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Phylogenetic relationships between *Oxytropis* DC. and *Astragalus* L. species native to an Old World diversity center inferred from nuclear ribosomal ITS and plastid *mat*K gene sequences

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Abstract: *Oxytropis* and *Astragalus* represent one of the largest angiosperm genera complexes. Although phylogenetic studies of this complex exist, the evolutionary relationships among *Astragalus* and *Oxytropis* species sharing similar habitats in the Old World have not been studied in detail. The phylogenetic relationships among 13 *Oxytropis* and 56 *Astragalus* species native to Turkey were inferred from nucleotide sequence variations in the nuclear ribosomal internal transcribed spacer (ITS) and chloroplast maturase-like protein (*mat*K) gene regions. In addition to our samples, 36 *Oxytropis* ITS and 6 *Oxytropis mat*K sequences were retrieved from GenBank and included in the analysis. Phylogenies derived from a maximum likelihood analysis of the sequences indicated that *Oxytropis* and *Astragalus* genera are more likely monophyletic. However, the results suggest that New World *Oxytropis* species did not evolve by a single adaptive radiation in the genus, but rather from different Old World lineages. The genetic divergence between genera was less when the *mat*K region was analyzed. Although the *Oxytropis* species did not show high genetic diversity, one subcluster of the genus was always distinctly separated in both trees. This subcluster was formed by the species *Oxytropis engizekensis* Duman & Vural and *O. persica* Boiss., which are also regarded as synonyms in regard to several morphological characters of the genus.

Key words: *Astragalus,* genetic diversity, ITS region, *mat*K region, molecular phylogeny, *Oxytropis*, synonym

1. Introduction

Oxytropis DC. (Fabaceae) is a taxonomically complex genus that includes about 330 species (Welsh, 2001) with the highest diversity in Central Asia (153–166 spp.; Malyshev, 2008). *Astragalus* L. is considered to be the most closely related genus to *Oxytropis* when their bulk morphology is considered (Wojciechowski, 1993). *Astragalus* is one of the largest flowering plant genera, with about 2500–3000 species (Podlech, 1999; Wojciechowski, 2005), and the geographic center of diversity is the Irano–Turkish phytogeographical region of Southwestern Asia, with 1000–1500 species (Polhill, 1981).

Cytogenetic evidence (Ledingham, 1960) and a number of morphological similarities (Barneby, 1952) illustrated that *Oxytropis* was derived from a Eurasian *Astragalus* genus. Not only morphologically, but also phylogenetically, high similarities were reported between these two genera (Kulshreshtha et al., 2004). Thus, the hypothesis of parallel evolution between *Astragalus* and *Oxytropis* is very meaningful (Wojciechowski et al., 1999). Even though these taxa share a number of common morphological features and evolved from the same ancestral species, molecular studies showed a striking separation between them in the phylogenetic tree (Sanderson and Wojciechowski, 1996; Wojciechowski, 2005).

Evaluation of the recent literature revealed that there are about 420 *Astragalus* (Hamzaoğlu and Kurt, 2002) and 13 *Oxytropis* species (Ozhatay, 2000) in Turkey. Anatomical and morphological characters of the two genera have been studied comparatively by several researchers (Duran and Aytaç, 2005; Podlech and Ekici, 2008; Özüdoğru et al., 2011). However, a delimitation of taxa based on only morphological features below the level of section cannot be done precisely. Thus, the total number of *Oxytropis* species is reported variably because of the inconsistency in taxonomic treatments (Ozhatay, 2000; Karaman Erkul and Aytaç, 2013).

The internal transcribed spacer region (ITS) of 18S–26S nuclear ribosomal DNA (nrDNA) is suitable for molecular systematic studies (Wojciechowski et al., 1993;

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Wojciechowski, 2005) due to its variability at the species level. In addition to the ITS region, the chloroplast DNA region *mat*K, a maturase-encoding gene located in the intron of the transfer RNA gene for lysine, is also widely used to resolve the taxonomic problems of closely related genera (Lavin et al., 2003; Steele and Wojciechowski, 2003). Therefore, these two regions were preferred to understand the phylogenetic relationships among/within *Astragalus* and *Oxytropis* species because they were already yielding comparable results when used for these genera.

In the literature, there are quite a number of valuable molecular studies (e.g., Wojciechowski et al., 1993; Sanderson and Wojciechowski, 1996; Wojciechowski et al., 1999; Dong et al., 2003b; Kazempour Osaloo et al., 2003, 2005; Wojciechowski, 2005; Scherson et al., 2008; Kazemi et al., 2009; Javanmardi et al., 2012; Dizkirici et al., 2014) indicating the phylogenetic relationships among *Astragalus* species, while there are few studies (Jorgensen et al., 2003; Gao et al., 2009; Archambault and Stromvik, 2012; Artyukova and Kozyrenko, 2012; Archambault, 2013) dealing with the molecular phylogeny of the genus *Oxytropis*. There is no study dealing with native *Oxytropis* species although Turkey is one of the diversity centers for both taxa. The current study comprises a great number of *Astragalus* and all *Oxytropis* species that are currently found naturally in Turkey. Except for *Oxytropis pilosa* L. and *O. aucheri* Boiss., none of the used native *Oxytropis* species had been previously subjected to molecular phylogenetic treatment.

The objectives of the current study were (i) to shed further light on the systematics and evolutionary structure of Old World *Oxytropis* and *Astragalus* genera complexes native to the *Astragalus* diversity center (Turkey), by using sequence diversity of the ITS and *mat*K regions; (ii) to rearrange the taxonomic rank of *Oxytropis* by using information taken from *mat*K and ITS sequence data; and (iii) to explore further the evolutionary relationships among Old and New World *Oxytropis* species.

2. Materials and methods

2.1. Plant samples and DNA isolation

Samples of both *Astragalus* and *Oxytropis* were collected throughout Turkey. Fifty-six *Astragalus* species with 168 accessions (Dizkirici et al., 2014), thirteen *Oxytropis* species with 45 accessions, *Caragana grandiflora* DC. as an outgroup [(AB051905 (ITS, Kazempour Osaloo et al., 2003), and AB854564 (*mat*K, Amirahmadi et al., 2014)] were utilized in the present study. Among the *Oxytropis* species, *O. kotschyana* Boiss. & Hohen., *O. pallasii* Pers., and *O. pilosa* are considered caulescent while the others are acaulescent species. The identity of individual species was determined based on their morphological features (Ekici et al., 2009; Karaman Erkul and Aytaç, 2013). During the field studies, at least two samples for each species were collected from the same or different geographical regions, depending on the size of the populations. Additionally, the ITS and *mat*K sequences of several Old and New World *Oxytropis* species, depending on availability, were obtained from the GenBank database to increase the interspecific sampling and to show the phylogenetic relationships between the Old World and New World *Oxytropis* species (Supplemental Data; Appendix I). Few *mat*K sequences were downloaded from the database since most of them were short (~700 bp) compared to the species native to Turkey (~1200 bp).

Total genomic DNA was isolated from fresh leaves or previously collected and deposited herbarium materials. Collected samples were kept in a small sandwich bags containing dry silica gel pellets during the field trip to keep the leaf samples fresh. A 2X CTAB (hexadecyl trimethyl-ammonium bromide) procedure described by Doyle and Doyle (1987) was used to isolate total DNA.

Nucleotide sequences of the nuclear ITS and chloroplast *mat*K regions of the *Astragalus* species and all other information were available from the previous phylogenetic study dealing with Old and New World *Astragalus* species (Dizkirici et al., 2014). The ITS and *mat*K sequence data of *Oxytropis* taken from the current study and those of *Astragalus* from the previous study were gathered to analyze together. The accession number, section name, and geographic origin of each studied *Oxytropis* and *Astragalus* species are provided in Table 1.

2.2. PCR amplification and DNA sequencing

The complete region of the ITS (ITS1 + 5.8S + ITS2) was amplified using primers ITSL (forward) and ITS4 (reverse) of Hsiao et al. (1995), while a large part of the *mat*K region (1191 bp) was amplified using primers F1 and R3 of Li et al. (1997). The DNA amplification for ITS was performed in a 50 µL volume containing 3 µL (10 ng/µL) of genomic DNA, 3 µL of 10X PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 3 µL of $MgCl_2$ (25 mM), 2 µL of dNTP mixture (10 mM), 2 µL of each primer (10 µM), 0.2 µL (5 u/µL) of Taq polymerase, and 34.8 µL of sterile water. Amplification was carried out using a DNA Thermal Cycler (Eppendorf Mastercycler 5333 v.2.30.33-09) with a program of 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 49 °C for 30 s, and elongation at 72 °C for 45 s, and then a final extension at 72 °C for 7 min to complete the primer-template extensions.

For the *mat*K region, DNA amplification was performed again in a 50 µL volume containing 4 µL (10 ng/µL) of genomic DNA, 5 µL of 10X PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 4 µL of $MgCl_2$ (25 mM), 3 µL of dNTP mixture (10 mM), 4 µL of each primer (10 µM), 0.3 µL (5 u/µL) of Taq

Table 1. Information on the *Oxytropis* and *Astragalus* species included in the study and NCBI accession numbers for both regions.

Section	Species	Number of genotypes sampled	Location (province in Turkey)[a]	Accession number (ITS)	Accession number (*mat*K)
Oxytropis					
Mesogaea	O. kotschyana Boiss. & Hohen.	2	Van /Hakkari	KM053394	KM387606
Protoxytropis	*O. savellanica* Boiss.	2	Niğde	KM053397	KM387612
	O. lupinoides Grossh.	7	Erzincan/Erzurum/Sivas	KM053393	KM387608
Janthina	*O. persica* Boiss.	2	Niğde/Van	KM053388	KM387609
	O. albana Steven	2	Kayseri/Kars	KM053386	KM387601
	O. karjaginii Grossh.	8	Erzurum/Van	KM053391	KM387605
	O. engizekensis Duman&Vural	3	Kahramanmaraş/Erzincan	KM053387	KM387603
Dolichocarpon	*O. fominii* Grossh.	4	Eskişehir/Ankara/Erzurum	KM053390	KM387604
	O. argyroleuca Bornm.	3	Ankara	KM053389	KM387600
Eumorpha	*O. aucheri* Boiss.	3	Ağrı	KM053385	KM387602
Chrysantha	*O. pallasii* Pers.	3	Erzurum	KM053395	KM387610
	O. pilosa L.	3	Artvin	KM053396	KM387611
Orobia	*O. lazica* Boiss.	3	Trabzon/Rize	KM053392	KM387607
Astragalus					
Incani	*A. achundovii* Grossh.	3	Hakkari	Dizkirici et al., 2014	KM387613
	A. ancistrocarpus Boiss.&Hausskn.	3	Şanlıurfa		KM387614
	A. brachycarpus M.Bieb.	3	Van/Erzurum		KM387615
	A. brevidentatus Podlech	3	Kastamonu /Ankara		KM387616
	A. campylosema Boiss.	3	Elazığ /Erzurum/Sivas		KM387617
	A. cariensis Boiss.	3	Muğla		KM387618
	A. cinereus Willd.	3	Erzincan /Erzurum		KM387619
	A. clavatus DC.	3	Mardin		KM387620
	A. czorochensis Charadze	3	Erzurum /Artvin		KM387621
	A. elongatus Willd.	3	Sivas/Şanlıurfa/Kastamonu		KM387622
	A. frickii Bunge	3	Artvin		KM387623
	A. germanicopolitanus Bornm.	3	Çankırı		KM387624
	A. glaucophyllus Bunge	3	Sivas		KM387625
	A. humillimus Freyn	3	Kastamonu		KM387626
	A. latifolius Lam.	3	Ağrı/Van/Erzurum		KM387627
	A. longisubulatus Podlech	3	Erzincan		KM387628
	A. micrancistrus Boiss. & Hausskn.	3	Van		KM387629
	A. nezaketae Duran & Aytac	3	Erzincan		KM387630
	A. olurensis Podlech	3	Artvin		KM387631
	A. polhillii Podlech	3	Van		KM387632
	A. sanguinolentus M.Bieb.	3	Sivas /Artvin/Yozgat		KM387633
	A. scabrifolius Boiss.	3	Malatya		KM387634
	A. schizopterus Boiss.	3	Denizli		KM387635
	A. sigmoideus Bunge	3	Kastamonu/Çankırı		KM387636
	A. spruneri Boiss.	3	Sivas/Çankırı/Kastamonu		KM387637
	A. robustus Bunge	3	Ağrı/Erzurum/Van		KM387638
	A. tigridis Boiss.	3	Erzincan		KM387639
	A. turkmenensis Dural	3	Konya		KM387640
	A. yildirimlii Aytac & Ekici	3	Ankara		KM387641
	A. zaraensis Podlech	3	Sivas		KM387642

Table 1. (Continued).

Section	Species	Number of genotypes sampled	Location (province in Turkey)[a]	Accession number (ITS)	Accession number (*mat*K)
Hypoglottidei	*A. akmanii* Aytac & Duman	3	Kahramanmaraş	Dizkirici et al., 2014	KM387643
	A. bachmarensis Grossh.	3	Artvin		KM387644
	A. cedreticola Duran & Podlech	3	Antalya		KM387645
	A. cicer L.	3	Erzurum		KM387646
	A. dasycarpus Chamb.	3	Van		KM387647
	A. viciaefolius DC.	3	Artvin/Trabzon		KM387648
	A. hartvigii Kit Tan	3	Antalya		KM387649
	A. lasioglottis M.Bieb.	3	Artvin		KM387650
	A. melanocarpus Bunge	3	Kahramanmaraş		KM387651
	A. oreades C.A.Mey.	3	Rize		KM387652
	A. ovatus DC.	3	Trabzon		KM387653
	A. saganlugensis Trautv.	3	Ağrı/Van		KM387654
	A. scholerianus Bornm.	3	Konya		KM387655
	A. vexillaris Boiss.	3	Şanlıurfa		KM387656
	A. viridissimus Freyn & Sint.	3	Trabzon		KM387657
Dissitiflori	*A. argyroides* Beck	3	Ağrı		KM387658
	A. aucheri Boiss.	3	Sivas/Erzincan		KM387659
	A. beypazaricus Podlech & Aytac	3	Ankara (Beypazarı)		KM387660
	A. cornutus Pall.	3	Ağrı		KM387661
	A. gladiatus Boiss.	3	Denizli		KM387662
	A. kastamonuensis Chamb. & Matthews	3	Kastamonu		KM387663
	A. nigrifructus Podlech & Aytac	3	Konya		KM387664
	A. nitens Boiss. & Heldr.	3	Sivas/Malatya		KM387665
	A. subulatus Pall.	3	Erzurum		KM387666
	A. taochius Woronov	3	Artvin/Erzurum		KM387667
	A. viridis Bunge	3	Kars		KM387668

[a]Each word after a '/' indicates a different location (province). If all specimens of a species were collected from same location, a '/' was not used.

polymerase, and 25.7 µL of sterile water. Amplification was carried out using a DNA Thermal Cycler with a program of 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and elongation at 72 °C for 90 s, and then a final extension at 72 °C for 10 min to complete the primer-template extensions.

A negative control was also included in the amplification procedure to be sure no contamination occurred. After the PCR amplification, the products of the ITS and *mat*K regions were run in a 1.5% and 1% agarose gel, respectively (using 1X TBE as the gel buffer). The PCR products were purified and sequenced from both ends using an ABI 310 Genetic Analyzer (PE Applied Biosystems) and an Automatic Sequencer (RefGen Biotechnology, Ankara). All ITS and *mat*K sequences and voucher information of the taxa included in the present study were deposited in the GenBank database (Table 1) (Dizkirici et al., 2014).

2.3. Phylogenetic analyses

The final ITS data set comprised 81 sequences of 49 *Oxytropis* species (13 from the current study and 36 from GenBank) and 168 sequences of 56 *Astragalus* species, while the *mat*K sequence data comprised 51 sequences of 19 *Oxytropis* species (13 from the current study and 6 from GenBank) and 168 sequences of 56 *Astragalus* species (Table 2). The nucleotide sequences of these regions were aligned using ClustalW (Thompson et al. 1994) using the following parameters: pairwise alignment gap opening = 15, gap extension = 6.6 and multiple alignment gap opening

Table 2. Estimated molecular diversity parameters for the genera *Oxytropis* and *Astragalus* based on ITS and *mat*K regions.

Parameters	ITS			*mat*K		
	Oxytropis(O)	*Astragalus*(A)	O+A	*Oxytropis*(O)	*Astragalus*(A)	O+A
# of species	13 (36[b])	56	69 (105[c])	13 (6[b])	56	69 (75[c])
# of sequences	45 (81[c])	168	213 (249[c])	45 (51[c])	168	213 (219[c])
Total length (bp)	641 (644[c])	634–639	643 (656[c])	1191	1191	1191
GC content (%)	55.5	53.6	53.9	31.6	31.2	31.3
P. I.[a] sites	11 (47[c])	56	88 (149[c])	10 (15[c])	15	38 (43[c])
# of deletions	– (5[c])	9	10 (17[c])	–	–	–
# of insertions	– (5[c])	9	10 (17[c])	–	–	–

[a] = parsimony informative, [b] = number of species taken from GenBank, and.[c] = calculated values with species retrieved from GenBank.

= 15, gap extension = 6.6, delay divergent sequences = 30% and transition weight = 0.5. All alignments used were checked and manually adjusted where necessary.

Alignment of the nrDNA ITS of the Turkish *Astragalus* and *Oxytropis* sequences revealed the presence of numerous single and multibase insertion/deletion events (indels). Five different indels ranging from one to six nucleotide lengths were observed in the aligned data of the ITS sequences. On the other hand, no indels were observed in the aligned data of the *mat*K sequences. During the analysis, the indels were coded as missing values. The coding of indels as missing or unknown data has the advantage that it retains information about substitutions that occur in the indel regions of other taxa (Wojciechowski et al., 1993).

For each region and genus, the basic sequence statistics including total nucleotide length (basepair; bp), % of guanine–cytosine (GC) content, number of deletions/insertions, and parsimony informative (variable) sites were computed using molecular evolutionary genetics analysis software (MEGA 5.0; Tamura et al., 2011) (Table 2). The phylogenetic analyses of the sequence data were carried out using the maximum likelihood (ML) method based on the Tamura–Nei model (1993) implemented with the MEGA software. An initial tree for the heuristic search was obtained automatically by applying the neighbor join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrapping was implemented in both data sets to quantify the support for clades. Supports for clades were evaluated by bootstrapping using 500 replicates (Felsenstein, 1985).

3. Results

Alignment of the ITS region of the Turkish *Astragalus* and *Oxytropis* species resulted in a data matrix of 643 bp characters, of which 88 (13.7%) sites were parsimony informative. The length of the region in the *Oxytropis* species was within the magnitude that was found in the *Astragalus* species. No variation was observed among the sequences of repeated individuals of each studied species, and so one representative sample for each species was used to construct a phylogenetic tree. There were no inserted or deleted nucleotide sites among the 641 bp length-sequences of the Turkish *Oxytropis* species. However, the length of the region in the investigated *Astragalus* species varied from 634 bp in *A. ovatus* DC. to 639 bp in *A. achundovii* Grossh. Five indel regions with different lengths were encountered in the sequences of the *Astragalus* species (Table 2). Four of these indels were composed of only one deleted\inserted nucleotide, while the fifth one included a deletion/insertion with a 6 bp length. The values for calculated GC content showed no remarkable differences between genera (Table 2). A high sequence identity of the ITS region was observed among the Turkish *Oxytropis* species and only 11 variable characters (1.7%) were detected within the genus, whereas genetic divergence was higher among the Turkish *Astragalus* species (56 variable sites, 8.8%) (Table 2). All of these parameters were also calculated when the new sequences downloaded from GenBank were included in the analysis. The calculated values are given in parentheses in Table 2. Nucleotide variation site was calculated as 47 when all of the *Oxytropis* species were considered in the analysis, which indicated a high diversity between the Turkish and the other *Oxytropis* species (Table 2).

In general, there is no information to clarify whether the length of ITS1 is longer or shorter than that of the ITS2 subunit in higher plants. In the current study, both of these

subunits had almost the same length (~235 bp for ITS1 and ~242 bp for ITS2) while the 5.8S subunit was shorter (~164 bp). A higher variability was observed in the ITS1 region. The sequence divergence in the aligned sequences of both genera from Turkey ranged from 0.0% to 18.3% (0–43 variable characters) in ITS1, from 0.0% to 3.0% (0–5 variable characters) in the 5.8S region, and from 0.0% to 16.5% (0–40 variable characters) in ITS2 (data available from authors). Less divergence of the 5.8S subregion was expected because this region is a transcribed area in the genome, and is responsible for the production of 5.8S rRNA.

Alignment of the *mat*K region resulted in a data matrix of 1191 bp nucleotides, of which 43 (3.6%) sites were parsimony informative. When only Old World species, native to Turkey, were analyzed, the number of variable sites was calculated as 10 and 15 for the genera *Oxytropis* and *Astragalus*, respectively. Indels were not observed among the 1191 bp length-sequences of the genera *Oxytropis* and *Astragalus*. The GC contents of the *mat*K region were almost the same for both genera (~31%). A higher similarity of sequences was observed in the DNA sequences of the *mat*K region (Table 2).

3.1. Genetic divergence

The genetic divergence (genetic distance, sequence diversity) between *Oxytropis* (New World and Old World) and *Astragalus* (Old World) genera was detected as 0.074 when a sequence of the ITS region was used. In the constructed phylogenetic tree, both *Oxytropis* and *Astragalus* appeared to be monophyletic clades supported with very high bootstrap values (Figure 1). The highest genetic divergence (0.090) was found between *Oxytropis aciphylla* (GQ422810) from GenBank database and *Astragalus gladiatus*, which is native to Turkey. Within *Oxytropis*, the highest genetic divergence was observed between *O. aciphylla* (GQ422810) and *O. caerulea* (HQ199316) (0.027). No genetic divergence was detected between native species *O. engizekensis* Duman & Vural and *O. persica* Boiss., nor between *O. argyroleuca* Bornm. and *O. fominii* Grossh.

Considering the *mat*K data, genetic divergence between the *Oxytropis* (including all species) and *Astragalus* genera was found to be low (0.018). Similar to the results from the ITS data, both *Oxytropis* and *Astragalus* were found to be monophyletic clades in the constructed phylogenetic tree (Figure 2). The highest genetic divergence (0.021) among the studied taxa was observed between several *Oxytropis* species [*Oxytropis lazica* Boiss., *O. campestris* (JQ669616), *O. parryi* (HQ293020)] and *Astragalus cicer*. Evolutionary divergence among the *Astragalus* species was also estimated based on the sequence of the *mat*K region. All species of the *Dissitiflori* section and *Astragalus cicer* (*Hypoglottidei*) demonstrated high divergence (0.008) with respect to species of the sections *Hypoglottidei* and *Incani*.

3.2. Evolutionary divergence

The analysis based upon variable characters yielded phylogenetic trees comprising two well supported major clades (Figures 1 and 2). In both of the constructed phylogenetic trees, all *Astragalus* species were included in the first clade and all *Oxytropis* species were assembled and comprised the second clade with 94%–100% bootstrap values. In the trees, only one representative was used to demonstrate a species since the sequences of replicate samples were identical. Moreover, sections of the genus *Astragalus* were simplified and indicated by schematic diagrams in the ITS tree because the evolutionary relationships among them were discussed in detail in a previous paper (Dizkirici et al., 2014).

The divergence of *Oxytropis* species from *Astragalus* species that is evident from the phylogenetic tree based on ITS region (Figure 1) was due to several substitutions located in the DNA sequence of the ITS1 and ITS2 subunits (Table 3). As expected, the DNA sequence of the 5.8S rDNA region was more conservative than those of the ITS1 and ITS2 subunits. In this region, only three substitutions were responsible for differentiating the genus *Oxytropis* from the genus *Astragalus* (Table 3).

Since phylogenetic relationships among *Astragalus* species based on ITS region were explained in detail in the previous study (Dizkirici et al., 2014), only relationships among the *Oxytropis* species are presented in depth here. Although relatively low genetic divergence among native *Oxytropis* species was present, the revealed phylogenetic relationships among them were meaningful. *Oxytropis kotschyana*, *O. pallasii*, and *O. pilosa*, Old World species native to Turkey, were separated from most of other species due to a few substitutions of DNA sequence in the ITS1 subunit (Table 3). *Oxytropis argyroleuca* – *O. fominii* as well as *O. engizekensis* – *O. persica* formed separate groups (Figure 1). Phylogenetic separation of *O. argyroleuca* and *O. fominii* species occurred due to substitutions observed at positions 34 (T–C), 54 (A–C), and 557 (T–C). On the other hand, the differentiation of *O. engizekensis* and *O. persica* from other related species of the genus was due to the presence of two nucleotide variations found at positions 118 (G–T) and 441 (G–A). As shown in Table 3, there was no substitution in the DNA sequence of the 5.8S subunit of the Turkish *Oxytropis* species, while a few substitutions in the same region were observed when *Oxytropis* and *Astragalus* species were analyzed together.

Even though monophyly of the genus *Oxytropis* was confirmed in the phylogenetic tree (Figure 1) by a branch supported with a 94% bootstrap value, relationships among *Oxytropis* species were poorly resolved and only seven branches received bootstrap values greater than 50%.

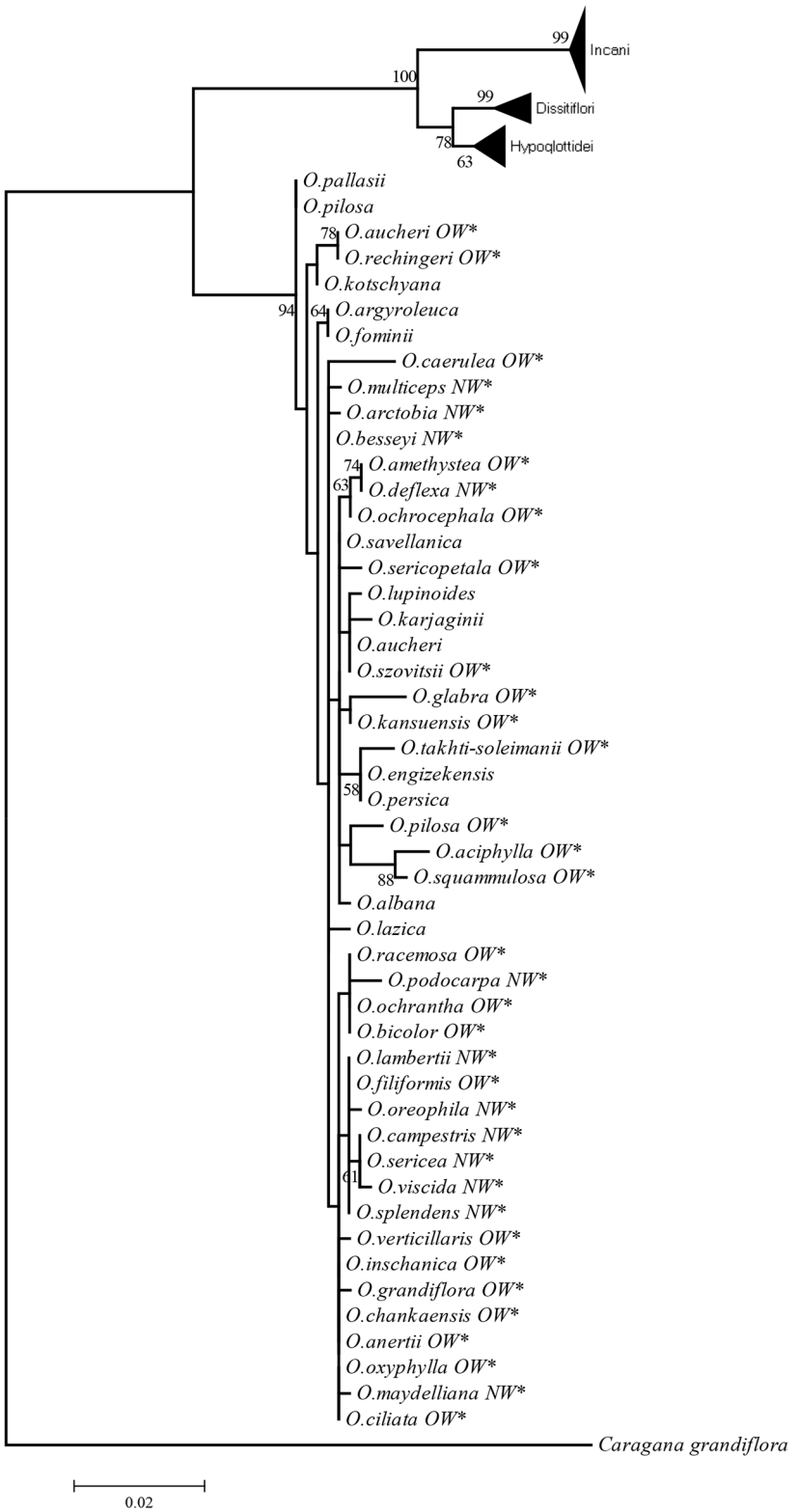

Figure 1. Phylogeny of the genera *Oxytropis* and *Astragalus* based on the DNA sequence of the nrDNA ITS region. Bootstrap values are indicated above the appropriate nodes for which support values were greater than 50%. *Astragalus* sections (*Incani, Hypoglottidei* and *Dissitiflori*) are indicated by symbols (Dizkirici et al., 2014). * = species was taken from the NCBI database, NW = New World, and OW = Old World.

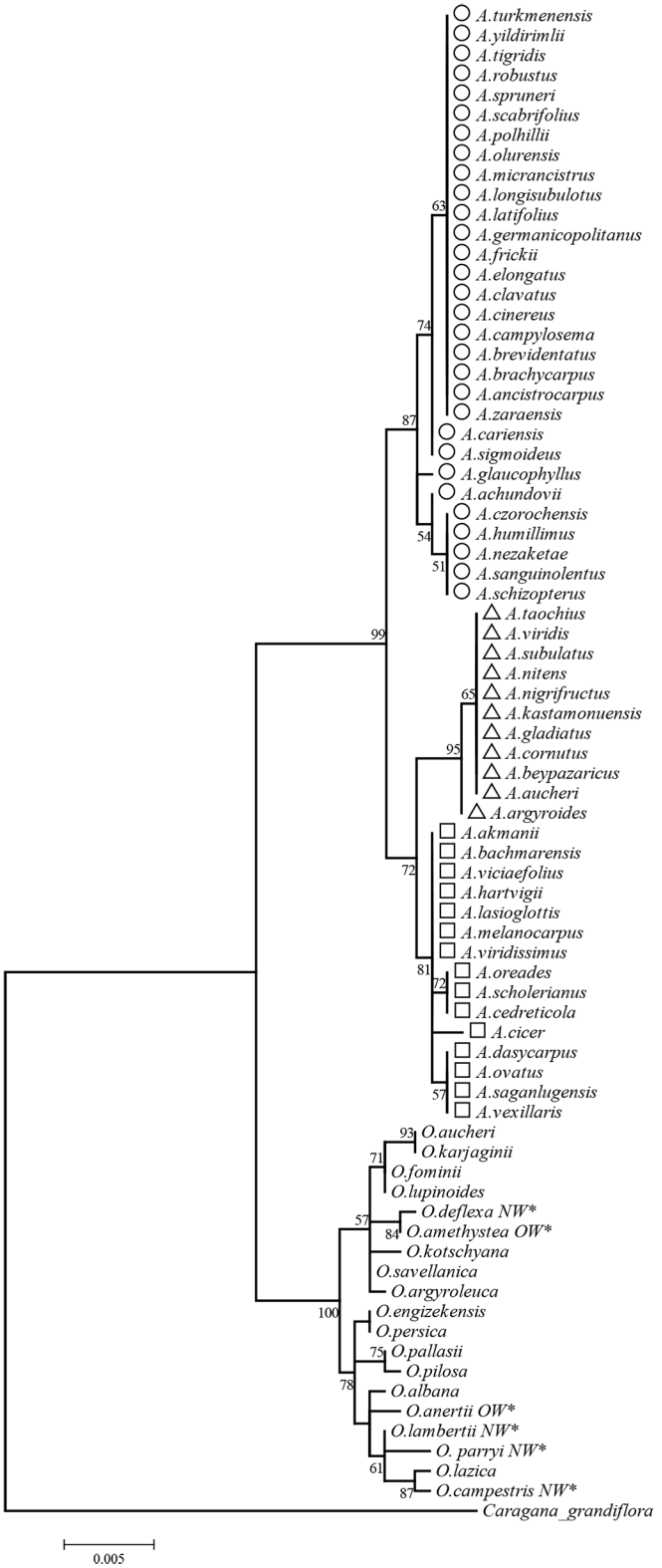

Figure 2. Phylogeny of the genera *Oxytropis* and *Astragalus* based on the DNA sequence of the plastid *mat*K gene region. Bootstrap values are indicated above the appropriate nodes for which support values were greater than 50%. Circles = *Incani,* squares = *Hypoglottidei*, and triangles = *Dissitiflori* sections of the genus *Astragalus*. * = species was taken from the NCBI database, NW = New World, and OW = Old World.

Table 3. Samples for substitution and indel observed in the DNA sequence of the aligned ITS regions for the Turkish genera *Oxytropis* and *Astragalus*. Absences of nucleotides are shown with a dash and substitutions are in bold. The numbers above each column depict the position of the corresponding nucleotide in the whole alignment (5' to 3') from the beginning of the ITS1 region to the end of the ITS2 region. (Not all species are shown in the table.)

	Variable nucleotide positions in the aligned DNA sequences of the ITS region																																					
		ITS1																					5.8S				ITS2												
	Species	34	41	54	55	64	67	69	70	71	73	74	107	108	109	110	111	112	113	118	180	209	238	362	368	369	427	428	441	449	494	519	537	544	557	558	559	582	589
Oxytropis	*O. aucheri*	C	C	A	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	C	G	C	T	C
	O. albana	C	C	C	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	C	G	C	T	C
	O. engizekensis	C	C	C	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	G	G	C	T	A	T	T	T	G	G	A	C	A	T	G	C	G	C	T	C
	O. persica	C	C	C	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	G	G	C	T	A	T	T	T	G	G	A	C	A	T	G	C	G	C	T	C
	O. argyroleuca	T	C	A	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	T	G	C	T	C
	O. fominii	T	C	A	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	T	G	C	T	C
	O. karjaginii	C	C	A	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	C	G	C	T	C
	O. lazica	C	C	C	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	T	G	C	T	C
	O. lupinoides	C	C	A	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	C	G	C	T	C
	O. kotschyana	T	C	C	G	T	G	G	T	G	T	T	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	T	G	C	T	C
	O. pallasii	T	C	C	G	T	G	G	T	G	T	T	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	C	G	C	T	C
	O. pilosa	T	C	C	G	T	G	G	T	G	T	T	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	C	G	C	T	C
	O. savellanica	C	C	C	G	T	G	G	T	G	T	C	G	C	G	C	A	T	T	T	G	C	T	A	T	T	T	G	A	A	C	A	T	G	C	G	C	T	C
Astragalus	*A. achundovii*	C	T	T	A	A	A	T	G	T	C	T	A	C	G	C	A	C	A	T	A	T	G	T	C	A	C	A	A	T	T	A	A	T	C	A	T	A	–
	A. cinereus	C	T	T	A	A	A	T	G	T	C	T	A	C	G	C	A	C	A	T	A	T	G	T	C	A	C	A	A	T	T	A	A	T	C	A	T	A	–
	A. zaraensis	C	T	T	A	A	A	T	G	T	C	T	A	C	G	C	A	C	A	T	A	T	G	T	C	A	C	A	A	T	T	A	A	T	C	A	T	A	–
	A. akmanii	C	T	T	A	A	G	T	G	T	T	T	T	–	–	–	–	–	–	C	A	C	G	A	C	A	C	A	A	T	C	A	A	T	C	A	T	A	–
	A. aucheri	C	T	T	A	A	G	T	G	T	T	T	T	–	–	–	–	–	–	C	A	G	G	A	C	A	C	A	A	T	C	G	A	T	C	A	T	A	–
	A. nitens	C	T	T	A	A	G	T	G	T	T	T	T	–	–	–	–	–	–	C	A	G	G	A	C	A	C	A	A	T	C	G	A	T	C	A	T	A	–
	A. viridis	C	T	T	A	A	G	T	G	T	T	T	T	–	–	–	–	–	–	C	A	G	G	A	C	A	C	A	A	T	C	G	A	T	C	A	T	A	–

The New and Old World *Oxytropis* species did not group separately in the phylogenetic tree and so a single adaptive radiation could not be considered for the evolution of the New World *Oxytropis* species (Figure 1). According to the tree constructed based on the sequence of the ITS region, the New World *Oxytropis* species evolved from the Old World group through at least two lineages. However, due to poor resolution in the phylogenetic tree, the ancestor of the New World group cannot be determined. All Turkish (Old World) *Oxytropis* species were clustered within the first clade including both Old and New World species. However, the majority of the New World *Oxytropis* species were included in the second cluster with a 61% bootstrap value (Figure 1).

Several nucleotide substitutions found in the DNA sequence of the *mat*K gene region (Table 4) caused the phylogenetic separation of the genus *Oxytropis* from *Astragalus* (Figure 2) with high bootstrap values. Similar to the ITS tree, the New World species were not separated from the Old World ones in the *mat*K tree. Therefore, we can say that the New World *Oxytropis* species may have followed different lineages while evolving from the Old World *Oxytropis* species. Although *Oxytropis kotschyana*, *O. pallasii*, and *O. pilosa* are known as caulescent species, *Oxytropis kotschyana* was phylogenetically different from *O. pallasii* and *O. pilosa* in the trees (Figures 1 and 2). *O. engizekensis* and *O. persica* were again grouped together without any genetic divergence in the *mat*K tree. However, *Oxytropis argyroleuca* and *O. fominii* were not grouped together in this tree even though they were clustered together in the ITS tree.

Three sections of the genus *Astragalus* were clearly separated from each other by forming different clusters in both the ITS and the *mat*K phylogenetic trees. The sections

Table 4. Samples for substitution observed in the DNA sequence of the aligned *mat*K region for the Turkish genera *Oxytropis* and *Astragalus*. Substitutions are shown in bold. The numbers above each column depict the position of the corresponding nucleotide in the whole alignment (5' to 3') from the beginning of the *mat*K region. (Not all species are shown in the table.)

	Variable nucleotide positions in the aligned DNA sequences of the *mat*K region																								
	Species	21	150	168	183	222	261	301	303	321	354	394	402	513	594	625	682	796	806	816	819	921	955	1091
Oxytropis	*O. aucheri*	A	T	T	T	T	T	G	C	C	T	G	T	C	T	T	A	G	T	G	C	T	C	C
	O. albana	A	G	T	T	T	G	G	C	C	T	G	C	C	T	T	A	G	T	G	C	T	T	C
	O. engizekensis	A	G	T	T	T	G	G	C	C	T	G	C	C	T	T	A	G	T	G	C	T	T	C
	O. persica	A	G	T	T	T	G	G	C	C	T	G	C	C	T	T	A	G	T	G	C	T	T	C
	O. argyroleuca	A	T	T	T	T	T	G	C	C	A	G	C	C	T	T	A	G	T	G	C	T	C	C
	O. fominii	A	T	T	T	T	T	G	C	C	T	G	T	C	T	T	A	G	T	G	C	T	C	C
	O. karjaginii	A	T	T	T	T	T	G	C	C	T	G	T	C	T	T	A	G	T	G	C	T	C	C
	O. lazica	A	G	T	T	T	G	G	C	C	T	G	C	C	T	T	A	G	T	G	C	T	C	C
	O. lupinoides	A	T	T	T	T	T	G	C	C	T	G	T	C	T	T	A	G	T	G	C	T	C	C
	O. kotschyana	A	T	T	T	T	T	G	C	C	T	G	C	C	T	T	A	G	T	G	C	T	C	C
	O. pallasii	A	G	T	T	T	G	G	C	C	T	G	C	C	T	T	A	G	T	G	C	T	C	C
	O. pilosa	A	G	T	T	T	G	G	C	C	T	G	C	C	T	T	A	G	T	G	C	T	C	C
	O. savellanica	A	T	T	T	T	T	G	C	C	T	G	C	C	T	T	A	G	T	G	C	T	C	C
Astragalus	*A. achundovii*	T	T	C	C	G	G	A	C	T	A	A	C	G	G	T	A	T	T	T	G	T	C	T
	A. cinereus	T	T	C	C	G	G	A	C	T	A	A	C	G	G	G	C	T	T	T	G	T	C	T
	A.olurensis	T	T	C	C	G	G	A	C	T	A	A	C	G	G	G	C	T	T	T	G	T	C	T
	A. zaraensis	T	T	C	C	G	G	A	C	T	A	A	C	G	G	G	C	T	T	T	G	T	C	T
	A. akmanii	T	T	T	C	G	G	A	T	T	A	A	C	G	G	T	A	T	T	T	G	C	C	T
	A. cedreticola	T	T	T	C	G	G	A	T	T	A	A	C	G	G	T	A	T	T	T	G	C	C	T
	A. cicer	T	T	T	C	G	G	A	T	T	A	A	C	G	G	T	A	T	T	T	G	C	C	T
	A.ovatus	T	T	T	C	G	G	A	T	T	A	A	C	G	G	T	A	T	T	T	G	C	C	T
	A. aucheri	T	T	T	T	G	G	A	T	T	A	A	C	G	G	T	A	T	C	T	G	T	C	T
	A. nitens	T	T	T	T	G	G	A	T	T	A	A	C	G	G	T	A	T	C	T	G	T	C	T
	A. viridis	T	T	T	T	G	G	A	T	T	A	A	C	G	G	T	A	T	C	T	G	T	C	T

Hypoglottidei and *Dissitiflori* were evolutionarily closer to each other than to the section *Incani* (Figures 1 and 2). Several positions were responsible for the phylogenetic separation of these *Astragalus* sections (Table 4; Figure 2). Since the evolutionary relationships among the *Astragalus* species were discussed in a previous paper (Dizkirici et al., 2014), details are not given here.

4. Discussion

Sequence data from both the ITS and the *mat*K regions were found to be useful to figure out the phylogenetic relationships among species within *Oxytropis* and *Astragalus* as well as the relationships between them. Although several studies (Dong et al., 2003a, 2003b; Yip and Kwan, 2006) found that the ITS (ITS1, 5.8S, and ITS2) region was more conservative than other regions such as 5S rRNA spacer, according to our results this region can provide accurate and reliable information to understand the phylogenetic relationships between and within these two genera.

The level of genetic variation (1.7% sequence divergence) observed in the ITS data of the native *Oxytropis* species was much lower than the intrageneric variation of the ITS sequences of the genus *Astragalus* (8.8% sequence divergence). Genetic divergence was still low even after new sequences of New and Old World *Oxytropis* species were added to the analysis. Low genetic divergence within the genus *Oxytropis* was also indicated by previous studies (Wojciechowski et al., 1993; Jorgensen et al., 2003; Mikhailova et al., 2008). Archambault and Stromvik (2012) used sequence data from the ITS region to understand phylogenetic relationships in *Oxytropis* and also added several *Astragalus* species to compare the genetic diversity between them. They concluded that the species of the genus *Astragalus* were more diverse than those of *Oxytropis*. Artyukova and Kozyrenko (2012) also reported similar results that the level of divergence of the species within and between the sections of the genus *Oxytropis* varies from 0% to 1.5% and from 0% to 3.7%, respectively. The low genetic variation of the ITS sequences characterizing most species of the genus *Oxytropis* may be regarded as an indication of their relatively recent and rapid divergence from a common ancestor, a high level of interspecific hybridization, and the reticulate pattern of evolution typical of the genus (Malyshev, 2008). The *mat*K region revealed a lower diversity than the ITS region. Similar results, that is, the finding of 0.8%–1.9% diversity among four *Astragalus* species, were also presented by Wojciechowski et al. (2004). Lower diversity was expected in the *mat*K than in the ITS region since this region is an exon and is responsible for production of a maturase-like protein (Neuhaus and Link, 1987). Even if the ITS region carries an exonic region (5.8S), it also carries two intronic areas (ITS1–2) on each side of the exonic area, and so a higher genetic divergence is expected in the ITS.

In both the genera *Astragalus* and *Oxytropis*, a high GC content of the ITS is meaningful because this region is responsible for maintaining the specific secondary structure of rRNA that provides functionality (Torres et al., 1990; Liu and Schardl, 1994; Schlotterer et al., 1994; Mai and Coleman, 1997). A high GC content leads to stability of the DNA and RNA secondary structures and is associated with the formation of stem-loop secondary structures. A high GC content of the ITS region was also reported for different species by several researchers (Wang et al., 2011; de Viana et al., 2014). Zheng et al. (2014) also found similar results to ours. They studied *Radix astragali* (*Astragalus membranaceus/A. mongholicus*) and its adulterants (about 29 species and 478 sequences) with DNA barcoding systems (ITS2, ITS, psbA-trnH, rbcL, *mat*K, and COI). They concluded that the average GC content from six barcodes was discrepant although the ITS and ITS2 regions from the nuclear ribosomal DNA revealed a higher GC content than the other barcodes did.

In the constructed phylogenetic trees, there were clear separations of all studied *Oxytropis* species from the *Astragalus* by the formation of two independent clusters with very high bootstrap values, suggesting that *Oxytropis* and *Astragalus* are monophyletic genera. These two genera were morphologically so close to each other that *Oxytropis* was considered to be part of the genus *Astragalus* in the past. However, in the present study, it was demonstrated that the molecular diversities of *Oxytropis* species regarding the sequence data from the ITS and *mat*K regions were quite different from those of *Astragalus* species. Kulshreshtha et al. (2004) used restriction fragment analyses to determine that the genus *Oxytropis* was monophyletic based on the phylogenetic data between the genera *Oxytropis* and *Astragalus*. The recent molecular studies based on nrDNA ITS and chloroplast *trn*L intron data clearly supported the hypothesis of parallel evolution of *Oxytropis* and *Astragalus* from a common proto-Astragalean ancestor (Wojciechowski et al., 1999; Wojciechowski, 2005).

In the ITS tree, the New World species did not form a monophyletic group within the genus *Oxytropis*. Four of the New World species (*O. multiceps*, *arctobia*, *besseyi*, and *deflexa*) separated and grouped with the Turkish and some other Old World *Oxytropis* species in the first clade while the remaining New World *Oxytropis* species grouped in the second clade with the other Old World species. A similar structure was also observed in the *mat*K tree topology: *O. deflexa* (NW) grouped with Old World species. This evolutionary relationship confirmed that the New World species did not evolve by a single adaptive radiation in the genus, but rather from different Old World lineages. These Old World *Oxytropis* species should be further investigated to find out if they migrated with human assistance or

originated from a common ancestral species. In the ITS tree, *Oxytropis aucheri** and *O. pilosa** obtained from GenBank did not group with their counterpart species in Turkey because of a high genetic divergence between the Turkish and the other *Oxytropis* species. This result was also supported by a study by Schlee et al. (2010). They used sequences of the ITS region to understand genetic diversity within the *Astragalus–Oxytropis* genus complex and concluded that the DNA sequences of the accessions of *O. pilosa* showed high interspecific and intraindividual genetic variations.

The phylogenetic trees proved that both *Astragalus* and *Oxytropis* are monophyletic genera. However, the relationships suggested by the nrDNA ITS and chloroplast *mat*K gene sequences of the different sections of *Oxytropis* did not agree with the traditional taxonomy. *Oxytropis* species of the same section were separated and did not form a monophyletic group in the dendrograms. Complex phylogenetic relationships within sections and subgenera may be caused by mutations, hybridizations, introgression, and unequal rates of molecular evolution owing to the diversity of ecological conditions. However, the DNA sequence data from both regions were quite useful to figure out the evolutionary relationships among sections of the genus *Astragalus*. All species from one section of the genus *Astragalus* grouped together and caused a separate subcluster, such as *Incani*, *Hypoglottidei*, and *Dissitiflori*.

No genetic divergence was observed between species *O. argyroleuca* and *O. fominii* as well as between *O. engizekensis* and *O. persica* when the ITS region was analyzed. However, only the *O. engizekensis* and *O. persica* species did not show any genetic divergence when the *mat*K region was studied. Karaman Erkul and Aytaç (2013) reported similar morphological structures between *O. argyroleuca* and *O. fominii* as well as between *O. engizekensis* and *O. persica*. As a result, they revised the status of species *O. engizekensis* to a synonym of *O. persica* and the species *O. fominii* to a synonym of *O. argyroleuca*. However, our results surely support treating only *O. engizekensis* and *O. persica* as synonyms.

Although the success of using the ITS and *mat*K regions to figure out phylogenetic relationships among *Astragalus* and *Oxytropis* has been demonstrated by several studies, the number of taxa can directly impact the outcome of the phylogenetic analyses. Therefore, the outcomes of this study could be valuable information for further studies dealing with the speciation in *Oxytropis* and *Astragalus*.

The ITS and *mat*K sequence phylogeny lead to the following conclusions: (i) knowledge of the degree of ITS and *mat*K sequence divergence between *Oxytropis* and *Astragalus* species was useful to demonstrate the phylogenetic relationship, especially at the generic level, (ii) sequence divergence was higher within the genus *Astragalus* compared to *Oxytropis* when the ITS region was analyzed, (iii) phylogenetic separation at the section level is very clear for the genus *Astragalus* whereas it is not clear for *Oxytropis*, (iv) New World *Oxytropis* species grouped with the Old World species into two different clusters, which showed that New World species did not evolve by a single adaptive radiation in the genus, but rather from different Old World lineages, (v) synonymous species *Oxytropis engizekensis* and *O. persica* were demonstrated by the sequence diversity of the ITS and *mat*K regions, and (vi) this study did not provide sufficient evidence that *Oxytropis fominii* is a synonym of *O. argyroleuca*.

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Supplemental Data

Phylogenetic relationships between *Oxytropis* DC. and *Astragalus* L. species native to an Old World diversity center inferred from nuclear ribosomal ITS and plastid *mat*K gene sequences

APPENDIX I. Species for which sequences were retrieved from GenBank:
ITS sequences: *Oxytropis arctobia* Bunge, *O. maydelliana* Trautv., *O. podocarpa* Gray, *O. deflexasubsp. Foliolosa* (Pall.) DC., *O. splendens* Douglas, *O. campestris* (L.) DC., *O. inschanica* Cheng, *O. filiformis* DC., *O. caerulea* (Pall.) DC., *O. grandiflora* DC., *O. squammulosa* DC. (HQ176487, HQ176485, HQ176483, HQ176481, HQ176479, HQ176475, HQ199322, HQ199321, HQ199316, HQ199315, HQ199318, Archambault, 2013), *O. lambertii* Pursh, *O. viscida* Nutt., *O. multiceps* Nutt., *O. sericea* Nutt., *O. szovitsii* Boiss & Buhse, *O. besseyi* Blank., *O. oreophila* Gray. (AF121753, AF121758, AF121760, AF121757, AF121754, AF121756, AF121755, Wojciechowski et al., 1999), *O. racemosa* Turcz (GQ422811, Gao et al., 2009), *O. bicolor* Bunge (HQ199317, Lu and Gao, 2010, unpublished), *O. chankaensis* Jurtz., *O. oxyphylla* DC. (FR839011, FR839000, Artyukova and Kozyrenko, 2012), *O. ciliata* Turcz (HQ199325, Lu and Gao, 2010, unpublished), *O. amethystea* Arv.-Touv. (GQ246045, Ahlquist and Wojciechowski, 2010, unpublished), *O. pilosa* (AF121759, Wojciechowski et al., 1999), *O. aucheri* (AB051908, Kazempour Osaloo et al., 2003), *O. anertii* Nakai (EF685971, Guo et al., 2010, unpublished), *O. ochrantha* Turcz., *O. aciphylla* Ledeb., *O. glabra* DC., *O. verticillaris* DC (GQ422820, GQ422810, GQ265961, GQ422818, Lu and Gao, 2010, unpublished), *O. sericopetala* Prain ex C.E.C. Fisch., *O. kansuensis* Bunge, *O. ochrocephala* Bunge (KJ143725, KJ143724, KJ143723, Cui and Li, 2014, unpublished), *O. takhti-soleimanii* Vassilcz., and *O. rechingeri* Vassilcz. (AB741306, AB741305, Javanmardi et al., 2012).
***mat*K sequences:** *O. anertii* Nakai (HM142266, Guo et al., 2010), *O. deflexa* (Pall.) DC. and *O. lambertii* Pursh (AY386878 and AY386915, Wojciechowski et al., 2004); *O. campestris* (L.) DC. and *O. amethystea* (JQ669616 and JQ669615, Wojciechowski, 2012, unpublished) and *O. parryi* A. Gray (HQ293020, Wojciechowski, 2011, unpublished).

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