

## REVISION AND DISTRIBUTION OF *LIPARIS* SPECIES (ORCHIDACEAE) IN AMUR REGION (RUSSIA)

Elena I. Terentjeva<sup>1</sup>, Tatyana I. Varlygina<sup>1</sup>, Galina F. Darman<sup>2</sup>,  
Galina V. Degtjareva<sup>1</sup>, Sergey V. Efimov<sup>1</sup>, Tagir H. Samigullin<sup>1</sup>

<sup>1</sup>Lomonosov Moscow State University, Russia

e-mail: [el.terenteva@mail.ru](mailto:el.terenteva@mail.ru), [tat-varlygina@yandex.ru](mailto:tat-varlygina@yandex.ru), [efimov-msu@yandex.ru](mailto:efimov-msu@yandex.ru)

<sup>2</sup>Botanical Garden-Institute of FEB RAS, Russia

e-mail: [gfdarman@yandex.ru](mailto:gfdarman@yandex.ru)

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In July 2018, in the Khingan State Nature Reserve and Muravyevskiy Park of Sustainable Development, we conducted the study of *Liparis* populations and preliminary determining the orchid individuals collected. In the family Orchidaceae, *Liparis* is one of the most difficult genera in terms of systematics. Observations in nature, research of herbarium specimens and literature demonstrate the considerable intraspecific variability in morphological traits. Therefore, to assess the taxonomic position of orchids from natural populations, we collected parts of the plants for further molecular-phylogenetic research. In total, we included 110 samples in the analysis. As a molecular marker, we used the internal transcribed spacers (ITS1–5.8S–ITS2) of 18S–26S nuclear ribosomal DNA. At the obtained phylogenetic tree, samples from the studied populations formed separate and clearly supported clades, that confirms their naturalness. ITS sequences of the analysed plants of each clade have group-specific substitutions, which mark their position on the phylogenetic tree. Analysis of both morphological traits and molecular-phylogenetic data allowed conducting species determination of plants from the genus *Liparis*. According to botanical literature, in the Amur region, two species were known: *Liparis japonica* and *L. makinoana*. The investigation of *Liparis* populations found in the field together with analysis results of morphological and molecular-phylogenetic data demonstrated that in the study area, besides *L. japonica* and *L. makinoana*, there are *L. kumokiri* and one more taxon, which could be a new species needed to be described in future. We clarified the distribution of these *Liparis* species in the study area by finding their new locations. The status of each population for studied species was appropriate at most of the locations. We noted the appropriate fruiting and offspring presence. At the studied location, permanent study plots could be established for conducting of further population-based studies. The obtained data on the status of plant populations will be used in the work on new editions of Red Data Books. In the study area, the presence of *Liparis makinoana* have not been confirmed by data of molecular-phylogenetic analysis.

**Key words:** molecular-phylogenetic study, morphological traits, phylogeny, population status, Protected Area, rare species, Red Data Book

### Introduction

The genus *Liparis* Rich. (tribe Malaxideae Lindl.) includes about 250–300 species distributed predominantly in the tropics of the Old World. In Russia, six *Liparis* species are known. *Liparis loeselii* (L.) Rich is distributed in European Russia and Siberia. *Liparis japonica* (Miq.) Maxim., *L. krameri* Franch. & Savat., *L. kumokiri* F. Maek., *L. makinoana* Schlechter and *L. sachalinensis* Nakai are known from the Russian Far East (Vakhrameeva et al., 2014).

This genus is still one of the most difficult Orchidaceae genera in terms of systematics. In-nature observations, investigation of data from herbaria and literature have demonstrated a considerable intraspecific variability in morphological parameters of *Liparis* species (Shibneva, 2007, 2011; Efimov, 2010). This makes it difficult or even impossible to distinguish certain species within this genus. In addition, the natural ranges of some *Liparis* species are overlapped, which additionally confuses their deter-

mination. The high degree of intraspecific polymorphism and a relatively small number of morphological traits significant for species determination make the limits between *Liparis* species by the discussion topic (Maekawa, 1971; Dressler, 1981; Kim & Kim, 1986; Shibneva, 2004, 2007, 2008; Tsutsumi & Yukawa, 2008; Efimov, 2010). The applying of molecular-phylogenetic methods together with morphological data allow approaching a new level in solving issues related to morphology, systematics and phylogeny of the genus *Liparis*. A worldwide active investigation of the genus *Liparis* is currently underway by comparing of sequences of rDNA internal transcribed spacers, as well as using other molecular markers, primarily encoding and non-coding regions of chloroplast DNA (genes *matK*, *ycf1*, spacers *trnL-trnF* and *trnS-trnG*) (Dressler, 1993; Cameron, 2005; Tsutsumi et al., 2007, 2008a,b; Lee et al., 2010; Li & Yan, 2013; Su et al., 2014, 2015; Terentjeva et al., 2016, 2018, 2019).

Previously (Vyshin, 1996; Starchenko & Darman, 2002; Vakhrameeva et al., 2014), only two *Liparis* species (*L. makinoana* Schlechter and *L. japonica* (Miq.) Maxim.) had been noted in the Amur region. They are included in the Red Data Book of the Russian Federation (2008) and the Red Data Book of the Amur region (2019). In addition, *L. japonica* is protected in the Khingan State Nature Reserve (Kudrin & Yakubov, 2013) and in the Amur Branch of the Botanical Garden-Institute of FEB RAS (Starchenko & Darman, 2002).

In July 2018, Terentieva et al. (2019) found for the first time *Liparis* plants in the forest area of the Muravyevskiy Park of the Sustainable Development during a botanical field trip. The found plants could not belong to any of the *Liparis* species known in the Amur region on the basis of morphological traits. In the field, the individuals, having green flowers, were preliminary named as *L. kumokiri*, while plants with coloured flowers left non-determined. The same situation occurred in the Khingan State Nature Reserve.

The present study aimed to clarify the list of *Liparis* species and their distribution in the Amur region using morphological data and molecular-phylogenetic methods. The molecular phylogenetic reconstruction is based on the previous phylogenetic analysis of Terentieva et al. (2019), being considerably supplemented by new samples and taxa from the GenBank database.

### Material and Methods

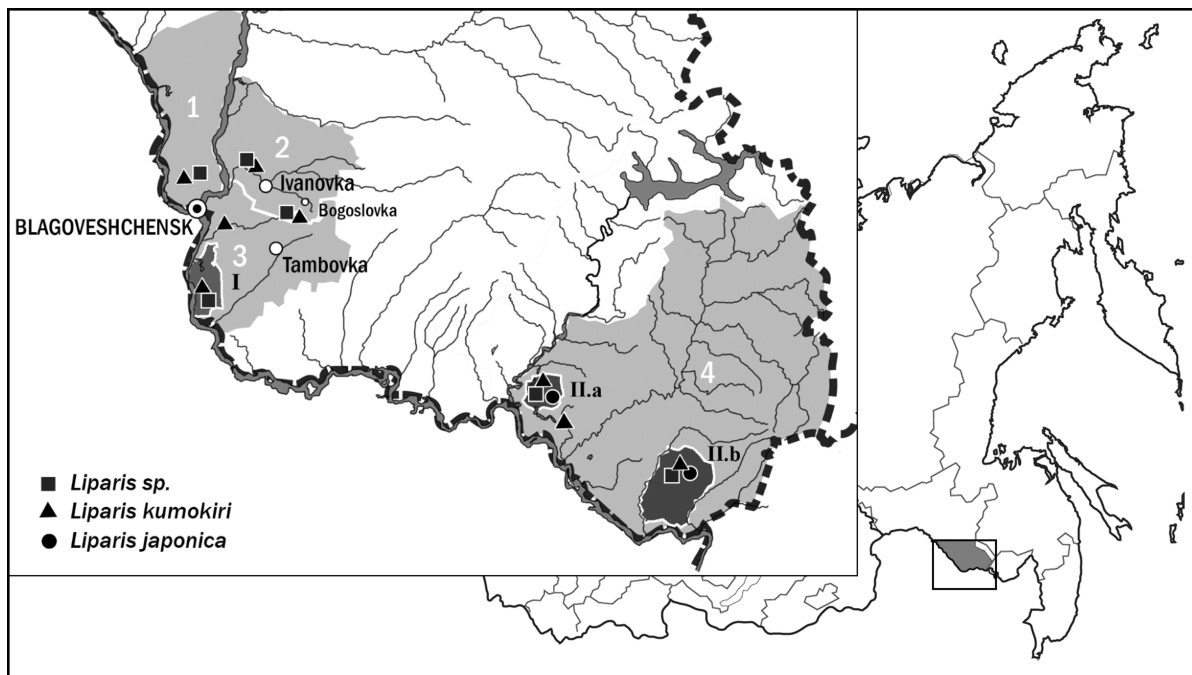
The material for the study was collected in July 2018 during a botanical field trip of the researchers of Moscow State University in two Protected Areas of the Amur region (Russia), the Muravyevskiy Park of the Sustainable Development (Tambovskiy district) and two sites of the Khingan State Nature Reserve (Arkharinskiy district). In addition, we collected samples outside Protected Areas: 1) in the surroundings of the villages Ivanovka and Bogoslovka (Ivanovskiy district); 2) on the gold slag disposals (Blagoveshchenskiy district); 3) in the forest massif near the Amur Branch of the Botanical Garden-Institute of FEB RAS (Fig. 1).

According to geobotanical zoning, the Muravyevskiy Park of the Sustainable Development belongs to the southern steppe zone and is represented by forests, shrubs, marshes and meadows. The flora of vascular plants includes 614 species (Darman, 2015). *Liparis* species have been studied in the forested part of the Protected Area.

In the Khingan State Nature Reserve, we conducted the research in the Antonovskoe forestry (surroundings of Lake Kleshinskoe) and in the Khinganskoe forestry (cordon Karapcha), and in the adjacent area, i.e. in the vicinity of Lake Dolgoe. Khingan State Nature Reserve is located at the southeastern edge of the Amur region, on the Khingan-Arkharinsk Lowland. According to Miskina (1971), the mountain area of the Protected Area (Khinganskoe forestry) belongs to the Far East broad-leaved landscape type, while its flat part (Antonovskoe forestry) belongs to the Far East forest-steppe landscape type.

In the study area, we surveyed all sites suitable for *Liparis* species using the route method. In the found populations, we measured morphological traits (which are significant for species determining) of plant individuals (Nevskiy, 1935; Vyshin, 1996; Shibneva, 2004, 2007, 2008; Efimov, 2010). We determined the plant height, length of inflorescences and ovaries, number of flowers, degree of the lip curling, length and width of leaf plate. Population-based studies have been carried out on the established transects (Rabotnov, 1950). Ontogenetic stages (age groups) of *Liparis* individuals were distinguished according to Tatarenko (1996) with our modifications. In the studied populations, we distinguished the following age groups: j – juvenile individuals; im – immature individuals; v – virginile (mature vegetative) individuals including non-flowering generative plants; g – generative individuals.

Since the known intraspecific variability in morphological traits of *Liparis* species does not allow a reliable identification of species in nature, we applied the molecular phylogenetic approach. Because all *Liparis* species are threatened, the collection of samples for the analysis was performed by harvesting only small parts of plants without digging the whole plant. For molecular-phylogenetic analysis, we used small parts of leaves from 32 samples of plant individuals originated from the studied *Liparis* populations. It was especially relevant for the populations where the species identification was almost impossible because generative individuals were absent, or in the stages of budding or fruiting. Some parts of vegetative and generative parts of plants were saved as herbarium vouchers with indication of the sampling locations (Electronic Supplement 1). In addition, the analysis included samples stored in herbariums (MW, LE, MAG, ABGI) and collected during 2015–2018 in the field trips through the Russian Far East (Primorsky Krai) (Electronic Supplement 1).



**Fig. 1.** Locations of *Liparis* species in the study area (Amur region, Russia). Designations: 1 – Blagoveshchenskiy district, 2 – Ivanovskiy district, 3 – Tambovskiy district, 4 – Arkharinskiy district; I – Muravyevskiy Park of the Sustainable Development; II – Khingan State Nature Reserve (a – Antonovskoe forestry, b – Khinganskoe forestry).

As a molecular marker in the molecular-phylogenetic analysis, we used internal transcribed spacers (ITS1–5.8S–ITS2) of 18S–26S nuclear ribosomal DNA. DNA extraction, amplification and sequencing the spacers ITS1–5.8S–ITS2 were conducted according to Valiejo-Roman et al. (2002). The obtained PCR products were sequenced on both strands. Then we investigated chromatograms of ITS1–5.8S–ITS2 region using software Chromas Lite v. 2.3, where for the analysed species we did not find double peaks and discrepancies through reads the nucleotide sequences from both forward and reverse primers.

We determined ITS sequences for 47 samples of the *Liparis* representatives (living plants collected in nature and herbarium specimens). To conduct the molecular-phylogenetic analysis, we additionally included 59 nucleotide ITS sequences of 22 (primarily closely related) *Liparis* species from the database GenBank (<https://www.ncbi.nlm.nih.gov/nucleotide/>). For this purpose, we used the results of other authors (Cameron, 2005; Tsutsumi et al., 2007; Chen et al., 2009; Lee et al., 2010; Li et al., 2013; Su et al., 2014, 2015). In addition, we included ITS sequences of three *Malaxis* species (*M. spicata* Sw., *M. brachypoda* Fernald, *M. monophyllos* (L.) Sw.), as this genus is closely related to the genus *Liparis*. In total, the data set of the molecular-phylogenetic analysis included 110 accessions from Russia, Korea, Japan, China, Hawaii Islands. The newly obtained nucleotide sequences

for the genus *Liparis* are annotated in the database GenBank (Electronic Supplement 1).

The nucleotide sequences of the analysed samples were aligned using the program MUSCLE (Edgar, 2004). The demonstration and manual adjustment of the aligned results were conducted using the program BioEdit v. 5.0.9. (Hall, 1999). The alignment of 110 nucleotide sequences of the ITS1–5.8S–ITS2 regions resulted in a matrix of 732 positions. Of them, 369 were constant, 254 parsimony informative, and 109 parsimony non-informative.

To construct the phylogenetic tree, we used the method of maximum parsimony realised using PAUP\* version 4.0b8 (Swofford, 2003). The heuristic search was used for finding the most parsimonious tree. The non-parametric bootstrap analysis (Felsenstein, 1985) was performed to assess the degree of support for particular clades (100 bootstrap replicates).

Bayesian analysis of molecular data was performed using the program MrBayes v. 3.2 (Ronquist et al., 2012). 5 000 000 generations were performed using Markov chain Monte Carlo. The number of simultaneously generated chains was four. A posterior probability of a tree node was calculated as the frequency of the corresponding node in the trees, which were obtained after the likelihood converged to equilibrium through the generation of Markov chains.

As an outgroup, we used *Dendrobium crumenatum* Sw., selected on the basis of research of Cameron (2005). Different loci obtained by two

methods are not similar, but congruent, in the main tree topologies. In the paper, we present a consensus phylogenetic tree constructed using Bayesian method (maximum parsimony tree is represented in Electronic Supplement 2: Fig. 1S).

## Results and Discussion

### *Morphological and population analysis*

Table 1 presents data on the morphological traits of three *Liparis* species according to the botanical literature (Nevskiy, 1935; Tatarenko, 1996; Vyshin, 1996; Shibneva, 2004, 2007, 2008; Efimov, 2010). Data for *Liparis* sp. were obtained by us during the field studies in the Amur region. Table 1 demonstrates that actually there are no clear traits allowing us to identify these *Liparis* species. All morphological traits are considerably variable. Their values could be overlapping for different species and depend on the habitat conditions.

### *Muravyevskiy Park of the Sustainable Development*

The orchid plants found in the forest of the Muravyevskiy Park of the Sustainable Development considerably differed from each other. On the basis of the morphology (Table 2), one of the specimens was identified as *Liparis kumokiri*, because values of its morphological traits (height is until 25 cm; there is no long petiole; relatively short inflorescence with yellow-greenish flowers, and narrow, strongly curled lip) were consistent with literature data (Shibneva, 2008, 2011; Efimov, 2010). Some individuals were named as *Liparis* sp., because their height, size, shape, denser texture of the leaf, and the form of the lip were different to other *Liparis* species. The populations of both species were located by dense groups being different in terms of abundance and area. At the same time, *L. kumokiri* occurs more commonly. In comparison with *L. kumokiri*, plants named as *Liparis* sp. had higher shoots, brownish or purple colouration of flowers, a higher number of flowers, ovary length, leaf plate size and darker colouration of leaves (Table 2).

Therefore, in the Muravyevskiy Park of the Sustainable Development, we found for the first time for

the Amur region the species *L. kumokiri* and plants treated by us as *Liparis* sp. We were going to test our assumptions using molecular-phylogenetic studies. *Liparis kumokiri* was noted on two sites of the Muravyevskiy Park of the Sustainable Development. The first of them is located in a planted pine (*Pinus koraiensis* Siebold & Zucc.) forb-grass forest (age of about 17 years) with sparse herb cover, located nearby administrative buildings. On this site, a small (three generative individuals) group of *L. kumokiri* was found. The main populations of both *Liparis* species have been noted on the second site, located 1 km from the first site. The first *L. kumokiri* micropopulation was located on the edge of pine (*Pinus koraiensis*) forbs grass forest and aspen (*Populus tremula* L.) forbs grass forest with 20% herb cover. This population covered an area of 8 m<sup>2</sup> with a density of 9.5 individuals per 1 m<sup>2</sup>. Its ontogenetic spectrum was represented by all age groups: 23.7% j + 18.4% im + 23.7% v + 34.2% g. Thus, this micropopulation was full-membered and normal (according to Rabotnov, 1950) with good reproduction due to young individuals (j + im) included 42.1% of all plants. In the sparse forbs dead-cover pine forest with 15% herb cover, we found other large micropopulations of *L. kumokiri* (more than 200 plants) and *Liparis* sp. (more than 100 plants). In these micropopulations, we found a good level of reproduction, i.e. 25% of young individuals for *L. kumokiri*, and 33.3% of young individuals for *Liparis* sp. The *Liparis kumokiri* density was 12.8 individuals per 1 m<sup>2</sup>, while *Liparis* sp. had 5.4 individuals per 1 m<sup>2</sup>. Therefore, all *Liparis* micropopulations are full-membered, sustainable and normal (according to Rabotnov, 1950), and the presented above parameters indicated a good vital status of them. During further studies in the Amur region, population of these two species were found at some locations in the Antonovskoe forestry and Khinganskoe forestry of the Khingan State Nature Reserve and in adjacent areas (Lake Dolgoe) (Arkharinskiy district). In addition, we registered them also outside Protected Areas, in some other districts of the Amur region (Ivanovskiy district and Blagoveshchenskiy district), and nearby the Botanical Garden in the town Blagoveshchensk (Fig. 1).

**Table 1.** Some morphological traits of generative individuals of *Liparis* species

Species	Height, cm	Inflorescence length, cm	Leaf length, cm	Leaf width, cm	Number of flowers	Leaf petiole	Flower colour
<i>Liparis japonica</i> *	25–35	until 20	until 10	5–7	9–25	8 cm, winged	greenish-yellow, rare brownish
<i>Liparis kumokiri</i> *	10–25	7	until 12	3–5	5–15	short, amplexicaul	greenish-yellow
<i>Liparis makinoana</i> *	15–25	10–17	6–8	3–5	20–25	short, amplexicaul	from greenish to purple
<i>Liparis</i> sp.	23–58	until 20	until 12	5–7	6–25	short, amplexicaul	from brownish to purple

Note: \* – literature data.

**Table 2.** Morphological traits for generative individuals of *Liparis* species in the Muravyevskiy Park of the Sustainable Development

Species	Traits						
	Height, cm		Number of flowers		Leaf size, cm		Ovary length, cm
	Average (n = 10)	min–max	Average (n = 10)	min–max	Leaf length	Leaf width	
<i>Liparis kumokiri</i>	18.60 ± 2.54	13–23	14.2 ± 2.2	9–20	10.20 ± 0.87	5.70 ± 0.74	0.70 ± 0.16
<i>Liparis</i> sp.	29.20 ± 2.64	23–36	20.5 ± 3.8	16–25	11.00 ± 1.10	5.30 ± 1.25	1.55 ± 0.22

### Khingan State Nature Reserve

Both species were registered on several sites in the vicinity of the Lake Kleshinskoe in the Antonovskoe forestry of the Khingan State Nature Reserve. Micropopulations were found in the sparse Siberian birch (*Betula platyphylla* Sukaczew (= *Betula pendula* subsp. *mandshurica* (Regel) Ashburner & McAll.) forbs forest, birch-oak (*Betula platyphylla*, *Quercus mongolica* Fisch. ex Ledeb.) forest with linden (*Tilia amurensis* Rupr.) undergrowth; willow (*Salix* sp.) thickets at the forest edge in the Borzi River valley, and other plant communities. Usually, *Liparis kumokiri* occurred in separate, sparse groups with a maximum density of 8.5 individuals per 1 m<sup>2</sup>. In the ontogenetic spectrum of micropopulations, young individuals often prevailed (j + im = until 54%). *Liparis* sp. has been found much rarer, in populations of which young individuals (j + im) reached until 17% in sum, while there were 50% of generative and 33% mature vegetative individuals (virginile and non-flowering generative individuals). A small *L. kumokiri* population, containing several low-number (1–7 individuals) groups, has been found nearby the Antonovskoe forestry of the Khingan State Nature Reserve in the Siberian birch (*Betula platyphylla*) forbs forest on the shore of Lake Dolgoe.

In the Khinganskoe forestry of the Khingan State Nature Reserve, we found two *Liparis* species inhabiting the low part of hillsides in the Karapcha River valley. The orchid populations were noted in linden (*Tilia amurensis*) sedge-forbs forest with a mixture of *Quercus mongolica* and *Acer pictum* subsp. *mono* (Maxim.) H. Ohashi with *Corylus heterophylla* Fisch. ex Trautv. undergrowth with 25–30% herb cover; in the *Acer pictum* subsp. *mono* forest with a mixture of *Quercus mongolica* and undergrowth of *Acer tegmentosum* Maxim. and *Acer pictum* subsp. *mono* with sparse herb cover containing species as *Hemerocallis middendorfii* Trautv. & C.A.Mey., *Carex* sp., *Phryma nana* Koidz., *Thalictrum aquilegifolium* var. *sibiricum* Regel & Tiling and others. As usual, we found small groups of *Liparis* individuals.

Sometimes, both species occurred in close proximity to each other. In addition, we noted that *Liparis* sp. prefers more moistured conditions than the other species. Its population (with a total number of 33 plants) is characterised by the predomination of mature individuals (45.5% g and 21.2% v), while young individuals were represented in sum about one-third of the total number of individuals. *Liparis kumokiri* often inhabited the more elevated sites at the middle parts of hillsides. The species grew sparsely, by single individuals or low-number groups (5–11 plants). The ratio of the mature (33.3% g and 16.7% v) and young (22.2% j and 27.8% im) individuals in a population was approximately equal. So, in both Antonovskoe forestry and Khinganskoe forestry of the Khingan State Nature Reserve, the populations of these species are full-membered, sustainable and normal (according to Rabotnov, 1950) having a good reproduction.

In addition, on the Khingan State Nature Reserve, we found *Liparis japonica*, which has not been previously known in the Amur region. Single individuals of this species were found in both forestries. In the Antonovskoe forestry, it was recorded in the vicinity of Lake Kleshinskoe, in sparse Siberian birch (*Betula platyphylla*) forbs forest, having 40% forest stand cover and 25% projective cover of the herb layer. Low-number groups of *L. japonica* were found in the Khinganskoe forestry (Fig. 1). At this location, the species inhabits broad-leaved forest with sparse herb cover on the hillside in the Karapcha River valley. *Liparis japonica* occurs considerably rarer than both previous species. Its populations are low in number, often non-full-membered; its reproduction was not found.

Table 3 presents morphological traits of all *Liparis* species found in the Khingan State Nature Reserve. We clearly see the differences in these data between species as well as between populations situated under different habitat conditions. For example, in the Khinganskoe forestry, the average height of generative individuals was more than in the Antonovskoe forestry. Perhaps, it is caused by higher values of soil moisture and richness.

**Table 3.** Morphological traits of generative individuals for the studied *Liparis* species in the Khingan State Nature Reserve

Species	Traits			
	Height, cm		Number of flowers	
	Average (n = 10)	min–max	Average (n = 10)	min–max
Antonovskoe forestry				
<i>Liparis kumokiri</i>	15.7 ± 2.75	8–23	6.75 ± 1.88	2–12
<i>Liparis</i> sp.	35.7 ± 7.9	23–42	12.1 ± 4.1	3–25
Khinganskoe forestry				
<i>Liparis kumokiri</i>	20.3 ± 2.67	16–25	6.7 ± 1.67	3–10
<i>Liparis japonica</i>	36 ± 2.5	31–40	15.8 ± 4.75	11–23
<i>Liparis</i> sp.	41.7 ± 9.9	23–58	14 ± 6.6	5–25

In addition, we found that the last year's upright old flowering stems of *L. kumokiri* with the remains of capsules remained. We did not note this for the other two *Liparis* species, perhaps, because they are characterised by higher peduncles, which fall and could not be found. The fruits' remains allowed us to conclude on the good level of the fruit productivity of *L. kumokiri*. According to Gap et al. (2001), the fruit productivity of this species is 86%, although it could be lower, until 10.2–12.2%. According to Varlygina et al. (2017), in the Kedrovaya Pad' State Nature Reserve, the fruit productivity of *Liparis* species varied from 30% to 60% for *L. japonica*, from 40% to 65% for *L. kumokiri*, and from 50% to 70% for *L. krameri*. This parameter considerably depends on the weather conditions and presence of pollinators.

We found *L. kumokiri* and *Liparis* sp. outside Protected Areas, too. In the vicinity of the village Ivanovka (Ivanovskiy district); planted pine (*Pinus koraiensis*) forbs forest with undergrowth of *Fraxinus mandshurica* Rupr. and *Betula platyphylla*, with *Malus baccata* (L.) Borkh. and *Rubus idaeus* subsp. *strigosus* (Michx.) Focke; forest stand cover is 50%; projective cover of the herb layer is 10–15%. The second found location was in the vicinity of the village Bogoslovka (Ivanovskiy district), in Siberian birch (*Betula platyphylla*) forbs forest with dense undergrowth from *Corylus heterophylla*; forest stand cover is 60%; the total projective cover of the herb layer is 10%. The third location was found on the gold slag disposals and in the oak-birch (*Quercus mongolica*, *Betula platyphylla*) forest in the Blagoveshchenskiy district. The fourth location was found in the forest of the town Blagoveshchensk, nearby the Amur Branch of the Botanical Garden-Institute of FEB RAS. The populations of these species were interspersed along the wide area.

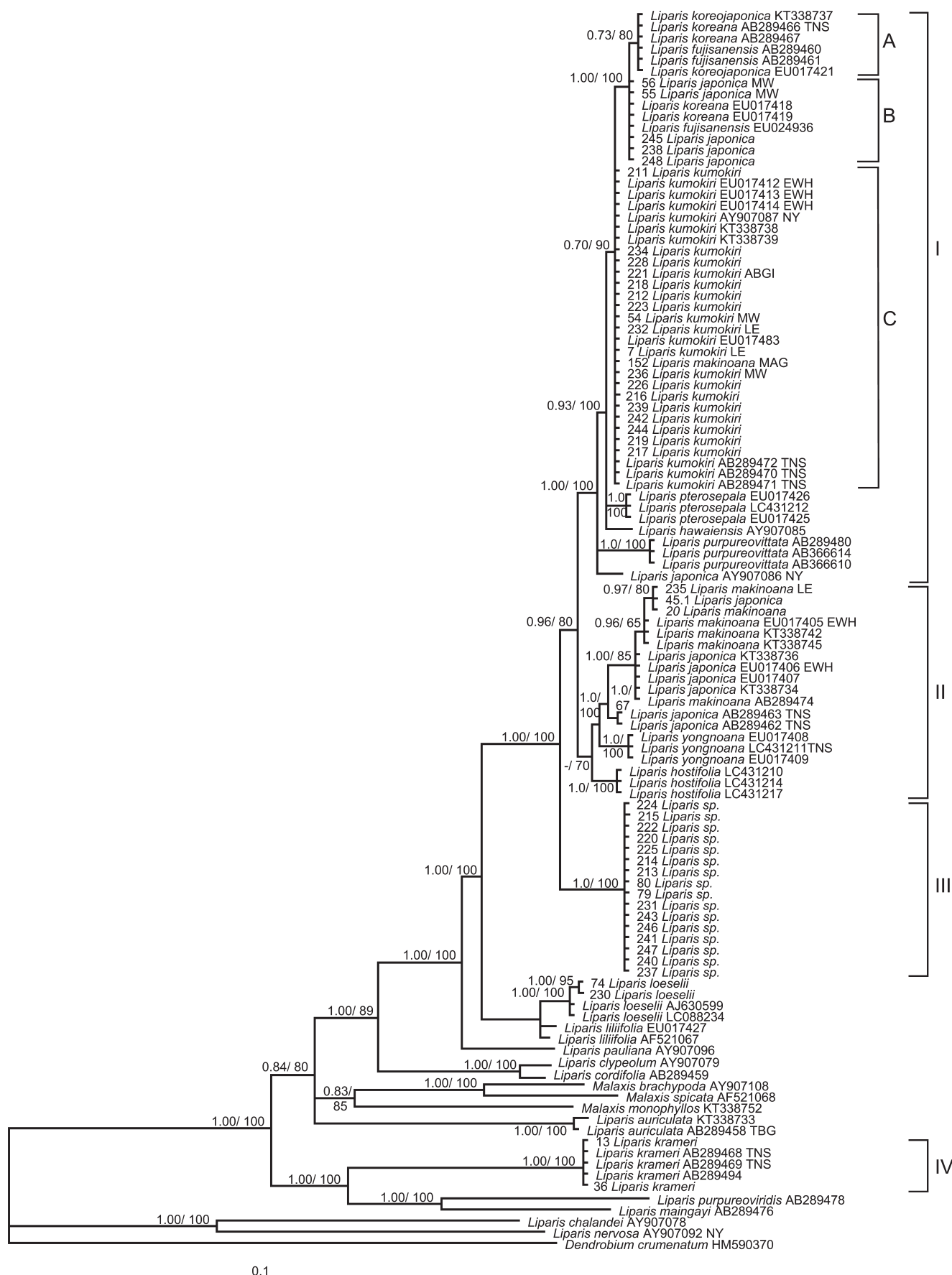
#### Molecular-phylogenetic analysis

In the obtained molecular-phylogenetic trees, the analysed samples of *Liparis* species formed four

highly supported clades (I, II, III, IV). This confirms their natural origin (Fig. 2).

Clade I consists of samples collected in the Muravyevskiy Park of the Sustainable Development, vicinities of town Blagoveshchensk, village Bogoslovka and village Ivanovka, in the Khingan State Nature Reserve (Antonovskoe and Khinganskoe forestries), in the vicinity of Lake Dolgoe, and, finally, samples of *L. kumokiri* and *L. japonica* extracted from herbarium collections (MW and LE). In addition, this clade includes taxa from the database GenBank: *L. kumokiri*, *L. koreana* (Nakai) Nakai, *L. fujisanensis* F. Kōta & S. Matsumoto, *L. hawaiiensis* H. Mann, and recently described species *L. pterosepala* N.S. Lee, C.S. Lee & K.S. Lee, *L. koreojaponica* Tsutsumi, T. Yukawa, N.S. Lee, C.S. Lee & M. Kato, and *L. purpureovittata* Tsutsumi, T. Yukawa & M. Kato, collected in Korea, Japan, and Hawaii Islands (Cameron, 2005; Tsutsumi et al., 2007, 2008a,b; Lee et al., 2010). Clade I is represented by a high number of difficult to distinguish species, which are characterised by polymorphism and blurred species boundaries. According to the analysis of ITS sequences, the samples collected in nature and originally identified as *L. kumokiri*, *L. japonica* and *L. makinoana* were located in different subclades of clade I, not always meeting their original identification on the basis of morphological traits.

Clade I consists of three subclades A, B and C. Inside the clade, all nodes have high values of bootstrap support and posterior probability. The species *L. pterosepala*, *L. hawaiiensis*, and *L. purpureovittata* occupy separate positions in the basal part of the clade that indicates the highest divergence level of these species compared to other representatives. In clade I, the divergence level between samples in ITS-sequences, expressed in *p*-distances, is low and equal to 0.0–1.7% indicating their closely relations and corresponding with other studies (Tsutsumi et al., 2007, 2008a,b; Lee et al., 2010; Su et al., 2015; Terentjeva et al., 2018).



**Fig. 2.** Phylogenetic relationship in *Liparis* as indicated by a majority-rule consensus tree of 5 000 000 trees from Bayesian analysis on the basis of nrDNA ITS1–5.8S–ITS2 dataset, using the TBR model. Numbers indicate node support (posterior probability values / maximum parsimony bootstrap values). Only bootstrap values > 50 and posterior probability values > 0.70 are shown.





Subclades C has a sister position with subclade B. Subclade C consists of samples collected in the Khingan State Nature Reserve (239, 242, 244), the Muravyevskiy Park of the Sustainable Development (211, 212, 217, 218, 219), in the vicinities of the town Blagoveshchensk (223), village Bogoslovka (234, 228), and samples of *L. kumokiri* from herbarium collections (MW, LE, EWH, NY). We selected samples of *L. kumokiri* from herbarium collections MW and LE on the basis of qualitative traits, which correspond to the species description accepted in Russian literature (Nevskiy, 1935; Vyshin, 1996). According to literature (Ohwi, 1965; Kim & Kim, 1986; Shibneva, 2004, 2007, 2008; Chen, 2009), values of morphological traits of *L. kumokiri* distributed in the south of the Russian Far East correspond to those in this species from Korea, Japan and China. Despite to some differences, our samples also correspond to the variation range in diagnostic traits of *L. kumokiri* confirming their presence in subclade C together with other samples from Russia and other countries.

Clade III is formed by samples of *L. makinoana*, *L. japonica*, *L. hostifolia* Koidz. & Nakai and *L. yongnoana* N.S. Lee, C.S. Lee & K.S. Lee from Japan, Korea and China (Genbank), herbarium specimen of *L. makinoana* (LE), plants collected by us in the Land of Leopard State Nature Reserve and Ussuri State Nature Reserve (Primorsky Krai, Russia) in 2015, and, finally, specimens collected in the Amur region in 2018. However, no one of the analysed samples from the Amur regions has been grouped with *L. makinoana*.

All samples of *Liparis* sp. from the Khingan State Nature Reserve (237, 240, 241, 243, 246, 247), Muravyevskiy Park of the Sustainable Development (213, 214, 215), vicinities of the town Blagoveshchensk (220, 227), village Ivanovka (222, 224), village Bogoslovka (225, 230, 231) with samples (79, 80) from Terneyskiy district and Lazovskiy district (Primorsky Krai, Russia) form a separate clade III with a high bootstrap support (100%) and a high posterior probability (1.0).

In the Terneyskiy district and Lazovskiy district, samples of *Liparis* (79, 80) were collected from fruiting plants, which made their identification difficult. Plants collected in the Amur region were flowering. But the set of their morphological traits (Table 1) did not allow us to identify them until the species level, either. The conducted molecular-phylogenetic analysis has demonstrated

that all samples in the clade III had identical ITS sequences with synapomorphic transitions (Table 4), which determine a position of taxa of this clade on the phylogenetic tree. Samples of clade III occupied a distant position from taxonomically closely related species *Liparis kumokiri*, *L. japonica*, *L. makinoana*, *L. koreojaponica*, *L. pterosepala*, *L. koreana*, *L. fujisanensis*, *L. hawaiiensis*, *L. hostifolia*, *L. purpureovittata*, and *L. yongnoana*, which were included into other clades.

The species *Liparis loeselii* (L.) Rich., *L. liifolia* (L.) A.Rich. & Lindl., *L. pauliana* Hand.-Mazz., *L. clypeolum* Lindl., *L. auriculata* Blume & Miq., *L. krameri* Franch. & Sav., *L. purpureoviridis* Burkill & Holttum, *L. maingayi* Ridl., *L. chalandei* Finet, *L. cordifolia* Hook.f., and *L. nervosa* (Thunb.) Lindl. occupy an isolated position or form separated clades in the basal part of the phylogenetic tree, which is consistent with both morphological and molecular-phylogenetic data of other studies (Cameron, 2005; Li & Yan, 2013; Su et al., 2014, 2015; Cheng et al., 2016).

Thus, the results of the molecular-phylogenetic analysis together with data of morphological studies allow us to assume that samples from *Liparis* population in clade III (so-called *Liparis* sp.) could have a species status, which requires additional investigations.

### Conclusions

According to the results of the molecular-phylogenetic analysis, *Liparis* samples collected in the Amur region and extracted from the database GenBank were arranged along the phylogenetic tree according to the species affinity. All closely related taxa are placed into sister clades and subclades which is in accordance with literature data.

The comparative-morphological studies with data of molecular-phylogenetic analysis allowed conducting taxonomic identification of *Liparis* plants collected in the Amur region. We have demonstrated the presence of *Liparis kumokiri* in the Amur region, as well as of presumably a new species, which we provisionally indicated as *Liparis* sp. A final conclusion for this issue requires further complex investigations.

We have confirmed the presence of *Liparis japonica* in the Khingan State Nature Reserve, and clarified the distribution of all found *Liparis* species in the Amur region. In addition, we revealed new locations of these species in the Muravyevskiy Park of the Sustainable Development, in the Antonovskoe and Khinganskoe

forests of the Khingan State Nature Reserve, in vicinities of Lake Dolgoe, village Ivanovka, village Bogoslovka, and nearby the town Blagoveshchensk. In most of the locations, the population status of the studied *Liparis* species was satisfactory. We noted a vital reproduction of the orchids. At the locations of *Liparis* populations, permanent study plots could be established for conducting long-term monitoring studies. The obtained data on the status of threatened plant populations will be used in the work on new editions of Red Data Books. On the studied sites of the Amur region, the presence of *Liparis makinoana* has not been confirmed by molecular-phylogenetic data.

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### Supporting Information

GenBank accession numbers and herbarium vouchers with indication of the sampling locations (Electronic Supplement 1: Voucher information and GenBank accession numbers for taxa used in this study), and a strict consensus tree derived using maximum parsimony analysis (Electronic Supplement 2: Phylogenetic relationship in *Liparis* as revealed by maximum parsimony analysis) may be found in the Supporting Information [here](#).

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## РЕВИЗИЯ И РАСПРОСТРАНЕНИЕ ВИДОВ РОДА *LIPARIS* (ORCHIDACEAE) В АМУРСКОЙ ОБЛАСТИ (РОССИЯ)

Е. И. Терентьева<sup>1</sup>, Т. И. Варлыгина<sup>1</sup>, Г. Ф. Дарман<sup>2</sup>,  
Г. В. Дегтярева<sup>1</sup>, С. В. Ефимов<sup>1</sup>, Т. Х. Самигуллин<sup>1</sup>

<sup>1</sup>Московский государственный университет имени М.В. Ломоносова, Россия  
e-mail: el.terenteva@mail.ru, tat-varlygina@yandex.ru, efimov-msu@yandex.ru

<sup>2</sup>Ботанический сад институт ДВО РАН, Россия  
e-mail: gfdarman@yandex.ru

В июле 2018 г. на территории государственного природного заповедника «Хинганский» и Муравьевского парка устойчивого развития было проведено обследование популяций видов рода *Liparis* и первичное определение растений. Род *Liparis* остается одним из наиболее сложных в систематическом отношении в семействе Orchidaceae. Наблюдения в природе, изучение гербарных сборов и литературных данных показывают значительную внутривидовую вариабельность морфологических признаков. Поэтому для оценки видовой принадлежности представителей природных популяций были взяты фрагменты растений для молекулярно-филогенетических исследований. Всего в анализ было включено 110 образцов. В качестве молекулярного маркера были выбраны ядерные последовательности межгенного участка (ITS1–5.8S–ITS2) транскрибируемого рибосомного оперона. На молекулярно-филогенетическом дереве образцы из исследуемых популяций формируют отдельные хорошо поддерживаемые клады, что подтверждает их естественность. ITS последовательности анализируемых растений каждой клады имеют группоспецифические замены, которые маркируют их положение на филогенетическом дереве. Анализ морфологических признаков в совокупности с молекулярными данными позволил провести видовую идентификацию растений из рода *Liparis*. На территории Амурской области, согласно флористическим сводкам, были отмечены два вида (*Liparis japonica* и *L. makinoana*). Изучение популяций *Liparis*, найденных в природе, а также результаты анализа морфологических и молекулярных данных показали, что на исследуемой территории, кроме *L. japonica* и *L. makinoana* произрастает *L. kumokiri*, а также еще один вид, возможно, являющийся новым для науки. Уточнено распространение этих видов на исследованных нами территориях Амурской области, а также выявлены их новые местонахождения. Состояние ценопопуляций изученных видов в большинстве мест обитания было удовлетворительным. Отмечено хорошее плодоношение и наличие возобновления. В местах их произрастания могут быть заложены пробные площади для проведения мониторинговых исследований. Полученные данные о состоянии популяций редких видов будут использованы при подготовке новых изданий красных книг. На обследованных нами территориях Амурской области наличие *Liparis makinoana* не подтверждено молекулярными данными.

**Ключевые слова:** Красная книга, молекулярные исследования, морфологические признаки, особо охраняемая природная территория, редкие виды, состояние ценопопуляций, филогения