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Re-assessment of subspecific taxa in *Astragalus* section *Anthylloidei* (Fabaceae) based on molecular evidence

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ABSTRACT

The taxonomic and phylogenetic status of several taxa previously recognized as subspecies in Astragalus sect. Anthylloidei is re-assessed based on DNA sequences and morphological features. We focused on Astragalus ebenoides (subsp. ebenoides and subsp. naghadehensis), Astragalus murinus (subsp. murins and subsp. bornmuelleri), Astragalus remotiflorus (subsp. remotiflorus and subsp. melanogramma), Astragalus nigrohirsutus (=Astragalus remotiflorus subsp. nigrohirsutus), Astragalus submitis (=Astragalus submitis subsp. submitis) and Astragalus yushensis (=Astragalus submitis subsp. maassoumii). A total of 15 accessions representing 14 ingroups and one outgroup were analysed for nrDNA ITS and plastid DNA, rpl32 gene and rpl32-trnL$_{(UAG)}$ intergenic spacer. Phylogenetic trees were constructed using neighbour joining, Bayesian and maximum parsimony methods. The phylogenetic analyses of both datasets revealed that the subspecies described formerly under each of the studied species are distinct and should be elevated to specific rank. The nucleotide sequence variations observed among different subspecies, along with morphological characters, provided appropriate criteria in setting the species boundaries. The new combinations and a diagnostic key to the studied species are provided.

Keywords: *Astragalus*, DNA barcoding, nrDNA ITS, plastid *rpl*32-*trn*L$_{(UAG)}$, section *Anthylloide*.

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Introduction

Astragalus L. (Fabaceae), as the largest genus of vascular plants, contains an estimated number of 3000 annual and perennial species (1, 2). *Astragalus* sect. *Anthylloidei* DC. includes 37 species (2) and is distributed in southwest Asia, and Iran with 28 species is the centre of diversity of this section. Some species are also found in Turkey (five) and Afghanistan (four). The members of this section, as one of the most heterogeneous groups of spiny *Astragalus,* are characterized by cushion forming habit, imparipinnate or paripinnate leaves, spiny rachises and inflated large fruiting calyces. The section has been revised taxonomically several times as a whole (1-7) and for the area of Flora Iranica (8-10). Tietz and Zarre (6) considered nine subspecies under *Astragalus ebenoides* Boiss., *Astragalus murinus* Boiss., *Astragalus remotiflorus* Boiss. and *Astragalus submitis* Boiss. & Hohen. in this section. Recently, Podlech (11) elevated *Astragalus submitis* subsp. *maassoumii* Tietz & Zarre to species level applying the name *Astragalus yushensis* T. Sabaii, Zarre & Podlech. Borjian *et al.* (12) promoted *Astragalus remotiflorus* subsp. *nigrohirsutus* Tietz & Zarre to species rank, because of the ploidy level of 2n= 6x= 48 in contrast to 2n= 2x= 16 reported for *Astragalus remotiflorus*. Sanderson and Wojchiekhowski (13) noted that "*Astragalus* possesses several features that have been postulated to promote diversification rates in angiosperms, including geographic population structures consisting of local isolates with restricted gene flow, the herbaceous habit (with associated reduced generation time; 14) exceptional chromosomal variability (15) and tendency toward parallelism and reversal associated with recurring ecological specializations (16, 17).

In this study, the nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) and chloroplast *rpl*32 gene plus *rpl*32-*trn*$L_{(UAG)}$ intergenic spacer (hereafter *rpl*32-*trn*$L_{(UAG)}$ region) were sequenced for phylogenetic reconstructions. The internal transcribed spacer (ITS) contain the signals needed to process the rRNA transcript (18) and have often been used for inferring phylogeny at inter- and intra-sectional levels in *Astragalus* (e.g., 19-26). The *rpl*32 gene and *rpl*32-*trn*$L_{(UAG)}$ spacer are located in the SSC region of the chloroplast genome. The average length of *rpl*32-*trn*$L_{(UAG)}$ spacer is 1018 bp., and it ranges from 543--1417 bp. This is one of the best non-coding regions for low-level molecular studies and barcoding (27, 28). *rpl*32-*trn*$L_{(UAG)}$ region has been rarely used in molecular phylogenetic investigations on *Astragalus* (24, 26).

The main goal of this paper is to evaluate the taxonomic and phylogenetic status of the above-mentioned subspecies and to provide a new taxonomic treatment for them in light of molecular phylogenetic findings.

Materials and Methods

Taxon sampling

Specimens deposited in the herbarium of TARI were used for DNA extraction. A total of 15 accessions representing 14 ingroups and one outgroup were analysed for nrDNA ITS, cpDNA *rpl*32 gene plus *rpl*32-*trn*$L_{(UAG)}$ and the combined sequences. Beside all subspecies to be re-assessed here (seven taxa), the known relative species (seven taxa) to each group have also been included in the analysis. A list of all taxa used in this study and the sources, as well as voucher specimen data, are given in Table 1.

Table 1. Taxa included in the nrDNA ITS, *rpl32-trn*L_{UAG} analyses

Species	Section	DNA source (location, voucher)	GenBank accession No. ITS/ rpl32-*trn*LUAG
Astragalus horridus Boiss.	*Acanthophace*	Iran: Mozaffarian 54874 (TARI)	AB052002*/AB908523
Astragalus bodeanus Fisch.	*Anthylloidei*	Iran: Mozaffarian 83758 (TARI)	AB908460/AB908501
Astragalus chardinii Boiss.	*Anthylloidei*	Iran: Sabeti 16064 (TARI)	AB908443/AB908482
Astragalus distans Fisch	*Anthylloidei*	Iran: Zarre 33641 (TUH)	AB908462/AB908504
Astragalus ebenoides Boiss.=*Astragalus ebenoides* subsp. *ebenoides*	*Anthylloidei*	Iran: Maassoumi & Mirhosseini 59421(TARI)	AB908445/AB908484
Astragalus naghadehensis (Tietz & Zarre) Naderi Safar & Maassoumi =*Astragalus ebenoides* ssp *naghadehensis* Tietz & Zarre	*Anthylloidei*	Iran: Siami 5413 (TARI)	AB999647/AB999652*
Astragalus murinus Boiss.=*Astragalus murinus* ssp. *murins*	*Anthylloidei*	Iran: Assadi & Abouhamzeh 46094 (TARI)	AB052008*/AB908487
Astragalus pseudomurinus Naderi Safar & Maassoumi =*Astragalus murinus* ssp. *bornmuelleri* Tietz & Zarre	*Anthylloidei*	Iran: Mozaffarian 63931 (TARI)	AB999648/AB999653*
Astragalus raswendicus Hausskn. & Bornm.	*Anthylloidei*	Iran: Babakhanloo & Amin 15647 (TARI)	AB908459/AB908500
Astragalus remotiflorus Boiss.=*Astragalus remotiflorus* subsp. *remotiflorus*	*Anthylloidei*	Iran: Assadi & Miller 25162 (TARI)	AB908446/AB908485
Astragalus melanogramma Boiss.=*Astragalus remotiflorus* ssp. *melanogramma* (Boiss.) Tietz & Zarre	*Anthylloidei*	Iran: Wendelbo & Assadi 11451 (TARI)	AB999649/AB999654*
Astragalus nigrohirsutus (Tietz & Zarre) Borjian, Maassoumi& Assadi = *Astragalus remotiflorus* ssp. *nigrohirsutus* Tietz & Zarre	*Anthylloidei*	Iran: Mozaffarian s.n. (TARI)	AB999650/AB999655*
Astragalus submitis Boiss. & Hohen.=*Astragalus submitis* ssp. *submitis*	*Anthylloidei*	Iran: Maassoumi & Shahsavari 80739 (TARI)	AB052009*/AB908486
Astragalus yushensis T. Sabaii, Zarre & Podlech.=*Astragalus submitis* ssp. *maassoumii* Tietz & Zarre	*Anthylloidei*	Iran: Maassoumi 55131 (TARI)	AB999651/AB999656*

Abbreviations for Herbaria: TARI, Herbarium of the Research Institute of Forests and Rangelands, Tehran, Iran; TUH, Tehran University Herbarium, Tehran, Iran. (*)These sequences were newly determined in this study and remaining ones were previously determined by us and obtained from GenBank.

DNA isolation, PCR and sequencing

Total genomic DNA was isolated from fresh or dried materials using modified CTAB method of Doyle and Doyle (29). The nrDNA ITS region was amplified using the primers ITS5m (30) and ITS4 (31) or AB101F and AB102R (32). The *rpl*32-*trn*$L_{(UAG)}$ region was amplified using the rpl32-F and trn$L_{(UAG)}$ primers (27). The PCR amplification was carried out in the volume of 20µL, containing 8µL deionized water, 10µL of the 2xTaq DNA polymerase master mix Red (Amplicon, Cat. No. 180301, 150 µM Tris-HCL pH 8.5, 40 µM $(NH_4)_2SO_4$, 3.0µM $MgCl_2$, 0.4µM dNTPs, 0.05 units μl^{-1}AmpliconTaq DNA polymerase, inert red dye and a stabilizer) 0.5µl of each primer (10 pmol/µl), and 1.0 µl of template DNA (20 ng/µl). PCR was carried out after following protocol: an initial 2.30 min pre-melting at 94ºC and 28 cycles of 50 s at 80ºC for *rpl*32-*trn*$L_{(UAG)}$ and 94ºC for nrDNA ITS for template denaturation, 40 s at 58ºC for primer annealing, and 55 s at 72ºC for primer extension, followed by 7 min at 72ºC for final extension. PCR products were separated by electrophoresis in 1% agarose gel stained with ethidium bromide and were photographed with a UVI 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 3730 xl DNA Analyzer (Applied Biosystems, USA).

Sequence alignment

Sequences for the above-mentioned taxa were edited using BioEdit ver. 7.0.9.0 (33) and aligned using MUSCLE under default parameters (34) followed by manual adjustment. The alignment of the datasets required the introduction of numerous single and multiple-base indels (insertions/deletions).

Phylogenetic analyses

Phylogenetic trees were constructed using three different methods: Neighbour Joining (NJ), Maximum Parsimony (MP) and Bayesian Inference (BI). NJ analyses were performed as performed in the software MEGA5 (35). For the NJ analysis, distance matrices were calculated using Kimura's two-parameter correction (36). Parsimony analyses were conducted using PAUP* version 4.0b10 (37). The heuristic search option was employed for each of the datasets, using tree bisection-reconnection (TBR) branch swapping, with 100 replications of random addition sequence and an automatic increase in the maximum number of trees. Uninformative characters were excluded from the analyses. Branch support values were calculated using a full heuristic search with 1000 bootstrap replicates (38) each with simple addition sequence.

The program MrBayes version 3.1.2 (39) was used for the Bayesian Inference (BI). On the basis of this analysis, datasets were analysed using the SYM+I+G model for nrDNA ITS, GTR+I+G for *rpl*32-*trn*$L_{(UAG)}$ and GTR+I for the combined dataset. Posteriors on the model parameters were estimated from the data, using the default priors. The analysis was carried out with 4 million 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% of 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. The convergence of MCMC chains was visualized with the Tracer program version 1.5 (40). The model of evolution employed for each dataset is the same as that of NJ analyses. Tree visualization was carried out using TreeView version1.6.6 (41).

The congruency of two single datasets (nrDNA ITS and cpDNA *rpl*32-*trn*L$_{(\mathrm{UAG})}$) was assessed using the partition homogeneity test or the incongruence length difference (ILD) test of Farris et al. (42) as implemented in PAUP* (37). The test was conducted with exclusion of invariant characters (43) using the heuristic search option involving 1000 replicates of the random addition sequence and TBR branch swapping with 1000 homogeneity replicates. The maximum number of trees was set to 1000.

Results and Discussion

As the focus of this paper, below we first discuss the phylogenetic status and inter-relationship of the analysed taxa in detail and compare diagnosing morphological characters among them. A brief note on the taxonomic treatment according to the obtained results and a key to the species is also presented at the end.

Phylogenetic analyses

The partition homogeneity test suggested that the nrDNA ITS and *rpl32-trn*L$_{\mathrm{UAG}}$ datasets were congruent ($P>0.05$); thus we combined these two datasets.

NJ analysis of two single and the combined datasets were topologically identical to that of BI and MP analyses. The trees resulting from three methods for the combined dataset were topologically similar to nrDNA ITS and *rpl*32-*trn*L$_{\mathrm{UAG}}$ but with higher supports.

The tree obtained from the NJ analysis of the combined dataset along with bootstrap and posterior probability values are displayed in Figure 1. The phylogram is composed of two main clades ("A" and "B"). Clade "A" comprises six taxa: *A. remotiflorus* subsp. *remotiflorus*, *A. remotiflorus* subsp. *melanogramma* (Boiss.) Tietz & Zarre, *A. nigrohirsutus* (=*Astragalus remotiflorus* subsp. *nigrohirsutus*), *A. murinus* subsp. *murinus*, *A. murinus* subsp. *bornmuelleri* Tietz & Zarre and *A. bodeanus* Fisch. Clade "B" is composed of *A. ebenoides* subsp. *ebenoides*, *A. ebenoides* subsp. *naghadehensis* Tietz & Zarre, *A. submitis*, *A. yushensis* (=*A. submitis* subsp. *maassoumii*), *A. chardinii* Boiss., *A. veiskaramii* Zarre, Podlech & T.Sabaii and *A. raswendicus* Hausskn. & Bornm. Within clade "A", *A. remotiflorus* subsp. *nigrohirsutus*, which recently promoted to species level based on cytological evidence ($2n= 6x = 48, 12$), is not directly allied with two other subspecies of *A. remotiflorus*. Thus, this new combination is confirmed by our analysis. *Astragalus murinus* subsp. *murinus* and *A. murinus* subsp. *bornmuelleri* are allied with a subclade comprising *A. remotiflorus* subsp. *melanogramma*, *A. remotiflorus* subsp. *remotiflorus* and *A. bodeanus*. Different placements of subspecies formerly assigned to a certain species on the obtained trees (Figure 1) may indicate different evolutionary pathways of these subspecies, and could be a signal for elevating them to species level. This pattern is observed in both *A. murinus* and *A. remotiflorus*. *Astragalus murinus* subsp. *murinus* and *A. murinus* subsp. *bornmuelleri* are differentiated by several characters. In the former, rachises are rigid and persistent for a long time; inflorescences are shorter than or at most as long as the leaves, with a peduncle 2–4cm and a loosely flowered raceme. In the latter, the rachises are more or less flexuous and deciduous; inflorescences are as long as or longer than the leaves, with a peduncle 7–16cm and a short raceme with crowded flowers (2, 6-8). Both subspecies are confined to west Iran. Two variable nucleotide sites, along with 16 indels in combined datasets, separate these taxa from a molecular view (Table 2).

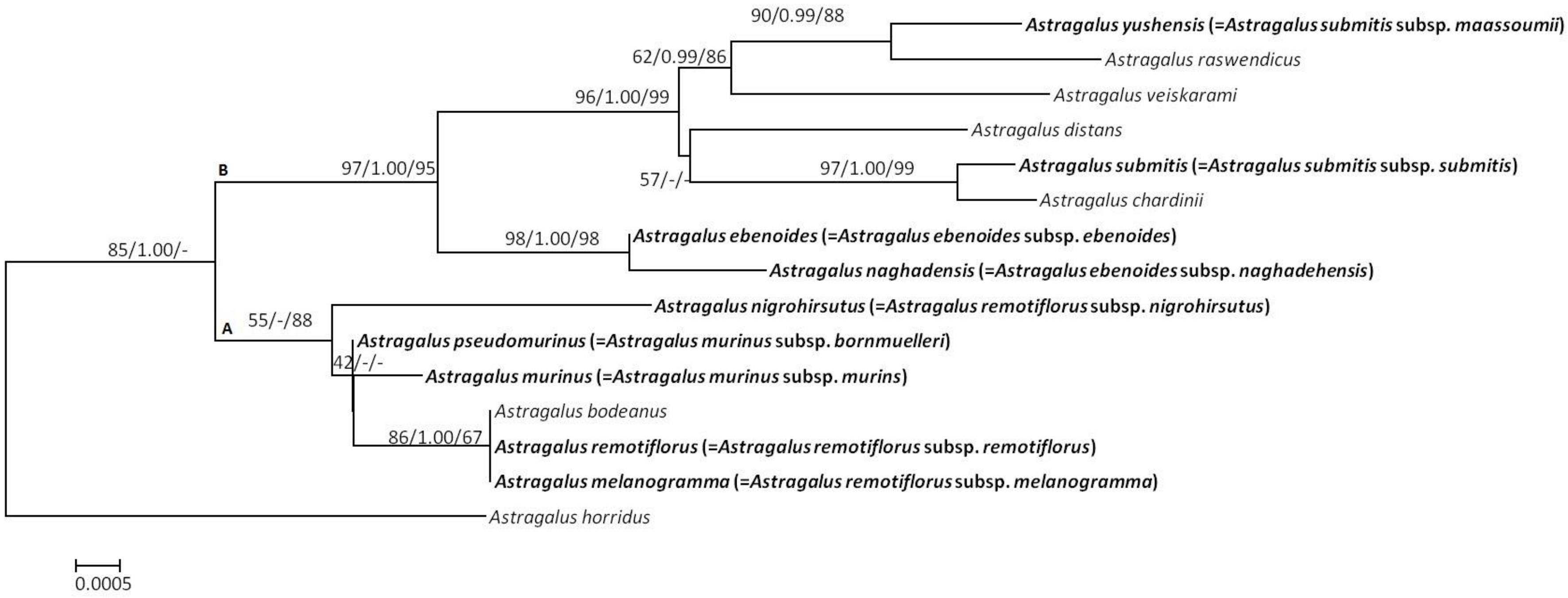

Figure 1. NJ tree derived from analysis of the combined nr DNA ITS and plastid *rpl32-trn*$L_{(UAG)}$ sequences. NJ/BI/MP supports was presented beside the branches

Table 2. Pair wise comparison of indels among analysed taxa

Taxa nodes	No. of indels
Astragalus ebenoides / Astragalus naghadehensis	1
Astragalus murinus / Astragalus pseudomurinus	16
Astragalus remotiflorus / Astragalus melanogramma	97
Astragalus remotiflorus / Astragalus nigrohirsutus	45
Astragalus melanogramma / Astragalus nigrohirsutus	60
Astragalus submitis / Astragalus yushensis	40

Table 3. Pair wise comparison of variable sites among analysed taxa (rDNA ITS/*rpl*32-*trn*L$_{UAG}$)

	1	2	3	4	5	6	7	8	9
1. *Astragalus ebenoides*									
2. *Astragalus naghadehensis*	1/1								
3. *Astragalus murinus*	8/6	9/7							
4. *Astragalus pseudomurinus*	7/4	8/5	0/2						
5. *Astragalus remotiflorus*	11/3	12/4	3/3	2/1					
6. *Astragalus melanogramma*	11/6	12/7	3/3	2/2	0/3				
7. *Astragalus nigrohirsutus*	12/4	13/5	6/2	5/0	9/2	9/2			
8. *Astragalus submitis*	9/2	10/3	14/6	13/4	17/3	17/6	18/4		
9. *Astragalus yushensis*	11/2	12/3	14/7	12/5	16/4	16/7	18/5	8/3	

A. remotiflorus subsp. *remotiflorus* and *A. remotiflorus* subsp. *melanogramma* are characterized by indumentum of white hairs opposed to *A. nigrohirsutus* (the former *A. remotiflorus* subsp. *nigrohirsutus*) with mixed black and white hairs on the inflorescence, calyx and legumes. *Astragalus remotiflorus* subsp. *remotiflorus* differs from *A. remotiflorus* subsp. *melanogramma* in having densely branched stems comprising several short and thick side-branches covered by persistent spines (not loosely branched stems, with slender side-branches and deciduous spines) (2, 6-8). In the combined dataset, these taxa differ by three nucleotide sites and 97 indels. *Astragalus nigrohirsutus* is also characterized by several nucleotide variations and indels compared with both other subspecies (Table 2 and 3). *Astragalus remotiflorus* subsp. *remotiflorus* and *A. remotiflorus* subsp. *melanogramma* are distributed in central and west Iran, respectively and *A. nigrohirsutus* is restricted to southwest Iran.

In the clade "B", *A. ebenoides* subsp. *ebenoides* and *A. ebenoides* subsp. *naghadensis* are united in a subclade. These taxa are known from west and northwest Iran, respectively. The difference in the branch lengths of the phylogram (Figure 1) indicates different evolutionary rates in the DNA sequences. *Astragalus ebenoides* subsp. *ebenoides* is characterized by fruiting calyx brownish yellow in colour and red striped, standard blade nearly quadrangular-orbicular, leaves with a terminal spine at most as long as the uppermost leaflets or often very short to nearly lacking. *Astragalus ebenoides* subsp. *naghadehensis* is distinct in having yellowish white fruiting calyx sometimes red at the teeth without red stripes, standard blade distinctly longer than wide, leaves with a terminal spine mostly distinct and up to one and half as long as the uppermost leaflets (2). Although the molecular differences between these two taxa are low, the morphological characters are adequate to separate them.

In the next subclades of clade “B” *A. distans* is sister to a clade of *A. chardinii* and *A. submitis*. *Astragalus veiskarami* is also sister to a clade of *A. raswendicus* and *A. yushensis* (= *Astragalus submitis* subsp. *maassoumii*). *Astragalus submitis* is characterized by fruiting calyx with white stripes and triangular teeth instead of red stripes and subulate teeth known in *A. yushensis* (1, 11). There are 11 variable nucleotide sites and 40 indels in the combined dataset separating these two taxa (Table 3).

Conclusions

nrDNA ITS and *trn*L$_{\mathrm{UAG}}$-*rpl*32 regions provided considerable resolution for species level phylogenetic reconstruction in *Astragalus*. A recent study of *A.* sect. *Anthylloidei* conducted by authors (26), indicated that these two markers as potential barcodes are useful to separate the species of this section. A low evolutionary rate of DNA sequences in *Astragalus* (13, 19, 20, and 23) may be caused by rapid species radiation along with local endemism. *Astragalus* possesses several features that have been postulated to promote diversification rates in angiosperms, including geographic population structures consisting of local isolates with restricted gene flow (13), the herbaceous habit (associated with reduced generation time, 14), exceptional chromosomal variability (15) and tendency toward parallelism and reversal associated with recurring ecological specializations (16, 17). Therefore, the small number of nucleotide sequence variations plus several multi-base indels (mostly more than one gap) among different subspecies of *A. murinus*, *A. remotiflorus* and *A. ebenoides* along with morphological characteristics could provide appropriate criteria in setting the species boundaries in this group (Table 2 and 3, Figure 1).

Taxonomic treatment

1. ***Astragalus ebenoides*** Boiss., Diagn. pl. orient., ser. 1, **2**: 70 (1843)
[≡ *Tragacantha ebenoides* (Boiss.) Kuntze, Revis. Gen. 2: 944 (1891)]

Holotype: Persia, circa Ispahan, *P.M.R.* Aucher-Eloy 4409 (G-BOIS!).

2. ***Astragalus naghadehensis*** (Tietz & Zarre) Naderi Safar & Maassoumi, comb. et stat. nov.
[≡ *Astragalus ebenoides* subsp. *naghadehensis* Tietz & Zarre, Sendtnera **2**: 304 (1994)]

Holotype: Iran, prov. W. Azarbaijan, Naghadeh, Mahabad road, 2.6.1974, Siami 5413 (TARI!).

3. ***Astragalus murinus*** Boiss., Diagn. pl. orient., ser. 1, **2**: 72 (1843)
[≡ *Tragacantha murina* (Boiss.) Kuntze, Revis. Gen. **2**: 946 (1891)]

Syntypes: Persia, prope Ispahan, P.M.R. Aucher-Eloy 440 (FI, FI-W, G: pp., G-BOIS!, M!, P, W!).

4. ***Astragalus pseudomurinus*** Naderi Safar & Maassoumi, comb. et nom. nov.
[≡ *Astragalus murinus* subsp. *bornmuelleri* Tietz & Zarre, Sendtnera **2**: 320 (1994), (non *A. bornmuelleri* Freyn 1890)]

Holotype: Iran, [prov. Markazi] in dit. urbis Sultanabad, m. Raswend, 4.6.1898, Th. Strauss (B). [Type with illustration: B! at: http://www.europeana.eu/portal/record/1160

5/BGBM___Herbarium_Berolinense___B_10_0277448.html?start=1&query=Astragalus+murinus+subsp.+bornmuelleri+&startPage=1&rows=24].

5. ***Astragalus remotiflorus*** Boiss., Diagn. pl. orient., ser. 1, **2**: 72 (1843)

[≡ *Tragacantha remotiflora* (Boiss.) Kuntze, Revis. Gen. **2**: 947 (1891)]

Holotype: Iran, In Persia australi, *P.M.R.* Aucher-Eloy 1325 (G-BOIS!)

6. ***Astragalus melanogramma*** Boiss., Fl. Or. Suppl.: 185 (1888)

[≡ *Astragalus remotiflorus* subsp. *melanogramma* (Boiss.) Tietz & Zarre, Sendtnera **2**: 331 (1994)]. Holotype: Iran, [prov. Hamadan] in jugus excelsis montis Elwend Persiae, [unde semina attulit], 28.5.1882, Th. Pichler 222 (G-BOIS), [isotype with illustration: B! at http://www.europeana.eu/portal/record/11605/E6628BA34C6477DEDD2EC4B11AED1EF0F5DE64D6.html?start=1&query=Astragalus+remotiflorus&startPage=1&rows=24]

7. ***Astragalus nigrohirsutus*** (Tietz & Zarre) Borjian, Maassoumi & Assadi, Iran. J. Bot, 18 (1): 102 (2012)

[≡ *Astragalus remotiflorus* subsp. *nigrohirsutus* Tietz & Zarre, Sendtnera **2**: 331 (1994)]

Holotype: Iran, Prov. Fars, Fasa, Kharman Kuh, 2900 m, 6.6.1983, V. Mozaffarian 47930 (TARI!).

8. ***Astragalus submitis*** Boiss. & Hohen. in schedis impr. ad Kotschy, Pl. pers. austr., ed. Hohenacker (1846) [et in Boissier, Diagn. pl. orient., ser. 1, **9**: 100 (1849)]

[≡ *Tragacantha submitis* (Boiss. & Hohen.) Kuntze, Revis. Gen. **2**: 948 (1891)]

Lectotype: Iran, in monte Elbrus supra pagum Passgala, 21.5.1943, Th. Kotschy 190 (G-BOIS!).

9. ***Astragalus yushensis*** T. Sabaii, Zarre & Podlech, Feddes Repert. **120**: 57 (2009)

[≡ *Astragalus submitis* Boiss. & Hohen. subsp. *maassoumii* Tietz & Zarre, Sendtnera 2: 337 (1994), non *Astragalus maassoumii* Podlech, Mitt. Bot. Staatssamml. München 25 (1): 280 (1988)]

Holotype: Iran, prov. Mazandaran, Pol-e Zangouleh to Doab, after Takour village, 2500 m, 3.6.1986, A.A. Maassoumi 55131 (TARI!).

Key to the species of sect. *Anthylloidei* [modified and adopted from (2)]

1. Leaves imparipinnate but terminal leaflet sometimes falling, and rachis becoming hard with age, but not spiny 2

– Leaves paripinnate, with spiny rachis (in *A. raddei* sometimes with a terminal leaflet) 14

2. Leaves 10–30 cm; standard 20–30 mm 3

– Leaves 6–20 cm; standard 14–22 mm (unknown in *A. daghestanicus*) 4

3. The whole plant subglabrous or partly sparsely hairy, only the calyx rather densely hairy at anthesis; calyx teeth 1–2 mm; standard 20–28 mm; wings distinctly longer than the keel 1. *A. halicacabus* Lam.

– At least petiole and rachis densely villous; calyx teeth 2–4 mm; standard 25–30 mm; wings scarcely longer than the keel 2. *A. belolipopovii* Kamelin

4. Plants with distinct stems 3–10 cm 5

– Plants acaulescent, or nearly so 6

5. Leaflets in 9–18 pairs, folded at the tip;

racemes 4–7 cm, densely many-flowered; calyx at anthesis 14–16 mm, in fruit bladdery, c. 20 mm long, teeth 5 mm; standard c. 20 mm

3. *A. willisii* Popov

– Leaflets in 10–12 pairs, flat; racemes 10–15 cm, loosely to remotely 18–25-flowered; calyx at anthesis 9–12 mm, in fruit 13–18 mm long, teeth 1.5–2 mm; standard 14–18 mm

4. *A. pseudanthylloides* Gontsch.

6. Leaflets in 9–13 pairs 7

– Leaflets in at least 12, but mostly in distinctly more, pairs 8

7. Stipules 4–5 mm, adnate to the petiole for 1–2 mm; calyx loosely covered with spreading hairs, with 5–12 reticulately connected reddish nerves; standard blade at the base hastate-auriculate

5. *A. khoshjailensis* Širj. & Rech.f.

– Stipules 8–9 mm, adnate to the petiole for 3–5 mm; calyx rather densely covered with ascending hairs, with 13–15 reticulately connected yellowish nerves; standard blade at the base not hastate-auriculate, subabruptly narrowed into the long claw

6. *A. wagneri* Bunge

8. Stipules 15–28 mm, with 6–8 distinct longitudinal nerves; leaves 17–28 cm calyx very soon globose inflated, c. 12 mm in diameter

7. *A. veiskaramii* Zarre, Podlech &T.Sabaii

– Stipules up to 12 mm, without distinct nerves; leaves up to 20 cm but mostly distinctly shorter; calyx at fruiting time ovoid-inflated, 15–20 mm long 9

9. Rachis and peduncles covered with very short hairs 0.1–0.3 mm 10

– Rachis and peduncles densely covered with spreading, tangled hairs 0.3–1 mm and often with fewer, straight hairs up 2 mm 11

10. Leaflets elliptic, 4–11 × 2–5 mm; calyx covered with appressed black hairs

8. *A. chardinii* Boiss.

– Leaflets widely elliptic to suborbicular, 2–4.5 × 1.6–4 mm; calyx covered with spreading white hairs

9. *A. surugensis* Boiss. & Hausskn.

11. Peduncles and calyx with white and black hairs; fruiting calyx with 20–30 parallel nerves

10. *A. anthylloides* Lam.

– Whole plant only with white hairs; fruiting calyx with reticulately connected nerves 13

12. Stipules 5–6 mm, adnate to the petiole for 2–3 mm; leaflets on upper side loosely or toward the margins rather densely hairy

11. *A. daghestanicus* Grossh.

– Stipules 8–12 mm, adnate to the petiole for 4–8 mm; leaflets on both sides densely to very densely hairy

12. *A. zederbaueri* Stadlm.

13. Ovary and legumes glabrous

13. *A. flexilipes* Bornm.

– Ovary and legumes hairy 14

14. Stipules connate behind the stem nearly for the whole length, the short free tips behind the stem 15

– Stipules connate for up to 1/2–3/4 of its length with free tips near to the petiole, more rarely free from each other 17

15. Hairs at calyx up to 0.8 mm; standard blade 5–6 mm long and wide

14. *A. pseudotortuosus* Tietz & Zarre

– Hairs at calyx up to 1.5(–2) mm; standard blade 8–11 × 6.5–10 mm 16

16. Inflorescences 5–17 cm, overtopping the leaves; racemes (3–) 5–15-flowered; fruiting calyx not red-striped
15. *A. tortuosus* DC.

– Inflorescences 2–5 cm, not overtopping the leaves; racemes (1–) 2–3-flowered; fruiting calyx red-striped 16. *A. coluteopsis* Parsa

17. Standard blade at the base rounded or slightly angularly passing into the claw, not or scarcely auriculate 18

– Standard blade at the base angular-auriculate to hastate 34

18. Leaflets in 4–11 pairs 19

Leaflets in at least 9–10, but mostly distinctly more and up to 16–35 pairs
23

19. Standard 9–12 mm 20

– Standard 17–26 mm 21

20. Calyx widely tubular, 7–8 mm, not or scarcely inflated at fruiting time, teeth 2–3 mm; bracts 1.5–3.5 mm, flat; bracteoles absent; petals purple
17. *A. fissicalyx* Sabaii, Zarre & Podlech

– Calyx tubular at anthesis, 8–13 mm, distinctly inflated at fruiting time, teeth 3–6 mm; bracts 3–8 mm, boat-shaped; barcteoles present, 2–4 mm; petals cream, pale lilac suffused
18. *A. diopogon* Bunge

21. Leaflets elliptic to obovate, 0.5–5 × 0.5–3 mm; calyx at anthesis tubular, 10–16 mm, at fruiting time inflated, 10–18 mm long 22

– Leaflets narrowly elliptic, 2.5–15 × 1–5 mm; calyx at anthesis 15–20 × 5–7 mm, at fruiting time 20–30 × 15 mm, white and black hairy
19. *A. ghashghaicus* Tietz & Zarre

22. Calyx only white hairy, teeth 2.5–6 mm; standard 17–20 mm; bracts 2.5–4.5 mm
20. *A. lalesarensis* Bornm.

Calyx with white and black hairs, teeth 2–3 mm; standard 20–24 mm; bracts 6–10 mm
21. *A. ermineus* V.A.Matthews

23. Calyx at beginning of anthesis 17–22 mm at fruiting time 25–35 × 15–22 mm
22. *A. fuhsii* Freyn & Sint.

– Calyx at beginning of anthesis at most up to 17 mm, at fruiting time at most up to 23 mm long 24

24. Calyx teeth triangular, 1–2 mm 25

– Calyx teeth subulate, (2–)2.5–8 mm
27

25. Calyx with 16–26 parallel nerves; axis of racemes sparsely appressed hairy
23. *A. distans* Fisch.

– Calyx with 9–17 reticulately connected nerves; axis of racemes with spreading or appressed and spreading hairs 26

26. Petiole 1/5–1/3 of rachis length; terminal spine up to twice as long as the uppermost leaflets; legumes compressed laterally
24 *A. submitis* Boiss. & Hohen.

– Petiole 1/3–1/2 of rachis length; terminal spine up to five times as long as the uppermost leaflets; legumes compressed dorsi-ventrally
25. *A. raswendicus* Hausskn. & Bornm.

27. Inflorescences not overtopping the leaves, with a peduncle 2–4 cm and a loosely 2–6 (–9)-flowered raceme

26. *A. murinus* Boiss.

– Inflorescences overtopping the leaves, more than ten-flowered; at fruiting time densely globose or loosely to densely cylindric 28

28. Calyx at beginning of anthesis 8–11 mm, at fruiting time 9–14 mm long; leaflets 1.5–4.5 mm; petals cream at the base, pink in the upper parts; legumes laterally compressed

27. *A. yushensis* T.Sabaii, Zarre & Podlech

– Calyx at beginning of anthesis 10–17 mm, at fruiting time 14–23 mm long; most leaflets distinctly longer; legumes compressed dorsi-ventrally 29

29. Racemes densely many-flowered, with more than 15 flowers, at fruiting time globose to shortly cylindric; fruiting calyx not red-striped, widest in upper half

28. *A. bodeanus* Fisch.

– Racemes at fruiting time loosely long cylindric, if densely shortly cylindric, then at most up to 15-flowered; fruiting calyx red-striped (only in *A. coluteoides* and *A. nigrohirsutus* unstriped), widest in lower half or in the middle 30

30. Rachises stout and rigid, persistent long time, the longer ones (13–) 15–25 cm; inflorescences distinctly overtopping the leaves; racemes at fruiting time loosely long cylindric, 10–25 cm 31

– Rachises slender, flexible, easily broken, the longer ones (8–) 10–17 cm; inflorescences only slightly longer than leaves; racemes at fruiting time shortly cylindric, 5–8 cm 32

31. Fruiting calyx merely white hairy, longer hairs up to 2.5 mm; legumes merely white hairy

29. *A. remotiflorus* Boiss.

– Fruiting calyx with white and black or with some grey hairs, longer ones at most up to 1 mm; legumes with white or with white and black hairs

30. *A. nigrohirsutus* (Tietz & Zarre) Borjian, Maassoumi & Assadi

32. Calyx covered with white and many dark grey or black hairs

31. *A. pseudomurinus* Naderi Safar & Maassoumi

– Calyx merely white hairy or with very few grey hairs mixed in 33

33. Fruiting calyx with red-stripes; racemes 3–8 cm, densely flowered

32. *A. melanogramma* Boiss.

– Fruiting calyx never red-striped; racemes 2–15 cm, loosely 3–13-flowered

33. *A. coluteoides* Willd.

34. Hairs in the inflorescence merely white 35

– Hairs in the inflorescence white and black 36

35. Calyx with parallel nerves, densely hairy, with many short hairs throughout; petals with dark purple blades 36

– Calyx with reticulately connected nerves, loosely hairy, with short hairs predominantly at the teeth; petals yellowish white, with blades at most pale purplish suffused

34. *A. szovitsii* Fisch. & C.A.Mey.

36. Fruiting calyx brownish yellow, with

red stripes; standard blade nearly square-orbicular, 5.5–8 × (4–)5–6.5 mm; plants with appressed or spreading hairs

35. *A. ebenoides* Boiss.

– Fruiting calyx yellow or red at the tip, without red stripes; standard blade oblong, (3.5–) 4–6 × 2–3 (–4) mm; plants always with appressed hairs

36. *A. naghadehensis* (Tietz & Zarre) Naderi Safar& Maassoumi

37. Rachises in the first year covered with spreading or recurved, tangled hairs 38

– Rachises in the first year covered with appressed to ascending straight hairs 40

38. Leaflets very densely hairy; bracts narrowly ovate to ovate

37. *A. megalocystis* Bunge

– Leaflets ± loosely hairy; bracts linear to subulate 39

39. Leaflets in 13–25 pairs, with a cusp up to 1.2 mm; keel blades with sigmoid upper edge

38. *A. lumsdenianus* Aitch. & Baker

– Leaflets in 5–18 pairs, with a minute cusp up to 0.3 mm; keel blades with convex upper edge

39. *A. raddei* Basil.

40. Inflorescences not overtopping the leaves; racemes loosely 3–7-flowered, its axis 2–5 cm

40. *A. rubrolineatis* Širj. & Rech.f.

– Inflorescences overtopping the leaves; racemes densely globose to shortly cylindrical, its axis 0.6–2 cm 41

41. Bracts 2–3.5 mm, ± persistent; bracteoles rarely present, 1.5–2.5 mm; fruiting calyx 10–15 × 6–9 mm

41. *A. keratensis* Bunge

– Bracts (3–)4–8 mm, soon falling; bracteoles always present, 3–7 mm; fruiting calyx 12–30 × 8–18 mm 42

42. Leaflets with a cusp up to 2 mm, on both sides densely grey-sericeous, on upper side with distinctly elevated nerves; keel blade with sigmoid upper edge; legumes 7–12 mm

42. *A. crassispinus* Bunge

– Leaflets obtuse or with a minute cusp up to 0.3 mm, loosely covered on both sides or on upper side only toward the margins with appressed to ascending hairy; keel blade with convex upper edge; legumes 4–5.5 mm

43. *A. raddei* Basil.

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