Molecular phylogeny and divergence times of *Astragalus* section *Hymenostegis*: An analysis of a rapidly diversifying species group in Fabaceae

Ali Bagheri1, Ali Asghar Maassoumi2, Mohammad Reza Rahiminejad2, Jonathan Brassac3 & Frank R. Blattner3,4

The taxa of *Astragalus* section *Hymenostegis* are an important element of mountainous and steppe habitats in Southwest Asia. A phylogenetic hypothesis of sect. *Hymenostegis* has been obtained from nuclear ribosomal DNA internal transcribed spacer (ITS) and plastid *ycf1* sequences of up to 303 individuals from 106 species, including all 89 taxa currently assigned to sect. *Hymenostegis*, 14 species of other *Astragalus* sections, and two species of *Oxytropis* and one *Biserrula* designated as outgroups. Bayesian phylogenetic inference and parsimony analyses reveal that three species from two other closely related sections group within sect. *Hymenostegis*, making the section paraphyletic. DNA sequence diversity is generally very low among *Hymenostegis* taxa, which is consistent with recent diversification of the section. We estimate that diversification in sect. *Hymenostegis* occurred in the middle to late Pleistocene, with many species arising only during the last one million years, when environmental conditions in the mountain regions of Southwest and Central Asia cycled repeatedly between dry and more humid conditions.

*Astragalus* is with about 2500–3000 species in 250 sections the largest genus of flowering plants1–7. It belongs to the legume family (Leguminosae or Fabaceae) that is, after Orchidaceae and Asteraceae, the third largest family of flowering plants consisting of about 730 genera and nearly 19,300 species. Also its subfamily Papilionoideae, with about 478 genera and 13,800 species, is very species rich. *Astragalus* belongs within this subfamily together with genera like *Oxytropis* and *Colutea* to the Astragalean clade of the so-called Inverted Repeat Lacking Clade (IRLC). Its members are all characterized by the loss of one copy of the two 25-kb inverted repeat regions in the chloroplast genome2,8–10.

Although species richness seems to be a general characteristic of the legumes, this feature is not evenly distributed among the groups within this family. Thus, Sanderson and Wojciechowski11 found that *Astragalus* itself is not significantly more speciose compared to its close relatives, but that species richness is a feature of the entire Astragalean clade in comparison to the other groups of the IRLC. High species numbers can originate through constant accumulation of diversity through time or following a punctuated pattern, where bursts of diversification rates alternate with times of stasis12. The latter pattern often results from the evolution of a key innovation that provides the possibility to fill a new ecological niche, or from a key opportunity, i.e. the colonization of a new, formerly not inhabited area and speciation therein12–15. For *Astragalus* and the entire Astragalean clade the reason(s) for the high species numbers are still unclear. In an effort to better understand reasons for and timing of species diversification in *Astragalus* we here analyze a large section of the genus, where we put some effort into arriving at a complete species sample.

1Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, 81746-73441, Iran.
2Botany Research Division, Research Institute of Forests and Rangelands, Agricultural Research, Education and Extension Organization (AREEEO), 13185–116, Tehran, Iran.
3Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), 06466, Gatersleben, Germany.
4German Centre of Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, 04103, Leipzig, Germany. Correspondence and requests for materials should be addressed to A.B. (email: a.bagheri@sci.ui.ac.ir)
Astragalus section Hymenostegis Bunge was validly published by Bunge \(^{16,17}\) including originally 23 species, and was typified with \textit{A. hymenostegis} Fisch. \& C.A.Mey. \(^{18}\). Section \textit{Hymenostegis} has been revised several times \(^{7,19–22}\). It is one of the largest sections of Old World \textit{Astragalus}, distributed mainly in Iran and Turkey \(^{23,24}\). Of the total number of 89 currently recognized species (Bagheri et al., unpublished data), 85 (95.5\%) occur in Iran (Fig. 1), from which 71 (79.8\%) are endemic to this country \(^{7,23–28}\). Members of sect. \textit{Hymenostegis} are morphologically characterized by an inflated calyx, basifixed white hairs, and wide and conspicuous bracts. The section was subdivided by Zarre and Podlech \(^{21}\) into two subsections: subsect. \textit{Hymenocoleus} (Bunge) Podlech \& Zarre, including only the Turkish endemic \textit{A. vaginans} DC., and subsect. \textit{Hymenostegis}. This division has been based on leaf and stem characters. Podlech and Zarre \(^{7}\) followed this treatment in their most recent account of Old World \textit{Astragalus}. Based on an analysis of the nuclear ribosomal DNA internal transcribed spacer (ITS) region of \textit{Astragalus}, sect. \textit{Hymenostegis} (represented by few taxa only) is nested within a clade including tragacanthic \textit{Astragalus} along with other major groups of spiny \textit{Astragalus} sections (including: \textit{Anthylloidei}, \textit{Tricholobus}, \textit{Acidodes}, \textit{Poterion}, \textit{Campylanthus} and \textit{Microphysa}) and seems to be non-monophyletic \(^{29–31}\). Up to now no detailed molecular phylogenetic analysis using multiple DNA loci and an exhaustive taxon sampling has been conducted for sect. \textit{Hymenostegis}. Here we describe a study of this taxon based on the nuclear ribosomal DNA (rDNA) internal transcribed spacer region (ITS) and chloroplast \textit{ycf1} sequences for phylogenetic reconstruction, two marker regions which are among the fastest evolving DNA parts found up to now in \textit{Astragalus}. The main purposes of our study are: (i) to assess the monophyly of sect. \textit{Hymenostegis}, (ii) to examine the evolutionary relationships within this section and to closely related taxa, and (iii) to roughly estimate diversification times for the species within the section and put this in the context of the generally high species diversity within the genus and family.

**Results**

**Phylogenetic analyses of ITS, \textit{ycf1}, and combined ITS + \textit{ycf1} sequences.** The alignment of the ITS dataset containing one individual each per species (ITS\textsubscript{106}). Had a length of 621 positions, with 124 variable characters of which 71 were parsimony informative. The parsimony analysis resulted in 359 trees of length 192 with a consistency index (CI) of 0.755 and a retention index (RI) of 0.880. The consensus tree is shown in Supplementary Fig. S1 (all figures indicated by “S” are provided as supplementary materials).

The two Bayesian phylogenetic (BI) analyses conducted with this dataset (\text{Nst} = 6 vs. \text{Nst} = 2) resulted in trees with identical topologies and very similar posterior probabilities (pp) for clades. Only one tree is therefore provided (Fig. 2). Also the maximum-likelihood (ML) analysis resulted in very similar species relationships (Supplementary Fig. S2). In these trees the assumed outgroup species \textit{Biserrula pelecinus} groups within \textit{Astragalus} with high support values (see Materials and Methods). Sequence differences among sect. \textit{Hymenostegis} taxa are generally low resulting also in low clade support values. Nine groups, consisting mainly of taxa with identical ITS sequences, are present, the largest with 36 species, smaller clades containing nine, five, four, three and two species sharing their ITS sequence. Three species (\textit{A. submittis}, \textit{A. tricholobus} and \textit{A. vaginans}), assumed to belonging to other sections of the genus, fall inside sect. \textit{Hymenostegis}.

The alignment of the ITS sequences for all individuals (ITS\textsubscript{303}). Included 282 individuals from sect. \textit{Hymenostegis} taxa, 19 from other \textit{Astragalus} sections, and two outgroup species. The alignment had a length of 628 positions, of which 506 characters were constant and 85 potentially parsimony informative. The 10,000 most
parsimonious trees (max tree limit) had a length of 185 steps (CI = 0.768, RI = 0.948). The ML analysis and BI analyses based on the two different models of DNA sequence evolution resulted in identical tree topologies and support values for the same clades. Therefore, only one tree is provided (Supplementary Fig. S3).

Figure 2. Bayesian phylogenetic tree based on nrDNA ITS sequences and including one individual per sect. *Hymenostegis* species. Clade support values (BI pp/MP bs) are given at the branches of the tree. Asterisks at branches indicate pp ≥ 0.98 and bs ≥ 80%, respectively. Blue numbers provide mean node ages in million years estimated through a Beast analysis. Asterisks behind species names indicate new species names currently in the process of valid publication, while species names given in bold face mark species formerly not included within sect. *Hymenostegis*. Colors of species names refer to distribution areas given in Fig. 3.
The monophyly of sect. *Hymenostegis* (including the three species mentioned above) is highly supported (pp 1, bs 100%) but taxon resolution within the section is relatively low, i.e. many species share identical ITS sequences, also in cases where these species are morphologically easily discernable. Some small subclades are present within sect. *Hymenostegis*. Regions of origin of the species are color-coded and the areas are provided in the inserted map.

Figure 3. Phylogenetic tree obtained by Bayesian phylogenetic inference of the combined nuclear rDNA ITS and chloroplast *ycf1* datasets of 64 *Astragalus* and one outgroup accession. Clade support values (BI pp/MP bs) are given at the branches of the tree. Asterisks at branches indicate pp ≥ 0.98 and bs ≥ 80%, respectively. Asterisks behind species names indicate new species names currently in the process of valid publication, while species names given in bold face mark species formerly not included within sect. *Hymenostegis*. Regions of origin of the species are color-coded and the areas are provided in the inserted map.

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the tree, although they rarely received reasonable clade support values. In this dataset we included between one and six accessions per species, which allows us to infer some cases of non-monophyly for species. Thus, in *A. chrysostachys*, taxa listed as varieties within the species (var. *parisienensis*, var. *dolichourus*, var. *khorasanicus*), group apart from the other *A. chrysostachys* accessions at two different positions in the tree. Varieties *dolichourus* and *khorasanicus* share ITS sequences and morphology with *A. conicus* where they might be taxonomically belonging. In case of var. *parisienensis* probably a new name has to be assigned.

The aligned data matrix for the ycf1. Data consisted of 1607 characters across 65 accessions including the outgroup species. 1571 characters were constant, and of the 36 variable characters 34 were potentially parsnomy informative. The 3648 most parsimonious trees had a length of 183 steps (CI = 0.934, RI = 0.882). Due to the small differences among the ycf1 sequences, phylogenetic resolution is very low in the MP tree as well as the BI analysis of this dataset. As we obtained for all these individuals also ITS sequences, we do not show these trees but use ycf1 together with the respective ITS sequences in a combined data matrix.

The partition-homogeneity test of ITS and ycf1 datasets. For the same 65 taxa carried out in Paup* 4.0a150 indicated no major conflicts between both loci. They were therefore combined in a concatenated data matrix for MP, ML and BI analyses.

The combined ITS + ycf1. Data matrix consists of 2222 characters across 65 accessions including the outgroup species. 1981 characters were constant, and of the 163 variable characters 78 were potentially parsnomy informative. The 216 most parsimonious trees had a length of 305 steps (CI = 0.934, RI = 0.882). Due to the small differences among the ycf1 sequences, phylogenetic resolution is very low in the MP tree as well as the BI analysis of this dataset. As we obtained for all these individuals also ITS sequences, we do not show these trees but use ycf1 together with the respective ITS sequences in a combined data matrix.

Age estimations for sect. Hymenostegis. The MCC tree (Supplementary Fig. S5) resulted in a topology similar to the ITS_106 tree inferred with MrBAYES except for the clade consisting of *A. vaginans*, *A. submittis* and 12 other species being sister to all other sect. *Hymenostegis* species. This specific topology is then in accord with the ITS + ycf1 topology. The analysis inferred a mean substitution rate of 2.44 × 10^{-9} (substitutions/site/year) and resulted in a crown group age of sect. *Hymenostegis* of 3.55 My (95% HPD = 2.13–5.08) with the majority of speciation events being younger than 1.5 My (Supplementary Fig. S5). The ages of the major nodes are given in the phylogenetic tree provided as Fig. 2.

Discussion

Monophyly of Astragalus and section Hymenostegis. In our analyses *Biserrula pelecinus* groups within *Astragalus*, which confirms Wojciechowski’s7 suggestion to treat it as *Astragalus pelecinus* (L.) Barneby as part of the genus and regard *Biserrula pelecinus* L. as a synonym of this taxon.

*Astragalus tricholobus*, *A. submittis* and *A. vaginans* from other closely related sections were placed among the species of sect. *Hymenostegis*. Thus, sect. *Hymenostegis* is paraphyletic when these three species are not included. This finding is consistent with the results of Kazempour Osaloo et al. (2003, 2005) and Naderi Safar et al.29–31. *Astragalus submittis* shares some morphological characters with members of sect. *Hymenostegis*, including the spiny form and inflated calyx at fruiting time. Although, due to its parapinate (not imparipinnate) leaflets and the possession of black and white (not just white) hairs it has been placed in sect. *Tricholobus* or sect. *Tricholobus* or sect. *Hymenocoleus* (L.) Barneby as a synonym of this taxon.

*Astragalus tricholobus* is nested in the main clade of sect. *Hymenostegis*, sharing its ITS sequence with many other core-*Hymenostegis* species. We could not obtain a ycf1 sequence for this species, which is the reason that it is lacking from the combined dataset. Morphologically *A. tricholobus* shares many characters like spiny forms, absence of black hairs, imparipinnate leaflets with sect. *Hymenostegis* taxa, although according to Zarre and Podlech (1996), Podlech and Maassoumi (2001) and Podlech and Zarre7,21,22 it is assumed to be member of sect. *Camptanthus* or sect. *Tricholobus*20,37. In contrast to the two former species, which show a mixture of typical and atypical characters of sect. *Hymenostegis*, the morphological and molecular characters unambiguously place *A. tricholobus* in sect. *Hymenostegis*.

Ages and relationships within section Hymenostegis. Generally, the DNA sequences analyzed provide relatively few differences for sect. *Hymenostegis* taxa (Figs 2 and 3). However, two major groups can be
Another four species represent sections not closely related to the taxon under study. These are *Hymenostegis*, *Microphysa* (sect. *Adiaspastus*) and *Incani* (sect. *Anthylloidei*). For the chloroplast *Hymenostegis* region the fastest evolving universal nuclear marker, and the *ycf1* gene that provided the best resolution in earlier *Astragalus* studies. Therefore, we assume that the *ycf1* gene is particularly useful for delimiting species and species groups which we were able to analyze in our study. Here using next-generation sequencing approaches to screen for genome-wide DNA differences seems to be a suitable approach to us for resolving such narrow sections. Moreover, these methods could simultaneously identify genome regions contributing to fast morphological changes. However, we were able to include ITS sequences for 303 individuals representing 89 species of sect. *Hymenostegis*. These species of other sections including ten species from the closely related and spiny sections. These are *Hymenostegis*, *Sesamei* (sect. *Sesamei*), *Asterias* (sect. *Asterias*), *Biserrula pelecinus* (sect. *Biserrula*), *Adiaspastus* (sect. *Adiaspastus*), *A. askius* (sect. *Incani*), *Asterias* (sect. *Sesamei*), *A. annularis* (sect. *Asterias*), *A. epiglottis* (sect. *Epiglottis*). Also, two species of *Oxytropis* (*O. karjaginii* and *O. kotschyana*) and *Biserrula pellicida* were included as outgroup taxa (but see below, 2.4). The herbarium specimens we used were up to 180 years old. Depending on storage conditions of the herbarium vouchers they were in diverse states of conservation, resulting also in rather diverse quality of extracted DNA. Therefore, not for all specimens both marker regions could be obtained. We complemented our datasets with sequences downloaded from the GenBank nucleotide database. Finally, we were able to include ITS sequences for 303 individuals representing 89 *Hymenostegis* species (some recognized as new taxa and up to now not formally described) plus 17 non-*Hymenostegis* taxa in our analysis. For the chloroplast *ycf1* dataset we included 65 individuals representing 54 sect. *Hymenostegis* species plus ten outgroup taxa. Taxa, origin and GenBank sequence identifiers are given in Supplementary Table S2.

Material and Methods

**Taxon sampling.** We sampled leaf material for DNA analyses from all species of sect. *Hymenostegis* derived from herbarium vouchers, including most of the type specimens, as well as materials collected during fieldwork. Thus, we checked all relevant collections of the herbaria AKSU, ANK, B, FAR, GAZI, HUI, M, MSB, TARI, W, and WU. All together we included 303 individuals representing 89 species of sect. *Hymenostegis* and 14 species of other sections including ten species from the closely related and spiny sections. These are *A. vaginans* (sect. *Hymenocoleus*), *A. submittis* (sect. *Anthylloidei*), *A. tricholobus* (sect. *Campylanthus*), *A. callistochys* (sect. *Microphysa*), *A. campylanthus* (sect. *Campylanthus*), *A. cephalanthus* (sect. *Microphysa*), *A. glaucanths* (sect. *Poterion*), *A. murmuns* (sect. *Anthylloidei*), *A. fasciculfolius* (sect. *Poterion*) and *A. leucargyreus* (sect. *Adiaspastus*). Another four species represent sections not closely related to the taxon under study. These are *A. askius* (sect. *Incani*), *A. asterias* (sect. *Sesamei*), *A. annularis* (sect. *Asterias*), *A. epiglottis* (sect. *Epiglottis*). Also, two species of *Oxytropis* (*O. karjaginii* and *O. kotschyana*) and *Biserrula pellicida* were included as outgroup taxa (but see below, 2.4). The herbarium specimens we used were up to 180 years old. Depending on storage conditions of the vouchers they were in diverse states of conservation, resulting also in rather diverse quality of extracted DNA. Therefore, not for all specimens both marker regions could be obtained. We complemented our datasets with sequences downloaded from the GenBank nucleotide database. Finally, we were able to include ITS sequences for 303 individuals representing 89 *Hymenostegis* species (some recognized as new taxa and up to now not formally described) plus 17 non-*Hymenostegis* taxa in our analysis. For the chloroplast *ycf1* dataset we included 65 individuals representing 54 sect. *Hymenostegis* species plus ten outgroup taxa. Taxa, origin and GenBank sequence identifiers are given in Supplementary Table S2.
accession numbers for materials in this study are listed in Supplementary Table S1 (all tables indicated by “S” are available as supplementary materials).

DNA extraction, amplification and sequencing.  Total genomic DNA was extracted from about 10 mg of herbarium or silica-gel dried material with the DNeasy Plant DNA Extraction Kit (Qiagen) according to the instructions of the manufacturer. DNA concentration and quality were afterwards checked on 0.8% agarose gels. The ITS region (ITS1, 5.8 S rDNA, ITS2) was amplified using the primers ITS-A and ITS-B62. ITS1 and ITS2 were amplified separately when DNA from very old herbarium sheets were used; in these cases, the primers ITS-C and ITS-D96 together with the primers ITS-A and ITS-B, were used. PCR was performed with 1.5 U Taq DNA Polymerase (Qiagen) in the supplied reaction buffer, 0.2 μM of each dNTP, 50 pmol of each primer, Q-Solution (Qiagen) with a final concentration of 20%, and about 20 ng of total DNA in 50 μl reaction volume in a GeneAmp PCR System 9700 (Perkin–Elmer). Amplification was performed with 3 min initial denaturation at 95 °C and 35 cycles of 30 s at 95 °C, 45 s at 56 °C and 30 s at 70 °C, followed by a final extension for 8 min at 70 °C. PCR products were purified using NucleoFast 96 PCR plates (Macherey-Nagel) following the manufacturer’s protocol, and eluted in 30 μl water. Both strands of the PCR products were directly sequenced with Applied Biosystems BigDye–Terminator technology on an ABI 3730xl automatic DNA sequencer using the primers from PCR amplifications. Forward and reverse sequences from each directly sequenced amplicon were inspected, manually edited where necessary in CHROMAS Lite 2.1 (Technelysium Pty Ltd), and assembled in single sequences.

To obtain sequence information from the chloroplast genome we sequenced the ycf1 gene. PCR amplification and sequencing followed Bartha et al.38. When due to low DNA quality not the entire gene region could be amplified by a single PCR, we used internal primers to amplify the locus in two separate parts. All primer sequences are provided in Supplementary Table S2. Forward and reverse sequences from each amplicon were inspected, manually edited where necessary in CHROMAS, and assembled in single sequences.

Sequence alignments and phylogenetic analyses.  ITS and ycf1 sequences were manually aligned. We assembled four different datasets: (i) including all ITS sequences from the 303 individuals available to us (named ITS_303); (ii) a second ITS dataset were we restricted the sample design to include one individual per species resulting in 106 sequences (ITS_106); (iii) a set of 65 sequences for the chloroplast gene ycf1; (iv) and a dataset derived from 65 individuals where ycf1 and ITS sequences could be obtained. To test different models of sequence evolution PAUP* 4.0a15097 was used. The Bayesian information criterion (BIC) chose the K80 + G model for the ITS datasets with one individual per species (ITS_106), whereas the Akaike information criterion (AICc) resulted in the SYM + G model. For the larger ITS dataset with multiple individuals for most Hymenostegies species (ITS_303) the respective models were JC (AICc) and TrNef + G (BIC). For the ycf1 dataset the model of sequence evolution was inferred as TVM + G (BIC, AICc), while for the ITS sequences, which were combined with the ycf1 data the models were TrNef + G (AICc) and HKY + G (BIC). In the cases were different models were inferred by AICc and BIC separate phylogenetic analyses were conducted and the trees were compared afterwards.

Potential conflict between the ITS and ycf1 loci was tested prior to combination by the partition-homogeneity test (ILD; 58) in PAUP*. This test was implemented with 100 replicates, using a heuristic search option with simple addition of taxa, TBR branch swapping and MaxTrees set to 1000.

Bayesian phylogenetic inference (BI) was conducted in MrBayes 3.2.696. In BI two times four chains were run for 20 million generations for all datasets specifying the respective model of sequence evolution. In all analyses we sampled a tree every 1000 generations. Converging log-likelihoods, potential scale reduction factors for each parameter and inspection of tabulated model parameters in MrBayes suggested that stationary had been reached in all analyses. The first 25% of trees of each run were discarded as burn-in.

For all datasets also parsimony analyses were conducted in PAUP* using a two-step heuristic search as described in Blattner94 for the ITS_303 dataset, including all 303 individuals, with the searches restricted to a maximum number of 10,000 trees. Heuristic searches with 25 random-addition-sequences were conducted for ITS_106, including one individual per species. Here as well a for ycf1 and the combined ITS + ycf1 datasets no maximum tree number restrictions were imposed. To test clade support bootstrap analyses were run on all datasets with re-sampling the large ITS_303 dataset 250 times, all other datasets 500 times with the same settings as before, except that we did not use random-addition sequences. Maximum-likelihood analyses of the ITS and combined ITS + ycf1 datasets were conducted in RAxML60 using the GTRGAMMA setting and 500 bootstrap re-samples to estimate branch support values.

Divergence time estimations.  To roughly estimate clade ages and divergence times within the analyzed taxa, we used a crown-group age of 12.4 My, given by Wojciechowski95, as secondary calibration point for Astragalus including Biserrula pelecinus. We used BEAST 2.4.341 to analyze the dataset of ITS sequences (ITS_106) with K80 + G as model of sequence evolution, a random local clock62, and the calibrated Yule prior63.64. The node age was defined as a normal-distributed prior (12.4 ± 1.45 My, 95% distribution interval). Monophyly was enforced for the Astragalus clade including Biserrula pelecinus, as this taxon according to Wojciechowski95, Podlech and Zarre9 and also the results of our study falls into Astragalus. Three independent analyses were run for 20 million generations each, sampling every 1000 generations. Effective sample size (ESS) and convergence of the analyses were assessed using TRACER 1.6 (part of the BEAST package). Appropriate burn-ins were estimated from each trace file, discarded and all analyses were combined with LOGCOMBINER (part of the BEAST package). A maximum clade credibility (MCC) tree was summarized with TREEANNOTATOR (part of the BEAST package) using the option “Common Ancestor heights” for the nodes.
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Author Contributions
A.B., A.A.M., M.R.R. and F.R.B. designed the study; A.B. and A.A.M. collected the samples; A.B., J.B. and F.R.B. performed all the experiments and statistical analyses; A.B. and F.R.B. wrote the paper; all authors reviewed the manuscript and approved the final version for publication.

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