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Phylogenetic relationships of *Onobrychis* Mill. (Fabaceae: Papilionoideae) based on ITS sequences of nuclear ribosomal DNA and morphological traits

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ABSTRACT

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The genus *Onobrychis* is subdivided into two subgenera: *Onobrychis* and *Sisyrosema*. Phylogenetic relationships of 19 species of *Onobrychis* (Fabaceae: tribe Hedysareae) and one representative each of genera *Eversmannia* and *Ebenus* were estimated from DNA sequences of the internal transcribed spacer (ITS) region. Parsimony analysis of the ITS region formed a dendrogram with strong bootstrap support from two groups: *Onobrychis* subgen. *Onobrychis* (except *O. laxiflora*) is in one group and *Onobrychis* subgen. *Sisyrosema* is in the other. Within group I, species of the *Onobrychis* section and species of the *Lophobrychis* and *Dendrobrychis* sections form well supported branches A and B, respectively (BP=99% and 91%). The close association between the *Onobrychis* section and *Lophobrychis* and *Dendrobrychis* indicated there is strong sequence homology among them, suggesting that these species are closely related in terms of phylogeny. Also there is strong sequence homology among sections of *Sisyrosema* subgen. (group II). Species of *Heliobrychis*, *Hymenobrychis* and *Afghanicae* form a branch (branch C) with 77% bootstrap support. Species of the *Hymenobrychis* section form a clade (clade F) with 82% bootstrap support, which indicates these species are closely related. The present nrDNA ITS data showed that *Onobrychis* subgen. *Sisyrosema* appears to be a well-supported monophyletic group (BP=77%), whereas the *Onobrychis* subgen. *Onobrychis* is not monophyletic due to the sister group relationship of one species of *Onobrychis* subgen. (*O. laxifolra*) to the subgen. *Sisyrosema*. Cluster analysis of morphological characters showed two major groups separating *Onobrychis* subgen. *Onobrychis* (except *O. laxiflora*) from *Onobrychis* subgen. *Sisyrosema*, which are in accordance with molecular phylogenetic groups.

Keywords: cluster analysis, Fabaceae, Hedysareae, nrDNA ITS, *Onobrychis*, phylogenetic analysis, *Sisyrosema*

INTRODUCTION

The Leguminosae are one of the largest families of flowering plants, with 18,000 species classified into around 650 genera. The family is usually divided into three sub-families: Papilionoideae, Caesalpinoideae and Mimosoideae (Polhill and Raven, 1981). The tribe Hedysareae was originally described as one of the tribes comprising the subfamily Papilionoideae (Bentham, 1865). Lock (2005) included in the Hedysareae many genera, such as: *Alhagi*, *Caragana*, *Corethrodendron*, *Ebenus*, *Eversmannia*, *Calophaca*, *Halimodendron*, *Hedysarum*, *Onobrychis*, *Sartoria*, *Sulla* and *Taverniera*. *Onobrychis* Mill. constitutes the second largest genus (after *Hedysarum*) of tribe Hedysareae in the sense adopted by Polhill (1981) (Mabberley, 1990; Lock, 2005).

The genus *Onobrychis*, represented by 170 perennial and annual species in the world, is densely distributed in the Anatolia-Iran-Caucasian triangle (Nixon, 2006; Emin and Kuddisi, 2010). Phylogenetic studies indicated that the primary center of genetic diversity of *Onobrychis* is in the Mediterranean region and that the ecological separation of this region into western and eastern sectors represents a main event in the evolution of the genus (Ashurmetov and Normatov, 1998). *Onobrychis* is a perennial and economically important genus used to improve the quality of the soil. It is also harvested as dried, fresh and purebred fodder (Emin and Kuddisi, 2010).

Yildiz *et al*. (1999) suggested that, based on fruit morphology, genus *Onobrychis* is subdivided into two subgenera, namely, *Onobrychis* with four

sections (*Dendrobrychis*, *Lophobrychis*, *Onobrychis* and *Laxiflorae*), and *Sisyrosema* with five sections (*Anthyllium*, *Afghanicae*, *Heliobrychis*, *Hymenobrychis* and *Insignes*) distinguished by different karyotypes, morphological features and geographical origins.

Typically, one of the areas utilized for phylogenetic inference at the generic and infrageneric levels in plants is the internal transcribed spacer (ITS) region of 18S-5.8S-26S nuclear ribosomal DNA (*nr*DNA). The advantages of this locus for phylogenetic reconstruction include: biparental inheritance, universal primers, intragenomic consistency and intergenomic variability (Baldwin *et al*., 1995). The internal transcribed spacer (ITS) mostly provides enough molecular markers which are acceptable for evolutionary studies at the species level (Ganzalo and Josep, 2007). It has been proved that ITS is suitable for studying interspecific relationships in many plant families (reviewed in Baldwin *et al.,* 1995), and particularly within the Fabaceae (Wojciechowski *et al.,* 1993; Sanderson *et al*., 1996; Downie *et al*., 1998).

The Hedysareae in general and *Hedysarum* (Chennaoui *et al*., 2007) and *Onobrychis* (Yildiz *et al*., 1999; Ahangarian *et al*., 2007) in particular remain under-sampled in molecular phylogenetic analyses. In a study by Yildiz *et al*. (1999), 40 species belonging to five sections of the *Onobrychis* genus and six species belonging to four sections of *Hedysarum* as an out-group were evaluated based on fruit morphology. Results showed there are no common traits for fruit, which supports the monophyly of the *Onobrychis* genus. The monophyly of *Onobrychis* and subgen. *Sisyrosema* has not been confirmed. Abou-El-Enain (2002) evaluated 22 taxa of six *Onobrychis* species belonging to the *Lophobrychis* section based on chromosome number, chromosome length and ploidy levels. His evaluation confirmed the monophyly of the *Onobrychis* genus. Ahangarian *et al*. (2007) estimated that there are 11 species of *Onobrychis* representing two subgenera and 8 sections. Results showed that *Onobrychis* subgen. *Onobrychis* is not monophyletic, whereas *Onobrychis* subgen. *Sisyrosema* forms a strongly supported clade. In contrast to *Onobrychis* section *Heliobrychis*, sections *Dendrobrychis* and *Onobrychis* are not monophyletic. Based on cytological evaluation of annual species of the *Onobrychis* genus in Iran, Ghanavati *et al*. (2012) reported that the basic chromosome number varied from x=7 to x=8.

Iran is one of the most important centers of *Onobrychis* genetic variation, for there are more than 69 annual and perennial species and subspecies of the genus distributed over different climatic regions. Because these species have high genetic variability, it is possible to use them as a rich and valuable genetic resource for breeding farm species. The range of the *Onobrychis* genus is very variable and the boundary between species is not clear. Molecular phylogenetic studies of *Onobrychis* species are limited to a small number of taxa (Wojciechowski *et al*., 2000; Ahangarian *et al*., 2007). Therefore, study of the reconstruction of the phylogenetic relationships and the boundary among species in this genus-using *nr*DNA ITS sequences are also considered. Determination of the phylogenetic relationships of these species, beside the introduction of new species and wild relatives of cultivated species of this genus would enhance the efficiency of *Onobrychis* breeding program.

The aim of this study was to investigate the relationships of taxa within two separate subgenera of *Onobrychis*, subgen. *Onobrychis* and subgen. *Sisyrosema*. To accomplish this goal, the internal transcribed spacer (ITS) region of the 18S-5.8S-28S nuclear ribosomal DNA (*nr*DNA) was sequenced. Morphological characteristics were studied and the resulting cluster compared with the molecular cluster.

MATERIALS AND METHODS

Taxon sampling

To conduct molecular phylogenetic studies during 2011-2012, leaf materials were sampled from herbarium specimens deposited in the Herbarium Research Center of Khorasan-e-Razavi Agricultural and Natural Resources Center (MRCH), Mashhad, Iran; the Herbarium of National Plant Gene Bank of Iran (HNPGBI); and the Herbarium of the Research Institute of Forests and Rangelands (TARI). In this study, 21 species including 19 species representing seven sections of the *Onobrychis* genus and two species of the other genus of tribe Hedysareae as an out-group, were selected for molecular phylogenetic reconstruction. Details of these species, including accession identities, geographical origins and genebank sequence accession numbers, are given in Table 1.

DNA extraction, PCR and sequencing

Total genomic DNA was isolated using a modified CTAB extraction method (Doyle and Doyle, 1987). The DNA concentration was determined with a Gene-Guant spectrophotometer (Pharmacia) and DNA quality was analyzed by agarose gel electrophoresis.

Table 1. Locality, voucher and sequence accession number of *Onobrychis* species.

Section-Species	Locality
***Ebenus stellata* Bioss.**	**Kerman: 27 Km from Jiroft towards Mahan, near Mohammadabad village, 1680 m.**
***Eversmannia subspinosa* (Fisch.) B. Fedtsch.**	**Semnan: 28 Km from Shahrood towards Azadshahr, 1500 m.**
Sect. *Onobrychis*: *Onobrychis cyri* var. *cyri* Grossh.	**West Azarbayjan: 75 Km from Khajeh towards Sonegoon cupper mine, 2340 m.**
Sect. *Onobrychis*: *Onobrychis shahpurensis* Rech. F.	**East Azarbaijan: Ouromie, Gardane Ghoshji**
Sect. *Onobrychis*: *Onobrychis persica* Sirj. & Rech. F.	**Ghazvin: Kohin cervix**
Sect. *Onobrychis*: *Onobrychis major* Boiss.	**Hamedan: Around of the Ekbatan massif**
Sect. *Dendrobrychis*: *Onobrychis elymaitica* Boiss. & Hausskn.	**Kohkiloye and Boyerahamd: Dehdasht, Lourab, Heidar abad, 1500 m.**
Sect. *Dendrobrychis*: *Onobrychis cornuta* (L.) Desv.	**Zanjan: Zanjan towards Ghorveh, first of Ghorveh road, Babarishani village, route of Hamzeh arab mountain, 1971 m.**
Sect. *Lophobrychis*: *Onobrychis pulchella* Schrenk	**Khorasan: Jangale Khaje**
Sect. *Lophobrychis*: *Onobrychis micrantha* Schrenk	**Khorasan: 75 Km from Mashhad, Kalat, 980 m.**
Sect. *Laxiflorae*: *Onobrychis laxiflora* Baker	**Khorasan: Birjand towards ghaenat**
Sect. *Afghanicae*: *Onobrychis tavernieraefolia* Stocks ex Boiss.	**Sistan and Baluchestan: 25 Km from Zahedan towards Khash, 1680 m.**
Sect. *Afghanicae*: *Onobrychis nummularia* Stocks ex Boiss.	**Hormozgan: 111 Km from Bandar abas towards Sirjan, 1000 m.**
Sect. *Heliobrychis*: *Onobrychis buhseana* Bung ex Bioss.	**East Azarbayjan: Bostan Abad, Sarab, 1800 m.**
Sect. *Heliobrychis*: *Onobrychis gaubae* Bornm.	**Tehran: the first of Damavand road, 20 Km from Bomhen, 1700 m.**
Sect. *Heliobrychis*: *Onobrychis mozaffarianii* Amirabadizadeh	**Esfahan: Semirum, Hanna, between Maurak and Khina to Khafr, 1900 m.**
Sect. *Heliobrychis*: *Onobrychis heliocarpa* Bioss.	**East Azarbayjan: Marand, Zenooz village**
Sect. *Hymenobrychis*: *Onobrychis ptolemaica* (Del.) DC.	**Khuzestan: road of Malek garden towards Izeh, 300m.**
Sect. *Hymenobrychis*: *Onobrychis galegifolia* Boiss.	**Kordestan: Marivan, Zarivar lake**
Sect. *Hymenobrychis*: *Onobrychis michauxii* DC.	**East Azarbayjan: Kalibar, Garmadooz section**
Sect. *Hymenobrychis*: *Onobrychis hohenackeriana* C. A. Mey.	**East Azarbayjan: Ahar, Varzatan village**

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

Table 2. Morphological characteristics of *Onobrychis* species.

Row	Plant characteristics	Score
1	Longevity	0= perennial; 1= annual or biennial
2	Vegetation form	0= shrubby; 1= suffrutescent; 2= herbaceous
3	Presence of prickle	0=prickly; 1= without prickle
	Stem	
4	Presence of stem	0=acaulescent; 1= having a stem
5	Presence of hair	0= glabrous; 1= glabrescent; 2= hairy
6	State of stem	0= procumbent-ascendent; 1= erect-strict
	Stipule	
7	Tissue	0= herbaceous; 1= membranous or searious
8	Position of stipule	0= sessile; 1= free-sessile; 2= free
9	Presence of hair	0= glabrous; 1= hairy or ciliate
10	Length of stipule	0≤6; 1>6
11	Shape of stipule	0= ovate-lanceolate or lanceolate-ovate; 1= triangular or subulate; 3= lanceolate-triangular or lanceolate; 4= lanceolate-subulate
	Leaf	
12	Number of basal leaflets	0≤3; 1>3
13	Number of cauline leaflet	0≤1; 1=1-4; 2=>4
14	Leaflet form	0= linear-elliptic or oblong elliptic-linear; 1= oblong-elliptic or elliptic-oblong; 2= oblong-ovate or ovate-oblong; 3= oblong-linear or linear-oblong; 4= elliptic-orbicular or orbicular-ovate or ovate-orbicular
15	Size of leaflet width	0= 5 mm; 1=5-8 mm; 2=5-15 mm; 3>15 mm
16	Length of leaflet	0<14 mm; 1=14-30 mm; 2>30 mm
17	State of tip of the leaflet	0= obtuse; 1=acute; 2= obtuse-acute
18	Presence of mucronate or apiculate	0= without mucronate-apiculate; 1= mucronate-apicolate
19	Presence of hair at upper surface of leaflet	0= glabrous; 1= hairy
20	Presence of hair at inferior surface of leaflet	0= glabrous; 1= hairy
21	Size of petiole	0= short; 1= long
	Peduncle	
22	Size of peduncle rather than leaves	0= shorter; 1= equal; 2= longer
23	State of peduncle at the end	0= spiny; 1= without spine
	Inflorescence	
24	Shape of raceme	0=sparse; 1= dense
25	Number of flowers per raceme	0>20; 1<20; 2<10
	Bract	
26	Figure of bract	0=lanceolate-subulate or lanceolate-linear or lanceolate; 1= subulate-lanceolate or subulate; 2= linear-lanceolate or linear or linear-subulate
27	Cover of bract	0= glabrous; 1= ciliate or hairy
28	Length of bract	0<5 mm; 1>5 mm
28	Length of bract	0<5 mm; 1>5 mm
	Calyx	
29	Teeth figure of calyx	0= lanceolate-linear or lanceolate-triangular or lanceolate or lanceolate-subulate; 1= linear-subulate or linear; 2= subulate; 3= triangular
Row	Plant characteristics	Score
30	Length of calyx	0<5 mm; 1=5-6 mm; 2>6
31	Teeth rather than tube	0=shorter; 1=equal; 2= hairy
32	Presence of hair	0=glabrous; 1= hairy
	Corolla	
33	Color of flower	0= red-pink-violet; 1= yellow-cream; 2= white-milky; 3= variegated
34	Appearance of corolla	0= concolorous; 1= concolorous or veined
	Standard	
35	Figure of standard	0= elliptic or elliptic-oblong; 1= obovate or obovate-elliptic or obovate-orbicular; 2= oblong or oblong-elliptic or oblong-ovate; 3= roundish or roundish-elliptic or roundish-ovate; 4= ovate or ovate-roundish or ovate-cuneate
36	Length of standard	0<7 mm; 1=7-10.5 mm; 2= 10.5-18 mm; 3>18 mm
37	State of tip	0= obtuse; 1= emarginated; 2= retuse or retuse-emarginate
38	Presence of claw	0=without claw; 1= with claw
39	Presence of hair	0= glabrous; 1= hairy
	Wing	
40	Length of wing	0= shorter than half the length of the keel; 1= shorter-equal with half the length of the keel; 2= longer than half the length of the keel; 3= equal with the length of the keel
41	Cover of wing	0= glabrous; 1= ciliate
42	State of tip	0= obtuse; 1= slightly acute; 2= acute; 3= very acute
43	Wing rather than calyx	0= shorter; 1= equal-subequal; 2= longer
44	Figure of wing	0= narrowly oblong or oblong-linear; 1= deltoid-oblong or ovate-rhomboid or ovate-triangular; 2= lanceolate; 3= falcate
	Keel	
45	Cover of keel	0= glabrous; 1= hairy
	Ovary	
46	Cover of ovary	0= glabrous; 1= hairy; 2= glabrous-hairy
47	Presence of stipe	0= sessile or subsessile; 1= short; 2= long
48	Number of ovule	0= 1; 1= 1 or 2; 2= 2 or 3; 3>3
	Pod	
49	Size of pod	0= small; 1= large
50	Figure of pod	0= suborbicular-semiorbicular; 1= lunate; 2= reniform; 3= orbicular; 4= linear or oblong
51	Appearance of pod	0= compressed; 1= convex
52	Presence of hair	0= glabrous or pubescence; 1= with bristle and plumose; 2= denselylanate; 3= cottony-wovwn together
53	Presence of stipe	0= non stipitate; 1= stipitate; 2= cuneate-winged
54	Presence of crest	0= without crest; 1= crestes
55	State of margin	0= without teeth; 1= with teeth
56	Number of seed	0= 1; 1= 1-2; 2= 2 or 3
57	State of dorsal suture of pod	0= erect-suberect; 1= curved
58	State of surface of disc	0= without spines; 1= spinous; 2= with or without spines; 3= with bristle
59	Number of loculus	0= uni locular; 1= bilocular; 2= multilocular
60	Shape of loculus at surface of disc	0= pitted; 1= foveolate and areolate; 2= smooth
61	curvature	0= circinate-incurved; 1= incurved; 2= without curvature

The complete *nr*DNA ITS region was amplified by standard double-stranded PCR (Eppendorf-Netheler-Hinz Gmbh, Germany) using primers ITS4 and ITS5 of White *et al*. (1990), and the following temperature regime: 3 min denaturation at 94 °C, followed by 30 30-sec cycles of denaturation at 94 °C, 45 sec primer annealing at 52 °C, primer extension for 1 min at 72 °C, and a final 10-min extension at 70 °C. 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 are 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.

Sequence alignment and data analysis

The *nr*DNA ITS sequences were aligned by Muscle and adjusted manually. Phylogenetic analysis was performed on the aligned data matrix using the maximum parsimony (MP) method as implemented in version 5 of MEGA (Tamura *et al*., 2011).

Morphological analysis

A total of 61 quantitative/qualitative traits related to vegetative and reproductive organs were studied in 19 species of the *Onobrychis* genus (Table 2). For statistical analysis, the qualitative traits were initially encoded according to the multi-state method, and the related means were considered for quantitative characters, which were standardized. Phenetic analysis was carried out using MVSP Vers. 3.2 (Kovach, 1985-2002) and the Centroid linkage method; phenograms of these species were prepared by analyzing morphological character variation in all species in each section.

RESULTS

The length of *nr*DNA ITS ranges from 684 base pairs (bp) in *O. ptolemaica* to 649 bp in *O. tavernierafolia.* Parsimony analyses of ITS sequences have provided consistency and retention indices of 0.71 and 0.78, respectively. The phylogenetic tree of *nr*DNA ITS sequences includes 19 *Onobrychis* species and 2 species of the other genus in the Hedysareae tribe as an out-group (see Fig. 1).

Parsimony analysis showed that all the species of the *Onobrychis* genus formed two main groups. Group I consisted of two branches belonging to different sections of the subgenus *Onobrychis*. The first branch, A, (BP=99%) consisted of the section *Onobrychis*. The second branch, B, (BP=91%) consisted of four species belonging to sections *Dendrobrychis* and *Lophobrychis.* In fact, this branch is composed of two well-supported clades (BP=100% and 93%, respectively). Clade (b_1) possesses two species of the *Dendrobrychis* section, whereas clade (b_2) includes two species of the *Lophobrychis* section. Branch A (species of *Onobrychis* section) is a sister group of branch B (species of the *Dendrobrychis* and *Lophobrychis* sections) with 88% bootstrap support.

Group I has successive sisters in group II, including representatives of one section of *Onobrychis* subgen. (*Laxiflorae*) and three sections of *Sisyrosema* subgen. (*Afghanicae*, *Heliobrychis* and *Hymenobrychis*), i.e., *O. laxiflora* through *O. michauxii*. In this group there is one monoclade (*O. laxiflora*) and a big branch, C. Relationships within this branch containing *O. nummularia* through *O. michauxii* were resolved properly. Branch C includes all sections of subgen. *Sisyrosema* or three well-supported clades (BP=96%, 99% and 82%, respectively). Clade D with good bootstrap support (99%) includes species of the *Heliobrychis* section. A single clade, E, (BP=96%) contains species of the *Afghanicae* section. In clade F, which includes species of the *Hymenobrychis* section, a strong phylogenetic affinity was observed between *O. michauxii* and *O. hohenackeriana*, as shown in subclade G.

For the morphological analysis, 19 species of the *Onobrychis* genus were analyzed based on 61 morphological traits. Figure 2 presents a Centroid linkage phenogram with percent similarity resulting from analyzing the morphological traits of 19 species. Apparently there are six separate groups, and each of the sections except *Laxiflorae* are separate from the other sections in a single group. Species of the *Hymenobrychis* section, including *O. ptolemaica*, *O. hohenackeriana*, *O. michauxii* and *O. galegifolia*, were placed in the first group, A, due to their common trait specifications (for example, herbaceous, pod with wide crest, stipitate, curved spinous surface of disc, etc.). Species of the *Afghanicae* section, *O. nummularia* and *O. tavernierafolia*, were placed in group B. Species of the *Heliobrychis* section, including *O. heliocarpa*, *O. mozaffarianii*, *O. gaubae* and *O. buhseana* and

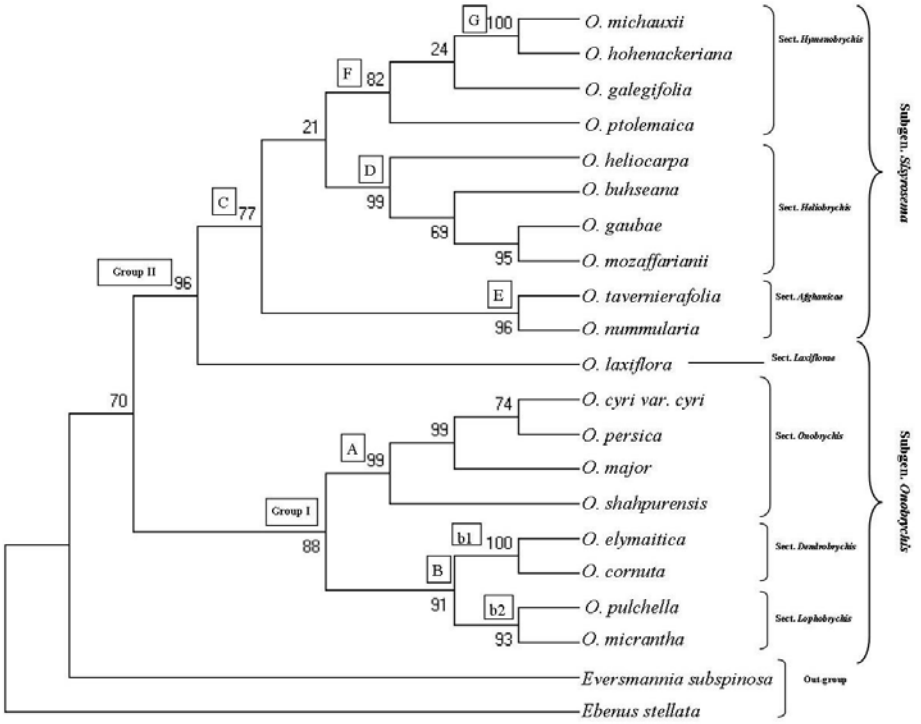

Fig. 1. Dendrogram generated from a phylogenetic analysis of DNA sequence data from internal transcribed spacers of the *nr*DNA of 19 *Onobrychis* species and two genera of the Hedysareae tribe as an out-group. Letters above the branches indicate clades. Bootstrap values are indicated above and below the branches.

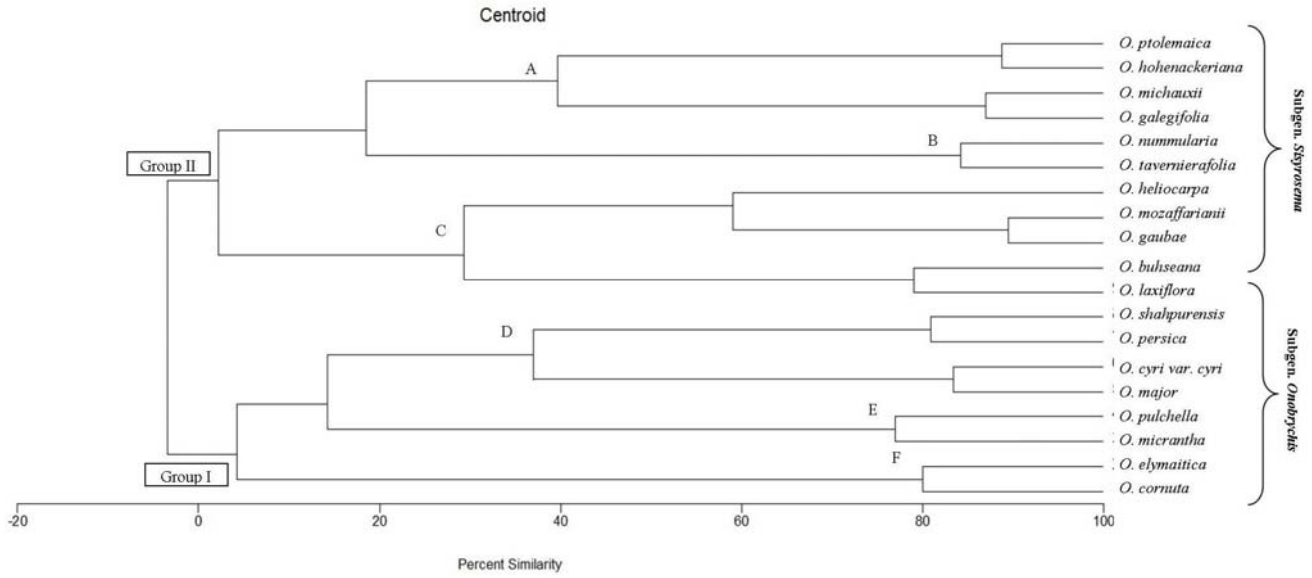

Fig. 2. Centroid linkage phenogram based on analyzing morphological data of 19 species of *Onobrychis* genus in Iran representing six groups (A, B, C, D, E and F).

one species of the *Laxiflorae* section (*O. laxiflora*), were placed in the third group, C, because of common characteristics such as perennial and semi-orbicular pod. Species of the *Onobrychis* section (*O. shahpurensis*, *O. persica*, *O. cyri* var. *cyri* and *O. major*) were clustered in group D. Species of the *Lophobrychis* section (*O. micrantha* and *O. pulchella*) were placed in the fifth group, E, and the last group, F, contained species of the *Dendrobrychis* section (*O. cornuta* and *O. elymaitica*).

Onobrychis subgen. *Onobrychis*, including *Onobrychis*, *Lophobrychis* and *Dendrobrychis* (except *Laxiflorae*), are positioned adjacently and completely separate from *Onobrychis* subgen. *Sisyrosema* including the *Afghanicae*, *Heliobrychis* and *Hymenobrychis* sections.

DISCUSSION

In this ITS analysis, all species of the *Lophobrychis*, *Onobrychis* and *Dendrobrychis* sections are clustered into a group separate from *Heliobrychis*, *Hymenobrychis*, *Afghanicae* (subgen. *Sisyrosema*) and *Laxiflorae* (subgen. *Onobrychis*) with good bootstrap support (88%). Based on its utility in numerical studies, the ITS is a useful marker for resolving phylogenetic relationships at various taxonomic levels, in particular the infrageneric level. However, caution is needed when analyzing ITS sequence data to avoid problems resulting from concerted evolution on the *nr*DNA arrays. Concerted evolution may homogenize different paralogous gene copies in a genome, leading to the loss of all but one of the copies, i.e., different copies may be present in different organisms by chance and this will create disagreement between the gene and species trees (Alvarez and Wendel, 2003).

A fundamental requirement for historical inference based on nucleic acid or protein sequences is that the genus compared is orthologous as opposed to paralogous. However, there are inherent risks in relying exclusively on rDNA sequences for phylogenetic inference, given the 'nomadic' nature of the *nr*DNA loci between inclusions of paralogous genes and exclusion of orthologous comparisons (Alvarez and Wendel, 2003).

In group I, species of the *Onobrychis* section (*O. cyri* var. *cyri*, *O. persica*, *O. major* and *O. shahpurensis*) form a branch (branch A) with 99% bootstrap support. This result indicates a close association among species within the *Onobrychis* section. The close association of the *Onobrychis* section with sections *Lophobrychis* (*O. pulchella* and *O. micrantha*) and *Dendrobrychis* (*O. elymaitica* and *O. cornuta*) indicates there is strong sequence homology among them, suggesting that these species are closely related in terms of phylogeny. Also there is strong sequence homology among sections of *Sisyrosema* subgen. (group II). The *Hymenobrychis* section is the sister group of the *Heliobrychis* section, and these sections are sister groups of the *Afghanicae* section. Species of *Heliobrychis*, *Hymenobrychis* and *Afghanicae* form a branch (branch C) with 77% bootstrap support. Species of the *Hymenobrychis* section (*O. michauxii*, *O. hohenackeriana*, O. *galegifolia* and *O. ptolemaica*) and species of the *Heliobrychis* section (*O. heliocarpa*, *O. buhseana*, *O. gaubae* and *O. mozaffarianii*) form two clades, F and D, with 82% and 99% bootstrap support, respectively, that predicate these species are closely related. The present study results are agreement with the findings from molecular phylogeny of the Hedysareae tribe with special reference to *Onobrychis* based on *nr*DNA ITS sequences (Ahangarian *et al*., 2007).

The present *nr*DNA ITS data show that the subgen. *Sisyrosema*, which is represented here by three of its five constitutive sections, appears to be a well-supported monophyletic group (BP=77%), whereas the subgen. *Onobrychis* is not monophyletic due to the sister group relationship of one species of *Onobrychis* subgen. (*O. laxiflora*) with the subgen. *Sisyrosema*. Our *nr*DNA ITS phylogeny (Fig. 1) shows that members of the subgen. *Sisyrosema* are derived from subgen. *Onobrychis*, recently. Ahangarian *et al*. (2007) also declared *Sisyrosema* subgen. as a monophyletic, whereas the subgen. *Onobrychis* is not monophyletic.

The *Laxiflorae* section is represented herein by a single species; hence the monophyly of this section cannot be addressed. All sections of *Onobrychis* and *Sisyrosema* subgen. appear to be monophyletic. Two species of *Dendrobrychis* (*O. elymaitica* and *O. cornuta*) are a sister group to a subclade that includes *O. pulchella* and *O. micrantha* (of section *Lophobrychis*).

In this study, cluster analysis of morphological characters in species of the *Onobrychis* genus showed two major groups separating *Onobrychis* subgen. *Onobrychis* from *Onobrychis* subgen. *sisyrosema*. Based on morphological variations, each of these sections, except *Laxiflorae*, separated from the other sections (groups A, B, C, D, E and F in Fig. 2). The distinguishing features of each section are presented below.

Onobrychis: Plant with hairs on stem, inferior surface of leaflet, ovary; number of basal leaflets >3 pairs; length of leaflet <14 mm; size of petiole is long; dense raceme; multiflowers per inflorescence, teeth longer than tube, length of standard=7-10.5 mm; length of the wing shorter than half the length of the keel; obtuse tip of the wing; wing shorter than calyx; number of ovules=1; small, semi-orbicular, non stipitate pod; convex appearance of pod; pod with crests, teeth, unilocular; pod without curvature; disc with spines.

Dendrobrychis: Plant with hairs on stem, upper and inferior surface of leaflet, bract, ovary; size of petiole is short; sparse raceme; <10 flowers per inflorescence; teeth shorter than tube; flower color is red- pink- violet; length of standard= 10.5-18 mm; standard with claw; length of the wing equal to the length of the keel; obtuse tip of the wing; wing longer than calyx, number of ovules=1; small, semi-orbicular, non stipitate pod; compressed appearance of pod; pod without crests, teeth, curvature; disc spineless; pod with unilocular.

Lophobrychis: Plant with hairs on stem, inferior surface of leaflet; length of leaflet <14 mm; size of petiole is large; sparse raceme; teeth longer than tube; concolorous appearance of corolla; length of standard <7 mm; standard without claw; length of the wing equal to the length of the keel; slightly acute tip of the wing; wing equal or sub-equal to calyx; number of ovules=1; stipitate pod; pod with crests, teeth, unilocular; disc with spines; incurved pod.

Laxiflorae: Plant with hairs on stem, upper and inferior surface of leaflet, bract, standard, ovary; length of leaflet= 14- 30 mm; sparse raceme; multiflowers per inflorescence; length of calyx <5 mm; teeth shorter than tube; flower color is yellow-cream; concolorous appearance of corolla; length of standard= 7- 10.5 mm; standard without claw; length of the wing equal to the length of the keel; very acute tip of the wing; wing longer than calyx; number of ovules=1; small, semibicular pod; compressed appearance of pod; non stipitate pod; pod with teeth, unilocular; disc with spines; pod without curvature.

Afghanicae: Plant with hairs on inferior surface of leaflet, bract, standard, ovary, pod; sparse raceme; <10 flowers per inflorescence; length of calyx >6 mm; veined appearance of corolla; teeth longer than tube; flower color is red- pink- violet; length of standard= 7-10.5 mm; standard without claw; length of the wing shorter than half the length of the keel; obtuse tip of the wing; wing shorter than calyx; number of ovules=2 or 3; large, orbicular pod; compressed appearance of pod; stipitate pod; pod without crests, teeth; pod with multilocular; disc with spines or spineless; circinate or incurved pod.

Heliobrychis: Plant with hairs on inferior surface of leaflet, bract, standard, ovary; length of stipule > 6 mm; number of basal leaflets ≤ 3 pairs; dense raceme; multiflowers per inflorescence; flower color is yellow-cream; length of the wing longer than half the length of the keel; very acute tip of the wing; number of ovules=1; large, semi-orbicular pod; convex appearance of pod; stipitate pod; pod without crests, teeth; pod with unilocular; disc with spines or spineless.

Hymenobrychis: Plant with hairs on inferior surface of leaflet, bract, standard, pod; dense raceme; multiflowers per inflorescence; length of calyx > 6 mm; teeth longer than tube; flower color is yellow-cream; standard with claw; length of the wing shorter than half the length of the keel; obtuse tip of the wing; wing shorter than calyx; number of ovules=2 or 3; large, reniform, stipitate pod; pod with crests, teeth, spineless, multilocular; incurved pod.

The subgen. *Sisyrosema* differs from the subgen. *Onobrychis* because of its large, crescent/kidney-shaped ovaries and pods, hairy vexillum, large persistent flowers and the epidermis of calyx without crystals (Rechinger, 1984; Yildiz *et al*., 1999). These features appear to be synapomorphics for the subgen. *Sisyrosema*.

Yildiz *et al*. (1999) suggested that monophyly of these two subgenera was not supported by a phylogenetic analysis of fruit characters. Ahangarian *et al*. (2007) concluded that subgen. *Onobrychis* is not monophyletic due to the sister group relationship of its two representative species to the subgen. *Sisyrosema* and the inclusion of two species of *Hedysarum* within it.

The ITS data analysis results showing two main groups, which are in accordance with morphological groups because it separates *Onobrychis* subgen. *Onobrychis* (*Onobrychis*, *Lophobrychis* and *Dendrobrychis*) with good bootstrap value of 88% from *Onobrychis* subgen. *Sisyrosema* (*Afghanicae*, *Heliobrychis* and *Hymenobrychis*) and the *Laxiflorae* section. Molecular phylogenetic and phenetic analyses confirmed that *Onobrychis* subgen. *Onobrychis* and *Onobrychis* subgen. *Sisyrosema* are closely related based on *nr*DNA ITS sequences and morphological characteristics.

This study shows that nucleotide sequence data from the internal transcribed spacer (ITS) of nuclear rRNA genes can be applied to investigate *Onobrychis* genetics, as indicated by other relevant research (e.g., Ahangarian *et al*., 2007). These data have high potential to reveal genetypic diversity and, in the longer term, to provide molecular markers that could be linked to phenotypic properties.

Iran is considered the center of origin and genetic diversity of the genus *Onobrychis*, because of different climatic conditions. Identification of these species facilitates selection of suitable and compatible genes. Breeders and biotechnology experts could transfer these genes to agronomic species and thereby develop drought tolerant varieties. Evaluation of phylogenetic relationships and traits can be a useful tool for determining the possibility of success in intergenomic crosses. Over the past few years, breeders have focused on annual species because they are reliable sources for improving perennial species. Phylogenetic surveys are thus essential for the stability of progenies of diploid and tetraploid hybridization.

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