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Assessment of genetic diversity of some Siberian and Far Eastern species of the genus *Spiraea* (Rosaceae) by newly developed multiplex panels of nuclear SSR loci

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Taxonomic and population genetic studies of the genus *Spiraea* (Rosaceae) species require new informative genetic markers. We screened 37 previously published heterologous oligonucleotide primer pairs for nuclear microsatellite loci and selected eight polymorphic and most reproducible of them for PCR multiplexing which substantially increases performance of routine mass genotyping. Three multiplex sets of 3, 3 and 2 loci, respectively, were developed and tested for ability to estimate the parameters of genetic variability and population structure in closely related species *Spiraea ussuriensis*, *S. flexuosa*, *S. chamaedryfolia* representing seven natural populations of the Russian Far East and Siberia. Allele number ranged among loci from twelve (*Spth20*) to three. Among 41 alleles found, 7 were unique in some species/populations. Analysis of parameters of genetic variability in *Spiraea* spp. showed similar values of allele number per locus and observed heterozygosity among populations and slightly greater estimates of expected heterozygosity in the samples of *S. flexuosa* ($N_A = 2.387$; $H_O = 0.387 \pm \pm 0.052$; $H_E = 0.540 \pm 0.055$) as compared to *S. ussuriensis* ($N_A = = 2.781$; $H_O = 0.385 \pm 0.079$; $H_E = 0.453 \pm 0.072$) and *S. chamaedryfolia* ($N_A = 2.875$; $H_O = 0.331 \pm 0.071$; $H_E = 0.505 \pm 0.069$). The observed values of genetic polymorphism parameters indicate the average level of genetic diversity of the studied species typical to previous studies in *Spiraea*. About 19 % of the observed variability occurred among populations ($F_{ST} = 0.191$) while 81 % of the total genetic variation concentrated within the populations. The loci *VS11*, *VS12*, *VS2*, and *VS6* contributed most to the observed differentiation. Nei genetic distances between populations ranged from 0.049 to 0.585. Genetic differentiation patterns among studied populations based on allele frequencies of nuclear microsatellite loci correspond with their geographical location. Genetic composition of some samples contradicted with their provisional species identification.

Kew words: *Spiraea ussuriensis*; *Spiraea flexuosa*; *Spiraea chamaedryfolia*; nuclear microsatellite loci; SSR; multiplex panels; genetic variability; population structure.

Оценка генетического разнообразия некоторых сибирских и дальневосточных видов рода *Spiraea* (Rosaceae) на основе разработанных мультиплексных панелей из ядерных микросателлитных локусов

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Для таксономических и популяционно-генетических исследований видов рода *Spiraea* (Rosaceae) требуются новые информативные генетические маркеры. Мы протестировали 37 ранее опубликованных гетерологичных пар олигонуклеотидных праймеров для ядерных микросателлитных локусов и отобрали 8, дающих полиморфные продукты амплификации и наиболее воспроизводимых для ПЦР-мультиплексирования, что существенно повышает эффективность рутинного массового генотипирования. Разработаны и апробированы три мультиплексных панели из трех, трех и двух локусов соответственно для оценки параметров генетической изменчивости и структуры популяции у близкородственных видов *Spiraea ussuriensis*, *S. flexuosa* и *S. chamaedryfolia* из семи природных популяций Дальнего Востока и Сибири. Число аллелей варьировало между локусами от двенадцати (в локусе *Spth20*) до трех. У некоторых видов/популяций из 41 выявленного аллеля 7 были уникальными. Анализ параметров генетической изменчивости в *Spiraea* spp. показывает схожие значения числа аллелей на локус и наблюдаемой гетерозиготности между популяциями и немногим более высокий уровень ожидаемой гетерозиготности в выборках *S. flexuosa* ($N_A = 2.387$; $H_O = 0.387 \pm 0.052$; $H_E = 0.540 \pm 0.055$) по сравнению с *S. ussuriensis* ($N_A = 2.781$; $H_O = 0.385 \pm 0.079$; $H_E = 0.453 \pm 0.072$) и *S. chamaedryfolia* ($N_A = 2.875$; $H_O = 0.331 \pm 0.071$; $H_E = 0.505 \pm \pm 0.069$). Выявленные значения параметров генетического полиморфизма свидетельствуют о среднем уровне генетического разнообразия изучаемых видов, характерном для ранее проведенных исследований в роде *Spiraea*. Около 19 % всей изучаемой изменчивости приходится на межпопуляционную ($F_{st} = 0.191$), в то время как 81 % общей генетической изменчивости сосредоточен в популяциях. Наибольший вклад в исследуемую дифференциацию вносят локусы *VS11*, *VS12*, *VS2*, *VS6*. Генетические расстояния Nei между популяциями варьировали от 0.049 до 0.585.

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Генетическая дифференциация исследуемых популяций, основанная на частотах аллелей ядерных микросателлитных локусов, соответствует географическому положению выборок. У некоторых образцов отмечается противоречие между аллельным разнообразием и их предварительной морфологической идентификацией.

Ключевые слова: *Spiraea ussuriensis*; *Spiraea flexuosa*; *Spiraea chamaedryfolia*; ядерные микросателлитные локусы; SSR; мультиплексные панели; генетическая изменчивость; популяционная структура.

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The study of biological diversity is one of the most important scientific directions in plant genetics. The nuclear microsatellite loci are highly variable codominant molecular markers widely used in population genetic studies, genetic identification of individual genotypes and clones, parentage analysis, and confirmation of the hybridity. The development of multiplex panels consisting of nuclear microsatellite loci is relevant for the genus *Spiraea* due to the complexity in the taxonomic identification of samples, the phenomenon of hybridization and polyploidy, and the clonal nature of the distribution of some species, so the fragment analysis based on the developed multiplex sets can substantially simplify the workflow.

The *Spiraea* species grow in temperate and boreal zones of the Northern Hemisphere. The southern border of the genus range in Asia passes through the Eastern and Northern Himalayas, in America the southern border crosses the Central part of Mexico. The genus *Spiraea* includes more than 100 taxa in the world flora and about 25 taxa in Russia.

There are few researches devoted to the analysis of population-genetic structure of species of *Spiraea* genus, and they are mainly associated with the development of primers for microsatellite analysis of specific species (Brzyski, 2010; Ashizawa et al., 2012; Khan et al., 2014). However, there are no publications on PCR multiplexing and development of multiplex assays for genotyping of *Spiraea* species. The population genetic studies in such species as *Spiraea ussuriensis*, *S. flexuosa*, *S. chamaedryfolia* have been conducted for the first time.

The goal of this research was to assess the genetic diversity of some Siberian and Far Eastern species of the genus *Spiraea* by newly developed multiplex panels of nuclear microsatellite markers able to raise efficiency and optimize population genetic studies, species and clone identification in the *Spiraea* taxa.

Materials and methods

The present study was carried out on 115 samples of *Spiraea* spp. collected by us in seven native stands located in Siberia and the Far East of Russia. While collecting samples, the ability of *Spiraea* to develop clonal offsprings was taken into account. The geographical location, code and sample size for the studied sites are listed below: *S. ussuriensis* – Ussuriysk (uss), Primorsky Krai, Ussuri district, 15 specimens; Ol'ga (olg), Primorsky Krai, Ol'ginsky district, 8 specimens; Gornovodnoye (gorn), Primorsky Krai, Ol'ginsky district, 18 specimens; Zeya (zey), Amurskaya oblast, Zeyskii district, 19 specimens; *S. flexuosa* – Shkotovo (shkt), Primorsky Krai, Shkotovsky district, 23 specimens; Buryat (ul), Buryatia, near Ulan-Ude city, 15 specimens; *S. chamaedryfolia* – Turochak (tur), Altayskiy Kray, Turochakskiy district, 17 specimens.

Total DNA was isolated from herbarium specimens by both the standard CTAB method (Doyle J.J., Doyle J.L., 1990) and the commercial kits for isolation of genomic DNA from plants – GeneJET Mini (Fermentas) and DNeasy Plant Mini Kit (Qiagen), MagMAX DNA Multi-Sample Kit (Invitrogene). The concentration and amount of DNA were measured in 0.8 % agarose gel and spectrophotometrically (NanoPhotometer P-Class P-360, Implen).

First of all, we carried out screening of primers designed for different species of *Spiraea* (Brzyski, 2010; Ashizawa et al., 2012; Khan et al., 2014). All 37 primer pairs previously published were selected for pre-screening. Each primer pair was first tested in a separate PCR following the original protocols. Loci that showed stable amplification were further combined into groups of three or two in order to develop PCR multiplex assays. If it was possible, primers with identical annealing temperature were combined into one set.

From the selected eight pre-screened loci three multiplex assays, each of them amplifying three or two loci, were developed (Table 1). DNA was diluted to a concentration of 10 ng/μl. A total PCR volume of 15 μl, containing 1.5 μl 10 × PCR Buffer, 0.75 μl 50 mM $MgCl_2$, 0.25 μl 10 mM dNTP mix, 1 μM of each primer 5 mM (the forward primer with a fluorescent label, linked to the 5′end; Evrogen, Russia), 0.2 μl 5 u/μl HS *Taq* DNA Polymerase (Evrogen, Russia), 8.3 μl ddH_2O, and 2 μl 10.0 ng DNA was used. The touchdown PCRs were run on DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad, USA) under the following conditions: 15 min of denaturation at 95 °C, 1 min at 94 °C, 1 min at 60–47 °C (temperature of primer annealing decreased in each cycle by 1 °C), 1 min at 72 °C (10 cycles); 1 min at 94 °C, 1 min at 47 °C and 1 min at 72 °C (25 cycles); terminal elongation at 72 °C for 20 min. The PCR products were diluted 1 : 10 or 1 : 50 times depending on the product concentration. For fragment analysis, 2 μl of diluted product was combined with 12 μl of total mixture of GeneScan 600 LIZ size standard (5 μl) and HiDi Formamide (190 μl) (Life Technologies). A fragment analysis was carried out on an ABI PRISM 3500 Genetic Analyzer (Life Technologies). Genotyping was performed using GeneMapper 5 software (Life Technologies).

The obtained multi-locus genotypes of the samples were analyzed in the program GenAlEx V.6.5 (Peakall, Smouse, 2012) in order to identify the main parameters of intra-popu-

Table 1. Characteristics of the eight loci used in three multiplex assays

Locus	Dye	Set	Repeat motif	Annealing temperature, °C	Size range, bp	Number of alleles
VS2	HEX	I	$(TC)_{14}$	53	86–90	3
VS6	FAM	I	$(TG)_{8}$	47	154–168	3
SA2	ROX	I	$(AG)_{17}$	51	134–168	4
VS11	FAM	II	$(CAG)_{4}$	57	146–164	7
Spth16	HEX	II	$(AC)_{6}(TC)_{8}$	54	73–81	3
SA4	ROX	II	$(AG)_{16}$	51	116–140	4
VS12	FAM	III	$(TGG)_{4}$	50	166–178	5
Spth20	HEX	III	$(AG)_{6}(AC)_{7}$	60	85–121	12

lation variability (an average and effective number of alleles per locus, expected and observed heterozygosity, inbreeding coefficient, etc.). The genotypes were tested in the program Micro-Checker v.2.2 (Van Oosterhout et al., 2004) in order to identify "null-alleles". The Ewens-Watterson tests for heterogeneity and for neutrality were made in the program PopGene32 (Yeh et al., 1999). Population genetic structure was inferred from multilocus microsatellite genotypes (K) using the Bayesian clustering algorithm in the program STRUCTURE v. 2.3.4 (Pritchard et al., 2000). For each number of inferred clusters (K) varied from 2 to 8. Five independent replicas of simulations with the number of iterations equal to 100000 with the previous heating period of 10.000 iterations were performed using the LOCPRIOR = 1 population data binding option. The most probable number of clusters was evaluated in the program Structure Harvester (Earl, von Holdt, 2012) using the method by G. Evanno et al. (2005) based on the selection of K with the highest likelihood ratio with the lowest standard deviation and the maximum increment (parameter DeltaK). Further processing of the results for the most probable K was performed in the program CLUMPP v.1.1.2 (Jakobsson, Rosenberg, 2007) and visualized in the program Distruct (Rosenberg, 2007).

Results

For microsatellite analysis of *Spiraea* species, 37 heterologous microsatellite loci were tested, initially designed to study the genetic variability of the rare North American species *S. virginiana* (Brzyski, 2010), the Japanese species *S. thunbergii* (Ashizawa et al., 2012) and the Asian species *S. alpine* and *S. mongolica* (Khan et al., 2014).

According to the results of testing 37 microsatellite nuclear loci 12 did not show specific amplification, 9 were monomorphic, 8 contained "null-alleles". Therefore, for further routine genotyping of *Spiraea* samples we selected eight pairs of primers demonstrating good reproducibility, stability and expressed polymorphism. Based on these loci, three multiplex panels (see Table 1) were designed in order to optimize routine by performing fragmented analysis on the capillary sequencer.

The selected loci were used to study the genetic polymorphism and population structure of closely related species *S. ussuriensis*, *S. flexuosa*, *S. chamaedryfolia* from seven Far Eastern and Siberian native populations. All the eight analyzed nuclear microsatellite loci in these *Spiraea* species were polymorphic. Most variable loci were *Spth20* and *VS11*, 12 and 7 alleles, respectively. The remaining loci (*VS2*, *VS6*, *SA2*, *Spth16*, *SA4*, and *VS12*) demonstrated lower allelic richness – from three to five alleles per locus. Among 105 individual specimens included in this study, homozygotes for "null-allele" were not found. A total of 41 allelic variants were identified, 7 alleles (17 %) of which were unique, occurring only in a single population. In the Shkotovsky population four unique alleles were detected. Based on the observed distributions of genotypes, the parameters of interpopulation variability were calculated (Table 2).

Analysis of genetic variability parameters in *Spiraea* spp. showed similar values of allele number per locus and observed heterozygosity among populations and slightly greater estimates of expected heterozygosity in the samples of *S. flexuosa* ($N_A = 2.387$; $H_O = 0.387 \pm 0.052$; $H_E = 0.540 \pm 0.055$) as compared to *S. ussuriensis* ($N_A = 2.781$; $H_O = 0.385 \pm 0.079$; $H_E = 0.453 \pm 0.072$) and *S. chamaedryfolia* ($N_A = 2.875$; $H_O = 0.331 \pm 0.071$; $H_E = 0.505 \pm 0.069$).

The values of the main parameters of genetic polymorphism estimated by us indicated the average level of genetic diversity of the studied *Spiraea* species to be within the limits of the values earlier revealed for populations of *S. virginiana* (Brzyski, 2010), *S. thunbergii* (Ashizawa et al., 2012), *S. alpina* and *S. mongolica* (Khan et al., 2014) for the corresponding loci. The comparison of observed and expected heterozygosity showed that all the loci indicated a deficit of heterozygous genotypes within samples (positive values of F_{IS}) for most of the studied microsatellite loci, except for the locus *Spth16*, which revealed a slight excess of heterozygotes (Table 3). Most genotype distributions within individual populations demonstrated also deficiency of heterozygotes (see Table 2). The Buryat population of *S. flexuosa* ($F = 0.371$) and the Turochak population of *S. chamaedryfolia* ($F = 0.340$) were distinguished by the increased values of Wright's fixation index, which can be explained by the low population sizes of these species, as well as by the probable self-pollination and/or consanguineous matings leading to a high degree of inbreeding. These observations showed the species of the genus *Spiraea* to be often reproduced not only sexually, but also through the root offspring. Thus, the observed deficit of heterozygotes may be caused by closely related crosses and vegetative reproduction prevailing in the species of section *Chamaedryon*.

The study of the population structure of the selected species of *Spiraea* using Wright's *F*-statistics (see Table 3) detected in

Table 2. Parameters of *Spiraea* genetic variability

Population code	N_A	N_E	H_O	H_E	F
uss	2.625	2.047	0.425 ± 0.079	0.466 ± 0.072	0.094
olg	2.000	1.679	0.297 ± 0.097	0.339 ± 0.083	0.156
gorn	3.000	2.005	0.396 ± 0.087	0.455 ± 0.072	0.144
zey	3.500	2.743	0.421 ± 0.054	0.561 ± 0.060	0.231
Average values for *S. ussuriensis*	2.781	2.119	0.385 ± 0.079	0.453 ± 0.072	0.156
shkt	3.750	2.488	0.424 ± 0.035	0.562 ± 0.050	0.231
ul	2.875	2.286	0.350 ± 0.069	0.518 ± 0.059	0.371
Average values for *S. flexuosa*	3.313	2.387	0.387 ± 0.052	0.540 ± 0.055	0.301
tur	2.875	2.265	0.331 ± 0.071	0.505 ± 0.069	0.390
Average values for all populations	2.946 ± 0.184	2.216 ± 0.112	0.378 ± 0.027	0.487 ± 0.026	0.239 ± 0.039

Note. N_A – average number of alleles per locus; N_E – effective number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; F – fixation index.

individuals of *Spiraea* spp. populations a 24 % deficit of heterozygotes (F_{IS} = 0.242) relative to the population and about 38 % being a deficit of heterozygous genotypes (F_{IT} = 0.383) relative to the species *S. chamaedryfolia* s.l. About 19 % of the total observed variability resulted from interpopulation variation (F_{ST} = 0.191). 81 % of all genetic polymorphism was concentrated within populations.

The loci with maximum differentiation of these populations were: *VS11*, *VS12*, *VS2*, and *VS6*. The test for heterogeneity of allele frequencies in geographically close samples from the Primorsky territory "olg" and "gorn" revealed significant differences between samples in allele frequencies in three loci: *VS11*, *VS2* and *SA4*, as well as in general (Table 4).

Based on the frequencies of alleles of the studied nuclear microsatellite loci the differentiation of the studied *Spiraea* spp. samples was analyzed. The standard genetic distances between populations range from 0.049 to 0.585. In general, genetic differentiation within the investigated populations corresponds to their geographical remoteness from each other.

Table 3. Values of Wright's *F*-statistics

Locus	F_{IS}	F_{IT}	F_{ST}
VS11	0.040	0.372	0.346
VS12	0.411	0.564	0.259
VS2	0.467	0.583	0.217
VS6	0.184	0.412	0.280
Spth20	0.323	0.454	0.194
Spth16	–0.051	–0.026	0.024
SA2	0.268	0.295	0.037
SA4	0.293	0.414	0.172
Average	0.242 ± 0.063	0.383 ± 0.067	0.191 ± 0.040

Note. F_{IS} – the inbreeding coefficient of an individual relative to the subpopulation to which it belongs; F_{IT} – the inbreeding coefficient of an individual relative to the whole population; F_{ST} – the coefficient of inbreeding of the subpopulation relative to the entire subdivided population.

Table 4. The results of the test for heterogeneity of allele frequencies in the populations "olg" and "gorn" of *S. ussuriensis* on the studied SSR loci

Locus	Value of χ^2	Number of degrees of freedom	Probability	Significance level
VS11	7.1633	1	0.0074	**
VS12	5.8196	3	0.1207	NS
VS2	11.2254	2	0.0037	**
VS6	0.8432	1	0.3585	NS
Spth20	10.2716	5	0.0679	NS
Spth16	0.9489	2	0.6222	NS
SA2	0.2941	2	0.8633	NS
SA4	28.5278	2	0.0000	***
In general	65.0938	18	0.0000	***

Note. The statistical significance of allele frequency shifts as determined by heterogeneity test is indicated by asterisks; p – the significance level; ** $p < 0.01$; *** $p < 0.001$; NS – not significant at the 5 % level.

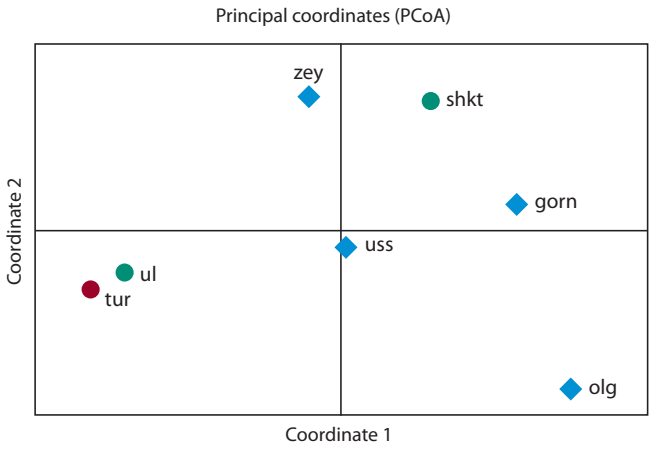

Fig. 1. Projection of the studied *Spiraea* populations on the two-coordinate system according to the PCoA-analysis of the Nei genetic distances matrix.

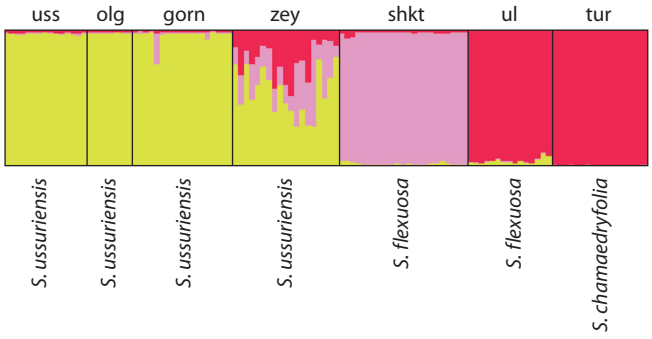

Fig. 2. Analysis of the population structure of *S. chamaedryfolia* s.l. with the assumed number of genetic clusters K = 3.

The Turochak and Buryat populations (the genetic distance is 0.049) were characterized by the lowest genetic differences. Previously, we showed strong affinity of *S. flexuosa* and *S. chamaedryfolia* species by morphological features. Analysis of genetic distances using multidimensional scaling (PCoA) demonstrated genetic differentiation of populations under the study (Fig. 1). Corresponding to their mutual geographical location, a grouping of samples with each other was observed. Based on the results of the main coordinates analysis two groups can be distinguished; one – more Western – combining the samples "tur" and "ul" from Altai and Buryatia, which belong to different species *S. chamaedryfolia* and *S. flexuosa*, respectively; while the second Eastern one was differentiated not so clearly and included the Far Eastern samples of *S. flexuosa* and *S. ussuriensis*. The samples of "shkt" population of *S. flexuosa* were among the populations of *S. ussuriensis*.

The analysis of population structure was also conducted in the program STRUCTURE v. 2.3.4 on the basis of multilocus genotypes. The most probable number of initial genetic clusters K = 3 was calculated corresponding to the provisional species – *S. chamaedryfolia*, *S. flexuosa*, *S. ussuriensis*. The identified clusters contribution to genotypes of populations and individuals as well as the distribution of individuals by population are visualized using different colors (Fig. 2). Taking into account the designation of clusters 1, 2 and 3 (K1, K2, K3), colors are yellow, pink and red, respectively. It is possible to note a clear predominance in the studied samples of *S. ussuriensis* genetic cluster K1, and K1 represents the most of the gene pool of *S. ussuriensis* from Primorsky Krai. In the "zey" sample from the Amur region, along with the predominance of K1, K2 and K3 clusters make a significant contribution to all individuals in the population. Genetic cluster K2 dominates the sample "shkt" *S. flexuosa* from Primorsky Krai. Cluster K3 prevails in samples "tur" and "ul" from Altai and Buryatia belonging to different species – *S. chamaedryfolia* and *S. flexuosa*, respectively, and slightly represented in all the other samples. The content of the genetic cluster K1 in small quantities is shown in samples *S. flexuosa* – "shkt" and "ul".

Discussion

The values of the main parameters of genetic polymorphism established by us indicated the average level of genetic diversity of the studied populations of *Spiraea* in the investigated regions and were within the same range as of similar values estimated for populations *S. virginiana* (Brzyski, 2010), *S. thunbergii* (Ashizawa et al., 2012), *S. alpina* and *S. mongolica* (Khan et al., 2014). A higher level of interpopulation variability (37.3 %) was observed in *S. prunifolia* for *simpliciflora* from Korea on the basis of analysis of ISSR repeats (Huh, 2009), and the highest (73.7 %) of the studied one – in *S. alpina* from the Tibetan upland in Central Asia based on variability of *trnL-trnF* sequences of chloroplast DNA (Zhang et al., 2012).

A similar pattern of heterogeneous structure of populations of species close to *S. chamaedryfolia* s.l. was shown by us earlier on a combination of morphological characteristics (Polyakova, 2004). *S. chamaedryfolia*, *S. flexuosa* and *S. ussuriensis* were not significantly different in morphometric characteristics meanwhile weak ones being distinguishable qualitative (within such features as: degree of the pubescence of the abaxial part of the leaf, the shape of the axillary buds, color of shoots, the nature of "jagged" edges of the leaf blade). Almost all signs of intermediate forms between these species have been found. The clustering of populations of these species by morphological features showed a single, structurally heterogeneous group. Most likely, it is necessary to consider these samples as one species – *S. chamaedryfolia*. A small number of relatively reliable indicators-discriminators in *S. flexuosa* and *S. ussuriensis* indicate their intraspecific rank. Probably, a separate taxonomic status should be considered for coastal populations of *S. flexuosa* and *S. ussuriensis* (*S. flexuosa* from the samples of population "shkt" formed a separate genetic cluster K2). The hybrids of *S. ussuriensis* with another close species *S. elegans* were described by A.I. Pojarkova (1939). According to the morphological characteristics of the hybrids such specimens were found by us in various parts of the Amur region. Probably, individuals from the population "zey" of *S. ussuriensis* from the Amur region have a hybrid origin, as indicated by the combination of contributions of different genetic clusters to this population (see Fig. 2).

Thus, the developed multiplex panels of eight nuclear microsatellite loci made it possible to study the genetic variability and population structure of close relatives of *S. chamaedryfolia* s.l., suggest hybrid origin of some specimens and populations. For the most accurate decisions on subspecies structure

of the *S. chamaedryfolia* s.l. complex and about the distribution of certain taxonomic units and the composition of genetically heterogeneous populations analysis of ecological, morphological and genetic data is required as well as samples should be more representative.

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Conflict of interest

The authors declare no conflict of interest.

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