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Molecular phylogeny of *Astragalus* section *Anthylloidei* (Fabaceae) inferred from nrDNA ITS and plastid *rpl*32-*trn*L$_{(UAG)}$ sequence data

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Abstract: The phylogeny of *Astragalus* L. section *Anthylloidei* DC. and its interrelationship with allies were examined. The study was conducted using nrDNA ITS and plastid *rpl*32-*trn*L$_{(UAG)}$ sequences. *Astragalus* sect. *Anthylloidei* is nonmonophyletic, and its members are scattered across the tree in 4 well-supported clades and intermixed with members of other spiny sections. All the multispecific informal groups of the section, with the exception of *A. murinus* Boiss. group, are not monophyletic. Morphological character evolution was mapped on the molecular tree. Our results suggest that morphology cannot elucidate infrageneric relationships in spiny *Astragalus* accurately; analyzed characters have evolved several times in sect. *Anthylloidei* and, thus, show high levels of homoplasy. Distribution of members of the section matches, more or less, certain geographic patterns, ranging from the Zagros mountains, Northwest Iran and Eastern Turkey, Central Iran to Northeast Iran, and Turkmenistan and Afghanistan. A new taxonomic system for this group of species is needed. The present study suggests that sect. *Halicacabus* Bunge, which has been merged with sect. *Anthylloidei*, should be resurrected since 8 species, including *A. halicacabus* Lam; *A. wagneri* Bartl. ex Bunge; *A. distans* Fisch.; *A. raswendicus* Hausskn. & Bornm.; *A. veiskaramii* Zarre, Podlech & T.Sabaii; *A. submitis* Boiss. & Hohen.; and *A. chardinii* Boiss. (sect. *Anthylloidei*) as well as *A. semnanensis* Bornm. & Rech. f. (sect. *Semnanenses* Podlech & Zarre), form a distinct clade. Sect. *Eriostoma* Bornm. is a distinct lineage from sect. *Anthylloidei*.

Key words: *Anthylloidei*, *Astragalus*, Leguminosae, nrDNA ITS, phylogeny

1. Introduction

The largest genus of vascular plants on earth, *Astragalus* L. (Fabaceae) contains an estimated 3000 annual and perennial species (Maassoumi, 1998; Podlech and Zarre, 2013). The greatest number of species is found in the cool temperate/semiarid and arid continental regions of the Old World (ca. 2400 spp.), western North America (ca. 450 spp.), and along the Andes in South America (ca. 100 spp.). The genus belongs to a large group of papilionoid legumes that lack the chloroplast DNA inverted repeat, the so-called inverted-repeat–lacking clade (IRLC) (Lavin et al., 1990; Wojciechowski et al., 1999, 2000, 2004; Wojciechowski, 2005). Within the IRLC, *Astragalus* together with *Biserrula* L., *Oxytropis* DC., and the Coluteoid clade comprises a well-supported monophyletic group, the so-called Astragalean clade (Sanderson and Liston, 1995; Sanderson and Wojciechowski, 1996; Wojciechowski et al., 1999, 2000, 2004; Wojciechowski, 2005). The bulk of *Astragalus* species, with the exception of some outliers including Old World euploids and neo-Astragalus, belong to *Astragalus* s. str. It composed of several clades that are named using letters A–I (Kazempour Osaloo et al., 2003, 2005). Clade G, a loosely resolved and weakly supported group, comprises spiny *Astragalus* species. The great majority of this group is characterized by cushion forming habit, paripinnate leaves, persistent spiny rachis, a nearly sessile inflated calyx, gum ducts, and ovoid unilocular (rarely semibilocular) pods with 1–4 seeds.

Astragalus sect. *Anthylloidei* DC. (Fabaceae) was established by De Candolle (1825). Without paying attention to De Candolle's classification, Bunge (1868) introduced 2 sections, *Halicacabus* Bunge and *Megalocystis* Bunge, and placed *Astragalus anthylloides* Lam., the lectotype of sect. *Anthylloidei*, in sect. *Halicacabus*. Subsequent regional flora (USSR: Gontscharov et al., 1965; Turkey: Chamberlin and Matthews, 1970) and a revision (Tietz and Zarre, 1994)

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followed Bunge's treatment. Recently, sect. *Anthylloidei* was resurrected and *Astragalus* sect. *Halicacabus* and *Astragalus* sect. *Megalocystis* merged in sect. *Anthylloidei* (Maassoumi, 1995; Podlech et al., 2001; Podlech and Zarre, 2013). This section, with 37 species worldwide (Podlech et al., 2012; Podlech and Zarre, 2013), has cushion-forming plants with imparipinnate or paripinnate leaves with spiny rachises and inflated fruiting calyces. They are distributed in several Southwest Asian countries. Iran, which has approximately 28 species, is the main biodiversity center of this section (Podlech et al., 2001; Sabaii et al., 2007; Podlech and Zarre, 2013; Maassoumi, 2014). Some species of the section also occur in Turkey (5 species) and Afghanistan (4 species). The section is one of the most heterogeneous and complicated groups of spiny *Astragalus* and has been revised several times (Bunge, 1868, 1869; Boissier, 1872; Tietz and Zarre, 1994; Maassoumi, 1995; Podlech and Zarre, 2013) including in the *Flora Iranica* area (Podlech et al., 2001). On the basis of floral and fruit characteristics the section has been divided into 6 informal species groups (Tietz and Zarre, 1994). However, its species relationships remained unresolved. *Astragalus eriostomus* Bornm., an endemic species to Iran, sometimes has been placed in sect. *Anthylloidei* (Maassoumi, 1995, 1998); however, it now has its own section, *Eriostoma* Bornm. (Podlech et al., 2001; Podlech and Zarre, 2013; Maassoumi, 2014).

Phylogenetic analysis based on nrDNA ITS sequences at the genus level, including a limited number of species of sect. *Anthylloidei* (only 3), indicated that this group is intermixed with tragacanthic *Astragalus* and does not form a monophyletic group (Kazempour Osaloo et al., 2003, 2005). No detailed phylogenetic analysis using multiple DNA sequence data in addition to adequate and balanced taxon sampling has been conducted on this section and its allies until now.

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) 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 (Wojchiekhowski et al., 1999; Kazempour Osaloo et al., 2003, 2005; Javanmardi et al., 2012; Taşci Margoz et al., 2013; İpek et al., 2014). The *rpl*32-*trn*$L_{(UAG)}$ region is 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 to 1417 bp. This is the best noncoding region for low-level molecular studies (Shaw et al., 2007; Dong et al., 2012). To our knowledge, the *rpl*32-*trn*$L_{(UAG)}$ region has rarely been used in molecular phylogenetic investigations on *Astragalus* (Bartha et al., 2013).

The main goals of the present study were:

(1) To evaluate the monophyly of sect. *Anthylloidei*; (2) to examine the evolutionary relationships within the section; (3) to investigatethe interrelationship between the section and its allies; (4) to determine the status of monotypic sections allied to sect. *Anthylloidei*, such as sect. *Eriostoma* and sect. *Semnanenses* Podlech and Zarre; and (5) to evaluate the evolutionary trends of several diagnostic morphological characters in the context of molecular phylogeny.

2. Materials and methods

2.1. Taxon sampling

Our sampling was focused on the Iranian spiny *Astragalus* with an emphasis on sect. *Anthylloidei*. A total of 56 species (52 ingroups and 4 outgroups) were sampled and analyzed for nrDNA ITS, cpDNA *rpl*32 gene, and *rpl*32-*trn*$L_{(UAG)}$ intergenic spacer. A combined dataset of both nuclear and plastid markers was also built including the same 56 taxa. We selected 24 species of sect. *Anthylloidei* mostly from Iran (out of 37 species) and representatives from its relative sections, i.e. sections *Campylanthus* Bunge, *Microphysa* Bunge, *Poterion* Bunge, *Macrophyllium* Boiss., *Macrosemium* Bunge, *Rhacophorus* Bunge, *Leucocercis* Bunge, *Polystegis* Boiss., *Acanthophace* Bunge, *Hymenostegis* Bunge, *Eriostoma*, and *Semnanenses*. Four nonspiny *Astragalus* species were chosen as outgroups. A list of all the taxa used in this study and the sources, voucher specimens, as well as GenBank accession numbers are given in Table 1.

2.2. DNA isolation, PCR, and sequencing

Total genomic DNA was isolated from fresh or dried materials using the modified CTAB method of Doyle and Doyle (1987). The nrDNA ITS region was amplified using the primers ITS5m (Sang et al., 1995) and ITS4 (White et al., 1990) or AB101F and AB102R (Douzery et al., 1999). The *rpl*32-*trn*$L_{(UAG)}$ region was amplified using the *rpl*32-F and trn$L_{(UAG)}$ primers (Shaw et al., 2007). The PCR amplification was carried out in a volume of 20 µL containing 8 µL of deionized water, 10 mL of the 2X Taq DNA polymerase Master Mix Red (Amplicon, cat. no. 180301; 150 µM Tris-HCl pH 8.5, 40 µM $(NH_4)2SO_4$, 3.0 µM $MgCl_2$, 0.4 µM dNTPs, 0.05 units μL^{-1} AmpliconTaq DNA polymerase, inert red dye, and a stabilizer), 0.5 µL of each primer (10 pmol/µL), and 1.0 µL of template DNA (20 ng/µL). PCR was carried out according to the following protocol: an initial 2.30-min premelting at 94 °C and 28 cycles of 50 s at 80 °C for *rpl*32-*trn*$L_{(UAG)}$ and 94 °C for nrDNA ITS for template denaturation; 40 s at 58 °C for primer annealing; and 55 s at 72 °C for primer extension, followed by 7 min at 72 °C for final extension. PCR products were separated by electrophoresis in 1% agarose gel stained with ethidium bromide and were

Table 1. Taxa included in the nrDNA ITS and *rpl*32-*trn*L_{UAG} analyses.

Species	Section	DNA source (location, voucher)	GenBank accession no. ITS/ *rpl*32-*trn*LUAG
A. horridus Boiss.	*Acanthophace*	Iran: Mozaffarian 54874 (TARI)	AB052002*/AB908523
A. aureus Willd.	*Adiaspastus*	Iran: Maassoumi 78452 (TARI)	AB908467/AB908518
A. brachycalyx Fisch.	*Adiaspastus*	Iran: Assadi & Mozaffarian 37096 (TARI)	AB052026*/AB908516
A. gevashensis D.F.Chamb. & V.A.Matthews	*Adiaspastus*	Turkey: Engel 41763 (MSB)	AB908468/AB908519
A. hystrix Fisch.	*Adiaspastus*	Iran: Maassoumi & Mozaffarian 78604 (TARI)	AB052014*/AB908515
A. ochrochlorus Boiss. & Hohen.	*Adiaspastus*	Iran: Shahsavari 69760 (TARI)	AB231086*/AB908517
A. anthyloides Lam.	*Anthylloidei*	Turkey: Nydegger 43063 (MSB)	AB908447/AB908488
A. bodeanus Fisch.	*Anthylloidei*	Iran: Mozaffarian 83758 (TARI)	AB908460/AB908501
A. chardinii Boiss.	*Anthylloidei*	Iran: Sabeti 16064 (TARI)	AB908443/AB908482
A. coluteopsis Parsa	*Anthylloidei*	Iran: Zarre et al. 39983 (TUH)	AB908461/AB908503
A. crassispinus Bunge	*Anthylloidei*	Iran: Anonymous 15394 (FUMH)	AB908453/AB908494
A. distans Fisch.	*Anthylloidei*	Iran: Zarre 33641 (TUH)	AB908462/AB908504
A. ebenoides Boiss.	*Anthylloidei*	Iran: Maassoumi & Mirhosseini 59421(TARI)	AB908445/AB908484
A. ghashghaicus Tietz & Zarre	*Anthylloidei*	Iran: Mozaffarian 57552(TARI)	AB908448/AB908489
A. halicacabus Lam.	*Anthylloidei*	Turkey: Aytac 8700 (GAZI)	AB908444/AB908483
A. keratensis Bunge	*Anthylloidei*	Iran: Maassoumi & Zarre 71945(TARI)	AB908454/AB908495
A. khoshjailensis Širj. & Rech.f.	*Anthylloidei*	Iran: Maassoumi. 47580 (TARI)	AB052010*/AB908502
A. lalesarensis Bornm.	*Anthylloidei*	Iran: Mirtajaddini s.n. (TARI)	AB908455/AB908496
A. lumsdenianus Aitch. & Baker	*Anthylloidei*	Iran: Mousavi & Hamidi 4260 (TARI)	AB908449/AB908490
A. megalocystis Bunge	*Anthylloidei*	Iran: Assadi & Mozaffarian 40389 (TARI)	AB908458/AB908499
A. murinus Boiss.	*Anthylloidei*	Iran: Assadi & Abouhamzeh 46094 (TARI)	AB052008*/AB908487
A. raddei Basil.	*Anthylloidei*	Iran: Maassoumi & Mozaffarian 79577 (TARI)	AB908452/AB908493
A. raswendicus Hausskn. & Bornm.	*Anthylloidei*	Iran: Babakhanlou & Amin 15647 (TARI)	AB908459/AB908500
A. remotiflorus Boiss.	*Anthylloidei*	Iran: Assadi & Miller 25162 (TARI)	AB908446/AB908485
A. rubrolineatus Širj. & Rech.f.	*Anthylloidei*	Iran: Assadi & Mozaffarian 40832 (TARI)	AB908456/AB908497
A. submitis Boiss. & Hohen.	*Anthylloidei*	Iran: Maassoumi & Shahsavari 80739 (TARI)	AB052009*/AB908486
A. szovitsii Fisch. & C.A.Mey.	*Anthylloidei*	Iran: Assadi 86737 (TARI)	AB908450/AB908491
A. tortuosus DC.	*Anthylloidei*	Iran: Fattahi & Khaledian 438 (TARI)	AB908451/AB908492
A. veiskaramii Zarre, Podlech & Sabaii	*Anthylloidei*	Iran: Veiskarami 23727 (TUH)	AB908463/AB908505
A. wagneri Bunge	*Anthylloidei*	Iran: Assadi 85298 (TARI)	AB908457/AB908498
A. campylanthus Boiss.	*Campylanthus*	Iran: Mozaffarian & Maassoumi 47790 (TARI)	AB052028*/AB908478
A. susianus Boiss.	*Campylanthus*	Iran: Mozaffarian 57270 (TARI)	AB908441/AB908479
A. tricholobus DC.	*Campylanthus*	Iran: Mozaffarian & Nowroozi 34005 (TARI)	AB052031*/AB908520
A. aegobromus Boiss. & Hohen.	*Caprini*	Iran: Maassoumi 55116 (TARI)	AB051953*/AB908469

Table 1. (Continued).

Species	Section	DNA source (location, voucher)	GenBank accession no. ITS/ *rpl*32-*trn*LUAG
A. eriostomus Bornm.	*Eriostoma*	Iran: Mozaffarian. 63794 (TARI)	AB052007*/AB908507
A. sciureus Boiss. & Hohen.	*Hymenostegis*	Iran: Mirfakhraiy 15594 (TARI)	AB231108*/AB908524
A. vaginans DC.	*Hymenostegis*	Turkey: Aytac 2440 (GAZI)	AB908466/AB908513
A. sinicus L.	*Lotidium*	Japan: Kazempour Osaloo 1999-01 (TARI)	AB051965*/AB908471
A. subsecundus Boiss.	*Laguropsis*	Iran: Maassoumi. 55105 (TARI)	AB051985*/AB908472
A. talimansurensis Sirj. & Rech. f.	*Leucocercis*	Iran: Assadi & Abouhamzeh 38835 (TARI)	AB231119*/AB908521
A. paradoxus Bunge	*Macrosemium*	Iran: Wendelbo & Assadi 19281 (TARI)	AB052001*/AB908514
A. oleifolius DC.	*Macrophyllium*	Iran: Maassoumi & Mozaffarian 79612 (TARI)	AB052019*/AB908511
A. dipodurus Bunge	*Macrophyllium*	Turkey: Akan & Mirdezlioglu 1098 Harran Univ. Herb.	AB908465/AB908512
A. cephalanthus DC.	*Microphysa*	Iran: Mozaffarian & Maassoumi. 47788 (TARI)	AB052027*/AB908481
A. microphysa Boiss.	*Microphysa*	Iran: Mozaffarian 57728 (TARI)	AB908442/AB908480
A. piptocephalus Boiss.	*Polystegis*	Iran: Maassoumi & Mozaffarian 76763 (TARI)	AB052018*/AB908522
A. fasciculifolius Boiss.	*Poterion*	Iran: Mozaffarian 49867 (TARI)	AB052016*/AB908508
A. glaucacanthos Fischer	*Poterion*	Iran: Assadi et al. 33356 (TARI)	AB052017*/AB908509
A. clusianus Boiss.	*Poterion*	Spain: Neydegger 35823 (MSB)	AB908464/AB908510
A. cymbostegis Bunge	*Rhacophorus*	Turkey: Duman 52699 (MSB) (3767 GAZI)	AB908439/AB908476
A. diphtherites Fenzl	*Rhacophorus*	Turkey: Mirdezlioglu 1332 (TARI)	AB908440/AB908477
A. echidna Bunge	*Rhacophorus*	Iran: Maassoumi & Zarre 71958 (TARI)	AB231133*/AB908474
A. stenolepis Fischer	*Rhacophorus*	Iran: Maassoumi 55128 (TARI)	AB052021*/AB908475
A. verus Oliver	*Rhacophorus*	Iran: Mozaffarian & Maassoumi 47797 (TARI)	AB052023*/AB908473
A. fragrans Willd.	*Synochreati*	Iran: Maassoumi. & Abouhamzeh 56916 (TARI)	AB051967*/AB908470
A. semnanensis Bornm. & Rech.f.	*Semnanenses*	Iran: Mozaffarian 58865 (TARI)	AB231118*/AB908506

Abbreviations for herbaria followed Holmgren and Holmgren (1998): FUMH, Ferdowsi University of Mashhad Herbarium, Mashhad, Iran; GAZI, Herbarium of Gazi University, Ankara, Turkey; MSB, Herbarium of Ludwig-Maximilians-Universität, Munich, Germany; TARI, Herbarium of the Research Institute of Forests and Rangelands, Tehran, Iran; TUH, Tehran University Herbarium, Tehran, Iran. (*) nrDNA ITS sequences for these taxa determined by Kazempour Osaloo et al. (2003, 2005) and obtained from GenBank; (–) not available in GenBank.

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, Foster City, CA, USA) with the appropriate primers in an ABI Prism 3730xl DNA Analyzer (Applied Biosystems).

2.3. Sequence alignment

Sequences for the above-mentioned taxa were edited using BioEdit version 7.0.9.0 (Hall, 1999) and were aligned with MUSCLE under default parameters (Edgar, 2004) followed by manual adjustment. The alignment of datasets required the introduction of numerous single- and multiple-base indels (insertions/deletions). Positions of indels were treated as missing data for all datasets.

2.4. Phylogenetic inferences

Maximum parsimony (MP), Bayesian inference (BI), and maximum likelihood (ML) were used for the phylogenetic analyses. The MP analyses were conducted using PAUP*

version 4.0b10 (Swofford, 2002). 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 (Felsenstein, 1985), each with a simple addition sequence.

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). On the basis of this analysis, datasets were analyzed using the SYM+I+G model for nrDNA ITS, GTR+I+G for *rpl*32-*trn*$L_{(UAG)}$, and HKY+I+G for the combined dataset. The program MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) was used for the BI. Posteriors on the model parameters were estimated from the data using the default priors. The analysis was carried out with 6 million generations using the Markov chain Monte Carlo (MCMC) search. MrBayes performed 2 simultaneous analyses starting from different random trees (nruns = 2) each with 4 Markov chains and trees sampled 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 (Rambaut and Drummond, 2009). Tree visualization was carried out using TreeView version 1.6.6 (Page, 2001).

The ML analyses for the datasets were performed in the programs GARLI (Zwickl, 2006) and raxmlGUI (Silvestro and Michalak, 2011). The model of evolution employed for each data set is the same as that of BI. Parametric bootstrap values for ML were calculated in raxmlGUI base on 1000 replicates with 1 search replicate per bootstrap replicate.

The congruency of 2 single datasets (nrDNA ITS and cpDNA *rpl*32-*trn*$L_{(UAG)}$) was assessed using the partition homogeneity test or the incongruence length difference (ILD) test of Farris et al. (1995), as implemented in PAUP* (Swofford, 2002). The test was conducted with exclusion of invariant characters (Cunningham, 1997) 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.

2.5. Analysis of morphological data

Character evolution was interpreted for 6 characters previously considered important diagnostic features in taxonomic treatments of spiny *Astragalus* (Maassoumi, 1995; Podlech et al., 2001; Podlech and Zarre, 2013). Likelihood mapping was performed using Mesquite v. 2.75 (Maddison and Maddison, 2011) on the obtained Bayesian tree based on the Mk1 model (Markov 1 parameter). The features were coded in a binary matrix and traced on the molecular tree. Characters are summarized in Table 2.

3. Results

3.1. Phylogenetic analyses

MP analyses of the 2 single and the combined datasets resulted in topologically identical trees to those of BI and ML. The length and composition of each DNA region sequenced, as well as tree statistics from the single and combined analyses of the 2 regions, are summarized in Table 3. The trees resulting from the 3 methods for the combined dataset were topologically similar to nrDNA ITS (tree not shown) but with high resolution and supports (Figure 1). The *rpl*32-*trn*$L_{(UAG)}$ dataset yielded trees with low resolutions and supports due to fewer informative characters (Table 3). This tree differed from the nrDNA ITS tree regarding the position of some taxa including *Astragalus khoshjailensis* Širj. & Rech.f. and *A. tortuosus* DC. (formed a subclade) as well as *A. lalesarensis* Bornm., *A. eriostomus*, and *A. semnanensis* Bornm. & Rech.f. (formed another subclade; tree not shown). These differences may be caused by hybridization or lineage sorting that took place a long time ago.

Table 2. Morphological characters traced on the molecular tree.

1. Leaves: imparipinnate = (0); paripinnate = (1)
2. Peduncle length: 0–3 cm = (0); >3 cm = (1)
3. Calyx shape: inflated = (0); campanulate = (1); tubular (2); tubular–turbinate = (3)
4. Hair size: <1.5 = (0); 1.5–3 = (1); >3 = (2)
5. Hair color: white = (0); black and white = (1)
6. Limb of standard: rounded = (0); hastate = (1)

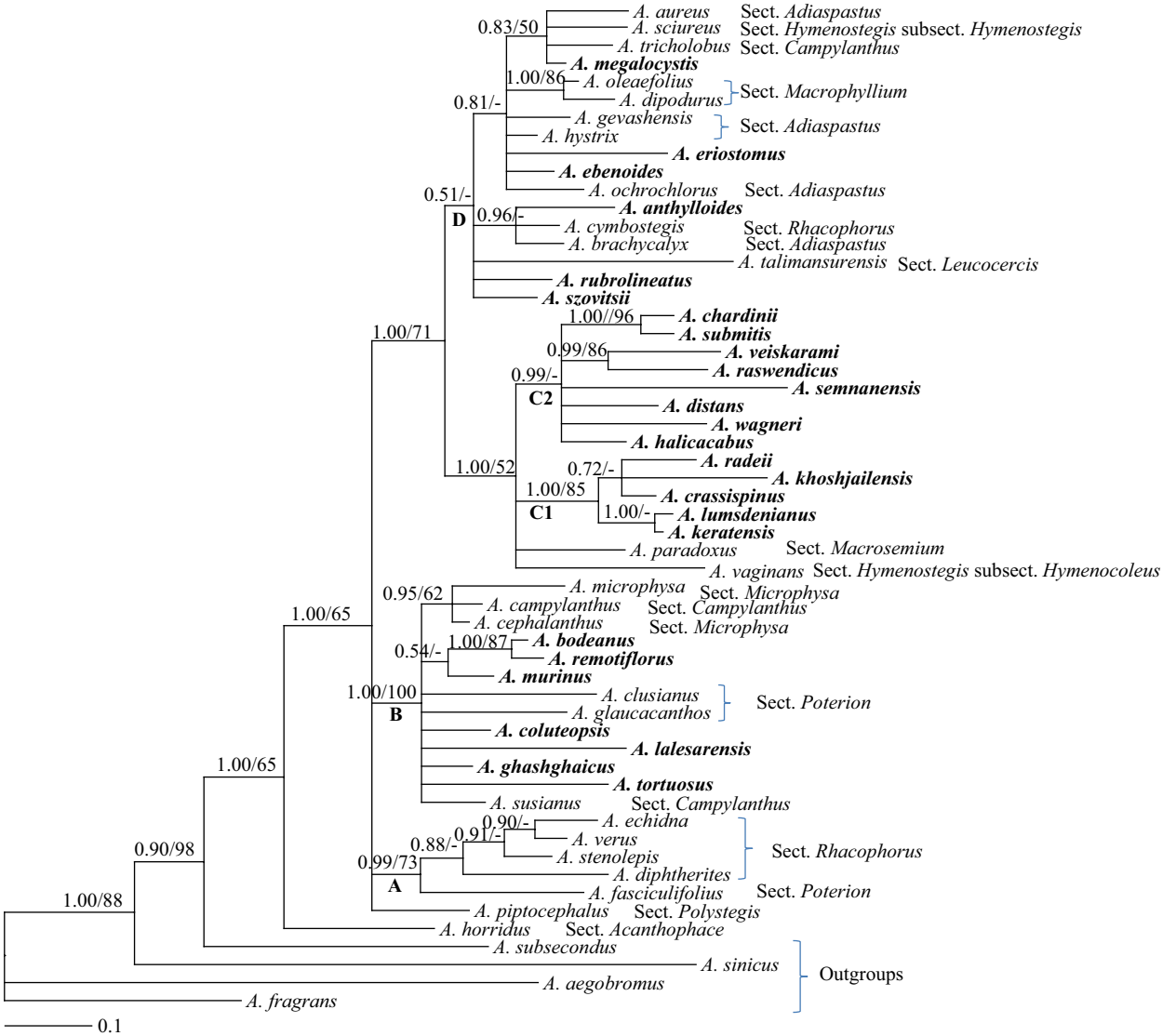

Figure 1. Fifty percent majority rule consensus tree resulting from Bayesian analysis of the combined nrDNA ITS and plastid *rpl*32-*trn*L$_{(UAG)}$ sequences. The members of sect. *Anthylloidei* are indicated in bold face.

Table 3. Dataset and tree statistics from separate and combined analyses of the nuclear and chloroplast regions.

	nrDNA ITS	cpDNA *rpl*32-*trn*L	Combined
Sequences (n)	56	56	56
Nucleotide sites	666	1185	1851
Informative characters	48	40	88
Uninformative characters	618	1145	1763
CI of MPTs	0.611	0.754	0.602
RI of MPTs	0.851	0.789	0.787
Number of MPTs	1000	1000	1000
Length of MPTs (steps)	108	65	191

CI, consistency index; MPTs, most parsimonious trees; RI, retention index.

The 50% majority rule tree gained from BI based on the combined dataset along with posterior probability and bootstrap values is displayed in Figure 1. *Astragalus horridus* Boiss. (sect. *Acanthophace*) is the first diverging branch (PP = 1.00) and sister to a large assemblage of spiny species (PP = 1). The assemblage is composed of 4 main clades and a single branch (*A. piptocephalus* Boiss. & Hausskn.: sect. *Polystegis*). The first clade (A) comprises 5 species from *A. fasciculifolius* Boiss. (sect. *Poterion*) through *A. echidna* Bunge (sect. *Rhacophorus*). The second clade (B) contains 13 species: a mixture of some members of sect. *Anthylloidei* (7 spp.) and representatives of sections *Poterion* (2 spp.), *Campylanthus* (2 spp.), and *Microphysa* (2 spp.). The third clade (C) with PP = 0.98 includes 15 species. The clade is, in turn, composed of 2 subclades (C1 and C2) that are mainly composed of members of sect. *Anthylloidei*.

The last clade (D) was the largest one with 17 species and included only 5 species of *Anthylloidei* and 12 species of 7 other spiny sections.

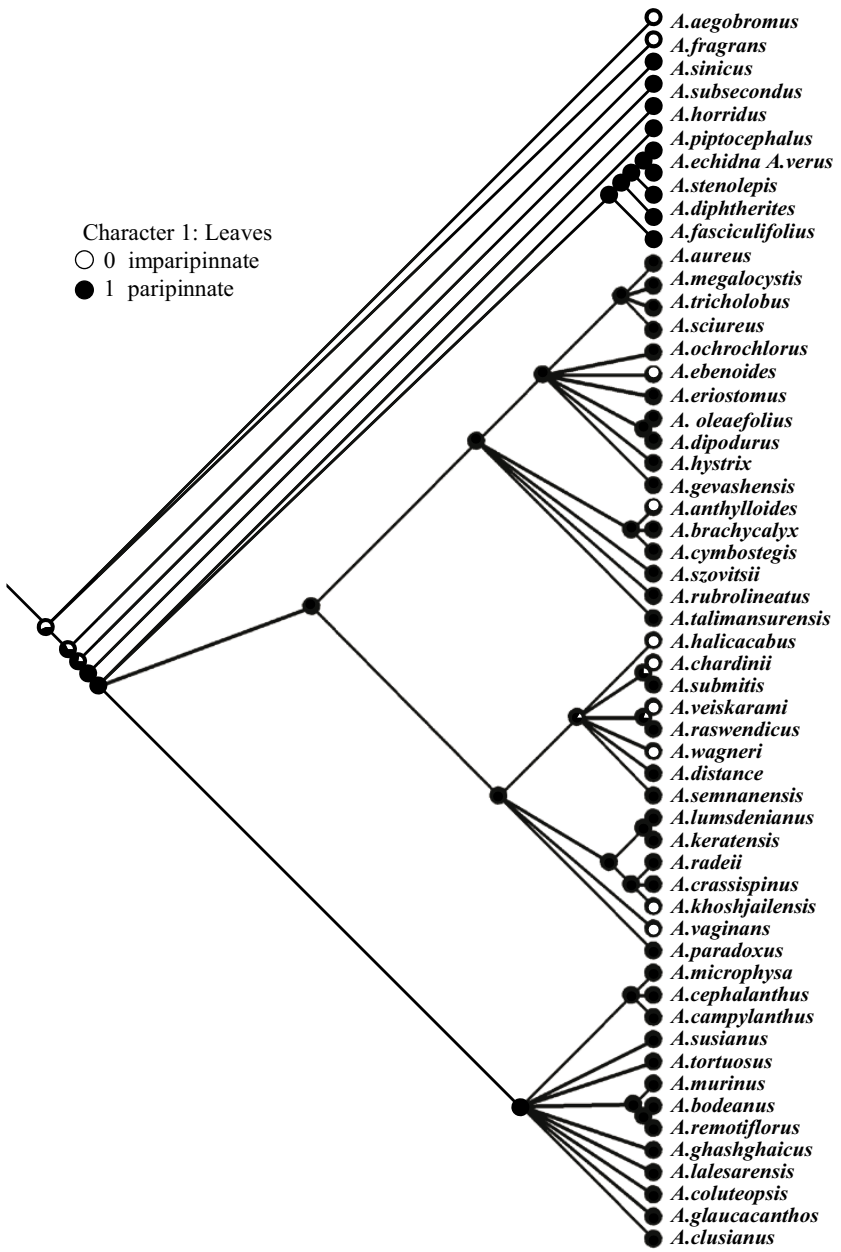

Figure 2. Evolutionary history of character 1 (leaves) mapped on the Bayesian tree obtained from the combined nr DNA ITS and plastid *rpl*32-*trn*$L_{(UAG)}$ sequences.

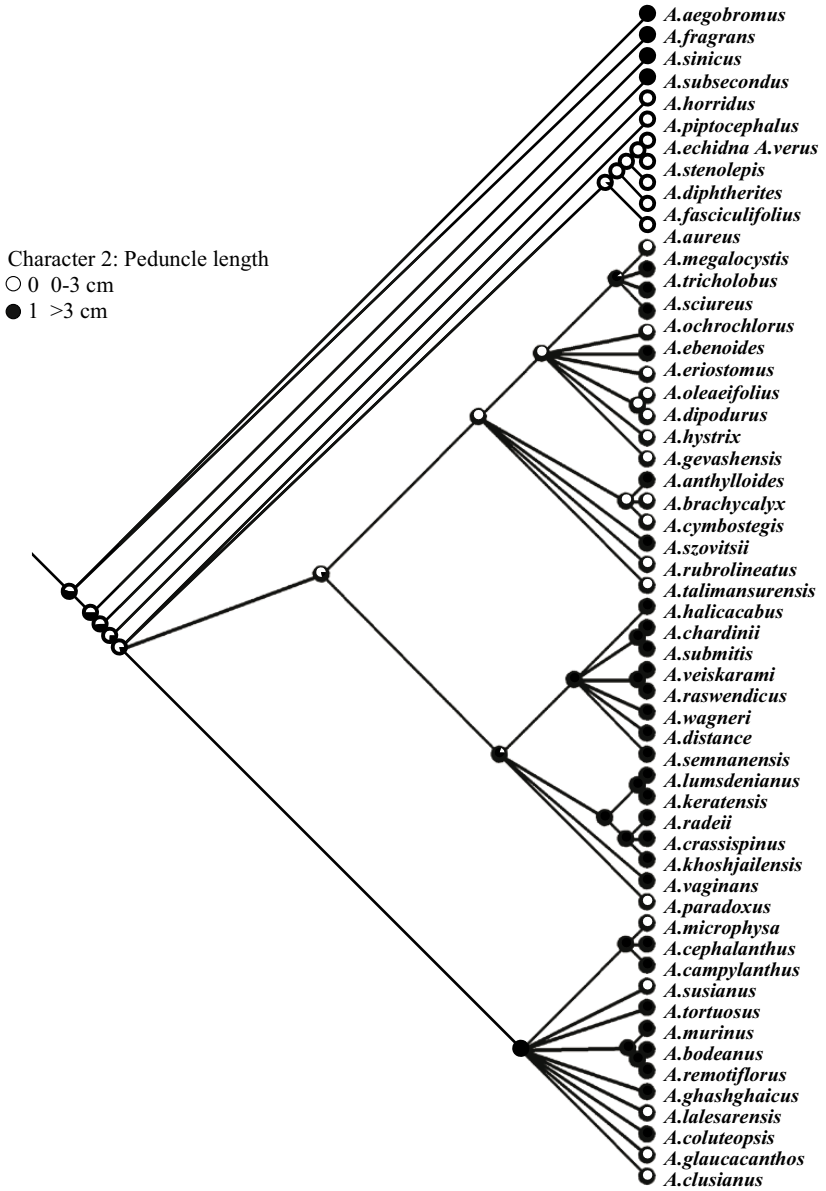

Figure 3. Evolutionary history of character 2 (peduncle length) mapped on the Bayesian tree obtained from the combined nr DNA ITS and plastid *rpl*32-*trn*$L_{(UAG)}$ sequences.

3.2. Morphological character evolution

The selected, traced characters on the BI tree gained from the combined dataset are presented in Figures 2–7. The patterns of homoplasy are shown using various colors.

4. Discussion

The present study indicates that spiny *Astragalus* forms a well-supported monophyletic group, which is generally consistent with findings of previous works (Kazempour Osaloo et al., 2003, 2005; Wojciechowski et al., 1999; Wojciechowski, 2005). This clade comprises members of 4 traditional subgenera of *Astragalus*: *Phaca* (L.) Bunge, *Calycophysa* Bunge, *Cercidotrix* Bunge, and *Tragacantha* Bunge (Bunge, 1868, 1869; Maassoumi, 1998). Thus, the monophyly of the ingroup is corroborated by our analyses. The following morphological features characterize the spiny *Astragalus*: mostly cushion-forming habit with paripinnate or imparipinnate persistent rachis, 1–4 seed pods, and adnation of wing and keel claws to the staminal tube to different extents (Bunge, 1868; Maassoumi, 1989, 1995, 2000; Zarre-Mobarakeh, 2000). *Astragalus horridus* (sect. *Acanthophace*) is sister to a large polytomy of the remaining spiny species, confirming its basal position as suggested in previous studies (Zarre and Podlech,

Figure 4. Evolutionary history of character 3 (calyx shape) mapped on the Bayesian tree obtained from the combined nr DNA ITS and plastid *rpl*32-*trn*L$_{(UAG)}$ sequences.

2001b; Zarre, 2003). The most important synapomorphy characterizing this sister clade is the presence of unilocular pods, which are otherwise bilocular in sect. *Acanthophace*.

Our phylogenetic analyses showed that many of the multispecific spiny sections analyzed herein, including the sections *Rhacophorus*, *Poterion*, *Campylanthus*, *Microphysa, Adiaspastus*, *Hymenostegis*, and *Anthylloidei*, are nonmonophyletic. As the focus of this paper, we first discuss the phylogenetic status and interrelationship of sect. *Anthylloidei* with its closest allies in detail below. Then we focus on the evolution of morphological characters and biogeography in the section. A brief note on possible taxonomic implications of our data is also presented.

4.1. Phylogenetic relationships

Astragalus sect. *Anthylloidei* has been considered one of the most complicated sections of spiny *Astragalus*, and its relationship with relatives was uncertain (Tietz and Zarre, 1994; Maassoumi, 1995). As noted above, the current

Figure 5. Evolutionary history of character 4 (hair size) mapped on the Bayesian tree obtained from the combined nr DNA ITS and plastid *rpl*32-*trn*L$_{(UAG)}$ sequences.

status of the section is not monophyletic. Here, we discuss this in light of the tree obtained from the combined analysis (Figure 1). The members of the section are scattered across the tree in 4 well-supported clades (B, C1, C2, and D) and intermixed with members of other allied sections (Figure 1).

Clade B contains 7 members of sect. *Anthylloidei* and 6 representatives of sections *Poterion, Campylanthus*, and *Microphysa*. The members of this clade are distinct in having a standard petal rounded at the base (Podlech and Zarre, 2013). Within this clade, 3 members of sect. *Anthylloidei*, i.e. *Astragalus remotiflorus* Boiss., *A. bodeanus* Fisch., and *A. murinus* Boiss., are closely related taxa, whereas, the other 4 species, i.e. *A. coluteopsis* Parsa, *A. lalesarensis*, *A. ghashghaicus* Tietz & Zarre, and *A. tortuosus*, group together in a polytomy (see also below).

Clade C1 contains 5 species of sect. *Anthylloidei*, i.e. *Astragalus crassispinus* Bunge, *A. khoshjailensis*, *A. raddei* Basil., *A. lumsdenianus* Aitch. & Baker, and *A. keratensis*

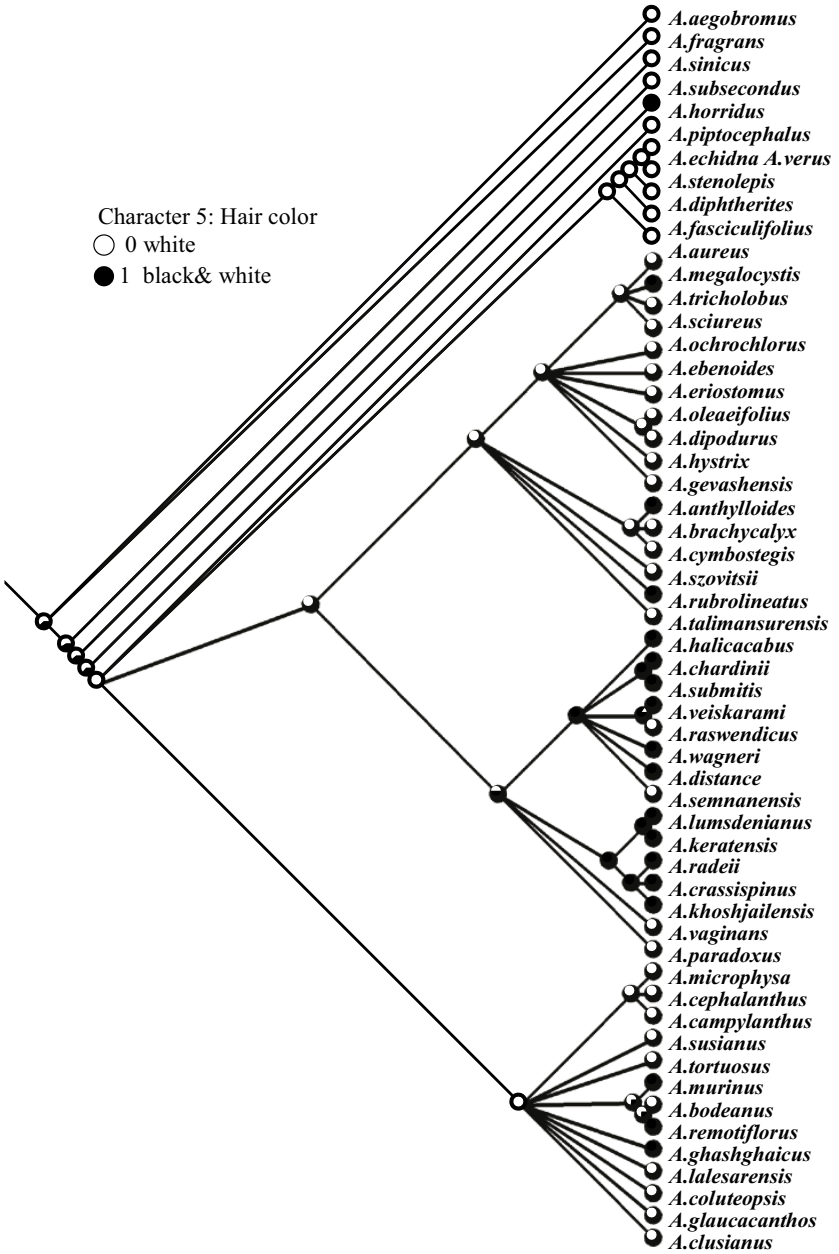

Figure 6. Evolutionary history of character 5 (hair color) mapped on the Bayesian tree obtained from the combined nr DNA ITS and plastid *rpl*32-*trn*$L_{(UAG)}$ sequences.

Bunge. These species do share several features including yellowish petals, auriculate standard at the base and keel blades with sigmoid/convex upper edge, densely multiflowered inflorescences, as well as a papery calyx with parallel nerves (except for *A. khoshjailensis*) (Podlech and Zarre, 2013). Seven other species of the section, including *A. wagneri* Bartl. ex Bunge; *A. distans* Fisch.; *A. halicacabus* Lam.; *A. raswendicus* Hausskn. & Bornm.; *A. veiskaramii* Zarre, Podlech & T.Sabaii; *A. submitis* Boiss. & Hohen.; and *A. chardinii* Boiss. (sect. *Anthylloidei*) as well as *A. semnanensis* (sect. *Semnanenses*), make a well-supported monophyletic clade (C2, PP = 1.00; Figure 1). All these taxa have a rounded standard without an auricle at the base (except *A. wagneri*) and an inflated calyx (except *A. semnanensis*) (Podlech et al., 2001; Podlech and Zarre, 2013). *Astragalus semnanensis*, a local endemic to gypsy substratum, Sorkheh, Semnan province, Iran, was originally described from sect. *Leucocercis* (Rechinger, 1940). It clearly differs, according to the simple hairs and hairy standard, from the remaining species of the section.

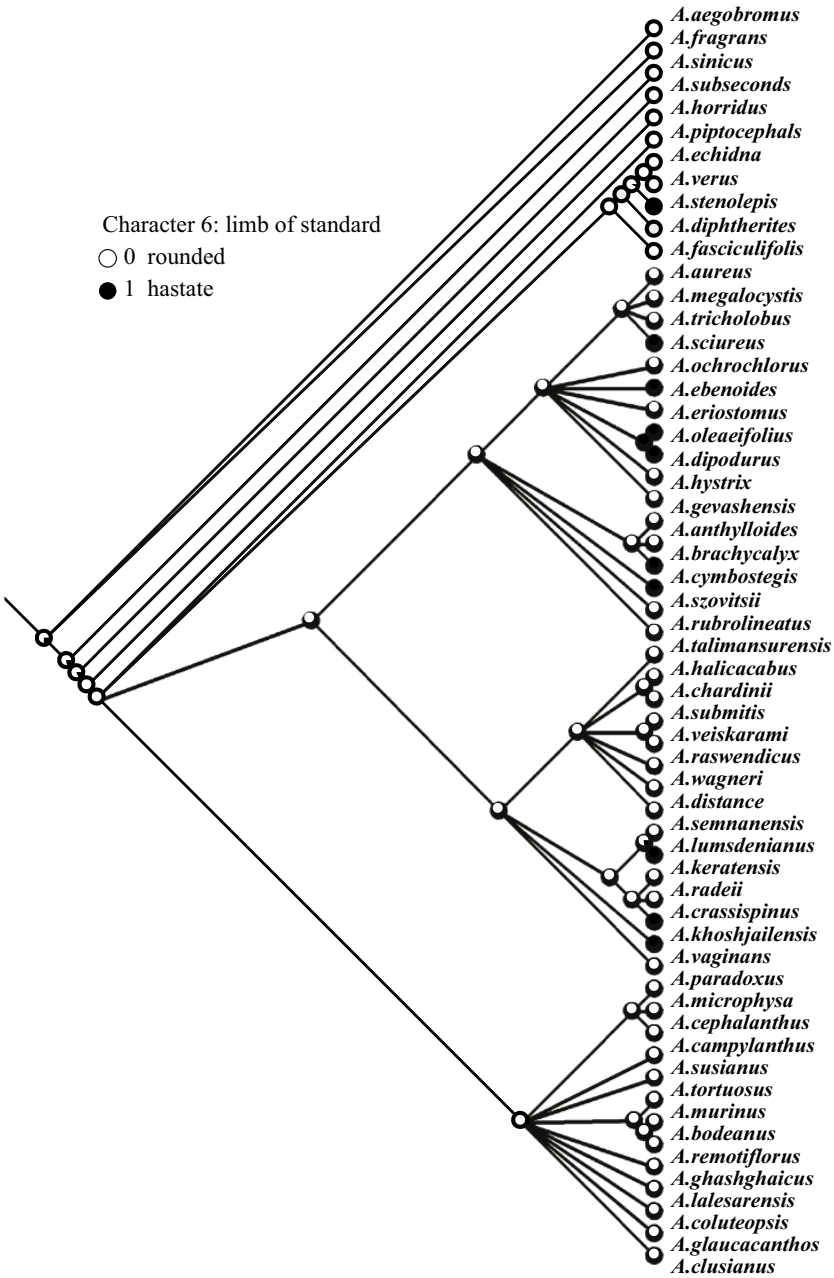

Figure 7. Evolutionary history of character 6 (limb of standard) mapped on the Bayesian tree obtained from the combined nr DNA ITS and plastid *rpl*32-*trn*$L_{(UAG)}$ sequences.

Simultaneously, Podlech et al. (2001) and Zarre and Podlech (2001a) placed this species in the new monotypic sect. *Semnanenses*. Zarre and Podlech (2001a), stating that based on morphological and micromorphological features this species is intermediate between sections *Acanthophace* and *Anthylloidei* (=sect. *Megalocystis*). Nevertheless, our data clearly placed it in a subclade of sect. *Anthylloidei* within clade C2. *Astragalus vaginans* DC. (sect. *Hymenostegis* subsect. *Hymenocoleus*) and *A. paradoxus* Bunge (sect. *Macrosemium*) are distinct lineages. The relatedness of these 2 taxa with sect. *Anthylloidei* has not been noted in previous studies (Tietz and Zarre, 1994; Maasoumi, 1995).

Five other species of sect. *Anthylloidei* are nested in clade D. *Astragalus rubrolineatus* is sister to the remaining species of the clade. *A. szovitsii* Fisch. & C.A.Mey., *A. anthylloides*, *A. ebenoides* Boiss., and *A. megalocystis* Bunge are distinct from each other within this clade. Surprisingly,

A. anthylloides, the lectotype of sect. *Anthylloidei* (Podlech et al., 2001), has no relative from its own section. Instead, it is well allied with *A. cymbostegis* Bunge (sect. *Rhacophorus*) and *A. brachycalyx* Fisch. ex Boiss. (sect. *Adiaspastus*). The only character linking *A. anthylloides* with both *A. cymbostegis* and *A. brachycalyx* is inclusion of the pod within the calyx (Podlech and Zarre, 2013).

As noted earlier, some authors (e.g., Maassoumi, 1995, 1998) classified *A. eriostomus* within sect. *Anthylloidei.* While it was originally placed in its own monotypic section, *Eriostoma* (Bornmüller, 1914, cited in Podlech et al., 2001), *Astragalus eriostomus* is distinguished from the members of sect. *Anthylloidei* in having emarginated leaflets and a glabrous to sparsely pilose calyx at the fruiting stage (Podlech et al., 2001; Podlech and Zarre, 2013). The present molecular data in corroboration with morphology confirm its sectional status distinct from sect. *Anthylloidei.*

Likewise, our analysis revealed that all of the multispecific, informal groups of sect. *Anthylloidei*, with the exception of the *A. murinus* group (Tietz and Zarre, 1994), are not monophyletic. The *A. murinus* group, composed of *A. murinus, A. bodeanus*, and *A. remotiflorus*, is characterized by leaflets of 10–20 pairs, hyaline membranous stipules, a calyx with parallel veins, standard petal rounded at the base, and pods dorsiventrally compressed. *Astragalus coluteoides* group comprises 4 species among which *A. coluteopsis* and *A. tortuosus*, analyzed herein, did not form a monophyletic group. Tietz and Zarre (1994) claimed that these 2 groups are closely related to members of sect. *Poterion* and named them the poterioid lineages. Although the 5 species of sect. *Anthylloidei* along with the 2 members of sect. *Poterion* (i.e. *A. clusianus* Soldano and *A. glaucacanthos* Fisch.) are nested within clade B, there is no direct link among them (Figure 1).

The *Astragalus szovitsii* group is characterized by small flowers, especially when addressing the calyx size, and includes 4 species (*A. keratensis*, *A. raddei*, *A. ebenoides*, and *A. szovitsii*) that were not retrieved in a single clade. The first 2 species were nested in a subclade (C1) with *A. crassispinus*, *A. khoshjailensis*, and *A. lumsdenianus*, whereas *A. ebenoides* and *A. szovitsii* were placed within clade D without a direct relationship. This group along with *A. lumsdenianus* was considered related to sect. *Microphysa*, the microphysoid lineage (Tietz and Zarre, 1994). However, our molecular data contradict this hypothesis. The *Astragalus megalocystis* group, sharing angular standards and represented by 3 species herein, did not form a single clade. One of its members, *A. ghashghaicus*, nested in clade B and is distantly related to *A. megalocystis* and *A. rubrolineatus.* Similarly the *Astragalus submitis* group with nonpungent or even nonindurated rachises as well as parallel calyx venation, includes *A. submitis*, *A. distans*, and *A. raswendicus* which did not retrieve a single clade, although these 3 species were nested within clade C2.

4.2. Character evolution

Most of the morphological features that have been used in delimitation of sections of spiny *Astragalus* show high levels of homoplasy (Zarre Mobarakeh, 2000). Our analysis indicates that only a few of these characters represent synapomorphy for the monophyletic groups on the molecular trees. Most of the characters traced diverged several times in the studied group. Our results suggest that morphology cannot elucidate infrageneric relationships in spiny *Astragalus* accurately. Evolutionary trends of some diagnostic morphological characters are discussed below (Figures 2–7).

Paripinnate versus imparipinnate leaves: Imparipinnate leaves appear multiple times in the tree. Within sect. *Anthylloidei* this trait occurs mainly in clade C1. However, 3 members of the clade, including *A. submitis*, *A. raswendicus*, and *A. distans* show paripinnate leaves. *A. ebenoides*, *A. anthylloides*, and *A. khoshjailensis* are other species of the section with imparipinnate leaves (Figure 2).

Peduncle length: Although all of the species of sect. *Anthylloidei* have a long peduncle (>3 cm), this trait has evolved several times in the tree (Figure 3).

Calyx shape: Mapping of this trait revealed that inflated calyx could occur in all of the spiny groups. Podlech (1982) suggested that this character could not determine the limitation of Bunge's subgenera. It also seems that it could not be used at a sectional level. This trait is a derived character state for the members of sect. *Anthylloidei*, in place of campanulate calyx; among outgroups that is the ancestral condition (Figure 4).

The size of calyx hairs: This feature shows some patterns of homoplasy, but it could describe some groups such as *A. murinus* informal group with medium hairs (1.5–3 mm). Medium hairs occurred in most members of spiny *Astragalus*, especially sect. *Anthylloidei*. However, this trait changed to short hairs (<1 mm) in clades B and C several times. Additionally, long hairs (>3 mm) occurred in clade D, with the exception of *A. megalocystis* and *A. eriostomus*. Short hairs should represent the most primitive character states for hair size, according to Zarre (2003) (Figure 5).

Hair color: In the micromorphological studies this character received a high weight and differs in some sections such as *Hymenostegis*, *Anthylloidei*, *Adiaspastus*, and *Rhacophorus* (Zarre, 2003), but our findings are incongruent with previous sectional circumscriptions. In the combined tree, 2 subclades of clade C show black and white hairs (except *A. raswendicus* and *A. semnanensis*, with only white hairs). White hair is the derived condition and is considered a synapomorphy for some groups including most species of clades B and D (Figure 6).

Standard base: The analyzed species mostly have a rounded standard limb that should be considered the ancestral condition. Hastate standard limb evolved several times in clades C and D, which contain *A. ebenoides*, *A. szovitsii*, *A. keratensis*, and *A. khoshjailensis* of sect. *Anthylloidei*. This could be considered a good character if we add more tragacanthic species (Figure 7).

4.3. Biogeography

Although the most tragacanthic species were not sampled and this study did not perform an unbiased biogeographic analysis, we can draw some conclusions about the biogeographic aspects of sect. *Anthylloidei*. As noted above, the members of sect. *Anthylloidei* are nested in 4 distinct clades (B, C1, C2, and D) which more or less match certain geographic distribution patterns (Figure 8). Clade B contains 7 species, mostly from the Zagros mountain range of Iran (NW and W Iran). Clade C1 includes 5 species is restricted to NE Iran, Turkmenistan, and Afghanistan. One of these 5 species, *A. khoshjailensis*, is endemic to Iran. The occurrence of these species at the easternmost range of the section indicates their unique and isolated position. The well-supported clade C2 shows an ambiguous and multiregional distribution pattern: *A. wagneri* and *A. chardinii* are restricted to NW Iran and E Turkey, *A. halicacabus* is endemic to Turkey, *A. veiskarami* and *A. raswendicus* are known from W Iran, *A. submitis* is distributed in N Iran (Alborz mountain range), and *A. distans* along with *A. semnanensis* are confined to central Iran. The distribution range of the members of clade D includes Turkey to N Iran along the Alborz mountain range: *A. ebenoides* is endemic to W Iran, *A. szovitsii* ranges from NW Iran to Turkey to Transcaucasia, *A. rubrolineatus* and *A. megalocystis* are both distributed in N Iran (eastern Alborz mountain range), and *A. anthylloides* is endemic to Turkey.

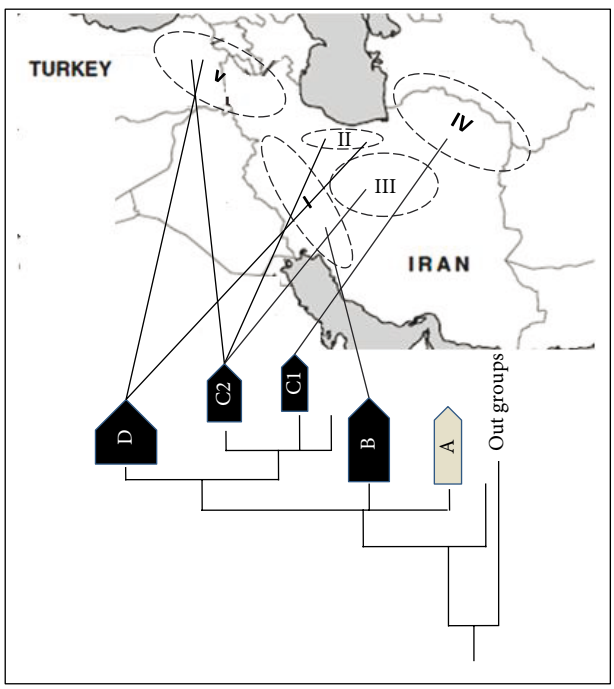

Figure 8. Distribution pattern of studied taxa in Iran. I: Zagros mountain range; II: Alborz mountain range (N. Iran); III: central Iran; IV: NE Iran; V: NW Iran and Turkey.

4.4. Taxonomic implications

Although our findings provide significant progress towards resolving the taxonomic problems of the heterogeneous sect. *Anthylloidei*, more exhaustive taxon sampling from other spiny sections and additional molecular sequence data are required to demonstrate the status of all species of sect. *Anthylloidei*. Nevertheless, a new taxonomic system for this group of species is needed. The present study suggests that sect. *Halicacabus*, which has been merged into sect. *Anthylloidei* (Maassoumi, 1995; Podlech et al., 2001; Podlech and Zarre, 2013), should be resurrected, since 8 species forming the clade C2 were united in a well-supported clade. Section *Semnanenses* should be reduced to synonymy of sect. *Halicacabus*. Maassoumi (2014) in *Flora of Iran* placed only *A. chardinii* and *A. veiskaramii* in sect. *Halicacabus*. The status of *A. anthylloides* (the lectotype of sect. *Anthylloidei*) indicates new circumscription for the section. More representatives from other spiny sections are required to determine the exact status of this species. *Astragalus megalocystis*, which is allied with several species from sections *Adiaspastus* and *Hymenostegis*, might be resurrected as sect. *Megalocystis* with new circumscription or merged into one of the allied sections. *Astragalus crassispinus*, *A. khoshjailensis*, *A. raddei*, *A. lumsdenianus*, and *A. keratensis* formed their own clade (C1) and probably represent a new section. For the remaining species of sect. *Anthylloidei* it is premature to suggest any taxonomic conclusions.

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