

## Genetic Diversity Within and Among Populations of Roseroot (*Rhodiola rosea* L.) Based on Molecular Markers

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### Abstract

*Rhodiola rosea* L. is a perennial adaptogenic medicinal plant found in cool climate of the northern hemisphere. The species is very diverse both in terms of morphological characteristics and in the content of the pharmacologically active substances. The genetic diversity of four geographically distant roseroot populations was studied with ISSR and SSR markers. Using 7 ISSR primers 64 DNA fragments were generated and 85,94% of those were found to be polymorphic, indicating high genetic variability at the species level (gene diversity = 0.33, Shannon index = 0.48). Lower level of diversity was detected at the population level (Shannon-index ranged from 0.2173 to 0.2696). Only four out of the eight SSR markers used were informative during this study. The primer pairs for these four SSR markers produced 25 fragments with an average of 6.25 putative alleles per locus. Observed heterozygosity ranged from 0.4 to 1.0, whereas expected heterozygosity ranged from 0.47 to 0.84. Cluster analysis based on both markers revealed the same groups, individuals clustered according to their geographic origin. The Southern-Uralian population was the most genetically isolated. ITS analysis was used for the determination whether these Southern-Uralian individuals belong to the same species.

**Keywords:** ISSR, ITS, molecular markers, *Rhodiola rosea*, SSR

### Introduction

*Rhodiola rosea* L., commonly known as golden root or roseroot is a traditional adaptogen medicinal plant. Scandinavian, Eastern-European and Asian people have used it for centuries as general immune-stimulant. Roseroot belongs to the family *Crassulaceae*. It is a herbaceous plant with thick rhizome, which contains pharmacologically important secondary metabolites (Brown *et al.*, 2002).

*Rhodiola rosea* displays a circumpolar distribution in the higher latitudes and elevations of the Northern hemisphere mainly in Asia and Europe (Brown *et al.*, 2002; Furmanowa *et al.*, 1995). According to Hegi (1963), its distribution in Europe extends from Iceland and the British Isles across Scandinavia as far south as the Pyrenees, the Alps, the Carpathian Mountains and other mountainous Balkan regions. Roseroot is highly variable both in phytochemical (Kurkin *et al.*, 1988, Wiedenfeld *et al.*, 2007) and in morphological aspects (Asdal *et al.* 2006; Ohba 1981, 1989).

According to *Flora Europaea* *Rhodiola rosea* has several synonyms like *Sedum rhodiola* DC., *Sedum rosea* (L.) Scop., *Rhodiola arctica* Boriss., *Rhodiola iremelica* Boriss., *Rhodiola scopolii* Simonk., *Sedum scopolii* Simonk. *Rhodiola iremelica* Boriss. is described in *Flora USSR* as an endemic species of Southern-Ural (1939). Analysis of the ITS (internal transcribed spacer) region is widely used in

taxonomy because it is easy to amplify and has high degree of variation even between closely related species. Mayazumi and Ohba (2004) have performed a large scale analysis of the *Cassulaceae* family, but no information is available about *R. iremelica*.

For genetic diversity studies mostly inter simple sequence repeats (ISSR) have been applied in the genus *Rhodiola*. Xia *et al.* (2005) studied the genetic variation within and among populations of *Rhodiola alsia* and later (Xia *et al.*, 2007) of *Rhodiola chrysanthemifolia* native to the Tibetan Plateau. Lei *et al.* (2006) explored the genetic variation in *Rhodiola crenulata* from the Hengduan Mountains. Finnish *Rhodiola rosea* populations were analysed by György *et al.* (2009). Yanbaev *et al.* (2007) studied *Rhodiola iremelica* populations in the Southern-Urals with isoenzymes. Elameen *et al.* (2008) investigated using AFLP (Amplified Fragment Length Polymorphisms) technique the genetic diversity in a Norwegian germplasm collection. Meng *et al.* (2007) used AFLP for analysing intra-specific genetic variation in the genus *Rhodiola* and the same method was used by Wang *et al.* (2009) for studying the genetic diversity of *Rhodiola rosea* populations from Tianshan Mountain. Recently Kozyrenko *et al.* (2011) analysed the genetic structure of *Rhodiola rosea* accessions mostly of Russian origin using ISSR technique.

In 2009 Zini *et al.* (2009) published eight microsatellite sequences (simple sequence repeats, SSR) of *Rhodiola*

*rosea* and flanking primer pairs specifically designed for this species. These primers were tested on two *Rhodiola rosea* populations from the Trentino Alps. Four out of these primers were also used by Kylin (2010) for evaluating genetic diversity of roseroot plants collected in Sweden, Greenland and Faroe Islands.

The aim of the present work was to characterize inter- and intrapopulation genetic variability among selected roseroot individuals from Northern-Urals, Southern-Urals, Novaya Zemlya and Altai Mountain populations using the eight available SSR markers and comparing these results with the results of ISSR method. The obtained data was compared against the genetic variability detected by the same markers in the roseroot plants studied from the Trentino region, Italy by Zini *et al.* (2009). An effort was made to clarify whether Southern-Uralian roseroot population considered to be *Rhodiola iremelica* is really a separate species or belongs to *Rhodiola rosea*.

## Materials and methods

### *Plant material and DNA preparation*

*Rhodiola rosea* plants were provided by the Institute of Biology, Komi Science Centre, Ural Division, Russian Academy of Sciences, Syktyvkar, Russia. Roseroot plants were collected in the Northern-Ural Mountains, Southern-Ural Mountains, in Novaya Zemlya and in the Altai Mountain (Fig. 1). The collected plants were cultivated in the experimental field of Syktyvkar University. Due to difficulties of transporting plant material only 5 plants were included in the study from each population, all together 20 individuals. The plant material was frozen in liquid nitrogen and was stored in -80°C until used. DNA was ex-

tracted from the frozen leaves according to a CTAB-based protocol (Pirttilä *et al.*, 2001). DNA concentration and quality was assessed using NanoDrop spectrophotometer (BioScience, Hungary) and visualized on 1% agarose gel.

### *PCR amplification of SSR and ISSR markers*

PCR was performed in 25 µl reaction volume containing 20-80 ng DNA, 10×PCR reaction buffer, 2.5 mM MgCl<sub>2</sub>, 2 mM dNTP mix, 2.5 µmol of each 5' and 3' end primers, 1 unit of *Taq* DNA polymerase (Fermentas, Szeged, Hungary) and sterile distilled water. Eight SSR primer pairs, designed specifically for roseroot by Zini *et al.* (2009) were used for the DNA amplification. The forward primers were fluorescently labelled (FAM). Seven ISSR primers from the UBC primer set #9 developed at the University of British Columbia, (Canada) (BC807, BC809, BC840, BC841, BC857, BC885 and BC888) were chosen based on preliminary experiments. PCR was carried out in a PTC 200 thermocycler (MJ Research, Budapest, Hungary) using touchdown strategy as described in the paper of Zini *et al.* (2009) in the case of SSR markers. For the amplification of ISSR fragments the following program was used: initial denaturation at 94°C for 4 min; followed by 40 cycles of 94°C for 60 s, 49°C for 90 s, 72°C for 90 s; and a final extension at 72°C for 7 min.

The PCR products were electrophoresed on a 1% (w/v) ethidium bromide-stained agarose gel (SeaKem LE Agarose, Lonza, Rockland, ME USA) in 1×TBE buffer with xylencyanol loading buffer to verify the occurrence of the amplification. For the ISSR analysis PCR products were separated for 2 h at 120 V. Amplified fragments were scored visually for presence (1) or absence (0) and the result were summarised in MS Excel table.



Fig. 1. Map showing the approximate locations of the four examined roseroot populations

The amplified SSR fragments were separated on 8% polyacrylamide gel at 80 W (55°C) for 4 h, and stained with a simple silver staining method (Bassam and Gresshoff, 2007). Amplified fragments were scored visually. To determine the exact size of different fragments, 3 from each detected fragment sizes were run in an automated sequencer ABIPRISM 3100 Genetic Analyzer (Applied Biosystems, Budapest, Hungary). Band scoring was analysed using Peak Scanner software 1.0 (Applied Biosystems, 2006).

Genetic relatedness among genotypes was studied by UPGMA (Unweighted Pair Group Method with Arithmetic averages) cluster analysis using Popgene version 1.32 (1997). Also Popgene was used to estimate expected ( $H_e$ ), observed ( $H_o$ ) heterozygosity, Nei's (1978) gene diversity and Shannon's Information Index (I) for co-dominant marker data (SSR). For dominant marker data (ISSR), Popgene was used to estimate number of polymorphic bands, percentage of polymorphic bands, Nei's gene diversity ( $h$ ) and Shannon's Information Index (I). For all individuals Hamming distance was calculated with PASSAGE 2 (Rosenberg and Anderson, 2011) in case of both marker method. The distance matrices were compared with Mantel test also performed with PASSAGE 2.

#### PCR amplification of ITS regions

The internal transcribed spacer region includes the two internal transcribed spacers (ITS1, ITS2) and the 5.8S gene of the 18S/26S nuclear ribosomal DNA (rDNA) repeats. This region was amplified using the primers ITS-LEU (Baum *et al.*, 1998) and ITS4 (White *et al.*, 1990). PCR mix was the same as described earlier. The PCR program started at 94°C for 4 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 1 min and 72°C for 1 min, followed by an additional 7 min extension at 72°C. The PCR products were separated on a 1% (w/v) ethidium bromide-stained agarose gel in 1×TBE buffer with xylencyanol loading buffer to verify the occurrence of the amplification. Fragment lengths were estimated by comparison with the 1-kb DNA ladder (Promega, Madison, USA).

The gel-purified PCR products (EZ-10 Spin Column DNA, Gel Extraction Kit, Bio Basic INC, Biocenter, Hungary) were directly used in the sequencing reaction. Sequencing used the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Warrington, UK) and was performed in an automated sequencer ABIPRISM 3100 Genetic Analyzer (Applied Biosystems, Budapest, Hungary). Both strands were sequenced and the same primers (ITS-LEU and ITS4) were used as in the PCR.

DNA sequences were compared using BLASTN at NCBI and ClustalW program (Thompson *et al.* 1994), and the aligned sequences were edited with BioEdit v.7.0.9.0.

## Results

#### SSR markers

Amplification was successful with 7 out of the 8 available roseroot SSR primers. The numbers of alleles per locus amplified in course of the study are presented in Tab. 1. Primers for marker RRE4 in most samples failed to amplify genomic DNA. Primer pair for marker RRF4 amplified mostly only one allele per locus indicating homozygosity for that allele or the presence of null alleles. The primers for RRE9 and RRF3 amplified monomorphic fragments for the tested plants (146 and 155; 133 and 143 bp respectively). Therefore only 4 (RRC10, RRD6, RRE2, RRE3) out of the 8 markers were informative during the study. The primer pairs for these four SSR markers produced 25 fragments. The number of alleles per locus ranged from 2 (RRE9 and RRF3) to 8 (RRC10). Sizes ranged from 103 (RRF4) to 185 bp (RRE2) (Tab. 2).

Genetic diversity parameters are presented in Tab. 2. Observed heterozygosity ( $H_o$ ) ranged from 0.4 to 1.0, whereas expected heterozygosity ( $H_e$ ) (genetic diversity) ranged from 0.47 at RRD6 for Altai to 0.84 at RRC10 for Altai population.

Genetic relationships among the studied populations of various origins are shown in Fig. 2. According to this dendrogram the S-Uralian population forms the most distinct group from the others.

Tab. 1. Obtained allele numbers and allele sizes at the examined loci. The brackets mean that amplification was not successful with 100% of the samples

Locus name	No. of alleles obtained in this study	Expected size range, based on Zini <i>et al.</i> 2009(in bp)	Obtained allele size (in bp)
RRC10	7	146-164	140-158
RRD6	6	168-186	166-182
RRE2	8	161-182	155-185
RRE3	4	173-183	173-183
RRE4	2 (-)	146-149	146-149
RRE9	2	143-161	146-155
RRF3	2	121-137	133-143
RRF4	2 (-)	103-118	103-115

Tab. 2. Genetic parameters assessed for the four roseroot population based on SSR markers

Locus	H <sub>o</sub>	H <sub>e</sub>	Nei	Ave Het	I
N-Urals					
RRC10	1.00	0.73	0.66	0.66	1.19
RRD6	0.40	0.62	0.56	0.56	0.95
RRE2	1.00	0.71	0.64	0.64	1.17
RRE3	1.00	0.73	0.66	0.66	1.19
Mean	0.85	0.70	0.63	0.63	1.13
SD	0.30	0.05	0.05	0.05	0.12
S-Urals					
RRC10	0.60	0.69	0.62	0.62	1.03
RRD6	1.00	0.73	0.66	0.66	1.22
RRE2	1.00	0.78	0.70	0.70	1.28
RRE3	1.00	0.73	0.66	0.66	1.19
Mean	0.90	0.73	0.66	0.66	1.18
SD	0.20	0.04	0.03	0.03	0.11
Altai					
RRC10	1.00	0.84	0.76	0.76	1.50
RRD6	0.60	0.47	0.42	0.42	1.61
RRE2	1.00	0.71	0.64	0.64	1.05
RRE3	1.00	0.56	0.50	0.50	0.69
Mean	0.90	0.64	0.58	0.58	0.97
SD	0.20	0.17	0.15	0.15	0.41
Novaya Zemlya					
RRC10	1.00	0.64	0.58	0.58	0.94
RRD6	1.00	0.64	0.58	0.58	0.94
RRE2	1.00	0.82	0.74	0.74	1.47
RRE3	1.00	0.69	0.62	0.62	1.03
Mean	1.00	0.70	0.63	0.63	1.10
SD	0.00	0.08	0.08	0.08	0.25

SD - standard deviation

*ISSR markers*

The selected 7 primers generated 64 bands, corresponding to an average of 9.14 bands per primer. An example for ISSR gel is presented in Fig. 3. The number of

population specific bands (either being specifically present or specifically missing) ranged from 1 to 7. The least variable band pattern was generated with primer BC857 (1 population-specific band out of 9), while the most variable band pattern was generated with primer BC840 (6 population-specific band out of 8). At the population level the Shannon-index was 0.48 and the percentage of polymorphic loci (PPL) was 85.94%. The comparison of the value of the Shannon-index and PPL in the 4 habitats is shown in Tab. 3. As indicated by these parameters, the highest level of variability occurred in New Land population (PPL = 46.88%; I = 0.2696), whereas the lowest level in the S-Uralian population (PPL = 37.5%; I = 0.2173). Genetic relationships among the studied individuals of various origins are shown in Fig. 4. Similarly to the dendrogram shown in Fig. 2, the S-Uralian population formed the most distinct group from the other populations.

*Comparison of the variability based on the two marker methods*

Mantel test ( $Z=84.59$ ) was performed for the comparison of the variability data gained with the two different marker system. The correlation was found to be 0.24 ( $p=0.001$ ), the Mantel test was highly significant. Based on this result the two marker methods found the same individuals to be close or to be distant. This confirms the two dendrograms (Fig. 2 and 4) being very similar.

*Amplification and sequencing of the ITS region*

The S-Uralian roseroot population clustered to a separate group in both dendrograms drawn based on the SSR and ISSR data (Fig. 2 and 3). Sequencing of the ITS region of all individuals from this population and also 2-3 individuals from the others was undertaken to clarify if the S-Uralian population belongs to *Rhodiola rosea* or possibly to *Rhodiola iremelica* (Yanbaev et al., 2007). The ITS-LEU and ITS4 primers amplified a 690 bp fragment from all samples. Fig. 4 shows the alignment of these se-

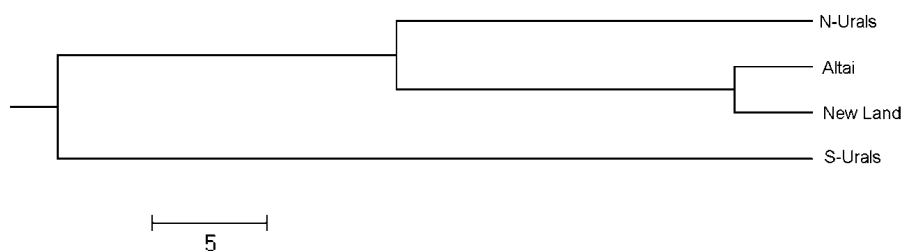


Fig. 2. Dendrogram of the four roseroot populations assayed in this study generated by UPGMA cluster analysis based on the similarity matrix obtained using Nei's genetic distance based on SSR data (Nei, 1978)

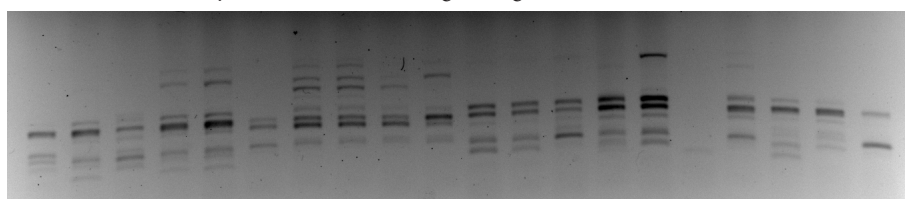


Fig. 3. Gel photo of the electrophoresis of the amplicons generated with the ISSR primer BC 885

Tab. 3. Genetic variability of roseroot populations based on 7 ISSR marker

Population	Number of polimorphic bands	% of polimorphic bands	Gene diversity (h)	Shannon-index (SI)	St. dev.
N-Urals	29	45.31%	0.18	0.26	0.30
S-Urals	24	37.50%	0.15	0.22	0.29
Altai	28	43.75%	0.17	0.25	0.29
Novaya Zemlya	30	46.88%	0.18	0.27	0.30
Total	55	85.94%	0.33	0.48	0.22

Tab. 4. Comparison of the obtained allele numbers at the examined locuses found by Zini *et al.* (2009), Kylin (2010) and in the present study. The brackets mean that amplification was not successful with 100% of the samples

Locus name	No. of alleles found by Zini <i>et al.</i> (2009)	No. of alleles found by Kylin (2010)	No. of alleles obtained in this study
RRC10	4	4	7
RRD6	5	4	6
RRE2	5	-	8
RRE3	3	2	4
RRE4	2	-	2 (-)
RRE9	3	2	2
RRF3	3	-	2
RRF4	3	-	2 (-)

quences to ITS sequence from other *Rhodiola* species (*R. rosea* GQ374198.1, *R. yunnanensis* AB088602.1, *R. wallichiana* AB088607.1, *R. macrocarpa* AB088590.1, *R. heterodonta* AB088596.1, *R. fastigiata* AB088594.1 and *R. chrysanthemifolia* AB088606.1) available in the NCBI genbank. The ITS sequences of the present study are almost identical as seen in Fig. 5.

## Discussion

The aim of the present study was to compare the SSR and ISSR methods for the analysis of the genetic diversity of *Rhodiola rosea*. Both SSR and ISSR markers revealed genetic diversity within the examined populations of roseroot.

Genetic variability within the populations detected with the ISSR method was somewhat higher than in the study of Kozyrenko *et al.* (2011) where eight primers were used. Shannon index ranged between 0.22-0.27 while in the study of Kozyrenko *et al.* (2011) it ranged between 0.16-0.26. The genetic diversity observed among the populations was much higher in the present study. Shan-

non index was 0.48 while in the study of Kozyrenko *et al.* (2011) it was just 0.29. This big alteration can be the result of the fact that the examined populations in this study are located at much diverse latitude, while Kozyrenko *et al.* (2011) took samples from more similar latitude, rather than the difference in the used markers, since half of the primers used are the same in the two studies. Earlier two Finnish roseroot populations were compared with five primers (György *et al.*, 2011) and Shannon-indexes of 0.3129 and 0.2316 were calculated within the two populations, while 0.3385 among the populations. Generally all these Shannon index values are similar to those of *R. crenulata* from the Hengdun Mountains (0.168-0.325, 12 primers), (Lei *et al.*, 2006) and somewhat higher than of *R. alsia* (0.0729-0.2235, 13 primers), (Xia *et al.*, 2005) and *R. chrysanthemifolia* (0.0833-0.2415, 13 primers), (Xia *et al.*, 2007) in the Tibetan Plateau.

Zini *et al.* (2009) developed the eight SSR markers available for *Rhodiola rosea*. The genetic diversity of two Italian roseroot populations was examined as validation of these markers. Kylin (2010) used four out of these SSR

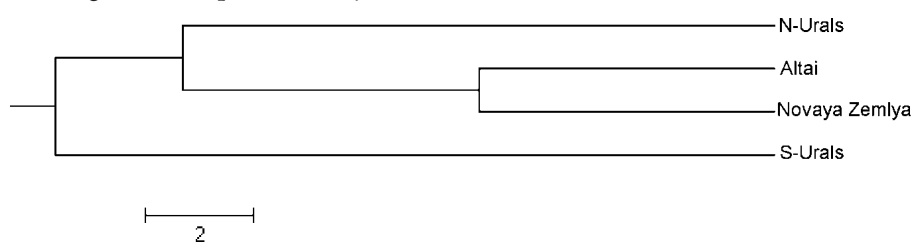


Fig. 4. Dendrogram of the 20 roseroot samples of 4 populations assayed in this study generated by UPGMA cluster analysis based on the similarity matrix obtained using Nei's genetic distance based on ISSR data (Nei, 1978)

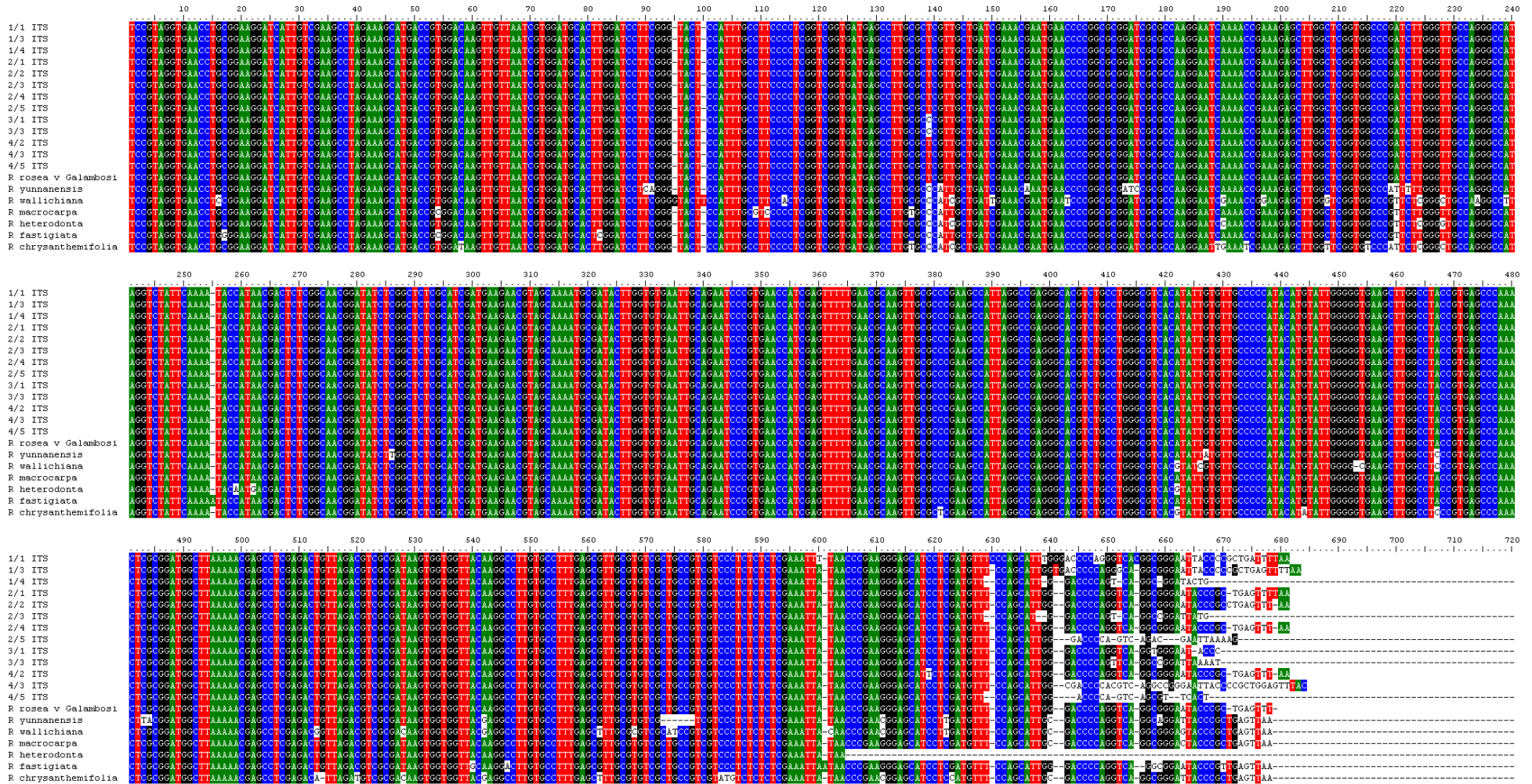


Fig. 5. Alignment of ITS sequences of 3 individuals from N-Uralian population (1/1, 1/3, 1/4), 5 individuals from S-Uralian population (2/1, 2/2, 2/3, 2/4, 2/5), 2 individuals from Altai population (3/1, 3/3), 3 individuals from New Land population and further *Rhodiola* ITS sequences found in the NCBI genbank: *R. rosea*, *R. yunnanensis*, *R. wallichiana*, *R. macrocarpa*, *R. heterodonta*, *R. fastigiata* and *R. chrysanthemifolia*

markers for exploring genetic diversity in the Swedish *Rhodiola rosea* collection (NordGen).

As it can be seen from Tab. 4, RRC10, RRD6 and RRE2 loci showed the highest polymorphism. RRE4, RRE9, RRF3 and RRF4 are either monomorphic (with two alleles) or many times even failed amplification. Higher number of alleles detected in this study indicates higher genetic diversity in the studied populations compared to the study of Zini *et al.* (2009) and Kylin (2010). Observed heterozygosity ( $H_o$ ) ranged from 0.4 to 1.0, whereas expected heterozygosity ( $H_e$ ) (genetic diversity) ranged from 0.4667 at RRD6 for Altai to 0.8444 at RRC10 for Altai. Lowest and highest observed heterozygosity for the same loci analysed by Zini *et al.* (2009) ranged from 0.091 at RRE3 to 0.760 at RRF3, and lowest value for expected heterozygosity was achieved at RRE3 (0.165) and highest value was at RRC10 (0.661). In the study of Kylin (2010) observed heterozygosity ranged from 0.0 at RRE9 to 1.0 at RRC10, while expected heterozygosity ranged from 0.198 at RRE3 to 0.733 at RRC10. Both expected and observed heterozygosity were higher in all four populations than reported previously for the same primer set, which indicates higher genetic variation in the studied roseroot populations.

The highest genetic diversity was found in the Novaya Zemlya population, while the lowest in the S-Uralian population both with the used SSR and ISSR markers (Tab. 3 and 4). The Novaya Zemlya population is the northernmost population examined so far. The optimal environmental circumstances may favour the maintenance of high genetic variability.

The S-Uralian population according to Yanbaev *et al.* (2007) is an endemic plant (*R. iremelica*). According to our results these plants form a clearly separated cluster on the dendrograms based both on SSR and ISSR markers. ITS sequence analysis was performed. Ishmuratova (2004) and Yanbaev *et al.* (2007) has written about these plants as *R. iremelica*. Borissova (1939) described *R. iremelica* in Flora USSR as new species. The difference between *R. iremelica* and *R. rosea* are the shape of the leaves and the size of the seeds. As mentioned earlier *R. rosea* displays high morphological variability. *Flora Europaea* states that *R. iremelica* is only a synonym for *R. rosea* (<http://rbg-web2.rbge.org.uk/FE/fe.html>). Mayuzumi and Ohba (2004) examined the phylogenetic position of Eastern-Asian *Sedoideae* and Gontcharova *et al.* (2006) has studied members of the *Sedoideae* subfamily based on ITS region. The ITS region sequences of different *Rhodiola* species gained in their studies were used in the alignment together with the sequences of the present study. Unfortunately no sequence data is available for *R. iremelica*. In those positions where *Rhodiola* species differ no alteration can be observed among the ITS sequences of the individuals from the four examined populations, which supports the S-Uralian population being *Rhodiola rosea* or a subspecies of it. Further studies are needed to clarify this issue.

In conclusion, using ISSR markers and SSR markers recently developed for roseroot we were able to assess genetic diversity of roseroot populations of different geographical origin. Both marker systems revealed similar results. However according to our results only four out of the eight SSR primers are feasible (RRC10, RRD6, RRE2, RRE3). Developing more roseroot specific SSR markers would be needed for more accurate studies.

#### Acknowledgement

This study was financed by Hungarian Scientific Research Fund (OTKA PD 83728) and the Hungarian National Development Agency with TÁMOP- 4.2.1/B-09/1/KMR-2010-0005 and TÁMOP -4.2.2/B-10/1-2010-0023 projects. Dr. Márta Ladányi is acknowledged for the assistance in performing the Mantel-test.

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