speciation, of which there is little proof to prove them (Lopez-Vinyallong *et al.* 2009), and incomplete lineage sorting (Zhang *et al.* 2015).

The divergence time of the studied species within the *Cynaroideae* and *Platyacanthae* sections based on ITS sequences was estimated to be around 3.5 mya. This result is in agreement with Lopez-Vinyallong *et al.* (2009), who also showed that the major radiation of the genus *Cousinia* has been estimated to have started ca. 8.7 mya.

According to our findings and previous authors (Susanna *et al.* 2003; Lopez-Vinyallong *et al.* 2009) phylogeny, evolutionary pathway and species relationships of the genus *Cousinia* are unclear and complicated. The genus *Cousinia* with its relatively young geological age (ca. 8.7 mya) and high number of taxa is unusual exposed to speciation. Djamali *et al.* (2012) showed that *Cousinia* consistently existed in the glacial age. They recorded an ~200,000-year pollen from Lake Urmia, northwest Iran. In contrast, the dispersal of its pollen grains was restricted. In the current results, all of the studied species except for *C. calocephala* had restricted geographical distributions and were isolated by geographical boundaries, which reduced the genflow. Therefore, geographical processes can be determining factors in the speciation of this genus. This result is entirely consistent with the results reported by Lopez-Vinyallong *et al.* (2009), as they also revealed that the dominant factor in speciation of the genus *Cousinia* is allopatric geographic speciation. These may partly justify the complexity and incongruence of the relationships in the studied species of the genus *Cousinia*.

As a general conclusion, based on the molecular studies of the observed specimens, it is suggested that both *Cynaroideae* and *Platyacanthae* sections are synonymous. To make a definitive decision on this, further molecular studies are necessary.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Neda Atazadeh, Masoud Sheidai and Farideh Attar. The first draft of the manuscript was written by Neda Atazadeh and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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