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Morphological and Molecular Cladistic Analyses of *Heliobrychis* Section (*Onobrychis* Genus)

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ABSTRACT

According to Flora Iranica, *Heliobrychis* section has 21 species. In this study, morphological and molecular cladistic analyses were performed for 29 *Onobrychis* taxa belong to *Heliobrychis* section. The result of morphological cladistic analysis indicate that the relationship of *O. aucheri* subsp. *aucheri* and *O. aucheri* subsp. *psammophila* were resolved (BP=52%), and *O. aucheri* subsp. *teheranica* was sister to them with 94% bootstrap support. In molecular cladistic analysis *O. aucheri* subsp. *psammophila* and *O. aucheri* subsp. *teheranica* were closest subspecies with high bootstrap support (BP=79%) but their relationships with *O. aucheri* subsp. *aucheri* were not resolved. In morphological and molecular cladistic analyses, there were no close relationships between *O. menaotricha* var. *melanotricha* and *O. melanotricha* var. *villosa* and each one formed sister group with other species. So contrast to Rechinger classification,*O. melanotricha* var. *melanotricha* and *O. melanotricha* var. *villosa* was raised to the rank of species as *O. melanotricha* and *O. villosa*.

Keywords*:* Cladistic analyses, *O. aucheri*, *O. psammophila*, *O. teheranica*, *O. melanotricha*, *O. villosa*.

INTRODUCTION

The genus *Onobrychis* Miller (Hedysareae, Papilionoideae, Leguminosae) with 130 perennial and annual species wordwide, is distributed mainly in the north temperate regions, but its centers of diversity are in the eastern Mediterranean area and western Asia (Yildiz et al. 1999). The genus was subdivided into two subgenera and nine sections (Rechinger 1984).

The taxonomy of the genus *Onobrychis* continues to be a subject of much confusion, mainly because of the different approaches to species delimitation, resulting in varying numbers of recognized species (Boissier 1872, Sirjaev 1925, Hedge 1970, Ball 1978,Rechinger 1984, Duman and Vural 1990, Aktoklu 2001). Inconsistency within the taxonomy of *Onobrychis* genus can be resolved by determining the phylogenetic relationships of *Onobrychis* species with morphological and molecular phylogenies. Defining of the

phylogenetic relationships of these species, beside the introduction of new species and determination of taxonomic position of the species would enhance the efficiency of *Onobrychis* breeding program.

One of the contradictions in taxonomy of *Onobrychis* genus is about *Heliobrychis* Bunge ex Boiss. section. This section was described by Rechinger (1984) which comprises 21 species. On the basis of Amirabadi-zadeh *et al.* (2007) results, general characteristics of the section are described as perennial or annual plants; crestless pods covered with pinnate bristles, orbicular, stipitate, along with a curved suture bearing seeds.

The aim of this research was to present result of phylogenetic studies on *Heliobrychis* section and to determine accurate taxonomic positions of species belong to this section.

MATERIAL AND METHODS

Taxon sampling

Twenty-nine taxa representing *Heliobrychis* section of *Onobrychis* genus were included as in-group taxa in the analyses. Based on molecular phylogenies using *nr*DNA ITS (SafaeiChaeiKar et al. 2012), two species of the other genus of tribe Hedysareae were selected as out-groups. Details of these taxa, including accession identities, geographical origins and gene bank sequence accession numbers, are given in Table 1.

Characters and characters states

Characters used in the cladistic analyses were obtained through examination of fresh materials in the field and herbarium specimens deposited at Herbarium of National Plant Gene Bank of Iran (NPGBI), Herbarium Research Center of Khorasan-e-Razavi Agricultural and Natural Resources center (MRCH), Mashhad, Iran and Herbarium of the Research Institute of Forests and Rangelands (TARI). Sixty-one characters which were used in the present analysis included: longevity, vegetation form, presence of prickle; stem (presence of stem, presence of hair, state of stem); stipule (tissue, position of stipule, presence of hair, length of stipule, shape of stipule); leaf (number of basal leaflets, number of cauline leaflet, leaflet form, size of leaflet width, length of leaflet, state of tip of the leaflet, presence of mucronateor apiculate, presence of hair at upper surface of leaflet, presence of hair at inferior surface of leaflet, size of petiole); peduncle (size of peduncle rather than leaves, state of peduncle at the end); inflorescence (shape of raceme, number of flowers per raceme); bract (figure of bract, cover of bract, length of bract); calyx (teeth figure of calyx, length of calyx, teeth rather than tube, presence of hair); corolla (color of flower, appearance of corolla); standard (figure of standard, length of standard, state of tip, presence of claw, presence of hair); wing (length of wing, cover of wing, state of tip, wing rather than calyx, figure of wing); keel (cover of keel); ovary (cover of ovary, presence of stipe, number of ovule); pod (size of pod, figure of pod, appearance of pod, presence of hair, presence of stipe, presence of crest, state of margin,

number of seed, state of dorsal suture of pod, state of surface of disc, number of loculus, shape of loculusat surface of disc, curvature).

DNA extraction, PCR and sequencing

Sequence data from nuclear ribosomal DNA internal transcribed spacers (*nr*DNA ITS) were obtained in order to analyze phylogenetic relationships of species. Total genomic DNA_S were isolated from dried leaf material using the modified CTAB (hexadecyltrimethyl ammonium bromide) method (Doyle & Doyle 1987). The complete *nr*DNA ITS region was amplified using ITS4 and ITS5 primers (White et al. 1990). PCR amplification were carried out on a thermal cycler using the following parameters: initial denaturation at $94^{\circ c}$ for 3 min followed by 30 cycles of denaturation at $94^{\circ c}$ for 30s, annealing at $52^{\circ c}$for 45s and extension at $72^{\circ c}$for 1 min and a final extension at $70^{\circ c}$ for 10 min.

Successfully amplified samples were purified using a gel purification kit (USA, Bioneer, Inc.). Nucleotide sequences of purified PCR products were determined using cycle sequencing and an automated DNA sequencer through Bioneer Co. The same *nr*DNA ITS primers ITS4 and ITS5 were utilized for cycle sequencing reactions. The sequences from the forward and reverse primers in each sample were aligned to generate a consensus sequence. As the sequences were of high quality, the forward and reverse sequences were identical, except for a few cases. These few discrepancies were resolved by repeated PCR and sequencing. Finally, each sequence related to each species was registered at the NCBI and a sequence accession number was obtained.

The *nr*DNA ITS sequence were aligned with Clustal W 1.8 (Thampson *et al*., 1994) and adjusted manually.

Cladistic analyses

Phylogenetic analyses were performed on the data matrix (morphological data) and aligned *nr*DNA ITS sequences using Maximum Parsimony method (MP) as implemented in the version 4.0b 10 of PAUP* (Swofford, 2002). All characters were considered as equally weighted. The heuristic search option was selected using 100 replications of random addition sequence with TBR (tree bisection reconnection) branch-swapping. In the analyses, supports for clades were evaluated by bootstrapping (Felsenstain, 1985) using 100 replications.

Table 1. locality, voucher and sequence accession number of *Heliobrychis* section of *Onobrychis* genus

Species	Locality	Voucher number	**Sequence accession number**
*Ebenusstellata*Bioss.	Kerman: 27 Km from Jiroft towards Mahan, near Mohammadabad village, 1680 m.	MRCH 7442	JX 426796
Eversmanniasubspinosa (Fisch.) B. Fedtsch.	Semnan: 28 Km from Shahrood towards Azadshahr, 1500 m.	MRCH 8983	JX 494755
*O. aurea*Ranjbar, Amirabadizadeh&Ghahremani	EasternAzarbayjan: Tabriz, Khajeh, Abkhandari research center, 1450 m.	MRCH 10037	JX 455139
*O. sojakii*Rech. F.	Kohkiloyeh and Boyerahmad: 45 Km from Khosravi towards Mamsani, near to Babameydan, 1800 m.	MRCH 5996	JX 426798
O. kermanensis (Sirj. &Rech. F.) Rech. F.	Kerman: Sirjan	NPGBI 6236	JX 426797
O. psoraleifolia var. *psoraleifolia*Boiss.	Esfahan: Ghomishloo, Baghak, 2000 m.	MRCH 1610	JX 290033
*O. andalanica*Bornm.	Sanandaj: Abidar park	NPGBI 6143	JX 455124
O. sylvatica	Khorasan: Mashhad	MRCH 5993	JX 290043
*O. szovitsii*Boiss.	Azarbayjan: 23 Km southeast of Khoy, SeyedHajin village, 1400-1450 m.	MRCH 6037	JX 455128
O. buhseana Bung ex Boiss.	EasternAzarbayjan: Boostanabad towards Sarab, 1800 M.	NPGBI 3875	JX 412238
O. melanotricha var. *villosa*Bornm.	Zanjan: Zanjan towards Ghorveh, Babarishani village, 1971 m.	NPGBI 6144	JX 455120
O. melanotricha var. *melanotricha*Boiss.	Hamedan: Nahavand, 1954 m.	NPGBI 6042	JX 290041
*O. oxyptera*Boiss.	Fars: 15 Km from Saadatshahr towards Arsanjan, 1800 m.	TAR I87626	JX 412235
*O. gypsicola*Rech. F.	Khozestan: 16 Km from BagheMalak towards Ramhormoz, 900 m.	MRCH 7757	JX 426799
*O. plantago*Bornm.	Kerman: Chopar mountain, 2600 m.	NPGBI 6237	JX 455140
*O. scrobiculata*Boiss.	Gharachaman	NPGBI 2823	JX 290034
*O. lunata*Boiss.	Hamedan:Malayer, 1994 m.	NPGBI 6041	JX 455127
*O. iranshahrii*Rech. F.	Kerman	MRCH 8265	JX 455125
O. depauperata var. *depauperata*Boiss.	EasternAzarbayjan: Marand towards Tabriz, Payam village, Mishoodagh hills	MRCH 8184	JX 455136
O. marandensis	EasternAzarbayjan: Marand towards Tabriz, Payam village, Mishoodagh hills, 1900 m.	MRCH 10052	JX 455119
*O. haussknechtii*Boiss.	Kermanshah	NPGBI 6145	JX 429954
O. *gaubae*Bornm.	Tehran: the first of Damavand road, 20 Km from Bomhen, 1700 m.	NPGBI 6146	JX 455118
O.*mozaffarianii*Amirabadi-zadeh	Esfahan: Semirum, Hanna, between Maurak and Khina to Khafr, 1900 m.	TARI 71263	JX 426794
*O. atropatana*Boiss.	Eastern Azarbayjan: Marand towards Zanooz, 1500 m.	NPGBI 3880	JX 412239
*O. argyrea*Boiss.	Azarbayjan: 2-12 Km west of Zenooz, 1500-1700 m.	NPGBI 10026	JX 412236
O. aucheri subsp. *aucheri*Boiss.	Ardabil: 15 Km Mianeh towards Ardabil, opposite of Ghazal-Ozan river, 110 m.	NPGBI 6147	JX 455117
O. aucheri subsp. *psammophila* (Bornm.) Rech. F.	Khorasan: TorbatHeydarieh, Kashmar, Baharieh, 1211 m.	NPGBI 6148	JX 455122
O. aucheri subsp. *teheranica* (Bornm.) Rech. F.	Khorasan: Neishaboor towards Sabzevar, after Baghjar	NPGBI 6149	JX 455123
*O. subacaulis*Boiss.	Eastern Azarbayjan: 8 Km Zenooz, Kanglomaraei, 1380 m.	MRCH 8478	JX 455135
O.*heliocarpa*Bioss.	East Azarbayjan: Marand, Zenooz village	NPGBI 6163	JX 429959
O. heterophylla C. A. Mey.	East Azarbayjan: Varzaghan towards Ahar, 20 Km remained to Satarkhan dam, 1721 m.	NPGB 6151	JX 455121

MRCH: Mashhad Research Center Herbarium.
NPGBI: National Plant Gene Bank of Iran.
TARI: Herbarium of the Research Institute of Forests and Rangelands.

RESULTS

Morphological cladistic analysis

The phylogenetic analysis based upon equally weighted characters yielded 524 most-parsimonious trees of 256 steps with CI of 0.38 and RI of 0.53. The strict consensus tree of these 524 trees is shown in Figure 1. Species belong to *Heliobrychis* section formed a strongly supported clade (BP=96%). Species relationships within this section were not properly resolved but *O. scrobiculata* and *O. atropatana* formed a weakly supported subclade (subclade a) (BP=60%) and species relationships within clade, b, are well resolved. Within this clade (clade b), *O. aucheri* subsp. *aucheri* and *O. aucheri* subsp. *Psammophila* were weakly allied taxa (BP=52%) and *O. aucheri* subsp. *teheranica* was sister to them (BP=94%). Also *O. heliocarpa*was sister to clade which include three subspecies belong to *O. aucheri* (BP= 84%). Some taxonomic distinctions among three subspecies of *O. aucheri* were mainly associated with state of stem; number of cauline leaflet; figure of wing; presence of stipe; figure of pod; number of ovule and number of seed (Table 2). Furthermore, in *Heliobrychis* section *O. melanotricha* var. *melanotricha* and *O. melanotericha* var. *villosa* were not closely related with each other. Some of their morphological differences considered in this study were summarized in Table 3.

Molecular cladistic analysis

Maximum parsimony (MP) analyze of the *nr*DNA ITS dataset (characters equally weighted) generated 825 most parsimonious tree with the length of 238 steps, CI=0.82 and RI=0.73. The 50% majority rule consensus tree from the phylogenetic analysis of *nr*DNA ITS sequences of 29 *Onobrychis*taxa (taxa belong to *Heliobrychis* section) and *Eversmannia* sub *spinosa* and *Ebenusstellata* as an out-group species is shown in Figure2.

Table 2. Comparison of morphological characters of three sub-species of *O. aucheri*

Sub-species	**Morphological characters**						
	Stem	**Leaf**	**Wing**	**Ovary**		**Pod**	
	State of stem	Number of cauline leaflet	Figure of wing	Number of ovule	Presence of stipe	Figure of pod	Number of seed
O. aucheri subsp. aucheri	procumbent	1-4 pairs	lanceolate	2 or 3	non stipitate	suborbicular	2 or 3
O. aucher subsp. teheranica	procumbent	> 4 pairs	narrowly oblong	1	stipitate	suborbicular	1
O. aucheri subsp. psammophila	erect	1-4 pairs	deltoid-oblong	2 or 3	stipitate	reniform	2 or 3

Table 3. Comparison of morphological characters of two varieties of *O. melanotricha*

varieties	**Morphological characters**						
	Stem		**Stipule**	**Leaf**		**Bract**	**Calyx**
	Presence of hair	State of stem	Position of stipule	Leaflet form	Presence of mucronate or apiculate	Length of bract	Teeth rather than tube
O. melanotricha var. melanotricha	glabrous	procumbent	sessile	oblong-ovate	without mucronate or apiculate	<5 m.m	equal
O. melanotricha var. villosa	glabrescent	erect	free	oblong-elliptic	mucronate-apiculate	>5 m.m	longer

Table 3. Continued

varieties	**Morphological characters**						
	Standard			**Wing**			**Pod**
	Figure of standard	State of tip	Presence of claw	Length of wing	State of tip	Figure of wing	Figure of pod
O. melanotricha var. melanotricha	ovate	emarginate	with claw	shorter-equal with half the length of the keel	very acute	deltoid-oblong	lunate
O. melanotricha var. villosa	roundish	obtuse	without claw	longer than half the length of the keel	acute	lanceolate	suborbicular

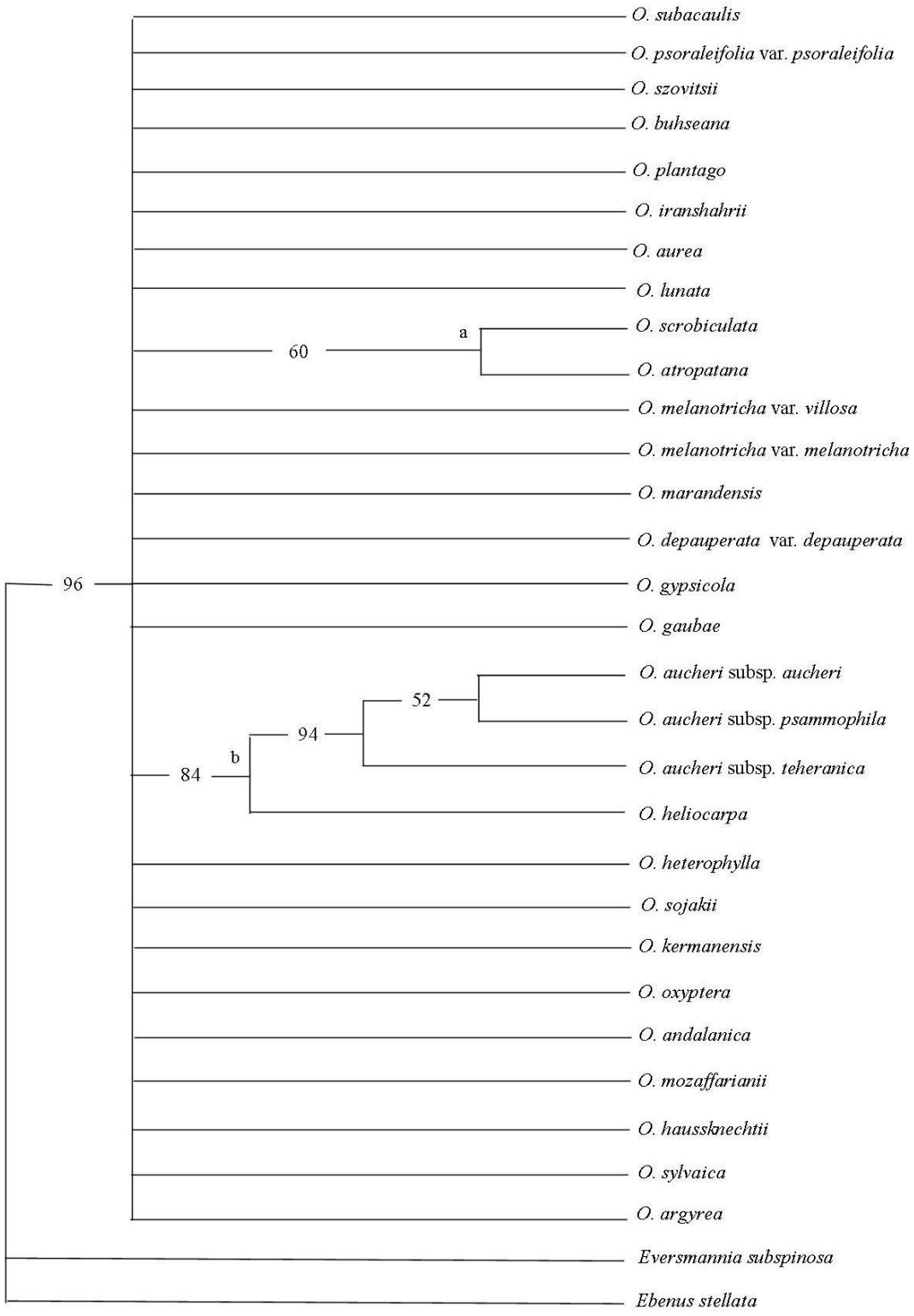

Figure 1. Maximum- parsimony 50% majority rule consensus tree generated from a phylogenetic analysis of morphological data of twenty-nine *Onobrychis* taxa (belong to *Heliobrychis* section) and two genus of tribe Hedysareae as an out-group. Bootstrap values of >50 % are indicated above the branches.

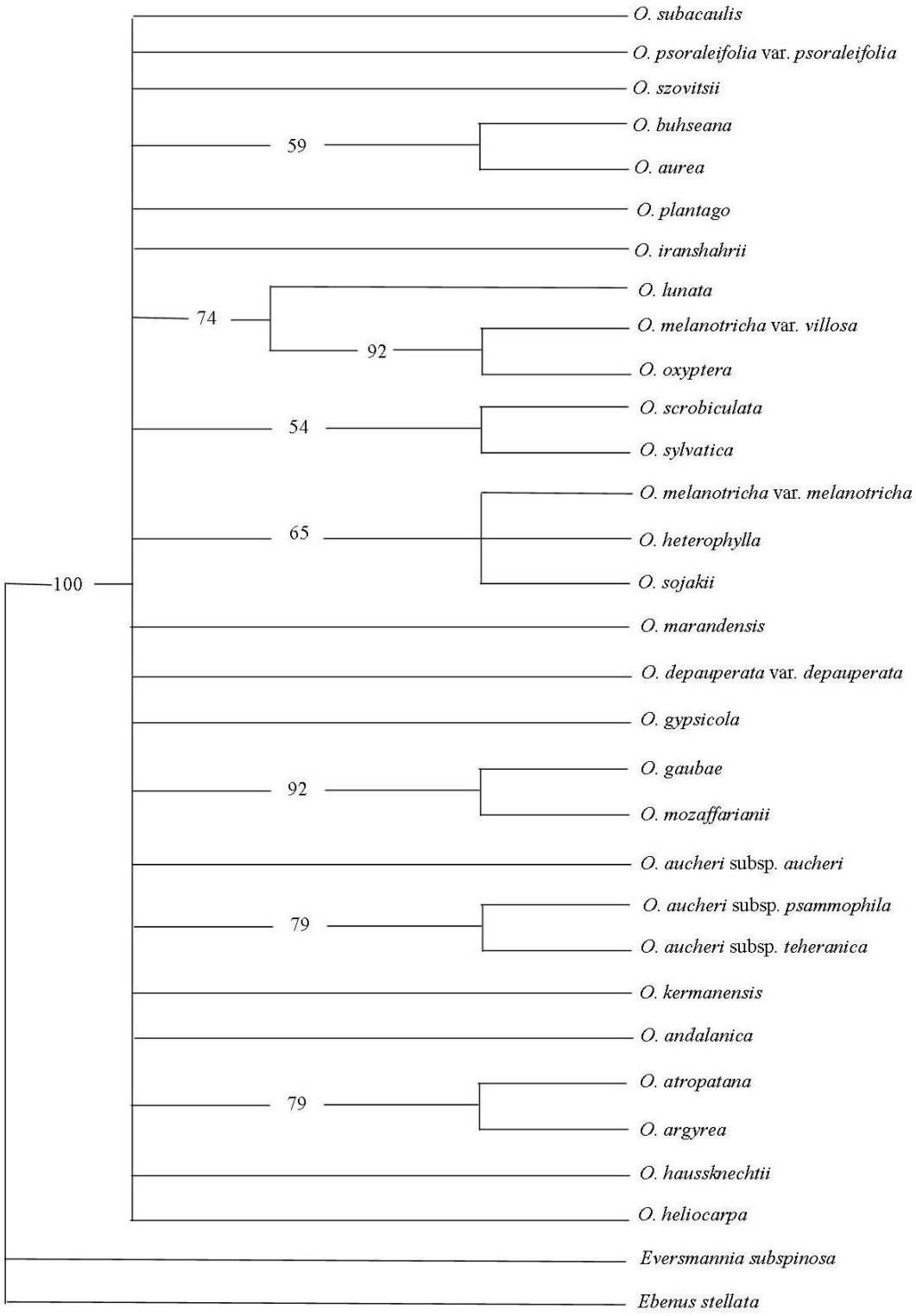

Figure 2. Maximum- parsimony 50% majority rule consensus tree generated from a phylogenetic analysis of DNA sequences data from internal transcribed spacers of the *nr*DNA of twenty-nine *Onobrychis* taxa (belong to *Heliobrychis* section) and two genus of tribe Hedysareae as an out-group. Bootstrap values of >50 % are indicated above the branches.

Species of *Heliobrychis* section made up a well-supported (BP=100%) and partially resolved clade, in which, there were a series of clades and subclades that will be explained about their phylogenetic relationships.

O. aucheri subsp. *teheranica* and *O. aucheri* subsp. *psammophila* formed a well-supported subclade (BP=79%), but these subspecies (*O. aucheri* subsp. *teheranica* and *O. aucheri* subsp. *psammophila*) were not close relatives to *O. aucheri* subsp. *Aucheri*. In other words *O. aucheri* subsp. *teheranica* and *O. aucheri* subsp. *psammophila*were closest species with high bootstrap support value of 79%, but they were not allied with *O. aucheri* subsp. *aucheri*,and *O. aucheri* subsp. *aucheri* was contained in a monoclade. *O. melanotricha* var. *melanotricha* and *O. melanotricha* var. *villosa* were not closely related with each other, so that *O. melanotricha* var. *melanotricha* formed a trichotomy with *O. heterophylla* and *O. sojakii* (BP=65%) and *O. melanotricha* var. *villosa* and *O. oxyptera* formed a well-supported subclade (BP=92%) that related to *O. lunata* (BP= 74%). This result indicates that there was no close association between the two varieties of *O. melanotricha*.

Also the phylogenetic relationships between *O. gaubae* and *O. mozaffarianii*; *O. atropatana* and *O. argyrea*; *O. buhseana* and *O. aurea*; *O. scrobiculata* and *O. sylvatica* was resolved (Figure 2).

DISCUSSION

Phylogenetic relationships of species belong to *Heliobrychis* section in molecular cladistic analysis resolved better than in morphological cladistic analysis because of the highest number of parsimony-informative characters in molecular cladistic analysis.

O. aucheri was for the first time identified by Boissier (1872). Bornmuller (1905) recognized two species of *Onobrychis* namely, *O. teheranica* and *O. psammophila*, and based on Rechinger (1984) taxonomic work, *O. aucheri* subsp. *aucheri*, *O. aucheri* subsp. *psammophila* and *O. aucheri* subsp. *teheranica* are morphologically similar to each other, and for this reason Rechinger (1984) introduced them as subspecies belong to *O. aucheri*.Thus, according to Flora Iranica (Rechinger 1984) *O. aucheri* from sect. *Heliobrychis* has three subspecies; *O. aucheri* subsp. *aucheri*, *O. aucheri* subsp. *teheranica* and *O. aucheri* subsp. *psammophila*.

Based on morphological and karyotypic studies done by Hatami and Nasirzadeh (2008), *O. aucheri* subsp. *teheranica* was diploid (2n=2x=16) but *O. auchri* subsp. *psammophila* was tetraploid (2n=4x=32). Karyotypic formula and karyotypic symmetry showed that *O. aucheri* subsp. *teheranica* was 7m+1sm, allocated to 1B class and *O. aucheri* subsp. *psammophila* was 12m+4m, allocated to 2B class. According to the morphological characteristics, two subspecies have important differences including: stem; number of leaflet; spines on pod, number of seed per pod, size of pod; sepal and vegetation form. So they concluded that due to morphological and karyotypic differences between *O. aucheri* subsp. *psammophila* and *O. aucheri* subsp. *teheranica*, could not be regarded as subspecies of *O. aucheri* and the suggestion of Bornmuller (1905) on regarding the two subspecies as two species is preferred.

In this research based on morphological cladistic analysis, relationship between *O. aucheri* subsp. *aucheri* and *O. aucheri* subsp. *psammophila* were resolved but statistically not well supported (BP=52%), and *O. aucheri* subsp. *teheranica* was sister to them with 94% bootstrap support. However, morphological cladistic analysis of this study indicated that these two subspecies (*O. aucheri* subsp. *aucheri* and *O. aucheri* subsp. *psammophila*) were weakly relative taxa, but they share consecutive morphological characteristics including; number of cauline leaflet, number of ovule, and number of seed. Morphological studies indicated that *O. aucheri* subsp. *aucheri* with procumbent stem; lanceolate wing; non-stipitate, suborbicular pod is distinguish from *O. aucheri* subsp. *psammophila*. Also, it differs from *O.aucheri* subsp. *teheranica* in having less cauline leaflets and 2 or 3 ovules and seeds. In molecular cladistic analysis *O. aucheri* subsp. *psammophila* and *O. aucheri* subsp. *teheranica* were closest subspecies with high bootstrap support (BP=79%), but their relationships with *O. aucheri* subsp. *aucheri* were not resolved. So we conclude that,due to phylogenetic relationships between three subspecies belong to *O. aucheri* based on morphological and molecular cladistic analyses, they will be considered as subspecies of *O. aucheri* and the suggestion of Rechinger (1984) is preferred and the Bornmuller (1905) and Hatami and Nasirzadeh (2008) opinions are rejected.

In addition to, Rechinger (1984) recommended *O. melanotricha* has two varieties namely: *O. melanotricha* var. *melanotricha* and *O. melanotricha* var. *villosa*. In morphological and molecular cladistic analyses, there were not close relationships between *O. menaotricha* var. *melanotricha* and *O. melanotricha* var. *villosa* and each one formed sister group with other species. The interspecies relationships of *Heliobrychis* section displayed by cladistic analyses of morphological and molecular phylogenies differed from those exhibited by traditional taxonomic classification (Rechinger 1984). Due to lake of close relationships between two varietis of *O. melanotricha* based on morphological and molecular analyses, we propose that this two varieties raised to the rank species as *O. melanotricha* and *O. villosa*.

CONCLUSION

Based on morphological and molecular cladistic results of this research, it is recommended that the two varieties belong to *O. melanotricha (O. melanotricha var. melanotricha and O. melanotricha var. villosa)* be introduce as species namely, *O. melanotricha* and *O. villosa* respectively.

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