

# Genetic Structure of Natural Populations of the Relict Species *Aristolochia manshuriensis* (Aristolochiaceae) in Disturbed and Intact Habitats

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**Abstract**—Genetic structure of natural populations of the rare relict plant Manchurian birthwort (*Aristolochia manshuriensis* Kom.) in the Russian part of its area was analyzed using allozyme markers. The studied *A. manshuriensis* populations differed in the degree of their intrapopulation differentiation. The populations Nezhinka and Anan'evka were more differentiated ( $F_{ST} = 0.1209$  and  $0.0576$ , respectively); these populations are located close in the regions of intense economic activity and are exposed to the strongest anthropogenic impact. A low degree of differentiation was detected in the population Malaya Borisovka ( $F_{ST} = 0.0393$ ), localized to intact habitats. The overall heterogeneity test has demonstrated that the population Malaya Anan'evka, exposed at present to small anthropogenic stress yet growing in disturbed habitats, displays no differentiation. These results suggest that at least three populations are influenced by genetic drift connected with a decrease in the reproductive and effective population sizes, which is caused, in particular, by anthropogenic impact. A high level of genetic similarity between the *A. manshuriensis* populations in Primor'e is discussed in connection with the evolutionary history of this species.

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## INTRODUCTION

The world science now pays considerable attention to the research into population genetic structure of rare and endemic species [1–3]. Despite that the genetic consequences of reduction in population sizes have been theoretically predicted, the corresponding empirical data are rather scarce, especially for relict plants. One of such plants, Manchurian birthwort (Dutchman's pipe) *Aristolochia manshuriensis* Kom., is a relict woody liana endemic to the Manchurian region [4]. This plant is used as a basic component in Chinese medicine. Its range is confined to North Korea, northeastern China, and southwestern Primorsky krai; however, this plant by now has almost disappeared from natural habitats due to intense harvesting and disturbance of its habitats [4–6].

In Russia, the species *A. manshuriensis* is in the Red List of the Russian Federation as an endangered species [7]. Several authors believe that the modern Manchurian birthwort populations in the South Primor'e flora are of autochthonous origin [6, 8, 9]. The known habitats of this plant in Primor'e are the basins of Borisovka, Nezhinka, and Anan'evka Rivers [10]. In populations, Manchurian birthwort grows with a nonuniform density as monodominant groups (patches) [4]. Individual plants are sometimes found at a distance of 0.5 km and more from each other; this is especially characteristic of the plants from the population of Borisovka River and its tributary, Malaya Borisovka River, where

the patches are weakly pronounced. The populations Nezhinka and Anan'evka consist of juvenile plants; reproductive plants have not been found. Presumably, this is connected with forest fires and uncontrolled cutting of this liana due to a high medicinal value of its extract [5]. Small Manchurian birthwort habitats with adult lianas have remained in the headstream of a tributary of the Anan'evka River, Malaya Anan'evka River, and, possibly, in the upper flow of the Nezhinka River, in hard-to-reach sites. The population Malaya Borisovka comprises plants of various ages. A more favorable state of this species in this habitat is explainable by a hard accessibility of this place. Overall, the present natural resources of this liana are so limited that its commercial use as a medicinal raw stuff is completely out of question, and the preservation and restoration of its natural populations (while it is still feasible) is an urgent issue.

Study of the population genetics of this rare plant is necessary to develop the strategy for preservation of its populations, especially taking into account that its area is fragmented and the exposure to anthropogenic factors constantly reduces its populations. No genetic description of *A. manshuriensis* populations is available, as well as of other members of the family Aristolochiaceae. We have earlier selected the allozyme markers for *A. manshuriensis* and described its polymorphism on the territory of Russia [11]. The goal of this work was to describe the *A. manshuriensis* inter-

**Table 1.** Wright's  $F$ -statistics for five polymorphic loci in four *Aristolochia manshuriensis* natural populations

Population	$F_{IS}$	$F_{IT}$	$F_{ST}$
Nezhinka (5 samples)	-0.1284	-0.0277	0.1209
Anan'evka (3 samples)	-0.1424	-0.0842	0.0576
Malaya Borisovka (5 samples)	-0.0972	-0.0620	0.0393
Malaya Anan'evka (3 samples)	-0.1862	-0.1627	0.0224
Total set of samples (16 samples)	-0.1285	0.0049	0.1465
Four populations (total samples for populations)	-0.0725	-0.0168	0.0650

Note:  $F_{IS}$  is subpopulation inbreeding coefficient;  $F_{IT}$ , overall inbreeding coefficient; and  $F_{ST}$ , the degree of genetic differentiation in subpopulations.

and intrapopulation differentiation using these allozyme markers. In addition, we attempted to analyze the genetic processes, primarily genetic drift and inbreeding, in connection with the degree of anthropogenic stress in the habitats of this plant in the Russian part of its area.

## MATERIALS AND METHODS

For electrophoretic analysis, *A. manshuriensis* plants were harvested in 2002–2007. Plant leaves were collected in 16 samples within 4 natural populations of Primorye located in the basins of Nezhinka River (five samples,  $N = 13, 12, 9, 13,$  and  $14$  plants; totally 61), Anan'evka River (three samples,  $N = 27, 9,$  and  $9$  plants; totally 45), Malaya Borisovka River (five samples,  $N = 21, 26, 25, 9,$  and  $28$  plants; totally 109), and Malaya Anan'evka River (three samples,  $N = 28, 15,$  and  $15$ ; totally 58 plants). Each sample is an individual group of plants of different ages. As both seed reproduction [6, 8] and vegetative reproduction [4, 9] have been described in *A. manshuriensis* natural populations, the sampled plants within one group were selected at a distance of at least 15 m between one another, and the groups within population were located at a distance of 300 m to 4 km.

Enzymes were fractionated by electrophoresis in 13% starch gel in three buffer systems—Tris–citrate (pH 6.2), Tris–citrate (pH 7.8), and Tris–EDTA–borate (pH 8.6)—as earlier described [11]. Enzymes were extracted from the leaves frozen in liquid nitrogen. In this work, we used five polymorphic loci described earlier: *Gpt* (glutamate-pyruvate transaminase, EC 2.6.1.2), *Gpi-2* (glucose phosphate isomerase, EC 5.3.1.9), *Acp-2* (acid phosphatase, EC 3.1.3.2), *Fe-2* (fluorescent esterase, EC 3.1.1.2), and *Pgm* (phosphoglucosmutase, EC 2.7.5.1).

Wright's  $F$ -statistics [12] (subpopulation inbreeding coefficient ( $F_{IS}$ ), overall inbreeding coefficient ( $F_{IT}$ ), and the degree of genetic differentiation in subpopulations ( $F_{ST}$ ) were calculated for each population to analyze their structure. In addition,  $F_{ST}$  was calculated for the populations. All coefficients were calculated for each individual locus and as a mean over all loci.

The program Bottleneck [13, 14] was used to detect the deviations from equilibrium state; this program makes it possible to analyze the probability of equilibrium between the gene drift and mutational process in each population based on the infinite allele mutation (IAM) model [13]. According to this model, the number of alleles in the populations after a recent reduction in their effective size decreases faster than their heterozygosity. For example, the observed gene diversity in the populations after a bottleneck is higher as compared with the expected values calculated according to the observed number of alleles with assumed equilibrium microevolutionary processes in the population [14].

To determine the genetic relationships between the *A. manshuriensis* samples and populations, we estimated their similarity based on Nei's genetic distances [15], calculated for nine loci (five polymorphic and four monomorphic) described earlier [11]. The dendrogram of population similarity was constructed from the coefficients of Nei's genetic distances using the unweighted pair group method with arithmetic mean (UPGMA).

## RESULTS

The *A. manshuriensis* samples studied differed in the allelic frequencies. The test for heterogeneity of allelic frequencies detected statistically significant differences between the samples in all populations except for the population Malaya Anan'evka. The population genetic structure of this species was analyzed using Wright's  $F$ -statistics (Table 1). The subpopulation inbreeding coefficient ( $F_{IS}$ ) values averaged over all loci suggested an excess heterozygotes within samples, namely, 12.8% in the samples of population Nezhinka, 14.2% in population Anan'evka, 9.7% in population Malaya Borisovka, and 18.6% in population Malaya Anan'evka. The mean  $F_{IT}$  values were also negative, thereby demonstrating an excess of heterozygotes within populations. The  $F_{ST}$  values demonstrated that the main part of genetic diversity in all populations is intrasample variation. The highest level of differentiation was observed in the population Nezhinka, where 12% of the overall variation was represented by intersample variation. As for the populations Anan'evka,

**Table 2.** Statistical significance of excess (+) or deficiency (–) of the diversity in *Aristolochia manshuriensis* populations according to three tests based on infinite allele mutation model [13]

Population	Sign test [35]	Standardized differences test [35]	Wilcoxon sign-rank test [36]	“Mode-shift” indicator
Nezhinka	+ 0.010*	+ 0.005*	+ 0.016	Presence of deviation
Anan’evka	+ 0.010*	+ 0.004*	+ 0.016	Presence of deviation
Malaya Borisovka	+ 0.284	+ 0.008*	+ 0.078	Equilibrium
Malaya Anan’evka	+ 0.031	+ 0.003*	+ 0.031	Presence of deviation

\* Significant at  $p > 0.010$ .**Table 3.** Matrix of mean genetic distances according to Nei [15]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	0	0.0101	0.0104	0.0544	0.0095	0.0298	0.0186	0.0414	0.0188	0.0145	0.0069	0.0095	0.0101	0.0174	0.0162	0.0187
2		0	0.0003	0.0351	0.0103	0.0331	0.0141	0.0603	0.0291	0.0330	0.0247	0.0273	0.0253	0.0343	0.0365	0.0379
3			0	0.0307	0.0085	0.0317	0.0156	0.0562	0.0266	0.0317	0.0236	0.0253	0.0247	0.0315	0.0351	0.0353
4				0	0.0303	0.0451	0.0445	0.0611	0.0518	0.0673	0.0594	0.0562	0.0552	0.0455	0.0641	0.0613
5					0	0.0107	0.0126	0.0209	0.0055	0.0102	0.0076	0.0072	0.0076	0.0087	0.0128	0.0103
6						0	0.0113	0.0112	0.0065	0.0111	0.0156	0.0162	0.0089	0.0107	0.0118	0.0116
7							0	0.0391	0.0208	0.0217	0.0206	0.0249	0.0133	0.0244	0.0233	0.0281
8								0	0.0078	0.0100	0.0160	0.0127	0.0131	0.0057	0.0128	0.0055
9									0	0.0033	0.0056	0.0044	0.0052	0.0047	0.0065	0.0022
10										0	0.0019	0.0020	0.0018	0.0040	0.0049	0.0014
11											0	0.0007	0.0017	0.0042	0.0041	0.0032
12												0	0.0030	0.0028	0.0055	0.0019
13													0	0.0035	0.0038	0.0040
14														0	0.0055	0.0020
15															0	0.0045
16																0

Note: samples 1–5, population Nezhinka; 6–8, population Anan’evka; 9–13, Malaya Borisovka; and 14–16, Malaya Anan’evka.

Malaya Borisovka, and Malaya Anan’evka, this value was smaller, amounting to 5.7, 3.9, and 2.4%, respectively. Table 1 lists also the values of hierarchical  $F$ -statistics for the set of samples (all studied samples) and populations (with the samples from each population pooled into a total sample). Interestingly,  $F_{IS}$  was negative at all levels, indicating an excess of heterozygotes in both samples and populations; note that it was the lowest at the population level (7.2%). The  $F_{IT}$  value at the population level was also negative; only for the overall set of samples, its value suggests an insignificant deficiency of heterozygotes (0.4%). The differentiation coefficient  $F_{ST}$  was 0.0650 between the populations and 0.1465 over the entire set of samples.

The results of the tests for shift in population equilibrium using the Bottleneck program are listed in Table 2. Two of the three tests used demonstrated a statistically significant excess of genetic diversity in the populations Nezhinka and Anan’evka and only one test, in the populations Malaya Borisovka and Malaya Anan’evka.

In addition, the mode-shift indicator, differentiating between the populations after a bottleneck and stable populations, demonstrates a shift in equilibrium in three populations—Nezhinka, Anan’evka, and Malaya Anan’evka. Thus, the analysis suggests that only the population Malaya Borisovka is in an equilibrium state and demonstrate that the population equilibrium in the remaining populations is shifted towards gene drift connected with a recent reduction in their size.

The similarity between the *A. manshuriensis* samples and populations studied was quantitatively assessed using the coefficients of Nei’s genetic distances [15] (Table 3). The calculated genetic distances ( $D_N$ ) between the samples fall into the range of 0.0003 (between samples 2 and 3 of the population Nezhinka) to 0.0673 (between samples 4 of the population Nezhinka and 10 of the population Malaya Borisovka). The mean genetic distances between the samples in the populations Nezhinka and Anan’evka were 0.0199 and 0.0205, respectively; in the populations Malaya Boris-

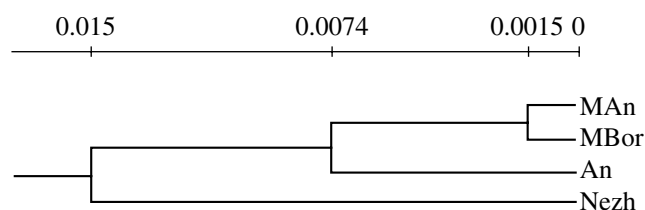
ovka and Malaya Anan'evka, this value was by one order of magnitude smaller, amounting to 0.0029 and 0.004, respectively.

The mean genetic distance between the Manchurian birthwort populations was 0.0096. The UPGMA dendrogram (figure) illustrates the genetic relationships between the studied populations. In this dendrogram, the populations Malaya Borisovka and Malaya Anan'evka cluster together with the minimal distance of 0.0015; the population Anan'evka is adjacent; and the most distant in the population Nezhinka.

## DISCUSSION

The analysis of *A. manshuriensis* population genetic structure has demonstrated that the studied populations differ in the degree of internal differentiation. Interestingly, rather compact populations Nezhinka and Anan'evka (Table 1) appeared the most heterogeneous, whereas the internal differentiation was absent in the population Malaya Anan'evka and low in the most extended population, Malaya Borisovka. The population genetic structure of a species is largely determined by several processes, such as gene drift, inbreeding, and selection [16–20]. In differentiated populations, the gene exchange between samples can withstand genetic erosion if its intensity is high enough [16, 17, 21]. The intensity of gene flow is determined by the presence of isolation barriers and the ability of a species to disseminate its genetic material [16, 17], which in plants is first and foremost connected with the mating system (vegetative or seed reproduction, self-pollination, cross-pollination, or apomixis) and the way of seed dissemination [18]. Like many members of the genus *Aristolochia*, Manchurian birthwort have special devices adapted for cross-pollination by the flower flies of the genus *Pegoplata* [22], which are able to provide an efficient pollen transport over only small distances. In the case of a patch population structure, this enhances the population differentiation into individual mating groups, where gene drift and inbreeding dominate among the microevolutionary processes.

Note that forest fragmentation changes the gene flow within populations with a patch structure, as has been illustratively demonstrated when comparing the sugar maple populations with patchy and continuous structures [23]. The studied Manchurian birthwort populations differ in their area, plant density and age composition, and the degree of anthropogenic stress. The largest in size and area is the population Malaya Borisovka; the plants in it are more uniformly distributed, and the patch structure is weakly pronounced. It is the only population that is almost unexposed to anthropogenic impact, inhabiting hard-to-reach sites. This population includes plants of various ages from seedlings to old lianas and contains many reproductive individuals. Presumably, a low degree of differentiation in this population is explained by the fact that the forest it inhabits is not fragmented but is rather a continuous



UPGMA dendrogram of the similarity between *Aristolochia manshuriensis* populations constructed based on the coefficients of Nei's genetic distances ( $D_N$ ): MAn, Malaya Anan'evka; MBor, Malaya Borisovka; An, Anan'evka; and Nezh, Nezhinka.

uninterrupted tract. The gene flow in such intact habitats is limited only by the ability of the species to reproduce.

The population Malaya Anan'evka is also rather remote; however, only one sample (15) grows in an intact habitat, where no tree felling has occurred. This is a very compact sample represented by adult reproductive lianas, young undergrowth, and seedlings. Two other samples (14 and 16) were collected at the site with previous intensive tree felling, where the forest cover is mainly replaced with secondary successions. Only young plants at the stage of vegetative development were detected in these samples. The absence of differentiation in this population can be explained by two reasons: either this population before tree felling was continuous as the population Malaya Borisovka or it restored after tree felling from the preserved individuals of sample 15, as we observed a founder effect in samples 14 and 16. Both reasons are equiprobable, as the  $F_{ST}$  value in long-lived species frequently reflects the intensity of gene exchange at the moment when the extant populations established [21]. On the other hand, the founder effect is favored by the data of Bottleneck analysis (Table 2), which demonstrates a shifted equilibrium in the population Malaya Anan'evka. The bottleneck effect in the population Malaya Anan'evka is also suggested by the mode-shift indicator, which does not show any deviation from equilibrium in another weakly differentiated population, Malaya Borisovka.

The populations Nezhinka and Anan'evka have been exposed to the strongest anthropogenic stress. Both populations inhabit the lower reaches of the Nezhinka and Anan'evka Rivers, close to the regions of intensive economic activity; in addition to tree felling and cutting of this liana itself, frequent ground fires destroy adult woody lianas. Both populations are small and compact, the samples are located close to one another and contain juvenile lianas; neither reproductive individuals nor seedlings were detected in these habitats. These populations, especially of the population Nezhinka (Table 1), has low genetic differentiation despite the absence of evident isolation barriers between the samples. A limited gene flow within the populations Nezhinka and Anan'evka can result from frequent fires and cuttings of lianas, which have led to

the loss of reproductive individuals in these habitats and increase in the stochastic processes in each patch with a recent effective size reduction in these populations. The coefficient  $F_{ST}$  depends on the intensity of gene exchange and is connected with the value of gene flow as  $N_{Em} = (1/F_{ST} - 1)/4$  [24]. The gene flow values ( $N_{Em}$ ) between *A. manshuriensis* samples calculated according to  $F_{ST}$  values amount to 1.82, 4.08, 6.10, and 10.91 migrants per generation for the populations Nezhinka, Anan'evka, Malaya Borisovka, and Malaya Anan'evka, respectively. According to the one-migrant-per-generation rule by Mills and Allendorf [25], the gene flow of up to ten migrants per generation [25] or more [26] is necessary to prevent the negative consequences of genetic drift. Evidently, the gene flow in the population Nezhinka (1.82) is insufficient to prevent the consequences of gene drift. Thus, a limited intersample gene exchange in the population Nezhinka along with the absence of reproductive individuals and small population size demonstrate that this population is extremely vulnerable, especially under a constant anthropogenic impact.

The effect of gene drift connected with a recent population reduction on the structure of the populations Nezhinka and Anan'evka confirms the results of Bottleneck analysis (Table 2). Two of the three tests as well as the mode-shift indicator reflect the shift from equilibrium in these populations in a statistically significant manner, thereby demonstrating the genetic consequences of population reduction.

In addition to gene drift, we cannot exclude the effect of vegetative reproduction on the structure of populations Nezhinka and Anan'evka. As mentioned above, neither reproductive plants nor even seedlings were found in these populations, despite that Manchurian birthwort mainly reproduces by seeds. *A. manshuriensis* has a weak ability to root [8]. However, the ground part of the liana is able to develop shoots and additional roots from lateral buds, which is sometimes erroneously regarded as vegetative reproduction. Such supplementary shoots of the same plant are likely to increase the plant density in a patch and contribute to accumulation of differences between individual patches.

Characteristic of the *A. manshuriensis* populations in Primor'e is a high level of genetic similarity (Fig. 2). The mean genetic distance  $D_N$  between the Manchurian birthwort populations amounted to 0.0096. As any data on the genetic distances between the members of the genus *Aristolochia* and even the family Aristolochiaceae are unavailable in the literature, it is difficult to say whether these  $D_N$  values are typical of the species belonging to this genus or reflect the specific features of the Russian part of *A. manshuriensis* area. The  $D_N$  values considerably vary in different taxa of angiosperms. For example, the interspecific genetic distances in the genus *Pittosporum* vary from 0.001 to 0.6578 [27]. The populations of *Cicer arietinum* display low  $D_N$  values (0.010–0.090) [28] as well as of *Lathyrus latifolius*

(0.010–0.150) [29]. Low values of interpopulation genetic distances of *A. manshuriensis* can result from both the balancing selection and its common gene pool in the recent past. The latter can be connected with the history of establishment and migration of this species, continuity of its area, and more tight connections between its populations in the past. Despite that all extant *A. manshuriensis* populations in the Russian part of its area are isolated from one another by a system of ridges, they all grow in the valleys of the rivers coming from the Borisovskoe Plateau. Presumably, this was the area of a single ancestral Manchurian birthwort population; the seeds could be transported from that site with water flow along a branch of the Plosky Ridge, giving rise to new populations located along the Nezhinka, Anan'evka, and Malaya Borisovka Rivers. Noteworthy that the populations Malaya Borisovka and Malaya Anan'evka, located in the headstream, are the closest and cluster together in the dendrogram with the minimal  $D_N$  of 0.0015 (figure). It is likely that the location of Borisovskoe Plateau in the southwestern Primor'e, the absence of glaciation on this territory [6, 30], and its altitude (the Pologaya Mountain is 741 m above sea level) enhanced the preservation of refugia of many thermophilic plants during Pleistocene–Holocene glaciation, which led to extinction of the thermophilic elements in northern Primor'e [31]. During the next period of sea and lake transgressions, the flooding of a vast territory within the Khankai Plain and coastal marine areas prevented the expansion of *A. manshuriensis* area. According to Nei's molecular clock [32], the theoretical time of divergence between the studied Manchurian birthwort populations is about 4800 years ago. Thus, the *A. manshuriensis* populations in the lower reaches of the Nezhinka, Anan'evka, and Malaya Borisovka Rivers were, presumably, established during the period of general recession of the Sea of Japan level during the last 5000 years [30]. Consequently, the range of this species expanded during the second half of Holocene and was possibly fragmented rather recently, during an increase in economic activities and forest fragmentation in the 20th century.

Thus, characteristic of the relict *A. manshuriensis* populations is a low level of genetic differentiation within the Russian part of its area. Genetic similarity of the studied populations is likely to be connected with the history of its spreading and interaction of various factors, such as migrations and selection. On the other hand, our results demonstrate that the present balance between the systematic and random microevolutionary factors in Manchurian birthwort populations is shifted, especially in the disturbed habitats, due to the genetic drift connected with the reduction in reproductive and effective population sizes, in particular, caused by intensive anthropogenic stress. This is evident by the example of the studied populations, two of which (Nezhinka and Anan'evka) are subject to a strong anthropogenic impact; the third one (Malaya Anan'evka) is exposed to a weaker anthropogenic

stress yet grows in disturbed habitats; and the fourth (Malaya Borisovka) is practically intact. The system of subpopulations connected by gene flow allows this species to remain more genetically stable in the successive generations as compared with an undifferentiated population [12, 33, 34], which is of the utmost importance for survival of an endangered species. Presumably, the population Malaya Borisovka of Manchurian birthwort is the only one in the Russian part of its area where a sufficiently intensive gene flow opposes the adverse consequences of gene drift and inbreeding. However, in the case of such structure, extinction of even one subpopulation can have a dramatic effect on the genetic diversity and survival of the overall population, as it has presumably occurred with the population Malaya Anan'evka. In the differentiated populations Nezhinka and Anan'evka, the influence of gene drift and inbreeding increases in each patch, whereas the gene flow between patches is ever weakening.

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