

# MOLECULAR PHYLOGENY OF THE TRIBE HEDYSAREAE WITH SPECIAL REFERENCE TO ONOBRYCHIS (FABACEAE) AS INFERRED FROM nrDNA ITS SEQUENCES

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A total of 53 species (54 sequence accessions) representing 28 species of the tribe *Hedysareae*, 5 species of *Vicioid* clade and 19 species of *Galegeae* including the two *Glycyrrhiza* species as outgroups was included in a phylogenetic analysis using nrDNA ITS sequences with maximum parsimony method. The analysis, based on successive reweighting by rescaled consistency index, revealed that *Vicioid* clade is the first branch followed by *Chesneya-Caragana* clade and *Astragalean* clade as successive sisters to *Hedysaroid* clade. The genus *Alhagi* is well allied, as the basal most taxon, with the remainder of *Hedysaroid* clade. *Ebenus* and *Taverniera* are each monophyletic and weakly allied taxa. *Hedysarum* is not monophyletic, its species are dispersed across *Hedysaroid* clade. *Sulla* a segregate of *Hedysarum*, forms a well supported monophyletic group with the inclusion of *H. humile*. The enigmatic genus *Eversmannia* is allied with *Onobrychis* clade. *Onobrychis* itself is not monophyletic due to inclusion of the two *Hedysarum* species, and *Onobrychis* subgenus *Onobrychis* is not monophyletic as well, whereas *Onobrychis* subgenus *Sisyrosema* forms a strongly supported clade. In contrast to *Onobrychis* section *Heliobrychis*, sections *Dendrobrychis* and *Onobrychis* appear not to be each monophyletic. *Hedysareae* is circumscribed herein to include only *Alhagi*, *Ebenus*, *Eversmannia*, *Hedysarum*, *Onobrychis*, *Sulla*, *Taverniera*, *Corethroedendron* and *Sartoria*.

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## فیلوژنی مولکولی قبیله Hedysareae با تأکید ویژه بر جنس Onobrychis بر اساس توالی های nrDNA ITS

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تعداد 53 گونه (54 توالی) شامل 28 گونه از قبیله *Hedysareae*، 5 گونه از کلاد *Vicioid* و 19 گونه از قبیله *Galegeae* از جمله دو گونه *Glycyrrhiza* به عنوان بیرون گروه با استفاده از توالی های nrDNA ITS در آنالیز فیلوژنی با روش بیشینه صرفه جویی قرار گرفت. آنالیز براساس وزن دهی مجدد پی در پی با شاخص سازگاری تصحیح شده فاش نمود که کلاد *Vicioid* اولین شاخه ای است که بدنیاال آن کلاد *Chesneya-Caragana* و کلاد *Astragalean* به عنوان گروه های خواهری کلاد *Hedysaroid* قرار دارند. جنس *Alhagi* به عنوان پایه ترین تاکسون با بقیه کلاد *Hedysaroid* به خوبی متحد می شود. هر کدام از جنس های *Ebenus* و *Taverniera* تک تبارند و به طور ضعیف با هم متحد هستند. جنس *Hedysarum* تک تبار نیست و گونه هایش در سراسر کلاد *Hedysaroid* پخش هستند. *Sulla* یک جنس جدا شده از *Hedysarum* با در بر گرفتن گونه *Hedysarum humile* گروه تک تبار به خوبی حمایت شده ای را تشکیل می دهد. جنس *Eversmannia* با کلاد *Onobrychis* خویشاوند است. خود جنس *Onobrychis* به علت قرار گرفتن دو گونه *Hedysarum* در میان گونه های تک تبار نیست و زیرجنس *Onobrychis* از جنس *Onobrychis* نیز تک تبار نیست، در حالیکه زیرجنس *Sisyrosema* یک کلاد قویاً حمایت شده ای را می سازد. در میان بخشه های چند گونه ای آنالیز شده از جنس *Onobrychis*، برخلاف بخشه *Heliobrychis*، بخشه های *Dendrobrychis* و *Onobrychis* ظاهراً تک تبار نیستند. در اینجا قبیله *Hedysareae* فقط مشتمل بر جنس های *Sartoria* و *Alhagi*, *Ebenus*, *Eversmannia*, *Hedysarum*, *Onobrychis*, *Sulla*, *Taverniera*, *Corethroedendron* می شود.

## INTRODUCTION

Since De Candolle (1825) onward, the taxonomic delimitation of the tribe *Hedysareae* has been

undergone several treatments by various authors (Bentham 1865; Hutchinson 1964; Polhill 1981a, 1994; Choi and Ohashi 2003; Lock 2005). Just recently, Lock

(2005) expanded the tribe to twelve genera including *Alhagi* Adans., *Calophaca* Fisch ex DC., *Caragana* Fabr. and *Halimodendron* Fisch ex DC., formerly treated in *Galegeae* (Polhill 1981b, 1994) in addition to *Corethroedendron* Basin., *Ebenus* L., *Eversmannia* Bunge, *Hedysarum* L., *Onobrychis* Mill., *Sartoria* Boiss. & Heldr., *Sulla* Medik. and *Taverniera* DC. Lock (2005) briefly overviewed the taxonomic history of some of controversial genera (e. g. *Hedysarum*, *Corethroedendron*, *Sulla* and *Taverniera*), and thus we do not repeat these again here. The tribe is a member of Polhill's temperate herbaceous group and has been traditionally placed close to the tribe *Galegeae*. Available molecular data (nrDNA ITS and *matK* sequences) showed that the Hedysaroid clade (composed of *Hedysarum boreale* Nutt., *Onobrychis montana* DC., *Alhagi camelorum* Fisch. and *A. maurorum* Medik.), is one of the three well-supported subclades, within the Inverted Repeat Lacking Clade (IRLC) (Sanderson and Wojciechowski 1996; Wojciechowski et al. 2000, 2004; Wojciechowski 2003, 2005, 2006). *Hedysareae* as a whole occurs in dry open localities with a continental temperate or mediterranean climate, and is restricted to Eurasia, North America and the Horn of Africa with Socotra. After *Hedysarum*, with ca. 160 species, *Onobrychis* is the second largest genus with ca. 130 species within the tribe (Mabberley 1990; Lock 2005). The genus is restricted only to Eurasia and well represented in the continental temperate and warm-temperate zones of the Irano-Turanian region. The Flora Iranica area is one of the main centers of diversity of the genus comprising over 75 species in two subgenera divided into nine sections (Rechinger 1984). *Hedysareae* in general and *Hedysarum* (cf. Chennaoui et al. 2007) and *Onobrychis* in particular remain under-sampled and hitherto no comprehensive molecular phylogenetic analysis including almost all the genera has been undertaken. The present work using nrDNA ITS sequences is the first report on the phylogeny of the tribe with relatively its broader taxonomic sampling. The goals of our paper are, therefore, to:

(1) Reconstruct the phylogenetic relationships of and delimit *Hedysareae* as a whole, (2) determine the generic relationships within it and (3) clarify infrageneric relationships within *Onobrychis*.

## MATERIALS AND METHODS

**Taxon Sampling.** The sampling in this study includes 28 species of the tribe *Hedysareae* (sensu Polhill 1981a), 5 species of the Vicioid clade (Sanderson and Wojciechowski 1996) and 19 species of the tribe *Galegeae* including two *Glycyrrhiza* as outgroups based on previous studies (Sanderson and Wojciechowski 1996; Hu et al. 2000; Wojciechowski et al. 2000, 2004; Wojciechowski 2003, 2005). The

complete nrDNA ITS (ITS1, 5.8S and ITS2) region for 19 taxa including *Alhagi pseudalhagi* (M. B.) Desv., *Onobrychis* (10 species of eight out of nine sections), *Hedysarum* (2 species), *Taverniera* (2 species), *Ebenus* (3 species), *Eversmannia subspinoso* Fisch., were determined first time by us. In the case of *Halimodendron halodendron* (Pall.) Voss, for which ITS1+ITS2 were previously sequenced by Sanderson and Wojciechowski (1996), nrDNA ITS sequences were also determined here again. For this and the remainder taxa, the sequences were obtained from gene bank (see Table 1).

**DNA Extraction.** Leaf materials were sampled from herbarium specimens deposited in the Herbarium of Research Institute of Forests and Rangelands (TARI), the Central Herbarium of University of Tehran (TUH), herbarium of Hacettepe University (HUB), and Herbarium of National Plant Gene Bank. Voucher specimens of those taxa collected from the natural habitats by us were deposited at the Tarbiat Modares University Plant Collection (see Table 1). Genomic DNAs were isolated using the modified CTAB methods of Doyle and Doyle (1987).

**Polymerase Chain Reaction (PCR).** The complete nrDNA ITS region was amplified using primers ITS4 and ITS5 of White et al. (1990). Amplification was done in a DNA thermal cycler (Primus 96, MWG, Germany). The quality of PCR products were checked by electrophoresis in a 0.8% agarose gel (using 1X TAE as the gel buffer) stained with ethidium bromide and then visualized under UV light. Nucleotide sequences of PCR products were determined using cycle sequencing and an automated DNA sequencer through Gen Fanavaran Co. The same nrDNA ITS primers ITS5 and ITS4 were used for cycle sequencing reactions.

**Phylogenetic analyses.** Sequences were aligned using clustal W 1.8 (Thompson et al. 1994) and adjusted visually. Individual Sequences from taxa included in the present study have been deposited in DDBJ (see Table 1. for accession numbers). Phylogenetic analyses were performed on the aligned data matrix using maximum parsimony method (MP) as implemented in the version 4.0b10 of PAUP\* (Swofford 2002) installed in a Macintosh computer. The heuristic search option was selected using 100 replications of random addition sequence and TBR branch-swapping with MulTrees on and steepest descent off. Analyses were conducted using a successive weighting (SW) strategy (Farris 1969). Weights were assigned to characters using the "reweight characters" option based on the rescaled consistency (RC) index (Farris 1989) with a base weight of 1. When the tree length and CI, RI and RC remained unchanged in successive rounds, these trees

were accepted as the SW trees. In both analyses, support for clades were evaluated by bootstrapping (Felsenstein 1985) using 1000 replications with the heuristic search option, simple addition sequence and TBR branch swapping.

## RESULTS

The aligned nrDNA ITS data matrix for 50 examined species are 715 nucleotide sites long of which 317 sites are potentially informative. The length of nrDNA ITS is ranging from 329 base pairs (bp) in *Halimodendron halodendron* (due to incomplete sequencing by Sanderson and Wojciechowski 1996) to 637 bp in *Ebenus stellata* Boiss. Maximum parsimony (MP) analysis of the nrDNA dataset (characters equally weighted) generated 2754 most parsimonious trees with a length of 1234 steps, CI= 0.459 and RI= 0.741 (with excluding autoapomorphies). MP analysis of the dataset after three rounds of successive weightings using rescaled consistency index (RC) yielded 27 most parsimonious trees with a length of 388.30 steps, a CI=0.625 and an RI=0.842. The strict consensus of these 27 trees with accompanying bootstrap values is given in Fig. 1. Here, we describe phylogenetic relationships of the studied taxa basing on the successive weighting analysis and mention the differences with the equally weighted one (the tree not shown) wherever is relevant.

In the tree (Fig. 1.), the well supported [bootstrap percentage (BP) of 100%] vicioid clade is the first branch followed by Chesneya-Caragana clade (BP=66%) and Astragalean clade (BP=100%) as successive sisters to Hedysaroid clade (85%) that includes representatives of 7 genera analyzed from *Alhagi* through *Onobrychis*. Bootstrap supports for sister group relationships of these grades are 100%, 91%, and 53%, respectively. Relationships among the last three clades were not resolved in the equally weighted analysis. Within the Hedysaroid clade, *Alhagi* represented herein with three species forms the basal most clade (BP=100%) followed by *Hedysarum papillosum* Boiss. and a weakly supported clade (BP=63%) composed of both monophyletic *Taverniera* and *Ebenus*, as successive sisters to an assemblage of remaining *Hedysarum* species, *Sulla*, *Eversmannia subspinoso* and *Onobrychis*. However, in the equally weighted analysis, relationships within an assemblage containing *Hedysarum papillosum* through *Onobrychis* were not resolved properly. *Sulla*, represented herein with 6 taxa, along with *H. humile* L., as nested within it, forms a well supported monophyletic group (BP=

100%) and for which, *H. membranaceum* Coss. & Bal. is a sister taxon. This clade is moderately united with *Eversmannia-Onobrychis* assemblage (BP= 71%). *Onobrychis* represented herein by 11 species, plus intermixed *H. boreale* and *H. wightianum* Aitch & Baker, forms a strongly supported clade (BP=98%). This clade is, in turn, composed of two well-supported subclades (BP= 98% and 100%, respectively), one of which comprising *Onobrychis* species solely. *Eversmannia* is well positioned as sister taxon to *Onobrychis* clade (BP= 96%).

## DISCUSSION

**Phylogenetic status and the delimitation of the tribe Hedysareae.** The tribe *Hedysareae* sensu Polhill (Polhill 1981a, 1994) along with tribes *Galegeae* (Wagstaff et al. 1999; Lock and Schrire 2005), *Cicereae*, *Trifolieae* and *Fabeae* (Lock and Maxted 2005) comprises the temperate herbaceous clade (THC, Sanderson and Wojciechowski 1996). The THC plus the three Millettoid genera *Wisteria*, *Callerya* and *Afgekia*, comprise IRLC (Wojciechowski et al., 2000, 2004). In "Advances in Legume Systematics", Polhill (1981a) pointed out that *Hedysareae* derived from the astragaloid part (subtribe *Astragalinae*) of the tribe *Galegeae*, without clarifying its closest relative genus/genera. Previous nrDNA ITS phylogenetic studies, with few taxon sampling of *Hedysareae* (*Hedysarum boreale* and *Onobrychis montana*), showed that these two genera are strongly allied with *Alhagi* (of *Galegeae*), the so called Hedysaroid clade (Sanderson and Wojciechowski 1996), and, in turn, are sister to either Vicioid-Astragalean clade (Sanderson and Liston 1995) or Vicioid clade alone (Sanderson and Wojciechowski 1996). Chloroplast gene *matK* (Wojciechowski et al. 2000, 2004) and combined nrDNA ITS-*matK* (Wojciechowski 2005) phylogenies, again with the same sampled taxa of the Hedysaroid clade, suggested a sister group relationship of it to *Caragana* of *Galegeae*. *Caragana*-Hedysaroid clade is allied with the Astragalean clade (Wojciechowski et al. 2000, 2004; Wojciechowski 2005), while their supertree analysis (Wojciechowski et al. 2000) positioned them outside both the Astragalean and Vicioid clades. Our nr DNA ITS-based phylogeny is not consistent with both *matK* and combined nrDNA ITS-*matK* phylogenies that the Hedysaroid, as a well supported clade (BP=85%), is allied solely with Astragalean clade, but the relationship is weakly supported (BP=53% , Fig. 1.).

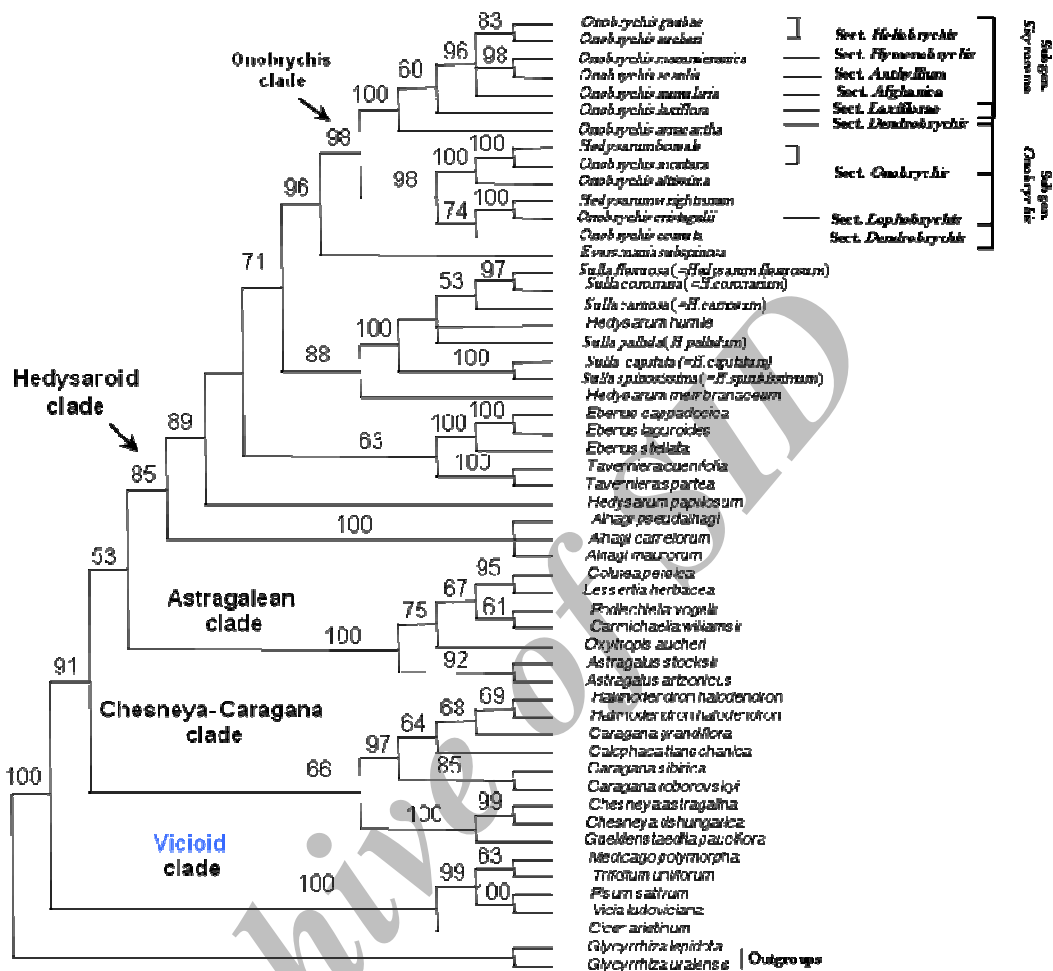


Fig. 1. Strict consensus tree of 27 parsimonious trees resulting from phylogenetic analysis of nrDNA ITS region after successive weighting with RC (length = 388.30 steps, CI= 0.625, RI= 0.842). Bootstrap values greater than 50% were shown above the branches.

Table 1. Taxa included in nrDNA ITS phylogenetic analyses.

Species	Voucher accession	Gene Bank accession numbers of ITS
<i>Alhagi camelorum</i> Fisch.	Adams 19-88 (ARIZ)	U50756, U50757
<i>Alhagi maurorum</i> Medik.	USDA 502281	U50486, U50487
<i>Alhagi pseudalhagi</i> (M. B.) Desv.	Iran: Kazempour Osaloo 2007-1 (TMUPC)	AB374181
<i>Astragalus arizonicus</i> Gray	USA:Sanderson 968	AF121690
<i>Astragalus stocksii</i> Benth. ex Bunge	Iran: Foroughi 10802 (TARI)	AB051966
<i>Calophaca tianschanica</i> (Fedtsch.) Boriss.	Former USSR : Skvortsov s.n. (A)	U51220, U51221
<i>Caragana grandiflora</i> (M.B.) DC.	Iran: Assadi & Shahsavari 65834 (TARI)	AB052035
<i>Caragana roborovskyi</i> Kom.	China: Zhang 200037 (PE)	AF521958
<i>Caragana sibirica</i> Medik.	Liu et al. <sup>a</sup>	AY626912
<i>Carmichaelia williamsii</i> Kirk	New Zealand Sanderson 1550	U50520, U50521
<i>Chesneya astragalina</i> Jaub. & Spach	Iran: Assadi & Maassoumi 55503 (TARI)	AB052036
<i>Chesneya dshungarica</i> Gobsk.	Former USSR:Goboskokov 5.29.55 (US)	U50350,U50351
<i>Cicer arietinum</i> L.	ICARDA, Flip82-150C	AJ237698
<i>Colutea persica</i> Boiss.	Iran: Foroughi 17434 (TARI)	AB052037
<i>Ebenus cappadocica</i> Hausskn. & Siehe	Turkey: Erik 2507 (HUB)	AB374182
<i>Ebenus laguroides</i> Boiss.	Turkey:Domez 5335 (HUB)	AB374183
<i>Ebenus stellata</i> Boiss.	Iran: Ahangarian & Kazempour Osaloo 2006-1 (TMUPC)	AB329691
<i>Eversmannia subspinosa</i> Fisch.	Iran: Freitag & Mozaffarian 28397 (TARI)	AB329692
<i>Glycyrrhiza lepidota</i> (Nutt.) Pursh.	Toolin 1572 (ARIZ)	U50758, U50759
<i>Glycyrrhiza uralensis</i> Fisch.	Hu 1142 Nursery specimen	AF467050
<i>Gueldenstaedtia pauciflora</i> (Pall.) Fisch. ex. DC.	Homepage of Matt Lavin Montana State University ( <a href="http://emini.Oscs.montana.edu/~mlavin/data/ITSmill.htm">http://emini.Oscs.montana.edu/~mlavin/data/ITSmill.htm</a> )	Not in gene bank
<i>Halimodendron halodendron</i> (Pall.) Voss.	Iran: Maassoumi et al. 86046 (TARI)	AB329693
<i>Halimodendron halodendron</i> (Pall.) Voss.	Stevens 2394 (US)	U56019, U56020
<i>Hedysarum boreale</i> Nutt.	USA: Wojciechowski & Sanderson 131 (ARIZ)	U50482 U50483,
<i>Hedysarum humile</i> L.	Morocco: THHU 0204 <sup>c</sup>	AY772227
<i>Hedysarum membranaceum</i> Coss. & Bal.	Algeria: THME 0202 <sup>c</sup>	AY772228
<i>Hedysarum papillosum</i> Boiss.	Iran: Assadi et al. 30175 (TARI)	AB329694
<i>Hedysarum wrightianum</i> Aitch. & Baker	Iran: Baghestani et al. 2006-2 (TMUPC)	AB329695
<i>Lessertia herbacea</i> DC.	Wojciechowski and Sanderson 299 (ARIZ)	AF121752
<i>Medicago polymorpha</i> L.	Jenkins 91-8 (ARIZ)	U50863, U50864
<i>Onobrychis acualis</i> Bornm.	Iran: Ebrahimi H-3260 (Iran Plant Gene Bank)	AB329696
<i>Onobrychis altissima</i> Gross.	Iran: Zarre et al. 2006.5.10 (TUH)	AB329697
<i>Onobrychis aucheri</i> Boiss.	Iran: Ahangarian & Kazempour Osaloo 2005-1(TMUPC)	AB329698
<i>Onobrychis cornuta</i> (L.) Desv.	Iran: Kazempour Osaloo 2006-3(TMUPC)	AB329699
<i>Onobrychis crista-galli</i> (L.) Lam.	Iran: Ahangarian & Kazempour Osaloo 2006-5(TMUPC)	AB329700
<i>Onobrychis gaubae</i> Bornm.	Iran: Ahangarian & Kazempour Osaloo 2005-2(TMUPC)	AB329701
<i>Onobrychis laxiflora</i> Baker	Iran: Aliyabadi 22172 (TUH)	AB329702
<i>Onobrychis mazanderanica</i> Rech. f.	Iran: Ahangarian & Kazempour Osaloo 2005-3 (TMUPC)	AB329703
<i>Onobrychis montana</i> DC.	Mason & Mason 3773 (ARIZ)	U50484, U504885
<i>Onobrychis nummularia</i> Boiss.	Iran: Mozaffarian 43904 (TARI)	AB329704

Species	Voucher accession	Gene Bank accession numbers of ITS
<i>Onobrychis arnacantha</i> Bunge	Iran: Assadi & Ranjbar 82292 (TARI)	AB329705
<i>Oxytropis aucheri</i> Boiss.	Iran: Maassoumi 55104 (TARI)	AB051908
<i>Pisum sativum</i> L.	USA: Wojciechowski 398 (ARIZ)	U50861, U50862
<i>Podlechiella vogelii</i> (Webb.) Maassoumi et Kazempour Osaloo	Iran: Mozaffarian et al. 39103 (TARI)	AB051911
<i>Sulla capitata</i> (Desf.) Choi & Ohashi (= <i>Hedysarum capitatum</i> Desf.)	Tunisia: THCP 0088 <sup>c</sup>	AY772223
<i>Sulla carnosus</i> (Desf.) Choi & Ohashi (= <i>Hedysarum carnosum</i> )	Algeria: THCR 0071 <sup>c</sup>	AY772224
<i>Sulla coronaria</i> (L.) Medik. (= <i>Hedysarum coronarium</i> )	Tunisia: THCO 0055 <sup>c</sup>	AY772225
<i>Sulla flexuosa</i> (L.) Medik	Morocco: THFL 0189 <sup>c</sup>	AY775312
<i>Sulla pallida</i> (Desf.) Choi & Ohashi (= <i>Hedysarum pallidum</i> )	Tunisia: THPA 0168 <sup>c</sup>	AY772229
<i>Sulla spinisissima</i> (L.) Choi & Ohashi (= <i>Hedysarum spinosissimum</i> L.)	Tunisia: THES 0121 <sup>c</sup>	AY772226
<i>Taverniera cuneifolia</i> (Roth.) Arn.	Iran: Ahangarian & Kazempour Osaloo 2006-4(TMUPC)	AB329706
<i>Taverniera sparta</i> (Burm. f.) DC.	Iran: Mozaffarian 49325 (TARI)	AB329707
<i>Trifolium uniflorum</i> L.	AZ4194, Margot Forde Forage Germplasm Center	AF0531798
<i>Vicia ludoviciana</i> Nutt.	McLaughlin & Bowers 3185 (ARIZ)	U51216, U51217

Abbreviations used in accession information: A, Arnold Arboretum/Gray Herbarium, Harvard University, Cambridge; ARIZ, University of Arizona Herbarium, Tucson; HUB, Herbarium of Hacettepe University, Ankara; ICARDA, International Center for Agricultural Research in the Dry Areas, Aleppo, Syria; PE, Herbarium of Institute of Botany, the Chinese Academy of Sciences, Beijing; TARI, Herbarium of the Research Institute of Forests and Rangelands, Tehran; TUH, Central Herbarium of University of Tehran, Tehran; TMUPC, Tarbiat Modares University Plant Collection, Tehran; US, National Museum of Natural History, Smithsonian Institution, Washington, D.C.; USDA, U.S. Department of Agriculture Plant Introduction accession numbers. Voucher accession for *Caragana sibirica* was not mentioned in the gene bank. <sup>b</sup>*Gueldenstaedtia pauciflora* was mistakenly written as *Spogiocarpella pauciflora* in the dataset appeared at the homepage of Mat Lavin. <sup>c</sup>Abbreviation used in accession information for these north African species were directly obtained from gene bank.

In Polhillian sense, (Polhill 1981a), *Hedysareae* forms a single complex dominated by *Onobrychis* and *Hedysarum* plus five segregates, *Eversmannia*, *Ebenus*, *Strachya*, *Sartoria* and *Taverniera*. Polhill excluded *Alhagi*, due to its some non-Hedysaroid characteristics including obtuse keel and fruit venation pattern, from *Hedysareae* (Hutchinson 1964), and placed it in *Galegeae*. But, he noted that the genus is relatively close to *Hedysareae*. The phylogenetic hypothesis presented here (Fig. 1.) confirms his view and that the genus is well positioned as a sister to the remainder of *Hedysareae* (see also Lock 2005). Lock (2005) with a speculation based on available molecular phylogenetic data (Wojciechowski et al. 2000, 2004) and morphology (Polhill 1981a; Choi and Ohashi 1996, 2003) expanded the tribe to comprise 12 genera with adding *Alhagi* plus *Caragana*, *Halimodendron* and

*Callophaca* formerly treated in *Galegeae* (Polhill 1981b, 1994). However, the present nrDNA ITS phylogeny is not concordant with Lock's view, since no direct relationship of *Caragana*, *Halimodendron* and *Callophaca* with *Hedysareae* is evident. *Caragana* and the two allies are morphologically so distinct from the Hedysaroid taxa (Polhill 1981a) to be classified within *Hedysareae*. On the other hand, these three genera plus *Chesneya* and *Gueldenstaedtia* were already classified in a newly established tribe *Caraganeae* (Ranjbar and Karamian 2003). But our nrDNA ITS (Fig. 1) and previous molecular phylogenetic studies, both nrDNA ITS (Sanderson and Wojciechowski 1996) and *matK* (Wojciechowski et al. 2000) show that monophyly of this new tribe at the current status is weakly supported.

Choi and Ohashi (2003), basing on gross morphological, palynological and cytological data, resurrected genera such as *Sulla* and *Corethroedendron* as segregates from *Hedysarum* but merged *Stracheya* into it and maintained *Taverniera* as a distinct genus. Our nrDNA ITS phylogenetic analysis of 7 genera (*Corethroedendron* and *Sartoria* not analyzed) of the *Hedysareae* united them in a well supported monophyletic group. Therefore, we suggest that the tribe in the present status comprises only *Alhagi*, *Hedysarum*, *Onobrychis*, *Eversmannia*, *Ebenus*, *Sartoria*, *Taverniera*, *Sulla* and *Corethroedendron*. The tribe is characterized by shorter or reduced wing petals, indehiscent (rarely dehiscent) jointed or 1(2)-seeded pods and chromosome base number of 7 or 8.

**Generic relationships within *Hedysareae*.** In the tree presented here (Fig. 1), the small SW Asian genus *Alhagi* with 3-5 species (Rechinger 1984; Lock and Simpson 1991; Lock 2005), as represented herein by three species and form its own strongly supported clade (BP=100%) positioned at the base of *Hedysareae*. As noted above, the sister group relationship of this genus with the rest of the tribe was already speculated (Polhill 1981a). Its obtuse keel and non-Hedysaroid fruit venation pattern as well as basic chromosome number of  $x=8$  are seemed to be symplesiomorphic characters. Recently, Awmack and Lock (2002) questioned the specific delimitation of this morphologically variable genus and dramatically reduced the number of its species to one, *A. maurorum*, with two subspecies, *A. maurorum* subsp. *maurorum* and *A. maurorum* subsp. *graecorum* (Boiss.) Awmack and Lock. According to seed protein data, the two species, *A. pseudalhagi* M. Bieb. and *A. graecorum* Boiss., appeared to be not distinct from each other (Sheidai et al. 2002). A comprehensive molecular systematic study of the genus with more geographic sampling of its representative taxa is, however, definitely necessary to evaluate the specific circumscription.

*Ebenus*, a genus of ca. 20 species growing in Eurasia with the main center of its diversity in Turkey (Huber-Morath 1971; Lock 2005), three of which analyzed here, is appeared to be monophyletic. Non-molecular characters including, one-segmented pods with 1 or 2 seeds (Huber-Morath 1971; Polhill 1981a), pollen grains with tricolpate apertures and reticulate exine sculpturing (Choi and Ohashi 1996; Perveen and Qaiser

1998) and a chromosome number of  $2n=14$  (Goldblatt 1981, Aytac 2000), suggested that *Ebenus* is apparently related to *Onobrychis*. However, the present nrDNA ITS phylogeny shows that the two genera are not closely related, indicating that these characters have been evolved in parallel among them.

*Taverniera*, a medium-sized genus of 16 species distributed in NE Africa and SW Asia (Tullin 1985; Mozaffarian 1988), two of which were included herein, is monophyletic and allied weakly with *Ebenus*. *Taverniera* is morphologically characterized by shrubby habit, leaves 1, 3-foliolate and short stipitate pods enclosed in the corolla (Polhill 1981a; Thulin 1985; Choi and Ohashi 2003). Gross morphological (Polhill 1981a; Thulin 1985; Choi and Ohashi, 2003), pollen morphological (Thulin 1985; Choi and Ohashi 1996, 2003) and nodal anatomical features altogether (Choi et al. 1998) suggested a close relationship of the genus with *Corethroedendron* (= *Hedysarum* sect. *Fruticosa*) of C. Asia. Our nrDNA ITS tree shows that *Taverniera* is distinct from *Hedysarum*.

In contrast to the monophyly of *Alhagi*, *Ebenus* and *Taverniera*, the genus *Hedysarum* is not monophyletic. One species of it, *H. papulosum*, is placed basally near *Alhagi*. The two of four remaining species, *H. membranaceum* and *H. humile* are allied with and nested within *Sulla*, respectively. While the other two, *H. boreale* and *H. wrightianum* are exclusively nested among *Onobrychis* species. Several workers (e.g. Polhill 1981a; Thulin 1985; Choi and Ohashi 1996) demonstrated that *Hedysarum*, a genus of ca. 160 species (Lock 2005), is not clearly delimited from the related genera *Corethroedendron*, *Stracheya* and *Taverniera*. As noted earlier, recently Choi and Ohashi (2003), based on the critical analysis of morphological data treated *Hedysarum* sect. *Spionsissima* Fedtsch. (= sect. *Hedysarum*, (Fedtschenko 1902; Chrtkova 1968) as a distinct genus *Sulla*, and transferred *H.* sect. *Fruticosa* Fedtsch. (Fedtschenko 1902, 1972) to the genus *Corethroedendron*; but reduced *Stracheya* Benth. to a section of *Hedysarum*. According to their classification, *Hedysarum* was currently circumscribed and composed of four sections *Hedysarum*, *Multicaulia* (Boiss.) Fedtsch., *Membranacea* Fedtsch. and *Stracheya* (Benth.) Choi & Ohashi.

*Sulla*, a genus of 7 species (Choi and Ohashi 2003), six of which were analyzed herein, is monophyletic with the inclusion of *H. humile*. The same conclusion was reached by Chennaoui et al. (2007). *Sulla* is distinct in the tribe by having a suite of some features including annual habit (except. *S. coronaria* (L.) Medik.), free stipules adnate at the base (Thulin 1985; Choi et al. 1999; Choi and Ohashi 2003) and colporate pollen grains (Ferguson and Skvarla 1981; Thulin 1985; Choi

and Ohashi 1996, 2003) as well as its unique distribution restricted to Mediterranean region. The sister group relationship of *Sulla* with *H.* sect. *Membranacea*, [a monotypic section represented by *H. membranaceum*, restricted to northernmost Africa, (Choi and Ohashi 2003)], revealed that it is a specialized taxon derived from the polyphyletic *Hedysarum*. Neither *Stracheya* nor *Corethroedendron* were sampled here to test their phylogenetic status within the tribe, but it can be speculated that the latter taxon may be closely related to *Taverniera* (see above discussion). An extensive species sampling covering all four sections of *Hedysarum* (sensu Choi and Ohashi 2003) plus *Corethroedendron* is definitely needed to evaluate their relationships and position more explicitly in the context of a molecular phylogeny.

*Eversmannia*, a small genus of four species, one of which, *E. subspinosa*, were analyzed herein, is closely allied with the *Onobrychis* clade. This is an unexpected result in that its relationship with *Onobrychis* has not been questioned. Fruit pericarp anatomy (Mironov and Sokoloff 2000) suggested that *E. subspinosa* is closely related to *Hedysarum*, while pollen morphology put it the sister to *Alhagi* (Choi and Ohashi 1996). In contrast to other members of the tribe, *Eversmannia* is characterized by non-lomentaceous and rather dehiscent fruits, that is, they do not break into segments in the manner typical of the tribe and transversal venation pattern of valves (Mironov and Sokoloff 2000). These characters are apparently seemed to be autapomorphies for this taxon. Until recently, *Eversmannia* was regarded as monotypic genus (*E. subspinosa*), but three local endemic species have been described from C. Asia (Yakovlev et al. 1996). It is represented here by only a single species, hence its monophyly can not be evaluated.

As mentioned above, *Onobrychis* with 11 sampled species plus the two intermixed *Hedysarum* species, forms a well-supported monophyletic group (BP=98%). According to a fruit morphology-based cladistic analysis, Yildiz et al. (1999) concluded that *Onobrychis* is not monophyletic due to inclusion of *O.* sect. *Dendrobrychis* DC. among *Hedysarum* sections. Although, our molecular phylogenetic study is partly congruent with this fruit morphological study; *O.* sect. *Dendrobrychis* is not placed outside *Onobrychis*, but the two sampled *Hedysarum* species are nested within *Onobrychis*. However, it is noteworthy that the present work is clearly in agreement with non-molecular studies (Polhil 1981b; Goldblatt 1981; Yildiz et al. 1999; Pavlova and Manova 2000) that these two genera are closely related taxa.

**Infrageneric Relationships within *Onobrychis*.** The genus *Onobrychis* comprises ca. 130 species

(Mabberley 1990) classified into two subgenera, and 9 sections (Rechinger 1984). The present nrDNA ITS data show that the subgen. *Sisyrosema* (Bunge) Grossh., which is represented here by four out of its five constitutive sections, appears to be a well supported monophyletic group (BP=96%), whereas the subgen. *Onobrychis* is not monophyletic due to the sister group relationship of its two representative species to the subgen. *Sisyrosema* and the inclusion of the two species of *Hedysarum* within it. Yildiz et al. (1999) suggested, however, that monophyly of these two subgenera was not supported by a phylogenetic analysis of fruit characters. The subgen. *Sisyrosema* differs from the subgenus *Onobrychis* by its large, crescent/kidney-shaped ovaries and pods, hairy vexillum, large persistent flowers and the epidermis of calyx without crystals (Rechinger 1984; Yildiz et al. 1999). These features appear to be synapomorphies for the subgen. *Sisyrosema*. Our nrDNA ITS phylogeny (Fig. 1) depicts that members of the subgenus *Sisyrosema* are derived from subgenus *Onobrychis*, recently.

Sections *Lophobrychis* Hand.-Mzt. [*Onobrychis crista-galii* (L.) Lam.] and *Laxiflorae* Baker (*O. laxiflora* Baker.) [both of subgen. *Onobrychis*], and *Anthyllium* Nab. (*O. acaulis* Bornm.), *Afghanicae* Sirj. (*O. nummularia* Boiss.) and *Hymenobrychis* DC. (*O. mazanderanica* Rech. f) [all three of subgen. *Sisyrosema*] are represented herein by only a single species; hence the monophyly of these sections cannot be addressed. The species-rich section *Heliobrychis* Bunge ex Boiss. (of subgen. *Sisyrosema*), represented here by two species *O. gaubae* Bornm. and *O. aucheri* Boiss., is monophyletic. In contrast, sections *Dendrobrychis* and *Onobrychis* (both of subgen. *Onobrychis*) appear not to be monophyletic. One species of *Dendrobrychis*, *O. cornuta* (L.) Desv., is the sister to a subclade of *O. crista-galii* (of sect. *Lophobrychis*) and *H. wrightianum*, whereas *O. arnacantha* Bunge is sister to *O. laxiflora* (of sect. *Laxiflorae*)-subgenus *Sisyrosema* clade. This is an unexpected finding that *Dendrobrychis* is a small and morphologically uniform section whose monophyly has not been questioned (Rechinger 1984; Yildiz et al. 1999). *Onobrychis cornuta* and *O. arnacantha* [as well as the five remainder species of the section (Rechinger 1984)] do share a suite of morphological features including shrubby cushion-forming habit, persistent spiny peduncle or petiole, wing petals two times longer than keel and unarmed pods. nrDNA ITS data indicate, however, that these two species are well separated phylogenetically, suggesting that these morphological characters have been evolved in parallel at least in these two species. The two members of sect.



*Onobrychis*, *O. altissima* and *O. montana* are, in order, sisters to *H. boreale*.

**Phylogenetic Conclusion.** The present analyses revealed that the well supported Vicioid clade is the first branch sister to the remaining taxa. On the tree based on equally weighting characters, Astragalean clade, *Chesneya-Caragana* clade and Hedysaroid clade form a large polytomy. Whereas, reweighting analysis showed that *Chesneya-Caragana* clade and Astragalean clade are successive sisters to Hedysaroid clade. This well supported clade comprises *Alhagi*, *Ebenus*, *Eversmannia*, *Hedysarum*, *Onobrychis*, *Sulla* and *Taverniera* solely. *Hedysareae* is circumscribed herein to include the above mentioned seven genera plus *Coretrodendron* and *Sartoria* (not sampled here) as putative relatives of *Hedysarum*. Within *Hedysareae*, the *Alhagi* is the first branch which is followed by *Hedysarum papillosum*, *Ebenus-Taverniera* clade, as successive sisters to the large clade composed of remaining *Hedysarum* species, *Sulla*, *Eversmannia subspinosa* and *Onobrychis*. Eleven species of *Onobrychis* plus *H. boreale* and *H. wightianum*, forms a well-supported clade (*Onobrychis* clade) as allied with *Eversmannia subspinosa*. Within *Onobrychis* clade, the *Onobrychis* subgenus *Sisyrosema* forms a monophyletic group, while the *Onobrychis* subgenus *Onobrychis* is not monophyletic. In contrary to section *Heliobrychis*, sections *Dendrobrychis* and *Onobrychis* appear not to be monophyletic. These data again illustrate the great potential of nrDNA ITS sequences for resolving relationship at a range of taxonomic levels, from closely related species, to sectional/subgeneric, to the generic and even tribal level. However, more taxon sampling and another source of DNA sequence, like chloroplast coding (e.g., *matK*, or *ndhF*) or noncoding (e.g., *trnL* intron and *trnL-trnF* intergenic spacer) regions, are definitely necessary to be analyzed in order to comparing and combination of produced gene phylogenies for the Hedysaroid taxa.

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