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ORIGINAL ARTICLE

The phylogeny and new classification of the genus *Onobrychis* (Fabaceae-Hedysareae): evidence from molecular data

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Abstract The present study was conducted to analyze the phylogenetic status of the genus *Onobrychis* and to evaluate the monophyly of its subgenera and sections and relationship among them. We sequenced the nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) and three chloroplast regions *trn*L-F, *rpl*32/*rpl*32-*trn*L$_{(UAG)}$ and *ndh*F-*rlp*32 for phylogenetic reconstruction of 51 species of *Onobrychis*. In all of our analyses, *Eversmannia subspinosa*, *Corethrodendron scoparium*, *Greuteria membranacea* and *G. argyrea* were chosen as outgroups. Phylogenetic analyses were performed by maximum parsimony, maximum likelihood and Bayesian methods. Our molecular data 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. Sections *Lipskyanae* and *Litvinovianae* are newly established and described, representing distinct lineages within the genus. *Onobrychis splendida*, a species hitherto without a sectional position, along with some members of sect. *Anthyllium* were retrieved representatives of section *Lipskyanae*. Sections *Afghanicae*, *Insignes, Heliobrychis* and *Hymenobrychis* (with the inclusion of two species of section *Anthyllium*) are monophyletic. Sections *Dendrobrychis* and *Lophobrychis* are reduced to synonymy of section *Onobrychis* and *Anthyllium* to synonymy of section *Hymenobrychis*. A taxonomic treatment for the genus is presented.

Keywords Classification · Molecular phylogeny · New sections · *Onobrychis* · Taxonomic treatment

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Introduction

Onobrychis Mill. with more than 130 species is ranked as the second largest genus of the tribe Hedysareae after *Hedysarum* (Mabberley 2008; Lock 2005; Amirahmadi et al. 2014a). The genus is distributed throughout temperate and subtropical regions of Eurasia, N and NE Africa (Townsend 1974). Most species of *Onobrychis* are distributed in the Flora Iranica area (77 species), the former USSR (62 species), Turkey (46 species) and Europe (23 species), respectively (Hedge 1970; Grossheim 1972; Ball 1978; Rechinger 1984). Recently, several new species of *Onobrychis* were described from Iran (e.g., Ranjbar et al. 2004, 2009; Amirabadizadeh et al. 2007, 2009; Amirahmadi et al. 2014b).

Širjaev (1925) based on floral characteristics divided the genus into two subgenera, *Onobrychis* (as *Euonobrychis* (Bunge ex Boiss.) Širj.) and *Sisyrosema* (Bunge ex Boiss.) Širj. each with four sections. Rechinger (1984) reclassified the two subgenera into four and five sections, namely *Onobrychis*, *Dendrobrychis* DC., *Lophobrychis* Hand.-Mazz., *Laxiflorae* (Širj.) Rech.f., *Anthyllium* Nábělek, *Afghanicae* Širj., *Heliobrychis* Bunge ex Boiss., *Hymenobrychis* DC. and *Insignes* (Širj.) Rech.f., respectively. However, the sectional positions of *O. splendida* Rech.f. & Podlech and *O. freitagii* Rech.f. (this species was not included in the present study) have remained hitherto uncertain. The infra-generic classification systems of *Onobrychis* are summarized in Table 1.

Several biosystematics studies ranging from non-molecular data to DNA sequences have been carried out on *Onobrychis* and allies (e.g., Yildiz et al. 1999; Pavlova and Manova 2000; Abou-El-Enain 2002; Ahangarian et al. 2007; Irfan et al. 2007; Hesamzadeh Hejazi and Ziaei Nasab 2010; Ranjbar et al. 2010, 2012; Arslan et al. 2012; Hayot Carbonero et al. 2012; Karamian et al. 2012; Avci et al. 2013, Lewke Bandara et al. 2013; Amirahmadi et al. 2014a; Safaei Chaei Kar et al. 2012, 2014; Duan et al. 2015; Zarrabian and Majidi 2015). But still, detailed phylogenetic analysis using multiple DNA sequence data and adequate taxon sampling of all recognized sections on the genus is lacking.

In this study, the nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) and three chloroplast regions, *trn*L$_{(UAA)}$ intron and *trn*L$_{(UAA)}$-*trn*F$_{(GAA)}$ intergenic spacer (hereafter *trn*L-F), *rpl*32 gene and *rpl*32-*trn*L$_{(UAG)}$ intergenic spacer (hereafter *rpl*32/*rpl32*-*trn*L$_{(UAG)}$) and *ndh*F-*rlp*32 intergenic spacer, were sequenced for phylogenetic reconstructions. The internal transcribed spacer (ITS) contains the signals needed to process the rRNA transcript (Baldwin et al. 1995) and has often been used for inferring phylogeny at intra- and intergeneric levels (e.g., Wojciechowski et al. 1999; Kazempour Osaloo et al. 2005; Amirahmadi et al. 2014a). The *trn*L-F and both *ndh*F-*rpl*32

Table 1 The infrageneric classification systems of the genus *Onobrychis*

Širjaev (1925, 1926)	Townsend (1974)	Rechinger (1984)	Present study
Subgenus *Euonobrychis* (Bunge ex Boiss.) Širj.	Subgenus *Onobrychis*	Subgenus *Onobrychis*	Subgenus *Onobrychis*
Sect. *Eubrychis* DC. (including subsects. *Macropterae* Hand.-Mazz., *Macrosemiae* Hand.-Mazz., *Brachysemiae* Hand.-Mazz., *albae* Hand.-Mazz., *Hispanicae* Širj. and *Vulgatae* Hand.-Mazz.)	Sect. *Onobrychis*	Sect. *Onobrychis*	Sect. *Onobrychis*
Sect. *Dendrobrychis* DC. (including sers. *Dielsianae* Širj. and *Litvinovianae* Širj.)	Sect. *Dendrobrychis* DC.	Sect. *Dendrobrychis* DC.	
Sect. *Lophobrychis* Hand.-Mazz. (including subsects. *Occidentales* Širj. and *Orientales* Širj.)	Sect. *Lophobrychis* Hand.-Mazz.	Sect. *Lophobrychis* Hand.-Mazz.	
Sect. *Hemicyclobrychis* Širj.			Sect. *Hemicyclobrychis* Širj.
		Sect. *Laxiflorae* (Širj.) Rech.f.	
Subgenus *Sisyrosema* (Bunge ex Boiss.) Širj.	Subgenus *Sisyrosema* (Bunge ex Boiss.) Širj.	Subgenus *Sisyrosema* (Bunge) Grossh[a]	Subgenus *Sisyrosema* (Bunge ex Boiss.) Širj.
Sect. *Afghanicae* Širj.		Sect. *Afghanicae* Širj.	Sect. *Afghanicae* Širj.
Sect. *Anthyllium* Nábělek (including subsects. *Fedtschenkoanae* Širj., *Lipskyanae* Širj., *Mirae* Širj. and *Nábělekianae* Širj.)	Sect. *Anthyllium* Nábělek	Sect. *Anthyllium* Nábělek	
			Sect. *Lipskyanae* (Širj.) Amirah. & Kaz. Osaloo
Sect. *Heliobrychis* Bunge (including subsects. *Szovitsianae* Širj., *Boissierianae* Širj. and *Persicae* Širj.)	Sect. *Heliobrychis* (Bunge ex Boiss.) Širj.	Sect. *Heliobrychis* Bunge ex Boiss.	Sect. *Heliobrychis* (Bunge ex Boiss.) Širj.
Sect. *Hymenobrychis* DC. (including subsects. *Insignes* Širj., *Modestae* Širj., *Pulcherrimae* Širj. and *Laxiflorae* Širj.)	Sect. *Hymenobrychis* DC.	Sect. *Hymenobrychis* DC.	Sect. *Hymenobrychis* DC.
		Sect. *Insignes* (Širj.) Rech.f.	Sect. *Insignes* (Širj.) Rech.f.
			Sect. *Laxiflorae* (Širj.) Rech.f.
			Sect. *Litvinovianae* (Širj.) Amirah. & Kaz. Osaloo

[a] The author name for the subgenus was illegitimately used

and *rpl*32/*rpl*32-*trn*$L_{(UAG)}$ are located in the large single copy and the small single copy regions of the chloroplast genome, respectively. The latest is the best non-coding region for low-level molecular studies (Shaw et al. 2007; Dong et al. 2012).

Our objectives in the present study are to use molecular sequence data, to generate hypotheses on the phylogenetic status of *Onobrychis* and relationships among its analyzed taxa herein (subgenera, sections and *O. splendida*) so to evaluate past classifications of the genus, to identify morphological features that characterize the main clades detected by the molecular analyses and to present a new phylogenetic classification of the genus.

Materials and methods

Taxon sampling

A total of 199 DNA sequences were generated from specimens deposited at the following herbaria: FUMH, GAZI, MSB, TARI, TUH and Tarbiat Modares Univ. Herb. or in several cases (especially species distributed in Iran) from silica-dried leaves. Two species of *Greuteria* (*G. membranacea* (Coss. & Bal.) Amirahm. & Kaz.Osaloo and *G. argyrea* (Greuter & Burdet) Amirahm.& Kaz.Osaloo), *Corethrodendron scoparium* (Fisch & Meyer) Fisch & Basiner and *Eversmannia subspinosa* (Fisch) B.Fedtsch. were selected as outgroups following our previous study (Amirahmadi et al. 2014a). The sampling strategy was to include representatives of all nine recognized sections of *Onobrychis* (sensu Rechinger 1984) including sects. *Heliobrychis* (9 species), *Hymenobrychis* (8 species), *Onobrychis* (9 species), *Lophobrychis* (5 species), *Dendrobrychis* (5 species), *Anthyllium* (5 species), *Afghanicae* (4 species), *Insignes* (3 species) and *Laxiflorae* (one species with two accessions), plus *O. splendida* (a species of unassigned section) which are listed in Table 2.

DNA extraction, amplification, and sequencing

Total genomic DNA was isolated from fresh or dried leaf material using modified CTAB method of Doyle and Doyle (1987). The nrDNA ITS region was amplified using the primers ITS5 m (Sang et al. 1995) and ITS4 (White et al. 1990) or AB101F and AB102R (Douzery et al. 1999). The *trn*L-F region was amplified using the universal “c” and “f” primers (Taberlet et al. 1991). The *rpl*32/*rpl*32–*trn*$L_{(UAG)}$ and *ndh*F-*rpl*32 regions were amplified using two primer pairs: *rpl*32-F/*trn*$L_{(UAG)}$ (Shaw et al. 2007) and *ndh*Fm/*rpl*32RR (both were designed in this study: 5′-AAGATTGATGTGTATATTC-3′ and 5′-TACGTTTTTTT GGAACTG-3′), respectively. The PCR amplification was carried out in the volume of 20 µl, containing 8 µl deionized water, 10 µl of the 2× Taq DNA polymerase master mix Red (Amplicon, Cat. No. 180301, 150 mM Tris–HCl pH = 8.5, 40 mM $(NH4)_2SO4$, 3.0 mM $MgCl_2$, 0.4 mM dNTPs, 0.05 units μl^{-1} Amplicon Taq DNA polymerase, inert red dye and a stabilizer) 0.5 µl of each primer (10 pmol/µl), and 1 µl of template DNA (20 ng/µl). PCR cycles consisted of pre-denaturation at 94 °C for 3 min followed by 28–35 cycles: denaturation at 94 °C for 1 min, annealing at a temperature depending on the region (55 °C for nrDNA and 58 °C for cpDNA) for 1 min and elongation at 72 °C for 1 min. A final elongation step of 7 min at 72 °C was performed. The quality of the PCR products were checked by electrophoresis on a 1 % (w/v) agarose gel (using 1× TBE as the gel buffer) stained with ethidium bromide and then was photographed with a UV gel documentation system (UVItec, Cambridge, UK). Each region was sequenced using the Big dye terminator cycle sequencing ready reaction kit (Applied Biosystems, USA) with the appropriate primers in an ABI Prism 3730XL DNA sequencer (Applied Biosystems, USA).

Alignment and phylogenetic analyses

Each of the single dataset was aligned using the web-based version of MUSCLE (Edgar 2004; at http://www.ebi.ac.uk/Tools/msa/muscle/) under default parameters followed by manual adjustment. Sequences of all datasets showed length variation (because of noncoding region), and it was necessary to introduce insertions/deletions in the alignment. Positions of indels were treated as missing data for all datasets. Phylogenetic analyses were performed based on the maximum parsimony (MP) and maximum likelihood (ML) methods as well as Bayesian inference (BI). Parsimony analyses were conducted using PAUP* version 4.0b10 (Swofford 2002). The heuristic search option was employed for each dataset, using tree bisection-reconnection (TBR) branch swapping, with 100 replications of random addition sequence and an automatic increase in the maximum number of trees. Branch support values were calculated using a full heuristic search with 1000 bootstrap replicates (Felsenstein 1985) each with simple addition sequence. In Bayesian method, models of sequence evolution were selected using the program MrModeltest version 2.3 (Nylander 2004) based on the Akaike information criterion (AIC) (Posada and Buckley 2004). This program indicated GTR + G for nrDNA ITS and plastid sequence datasets (*trn*L–F, *rpl*32/*rpl*32-*trn*$L_{(UAG)}$ and *ndh*F-*rpl*32) and GTR + G+I for the combined dataset, as the best model for nucleotide substitution. The program MrBayes version 3.2.4 (Ronquist et al. 2012) was used for the Bayesian phylogenetic analyses. Posteriors on the model parameters were estimated from the data, using the default priors. The analysis was carried out with 6 million

Table 2 Taxa included in the nrDNA ITS, *trn*L-F, *rpl*32-*trn*L_{UAG} and *ndh*F-*rpl*32 analyses

Taxa	Source, voucher	GenBank Accession Number			
		nrDNA ITS	*trn*L-F	*rpl*32-*trn*L_{UAG}	*ndh*F-*rpl*32
Corethrodendron scoparium (Fisch. & Meyer) Fisch. & Basiner	China: *Xu* et al., *86862* (MSB)	AB854478[a]	AB854521[a]	LC137101	LC137153
Eversmannia subspinosa (Fisch.) B.Fedtsch.	Iran: *Freitag* and *Mozaffarian*, *28397* (TARI)	AB329692[a]	AB854527[a]	LC137102	LC137155
Greuteria membranacea (Coss. & Bal.) Amirahm. & Kaz. Osaloo (= *H. membranaceum*)	*Podlech 49070* (MSB)	AB854486[a]	AB854530[a]	LC137103	LC137156
Greuteria argyrea (Greuter & Burdet) Amirahm. & Kaz.Osaloo (= *H. argyreum*)	*Podlech 48626* (MSB)	AB854487[a]	AB854531[a]	LC137104	–
Onobrychis acaulis Bornm.	Iran: *Kazempour Osaloo* et al. *2012-1* (TMUH)	LC137019	LC137060	LC137105	LC137157
O. aequidentata d'Urv.	France: *Auriault, 16177* (MSB)	LC137020	LC137061	LC137106	LC137158
O. afghanica Širj. & Rech.f.	Afghanistan: *Podlech 15931* (MSB)	AB854501[a]	AB854544[a]	LC137107	LC137159
O. altissima Grossh.	Iran: *Zarre* et al. s.n. (TUH)	LC137021	LC137062	LC137108	LC137160
O. amoena ssp. *meshedensis* Širj. & Rech.f.	Iran: *Kazempour Osaloo* et al. *2011-2* (TMUH)	LC137022	LC137063	LC137109	LC137161
O. argentea Boiss.	Algeria: *Podlech, 96963* (MSB)	LC137023	LC137064	LC137110	LC137162
O. arnacantha Bunge ex Boiss.	Iran: *Faghihnia* and *Zangooii 26074* (FUMH)	LC137024	LC137065	LC137111	LC137163
O. atropatana Boiss.	Iran: *Zehzad 2844* (Urmia University Herbarium)	LC137025	LC137066	LC137112	LC137164
O. bungei Boiss.	Iran: *Rechinger 43484* (MSB)	LC137026	LC137067	LC137113	LC137165
O. caput-galli (L.) Lam.	Iran: *Kazempour Osaloo* et al. *2012-4* (TMUH)	LC137027	LC137068	LC137114	LC137166
O. cornuta (L.) Desv.	Iran: *Kazempour Osaloo* et al. *2012-2* (TMUH) (TMUH)	LC137028	LC137069	LC137115	LC137167
O. crista-galli (L.) Lam.	Iran: *Kazempour Osaloo* et al. *2012-3* (TMUH)	LC137029	LC137070	LC137116	LC137168
O. dealbata Stocks	Afghanistan: *Podlech 30864* (MSB)	LC137030	LC137071	LC137117	LC137169
O. echidna Lipsky	Tajikistan: *Kaletkina* s.n. (TARI)	LC137031	LC137072	LC137118	LC137170
O. elymaitica Boiss. & Hausskn.	Iran: *Mozaffarian 71259* (TARI)	LC137032	LC137073	LC137119	LC137171
O. eubrychidea Boiss.	Afghanistan: *Poldelch 17583* (MSB)	LC137059	LC137100	LC137150	LC137204
O. gaubae Bornm.	Iran: *Kazempour Osaloo* et al. *2011-4* (TMUH)	LC137033	LC137074	LC137120	LC137172
O. gypsicola Rech.f.	Iran: *Mozaffarian 70189* (TARI)	LC137034	LC137075	LC137121	LC137173
O. heliocarpa Boiss.	Iran: *Assadi 86772* (TARI)	LC137035	LC137076	LC137122	LC137174
O. heterophylla C.A.Mey.	Iran: *Assadi 86659* (TARI)	LC137036	LC137077	–	LC137175
O. iranensis Amirab. & Ghanavati	Iran: *Assadi* and *Amriabadizadeh 84707* (TARI)	LC137037	LC137078	LC137123	LC137176
O. laxiflora Baker	Afghanistan: *Lamond 1982* (MSB)	AB854505[a]	AB854548[a]	–	LC137177
O. laxiflora Baker ssp. *taftanica* Rech.f.	Iran: *Ayatollahi* and *Zangooii 14315* (FUMH)	LC137038	LC137079	LC137124	LC137178
O. lunata Boiss.	Iran: *Rechinger 42530* (MSB)	LC137039	LC137080	LC137125	–
O. mazanderanica Rech.f.	Iran: *Kazempour Osaloo* et al. *2011-3* (TMUH)	LC137040	LC137081	LC137126	LC137179
O. melanotricha Boiss. var. *villosa* Bornm.	Iran: *Nasirizadeh* and *Hatami* s.n (TARI)	LC137041	LC137082	LC137127	LC137180
O. merxmuelleri Podlech	Afghanistan: *Podlech 10621* (MSB)	AB854506[a]	AB854549[a]	–	LC137181
O. micrantha Schrenk	Iran: *Ayatollahi* and *Joharchii 13063* (TARI)	LC137042	LC137083	LC137128	LC137182
O. michauxii DC.	Iran: *Assadi 86612* (TARI)	LC137043	LC137084	LC137129	LC137183
O. microptera Baker ex Aitch.	Afghanistan: *Podlech 288872* (MSB)	LC137044	LC137085	LC137130	LC137184
O. nummularia Stocks	Iran: *Mozaffarian 10097* (TARI)	LC137045	LC137086	LC137131	LC137185
O. ptolemaica DC.	Iran: *Salehi* and *Zahrabi 395* (HKNRRC)	AB854507[a]	AB854550[a]	LC137132	LC137186
O. pulchella Schrenk	Iran: *Ghahraman* et al. *27318* (TUH)	AB854508[a]	AB558519[a]	LC137133	LC137187
O. radiata (Desf.) M.Bieb.	Azerbaijan: *Kuorkoba 374* (TARI)	LC137046	LC137087	LC137134	LC137188

Table 2 continued

Taxa	Source, voucher	GenBank Accession Number			
		nrDNA ITS	*trn*L-F	*rpl*32-*trn*L_{UAG}	*ndh*F-*rpl*32
O. rechingerorum Wendelbo	Iran: *Mozaffarian* et al. *39396* (TARI)	LC137047	LC137088	LC137135	LC137189
O. schugnanica B.Fedtsch.	Tajikistan: *Nepli 859* (TARI)	LC137048	LC137089	LC137136	LC137190
O. shahpurensis Rech.f.	Iran: *Rechinger* and *Renz 49668* (TARI)	LC137049	LC137090	LC137137	LC137191
O. sintenisii Bornm.	Iran: *Joharchii* and *Mohabbat 33132* (FUMH)	LC137050	LC137091	LC137138	LC137192
O. sosnowskyi Grossh.	Iran: *Mozaffarian 93762* (TARI)	LC137051	LC137092	LC137139	LC137193
O. splendida Rech.f. & Podlech	Afghanistan: *Podlech 21892* (MSB)	LC137052	LC137093	LC137140	LC137194
O. stewartii Baker	Pakistan: *Rechinger 30066* (MSB)	AB854509[a]	AB854551[a]	LC137141	LC137195
O. susiana Nábělek	Iran: *Jamzad* and *Morid 79206* (TARI)	LC137053	LC137094	LC137142	LC137196
O. szovitsii Boiss.	Iran: *Kazempou Osaloo* et al. *2012-5* (TMUH)	LC137054	LC137095	LC137143	LC137197
O. supina (Vill.) DC.	France: *Podlech 57824* (MSB)	LC137055	LC137096	LC137144	LC137198
O. tanaitica Spreng.	China: *Xu* et al. *87212*(MSB)	LC137056	LC137097	LC137145	LC137199
O. talagonica Rech.f.	Iran: *Charkhchian 1708* (HQNRRC)	LC137057	LC137098	LC137146	LC137200
O.tavernieraefolia Stocks ex Boiss.	Afghanistan: *Podlech 97568* (MSB)	AB854510[a]	AB854552[a]	LC137147	LC137201
O.teheranica Bornm.	Iran: *Ahangarian* and *Kazempour Osaloo 2005-1* (TMUH)	AB329698[a]	AB854553[a]	LC137148	LC137202
O. tournefortii (Willd.) Desv.	Turkey: *Celik 33263* (TARI)	LC137058	LC137099	LC137149	LC137203
O. verae Širj.	Iran: *Kazempour Osaloo 2011-1* (TMUH)	AB854511[a]	AB854554[a]	LC137151	LC137205
O. viciifolia Scop.	Spain: *Podlech 6892* (MSB)	AB854512[a]	AB854555[a]	LC137152	LC137206

FUMH, Ferdowsi University of Mashhad Herbarium, Mashhad, Iran; MSB, Herbarium of Ludwig-Maximilians-Universität, München, Germany; TARI, Herbarium of the Research Institute of Forests and Rangelands, Tehran, Iran; TMUH, Tarbiat Modares University Herbarium, Tehran, Iran; TUH, Tehran University Herbarium, Tehran, Iran; HKNRRC, Herbarium of Khozestan Natural Resources Research Center Herbarium; HQNRRC, Herbarium of Qazvin Natural Resources Research Center

[a] nrDNA ITS and *trn*L-F sequences for those taxa were retrieved from GenBank. – sequences of those regions were missing

generations, using the Markov chain Monte Carlo (MCMC) search. MrBayes performed two simultaneous analyses starting from different random trees (Nruns = 2) each with four Markov chains and trees sampled at every 100 generations. The first 25 % trees were discarded as the burn-in. The remaining trees were then used to build a 50 % majority rule consensus tree accompanied with posterior probability (PP) values. Tree visualization was carried out using TreeView version 1.6.6 (Page 2001). Also ML analyses were performed for the datasets in the program GARLI (Zwickl 2006) and raxmlGUI (Silvestro and Michalak 2012). The model of evolution employed for each dataset is the same as that of Bayesian analyses. Parametric bootstrap values for ML were calculated in GARLI and raxmlGUI based on 1000 replicates with one search replicate per bootstrap replicate.

Overall mean *p*-distance for each dataset was computed using MEGA6 (Tamura et al. 2013).

ILD test

Combinability of these datasets was assessed by use of the partition homogeneity test [the incongruence length difference test (ILD) of Farris et al. (1995)] as implemented in PAUP* (Swofford 2002). The test was conducted with the exclusion of invariant characters (Cunningham 1997) using the heuristic search option involving simple addition sequence and TBR branch swapping with 1000 homogeneity replicates.

Results

Detailed information about alignment characteristics and statistics from the analyses is presented in Table 3. The Parsimony, Likelihood and Bayesian analyses of nrDNA ITS and plastid datasets, *trn*L–F, *rpl*32/*rpl*32-*trn*$L_{(UAG)}$ and *ndh*F-*rpl*32 (as Online resources 1, 2, 3, 4) as well as the combined nuclear-plastid dataset (Fig. 1), produced congruent trees without any major difference. The ILD test did not reveal significant incongruence ($p < 0.07$) between the individual datasets. Therefore, only the results of the Bayesian inference of the combined dataset are discussed below (Fig. 1). The monophyly of *Onobrychis* was well supported (PP = 1.0, ML/BS = 100/96), and two main lineages could be identified within the genus, clades "A"

Table 3 Alignment characteristics and statistics of maximum parsimony analysis for ITS, *trn*L-F, *rpl*32-*trn*L, *ndh*F-*rpl*32 and combined dataset

	NrDNA		CpDNA		Nr + cp
	ITS	*trn*L-F	*rpl*32-*trn*L^{UAG}	*ndh*F-*rpl*32	Combined
Sequences (n)	55	55	52	55	55
Nucleotide sites	675	772	944	731	3122
Informative characters	176	114	109	64	463
Uninformative characters	499	658	835	667	2659
CI of MPTs	0.612	0.822	0.735	0.737	0.658
RI of MPTs	0.872	0.957	0.937	0.948	0.905
RI—CI MPTs	0.260	0.135	0.202	0.211	0.247
Number of MPTs	348	2	10,000	10,000	108
Length of MPTs	423	169	185	99	925
Mean p-distance	0.062	0.022	0.040	0.031	0.045
Evolutionary model selected (under AIC)	GTR + G	GTR + G	GTR + G	GTR + G	GTR + I+G

and "B" (Fig. 1). The clade "A" is composed of members of sects. *Onobrychis*, *Lophobrychis* and some members of sect. *Dendrobrychis*; the clade "B" comprised members of sects. *Anthyllium*, *Insignes*, *Laxiflorae*, *Afghanicae*, *Heliobrychis*, *Hymenobrychis* and some members of sect. *Dendrobrychis* plus *O. splendida*. Sections *Dendrobrychis*, *Lophobrychis*, *Onobrychis*, *Anthyllium*, *Laxiflorae* and *Hymenobrychis* in all analyses are non-monophyletic. Conversely, sections *Afghanicae*, *Insignes* (except in nrDNA ITS) and *Heliobrychis* (except in *rpl*32/*rpl*32-*trn*L$_{(UAG)}$, *ndh*F-*rpl*32) are monophyletic. The aligned data matrix used in this study is presented as Online Resource 5.

Discussion

Phylogenetic status and composition of *Onobrychis*

Recent phylogenetic studies (Amirahmadi et al. 2014a; Safaei Chaei Kar et al. 2012, 2014; Duan et al. 2015; Zarrabian and Majidi 2015) retrieved all *Onobrychis* species in a well-supported monophyletic group with *Eversmannia subspinosa* and allied genera (*Corethrodendron*, *Greuteria*; Amirahmadi et al. 2014a) showing close relationship. Nonetheless, based on only nrDNA ITS data, few studies (Hayot Carbonero et al. 2012; Lewke Bandara et al. 2013) suggested that *Onobrychis*, due to the inclusion of *Eversmannia* within it, is not monophyletic. The present results confirm, however, the monophyly of *Onobrychis* as a well-supported clade (PP = 1.00, ML/BS = 100/96). Our analyses are in agreement with recent findings (Carbonero et al. 2012; Lewke Bandara et al. 2013; Amirahmadi et al. 2014a; Safaei Chaei Kar et al. 2014; Duan et al. 2015) which reveal that *Onobrychis* is divided into two strongly supported clades. As shown in Fig. 1, the clade "A" includes the great majority of subgen. *Onobrychis* sensu Rechinger (1984), comprising sects. *Lophobrychis* and *Onobrychis* as well as some members of sect. *Dendrobrychis* (*O. cornuta* (L.) Desv. and *O. elymaitica* Boiss. & Hausskn. ex Boiss.). The clade "B" includes the remaining members of subgen. *Onobrychis* comprising other members of sect. *Dendrobrychis* (*O. arnacantha* Bunge ex Boiss., *O. afghanica* Širj. & Rech f. and *O. echidna* Lipsky) and sect. *Laxiflorae* along with all members of subgen. *Sisyrosema* sensu Rechinger (1984) comprising sects. *Anthyllium*, *Afghanicae*, *Heliobrychis*, *Hymenobrychis* and *Insignes* as well as *O. splendida*.

New subgeneric concept in *Onobrychis*

As noted above, our tree topologies (Fig. 1, Online Resources 1, 2, 3, 4) indicate that some members of subgen. *Onobrychis* are nested within subgen. *Sisyrosema,* and thus they are not monophyletic at the current status. This conclusion was already reached by several studies (Yildiz et al. 1999; Hayot Carbonero et al. 2012; Amirahmadi et al. 2014a; Safaei Chaei Kar et al. 2014; Duan et al. 2015; Zarrabian and Majidi 2015). Molecular results presented by us and recent authors (Hayot Carbonero et al. 2012; Lewke Bandara et al. 2013; Amirahmadi et al. 2014a; Safaei Chaei Kar et al. 2014) revealed inconsistencies with the two traditionally recognized subgenera (Širjaev 1925, 1926; Rechinger 1984) based on morphological characters. For example, sect. *Laxiflorae* with a semi-curved suture pod was treated by Širjaev (1926) as subgen. *Sisyrosema* sect. *Hymenobrychis* subsect. *Laxiflorae,* while Rechinger (1984) transferred this subsection to subgen. *Onobrychis* and treated it as sect. *Laxiflorae* without any clear explanation. According to this study, the clade "A" includes all members of subgen. *Onobrychis* (sensu Rechinger 1984) with the exception of members of sect. *Laxiflorae* and some members of sect. *Dendrobrychis* (*O. arnacantha*, *O.*

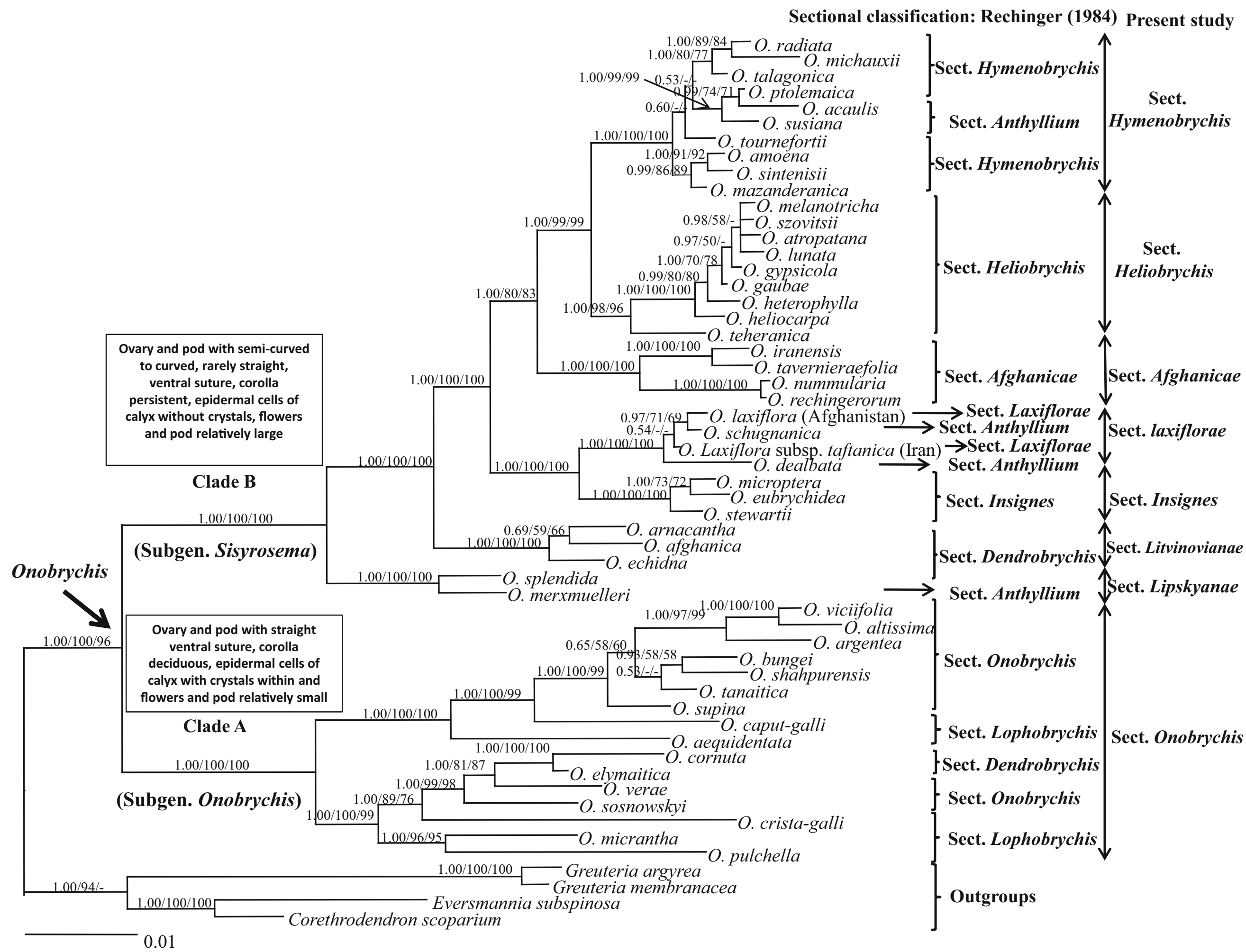

Fig. 1 Fifty percent majority rule consensus tree resulting from Bayesian inference of the combined nrDNA ITS-plastid (*trn*L-F, *rpl*32/*rpl*32-*trn*L$_{UAG}$ and *ndh*F-*rpl*32) dataset. *Numbers* above branches are posterior probability and likelihood as well as parsimony bootstrap values, respectively. Values <50 % were not shown. Subgen. *Onobrychis* and subgen. *Sisyrosema,* corresponding to the clades "*A*" and "*B*", respectively, were redefined at the present study. Characters distinguishing the clades "*A*" and "*B*" were mapped as *boxed* above them. The sectional classification for the genus *Onobrychis* based on Rechinger (1984) and the present study was given at the *right side* of the tree

afghanica and *O. echidna*). We concluded that the clade "A" based on our phylogenetic hypothesis is presented as subgen. *Onobrychis* in emend us and recognized by the pod with straight ventral suture, the deciduous corolla, the presence of crystal in the calyx epidermal cells and relatively small flowers and pods. The clade "B" includes all members of subgen. *Sisyrosema* (sensu Rechinger 1984) along with sect. *Laxiflorae*, other members of sect. *Dendrobrychis* (*O. arnacantha*, *O.afghanica* and *O.echidna*) and *O. splendida*. The synapomorphies for members of the clade "B" are the pod with semi-curved to curved, rarely straight ventral suture, the persistent corolla, the absence of crystal in the calyx epidermal and relatively large flowers and pods. The clade "B" is presented here as subgen. *Sisyrosema* in emend us (Table 1 and "Taxonomic treatment").

Monophyly and relationships of sections

Members of sects. *Onobrychis*, *Lophobrychis* and some members of sect. *Dendrobrychis* (*O. elymaitica* and *O. cornuta*) analyzed herein are intermixed with each other (Fig. 1; PP = 1.0 ML/BS = 100/100). They are gathered in two subclades of the clade "A" (subgen. *Onobrychis*). In the first subclade, members of sect. *Onobrychis* from *O. viciifolia* Scop. (type species of sect. *Onobrychis*) through *O. supine* (Vill.) DC. form a monophyletic group. In addition, *O. aequidentata* d'Urv. and *O.caput-galli* (L.) Lam. (type species of sect. *Lophobrychis*) are successive sisters to this group. In the second subclade, *O. elymaitica* and *O. cornuta* (type species of *Dendrobrychis*) are sister taxa for which *O. verae* Širj. and *O. sosnovskyi* Grossh. from sect. *Onobrychis*, and *O. crista-galli* (L.) Lam. from sect. *Lophobrychis* are successive grades, respectively. Finally, this newly formed group is sister to a branch including *O. micrantha* Schrenk and *O. pulchella* Schrenk from sect. *Lophobrychis*. These findings are congruent with previous studies (Hayot Carbonero et al. 2012; Lewke Bandara et al. 2013; Safaei Chaei Kar et al. 2014) which showed that sects. *Onobrychis, Lophobrychis* and *Dendrobrychis* are closely related, but none are monophyletic. Limited numbers of characters have been used for delimitation of the sections in the literature. Section *Onobrychis* (sensu Širjaev 1925; Rechinger 1984) comprises perennial herbs, rarely woody at the base, with many flowered, long or short wings and 1-seeded pod as opposed to annual herbs, with few flowered, long wings and 1 or 2-seeded pods. However, there are some morphological similarities between sects. *Onobrychis* and *Lophobrychis,* particularly in shape of pods and the teeth of the crest and having long-wing petals. It seems that annual or perennial habit at least in the studied genus should not be diagnostic features to separate species as two different sections, namely *Lophobrychis* and *Onobrychis*. Consequently, based on the present molecular study, sect. *Lophobrychis* is considered as a synonym of sect. *Onobrychis* (see "Taxonomic treatment"). Section *Dendrobrychis* only includes the spiny cushion-forming species. Širjaev (1925) subdivided the section into the two series, *Dielsianae* Širj. and *Litvinovianae* Širj., and distinguished them with the spiny peduncle and petiole, respectively. Phylogenetic analysis showed that *O. cornuta* and *O. elymaitica* from series *Dielsianae* (with the spiny peduncle, semi-orbicular legume, and crystal in the calyx epidermal cells) are nested within the clade "A". *Onobrychis afghanica*, *O. arnacantha* and *O. echidna* from series *Litvinovianae* are nested within the clade "B" and comprise a distinct lineage in *Onobrychis*. On the basis of our phylogenetic results and morphological similarities (e.g., having long-wing petals and pod shape), sect. *Dendrobrychis* is also treated as a synonym of sect. *Onobrychis* (see "Taxonomic treatment").

It should be noted that although *O. hemicycla* C.I. Blanche ex Boiss., a sole member of sect. *Hemicyclobrychis,* restricted to Syria, was not analyzed here, we followed Širjaev's treatment (1925) to retain the species in its own section as a member of subgen. *Onobrychis* (see Table 1 and "Taxonomic treatment").

Sections *Anthyllium*, *Insignes*, *Laxiflorae*, *Afghanicae*, *Heliobrychis* and *Hymenobrychis* along with the rest of sect. *Dendrobrychis* and *O. splendid* are members of the clade "B" (subgen. *Sisyrosema*). The present phylogenetic study revealed that sect. *Anthyllium* with 8 species, represented herein by 5 species, is not monophyletic, indicating that the delimitation of *Anthyllium* on the basis of morphological features is artificial and the diagnostic features of it overlap with other sections/species. Its representatives were placed in three distinct subclades. One of which, *O. merxmuelleri* Podlech was well united with *O. splendida* (a species of unassigned section) and, in turn, both formed the basal lineage in the clade "B". This relationship has never been mentioned in earlier studies (e.g., Rechinger 1984). Podlech (1967) established *O. merxmuelleri* as a new species and designated it as the closest relative of *O. grandis* Lipsky of *Anthyllium* subsect. *Lipskyanae* and placed it in this subsection. This relationship was also retrieved in cpDNA tree of Duan et al. (2015). These two species and *O. splendida* do share several morphological characteristics such as taller perennial habit, leaves with remote leaflets, lax inflorescence, legume with straight ventral suture and 1-seeded (Rechinger 1984). Hence, based on the molecular data, we excluded the two species from sect. *Anthyllium* and along with *O. splendida* placed them in the newly erected section *Lipskyanae* (see "Taxonomic treatment"). Two other species of sect. *Anthyllium*, *O. dealbata* Stocks and *O. schugnanica* B.Fedtsch. with the inclusion of two taxa of *O. laxiflora* Baker of sect. *Laxiflorae* (typified by *O. laxiflora*) formed a monophyletic group. The placement of *O. dealbata* with *O. laxiflora* corroborates Lewke Bandara et al. (2013), although they analyzed *O. dasycephala* Baker (a synonym of *O. dealbata*) and did not mention such close species relationship. Hence, these two species are considered to be members of sect. *Laxiflorae.* Two another species of sect. *Anthyllium* (*O. acaulis* Bornm. and *O. Susiana* Nábělek) were nested within sect. *Hymenobrychis* and united with *O. ptolemaica* (Del.) DC. This is consistent with findings of previous studies (Ahangarian et al. 2007; Hayot Carbonero et al. 2012; Lewke Bandara et al. 2013; Safaei Chaei Kar et al. 2014), which found *O. acualis* within sect. *Hymenobrychis*. The characteristics of the two species are well suited with that of members (in particular *O. ptolemaica*) of sect. *Hymenobrychis*. Due to nomenclatural priority, we merged sect. *Anthyllium,* typified here by *O. susiana*, in the sect. *Hymenobrychis,* (see "Taxonomic treatment"). *Onobrychis arnacantha*, *O. afghanica* and *O. echidna*, which are here recognized as members of new section *Litvinovianae* (type species: *O. arnacantha*), form the second lineage within Clade "B". They are distinct in having cushion-forming habit, spiny petiole, semi-lunar pod and without crystal in the calyx epidermal cells. The placement of *O. arnacantha* in subgen. *Sisyrosema* (Clade "B") is congruent with the analyses of Hayot Carbonero et al. (2012), Lewke Bandara et al. (2013) and Safaei Chaei Kar et al. (2014). Section *Insignes,* represented with three species, is monophyletic and along with *Anthyllium* + *Laxiflorae* formed the third diverging subclade. Both sections *Afghanicae* and *Heliobrychis* comprise independent lineages and are monophyletic. The monophyly of sect. *Heliobrychis* was supported by Ahangarian et al. (2007), Hayot Carbonero et al. (2012) and Lewke Bandara et al. (2013), whereas the monophyly of both sections was questioned by nrDNA ITS data of Safaei Chaei Kar et al. (2014). This discrepancy might be because of PCR contamination of *O. iranenesis* Amirab. & Ghanavati with *O. aucheri* Boiss. in the study of Safaei Chaei Kar et al. It merits to note that in this and previous works, *O. teheranica* Bornm. (= *O. aucheri* ssp. *teheranica* (Bornm.) Rech. f.), an annual species, is sister to the remaining of *Heliobrychis*. This indicates that annual habit in the section might be an ancestral state. Analyses of molecular data showed that sect. *Hymenobrychis* with the inclusion of two members of *Anthyllium* (*O. acualis* and *O. susiana*) formed a distinct lineage and, in turn, well united with sect. *Heliobrychis*.

Conclusions

The results of the present analysis based on multiple DNA regions provide more convincing evidence as to the phylogenetic relationships among *Onobrychis* taxa examined. The present molecular study illustrated that the current infrageneric classification of the genus is no longer tenable. A new taxonomic classification of the genus at both subgeneric and sectional levels along with the typification is hereby presented. An inclusive phylogenetic study dealing with species rich sections of *Onobrychis* such as *Onobrychis* (sensu us), *Heliobrychis* and *Hymenobrychis* using several DNA markers and comprehensive taxon sampling is especially needed to clarify their evolutionary history as we have already progressed in this regard.

Taxonomic treatment

Onobrychis Mill., Gard. Dict. Abr. Ed. 4. 1754

= *Onobruchus* Medik., Vorles. Churpfälz. Phys.-Öcon. Ges. 2:372. 1787

= *Eriocarpaea* Bertol., Nov. Comm. Acad. Bonon. 6:234. 1843.—TYPE (designated by Hanelt 2001): *Onobrychis viciifolia* Scop.

Subg. *Onobrychis* emend Amirahm. & Kaz. Osaloo ≡ Sect. *Euonobrychis* Bunge ex Boiss., Fl. Or. 2:526. 1872 ≡ Subgen. *Euonobrychis* (Bunge ex Boiss.) Širj., Spisy Přír. Fak. Masarykovy Univ. 56:18. 1925.—TYPE: *Onobrychis viciifolia* Scop.

Sect. *Onobrychis* emend Amirahm. & Kaz. Osaloo ≡ Sect. *Eubrychis* DC. Prodr. 2: 344. 1825.—TYPE: *Onobrychis viciifolia* Scop.

= Sect. *Dendrobrychis* DC., Prodr. 2:347. 1825. **syn. nov.**—TYPE (**designated here**): *O. cornuta* (L.) Desv.

= Sect. *Dendrobrychis* DC. ser. *Dielsianae* Širj., Spisy Přír. Fak. Masarykovy Univ. 56:22. 1925.—TYPE (**designated here**): *O. cornuta* (L.) Desv.

= Sect. *Lophobrychis* Hand.-Mazz., Osterr. Bot. Zeitschr. 59:373. 1909. **syn. nov.**—TYPE (**designated here**): *O. caput-galli* (L.) Lam.

= Sect. *Lophobrychis* Hand.-Mazz. subsect. *Occidentales* Širj., Spisy Přír. Fak. Masarykovy Univ. 56:34. 1925. **syn. nov.**—TYPE (**designated here**): *O. caput-galli* (L.) Lam.

= Sect. *Lophobrychis* Hand.-Mazz. subsect. *Orientales* Širj., Spisy Přír. Fak. Masarykovy Univ. 56:51. 1925. **syn. nov.**—TYPE (**designated here**): *O. micrantha* Schrenk.

Note The original publication is sometimes cited as Flore Française 4: 511. 1805, but this page deals with *Ononis* L. *Onobrychis* can be found on page 611, but *Dendrobrychis* is not mentioned here (Lamarck and De Candolle 1805).

Description: Annual or perennial herbs, sometimes cushion-forming shrublets. Peduncle rarely spiny. Flowers relatively small. Epidermal cells of calyx with crystal. Corolla deciduous, glabrous, wings longer or shorter than keel. Legume relatively small, semi-orbicular, with 1, 2 seeds, sessile or rarely stipitate, with straight ventral (seminiferous) suture, with or without crest, disk and crest smooth, spiny or dentate.

Sect. *Hemicyclobrychis* Širj., Spisy Přír. Fak. Masarykovy Univ. 56:56. 1925.—TYPE (**designated here**): *O. hemicycla* C.I.Blanche ex Boiss.

Description: Perennial herbs. Wings shorter than keel. Legume semi-orbicular, 1-seeded, sessile, with straight ventral suture, crest broad, dentate.

Subg. *Sisyrosema* (Bunge ex Boiss.) Širj., Spisy Přír. Fak. Masarykovy Univ. 76:5. 1926. emend. Amirahm. & Kaz. Osaloo ≡ Sect. *Sisyrosema* Bunge ex Boiss., F1. Orient. 2:526. 1872.—TYPE (**designated here**): *O. radiata* (Desf.) M.Bieb.

Description: Perennial, rarely annual herbs, sometimes cushion-forming shrublets. Flowers relatively large. Epidermal cells of calyx without crystal. Corolla persistent, more or less pubescent. Legume relatively large, with semi-curved to fully curved, rarely straight ventral (seminiferous) suture, with or without crest, disk and crest spineless or spiny or dentate.

Sect. *Afghanicae* Širj., Spisy Přír. Fak. Masarykovy Univ. 76:18. 1926.—TYPE (**designated here**): *O. tavernieraefolia* Stocks ex Boiss.

Description: Annual herbs. Leaves with 1-2 pairs of leaflets. Legume 2 (-1)-locular, 2(-1)-seeded, coiled inwards from the tip, flattened out, bristled or sometimes cottony-woven together at margins; disk with distinct pits, rarely spiny.

Sect. *Laxiflorae* (Širj.) Rech.f., Fl. Iranica 157:415. 1984. emend. Amirahm. & Kaz. Osaloo ≡ Sect. *Hymenobrychis* DC. subsect. *Laxiflorae* Širj., Spisy Přír. Fak. Masarykovy Univ. 76:106. 1926.—TYPE (designated by Rechinger 1984): *O. laxiflora* Baker.

= Sect. *Anthyllium* Nábělek subsect. *Fedcenkoanae* Širj., Spisy Přír. Fak. Masarykovy Univ. 76:8. 1926. **syn. nov.**—TYPE **(designated here)**: *O. schugnanica* B.Fedtsch.

= Sect. *Anthyllium* Nábělek subsect. *Mirae* Širj., Spisy Přír. Fak. Masarykovy Univ. 76:13. 1926. **syn. nov.**—TYPE (**designated here**): *O. dealbata* Stocks.

Description: Perennial herbs, caulescent or acaulescent. Stipules mostly free rarely connate. Wings shorter, sometimes longer than keel. Legume semiglobular, straight or slightly curved at ventral suture, with narrow to rather broad crest and dentate.

Sect. *Lipskyanae* (Širj.) Amirah. & Kaz. Osaloo, **stat. nov.** ≡ Sect. *Anthyllium* Nábělek subsect. *Lipskyanae* Širj., Spisy Přír. Fak. Masarykovy Univ. 76:11. 1926.—TYPE (**designated here**): *O. grandis* Lipsky.

Description: Perennial herb, caulescent. Leaves with remote leaflets. Inflorescence lax, peduncle curved after flowering; Legume semiglobular, 1-seeded, with straight ventral suture and dentate crest.

Sect. *Heliobrychis* (Bunge ex Boiss.) Sirj., Spisy Přír. Fak. Masarykovy Univ. 76:19. 1926 ≡ Sect. *Sisyrosema* Bunge ex Boiss. subsect. *Heliobrychideae* Bunge ex Boiss., Fl. Orient. 2:527. 1872.—TYPE (**designated here**): *O. heterophylla* C.A.Mey.

Description: Perennial rarely annual herbs, caulescent or almost acaulescent. Wings shorter than keel. Legume suborbicular, covered with pinnate bristles, stipitate, with curved ventral suture and without crest.

Sect. *Hymenobrychis* DC., Prodr. 2:346. 1825. emend. Amirahm. & Kaz. Osaloo.—TYPE (**designated here**): *O. radiata* (Desf.) M. Bieb.

= Sect. *Anthyllium* Nábělek, Spisy Přír. Fak. Masarykovy Univ. 35:96. 1923. **syn. nov**.—TYPE (**designated here**): *O. susiana* Nábělek

= Sect. *Anthyllium* Nábělek subsect. *Nabélekianae* Širj., Spisy Přír. Fak. Masarykovy Univ. 76:14. 1926. **syn. nov.**—TYPE **(designated here)**: *O. susiana* Nábělek.

Description: Perennial rarely biennial herbs, caulescent rarely acaulescent. Wings short. Legume stipitate, with semi-curved to curved ventral suture; crest well-developed, more or less dentate, disk hairy, bristly or rarely glabrous.

Sect. *Insignes* (Širj.) Rech.f., Fl. Iranica 157: 460. 1984 ≡ Sect. *Hymenobrychis* DC. subsect. *Insignes* Širj., Spisy Přír. Fak. Masarykovy Univ. 76:58. 1926.—TYPE (designated by Rechinger 1984): *O. eubrychidea* Boiss.

Description: Perennial or annual herb, more or less caulescent, Wings shorter than keel. Legume orbicular-reniform with curved ventral suture; crest membranous, irregularly dentate.

Sect. *Litvinovianae* (Širj.) Amirahm. & Kaz. Osaloo, **stat. nov.** ≡ Sect. *Dendrobrychis* DC. ser. *Litvinovianae* Širj., Spisy Přír. Fak. Masarykovy Univ. 56: 29. 1925.—TYPE (**designated here**): *O. arnacantha* Bunge ex Boiss.

Description: Cushion-forming, strongly branching shrublets. Petioles spinescent. Legume semi-lunar.

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Information on Electronic Supplementary Material

Online Resource 1. Fifty percent majority rule consensus tree resulting from Bayesian inference of the nrDNA ITS dataset.
Online Resource 2. Fifty percent majority rule consensus tree resulting from Bayesian inference of the plastid *trn*L-F dataset.
Online Resource 3. Fifty percent majority rule consensus tree resulting from Bayesian inference of the plastid *rpl*32/*rpl*32- *trn*L_{UAG} dataset.
Online Resource 4. Fifty percent majority rule consensus tree resulting from Bayesian inference of the plastid *ndh*F-*rpl*32 dataset.
Online Resource 5. The aligned data matrix used in this study.

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