

Molecular systematics of the Old World *Astragalus* (Fabaceae) as inferred from nrDNA ITS sequence data

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Kazempour Osaloo, S. (Department of Plant Biology, Tarbiat Modares University, Tehran 14115-175, Iran; e-mail: skosaloo@yahoo.com), A. A. Maassoumi (Department of Botany, Research Institute of Forests and Rangelands, Tehran 13185-116, Iran) & N. Murakami (Department of Botany, Graduate School of Science, Kyoto University, Kyoto, 606-8502, Japan). Molecular systematics of the Old World *Astragalus* (Fabaceae) as inferred from nrDNA ITS sequence data. *Brittonia* 57: 367–381. 2005.—This study represents a nuclear rDNA ITS-based phylogenetic analyses of a greater sampling of the Old World *Astragalus* compared to our previous work (212 vs. 134 taxa). Phylogenetic relationships among 212 species (213 accessions) of the Old World *Astragalus*, including newly segregated monotypic genus *Podlechiella*, the two aneuploid New World *Astragalus*, and five related genera, were inferred from analyses of nuclear rDNA ITS sequences using maximum parsimony. A total of 658 nucleotide sites and four binary characters for indels were analyzed. The results of phylogenetic analyses suggest sect. *Phyllobium*, comprising mostly the Chinese species, is placed outside of the so-called *Astragalus* s. str. and is a well-supported monophyletic group. The monotypic annual segregate genus *Thlaspidium* (\equiv *Astragalus* sect. *Thlaspidium*, *A. thlaspi*), is clearly nested within *Astragalus* s. str. Among the many sections analyzed here, only sects. *Cenanthrum*, *Caraganella*, *Eremophysa*, *Incani*, *Laxiflori*, and *Lotidium* are strongly supported as monophyletic. Our analysis, in agreement with previous studies, shows that the North American euploid *Astragalus* species are scattered throughout the Old World groups of the genus.

Key words: *Astragalus*, Fabaceae, nrDNA ITS, *Phyllobium*, phylogeny, *Podlechiella*, *Thlaspidium*.

Astragalus L. (Fabaceae) is the largest genus of flowering plants, with some 3000 species. Almost all species are herbs, most are perennial, and none climb. The bulk of diversity in *Astragalus* is centered in southwestern and central Asia and there may well be more than 800 species in Iran alone (Lock & Simpson, 1991; Podlech, 1986, 1998, 1999; Maassoumi, 1998, 2000, unpubl. data, Maassoumi et al., 1999). Recently, Wojciechowski et al. (1999), Kazempour Osaloo et al. (2003) and Kang et al. (2003), using nuclear ribosomal DNA

internal transcribed spacer and 5.8S region sequences (nrDNA ITS) demonstrated that all but a dozen of species, including three annuals and eight perennials (taxonomic outliers), are united in a well-supported clade, so-called *Astragalus* s. str. Within this assemblage, the Old World species, mostly having euploid series of $n = 8, 16, 32, \dots$, form several successive clades. The New World aneuploids, known as “Neo-*Astragalus*,” are nested within one of these Old World clades, and they possess chromosome numbers of $n = 11, 12, 13, 14, \text{ or } 15$ (Wojciechowski et al., 1993,

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1999; Sanderson & Liston, 1995; Sanderson & Wojciechowski, 1996).

The results of these molecular phylogenetic studies showed that the traditional subgenera of *Astragalus* (*Epiglottis*, *Trimeniaeus*, *Phaca*, *Hypoglottis*, *Calycophyssa*, *Tragacantha*, *Cercidothrix*, *Calycocystis*, and *Pogonophace*), recognized by Bunge (1868, 1869) and later modified by Maassoumi (1998), are not monophyletic. Likewise, monophyly of Podlech's (1982, 1991, 1994) three new subgenera (*Trimeniaeus*, *Astragalus*, and *Cercidothrix*) as well as the recognition of *Astracantha* Podl. (Podlech, 1983) as separate from *Astragalus* were not supported (see detail discussion in Kazempour Osaloo et al., 2003). More recently, following the molecular phylogenetic data, the traditionally recognized subgenera and Podlech's subgenera along with the segregate genera *Barnebyella* Podl. and *Ophiocarpus* (Bunge) Ikonn were synonymized with *Astragalus* (Maassoumi, 2003). Our previous work (Kazempour Osaloo et al., 2003), however, indicated that a number of species-rich sections such as *Cenanthrum*, *Chronopus*, *Laxiflori*, *Lotidium*, *Incani*, and *Ammodendron* are monophyletic.

Based on nrDNA ITS and chloroplast gene *ndhF* sequences as well as morphological features, *Astragalus vogelii* (Webb) Bornm., one of the outliers, was elevated to the new generic rank, *Podlechiella* Maassoumi & Kazempour Osaloo and the other outlier, *A. sinicus* L., was nested within "Astragalus s. str. clade" (Kazempour Osaloo et al., 2003). Others of the annual monotypic segregate genera that were nested within *Astragalus* s. str. (Kazempour Osaloo et al., 2003) include *Barnebyella* (*B. calycina* (Stocks) Podl.; =*Astragalus migpo* Kamelin, Podlech 1994) and *Ophiocarpus* (*O. aitchisonii* (Baker) Podl.; =*A. ophiocarpus* Bunge, Ikonnikov 1977), along with peculiar annual species such as *A. dipelta* Bunge (= *Didymopelta*, see Hutchinson, 1964), *A. schmalhauseni* Bunge, and *A. vicarius* Lipsky (= *Sewerzowia*, see Hutchinson, 1964; Rassulova, 1978 cited in Podlech, 1994). *Thlaspidium* (Lipsky) Rassulova (*Th. thlaspi* (Lipsky) Rassulova; = *A. thlaspi* Lipsky, Rassulova, 1978 cited in

Podlech, 1994) was not sampled in our previous study.

This study represents a significant increase in sampling of the Old World species of *Astragalus* for nrDNA ITS sequencing (134 versus 212 taxa) compared to our previous work (Kazempour Osaloo et al., 2003), including a sampling from a few new groups (sections and the segregate genus *Thlaspidium*) from the Old World. We here reconstruct nrDNA ITS phylogeny to clarify the position of *Thlaspidium* and the placement of the North American euploid species of the Old World *Astragalus* and to test the monophyly and position of section *Phyllolobium* (which was first reported by Kang et al., 2003) and other sections that were not included in our previous paper.

Materials and Methods

DNA ISOLATION, PCR, AND SEQUENCING

Total genomic DNA was extracted from dry leaves from individual plants following the modified 2 × CTAB procedure of Doyle and Doyle (1987). nrDNA ITS sequences were obtained for 220 species (221 accessions). Eighty-one *Astragalus* species, including *Thlaspidium* (= *A. thlaspi*), were newly sampled for this study (see Table I for accession numbers and information on vouchers), and the other 139 taxa were the same ones included in our previous paper (Kazempour Osaloo et al., 2003). The PCR amplifications and nrDNA ITS sequencing followed methods described in Kazempour Osaloo et al. (2003).

PHYLOGENETIC ANALYSIS

Alignment of the nrDNA ITS sequences required the introduction of numerous single and multibase insertion/deletion events (indel). Only four indels ranging from 3 to 36 nucleotides were included as recoded binary characters in the nrDNA ITS data set; these represent cases in which alignment was absolutely unambiguous. *Caragana grandiflora* (M. B.) DC. and *Chesneya astragalina* Jaub. & Spach. were defined as outgroups according to previous studies (Sanderson and Wojciechowski, 1996; Wojciechowski et al., 1999; Kazempour Osa-

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TABLE I
Astragalus SPECIES INCLUDED IN THE PRESENT nrDNA ITS PHYLOGENETIC ANALYSES^a

Species	Section ^b	DNA source (location, voucher) ^c	GenBank accession no.
<i>A. chartostegius</i> Boiss.	<i>Acidodes</i>	Iran: <i>Assadi & Mozaffarian 31416</i> (TARI)	AB231083
<i>A. icmadophilus</i> Hand.-Mazz.	<i>Acidodes</i>	Iran: <i>Zehzad 2718</i> (TARI)	AB231084
<i>A. leiophyllus</i> Freyn & Bornm.	<i>Acidodes</i>	Iran: <i>Maassoumi & Mozaffarian 78587</i> (TARI)	AB231085
<i>A. ochrochlorus</i> Boiss. & Hohen.	<i>Acidodes</i>	Iran: <i>Shahsavari 69760</i> (TARI)	AB231086
<i>A. hololeios</i> Bornm.	<i>Aegacantha</i>	Afghanistan: <i>Rechinger 17007</i> (TARI)	AB231087
<i>A. tarumensis</i> Sirj. & Rech. f.	<i>Ammodendron</i>	Iran: <i>Wendelbo & Foroughi 15851</i> (TARI)	AB231088
<i>A. cymbicarpos</i> Brot.	<i>Bucerates</i>	Spain: <i>W & S 410</i> (ARIZ)	AF121678
<i>A. pellitus</i> Bunge	<i>Caprini</i>	Iran: <i>Assadi & Maassoumi 55884</i> (TARI)	AB231089
<i>A. koschukensis</i> Boiss.	<i>Caraganella</i>	Pakistan: <i>Anonymous 29071</i> (TARI)	AB231090
<i>A. aksuensis</i> Bunge	<i>Cenanthrum</i>	Former USSR: <i>Anonymous 108</i> (TARI)	AB231091
<i>A. americanus</i> (Hook.) Jones	<i>Cenanthrum</i>	USA: <i>Nelson 6870</i> (RM)	U50492, U50493 ^d
<i>A. frigidus</i> (L.) Gray	<i>Cenanthrum</i>	Former USSR: <i>Anonymous 5732</i> (TARI)	AB231092
<i>A. coluteocarpus</i> Boiss.	<i>Chlorostachys</i>	Afghanistan: <i>Rechinger s.n.</i> (TARI)	AB231093
<i>A. dshimensis</i> Gontsch.	<i>Chlorostachys</i> (<i>Sesbanella</i>)	China: <i>Dong et al. 99-04-438</i> (DBHKUST)	AF359755 ^d
<i>A. hoantchy</i> Franch.	<i>Chlorostachys</i> (<i>Sesbanella</i>)	China: <i>Kang 01-01</i> (PE)	AF521952 ^d
<i>A. austriacus</i> L.	<i>Craccina</i>	Former USSR: <i>Anonymous 313</i> (TARI)	AB231094
<i>A. xanthomeloides</i> Korovin & Popov	<i>Cysticalyx</i>	Former USSR: <i>Anonymous 4476</i> (TARI)	AB231095
<i>A. ruscifolius</i> Boiss.	<i>Dissitiflori</i>	Iran: <i>Mozaffarian 54348</i> (TARI)	AB231096
<i>A. kahircicus</i> DC.	<i>Eremophysa</i>	Iran: <i>Mozaffarian 67574</i> (TARI)	AB231097
<i>A. lehmannianus</i> Bunge	<i>Eremophysa</i>	China: <i>Dong et al. 99-08-103</i> (DBHKUST)	AF359756 ^d
<i>A. catacamptus</i> Bunge	<i>Erioceras</i>	Iran: <i>Wendelbo et al. 11165</i> (TARI)	AB231098
<i>A. daenensis</i> Boiss.	<i>Hemiphaca</i>	Iran: <i>Mozaffarian 58171</i> (TARI)	AB231099
<i>A. penetratus</i> Maassoumi	<i>Hemiphaca</i>	Iran: <i>Wendelbo & Foroughi 17061</i> (TARI)	AB231100
<i>A. campylotrichus</i> Bunge	<i>Heterodontus</i>	Iran: <i>Assadi & Maassoumi 50864</i> (TARI)	AB231101
<i>A. glumaceus</i> Boiss.	<i>Hymenostegis</i>	Iran: <i>Mozaffarian 65006</i> (TARI)	AB231102
<i>A. hymenostegis</i> Fisch. & C.A. Meyer	<i>Hymenostegis</i>	Iran: <i>Maassoumi 64887</i> (TARI)	AB231103
<i>A. kohrudicus</i> Bunge	<i>Hymenostegis</i>	Iran: <i>Pabot 28694</i> (TARI)	AB231104
<i>A. leptynticus</i> Maassoumi	<i>Hymenostegis</i>	Iran: <i>Assadi & Mozaffarian 35301</i> (TARI)	AB231105
<i>A. paralurges</i> Bunge	<i>Hymenostegis</i>	Iran: <i>Fattahi & Khaledian 1221</i> (TARI)	AB231106
<i>A. recognitus</i> Fisch.	<i>Hymenostegis</i>	Iran: <i>Mozaffarian & Nowroozi 34544</i> (TARI)	AB231107
<i>A. sciureus</i> Boiss. & Hohen.	<i>Hymenostegis</i>	Iran: <i>Mirfakhraiy 15594</i> (TARI)	AB231108
<i>A. agrestis</i> Dougl. ex G. Don.	<i>Hypoglottidei</i>	USA: <i>Sanderson 917</i> (ARIZ)	L10758, L10759 ^d
<i>A. atricapillus</i> Bornm.	<i>Hypoglottidei</i>	Iran: <i>Assadi & Mozaffarian 40879</i> (TARI)	AB231109
<i>A. cicer</i> L.	<i>Hypoglottidei</i>	Iran (adventive): <i>Maassoumi 5076</i> (TARI)	AB231110
<i>A. glaux</i> L.	<i>Hypoglottidei</i>	Morocco: <i>Podlech 46544</i> (TARI)	AB231111
<i>A. hystrix</i> Bunge	<i>Hystrix</i>	Iran: <i>Youssefy 1101</i> (TARI)	AB231112

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Species	Section ^a	DNA source (location, voucher) ^b	GenBank accession no.
<i>A. yueksekovae</i> Matthews ^c	<i>Hystrix</i>	Iran: <i>Maassoumi & Mozaffarian</i> 78604 (TARI)	AB052014
<i>A. askius</i> Bunge	<i>Incani</i>	Iran: <i>Riazi</i> 6344 (TARI)	AB231113
<i>A. cyclophyllon</i> G. Beck	<i>Incani</i>	Iran: <i>Assadi</i> 60580 (TARI)	AB231114
<i>A. refractus</i> C.A. Meyer	<i>Incani</i>	Iran: <i>Maassoumi & Abouhamzeh</i> 56920 (TARI)	AB231115
<i>A. supervisus</i> Sheld.	<i>Incani</i>	Iran: <i>Wendelbo et al.</i> 10844 (TARI)	AB231116
<i>A. alpinus</i> L.	<i>Komaroviella</i>	USA: <i>W & S</i> 183 (ARIZ)	L10760, L10761 ^d
<i>A. pseudonobilis</i> Bunge ex Fedtsch.	<i>Laguropsis</i>	Former USSR: <i>Anonymous s.n.</i> (TARI)	AB231117
<i>A. semnanensis</i> Bornm. & Rech. f.	<i>Leucocercis</i> (<i>Semnanenses</i>)	Iran: <i>Mozaffarian</i> 58865 (TARI)	AB231118
<i>A. talimansurensis</i> Sirj. & Rech. f.	<i>Leucocercis</i>	Iran: <i>Assadi & Abouhamzeh</i> 38835 (TARI)	AB231119
<i>A. krauseanu</i> Regel	<i>Macrocystis</i>	Former USSR: <i>Anonymous s.n.</i> (TARI)	AB231120
<i>A. neurophyllus</i> Franch.	<i>Macrocystis</i>	Former USSR: <i>Anonymous</i> 6225 (TARI)	AB231121
<i>A. anserinaefolius</i> Boiss.	<i>Malacothrix</i>	Iran: <i>Assadi</i> 23234 (TARI)	AB231122
<i>A. laristanicus</i> Bornm. & Gauba	<i>Malacothrix</i>	Iran: <i>Foroughi</i> 10790 (TARI)	AB231123
<i>A. macrourus</i> Fisch. & C.A. Meyer	<i>Malacothrix</i>	Iran: <i>Maassoumi & Mirhosseini</i> 59390 (TARI)	AB231124
<i>A. pauperiflorus</i> Bornm.	<i>Malacothrix</i>	Iran: <i>Riazi</i> 7496 (TARI)	AB231125
<i>A. podocarpus</i> C.A. Meyer	<i>Malacothrix</i>	Iran: <i>Mozaffarian & Maassoumi</i> 78537 (TARI)	AB231126
<i>A. callistachys</i> Buhse	<i>Microphysa</i>	Iran: <i>Zarre</i> 69594 (TARI)	AB231127
<i>A. adsurgens</i> Pallas	<i>Onobrychoidei</i>	China: <i>W & S</i> 267 (ARIZ)	AF121674
<i>A. brevidens</i> Freyn & Sint.	<i>Onobrychoidei</i>	Iran: <i>Mozaffarian</i> 48723 (TARI)	AB231128
<i>A. lilacinus</i> Boiss.	<i>Onobrychoidei</i>	Iran: <i>Assadi & Mozaffarian</i> 33157 (TARI)	AB231129
<i>A. glochideus</i> Boriss.	<i>Ornithopodium</i>	Iran: <i>Maassoumi & Abouhamzeh</i> 56871 (TARI)	AB231130
<i>A. stevenianus</i> DC.	<i>Ornithopodium</i>	Iran: <i>Wendelbo & Assadi</i> 28016 (TARI)	AB231131
<i>A. complanatus</i> R.Br. ex Bunge	<i>Phyllolobium</i> (<i>Phyllolobium</i>)	China: <i>Kang</i> 01-03 (PE)	AF521950 ^d
<i>A. balfourianus</i> Simps.	<i>Phyllolobium</i> (<i>Bibracteola</i>)	China: <i>Zhang</i> 99-303 (PE)	AF521951 ^d
<i>A. tribulifolius</i> Benth. ex Bunge	<i>Phyllolobium</i> (<i>Bibracteola</i>)	China: <i>Qin et al.</i> 608 (PE)	AF521953 ^d
<i>A. milingensis</i> Ni & Li	<i>Phyllolobium</i> (<i>Bibracteola</i>)	China: <i>Qin et al.</i> 608 (PE)	AF521954 ^d
<i>A. yatungensis</i> Ni & Li	<i>Phyllolobium</i> (<i>Bibracteola</i>)	China: <i>Qin et al.</i> 701 (PE)	AF521955 ^d
<i>A. hendersonii</i> Baker	<i>Phyllolobium</i> (<i>Trichostylus</i>)	China: <i>Qin et al.</i> 644 (PE)	AF521957 ^d
<i>A. plagiophacos</i> Maassoumi & Podl.	<i>Plagiophaca</i>	Iran: <i>Assadi & Mozaffarian</i> 40861 (TARI)	AB231132
<i>A. echidna</i> Bunge	<i>Platonychium</i>	Iran: <i>Maassoumi & Zarre</i> 71958 (TARI)	AB231133
<i>A. gummifer</i> Labill.	<i>Platonychium</i>	Iran: <i>Maassoumi</i> 76792 (TARI)	AB231134
<i>A. leucocephalus</i> Graham ex Benth	<i>Poliothrix</i>	Pakistan: <i>Anonymous</i> 30600 (TARI)	AB231135
<i>A. compactus</i> Lam.	<i>Rhacophorus</i>	Iran: <i>Mozaffarian & Maassoumi</i> 47789 (TARI)	AB231136
<i>A. eriosphaerus</i> Boiss.	<i>Rhacophorus</i>	Iran: <i>Maassoumi & Mozaffarian</i> 78563 (TARI)	AB231137
<i>A. gossypinus</i> Fisch.	<i>Rhacophorus</i>	Iran: <i>Maassoumi</i> 50973 (TARI)	AB231138

TABLE I
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Species	Section ^b	DNA source (location, voucher) ^c	GenBank accession no.
<i>A. meyeri</i> Boiss.	<i>Rhacophorus</i>	Iran: <i>Maassoumi & Abouhamzeh</i> 56950 (TARI)	AB231139
<i>A. paralipomenus</i> Bunge	<i>Rhacophorus</i>	Iran: <i>Maassoumi</i> 78617 (TARI)	AB231140
<i>A. pycnocephalus</i> Fisch.	<i>Rhacophorus</i>	Iran: <i>Maassoumi & Mozaffarian</i> 78615 (TARI)	AB231141
<i>A. saxifractor</i> Rech. f. & Gilli	<i>Saxifractor</i>	Afghanistan: <i>Rechinger</i> 34357 (TARI)	AB231142
<i>A. kerkukiensis</i> Bornm.	<i>Sesamei</i>	Iran: <i>Riazi</i> 9112 (TARI)	AB231143
<i>A. floccosus</i> Boiss.	<i>Stenonychium</i>	Iran: <i>Mozaffarian & Maassoumi</i> 47704 (TARI)	AB231144
<i>A. sphaeranthus</i> Boiss.	<i>Stereothrix</i>	Iran: <i>Riazi</i> 10230 (TARI)	AB231145
<i>A. ulodjensis</i> Sirj. & Rech. f.	<i>Stereothrix</i>	Iran: <i>Babakhanlou & Amin</i> 15417 (TARI)	AB231146
<i>A. depressus</i> L.	<i>Tapinodes</i>	Germany: <i>Podlech</i> 39745 (TARI)	AB231147
<i>A. botryophorus</i> Maassoumi & Podl.	<i>Theiochrus</i>	Iran: <i>Maassoumi</i> 59350 (TARI)	AB231148
<i>A. thlaspi</i> Lipski = <i>Thlaspidium thlaspi</i> (Lipski) Rassulova	<i>Thlaspidium</i>	Tadzhikstan: <i>Anonymous s.n.</i> (TARI)	AB231149

^a Information on the remaining taxa analyzed here can be found in Kazempour Osaloo et al. (2003).

^b Section names for *Astragalus* species follow the treatments of Maassoumi (1998, 2000), Maassoumi et al. (1999), and Podlech (1999); those in parenthesis are based on Fu (1993) and Zarre and Podlech (2001).

^c Abbreviations used in DNA source information: ARIZ, University of Arizona Herbarium, Tucson; DBHKUST, Department of Biology, The Hong Kong University of Science and Technology; PE, Herbarium of Institute of Botany, the Chinese Academy of Sciences, Beijing; TARI, Herbarium of the Research Institute of Forests and Rangelands, Tehran; W & S, Wojciechowski & Sanderson.

^d nrDNA ITS sequences for these taxa obtained from GenBank [DNA Data Bank of Japan (DDBJ)].

^e The voucher specimen for *A. yueksekovae* Matthews was identified as *A. hystrix* Bunge in Kazempour Osaloo et al. (2003).

loo et al., 2003). The ingroup contained two *Oxytropis* species, *Colutea persica* Boiss., *Podlechiella vogelii* (Webb) Maassoumi & Kazempour Osaloo (= *A. vogelii* (Webb) Bornm.), *Biserrula pelecinus* L. (= *A. pelecinus* (L.) Barneby, sect. *Biserrula*), and 213 *Astragalus* species including segregate annual genera.

The data were analyzed in PAUP* 4.0b10 (Swofford, 2002) using maximum parsimony (MP). The heuristic MP search employed random addition of sequences for 1000 replicates with tree-bisection-reconnection (TBR) branch swapping on 10 trees per replicate with the maximum number of saved trees set at 30,000. Then, a reverse-constraint analysis with the strict consensus of the 30,000 shortest trees saved was run, using the same search strategy of the unconstrained analysis, as suggested by Catalán et al. (1997) and Downie et al. (1998). That is, PAUP* searched for the shortest trees that were not congruent with the strict consensus of the 30,000 trees saved in the

first search. No additional trees were found at the length of the initial 30,000 trees, which suggests that the strict consensus tree adequately summarizes the available evidence, even though the exact number of trees at that length is not known. Bootstrap values were calculated as measures of branch support (Felsenstein, 1985) using 1000 replicates with a simple addition sequence, nearest-neighbor interchange (NNI) branch swapping, and the maximum number of saved trees set at 500.

Results

The nrDNA ITS data set comprised 654 nucleotide sites, 211 (32.3%) of which were parsimony informative. The length of the nrDNA ITS region ranged from 567 to 613 base pairs. We included four informative indel characters in the nrDNA ITS data set (numbers 655–658 in data set). MP analysis of the full nrDNA ITS data set, including indel characters, resulted in 30,000 equally

parsimonious trees of 994 steps (CI = 0.497, RI = 0.846, RC = 0.420). The strict consensus of 30,000 of these trees, with accompanying bootstrap values, is presented in Figure 1. The nrDNA ITS data reveal that *Astragalus* species are assembled in three well-supported clades (Fig. 1), which include (1) the so-called "*Astragalus* s. str. clade," comprising the vast majority of the species, (2) clade "J" composed of six species of sect. *Phyllobium*, and (3) clade "K" containing *A. epiglottis* L. (sect. *Epi-glottis*) and *A. annularis* Forssk. (sect. *Annulares*) plus *Biserrula pelecinus*. *Podlechiella vogelii* and *Colutea persica* Boiss. form a grade, within which sect. *Phyllobium* (clade "J") is derived. The two species of *Oxytropis* (*O. szovitsii* Boiss. & Buhse and *O. aucheri* Boiss.) form their own clade. Relationships between *Oxytropis*, clade "K," and an assemblage of *Phyllobium-P. vogelii-C. persica* are not resolved; however, this entire clade is sister group to *Astragalus* s. str.

Astragalus s. str. is composed of two large clades. One clade (clade "A"), which comprises solely perennial species, is strongly supported (97% bootstrap). This clade and remaining ones (clades "B," "C," "D," "E," "F," and "H"), with the exception of "G1," "G2," and "I," correspond to those identified in our previous nrDNA ITS study (Kazempour Osaloo et al., 2003). Clades "G1" and "G2" are the relabeling of subclades "G1," "G2," "G3," and "G4" of clade "G" identified in our previous study, and clade "I" is identified here. Relationships within large clade "A" (containing 38 species, of which eleven species were newly sampled) almost the same that indicated in our previous analysis. Three of the 11 species are *A. aksuensis* Bunge, *A. americanus* (Hook.) Jones, and *A. frigidus* (L.) Gray, belonging to the well-resolved and well-supported (96% bootstrap) monophyletic sect. *Cenanthrum*, consistent with Wojciechowski et al. (1999) and Dong et al. (2003). *Astragalus koschukensis* Boiss. is strongly allied with *A. stoksii* Benth. ex Bunge both of sect. *Caraganella*. *Astragalus hoantchy* Franch. and *A. dshimensis* Gontsch. form a clade, which is in turn sister to *A. chinensis*

L. f. of sect. *Nuculiella*. *Astragalus coluteocarpus* is sister to an assemblage containing members of sects. *Pelta*, *Aegacantha*, *Pendulina*, and *Caprini*. *Astragalus hololeios* Bornm. (of *Aegacantha*) and *A. pellitus* Bunge (of *Caprini*) are newly sampled species whose relationships are unresolved. Finally, *A. kahiricus* DC., *A. lehmannianus* Bunge, and *A. chiwensis* Bunge (all of sect. *Eremophysa*) form a well-supported clade (92% bootstrap) that is marked by a four-base indel in ITS1.

The second clade is a larger assemblage with a trichotomy among three moderately to well-supported clades ["B" (69%), "C" (85%), and "D" (55% bootstrap)] of both annual and perennial species. Clade "B" includes, in addition to 12 species from our previous study, three newly sampled species (*Astragalus botryophorus* Maassoumi & Podl., *A. kerkukiensis* Bornm. and *Thlaspidium thlaspi* = *A. thlaspi*). *Astragalus botryophorus*, along with *A. siliquosus* Boiss. (both of sect. *Theiochrus*) and *A. zerdanus* Boiss. (of sect. *Hemiphaca*), make up a strongly supported trichotomy (95% bootstrap), which is the sister group of annual species *A. campylorrhynchus* Fisch. & Meyer (sect. *Annulares*). *Thlaspidium thlaspi* is unresolved and part of a weakly supported polytomy and *A. kerkukiensis* (sect. *Sesamei*) is a branch of the next polytomy containing its consectional taxa (*A. coronilla* Bunge and *A. persepolitans* Boiss.) and some members of sect. *Oxyglottis*. Clade "C" contains 10 species, of which *A. depressus* L. (sect. *Tapinodes*) and *A. cymbicarpos* Brot. (sect. *Bucerates*) were newly sampled. The former species is a branch of a moderately supported trichotomy (73% bootstrap) and the latter one is allied with *A. hamosus* L. Clade "D" is the largest assemblage of *Astragalus* s. str. and comprises six clades: "E," "F," "G1," "G2," "H," "I," in addition to *A. leucocephalus* Graham ex Benth. (sect. *Poliothrix*). Clade "E" is sister group to the remaining clades and contains four species, including the newly-sampled circumboreal *A. alpinus* L. (sect. *Komaroviella*). Clade "H" has a high bootstrap value (100%), but in spite of adding four more species to this group (*A. askius* Bunge, *A. cyclophyllon*

Text continues on page 376

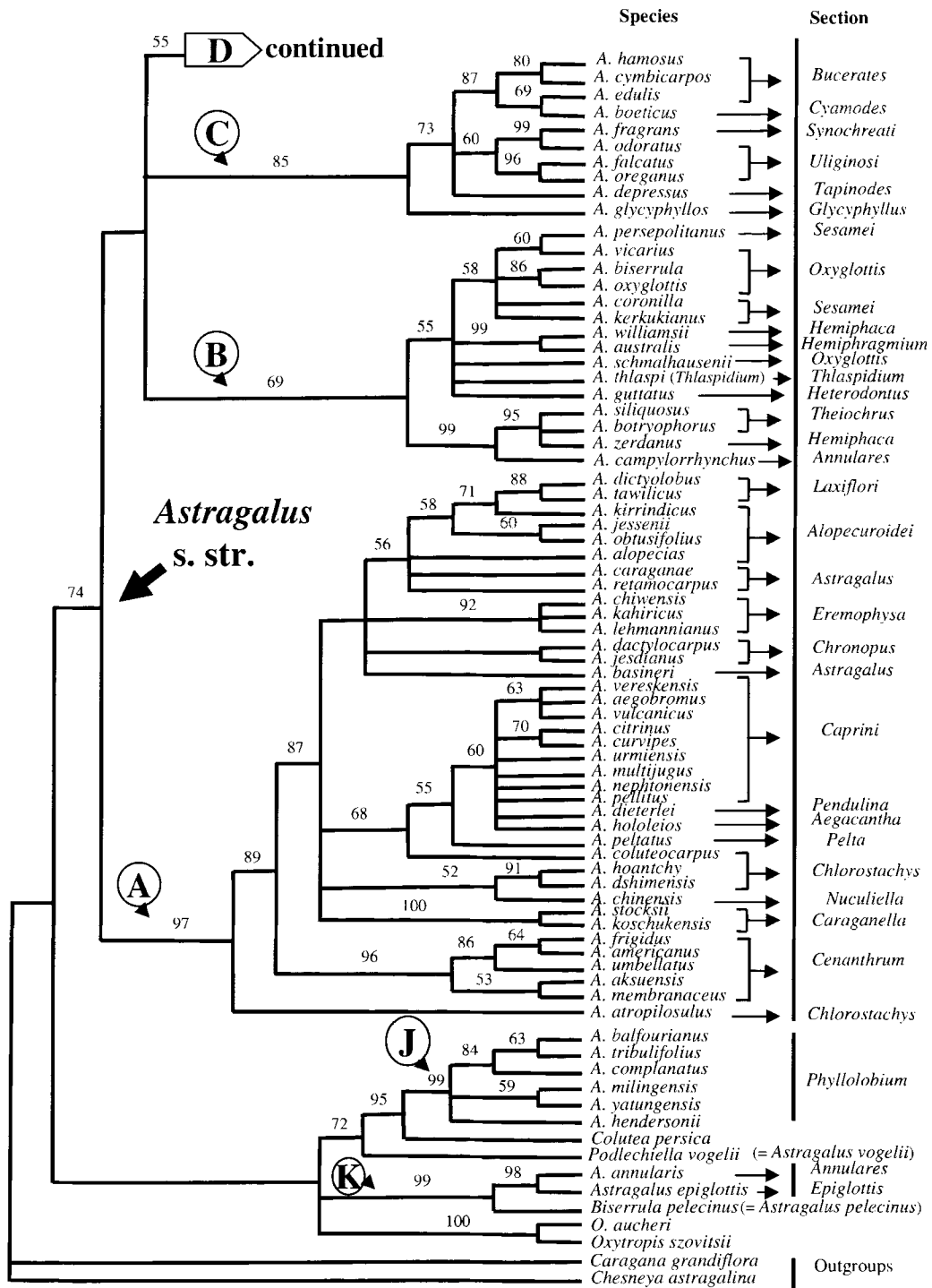


FIG. 1. Strict consensus of 30,000 most parsimonious trees resulting from phylogenetic analysis of 221 complete nrDNA ITS sequences for *Astragalus* and related genera (length = 994 steps, CI = 0.497, RI = 0.846, and RC = 0.420). Bootstrap values are indicated above branches for values >50%. Clades within and outside of *Astragalus* s. str. identified by letters "A"–"I" and "J"–"K," respectively, are discussed in the text. New World aneuploid *Astragalus* taxa (species and section) are indicated by asterisks.

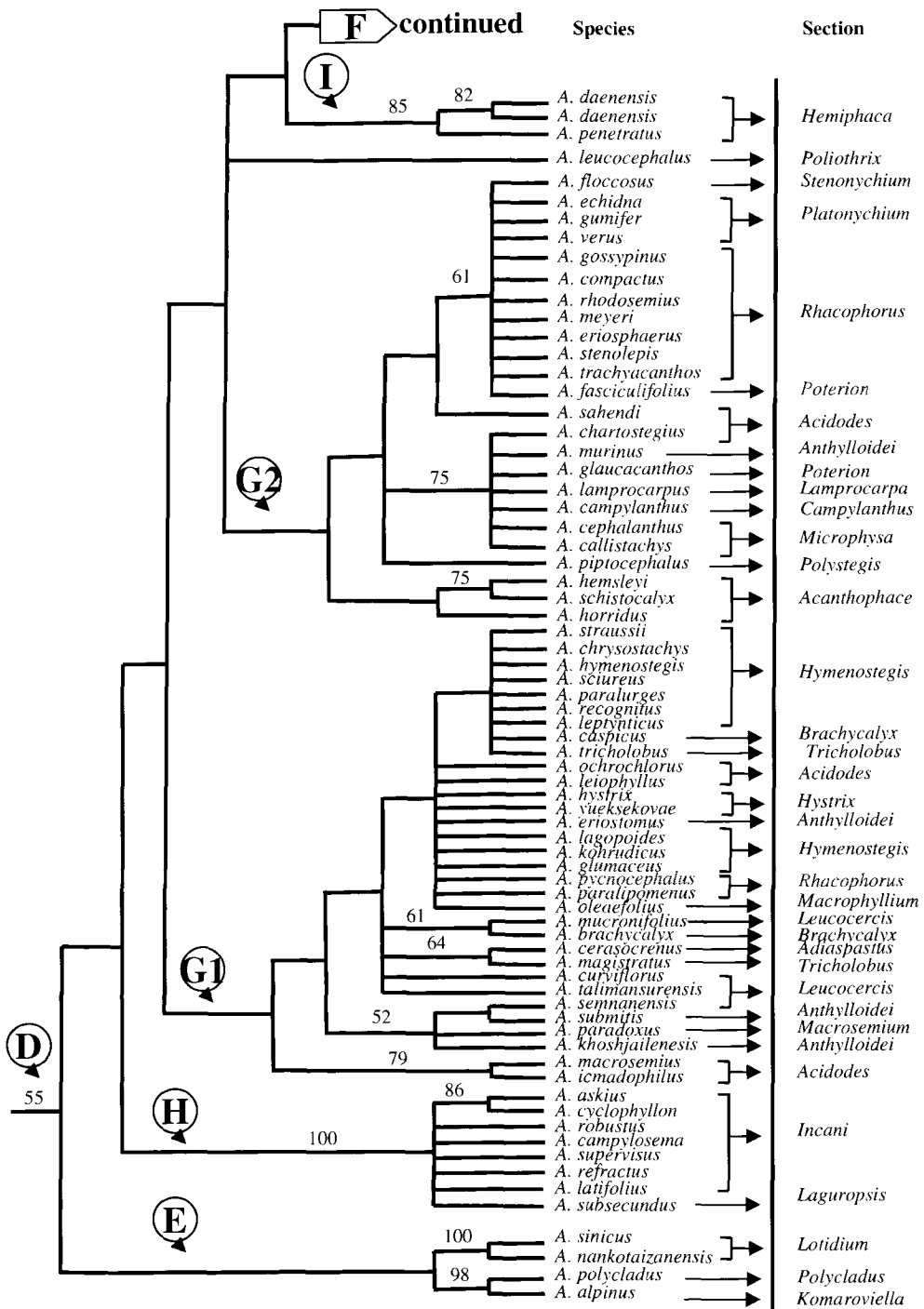


FIG. 1. Continued.

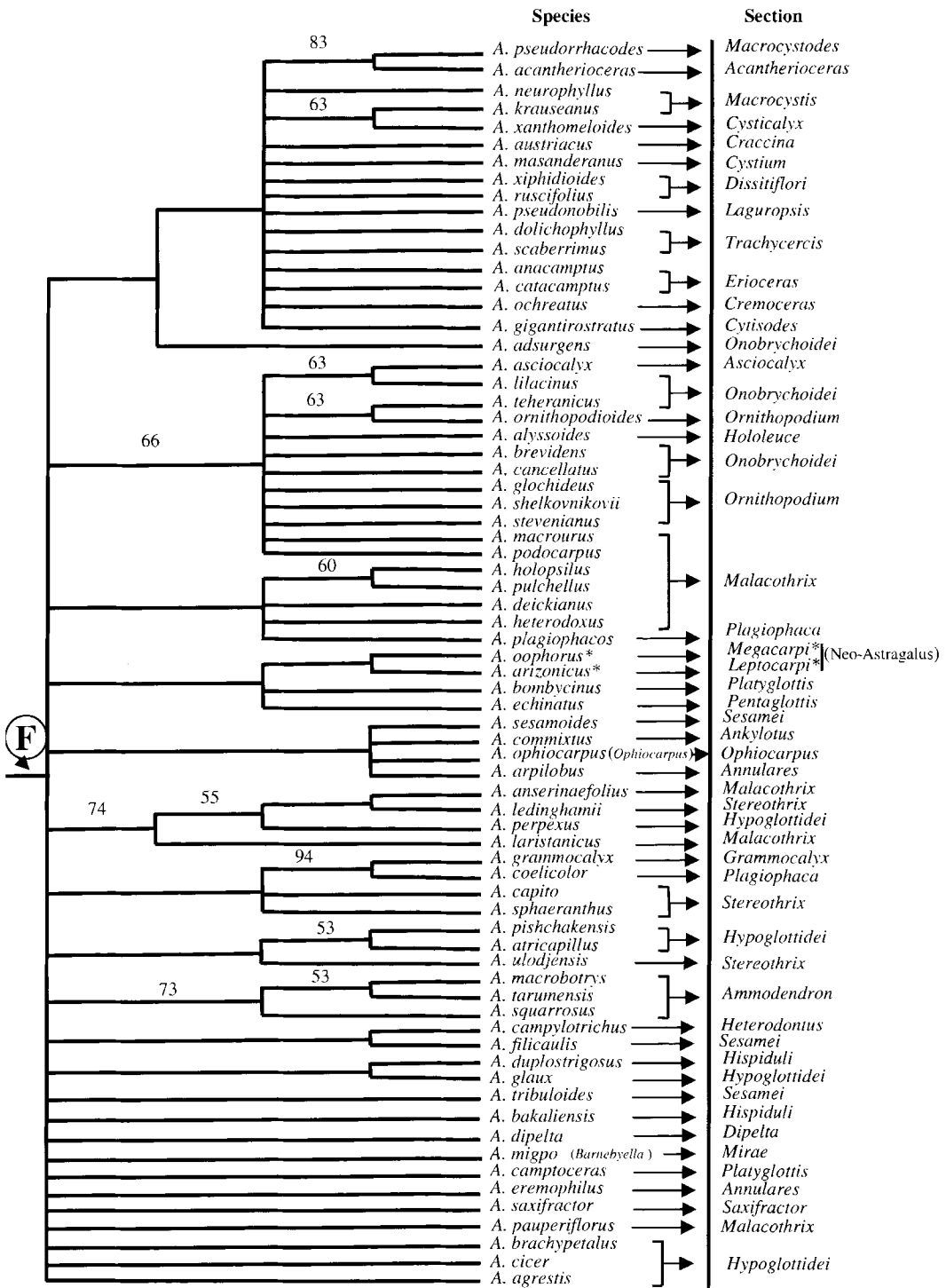


FIG. 1. Continued.

Beck, *A. refractus* Meyer and *A. supervisus* Sheld.; all of sect. *Incani*), relationships are not resolved within it. Clade "G1" comprises 31 exclusively thorny cushion-forming taxa (including 15 newly sampled ones) plus non-thorny *A. paradoxus* Bunge (of sect. *Macrosemium*). There are four weakly/moderately supported subclades of two or more species and several unresolved species within this clade. Clade "G2" contains 24 other thorny cushion-forming species (including nine newly sampled ones). Within this clade, three weakly to moderately supported subclades of two or more species can be found. Interestingly, species of sections *Acidodes*, *Anthylloidei*, and *Rhacophorus* are nested in both clades "G1" and "G2," making these sections polyphyletic. Clade "I" is the next group that is strongly supported (85% bootstrap) and marked by a three-base indel in ITS2. This clade includes *A. daenensis* (two accessions sampled) Boiss. and *A. penetratus* Maassoumi (both of sect. *Hemiphaca*). Clade "F" contains 71 species (2 New World aneuploid species and 69 Old World euploid species), of which 28 Old World species were newly sampled. This clade is marked by a multi-base indel in ITS1 that was identified in previous studies (Wojciechowski et al., 1993, 1999; Kazempour Osaloo et al., 2003). Many species within this group are unresolved, with the exception of two highly supported subclades (>80% bootstrap) of two species, and several weakly to moderately supported subclades that contain two or more species.

Discussion

THE PHYLOGENETIC POSITION OF THE SEGREGATE GENUS THLASPIDIUM

As noted in the introduction, a number of monotypic and polytypic annual genera have been considered allied with, or part of, *Astragalus*. Most notable of these are *Barnebyella*, *Ophiocarpus*, *Didymopelta*, *Sewerzowia*, and *Thlaspidium*. Recently, the status of these, except *Thlaspidium*, was assessed with molecular data (Kazempour Osaloo et al., 2003). In these analyses and in the present study, *Barnebyella*, *Ophiocarpus*, and *Didymopelta* were clearly nested

in clade "F" and *Sewerzowia* in clade "B" within *Astragalus* s. str. (Fig. 1).

Rassulova (1978 cited in Podlech, 1994) treated *Astragalus thlaspi* (sect. *Thlaspidium*) as the new separate genus *Thlaspidium* (*Th. thlaspi*). This species is characterized by fruits that are elliptic, strongly dorsally compressed, broadly winged along the margins, bilocular, and oligo-seeded. The genus/section name *Thlaspidium* and that species name were taken from the genus name *Thlaspi* (Brassicaceae) because of the similarity in the fruit features. Podlech (1991, 1994) postulated that the species has evolutionarily arisen from sect. *Oxyglottis*. Our molecular data clearly place *Thlaspidium* within *Astragalus* s. str. (clade "B"), but do not ally it with any of a number of species (among members of sect. *Oxyglottis*), in clade "B."

THE PLACEMENT OF THE NORTH AMERICAN EUPLOID ASTRAGALUS

Eight species out of the recognized eleven North American *Astragalus* that have euploid chromosome numbers based on $n = 8$ (Barneby, 1964) were sampled in the present study, which doubles the sampling relative to our previous study. These species are scattered among the Old World groups of *Astragalus* s. str., as indicated in previous studies (Wojciechowski et al., 1993, 1999; Kazempour Osaloo et al., 2003). *Astragalus umbellatus* Bunge and *A. americanus* (sect. *Cenanthrum*, sensu Maassoumi, 1998) are allied with the three Old World members of the section in a well-supported clade within the large clade "A" (Fig. 1). *Astragalus australis* (L.) Lam. (sect. *Hemiphragmium*) and *A. williamsii* Rydb. (sect. *Hemiphaca*), as well allied sister taxa, are placed along with the Old World annuals in a weakly supported polytomy within clade "B." The following two species are more closely related to Asian counterparts: North American *A. oregonus* Nutt. ex T. & G. is the sister to Asian *A. falcatus* Lam. (both of sect. *Uliginosi*) within clade "C," and *A. alpinus* (sect. *Komaroviella*) is the sister group to Asian *A. polycladus* Bureau & Franch. (sect. *Polycladus*) in clade "E." Finally, *A.*

agrestis Dougl. ex G. Don. (sect. *Hypoglottidei*) and *A. adsurgens* Pallas (sect. *Onobrychoidei*) are found in a large polytomy of clade "F."

PHYLOGENETIC STATUS OF SECT.
PHYLLOLOBIUM AND OTHER NEWLY
SAMPLED SECTIONS OF THE OLD
WORLD ASTRAGALUS

Our previous study (Kazempour Osaloo et al., 2003) revealed that the delimitation of many taxa of the Old World *Astragalus* (subgenera and sections) based solely on morphological characters is questionable. The study demonstrated non-monophyly of both the traditionally recognized subgenera [*Epiglottis*, *Trimeniaeus*, *Phaca*, *Hypoglottis*, *Calycophysa*, *Tragacantha*, *Cercidothrix* and *Calycocystis* (Bunge, 1868, 1869; Maassoumi, 1998)] and Podlech's (1982, 1991, 1994) new subgenera (*Trimeniaeus*, *Astragalus* and *Cercidothrix*). Simultaneously, Kang et al. (2003) suggested that Bunge's subgen. *Pogonophace* (sensu Fu, 1993) is not also a monophyletic group based on nrDNA ITS sequences. Among the many species-rich sections sampled in our previous analysis, only *Cenanthrum*, *Chronopus*, *Laxiflori*, *Lotidium*, *Incanti*, and *Ammodendron* were monophyletic. Here, we briefly examine phylogenetic status and monophyly of sect. *Phyllobium* and any other sections as well as those previously analyzed ones with more representatives that were newly included in this study.

The members of sect. *Phyllobium* (sensu Wenninger, 1992) are characterized by non-interconnecting keel and wing petals and a ciliate style similar to the diagnostic pollen brushes of subtribe Coluteinae (Barneby, 1964; Lavin & Delgado, 1990; Fu, 1993; Kang & Zhang, 2004). Some authors (e.g., Fu, 1993; Kang et al., 2003; Kang & Zhang, 2004) have accepted three sects. (*Phyllobium*, *Bibracteolati*, and *Trichostylus*) instead of a single sect. *Phyllobium* (sensu Wenninger, 1992; as we adopt here). Most of ca. 30 species of the sect. *Phyllobium* are distributed in China (Fu, 1993; Maassoumi, 1998). More recently, Kang et al. (2003), using nrDNA ITS data, revealed that seven sampled species of sects. *Phyl-*

lobium, *Bibracteolati*, and *Trichostylus* (=Wenninger's sect. *Phyllobium*) form a strongly supported clade (99% bootstrap value) with a closer relationship to Galegeae subtribe Coluteinae (sensu Polhill, 1981) than to *Astragalus* s. str. Our molecular phylogenetic analysis of nrDNA ITS data clearly confirms the finding that sect. *Phyllobium* (six species included) is a well-supported monophyletic group (99% bootstrap, Fig. 1, clade "J"), which is placed outside of *Astragalus* s. str., as the sister group of *Colutea persica*, a representative of the subtribe Coluteinae, with high bootstrap support (95%). Liston and Wheeler (1994) and Sanderson and Wojciechowski (1996), using chloroplast *rpoC* gene restriction site data and nrDNA ITS sequences, respectively, first indicated the close relationship of sect. *Phyllobium*, represented in their analysis by *A. complanatus* R. Br. ex Bunge (the type of the sect. *Phyllobium*), with subtribe Coluteinae (see also Sanderson & Liston, 1995; Wojciechowski et al., 1999; Kang et al., 2003). According to the molecular and morphological (especially pollen brush) data, Kang and Zhang (2004) suggested that the three sects. *Phyllobium*, *Bibracteolati*, and *Trichostylus* (=Wenninger's sect. *Phyllobium*) be treated as a distinct genus, *Phyllobium* Fisch., a treatment that differs from the concept of Barneby (1964). In fact, this genus was first established by Fischer (1818) based on *P. chinense* Fisch. Barneby (1964:1164) suggested that the morphological similarity (non-interconnecting keel and wing petals) of sect. *Phyllobium* (represented by *A. complanatus*) and the other barbistyled members of subgen. *Pogonophace*, along with the Coluteinae genera *Sphaerophysa* DC. (*S. salsula* (Pall.) DC.), *Swainsona* Salisb., and *Lessertia* DC., might indicate that together they form a single large genus, for which the suitable name is *Phyllobium*. Barneby's concept of *Phyllobium* is not, however, supported by molecular data, which suggest that sect. *Phyllobium* (sensu Wenninger, 1992) is an isolated group within the Colutoide clade without having any close affinity to either *Sphaerophysa*, *Swainsona*, or *Lessertia* (Kang et al., 2003). We are here in agree-

ment with Kang and Zhang's (2004) suggestion to resurrect the genus *Phyllolobium* and make the nomenclatural transfers in the context of a taxonomic revision. Section *Chlorostachys*, as currently circumscribed (Weninger, 1992; Maassoumi, 1998), is not monophyletic. The four sampled species of this section are found in clade "A," which is sister to the rest of *Astragalus* s. str. The east African *A. atropilosulus* (Hochst.) Bunge is supported as first branch of the clade "A," consistent with previous works (e.g., Wojciechowski et al., 1999; Kazempour Osaloo et al., 2003). Three other species of sect. *Chlorostachys*, *A. hoantchy*, *A. dshimensis* (both from east Asia), and *A. coluteocarpus* (west Asian), are not closely related to *A. atropilosulus*. The first two are sister taxa and are allied weakly with another east Asian species, *A. chinensis* (sect. *Nuculiella*), and *A. coluteocarpus* is allied with west Asian sects. *Pelata*, *Aegacantha*, *Pendulina*, and *Caprini*.

Astragalus sect. *Caraganella* (subgenus *Cercidothrix*), which consists of four species (Maassoumi, 1998), represented in this study by *A. stocksii* and newly sampled *A. koschukensis*, is monophyletic (100% bootstrap). It has been considered that this spiny subshrubby section (covered by both medifixed and basifixed hairs) is a very ancient palaeoxeromorphic and an isolated taxon that has no close relative within *Astragalus* (Podlech, 1975, 1998). In contrast, Ghahremani-nejad (2004) claimed that the section is close to the woody sections with medifixed hairs (subgen. *Cercidothrix* sensu Podlech, 1982). However, the present nrDNA ITS data, like our previous study including both nrDNA ITS, *ndhF* and the combined data (Kazempour Osaloo et al., 2003), explicitly show that the section is solely along with basifixed hair sections nested in an unresolved assemblage within clade "A." Likewise, sect. *Eremophysa*, represented here by three species, is a monophyletic group, but its relationships with sects. *Astragalus*, *Chronopus*, *Alopeuroidei*, and *Laxiflori* is unresolved.

The phylogenetic placement of *A. hololeios* (sect. *Aegacantha*: Maassoumi, 1998; Podlech et al., 2001), in an unresolved clade comprising members of sect. *Caprini*

and *Pendulina* shows that the species could be placed in its own section (*Hololeios*, Rechinger, 1958; Deml, 1972). Some of its morphological characters such as glabrous form (except for the fruit), short campanulate-cylindrical calyx, and long-pedunculate racemes are different from that of sect. *Aegacantha* (Podlech et al., 2001). However, more species are needed to be analyzed to clarify the phylogenetic status of *Aegacantha*. *Astragalus* sect. *Theiochrus* is a small section with two species, *A. siliquosus* Boiss. and *A. botryophorus*. These species, along with *A. zerdanus* of sect. *Hemiphaca*, form a well-supported trichotomy within clade "B." These taxa share certain morphological features, including the membranous stipules, short calyx teeth, and glabrous, sparsely spotted, minutely stipitate fruits (Maassoumi, 1989; Podlech, 1999). The monophyly of the small Mediterranean sect. *Tapinodes*, represented herein by *A. depressus*, cannot be evaluated and the position of the species is not resolved within clade "C." The species-rich sect. *Incani*, represented here by seven species (including four newly sampled ones plus *A. subsecundus* of sect. *Laguropsis*) is a well-supported (100% bootstrap) monophyletic group, as suggested in our earlier study (Kazempour Osaloo et al., 2003). The close affinity of *A. subsecundus* with members of *Incani* was recently determined with a careful examination of morphological features and considered to be a member of this section (Maassoumi, unpubl. data). Thorny-cushion forming sects. *Acidodes*, *Hymenostegis*, *Leucocercis*, and *Rhacophorus*, despite the extensive sampling in this study, are not each monophyletic (Fig. 1, clades "G1" and "G2"). This conclusion was first reached in our previous work (Kazempour Osaloo et al., 2003). Species of these sections are intermixed with the other thorny-cushion forming taxa, indicating that the delimitation of these taxa, solely based upon morphological characters, is artificial. Likewise, sect. *Hystrix*, with only two species, is not monophyletic, and monophyly of sect. *Stenonychium*, represented here with one species (*A. floccosus* Boiss.), cannot be addressed. Similarly, section *Leucocercis*, which is endemic to Iran (Lock &

Simpson, 1991; Maassoumi, 1998), is not monophyletic. In all morphological studies, this section, due to mainly having medifixed hairs, has been considered to be related solely with the medifixed hair sections (Bunges' or Podlech's subgenus *Cercidothrix*; Podlech, 1982; Maassoumi, 1998; Zarre, 2003; Ghahremani-nejad, 2004). Our molecular data revealed that the members (four species analyzed here) of *Leucocercis* are related with only the other thorny-cushion forming taxa, covered by medifixed hairs, nested in clade "G1," as suggested first in our previous study (Kazempour Osaloo et al., 2003). Recently, one of the species of sect. *Leucocercis*, *A. semnanensis* Bornm. & Rech. f., was transferred to the newly monotypic section *Semnanenses* based upon basifixed hairs and the hairy standard (Zarre & Podlech, 2001; Podlech et al., 2001). These authors suggested that the species is intermediate between sects. *Acanthophaea* and *Anthylloidei* (= *Megalocystis*), while our data weakly ally it with *A. submitis* Boiss. & Hohen. of the latter section. In our tree (Fig. 1), like that of the previous work, resolution among thorny-cushion forming taxa is very poor, however, and only a few small groups have high bootstrap support. This lack of resolution is a reflection of the extremely low sequence divergence values for nrDNA ITS across the thorny-cushion forming taxa (less than 0.03 substitutions per site for pairs of the taxa).

Astragalus daenensis and *A. penetratus* (both of sect. *Hemiphaca*) are well supported sister species (clade "I") which have no relationship with either the other species of the section or any other species of *Astragalus*. The phylogenetic status of sects. *Malacothrix*, *Hypoglottidei*, *Stereothrix*, and *Plagiophaca*, was evaluated in our earlier study (Kazempour Osaloo et al., 2003). These results and the present analysis, with expanded sampling, showed that none of these sections is monophyletic as currently circumscribed. Some of the species are intermixed in three distinct subclades, while the rest either are unresolved branches or allied with the representatives of other sections within clade "F." Oskoueiyan et al. (unpubl. data), using the cladistic analyses

of morphological characters of *Malacothrix* and its allied sections, came to the same conclusion. In our nrDNA ITS tree, *A. macrocrous* Fisch. & Meyer and *A. podocarpus* Meyer (both of *Malacothrix*, Maassoumi, 1993), which have an indumentum of basifixed hairs mixed with extremely asymmetrical medifixed hairs, are nested in a polytomy comprising sects. *Onobrychoidei*, *Ornithopodium*, *Hololeuce*, and *Asciocalyx* with medifixed hairs (see Fig. 1). This is an unexpected result that has never been suggested in the *Astragalus* literature up to now (e.g., Maassoumi, 1989; 1993). Our nrDNA ITS data suggest that delimitation of these sections, like many others, based upon morphological characters (Maassoumi, 1989; Podlech, 1986), is artificial and needs reclassification based on molecular (or combined with morphology) phylogenetic results. Indeed, we are now conducting the nrDNA ITS phylogenetic analysis of *Malacothrix* and its allies, with the extensive sampling, to clarify the taxonomic status of these groups (Kazempour Osaloo & Maassoumi, unpubl. data). The large sect. *Ammodendron*, represented here by three species, forms a moderately supported clade (73% bootstrap). The analyzed members of the section are marked by a multi-base indel (a 36 base deletion) in ITS1, which was first identified in our earlier work (Kazempour Osaloo et al., 2003). The closest relative of sect. *Ammodendron* is not evident within clade "F" in our tree. Section *Heterodontus*, represented here by *A. guttatus* Banks & Soland and *A. campylotrichus* Bunge, is not monophyletic group. The former species was first analyzed in our previous study and nested within clade "B." In contrast, the latter is weakly allied with *A. filicaulis* Kar. & Kir. of sect. *Sesamei* within clade "F." Other newly analyzed sections here are *Saxifactor*, *Cysticalyx*, *Craccina*, *Macrocystis*, and *Laguropsis*, of which the first three are represented by a single species and hence their monophyly cannot be evaluated. The last two sections, as well as sects. *Dissitiflori* and *Erioceras*, sampled with two species, are probably not monophyletic. Furthermore, relationships among those sections (except *Saxifactor*) are unresolved.

In short, our present phylogenetic study of Old World *Astragalus*, as a summary of previous work, provides a framework for future studies to deal with the reclassification of some well resolved and supported groups. It is now time to examine the evolution of morphological characters in order to find synapomorphies supporting some well-resolved and -supported clades identified in the present study. However, some parts of our phylogenetic tree are not well-resolved and supported, and it would be best to look for alternative nuclear markers with higher molecular divergence than nrDNA ITS.

Acknowledgments

This work was supported by research council of Tarbiat Modares University and a JSPS Fellowship to S. Kazempour Osaloo. We thank A. Liston and M. F. Wojciechowski for inviting us to contribute to the Rupert Barneby Legume symposium. We also thank two anonymous reviewers for helpful comments on the manuscript.

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