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Article in *Botanica Pacifica* · January 2022

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Genetic diversity of *Rhododendron redowskianum* Maxim., a rare species of Siberian and Far Eastern rhododendrons, based on plastid and nuclear DNA markers

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Manuscript received: 27.09.2022

Review completed: 16.10.2022

Accepted for publication: 20.00.2022

Published online: 21.10.2022

ABSTRACT

The genetic diversity of 78 individuals of *Rhododendron redowskianum* Maxim. from five natural populations of different territories of the Far East – Magadan Region, Sakhalin Region, Primorye Territory and Northeast China was studied. Variability of the species according to five markers of plastid DNA was very low. The only one mutation was detected in one of the markers in a single individual from Sikhote-Alin population. Analysis of the polymorphism of eight nuclear microsatellites, on the contrary, revealed a high level of diversity in all samples (mean values: $A_a = 6.44$, $A_e = 4.43$, $H_o = 0.514$, $H_e = 0.523$) and high interpopulation differentiation ($F_{ST} = 0.299$, $p < 0.001$). Population structure analysis identified four genetic clusters that correspond to the four geographic areas represented. Relationship analysis based on plastid DNA data revealed the ancestral position of *R. redowskianum* in relation to the closely related species *R. camtschaticum* Pall. s.l.

Keywords: Far East, plastid DNA, genetic variability, nuclear microsatellites, population structure

РЕЗЮМЕ

Полежаева М.А., Модоров М.В., Мочалова О.А., Хорева М.Г., Колдаева М.Н., Марчук Е.А. Генетическое разнообразие редкого вида рододендрона Сибири и Дальнего Востока *Rhododendron redowskianum* Maxim. по маркерам хлоропластной и ядерной ДНК. Изучено генетическое разнообразие 78 образцов редкого вида *Rhododendron redowskianum* Maxim. из пяти природных популяций различных регионов Дальнего Востока – Магаданской и Сахалинской областей, Приморского края и северо-восточного Китая. Изменчивость вида по пяти маркерным фрагментам плазмидной ДНК оказалась очень низкой. По одному из маркеров выявлена единичная мутация в одном образце из Сихотэ-Алиня. Анализ полиморфизма восьми ядерных микросателлитов, напротив, показал высокий уровень разнообразия во всех выборках (средние значения числа аллелей $A_a=6.44$, эффективного числа аллелей $A_e=4.43$, наблюдаемой и ожидаемой гетерозиготности $H_o=0.514$, $H_e=0.523$, соответственно) и высокую межпопуляционную дифференциацию ($F_{ST} = 0.299$, $p < 0.001$). Анализ популяционной структуры выявил четыре генетических кластера, которые соответствуют четырем представленным географическим районам. Анализ родства, проведенный по данным хлоропластной ДНК, выявил сестринское положение *R. redowskianum* по отношению к близкородственному виду *R. camtschaticum* Pall. s.l.

Ключевые слова: генетическая изменчивость, Дальний Восток, популяционная структура, плазмидная ДНК, ядерные микросателлиты

The problem of protecting biodiversity has received a lot of attention in recent decades. Among endangered plants, species with medicinal and ornamental properties are most often found. A special position is held by species with relict and endemic habitats, as well as with the sporadic nature of distribution. Rare distribution can be caused by the loss of suitable habitats due to natural historical processes, including changing climate, as well as due to anthropogenic and technogenic transformation. All representatives of the genus *Rhododendron* have ornamental and pharmacological properties, many of them have narrow ranges, so it is not surprising that in 2003 the world's "The Red List of Rhododendrons" was created, which has undergone more than 8 reprints (Gibbs et al. 2011). All

species of rhododendrons of Russia, about 12–16 species (Aleksandrova 1975), are also included in the protection lists of federal and regional levels. The least studied of them is *Rhododendron redowskianum* Maxim. It is one of the smallest rhododendrons, nevertheless, having wide distribution area in Eastern Siberia and sporadic distribution in the Far East. *R. redowskianum* is a deciduous shrub 8–20 cm tall, with leaves up to 2 cm long and a corolla about 1.5–2 cm in diameter. As a rule, it grows in small thickets and is confined to mountain ridges, damp stony screes, and mountain tundras. Its range includes the mountains of the Primorye, Sakhalin, Magadan Regions, Transbaikalia and Khabarovsk Territories, Sakha-Yakutia Republic, not reaching Chukotka. Outside Russia, it occurs in China and

the Korean Peninsula (Aleksandrova 1975). The species is included in the Red Data Books of the Republics of Buryatia and Sakha-Yakutia, Transbaikalia Territory, Irkutsk and Sakhalin Regions (Plantarium 2007–2022). It is poorly studied: mainly the information related to the study of onto- and morphogenesis (Mazurenko 1980) and introduction of the species into culture (Petukhova 2005) was found. Studies of pollen shape (Sarwar & Takahashi 2013), seed surface (Wang et al. 2007a), leaf epidermal features (Wang et al. 2007b), as well as chromosome number, and analysis of some molecular markers (Gao et al. 2002, Goetsch et al. 2005) all support the separate position of the *Therorhodion* section, to which *R. redowskianum* and *R. camtschaticum* Pall. s.l. belong. This small section periodically changes its status to genus, but the combination *Therorhodion redowskianum* Maxim. is rarely found in both Russian and foreign literature. Along with classical methods for accounting and monitoring of biodiversity, methods of analyzing the population structure of species by means of molecular genetic markers have been actively involved in recent decades.

The aim of this work was to characterize the level of genetic variability of *R. redowskianum* in the Far East region, as well as to clarify its phylogenetic relationships with closely related *R. camtschaticum* s.l. For this purpose, the variability of five plastid DNA loci and ten nuclear microsatellite loci was analyzed in 78 specimens of *R. redowskianum* from the natural populations from the territory of the Magadan Region (Okhotsk-Kolyma watershed), Sakhalin Region,

Primorye Territory (Sikhote-Alin) and China (Changbai Mountains) (Table 1, Fig. 1A).

MATERIAL AND METHODS

Plant material (leaves) was collected from five populations in the natural habitat of the species. The places of collection and number of samples are shown in the Table 1. DNA was extracted by the CTAB method (Devey et al. 1996) from silica gel-dried leaves. Amplification of plastid intergenic spacers DNA was performed according to the protocol and PCR temperature profile recommended by the authors for five marker sites: *trnK-matK* (Johnson & Soltis 1995); *trnF-trnV* (Dumolin-Lapegue et al. 1997); *trnH-psbA*, *trnS-trnG* (Hamilton 1999), and one part (TabCD) of the *trnT-trnF* spacer (Taberlet et al. 1991). The sequences of these fragments for six individuals from each population were obtained by NANOFOR 05 genetic analyzer (Syntol, Russia). Sequence alignment and accounting for variant sites were performed using BioEdit software (Hall 1999). The obtained fragment sequences were placed in GenBank under accession numbers (ON508844 – ON508850). Construction of the phylogenetic tree using sequences of plastid DNA regions that we sequenced now for *R. redowskianum* and earlier for *R. camtschaticum* and *R. glandulosum* Standl. ex Small (Polezhaeva et al. 2020), GenBank numbers MN125545 – 125551, was performed using Mr. Bayes software (Ronquist & Huelsenbeck 2003). For the primary selection of nuclear microsatellite markers, amplification

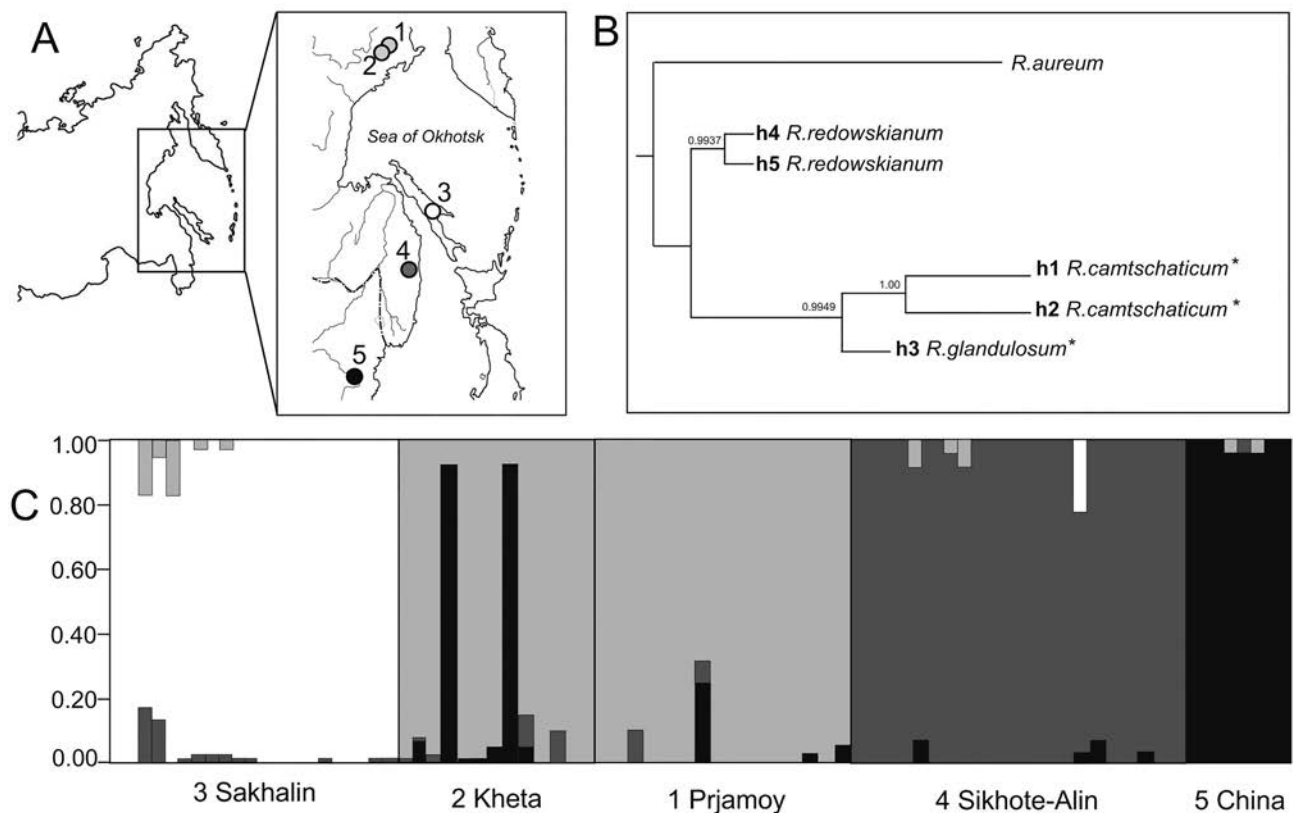


Figure 1 Geographic location of the studied populations of *Rhododendron redowskianum* Maxim. (A); numbers correspond to the Table 1; color of the circle corresponds to the color of the genetic cluster see part of figure "C". B – phylogenetic tree of the individuals, constructed on the basis of variability of plastid DNA markers in the MrBayes prigram. (*) marked specimens for which the sequences were taken from Genebank. C – the probability of assigning 78 individuals to one of the four clusters on the basis of variability of nuclear microsatellites in the "admixture" analysis in the STRUCTURE program

with primers developed for other species, *R. aureum* Georgi (Kwak et al. 2015) and *R. ferrugineum* L. (Charrier et al. 2014), was performed with polymerase chain reaction conditions described by the authors. Ten loci showed stable amplification: RA_10, RA_14, RA_137, RA_138, RA_148; RF_6P, RF_14, RF_46, RF_75, RF_113. The lengths of the amplified fragments were determined on a NANOFOR 05 genetic analyzer (Syntol, Russia) in the presence of molecular weight marker S-450 (Gordiz, Russia). Chromatograms were interpreted in GeneMapper v. 4.0. Parameters of genetic diversity and interpopulation differentiation, as well as population structure were estimated using the Principal Coordinate Analysis (PCoA) based on paired Nei's distances in the GenAlEx 6.5 program (Peakall & Smouse 2006). Phylogenetic analysis by Bayesian clustering was performed in Structure 2.2 (Pritchard et al. 2000). The algorithm was applied in five replicates for each of the K values from 2 to 4 using the admixture model (which takes into account the probable mixed origin of samples under the condition of independence of allele frequencies between clusters) with the number of iterations of 100,000. The optimal number of groups was selected using the STRUCTURE harvester program (Earl & von Holdt 2012).

RESULTS

The variability of the plastid DNA markers was very low: when five marker fragments were sequenced in six individuals from each population, only one, *trnH-psbA*, showed a ten base pairs deletion in one individual from the population from the Primorye Territory. This fragment was sequenced in all 78 individuals, but the deletion was detected only in this one. No polymorphism was detected at two nuclear microsatellite loci – RF_46 and RF_6P, so they were excluded from the further analysis. The polymorphism of the remaining eight loci in the populations ranged from 60 to 80%. The number of alleles detected at the loci in all populations varies from 2 to 39: locus RA_10 revealed 17 alleles, RA_14-33 alleles, RA_137-10 alleles, RA_138-19 alleles, RA_148-28 alleles, RF_14-2 alleles, RF_75-39 alleles, RF_113-6 alleles. The indices of genetic diversity are presented in the Table 1. The mean number of alleles per locus (Aa) varies in the populations from 3.2 to 8.2, the effective number of alleles (Ae) from 2.27 to 6.10. The values of observed (Ho) and expected heterozygosity (He)

ranged from 0.386 to 0.615 and from 0.372 to 0.628, respectively. The mean values of these indices are: Aa = 6.44, Ae = 4.43, Ho = 0.514, He = 0.523. The average value of the fixation index F is 0.034, which indicates a small deficiency of heterozygous genotypes. A large number of unique haplotypes are observed in all samples: from 4 to 20.

The genetic differentiation between the analyzed populations was about 30 % ($F_{ST} = 0.299$, $p < 0.001$). The main contribution to the interpopulation differentiation is made by loci RA_138 and RA_137 (32.4 and 47.2 %, respectively). The genetic distances D Nei (Nei 1972) between populations ranged from 0.062 to 0.571. Based on their values, the population structure of the species was analyzed by the PCoA. On the ordination populations are located according to geographical position: populations from China and Sakhalin Island are considerably removed from Magadan Region population, a little less distanced population from Primorye Territory population. The first axis accounts for 42.49 % of the variability, the second accounts for 32.54 % (figure is not shown). Similar results were obtained using the Bayesian algorithm. The maximum value of the logarithm of the posterior probability was obtained for four groups (K = 4). This means that the total sample can be divided into four genetic clusters (Fig. 1B): two populations from the Magadan Region are assigned to one of them; the remaining populations represent independent genetic clusters. In several individuals, mixed ancestry was revealed.

DISCUSSION

When analyzing the genetic diversity of *R. redowskianum*, nuclear microsatellites were much more effective than plastid DNA markers. The level of variability of the former is high and comparable with that of other representatives of the genus distributed in the Far East territory. For example, in the alpine species, *R. aureum*, the number of alleles per locus varied from 4 to 29, the average diversity values were Aa = 5.270; Ae = 3.37; He = 0.615; Ho = 0.595 $F_{IS} = 0.037$ (Polezhaeva et al. 2021). Similar values were found in rhododendrons common in China, *R. decorum*, *R. ovatum*, and *R. simsii* (Wang et al. 2013, Tan et al. 2009). Along with high allelic diversity, there is high interpopulation differentiation ($F_{ST} = 0.299$). Although this species, like all rhododendrons, has small seeds that are easily dispersed by the wind, it does not form mass thickets, grows sporadically, and begins to

Table 1. Geographic coordinates of collection sites and indices of genetic diversity of nuclear microsatellites in the studied samples of *Rhododendron redowskianum*

Population	Coordinates	N	Aa	Ae	Ap	Ho	He	Fis
1. Magadan Region, Khasynsky District, in the Kheta River	61°36'N 152°09'E	13	7.7	5.82	10	0.615	0.628	0.033
2. Magadan region, Khasynsky district, Creek Prjamoy	61°55'N 151°25'E	17	8.2	6.10	20	0.606	0.609	0.021
3. Sakhalin Region, Smirnykh District, Vaida Mountain	49°30'N/142°29'E	19	6.1	3.53	18	0.411	0.461	0.150
4. Primorye Territory, Sikhote-Alin, Krasnoarmeisky District, Ozernoye Plateau	45°28'N/136°21'E	22	7.0	4.42	14	0.555	0.544	0.038
5. China, Changbai–Korean Autonomous District, Changbaek-Sanjulgi Mountain	41°45'N/127°57'E	7	3.2	2.27	5	0.386	0.372	-0.090
Mean		15.6	6.44	4.43	13.4	0.514	0.523	0.034

N – total number of individuals; mean indices for 8 loci in the population: Aa – number of alleles; Ae – effective number of alleles; Ap – number of unique alleles; Ho – observed heterozygosity; He – expected heterozygosity; Fis – inbreeding coefficient

flower and bear fruit in nature according to various data by the 10–40th year of life (Mazurenko 1980), which obviously complicates the interpopulation genetic flow. In this study, the samples represented rather distant geographically, so this level of differentiation is natural. During clustering, the program interprets some individuals as having a mixed genetic nature: thus, in the populations from the Magadan Region, three individuals with a high degree of probability are attributed to the China cluster. This can be explained by the fact that a larger population sample made it possible to capture the ancestral polymorphism, since the center of species diversity and dispersal of rhododendrons is considered the southern regions of Asia (Irving & Hebda 1993). A higher level of heterozygosity is typical of populations from the Magadan Region, where *R. redowskianum* is more abundant. Slightly less heterozygosity is in population from the Primorye Territory and Sakhalin (Table 1). The decrease in the level of variability in China, cannot be interpreted clearly because of the small population sample size. The low genetic diversity of plastid DNA markers with high diversity of nuclear microsatellites may be the result of a decrease in the number of the species in the distant past followed by the long-term existence of populations without sharp fluctuations in abundance. A high number of unique alleles in the population samples confirm this assumption. This phenomenon is related to the effective population size for markers having uniparental and biparental types of inheritance and to their mutation rate. The probability of a decrease in the variability for slowly mutating plastid DNA markers having maternal inheritance and spreading with seeds is much higher when the population size decreases. The effective population size for biparentally inherited rapidly mutant nuclear microsatellites is twice as large, hence, the probability of diversity conservation is higher.

Of particular interest is the question of the origin of *R. redowskianum*. In the monograph "Flora of Suntar-Khayata", Yurtsev (1968) suggested the origin of *R. redowskianum* as a derivative of the oceanic mountainous species *R. camtschaticum*. He suppose that its isolation was related to the increasing continental climate of the highlands remote from the sea, and possibly to the glaciation of coastal mountain ranges (separation of coastal and inland populations). Note that *R. redowskianum* is really confined to continental mountain areas and has a range extending westward to Lake Baikal, it is absent in Chukotka and did not cross the Beringian bridge in the past, unlike *R. camtschaticum* (in its broad sense), which has a priocceanic amphiberingian distribution. We previously studied the variability of three plastid DNA markers (*trnK-matK*; *trnF-trnV*; *trnH-psbA*) in *R. camtschaticum* and its subspecies *R. camtschaticum* ssp. *glandulosum* (Standl.) Hult. in Kamchatka, which yielded three haplotypes h1 and h2 in the former and h3 in the latter taxon (Polezhaeva et al. 2020). For *R. redowskianum*, from the sequences of these three plastid DNA markers, the haplotypes h4 (common) and h5 (identified in the only one individual from Sikhote-Alin) were compiled. On the phylogenetic tree (Fig. 1B), constructed on the basis of the variability of these markers, the haplotype branch of *R. redowskianum* occupies a sister position, i.e., indicating that

the separation of this species preceded the diversification within the *R. camtschaticum* s.l. clade. The same position was previously obtained using another set of genetic markers of plastid and nuclear DNA (Oliver et al. 2014). Thus, the assumption about the origin of *R. redowskianum* from *R. camtschaticum* is not confirmed.

CONCLUSION

The observed pattern of different levels of variability for different types of markers indicates the importance of selecting suitable tools when assessing the genetic diversity of species for which biodiversity conservation programs or management planning are being developed. Even among the many nuclear microsatellite loci successfully amplified at the selection stage, not all were highly polymorphic, and only a few with high differentiation power. In general, we can conclude that *R. redowskianum* in the Far East has a high genetic diversity, despite its sporadic distribution. Clarification of phylogenetic relationships in the *Therorhodon* section requires further study with involvement of more specimens of other species of the section.

ACKNOWLEDGEMENTS

The authors are grateful to S. Ickert-Bond for providing samples from China and to I.V. Frolov for some of the samples from the Sakhalin Island. The work (partial collection of samples and its identification) was performed under the state assignment of the Institute of Plant and Animal Ecology UB RAS (No. 122021000090-5) and Botanical Garden-Institute FEB RAS (No. 122040800085-4); the molecular genetic analysis was supported by a grant from the Russian Foundation for Basic Research (No. 20-04-00417a).

The authors declare that they have no conflict of interest.

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