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SHORT COMMUNICATIONS

Genetic Identification of Closely Related Endangered *Rhododendron* Species from East Asia

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Abstract—In the present study, two chloroplast DNA markers (*trn*H-*psb*A, TabCD) were used to analyze genetic differentiation of six populations of *Rhododendron adamsii* from Sakhalin, Yakutia, Buryatia, Western Sayan, and Magadan oblast and two populations of *Rhododendron parvifolium* from Buryatia and Khabarovsk krai. A total of six haplotypes were found. While *R. parvifolium* with a single unique haplotype did not possess any genetic variability, *R. adamsii* exhibited a geographically determined distribution of cpDNA haplotypes. The samples from Buryatia were grouped into one homogeneous clade and the samples from the Far Eastern part of the range were grouped into another, showing a moderate genetic diversity within populations. Analysis of molecular variance (AMOVA) detected high genetic differentiation among *R. adamsii* populations (F_{ST} = 0.98, $p < 0.001$). GenBank sequences of closely related species were obtained for the estimation of the phylogenetic relationships of these species. We suggest that *trn*H-*psb*A and TabCD markers may be applicable for the species identification and for the elucidation of the population structure of rare *R. adamsii*.

Keywords: genetic differentiation, population structure, cpDNA, *Rhododendron adamsii*, *Rhododendron parvifolium*, *trn*H-*psb*A, TabCD

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The dynamics of the composition of plant communities and areas of individual plant species is determined by various factors, including human economic activity, climatic fluctuations, and specific requirements of species for habitat conditions. All this is reflected in the genetic diversity of populations—the basis of their evolutionary potential and stability. Thus, effective strategies for the assessment of biological diversity and the conservation of rare species should be based on knowledge of their genetic structure. This is especially true for medicinal plants. Even being included in the Red Book register, they are under the threat of population decline because of haphazard procurement of raw materials. The development of species-specific genetic markers is a tool that makes it possible not only to identify a plant and to determine the place of collection of a rare species in the event of illegal harvesting but also to study its genetic structure throughout its range.

Rhododendron adamsii Rehd. is a low-growing evergreen shrub that forms thickets in the alpine and subalpine belts at an elevation of 1200–2500 m above sea level in the mountains of Eastern Siberia and the Far East, as well as Mongolia [1]. The species is listed in the red books in all growing regions [2]. This plant is widely used in Indo-Tibetan, Mongolian, and Buryat medicine. Currently *R. adamsii* is not included in the official list of pharmacopoeial plants in Russia; however, the species is considered promising for introduction into medical practice and is being actively studied from a biochemical point of view [3–5]. Uncontrolled collection as food and medicinal raw materials, confinement to special growing conditions (facultative calcephilia), and slow growth [1]—all this threatens the existence of some populations of the species. A special anthropogenic press turns out to be in the Baikal regions, where harvesting is carried out for commercial purposes.

Few works have been devoted to the morphological variability of the species [3, 6], and population genetic studies have not been carried out at all. Obviously, it is necessary to develop markers for the purposes of environmental monitoring, as well as allowing for effective identification of raw materials: to distinguish it from a closely related morphologically similar species *R. parvifolium*, with which it often coexists in the same habitats and even forms hybrids (×*R. burjaticum*) [1, 7]. In addition to practical application, knowledge about

Table 1. Geographic coordinates of collection sites and identified cpDNA haplotypes in the studied samples of *R. adamsii* and *R. parvifolium*

	Populations	Coordinates, N/E	Number of individuals	Haplotype (*N*)
1	*R. adamsii*. Magadan oblast (specimens from the herbarium with the MAG index)	–	12	h1 (11) h3 (1)
2	*R. adamsii*. Sakhalin oblast, Smirnykhovsky district, Mount Vaida	49°52′/143°27′	10	h4 (10)
3	*R. adamsii*. Sakha Republic (Yakutia), Ust-Maisky district, village of Ust-Yudoma, Ichene Mountain	59°24′/135°06′	9	h1 (8) h2 (1)
4	*R. adamsii*. Buryatia, Tunkinsky district, outskirts of village of Arshan	51°55′/102°24′	4	h5 (4)
5	*R. adamsii*. Buryatia, Tunkinsky district, outskirts of village of Mondy	51°44′/101°04′	9	h5 (9)
6	*R. adamsii*. Krasnoyarsk krai, Ermakovsky district, Mount Tushkanchik (Western Sayan)	52°49′/93°23′	6	h5 (6)
7	*R. parvifolium*. Khabarovsk krai, Nanaysky district, southern slope of Tordoki-Yani	48°52′/138°02′	11	h6 (11)
8	*R. parvifolium*. Buryatia, Tunkinsky district, outskirts of village of Mondy	51°44′/101°04′	12	h6 (12)

the genetic structure of the species is of fundamental interest aimed at identifying the Central Asian floristic relationships of the high-mountain flora of Eastern Siberia. Species closely related to *R. adamsii* grow in the Himalayas (*R. anthopogon* D. Don), in Afghanistan (*R. colletianum* Aith. et Hemsl.), and in Central China (*R. anthopogonoides* Maxim., *R. cephalanthum* Franch.) [1].

The purpose of this work is to study the level of genetic diversity and population genetic structure of *R. adamsii*, as well as to identify a marker suitable for the identification of raw materials.

We analyzed 50 plants of *Rhododendron adamsii* from natural populations from the territory of the Western Sayan, Buryatia, Yakutia, Magadan oblast, and Sakhalin Island, as well as 23 *Rhododendron parvifolium* plants from Buryatia and Khabarovsk krai (Table 1). DNA was isolated according to the standard protocol for plant tissues (CTAB method) [8] from leaves dried in silica gel. DNA of the samples from Magadan oblast was isolated from herbarium specimens of the Institute of Biological Problems of the North (herbarium index MAG, numbers 0009893, 0009891, 0009902, 0009897, 0009898, 0009909, 0009903, 0009904, 0009905, 009910, 0009915, 0009920). At the first stage, amplification was carried out on several specimens with universal primers for nine marker chloroplast fragments. According to the protocol and temperature profile of PCR recommended by the authors, we carried out amplification of some of the fragments *trn*K–*mat*K [9] and *psb*C–*trn*S [10]; three sites *trn*T–*trn*F (TabAB, TabCD, TabEF) [11]; *trn*F-*Trn*V [12]; *trn*H-*psb*A and *trn*S-*trn*G [13]; and *atp*H-*atp*I [14]. For fragments with stable amplification by sequencing on an ABI 3130 genetic analyzer (Applied Biosystems, United States), the sequences were determined and variable sites were identified. For the analysis of all material, marker fragments were selected in which more than three variable sites were found: *trn*H-*psb*A and TabCD. The sequences were aligned manually with the BioEdit software [15]. The calculation of the level of differentiation and the distribution of genetic variability within and between populations (analysis of molecular variance, AMOVA) was performed using the Arlequin v. 3.5.1.2 program [16]. Maximum parsimony network of cpDNA haplotypes was constructed in Network v. 4.6.1.2 [17]. Each mutation (mononucleotide substitution or indel, regardless of size) was coded as a single mutational event. The phylogenetic tree for all samples was constructed using Bayesian analysis in MrBayes v. 3.1.2 [18] on the basis of the model of nucleotide substitutions GTR + G + I. To construct the tree, we included data from the GenBank for closely related species: *trn*H-*psb*A intergenic spacer of *R. anthopogon* (GenBank numbers KM605665.1, KM605664.1, KM605603.1) and *R. cephalantum* (GenBank numbers JN046777.1, JN046776.1, KM605536.1, JN046775.1); TabCD intron of *R. lapponicum* (GenBank numbers GQ245396.1, DQ860601.1). A specimen of the subgenus Terorhodion was used as an outgroup—*Rhododednron camtschaticum*.

The length of the combined sequence of two fragments was 755 bp (TabCD 1–387; *trn*H-*psb*A 388–755). Ten variable sites were identified (Table 2); there were three mononucleotide substitutions and two microsatellites in the fragment TabCD and four mononucleotide substitutions and one indel in the fragment *trn*H-*psb*A. The variability was grouped into

Table 2. Segregating sites for seven cpDNA haplotypes. Reference haplotype h1

Haplotype	Nucleotide position									
	*trn*L-*trn*F (TabCD)					*trn*H-*psb*A				
	54	68	80	151	258	515	548	542–557	622	702
h1	T	A	A	C	–	A	T	–	T	T
h2	T	A	A	C	–	A	G	–	T	T
h3	G	–	A	C	–	A	G	–	T	T
h4	T	A	A	C	–	A	T	–	T	C
h5	G	–	A	C	T	A	G	–	T	T
h6	T	–	G	T	–	C	–	a	A	T

a—GAAAGGTATATAAATT.

six haplotypes (h1–h6). The sequences of fragments for each haplotype are deposited in GenBank with accession numbers MW888399–MW888409. GenBank samples are coded as haplotype h8 for *R. lapponicum* and h9–h11 for *R. cephalantum* and *R. anthopogon*.

The distribution of haplotypes within the samples is shown in Fig. 1b. In samples of *R. parvifolium* there is one species-specific haplotype h6. In *R. adamsii* five haplotypes were discovered and their distribution is structured geographically. Haplotype h1 is widespread in the east of the range in samples from Magadan oblast and Yakutia. Rare haplotypes h2 and h3 were also identified there. In the sample from the central part of Sakhalin the h4 haplotype was identified. Haplotype h5 was recorded in the western part of the range on the territory of Buryatia. To determine the relationships within the genus the studied samples were grouped with those from GenBank. The statistical parsimony network of cpDNA haplotypes (Fig. 1a) resulted in two haplogroups corresponding to the two sections. *R. parvifolium* and *R. lapponicum* (h6, h8) belong to the Rhododendron section. *R. adamsii*, *R. anthopogon*, and *R. cephalantum* (h1–h5, h9–h11) belong to the Pogonanthum section [19]. In the Bayesian tree (Fig. 1c), specimens from different populations are grouped according to Rhododendron sections and well-supported (*PP*-value = 1.00). Within the clade of the Pogonathum section, the subclads are less-supported (*PP*-value = 0.72–0.93), but *R. adamsii* haplotypes have a distinct regional structure. According to the AMOVA results, the variation among species *R. parvifolium* and *R. adamsii* was $F_{ST} = 0.985$ ($P < 0.0001$), within *R. adamsii* populations $F_{ST} = 0.923$ ($P < 0.0001$), and even higher taking into account the relationship of haplotypes, $F_{ST} = 0.961$ ($P < 0.0001$).

Thus, the observed cpDNA variation corresponds to the two described species. Earlier for *R. parvifolium* and *R. adamsii* no cpDNA variation was revealed using the internal transcribed spacer (ITS) nuclear marker [20], which is widely used for species identification. The chloroplast DNA markers that we used occured to be more efficient. Each fragment could be successfully applied to define the species of *R. parvifolium* and *R. adamsii* by PCR diagnostics. Although these species have distinct morphological features (the most striking of which are inflorescences)—from 5–10 funnel-bell-shaped flowers with a pronounced tubular part in *R. adamsii* and from 2–5 wide-bell-shaped flowers in *R. parvifolium*—the color of the corolla in both species varies from white to pink and, in general, they have a similar appearance [1, 3, 21, 22]. The morphological similarity, the ability to cross, and the genetic affinity for the ITS marker put in question the legitimacy of the separation of these species into different subsections [23]. Upon species identification in the absence of generative organs, such as dried leaves, representing food raw materials, or in a herbarium without flowers, they can often be confused. Although there is a well-known work on determining the authenticity of raw materials on the basis of the anatomical features of leaves and histochemical reaction to the localization of active substances [3]. Diagnostics using a genetic marker is the simplest and most optimal solution to the problem of species identification.

The use of both fragments makes it possible, with a high probability, to clarify the growing area of *R. adamsii*. According to our data, the population-genetic structure of this species represents two clusters (Fig. 1): monomorphic western and more diverse eastern. Differences between the western and eastern parts of the range are also observed according to the data on morphological variability. The linear parameters of leaf blades and petioles in the population from Tuva (western part of the range) differed in a low level of variability compared to the population from Yakutia (eastern part of the range); specimens from these populations had a clear division not only by the linear dimensions of the leaf but also by the color of the corolla [21].

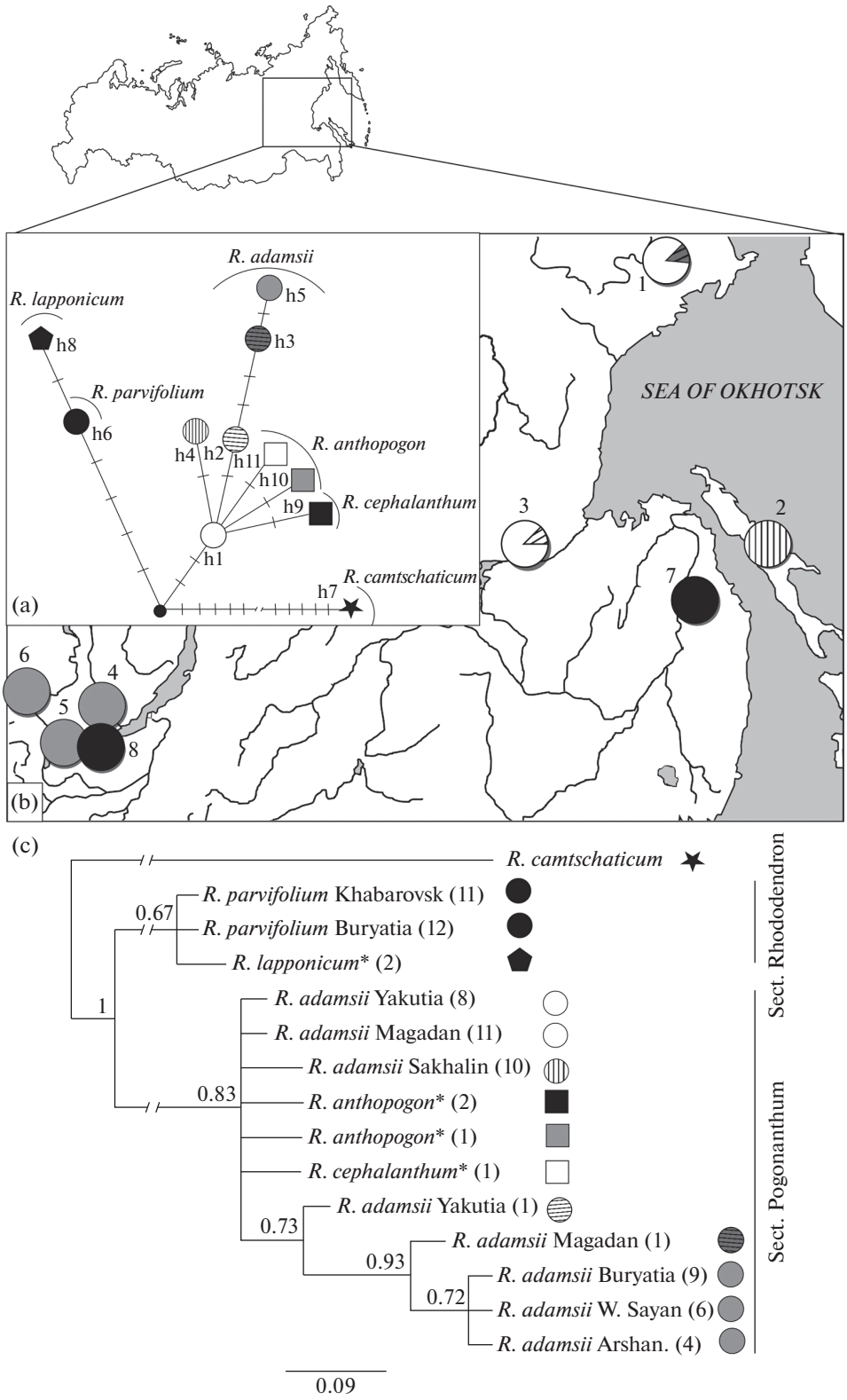

Fig. 1. (a) Population codes follow Table 1. (b) (c) Phylogenetic tree of the studied samples. (*) Samples mark the sequences of GenBank accessions; the frequency of the haplotype within the sample is indicated in parentheses.

Comparison of the studied species with closely related species according to data from GenBank deserves special discussion. *R. parvifolium* and *R. lapponicum* are often indicated as synonyms [1, 24]. These species are poorly distinguishable morphologically and, rather, stand out solely because of geographical remoteness. In a broad sense, *Rhododendron parvifolium* has an extensive range, covering, not only Siberia and the Far East but also Scandinavia, Greenland and North America [24]. Obviously, in such a vast area, it is unlikely to be homogeneous, and the question of its genetic structure and taxonomic division remains open. In our study, no differences were found between the samples from Buryatia and Khabarovsk krai; two samples from GenBank originating from Norway also did not differ from each other, but differed from our samples by one mutation in the TabCD fragment.

For similar morphology, specimens of *R. adamsii* from Siberia were originally classified as *R. anthopogon* growing in the Himalayas, which was later found to be erroneous [26]. Comparison of our samples with several sequences *trn*H-*psb*A of two closely related species—*R. anthopogon* and *R. cephalantum*—indicates their close relationship. These species were not previously compared by genetic markers. In the phylogenetic tree (Fig. 1c) they form branches with supports at the level of intraspecific variability. On the one hand, this may be due to uneven sampling, since even if several specimens from GenBank had different haplotypes, then a comparison of full-size samples would most likely reveal a clearer structure. On the other hand, the reason may be that this type of marker has insufficient resolution within the Pogonathum section.

The obtained molecular genetic data can be used to develop programs for monitoring and preserving these rare species, as well as to develop measures for the rational use of natural resources. Chloroplast DNA fragments *trn*H-*psb*A and TabCD are suitable for distinguishing species *R. adamsii* and *R. parvifolium* when it is difficult to do this by morphological characteristics. To identify the clearest genetic structure within the western part of the range *R. adamsii*, where anthropogenic pressure is expressed, further research is needed using multilocus genetic analysis. The relationship of *R. adamsii* to other species (*R. anthopogon* and *R. cephalantum*) of section Pogonanthum is confirmed.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest.

This article does not contain any research using animals or people as objects of research.

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