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Phylogenetic Relationships of the Species of Asian Russia of the Subgenera *Phacoxytropis* and *Tragacanthoxytropis* Genus *Oxytropis* Based on the Polymorphism of Markers of the Chloroplast and Nuclear Genomes

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Abstract—On the basis of the analysis of the nucleotide polymorphism of the intergenic spacers *psbA−trnH*, *trnL−trnF*, and *trnS−trnG* of chloroplast DNA of the *Oxytropis* species from Asian Russia, *O. tragacanthoides* section *Hystrix* subgenus *Tragacanthoxytropis*, *O. coerulea*, *O. filiformis*, and *O. mandshurica* sect. *Janthina*, and *O. deflexa* and *O. glabra* sect. *Mesogaea* subg. *Phacoxytropis*, it was found that all populations are characterized by high haplotype diversity (*h* varies from 0.676 to 1.000), except for species of the sect. *Mesogaea* (*h* varies from 0 to 0.333). Species-specific markers were found for *O. tragacanthoides*, *O. deflexa*, *O. glabra*, and *O. mandshurica*, as well as specific markers for the sect. *Mesogaea*. Reconstruction of the phylogenetic relationships of the chlorotypes of the species of the subg. *Phacoxytropis*, *Tragacanthoxytropis*, and *Oxytropis* showed that the species of the sect. *Janthina* are combined into one well-supported clade with the species of the subg. *Tragacanthoxytropis* and *Oxytropis*, but their relationships remained unresolved. An analysis of the genealogical relationships of the ribotypes of the ITS of nuclear DNA revealed a common ribotype for the species *O. tragacanthoides*, *O. coerulea*, *O. lanata*, *O. chankaensis*, *O. oxyphylla*, and *O. triphylla*, belonging to three subgenera. The revealed genetic affinity with clear morphological differences is characteristic of taxa with a common origin that have experienced relatively recent rapid adaptive radiation. The obtained data on the variability of markers of the nuclear and chloroplast genomes confirm the status of *O. coerulea*, *O. filiformis*, and *O. mandshurica* as three separate species.

Keywords: *Oxytropis*, Fabaceae, genetic diversity, phylogenetic relationships, chloroplast DNA, ITS

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INTRODUCTION

The genus *Oxytropis* DC. (Fabaceae) presumably formed about 5.6 million years ago at the border of the Miocene and Pliocene [1, 2] during the evolution of species of the genus *Astragalus* L., subgenus *Phaca* (L.) Bunge [2, 3]. Species *Oxytropis deflexa* (Pall.) DC. and *O. glabra* (Lam.) DC. section *Mesogaea* Bunge subg. *Phacoxytropis* Bunge are believed to be morphologically and ecologically close to them [2−4]. Only these two species of the section are represented among the flora of Asian Russia [5]. *O. deflexa* is an Eastern Siberian and North American species, which is characterized by significant polymorphism throughout a wide discontinuous range [4−6]. It is believed to be a relict and the most ancient species of the section [3],

and it is included in the regional Red Books as it exists in small isolated populations. *O. glabra* is a polymorphic species, which is widespread in European Russia (Bashkortostan, Ural River), Middle and Central Asia, Mongolia, and Northwestern China [5−7].

The subg. *Phacoxytropis* includes also the sect. *Janthina* Bunge, the species of which are morphologically similar to the species of the sect. *Mesogaea* [5]. In the Asian part of Russia, the sect. *Janthina* is represented by six species: *O. kaspensis* Krasnob. et Pschen., *O. ladyginii* Kryl., *O. saposhnikovii* Kryl., *O. coerulea* (Pall.) DC., *O. filiformis* DC., and *O. mandshurica* Bunge. The relationships between the last three species are rather complicated [5]. Malyshev [5] believed that *O. filiformis* poorly differs from *O. coerulea* and

introgresses with it in Cis- and Transbaikalia. *O. filiformis* and *O. mandshurica* are independent species, while *O. caerulea* (Pall.) DC. and *O. coerulea* Turcz. are the synonyms of *O. coerulea* (Pall.) DC. The authors of *The Flora of China* [6] mention *O. coerulea* Turcz. as the synonym of *O. filiformis* and *O. mandshurica* as the synonym of *O. caerulea* (Pall.). They also include *O. filiformis* and *O. caerulea* in the sect. *Eumorpha* (Bge.) Abduss., which they include in the subg. *Oxytropis* ex genere *Oxytropis* DC. together with the sect. *Mesogaea* and *Janthina*. Further studies of the nucleotide polymorphism of the chloroplast genomes of the species from Asian Russia, the subg. *Oxytropis* and *Phacoxytropis* [8], showed that only the sect. *Mesogaea* corresponds to the subg. *Phacoxytropis*, whereas the sect. *Janthina* of the same subgenus is united with the sect. *Orobia* Bunge, *Verticillares* DC., and *Xerobia* Bunge of the subg. *Oxytropis*. Moreover, numerous molecular differences between the *O. coerulea* and *O. mandshurica* were revealed, indicating independence of these taxa. Nevertheless, contradictions and controversies still exist.

The subg. *Tragacanthoxytropis* Vass. is another ancient branch of the genus *Oxytropis*. The species of this subgenus are represented by shrublet forms and are sharply different morphologically from the species of other subgenera [3, 9]. One of the most interesting species of the subg. *Tragacanthoxytropis* is *O. tragacanthoides* Fisch. ex DC. sect. *Hystrix* Bunge. This mountain-steppe species is characterized by a discontinuous range and may be found in Central and Southeastern Altai and Mongolia; it is seldom found in the Tuva Hollow and Khakassia, where the northern border of the area passes, as well as in several regions of Cisbaikalia [5, 9]. *O. tragacanthoides* is considered as a relict of the Miocene and Pliocene flora [3, 9]. It is included in the regional Red Books as a vulnerable species under the threat of extinction, as well as a probably extinct species.

This work is the prolongation of population studies of the endemic *Oxytropis* species [10−14], as well as phylogenetic relationships between the species sect. *Verticillares* [15], *Orobia* [16], and *Arctobia* [17] and subgenera of the genus *Oxytropis* [8], which was carried out by the sequencing of the *psbA−trnH, trnL−trnF,* and *trnS−trnG* intergenic spacers of the chloroplast DNA (cpDNA) and the ITS of nuclear DNA (nrDNA).

The goal of this work was to study the genetic diversity and population structure of the *Oxytropis* species of the Asian Russia of the sect. *Mesogaea* and *Janthina* subg. *Phacoxytropis* and the sect. *Hystrix* subg. *Tragacanthoxytropis*, as well as to reconstruct the phylogenetic relationships of the species of the subg. *Phacoxytropis*, *Tragacanthoxytropis*, and *Oxytropis* based on the analysis of nucleotide sequence variability of the *psbA−trnH, trnL−trnF,* and *trnS−trnG* intergenic spacers of cpDNA and the ITS nrDNA.

MATERIALS AND METHODS

The objects of the study were 124 plants belonging to six species: *O. tragacanthoides* (20 samples) sect. *Hystrix* subg. *Tragacanthoxytropis*; *O. coerulea* (18), *O. filiformis* (20), and *O. mandshurica* (34) sect. *Janthina*; and *O. deflexa* (25) and *O. glabra* (7) sect. *Mesogaea* subg. *Phacoxytropis*, which were obtained from 22 wild populations (Table 1, Fig. 1). Names of the species, sections, and subgenera of the genus *Oxytropis* are given in accordance with [5].

DNA was extracted from lyophilized leaves. The extraction buffer contained 100 mM Tris-HCl (pH 8.0), 0.7 M NaCl, 40 mM EDTA, 1% CTAB (hexadecyltrimethylammonium bromide), and 10 mL/L β-mercaptoethanol. The extract was incubated at 65°C for 40 min. The DNA was deproteinized with chloroform : octanol (24 : 1) mixture and precipitated with equal volume of isopropanol in the presence of 0.3 M sodium acetate. DNA was washed with 75% ethanol and dissolved in the buffer, which contained 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The amount of DNA in a sample was estimated by comparison with the λ phage DNA of known concentration by electrophoresis in 1.4% agarose gel [10]. Amplification of the *psbA−trnH, trnL−trnF,* and *trnS−trnG* intergenic spacers was performed as described in [8, 12, 13]. The ITS region of nrDNA was amplified with the ITS1 and ITS4 primers under the reaction conditions and temperature described in [18]. Cyclic sequencing of both chains of DNA fragments was performed with the Big Dye Terminator v. 3.1 kit (Applied Biosystems, USA). The nucleotide sequences of the direct and reverse chains were determined with an ABI 3500 genetic analyzer (Applied Biosystems, USA) and then edited and assembled with the Staden Package v. 1.5 program package [19]. Region sequences were aligned with the SeaView v. 4.7 program for each sample [20].

The matrix of concatenated sequences of three cpDNA spacers was used for calculation of the haplotype (h) and nucleotide (π) diversity (for the populations with the number of samples no less than 5) and the degree of divergence (Dxy) between the populations/species based on nucleotide substitutions, for the analysis of molecular variance (AMOVA), and for the haplotype identification using the Arlequin v. 3.5 [21] and DnaSP v. 5.0 [22] programs. The statistical significance (P) of the fixation indices (F_{ST}) was assessed based on 1023 permutations. The phylogenetic analysis was performed by the ML, MP, and NJ methods using the PAUP v. 4.0b10 program [23].

The optimal model of the nucleotide substitution evolution for the ML analysis was chosen with the Modeltest v. 3.06 program [24] using the hierarchical tests. To perform the ML and MP analyses, the heuristic search for an optimal topology with the Tree Bisection-Reconnection (TBR) algorithm was used. The statistical confidence of the degree of branching was assessed by the bootstrap analysis of 1000 alterna-

Table 1. The studied populations of the *Oxytropis* species, subgenera *Tragacanthoxytropis* and *Phacoxytropis*, and parameters of genetic diversity obtained by the data of cpDNA analysis

Species, location (number of specimens)	Coordinates N, E	Code	Haplotype	Diversity	
				h (SD)	π (SD)
Subgenus Tragacanthoxytropis					
Sect. Hystrix					
O. tragacanthoides					
Altai Republic, Ukok Plateau, right bank of the Zhumaly River, elevation 2422 m (10)	49.51°, 88.06°	TRA1	P1–P7	0.867 (0.107)	0.0048 (0.0027)
Altai Republic, outskirts of the village of Chagan-Uzun, elevation 1780 m (5)	50.10°, 88.38°	TRA2	P8–P10	0.700 (0.218)	0.0008 (0.0006)
Mongolia, Central Aimag, environs of Öndörshireet somon, elevation 1282 m (5)	47.55°, 105.11°	TRA3	P11–P13	0.800 (0.164)	0.0005 (0.0004)
Subgenus Phacoxytropis					
Sect. Janthina					
O. coerulea					
Republic of Buryatia, outskirts of the village of Zaigraevo, elevation 675 m (5)	51.87°, 108.24°	COE1	P14–P17	0.900 (0.161)	0.0017 (0.0012)
Irkutsk oblast, outskirts of the village of Sarma, elevation 476 m (12)	53.12°, 106.85°	COE2	P18–P27	0.970 (0.044)	0.0046 (0.0025)
Irkutsk oblast, western coast of Lake Baikal, outskirts of the village of Sakhyurta (1)	53.01°, 106.89°	COE3	P28	–	–
O. filiformis					
Transbaikalia, environs of Lake Nozhiy, elevation 686 m (4)	50.82°, 114.72°	FIL1	P29–P32	–	–
Mongolia, Central Aimag, environs of Argalant somon, elevation 1488 m (5)	47.77°, 105.90°	FIL2	P29, P33–P36	1.000 (0.126)	0.0048 (0.0031)
Mongolia, Eastern Aimag, environs of Gurvanzagal somon, elevation 837 m (2)	48.86°, 115.11°	FIL3	P37, P38	–	–
Mongolia, Eastern Aimag, environs of Gurvanzagal somon, elevation 797 m (9)	49.27°, 114.71°	FIL4	P30, P37, P39–P41	0.722 (0.159)	0.0012 (0.0008)

Table 1. (Contd.)

Species, location (number of specimens)	Coordinates N, E	Code	Haplotype	Diversity	
				h (SD)	π (SD)
O. mandshurica					
Primorsky krai, Sikhote-Alinsky Reserve, Udobnaya Bay (15)	44.95°, 136.55°	MAN1	P42–P47	0.838 (0.061)	0.0015 (0.0009)
Primorsky krai, Sikhote-Alinsky Reserve, Blagodatnoe Cordon (4)	45.21°, 136.53°	MAN2	P45	–	–
Primorsky krai, outskirts of Dalnegorsk, Barachnaya Deep (15)	44.57°, 135.62°	MAN3	P42, P45, P48, P49	0.676 (0.101)	0.0004 (0.0003)
Sect. *Mesogaea*					
O. deflexa					
Republic of Buryatia, Dzherginsky Reserve, Birankur Tract (2)	55.11°, 111.46°	DEF1	P50, P51	–	–
Tuva Republic, Sayan Pass (1)	51.43°, 89.53°	DEF2	P52	–	–
Magadan oblast, outskirts of Susuman (9)	62.83°, 148.22°	DEF3	P53, P54	0.222 (0.166)	0.0098 (0.0054)
Magadan oblast, environs of the village of Orotuk, terrace above the floodplain of the Kolyma River (9)	62.10°, 148.49°	DEF4	P54	0.000 (0.000)	0.0000 (0.0000)
Magadan oblast, Pyagin Peninsula, the Middle Cape, near the spring (1)	59.32°, 154.57°	DEF5	P55	–	–
Magadan oblast, village of Seymchan (1)	62.93°, 152.39°	DEF6	P55	–	–
Eastern Taimyr, confluence of the Bolshaya Lesnaya Rassokha River and the Novaya River (2)	72.60°, 101.26°	DEF7	P55, P56	–	–
O. glabra					
Republic of Buryatia, outskirts of the village Orongoy, elevation 528 m (6)	51.55°, 107.03°	GLA1	P57, P58	0.333 (0.215)	0.0001 (0.0002)
Krasnoyarsk krai, outskirts of the village of Temra, the coast of Lake Gniloe (1)	55.43°, 89.27°	GLA2	P59	–	–

SD—standard deviation.

Fig. 1. Schematic map of 22 places from which samples of *Oxytropis tragacanthoides* sect. *Hystrix* subg. *Tragacanthoxytropis*, *O. coerulea*, *O. filiformis*, and *O. mandshurica* sect. *Janthina*, and *O. deflexa* and *O. glabra* sect. *Mesogaea* subg. *Phacoxytropis* were collected. See Table 1 for the population code.

tive trees (BP, %). Moreover, the Bayesian inference method (BI) was applied using the MrBayes 3.1.6 program [25] on the CIPRES portal (http://www.phylo.org/) [26]. The posterior probabilities (PP) were estimated in order to assess the confidence. The BP < 50% and PP < 0.95 values were not considered. Previously obtained nucleotide sequences of the same spacers (LM653198, LM653161, LM653235) of the *A. davuricus* (Pall.) DC. were used as the outgroup [8]. The genealogical relationships of the ITS nrDNA ribotypes were analyzed by the median-joining (MJ) method in the Network v. 5.0.1.1 program [27], encoding each deletion/insertion as a single mutation event regardless of its size. The previously obtained ITS (LM653272) sequence of the *A. davuricus* was used as the outgroup.

RESULTS

The nucleotide sequences of each of the regions *psbA−trnH*, *trnL−trnF*, and *trnS−trnG* cpDNA of 124 samples of species studied were characterized by relatively low nucleotide variability and different length owing to the presence of short (1−8 bp) and long (52−168 bp) insertions/deletions (indels), as well as mono- and dinucleotide repeats. After the alignment, the length of the combined matrix of three regions was

2769 sites. We identified 42 variable nucleotide substitutions, 38 of which were parsimony informative and 4 were single substitutions. We revealed 59 chlorotypes (P1−P59), 40 (67.8%) of which were unique ones. The species studied contained no shared chlorotypes (Table 1). The chlorotypes sequences were deposited in the GenBank/ENA/EMBL-EBI databases, and the accession numbers are given in Table 2. All species studied, except *O. coerulea* and *O. filiformis*, has species-specific markers. For example, *O. mandshurica* and *O. tragacanthoides* has the A nucleotide in the positions 862 and 1658, respectively. *O. deflexa* was shown to contain four nucleotide substitutions (T in the positions 332 and 1406, C in the position 2105, and G in the position 1410) and the insertion of 97−110 bp (the positions 684−793). *O. glabra* has six nucleotide substitutions (G in the positions 261, 332, 1935, 2099, and 2597 and T in the position 2346) and the deletion of the A nucleotide in the position 861. Moreover, marker nucleotide substitutions specific to the sect. *Mesogaea* were identified. All sequences of this section representatives has six substitutions (G in the position 136, T in the position 618, C in the positions 650 and 1087, and A in the positions 1393 and 1394) and six indels of 1−6 bp, which are absent in other species. Populations of *O. tragacanthoides* subg.

Tragacanthoxytropis, as well as *O. coerulea*, *O. filiformis*, and *O. mandshurica* sect. *Janthina* subg. *Phacoxytropis*, are characterized by high haplotype and low/medium nucleotide diversity, whereas the *O. glabra* population demonstrated both low haplotype and low nucleotide diversity. The population DEF3 of *O. deflexa* was characterized by a low haplotype and quite high level of nucleotide diversity, whereas the DEF4 population was monomorphic (Table 1).

The AMOVA analysis showed the highest and statistically significant differences between the populations of *O. deflexa* and *O. tragacanthoides* (F_{ST} = 0.81602 and F_{ST} = 0.75975 respectively, $P < 0.0001$). It was shown that about 35% of total genetic variability refers to the interpopulation component of *O. coerulea* and *O. mandshurica* (F_{ST} = 0.34055, $P < 0.05$ and F_{ST} = 0.33889, $P < 0.0001$, respectively). A statistically insignificant though high differentiation was observed between the *O. glabra* populations (F_{ST} = 0.71429, $P > 0.05$), whereas no differentiation was found between the populations of *O. filiformis* (F_{ST} = −0.08489, $P > 0.05$). Another indicator of the level of genetic disjunity between the populations is the divergence of nucleotide sequences (*D*xy). The *D*xy values between the *O. tragacanthoides* populations (i.e., the mean number of nucleotide substitutions per one site and the mean number of nucleotide differences (the number of fixed differences)) varied from 0.00142 to 0.00226 and from 3.400 (3) to 5.400 (2) respectively. The lowest *D*xy values were obtained between the *O. mandshurica* populations, as well as between *O. filiformis* populations (Table 4). Conversely, the highest values were observed between the *O. deflexa* populations. No divergence was observed between two *O. glabra* populations (Table 5).

The *D*xy values between the species are shown in Table 6. Low divergence was observed between *O. coerulea* and *O. filiformis*, the values of which were lower than those estimated between the populations of *O. coerulea* (Table 4). The divergence between *O. filiformis* and *O. mandshurica* corresponded to the interpopulation values, while the divergence between the *O. coerulea* and *O. mandshurica* was almost 1.4 times higher than the interpopulation divergence. The nucleotide divergence of *O. tragacanthoides* from the species of the sect. *Janthina* was almost 1.8 times higher than interpopulation values and almost 5 times higher than that from the species of the sect. *Mesogaea* (Table 6). The hierarchical AMOVA showed that the differentiation between *O. coerulea* and *O. filiformis* was low and insignificant (F_{CT} = 0.19727, $P > 0.075$), whereas the differentiation between the three species of the sect. *Janthina* was more than 57% (F_{CT} = 0.57412, $P < 0.0001$). The biggest differences were observed between the sections *Mesogaea* and *Janthina* (F_{CT} = 0.89754, $P < 0.0001$), and there were slightly smaller differences between *O. tragacanthoides* and section *Mesogaea* (F_{CT} = 0.79942, $P < 0.004$). The genetic differences between *O. tragacanthoides* and section *Janthina* were lower than the population values (F_{CT} = 0.32227, $P < 0.009$).

To reconstruct the phylogenetic relationships, the matrix, which included 46 out of 59 chlorotypes identified in the species of two subgenera (some unique chlorotypes were excluded because of higher representativeness), was replenished with previously obtained chlorotype sequences of five species of subg. *Oxytropis*: *O. triphylla* (Pall.) Pers. (8 chlorotypes) sect. *Xerobia* Bunge, *O. lanata* (Pall.) DC. (10) and *O. gracillima* Bunge (5) sect. *Verticillares* DC., *O. sordida* (Willd.) Pers. (5) and *O. ochotensis* Bunge (10) sect. *Orobia* Bunge (Table 2). The length of the combined matrix of 84 chlorotype sequences of three regions after alignment was 2790 sites. The analysis revealed 48 variable nucleotide substitutions, 43 of which were parsimony informative and 5 were considered as single ones. No shared chlorotypes were found among the species. The phylogenetic analysis carried out by the MP, NJ, and ML methods provided phylogenetic trees of similar topology. Figure 2 shows the MP tree (consensus of 10000 trees: length of 413 steps, CI = 0.5617, HI = 0.4383, RI = 0.8002), on which chlorotypes of the *Oxytropis* species form two sister clades. The clade I with high support only in the MP analysis united the chlorotypes of *O. deflexa* and *O. glabra* sect. *Mesogaea* subg. *Phacoxytropis*. Chlorotypes of each species formed highly supported groups (Fig. 2). The clade II united the chlorotypes of the species of the subg. *Tragacanthoxytropis* and *Oxytropis* and the section *Janthina* subg. *Phacoxytropis* with a high level of support in all analyses. However, the relationships among them remained unresolved. The chlorotypes of the *O. mandshurica*, *O. sordida*, *O. gracillima*, and *O. lanata* formed well-supported groups. It is noteworthy that three chlorotypes of *O. tragacanthoides* (P1, P3, and P4) subg. *Tragacanthoxytropis* were united into one group with the chlorotypes of *O. triphylla* sect. *Xerobia* subg. *Oxytropis* with a medium level of support in the MP, ML, and NJ analyses and a high level of support in the BI analysis (Fig. 2).

The ITS nrDNA were sequenced in 49 samples: *O. tragacanthoides* (13), *O. coerulea* (11), *O. filiformis* (7), *O. mandshurica* (9), *O. deflexa* (6), and *O. glabra* (3). These specimens represented the majority of cpDNA chlorotypes revealed in this study. The ITS region is characterized by the same length (603 bp) and low nucleotide variability: 592 monomorphic sites and 11 variable sites, which were parsimony informative. Six substitutions (the positions 57, 68, 90, 122, 200, and 201) were found in the ITS1 spacer and five substitutions (the positions 422, 427, 513, 548, and 555) were found in the ITS2 spacer. We revealed six ribotypes: two ribotypes (RP1 and RP2) belonged to *O. coerulea*; all other species contained one of the ribotypes: *O. filiformis*—RP3, *O. mandshurica*—RP4,

Table 2. Chlorotypes of the *Oxytropis* species and the GenBank/ENA/EMBL-EBI accession numbers for the nucleotide sequences of the *psbA−trnH*, *trnL−trnF*, and *trnS−trnG* intergenic spacers of cpDNA

Chlorotype	Accession number		
	psbA−trnH	*trnL−trnF*	*trnS−trnG*
O. tragacanthoides			
P1	MW172222	MW177548	MW177535
P2	MW172223	MW177549	MW177536
P3	MW172224	MW177550	MW177537
P4	MW172225	MW177551	MW177538
P5	MW172226	MW177552	MW177539
P6	MW172227	MW177553	MW177540
P7	MW172228	MW177554	MW177541
P8	MW172229	MW177555	MW177542
P9	MW172230	MW177556	MW177543
P10	MW172231	MW177557	MW177544
P11	MW172232	MW177558	MW177545
P12	MW172233	MW177559	MW177546
P13	MW172234	MW177560	MW177547
O. coerulea			
P14	LR898256	LR898302	LR898413
P15	LR898257	LR898303	LR898414
P16	LR898258	LR898304	LR898415
P17	LR898259	LR898305	LR898416
P18	LR898260	LR898306	LR898417
P19	LR898261	LR898307	LR898418
P20	LR898262	LR898308	LR898419
P21	LR898263	LR898309	LR898420
P22	LR898264	LR898310	LR898421
P23	LR898265	LR898311	LR898422
P24	LR898266	LR898312	LR898423
P25	LR898267	LR898313	LR898424
P26	LR898268	LR898314	LR898425
P27	LR898269	LR898315	LR898426
P28	LR898270	LR898316	LR898427
O. filiformis			
P29	LR898271	LR898317	LR898428
P30	LR898272	LR898318	LR898429
P31	LR898273	LR898319	LR898430
P32	LR898274	LR898320	LR898431
P33	LR898275	LR898321	LR898432
P34	LR898276	LR898322	LR898433
P35	LR898277	LR898323	LR898434
P36	LR898278	LR898324	LR898435
P37	LR898278	LR898325	LR898436
P38	LR898280	LR898326	LR898437
P39	LR898281	LR898327	LR898438
P40	LR898282	LR898328	LR898439
P41	LR898283	LR898329	LR898440

Table 2. (Contd.)

Chlorotype	Accession number		
	psbA−trnH	*trnL−trnF*	*trnS−trnG*
O. mandshurica			
P42	LR898284	LR898330	LR898441
P43	LR898285	LR898331	LR898442
P44	LR898286	LR898332	LR898443
P45	LR898287	LR898333	LR898444
P46	LR898288	LR898334	LR898445
P47	LR898289	LR898335	LR898446
P48	LR898290	LR898336	LR898447
P49	LR898291	LR898337	LR898448
O. deflexa			
P50	LR898292	LR898338	LR898449
P51	LR898293	LR898339	LR898450
P52	LR898294	LR898340	LR898451
P53	LR898295	LR898341	LR898452
P54	LR898296	LR898342	LR898453
P55	LR898297	LR898343	LR898454
P56	LR898298	LR898344	LR898455
O. glabra			
P57	LR898299	LR898345	LR898456
P58	LR898300	LR898346	LR898457
P59	LR898301	LR898347	LR898458
O. triphylla*			
H1	*LT856461*	*LT856494*	*LT856527*
H2	*LT856462*	*LT856495*	*LT856528*
H3	*LT856463*	*LT856496*	*LT856529*
H4	*LT856464*	*LT856497*	*LT856530*
H5	*LT856465*	*LT856498*	*LT856531*
H6	*LT856466*	*LT856499*	*LT856532*
H7	*LT856467*	*LT856500*	*LT856533*
H10	*LT856472*	*LT856505*	*LT856538*
O. lanata**			
V1	*LT994841*	*LT994895*	*LT994949*
V3	*LT994843*	*LT994897*	*LT994951*
V4	*LT994844*	*LT994898*	*LT994952*
V5	*LT994845*	*LT994899*	*LT994953*
V7	*LT994847*	*LT994901*	*LT994955*
V9	*LT994849*	*LT994903*	*LT994957*
V13	*LT994853*	*LT994907*	*LT994961*
V14	*LT994854*	*LT994908*	*LT994962*
V16	*LT994856*	*LT994910*	*LT994964*
V18	*LT994858*	*LT994912*	*LT994966*

Table 2. (Contd.)

Chlorotype	Accession number		
	psbA−trnH	*trnL−trnF*	*trnS−trnG*
O. gracillima*			
V58	*MH174938*	*LT996062*	*LT996067*
V59	*LT996058*	*LT996063*	*LT996068*
V60	*LT996059*	*LT996064*	*LT996069*
V61	*LT996060*	*LT996065*	*LT996070*
V62	*LT996061*	*LT996066*	*LT996071*
O. sordida**			
H1	*LS991870*	*LS991896*	*LS991922*
H2	*LS991871*	*LS991897*	*LS991923*
H3	*LS991872*	*LS991898*	*LS991924*
H4	*LS991873*	*LS991899*	*LS991925*
H5	*LS991874*	*LS991900*	*LS991926*
O. ochotensis***			
H1	*MK806162*	*MK806201*	*MK806240*
H2	*MK806163*	*MK806202*	*MK806241*
H3	*MK806164*	*MK806203*	*MK806242*
H4	*MK806165*	*MK806204*	*MK806243*
H5	*MK806166*	*MK806205*	*MK806244*
H7	*MK806168*	*MK806207*	*MK806246*
H9	*MK806170*	*MK806209*	*MK806248*
H11	*MK806172*	*MK806211*	*MK806250*
H12	*MK806173*	*MK806212*	*MK806251*
H14	*MK806175*	*MK806214*	*MK806253*

The accession numbers in italics are available in the previous studies: *—[12], **—[15], ***—[14], ****—[16].

O. deflexa—RP5, *O. glabra*—RP6, and *O. tragacanthoides*—RP1, which was shared with *O. coerulea*. The ribotype sequences of the species were deposited in the GenBank/ENA/EMBL-EBI databases under the accession numbers MW186811, LR898459−LR898464. To reveal the genealogical relationships, the ribotype matrix of the species of two subgenera was replenished with previously obtained ITS sequences of the species of subg. *Oxytropis*: *O. triphylla* (MW015143) sect. *Xerobia*, *O. lanata* (LM653259, LM653260), *O. chankaensis* Jurtz. (FR839001, FR839010), and *O. oxyphylla* (Pall.) DC. (FR839000) sect. *Verticillares*, and *O. ochotensis* (MK795939, MK795941−MK795943) sect. *Orobia*, as well as *A. davuricus* as the outgroup. The median network constructed is shown in Fig. 3. The closest to *Astragalus* was *O. deflexa*, the ribotype of which was connected with the *A. davuricus* ribotype via 42 mutational steps and a hypothetical ribotype, which was extinct or not revealed in this study. Five mutational steps and a hypothetical ribotype separate *O. deflexa* and *O. glabra* sect. *Mesogaea* subg. *Phacoxytropis*. Close to them are the ribotypes of *O. mandshurica* and *O. filiformis* sect. *Janthina* of the same subgenus. The most widespread and shared by six species, belonging to three subgenera, the RP1 ribotype, occupied the central position in the network and formed the star-like structure with other ribotypes, which were connected by single-mutation transitions (Fig. 3).

DISCUSSION

The genetic diversity of the populations of *O. tragacanthoides* subg. *Tragacanthoxytropis* and *O. coerulea*, *O. filiformis*, and *O. mandshurica* sect. *Janthina* subg. *Phacoxytropis* was characterized by a combination of a high level of haplotype diversity and low/medium level of nucleotide diversity (Table 1). This situation was observed for several endemic *Oxytopis* species [12], some populations of *O. glandulosa* [13] and *O. ruthenica* [16], and some other representatives of the fam. Fabaceae, such as *Astragalus onobrychis* L. [28] and *Sophora* L. species [29]. This indicated rapid growth of a population after its decrease, which was followed by recovery of haplotype diversity on account of the mutation process [28, 30]. Low haplotype diver-

Table 3. Distribution of genetic variability (AMOVA) among the *Oxytropis* groups

Dispersion source	Genetic difference (%) between		
	groups	populations within groups	specimens in a population
Populations of *Oxytropis* species			
One group: (populations of *O. tragacanthoides*)	–	75.97*	24.03
One group: (populations of *O. coerulea*)	–	34.05**	65.95
One group: (populations of *O. filiformis*)	–	–8.49 ns	108.49
One group: (populations of *O. mandshurica*)	–	33.89*	66.11
One group: (populations of *O. deflexa*)	–	81.60*	18.4
One group: (populations of *O. glabra*)	–	71.43 ns	28.57
Two groups: (populations of *O. coerulea*) and (populations of *O. filiformis*)	19.72 ns	16.51**	32.98*
Three groups: (populations of *O. coerulea*), (populations of *O. filiformis*), and (populations of *O. mandshurica*)	57.41*	10.03**	32.56*
Two groups: (populations of *O. deflexa*) and (populations of *O. glabra*)	74.74**	21.17*	4.09*
Two groups: (populations of the sect. *Janthina* species) and (populations of the sect. *Mesogaea* species)	89.75*	8.91*	1.33*
Two groups: (populations of *O. tragacanthoides*) and (populations of the sect. *Janthina* species)	32.23**	44.68*	23.09*
Two groups: (populations of *O. tragacanthoides*) and (populations of the sect. *Mesogaea* species)	79.94**	18.32*	1.74*

* *P* < 0.0001; ** *P* < 0.05; ns—not significant. The confidence level was assessed on the basis of 1023 permutations.

Table 4. Nucleotide divergence between the populations of *Oxytropis coerulea*, *O. filiformis*, and *O. mandshurica* sect. *Janthina* subg. *Phacoxytropis*

Population	COE1	COE2	COE3	FIL1	FIL2	FIL3	FIL4	MAN1	MAN2	MAN3
COE1	–	3.917 (2)	4.000 (4)	0.000 (0)	0.200 (0)	0.000 (0)	0.000 (0)	4.067 (4)	4.000 (4)	4.000 (4)
COE2	0.00164	–	4.250 (2)	3.917 (2)	4.117 (2)	3.917 (2)	3.917 (2)	6.317 (4)	6.250 (4)	6.250 (4)
COE3	0.00168	0.00178	–	4.000 (4)	4.200 (4)	4.000 (4)	4.000 (4)	4.067 (4)	4.000 (4)	4.000 (4)
FIL1	0.00000	0.00164	0.00168	–	0.200 (0)	0.000 (0)	0.000 (0)	4.067 (4)	4.000 (4)	4.000 (4)
FIL2	0.00008	0.00173	0.00176	0.00008	–	0.200 (0)	0.200 (0)	4.267 (4)	4.200 (4)	4.200 (4)
FIL3	0.00000	0.00164	0.00168	0.00000	0.00008	–	0.000 (0)	4.067 (4)	4.000 (4)	4.000 (4)
FIL4	0.00000	0.00164	0.00168	0.00000	0.00008	0.00000	–	4.067 (4)	4.000 (4)	4.000 (4)
MAN1	0.00170	0.00265	0.00170	0.00170	0.00179	0.00170	0.00170	–	0.067 (0)	0.067 (0)
MAN2	0.00167	0.00262	0.00167	0.00167	0.00176	0.00167	0.00167	0.00003	–	0.000 (0)
MAN3	0.00167	0.00262	0.00167	0.00167	0.00176	0.00167	0.00167	0.00003	0.00000	–

The mean number of nucleotide differences (the number of fixed differences) is shown above the diagonal; the mean number of nucleotide substitutions per one site is shown below the diagonal. See Table 1 for the population code.

sity in the populations of *O. deflexa* and *O. glabra* sect. *Mesogaea* subg. *Phacoxytropis* (Table 1) may be due to the gene drift in small isolated populations, as well as to the effect of selection under severe environmental conditions. The high level of interpopulation differentiation of *O. deflexa* (Table 3) may be explained, first of all, by significant geographical distance between their locations (Southern Siberia, Magadan oblast, Taimyr) (Fig. 1). Absence of nucleotide divergence between the geographically distant populations of the *O. glabra* (Table 5) may be considered as a demonstration of the ancestral polymorphism of the widespread species. Both the *O. deflexa* and *O. glabra* carried the species-specific marker nucleotide substitutions in the cpDNA, as well as marker substitutions for the sect. *Mesogaea*. The *Mesogaea* species were significantly divergent from the species sect. *Janthina* of the same subgenus and *O. tragacanthoides* subg. *Tragacan-*

Table 5. Nucleotide divergence between the populations of *Oxytropis deflexa* and *O. glabra* sect. *Mesogaea* subg. *Phacoxytropis*

Population	DEF1	DEF2	DEF3	DEF4	DEF5	DEF6	DEF7	GLA1	GLA2
DEF1	–	4.000 (2)	4.000 (2)	4.000 (2)	6.000 (4)	6.000 (4)	6.000 (4)	15.000 (13)	15.000 (13)
DEF2	0.00175	–	0.000 (0)	0.000 (0)	3.000 (3)	3.000 (3)	3.000 (3)	14.000 (14)	14.000 (14)
DEF3	0.00167	0.00000	–	0.000 (0)	3.000 (3)	3.000 (3)	3.000 (3)	16.000 (16)	16.000 (16)
DEF4	0.00167	0.00000	0.00000	–	3.000 (3)	3.000 (3)	3.000 (3)	16.000 (16)	16.000 (16)
DEF5	0.00263	0.00131	0.00131	0.00131	–	0.000 (0)	0.000 (0)	12.000 (12)	12.000 (12)
DEF6	0.00263	0.00131	0.00131	0.00131	0.00000	–	0.000 (0)	12.000 (12)	12.000 (12)
DEF7	0.00263	0.00131	0.00131	0.00131	0.00000	0.00000	–	12.000 (12)	12.000 (12)
GLA1	0.00661	0.00639	0.00705	0.00705	0.00547	0.00547	0.00547	–	0.000 (0)
GLA2	0.00661	0.00638	0.00705	0.00705	0.00547	0.00547	0.00547	0.00000	–

See the note for Table 4.

Table 6. Nucleotide divergence between the *Oxytropis* species, subgenera *Tragacanthoxytropis* and *Phacoxytropis*

| Species	O. tragacanthoides	O. coerulea	O. filiformis	O. mandshurica	O. deflexa	O. glabra
O. tragacanthoides	–	7.939 (2)	7.600 (5)	7.579 (5)	22.910 (14)	22.800 (20)
O. coerulea	0.00334	–	2.883 (0)	5.529 (2)	24.829 (16)	24.389 (21)
O. filiformis	0.00320	0.00121	–	4.079 (4)	23.490 (18)	23.050 (23)
O. mandshurica	0.00318	0.00232	0.00171	–	21.549 (17)	22.029 (22)
O. deflexa	0.01097	0.01193	0.01128	0.01034	–	13.600 (10)
O. glabra	0.01031	0.01106	0.01044	0.00994	0.00620	–

See the note for Table 4.

thoxytropis (Table 6). The basal position of the ITS nrDNA ribotypes of *O. deflexa* and *O. glabra* in the genealogical network demonstrated their more ancient origin (Fig. 3). Previously, the analysis of the TRPT markers of the nuclear genome and the *matK* markers of the chloroplast genome revealed a separate location for *O. deflexa* [31]. A similar situation was shown for *O. deflexa* and *O. glabra* by the analysis of the ITS nrDNA variability [32]. Moreover, study of the polymorphism of the *trnL−trnF* intergenic spacer and the *trnL* intron cpDNA of the *Oxytropis* species from Turkey showed that *O. kotschyana* sect. *Mesogaea* was significantly divergent from both the species of the sect. *Janthina* and the species of other subgenera [33].

In the present study, the sect. *Janthina* is represented by three species. *O. filiformis* lacks interpopulation differentiation (Table 3). It was also characterized by a low level of nucleotide divergence, which is probably due to the active gene exchange between closely located populations (Table 4). The *O. coerulea* populations were shown to be significantly divergent from each other (Table 4). The same level of nucleotide divergence was observed between the populations of *O. coerulea* and *O. filiformis*. No nucleotide divergence was observed between the population COE1 of *O. coerulea*, which is located eastward of Lake Baikal (Buryatia), and the populations FIL1, FIL3 and FIL4 of

O. filiformis from Transbaikalia and Mongolia (Table 4). Data of the polymorphism analysis of the cpDNA intergenic spacers (absence of the species-specific markers in the cpDNA and significant genetic differentiation between *O. coerulea* and *O. filiformis*, and combining of chlorotypes into one, though poorly supported, phylogroup) (Fig. 2) indicated close genetic proximity of these two species. Nevertheless, absence of common chlorotypes and presence of different ribotypes ITS nrDNA confirmed independence of *O. coerulea* and *O. filiformis*. *O. mandshurica* was shown to carry a species-specific marker in the *trnL−trnF* spacer of cpDNA. Its chlorotypes were shown to form a highly supported phylogroup, and an individual ribotype was identified (Fig. 2). These data, as well as a high level of differentiation between the species of the sect. *Janthina*, confirm, on the whole, *O. mandshurica* as an independent species (Table 3).

A considerable nucleotide divergence and high interpopulation differentiation was observed between the populations of *O. tragacanthoides*. This may be due to their isolation and cofinement of the species to specific ecotopes, as well as to the presence of karyological races. For example, it was shown that *O. tragacanthoides* contains both diploid ($2n = 16$) and tetraploid ($2n = 32$) representatives [5, 9, 34]. Chlorotypes revealed in *O. tragacanthoides* were divided into two
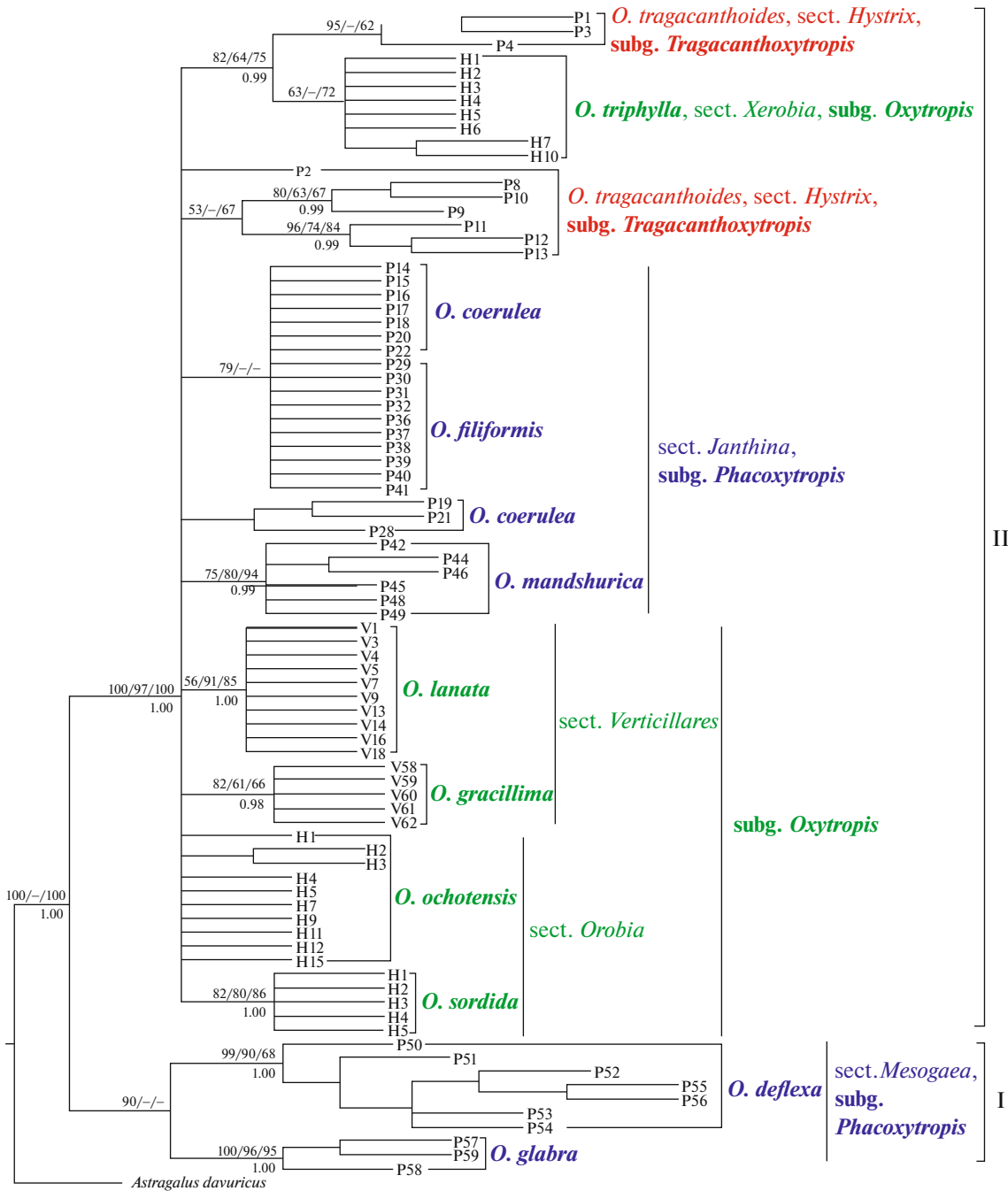
Fig. 2. The MP tree of the phylogenetic relationships of the cpDNA chlorotypes of the *Oxytropis* species, subgenera *Tragacanthoxytropis*, *Phacoxytropis*, and *Oxytropis*. The numbers above the branches indicate the bootstrap indices calculated for the MP/NJ/ML methods (>50%); the numbers below the branches indicate the a posteriori probabilities calculated for the BI analysis (>0.95). Names of the species, sections, and subgenera of the *Oxytropis* genus are given in accordance with [5].

groups in the MP tree. In one of these groups, which was characterized by a medium level of support in the MP, ML, and NJ analyses and high level of support in the BI analysis, three chlorotypes were united with the chlorotypes of *O. triphylla* sect. *Xerobia* subg. *Oxytropis*, which was characterized by a diploid (2*n* = 16) karyotype (Fig. 2) [35]. Moreover, these species were

characterized by one common ITS ribotype (Fig. 3). However, morphologically, *O. tragacanthoides* and *O. triphylla* are sharply different both from each other and from other *Oxytropis* species. The representatives of the subg. *Tragacanthoxytropis* are cushion-formed thorn shrubs. The characteristic feature of the *O. triphylla* is the presence of leaves with one or rarely

two pairs of liflets. On the whole, the species is morphologically isolated into the special oligotype series *Triphyllae* within the sect. *Xerobia* [5]. One may suggest that genetic closeness of these relict species from Southern Siberia is due to the wide distribution of the ancestral forms of the *Oxytropis* on this territory [9].

The phylogenetic relationships of chlorotypes of the *Oxytropis* species from the Asian Russia, the subgenera *Tragacanthoxytropis* and *Oxytropis*, and the sect. *Janthina* subg. *Phacoxytropis*, which form the highly sustained clade II (Fig. 2), remained unresolved. Moreover, the analysis of genealogical relationships of the ITS nrDNA ribotypes of *O. tragacanthoides*, *O. coerulea*, *O. lanata*, *O. chankaensis*, *O. oxyphylla*, and *O. triphylla*, which belong to these three subgenera, revealed the common RP1 ribotype (Fig. 3). All these phenomena may be considered as a demonstration of the reticulate evolution observed for the *Oxytropis* species [5]. The revealed genetic relationships at clear morhological differences are typical of the taxa which are characterized by a common origin and underwent recently fast adaptive radiation, as was shown for several genera of the fam. Fabaceae [1]. Considering the isolation of the legume group, which included both *Astragalus* and *Oxytropis*, took place about 39 million years ago [36], and the genus *Oxytropis* appeared at the border between the Miocene and Pliocene about 5.6 million years ago, one may suggest that *Oxytropis* species divergence was relatively recent. Fast adaptive radiation was shown for the *Oxytropis* species [1], as well as for the other genera of the fam. Fabaceae, including *Pultenaea* [37], *Astragalus* [38], *Sophora* [29], etc. A certain contribution to the observed relationships between the *Oxytropis* species of the three subgenera might have been made by hybridization between the incompletely specialized taxa after fast radiation, which took place at the early stages of the evolution of the genus, but prior to the divergence of genealogical lines, as was shown for the *Pultenaea* species [37].

Therefore, the analysis of nucleotide polymorphism of the *psbA−trnH*, *trnL−trnF*, and *trnS−trnG* intergenic spacers of cpDNA and the ITS nrDNA in a wide sample of specimens obtained from different populations of *O. coerulea*, *O. filiformis* and *O. mandshurica* sect. *Janthina* subg. *Phacoxytropis* confirmed these three taxa to be independent species. Reconstruction of phylogenetic relationships of the chlorotypes and analysis of genealogical relationships of the ITS ribotypes of the species of the subg. *Phacoxytropis*, *Tragacanthoxytropis*, and *Oxytropis* are consistent with the data obtained by Zhu et al. [6], which demonstrated that *O. coerulea*, *O. filiformis*, and *O. mandshurica* belong to the subg. *Oxytropis*. Nevertheless, further studies that would include other species of this section are required.
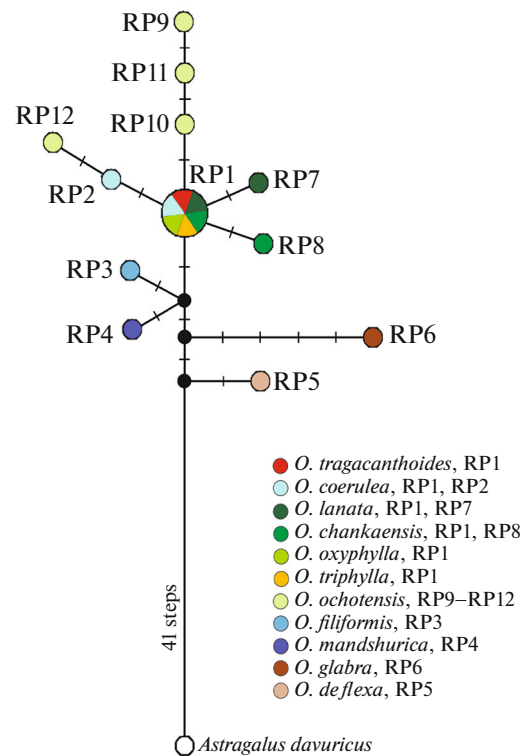
Fig. 3. The genealogical network of the ITS nrDNA ribotypes of the *Oxytropis* species, subgenera *Tragacanthoxytropis* (RP1), *Phacoxytropis* (RP1−RP6), and *Oxytropis* (RP1, RP7−RP12), constructed by the MJ method. The circle size shows the ribotype frequencies; small black circles correspond to the hypothetical haplotypes; thin crosscuts on the branches indicate the mutation events.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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