

ISSR and RAPD analyses of species and their relationships in the genus *Cirsium* (Asteraceae) in Iran

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Abstract. The species relationships of *Cirsium* species were studied by using RAPD and ISSR molecular markers, as well as other data provided by cytology, genome size and morphological studies. A total of 428 RAPD bands were obtained, with 236 polymorphic bands. Several specific RAPD bands were obtained for the studied species. Similarly, out of 10 ISSR primers used, six primers produced reproducible bands. A total of 90 ISSR bands were obtained, out of which 19 bands were specific bands and one band was present in all species, except one. The NJ and parsimony trees obtained on the basis of a combined data set separated the species of different sections according to the taxonomic treatment of the genus in *Flora Iranica*.

Key words: *Cirsium*, ISSR, RAPD, taxonomy

Introduction

About 250 perennial, biennial, or seldom annual and spiny species are known from the genus *Cirsium* Mill. (Asteraceae). These species grow mainly in the Northern Hemisphere, Europe, North Africa, Siberia, Central Asia, West and East Africa, and Central America (Zomlefer 1994; Bureš & al. 2004; Segarra-Moragues & al. 2007).

The *Cirsium* species are annual, biennial or perennial, 50–200 cm tall, with erect spiny stems. The stems are branched or simple, occasionally narrowly spiny-winged. The leaves are basal and cauline; finely bristly-dentate to coarsely dentate, or 1–3 times pinnately lobed. The teeth and lobes are bristly-tipped, with green faces, which are glabrous or densely gray-canesc-

cent, usually eglandular. Their flower heads are discoid, borne singly, terminal and in distal axils, or in racemiform, spiciform, subcapitate, paniculiform, or corymbiform arrays. The involucre are cylindrical to ovoid, or spherical. The phyllaries are many in series of 5–20, subequal, or weakly to strongly, outer and middle, with bases appressed and apices spreading to erect, usually spine-tipped (Zomlefer 1994; Bureš & al. 2004; Segarra-Moragues & al. 2007).

The receptacles are flat to convex, epaleate, covered with tawny to white bristles or setiform scales. The florets are 25–200; corollas white to pink, red, yellow or purple, bilateral; the tubes long, slender, distally bent; the throats short, abruptly expanded.

The genus is well known for occurrence of interspecific hybridization resulting from the complex

nature of the genus showing extensive morphological variability. Such great morphological variation of the *Cirsium* is also partly due to its highly variable germplasm interacting with different environmental conditions. The occurrence of frequent hybrids in the genus *Cirsium* indicates lack of breeding barriers and, therefore, new forms may arise in nature, due to interspecific hybridization (Ownbey 1951; Moore & Frankton 1974; Charadze 1998; Bureš & al. 2004).

Extensive biosystematic, molecular and systematic studies have been performed on the genus *Cirsium* (e.g., Moore & Frankton 1969; Tonian 1982; Bare & al. 1998; Kelch & Baldwin 2003; Bureš & al. 2004; Ozcan & al. 2008).

Molecular phylogenetic studies by Kelch & Baldwin (2003) indicated that the great diversity of the New World *Cirsium* taxa is due to rapid evolutionary diversification based on a single initial introduction from Eurasia. Such remarkable evolutionary and morphological diversification in the North American *Cir-*

sium has not been accompanied by very great divergence in the base sequences of the genes, suggesting that either diversification has been very rapid, or that genetic markers in the North American *Cirsium* mutate slower than in most other lineages. Similar studies were limited to the species growing in Iran and only few cytological reports have been available so far (Ghaffari 1999; Nouroozi & al. 2010, 2011). About 36 *Cirsium* species have been reported in *Flora Iranica* (Petrak 1979) and have been classified in five sections. The present study deals with the molecular analyses of *Cirsium* species of Iran in an attempt to use the obtained data, so as to study the relationships between the species.

Material and methods

Plant material

The *Cirsium* species belonging to five sections (Table 1) growing in Iran were used in the present

Table 1. *Cirsium* species and locality and voucher specimens of the populations.

Species	Localities
Section <i>Pseudoepitrachys</i> Petrak	
<i>C. pyramidale</i> Bornm.	Kerman: Baft, Honkai, 32704-TUH.
<i>C. spectabile</i> DC.	Kerman: Baft, Gugher, Gonbadan 32098-TUH.
<i>C. congestum</i> Fisch & C.A.Mey	Azarbaijan: Khoy to Salmas, Ghooshchi, 2050 m, Nouroozi, 8600181-SBUH.
Section – <i>Epitrachys</i> DC.	
<i>C. bracteosum</i> DC.	Boyerahmad: 5 km from Kumehr to Ardakan, 2750 m, Ghahreman & Attar, 25211-TUH.
<i>C. bornmülleri</i> Sint. ex Bornm.	Khorassan: 47 km from Shirvan to Sarani, 1740 m, Ghahreman & Attar, 27658-TUH.
<i>C. strigosum</i> (M.Bieb.) M.Bieb.	Tehran: Polour, Nava village road, 2500 m, Ghahreman & Attar, 21972-TUH.
<i>C. ciliatum</i> (Murray) Moench	Azerbaijan: Road of Salmas to Khoy 1437 m, Nouroozi & Ghorbani, 8600160-HSBU.
<i>C. osseticum</i> (Adams) Petrak	Mazandaran: Royan to Baladeh, 2225 m, Nouroozi, 8600198-HSBU.
<i>C. aduncum</i> Fisch & C.A. Mey	Azerbaijan: Meshkinshahr, Mazraejahan, 1169 m, Nouroozi & Fathollahi, 8600170-HSBU.
<i>C. haussknechtii</i> Boiss.	Azerbaijan: Tabriz, Kandavan, Hilevar village, 1600 m, Nouroozi, 8600187-HSBU.
<i>C. lappaceum</i> M.Bieb.	Azerbaijan: Meshkinshahr, Lahrood, Sabalan, Shabil, 2850 m, Mozaffarian, 70123-TUH.
<i>C. turkestanicum</i> (Regel) Petrak	Khorassan: between Shirvan and Sarani, after Zivar village, 2130 m, Ghahreman & Attar, 27661-TUH.
<i>C. vulgare</i> (Savi) Ten.	Azerbaijan: Astara to Ardebil, Heiran, 1500 m, Nouroozi, 8600120-HSBU.
Section – <i>Echenais</i> (Cass.) Petrak	
<i>C. echinus</i> (M.Bieb.) Hand-Mzt.	Azerbaijan: Meshkinshahr, Mazraejahan, 1169 m, Nouroozi & Fathollahi, 8600211-HSBU.
Section-<i>Cirsium</i>	
<i>C. obvallatum</i> (M.Bieb.) M.Bieb.	Azerbaijan: Meshkinshahr, Sabalan, Shabil, 2600 m, Nouroozi, 8600227-HSBU.
<i>C. alatum</i> (S.G.Gomelin) Bobrov	Azerbaijan: Uremia, Ghasemloo, 1300 m, Nouroozi, 8600228-HSBU.
<i>C. elodes</i> M.Bieb.	Tehran: Haraz road, Lasem village, 1700 m, Nouroozi & Fathollahi, 8600235-HSBU.
<i>C. rhizocephalum</i> C.A.Mey	Kerman: Baft, Hankai, 2300 m, Mirtadhedini, 28722-TUH.
<i>C. rhizocephalum</i>	Azerbaijan: ca. 22 km from Ahar to Tabriz, Goijabel Mts. 1700 m, Ghahreman & Mozaffarian, 17563-TUH.
Section – <i>Cephalonoplos</i> (Necker) DC.	
<i>C. arvense</i> (L.) Scop.	Azerbaijan: Meshkinshahr, Khiavchay, 1527 m, Nouroozi & Fathollahi, 8600194-HSBU.
Out-group Species	
<i>Carduus hamulosus</i> Ehrh.	Azerbaijan: Ardebil to Astara, Fandoghloo forests, 1500 m, Nouroozi, 8700116-HSBU

study. The plant material was determined according to *Flora Iranica* (Petraik 1979). The voucher specimens are deposited in the Herbarium of Shahid Beheshti University (HSBU) and the Herbarium of Tehran University (TUH).

RAPD analysis

Thirty RAPD decamer primers of the Operon technology (Alameda, Canada) belonging to OPA, OPH, OPC, OPR & OPM sets were used in molecular study of the *Cirsium* species. DNA extraction followed the CTAB method (Murray & Thompson 1980). The PCR reaction mixture consisted of 1 ng template DNA, 1 × PCR buffer (10 mM Tris-HCL pH 8.8, 250 mM KCL), 200 μM dNTPs, 0.80 μM 10-base random primers, and 1 unit of *Taq* polymerase, in a total volume of 25 μL. DNA amplification was performed on a GP-001 palm cycler (Corbet, Australia). Template DNA was initially denatured at 92 °C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 1 min at 92 °C, primer annealing for 1 min at 36 °C, and primer extension for 2 min at 72 °C. A final incubation for 10 min at 72 °C was performed to ensure that the primer extension reaction proceeded to completion. The PCR amplified products were separated by electrophoresis on a 2% agarose gels using 0.5 X TBE buffer (44.5 Mm Tris/Borate, 0.5 Mm EDTA, pH 8.0). The gels were stained with ethidium bromide and visualized under UV light. A 100 bp DNA ladder (GeneRuler, Fermentas) was used as a molecular standard, in order to confirm the appropriate RAPD markers. These markers were named according to the primer origin, followed by the primer number and the size of amplified products in base pairs. The RAPD experiment was repeated thrice and reproducible bands were used for further studies. RAPD bands were treated as binary characters and coded accordingly (presence = 1, absence = 0).

ISSR assay

Six ISSR primers were used: (GA)₉T, UBC810, UBC811, UBC834, UBC849, and (CA)₇GT commercialized by UBC (the University of British Columbia). PCR reactions were performed in 25 μL volume containing 10 mM Tris-HCL buffer at pH 8; 50 mM KCl; 1.5 mM MgCl₂; 0.2 mM of each dNTP; 0.2 μM of a single primer; 20 ng genomic DNA, and 3 units of *Taq* DNA polymerase (Bioron, Germany). Ampli-

fication reactions were performed in a Techne Thermocycler (Germany) according to the following program: 5 min initial denaturation step 94 °C, 30 s at 94 °C; 1 min at 50 °C, and 1 min at 72 °C. The reaction was completed by final extension step of 7 min at 72 °C. Amplification products were visualized by running on 2% agarose gel after ethidium bromide staining. Fragment size was estimated by using a 100-base-pairs (bp) molecular size ladder (Fermentas, Germany).

Data analyses

The obtained RAPD and ISSR bands were treated as binary characters and coded accordingly (presence = 1, absence = 0). Jaccard's similarity and Nei's genetic distance (Nei 1972) were determined among the species, according to the Neighbor Joining (NJ) clustering method (Podani 2000). NTSYS Ver. 2.02 (1998) was used for clustering.

For both RAPD and ISSR data, genetic diversity of the species in each section was determined by percentage of the polymorphic alleles, Nei's Expected Heterozygosity (He), and the Shannon Information Index (I) (Nei 1972). Association between the genetic distance of species based on RAPD and ISSR markers was determined by the Mantel test, after 999 permutations (Podani 2000).

On the basis of clustering results, different groups (sections) were further investigated for their molecular difference by the AMOVA (Analysis of Molecular Variance) test using GenAlex 6.4 (Peakall & Smouse 2006).

Results

A total of 428 RAPD bands were obtained (Fig. 1), of which only four bands were common for all studied species and 238 bands were polymorphic. Several specific RAPD bands were also obtained for the studied species, for example, 15 unique bands occurred in *C. strigosum*, 14 unique bands occurred in *C. pyramidale*, while *C. congestum* & *C. obvallatum* had only one unique band. For example, a band with 500 bp molecular weight of OPA-12 and bands with 1270, 1480 and 2700 bp of the primer OPC-09 occurred only in *C. pyramidale*, while bands with 700 bp of OPA-05, 2800 bp

of OP0-9 and 9700 bp of OPM-10 occurred only in *C. spectabile*.

C. arvense showed some specific RAPD bands of 3000 bp, which were produced by primer OPA4; *C. congestum* had two specific RAPD bands of OPA3 (2000 bp and 1450 bp, respectively) by OPA-5, while *C. haussknechtii* showed a specific band of 950 bp by OPA-3 primer and *C. turkestanicum* showed a specific band of 1150 bp by primer OPA5.

The highest percentage of RAPD loci polymorphism (40%), Shannon's Information Index (14.7%) and Expected Heterozygosity (8.9%) occurred in the species of sect. *Epitrachys* (Fig. 2).

Out of the 10 used ISSR primers, six produced reproducible bands (Fig. 3). A total of 90 ISSR bands were obtained, of which 19 bands were specific and one band was present in all species, except one. For example, in the ISSR primer (CA)7GT, band 13 000 bp

occurred only in *C. lappaceum*, band 2800 bp was specific for *C. obvallatum*, bands 1600 bp and 1800 occurred only in *C. spectabile*, while band 1500 bp occurred in *C. echinus*. In the ISSR primer UBC835, band 100 bp occurred only in *C. haussknechtii*, band 750 bp was specific for *C. turkestanicum*, band 700 bp occurred in *C. strigosum*, and band 680 bp was specific for *C. vulgare*.

C. arvense showed three specific ISSR bands (430, 750 and 1000 bp, respectively) in the primer UBC834. *C. pyramidale* showed one specific band (920 bp) of the ISSR primer UBC807.

The highest percentage of ISSR loci polymorphism (75.5%), Shannon's Information Index (30.0%) and Expected Heterozygosity (18.0%) occurred in the species of sect. *Epitrachys* (Fig. 4).

A Mantel test was performed to show association between the obtained RAPD and ISSR genetic dis-

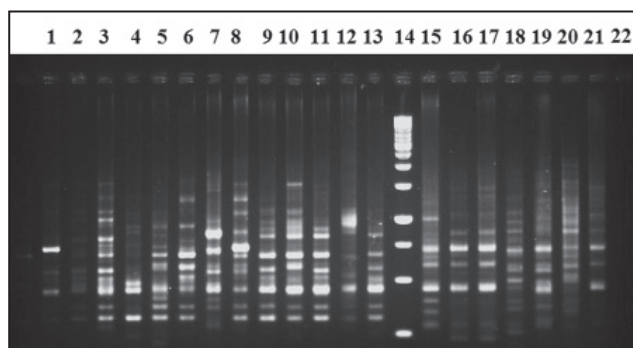


Fig. 1. Profile of the RAPD primer OPR-02. The lanes from 1 to 21 are: DNA No, *C. pyramidale*, *C. spectabile*, *C. congestum*, *C. bracteosum*, *C. bornmülleri*, *C. strigosum*, *C. ciliatum*, *C. osseticum*, *C. aduncum*, *C. haussknechtii*, *C. lappaceum*, *C. turkestanicum*, *C. vulgare*, *C. echinus*, molecular ladder, *C. obvallatum*, *C. alatum*, *C. elodes*, *C. rhizocephalum*, *C. arvense*, *Carduus hamulosus* and DNA No.

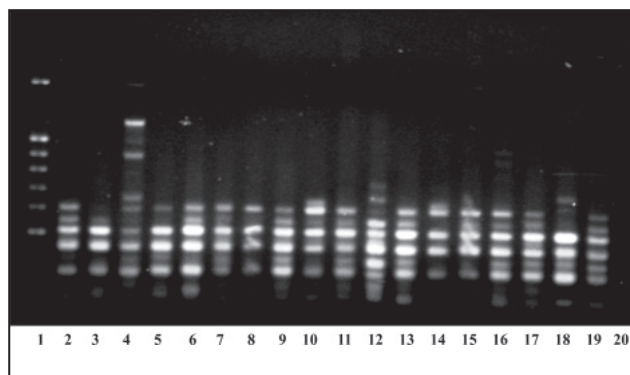


Fig. 3. Profile of ISSR primer CA7GT. The lanes from 1 to 20 are: molecular ladder, *C. pyramidale*, *C. strigosum*, *Carduus hamulosus*, *C. osseticum*, *C. vulgare*, *C. elodes*, *C. aduncum*, *C. ciliatum*, *C. echinus*, *C. lappaceum*, *C. spectabile*, *C. rhizocephalum*, *C. alatum*, *C. turkestanicum*, *C. obvallatum*, *C. congestum*, *C. haussknechtii*, *C. arvense* and DNA No.

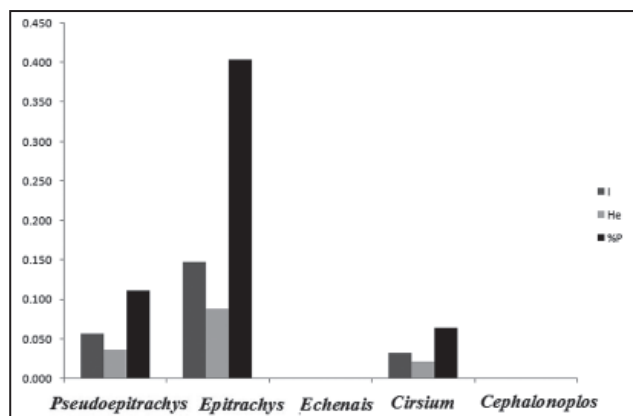


Fig. 2. Percentage of polymorphic loci (%P), Shannon's Information Index (I) and Expected Heterozygosity (He) determined for RAPD data in the studied *Cirsium* sections.

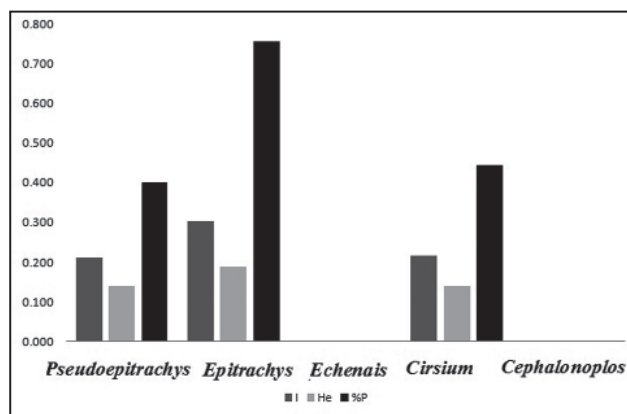


Fig. 4. Percentage of polymorphic loci (%P), Shannon's Information Index (I), and Expected Heterozygosity (He) determined for ISSR data in the studied *Cirsium* sections.

tances among the studied species after 999 permutations, which resulted in an association value of $r = 0.99$ ($p < 0.001$), indicating an almost complete significant association between the two molecular markers.

The grouping of the species based on combined ISSR and RAPD data according to the NJ method divided the species from different sections into separate cluster groups (Fig. 3). *Carduus hamulosus* L. as the out-group species was separated from the in-group species and the species of *Cirsium* from different sections.

C. congestum, *C. spectabile* and *C. pyramidale* of sect. *Pseudoepitrachys* comprised the first major cluster in which the first two species showed affinity and were joined together.

The species of sect. *Epitrachys* formed the second major cluster. Three species of *C. strigosum*, *C. ciliatum* and *C. osseticum* showed more affinity and formed the first subcluster, while *C. aduncum* and *C. haussknechtii* comprised the second subcluster. *C. lappaceum* joined these two species at some distance. *C. turkestanicum* and *C. vulgare* showed some genetic difference from the other species of this section and formed a separate subcluster slightly distanced from the others.

C. echinus, the only studied species from *C. sect. Echenais* (Cass.) Petrak, also joined these species at some distance, with a position in-between the members of sect. *Epitrachys* and sect. *Cirsium*. The species of sect. *Cirsium* comprised the third major cluster with *C. alatum* and *C. rhizocephalum*, showing higher similarity and joining together. *C. obvallatum* and *C. elodes* of the same sect. joined these two species at some distance. *C. arvense* of sect. *Cephalonoplos* (Necker) DC. showed affinity for the members of sect. *Cirsium* and joined them at some distance.

Among molecular markers and morphological characters, Spearman Correlation showed a positive significant correlation between some of the RAPD and ISSR loci with phyllary margin, exine texture and spine, as well as stem spine.

The AMOVA analysis showed that 36% of total variance was within sections, while 64% of variation was among the sections variation. A significant molecular difference ($F = 0.64$, $P < 0.01$) occurred among the studied sections.

Genetic polymorphism and diversity could only be determined in the three sections of *Pseudoepitrachys*, *Epitrachys* and *Cirsium*, which had more than one species in the present study (Fig. 3), indicating the higher value of polymorphic loci (40.7%), Shannon's Information Index (14.8%) and Expected Heterozygosity (11.7%) in sect. *Pseudoepitrachys*.

Genetic distinctness of the studied *Cirsium* sections was further supported by the obtained PCoA and PCA plots based on the most variable RAPD and ISSR loci, which both produced similar results; therefore, only the PCo plot is discussed here (Fig. 5). The first two PCO axes comprised about 67% of total variance. The species of sect. *Cirsium* and *Cephalonoplos* were separated from the other sections, along with the first factor with about 47% of total variance. However, the only studied species from sect. *Cephalonoplos* was placed in the lower angle of this axis, while the species of sect. *Cirsium* were placed to the opposite side of the first axis.

The species of sect. *Pseudoepitrachys* were placed in the lower part of the second axis, well separated from the other species due to their genetic difference. The species of sections *Epitrachys* and *Echenais* were placed in the upper part of the second axis and slightly distanced from each other.

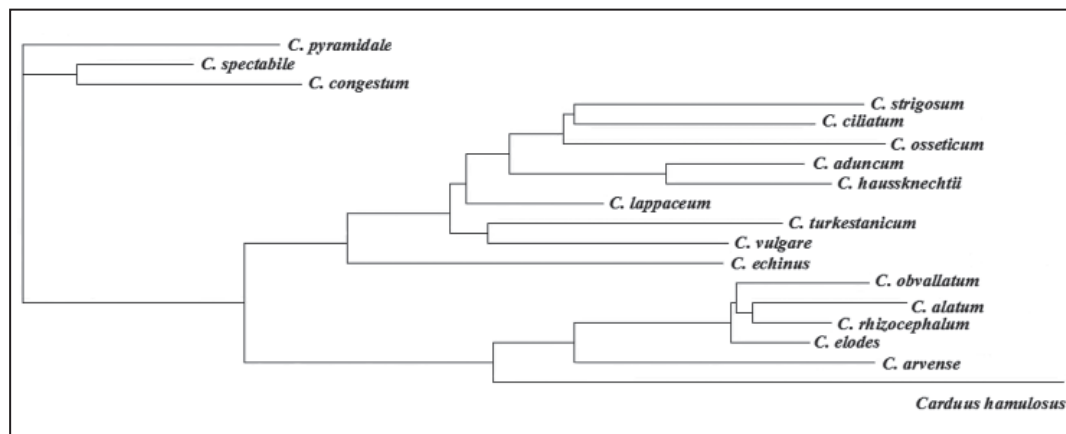


Fig. 5. NJ tree of combined RAPD and ISSR data.

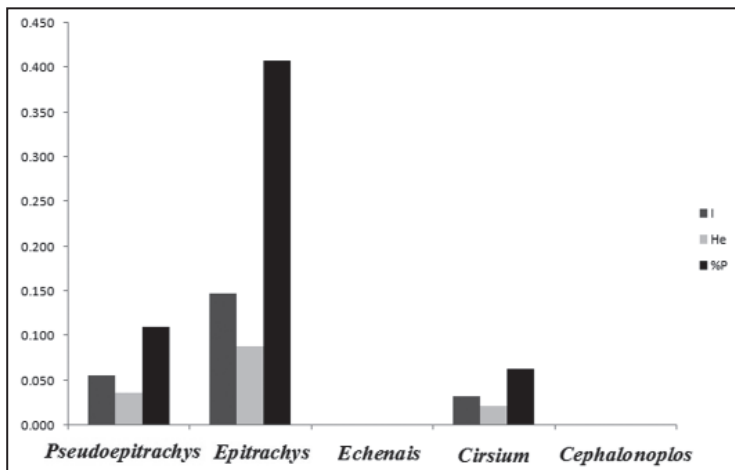


Fig. 6. Percentage of polymorphic loci (%P), Shannon's Information Index (I), and Expected Heterozygosity (He) determined for combined ISSR and RAPD data in the studied *Cirsium* sections.

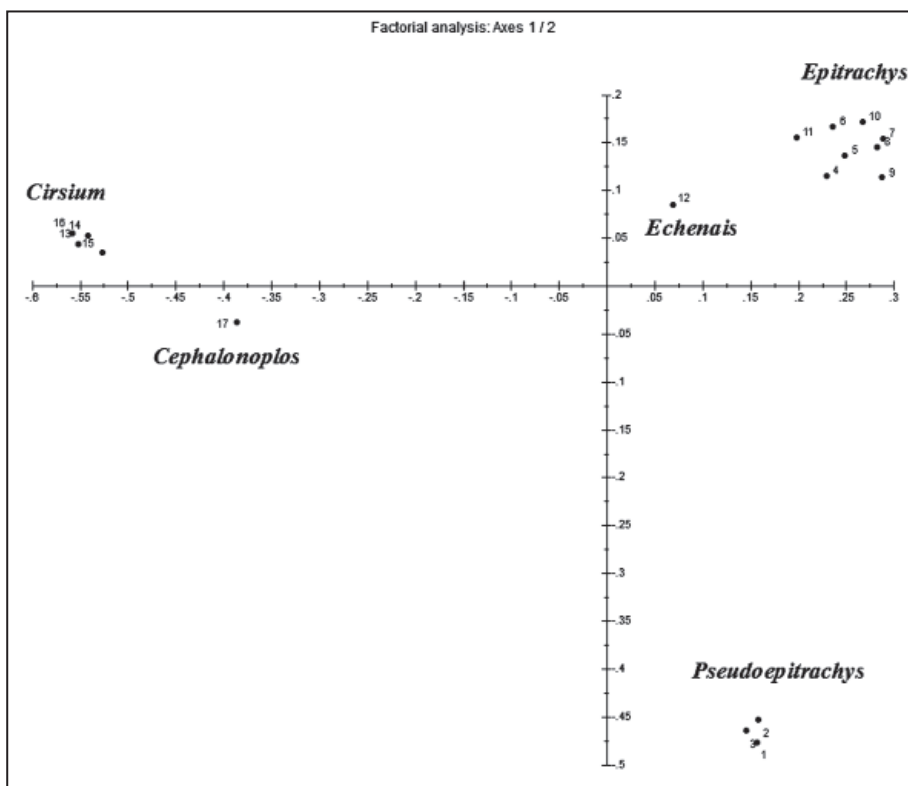


Fig. 7. PCA ordination of the *Cirsium* sections.

Discussion

The species investigated in the present study are from all sections of the Iranian *Cirsium*. According to Petrak (1979), the genus *Cirsium* has five sections in Iran, including *Pseudoepitrachys*, *Epitrachys*, *Echenais*, *Cirsium*, and *Cephalonoplos*.

The specific RAPD and ISSR bands obtained in the studied species indicate the genomic changes occurring during the species diversification and that these apomorphic characters may be used also in species

delimitation. This is supported by the AMOVA analysis showing significant molecular difference among the studied sections.

Separation of the *Cirsium* sections from each other in the NJ tree indicates that molecular data could be used in the genus taxonomy. The obtained results agree with the species affinities suggested in *Flora Iranica* (Petrak 1979). For example, *C. strigosum* from sect. *Epitrachys*, subsect. *Bracteosa* Petrak shows affinity for *C. ciliatum* and *C. osseticum* of subsect. *Ciliata* Petrak. Similarly, *C. aduncum* and *C. haussknechtii*

of subsect. *Microcephala* Petrak form a distinct cluster and show affinity for *C. lappaceum* of subsect. *Lappacea* Petrak,

Two species of *C. turkestanicum* from subsect. *Turkestanica* Petrak and *C. vulgare* from subsect. *Lanceolata* Petrak are placed close to each other, joining with the previous subsections similar to their taxonomic treatment in *Flora Iranica*. The same holds true for *C. echenais* from the sect. *Echenais* (Cass.) Petrak placed close to the species from sect. *Epitrachys*. These sections are considered close to each other in *Flora Iranica* (Petrak 1979).

According to *Flora Iranica*, in sect. *Cirsium*, *C. obvallatum* and *C. alatum* are closer to each other, while in the obtained NJ tree, *C. rhizocephalum* shows closer affinity for *C. alatum*, followed by *C. obvallatum*. Therefore, further studies are needed to determine the relationships between these species.

C. arvense of sect. *Cephalonoplos* (Necker) DC. shows affinity for the species of sect. *Cirsium* Petrak and joins them at some distance. This also agrees with the taxonomic treatment in *Flora Iranica*.

The Mantel test shows association between the obtained RAPD and ISSR genetic distances among the studied species, indicating an almost complete significant association between the two molecular markers and the fact that the trend of RAPD loci change/mutation during the species diversification is similar to the trend occurring in ISSR loci. This is further supported by the percentage of polymorphic loci obtained for the two molecular markers (Figs 4 and 5), as well as the Shannon's Information Index (I) and Expected Heterozygosity (He) determined for RAPD data in the studied *Cirsium* sections. In all these genetic diversity values, the highest values occurred in the species of sect. *Epitrachys*, followed by the species of sect. *Pseuduepitrachys* and sect. *Cirsium*.

While studying genome size variation in the genus *Cirsium*, Bureš & al. (2004) pointed out the relationship between the genome size and certain morphological characters in these taxa, including flower head diameter, involucre length, involucre width, corolla length, pappus length, and achene length. Furthermore, our own study of the genome size of the Iranian *Cirsium* taxa (unpubl. data) has shown a relationship between the genome size and the degree of spinosity (presence of stiff spines or barbs on leaves and stems). The present study also shows a positive significant correlation between some of the ISSR and

RAPD loci and the morphological characters, such as phyllary features and stem spine. Moreover, our earlier cytological study (Nouroozi & al. 2010, 2011) performed on the *Cirsium* species growing in Iran has shown that the species of *C. spectabile*, *C. congestum*, *C. strigosum*, *C. ciliatum*, *C. osseticum*, *C. aduncum*, *C. haussknechtii*, *C. turkestanicum*, *C. echinus*, *C. obvallatum*, *C. libanoticum*, *C. alatum*, *C. echinus*, *C. obvallatum*, *C. libanoticum*, *C. hygrophilum*, and *C. arvense* had $2n=2x=34$ chromosome number, while the species of *C. lappaceum*, *C. vulgare* and *C. elodes* have $2n=4x=68$. These species differed in their chromosome pairing behaviour and some of them manifested occurrence of heterozygote translocations and B-chromosomes (Nouroozi & al. 2010, 2011).

Therefore, the present study indicates that both quantitative (change in genome size/C-value) and qualitative (changes in DNA sequences revealed by ISSR and RAPD analyses) changes in the genome, along with polyploidy, structural changes in the chromosomes (heterozygote translocations), as well as occurrence of B-chromosomes have played a part in the genus *Cirsium* speciation and that use of these data (morphological, cytological, molecular and genome size) is valuable in the genus taxonomy and can also help in inferring the phylogenetic relationships of the species.

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