Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: N. Atazadeh, M. Sheidai, F. Attar, F. Koohdar (2020) Species delimitation in the genus *Cousinia* Cass. (Family Asteraceae), sections *Cynaroideae* Bunge and *Platyacanthae* Rech. f.: morphometry and molecular analysis. *Caryologia* 73(3): 3-12. doi: 10.13128/caryologia-275

Received: May 30, 2019

Accepted: April 05, 2020

Published: December 31, 2020

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Species delimitation in the genus *Cousinia* Cass. (Family Asteraceae), sections *Cynaroideae* Bunge and *Platyacanthae* Rech. f.: morphometry and molecular analysis

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Abstract. The genus *Cousinia* of the tribe Cardueae with about 700 species is one of the most diverse genera in Central and southwest Asia. The section *Cynaroides* with 89 species is the largest section of the genus. Due to the controversy in the number of *Cousinia* species and their delineation, the first step in studying the genus is to identify and delimit presumed species. Species delimitation is usually difficult in the species with overlaps in their morphological features. Therefore, we used a combination of morphological and molecular markers (ISSRs) to carry out delimitation in 204 taxa of 68 *Cousinia* species whitin the *Cynaroideae* and *Platyacanthae* sections. The species delineation based on morphometry and ISSR data were done by UPGMA clustering. The samples of each species were placed close to each other and formed a single sub-cluster, separated from the other studied *Cousinia* species. In the present study the studied *Cousinia* species within *Cynaroideae* and *Platyacanthae* sections could be delimited from each other based on ISSR and morphological data. Therefore, using ISSR and morphological data can be useful in identifying and delineating crucial species. The Mantel test performed between morphological distance and Nei genetic distance produced non-significant correlation. This result also supports distance analyses of the trees and reveals that the two dendrograms are not correlated. Some possible reasons for this incongruence are proposed: the high number of taxa in the genus *Cousinia,* morphological traits homoplasious, convergent evolution and incomplete lineage sorting.

Keywords: *Cousinia, Cynaroideae,* ISSR, morphometry.

INTRODUCTION

The genus *Cousinia* Cass. of the tribe Cardueae (Family Asteraceae) with about 700 species is one of the most diverse genera in Central and southwest (SW) Asia After *Senecio* L. (c. 1500 species) and *Vernonia* Schreb. (c. 1000 species) (Tscherneva 1962; Rechinger 1972, 1979; Frodin 2004; Attar and

Ghahreman 2006; Susanna and Garcia-Jacas 2006; Attar and Djavadi 2010; Mehregan and Assadi 2016; Minaeifar *et al.* 2016; Rastegar *et al.* 2017, 2018). Due to the extensive morphological variability in the genus, *Cousinia* taxonomy is complicated and controversial (Mabberley 1990; Haffner 2000; Susanna *et al.* 2003). The genus *Cousinia* contains more than 400 species in SW Asia, with the highest number of species in the Flora Iranica area, out of which 379 are endemic. These species are distributed in mountainous regions of Iran, Afghanistan and Turkmenistan (Rechinger 1986; Knapp 1987). Although the exact number of *Cousinia* species in Iran is still unknown, about 270 species have been reported till now (Assadi 2009; Attar and Djavadi 2010). Out of these, nearly 200 endemic *Cousinia* species occur in Iran (Djavadi *et al.* 2007; Zare *et al.* 2013). The *Cousinia* species are distributed in 70 sections (Rechinger 1986). The section *Cynaroideae* Bunge with 89 species is the largest section of the genus and contains Irano-Turkestanian elements (Tscherneva 1962; Rechinger 1972, 1979; Huber- Morath 1975; Attar and Djavadi 2010; Rastegar *et al.* 2017, 2018). This sect. includes those species consisting of decurrent leaves and appendiculate bracts (Tscherneva 1962; Rechinger 1972, 1979; Huber- Morath 1975). Iran with 77 taxa, of which 66 are endemic, seems to be the centre of diversity of the section *Cynaroideae* (Attar and Ghahreman 2006). The section *Platyacanthae* Rech. f. has 6 species in Flora Iranica of which 5 species are endemic in Iran (Rechinger 1972).

Due to the controversy in the number of *Cousinia* species and their delineation, the first step in studying the genus is to identify and delimit presumed species. Species delimitation is usually difficult in the species with overlaps in their morphological features (Wiens 2007). In such cases, combined morphological and molecular data have been used to delimit these taxonomic identities (Duminil and Di Michele 2009; Minaeifar *et al.* 2016; Hassanpour *et al.* 2018; Eftekharian *et al.* 2018).

Different molecular markers have been used in plant taxonomy and phylogeny, but some of them such as inter-simple sequence repeats (ISSRs) seems to be very efficient in delineating species, varieties, ecotype and even genotypes of a single species (See for example, Sheidai *et al.* 2012, 2013; Safaei *et al.* 2016; Eftekharian *et al.* 2018). Therefore, we used a combination of morphological and molecular markers (ISSRs) to carry out *Cousinia* species delimitation in sections *Cynaroideae* and *Platyacanthae.*

MATERIALS AND METHODS

Plant material

The data investigated and discussed in the present study are based on 204 samples of 68 species in of the sections *Cynaroideae* and *Platyacanthae*. Sixty-three species (189 specimens) of *Cynaroideae* and five species (15 specimens) of *Platyacanthae* were selected. The plant samples were collected from Iran (Table 1). The vouch-

Table 1. Investigated *Cousinia* species and their voucher information.

No	Taxa	Section	Locality	Voucher no.
1	*C. keredjensis* Bornm. & Gauba	*Cynaroides* Bunge	Tehran	21807(TUH)
2	*C. elwendensis* Bornm.	*Cynaroides* Bunge	Hamadan-Alvand Mountains	20566(TUH)
3	*C. grandis* C. A. Mey.	*Cynaroides* Bunge	Azarbaijan	21343(TUH)
4	*C. disfulensis* Bornm.	*Cynaroides* Bunge	Lorestan- Khorram Abad	27589(TUH)
5	*C. bornmulleri* C. Winkl.	*Cynaroides* Bunge	Esfahan	22532(TUH)
6	*C. behboudiana* Rech. f. & Esfand.	*Cynaroides* Bunge	Ghazvin	27629(TUH)
7	*C. inflata* Boiss. & Hausskn.	*Cynaroides* Bunge	Kurdestan	39552(TUH)
8	*C. eriocephala* Boiss. & Hausskn.	*Cynaroides* Bunge	Azarbaijan	22442(TUH)
9	*C. calocephala* Jaub. & Spach	*Cynaroides* Bunge	Azarbaijan-Mianeh	46276(TUH)
10	*C. farsistanica* Bornm.	*Cynaroides* Bunge	Kerman	28636(TUH)
11	*C. jaccobsii* Rech. f.	*Cynaroides* Bunge	Ilam	22370(TUH)
12	*C. denaensis* Attar & Djavadi	*Cynaroides* Bunge	Boyer-Ahmad	22495(TUH)
13	*C. concinna* Boiss. & Hausskn.	*Cynaroides* Bunge	Kurdestan	20562(TUH)
14	*C. grantii* Rech. f.	*Cynaroides* Bunge	Azarbaijan	22490(TUH)
15	*C. bobeckii* Rech. f.	*Cynaroides* Bunge	Ardabil	46221(TUH)
16	*C. barbeyi* C. Winkl.	*Cynaroides* Bunge	Boyer-Ahmad	22494(TUH)
17	*C. kirrindica* Bornm. & Rech. f.	*Cynaroides* Bunge	Ilam	19711(TUH)

No	Taxa	Section	Locality	Voucher no.
18	*C. khorramabadensis* Bornm.	*Cynaroides* Bunge	Lorestan	21851(TUH)
19	*C. lactiflora* Rech. f.	*Cynaroides* Bunge	Lorestan	46299(TUH)
20	*C. phyllocephala* Bornm. & Gauba	*Cynaroides* Bunge	Lorestan- Khorram Abad	46292(TUH)
21	*C. lurorum* Bornm.	*Cynaroides* Bunge	Kermanshah- Mahidasht	20568(TUH)
22	*C. verbascifolia* Bunge	*Cynaroides* Bunge	Khorasan-Mashhad	43013(TUH)
23	*C. monocephala* Bunge	*Cynaroides* Bunge	Khorasan- Ghouchan	21931(TUH)
24	*C. shebliensis* Ghahreman	*Cynaroides* Bunge	Azarbaijan- Tabriz	20580(TUH)
25	*C. millefontana* Rech. f.	*Cynaroides* Bunge	Kurdestan-Marivan	20227(TUH)
26	*C. sanandajensis* Rech. f.	*Cynaroides* Bunge	Hamadan	46287(TUH)
27	*C. zardkuhensis* Attar & Ghahreman	*Cynaroides* Bunge	Chahar Mahal& Bakhtiari	21887(TUH)
28	*C. pergamacea* Boiss. & Hausskn.	*Cynaroides* Bunge	Kurdestan	22571(TUH)
29	*C. macrocephala* C. A. Mey.	*Cynaroides* Bunge	Ardebil- Meshkin shahr	42925(TUH)
30	*C. onopordioides* Ledeb.	*Cynaroides* Bunge	Khorasan: Kashmar	28685(TUH)
31	*C. aligudarzensis* Attar & Ghahreman	*Cynaroides* Bunge	Lorestan-Aligudarz	27613(TUH)
32	*C. dalahuensis* Attar & Ghahreman	*Cynaroides* Bunge	Kermanshah- Mahidasht	19929(TUH)
33	*C. carolihenrici* Attar & Ghahreman	*Cynaroides* Bunge	Kurdestan	22455 (TUH)
34	*C. khansarica* Attar & Ghahreman	*Cynaroides* Bunge	Esfahan: Khansar	20037(TUH)
35	*C. lurestanica* Attar & Djavadi	*Cynaroides* Bunge	Lorestan	21824(TUH)
36	*C. parsana* Ghahreman	*Cynaroides* Bunge	Hamadan	20553(TUH)
37	*C. pasargadensis* Attar	*Cynaroides* Bunge	Fars: Dashte Arjan	36294(TUH)
38	*C. perspolitana* Attar & Ghahreman	*Cynaroides* Bunge	Fars: Abadeh	22509(TUH)
39	*C. silvanica* Attar	*Cynaroides* Bunge	W Azarbaijan: Urmie	24064(TUH)
40	*C. shulabadensis* Attar & Ghahreman	*Cynaroides* Bunge	Lorestan- Shul Abad	21874(TUH)
41	*C. algurdina* Rech. f.	*Cynaroides* Bunge	Azarbaijan- Tabriz	30533(TUH)
42	*C. mobayenii* Ghahreman & Attar	*Cynaroides* Bunge	Kermanshah- Eslamabad	20569(TUH)
43	*C. sabalanica* Attar	*Cynaroides* Bunge	Ardebil	22570(TUH)
44	*C. kurdistanica* Attar	*Cynaroides* Bunge	Kurdestan- Maryvan	3232(TUH)
45	*C. gaharensis* Attar & Djavadi	*Cynaroides* Bunge	Lorestan- Shulabad	38259(TUH)
46	*C. kermanshahensis* Attar	*Cynaroides* Bunge	Kermanshah: Eslam-Abad	19810(TUH)
47	*C. fursei* Rech. f.	*Cynaroides* Bunge	Kurdestan-Marivan	18314(TUH)
48	*C. chlorosphaera* Bornm.	*Cynaroides* Bunge	Chahar Mahal& Bakhtiari: Soreshjan	26244(TUH)
49	*C. cynaroides* C. A. Mey	*Cynaroides* Bunge	Ardebil	22581(TUH)
50	*C. gilliatii* Rech. f.	*Cynaroides* Bunge	Azarbaijan	21967(TUH)
51	*C. iranica* C. Winkl. & Strauss.	*Cynaroides* Bunge	Arak	21881(TUH)
52	*C. kotschyi* Boiss.	*Cynaroides* Bunge	Azarbaijan	46244(TUH)
53	*C. kopikaradaghensis* Rech. f.	*Cynaroides* Bunge	Kurdestan: Saqqez	(TUH)
54	*C. sagittata* C. Winkl. & Strauss.	*Cynaroides* Bunge	Arak	21822(TUH)
55	*C. nana* Attar	*Cynaroides* Bunge	Arak	14347(TUH)
56	*C. sahandica* Attar & Djavadi	*Cynaroides* Bunge	Azarbaijan	46272(TUH)
57	*C. lordeganensis* Mehregan	*Cynaroides* Bunge	Chahar Mahal& Bakhtiari	46301(TUH)
58	*C. hamadanensis* Rech. f.	*Cynaroides* Bunge	Hamadan- Malayer	46290(TUH)
59	*C. subinflata* Bornm.	*Cynaroides* Bunge	Kermanshah	(TUH)
60	*C. kornhuberi* Heimerl	*Cynaroides* Bunge	Hamadan	22372(TUH)
61	*C. sardashtensis* Rech. f.	*Cynaroides* Bunge	Chahar Mahal& Bakhtiari	20073(TUH)
62	*C. sefidiana* Rech. f.	*Cynaroides* Bunge	Lorestan	21861(TUH)
63	*C. platyacantha* Bunge	*Platyacanthae* Rech. f.	Khorasan	43212(TUH)
64	*C. freynii* Bornm.	*Platyacanthae* Rech. f.	Semnan- Shahrud	27675(TUH)
65	*C. reshingerorum* Bornm.	*Platyacanthae* Rech. f.	Khorasan-Torbate Jam	39729(TUH)
66	*C. bienerti* Bunge	*Platyacanthae* Rech. f.	Khorasan-Neyshabur	28682(TUH)
67	*C. trachyphyllaria* Bornm. & Rech. f.	*Platyacanthae* Rech. f.	Khorasan- Ghouchan	21932(TUH)
68	*C. ecbatanensis* Bornm.	*Cynaroides* Bunge	Hamadan	22371(TUH)

er specimens have been deposited in The Herbarium of Tehran University (TUH) (Table 1).

DNA extraction and PCR amplification

Total genomic DNA was extracted from leaf tissue using protocol of the CTAB-activated charcoal and Polyvenyl Pyrrolidone (PVP) method (Murray and Thompson 1980). Quality of extracted DNA was examined by running on 0.8% Agarose gels. Each 20 ml PCR mixture contained 10 ml of 2_ PCR buffer, 0.5 mM of each primer, 200 mM of each dNTP, 1 Unit of Taq DNA polymerase (Bioron, Ludwigschafen, Germany), and 1 ml of template genomic DNA at 20 ng mle1. The PCR amplification program was performed in a Techne thermocycler (Germany) with the following program: 5 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 54.6 °C, and 2 min at 72 °C, with a final extension step of 10 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. Ten ISSR primers, UBC 807, UBC 810, UBC 811, UBC 834, 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).

Morphological analysis

In total, 19 morphological characters (quantitative and qualitative) were studied (Table 2). Morphological characters were coded accordingly. Data were standardized (mean = 0, variance = 1) and used for multivariate analyses. UPGMA (Unweighted paired group using average), and Ward (Minimum spherical variance) clustering based on Euclidean distance and Gower distances as well as principal coordinate analysis (PCoA) and multidimensional scaling (MDS) methods were used for grouping of the species. Principal components analysis (PCA) was used to identify the most variable morphological characters. (Podani 2000; Safaei *et al.* 2016). Data analyses were performed by PAST ver. 2.17 (Hammer *et al.* 2012).

Molecular analysis

The obtained ISSR bands were treated as binary characters (presence = 1, absence = 0). The number of

Table 2. Morphological characters and their code.

Character	Code				
Head diameter	x<3	3≤x≤6	x>6		
Flower 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		
Crolla length	x< 20	20≤x≤25	x>25		
Habitate	Woodland	Alpine	Stepp		
Leaves indumentum	Present	Absent			
Stem leaves	Interruptedly decurrent	Countinuously decurrent	Undecurrent		
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
Appendages shape of median bracts	Sagitate	Triangular	Rhombic	Ovate	Lanceolate
Appendages margin of median bracts	Smooth	1-2 spins	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	

private bands versus common bands was determined. The genetic diversity parameters like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Freeland *et al.* 2011) were determined for each population. Nei's genetic distance was used for clustering (Weising *et al.* 2005). Neighbor Joining (NJ) and UPGMA (Unweighted paired group using average) clustering were used for the species grouping after 100 times bootstrapping/permutations (Freeland *et al.* 2011). The consensus tree was constructed from the obtained morphological and ISSR trees. Similarly, tree distance was estimated accordingly. The Mantel test between dendrograms was performed to check their agreement. PAST ver. 2.17 (Hammer *et al.* 2012) and DARwin ver. 5 (Perrier & Jacquemoud-Collet 2006) programs were used for these analyses. AMOVA (analysis of molecular variance) (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006) was used to determine species genetic differentiation. Gene flow was determined by: (1) calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm 1/4 0.5(1 e Gst)/Gst, (2) reticulation analysis that is based on the least square method as performed in T-REX (Boc *et al.* 2012).

RESULTS

Morphometry

UPGMA dendrogram of the studied *Cousinia* species based on morphological characters (Figure 1) placed the studied samples of most of the species together and in a separate sub-cluster. This indicates that *Cousinia* species can be differentiated by the used morphological features. UPGMA dendrogram also separated *Cousinia* species of the two sections *Cynaroideae* and *Platyacanthae*.Therefore, the morphological characters studied can delimit these sections too.

PCA analysis of morphological characters revealed that the first two PCA components comprised about 79% of total variation. Morphological characters like shape and length of the appendages of the median bracts, diameter of the heads, the No. of flowers and length of the corolla had the highest value of correlation with these components and are the most variable morphological features among the studied plants. In fact, these morphological features are of taxonomic value in the two sections *Cynaroideae* and *Platyacanthae*.

ISSR assay

The used ISSR primers produced 36 reproducible bands/loci, out of which only 1 band was monomorphic, while the others were polymorphic bands. The highest number of ISSR bands occurred in *C. keredjensis* Bornm. & Gauba (20), while *C. cynaroides* C. A. Mey had the lowest number of bands (5). A single private ISSR band occurred in *C. keredjensis*, while the other bands were common among the *Cousinia* species.

Discriminating power of ISSR loci as determined by Gst against Nm (migration) analysis (Table 3), revealed that almost all ISSR loci have excellent discriminating power (>0.95). Therefore, ISSR markers are efficient in differentiating *Cousinia* species studied.

The highest value for Nei genetic distance (0.87) occurred between *C. bienerti* Bunge and *C. elwendensis* Bornm., followed by *C. freynii* Bornm. and *C. elwendensis* (0.81). Similarly, the lowest value for the same (0.02) was observed between *C. reshingerorum* Bornm. and *C. bienerti*.

UPGMA dendrogram of the studied *Cousinia* species based on ISSR data (Figure 2) separated these species in distinct sub-clusters. Therefore, ISSR molecular markers can be used in taxonomy of the genus. These molecular markers can also differentiate two sections of *Cynaroideae* and *Platyacanthae*.

AMOVA produced significant genetic difference among the studied *Cousinia* species (P = 0.001), which indicates that the studied species are genetically differentiated. AMOVA revealed that 99% of total genetic difference was due to among species genetic differentiation, while 1% was due to within species genetic variability.

The species relationship illustrated by UPGMA dendrograms based on morphological features and molecular data were not congruent. It was also illustrated in the consensus tree of these dendrograms (Figure 3). This tree revealed that only in some cases the studied *Cousinia* species show the same relationship in both morphological and molecular trees. For instance, *C. zardkuhensis* Attar & Ghahreman (No. 27 in Figure 3) and *C. chlorosphaera* Bornm. (No. 48 in Figure 3) were placed close to each other. The same applied for for *C. platyacantha* Bunge (No. 63 in Figure 3) and *C. freynii* (No. 64 in Figure 3). Similarly, three species of *C. reshingerorum*, *C. bienerti* and *C. trachyphyllaria* Bornm. & Rech. f. (No. 65-67 in Figure 3) formed a distinct cluster in the obtained consensus tree. The rest of *Cousinia* species studied were placed together in an unresolved cluster. This means that, their relationship is differently pictured in the obtained morphological and molecular dendrograms. Tree distance between the obtained morpho-

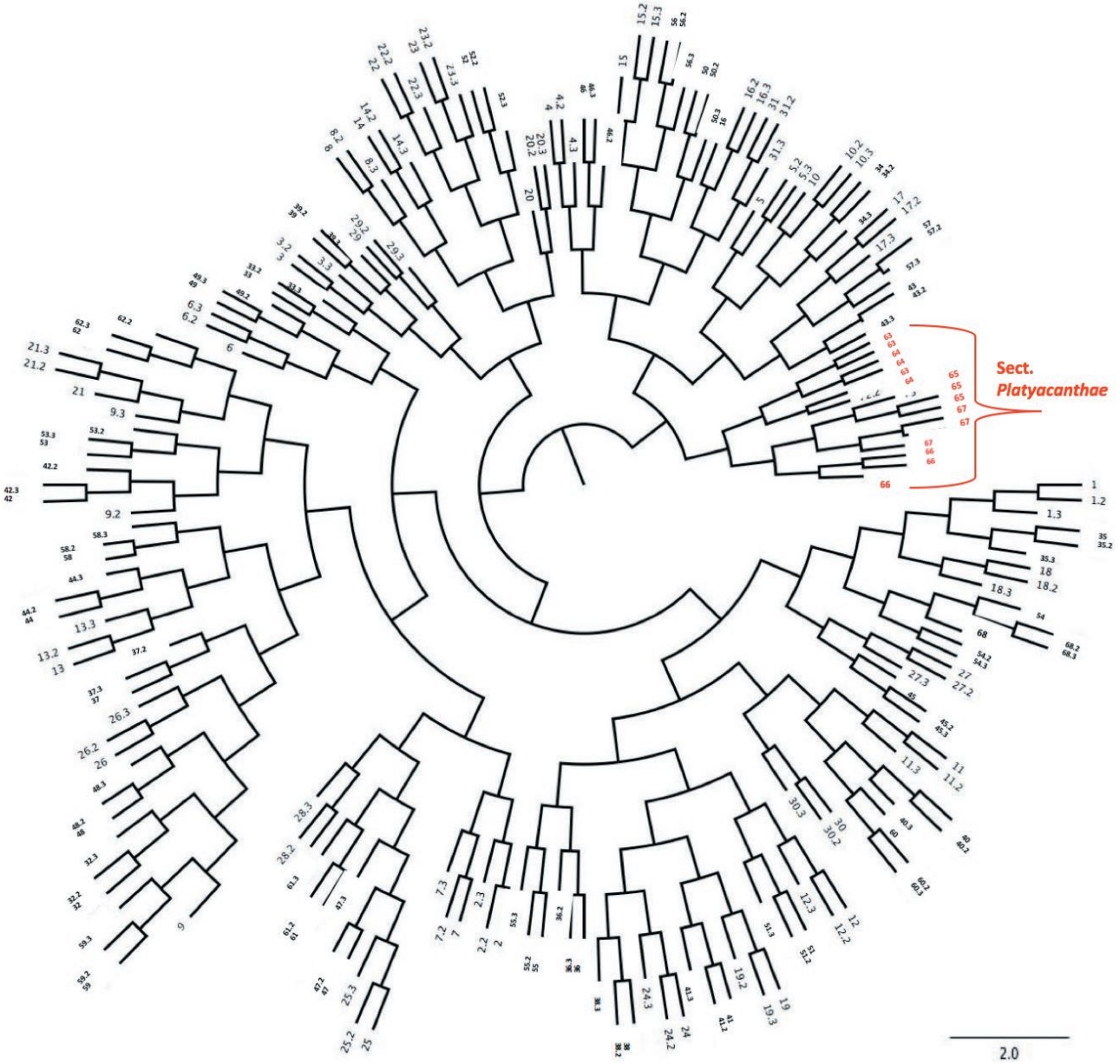

Figure 1. UPGMA dendrogram of the studied *Cousinia* species based on morphological data. (The specie 1-68 are according to Table 1).

logical and ISSR dendrograms after adjusting the edges in each dendrogram was 0.36. Similarly, comparison of these two dendrograms based on Quartet tree distance method, produced 0.64 difference. Both these results indicate that morphological relationship of the studied *Cousinia* species, differed in great extent with ISSR based species relationship.

The performed Mantel test between morphological distance and Nei genetic distance produced non-significant correlation (Correlation R = 0.06, p = 0.113). This indicates that morphological divergence in the studied species is not correlated with genetic distance.

DISCUSSION

As mentioned by the authors, taxonomy and molecular phylogeny of the genus *Cousinia* is complicated and unresolved mainly due to disagreement between the morphological and molecular phylogenetic studies (See

Table 3. Discrimination power of ISSR loci in studied Cousinia species.

Locus	Sample Size	Ht	Hs	Gst	Nm*
Locus1	204	0.1107	0.0000	1.0000	0.0000
Locus2	204	0.4027	0.0000	1.0000	0.0000
Locus3	204	0.4931	0.0000	1.0000	0.0000
Locus4	204	0.2712	0.0000	1.0000	0.0000
Locus5	204	0.3893	0.0000	1.0000	0.0000
Locus6	204	0.0843	0.0000	1.0000	0.0000
Locus7	204	0.0571	0.0000	1.0000	0.0000
Locus8	204	0.3599	0.0000	1.0000	0.0000
Locus9	204	0.3270	0.0000	1.0000	0.0000
Locus10	204	0.4961	0.0000	1.0000	0.0000
Locus11	204	0.3655	0.0088	0.9759	0.0124
Locus12	204	0.2297	0.0000	1.0000	0.0000
Locus13	204	0.4650	0.0000	1.0000	0.0000
Locus14	204	0.2297	0.0000	1.0000	0.0000
Locus15	204	0.3270	0.0000	1.0000	0.0000
Locus16	204	0.1454	0.0088	0.9394	0.0323
Locus17	204	0.2712	0.0000	1.0000	0.0000
Locus18	204	0.1499	0.0132	0.9118	0.0484
Locus19	204	0.0394	0.0088	0.7763	0.1441
Locus20	204	0.0107	0.0088	0.1791	2.2922
Locus21	204	0.0571	0.0000	1.0000	0.0000
Locus22	204	0.1107	0.0000	1.0000	0.0000
Locus23	204	0.2712	0.0000	1.0000	0.0000
Locus24	204	0.4377	0.0000	1.0000	0.0000
Locus25	204	0.4983	0.0000	1.0000	0.0000
Locus26	204	0.1609	0.0000	1.0000	0.0000
Locus27	204	0.2297	0.0000	1.0000	0.0000
Locus28	204	0.2712	0.0000	1.0000	0.0000
Locus29	204	0.0843	0.0000	1.0000	0.0000
Locus30	204	0.2297	0.0000	1.0000	0.0000
Locus31	204	0.2076	0.0000	1.0000	0.0000
Locus32	204	0.0843	0.0000	1.0000	0.0000
Locus33	204	0.0290	0.0000	1.0000	0.0000
Locus34	204	0.0571	0.0000	1.0000	0.0000
Locus35	204	0.1609	0.0000	1.0000	0.0000
Locus36	204	0.0571	0.0000	1.0000	0.0000
Mean	204	0.2270	0.0013	0.9941	0.0030

Nm = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst.

for example, Sausana *et al.* 2003; Lopez-Vinyallonga *et al.* 2009). Moreover, several overlapping morphological characteristics at the species level makes the species identification and delineation difficult (Attar and Djavadi 2010). In the present study, we could delimit the studied *Cousinia* species based on both the used morphological and molecular data. We suggest that certain morphological characters like shape and the length of the appendages of the median bracts, diameter of the heads, the No. of flowers and the length of the corolla are taxonomically useful at the species level.

Interesting enough, the both sections *Cynaroideae* and *Platyacanthae* are separated from each other due to the difference in traits such as stem leaves and appendages of median bracts. Therefore, these characters are of more practical utility, particularly in sectional level classification in the genus *Cousinia*.

The obtained species relationship based on morphological features are in agreement with previous studies. For example, within the section *Platyacanthae*; *C. Platyacanthae* and *C. freynii* were placed close each other due to the similarity in all features except color of corolla. Their close affinity to each other was also noticed by Asaadi and Mehregan (Flora of Iran 2017). Similarly, *C. reshingerorum*, *C. bienerti* and *C. trachyphyllaria* showed morphological resemblance due to the traits such as No. of the flowers, the length of the corolla, the color of the anther tube, inner bracts, receptacle bristles, diameter of head, ratio of limb/tube. Close morphological affinity among these species was also illustrated by Asaadi and Mehregan (Flora of Iran 2017).

In the section *Cynaroideae*, ISSR data showed genetic affinity between *C. grantii* Rech. f. and *C.grandis* C. A. Mey., and also between *C. verbascifolia* Bunge and *C. monocephala* Bunge. These species also showed morphological similarities. The same holded true for *C. pergamaceae* Boiss. & Hausskn., *C. millefontana* Rech. f., and *C. carolihenrici* Attar & Ghahreman; as well as for *C. zardkuhensis* and *C. chlorosphaera*. ISSR data revealed close affinity between *C. disfulensis* Bornm., *C. jaccobsii* Rech. f. and *C.kermanshahensis* Attar; which is almost in agreement with the morphological data. The same applied for *C. nana* Attar and *C. kotschyi* Boiss. These results are almost in agreement with the taxonomic treatment of the section *Cynaroideae* (Attar and Djavadi 2010). The other studied *Cousinia* species in the section *Cynaroideae* differed in their affinity in genetic tree versus morphological tree. This is in agreement with results of Lopez-Vinyallonga *et al.* (2009), as they also indicated that morphological traits are highly incongruent with molecular data in *Arctium-Cousinia* complex and considered morphological characters homoplasious.

In general, various reasons were suggested for this incongruence between molecular and morphological analyses: the high number of taxa in the genus *Cousinia,* homoplasious of the morphological traits, convergent evolution (Susanna *et al.* 2003; Lopez-Vinyallonga *et al.* 2009), incomplete lineage sorting (Zhang *et al.* 2015); as well as the occurrence of intermediate forms and

Figure 2. UPGMA dendrogram of the studied *Cousinia* species based on ISSR data.

homoploid hybrid speciation, which there is little proof to prove them (Mehregan & Kadereit 2009; Lopez-Vinyallonga *et al.* 2009). Furthermore, the genus *Cousinia* with its relatively young geological age (ca. 8.7 mya) and high number of taxa is thoroughly unusually exposed to speciation (Lopez-Vinyallong *et al.* 2009). This is entirely consistent with the results reported by Lopez-Vinyallonga *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*.

In conclusion, along with confirmation by the published literature, the current study proved that the morphological characters and ISSR molecular data are useful for the correct identification and species delimitation within the *Cynaroideae* and *Platyacanthae* sections. Both quantitative and qualitative morphological characteristics are important and suitable for the identification of species of the genus *Cousinia*.

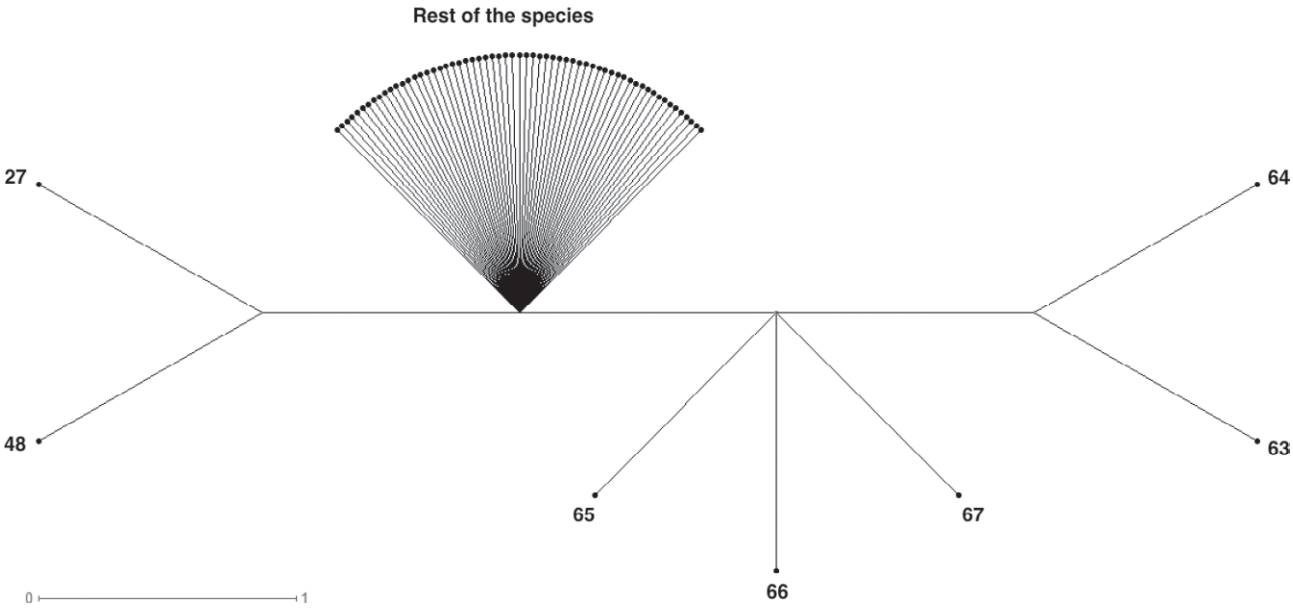

Fig. 3. Consensus tree based on morphological and ISSR dendrograms in studied *Cousinia* species.

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