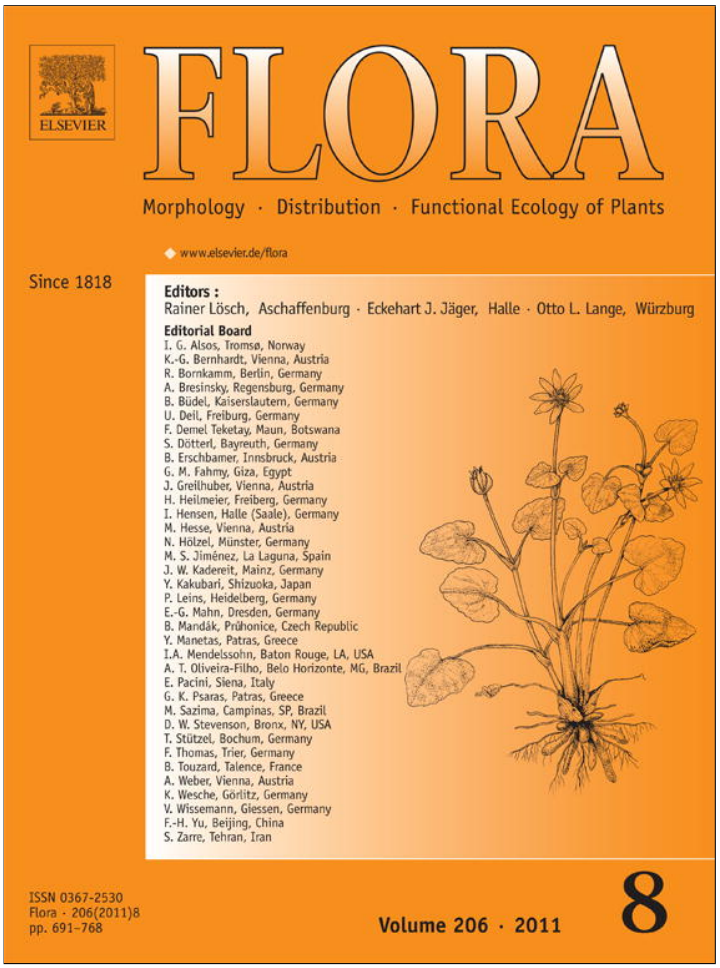

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Analysis of the genetic structure of *Rhodiola rosea* (Crassulaceae) using inter-simple sequence repeat (ISSR) polymorphisms

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ABSTRACT

The genetic diversity and differentiation of eleven *R. rosea* populations from different parts of its wide area of occurrence were studied by ISSR markers. Using eight primers, 252 DNA fragments were generated, and 243 of those DNA fragments were found to be polymorphic, indicating a high genetic variability at the species level (P = 96.4%, h = 0.176, SI = 0.291). Relatively low levels of diversity were determined at the population level (P 30.6–59.1%, h 0.088–0.165, SI 0.137–0.257). AMOVA analysis revealed that the majority of the genetic variation was within populations (65.42%), and the variance among populations was 34.58%. Cluster analysis revealed two groups of *R. rosea* populations; these groups likely represent distinct evolutionary lines in the species, which are different in genetic structure, evolutionary history and chorological migration routes.

Introduction

Rhodiola rosea L. (golden root or roseroot; Crassulaceae) is a popular medicinal plant that has an adaptogenic effect (Brown et al., 2002; Gregory and Kelly, 2001; Panossian et al., 2010; Spasov et al., 2000). The species was first described by Carl v. Linné (Linnaeus, 1753); however, his description was not linked to a type specimen and was typified later by Ohba (1993). The habitat of the type specimen is not known with certainty. According to the herbarium label, the species "occurs in mountains of Lapland, Austria, Switzerland, [and] Britain". Thus, typification of *R. rosea* was unclear from the first description and remains so until now. It is broadly acknowledged that the species is highly polymorphic, and confident differentiation of *R. rosea* from related taxa is problematic due to a variety of phenotypic features in members of the section *Rhodiola* appears superfluous. Numerous ecological and/or geographical races of *R. rosea* are recognised as distinct species (e.g., *R. sachalinensis* Boriss., *R. arctica* Boriss., *R. iremelica* Boriss. and some others) by some authors, and others regard them as infraspecific taxa or even synonyms (Ohba, 1981, 2005). Ohba (1981) listed ca. 50 synonyms attributed to *R. rosea*, which fact clearly reflects the complexity of the species taxonomy.

R. rosea has a broad circumboreal distribution (Ohba, 1989); it occurs in alpine habitats of the boreal zone of Eurasia and Appalachia, as well as in most parts of the Arctic, the Far Eastern coasts of Eurasia and North-Atlantic coast of N. America (Clausen, 1975; Ohba, 1989). The current geographic range of *R. rosea* (Fig. 1) mostly spans historically glaciated areas with multiple Pleistocene refugia.

In Russia, this species is common in the Southern Siberian mountains (Saratikov, 1974). Most botanists agree that *R. rosea* originated in this area and then migrated along the mountain ranges latitudinally and northwards to the Arctic (Polozij et al., 1985). In the Russian Far East, *R. rosea* and/or its subspecies occur in Primorye, Sakhalin Island, and the Amur region. Its occurrences are also particularly common and abundant in Kamchatka (Borissova, 1939; Frolov and Poletaeva, 1998; Kozevnikov, 1988; Krasnov et al., 1979). According to preliminary estimates, the stock of *R. rosea* in Kamchatka is comparable with that in Altai Mts.

Despite a wide distribution, the species needs to be protected; over-harvesting has caused *R. rosea* populations to decline and even become extinct in some areas. Aridification and climate warming observed in recent years also negatively affects the species and reduces its populations, even those that are not affected directly by humans. Due to these factors, *R. rosea* has been enlisted in the Red Data Book of Russia and many regional Red Data Books.

Although *R. rosea* is an economically and pharmaceutically important species, very little information on its affinity with other species, genetic diversity and population structure exists (Elameen et al., 2008; Gontcharova et al., 2006; Mayuzumi and Ohba, 2004; Uvarova et al., 2009). The purpose of our study was to analyse the genetic variability of *R. rosea* and to identify its population

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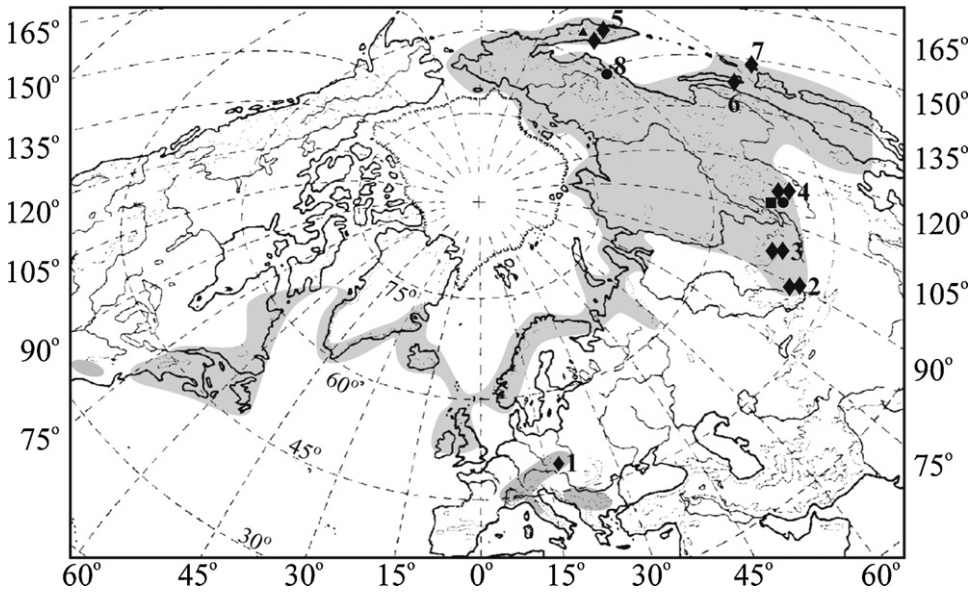

Fig. 1. A map showing distribution range of *Rhodiola rosea* (grey shadow) and location of the populations sampled for this study. ♦ – *Rhodiola rosea*; ▲ – *R. integrifolia*; • – *R. stephanii*; ■ – *R. pinnatifida*. (1) Poland; (2) Altai Mts.; (3) Sajan Mts.; (4) Zabaikal'e; (5) Kamchatka; (6) Sakhalin; (7) Iturup; (8) Khabarovsk.

structure on the major portion of its wide distribution area from Europe to the Far East, using inter-simple sequence repeat (ISSR) markers. ISSR markers are a multilocus system of a dominant type. ISSR analysis is a sensitive method with some advantages over other genetic tools and often provides sufficient information for studying the genetic diversity in plant populations (Ayres and Strong, 2001; Godwin et al., 1997; Goulao et al., 2001; Wolfe, 2005; Zietkiewicz et al., 1994).

Material and methods

One hundred forty seven plants of *Rhodiola rosea* were collected from eleven populations (Fig. 1). In addition, one population of *R. pinnatifida* Boriss. (15 specimens), two populations of *R. stephanii* (Cham.) Trautv. et C.A. Meyer (31 specimens) and one population of *R. integrifolia* Raf. (13 specimens; Table 1) were sampled for comparison. Altogether, 206 DNA samples were used for this study. Voucher specimens (VLA; 1–3 plants per population) are deposited at the Herbarium of Institute of Biology and Soil Science, Vladivostok. All specimens used for DNA extraction were stored at −70 °C in the IBSS.

Total genomic DNA was isolated from 150 to 200 mg of silica-dried leaves following the protocol established by Isabel et al. (1993). DNA quality and quantity was determined by electrophoresis on a 1.0% agarose gel in TBE buffer and visualised with ethidium bromide using lambda phage DNA (Sigma, USA) as a standard.

PCR amplification of the total genomic DNA with ISSR primers was carried out on a UNO II 48 thermocycler (Biometra, Germany) using 25 μl of reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.001% gelatine, 0.250 mM of each dNTP, 0.2–0.4 mM primer, 30–50 ng of template DNA and 0.8 U *Taq* polymerase. The thermocycler program was 5 min at 94 °C, followed by 40 cycles of 45 s at 94 °C, 45 s annealing at 48 or 58 °C (Table 2) and 45 s extension at 72 °C, and a final 5 min extension at 72 °C. The PCR products were separated on a 1.4% agarose gel in TBE buffer and visualized with ethidium bromide. Molecular weights were estimated using lambda DNA/*Eco*RI + *Hin*dIII markers (Fermentas, Lithuania).

Table 1
Geographical location and sample size (*n*) of *Rhodiola rosea, R. pinnatifida, R. stephanii* and *R. integrifolia* populations.

Population	Location	*n*
Rhodiola rosea		
Altai-1	Russia, Altai Republic, Ulagan Dist., upper current of Jarlinri stream, 2500 m asl	12
Altai-2f	Russia, Altai Republic, Kosh-Agach Distr., Southern Chujski Mt. Range, Lake Karakul vicinity, 2700 m asl	13
Sayan-1	Russia, Krasnojarsk Territory, Western Sajan Mts., Egarki Nature Park, Mt. Tushkanchik	16
Sayan-2	Russia, Krasnojarsk Territory, Western Sajan Mts., Egarki Nature Park, vicinity of Lake Circus	15
Sakhalin	Russia, Sakhalin Territory, Holmsk Distr., vicinity of town Chehow, sea cliffs	11
Iturup	Russia, Sakhalin Territory, Kurilsky Distr., Iturup Island, vicinity of Burevestnic settlement	16
Kamchatka-1	Russia, Kamchatski Kray, Ust-Kamchatsky Distr., 1000 m asl	12
Kamchatka-2	Russia, Kamchatski Kray, Bistrinski Distr., Esso settlement vicinity, Sredinni Mt. Range, 1000 m asl	12
Zabaikal'e-1	Russia, Zabaikalski Kray, Kirinsky Distr., Sokhondo Nature Reserve, Tcagan-Ula Mt., 2200 m asl	15
Zabaikal'e-2	Russia, Zabaikalski Kray, Kirinsky Distr., Sokhondo Nature Reserve, Bolshoj Sokhondo Mt., 2300 m asl	11
Poland	Poland, Tatrzanski Park Narodowy, a ridge between Kondracka Kopa Mt. and Kondracka Przelcz Pass, 1700 m asl	14
Rhodiola stephanii		
Khabarovsk	Russia, Khabarovski Kray, Okhotsky Distr., Okhotsk town vicinity, Laptinskie Mts., Gadatelni stream valley	17
Zabaikal'e-1	Russia, Zabaikalski Kray, Kirinsky Distr., Sokhondo Nature Reserve, Lake Bukukunskoe shore, 2090 m asl	14
Rhodiola pinnatifida		
Zabaikal'e-2	Russia, Zabaikalski Kray, Kirinsky Distr., Sokhondo Nature Reserve, Bukukun stream upper current, 1700 m asl	15
Rhodiola integrifolia		
Kamchatka	Russia, Kamchatski Kray, Ust-Kamchatsky Distr., Sopka Ploskaja volcano, 1000 m asl	13

Table 2
ISSR primers used for analysis of *Rhodiola* populations, primer annealing temperatures and number of bands scored.

ISSR primer	Primer sequence (5′ → 3′)	Annealing temperature (°C)	Number of bands scored
807	$(AG)_8T$	48	28
809	$(AG)_8G$	48	27
812	$(GA)_8T$	48	32
834	$(AG)_8(CT)T$	48	35
841	$(GA)_8(CT)C$	48	34
868	$(GAA)_6$	48	29
888	$(CGT)(ACT)(CGT)(CA)_7$	48	29
C4	$(AGC)_6G$	58	38
Total			252

Initially, 18 ISSR primers (Syntol, Moscow, Russia) were tested with three *R. rosea* DNA samples representing three populations. On the basis of reproducibility and the maximum number of distinctly scorable polymorphic bands, eight ISSR primers complimenting di- and tri-nucleotide microsatellite repeats were selected for further study (Table 2).

For each primer, bands were scored as either present (1) or absent (0). POPGENE (Yeh and Boyle, 1997) and TFPGA (Miller, 1997) software packages were used to estimate the frequency of each band, the percentage of polymorphic loci (*P*), Nei's (1973) gene diversity (*h*), Shannon's index of phenotypic diversity (*SI*, Lewontin, 1972), genetic differentiation (G_{ST}) and gene flow ($Nm = 0.5(1 - G_{ST})/G_{ST}$). Dendrograms of genetic relationships between the populations and between the individuals were constructed by applying the unweighted pair group method (UPGMA) using TFPGA and TREECON (Van de Peer and De Wachter, 1994) software packages, respectively.

A binomial ISSR matrix was also used for the analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.01 (Excoffier et al., 2005). This analysis was performed to estimate the partition of the genetic variation within and between populations. The significance of the variance components was determined with a permutation test (10,000 replicates).

Results

Using eight primers, ISSR analyses were conducted on 206 plants from fifteen *Rhodiola* populations and produced 252 DNA fragments. In 147 plants from eleven populations of *R. rosea,* 243 loci (96.4%) were polymorphic. The fragments ranged in size from 300 to 2000 bp, and their number was primer-specific, from 27 to 38 bands, with 31.5 loci per primer on average (Table 2). The genetic diversity estimates of population and species levels are presented in Table 3.

Table 3
Genetic variability of *Rhodiola* populations based on 252 ISSR markers.

Population	*P*, %	*h*	*SI*
Rhodiola rosea			
Altai-1	42.5	0.124	0.191
Altai-2	42.1	0.125	0.192
Sayan-1	40.5	0.103	0.164
Sayan-2	38.9	0.102	0.162
Sakhalin	35.7	0.117	0.177
Iturup	59.1	0.165	0.257
Kamchatka-1	30.6	0.088	0.137
Kamchatka-2	39.3	0.115	0.178
Zabaikal'e-1	52.4	0.132	0.211
Zabaikal'e-2	59.1	0.142	0.230
Poland	46.8	0.094	0.157
All populations	96.4	0.176	0.291
R. stephanii (Khabarovsk)	55.9	0.141	0.226
R. stephanii (Zabaikal'e)	47.2	0.118	0.187
R. pinnatifida (Zabaikal'e)	37.3	0.087	0.141
R. integrifolia (Kamchatka)	38.5	0.111	0.171

In *R. rosea,* the highest values of *P*, *h* and *SI* were observed in a population from Iturup Island (59.1%, 0.165 and 0.257, respectively), and the lowest in a population from Kamchatka-1 (30.6%, 0.088 and 0.137, respectively; Table 3). The proportion of genetic diversity between populations (G_{ST}) was 0.325, indicating that the variations between populations and those within populations contributed 67.5% and 32.5%, respectively. The estimated average of gene flow (*Nm*) was 1.0. Populations from the same geographic area had a low genetic differentiation. In Zabaikal'e, it accounted for 0.055, in Altai – 0.098, in the Sajan Mts. – 0.108 and in Kamchatka – 0.166. At the same time, these populations were characterised by a relatively high estimated gene flow: 8.5, 4.6, 4.1 and 2.5, respectively.

The genetic distances for pairs of populations varied to a considerable degree with an eightfold maximum difference. The Altai-1 population was the most genetically distant from the Kamchatka-1 population (0.0976), while the Zabaikal'e-1 and Zabaikal'e-2 populations were closest to each other (0.0122).

The lowest values of interspecific genetic distances were found between populations of *R. pinnatifida* and *R. rosea* collected

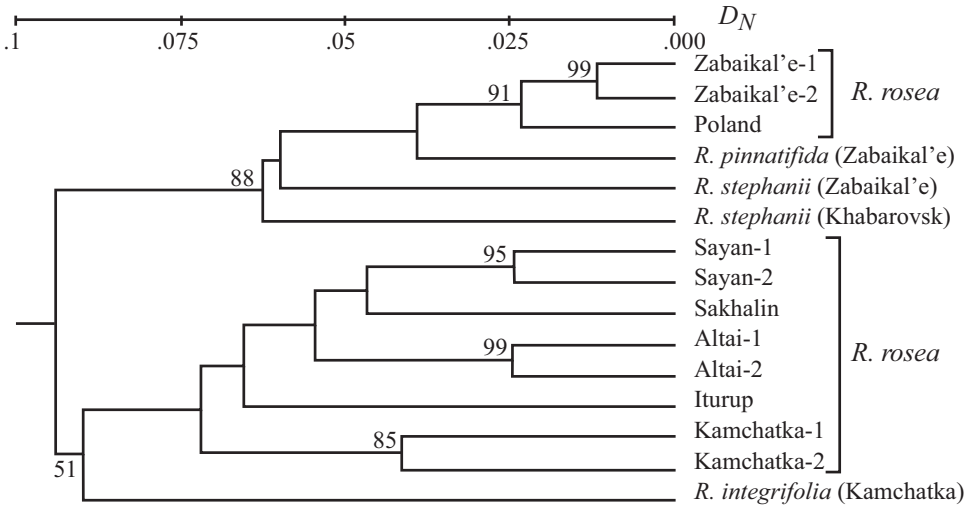

Fig. 2. UPGMA dendrogram of eleven *Rhodiola rosea*, two *R. stephanii*, *R. integrifolia* and *R. pinnatifida* populations constructed based on Nei's (1973) genetic distances calculated from 252 ISSR markers. The numbers on the branches indicate the bootstrap support (only those above 50% are shown).

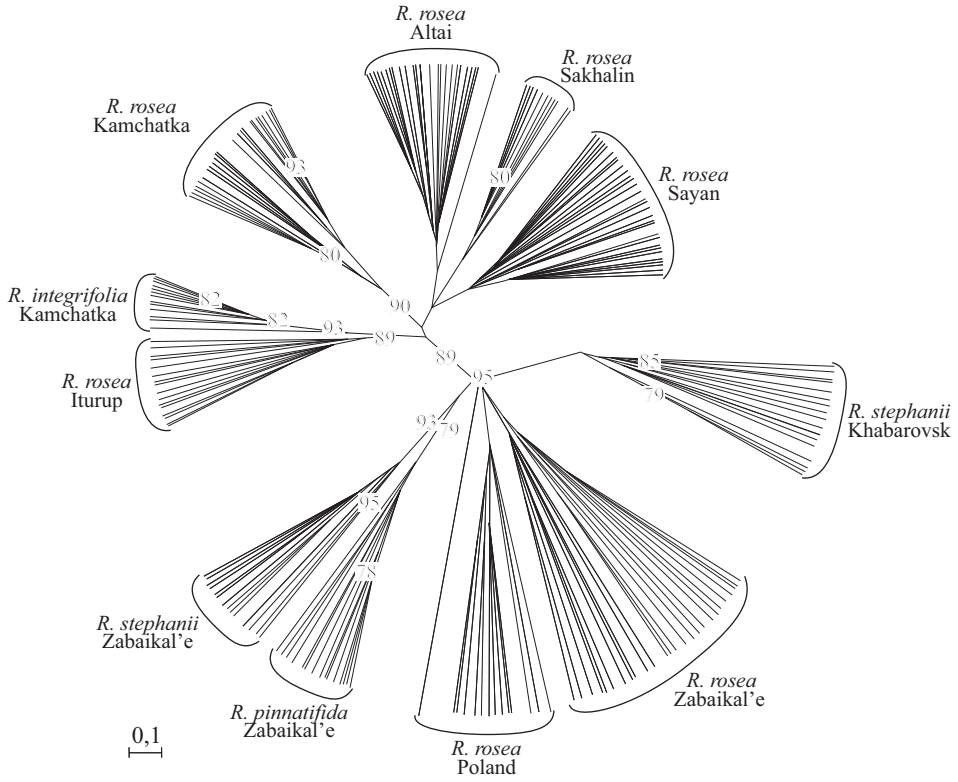

Fig. 3. Unrooted UPGMA dendrogram showing genetic relationships between the individuals in the population studied constructed based on Nei and Li's (1979) genetic distances from 252 ISSR markers. The numbers on the branches indicate the bootstrap support (≥50%).

from Zabaikal'e-1 (0.0375), and the highest (0.1405) were found between *R. stephanii* (Khabarovsk) and *R. rosea* (Kamchatka-1).

Using an UPGMA cluster analysis, populations of four *Rhodiola* species were grouped into two supported clades (Fig. 2). One clade (BP 88%) comprised three populations of *R. rosea* (Zabaikal'e-1, Zabaikal'e-2 and Poland) together with populations of *R. pinnatifida* and *R. stephanii*. The second clade (BP 51%) included all remaining populations of *R. rosea* and *R. integrifolia*. The genetic distance between the two groups was 0.0305. In the second clade, populations of *R. rosea* from the same geographic region formed respective regional clades supported by high bootstrap values (BP 85–99%; Fig. 2). The population of *R. rosea* from Sakhalin was genetically closer to populations from the Sayan Mts. (D_N 0.0427 and 0.0526) than to those from Kamchatka (D_N 0.0602 and 0.0652) or to a geographically adjacent population from Iturup Island (D_N 0.0611).

Fig. 3 presents an unrooted dendrogram showing relationships within the populations. The branching pattern between the populations is generally the same as in Fig. 2, and the branch separating the two groups of *R. rosea* populations again attained high support (BP 89%). Genetic differences between the plants were relatively high and exceeded those between populations. UPGMA analyses revealed that clustering of specimens generally corresponded to their geographic origin.

AMOVA analysis of eleven *R. rosea* populations (Table 4) showed that most molecular variation was found within populations, and there was less than 35% of total genetic variance among the populations (Φ_{ST} = 0.346). When the populations were analysed in two groups according to their clustering in the UPGMA dendrogram (Fig. 2), the majority of molecular variance was observed among individuals within populations (59.57%). Furthermore, significant parts of genetic variation were due to differences among groups (15.86% of the total) and among populations within groups (24.57% of the total; Table 4).

Discussion

The present study is the first to use inter-simple sequence repeat (ISSR) data to assess genetic variability and differentiation in populations of *R. rosea*, a valuable medicinal plant with a wide distribution in the circumboreal zone. ISSR analysis revealed a relatively low percentage of ISSR polymorphisms in *R. rosea*

Table 4
Results of molecular variance (AMOVA) for *Rhodiola rosea* populations based on ISSR markers.

Source of variation	d.f.	CV	% Total	Fixation index
Among populations (total)	10	10.32	34.58	Φ_{ST} = 0.346**
Within populations	136	19.53	65.42	
Between groups: (Altai Mts., Sayan Mts., Sakhalin, Iturup, Kamchatka) vs. (Poland, Zabaikal'e)	1	5.20	15.86	Φ_{CT} = 0.159*
Between population within groups	9	8.05	24.57	Φ_{SC} = 0.292**
Within population	136	19.53	59.57	Φ_{ST} = 0.404**

d.f., degrees of freedom; CV, variance-component estimates; % Total, percentage of total variance contributed by each component; Φ_{CT}, correlation of individuals within groups relative to the total; Φ_{SC}, correlation within populations relative to groups; Φ_{ST}, correlation within populations relative to the total. Significance levels are based on 1000 permutations.

* Statistical significance at $p < 0.001$.

** Statistical significance at $p = 0.000$.

populations (*P* 30.6–59.1%). These values were lower than those in *R. rosea* germplasm collections in Norway (77.1–86.2%, Elameen et al., 2008), in *R. angusta* (69.6–78.8%, Yan et al., 1999) and *R. alsia* (63.4–88.6%, Xia et al., 2005), but comparable with those observed in populations of *R. chrysanthemifolia* (22.0–48.8%, Xia et al., 2007). In general, *R. rosea* is characterised by a high genetic variability (96.4%) that is typical for a species with wide distribution ranges due to local short-scale evolutionary processes (Hamrick and Godt, 1989; Serebryanaya and Shipunov, 2009). As in most out-crossing plant species, genetic variability was found inside populations of *R. rosea* (Table 4).

It should be noted that geographically adjacent populations of *R. rosea* were characterised by similar values of genetic diversity that, in our opinion, allows their classification as subpopulations of a macroregion (e.g., Altai, Sayan Mts., Zabaikal'e and Kamchatka). The results of UPGMA cluster analysis supported this conclusion and resolved distinct clades of regional macropopulations of *R. rosea* from Altai, Sayan Mts., Zabaikal'e and Kamchatka with high support (≥85%; Fig. 2). In general, the subpopulations that form regional macropopulations of *R. rosea* show few genetic differences between one another. However, it is notable that the geographically distant European population of *R. rosea* from Poland showed a close affinity to two populations from Zabaikal'e (95%; Fig. 2). In fact, the genetic distance between the Polish population and any of the Zabaikal'e populations was lower than the genetic distance between subpopulations of any of the macroregions (i.e., Altai, Sayan Mts. and Kamchatka).

Our study revealed a significant differentiation between eleven studied populations of *R. rosea* (G_{ST} = 0.325, Φ_{ST} = 0.346). However, the differentiation between subpopulations of one macroregion (e.g., Kamchatka, Sayan Mts., Altai Mts.) was two to six times lower (G_{ST} = 0.055–0.166) than between all populations. Additionally, the gene flow (average value for four regions, 4.9) was comparable with that in a pool of *R. rosea* plants from Norway (0.0797 and 5.564, respectively; Elameen et al., 2008), which also could be regarded as a macropopulation. Thus, high intrapopulation differentiation and low estimated average gene flow (Nm = 1.0) for the set of *R. rosea* specimens analysed suggests that the species comprises several regional macropopulations that are genetically isolated. The distinctness of these macropopulations suggests their long-term residence in the respective region. A similar phylogeographic pattern was revealed in North American populations of *R. integrifolia* (Guest, 2001).

UPGMA analysis resolved two clusters of *R. rosea* populations; one highly supported cluster (95%) was comprised of populations from Zabaikal'e and Poland, and the second weakly supported cluster (51%) included populations from Altai, Sayan Mts., Sakhalin, Kamchatka and Iturup (Figs. 2 and 3). Thus, our data suggest that *R. rosea* is composed of at least two evolutionary lines, and these lines may be distinct in genetic structure, evolutionary history and migration routes. We can conclude that the differentiation within the smaller cluster (the Polish and Zabaikal'e populations) is much less pronounced than between geographically adjacent subpopulations of any macroregion from the second cluster.

The genetic affinity of the European and Zabaikal'e *R. rosea* populations to those of *R. pinnatifida* and *R. stephanii* (88% support; Fig. 2) suggests a genetic split in *R. rosea*. Both *R. pinnatifida* (sect. *Rhodiola*) and particularly *R. stephanii* (sect. *Chamaerhodiola*) are distinct from *R. rosea* phenotypically. However, molecular phylogenetic studies have shown that the features of vegetative morphology that are traditionally used in the systematics of the genus *Rhodiola* do not reflect species relatedness in this genus (Mayuzumi and Ohba, 2004; Gontcharova et al., 2006, 2009). Additionally, a recent study on the ultrastructure of *R. rosea* seeds collected from different parts of the species' distribution range also suggested the presence of two phenotypes in this species (Gontcharova et al., 2009): Seeds with a laticostate surface are typical of European populations, while seeds with laticostate and tenuicostate surfaces are typical of Asian populations. Thus, it is possible that these two phenotypes correspond to the evolutionary lines of *R. rosea* revealed by ISSR markers in the present study.

Most authors agree that the Himalayas, the Pamiro-Alaj Mts. and the Tian Shan Mts. are the centres of origin and distribution of the genus *Rhodiola* (Borissova, 1939; Frolov and Poletaeva, 1998; Kozevnikov, 1988; Krasnov et al., 1979; Ohba, 1989). However, *R. rosea* is typically found in more boreal regions, and most of its current distribution range covers previously glaciated areas that are rich in former refugia. The southern boundary of the species distribution is limited by the Altai and Sayan Mts. and southern Zabaikal'e; it reaches south of 40°N only in the Far East. It was hypothesised that *R. rosea* originated in the Southern Siberia highlands during the Pliocene and then migrated westwards to southern Ural during the Pleistocene maximal glaciation. Further distribution of *R. rosea* northwards to the Arctic was possible through two alpine corridors: along the Ural Mts. and by mountain ridges of Eastern Siberia (Frolov and Poletaeva, 1998). It is likely that the two clusters of *R. rosea* populations established here represent the evolutionary lines that diverged earlier within this species. However, the affinity of European and Zabaikalian populations of *R. rosea* to *R. pinnatifida* and particularly to *R. stephanii* could also indicate that genetic differences between the two groups of *R. rosea* populations (Fig. 2) reflect the artificial nature of the species, masked by homoplasy of phenotypic characters. This must be investigated further with broader sampling of populations and molecular markers, in order to resolve finally the genetic structure of *R. rosea* and clarify its taxonomy.

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