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Genetic diversity and relationships within and among *Onobrychis* species using molecular markers

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Abstract: Little is known about the taxonomic relationships of the genus *Onobrychis* (Fabaceae). To study inter- and intraspecific variations and the relationships among the species, 102 accessions (33 species) of the genus *Onobrychis* were evaluated using 22 inter-simple sequence repeat (ISSR) markers. Almost all the species belonging to the section *Onobrychis* clustered together. A similar pattern was found with the species belonging to the section *Hymenobrychis*, while those of the section *Lophobrychis* did not cluster together. The results indicate that the section *Lophobrychis* has a comparatively derived organization that can be attributed to the differences in their taxonomic delimitation. Geographical pattern was found to be associated with the genetic diversity within the species. Principal coordinates analysis (PCoA) indicated a high association among the sections *Lophobrychis*, *Heliobrychis*, and *Hymenobrychis*. Thus, the present subgeneric classification of *Onobrychis* (*Onobrychis* and *Sisyrosema*) was not supported. The results based on Nei's similarity suggest that some species in the genus *Onobrychis* (especially *O. altissima*) might have been outcrossing with reproductive ability with *O. viciifolia*. Our results suggest that species with x = 7 chromosome are closer to *O. viciifolia* than to those with x = 8. Therefore, a base number n = 8 can be assumed for this genus (within *Onobrychis* genus basal), changing to 7 through aneuploid loss.

Key words: Phylogeny, *Onobrychis*, ISSR, diversity, genetic similarity

1. Introduction

The genus *Onobrychis* Miller (tribe Hedysareae, family Leguminoseae) comprises about 170 species in two subgenera with 9 sections (the subgenus *Onobrychis* including the sections *Dendrobrychis*, *Lophobrychis*, *Onobrychis*, and *Laxiflorae* as well as the subgenus *Sisyrosema* including the sections *Anthyllium*, *Afghanicae*, *Heliobrychis*, *Hymenobrychis*, and *Insignes* (Ranjbar et al., 2010)). This genus contains various annual and perennial species that can be distinguished mostly by their morphology and geographical distribution extending from the western Himalayas to Caucasia, Eurasia, North America, and Africa (Pavlova and Monova, 2000). However, almost all the *Onobrychis* species are restricted to northwestern Asia, especially to Iran and Anatolia, making this area the specific home of this genus diversity (Yildiz et al., 1999; Zarrabian et al., 2013).

The evolutionary trend in *Onobrychis* has been briefly explained with respect to chromosome number. Goldblatt (1981) suggested that x = 8 is the ancestral chromosome number and that the species with x = 7 are derived through aneuploid loss. However, Falistocco (1991) and Gomurgen (1996) claimed that evaluation within the genus took place by increasing basic chromosome number. Abou-El-Enain (2002) showed that the chromosome type of the genus varied between metacentric and submetacentric, ranging from 1.6 µm (small-medium) to 2.6 µm (medium) in length. He also detected five ploidy levels [(2n = 2x = 14), (2n = 4x = 28), (2n = 2x = 16), (2n = 4x = 32), and (2n = 8x = 56)] in the genus. Sepet et al. (2011) reported that the mean chromosome length in eight species ranged from 1.54 µm to 4.21 µm.

A number of studies mainly dealing with cytogenetics and seed storage proteins have been conducted to evaluate the phylogenetic relationships in the genus *Onobrychis*. Abou-El-Enain (2002) suggested that the section *Lophobrychis* has a comparatively highly derived organization and can be considered as a heterogeneous unit in the genus *Onobrychis*. Their hypothesis was not, however, confirmed by Emer et al. (2007), who reported that the species belonging to the section *Lophobrychis* had similar band profiles based on seed storage proteins. Arslan and Ertuğrul (2010), judging on the basis of seed storage proteins, indicated that the section *Heliobrychis* had a higher similarity to *Hymenobrychis* than to *Onobrychis*. Various studies have shown that DNA markers

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are phenotypically neutral, abundant, and less subject to environmental effects. Moreover, they can be useful not only in resolving complex phylogenetic problems but also for discovering new phylogenetic relationships in many plant species (Fang et al., 1998). There are numerous DNA-based marker systems suitable for phylogenetic and genetic diversity assessments. The inter-simple sequence repeat (ISSR) marker is one such technique that can rapidly differentiate closely related individuals (Zietkiewicz et al., 1994). The advantages that make the ISSR marker an unbiased tool for evaluating phylogeny in plant genera include: high polymorphism, reproducibility, and cost effectiveness, while it requires no prior information about the sequence (Bornet et al., 2002).

The genus *Onobrychis* has a wide geographical dispersion in the world (Zarrabian et al., 2013). Therefore, its phylogenetic analysis will not only be helpful for the taxonomy of this genus but will also promote the efficient use of genetic variation in breeding programs (Sikdar et al., 2010). The present study was designed to assess the genetic diversity and the relationships within and among *Onobrychis* species through ISSR markers, which can be used to identify the basis for the classification of this genus.

2. Materials and methods

2.1. Plant materials

One hundred and two accessions belonging to 33 species of the genus *Onobrychis* were used in this study (Table 1). The Iranian accessions were collected from different geographical regions nationwide. The exotic accessions were obtained from the Gene Bank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) and the United States Department of Agriculture (USDA). All the 102 accessions were germinated and grown in a greenhouse in January of 2011 and used for DNA extraction.

2.2. DNA extraction and PCR amplification

The genomic DNA was isolated from young leaves of 10 plant tissues using the method described in Murray and Thomson (1980). Agarose gel (0.7%) electrophoresis was used for the DNA qualitative and quantitative determinations. Of the 47 ISSR primers screened, 22 produced a higher number of reproducible bands, which were selected for ISSR analysis (Table 2). PCR was performed for a total volume of 15 µL of a solution containing 20 ng of total DNA, 1.5 10X PCR buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 2 pM of each primer, and 1 U *Taq* DNA polymerase. Amplification was accomplished in a thermocycler (Bio-Rad) according to the following program: initial denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 1 min, appropriate annealing temperature (Table 2) for 45 s, 72 °C for 2 min, and a final extension step at 72 °C for 7 min. Amplified DNA fragments were separated in a 1.5% agarose gel at 100 W for 2 h in 1X TBE buffer (100 mM Tris–Borate, pH 8.0, 2 mM EDTA) and stained with ethidium bromide.

2.3. Statistical analysis

Only the sharp and precise bands were scored as 1 for presence and 0 for absence to create the data matrix of computation. The information content (PIC), resolving power (RP), and marker index (MI) of each ISSR marker were computed using the following formulae:

$PIC_i = (2f_i \times (1 - f_i))$ (Roldan-Ruiz et al., 2000)

$RP_i = \Sigma(1 - (2 \times | 0.5 - f_i |))$ (Prevost and Wilkinson, 1999)

$MI_i = PIC_i \times N_i \times \beta_i$ (Powell et al., 1996),

where the *subscript i* represents the *ith* primer, f_i is the frequency of the amplified allele, $(1 - f_i)$ is the frequency of the null allele, PIC_i is the information content of the *ith* primer, N_i is the total band for the *ith* primer, and β_i is the percentage of the *ith* primer's polymorphic band. A phylogeny dendrogram, with 1500 replicates, was constructed based on the P-distance methods within the neighbor joining (NJ) model using the program MEGA (ver. 5.05). Popgene (ver. 1.32) (Yeh et al., 1999) was used to evaluate Nei's genetic similarity (Nei, 1972) among the 33 species. Moreover, Nei's genetic similarity was used to perform the principal coordinates analysis (PCoA) using NTSYS (ver. 2.02) (Rohlf, 1998).

3. Results

From the 47 primers, 22 were chosen for phylogeny evaluation in the genus *Onobrychis* according to primary screening (Table 2; Figure 1). The remaining primers did not produce any reliable or reproductive bands. The 22 primers in this study produced 243 bands, of which 235 (96.7%) were polymorphic (Table 2). The amplified bands ranged between 200 bp and 1400 bp. The highest number of bands was amplified with $(CA)_8$-G and the lowest was observed for $(CA)_8$-RT primers. The value of polymorphism information content (PIC) ranged from 0.34% to 0.47% with an average of 0.41% (Table 2). Although several primers had the highest percentage of polymorphic loci, the lowest was observed for $(GA)_8$-RT with 83.33% (Table 2). The highest and lowest RPs were observed with $(GA)_8$-SG and $(CA)_8$-RT primers, respectively. The maximum MI was estimated for $(TC)_8$-G and the minimum was observed for $(CA)_8$-RT primer. For the number of polymorphic loci among accessions per species, the highest and lowest were calculated for *O. vassilczenkoi* (Soviet Union, VASSOM1) and *O. viciifolia* (Iran, Esfahan, VICESfS9) accessions, respectively (Table 1). Moreover, the highest number of polymorphic loci for the species was calculated for *O. arenaria*, while the lowest was observed for *O. melanotricha* (Table 1). Nei's average genetic similarity of 0.407 ranged from 0.11 (between

Table 1. Information on the species and accessions investigated in this study.

No.	Code	Origin	Subgenus	Section	Species	TNB	NSPB	NSMB	C
1	TRAARZ1	Armenia	*Onobrychis*	*Onobrychis*	*O. transcaucasica*	80	-	-	A_I
2	TRAARZ11	Armenia	*Onobrychis*	*Onobrychis*	*O. transcaucasica*	80	-	-	A_I
3	TRAARZ12	Armenia	*Onobrychis*	*Onobrychis*	*O. transcaucasica*	73	-	-	A_I
4	TRAIRZ5	Iran	*Onobrychis*	*Onobrychis*	*O. transcaucasica*	78	-	-	A_I
5	TRAIRZ4	Iran	*Onobrychis*	*Onobrychis*	*O. transcaucasica*	82	-	-	A_I
6	TRATUZ8	Turkey	*Onobrychis*	*Onobrychis*	*O. transcaucasica*	73	-	-	A_I
7	TRAGEZ6	Georgia	*Onobrychis*	*Onobrychis*	*O. transcaucasica*	77	-	-	A_I
8	TRAGEZ7	Georgia	*Onobrychis*	*Onobrychis*	*O. transcaucasica*	76	-	-	A_I
9	TRAUZZ10	Uzbekistan	*Onobrychis*	*Onobrychis*	*O. transcaucasica*	83	-	-	A_I
							88	26	
10	ARESOT1	Soviet Union	*Onobrychis*	*Onobrychis*	*O. arenaria*	74	-	-	A_I
11	ARESOT5	Soviet Union	*Onobrychis*	*Onobrychis*	*O. arenaria*	83	-	-	A_I
12	ARESOT6	Soviet Union	*Onobrychis*	*Onobrychis*	*O. arenaria*	88	-	-	A_I
13	ARESOT11	Soviet Union	*Onobrychis*	*Onobrychis*	*O. arenaria*	73	-	-	A_I
14	ARESOT12	Soviet Union	*Onobrychis*	*Onobrychis*	*O. arenaria*	75	-	-	A_I
15	ARERUT7	Russia	*Onobrychis*	*Onobrychis*	*O. arenaria*	90	-	-	A_I
16	ARERUT2	Russia	*Onobrychis*	*Onobrychis*	*O. arenaria*	85	-	-	A_I
17	ARERUT3	Russia	*Onobrychis*	*Onobrychis*	*O. arenaria*	73	-	-	A_I
18	ARETOT8	Romania	*Onobrychis*	*Onobrychis*	*O. arenaria*	86	-	-	A_I
19	AREAZT13	Azerbaijani	*Onobrychis*	*Onobrychis*	*O. arenaria*	70	-	-	A_I
							104	25	
20	IBESOC2	Soviet Union	*Onobrychis*	*Onobrychis*	*O. iberica*	93	-	-	A_I
21	IBEPAC3	Pakistan	*Onobrychis*	*Onobrychis*	*O. iberica*	80	-	-	A_I
							66	52	
22	CYRRUV2	Russia	*Onobrychis*	*Onobrychis*	*O. cyri*	78	-	-	A_I
23	CYRSOV3	Russia	*Onobrychis*	*Onobrychis*	*O. cyri*	81	-	-	A_I
							95	27	
24	ALTSON1	Soviet Union	*Onobrychis*	*Onobrychis*	*O. altissima*	81	-	-	A_I
25	ALTSON2	Soviet Union	*Onobrychis*	*Onobrychis*	*O. altissima*	80	-	-	A_I
26	ALTRUN4	Russia	*Onobrychis*	*Onobrychis*	*O. altissima*	83	-	-	A_I
27	ALTAZN3	Azerbaijan	*Onobrychis*	*Onobrychis*	*O. altissima*	78	-	-	A_I
28	ALGEN5	Georgia	*Onobrychis*	*Onobrychis*	*O. altissima*	69	-	-	A_I
29	ALIRN6	Iran	*Onobrychis*	*Onobrychis*	*O. altissima*	81	-	-	A_I
30	ALIRN7	Iran	*Onobrychis*	*Onobrychis*	*O. altissima*	78	-	-	A_I
							81	50	
31	ALBBUG3	Bulgaria	*Onobrychis*	*Onobrychis*	*O. alba*	90	-	-	A_{IV}
32	ALBBUG4	Bulgaria	*Onobrychis*	*Onobrychis*	*O. alba*	87	-	-	A_{IV}
							50	61	
33	ARGMOA1	Morocco	*Onobrychis*	*Onobrychis*	*O. argentea*	64	-	-	A_{II}
34	ARGMOA2	Morocco	*Onobrychis*	*Onobrychis*	*O. argentea*	69	-	-	A_{II}
35	ARGSPA4	Spain	*Onobrychis*	*Onobrychis*	*O. argentea*	57	-	-	A_{II}
							68	33	
36	INERUI1	Russia	*Onobrychis*	*Onobrychis*	*O. inermis*	75	-	-	A_I
37	INERUI2	Russia	*Onobrychis*	*Onobrychis*	*O. inermis*	73	-	-	A_I
38	INERUI5	Russia	*Onobrychis*	*Onobrychis*	*O. inermis*	70	-	-	A_I
							48	47	
39	BIEHUO3	Soviet Union	*Onobrychis*	*Onobrychis*	*O. biebersteinii*	83	-	-	A_I
40	BIERUO6	Russia	*Onobrychis*	*Onobrychis*	*O. biebersteinii*	84	-	-	A_I
							40	55	

TNB = Total number of bands NSPB = Number of species polymorphic bands NSMB = Number of species monomorphic bands
C = cluster analysis

Table 1. (Continued).

No.	Code	Origin	Subgenus	Section	Species	TNB	NSPB	NSMB	C
41	PETIRD1	Iran	*Onobrychis*	*Onobrychis*	*O. petraea*	77	-	-	A_I
42	PETIRD4	Iran	*Onobrychis*	*Onobrychis*	*O. petraea*	77	-	-	A_I
43	PETGED2	Germany	*Onobrychis*	*Onobrychis*	*O. petraea*	74	-	-	A_I
44	PETRUD3	Russia	*Onobrychis*	*Onobrychis*	*O. petraea*	67	-	-	A_I
45	PETRUD5	Russia	*Onobrychis*	*Onobrychis*	*O. petraea*	83	-	-	A_I
							61	54	
46	OXYSOAF	Soviet Union	*Onobrychis*	*Onobrychis*	*O. oxyodonta*	73	-	-	A_I
							-	-	
47	GRABUAC	Bulgaria	*Onobrychis*	*Onobrychis*	*O. gracilis*	51	-	-	A_{III}
							-	-	
48	PERIRX2	Iran	*Onobrychis*	*Onobrychis*	*O. persica*	51	-	-	A_{III}
							-	-	
49	HAJSOAD	Soviet Union	*Onobrychis*	*Onobrychis*	*O. hajastana*	76	-	-	A_I
							-	-	
50	MEGTUJ1	Turkey	*Onobrychis*	*Onobrychis*	*O. megataphrose*	45	-	-	A_{III}
							-	-	
51	MONFEAH	France	*Onobrychis*	*Onobrychis*	*O. Montana*	40	-	-	A_{VI}
							-	-	
52	VICAZmS1	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	50	-	-	C_I
53	VICAZaS2	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	54	-	-	C_I
54	VICKES3	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	52	-	-	C_I
55	VICLOaS4	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	53	-	-	C_I
56	VICHAazS5	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	52	-	-	C_I
57	VICTEdS6	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	50	-	-	C_I
58	VICMAkS7	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	52	-	-	C_I
59	VICKOdS8	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	53	-	-	C_I
60	VICESfS9	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	59	-	-	C_I
61	VICESKS10	Iran	*Onobrychis*	*Onobrychis*	*O. viciifolia*	60	-	-	C_I
62	VICCHS101	China	*Onobrychis*	*Onobrychis*	*O. viciifolia*	69	-	-	C_{II}
63	VICAMS102	America	*Onobrychis*	*Onobrychis*	*O. viciifolia*	68	-	-	C_{II}
64	VICCHS103	Czech Republic	*Onobrychis*	*Onobrychis*	*O. viciifolia*	60	-	-	C_{II}
65	VICGES104	Kyrgyz Republic	*Onobrychis*	*Onobrychis*	*O. viciifolia*	66	-	-	C_{II}
66	VICRUS105	Spain	*Onobrychis*	*Onobrychis*	*O. viciifolia*	68	-	-	C_{II}
67	VICUNS106	Unknown	*Onobrychis*	*Onobrychis*	*O. viciifolia*	66	-	-	C_{II}
68	VICENS107	England	*Onobrychis*	*Onobrychis*	*O. viciifolia*	63	-	-	C_{II}
69	VICGES108	Kyrgyz Republic	*Onobrychis*	*Onobrychis*	*O. viciifolia*	74	-	-	C_{II}
70	VICOKS109	Ukraine	*Onobrychis*	*Onobrychis*	*O. viciifolia*	84	-	-	C_{II}
71	VICENS110	England	*Onobrychis*	*Onobrychis*	*O. viciifolia*	79	-	-	C_{II}
72	VICMOS111	Morocco	*Onobrychis*	*Onobrychis*	*O. viciifolia*	63	-	-	C_{II}
73	VICRUS112	Russia	*Onobrychis*	*Onobrychis*	*O. viciifolia*	69	-	-	C_{II}
74	VICRUS113	Russia	*Onobrychis*	*Onobrychis*	*O. viciifolia*	66	-	-	C_{II}
75	VICROS114	Romania	*Onobrychis*	*Onobrychis*	*O. viciifolia*	67	-	-	C_{II}
							90	40	
76	CAPTUB2	Turkey	*Onobrychis*	*Lophobrychis*	*O. caput-galli*	73	-	-	A_I
77	CAPISB5	Israel	*Onobrychis*	*Lophobrychis*	*O. caput-galli*	70	-	-	A_I
78	CAPUNB6	Unknown	*Onobrychis*	*Lophobrychis*	*O. caput-galli*	61	-	-	A_I
							73	27	

TNB = Total number of bands NSPB = Number of species polymorphic bands NSMB = Number of species monomorphic bands
C = cluster analysis

Table 1. (Continued).

No.	Code	Origin	Subgenus	Section	Species	TNB	NSPB	NSMB	C
79	CRIIRP2	Iran	*Onobrychis*	*Lophobrychis*	*O. crista-galli*	76	-	-	A_V
80	CRIISP5	Israel	*Onobrychis*	*Lophobrychis*	*O. crista-galli*	73	-	-	A_V
81	CRIUNP8	Unknown	*Onobrychis*	*Lophobrychis*	*O. crista-galli*	67	-	-	A_V
							41	47	
82	AEQFEAG	France	*Onobrychis*	*Lophobrychis*	*O. aequidentata*	64	-	-	A_{IV}
							-	-	
83	PULTOAI	Turkmenistan	*Onobrychis*	*Lophobrychis*	*O. pulchella*	55	-	-	B_{II}
							-	-	
84	MOLIRFEAL1	Iran	*Sisyrosema*	*Heliobrychis*	*O. melanotricha*	66	-	-	B_{II}
85	MALIRSEAL2	Iran	*Sisyrosema*	*Heliobrychis*	*O. melanotricha*	64	-	-	B_{II}
							19	46	
86	ARGTUAB	Turkey	*Sisyrosema*	*Heliobrychis*	*O.argyrea*	74	-	-	A_I
							-	-	
87	PTOPIL1	Unknown	*Sisyrosema*	*Hymenobrychis*	*O. petolemaica*	72	-	-	A_{IV}
88	PTOIQL2	Iraq	*Sisyrosema*	*Hymenobrychis*	*O. petolemaica*	79	-	-	A_{IV}
							46	49	
89	HYPTUW1	Turkey	*Sisyrosema*	*Hymenobrychis*	*O. hypargyrea*	80	-	-	A_{IV}
90	HYPTUW2	Turkey	*Sisyrosema*	*Hymenobrychis*	*O. hypargyrea*	77	-	-	A_{IV}
							23	65	
91	VASSOM1	Soviet Union	*Sisyrosema*	*Hymenobrychis*	*O. vassilczenkoi*	96	-	-	A_{IV}
92	VASRUM2	Russia	*Sisyrosema*	*Hymenobrychis*	*O. vassilczenkoi*	91	-	-	A_{IV}
							94	22	
93	MICTUR1	Turkey	*Sisyrosema*	*Hymenobrychis*	*O. michauxii*	77	-	-	A_{IV}
94	MICIRR2	Iran	*Sisyrosema*	*Hymenobrychis*	*O. michauxii*	74	-	-	A_{IV}
							43	56	
95	SINSOY1	Soviet Union	*Sisyrosema*	*Hymenobrychis*	*O. sintenisii*	78	-	-	A_{IV}
96	SINIRY2	Iran	*Sisyrosema*	*Hymenobrychis*	*O. sintenisii*	71	-	-	A_{IV}
97	SINIRY3	Iran	*Sisyrosema*	*Hymenobrychis*	*O. sintenisii*	68	-	-	A_{IV}
							61	35	
98	CHOSOH1	Soviet Union	*Sisyrosema*	*Hymenobrychis*	*O. chorassanica*	56	-	-	A_{III}
							-	-	
99	VAGRUF1	Soviet Union	*Sisyrosema*	*Hymenobrychis*	*O. vaginalis*	76	-	-	A_{IV}
							-	-	
100	KEMSOAE	Soviet Union	*Sisyrosema*	*Hymenobrychis*	*O. kemulariea*	75	-	-	A_I
							-	-	
101	BOBRUAJ	Russia	*Sisyrosema*	*Hymenobrychis*	*O. bobrovii*	57	-	-	B_{II}
							-	-	
102	RADARAK	Armenia	*Sisyrosema*	*Hymenobrychis*	*O. radiate*	51	-	-	B_I

TNB = Total number of bands NSPB = Number of species polymorphic bands NSMB = Number of species monomorphic bands
C = cluster analysis

Table 2. ISSR primers used in this study.

Num.	Sequence (3'-5')	T_a (°C)	Size range	NPB/NB	PPB%	PIC	MI	RP
1	$(CA)_8G$	52	400–1300	14/14	100	0.35	4.93	8.2
2	$(TC)_8C$	56	350–1400	15/15	100	0.38	5.76	8.1
3	$(TC)_8G$	54	300–1100	12/12	100	0.42	4.11	5.8
4	$(AC)_8G$	48	400–1100	9/10	90	0.42	3.78	5.25
5	$(CA)_8$-RT	46	300–1300	5/6	83.33	0.42	2.09	3.15
6	$(GA)_8$-RT	51	250–1350	11/11	100	0.45	4.93	7.97
7	$(AC)_7$-DBD	50	200–1300	11/12	91.66	0.37	4.11	6.43
8	$(AG)_7C$	52	200–1100	9/9	100	0.4	3.63	5.84
9	$(GA)_8$-SC	57	200–1200	12/12	100	0.41	4.94	7.27
10	$(AC)_8C$	48	250–1400	7/7	100	0.43	3.03	4.9
11	$(AG)_8$-SG	56	300–1100	11/12	91.66	0.38	4.19	5.19
12	$(GA)_8$-SG	58	200–1100	13/14	92.85	0.47	5.65	8.61
13	$(GA)_8$-WT	47	350–1000	10/11	90.9	0.43	4.29	7.4
14	$(CT)_8$-RG	51	250–1100	10/10	100	0.34	3.44	5.25
15	$(GA)_8C$	50	200–1300	10/10	100	0.48	4.78	8.54
16	$(AC)_8C$	54	200–1350	12/13	92.3	0.46	5.55	7.81
17	$(GA)_8$-YT	52	200–800	11/11	100	0.44	4.81	6.81
18	$(GA)_8$-YC	54	150–1100	12/12	100	0.4	4.85	6.47
19	$(AG)_8$-YT	54	150–1200	9/9	100	0.35	3.12	5.11
20	$(GACA)_4$	50	300–1300	11/12	91.66	0.43	4.75	5.88
21	$(GA)_8$-RC	51	300–1400	10/10	100	0.46	4.55	8.22
22	$(GACA)_5$	55	300–1400	11/11	100	0.43	4.75	6.72

T_a = Annealing temperature, NPB = Number of polymorphic bands, NB = Number of total bands, PPB = Percentage of polymorphic bands, PIC = Polymorphism information content, MI = Marker index, RP = Resolving power

O. megataphrose and *O. pulchella*) to 0.79 (between *O. transcaucasica* and *O. arenaria*) (data not shown).

Phylogenetic analysis performed by the P-distance method based on the NJ model is presented in Table 1 and Figure 2. Our results indicated high intra- and interspecies genetic variations among the *Onobrychis* species. The dendrogram indicated that all the accessions belonging to each species clustered together, except for *O. transcaucasica.* Cluster analysis separated all the 102 accessions (33 species) into three groups. Group A was further divided into six subclusters that contained different species. Subgroup A_I contained 13 species (*O. arenaria*, *O. altissima*, *O. caput-galli*, *O. biebersteinii*, *O. cyri*, *O. inermis*, *O. transcaucasica*, *O. petraea*, *O. iberica*, *O. hajastana*, *O. argyrea*, *O. oxyodonta*, and *O. kemulariea*). The second subgroup (A_{II}) contained only one species (*O. argentea*), while the third (A_{III}) contained 4 species (*O. gracilis*, *O. persica*, *O. megataphrose*, and *O. chorassanica*). Subcluster four (A_{IV}) consisted of the greatest number of species belonging to the section *Hymenobrychis* (i.e. *O. hypargyrea*, *O. petolemaica*, *O. michauxii*, *O. sintenisii*, *O. vassilczenkoi*, and *O. vaginalis*). Moreover, this subcluster contained the two species *O. alba* (section *Onobrychis*) and *O. aequidentata* (section *Lophobrychis*). Only one species was dropped in each of the subclusters five (V) and six (VI), which were *O. crista-galli* and *O. montana*, respectively. The second group (B) consisted of 4 species (*O. melanotricha*, *O. bobrovii*, *O. pulchella*, and *O. radiata*),

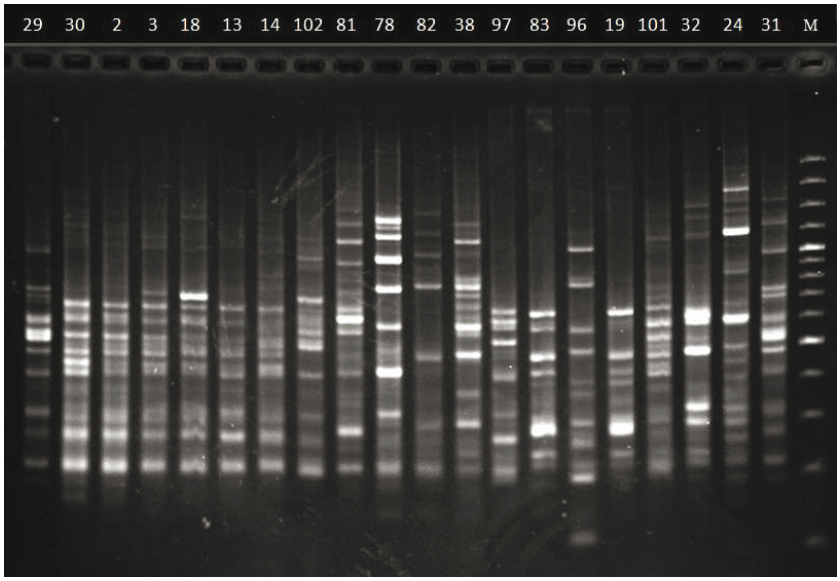

Figure 1. ISSR marker pattern for primer (GA)8-YT in different *Onobrychis* species (number above each gel described in Table 1).

but noticeably *O. radiata* was separated from the other species and categorized in a distinct subgroup (B_I). All the *O. viciifolia* accessions were grouped in the last cluster (C), which was further divided into two subclusters. Subcluster C_I contained all the Iranian accessions, while C_{II} included all the exotic ones (all derived from Europe, except for VICCHS101 from China and VICAMS102 from the USA).

PCoA was performed for the 33 species (Figure 3). The first three principal components explained 32.1% of all the variation. Based on PCoA, four district groups were found, the first (A) and the second (B) groups of which contained all the species of the section *Onobrychis*. Moreover, *O. caput-galli* was clustered in the section *Onobrychis* (group A). Group C contained 8 species from which 4 belonged to the section *Hymenobrychis*, 2 to *Heliobrychis*, and 2 to *Lophobrychis*. Group D contained 8 species, all of which, except for *O. pulchella*, belonged to the section *Hymenobrychis* (Figure 3).

4. Discussion

In this study, 22 ISSR primers were successfully used to investigate the genetic variation and phylogeny of the species of *Onobrychis*. Based on the genetic indices (polymorphism information content, marker index, and resolving power) used, the $(GA)_8$-SG sequence of ISSR was identified as the best informative primer. As Wang et al. (2006) maintained, dinucleotide motifs in high plant genomes are more common than the tri-, tetra-, or panta-nucleotides and, within these dinucleotides, poly (GA) is more variable than the others. Our results indicate that the poly GA-anchored ISSR primer produces more bands. Therefore, the frequency of poly (GA) in the *Onobrychis*' genome is higher than that of the other dinucleotide motifs.

4.1. Within species diversity

ISSR data have been used in detecting genetic diversity in many species (e.g., Wang et al., 2006; Fu et al., 2008). In our study, the ISSR marker was able to separate completely all the accessions belonging to different species, except for *O. transcaucasica*. Moreover, the diversity within species (among the accessions) was mainly supported by geographical patterns. For example, the pattern of diversity in cultivated sainfoin roughly corresponded to geographical origin. The two major subclusters observed in *O. viciifolia* consisted of one comprising all the Iranian accessions and the other including all the exotic ones. We assumed that the high polymorphism observed in *O. viciifolia* was related to the wide area of the collection site. However, this distinction may be the reflection of different domestication routes with different ancestors. Using morphological, anatomical, and ISSR traits on 80 accessions of *O. viciifolia*, Zarrabian et al. (2013) showed that the high level of population differentiation may comply with the theoretical prediction from an "isolation by distance" model. In this model, total population is assumed to be divided into subgroups, each breeding at random within itself.

A geographical pattern was also observed in such other species as *O. altissima* (Figure 2, Group A_I), *O. sintenisii* (Figure 2, Group A_{IV}), and *O. transcaucasica* (Figure 2, Group A_I). Even though two distinct groups were identified for *O. transcaucasica* in the dendrogram (Figure 2, Group

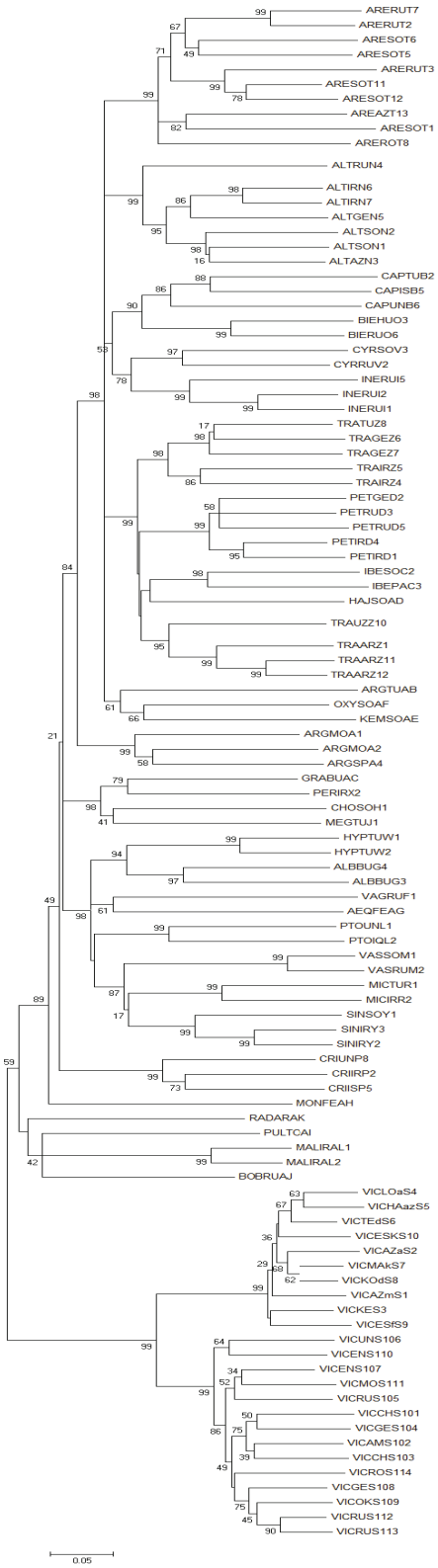

Figure 2. Relationships between *Onobrychis* accessions based on NJ tree using the P-distance method.

A_1), almost all the accessions belonging to the same latitude clustered together, except for the Georgian accession, which clustered with the Iranian and Turkish accessions. This misclassification might be due to the high diversity in the species *O. transcaucasica*, the dominant nature of ISSR markers, or the heterogeneity of this species. Overall, the accessions from geographically similar locations tend to be closer to each other, leading to a high association between genetic diversity (within species level) and geographical pattern. The level of genetic diversity in plant species is affected by a variety of factors including breeding systems, seed dispersal mechanisms, geographic ranges, life forms, and natural selection (Su et al., 2009), among which the geographic range possibly plays a major role in the maintenance of the genetic variation in *Onobrychis*. Budak et al. (2004) reported that the study of genetic diversity in buffalograss led to the establishment of groups consisting of germplasms from different geographical regions. They attributed these to germplasm exchanges and ecotype selection.

4.2. Among species diversity

As mentioned earlier, the genus *Onobrychis* consists of 2 subgenera and 9 sections. In this study, 4 sections [*Onobrychis* (17 species), *Lophobrychis* (4 species), *Hymenobrychis* (10 *species*), and *Heliobrychis* (2 species)] were investigated. Most of the species belonging to the section *Onobrychis* clustered together in subgroup A_1, in which two species (*O. arenaria* and *O. altissima*) clustered separately and far from the other members in this group. Arslan and Ertuğrul (2010) reported that *O. altissima* clustered far from the other members of the section *Onobrychis* based on seed storage proteins.

In group A_1, most of the species belonged to the section *Onobrychis*, while other noticeable members were three accessions of *O. caput-galli* (section *Lophobrychis*), which shared a node with 90% similarity level with *O. biebersteinii* (Figure 2). According to the *Flora of Turkey* (Davis et al., 1988) and *Flora Europaea* (Ball, 1968), the section *Lophobrychis* is closer to *Onobrychis* than it is to *Heliobrychis* or *Hymenobrychis*. This finding is confirmed by Yildiz et al. (1999), who studied fruit morphology in 40 *Onobrychis* species. Emre et al. (2007) studied seed storage proteins in 8 species of the genus *Onobrychis* and showed that the species in the sections *Lophobrychis* and *Onobrychis* clustered together. However, Aboul-El-Enain (2002) suggested that the section *Lophobrychis* was a comparatively derived organization that could be referred to a difference in their taxonomic delimitation. Moreover, very variable chromosome numbers have been documented in this section, e.g., *O. aequidentata* (2n = 14, 16, and 28) and *O. caput-galli* (2n = 14) (Abou-El Enain, 2002). Lewke Bandara et al. (2013) stated that such variation (even apparently with different base chromosome numbers) may suggest the presence of a different species

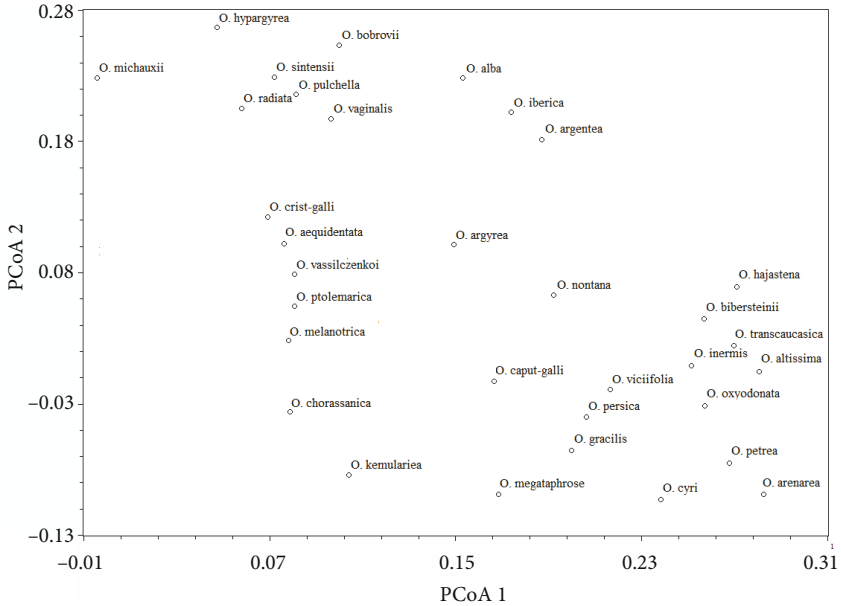

Figure 3. Two-dimensional representation of PCoA for 33 *Onobrychis* species determined on the basis of ISSR markers.

poorly characterized from a morphological point of view, or even the presence of hybrids or species of hybrid origin, under the names *O. aequidentata* and *O. caput-galli*. Our results indicate that the species that belong to the section *Lophobrychis* were not coherent in one cluster but clustered with other sections, i.e. *O. aequidentata* and *O. pulchella* clustered with the species in the sect. *Hymenobrychis* (Figure 2, Groups A_{III} and B, respectively) and *O. crista-galli* clustered in a distinct group (Group A_V). Thus, the conception of *Lophobrychis* as a section might be meaningless and flawed.

Moreover, the results of PCoA analysis showed that *O. caput-galli* was the only species of the section *Lophobrychis* that grouped with the section *Onobrychis*, while the remaining species in this section (i.e. *O. pulchella*, *O. aequidentata*, and *O. crista-galli*) clustered with the sections *Hymenobrychis* and *Heliobrychis*, indicating no strong relationship between the sections *Onobrychis* and *Lophobrychis* (Figure 3).

Our results also show that the present subgeneric classification of the genus *Onobrychis* (*Onobrychis* and *Sisyrosema*) cannot be supported. A number of studies have been conducted so far on the validity of the *Onobrychis* subgeneric classification. Yildiz et al. (1999) used the fruit morphology of some *Onobrychis* species and Emre et al. (2007) used seed protein profile to show that the subgenera *Onobrychis* and *Sisyrosema* cannot be confirmed. However, Ahangarian et al. (2007) studying the internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA), and Arslan and Ertuğrul (2010), investigating seed storage proteins, have suggested that the subgenus *Sisyrosema* can be separated from the subgenus *Onobrychis*. Lewke Bandara et al. (2013), based on nuclear (ITS) and chloroplast (matK) markers, and Safaei Chaei Kar et al. (2014), based on ITS and trnL–trnF DNA sequence data, reported that *Sysirosema* was resolved as monophyletic with high support and should therefore be maintained. As ISSR has a different nature from ITS, further studies with other markers may be needed to reject or accept this hypothesis in future.

The subgroup A_{IV} consisted of most of the species belonging to the section *Hymenobrychis*. Furthermore, two species, *O. alba* (section *Onobrychis*) and *O. aequidentata* (section *Lophobrychis*), have been placed in this subgroup. A similar misclassification observed for *O. alba* (in the subgroup A_{IV}), *O. chorassanica* (in the subgroup A_{III}), and *O. argyrea* (in the subgroup A_I) might have been due to the small size of the samples, inadequate number of individuals per population, and lack of ISSR loci in some species (Wolfe et al., 2001). Another possible explanation for this misgrouping of some species is suggested by Hayot Carbonero et al. (2012). Based on ITS sequence data they suggested that the *Onobrychis* taxonomy is overcomplicated by the existence of synonyms and spurious subspecies. They also reported that *O. pyrenaica* (Sennen), *O. altissima* Grossh., *O. arenaria* (Kit.) DC., *O. inermis* Steven, and *O. montana* DC. might all be synonyms for *O. viciifolia*, that *O. pulchella* Schrenk ex Fisch. et C.A.Mey. is the same as *O. alba* (Waldst. et Kit.) Desv., and that *O. antasiatica* hort., nom. inval. is synonymous with *O. ranscaucasica* Grossh.

The subgroup A_{VI} only consists of one species, namely *O. montana* (Figure 2). Pavlava and Monova (2000)

explained that the genus *Onobrychis* is an open pollinated plant so that certain sections such as *Onobrychis* in the genus were expected to be nonmonophyletic.

Group B consisted of 4 species classified into 2 subclusters. Subcluster B_I consisted of *O. pulchella* (sect. *Lophobrychis*), *O. melanotricha* (sect. *Heliobrychis*), and *O. bobrovii* (sect. *Hymenobrychis*), while subcluster B_{II} consisted of *O. radiata* (sect. *Hymenobrychis*). The main difference between these two subclusters lies in the base number of their chromosomes. Subcluster B_I had 7 basic chromosomes (x = 7), while subcluster B_{II} contained 8 (Aboul-El-Enain, 2002; Hesamzadeh Hejazi and Ziaei Nasab, 2010). For group C, the cultivated species form a unique group with all *O. viciifolia* accessions (Figure 2, Group C). We assume that the geographic isolation, ecological adaption (especially the long-term selection by humans for better performance of cultivated sainfoin), and the likelihood of DNA mutation or recombination led to the diversification and the high genetic polymorphism in this species as compared to others.

As mentioned before, Gömürgen (1996) suggested that the basic chromosome count, x = 8, is associated with the annual species while x = 7 is more frequent in perennial *Onobrychis* species. This suggestion was confirmed by Abou-El-Enain (2002) in three annual *Onobrychis* species. However, Arslan et al. (2012) disagreed with this suggestion because some perennial *Onobrychis* species have two basic chromosome counts x = 8 and 7 as *O. tournefortii.* In this study, no differences were observed in ISSR marker patterns among the annual and perennial species, except in *O. crista galli*, which clustered in a distinct group (Figure 2). Pavlova and Monova (2000) also found no differences in pollen morphology among the annual or perennial species in the genus *Onobrychis*.

Nie's (1972) similarity matrix showed that *O. viciifolia* has a high similarity to each of the species *O. altissima* (0.59), *O. inermis* (0.58), *O. transcaucasica* (0.56), and *O. arenaria* (0.52). Wolf and Randle (2001) suggested that species with a high genetic similarity may also have combining ability. They suggested that cloned species exhibited similarity rates ranging between 0.96 and 0.97 and percentages of polymorphic loci ranging from 10.4 to 20.8; however, the similarity rates for outcrossing species ranged between 0.50 and 0.53 and percentages of polymorphic loci ranged from 88 to 95. For self-pollinated species, they estimated an average similarity value between those for the cloned and the outcrossing species. The same pattern has been shown in *Penstemon* spp. (Wolf et al., 1998) and *Hyobanche* spp. (Wolf and Randle, 2001). Our results probably indicate that some species in the genus *Onobrychis* have an outcrossing reproductive ability, which gives it the potential for hybridization with *O. viciifolia*. On the other hand, a variation in chromosome number and ploidy level is known for some species, for example *O. altissima* (2n = 14, 28) and *O. viciifolia* (2n = 22, 27, 28, 29) (Hesamzadeh Hejazi and Ziaei Nasab, 2010; Arslan et al., 2012). After Ranjbar et al. (2010), *O. altissima* is considered to be closely related to *O. viciifolia* and may be a progenitor of it, while, based on morphological similarity, a close relationship between the 2 species was postulated by Hedge (1970). Therefore, based on our similarity matrix results, it seems that these two species may be useful for inter-specific hybridization programs. However, this is only a hypothesis and further studies with larger samples of species and populations are required for validation.

A bibliographical search reveals *Onobrychis* species experienced descending aneuploidy during their evolutionary history. Ahangarian et al. (2007) reported that basic chromosome numbers (x = 8) are maintained in basal taxa of the tribe Hedysareae (Khatoon and Ali, 2006), whereas smaller numbers (x = 7) are found in terminal genera such as *Onobrychis*. On the other hand, within the *Onobrychis* genus basal, sections such as *Dendrobrychis* and *Lophobrychis* have x = 8 as the basic chromosome number, followed by the section *Onobrychis*, which has two basic chromosome number (x = 7 and x = 8) and polyploidy (Ranjbar et al., 2012). Therefore, aneuploidy is an evolutionary process in this genus that was followed by polyploidy in some sections (i.e. sect. *Onobrychis*), and so based on our results the species with x = 7 chromosome are closer to *O. viciifolia* (such as *O. altissima* (with 59% similarity) and *O. transcaucasica* (with 56% similarity)) than to those with x = 8 chromosome (such as *O. petolemaica* (with 38% similarity) and *O. melanotricha* (with 37% similarity)). Our results are in agreement with Goldblatt (1981) and Ranjbar et al. (2012), in which a base number n = 8 can be assumed for this genus (present in the more basal genera), changing to 7 through aneuploid loss.

In summary, ISSR markers have been successfully used to detect genetic diversity not only among species but also within certain species (e.g., *O. viciifolia*, *O. altissima*, and *O. transcaucasica*). A high association was observed to exist between geographical patterns and genetic diversity within species.

Another finding of the present study is the close relationship detected between the section *Lophobrychis*, on the one hand, and *Hymenobrychis* and *Heliobrychis*, on the other. Therefore, the present subgenus classification of the genus *Onobrychis* is not supported. However, the findings of the present study are not adequate to lead to a satisfactory improvement in the *Onobrychis* classification on the basis of ISSR markers, and more in-depth research is needed and an adequately comprehensive and sophisticated molecular marker system is required to gain better results on the phylogeny and genetic diversity of the genus *Onobrychis*.

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