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Phylogenetic relationships among native *Oxytropis* species in Turkey using the trnL intron, trnL-F IGS, and trnV intron cpDNA regions

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Abstract: We tested the phylogenetic utility of three chloroplast DNA loci, i.e. the trnL$^{(UAA)}$ intron, trnL$^{(UAA)}$-F$^{(GAA)}$ intergenic spacer (IGS), and trnV$^{(UAC)}$ intron, across thirteen native *Oxytropis* species. Our objective was to determine whether any of these chloroplast DNA markers could be beneficial to figure out phylogenetic relationships among *Oxytropis* species. To increase the interspecific sampling, nine sequences of the trnL intron and trnL-F regions were retrieved from GenBank and included in the analyses. No sequence of the trnV intron region was available in the database and so only sequences of the native species were used for the analyses. Phylogenies derived from maximum likelihood and maximum parsimony analyses indicated that the trnL intron and trnV intron regions provided better resolution for relationships among species with respect to the trnL-F region. The highest variable and parsimony informative sites were observed in the trnL intron region, while the lowest sites were seen in the trnV intron. Less variable sites for the trnV intron region were expected since no foreign sequences could be included in the analysis. *Oxytropis lazica* was phylogenetically separated from native species and clustered with foreign ones when the trnL intron and trnL-F regions were analyzed. The result obtained from the trnV intron region proved that *Oxytropis engizekensis* Duman and Vural may be a synonym of *O. persica* Boiss. A previous study that used morphological characters arrived at a similar conclusion.

Key words: *Oxytropis*, phylogeny, trnL intron, trnL-F IGS, trnV intron

1. Introduction

Oxytropis D.C. (Fabaceae) comprises about 330 species (Welsh, 2001), with the highest diversity in Central Asia (153–166 spp.; Malyshev, 2008). Representatives of the genus are spread in temperate and cold regions of the northern hemisphere, especially in the mountains of Asia. In the *Flora of Turkey*, 13 *Oxytropis* species have been described and three of them are considered endemic (Chamberlain and Matthews, 1970; Ozhatay, 2000). Karaman Erkul and Aytaç (2013) revised the taxonomical status of the genus using morphological characters such as stipule structure, leaf length, inflorescence shape, and fruit structure and some species were regarded as synonyms. Therefore, they reduced the number of species in Turkey from 13 to 11. In their study, *O. engizekensis* Duman and Vural was treated as a synonym of *O. persica* Boiss. and *O. fominii* Grossh. as a synonym of *O. argyroleuca* Bornm. However, in the current study 13 candidate species were analyzed based on DNA sequences of three different chloroplast DNA regions to figure out the phylogeny of the genus and prove synonymous relationships between the mentioned species.

Oxytropis species are morphologically diverse, which may be because of their various habitats. Relationships among species of the genus have been studied based on anatomical and morphological characters such as pollen morphology, seed morphology, stomata, trichome, leaf surface morphology, fruit, and flower structures by several researchers (Dickoré and Kriechbaum, 2006; Kholina and Kholin, 2008; Karaman et al., 2009; Ceter et al., 2013; Erkul et al., 2014). Even though there are numerous studies indicating morphological variations in *Oxytropis* species, the number of studies in the literature concentrating on phylogenetic relationships among them is limited (Jorgensen et al., 2003; Gao et al., 2009; Archambault and Stromvik, 2012; Artyukova and Kozyrenko, 2012). Furthermore, none of these studies covers all of the Turkish species. Therefore, we decided to investigate the phylogenetic relationships among 13 Turkish *Oxytropis* species using molecular markers in the present study.

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In the current study, we wanted to use DNA barcoding regions, which are both variable and have highly conserved primer sequences. All studied regions were selected from chloroplast DNA. The first one was the *trn*L$^{(UAA)}$ intron, whose sequences have been commonly used for evolutionary studies among related species (Taberlet et al., 1991; Gielly et al., 1996; Zhang et al., 2004; Chen et al., 2005; Dizkirici et al., 2013; Saha et al., 2014). The second region, the *trn*L$^{(UAA)}$-F$^{(GAA)}$ intergenic spacer (IGS), was selected due to its utility at the generic and infrageneric level as a former region (Taberlet et al., 1991; Gielly and Taberlet, 1994; Bakker et al., 1999; Kores et al., 2001; Hoggard et al., 2004). These two regions were selected as DNA barcodes, since amplification of them across a wide taxonomic range is easy due to the universal primers designed by Taberlet et al. (1991). The last region, again a noncoding region, the trnV$^{(UAC)}$ intron, was selected since this region tends to evolve faster than coding sequences. The trnV intron has also been used before in different angiosperm taxa such as *Nicotiana*, *Zea mays*, *Hordeum vulgure*, and *Pisum sutivum* (Learn et al., 1992).

The aims of the present study were (i) to figure out phylogenetic relationships among *Oxytropis* species that are native to Turkey using three different cpDNA regions, (ii) to determine which region (trnL intron, trnL-F IGS, or trnV intron) was more powerful to understand evolutionary relationships among *Oxytropis* species, (iii) and to rearrange and prove the taxonomic rank of the species that was determined by another study that used morphological characters (Karaman Erkul and Aytaç, 2013).

2. Materials and methods

2.1. Plant samples and DNA isolation

Samples of the genus *Oxytropis* were collected from different parts of Turkey. Thirteen *Oxytropis* species with different numbers of representative samples were used to get more reliable results (Table 1). *Caragana grandiflora* DC. (trnL intron + trnL-F IGS; AB287412, Kazempour Osaloo et al., 2006) and *Lathyrus ochroleucus* Hook. (trnV intron; KJ806198, Sveinsson and Cronk, unpubl. data) were utilized as outgroups. Nine sequences of the trnL-F region (trnL intron + trnL-F IGS) [*Oxytopis oxyphylla* DC.* FR694956 (Artyukova and Kozyrenko, 2012); *O. glabra* DC.* KC936890, *O. ciliata* Turcz.* KC936889, *O. inschanica* H.C.Fu and Cheng f.* KC936888, *O. aciphylla* Ledeb.* JX878501, *O. ochrantha* Turcz.* JX893515, *O. verticillaris* DC.* JX893514 (Lu and Gao, unpubl. data); *O. chankaensis* Jurtzev* FN808331 (Artyukova et al., 2011); *O. aucheri* Boiss.* AB287416 (Kazempour et al., 2006)] retrieved from GenBank were combined with our data to increase the interspecific sampling and show

Table 1. List of studied taxa with their collection locality and NCBI GenBank accession numbers for the trnL-F and trnV intron regions.

Section	Species	Number of genotypes sampled	Location (province in Turkey)	Accession number trnL-F (*trnL* intron+trnL-F IGS)	Accession number (*trnV*)
Oxytropis					
Mesogaea	*O. kotschyana* Boiss. & Hohen.	2	Van/Hakkari	KR908676	KR908689
Protoxytropis	*O. savellanica* Bunge ex Boiss.	2	Niğde	KR908677	KR908690
	O. lupinoides Grossh.	7	Erzincan/Erzurum/Sivas	KR908678	KR908691
Janthina	*O. persica* Boiss.	2	Niğde/Van	KR908679	KR908692
	O. albana Steven	2	Kayseri/Kars	KR908680	KR908693
	O. karjaginii Grossh.	8	Erzurum/Van	KR908681	KR908694
	O. engizekensis Duman &Vural	3	K.maraş/Erzincan	KR908682	KR908695
Dolichocarpon	*O. fominii* Grossh.	4	Eskişehir/Ankara/Erzurum	KR908683	KR908696
	O. argyroleuca Bornm.	3	Ankara	KR908684	KR908697
Eumorpha	*O. aucheri* Boiss.	3	Ağrı	KR908685	KR908698
Chrysantha	*O. pallasii* Pers.	3	Erzurum	KR908686	KR908699
	O. pilosa DC.	3	Artvin	KR908687	KR908700
Orobia	*O. lazica* Boiss.	3	Trabzon/Rize	KR908688	KR908701
Outgroup					
	C. grandiflora	1	-	AB287412	-
	L. ochroleucus	1	-	-	KJ806198

the phylogenetic relationships among native and foreign species of the genus (* indicates species retrieved from the GenBank database). DNA sequences of the trnV intron region were not found in GenBank and so only sequences of native samples were used in the analysis.

Total genomic DNA was extracted from fresh or silica-dried plant material using a standard hexadecyltrimethylammonium bromide (CTAB) protocol with minor modifications (Doyle and Doyle, 1987). Quality of DNA for each sample was determined by running them on a 1.0% 1X-TAE (Tris base, glacial acetic acid, and 0.5 M EDTA, pH 8.0) agarose gel stained with EtBr. DNA concentration was measured using a Hoefer DyNA QuantTM 200 Fluorometer, and DNA stocks were diluted to 10 ng/µL for PCR studies.

2.2. PCR amplification, sequencing, sequence alignment, and phylogenetic analyses

PCR reactions were done in 25-µL volumes with the following reaction components: genomic DNA (10 ng/µL), 10X PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], $MgCl_2$ (25 mM), dNTP mixture (10 mM), a selected primer pair (10 µM), Taq polymerase (5 U/µL), and sterile water. Volumes of them for each region were decided on after optimization studies. The PCR reaction of each region consisted of almost the same amount of buffer (2.5 µL), $MgCl_2$ (2 µL), dNTP (1 µL), and Taq polymerase (0.1 µL). The amount of each primer was 0.5 µL for trnL-F IGS and 1 µL for both the trnL and trnV intron regions, and 0.5, 1, and 2 µL of diluted DNA was added to PCR mixtures of the trnL-F IGS, trnL, and trnV intron regions, respectively. PCR amplification of the trnL intron, trnL-F IGS regions was done by using primer pairs c (forward)/d (reverse) and e/f, respectively (Taberlet et al., 1991). Primer pair trnVF and trnVR was used to amplify the trnV intron region (Wang et al., 1999). PCR amplification protocols always started with 2 min initial denaturation at 94 °C and terminated with 10 min at 72 °C. Each reaction ended with a final 4 °C hold step and consisted of 30 cycles. The length of time for denaturation was 30 s, primer annealing was 25 s, and extension step was 30 s for each region. While 94 °C was used for the denaturation step, 72 °C was used for extension. Annealing temperature was changed according to the optimization studies; 53 °C, 54 °C, and 57 °C were used for the trnL-F IGS, trnV, and trnL intron, respectively. Purified PCR products were sequenced in both directions using an ABI 310 Genetic Analyzer (PE Applied Biosystems) and an Automatic Sequencer (RefGen Biotechnology, Ankara, Turkey).

Completed sequences were aligned with the aid of the program ClustalX (Thompson et al., 1997). The following parameters were used during the alignment procedure: pairwise alignment gap opening = 15, gap extension = 6.6 and multiple alignment gap-opening = 15, gap extension = 6.7, delay divergent sequences = 30%, and transition weight = 0.5. Alignments were checked and manually adjusted where necessary. All sequences were combined with the sequences of used regions downloaded from GenBank and analyzed together. Sequence data have been deposited in GenBank (accession numbers are indicated in Table 1). Borders of the trnL intron and trnL-F IGS regions were decided on by using different sequences downloaded from GenBank (Wojciechowski et al., 1999; Jansen et al., 2008; Artyukova et al., 2011). No sequences of the trnV intron region for *Oxytropis* samples were found in GenBank. Thus, complete chloroplast DNA sequences of different species were used to decide on the border of the region (Magee et al., 2010; Sabir et al., 2014). Prior to the construction of phylogenetic trees, number of used taxa/samples, total nucleotide length (bp), G/C content (%), number of deletions/insertions (indel), variable/parsimony informative sites, and evolutionary divergence over all sequence pairs (mean distance) for each region (trnL intron, trnL-F IGS, and trnV intron; Table 2) were calculated using Molecular Evolutionary Genetics Analysis software (MEGA 5; Tamura et al., 2011).

A phylogenetic tree of each studied region (trnL intron, trnL-F IGS, and trnV intron) was constructed using two different methods: maximum likelihood (ML) and maximum parsimony (MP). The Tamura–Nei model (1993) and bootstrap analysis with 500 replications (Felsenstein, 1985) were selected to construct the ML tree. In the ML method, initial tree(s) for the heuristic search were 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 the superior log likelihood value. The tree bisection reconnection (TBR) search method was employed with 100 random addition replications to construct the MP trees and the consensus tree inferred from the 10 most parsimonious trees.

3. Results

Among the cpDNA regions examined, the most variable region was the trnL intron. This region is composed of 45 accessions of 13 native *Oxytropis* taxa and 9 foreign accessions downloaded from GenBank. The length of the trnL intron region in the native taxa varied in length from 582 (*O. fominii* and *O. argyroleuca*) to 602 bp (*O. lazica* Boiss.). Eleven polymorphic sites were detected in 607 base pair sequences and the number of these sites increased (17 bp sites) when foreign sequences downloaded from GenBank were added to the data. Using the parsimony criterion, 15 of the 17 variable sites were parsimoniously informative for the trnL intron (Table 2). The trnL-F IGS

Table 2. Estimated molecular diversity parameters for the trnL intron, trnL-F IGS, and trnV intron cpDNA regions.

	trnL intron	trnL-F IGS	trnV intron
Number of taxa	13 (22)[a]	13 (22)[a]	13
Number of sequences	45 (54)[a]	45 (54)[a]	45
Total length (bp)	607 (618)[a]	113 (114)[a]	609
Variable sites	11 (17)[a]	6 (6)[a]	3
P. informative sites	11 (15)[a]	6 (6)[a]	3
Number of indels (bp)	25 (37)[a]	3 (9)[a]	20
G/C content (%)	32.2 (32.1)[a]	28 (27.8)[a]	31.3
Mean distance	0.002 (0.006)[a]	0.007 (0.025)[a]	0.002

[a] values were calculated with data including taxa retrieved from GenBank

region, located near the trnL intron, was less variable (6 sites) whether sequences retrieved from the database were added to the data or not (Table 2). The length of the region in the native taxa varied in length from 109 (*O. persica* and *O. engizekensis*) to 113 bp (*O. argyroleuca*). All sites were parsimoniously informative, not including the polymorphisms introduced by insertions/deletions (Table 2). The trnV intron region was the most conserved area; only 3 substitutions were detected (Table 2). The length of the region in the native taxa varied in length from 591 (*O. engizekensis*) to 604 bp (*O. pallasii* Pers. and *O. pilosa* DC). Variations among native and foreign species could not be seen for this region because no accessible sequence was present in the GenBank database. The lower variation in this region may originate from missing foreign sequences of the region in the database.

The average GC content varied little among regions (about 30%). A great number of "AT" repeats located between the 281st and 310th bases (trnL intron) and "T" repeats located between the 669th and 681st bases in the total aligned data (trnL-F) caused intricate indels among taxa. A similar situation was also observed in the sequence of the trnV intron region; the first indel comprised an insertion of "AGCAAAGGATTT" bases (between the 120th and 133rd bases) and the second one comprised polyT bases (293rd–303rd). Therefore, indels were not included when the phylogenetic trees were constructed. No substitution and/or indels among sequences of repeated samples of each taxon were observed. Therefore, only one representative sequence was used for each taxon to construct the trees.

The topologies of the MP and ML phylogenetic trees had no considerable differences, and so only one tree (ML) was presented for each region. The analysis based upon variable characters yielded phylogenetic trees comprising two well-supported major clades (Figures 1 and 2). The ML tree of the trnL intron region differentiated almost all native *Oxytropis* species from the foreign ones retrieved from GenBank except *O. glabra** and *O. aucheri** (Figure 1). *Oxytropis lazica* did not group with native species because of few substitutions. Native species did not differentiate well when the trnL intron region was used due to low genetic variations among species. Only *O. kotschyana* Boiss. and Hohen., *O. fominii*, and *O. savellanica* Bunge ex Boiss. separated from the others in the cluster (Figure 1). The tree constructed using the trnL-F IGS region was less informative; there were two clusters without subclusters. Almost all native species grouped within the first cluster and most of the foreign species grouped within the second cluster (Figure 2). There was no subcluster in the clusters because of the small number of variations through the sequence. *Oxytropis lazica* was again separated from the remaining native species, and *O. glabra** and *O. aucheri** grouped with native species. When we compare the trnL intron with the trnL-F IGS region, it is safe to say that the trnL intron is more useful for phylogenetic studies of the genus *Oxytropis*. Sequences of these two regions were combined and a new tree was constructed. It showed the same topology as the tree constructed based on the trnL intron (Figure 1).

Oxytropis engizekensis and *O. persica* remained distinct from other native species in the tree constructed using the sequence of the trnV intron cpDNA region (Figure 3) and *O. kotschyana* connected to the tree distantly as observed in the former tree (Figure 1). Phylogenetic separation of *O. kotschyana* occurred due to substitutions observed at position 292 (T-C). Differentiation of *O. engizekensis* and *O. persica* from other related species of the genus was due to the presence of one nucleotide variation found at position 541 (A-C). Even though native species are found in different sections, most of them, i.e. *O. pilosa*, *O. pallasii*, and *O. lupinoides* Grossh., were not separated from each

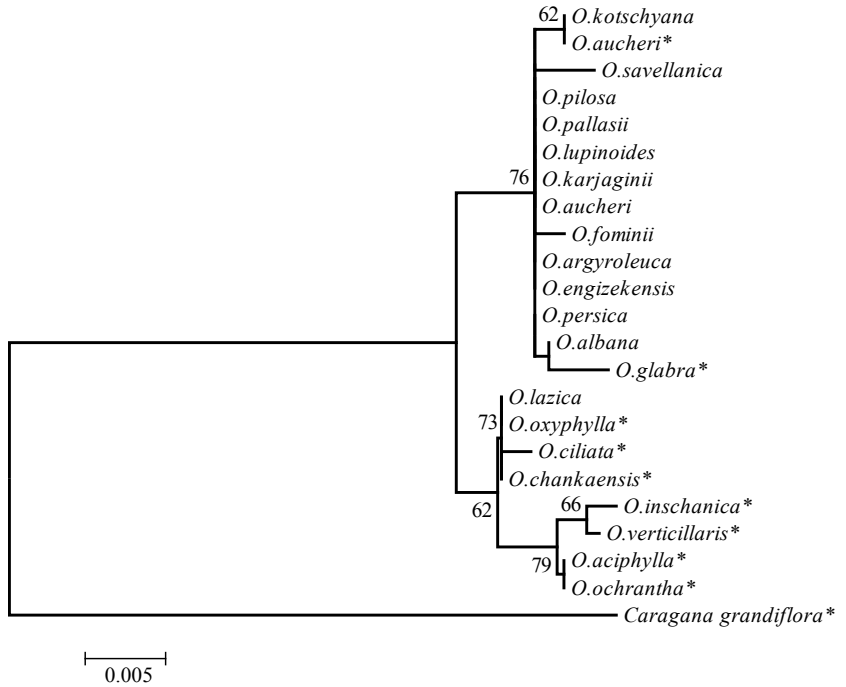

Figure 1. Phylogenetic tree for representatives of the genus *Oxytropis* constructed on the basis of comparison of the trnL intron and combined (trnL intron + trnL-F IGS) sequences by the ML (MP) method. The numerals are the bootstrap indices calculated using 500 pseudoreplicates; the asterisks designate the sequences obtained from GenBank.

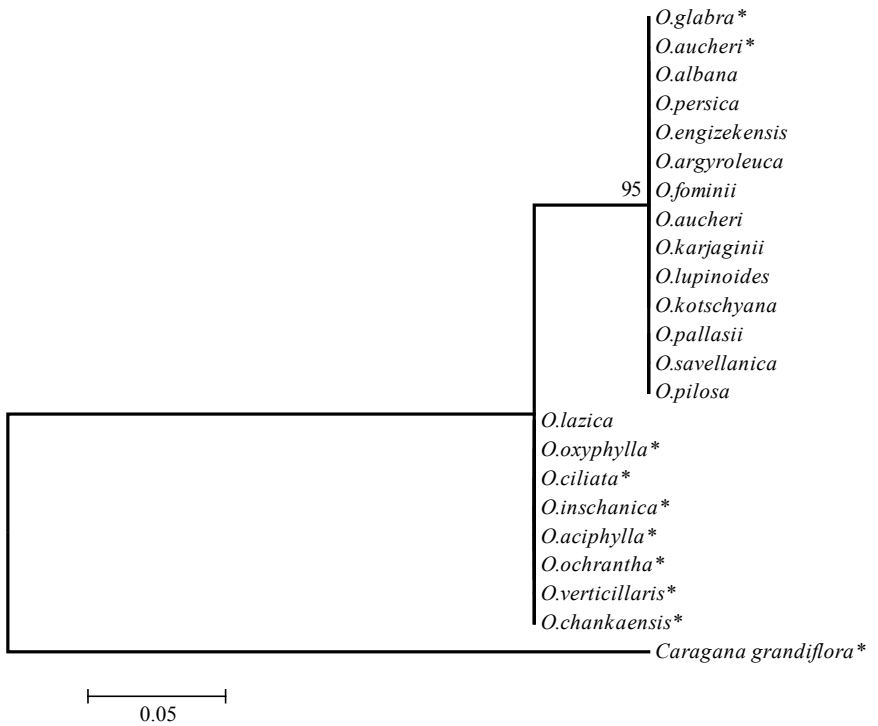

Figure 2. Phylogenetic tree for representatives of the genus *Oxytropis* constructed on the basis of comparison of the trnL-F IGS region sequences by the ML (MP) method. The numerals are the bootstrap indices calculated using 500 pseudoreplicates; the asterisks designate the sequences obtained from GenBank.

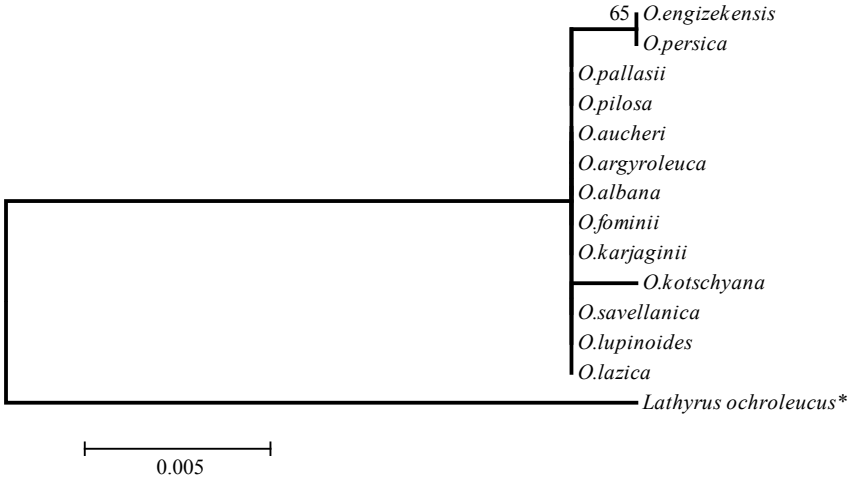

Figure 3. Phylogenetic tree for representatives of the genus *Oxytropis* constructed on the basis of comparison of the trnV intron region sequences by the ML (MP) method. The numerals are the bootstrap indices calculated using 500 pseudoreplicates.

other in either the trnL-F (trnL intron and trnL-F IGS) or the trnV intron tree.

4. Discussion

In the family Fabaceae, variation of the chloroplast genome is expected to be less than that of the nuclear genome because cpDNA is maternally inherited in this family (Doyle et al., 2004). Although a small amount of variation was detected in the examined cpDNA regions, *O. lazica* separated from native species and grouped with foreign ones when the trnL intron and trnL-F IGS regions were analyzed (Figures 1 and 2). Karaman et al. (2009) studied leaflet micromorphology of native *Oxytropis* species and indicated that most of studied species had xeromorphic stomata while *O. kotschyana*, *O. pallasii*, *O. pilosa*, and *O. lazica* had mesomorphic ones. They also indicated that *O. kotschyana*, *O. pallasii*, and *O. pilosa* bloom earlier (April/May) compared to *O. lazica* (July/August). Phylogenetic separation of *O. lazica* is meaningful because this species is found in a different subgenus, *Euoxytropis* (Boiss.) Bunge, while other studied species are found in either the subgenus *Phacoxytropis* Bunge or *Oxytropis* DC. Phylogenetic separation of this species may also be caused by the natural habitat (Trabzon/Rize), which is isolated from the others. Pollen morphology of native *Oxytropis* species was studied by Ceter et al. (2013) and they also proved differences between the pollen structure of *O. lazica* and that of the other species. They indicated that dimensions of the mesocolpium, colpus length (clg), colpus width (clt), porus length (plg), and porus width (plt) of *O. lazica* were very different compared to the others.

Among the *Oxytropis* species, *O. kotschyana*, *O. pallasii*, and *O. pilosa* are called caulescent while the others are acaulescent species. Within caulescent species, only *Oxytropis kotschyana* showed high divergence and separated from both caulescent and acaulescent species in the trees constructed by the trnL and trnV intron regions. *Oxytropis kotschyana* is closely related to *O. pallasii* but distinguished by its lavender–blue corolla and pendant legume. *Oxytropis pallasii* is related to *O. pilosa*, but Karaman Erkul and Aytaç (2013) separated them by using some morphological characters such as bract, leaflet apex, peduncle, raceme, keel structures, and corolla color. Even though there are some morphological difference between samples of *O. pallasii* and *O. pilosa*, the DNA markers used were not sufficient or suitable to separate them evolutionarily. Phylogenetic separation of *O. kotschyana* from other caulescent species was also proved by a previous study in which nuclear ribosomal ITS and plastid *mat*K gene sequences were used (Dızkırıcı Tekpınar et al., 2016). *Oxytropis pallasii* and *O. pilosa* are found in the section *Chrysantha* while *O. kotschyana* is in Mesogaea even if they are called caulescent species. Therefore, phylogenetic separation of *O. kotschyana*, located in a different section, can be expected.

Oxytropis savellanica and *O. fominii* showed higher genetic variation when only the trnL intron region was analyzed (Figure 1). Ceter et al. (2013) indicated that the shortest porus length (2.82 µm) was seen in *O. savellanica*, whereas the longest (5.04 µm) one was in *O. lazica* within the native species studied. Hence, the distant location of these two species in the trnL intron tree may be meaningful. Karaman Erkul and Aytaç (2013) proved morphological structure similarities between *O. argyroleuca* and *O. fominii* as well as between *O. engizekensis* and *O. persica*. As a result, they revised the status of the species *O. engizekensis* to a synonym of *O. persica* and the species *O. fominii* to a synonym of *O. argyroleuca. Oxytropis fominii*

separated from *O. argyroleuca* when the trnL intron region was analyzed (Figure 1) and also these two species did not separate from other native species in other trees (Figures 2 and 3). Therefore, we cannot phylogenetically support the idea expressing synonymity of *O. fominii* with *O. argyroleuca*. However, a close relationship between *O. engizekensis* and *O. persica* was observed in the trnV intron tree; these two species are genetically the same and are separated from the other native species (Figure 3). A similar result was also seen in our previous study (Dızkırıcı Tekpınar et al., 2016); genetic divergence was not observed between species *O. argyroleuca* and *O. fominii* or between *O. engizekensis* and *O. persica* when the ITS region was analyzed. In addition, *O. engizekensis* and *O. persica* species did not show any genetic divergence when the *mat*K region was studied either. When all these results are considered, we can say that *O. engizekensis* is a synonym of *O. persica* but synonymity of *O. fominii* with *O. argyroleuca* is still questionable.

When three chloroplast DNA markers were considered, low genetic variation was observed among species. A high level of similarity of the regions 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). However, the trnL intron region will be the best choice within the studied regions due to high genetic diversity. The trnL-F IGS region may not be a good choice because of its short length and low resolution in the tree. We could not reach a conclusive result for the last region, the trnV intron. Its genetic variation was low but this might be due to missing foreign species in the database. The sequences of the trnV intron region obtained from the current study would be valuable because this is the first time these sequences were submitted to GenBank so that they can be used by other researchers.

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