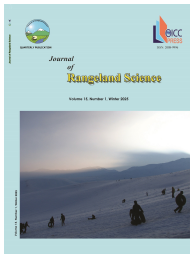

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Assessment of the relationship between *Onobrychis* species through morphological and molecular perspectives

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Abstract:

The genus *Onobrychis*, a prominent member of the Fabaceae family, holds pivotal agricultural significance in Iran, particularly in pastures and croplands. This study, conducted in 2020, undertook a comprehensive re-evaluation of the phylogeny of *Onobrychis*. Our approach encompassed an examination of seed and fruit macro- and micro-morphological traits, coupled with the utilization of ISSR molecular markers. The investigation revealed notable diversity in both seed and fruit morphological traits. Notably, we identified a novel fruit type, the "*O. crista-galli* type," in addition to the three established fruit types (*O. radiata*, *O. beata*, and *O. ornata*). Moreover, molecular analyses, employing a set of 22 ISSR primers, demonstrated robust efficiency in genetically discriminating among the various species. Contrasting traditional taxonomic classifications, our Maximum Likelihood phylogenetic tree analysis validated the subgenus classification (*Onobrychis* and *Sisyrosema*). Intriguingly, it diverged from conventional wisdom in the sections of the *Onobrychis* subgenus (*Lophobrychis* and *Onobrychis*), challenging their monophyletic nature. This discrepancy suggests that these sections might not be cohesive, particularly highlighting the non-monophyly of the *Lophobrychis* section. This insight prompts consideration of reclassifying *Lophobrychis* as a heterogeneous unit, potentially integrated into the *Onobrychis* section. In summary, this study provides a nuanced exploration of the phylogeny of the *Onobrychis* genus, employing a dual morphological and molecular perspective. The findings challenge traditional taxonomic assumptions, opening avenues for a refined understanding of the evolutionary relationships within *Onobrychis* and offering insights that could influence future taxonomic classifications.

Keywords: Phylogeny; Seed type; Species; Seed coat; SEM study

Introduction

Sainfoin is a highly palatable and nutritious forage legume used for hay and pasture production (Bhattarai et al., 2015). As a member of Legumes, it convert atmospheric nitrogen into bioavailable compounds (Kar et al., 2014). Moreover, it has unique tannin and polyphenol compositions giving those features that reportedly increase protein utilization and prevent bloating (Hayot-Carbonero et al., 2012). Recent studies revealed that tannin composition has methane-control potential from ruminants, anthelmintic properties, and protein-protection capability from early degradation in the rumen when used as a forage crop in the diet of ruminant animals (Mora-Ortiz and Smith, 2018). So, sainfoin's environmental and nutraceutical attributes make it a valuable plant for mono culture in pastures or grass-legume mix cropping.

The genus *Onobrychis* Mill. includes about 170 species around the world. This genus is widely distributed in northern temperate regions of the world whereas Iran and Turkey are considered to be the center of diversity for this species. (Rechinger, 1969; Yildiz et al., 1999; Hayot-Carbonero et al., 2012). This genus is divided into two subgenera: *Onobrychis* and *Sisyrosema*, which are distinguished from each

other through deciduous flowers, glabrous corollas, erect fruits, and the epidermis of a calyx with crystals within (Yildiz et al., 1999). Moreover, the subgenus *Onobrychis* includes four sections (*Dendrobrychis* DC, *Lophobrychis*, *Onobrychis*, and *Laxiflorae*) while the subgenus *Sisyrosema* comprises five sections (*Anthyllium*, *Afghanicae*, *Heliobrychis*, *Hymenobrychis*, and *Insignes*) (Rechinger, 1969). Up until now, several approaches have been adopted to provide an appropriate classification of the *Onobrychis* genus. Yildiz et al. (1999) recognized 170 species based on fruit morphological characters and re-evaluated the sectional delimitation of the *Onobrychis* genus. However, later studies suggested that it would be necessary to re-examine the taxonomic position of the *Onobrychis* taxa (Cenci et al., 2000; Abou-El-Enain, 2002). A karyological study within *Onobrychis* clade showed that sub-genus *Sisyrosema*, unlike subgenus *Onobrychis*, is monophyletic (Hejazi and ZiaeiNasab, 2010). Arslan and Ertugrul (2010) maintained that a greater similarity could be was observed on the basis of seed storage protein between the *Heliobrychis* and *Hymenobrychis* sections rather than the *Onobrychis* section. However, the low number of species in their studies makes it hard to draw clear conclusions about the genus.

This genus has been the subject of several DNA studies and unlike seed storage protein, DNA fingerprinting revealed that a classification of the *Onobrychis* genus weakly supported the taxonomical sections and many species would be synonyms (Hayot-Carbonero et al., 2012; Kar et al., 2014; Duan et al., 2015; Zarrabian and Majidi, 2015; Amirahmadi et al., 2016). Kar et al. (2014) reported the paraphyly of *Onobrychis* and *Sisyrosema* subgenera and they proposed sections *Onobrychis*, *Dendrobrychis*, *Heliobrychis*, and *Hymenobrychis* had the paraphyletic nature. Which confirmed in later studies by Amirahmadi et al. (2016) based on nrDNA ITS, plastid trnL-F, and matK sequences. However, later studies indicate that *Onobrychis* is monophyletic and composed of two main clades, each corresponding to the redefined subgenus *Onobrychis* (including sections *Onobrychis* and *Hemicyclobrychis*) and subgenus *Sisyrosema* (including sections *Afghanicae*, *Laxiflorae*, *Heliobrychis*, *Hymenobrychis*, *Insignes*, *Lipskyanae*, and *Litvinovianae*), respectively. They also declared that sections *Dendrobrychis* and *Lophobrychis* are reduced to synonymy of section *Onobrychis* and *Anthyllium* to synonymy of section *Hymenobrychis*. The other studies based on three non-coding chloroplast sequences and the nuclear ribosomal DNA internal transcribed spacer revealed that section *Heliobrychis* was retrieved as a well-supported monophyletic group sister to *Hymenobrychis* (Kaveh et al., 2019).

So, to expand our knowledge of the classification and genetic structure of the *Onobrychis* genus, the present study was conducted with the following objectives: 1) To illustrate the structures of the fruit surface and the external morphology of mature seeds, 2) To evaluate the diagnostic value of the investigated characters in terms of their systematic implications, and 3) To clarify phylogenetic relationships within the genera.

Martials and methods

Plant material

Eighty-eight accessions belonging to 31 species of the genus *Onobrychis* were used in this study (Table 1). The seeds of germplasms were obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), the United States Department of Agriculture (USDA), and some species were collected from different geographical regions nationwide by the authors. All the accessions used for molecular experiments were germinated and grown at 25°C with a 16/8 h day/night cycle in the greenhouse.

Seed micro and morphological analysis

For seed Macro-morphological observations, seven seeds and legumes were placed under a binocular stereoscopic microscope (Tables 1 and 2). Moreover, 15 seeds and fruit of each accession were measured for dimensions using a Digital Caliper, and their average was used for each species (Table 1 and figure 1). For the cross-section of seed coats, three seeds were randomly selected from each accession, and the seed coats were separated before they were soaked in distilled water for 45 min. For convenience and uniformity of the cuts, a razor blade was used for making cross-sections. Transverse sections were cleaned with sodium hypochlorite for 1 min and slides were examined under a Nikon ECLIPSE E600 microscope (×400). For the seed coat cross-sections, the Palisade layer (or Malpighian cells), osteosclereids, inner parenchyma tissue, and total seed coat thickness was measured with a Zoom browser ex. Ink program (Tables 1 and 2; figure 2). Also, mature seeds of the species were selected for the Scanning electron microscopy (SEM) study. For this purpose, the seeds were mounted on stubs using a double-face carbon tape and covered with gold (Au) at 15 mA for 3 min under a high vacuum in an Ion Sputter Coating Unit (Baltek SCD-005). The samples were subsequently detected and photographed using SEM (Philips XL 130). The terminology of Botanical Latin Stearn (Stearn, 1983; Barthlott, 1984; Punt et al., 1994) was used to describe the SEM aspects of seed coat ornamentations (Figs. 3 and 4).

ISSR analysis

For ISSR analysis, seeds of 31 species were sown and young leaves (0.5 g) from each accession were chosen. Total genomic DNA was extracted according to the Murray and Thompson (1980) method and the extracts from all the accessions belonging to one species were mixed. Moreover, the quality and quantity of DNA were checked by agarose gel (0.7%) electrophoresis. 45 ISSR primers were screened, 22 produced a higher number of reproducible bands, which were selected for ISSR analysis (Table 2). The ISSR reaction mixture (total volume = 15 ml) contained 20 ng total DNA, 1.5 mM 10x PCR buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 2 pM of each primer, and 1 U Taq DNA polymerase. Amplifications were performed using a Bio-Rad Thermocycler (58BR 08334) programmed for 4 min at 94 °C as the initial denaturation temperature followed by 35 cycles at 94 °C for 1 min, 45 S at an appropriate annealing temperature (Table 2), 2 min at 72 °C, and 7 min at 72 °C as the last

Table 1. Information of species and accessions investigated in this study.

Num.	Sub-genus	Section	Species	No.	Origin
1	*Onobrychis*	*Onobrychis*	*O. transcaucasia*	9	Armenia, Iran, Turkey, Georgia, and Uzbekistan
2	*Onobrychis*	*Onobrychis*	*O. arenaria* (Kit.) DC.	10	Soviet Union, Russia, Romania, and Azerbaijani
3	*Onobrychis*	*Onobrychis*	*O. iberica*- Grossh	3	Soviet Union and Pakistan
4	*Onobrychis*	*Onobrychis*	*O. cyri*- Grossh	2	Russia
5	*Onobrychis*	*Onobrychis*	*O. altissima*	7	Soviet Union, Russia, Azerbaijani, Georgia, Iran
6	*Onobrychis*	*Onobrychis*	*O. alba*	3	Bulgaria and Soviet Union
7	*Onobrychis*	*Onobrychis*	*O. inermis* Steven	4	Russia
8	*Onobrychis*	*Onobrychis*	*O. petraea*	5	Germany, Iran, and Russia
9	*Onobrychis*	*Onobrychis*	*O. oxyodonta* Bioss.	1	Soviet Union
10	*Onobrychis*	*Onobrychis*	*O. gracilis* Besser	1	Bulgaria
11	*Onobrychis*	*Onobrychis*	*O. persica*	1	Iran
12	*Onobrychis*	*Onobrychis*	*O. peduncularis* (Cav) DC.	1	Spain
13	*Onobrychis*	*Onobrychis*	*O. hajastana*- Grossh	1	Soviet Union
14	*Onobrychis*	*Onobrychis*	*O. megataphros* Bioss.	1	Turkey
15	*Onobrychis*	*Onobrychis*	*O. montana* DC.	1	France
16	*Onobrychis*	*Onobrychis*	*O. viciifolia*	10	Iran, China, USA, Czech Republic, Kyrgyz, Spain, Ukraine, England, Morocco, and Russia
17	*Onobrychis*	*Onobrychis*	*O. kemulariae*	1	Soviet Union
18	*Onobrychis*	*Lophobrychis*	*O. caput-galli*	3	Turkey, Israel, and Unknown
19	*Onobrychis*	*Lophobrychis*	*O. crista-galli*	4	Iran, Israel, and Unknown
20	*Onobrychis*	*Lophobrychis*	*O. aequidentata*	3	France
21	*Onobrychis*	*Lophobrychis*	*O. pulchella*	1	Turkmenistan
22	*Sisyrosema*	*Heliobrychis*	*O. melanotricha*	2	Iran
23	*Sisyrosema*	*Heliobrychis*	*O. argyrea*	1	Turkey
24	*Sisyrosema*	*Hymenobrychis*	*O. ptolemaica*	2	Iraq and unknown
25	*Sisyrosema*	*Hymenobrychis*	*O. hypargyrea*	2	Turkey
26	*Sisyrosema*	*Hymenobrychis*	*O. michauxii*	2	Turkey and Iran
27	*Sisyrosema*	*Hymenobrychis*	*O. chorassanica*	1	Soviet Union
28	*Sisyrosema*	*Hymenobrychis*	*O. sintenisii*	3	Soviet Union and Iran
29	*Sisyrosema*	*Hymenobrychis*	*O. bobrovii* Grossh	1	Russia
30	*Sisyrosema*	*Hymenobrychis*	*O. pallasii*	1	Soviet Union
31	*Sisyrosema*	*Hymenobrychis*	*O. radiata*	1	Armenia

Table 2. ISSR primers information used in this study.

Num.	Sequence (3′-5′)	T_a	NB	NPB	PP%	H	PIC	E	H.av	MI	D	Rp
1	(CA)8G	52	14	11	0.79	0.494	0.372	6.097	0.0014	3.23	0.694	7.419
2	(TC)8C	56	15	15	1.00	0.484	0.367	6.161	0.0010	5.51	0.832	10.000
3	(TC)8G	54	14	12	0.86	0.472	0.361	4.581	0.0013	3.73	0.855	7.548
4	(AC)8 G	48	10	9	0.90	0.499	0.375	4.355	0.0018	3.04	0.767	7.032
5	(CA)8- RT	46	7	5	0.71	0.487	0.368	2.903	0.0031	1.31	0.664	3.161
6	(GA)8- RT	51	14	11	0.79	0.500	0.375	5.452	0.0015	3.26	0.755	7.097
7	(AC)7 DBD	50	12	10	0.83	0.475	0.362	6.742	0.0014	3.66	0.625	7.226
8	(AG)7C	52	9	9	1.00	0.487	0.368	3.774	0.0017	3.31	0.825	4.000
9	(GA)8SC	57	12	11	0.92	0.494	0.372	6.097	0.0014	3.76	0.694	7.613
10	(AC)8C	48	8	7	0.88	0.500	0.375	3.548	0.0023	2.31	0.744	4.258
11	(AG)8 SG	56	10	10	1.00	0.500	0.374	4.903	0.0016	3.74	0.760	6.387
12	(GA)8 SG	58	14	12	0.86	0.483	0.366	7.710	0.0012	4.42	0.649	8.065
13	(GA)8 WT	47	11	11	1.00	0.486	0.368	6.419	0.0014	4.05	0.660	8.129
14	(CT)8-RG	51	10	9	0.9	0.487	0.368	4.194	0.0016	3.68	0.825	5.548
15	(GA)8-C	50	12	10	0.83	0.437	0.342	6.774	0.0014	2.84	0.542	5.677
16	(AC)8-T	54	13	10	0.77	0.423	0.333	6.968	0.0014	2.56	0.515	5.871
17	(GA)8-YT	52	11	10	0.91	0.490	0.370	5.710	0.0016	3.37	0.675	7.032
18	(GA)8-YC	54	12	11	0.92	0.473	0.361	4.226	0.0014	3.65	0.853	6.968
19	(AG)8-YT	54	10	10	1.00	0.485	0.367	4.129	0.0016	3.67	0.830	6.000
20	(GACA)4	50	12	9	0.75	0.498	0.374	4.774	0.0018	2.52	0.720	7.355
21	(GA)8- RC	51	12	11	0.92	0.497	0.373	5.935	0.0015	3.77	0.710	8.710
22	(GACA)5	55	10	10	1.00	0.496	0.373	5.419	0.0016	3.73	0.707	5.742

T_a = Annealing temperature, NB: Number of total bands, NPB: Number of polymorphic bands,
PP%: Percentage of the polymorphic band, H: Heterozygosity index, PIC: Polymorphic Information Content,
E: Effective multiplex ratio, H.av: Arithmetic mean of Heterozygosity index,
MI: Marker Index, D: Discriminating power, and R: Resolving power.

synthesis step. Amplified DNA fragments were separated in a 1.5% agarose gel at 100 W for 2 h in 1 × TBE buffer (100 mM Tris–Borate, pH 8.0, 2 mM EDTA) and stained with ethidium bromide.

Statistical analysis

The morphological and anatomical characteristics of fruit and seeds were coded on a scale from 0 (absent) to 1 (present) to create a data matrix for analysis (Table 2). Molecular analysis considered well-resolved fragments as 1 for 'present' and others as 0 in a binary matrix (Table S3). Out of 252 bands, 223 were used for analysis to ensure accuracy. Parameters like PIC, Rp, Dp, H, H.avp, and E were calculated using the iMEC program (Amiryousefi et al., 2018) (Table 2). Genetic similarity was assessed using the Jaccard index in NTSYS (Rohlf, 1998) (Table 3), and PCA was conducted with NCSS software (2021). Genetic divergence between *Onobrychis* species was measured using Euclidean genetic distances in R (R Project for Statistical Computing). A phylogenetic tree based on combined seed morphological traits and ISSR marker data was constructed using Maximum Likelihood methods in MEGA (Tamura et al., 2013). Genetic structure parameters were evaluated with the Popgene program (Yeh et al., 1999). Arlequin (Excoffier and Lischer, 2010) assessed diversity between subgroups and sections. Structure (Pritchard et al., 2010) detected genetic structure and admixture using Bayesian clustering. The most likely number of genetic groups (K) was estimated using Structure Harvester (ver. 0.6.93) (http://taylor0.biology.ucla.edu/structure) (Earl, 2012).

Results

Seed and fruit morphological study

Seed and fruit morphological characteristics of the species are shown in Tables 1 and 2. The fruit was acanaceous in all the species, except in *O. pulchella*. On the other hand, the only species with short teeth on spines (component spines) was *O. crista-galli* while other species had simple spines (Table 2). Among the members of the *Onobrychis* genus only two species (*O. hajastana* and *O. crista-galli*) had thorns with triangular cross-sections on the disc, while other species had conical or both conical and triangular cross-section spines on the disc (Table S2 and Figure S1). The marginal spine cross-sections in all the species were conical except those belonging to the *Lophobrychis* and *Onobrychis* sections. The surface of the fruit in all the species was villous except for *O. persica*, *O. pulchella*, and *O. crista-galli*, while the only species with silky hair was *O. melanotricha* (Table 2). The fruit morphological result showed that all the species belonging to the *Hymenobrychis* section had sub-marginal veins and borders (Table 2 and figure 1). On the other hand, only two species (*O. melanotricha* and *O. argyrea*) had no crest on fruits (Table 2 and figure 1).

Furthermore, all the species of the *Hymenobrychis* sections had fully-curved crests. The size of the legumes ranged between 1.49 mm (*O. pulchella*) and 2.91 mm (*O. arenaria*) in length, 9.74 mm (*O. pulchella*) and 1.98 mm (*O. Arenaria*) in width, and 2.37 (*O. caput-galli*) and 1.125 (*O. petolemaica*) in length/width ratio. Among the members of the *Onobrychis* genus, only the *O. crista-galli* and species belonging to the *Hymenobrychis* section were found to have two seeds in the fruit, while the other species contained a single seed in the fruit (Table S2). The seed shapes were distinguished as Trapeziform, Orbiculate, Rhombic, Reniform, and Ovate. However, the general shape of the seeds in the four sections was reniform (Table 2).

Seed micro-morphological study

Light microscopy study

The general anatomy of the *Onobrychis* seed coat was found to be similar to that described by Cenci et al. (2000) who reported the outer layer represented by radially elongated cells to form a palisade-like (Malpighian cells) layer that constitutes the outer epidermis. Below the Malpighian cells, there were bone-shaped and thick-walled cells, termed 'osteosclereids', which underlie the inner tissue with thin-walled parenchyma cells which are followed by the cuticle layer (figure 2). In this study, *O. pulchella* showed the highest values for the Malpighian cells while *O. melanotricha* had the lowest values for the osteosclerosis layer. Significant differences were also observed in the total thickness of the parenchyma layer, with *O. michauxii* having the highest (0.88 mm) and *O. pallasii* having the lowest value (0.024 mm). The total seed coat thickness ranged between 0.142 mm and 0.052 mm in *O. pulchella* and *O. melanotricha*, respectively.

Scanning electron microscopy study (SEM)

The seed coat sculpture patterns were classified into six types: Reticulate (having a network wrinkle), Undulate (having wavy wrinkles), Druse (having aggregation of ir-

Table 3. Genetic parameter of *Onobrychis* subgenera and sections.

Subgenus	Section	No. of species	Na	Ne	H	I	No. polymorphic bands	PPB%
midrule *Onobrychis*		21	1.84	1.54	0.31	0.47	189	84.75
	Onobrychis	17	1.80	1.51	0.3	0.44	178	79.82
	Lophobrychis	4	1.68	1.47	0.27	0.4	153	68.61
Sisyrosema		10	1.86	1.57	0.33	0.49	192	86.1
	Heliobrychis	2	1.34	1.24	0.14	0.21	77	34.53
	Hymenobrychis	8	1.81	1.5	0.3	0.44	182	81.61

Na: Observed number of alleles, Ne: Effective number of alleles, H: gene diversity,
I: Shannon's index, PPB%: The percentage of polymorphic loci.

regular wrinkles), Convolutus (having tortuous wrinkles), Flat (having flat wrinkles), and Stellate (Asteroid elevations) (Table 1 and figures 3 and 4). The majority of the studied species showed the reticulate (14 species) followed by Convolutus (8 species), Undulate (5 species), flat (2 species), and the remaining two types each represented by only one single species (Table 2 and figure 4).

Cluster analysis of seed and fruit morphological data

Based on 75 seed morphological traits (Tables 1 and 2), the species were divided into two main groups (figure 1A). Group A consisted of all the species from the *Onobrychis*, *Lophobrychis* sections except *O. aequidentata* while the second group contains all the species from the *Hymenobrychis* and *Heliobrychis* sections along with *O. pulchella* (figure 1A). As a complementary test in grouping species with minimal data loss, we performed principal components analysis in which the first three principal components covered 41.79% of the cumulative variation. Two-dimensional visual illustrations of the first three PCA are shown in figure 2A. Based on the first two components, two district groups were found. The first group contains all the *Onobrychis* subgenera species, despite the small distance of the *O. pulchella* due to the first PC. The second group contains all *Sisyrosema* subgenus species in which almost all the species related to the section *Heliobrychis* were separated from the section *Hymenobrychis* according to the second PC (figure 2A).

ISSR analysis

Marker efficiency

The 22 selected primers amplified 252 bands, of which 223 (88.5%) were polymorphic among 31 *Onobrychis* species

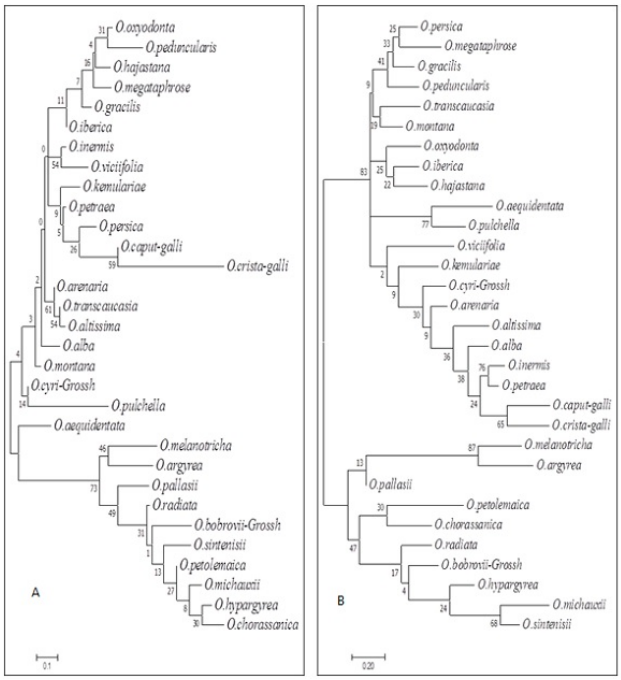

Figure 1. Association between *Onobrychis* species revealed by complete linkage model and Euclidean's genetic distance based on seed and fruit macro & micro-morphological traits (A) and ISSR marker (B).

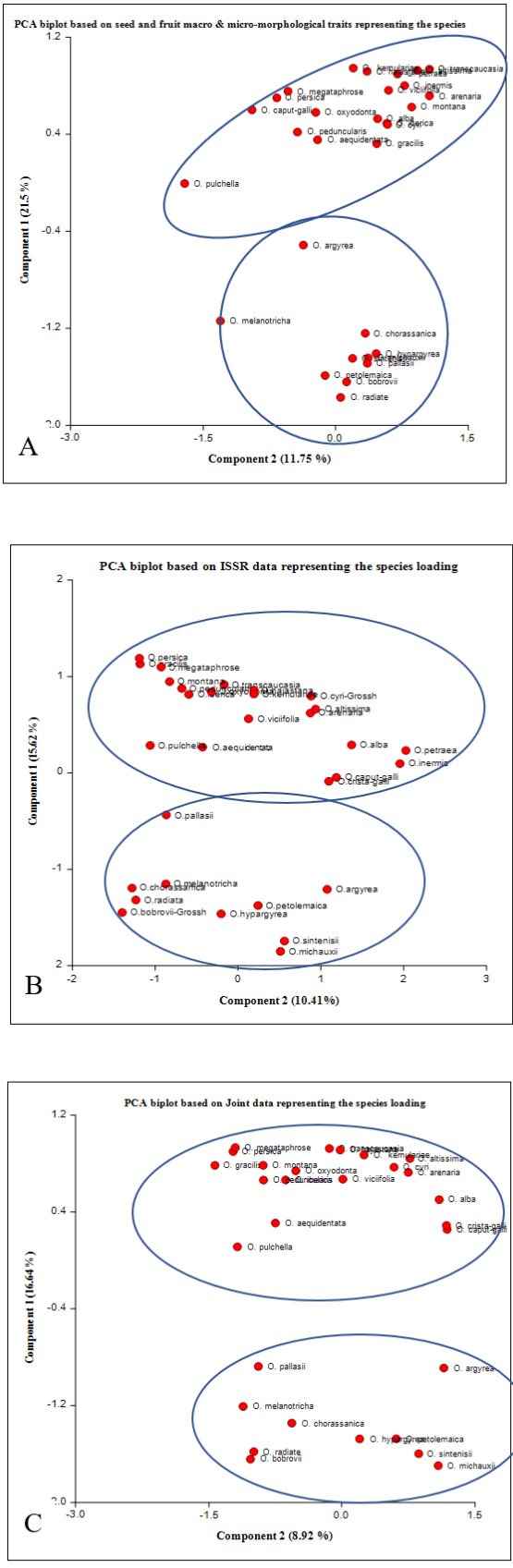

Figure 2. PCA (correlation's measure) case scores using NCSS program. A: Seed and Fruit macro & micro-morphological traits, B: ISSR marker, and C: Joint data.

Table 4. The Analysis of Molecular Variance (AMOVA) among 31 *Onobrychis* species.

Source of variation	Df	SS	Variance components	Var %	p-value
Among subgenus	1	416.42	3.87	7.08	<0.001
Among sections within the subgenus	2	355.79	13.39	24.51	0.0001
Among species within sections	59	2205.56	37.38	68.41	0.0001
Total	62	2977.77	54.64		

(Table 3). The amplified DNA ranged from 150 to 1400 bp. The maximum and minimum numbers of bands were observed by the primers (TC)8-C and (CA)8-RT, respectively. The percentage of polymorphic bands ranged from 75% to 100% (Table 2). The heterozygosity (H) varied from 0.423 (ISSR 16) to 0.5 for ISSR 06&10&11 with a mean of 0.484 per primer. The polymorphism information content (PIC) value ranged from 0.33% ((AC)8-T) to 0.38% ((GA)8- RT) with an average of 0.37%. Moreover, the marker index (MI) with an average value of 0.008 ranged from 0.006 to 0.01 (Table 2). For resolving power index (Rp), the lowest and highest values belonged to (CA)8- RT (3.16) and (TC)8C (10), respectively (Table 2). The arithmetic means of heterozygosity (H.av) ranged between 0.001 and 0.0031 with a mean of 0.0016 per primer. To determine the judicious profundity of primer, we calculate discriminative power (D) with a mean index of 0.723 and extend it from 0.515 ((AC)8-T) to 0.855 ((TC)8G). The effective multiplex ratio (E), which is a conditional factor on the magnitude of primer polymorphism, is spanned from 2.9 ((CA)8- RT) to 7.71 ((GA)8 SG) with an average of 5.312 per primer.

ISSR-based genetic similarity (GS)

Genetic distances and similarity coefficients for different species (Jaccard methods) and sections (Ni & Li method) are calculated and are shown in Table 3, respectively. The findings of the present study revealed the significant genetic differentiation among the *Onobrychis* species which ranged from 0.199 (*O. bobrovii* vs. *O. megataphros*) to 0.833 (*O. petraea* vas *O. inermis*) (Table 4). Moreover, from a sectional classification point of view, the highest similarity was observed between *Onobrychis* and *Lophobrychis* sections (0.87) while the lowest similarity was calculated for *Lophobrychis* and *Heliobrychis* (0.61) (Table 4).

ISSR cluster analysis

The cluster analysis based on the ISSR marker was performed for the 31 species as shown in figure 1B. The species were divided into two main groups in which all species belong to *Onobrychis* and *Sisyrosema* subgenus grouped in the first and second clades, respectively (figure 1B); however, in the first group, all species belonging to the *Onobrychis* subgenera are mixed, while in the second group *Hymenobrychis* and *Heliobrychis* sections are mostly separated (figure 1B). For more validation, the principal component analysis was run. The first three principal components cumulatively explained 32.36% of all the variation (Table 4). Like the cluster analysis, we observed complete discrimination of subgenus. However, unlike cluster analysis, *Sisyrosema* sectional classification was not observed, and also, based on the first component *O. pallasii*, were grouped far from their other subgenus member (figure 2B).

Seed and fruit morphological and ISSR combination analysis

To better discriminate between the *Onobrychis* species, both seed morphological traits and ISSR marker data were combined. So, based on joint data, Jaccard's Genetic similarity coefficients (GS) between the studied species were calculated and were in the range between 0.185 (*O. melanotricha* vs *O. persica*) and 0.806 (*O. inermis* vs *O. petraea*) (Table 4). The GS mean value for each section was 0.5, 0.43, 0.52, and 0.49 for *Onobrychis*, *Lophobrychis*, *Heliobrychis*, and *Hymenobrychis*, respectively.

The hierarchical clustering analysis based on Joint data is shown in Figure 4. The dendrogram illustrated that all 31 species were clustered into two main groups. Group A consisted of all *Onobrychis* subgenus species in which *O. arenaria* was classified in a separate subgroup. Furthermore, all the *Hymenobrychis* and *Heliobrychis* section species were clustered into the second group with complete separation (figure 4).

To lead the clustering investigation, principal component analysis was used. The distribution of eigenvalues, percentage of genetic variation, and cumulative percentage of genetic variation were displayed in Table S6. In the case of joint PCA analysis from the first principal component, the highest value was for *O. megataphros* and *O. transcaucasia* (0.93 and 0.92 respectively) while the least value (-1.96) was found for *O. michauxii* which has the least contribution to total diversity (Table 4). The first two PC bi-plots showed the complete separation of the subgenus classification (figure 2C). However, as far as the sections are concerned, this discrimination has not happened.

Another noteworthy outcome derived from our PCA analysis indicates the absence of differentiation between annual and perennial species within the *Onobrychis* genus. Despite the botanical classifications presented in Flora Iranica (Rechinger, 1969), Flora of Turkey (Davis et al., 1988), and Flora Europaea (Ball, 1968), which designate all species in the *Lophobrychis* section as annual and the rest as perennial, our study found no significant distinctions. This observation holds true for both seed anatomical and fruit morphological traits, as well as ISSR markers (figures 1 & 2). Our findings, consistent with the work of Pavlova and Manova (2000), underscore the lack of discernible differences in pollen morphology among annual and perennial species within the *Onobrychis* genus.

Genetic structure

Estimates of population parameters in each subgroup are shown in Table 3. The results showed that there was a

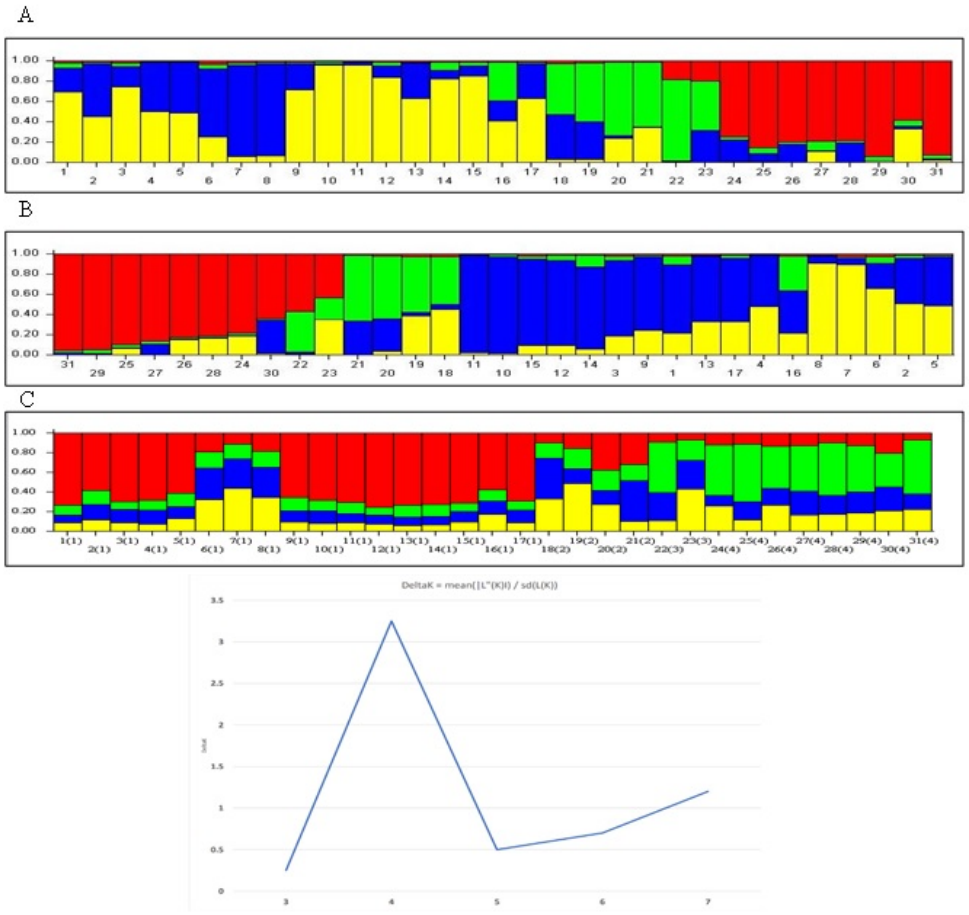

Figure 3. Structure harvester and Delta K value Bayesian model-based assessment of population structure for 31 *Onobrychis* species based on ISSR markers. A: Bar Plot based on original population order, B: Bar plot based on estimated membership coefficients values, C: Bar Plot based on species ID (population) order, D: ΔK = mean (|L''(K)|)/sd(L(K)) and ΔK = 4 indicates the best K value.

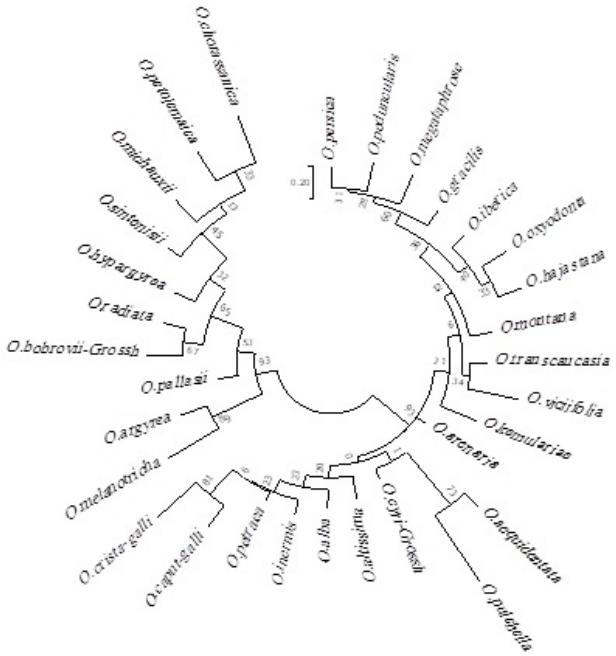

Figure 4. The Maximum Likelihood phylogenetic tree illustrating the genetic relationship among *Onobrychis* species based on 1000 bootstrap based on the Maximum Likelihood model.

slight difference between the calculated parameters for both subgenera. However, the calculation of the parameter for each section showed that the greatest variation was related to *Onobrychis* and *Hymenobrychis* sections equally (Na: 1.8, Ne: 1.5, H: 0.3, and I: 0.44) while the lowest variation belongs to *Heliobrychis* section (Na: 1.34, Ne: 1.24, H: 0.14, and I: 0.21).

In addition, to study the structure of the existing diversity in more detail, molecular analysis of variance (AMOVA) was used (Table 4). Based on the AMOVA result, 7.8% of the total variation was related to the subgenus difference, while 24.51% of the diversity was related to the differences between the sections in each subgenus, and 68.41% of the variations were related to the differences between the species in each section.

For evaluation of population structure and assigning species to specific populations based on ISSR marker, Structure version 2.2.3 was used based on a Bayesian model. The structure analysis was initially performed based on the maximum number of (K = 1 to 7), as the original population order and the most probable value of population were calculated to the maximum peak at ΔK=4 (K value = 3.25; Lnprob (K) = -3669.8). The rate of change of the likelihood distribution (mean), and the absolute value of the 2nd order of the change of the likelihood distribution (mean) for ΔK = 4 are shown in figure 3. Based on best K = 4 (figure 3d), it was determined that all the evaluated species could be positioned into four major groups visualized with four distinct colors of blue, green, yellow, and red. Each individual is represented by one vertical bar plot and the labels below the bar plots are the corresponding numbers for each species in Table 1. More than one color in each bar plot reveals the genetic complexity of that individual that, in this case, the species belongs to a group that has the largest color width of that cluster (figure 3).

Discussion

Seed and fruit macro-morphological characteristics

Morphological characteristics, seed-coat features, and seed-coat anatomy have proven useful for identifying species in the *Onobrychis* genus. Also highlighted the significance of fruit characteristics in species differentiation within this genus. While these characteristics vary considerably, they are valuable for classification and identification. Although no single fruit trait is exclusive to a subgenus or section, Yildiz et al. (1999) proposed three fruit morphology types (*O. radiata* type, *O. beata* type, and *O. ornata* type) for the *Onobrychis* genus. However, our study reveals that species resembling the *O. beata* type exhibit diversity in legume shape, spine density, crest length, and spine type. Thus, we propose recognizing a new fruit type, the *O. crista galli* type, in addition to the existing three. We provide a key for distinguishing between these fruit types. Type 1 (*O. beata* type) includes species with specific traits observed in the *Onobrychis* and some *Lophobrychis* sections. Type 2 (*O. radiata* type) characterizes species in the *Hymenobrychis* section. Type 3 (*O. crista galli* type) is unique to *O. crista-galli*. Type 4 (*O. ornate* type) is found in the *Heliobrychis* section.

Seed micro-morphological characteristics

Seed coat patterns have various applications, including classification, evolutionary relationships, and genetic marker identification (Wang et al., 2013). These patterns are typically species-specific, consistent across populations (Koul et al., 2000; Moazzeni et al., 2007). In this study, we examined seed coat microsculpturing in the *Onobrychis* genus, identifying six main patterns, with the reticulate pattern being the most common. However, these patterns didn't align with sections or subgenera, likely due to complex inheritance, maternal effects, and environmental factors (Deynze et al., 1995). To find more reliable taxonomic traits, we analyzed seed coat anatomy. We found that variations in seed coat thickness were mainly due to Malpighian cell length and inner parenchyma tissues. Notably, all *siserosema* subgenus species had shorter Malpighian cells (<0.034 mm) except for *O. chorassanica*, while most subgenus *Onobrychis* species had longer cells (>0.034 mm). This result aligns with Cenci et al. (2000) findings. Contrary to their study, we observed no consistent trend in seed coat thickness among diploid species, possibly due to the limited species they studied.

ISSR marker

ISSR markers are widely recognized for their high polymorphism, making them valuable tools in genetic diversity analysis, phylogenetics, gene tagging, genome mapping, and evolutionary biology (Pradeep et al., 2002). Our study's ISSR marker results indicate a significant level of polymorphism, underscoring their effectiveness in assessing the phylogenetic relationships among *Onobrychis* species. Several crucial factors influence primer efficacy, with two primary measures being expected heterozygosity (H) and polymorphic information content (PIC). H and PIC quantify a marker's ability to detect genetic polymorphisms, making them pivotal in marker selection for genetic studies (Khan et al., 2021). Regarding H and PIC values, within the studied population, they exceeded the average range for a dominant marker, suggesting an advanced discriminatory capacity of this marker system. Furthermore, the MI index, which factors in total band count and polymorphic ratio, illustrating each primer's potential to generate more bands, and Rp, assessing primers' ability to distinguish individuals, all exhibited high polymorphism and efficiency in genetic discrimination analysis for this genus. In summary, higher RP and MI indices correspond to more effective primers (Zarei and Erfani-Moghadam, 2021). Therefore, the most effective primers are (TC)8C (Rp = 10, MI = 5.51), (GA)8-RC (Rp = 8.7, MI = 3.77), (GA)8 WT (Rp = 8.12, MI = 4.05), and (GA)8 SG (Rp = 8, MI = 4.42).

Genetic structure based on ISSR marker

The population structure of the *Onobrychis* genus assessed by Bayesian admixture analysis indicated 4 clusters. Based on Q > 0.60, out of 31 *Onobrychis* species 15 were almost pure and the other species were highly complex, indicating these species were genetically admixture. Interestingly all the species belonging to the *Lophobrychis* section (numbers 18, 19, 20, and 21) have a different color in each bar

plot which reveals their genetic complexity. Moreover, the bar related to *O. viciifolia* had three colors (red, blue, and yellow) in almost equal proportions, which indicates the genetic complexity of this species too. Perhaps, the cross-pollinated nature of cultivated sainfoin (*O. viciifolia*) as well as the vast diversity regain make this species so complex.

Sectional and subgenus classification

In traditional species classification, morphological traits such as flower and leaf characteristics, seeds, and fruits have historically been used to categorize and group species into sections or subgenera. Some past studies (Yildiz et al., 1999) argued against the existence of evidence supporting subgenera classification while endorsing sectional discrimination based on seeds and fruits morphology. Conversely, phylogenetic studies relying on molecular markers (Amirahmadi et al., 2016; Kar et al., 2014) produced opposite results, supporting subgenera classification and failing to verify sectional discrimination, particularly in the *Onobrychis* subgenus. Our research, despite providing identification keys for species' fruits and seeds, did not confirm sectional discrimination within the *Onobrychis* subgenera. This discrepancy arises from factors such as open pollination, high allogamy rates (Avci et al., 2016), extensive geographical dispersion (Zarrabian et al., 2013), hybridization, and introgression events (Bandara et al., 2013) among *Onobrychis* taxa. These factors make morphological traits more susceptible to environmental influences. In contrast to morphological traits, most molecular data supported subgenera classification but did not uphold sectional discrimination within the *Onobrychis* subgenus (Amirahmadi et al., 2016; Kar et al., 2014; Duan et al., 2015). To enhance the accuracy of species relationships, especially between sections and subgenera, we used combined data and conducted downstream analysis. Our comprehensive species sampling suggests that *Onobrychis* and *Sisyrosema* are distinct and can be considered monophyletic subgenera. Unlike *Onobrychis*, *Sisyrosema* aligns with its traditional sectional classification and should be retained. However, none of the sections within the *Onobrychis* subgenera appear monophyletic, as *O. caput-galli* and *O. crista-galli* cluster separately from other *Lophobrychis* species (*O. aequidentata* and *O. pulchelle*) and intermingle with species from the *Onobrychis* subgenus. Other researchers have suggested that the subgenus *Sisyrosema* might have originated from the subgenus *Onobrychis* based on seed storage protein (Arslan and Ertugrul, 2010) and sequence data (Duan et al., 2015). In summary, our study highlights a disconnect between morphological and molecular data, suggesting the need for a combined approach to understand species relationships within the *Onobrychis* subgenus.

Lophobrychis section as a heterogeneous unit

In our phylogenetic analysis, species placement on the tree indicated genetic similarity, with closer positioning implying greater genetic likeness (Khan et al., 2021). Such a trend has not been observed for the species related to *Lophobrychis* section. In this study, no independent clade containing all species of the *Lophobrychis* section was found, neither through molecular markers nor with morphological traits. Therefore, in contrast to the traditional taxonomic classification, species belonging to the *Lophobrychis* section were not coherently classified into one group (figure 1 and figure 3), indicating the *Lophobrychis* section not only has a comparatively highly derived organization but also can be considered as a heterogeneous unit in the *Onobrychis* genus (Abou-El-Enain, 2002, 2004). Although a previous study (Kar et al., 2014) showed *Onobrychis* and *Lophobrychis* sections were separated, the high similarity found in this study between *Lophobrychis* and other sections, especially the *Onobrychis*, gives rise to the probability that the concept of section applied to *Lophobrychis* is meaningless or unrealistic. Bandara et al. (2013), based on ITS sequences, supported the sectional treatment and may require some changes. They assumed that, in order to form a monophyletic clade corresponding to a section, part of *Lophobrychis*, *Dendrobrychis* and the subsection *Macropterae* of *Onobrychis* section should be kept together. Hayot-Carbonero et al. (2012) declared the *Lophobrychis* and *Onobrychis* sections were less clearly distinct. By looking at the similarity matrix based on the joint data. *O. caput-galli* had a higher similarity to the species belonging to the *Onobrychis* section rather than the rest of the *Lophobrychis* species. Elena (2006) reported that *O. caput-galli* was cytotaxonomically closer to the section *Onobrychis* rather than other species of its own section. Overall, discriminating between *Lophobrychis* and *Onobrychis* sections poses challenges, possibly due to a relatively recent common ancestor (Hayot-Carbonero et al., 2012).

Conclusion

Investigations of seed macro- and micro-morphology of the genus *Onobrychis* showed that it could be a useful tool (especially macro-morphological traits) for differentiating species. Hence, these data alone are insufficient for phylogenetic analysis. Such conflict may be due to the fact that species were assigned to different sections based on only one or few morphological characters and some characters may have converged in the course of evolutionary history (Duan et al., 2015). The results obtained from this study support the subgenus (*Onobrychis* and *Sisyrosema*) classification while the monophyly of sections belonging to the *Onobrychis* subgenus was not supported. Moreover, it seems that the section *Lophobrychis* has a comparatively highly derived organization and can be considered as a heterogeneous unit in the Onobrychis genus. Our results also showed that the combination of seed macro- and micro-morphological traits with ISSR data provides complementary information for the *Onobrychis* genus classification. However, more studies with a larger number of molecular markers will be needed to obtain an appropriate phylogenetic classification.

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Authors contributions
Authors have contributed equally in preparing and writing the manuscript.

Availability of data and materials
The authors declare that the data supporting the findings of this study are available within the paper.

Conflict of interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Abou-El-Enain M.M. (2002) Chromosomal criteria and their phylogenetic implications in the genus *Onobrychis* Mill, Sect. *Lophobrychis* (Leguminosae) with special reference to Egyptian species. *Botanical Journal of the Linnean Society* 139:409–414.

——— (2004) SDS-PAGE of seed protein criteria in relation to taxonomy of *Onobrychis* sect. *Lophobrychis* s. str. and the Egyptian species. *Cytologia* 69:351–358.

Amirahmadi A., Kazempour-Osaloo S., Kaveh A., Maassoumi A.A., Naderi R. (2016) The phylogeny and new classification of the genus *Onobrychis* (*Fabaceae-Hedysareae*): evidence from molecular data. *Plant Systematics and Evolution* 302:1445–1456.

Amiryousefi A., Hyvönen J., Poczai P. (2018) iMEC: Online Marker Efficiency Calculator. *Applications in Plant Sciences* 6 (6): e1159.

Arslan E., Ertugrul K. (2010) Genetic relationships of the genera *Onobrychis*, Hedysarum, and Sartoria using seed storage proteins. *Turkish Journal of Biology* 34:67–73.

Avci S., Tekin N., Sancak C., Özcan S., Marang A.O. (2016) Phylogenetic relationship of some *Onobrychis* taxa naturally grown in Turkey based on morphology and nuclear ribosomal DNA ITS Sequences. *Legume Research* 39 (5): 665–67.

Ball P.W. (1968) *Onobrychis* Miller. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentin SM (ed) Flora Europaea. *Cambridge: Cambridge University* 2:187–191.

Bandara N.L., Papini A., Mosti S., Brown T., Smith L.M.J. (2013) A phylogenetic analysis of genus *Onobrychis* and its relationships within the tribe Hedysareae (Fabaceae). *Turkish Journal of Botany* 37:981–992.

Barthlott W. (1984) Microstructural features of seed surfaces. *In: Heywood VH, Moore DM (Ed) Current concepts in plant taxonomy* (London)

Bhattarai S., Coulman B., Biligetu B. (2015) Sainfoin (*Onobrychis viciifolia* Scop.): renewed interest as a forage legume for western Canada. *Canadian Journal of Plant Science* 96 (5): 748–756.

Cenci C.A., Bassi G., Ferranti E., Romano B. (2000) Some morphometric, anatomical and biochemical characteristics of fruits and seeds of *Onobrychis* spp. in Italy. *Plant Biosystems* 134:91–98.

Davis P.H., Mill R.R., Tan K. (1988) Flora of Turkey and the East Aegean Islands (Supplement). *Edinburgh: Edinburgh University* 10:561–588.

Deynze A.E. Van, Landry B.S., Pauls K.P. (1995) The identification of restriction fragment length polymorphisms linked to seed colour genes in *Brassica napus*. *Genome* 38:534–542.

Duan L., Wen J., Yang X., Liu P.L., Arslan E. (2015) Phylogeny of *Hedysarum* and tribe Hedysareae (Leguminosae: Papilionoideae) inferred from sequence data of ITS, matK, trnL-F and psbA-trnH. *Taxon* 64 (1): 49–64.

Earl D.A. (2012) Structure Harvester: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* 4 (2): 359–361.

Elena T. (2006) Cytological aspects of the Onobrychis genus. *Buletin USAMV* 62:154–158.

Excoffier L., Lischer H.E.L. (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10:564–567.

Hayot-Carbonero C., Carbonero F., Smith L.M.J., Brown T.A. (2012) Phylogenetic characterisation of Onobrychis species with special focus on the forage crop Onobrychis viciifolia Scop. *Genetic Resources and Crop Evolution* 59:1777–1788.

Hejazi H., ZiaeiNasab M.Z. (2010) Cytotaxonomy of some Onobrychis (Fabaceae) species and populations in Iran. *Caryologia* 63 (1): 18–31.

Kar S. Safaei Chaei, Ghanavati F., Naghavi M.R., Amirabadi-zade H., Rabiee R. (2014) Molecular phylogenetics of the Onobrychis genus (Fabaceae: Papilionoideae) using ITS and trnL–trnF DNA sequence data. *Australian Journal of Botany* 62:235–250.

Kaveh A., Kazempour S., Amirahmadi A., Maassoumi A., Schneeweiss G.M. (2019) Systematics of Onobrychis sect. Heliobrychis (Fabaceae): morphology and molecular phylogeny revisited. *Plant Systematics and Evolution* 305:33–48.

Khan M.M.H., Rafii M.Y., Ramlee S.I., Josoh M., Mamum D.A. (2021) DNA fingerprinting, fixation-index (Fst), and admixture mapping of selected Bambara groundnut (*Vigna subterranea* [L.] Verdc.) accessions using ISSR markers system. *Scientific Reports* 11:14527. DOI: https://doi.org/10.1038/s41598-021-93867-5.

Koul K.K., Nagpal R., Raina S.N. (2000) Seed Coat Microsculpturing in Brassica and Allied Genera (Subtribes Brassicinae, Raphaninae, Moricandiinae). *Annals of Botany* 86:385–397.

Moazzeni H., Zarre S., Al-Shehbaz I.A., Mummenhoff K. (2007) Seed-coat microsculpturing and its systematic application in Isatis (Brassicaceae) and allied genera in Iran. *Flora* 202:447–454.

Mora-Ortiz M., Smith L. (2018) *Onobrychis viciifolia*; a comprehensive literature review of its history, etymology, taxonomy, genetics, agronomy and botany. *Plant Genetic Resources* 16 (5): 403–418.

Murray M.G., Thompson W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* 8:4321–4325.

Pavlova D.K., Manova V.I. (2000) Pollen morphology of the genera *Onobrychis* and *Hedysarum* (Hedysarea, Fabaceae) in Bulgaria. *Annales Botanici Fennici* 37:207–217.

Pradeep R.M., Sarla N., Siddiq E. (2002) Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica* 128:9–17. DOI: https://doi.org/10.1023/A:1020691618797.

Pritchard J.K., Wen W., Falush D. (2010) Documentation for STRUCTURE Software: Version 2 (University of Chicago).

Punt W., Blackmore S., Nilsson S., Thomas A. (1994) Glossary of pollen and spore terminology. *LPP Foundation, Utrecht*

Rechinger K.H. (1969) Flora Iranica. In: Reshinger KH (ed). *Akademische Druck- und Verlgsantalt* (Austria) 157:387.

Rohlf F.J. (1998) NTSYS-pc numerical taxonomy and multivariate analysis system.Version 2.00 *Exeter Software, Setauket, NY.*

Stearn W.T. (1983) Botanical Latian. (London)

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30 (12): 2725–2729.

Wang Y. y., Wei W., Kang D. m., Ma K. p. (2013) Seed Coat Microsculpturing Is Related to Genomic Components in Wild Brassica juncea and Sinapis arvensis. *PLoS ONE* 8 (12): e83634. DOI: https://doi.org/10.1371/journal.pone.0083634.

Yeh F.C., Yang R., Boyle T. (1999) Popgene version 1.32: Microsoft Windows-based freeware for population genetic analysis. *University of Alberta, Edmonton*

Yildiz B., Ciplak B., Aktoklu E. (1999) Fruit morphology of sections of the genus *Onobrychis* Miller (Fabaceae) and its phylogenetic implications. *Israel Journal of Plant Sciences* 47:269–282.

Zarei A., Erfani-Moghadam J. (2021) SCoT markers provide insight into the genetic diversity, population structure and phylogenetic relationships among three Pistacia species of Iran. *Genetic Resources and Crop Evolution* 68 (4): 1625–1643.

Zarrabian M., Majidi M.M. (2015) Genetic diversity and relationships within and among *Onobrychis* species using molecular markers. *Turkish Journal of Botany* 39:681–692.

Zarrabian M., Majidi M.M., Ehtemam M.H. (2013) Genetic Diversity in a Worldwide Collection of Sainfoin Using Morphological, Anatomical, and Molecular Markers. *Crop Science* 53:1–14.