Chinese Medicine, 2014, 5, 231-243
Published Online December 2014 in SciRes. http://www.scirp.org/journal/cm
http://dx.doi.org/10.4236/cm.2014.54028

Scientific Research

Microsatellite Analysis of *Panax ginseng* Natural Populations in Russia

Galina D. Reunova*, Olga G. Koren, Tamara I. Muzarok, Yury N. Zhuravlev

Department of Biotechnology, Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia
Email: *reunova@ibss.dro.ru, academic.secretary@biosoil.ru, muzarok@ibss.dro.ru, zhuravlev@biosoil.ru

Received 9 September 2014; revised 26 October 2014; accepted 13 November 2014

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Abstract

***Panax ginseng* C.A. Meyer is an endangered species in Russia. To restore this species, effective protective measures, including the reintroduction into favorable habitats, must be worked out considering the specificity of genetic structure of ginseng populations. One hundred and thirty-nine *P. ginseng* plants were collected from the forests of nine administrative areas of Primorsky Territory of Russia and transferred to a collection nursery for further investigation. Microsatellite markers were used to study the genetic diversity and the genetic structure of ginseng populations. For populations studied with SSR, the number of observed alleles was ranging from 15 to 25, allelic richness from 1.83 to 3.04, polymorphic loci from 62.5% to 87.7%, observed heterozygosity from 0.410 to 0.512 (an average of 0.453) and expected heterozygosity from 0.304 to 0.479, with an average of 0.393. The values of the inbreeding coefficient within populations (F_{is}) ranged from −0.447 to 0.056, and their average value was −0.296. Genetic differentiation among populations was significant (F_{st} = 0.115) but an isolation-by-distance pattern was not detected. UPGMA and MS-tree confirmed the presence of genetic structure within *P. ginseng* and visualized genetic relationships of populations with similar pattern. STRUCTURE analysis revealed the genetic admixture between different ginseng populations. It was established with SSR markers that *P. ginseng* still preserves substantial genetic resources although all populations are largely exhausted. Because ginseng populations are significantly differentiated all of them should be restored. Considering the admixture of ginseng populations it would be advisable to apply the individual assignment test to verify the content of indigenous populations and to identify the "true" population plants to serve as stock material for reintroduction.**

Keywords

***Panax ginseng*, Genetic Diversity, Genetic Structure, Microsatellites**

*Corresponding author.

How to cite this paper: Reunova, G.D., Koren, O.G., Muzarok, T.I. and Zhuravlev, Y.N. (2014) Microsatellite Analysis of *Panax ginseng* Natural Populations in Russia. *Chinese Medicine*, **5**, 231-243. http://dx.doi.org/10.4236/cm.2014.54028

1. Introduction

The species *Panax ginseng* Meyer (Korean ginseng) belongs to the family Araliaceae and is one of the most important medicinal plants in East Asia. Ginseng represents the herbaceous perennial plant, which prefers to grow in the ripe and dense Korean pine, Korean pine-black-fir-broad-leaved and white beech forests [1] [2]. In nature, ginseng grows singly or in groups ("families"). The lifetime of ginseng plants is approximately 7 - 10 years in plantation and 60 - 100 years in nature. Some authors speculate that ginseng is able to live 400 years. Reproduction of *P. ginseng* occurs exclusively by seeds, which are carried by birds (the hazel grouse, the jay, the cedar bird, etc.) and rodents [1] [2].

In the first half of the 20th century, wild-growing ginseng plants could be found on the large forestlands of China, Korea and the Russian Primorye, but currently, there are almost no wild ginseng plants remaining in China and Korea [2] [3]. Consequently, ginseng is being developed in these countries as a horticultural crop [4]. At the same time, Russia is the only country where *P. ginseng* is still growing in its natural habitat. Because all natural populations of *P. ginseng* are largely exhausted, reintroduction is essential to restore this plant to its former range.

The theoretical aspects of species reintroduction were discussed in the literature [5] [6]. Most researchers agree that for successful reintroduction, a set of rules must be followed to provide the species with the genetic and ecological prosperity inherent in the species' habitat [7]. Genetic variability is critical for a species to adapt to environmental changes and survive in long term [8]. It is important to acknowledge that the genotypic structure of populations is an evolutionary determined characteristic, which, if ignored, would lead to irreparable consequences [9]. Therefore, the assessment of genetic variation and partitioning within and between populations are principal constituents in a species conservation program [9] [10]. The reintroduction strategy must assume the reinstatement of allelic frequencies typical for the population under study [11]. An essential step of reintroduction is the identification of plants intended to be used for the reintroduction in regard of their conformity to ancestral local population. The genetic admixture in local populations of *P. ginseng* might be a consequence; the special care for ginseng among harvesters established a practice of cultivating ginseng on "forest ginseng plantations" where the seeds from wild plants collected in distant populations were used. This practice most likely ensured the sustainable management of ginseng populations; simultaneously, genetic material from different populations was mixed. An immigration rate in the populations of *P. ginseng* could also be provided through effective seed dispersal across the landscape by birds and mammals. Therefore, verifying whether the "indigenous" populations of *P. ginseng* are mixed is necessary.

In recent years, attention has focused mainly on the development of various chemical and molecular techniques for differentiating between samples of *P. ginseng* and authenticating different *Panax* species [12]. However, not much has been reported about genetic variation and the population structure of *P. ginseng* [13]-[16]. In our studies of wild *P. ginseng* populations, a low level of genetic polymorphisms was detected by allozyme and RAPD markers [17] [18], which means effective conservation strategies would be difficult to design using these markers. Fluorescently labeled AFLP markers were highly effective in determining the genetic diversity and genetic structure of *P. ginseng* [19]-[23]. However, because of the dominance of AFLP markers, it was impossible to calculate the allelic frequencies directly to obtain unbiased estimates of population diversity and differentiation. Here, we report the microsatellite analysis of *P. ginseng* populations previously studied with AFLPs [19]-[23].

Among the various molecular markers, microsatellite markers (SSRs) have been successfully used to describe population genetic diversity, because they are codominant and highly polymorphic [24]. Studies using SSR markers in the *Panax* genus have only recently been initiated. Sets of microsatellite markers produced from SSR-enriched libraries [4] [25], bacterial artificial chromosome (BAC) end sequences [26], or expressed sequence tags (EST) [27] were employed to characterize the genetic diversity of ginseng collections.

In this study, our purposes were: 1) to complete previous AFLP study on genetic diversity of *P. ginseng* by estimating the within-population fixation index (F_{is}) with codominant markers; 2) to compare SSR estimates of population diversity and differentiation of *P. ginseng* with those previously obtained in our studies with AFLP markers; and 3) to verify the utility of microsatellites for assigning *P. ginseng* individuals to their population of origin.

2. Materials and Methods

2.1. Sampled Populations

One hundred and thirty-nine (139) *P. ginseng* plants were collected from the forests of nine administrative areas

of Primorsky Territory (Russia) and transferred to a collection nursery for further analyses. Geographic locations of sampled populations are shown in **Figure 1** where the populations were coded with the names of the areas. Twenty (20) *P. ginseng* plants were collected from the Chuguevsk area (*Chu*), 22 from the Spassk area (*Spa*), 17 from the Ussuriisk area (*Uss*), 12 from the Dalnerechensk area (*Drech*), 16 from the Dalnegorsk area (*Dgor*), 12 from the Olginsk area (*Olg*), 14 from the Pozharsk area (*Pozh*), 16 from the Partizansk area (*Part*) and 10 from the Yakovlevsk area (*Yak*).

2.2. DNA Extraction and SSR Procedure

Genomic DNA was extracted from fresh leaf tissue according to Zhuravlev *et al.* [20]. Each leaf sample was frozen in liquid nitrogen and then grounded into a fine powder using a mortar and pestle.

Polymerase chain reactions were performed in 12 μL reaction mixtures containing 15 ng of genomic DNA, 0.3 mM dNTP, 5 pM of each primer, 2.8 mM $MgCl_2$, 0.5 unit of *Taq* DNA polymerase and 1× supplied reaction buffer (Fermentas, Canada). The PCR profile using the MyCycler^{TM} (Bio-Rad, Germany) thermocycler program consisted of 1 cycle at 95˚C for 5 min, followed by 34 cycles at 94˚C for 30 s, 56˚ - 60˚ (depending on primer used) for 30 s, and 72˚C for 1 min. PCR concluded with a final extension at 72˚C for 15 min. Eleven SSR polymorphic markers previously developed by Kim *et al*. [25] from *P. ginseng* were analyzed. The primers were synthesized by Syntol (Russia). Forward primers were labeled at the 5'-end with 6-carboxyfluorescein (6-FAM) (Applied Biosystems, USA). The fluorescently labeled PCR products were mixed with the GeneScan-500 LIZ size standard (Applied Biosystems, USA) and separated by capillary electrophoresis using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA) for allele detection. Alleles were visualized and sized using the GeneScan 3.7.1 software (Applied Biosystems, USA); alleles were designated by the number of their repeats. All PCRs contained negative and positive controls that were genotyped and analyzed alongside the samples. Genomic DNA was re-extracted for 10% of the individuals and genotyped a second time to check for consistency of allele-calling.

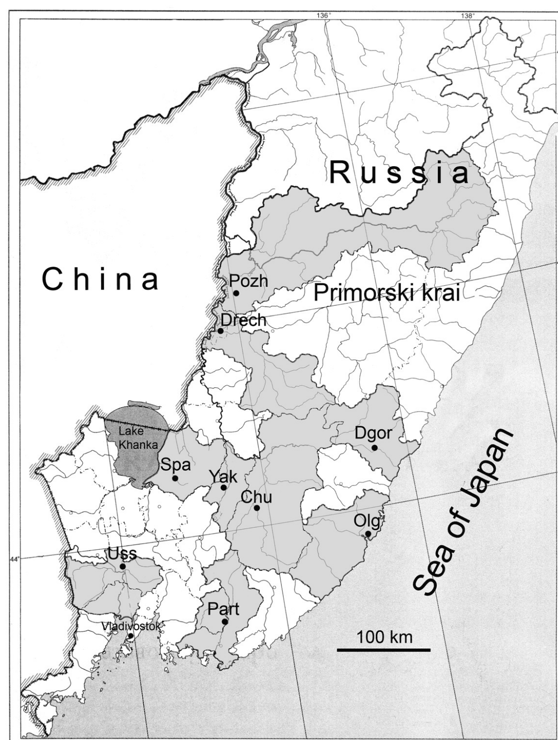

Figure 1. The administrative areas of Primorsky Territory of Russia where *P. ginseng* plants were collected. Names of the areas: Chuguevsk—*Chu*, Spassk—*Spa*, Ussuriisk—*Uss*, Dalnerechensk—*Drech*, Dalnegorsk—*Dgor*, Olginsk—*Olg*, Pozharsk—*Pozh*, Partizansk—*Part*, Yakovlevsk—*Yak*. The administrative centers of the areas are marked with dots.

2.3. Data Analysis

The POPGENE 1.31 [28] software was applied to estimate the frequencies of alleles and to determine the genetic variation parameters, such as the observed number of alleles, percentage of polymorphic loci (P), observed heterozygosity (H_0), expected panmictic heterozygosity (H_E), and F-statistics of Wright (F_{it}, F_{is}, F_{st}) for loci. The inbreeding coefficient F_{is} [29] for populations was computed using FSTAT 2.9.3 [30]. Allelic richness (A_R) was estimated based on a fixed sample size of 10 plants with FSTAT 2.9.3. GENEPOP [31] online version (http:www.genepop.curtin.edu.au/) and ARLEQUIN 3.1 [32] were used to assess Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD), respectively. An analysis of molecular variance (AMOVA, [33]) was performed with GENALEX 6.0 [34] to estimate the variance components attributable to inter-individual and inter-population differences. Genetic divergence between populations was quantified by estimating pairwise F_{ST} values and calculating their significance by bootstrapping analysis using the ARLEQUIN 3.1 software. To test possible correlations between pairwise genetic and geographic distances, the ARLEQUIN 3.1 software was used to conduct a Mantel test with 5000 permutations by regression analysis $F_{ST}/(1 - F_{ST})$ against the natural logarithm of geographic distance (km) between populations.

Cavalli-Sforza and Edwards' [35] chord distance, D_c, was calculated by the PHYLIP program (online version, http://evolution.gs.washington.edu/phylip/software.html), and tree was constructed among populations with the UPGMA clustering method using NTSYSpc 2.0 [36]. A computer simulation study of various genetic distance measurements found D_c to be the most suitable in obtaining the correct tree topology based on a microsatellite analysis [37]. Using a matrix of Cavalli-Sforza and Edwards' chord distances, the minimum spanning tree (MS-tree) between populations was reconstructed using NTSYSpc 2.0. In addition, the population genetic structure was evaluated by a model-based Bayesian clustering method [38] implemented in STRUCTURE 2.3.2 [39]. To test whether any cluster could be found in studied material, three independent runs with 100-300,000 Markov chain Monte Carlo (MCMC) iterations each and 10-30,000 burn-in periods with K between 1 and 10 were performed. In simulations, the admixture model with correlated allele frequencies was used. SSR data were also subjected to individual-based assignment tests by applying the maximum likelihood method with STRUCTURE 2.3.2 [39] to verify the population belonging of every *P. ginseng* individual. Our goal was to detect genetic outliers (*i.e.* migrant genotypes) in the sampled populations.

3. Results

3.1. Genetic Diversity

In this study 139 individual plants were analyzed using 11 SSR primers developed by Kim *et al.* [25] from *P. ginseng* (**Table 1**). One primer pair was monomorphic (PG22) and two primer pairs (PG770 and PG946) did not synthesize fragments. In all populations studied, an aggregate number of 27 alleles across eight SSRs were detected with an average of 3.4 alleles per locus. Approximately 36% of alleles were shared by all *P. ginseng* populations studied. The remaining portion of alleles (64%) was detected in groups consisting of 2 - 8 populations. Every population (except *Yak*) contained some rare alleles (from 1 to 4) occurring with frequencies lower than 0.05. Two private alleles were detected only in *Pozh.* A high proportion of polymorphic loci pairs were found to exhibit LD in *P. ginseng*. Of the examined unique combinations in the nine populations, 53.5% were statistically significant ($p = 0.05$).

In studied populations the number of observed alleles ranging from 15 to 25, allelic richness from 1.83 to 3.04, polymorphic loci from 62.5% to 87.7%, observed heterozygosity from 0.410 to 0.512 (an average of 0.453) and expected heterozygosity from 0.304 to 0.479, with an average of 0.393 (**Table 2**). The values of the inbreeding coefficient within populations (F_{is}) ranged from −0.447 to 0.056, and their average value was −0.296. Generally, deviation from HWE was due to excessive heterozygosity, which was significant in six of the nine populations (**Table 2**).

3.2. Population Structure

The coefficient of population differentiation, F_{st} determined by POPGENE was 0.115. An AMOVA analysis revealed that 9% of the total genetic variation was attributed to the variability among the populations, whereas 91% of the total genetic variation was represented within populations (**Table 3**). The fixation index (F_{ST}) was 0.09 ($p = 0.001$). Moreover, pairwise estimates of F_{ST} ranged from 0.001 to 0.211 (**Table 4**), and only 6 pairs (*Drech*

Table 1. Oligonucleotide primer sequences and annealing temperatures of the 11 microsatellite loci developed by Kim *et al.* [25] from *P. ginseng* and tested in this study.

Locus	Primer sequence, 5'-3'	Annealing temperature, ˚C
PG22	F: CTGTCTATGCAAGTTGCGGCTG R: ATCAAGTTGGAAATCAGGTGGG	58
PG29	F: AATCAGAAACAAAGAAAGCTAAAAC R: CTCTCTCATCTCTCTCTCTTCC	60
PG281	F: GTAGTAGTAGTAAAAACTTTGCTAACG R: ATTTACAACTCTCTTCTTCCTCTAC	60
PG287	F: GTGGGACTGGTATACAATAAGA R:GTGTTCTTAGTTGCCCATTTG	60
PG668	F: CTGGCATCGAAGTTTCTCCATTC R: TGCATAGCACAGAGAGGAGG	60
PG770	F: CCTCTTTGGGGCAGGGATATTTG R: CCAGCAAACCCAAACCCTCCTC	60
PG946	F: GAATCGAAGTGTTAAGTTGAT R: CTTAAATCGATGATAACACC	56
PG1319	F: GCATGAACGGATACACCTTGAGG R: GGTATGCACCAGAAACGGACTGG	58
PG1419	F: ACTCAAAATTCTACAGCTTCCTC R: GATACCCCAG GCAGTCTGATGAC	56
PG1481	F: GGAGGTGATTGATGTAGTGGAATCC R: GGCTCTCCTATACTCACTATTTCCC	58
PG1663	F: CTACACGCTTTTTCATAGCTTACA R: TGTCTGCATAAAAGAGTTCGAGGC	60

Table 2. Gene diversity statistics in *Panax ginseng* populations estimated based on the analysis of eight polymorphic microsatellite loci.

Population	Number of alleles	A_R	P (%)	H_0	H_e	F_{is}	p (HWE)
Uss	17	2.01	75.35	0.441	0.313	−0.430	0.0001
Spa	17	1.92	87.50	0.457	0.319	−0.447	0.0031
Drech	18	2.16	87.20	0.417	0.342	−0.231	0.1686
Chu	16	1.91	62.50	0.481	0.337	−0.446	0.0001
Dgor	21	2.63	87.70	0.453	0.479	0.056	0.7005
Olg	19	2.30	76.42	0.447	0.352	−0.286	0.0404
Pozh	25	3.04	87.42	0.455	0.424	−0.075	0.7957
Part	15	1.83	62.65	0.410	0.304	−0.366	0.0009
Yak	17	2.12	75.15	0.512	0.359	−0.436	0.0001
Mean	18.33	2.49	77.99	0.453	0.393	−0.296	
Species-level value			100.00				

A_R, allelic richness; P, percent of polymorphic loci; H_0, observed heterozygosity; H_e, expected panmictic heterozygosity; F_{is}, the inbreeding coefficient within population; p (HWE), p value for significance of deviation from Hardy-Weinberg equilibrium.

Table 3. Analysis of molecular variance (AMOVA) for populations of *Panax ginseng*. Level of significance is based on 1023 iterations.

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage variation
Among populations	8	5644.750	17.747	9.0
Within populations	265	44915.278	169.492	91.0
Total	273	50560.028	187.239	

Fixation index $F_{ST} = 0.09$; p value = 0.001.

Table 4. Pairwise genetic distance matrix based on genetic differentiation (F_{ST}) (lower diagonal) and geographic distance (km, upper diagonal) for populations of *Panax ginseng*.

Population	*Uss*	*Spa*	*Drech*	*Chu*	*Dgor*	*Olg*	*Pozh*	*Part*	*Yak*
Uss	0.000	115	275	165	325	275	325	125	140
Spa	0.178	0.000	170	110	240	220	215	165	60
Drech	0.138	0.005	0.000	210	230	265	50	310	175
Chu	0.127	0.007	0.035	0.000	155	115	245	120	50
Dgor	0.159	0.211	0.171	0.196	0.000	90	245	255	184
Olg	0.135	0.084	0.060	0.069	0.187	0.000	290	190	160
Pozh	0.020	0.126	0.077	0.101	0.065	0.055	0.000	350	220
Part	0.107	0.127	0.063	0.117	0.201	0.032	0.051	0.000	135
Yak	0.063	0.024	0.011	0.014	0.157	0.002	0.024	0.001	0.000

and *Spa*, *Chu* and *Spa*, *Yak* and *Drech*, *Yak* and *Chu*, *Yak* and *Olg*, *Yak* and *Part*) gave nonsignificant results (with $p > 0.05$) after the permutation procedure.

Cavalli-Sforza and Edwards' chord distances among populations ranged from 0.027 to 0.301 (**Table 5**). A UPGMA dendrogram constructed on the basis of the interpopulation chord genetic distances, divided all populations into two main clusters with sub-clusters. One cluster contained *Dgor*, *Pozh* with distant from them *Uss* population and the second cluster contained *Spa*, *Chu*, *Drech*, *Yak* with *Olg* and *Part* being distant from other populations in this cluster (**Figure 2**). The MS-tree similarly divided all populations into two main clusters to visualize the genetic relationships among the *P. ginseng* populations (**Figure 3**). The way the populations tended to cluster using UPGMA (**Figure 2**) and MS-tree (**Figure 3**) appeared to be independent on their geographical origin (**Table 4**). The correlation analysis did not reveal any association between pairwise F_{ST} values and geographical distances among the populations ($r = 0.078$; $p = 0.374$).

The STRUCTURE results revealed that ln(*P*) is maximized at $K = 4$, suggesting the presence of four genetically distinct groups (**Figure 4**). According to results of STRUCTURE, the group I (red) contained 12 of the 15 *Part*, eight of the 12 *Olg* plants, six of the 10 *Yak* plants, four of the 12 *Drech* plants, and one plant of the other populations, excluding the *Uss*. The STRUCTURE group II (green) included 11 of the 16 *Dgor* plants, six of the 14 *Pozh* plants, and one plant from each of the *Olg* and *Uss* populations. The STRUCTURE group III (blue) contained 17 of the 22 *Spa* plants, 14 of the 20 *Chu* plants, five of the 12 *Drech* plants, three of the 10 *Yak* plants, three of the 12 *Olg* plants, and one plant of the *Part* and *Dgor* populations. The STRUCTURE group IV (yellow) included all of the *Uss* plants (excluding one plant), five of the 14 *Pozh* plants, three of the 16 *Dgor* plants, and five, four, three, two, and one of the *Chu*, *Spa*, *Drech*, *Part* and *Yak* plants, respectively. The individual assignment test with SSR results revealed that only 58 samples out of 139 studied *P. ginseng* samples (42.0%) were assigned to their original populations (results not shown).

4. Discussion

4.1. Genetic Diversity

The populations of *P. ginseng* growing on Primorsky Territory of Russia are characterized by a lower level of allozymes and dominant markers variation than most species with restricted ranges [40]. Our earlier studies demonstrated low levels of genetic variability in *P. ginseng*, as detected by allozymes ($P = 7.6\%$, $H_e = 0.022$), RAPD ($P = 4.0\%$, $H_e = 0.013$) and ISSR ($P = 9.3\%$, $H_e = 0.014$) [17] [19] [21]. These values are consistent with the data from the genetic studies of cultivated ginseng sampled from China and Korea (e.g., [14]). The current study with SSR markers demonstrated a higher genetic diversity in *P. ginseng* comparatively with that obtained with AFLP markers [20] [22] [23]. The polymorphism in *P. ginseng* was 77.99% and 100% at population and species levels, respectively, as estimated by the SSR method. The mean expected heterozygosity (H_e) was 0.393, which is slightly different from those revealed in other studies of *P. ginseng* with SSR markers (0.502, [4];

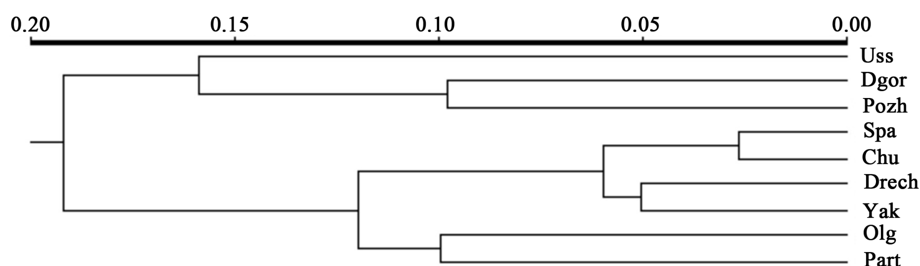

Figure 2. UPGMA dendrogram based on Cavalli_Sforza and Edwards' chord distances among nine *P. ginseng* populations. Population abbreviations are as in "Materials and Methods".

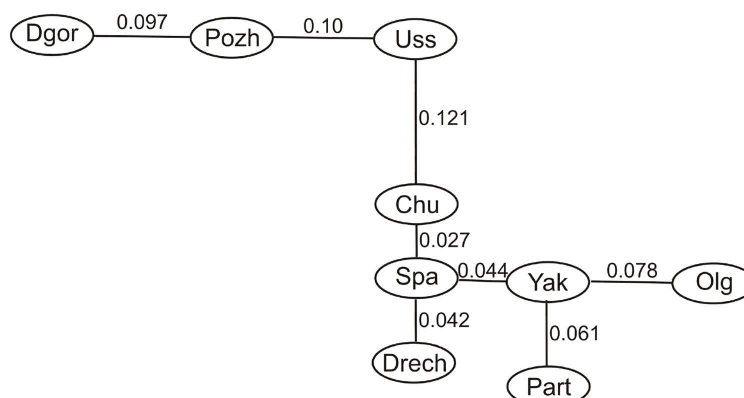

Figure 3. MS-tree representing phylogenetic relationships among nine *P. ginseng* populations. The numbers on lines show Cavalli_Sforza and Edwards' chord distances among populations. Population abbreviations are as in "Materials and Methods".

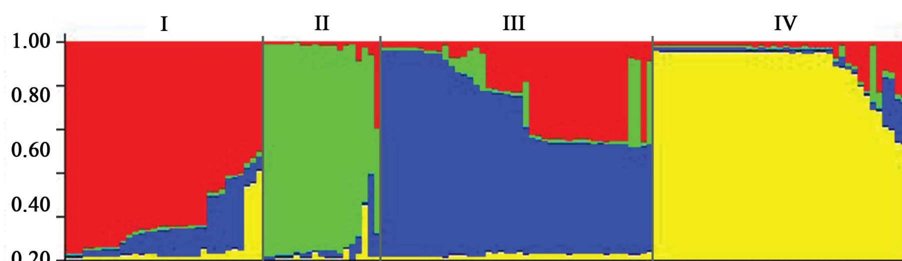

Figure 4. Genetic structure across 139 individuals from nine *P. ginseng* populations using the model-based Bayesian algorithm implemented in the program STRUCTURE. A panel corresponds to K = 4 groups. Fractional membership in each of the clusters (I, II, III, IV) is indicated by *colour*. The populations which created every cluster are described in "Results".

Table 5. Cavalli_Sforza and Edwards' chord distance among populations of *Panax ginseng*.

Population	*Uss*	*Spa*	*Drech*	*Chu*	*Dgor*	*Olg*	*Pozh*	*Part*	*Yak*
Uss	0.000								
Spa	0.172	0.000							
Drech	0.204	0.042	0.000						
Chu	0.121	0.027	0.096	0.000					
Dgor	0.216	0.230	0.205	0.240	0.000				
Olg	0.224	0.142	0.134	0.141	0.246	0.000			
Pozh	0.100	0.163	0.147	0.159	0.098	0.141	0.000		
Part	0.199	0.143	0.105	0.152	0.301	0.099	0.156	0.000	
Yak	0.155	0.044	0.050	0.025	0.251	0.078	0.137	0.061	0.000

0.460, [41]; 0.313, [42]). These differences may be attributed to the source of plant samples, as *P. ginseng* was mainly represented in cultivated forms subjected to artificial breeding and selection. In this study, only wild ginseng was investigated. Furthermore, the populations studied inhabit the northeastern margin of the former range of ginseng, which may be one of the reasons for the observed reduction in the level of genetic variability [40]. Similar values of SSR genetic diversity were observed in two related species, *P. notoginseng* (H_e = 0.350, [43]), and *P. vietnamensis* (H_e = 0.550, [44]).

Our studies with SSR showed that *P. ginseng* is significantly differentiated (F_{ST} = 0.09; p = 0.001). The significant differentiation of the ginseng population was also demonstrated in the studies with AFLP markers [20] [22]. The observed genetic differentiation among populations indicates management for the conservation of genetic variability in *P. ginseng* should aim to preserve all investigated populations with the allelic frequencies determined in this study. The populations which exhibited high allelic richness, such as *Pozh* (A_R = 3.04), *Dgor* (A_R = 2.63), *Olg* (A_R = 2.30) and *Drech* (A_R = 2.16), should be a priority, as allelic richness was highly recommended as an appropriate measure in the context of genetic conservation [45].

The results obtained indicated an excess of heterozygotes in all populations, except for *Dgor*. The mean observed heterozygosity in populations was 0.453, whereas the mean expected heterozygosity was 0.393. This result is rather unexpected for a species with a restricted and fragmented range. Despite the fact that in the past ginseng was widely distributed in the Far Eastern forests covering present-day territories of northern-east China, Korea and the Russian Primorye, it has been described as a rare plant even in ancient manuscripts [2]. In conditions of low plant density, one would expect a high level of inbreeding within populations, especially considering the peculiarities of the ginseng mating system, such as floral phenology and the inability of insect pollinators (syrphids) to fly long distances [46]. In contrast, the inbreeding coefficient, F_{is}, was negative within almost all populations studied. Additionally, an excess of heterozygotes was shown for *P. ginseng* by Ma *et al*. [4] with SSR markers.

An excess of heterozygotes was found in some long-lived perennial plants with codominant markers, such as those used in the SSR [47] and allozyme analyses [48] [49]. In contrast to well-described reasons causing the deficit of heterozygotes, little is known about the causes of excess of heterozygosity in natural plant populations. Presumably, excess of heterozygosity in populations may be related to overdominance and selection as well as agamospermy [47]. Facultative apomixis was found in *P. ginseng* [50]. However, it has been shown that a type of ginseng agamospermy is similar to diplospory, which can impede inbreeding but not prevent it [19]. Overdominance and selection against homozygotes can play a significant role in the survival of ginseng under severe climate conditions on the northeastern margin of the range. Actually, we observed a high mortality of seedlings during germination experiments. Nevertheless, the existence of the overdominance phenomenon in *P. ginseng* populations requires careful investigation, including an evaluation of the observed heterozygosity and F_{is} indices across different developmental stages (seeds, seedlings and adults).

Another reason for the observed excess of heterozygotes in ginseng populations may be their tetraploid origin. Presumably, *P. ginseng* is allotetraploid, as was demonstrated in comparative studies of the allozyme banding patterns of the two closest species, *P. ginseng* and *P. quinquefolius* [50]. A number of duplicated loci and fixed heterozygotes detected in *P. ginseng* denoted an allotetraploid origin of this species. Allotetraploids usually demonstrate lower levels of allozyme variability than autotetraploids. At the same time, the low variability of allotetraploids is compensated for by the variety of parental genomes that have formed this species. Differences between parental genomes impede free gene recombination during meiosis but increase the species genomic diversity due to fixed heterozygosity, which can be detected with codominant markers. Nevertheless, high levels of observed heterozygosity may be associated with the population structure and reflect the history of its formation under the influences of global climatic and local human-induced impacts.

4.2. Population Structure

To study the genetic structure of *P. ginseng* populations, we used a wide range of analytical methods. The *F*-statistics of Wright (F_{st}) and AMOVA (F_{ST}) revealed genetic structure within *P. ginseng* and showed a moderate population differentiation (F_{st} = 0.115; F_{ST} = 0.09). The value of F_{ST} was lower than the inter-population variability obtained with AFLP markers (F_{ST} = 0.36) [20] [22]. Differentiation is expected to be lower when assessed through microsatellites compared with more slowly evolving markers [51]. This is a consequence of both the higher number of alleles in microsatellites and their higher probability of homoplasy, further decreasing genetic

divergence between populations [51].

UPGMA and MS-tree confirmed the presence of genetic structure within *P. ginseng* and visualized genetic relationships of populations with similar pattern. Two main clusters with sub-clusters were obtained (**Figure 2** and **Figure 3**). The Ms-tree (**Figure 3**) did not only confirm the UPGMA data about genetic structure of *P. ginseng* but also allowed discuss the ginseng evolutionary history. The "knot" state of the genetically distant *Uss* and *Chu* populations on Ms-tree (**Figure 3**) testifies that the *P. ginseng* could disperse across the Russian Primorye from south and west. The similar supposition was made in our studies of *P. ginseng* using AFLP markers [20] [22].

The present population relationships inferred from UPGMA and MS-tree were not always consistent with the geographical distribution of the *P. ginseng* populations. For instance, the geographically distant *Uss* and *Pozh* populations were genetically very close but the geographically nearest *Drech* and *Pozh* populations were genetically distant enough (**Table 4**). The population genetic structure of *P. ginseng* derived from SSR analysis was not consistent with a pattern of isolation by distance. Correlation analysis showed that the differences in genetic diversity among the ginseng populations were not related to their geographical distances ($r = 0.078$; $p = 0.374$). This means there was no progressive increase of genetic differentiation with geographic distance, which should be expected if pollen and seed dispersal modes were the main factors influencing the population structure. A negative and nonsignificant ($r = -0.174$; $p = 0.817$) correlation between genetic differentiation and geographic distance was also detected in our study of *P. ginseng* with AFLP markers [22] [23]. The suggestion could be made that, in addition to the effect of species traits, other factors played a very important role in shaping the population genetic structure of *P. ginseng* in Russia.

Tree-based methods that use genetic distances between individuals to discover the species genetic structure are only loosely connected to statistical procedures that allow for the identification of homogeneous clusters of individuals [52]. A Bayesian algorithm implemented in the STRUCTURE program [38] identifies such groups without prior information about number of locations at which the individuals were sampled and also calculates the likelihood of plant belonging to specific group. As detailed in the results, the data obtained by STRUCTURE program are in agree with that obtained using UPGMA (**Figure 2**) and Ms-tree (**Figure 3**). The STRUCTURE analysis defined four homogeneous groups of *P. ginseng* populations (**Figure 4**). The number of STRUCTURE groups was the same as the number of sub-clusters of UPGMA and Ms-tree. The content of "green" and "yellow" STRUCTURE groups corresponded to two sub-clusters of the first main UPGMA and Ms-tree cluster and the plants of "red" and "blue" STRUCTURE groups consisted of plants that could be found in the two sub-clusters of second main cluster of these trees. However, with STRUCTURE the information was revealed that was not evident using UPGMA and Ms-tree. It became clear that none of the studied populations formed a distinct cluster (**Figure 4**). The admixture of ginseng plants belonging to different STRUCTURE clusters was shown. Some individuals showed a stronger genetic resemblance to populations other than their own putative populations. It is unlikely that effective pollen flow could generate such a high genetic resemblance to another population because the male gamete contains only half the genetic load of an individual. Therefore, the outlier genotypes detected by the assignment test in STRUCTURE can be considered the products of successful seed dispersal events, *i.e*., migrants [53]. The long-distance seed dispersal by birds, animals and also humans could result in the mixing of ginseng plants. Considering the history of controlled and uncontrolled ginseng cultivation in the fields and, more importantly, in the forest farms, we believe that human activity especially influenced on population genetic structure of *P. ginseng* in Russia. The low bootstrap values of the UPGMA dendrogram obtained in this study could be the result of the ginseng plant mixing phenomenon. The high amounts of linkage disequilibrium within *P. ginseng* populations could also be explained by population admixture [54]. The absence of private alleles in most *P. ginseng* populations also testifies to population admixture. At the same time, the genetic similarity of geographically distant ginseng populations may have been due to converging common selection forces in geographically disparate populations [55].

The individual assignment test revealed that only 58 samples out of 139 studied *P. ginseng* samples (42.0%) were assigned to their original populations (*i.e.* having the highest likelihood value of belonging to the populations they came from). The estimated immigration rate in the *P. ginseng* populations, analyzed with AFLP markers was considerably higher and equal 77.7% [22]. Some studied with AFLPs ginseng populations had 100% assignment success [22]. The comparative studies by other authors have also highlighted the higher discriminatory power of AFLP markers over microsatellites in population assignment tests [56]. Thus, in our study, SSR markers demonstrated low efficiency in assigning individuals to their population of origin to be chosen to serve as stock material for *P. ginseng* reintroduction.

5. Conclusion

In the present study using SSR markers, we discovered a high level of genetic diversity and obtained the main genetic characteristics for populations of wild *P. ginseng*. Ginseng in Russia does not require measures such as genetic rescue for species persistence but is in need of recovery measures because natural populations are largely exhausted as a result of the excessive harvesting of ginseng roots. Because ginseng populations are significantly differentiated all of them should be restored with the allelic frequencies determined in this study. Populations with high allelic richness should be granted priority consideration. Due to the mixing of ginseng plants, the *P. ginseng* populations should be checked with individual assignment test to verify the content of indigenous populations and to identify the "true" plants to serve as stock material for reintroduction. Considering the higher discriminatory power of AFLP markers over microsatellites in individual assignment tests, we recommend that the plants which were correctly assigned to their population of origin with AFLP markers should be chosen as material for *P. ginseng* reintroduction.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

YNZ and GDR designed research. GDR and ILK performed the research and analyzed the data. TIM collected the plants. GDR and OGK wrote the manuscript. YNZ helped in writing the manuscript and coordinating the study. All authors read and approved the final version of the manuscript.

Acknowledgements

This study was supported by the Russian Academy of Sciences, research projects No. 12-I-P30-02, No. 12-I-OBN-06 and by RFBR, research project No. 11-04-98515-r_vostok_a. We thank ever so much Irina Kats for participation in this study and mourn over her timeless decease.

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