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PLANT GENETICS

Phylogenetic Relationships of *Oxytropis* Section *Arctobia* of Northeast Asia according to Sequencing of the Intergenic Spacers of Chloroplast and ITS of Nuclear Genomes

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Abstract—Three intergenic spacers (*psbA–trnH*, *trnL–trnF*, *trnS–trnG*) of chloroplast DNA (cpDNA) and an internal transcribed spacer of nuclear ribosomal DNA (ITS rDNA) were used to study the genetic diversity and phylogenetic relationships of the species *Oxytropis czukotica*, *O. exserta*, *O. gorodkovii*, *O. kamtschatica*, *O. mertensiana*, *O. nigrescens*, *O. pumilio*, *O. revoluta*, and *O. susumanica* of the section *Arctobia* of the genus *Oxytropis*. According to cpDNA data, most populations are characterized by a low and medium haplotype (*h* varies from 0.154 to 0.583) and low nucleotide (π varies from 0.0002 to 0.0050) diversity. An analysis of the genealogical relationships of chlorotypes showed a clear separation of the studied taxa and the genetic proximity of *O. nigrescens* to *O. susumanica* and *O. kamtschatica* to *O. exserta*, the last two being the most diverged from all the others. In *O. czukotica*, three ITS rDNA ribotypes were detected; in *O. nigrescens* and *O. susumanica*, one common ribotype was found; and in all the others, one individual ribotype was identified for each species. According to the data of nucleotide polymorphism of markers of two genomes, the status of *O. czukotica*, *O. gorodkovii*, and *O. pumilio* taxa as a three separate species was confirmed. We suggest that *O. susumanica* is an intraspecific taxon of *O. nigrescens*. The revealed genetic similarities and differences of *O. revoluta*, *O. exserta*, and *O. kamtschatica* and their phylogenetic relationships do not correspond to the division of the *Arctobia* section into subsections; therefore, additional comprehensive studies are needed.

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

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INTRODUCTION

Metaarctic, amphi-Beringian section *Arctobia* Bunge, subgenus *Oxytropis*, genus *Oxytropis* DC. includes 16 species and subspecies in North Asia and North America [1–7]. The first description of the section, which included the species *O. nigrescens* (Pall.) Fisch., *O. pumilio* (Pall.) Ledeb., and *O. arctobia* Bunge, was presented by Bunge [8]. Later, the section was revised by Yurtsev [2], who divided it into five subsections: *Arctobia*, *Kamtschaticae*, *Revolutae*, *Podocarpae*, and *Mertensiana* [2, 3, 5]. The largest *Arctobia* subsection with the central species *O. nigrescens*, which together with closely related species *O. czukotica* Jurtz., *O. gorodkovii* Jurtz., *O. pumilio*, and *O. bryophila* (Greene) Jurtz. constitutes the *O. nigrescens* s. l. complex, is believed to be the most taxonomically complicated [3, 5, 7]. The borders between the species may sometimes be unclear because of their high phenotypic plasticity, resulting in contradictory assessments of the taxonomic ranks. For example, most American researchers, including Barneby [1] and Welsh [4], assign all North American plants of the *O. nigrescens* s. l. complex to the *O. nigrescens*. Swedish botanist Hulten assigns them to the *O. nigrescens* with three subspecies (*bryophila*, *pygmaea*, *arctobia*), suggesting that their status needs verification [9]. Yurtsev [3, 5], the authors of the "Annotated Checklist of the Panarctic Flora (PAF) Vascular Plants" [7], and other botanists [6, 10] accept all taxa of the complex in the rank of closely related species, as they have some differences in morphology and ploidy. Moreover, Yurtsev [3, 5] notes that most species have parapatric ranges: *O. nigrescens* s. str. distributed from Yenisei to Kolyma; *O. czukotica* distributed from Kolyma to the Bering Strait; *O. gorodkovii* distributed near the Bering Strait and further eastwards; *O. pumilio* grows on the Kamchatka Peninsula and on the Northern and Middle Kuril Islands; *O. bryophila* distributed mostly on the

territories of Yukon and Alaska. Malyshev in his later study [11] treats, following Hulten [9], *O. czukotica* as a synonym of *O. pumilio*. Integrated analysis of morphological traits, seed anatomy, and nucleotide polymorphism of the markers of nuclear (*CNGC5*, *LE*, and *TRPT*) and chloroplast (*matK*) genomes of the species of the section *Arctobia* from North America [12] showed the presence of two species of the *O. nigrescens* s. l. complex: *O. gorodkovii* and *O. bryophila*. The author cites *O. czukotica* as the synonym of *O. bryophila*. However, the resolving power of the molecular markers used in this study was rather poor.

To reconstruct the phylogenetic relationships between the *Oxytropis* species, the internal transcribed spacer of nuclear ribosomal DNA (ITS rDNA) [13], the ITS rDNA + *matK* of the chloroplast DNA (cpDNA) [14], and the ITS rDNA + *trnL–trnF* cpDNA [15] were also used. This, however, did not lead to the resolution of species relationships even at the section level. We carried out a series of studies on the basis of comparative analysis of the nucleotide polymorphism of the *psbA–trnH* + *trnL–trnF* + *trnS–trnG* intergenic spacers of cpDNA, that confirmed the status of the *O. czukotica* as a separate species [16], allowed to reveal the genetic diversity and population structure of rare and endemic *Oxytropis* species *O. chankaensis* Jurtz. [17], *O. triphylla* (Pall.) Pers., *O. bargusinensis* Peschkova, and *O. interposita* Sipl. [18], and *O. glandulosa* Turcz. [19] and five species of the section *Orobia* Bunge [20], and to reconstruct the phylogenetic relations within the sections *Xerobia* Bunge [21] and *Verticillares* DC. [22]. We revealed significant differences in the chloroplast and nuclear (ITS rDNA) genomes of the *O. ruthenica* Vass. and *O. kunashiriensis* Kitam. of section *Orobia*, which confirmed independence of these species. It was shown that *O. erecta* Kom. and *O. litoralis* Kom., which belong to the same section, are local phenotypes of the widespread polyploid species *O. ochotensis* Bunge [20]. The analysis of genealogical relationships of the haplotypes revealed clearly isolated evolutionary lines in *O. glandulosa* [19] and in *O. ruthenica* [20], which may point to active microevolutionary processes and/or the presence of cryptic species.

Malyshev [6] cited nine species of the section *Arctobia* for the Asian Russia: *O. bryophila*, *O. czukotica*, *O. exserta* Jurtz., *O. gorodkovii*, *O. kamtschatica* Hult., *O. mertensiana* Turcz., *O. nigrescens*, *O. pumilio*, and *O. revoluta* Ledeb.; apart from this, he mentions three more taxa: *O. siegizmundii* N.S. Pavlova, *O. susumanica* Jurtz., and *O. czerskii* Jurtz. The first one does not possess a wide range (it is represented only in Koryakia, upper reaches of the Aynyn River, and in the north of Penzhina Bay), whereas the second and the third ones were found just once in the Susumansky district of Magadan oblast and in the Chersky Range in the upper reaches of the Indigirka River, respectively. Therefore, these taxa need additional study to be recognized as independent species. Most species of this section are diploid: in *O. exserta*, *O. mertensiana*, *O. nigrescens*, *O. pumilio*, and *O. revoluta*, $2n = 16$; in *O. czukotica* and *O. gorodkovii*, $2n = 16$ and $2n = 32$; in *O. kamtschatica*, $2n = 16$ and $2n = 96$ [6]. No data are available for *O. bryophila*, *O. siegizmundii*, *O. susumanica*, and *O. czerskii*.

The goal of this work was to study the genetic diversity and population structure as well as to reconstruct the phylogenetic relationships of the *Oxytropis* species of the section *Arctobia* in Northeast Asia according to the data of nucleotide polymorphism of the *psbA–trnH*, *trnL–trnF*, and *trnS–trnG* intergenic spacer sequences of cpDNA and the ITS rDNA.

MATERIALS AND METHODS

The material of this study was 170 plants of *O. czukotica* (86 specimens), *O. exserta* (13 specimens), *O. gorodkovii* (3 specimens), *O. kamtschatica* (9 specimens), *O. mertensiana* (22 specimens), *O. nigrescens* (3 specimens), *O. pumilio* (17 specimens), *O. revoluta* (11 specimens), and *O. susumanica* (6 specimens) of section *Arctobia* of subgenus *Oxytropis* from 26 natural locations (Fig. 1). In the present study, we used the taxonomic classification of the section *Arctobia* proposed by Malyshev [6]. The sample size, the population code, and geographical coordinates are given in Table 1. The methods of DNA isolation, amplification, and sequencing of the *psbA–trnH, trnL–trnF*, and *trnS–trnG* intergenic spacers were previously published in [16, 18, 19]. The nucleotide sequences of the direct and reverse chains were determined with an ABI 3500 genetic analyzer (Applied Biosystems, United States) and then edited and assembled with the Staden Package 1.5 program package [23].

For each region, sequences were aligned with the SeaView 4.7 program [24] for each specimen. The matrix of combined sequences was used to calculate the number of haplotypes, as well as the haplotype (h) and nucleotide (π) diversity (for populations of five or more specimens), and for molecular dispersion analysis (AMOVA) using the Arlequin 3.5 program [25]. The statistical confidence (P) was assessed on the basis of 1023 permutations. The level of divergence based on nucleotide substitution (Dxy) was assessed with the DnaSP 5.0 program [26]. The genealogical relationships of the cpDNA haplotypes were analyzed by the median-joining (MJ) method using the Network 5.0 [27] by coding each deletion or insertion regardless of their size as a single mutational event. The previously obtained [18] nucleotide sequences of the *psbA–trnH*, *trnL–trnF*, and *trnS–trnG* intergenic spacers of cpDNA (GenBank accession numbers LT856572, LT856585, LT856598, respectively) of *O. glabra*, section *Mesogaea* Bunge of the subgenus *Phacoxytropis* Bunge were used as out group.

The ITS rDNA region was amplified for 87 specimens (*O. czukotica* (33 specimens), *O. exserta*

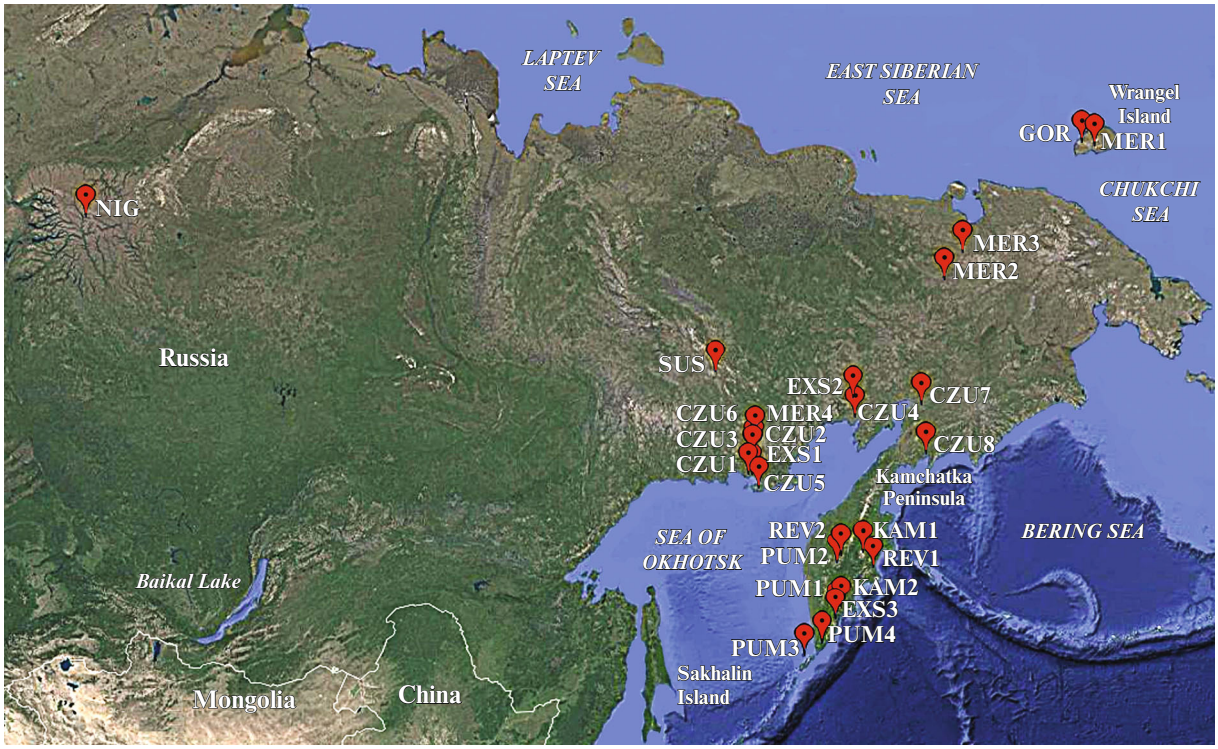

Fig. 1. Map showing the places where the *Arctobia* plants were collected. See Table 1 for the population code.

(10 specimens), *O. gorodkovii* (3 specimens), *O. kamtschatica* (6 specimens), *O. mertensiana* (10 specimens), *O. nigrescens* (2 specimens), *O. pumilio* (13 specimens), *O. revoluta* (6 specimens), and *O. susumanica* (4 specimens)), which represented all cpDNA haplotypes revealed in this study, with the ITS1 and ITS4 primers at the reaction conditions described in [28]. Editing, assembly, alignment, and analysis of the ITS sequences were performed with the programs mentioned above. Phylogenetic analysis of sequences was carried out by the method of maximum parsimony (MP), using a heuristic search for optimal topology with the PAUP 4.0b10 program package [29]. Confidence of the branching order was assessed by the bootstrap analysis of 1000 alternative phylogenetic trees (bootstrap percentage, BP, %). The sequence of the *O. glabra* retrieved from GenBank (LC213354) was used as out group.

The study was carried out using the equipment of the Joint-Use Center "Biotechnolohy and Genetic Engineering," Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences.

RESULTS

The *psbA–trnH*, *trnL–trnF*, and *trnS–trnG* intergenic spacers of cpDNA were successfully amplified and sequenced for all 170 specimens of nine taxa. The nucleotide sequences were characterized by relatively low nucleotide variability and different length owing to the presence of mono- and dinucleotide repeats and short (4–7 nucleotides) and long (up to 188 nucleotides) indels. The total length of the combined sequences of three regions was 2579 sites (1–472, 473–1259, and 1260–2579 respectively), 2192 of which were monomorphic, 367 sites corresponded to the indels and 20 sites were variable. Eighteen nucleotide substitutions (the positions 214, 389, 509, 702, 1160, 1314, 1437, 1534, 1680, 1947, 2008, 2012, 2024, 2025, 2027, 2215, 2403, and 2411) were parsimony informative and two substitutions (the positions 1919 and 2176) were single.

Most populations are characterized by low and medium haplotype diversity ($h = 0.154–0.583$), except the CZU5 ($h = 0.743$) and CZU6 ($h = 0.700$) of *O. czukotica* and SUS ($h = 0.800$) of *O. susumanica*, and low nucleotide diversity ($\pi = 0.0002–0.0050$) (Table 1). The KAM2 population of *O. kamtschatica* and the MER3 population of *O. mertensiana* were monomorphic. No divergence of the nucleotide sequences (*D*xy) were found between the populations of each species: *O. kamtschatica*, *O. mertensiana* and *O. revoluta*. In *O. czukotica*, the highest *D*xy values (the mean number of nucleotide differences (the number of fixed differences) and the mean number of nucleotide substitutions per site) were found between the CZU1–CZU6 and CZU5–CZU6 population pairs (1.600 (1) and 0.00067, respectively). In *O. exserta*, these values were 3.286 (3) and 0.00132 between the

Table 1. The populations studied of the *Oxytropis* section *Arctobia* and the parameters of genetic diversity according to cpDNA data

Species, location (number of samples)	Latitude, longitude	Code	Haplotypes (number of samples)	Diversity (standard deviation)	
				haplotype	nucleotide
O. czukotica					
Magadan oblast, near Magadan, the Medvezhka River (13)	59.58°, 150.86°	CZU1	A1 (12), A2 (1)	0.154 (0.126)	0.0004 (0.0003)
Magadan oblast, Khasynsky region, the Nukh Mountain (15)	60.36°, 151.32°	CZU2	A3 (11), A4 (1), A5 (2), A6 (1)	0.467 (0.148)	0.0023 (0.0013)
Magadan oblast, Khasynsky region, Sopka 1200 (7)	60.32°, 151.25°	CZU3	A7 (5), A8 (2)	0.476 (0.171)	0.0012 (0.0008)
Magadan oblast, North-Evensky region, east of the M. Varkhalam River (7)	61.95°, 159.96°	CZU4	A9 (3), A10 (4)	0.571 (0.119)	0.0002 (0.0002)
Magadan oblast, Olsky region, Koni Peninsula, Cape Alevin (17)	58.97°, 151.81°	CZU5	A11 (2), A12 (1), A13 (4), A14 (8), A15 (1), A16 (1)	0.743 (0.089)	0.0011 (0.0007)
Magadan oblast, Khasynsky region, the Medvezhya Mountain (5)	61.13°, 151.47°	CZU6	A6 (1), A7 (3), A17 (1)	0.700 (0.218)	0.0050 (0.0032)
Kamchatsky krai, Penzhinsky region, the lower reaches of the Penzhina River (14)	62.44°, 165.63°	CZU7	A7 (9), A18 (3), A19 (2)	0.560 (0.124)	0.0003 (0.0002)
Kamchatsky krai, Olutorsky region, near the village Tilichki (8)	60.44°, 166.03°	CZU8	A19 (7), A20 (1)	0.250 (0.180)	0.0012 (0.0008)
O. exserta					
Magadan oblast, Olsky region, near the village Nukla (7)	59.57°, 151.23°	EXS1	A21 (5), A22 (1), A23 (1)	0.524 (0.209)	0.0020 (0.0013)
Magadan oblast, North-Evensky region, upper reaches of the Gizhiga River (2)	62.72°, 159.83°	EXS2	A21 (1), A24 (1)	–	–
Kamchatsky krai, Elizovsky region, Viluchinskaya Sopka Volcano (4)	52.71°, 158.28°	EXS3	A25 (1), A26 (3)	–	–
O. gorodkovii					
Chukotka, Wrangel Island, Somnitelnaya Bay (3)	71.15°, 179.29°	GOR	A27 (1), A28 (1), A29 (1)	–	–
O. kamtschatica					
Kamchatsky krai, Kluchevskaya Sopka Volcano (4)	56.06°, 160.62°	KAM1	A30 (1), A31 (3)	–	–
Kamchatsky krai, Avachinskaya Sopka Volcano (5)	53.26°, 158.84°	KAM2	A32 (5)	0.000 (0.000)	0.0000 (0.0000)

Table 1. (Contd.)

Species, location (number of samples)	Latitude, longitude	Code	Haplotypes (number of samples)	Diversity (standard deviation)	
				haplotype	nucleotide
O. mertensiana					
Chukotka, Wrangel Island, Somnitelnaya Bay (2)	71.06°, 179.63°	MER1	A33 (1), A34 (1)	–	–
Chukotka, Bilibinsky region, near Lake Lipchikvygytyn (9)	67.00°, 167.58°	MER2	A34 (7), A35 (2)	0.389 (0.164)	0.0003 (0.0003)
Chukotka, Bilibinsky region, the Maly Anuy River (10)	67.90°, 169.14°	MER3	A35 (10)	0.000 (0.000)	0.0000 (0.0000)
Magadan oblast, Khasynsky region, Ola Plateau, upper reaches of the Ola River (1)	60.63°, 151.37°	MER4	A33 (1)	–	–
O. nigrescens					
Krasnoyarsk krai, Putorana Plateau, near Lake Ayan (3)	69.01°, 94.25°	NIG	A36 (1), A37 (1), A38 (1)		
O. pumilio					
Kamchatsky krai, Avachinskaya Sopka Volcano (9)	53.12°, 158.47°	PUM1	A39 (6), A40 (1), A41 (1), A42 (1)	0.583 (0.183)	0.0005 (0.0004)
Kamchatsky krai, near the village Esso, Kozyrevsky Mountain Ridge (4)	55.53°, 158.47°	PUM2	A43 (1), A44 (1), A45 (1), A46 (1)	–	–
Kuril Archipelago, Atlasov Island, Alaid Volcano (3)	50.86°, 155.56°	PUM3	A47 (3)	–	–
Kamchatsky krai, Ilyinsky Volcano (1)	51.50°, 157.20°	PUM4	A48 (1)	–	–
O. revoluta					
Kamchatsky krai, Tolbachik, near the Vysokaya Mountain (3)	55.30°, 161.51°	REV1	A49 (3)	–	–
Kamchatsky krai, near the village Esso, Kozyrevsky Mountain Ridge (8)	55.88°, 158.80°	REV2	A50 (7), A51 (1)	0.250 (0.180)	0.0002 (0.0002)
O. susumanica					
Magadan oblast, Susumansky region, Lake Momontay (6)	63.72°, 148.12°	SUS	A52 (3), A53 (1), A54 (1), A55(1)	0.800 (0.172)	0.0006 (0.0005)

Table 2. Distribution of the genetic variation between groups of *Oxytropis* section *Arctobia* according to cpDNA data

Group	Source of variation	*D.f.*	Variation, %	Fixation indices
O. czukotica	Between the populations	7	75.50	F_{ST} = 0.75503*
	Inside the populations	78	24.50	
O. exserta	Between the populations	2	70.31	F_{ST} = 0.70314**
	Inside the populations	10	29.69	
O. kamtschatica	Between the populations	1	84.64	F_{ST} = 0.84642***
	Inside the populations	7	15.36	
O. mertensiana	Between the populations	3	69.97	F_{ST} = 0.69973**
	Inside the populations	18	30.03	
O. pumilio	Between the populations	3	97.62	F_{ST} = 0.97624*
	Inside the populations	13	2.38	
O. revoluta	Between the populations	1	93.86	F_{ST} = 0.93857***
	Inside the populations	9	6.14	
O. nigrescens and *O. susumanica*	Between the populations	1	91.41	F_{ST} = 0.91413***
	Inside the populations	7	8.59	
O. exserta and *O. kamtschatica*	Between the groups	1	54.03	F_{CT} = 0.54029 ns
	Between the populations inside the groups	3	34.06	F_{SC} = 0.74085*
	Inside the populations	17	11.91	F_{ST} = 0.88087*
Nine species under study	Between the groups	8	80.73	F_{CT} = 0.80735*
	Between the populations inside the groups	17	17.00	F_{SC} = 0.88218*
	Inside the populations	144	2.27	F_{ST} = 0.97730*

D.f.—degrees of freedom; * $P = 0.0000$; ** $0.0009 < P < 0.001$; *** $0.003 < P < 0.01$; ns—not significant. The confidence level is estimated on the basis of 1023 permutations.

EXS1–EXS3 populations; in *O. pumilio*, they were 1.250 (1) and 0.00056 between the PUM2 population and each of the PUM1, PUM3 and PUM4 populations. The AMOVA analysis showed that, in each of the *O. czukotica*, *O. exserta* and *O. kamtschatica* species, the main part of all genetic variation (70–85%) is due to interpopulation differences, which reach 94% in *O. revoluta* and more than 97% in *O. pumilio* (Table 2). The hierarchical AMOVA assay of nine species studied revealed high differentiation between the species, more than 80% of variability was due to the interspecies differences (Table 2). The divergence of nucleotide sequences between the species is shown in Table 3. The maximum divergence was observed between pairs formed by each of the *O. kamtschatica* and *O. exserta* species with all the others. The minimum divergence was observed between *O. nigrescens* and *O. susumanica*.

The analysis of 170 sequences of the combined matrix revealed 55 haplotypes (chlorotypes), 30 of which (54.5%) were unique (Table 1). The sequences of three regions of cpDNA of each chlorotype were deposed in GenBank (LR742876–LR743040). It was found out that 20 chlorotypes belonged to *O. czukotica* (A1–A20), 6 to *O. exserta* (A21–A26), 3 to *O. gorodkovii* (A27–A29), 3 to *O. kamtschatica* (A30–A32), 3 to *O. mertensiana* (A33–A35), 3 to *O. nigrescens* (A36–A38), 10 to *O. pumilio* (A39–A48), 3 to *O. revoluta* (A49–A51), and 4 to *O. susumanica* (A52–A55). No common chlorotypes were found among the taxa (Table 1). Chlorotypes form seven haplogroups (I–VII) in the median network of genealogical relationships (Fig 2a). Haplogroups I, II, V, and VII are formed by the chlorotypes in accordance with the species to which they belong. Haplogroup III includes chlorotypes of *O. nigrescens* and *O. susumanica*, and haplogroup IV includes chlorotypes of *O. kamtschatica* and *O. exserta*. Each of the two last haplogroups has marker nucleotide substitutions and indels: haplogroup III has C in the position 1160 of the combined matrix; haplogroup IV has six nucleotide substitutions (G in the positions 509 and 2024, T in the positions 1437 and 1680, A in the position 2012, and C in the position 2025) and five indels, four of which are short (1–7 nucleotides) and one long (up to 100 nucleo-

Table 3. Nucleotide divergence (*D*xy) between the *Oxytropis* species of the section *Arctobia* according to cpDNA data

Species	*O. czukotica*	*O. exserta*	*O. gorodkovii*	*O. kamtschatica*	*O. mertensiana*	*O. nigrescens*	*O. pumilio*	*O. revoluta*	*O. susumanica*
O. czukotica	–	9.549 (8)	3.395 (2)	11.395 (10)	2.395 (1)	2.729 (1)	2.506 (0)	2.395 (1)	3.372 (1)
O. exserta	0.00402	–	8.154 (8)	6.462 (5)	9.154 (9)	9.487 (9)	9.330 (8)	9.154 (9)	10.154 (10)
O. gorodkovii	0.00142	0.00342	–	10.000 (10)	3.000 (3)	3.333 (3)	2.176 (1)	3.000 (3)	4.000 (4)
O. kamtschatica	0.00480	0.00260	0.00420	–	11.000 (11)	11.333 (11)	11.176 (10)	11.000 (11)	12.000 (12)
O. mertensiana	0.00100	0.00386	0.00126	0.00463	–	2.333 (2)	3.176 (2)	2.000 (2)	3.000 (3)
O. nigrescens	0.00114	0.00399	0.00139	0.00476	0.00098	–	3.510 (2)	2.333 (2)	1.333 (1)
O. pumilio	0.00112	0.00421	0.00098	0.00504	0.00143	0.00158	–	3.176 (2)	4.176 (3)
O. revoluta	0.00100	0.00384	0.00125	0.00461	0.00084	0.00097	0.00143	–	3.000 (3)
O. susumanica	0.00141	0.00426	0.00167	0.00504	0.00126	0.00056	0.00187	0.00125	–

Values above the diagonal are the mean number of nucleotide differences between species (the number of fixed differences); values below the diagonal are the mean number of nucleotide substitutions per site.

tides). In these haplogroups, chlorotypes form groups according to taxonomic affiliation. For example, groups of chlorotypes of *O. nigrescens* and *O. susumanica* are separated by eight mutational steps from each other, whereas *O. kamtschatica* and *O. exserta* are separated by seven mutational steps. The AMOVA analysis showed that the main part of genetic variability is due to the variability between *O. nigrescens* and *O. susumanica* (more than 91%) (Table 2). The hierarchical AMOVA of the haplogroups of *O. kamtschatica* and *O. exserta* showed that about 34% of the variability is due to the interpopulation component within the species, about 12% is due to the intrapopulation component, and 54% of the variability is due to the differences between the species, though these differences are statistically insignificant (Table 2). Haplogroup IV is most genetically distant from all the others haplogroups due to high nucleotide divergence of *O. exserta* and *O. kamtschatica* from all other species (Table 3). For example, it is separated from the closest chlorotype A6 of *O. czukotica* by 20 mutational steps and five indels (Fig. 2a).

We revealed the species-specific molecular markers in *O. revoluta* (A in the position 214), *O. gorodkovii* (T in the position 721), *O. mertensiana* (A in the position 2411 and three short indels of 4–6 nucleotides), *O. kamtschatica* (G in the positions 1314 and 2048, A in the positions 2073 and 2075, C in the position 1534, and the deletion of three nucleotides), and *O. exserta* (T in the positions 2048, 2073, and 2075 and the insertion of three nucleotides). As noted above, the taxa *O. nigrescens* and *O. susumanica* have one common substitution. *O. czukotica* and *O. pumilio* possess no species-specific markers. However, it is noteworthy that some populations of *O. pumilio* have specific markers. For example, the insertion of seven nucleotides was found in PUM1 (AAGTATT, in the position 400–406); a nucleotide substitution was found in PUM2 (C in the position 828, instead of A in all other populations); the deletion of 188 nucleotides was found in PUM1, PUM3, and PUM4 (the position 707–894). The distribution of chlorotypes of *O. pumilio* in the median network corresponds to the population affiliation, though no such correlation was observed for *O. czukotica* (Fig. 2a). Hence, the analysis of genealogical relationships of chlorotypes revealed clear separation of nine *Oxytropis* species of the *Arctobia* section and showed the genetic proximity between *O. nigrescens* and *O. susumanica*, as well as between *O. kamtschatica* and *O. exserta*.

The nucleotide sequences of the ITS rDNA were obtained for 87 samples, which represented all cpDNA haplotypes revealed in this study. The length of the ITS was 603 bp except for the representatives of *O. exserta*, *O. kamtschatica*, and *O. revoluta* (602 bp), the sequences of which carried the deletion of one nucleotide (G in the position 173). It was shown that 11 out of 603 sites were variable and parsimony informative: six substitutions in ITS1 (the positions 76, 103, 116, 119, 160, and 200) and five substitutions in ITS2 (the positions 418, 427, 458, 484, and 555). We revealed ten haplotypes (ribotypes), the sequences of which were deposited in GenBank (MN784407–MN784416). Three ribotypes (R1–R3) belong to *O. czukotica*, while *O. nigrescens* and *O. susumanica* share one common ribotype (R8). All other species have one individual ribotype: *O. gorodkovii*—R4, *O. exserta*—R5, *O. kamtschatica*—R6, *O. mertensiana*—R7, *O. pumilio*—R9, and *O. revoluta*—R10. Phylogenetic analysis of ribotypes by the MP method revealed five similarly parsimonious trees, the strict consensus of which is shown in the Fig. 2b (tree length of 20 steps; CI = 0.8500; HI = 0.1500; RI = 0.7000). Ribotypes of the species of section *Arctobia* differ from the *O. glabra* ribotype, section *Mesogaea*, by four marker substitutions. However, the monophyly of sec-

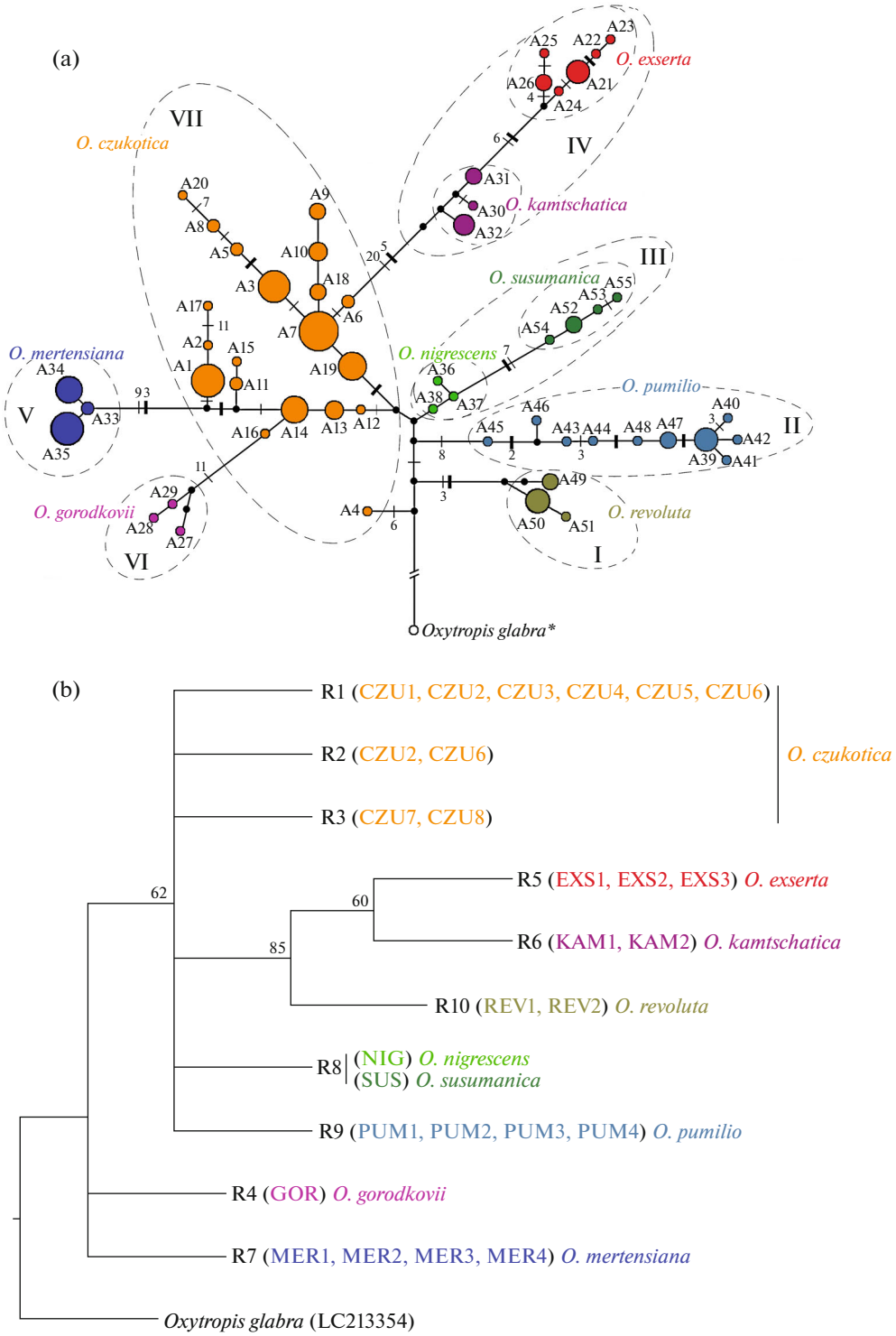

Fig. 2. Phylogenetic relationships between the *Oxytropis* section *Arctobia*. (a) Genealogical network of the cpDNA haplotypes (A1–A55) constructed by the MJ method. The circle size represents the haplotype frequency; small black circles represent the median vectors; thin transverse lines on the branches correspond to the mutational events; thick black lines indicate the indels. Numerals near the thin and thick lines show the number of polymorphic sites and indels. The dotted line shows the haplogroups I–VII and the groups of haplotypes of species forming the single haplogroup. * Mutations of *O. glabra*, used as an out group, are not shown and have not been considered. (b) Phylogenetic tree of the ITS rDNA ribotypes (R1–R10) constructed by the MP method. Numerals in the branching nodes show the bootstrap values (>50%). See Table 1 for the population code. The ITS sequence of *O. glabra* (GenBank LC213354) was used as an out group.

tion *Arctobia* is not statistically supported. The ribotype R4 of *O. gorodkovii* and the ribotype R7 of *O. mertensiana* occupy isolated position. The ribotypes of other species form a poorly supported clade (BP 62%), in which only the ribotypes R10 of *O. revoluta*, R5 of *O. exserta*, and R6 of *O. kamtschatica* form a highly supported phylogroup (BP 87%). The ribotype of *O. revoluta* is the sister one to the poorly supported ribotype group of *O. exserta* and *O. kamtschatica* (BP 60%). The phylogenetic relationships of the R1–R3 ribotypes of *O. czukotica* and the R9 ribotype of *O. pumilio* between each other, as well as between them and all the other ribotypes in this clade, remained unresolved (Fig. 2b).

DISCUSSION

The analysis of nucleotide polymorphism of the *psbA–trnH* + *trnL–trnF* + *trnS–trnG* intergenic spacers of cpDNA revealed that most populations of the species of section *Arctobia* studied are characterized by low and medium haplotype diversity and low nucleotide diversity, while two populations were monomorphic (Table 1).

The low level of genetic diversity may be due to the mutual influence of several factors, such as ecological confinement, fluctuations of the population size and gene drift associated with it and evolutionary history of species. For example, *O. czukotica* plants are acidophilic, inhabited on acidic mountain soils, whereas *O. gorodkovii* plants are calciumphilic, typical for carbonate soils [2, 3]. This results in fragmentation of populations. Most species of the section *Arctobia* inhabit territories which underwent intense glaciation in the Pleistocene [3]. Therefore, these populations survived a sharp decrease in their size followed by the recovery from a small number of founders. The observed high level (70–98%) of interpopulation variability in *O. czukotica*, *O. exserta*, *O. kamtschatica*, *O. revoluta*, and *O. pumilio* may be explained by isolation of populations together with the gene drift.

The level of haplotype diversity in the populations of *O. czukotica* varies from low to high (Table 1). This predominately tetraploid species (in 28 locations on the Chukotka, Wrangel Island, and the Kolyma Upland, $2n = 32$ [30], and only on the Prodolgovataya Mountain and Kluchevskaya Sopka Volcano of Kamchatsky Krai, $2n = 16$ [31]) is characterized by a high level of morphological variability [3]. The majority of polyploid species are characterized by a higher level of genetic polymorphism and, therefore, higher resistance to severe environmental conditions and increased adaptation capacity [32]. Apparently, polyploidization of *O. czukotica* made it quite resistant to the climatic fluctuations of the Pleistocene [33], allowing it to spread on an enormous territory and keep the level of polymorphism typical of its ancestral species. The distribution of the *O. czukotica* chlorotypes, which does not correspond to the population belonging, is most probably due to the continuous gene flow between the populations via a chain of intermediate local habitats (Fig. 2a). Both intra- and interspecies phylogenetic relationships of the ITS rDNA of *O. czukotica* have remained unresolved (Fig. 2b). This may be due to the generally longer period of lineage sorting for nuclear DNA because of its biparental inheritance and larger effective population size [34].

The *O. nigrescens* and *O. susumanica* taxa have different chlorotypes (A36–A38 and A52–A55, respectively) and the common R8 ribotype (Table 1, Fig. 2) and are considered to the descendants of the same ancestral form. Bearing in mind minimal nucleotide divergence (Table 3) and the presence of the same marker nucleotide substitution (C in the position 1160), one may suggest that narrow local endemic *O. susumanica* is an intraspecific taxon of widespread *O. nigrescens*. Based on the morphological traits, Malyshev believed *O. susumanica* to be an aberrant of *O. nigrescens* [6]. To clarify the taxonomic status of *O. susumanica*, additional morphological and genetic studies of the extended sample are required.

Clear divergence of the chlorotypes of *O. nigrescens*, *O. czukotica*, *O. gorodkovii*, and *O. pumilio* of the *O. nigrescens* s. l. complex of Northeast Asia (Fig. 2a), the absence of common haplotypes, the revealed markers specific to some populations of *O. pumilio* and species-specific for *O. nigrescens* and *O. gorodkovii*, and the presence of individual ITS rDNA ribotypes of all species are consistent with the opinions of many botanists [3, 5–7, 10] about species independence of the *O. gorodkovii*, *O. pumilio*, and *O. czukotica* taxa, as well as with the data of our previous molecular genetic studies [16].

The revealed distribution of the *O. revoluta*, *O. exserta*, and *O. kamtschatica* chlorotypes in the median network (Fig. 2a) does not correspond to the division of the *Arctobia* section into the subsections: The *O. revoluta* and *O. exserta* refer to the subsection *Revolutae*, whereas the latter belongs to the subsection *Kamtschaticae* [2, 3]. However, it was previously shown that, according to the degree of separation, *O. revoluta* and *O. exserta* could be considered as two monotypic series [2], and that the *O. exserta* and *O. kamtschatica* species are morphologically very close to each other and sometimes indistinguishable at the flowering stage [3]. Our comparative analysis of morphological traits given by Pavlova for these species [10] showed that by most of them *O. exserta* is closer to *O. kamtschatica* rather than *O. revoluta*. For example, *O. exserta* and *O. kamtschatica* plants are characterized by a tall of 15–20 cm, dense pubescence, multiflower inflorescences (up to 5–6 flowers), and legumes covered with white hairs and with wide ventral septum. *O. revoluta* plants are slightly pubescent or glabrous, they are characterized by a tall of 10 cm, inflorescences with 2–3 flowers, legumes covered with black or red hairs and with narrow ventral septum. The

revealed high nucleotide divergence of cpDNA of *O. exserta* and *O. kamtschatica* from all other species (Table 3) and also common marker nucleotide substitutions and indels, connection into the same haplogroup along with preservation of species independence (Fig. 2a), and proximity of the ITS rDNA ribotypes (Fig. 2b) taken together point to the necessity to revise the division of the section *Arctobia* into subsections. In addition, the *O. revoluta* ribotype forms one statistically highly supported phylogroup with the *O. exserta* and *O. kamtschatica* ribotypes, though it separates earlier (Fig. 2b). This contradicts the opinion of Yurtsev [2, 5], who believed *O. kamtschatica* to be the closest species to the ancestral type of the section *Arctobia*, and that *O. revoluta* originated from *O. exserta*. The data obtained in our study showed that *O. kamtschatica* and *O. exserta* are more younger species, whereas the *O. czukotica*, *O. pumilio*, and *O. revoluta* are conversely more ancient.

Therefore, the data of marker polymorphism of the chloroplast and nuclear genomes are consistent with the existing views on the status of the *O. czukotica*, *O. gorodkovii*, and *O. pumilio* taxa as a separate species. The division of the section *Arctobia* into subsections obviously needs a revision, and additional complex morphological and genetic studies of an extended samples of *O. susumanica* are required for more correct establishment of the status of this taxon.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare 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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