ISSN 1022-7954, Russian Journal of Genetics, 2018, Vol. 54, No. 2, pp. 262–265. © Pleiades Publishing, Inc., 2018.
Original Russian Text © E.A. Vasyutkina, I.Yu. Adrianova, G.D. Reunova, T.P.T. Nguyen, Yu.N. Zhuravlev, 2018, published in Genetika, 2018, Vol. 54, No. 2, pp. 266–270.

SHORT COMMUNICATIONS

A Comparative Analysis of Genetic Variability and Differentiation in *Panax vietnamensis* Ha et Grushv. and *P. ginseng* C.A. Meyer Using ISSR Markers

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Received March 22, 2017; in final form, May 10, 2017

Abstract—A comparative analysis of the genetic variability and differentiation of rare medicinal ginseng species, *Panax vietnamensis* Ha et Grushv. and *P. ginseng* C.A. Meyer, was carried out using inter-simple sequence repeat markers. It was demonstrated that all the genetic diversity parameters of Vietnamese ginseng were high and considerably exceeded those of *P. ginseng*. On the contrary, the level of genetic differentiation was higher in true ginseng. It is suggested that the differences in the levels of genetic variability and differentiation of the two ginseng species were influenced by the demographic history, peculiarities of the reproductive system, and human activity.

Keywords: Panax vietnamensis, Vietnamese ginseng, *P. ginseng*, rare plants, genetic variability, ISSR

DOI: 10.1134/S1022795418020163

All representatives of the genus of herbaceous perennials *Panax* L. are of medical importance. One of these is *Panax vietnamensis* Ha et Grushv., found in the highlands of central Vietnam, at the southern border of the genus range [1–3]. The commercial success of Vietnamese ginseng arose in the mid-1970s with the beginning of pharmacological studies. Currently, *P. vietnamensis* grows only in two protected areas in the Quang Nam and Kon Tum provinces, located on the slopes of Ngoc Lin Mountain at an altitude of more than 1500 m above sea level and is listed in the Red Data Book of Vietnam [2]. Another representative of this genus is true ginseng, *P. ginseng* C.A. Meyer, which is found in natural habitats only in the south of the Russian Far East, although until the middle of the last century it was also distributed in northeast China and the north of the Korean Peninsula [3]. In the Red Data Book of the Russian Federation, it has the status of endangered species [4]. The main factors influencing the range size reduction of both species are their excessive exploitation owing to high medicinal value, as well as the destruction of natural habitats resulting from human economic activity. These factors contribute to reducing the genetic diversity level of ginseng populations, which in turn can lead to a considerable depletion of the species gene pool and, in the future, to its extinction. Genetic variability is critical for adaptation of a species to environmental changes and for its long-term survival. This study presents the results of a comparative analysis of the genetic variability and genetic differentiation of *P. vietnamensis* and *P. ginseng* using inter-simple sequence repeat markers (ISSR).

Fresh leaves of Vietnamese ginseng were sampled from two populations in the Quang Nam and Kon Tum provinces (Vietnam). Young leaves of *P. ginseng* were sampled from the populations of Spasskii and Nadezhdinskii administrative regions of Primorsky Krai (Russia). The material was stored in silica gel at 4°C. Genomic DNA was isolated according the method of Ehta et al. [5]. Primers for ISSR analysis were synthesized by Syntol Company (Moscow, Russia) in accordance with a set of primers developed at the University of British Columbia (UBC, Canada). Of the 35 primers, 13 were selected, which gave clearly visible and reproducible fragments with the ginseng DNA (Table 1). PCR was performed in a final volume of 20 μL, containing 10–20 ng DNA, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 1× *Taq* buffer with KCl, 0.7 units of recombinant *Taq* DNA polymerase (Thermo Scientific), and 0.1 μM primer. The PCR temperature profile consisted of denaturation at 94°C for 5 min, followed by 40 cycles, with three steps in each cycle, consisting of 45 s at 94°C, 45 s at 55°C, 45 s at 72°C; and final elongation at 72°C for 10 min. The PCR reaction with each primer was repeated at least twice. The PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide.

Table 1. List of ISSR primers used in the study and amplified DNA fragments

Primer	Sequence 5' → 3'	Fragment size, bp	Number of fragments/number of polymorphic fragments	Number of polymorphic fragments for a species	
				P. vietnamensis	*P. ginseng*
UBC807	$(AG)_8T$	213–1674	29/25	21	5
UBC808	$(AG)_8C$	252–2010	32/29	28	2
UBC809	$(AG)_8G$	200–1056	27/20	12	1
UBC811	$(GA)_8C$	389–2045	25/23	20	4
UBC826	$(AC)_8C$	424–1379	17/14	13	2
UBC836	$(AG)_8YA$*	203–1318	25/24	22	6
UBC842	$(GA)_8YG$*	334–1423	24/22	16	3
UBC855	$(AC)_8YT$*	306–2500	26/26	21	5
UBC856	$(AC)_8YA$*	314–1408	22/20	17	9
UBC857	$(AC)_8YG$*	420–2124	21/17	15	3
UBC866	$(CTC)_6$	549–1905	14/12	8	3
UBC873	$(GACA)_4$	313–1356	27/26	22	13
UBC880	$(GGAGA)_3$	323–1257	16/13	5	6
Mean			23.5/20.9	16.9	4.8
Total			305/271	220	62

* Y = (C, T).

The analysis was performed on the basis of a binary matrix in which the presence or absence of fragments was designated as “1” or “0.” Since the ISSR markers are dominant, the calculations were carried out under the assumption that the populations were in the Hardy–Weinberg equilibrium. The parameters of genetic variability and its distribution within and between populations (AMOVA) were obtained using the GenAlEx 6.2 add-in for the MS Excel spreadsheet [6]. To exclude the influence of the sample size in calculating the proportion of polymorphic loci, the 95% criterion (P_{95}) in the TFPGA software program was applied [7]. The gene flow was calculated using the formula $Nm = (1 - \Phi_{ST})/4\Phi_{ST}$ [8].

Since the use of the Bayesian approach avoids the limitation of dominant markers, a Bayesian parameter of genetic differentiation θ^{II} in the Hickory v1.1 software program was obtained [9]. Of the four Bayesian models studied, the best fit for both ginseng species was the full model with the lowest deviance information criterion (DIC).

Population structure of *P. vietnamensis* and *P. ginseng* was analyzed using the STRUCTURE v2.3.2 software program [10]. The following parameters were set: burn-in period 300000, iterations 500000, correlated allele frequencies, admixture model. The number of putative clusters (K) was in the range from 1 to 5 for each species. Three runs of the analysis were performed for each value of K. The most probable number of K was determined in the Web program Structure Harvester [11].

The analysis of the entire set of data for two ginseng species yielded 305 ISSR markers, of which 271 (89%) fragments were polymorphic (Table 1). Depending on the used primer and the species assignment, the number of polymorphic bands varied. More than 53% of all scored fragments were common to both species. One hundred fifteen amplicons were characteristic only for *P. vietnamensis*, and 28 amplicons belonged to *P. ginseng*. Each ginseng species had 21 marker bands.

Table 2 shows the genetic variability indices of the species under study. Contrary to expectations, the level of genetic diversity of Vietnamese ginseng was high and comparable to that of cultivated *P. ginseng* from northeastern China, as well as to that of wild and cultivated *P. quinquefolius* L. from eastern North America, analyzed with ISSR and RAPD markers, respectively [12, 13]. In contrast to *P. vietnamensis*, true ginseng from Primorskii Krai has low genetic variability both at the species level and at the population level (Table 2). The differences in the levels of genetic diversity of the species analyzed can be the result of their different demographic history associated with the geological formation of the territories on which they grow. Previously, it was demonstrated that *P. ginseng* had experienced a bottleneck [14]. Thus, the absence of refugia for true ginseng during the last Pleistocene–Holocene cold spell in the south of the

Table 2. Genetic variability and differentiation parameters for *P. vietnamensis* and *P. ginseng*

Population/species	N	I	H_e	P_{95}, %	Φ_{ST}	θ^{II} (2.5%–97.5%)	Nm
Quang Nam	35	0.333 (0.017)	0.221 (0.012)	61.3	–	–	–
Kon Tum	23	0.367 (0.016)	0.244 (0.011)	65.6	–	–	–
P. vietnamensis	58	0.381 (0.015)	0.251 (0.011)	70.9	0.113	0.123 (0.083–0.159)	1.962
Spasskaya	15	0.090 (0.012)	0.059 (0.010)	14.4	–	–	–
Nadezhdinskaya	15	0.112 (0.16)	0.074 (0.011)	18.9	–	–	–
P. ginseng	30	0.139 (0.017)	0.091 (0.011)	24.9	0.269	0.268 (0.147–0.425)	0.679

N, sample size; I, Shannon index; H_e, expected heterozygosity, standard deviations are given in brackets; P_{95}, the proportion of polymorphic loci at 95% criterion; Φ_{ST}, genetic differentiation index (AMOVA, $P < 0.001$, 999 permutations); θ^{II}, Bayesian genetic differentiation parameter, in brackets is the 95% confidence interval; Nm, gene flow.

Russian Far East led to a sharp reduction in its abundance. Then, in the Early Holocene, the climate warming facilitated the restoration of heat-loving ginseng from a small number of surviving individuals and its northward dispersal along the Ussuri River. Further centuries-old existence of populations with low plant density created conditions for inbreeding, which obviously did not provide the restoration of genetic diversity. The situation was aggravated by active removal of ginseng roots in the forests of Primorsky Krai which happened at the beginning of the 20th century and led to even more severe depletion of the species gene pool [15]. In contrast, Vietnamese ginseng is located on the southern border of the *Panax* range, in a region that was not affected by glaciation. Therefore, it is suggested that *P. vietnamensis* was not subjected to a bottleneck. Owing to the fact that Vietnamese ginseng grows in hard-to-reach areas, namely, in humid tropical high-mountain forests, its medicinal properties for a long time were known only to small ethnic groups, so the consequences of anthropogenic pressure, probably, did not have time to affect its genetic diversity.

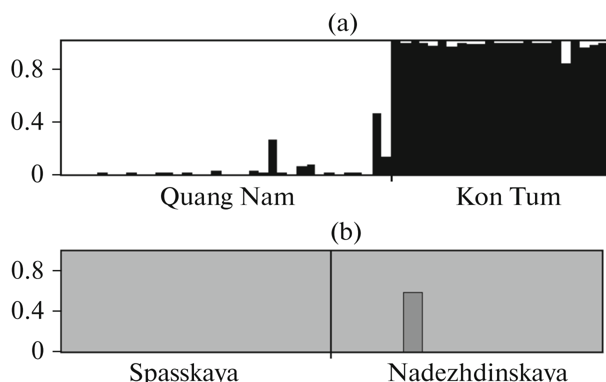

Fig. 1. The genetic structure of *P. vietnamensis* (a) and *P. ginseng* (b) obtained using the Bayesian algorithm in the STRUCTURE v2.3.2 software program. The scale to the left shows the probability of assignment of each plant to one of the genetic clusters (white and black color for *P. vietnamensis*, light and dark gray color for *P. ginseng*).

Analysis of the genetic structure in each species using AMOVA and the Bayesian approach showed that the values of the parameters of genetic differentiation Φ_{ST} and θ^{II} were similar (Table 2). It was demonstrated that, in Vietnamese ginseng, the interpopulation component accounted for 11.3% of total genetic variability, whereas in true ginseng, the value of this parameter was 26.9%. The low level of genetic differentiation in *P. vietnamensis*, compared to *P. ginseng*, is determined by higher gene flow (1.96 vs. 0.68, Table 2), which can be associated with both geographical closeness of Vietnamese ginseng populations and the presence of a higher number of insect pollinators, as well as birds and small animals that promote the seed dispersal over its range. In our opinion, the main factor influencing the genetic differentiation of *P. ginseng*, in addition to geographic remoteness, is the reproductive system, since it was demonstrated that the formation of seeds in this species occurred mainly by self-pollination and agamospermy and less often by cross pollination [15, 16]. Similar studies of the *P. vietnamensis* reproduction system are absent from the available literature; therefore, it is not possible to evaluate the effect of this factor on the genetic differentiation in Vietnamese ginseng.

Bayesian analysis of the genotype distributions in the STRUCTURE software program showed the most probable segregation of the *P. vietnamensis* specimens into two genetic clusters ($K = 2$), which corresponded to the population assignment (Fig. 1a). However, in both populations, there were plants that to different extent belonged to another cluster, pointing to the gene exchange between them. At the same time, for *P. ginseng*, the probability of assigning both populations to a single genetic cluster (indicated in light gray) was demonstrated, and only one plant accession from the Nadezhdinskaya population by 60% belonged to the second genetic cluster (shown in dark gray in Fig. 1b, $K = 2$). The reason for this clustering pattern of true ginseng is probably associated with mixing of plants belonging to different populations following the controlled and uncontrolled cultivation of *P. ginseng* in the

forests of Primorsky Krai which took place in the middle of the 19th century [3].

Thus, a comparative population genetic analysis of two ginseng species carried out using ISSR markers revealed that *P. vietnamensis* and *P. ginseng* were characterized by different levels of genetic variability and differentiation that reflected the features of their historical past and the reproductive system. Vietnamese ginseng, which has a limited natural distribution and rare occurrence in nature, is characterized by high genetic diversity along with low genetic differentiation, which confirms our supposition that human activity had no considerable effect on its genetic diversity, in contrast to *P. ginseng*. Nevertheless, given the narrow localization and high medicinal value of *P. vietnamensis*, the need to develop a conservation strategy for this species remains urgent.

ACKNOWLEDGMENTS

We thank Cand. Sci. (Biol.) T.I. Muzarok for assistance in the collection of material in Primorsky Krai.

This study was supported by the Presidium of the Russian Academy of Sciences (grant nos. 15-I-6-029, 15-I-6-030 and VANT14-005) and by the Vietnam Academy of Science and Technology (VAST) (grant no. VAST.HTQT.Nga 10/15-16).

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Translated by N. Maleeva