Taxonomic assessment of the Oxytropis species from South-East of Kazakhstan

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The genus Oxytropis DC. is one of the largest genera in the Fabaceae family. The most plant species belonging to the Oxytropis genus have an important medicinal value. Currently the botanical taxonomy of the genus is complicated due to existence of many subgenera and sections that developed based on morphological traits. Also, in the literature there is lack of knowledge on phylogeny of Oxytropis species from Central Asian region. Therefore, the purpose of the present study was the clarification of taxonomic relationship of two Oxytropis species from South-East of Kazakhstan (O. almaatensis Bajt. and O. glabra DC.). The study was based on using phylogenetic analysis and haplotype network assessment based on sequences ITS (internal transcribed spacers), which is DNA marker of nuclear genome. Plant materials of O. almaatensis were collected from 2 populations in two neighboring Gorges in Trans Il Alatau Mountains, O. glabra plant material was obtained from Herbarium of the Department of Biodiversity and Bioresources, Al-Farabi Kazakh National University. Based on DNA sequences of ITS the phylogenetic and network relationships were investigated by using Neighbor Joining and Median Joining methods, respectively. The nucleotide sequences of ITS of O. almaatensis and O. glabra were aligned with sequences of 29 Oxytropis references found in the NCBI database. Out of the 601 aligned positions of ITS 33 (5.6 %) sites were found to be polymorphic nucleotides and used in evaluation of the genetic relationship of species. Constructed MJ haplotype network showed a very high congruence with the NJ phylogenetic tree. MJ network provided valuable additional hints in clarification of the taxonomic relationship among species involved in the analysis. In this study phylogenetic NJ tree and MJ network based on the variation of ITS sequences confirmed the monophyletic origin of the genus. The ITS haplotype network suggested that O. glabra is very diverse species and possibly played important role in the evolutionary processes of the genus in Central Asian region. The study is additional contribution in the molecular taxonomy of complex Oxytropis genus.

Key words: Oxytropis; Oxytropis almaatensis; Oxytropis glabra; DNA barcoding; haplotype network.

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Oxytropis DC. with approximately 450 species, most of which are hairy perennial plants, is one of the largest genera in the family Fabaceae (Malyshev, 2008a). Oxytropis species are well distributed in Central Asia and rich in endemics, especially in mountain systems of Mongolian Altay, Tien Shan, Nanshan and Himalayas (Grubov, 2003). Grubov (2003) reported that Central Asia, along with West Asia, is the most important center of the speciation of genus Oxytropis. In Central Asia the genus consists of all the six subgenera and sixteen sections (Grubov, 2003). In northern Tien Shan the species composition of the genus Oxytropis has been studied by Abdulina (1978). Morphological studies of the species found in the northern Tien Shan region were carried out, the most convenient traits for diagnostics of taxa were identified, areas of endemic species and maps of their distribution have been specified (Abdulina, 1978). According to Malyshev (2008b) the genus is represented by 6 subgenera and 25 sections. Author clustered 25 sections according to the variability of the ITS nucleotide sequences. The study was conducted in the frame of the nation-wide research project DNA barcoding of wild flora of Kazakhstan (Turuspekov, Abugalieva, 2015) that combined efforts of local botanists and geneticists from Biotechnology Research Organizations, Botanical Gardens, National Nature Parks and Reserves as well as project “Informational system for molecular genetic and botanical documentation of wild flora in Kazakhstan”. It is another contribution to the description of the genetic variation of wild flora in Kazakhstan (Adams, Turuspekov, 1998; Turuspekov et al., 2002, 2014; Genievskaya et al., 2017).

Materials and methods
Sample collections and DNA extraction. Samples of leaves from O. almaatensis were collected from 2 populations in two different Gorges in Trans Ili Alatau Mountains (Big Almaty gorge and Small Almaty gorge) in 2015 and 2016, five plant samples from each population were chosen for the genetic analysis. O. glabra plant material was obtained from Herbarium of the Department of Biodiversity and Bioresources, al-Farabi Kazakh National University. For the construction haplotype network and phylogenetic tree ITS sequences were taken from NCBI (https://www.ncbi.nlm.nih.gov/genbank/). DNA was extracted using CTAB protocol (Doyle J.J., Doyle J.L., 1987) and stored at –20 °C until use.

DNA amplification and sequencing. PCR fragments were amplified from nuclear ribosomal complex including ITS1 and ITS2 (White et al., 1990). PCR was performed by using Veriti Thermo cycler (Applied Biosystems, Foster City, CA, USA). PCR reaction (total volume 16 µl) contained 4 mM of each dNTP, 6.4 mM of primer mix, 1.6 U of Taq DNA polymerase and 80 ng of total genomic DNA. The entire ITS-1, 5.8S, and ITS-2 region was polymerase chain reaction (PCR)-amplified using primers ITS1nF (5’-AGAAGTCGTAACAGGTTTC CGTAGG-3’) and ITS4nR (5’-TCTCCGCTTATGATGC-3’) with annealing temperature 58 °C (White et al., 1990). PCR products were run in 1.5 % agarose gel electrophoresis at 80 V voltage for 40 min. Single bands with expected sizes around 650 bp were cut out from gels and purified using ULTRAprep® Agarose Gel Extraction Mini Prep Kit (AHN Biotechnologie GmbH, Nordhausen, Germany) according to the protocol provided by the company. Purified DNA amplicons were used for the sequence reactions with forward and reverse primers separately. All reactions were performed in the frame of the nation-wide research project DNA barcoding of wild flora of Kazakhstan (Turuspekov, Abugalieva, 2015) that combined efforts of local botanists and geneticists from Biotechnology Research Organizations, Botanical Gardens, National Nature Parks and Reserves as well as project “Informational system for molecular genetic and botanical documentation of wild flora in Kazakhstan”. It is another contribution to the description of the genetic variation of wild flora in Kazakhstan (Adams, Turuspekov, 1998; Turuspekov et al., 2002, 2014; Genievskaya et al., 2017).
Haplotype Network was reconstructed using the Median Joining method (Bandelt et al., 1999) in PopART v.1.7 (Leigh, Bryant, 2015). The aligned sequences were converted into Nexus file format in DNASP v5.10 (Librado, Rozas, 2009) for the operations in the PopART software (version 1.7).

Results

Phylogenetic tree analyses based on ITS sequences

The DNA sequences of ITS of *O. almaatensis* and *O. glabra* were aligned with sequences of 29 *Oxytropis* references extracted from NCBI, and *Astragalus polaris* and *Astragalus mollissimus* were chosen as the outgroup taxa. The length of ITS (including ITS1, 5.8S, and ITS2) region for *Oxytropis* was...
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601 bp. 33 (5.6 %) sites out of the 601 aligned positions of ITS were polymorphic without outgroup. Singleton variable sites was 16, parsimony informative sites was 17.

The ITS nucleotide dataset consisted from O. almaatensis, O. glabra sequenced in this study, as well as 29 Oxytropis species and two outgroup species (A. polaris, A. mollissimus) collected from the NCBI database. The NJ tree clustered all Oxytropis accessions into four clusters and separated from the outgroup (Fig. 1). Sequences of O. almaatensis and O. glabra grouped with five references of O. glabra from NCBI in Cluster I. The Cluster II grouped together O. pallasii, O. pilosa, O. kansuensis, O. deflexa, and O. aciphylla. The Cluster III was represented by O. oxyphylla, O. intermedia, O. inschanica, O. microphylla, O. maydelliana, O. filiformis, O. evenorum, O. lanata, O. racemosa, O. ochrantha, O. chankaensis, O. campestris, O. caerulea, O. borealis, O. mandshurica. The Cluster IV was represented by following species from NCBI: O. kamtschatica, O. intermedia, O. inschanica, O. microphylla, O. maydelliana, O. filiformis, O. evenorum, O. lanata, O. racemosa, O. ochrantha, O. chankaensis, O. campestris, O. caerulea, O. borealis, O. mandshurica. The Cluster IV was represented by following species from NCBI: O. kamtschatica, O. intermedia, O. inschanica, O. microphylla, O. maydelliana, O. filiformis, O. evenorum, O. lanata, O. racemosa, O. ochrantha, O. chankaensis, O. campestris, O. caerulea, O. borealis, O. mandshurica.

Discussion

The traditional taxonomy of the genus Oxytropis is still unresolved and has many difficulties. Therefore the application of haplotype network and phylogenetic tree methods using polymorphic molecular markers is essential additional asset molecular taxonomy analyses of complicated genera. In this study phylogenetic NJ tree and MJ network based on the variation of ITS sequences confirmed the monophyletic origin of the genus (see Figs. 1, 2). This result is well in line with previously published results (Archambault, Strömvik, 2012;...

Haplotype network analyses based on ITS sequences

Twenty-nine haplotypes were identified for the ITS region in 33 accessions of Oxytropis genus and outgroup species in the network association analysis (Fig. 2). The results suggested that Hd = 0.991 (haplotype diversity), π = 0.01498 (nucleotide diversity), and k = 8.86553 (average number of nucleotide differences). The 29 haplotypes generated four haplogroups that corresponded to the NJ phylogenetic tree.

The largest haplotype H6 included O. oxyphylla, O. intermedia, O. inschanica (from NCBI) in haplogroup III. The next largest haplotype H3 contained O. racemosa, O. ochrantha from NCBI from the same haplogroup III. Haplotype 24 included two references of one species O. glabra in haplogroup I. Local species O. glabra and O. almaatensis generated two different haplotypes, H26 and H27, respectively, in haplogroup I (see Fig. 2, Table).

Fig. 1. Neighbor Joining phylogenetic tree reconstructed from the analysis of ITS sequences of Oxytropis and outgroup species.
The phylogenetic tree showed that *O. glabra* (section *Meso-gaea*) and *O. almaatensis* (section *Eumorpha*) are genetically close to each other within the genus *Oxytropis*. This result is suggesting that there is a possibility of existence of extinct or extant group of relative species that can be evolutionary closely associated with both *O. glabra* and *O. almaatensis*. Therefore, additional studies should be done to clarify this hypothesis.

The ITS network suggested that *O. glabra* is highly polymorphic species and one of their haplotype (Hap_26) is the closest point to two outgroup species of *Astragalus* (Hap_26 and Hap_29) (see Fig. 2). As both haplotypes, Hap_26 and Hap_27, represented two genetically close species sampled in southeast Kazakhstan, it can be speculated that these regions might associate with one of the centers of diversification for this genus.

The second group of species consisted of five following species – *O. pilosa*, *O. pallasii*, *O. kansuensis*, *O. deflexa*, and *O. aciphylla*. In previously published articles the majority of these species was often clustered together with *O. glabra* (Archambault, Strömvik, 2012; Artyukova, Kozyrenko, 2012; Kholina et al., 2016). In this study, the haplotype network separated these two groups as all five species of the second cluster were bound to the same median vector (mv) (see Fig. 2). Thus, it is a possibility that species may have the same extinct or extant predecessor, which is genetically close to *O. kansuensis*, *O. deflexa*, and *O. aciphylla*. Most populated groups of *Oxytropis* species formed the third cluster (haplogroup III) that has a connection to the *O. mandshurica* via a common mv in the network (Fig. 2). Similarly, *O. mandshurica* (Hap_9) using the same mv was also connected to haplogroup IV, represented by four Far East species (Kholina et al., 2016). It is interesting that the network is suggesting a close genetic relationship between *O. filiformis* (haplogroup III) and *O. hidakamontana* (haplogroup IV) despite their clusterization in different sub clades (see Fig. 2).

In general, the constructed haplotype network showed a very high congruence with the NJ phylogenetic tree. As generated NJ dendrogram showed a relatively low bootstrap value indices; the network provided valuable additional hints in clarification of the taxonomic relationship among species involved in the analysis. The study is another contribution in the molecular taxonomy of complex *Oxytropis* genus.

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**Conflict of interest**
The authors declare no conflict of interest.

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**Fig. 2.** The Median-joining haplotype network of ITS of *Oxytropis* and outgroup species.
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