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PLANT GENETICS

Molecular Phylogenetic Analysis of the Endemic Far Eastern Closely Related *Oxytropis* Species of Section *Orobia* (Fabaceae)

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Abstract—The questions about the taxonomic status and phylogenetic relationships of the Far Eastern closely related species *Oxytropis ochotensis*, *O. litoralis*, *O. erecta*, *O. ruthenica*, and *O. kunashiriensis* of the section *Orobia* of the genus *Oxytropis* still remain unresolved. The study of the polymorphism of nucleotide sequences of the *psbA–trnH*, *trnL–trnF*, and *trnS–trnG* cpDNA intergenic spacers showed that populations of *O. ochotensis* and *O. erecta* are characterized by a low (0.378–0.495) haplotype (*h*) and a low (0.0006–0.0009) nucleotide (π) diversity, and in populations of *O. ruthenica*, *h* varies from 0.154 to 0.872 and π varies from 0.0002 to 0.0016. One *O. ochotensis* population from Magadan oblast and one *O. ruthenica* population from Russky Island (Primorsky krai) are monomorphic. Low nucleotide divergence of cpDNA between species *O. ochotensis*, *O. erecta*, and *O. litoralis* and also statistically insignificant genetic differentiation between them, the formation of a single haplogroup in the phylogenetic network, and the absence of species-specific molecular markers indicate the unity of their gene pool. A study of the ITS rDNA polymorphism revealed private ribotypes in *O. ruthenica* and *O. kunashiriensis*, the presence of a common ribotype in *O. ochotensis*, *O. erecta*, and *O. litoralis*, and the intraspecific polymorphism in *O. ochotensis* and *O. erecta*. The differences revealed in the chloroplast and nuclear genomes confirm the independence of *O. ruthenica* and *O. kunashiriensis* and suggest that *O. erecta* and *O. litoralis* are local phenotypes of the widespread polyploid species *O. ochotensis*. An analysis of the phylogenetic relationships of the cpDNA haplotypes showed a clear separation of *O. ruthenica* populations into two evolutionary lineages, but with a single ITS ribotype.

Keywords: Oxytropis, *Orobia*, Fabaceae, genetic diversity, phylogenetic relationships, intergenic spacers, chloroplast DNA, ITS rDNA

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INTRODUCTION

A key step in the study of biodiversity is the determination of species boundaries in evolutionarily young plant groups and the establishment of their affinity. The absence of such information can lead to an erroneous sampling of taxa, an incorrect assessment of biodiversity, and other negative consequences; therefore, the distinction between species within closely related groups is very important for systematics and evolutionary biology [1, 2]. Until now, questions about the taxonomic status and phylogenetic relationships of the Far East closely related endemic species *Oxytropis ochotensis* Bunge, *O. litoralis* Kom., *O. erecta* Kom., *O. ruthenica* Vass., and *O. kunashiriensis* Kitam. of the section *Orobia* Bunge of the subgenus *Oxytropis* of the large polymorphic genus *Oxytropis* DC. (Fabaceae) remain open. The names of species, sections, and subgenera are given according to N.S. Pavlova [3]. *O. ochotensis* is an East Siberian metaarctic mountain species, endemic to Northeast Asia; it is distributed in the Far East sector of the Arctic (Lower Kolyma and Anyuiskoe and Chukotskoe highlands) and beyond (Suntar-Khayat Ridge, Nersky Plateau, Chersky Ridge (East), Kamchatka Peninsula, and the northwest coast of the Sea of Okhotsk) [3–6]. The *O. litoralis* and *O. erecta* species are endemic to the Kamchatka Peninsula [3, 5]. Thus, the habitats of three species *O. ochotensis, O. litoralis*, and *O. erecta* overlap in the eastern part of the Kamchatka Peninsula. *O. ruthenica* is an endemic to the Ussuriysk floristic region of the Russian Far East [3, 7]. There are conflicting opinions about the taxonomic status of *O. erecta*, *O. litoralis*, and *O. ruthenica*. So, V.N. Voroshilov [8] considered *O. litoralis* a synonym for *O. erecta*; V.V. Yakubov and O.A. Chernyagina [5] suggested *O. erecta* and *O. litoralis* as subspecies of *O. ochotensis*. According to N.S. Pavlova [3], *O. erecta*, *O. litoralis*, and *O. ochotensis*

are independent species. Later L.I. Malyshev [6, 9, 10] discovered the phenetic affinity of *O. litoralis* and *O. ruthenica* and lowered the rank of the latter to a subspecies—*O. litoralis* subsp. *ruthenica* (Vass.) Malyshev. *O. kunashiriensis* is endemic to Kunashir Island of the Kuril Archipelago [3, 10, 11]. N.S. Pavlova noted [3] that the morphology of *O. kunashiriensis* is close to *O. erecta* and *O. litoralis*, and L.I. Malyshev [9] noted that the independence of the *O. kunashiriensis* species is doubtful.

The reason for this taxonomic uncertainty is the lack of reliable morphological diagnostic features, which is associated with recent speciation, a high level of interspecific hybridization, and polyploidy among representatives of *Oxytropis*. So, in *O. ochotensis*, depending on the habitat, two variations of cytotypes were found: $2n = 64$ in populations from the Lower Kolyma, Anyuiskoe Highlands, and Northeastern Yakutia [12]; $2n = 48$ in a sharply isolated population in the downstream of the Alyarmagtyn River in the Chukotskoe Highlands [4]; the number of chromosomes of *O. erecta* $2n = 48$ [12] and *O. ruthenica* $2n = 16$ [13]. The number of chromosomes of *O. litoralis* and *O. kunashiriensis* is currently unknown.

In modern genetic studies of plants, markers of the nuclear and chloroplast genomes are successfully used. These include full or partial sequences of the ITS region (ITS1–5.8S rRNA–ITS2) of the ribosomal nuclear DNA operon (rDNA) and noncoding regions (rapidly evolving introns and intergenic spacers) of chloroplast DNA (cpDNA), the combined use of which is especially effective at the level of closely related species. Thus, according to the sequencing of markers of two genomes, the evolutionary relations of closely related species of *Paspalum* (Poaceae) with various cytotypes [14] and 30 species of woody angiosperms from southern Brazil [15] were clarified, and the state of natural populations of a number of rare and endemic species of the genus *Oxytropis* DC. was estimated, the sectional affiliation and taxonomic status of some species were clarified, and phylogenetic relationships in some groups of the genus were reconstructed [16–25].

The present work is devoted to the study of genetic diversity, population structure, and the assessment of the degree of divergence of the chloroplast genome of the Far East closely related species *O. ochotensis*, *O. erecta*, *O. litoralis*, *O. ruthenica*, and *O. kunashiriensis* with the aim of clarifying the taxonomic status of the latter four according to the data of polymorphism of nucleotide sequences of *psbA–trnH, trnL–trnF*, and *trnS–trnG* intergenic spacers of cpDNA and ITS rDNA.

MATERIALS AND METHODS

The material includes 177 plants from natural populations of *O. ochotensis* (55 samples), *O. erecta* (19), *O. litoralis* (2), *O. ruthenica* (99), and *O. kunashiriensis* (2) (Table 1, Fig. 1).

The methods of DNA extraction, amplification, and sequencing of the *psbA–trnH, trnL–trnF*, and *trnS–trnG* intergenic spacers were presented in our previous works [22, 24–26]. The nucleotide sequences of the forward and reverse chains were determined on an ABI 3500 genetic analyzer (Applied Biosystems, United States), then edited and assembled using the Staden Package v. 1.5 [27]. For each sample, the sequence of regions was aligned manually in SeaView v. 4.7 [28]. The matrix of combined sequences was used to calculate the number of haplotypes and their frequency in populations, haplotype (*h*) and nucleotide (π) diversity (for populations with five or more samples), and levels of differentiation and distribution of genetic variation within and between populations and/or groups of populations (molecular dispersion analysis, AMOVA) in the Arlequin v. 3.5 package [29]. The statistical significance (*P*) of fixation indices (Φ_{ST}) was evaluated on the basis of 1023 permutations. The gene flow (*Nm*) and the degree of divergence between populations/species (*D*xy) based on nucleotide substitutions were determined in DnaSP v. 5.0 [30]. Genealogical relationships of cpDNA haplotypes were analyzed by the median-joining (MJ) method in Network v. 5.0.1.1 [31], encoding each deletion or insertion, regardless of their size, as a single mutational event. The nucleotide sequences of the *psbA–trnH*, *trnL–trnF*, and *trnS–trnG* cpDNA intergenic spacers of the *O. glabra* section *Mesogaea* Bunge of the subgenus *Phacoxytropis* Bunge that we obtained earlier [24] were used (accession numbers in GenBank LT856572, LT856585, and LT856598, respectively).

The ITS rDNA region was amplified in 62 samples: *O. ochotensis* (20 samples), *O. erecta* (13), *O. litoralis* (2), *O. ruthenica* (25), and *O. kunashiriensis* (2) representing all cpDNA haplotypes identified in this work, with primers ITS1 and ITS4, under the reaction conditions and temperature conditions given in [32]. Editing, assembling, aligning, and analysis of ITS sequences were performed using the software described above. The phylogenetic analysis of the sequences was performed by the maximum parsimony (MP) method, using a heuristic search for the optimal topology, in the PAUP v. 4.0b10 software package [33]. The statistical significance of the branching order was evaluated using bootstrap analysis of 1000 alternative trees (bootstrap percentage, BP, %).

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

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

Location (number of samples)	Code	Diversity (standard deviation)		Haplotype (number of samples)
		haplotype	nucleotide	
O. ochotensis				
Kamchatka, Klyuchevskaya Sopka Volcano, hillside (10)	OKAK	0.378 (0.181)	0.0009 (0.0006)	H1 (8) H2 (1) H3 (1)
Kamchatka, Ploskaya Sopka Volcano, southeast macro hillside (13)	OKAP	0.385 (0.132)	0.0006 (0.0005)	H1 (10) H2 (3)
Kamchatka, Avachinskaya Sopka Volcano, south macro hillside (14)	OKAA	0.495 (0.151)	0.0008 (0.0006)	H4 (10) H5 (2) H6 (1) H7 (1)
Kamchatka, near Ust-Kamchatsk, hillside of Uval'naya Mountain (1)	OKAU	–	–	H8 (1)
Kamchatka, average course of the Raduga River (1)	OKAR	–	–	H9 (1)
Kamchatka, mountains of the Kamchatka Cape, springhead of Uglovaya River (1)	OKAM	–	–	H10 (1)
Magadan oblast, near Orotuk (10)	OMAO	0.000 (0.000)	0.0000 (0.0000)	H11 (10)
Magadan oblast, near Burkand'ya (3)	OMAB	–	–	H12 (2) H13 (1)
Magadan oblast, near Shturmovoi (1)	OMAS	–	–	H14 (1)
Magadan oblast, near Madaun, Lebedinaya Mountain, hillside (1)	OMAM	–	–	H15 (1)
O. erecta				
Kamchatka, Avachinskii Bay, coastal sand shafts (16)	EKAZ	0.425 (0.133)	0.0009 (0.0006)	H16 (3) H17 (12) H18 (1)
Kamchatka, Ploskaya Sopka Volcano hillside (1)	EKAP	–	–	H19 (1)
Kamchatka, Avachinskaya Sopka Volcano, near Petropavlovsk-Kamchatsky (1)	EKAA	–	–	H17 (1)
Kamchatka, Lake Tolmachev, coastal terrace (1)	EKAT	–	–	H20 (1)
O. litoralis				
Kamchatka, southwestern part, sea terrace (1)	LKAM	–	–	H10 (1)
Kamchatka, near Krutoberegovo, Lake Nerpich'e, coastal terrace (1)	LKAN	–	–	H10 (1)
O. ruthenica				
Primorsky krai, Russky Island, Cape Tobizina, coastal cliffs (17)	RRUT	0.000 (0.000)	0.0000 (0.0000)	H21 (17)
Primorsky krai, Russky Island, Cape Vyatlina, sea terrace (17)	RRUV	0.228 (0.129)	0.0002 (0.0002)	H21 (15) H22 (1) H23 (1)
Primorsky krai, Popov Island, Pogranichnaya Bay, sea terrace (14)	RPOP	0.363 (0.130)	0.0002 (0.0002)	H23 (11) H24 (3)
Primorsky krai, Putyatin Island, west coast, coastal cliffs (19)	RPUZ	0.637 (0.104)	0.0011 (0.0007)	H25 (1) H26 (2) H27 (11) H28 (1) H29 (4)

Table 1. (Contd.)

Location (number of samples)	Code	Diversity (standard deviation)		Haplotype (number of samples)
		haplotype	nucleotide	
Primorsky krai, Putyatin Island, east coast, coastal cliffs (13)	RPUV	0.154 (0.126)	0.0004 (0.0003)	H27 (12) H30 (1)
Primorsky krai, near Amgu, coastal cliffs (6)	RAMG	0.600 (0.129)	0.0002 (0.0002)	H31 (3) H32 (3)
Primorsky krai, near Vrangel, Cape Kamensky, hillside by the sea (13)	RVRA	0.872 (0.067)	0.0016 (0.0010)	H32 (1) H33 (1) H34 (2) H35 (4) H36 (3) H37 (1) H38 (1)
O. kunashiriensis				
Kuril Islands, Kunashir Island, Lovtsov Cape (2)	KUN	–	–	H39 (1)

RESULTS

The nucleotide sequences of each of the *psbA–trnH, trnL–trnF*, and *trnS–trnG* cpDNA regions in 177 samples of *O. ochotensis*, *O. erecta*, *O. litoralis*, *O. ruthenica*, and *O. kunashiriensis* are characterized by relatively low nucleotide variability and different lengths owing to the presence of insertions/deletions (indels) and mono- and dinucleotide repeats. The length of the combined matrix of the three regions after alignment was 2476 sites (1–484, 485–1270, and 1271–2476, respectively), of which 2359 were monomorphic and 14 were variable. Twelve nucleotide substitutions at positions 240, 281, 414, 1269, 1270, 1313, 1557, 1631, 1804, 1848, 2244, and 2300 were parsimony informative. An analysis of the obtained matrix revealed 39 haplotypes (H1–H39), of which 18 (46.1%) were unique (Table 1). The sequences of the three cpDNA regions were deposited in GenBank under accession numbers MK806162–MK806278. *O. ochotensis* has 15 haplotypes (H1–H15), *O. erecta* has 5 (H16–H20), *O. litoralis* has 1 (H10), *O. ruthenica* has 18 (H21–H38), and *O. kunashiriensis* has 1 (H39). A common haplotype (H10) was found in *O. ochotensis* and *O. litoralis* (Table 1).

The populations of *O. ochotensis* and *O. erecta* are characterized by a low (0.378–0.495) haplotype and low (0.0006–0.0009) nucleotide diversity, while in the populations of *O. ruthenica*, *h* varies from 0.154 to 0.872 and π varies from 0.0002 to 0.0016 (Table 1). The OMAO *O. ochotensis* and RRUT *O. ruthenica* populations were monomorphic (Table 1). One of the indicators of the degree of genetic fragmentation between populations is the divergence of nucleotide sequences (*D*xy). In *O. ochotensis*, the highest *D*xy values were determined between the Kamchatka OKAK and OKAP populations and the Magadan OMAB population, as well as between the Magadan OMAB and OMAM populations (Table 2). The nucleotide divergence between the populations of *O. erecta* and *O. litoralis*, as well as between the populations of each species, is absent or very low (Table 2). It should be noted that the *D*xy values between most populations of *O. ochotensis*, *O. erecta*, and *O. litoralis* are low and correspond to the intraspecific level of *O. ochotensis*. In *O. ruthenica*, the RRUT, RRUV, and RPOP populations from Russky and Popov islands are significantly diverged from all others (Table 3). A high degree of differentiation of species populations is also indicated by the results of molecular dispersion analysis. According to AMOVA (Table 4), in *O. ochotensis*, *O. ruthenica*, and *O. erecta*, the main part of all genetic variation is due to interpopulation differences (Φ_{ST} = 0.8703, $P < 0.0001$; Φ_{ST} = 0.8898, $P < 0.0001$; Φ_{ST} = 0.8215, $P > 0.05$, respectively). The value of the fixation index in *O. erecta* is statistically insignificant, which is associated with the small size of three of the four studied samples. The gene flow (*Nm*) between the populations of *O. ochotensis* and *O. ruthenica* was 0.11 and 0.09 migrants per generation, respectively. Hierarchical AMOVA showed that there are no statistically significant genetic differences between *O. erecta* and *O. litoralis* or between *O. ochotensis*, *O. erecta*, and *O. litoralis* (Table 4). Divergence of nucleotide sequences between pairs of *O. ochotensis–O. erecta*, *O. ochotensis–O. litoralis*, and *O. erecta–O. litoralis* is extremely low (Table 5) and corresponds to the interpopulation level of *O. ochotensis* and *O. ruthenica* (Tables 2, 3). The highest *Dxy* values are determined between *O. kunashiriensis* and all other species (Table 5). According to AMOVA results, 35.2% of the variability is due to differences between species, while the inter-

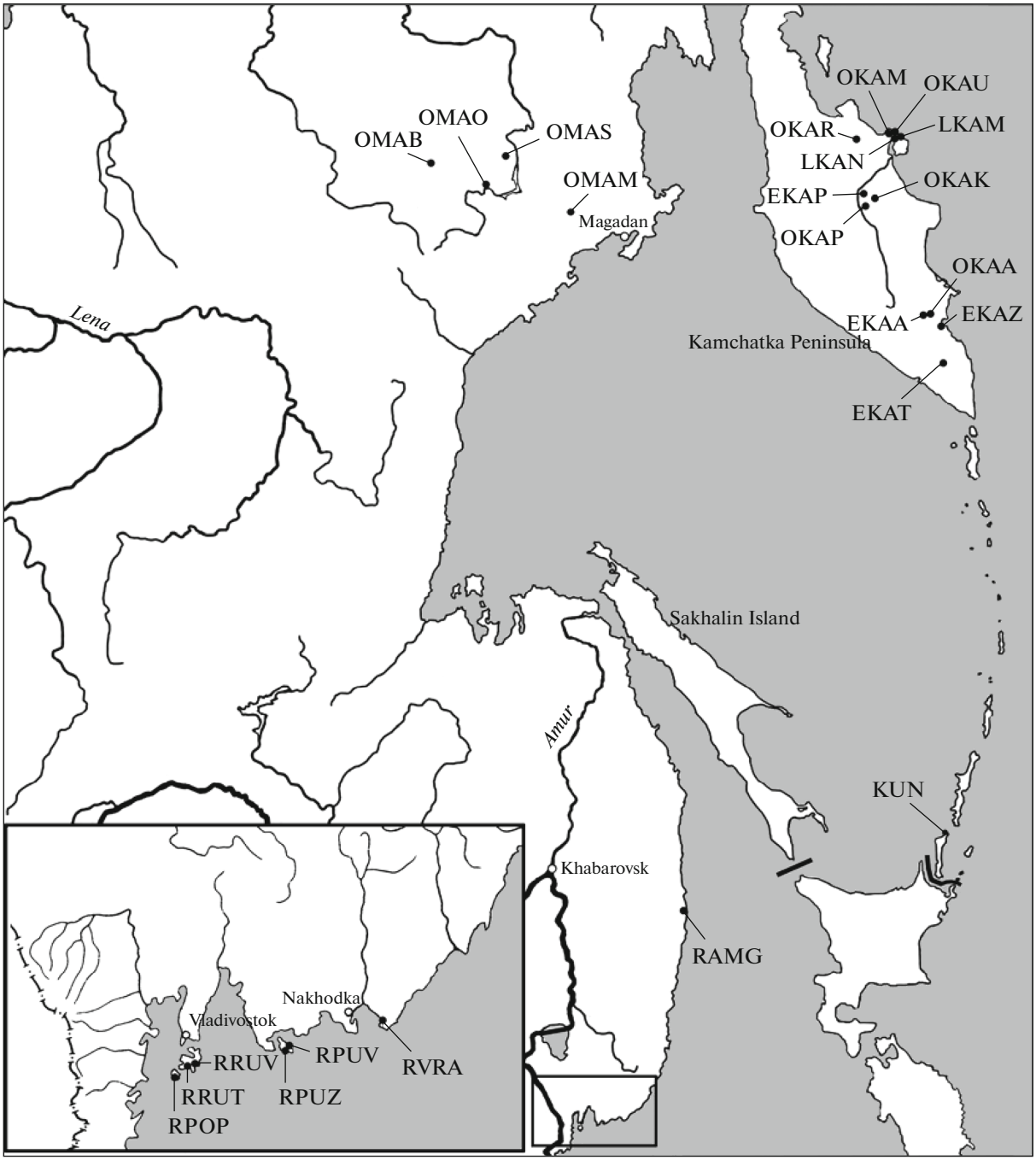

Fig. 1. Schematic map showing the collection sites of *Oxytropis ochotensis*, *O. erecta*, *O. litoralis*, *O. ruthenica*, and *O. kunashiriensis*. Population code, see Table 1.

and intrapopulation components account for 56.9 and 7.9%, respectively (Table 4).

To clarify the phylogenetic relations between the studied species, a median network of genealogical relationships of cpDNA haplotypes was constructed (Fig. 2a). The identified haplotypes form four haplogroups. Haplogroup I included all the haplotypes of *O. ochotensis* (H1–H15), *O. erecta* (H16–H20), and *O. litoralis* (H10); haplogroups II and III included haplotypes of *O. ruthenica*: H21–H24 (Russky and Popov Islands) and H25–H38 (continent and Putyatin Island), respectively; and haplogroup IV is represented only by the haplotype of *O. kunashiriensis* (H39). Haplogroups I–III diverge from a hypothetical haplotype (undetected in our study or an extinct ancestral one) and are separated by 5–7 mutational steps (Fig. 2a). Haplotype H39 of *O. kunashiriensis* is

Table 2. Nucleotide divergence between populations of *Oxytropis ochotensis*, *O. erecta*, and *O. litoralis* according to cpDNA

Code	OKAK	OKAP	OKAA	OKAU	OKAR	OKAM	OMAO	OMAB	OMAS	OMAM	EKAZ	EKAP	EKAA	EKAT	LKAM	LKAN
OKAK	–	0.00 (0)	1.43 (0)	3.00 (3)	3.00 (3)	1.00 (1)	3.00 (3)	4.00 (4)	1.00 (1)	0.00 (0)	0.75 (0)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
OKAP	0.000	–	1.43 (0)	3.00 (3)	3.00 (3)	1.00 (1)	3.00 (0)	4.00 (4)	1.00 (1)	0.00 (0)	0.75 (0)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
OKAA	0.060	0.060	–	1.57 (1)	1.57 (1)	2.43 (1)	1.57 (1)	2.57 (2)	2.43 (1)	1.43 (0)	2.18 (0)	2.43 (1)	2.43 (1)	2.43 (1)	2.43 (1)	2.43 (1)
OKAU	0.125	0.125	0.066	–	0.00 (0)	2.00 (2)	0.00 (0)	1.00 (1)	2.00 (2)	3.00 (3)	2.25 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)
OKAR	0.125	0.125	0.066	0.000	–	2.00 (2)	0.00 (0)	1.00 (1)	2.00 (2)	3.00 (3)	2.25 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)
OKAM	0.042	0.042	0.101	0.083	0.083	–	2.00 (2)	3.00 (3)	0.00 (0)	1.00 (1)	0.25 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
OMAO	0.125	0.125	0.066	0.000	0.000	0.083	–	1.00 (0)	2.00 (2)	3.00 (3)	2.25 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)
OMAB	0.167	0.167	0.107	0.042	0.042	0.125	0.042	–	3.00 (3)	4.00 (4)	3.25 (3)	3.00 (3)	3.00 (3)	3.00 (3)	3.00 (3)	3.00 (3)
OMAS	0.042	0.042	0.101	0.083	0.083	0.000	0.083	0.125	–	1.00 (1)	0.25 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
OMAM	0.000	0.000	0.060	0.125	0.125	0.042	0.124	0.167	0.041	–		1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
EKAZ	0.031	0.031	0.091	0.094	0.094	0.010	0.094	0.136	0.010	0.031	–	0.25 (0)	0.25 (0)	0.25 (0)	0.25 (0)	0.25 (0)
EKAP	0.042	0.042	0.101	0.083	0.083	0.000	0.083	0.125	0.000	0.042	0.010	–	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
EKAA	0.042	0.042	0.101	0.083	0.083	0.000	0.083	0.125	0.000	0.042	0.010	0.000	–	0.00 (0)	0.00 (0)	0.00 (0)
EKAT	0.042	0.042	0.101	0.083	0.083	0.000	0.083	0.125	0.000	0.042	0.010	0.000	0.000	–	0.00 (0)	0.00 (0)
LKAM	0.042	0.042	0.101	0.083	0.083	0.000	0.083	0.125	0.000	0.042	0.010	0.000	0.000	0.000	–	0.00 (0)
LKAN	0.042	0.042	0.101	0.083	0.083	0.000	0.083	0.125	0.000	0.042	0.010	0.000	0.000	0.000	0.000	–

Above the diagonal is the average number of nucleotide differences (the number of fixed differences); below the diagonal is the average number of nucleotide substitutions per site $\times 10^{-2}$.
Population code see in Table 1.

Table 3. Nucleotide divergence between populations of *Oxytropis ruthenica* according to cpDNA

Population code	RRUT	RRUV	RPOP	RPUZ	RPUV	RAMG	RVRA
RRUT	–	0.294 (0)	2.000 (2)	2.895 (2)	2.923 (2)	2.500 (2)	2.000 (2)
RRUV	0.012	–	1.824 (0)	3.189 (2)	3.217 (2)	2.794 (2)	2.294 (2)
RPOP	0.083	0.076	–	4.895 (4)	4.923 (4)	4.500 (4)	4.000 (4)
RPUZ	0.121	0.133	0.205	–	0.263 (0)	1.395 (0)	0.895 (0)
RPUV	0.122	0.135	0.206	0.011	–	1.423 (0)	0.923 (0)
RAMG	0.104	0.117	0.188	0.058	0.059	–	0.500 (0)
RVRA	0.084	0.096	0.167	0.037	0.039	0.021	–

Above the diagonal is the average number of nucleotide differences (the number of fixed differences); below the diagonal is the average number of nucleotide substitutions per site $\times 10^{-2}$. Population code see in Table 1.

Table 4. Distribution of genetic variation (AMOVA) between *Oxytropis* groups according to cpDNA

Dispersion source	Genetic variation (%) between		
	groups	populations within groups	individuals in a population
Populations of *Oxytropis* species			
One group: (all populations of *O. ochotensis*)	–	87.03*	12.97
One group: (all populations of *O. erecta*)	–	82.15 ns	17.85
One group: (all populations of *O. ruthenica*)	–	88.98*	11.02
Two groups: (all populations of *O. erecta*) and (*O. litoralis* population)	–69.53 ns	133.63 ns	35.90**
Two groups: (*O. litoralis* population) and (all populations of *O. ruthenica*)	42.54 ns	51.06*	6.41*
Three groups: (all populations of *O. ochotensis*), (all populations of *O. erecta*), and (*O. litoralis* population)	8.73 ns	78.11*	13.16*
Five groups: (all populations of *O. ochotensis*), (all populations of *O. erecta*), (*O. litoralis* population), (all populations of *O. ruthenica*), and (*O. kunashiriensis*)	35.20**	56.90*	7.90*
Haplogroups identified in Network analysis			
One group: (haplogroup I)	–	86.22*	13.78
Two groups: (haplogroup II) and (haplogroup III)	84.64**	8.16*	7.20*
Four groups: (haplogroup I), (haplogroup II), (haplogroup III), and (haplogroup IV)	62.86**	29.69*	7.46*

* $P < 0.0001$; ** $P < 0.05$; ns—nonsignificant. The significance level is determined on the basis of 1023 permutations.

Table 5. Nucleotide divergence between the species *Oxytropis ochotensis*, *O. erecta*, *O. litoralis*, *O. ruthenic*, and *O. kunashiriensis* according to cpDNA

Species	*O. ochotensis*	*O. erecta*	*O. litoralis*	*O. ruthenica*	*O. kunashiriensis*
O. ochotensis	–	1.576 (0)	1.655 (0)	4.017 (1)	5.655 (4)
O. erecta	0.066	–	0.211 (0)	2.867 (1)	4.211 (4)
O. litoralis	0.069	0.009	–	2.657 (1)	4.000 (4)
O. ruthenica	0.169	0.120	0.111	–	5.626 (4)
O. kunashiriensis	0.237	0.176	0.167	0.236	–

Above the diagonal is the average number of nucleotide differences (the number of fixed differences); below the diagonal is the average number of nucleotide substitutions per site $\times 10^{-2}$.

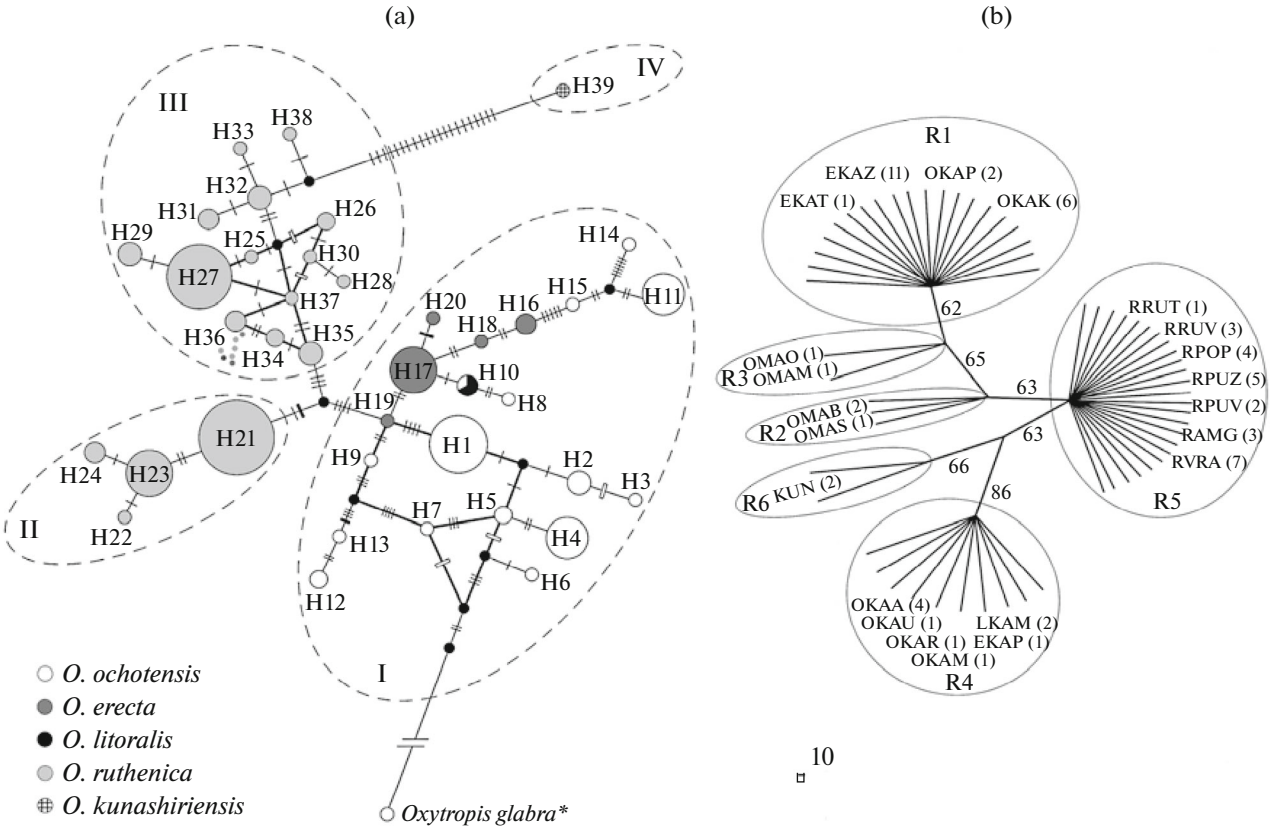

Fig. 2. Phylogenetic relationships of closely related species *Oxytropis ochotensis*, *O. erecta*, *O. litoralis*, *O. ruthenica*, and *O. kunashiriensis*. (a) Genealogical network of cpDNA haplotypes (H1–H39) constructed using the MJ method. The size of the circles reflects the frequency of occurrence of haplotypes, small black circles indicate hypothetical haplotypes, transverse thin dashes on the branches mark mutation events, black thick dashes mark insertion of nucleotides, white thick dashes mark deletion of nucleotides, and the dotted line shows haplogroups I–IV. * Mutations for *O. glabra*, used as an out group, are not indicated and are not considered. (b) Phylogenetic unrooted MP tree (tree length of 12 steps, CI = 1.0, HI = 0.0, RI = 1.0) based on the analysis of ITS rDNA sequences of 62 samples. Numbers indicate bootstrap index values (above 50%). At the bottom of the figure, the unit of measure of the length of the branches is indicated—10 steps. The solid line indicates the clusters combining the samples with the same ribotype (R1–R6). Population code, see Table 1; the number of samples of this population is given in parentheses.

connected with haplotypes H32 and H38 of *O. ruthenica* of haplogroup III through 22 mutational steps and a hypothetical haplotype. Such a genetic separation of *O. kunashiriensis* from all other species is due to high nucleotide divergence (Table 5). The distribution of the haplotypes of *O. ochotensis*, *O. erecta*, and *O. litoralis* in haplogroup I does not correspond to either population or taxonomic affiliation, which together with low nucleotide divergence between most populations of these species (Table 2) indicates the genetic homogeneity of this group. According to AMOVA (Table 4), the main part of genetic variation in haplogroup I falls in variability between populations (Φ_{ST} = 0.8621, $P < 0.0001$). A high degree of divergence (about 85%) between haplogroups II and III of *O. ruthenica* (Table 4) indicates a significant genetic isolation of populations from Russky and Popov islands from all others. The presence of alternative links (loop structures in the network) between the haplotypes in haplogroups I and III (Fig. 2a) does not allow us to unambiguously establish the relationship between the Kamchatka populations of *O. ochotensis* and *O. erecta* or between populations of *O. ruthenica* from Putyatin Island and the population from the vicinity of the village of Vrangel.

In haplogroups I, II, and IV, marker nucleotide substitutions and indels were revealed: in I—T at position 2300; in II—T at position 1631 and an insertion of 10 nucleotides (TTTTTATATC, positions 1381–1390); in IV—a deletion of four nucleotides (GAAA, positions 302–305) and C at positions 1557, 1804, and 1848. Haplogroups II and III, combining *O. ruthenica* haplotypes, have a common marker substitution (A at position 2300); haplogroups III and IV, C at position 2244. The nucleotide divergence of sequences between haplogroups I–IV (Table 6) is comparable to that between the *O. ochotensis*–*O. ruthenica* and *O. ochotensis*–*O. kunashiriensis* species (Table 5). Hierarchical AMOVA showed that about 63% of the variability is due to differences between haplogroups (Table 4).

Table 6. Nucleotide divergence between haplogroups identified in Network analysis of cpDNA haplotypes of *Oxytropis ochotensis*, *O. erecta*, *O. litoralis*, *O. ruthenica*, and *O. kunashiriensis*

Haplogroup	I	II	III	IV
I	–	4.050 (2)	3.557 (2)	5.200 (4)
II	0.170	–	4.107 (2)	7.750 (6)
III	0.150	0.172	–	4.357 (4)
IV	0.218	0.324	0.183	–

Above the diagonal is the average number of nucleotide differences (the number of fixed differences); below the diagonal is the average number of nucleotide substitutions per site $\times 10^{-2}$. Haplogroups I–IV—see Fig. 2a and Results.

Thus, genealogical analysis revealed a clear separation of cpDNA haplotypes of five closely related *Oxytropis* species into four evolutionary branches: (1) *O. ochotensis*–*O. erecta*–*O. litoralis*; (2) *O. ruthenica* from Russky and Popov islands; (3) *O. ruthenica* from Putyatin Island and the continental part of Primorsky Krai; (4) *O. kunashiriensis*.

The nucleotide sequences of ITS rDNA of 62 samples of the studied species of *Oxytropis*, representing all the cpDNA haplotypes identified in this study, are characterized by the same length (603 bp) and low nucleotide variability. The boundaries of the three parts of region were determined by comparison with the sequence of ITS *O. viscida* Nutt. from the GenBank database under accession number AF121758 [34]. The sequence sizes of the ITS1, 5.8S rDNA gene, and ITS2 were 227, 164, and 212 bp, respectively. Of the 603 sites, seven were variable and parsimony informative: four substitutions (positions 68, 119, 122, and 227) in ITS1 and three (positions 466, 523, and 564) in ITS2. In 62 sequences, six ribotypes (R1–R6) were identified, the sequences of which were deposited in GenBank under accession numbers MK795939–MK795946. Four ribotypes belong to *O. ochotensis* (R1–R4), R1 was also detected in 12 samples of *O. erecta*, and R4 was detected in one sample of *O. erecta* and in *O. litoralis*. Only one ribotype (R5) was found in 25 samples of *O. ruthenica*, representing 18 cpDNA haplotypes; and R6 was found in *O. kunashiriensis*. The phylogenetic analysis of ITS sequences by the MP method yielded one tree (tree length of 12 steps, CI = 1.0, HI = 0.0, RI = 1.0). Samples of different populations and species are grouped in accordance with a specific ribotype with moderate (BP 62–86%) statistical support (Fig. 2b). Plants from Kamchatka populations of the *O. ochotensis* and *O. erecta* species are distributed in two clusters with R1 and R4 ribotypes, and samples from Magadan populations of *O. ochotensis* are distributed in two clusters with R2 and R3 ribotypes. Samples of *O. litoralis* were combined with Kamchatka samples of *O. ochotensis* and *O. erecta*, which have the R4 ribotype. Separate clusters are formed by the samples of *O. ruthenica* and *O. kunashiriensis* with ribotypes R5 and R6, respectively.

DISCUSSION

An analysis of the variability of the nucleotide sequences of the *psbA–trnH*, *trnL–trnF*, and *trnS-trnG* intergenic spacers of the chloroplast genome in representatives of 12 populations of Far East endemic species *O. ochotensis*, *O. erecta*, *O. litoralis*, and *O. ruthenica* revealed that seven of them are characterized by a low haplotype (*h*) diversity, three are characterized by high haplotype diversity and low nucleotide (π) diversity, and two populations are monomorphic (Table 1). Low values of genetic diversity are usually associated with the passing of a population through a so-called bottleneck—a sharp decrease in numbers with its subsequent restoration; high *h* and low π values are characteristic of populations with a rapid growth in numbers from a small number of founders, when sufficient time has passed for restoration of haplotype variability due to the mutation process, but not sufficient for accumulation of significant nucleotide differences in DNA sequences [35]. The lack of genetic diversity in the Magadan OMAO population of *O. ochotensis* and in RRUT population of *O. ruthenica* from Russky Island may indicate their origin from a small group of closely related plants. So, the island endemics *Eriogonum arborescens* Greene, *E. giganteum* S. Watson [36], and *Astragalus edulis* Bunge [37] are characterized by the presence of only one haplotype on each of the islands, which is explained by their single colonization, isolation, and reduction or complete cessation of gene exchange between island and parental populations [36, 37]. In general, the genetic diversity of the studied populations is lower than in the populations of endemic species *O. chankaensis* Jurtz. [16], *O. bargusinensis* Peschk., *O. interposita* Sipl., and *O. triphylla* (Pall.) Pers. [24] and in the Barguzin populations of *O. glandulosa* Turcz. [25].

Low nucleotide divergence and statistically insignificant genetic differentiation between *O. ochotensis*, *O. erecta*, and *O. litoralis* (Tables 4, 5), the absence of specific molecular markers, the presence of a single marker nucleotide substitution (T at position 2300), and the formation of a single haplogroup in the median network (Fig. 2a) suggest that they all belong to the same species—*O. ochotensis*. This confirms the previously made assumptions about the synonymy of *O. erecta* and *O. litoralis* [8] and about the need to con-

sider them as subspecies or varieties of *O. ochotensis* [5]. Significant nucleotide divergence of the *O. litoralis* and *O. ruthenica* species (Table 5), the belonging of haplotypes to different haplogroups (Fig. 2a), and the absence of common molecular markers cast doubt on the conclusion of L.I. Malyshev about their taxonomic proximity [9]. On the dendrogram of differences based on the analysis of 47 qualitative morphological characters, *O. litoralis* and *O. ruthenica* were in the same cluster; the difference was 22%; on the basis of this, the author lowered the rank of the latter to a subspecies of *O. litoralis*. At the same time, considering the moderately different species *O. alpestris* Schischkin and *O. helenae* N.S. Pavlova (24% difference), L.I. Malyshev suggested: "they can be separate species, because they have a far separated range: one inhabits the west of Gorny Altai and the other inhabits Sakhalin, and their morphological similarity may be a manifestation of parallel (homological) evolution" [9]. Returning to *O. litoralis* and *O. ruthenica*, one should note that the ranges of these species are also geographically significantly separated (Fig. 1); they are Northeast Kamchatka and Primorye, respectively [3], and no intermediate forms were found. Thus, on the basis of the foregoing and our molecular genetic data on the variability of the chloroplast genome, it can be argued that *O. litoralis* and *O. ruthenica* are different species, as was suggested from the first description of *O. ruthenica* [3, 7, 8].

High (about 89%) statistically significant population differentiation in *O. ruthenica* indicates actively ongoing speciation processes. The formation of two clearly isolated evolutionary lines in *O. ruthenica* is confirmed by the absence of common haplotypes, pronounced differentiation (about 85%) of the two haplogroups (Fig. 2a), and the identified molecular markers in each of them. The presence of two lines—one includes haplotypes of populations of Russky and Popov islands, and the other includes haplotypes of populations of the continental part of Primorsky krai and Putyatin Island—may be the result of a lack of genetic exchange between them over the years. So, the isolation time of Russky and Popov islands is about 8500 years, and that of Putyatin Island is 7000 years [38]. Three evolutionary lines were identified earlier in *O. glandulosa* [28], but, unlike *O. ruthenica*, in populations with different ploidy, and the existence of cryptic species that replace or coexist with *O. glandulosa* was suggested, the appearance of which in plants is associated primarily with polyploidy. The analysis of polymorphism of marker regions of cpDNA revealed a high nucleotide divergence of the chloroplast genome of *O. kunashiriensis* from other taxa (Tables 5, 6; Fig. 2a), which confirms its species status.

Previously, we [17, 39] and other researchers [18, 20, 23] showed that, in *Oxytropis* species, both close related and from different sections, despite morphological differentiation, the ITS rDNA sequences are identical or have slight differences. In *O. ruthenica* and *O. kunashiriensis*, individual R5 and R6 ribotypes were detected, respectively, which indicates their independence, and the presence of a common R4 ribotype in *O. ochotensis*, *O. erecta*, and *O. litoralis* confirms their genetic similarity. The observed intraspecific ITS polymorphism in *O. ochotensis* and *O. erecta*—four (R1–R4) and two (R1 and R4) ribotypes, respectively—apparently reflects the allopolyploid origin of the hexaploids *O. ochotensis* and *O. erecta* and the octoploid *O. ochotensis* as a result of hybridization of polymorphic diploid ancestors.

Thus, the analysis of the polymorphism of the intergenic spacers of cpDNA and ITS rDNA of closely related species of the section *Orobia* confirms the species status of *O. ruthenica* and *O. kunashiriensis*; *O. erecta* and *O. litoralis* are most likely local phenotypes of the widespread polyploid species *O. ochotensis*. For the final establishment of the status of *O. erecta*, *O. litoralis*, and two evolutionary lines of the chloroplast genome of *O. ruthenica*, additional genetic, morphological, and cytological studies of an extended sample of plants covering the entire range of these taxa are necessary.

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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