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PLANT GENETICS

RAPD and Allozyme Analysis of Genetic Diversity in *Panax ginseng* C.A. Meyer and *P. quinquefolius* L.

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Abstract—Inter- and intraspecific variation of two ginseng species *Panax ginseng* and *P. quinquefolius* was estimated by studying 159 RAPD and 39 allozyme loci. Parameters of polymorphism and genetic diversity were determined and a tree was constructed to characterize the differences between individual plants, samples, and species. Genetic variation in *P. ginseng* proved to be lower than in *P. quinquefolius.* Gene diversity in the total *P. ginseng* sample was comparable with the mean expected heterozygosity of herbaceous plants. This suggests that wild *P. ginseng* plants in various areas of the currently fragmented natural habitat and cultivated plants of different origin have retained a significant proportion of their gene pool. The mean heterozygosity calculated per polymorphic locus for the RAPD phenotypes is similar to that of the allozyme loci and may be helpful in estimating gene diversity in populations of rare and endangered plant species.

INTRODUCTION

Seven [1] to fourteen [2] species of perennial herbaceous medicinal plants are assigned to the genus *Panax* L. (Araliaceae) by different authors. Most of these plants occur in Asia except for two species, *Panax trifolius* and *P. quinquefolius,* found in the east of North America [3]. Only *P. ginseng* C.A. Meyer (Oriental ginseng) and *P. quinquefolius* L. (American ginseng) are currently extensively cultivated, because the medicinal substances obtained from their roots are used in pharmaceutical, cosmetic, and food industry [1, 3]. Both species are natural tetraploids with chromosome number $2n = 4x = 48$ [4-7]. Despite morphological and anatomical similarity of these species, their hybrids are sterile [5, 8], and they are readily genetically distinguishable [9-11], which confirms their status of independent species.

Panax ginseng is currently represented by relatively small, considerably depleted natural localities restricted to Primorsky Krai of the Russian Far East and by "domesticated" forms cultivated for different periods [9, 12]. These half-wild cultivated ginseng plants retain all major properties of the wild plants. Note that *P. ginseng* is cultivated since 1st century B.C., whereas the plants from various forest plantation fields have a far shorter history of cultivation [1].

The history of *P. guinquefolius,* a representative of Northern American flora, is essentially similar to that of *P. ginseng.* The uncontrolled exploitation of the resources resulted in a reduction of the natural range of this plant, was depletion of its gene pool, and even a hazard of its extinction. *Panax quinquefolius* is cultivated since the late 19th century, and the plantation fields of cultivated American ginseng represent unimproved forms with an almost century-long history of cultivation [5].

Preliminary study of genetic diversity of the existing populations is required for the purpose of conservation and reintroduction of rare and endangered species. In recent years, the method of polymerase chain reaction with random primers (RAPD) is widely applied for specific, population, and individual identification of organisms with undetermined DNA nucleotide sequences [10,11,13, 14]. By this method, intervarietal and interlinear polymorphism was established in ginseng cultivated in Korea [10, 11] and the genetic distances were determined between natural and cultivated populations of P. *quinquefolius* [15].

We have previously studied genetic variation of wild and cultivated *P. ginseng* using RAPD and allozyme analyses [12, 16, 17]. In this study, genetic diversity of populations *P. ginseng* and *P. quinquefolius* was estimated by these two methods.

MATERIALS AND METHODS. We

used *P. ginseng* and *P. quinquefolius* plants

Panax ginseng: 15 plants of Spassk population, which were transferred from taiga of Spassk rayon and grown in a "forest farm," and 23 plants from a plantation field set up from roots of unknown origin purchased from amateur ginseng breeders. All cultivated plants were subdivided into two morphotypes, MI (14 plants) and MII (9 plants), which differed in their habitus and leaf plate form and color.

Panax quinquefolius: 22 plants grown from seeds of American cultivated ginseng.

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DNA isolation and amplification with decamer primers (Kit A-F, Operon Technologies, United States) was conducted as described previously [16,18]. Amplification products were fractionated by electrophoresis in 2% agarose containing ethidium bromide and photographed in UV-light. Only well discernable and reproducible bands were scored.

Statistical treatment was based on binary matrices, in which presence-absence of the same fragments was designated as 1 or 0. In compared samples, different intensity of bands corresponding to amplicons of the same size was not taken into account. The bands with significantly lower intensity were also designated as 0. Based on matrices and using POPGENE [19] and TFPGA [20] software packages, the proportion of polymorphic loci at 95% criterion *(P_{95})*, the number of alleles per locus (A), effective number of alleles per locus *(A_e)*, allele frequency (as in [21]), and mean expected heterozygosity *(H_e)* were calculated for all samples [22]. The populations were also tested for differentiation [23].

Coefficient of pairwise similarity between individuals was calculated from the formula: $S = 2N_{ab}/(N_a + N_b)$, where N_{ab} is the number of identical fragments in individuals *a* and *b;* N_a and N_b are the numbers of fragments in plants *a* and *b*, respectively [24]. Based on the *S* values, the mean intragroup similarity between individuals (S_{av}) was calculated for each sample. Based on pairwise matrices of genetic similarity (divergence), trees of genetic relationships between plants were constructed using UPGMA with bootstrap estimates for reliability of the branching order (TREECON software package [25]).

Mean sample gene diversity H_s, total gene diversity in the total sample H_T, and subdivision index G_{ST} were calculated as in [26].

Allozyme analysis was conducted as described previously [17] using leaves from live plants. Electrophoresis was performed in 13% starch gel in three buffer systems: Tris—citrate, pH 6.2; Tris-EDTA-borate, pH 8.6; and lithium-borate, pH 8.1/8.5. The following gene-enzyme systems were used: alcohol dehydrogenase (EC 1.1.1.1), isocitrate dehydrogenase (EC 1.1.1.42), shikimate dehydrogenase (EC 1.1.1.25), glutamate dehydrogenase (EC 1.4.1.2), formiate dehydrogenase (EC 1.2.1.2), sorbitol dehydrogenase (EC 1.1.1.14), malate dehydrogenase (EC 1.1.1.37), malic enzyme (EC 1.1.1.40), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucosephosphate isomerase (EC 5.3.1.9), triosephosphate isomerase (EC 5.3.1.1), phosphoglucomutase (EC 2.7.5.1), glutamate-pyruvate transaminase (EC 2.6.1.2), aspartate aminotransferase (EC 2.6.1.1), leucine aminopeptidase (EC3.4.11.1), uridinephosphate-glucose pyrophosphorylase (EC 2.7.7.9), hexokinase (EC 2.7.1.1), aconitase (EC 4.2.1.3), diaphorase (EC 1.6.4.3), aldolase (EC 4.1.2.13), acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.3.1), esterase (EC 3.1.1.1), and fluorescent esterase (EC 3.1.1.2).

Estimates of polymorphism, the number of alleles per locus, and mean heterozygosity were calculated by standard methods [27]; genetic distances between species were determined as described in [28].

RESULTS AND DISCUSSION

As shown previously [16], 84 out of 120 examined commercial decamer primers were efficient in polymerase chain reaction (PCR) with DNA from *P. ginseng.* Some of these randomly chosen efficient primers were used in this study (Table 1).

The use of 15 instead of 9 primers in RAPD analysis of two ginseng species increased the total number of tested loci by more than 1.5 times (260 instead of 159), whereas the average number of fragments per primer (17.67 and 17.33) and a proportion of polymorphic loci (0.7577 and 0.7673) were insignificantly altered. Among all scored fragments, at least 20% were shared by *P. ginseng* and *P. quinquefolim*, probably being marker fragments for the genus *Panax*. About 18% of fragments common for all 22 representatives of American ginseng, and about 30% of those common for 38 plants of Oriental ginseng, may be considered species-specific. The remaining amplicons were polymorphic. RAPD analysis with nine primers showed that in the total sample, *P. ginseng* polymorphism reached 35.48%, though in the individual samples, it varied from 9.68 to 14.52% (Table 2). These values are somewhat higher than those obtained earlier with a lower number of primers for the cultivated and wild ginseng [12] and by allozyme analysis [29]. In a sample of P. *quinquefolius*, polymorhism reached 41.28%, which is in agreement with data published on American ginseng (45.7%) [5] and testifies to a higher gene diversity of this species as compared to *P. ginseng*. The same is confirmed by the values of the effective number of alleles per locus A_e. For example, in a studied sample of *P. quinquefolius*, A_e was on average about 9% higher than in either of the examined samples of *P. ginseng* (Table 2), which is consistent with the results of allozyme analysis.

Coefficient of pairwise similarity 5 between *P. quinquefolius* plants varied from 0.8696 to 0.9849; in the total sample of Oriental ginseng, it varied from 0.8529 to 1.0000. The mean group similarity in *P. quinquefolius* plants was close to that in the total sample of *P. ginseng* but lower than in either individual sample, where the most similar were the plants of MII and MI morphotypes, and the least similar were individuals from the Spassk population (Table 2).

The figure shows a tree of genetic relationships between the examined plants, which is constructed from matrices of Nei's genetic distances (D_N) using UPGMA. All plants group into two clusters according to their species affiliation; D_N between these clusters is equal to 0.6147, which is similar to that between species of

Table 1. Primers used for studying genetic diversity of *P. ginseng* and *P. quinquefolius*

Primer	Nucleotide sequence (5' —— 3')	Number of polymorphic fragments/number of fragments taken into account		Total number of fragments taken into account
		P. ginseng	*P. quinquefolius*	
OPB-12	CCTTGACGCA	2/13	3/9	17
OPC-02*	GTGAGGCGTC	1/8	10/14	17
OPC-5*	GATGACCGCC	1/8	0/9	15
OPC-08	TGGACCGGTG	2/10	2/8	13
OPC-14*	TGCGTGCTTG	1/7	4/10	15
OPC-15	GACGGATCAG	2/13	11/17	22
OPD-02	GGACCCAACC	4/22	4/17	26
OPD-07	TTGGCACGGG	5/21	7/18	25
OPD-11	AGCGCCATTG	2/13	6/12	16
OPD-13	GGGGTGACGA	0/8	6/10	13
OPD-20	ACCCGGTCAC	1/12	6/11	16
OPE-11	GAGTCTCAGG	2/11	0/6	11
OPE-18*	GGACTGCAGA	1/10	6/17	22
OPF-04*	GGTGATCAGG	4/10	3/6	16
Total		29/175	71/178	260

* Primers additionally used for comparison *of P. quinquefolius* and the Spassk sample of *P. ginseng.*

Table 2. Parameters of genetic variation for *P. ginseng* and *P. quinquefolius*

Sample	*A*	Ae	P_{95}, %	S_{av}	H_e	$H_{e,}$ $p_k < 1 - 3/n$
P. ginseng (*n* = 14), morphotype I	1.0943	1.0583	12.20	0.9760 (0.0097)	0.0479 (0.0113)	0.0260 (0.0085)
P. ginseng (*n* = 9), morphotype II	1.0755	1.0548	9.68	0.9755 (0.0105)	0.0408 (0.0114)	0.0282 (0.0095)
P. ginseng (*n* = 23), plantation field	1.1698	1.0936	21.14	0.9592 (0.0198)	0.0783 (0.0129)	0.0597 (0.0116)
P. ginseng (*n* = 15), Spassk	1.1258	1.0799	14.52	0.9636 (0.0148)	0.0600(0.0128)	0.0486 (0.0117)
P. ginseng (*n* = 15), Spassk*	1.1115	1.0751	15.43	0.9671 (0.0129)	0.0648 (0.0110)	0.0514 (0.0101)
P. ginseng (*n* = 38), total sample	1.2830	1.1715	35.48	0.9236 (0.0406)	0.1348 (0.0142)	0.1133 (0.0144)
P. quinquefolius (*n* = 22)	1.2893	1.1831	41.28	0.9393 (0.0219)	0.1637(0.0159)	0.0897 (0.0153)
*P. quinquefolius** (*n* = 22)	1.2731	1.1776	39.33	0.9328 (0.0184)	0.1569 (0.0125)	0.1019 (0.0123)

Notes: *n*, the number of plants in a sample; *A*, the number of alleles per locus; A_e, effective number of alleles per locus; P_{95}, proportion of polymorphic loci at 95% criterion; S_{av}, mean intragroup similarity; H_e, mean expected heterozygosity for all loci; H_e, p_k <1 - 3/n, mean expected heterozygosity for all loci taking the fragments with the frequency lower than 1 - *3/n* as polymorphic. Standard deviations are given in parentheses. * Data obtained with 15 primers.

the genus *Aralia* (0.6451) that belongs to Araliaceae family (unpublished data). Note that the RAPD-based genetic distance between studied species of ginseng is higher than the distance calculated from analysis of 25 enzyme systems (D_N = 0.306). This can be explained by a lower number of loci studied by allozyme analysis and by the fact that the total variation of the studied genes is not reflected in this analysis, since electrophoresis does not detect all mutations [29, 30]. All *P. ginseng* representatives form a cluster, which is reliably (bootstrap index 99%) subdivided into two groups: one of them is formed by plants of the Spassk population, whereas cultivated plants are combined in the second group. The cultivated plants are in turn sub-

Table 3. Values of expected heterozygosity based on RAPD and allozyme analyses

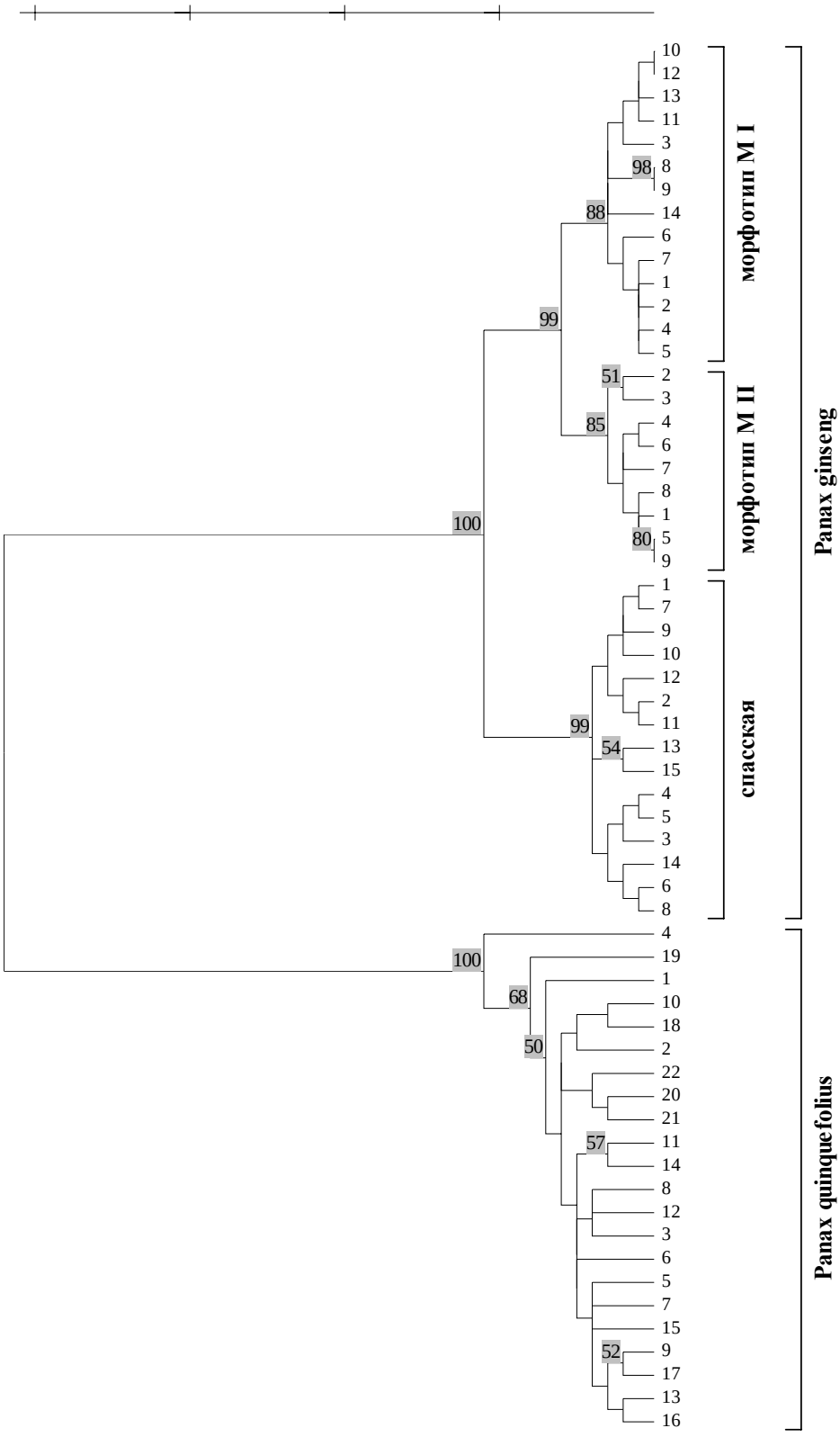
Parameter	*P. ginseng*				*P. quinquefolius*	
	plantation field		Spassk			
	RAPD (n = 23)	allozyme (*n* = 37)	RAPD (*n* = 15)	allozyme (*n* = 49)	RAPD (*n* = 22)	allozyme (*n* = 44)
Mean heterozygosity	0.0783 (0.0027)	0.0200 (0.0014)	0.0600 (0.0026)	0.0250 (0.0011)	0.1637 (0.0038)	0.06655 (0.00001)
Mean heterozygosity*	0.0597 (0.0023)		0.0486 (0.0024)		0.0897 (0.0026)	
Mean heterozygosity per polymorphic locus*	0.3674 (0.0141)	0.2600 (0.0230)	0.3515 (0.0177)	0.3300 (0.0220)	0.3762 (0.0110)	0.3660 (0.0010)
The number of polymorphic loci	20	3	17	3	26	6

Notes: *n*, the number of individuals in a sample; standard deviations are in parentheses. * The fragments with frequency lower than 1 - (3/n) were considered as polymorphic.

divided into two branches (bootstrap index 85 and 88%) with insignificant genetic distinction (D_N = 0.0593); each branch combines individuals of a certain morphotype. With five primers, it was also shown that the cultivated plants formed two clusters according to their morphotypes [12]. All examined wild plants from different areas of Primorsky Krai contribute to the same cluster with the morphotype I plants. In this study, the plants of the Spassk population from the forest farm formed a separate cluster (figure) and exhibit higher polymorphism than the previously studied wild plants from Spassk rayon.

In the studied *P. ginseng* samples, polymorphic fragments occur at different frequencies. The significance of differences in allele frequencies between samples was not similar. Differentiation test for all loci [23] showed heterogeneity of total *P. ginseng* sample and differentiation between plants of the Spassk population taken from the forest farm and cultivated plants (X^2 = 480.7248, *d.f.* = 318, *p* = 0.0000). Conversely, insignificant differences were determined between the frequencies of polymorphic fragments in morphotypes MI and MII (x^2 = 180.7422, *d.f.* = 318, *p* = 1.0000), though the intragroup similarity values were virtually the same and expected heterozygosities H_e were low (Table 2). The plants of these morphotypes may represent groups originating from a small number of closely related ancestors. Their progeny may have been formed mostly by self-pollination and apomixes, which were described for *P. ginseng* along with cross-pollination [31]. Thus, we consider all cultivated plants as those forming a single sample, in which all parameters of genetic variation are somewhat higher than in samples of individual morphotypes. Spassk population from the forest farm exhibits a lower intragroup similarity and higher expected heterozygosity, than the plants of two morphotypes, and therefore, the former may be considered as an isolated group in the total sample of *P. ginseng* plants.

In an earlier study estimating genetic variation for 25 enzyme loci in wild and cultivated plants of *P. ginseng*, 36 monomorphic and 3 polymorphic genes were identified and "damaged" genetic structure of populations of this endangered species was determined from allozyme heterozygosity [29]. Average heterozygosity of the wild ginseng (0.03 and lower) does not much differ from that of cultivated ginseng. These values are low as compared to mean heterozygosity of herbaceous plants (0.13 [32]), but similar to the estimates obtained by allozyme analysis for many rare and endangered species with restricted ranges [33-37]. Note that the allozyme markers incompletely reflect genomic variation of a species, because it can be determined only for expressed genes, which are not always selectively neutral. With RAPD markers genetic variation can be evaluated more completely, because the noncoding DNA sequences that are under less strong selection also can be analyzed with these markers [38, 39]. However, they are dominant and, therefore, it is impossible to distinguish the dominant homozygotes from heterozygotes and, consequently, to calculate such parameters of genetic variation as F_{IS} and F_{IT}. In recent years, authors who compared parameters of genetic variation in the same samples of plant species revealed positive correlation between parameters based on allozyme and RAPD markers [39-41]. However, the expected heterozygosity and F_{ST} calculated from RAPD phenotypes were inconsistent with those based on RAPD genotypes and allozyme analysis. Deviation in these parameters is reduced if polymorphic fragments with the frequency of Pt > 1 - (3/n), where *n* is the sample size [21, 40], are excluded (Table 3). With this approach used to calculate mean heterozygosity per polymorphic locus, the deviations are not so significant and seem to reflect differences in the number of analyzed loci and in variation of individual RAPD and allozyme loci in the samples examined. The H_e values calculated for all RAPD loci in both total sample of *P. ginseng* and in each sample (Spassk and plantation field) individually are lower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Panax ginseng
Panax quinquefolius

than the H_e value for the sample of plantation field *P. quinquefolius* (Table 2). Mean heterozygosity per polymorphic locus of P. *quinquefolius* is close to that in the total sample of *P. ginseng* (0.3767), but is by 7% higher than in plants of the Spassk population and by 2.4% higher than in the cultivated plants of *P. ginseng* (Table 3). This data testify to higher genetic variation of American ginseng. If monomorphic loci are excluded, average heterozygosity of the total *P. ginseng* sample is higher than in the *P. quinquefolius* sample (Table 2), because in the latter, over 40% of the polymorphic loci have low frequencies of the null allele.

Table 4 shows parameters of gene diversity in *P. ginseng* samples, which are calculated for each primer and all loci as a whole. The primers differed in their ability to reveal gene diversity. For some primers, high values of G_{ST} (a measure of population differentiation) calculated for polymorphic loci and for all loci suggest the presence of fixed alleles in the samples. The G_{ST} parameter calculated for polymorphic loci for all primers is equal to 0.4331. Hence, at least 40% of the total variation of this species is accounted for by the interpopula-tion component, whereas variation in the samples themselves was not very high. The G_{ST} calculated as described in [26] is higher and more sensitive to differences in allele frequency in the samples [42]. High values of G_{ST} are characteristic only of self-pollinating plants [43]. Therefore, low variation of *P. ginseng* within the samples may result from inbreeding and subdivision at the family level. In the total sample, low mean sample gene diversity and far higher general gene diversity of the total sample may reflect the founder effect, i.e., the origin of the cultivated plants from one or several closely relative individuals.

Thus, our results for both examined *Panax* species show that the values of mean expected heterozygosity for the polymorphic loci inferred from on allozyme and RAPD analysis differ insignificantly. RAPD analysis is preferable for estimating genetic variability and differentiation of rare species, especially when allozyme locus variation is low, because this method requires no high DNA quality and preliminary information on the primary DNA structure, and polymorphism and selective neutrality of RAPD loci are higher than those of the functional loci, for example, allozymes [38,41].

These data suggest that genetic variation in *P. ginseng* is lower than in *P. quinquefolius.* Mean expected heterozygosity of the total *P. ginseng* sample based on RAPD phenotypes is 0.1348, which is similar to the corresponding parameter assessed by allozyme analysis for herbaceous plants [32]. Note that each studied sample (Spassk and plantation field) show high mean sample similarity and low gene diversity. This suggests that wild *P. ginseng* plants inhabiting various localities of a currently fragmented areal and cultivated plants of different origin retained a large proportion of gene pool. However, recurrent inbreeding and restricted cross-pollination in ginseng [31] have probably led to low sample variation and homozygous samples. Therefore, while developing the artificial reserves for preservation and reintroduction of this endangered species, the plants from a higher number of natural habitats (localities) and private plantation fields should be used. Preliminary estimation of genetic diversity by the RAPD method is also required.

Table 4. Parameters of gene diversity in *P. ginseng* samples

Primers	Hs	H_T	G_{ST}	G_{ST}	G_{ST}
OPC-08	0.0592	0.0648	0.0056	0.0857	0.0698
OPD-11	0.0850	0.1763	0.0914	0.5180	0.4259
OPD-13	0.0000	0.0000	0.0000	0.0000	0.0000
OPD-02	0.0372	0.1211	0.0839	0.6931	0.6213
OPD-07	0.0840	0.1048	0.0208	0.1985	0.1763
OPD-20	0.0162	0.1407	0.1245	0.8849	0.7426
OPC-15	0.0387	0.1916	0.1529	0.7981	0.6744
OPB-12	0.0706	0.0850	0.0147	0.1688	0.1728
OPE- 11	0.0663	0.0853	0.0190	0.2222	0.2356
Mean	0.0539	0.1141	0.0602	0.5278	0.4321

H_S, mean sample gene diversity for all loci; H_T, total gene diversity in total sample *of P. ginseng;* D_{ST}, total diversity between samples; G_{ST}. subdivision parameter calculated as described in [26].

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