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Genetic variation and hybridization between *Lotus corniculatus* L. and *L. stepposus* Kramina (Leguminosae) in Russia and Ukraine: evidence from ISSR marker patterns and morphology

Tatiana E. Kramina

Summary: A study of genetic diversity of inter-simple sequence repeat (ISSR) markers and morphological variation was conducted in 24 populations of *Lotus corniculatus* L. (Co), *L. stepposus* Kramina (S) and a species of putative hybrid origin *L. ucrainicus* Klok. (U) from European Russia and Ukraine. The study revealed that individuals of *L. ucrainicus* are characterized by a transitional morphology between *L. corniculatus* and *L. stepposus*. Analysis of ISSR banding patterns demonstrated that *L. ucrainicus* shared the majority of species-exclusive bands (i.e. those presented in one parental species only) from both presumed parental species and mainly had an intermediate band frequency of species-typical markers (i.e. those with differentiated band frequencies between parental species) as compared to *L. corniculatus* and *L. stepposus*. Bayesian analysis by Structure program suggested a presence of two (Co and S) or three species (Co, S and U) as the most probable population structure. In the first case, individuals of *L. ucrainicus* were genetically admixed. Analysis of molecular variance of ISSR data indicated highly significant genetic differentiation among species and among as well as within populations ($p = 0.00000$). A gene flow among studied *Lotus* species is more restricted than within each of them. As assessed by Mantel test, geographic component in genetic variation was prominent in *L. stepposus* ($r = 0.77$) and *L. corniculatus* ($r = 0.51$), while for *L. ucrainicus* it was not significant ($r = 0.17$, $p = 0.188$). Revealed patterns of molecular and morphological variation mainly support a hybrid origin of *L. ucrainicus* from parental species *L. corniculatus* and *L. stepposus*. A partially restricted bidirectional gene flow between *L. corniculatus* and *L. stepposus* through introgressive hybridization with *L. ucrainicus* is supposed.

Keywords: *Lotus*, Loteae, Leguminosae, ISSR markers, hybridization, genetic variability, population, Russia, Ukraine

Lotus corniculatus L. and *L. stepposus* Kramina are members of the section *Lotus* of the genus *Lotus* L. and the *Lotus corniculatus* species complex, which aggregates a number of diploid and tetraploid species (Ball & Chrtková-Žertová 1968), including a model species *L. japonicus* (Barykina & Kramina 2006). Species diagnostics in this group is not very clear, and some of the species are poorly delimited morphologically. *Lotus stepposus* was described from the eastern part of Ukraine (Kramina 2000). It is distributed in the steppe zone of Eastern Europe, north Kazakhstan and south Siberia. The species is characterized by a diploid chromosome number $2n = 12$, which was demonstrated by both direct counting (Kramina 1999) and flow cytometry (Kramina et al. 2012), while *L. corniculatus* is predominantly tetraploid with $2n = 24$ (Grant 1995). Molecular phylogenetic studies of the genus *Lotus* revealed close relationships between *L. corniculatus* and *L. stepposus* (Degtjareva et al. 2006, 2008), morphological differences between these two species are not also too big, however, they can be distinguished by a set of morphological characters (Kramina 2000; Kramina et al. 2012). Earlier, the presence of a transitional zone was demonstrated in areas of contact or overlapping of distribution ranges of *L. corniculatus* and *L. stepposus*. Morphologically intermediate specimens occur in this zone, and

one of them had been described in the middle of 20th century as *L. ucrainicus* Klok. (Klokov 1961), and later its hybrid origin was hypothesized (Kramina 2000).

Inter-simple sequence repeat (ISSR) markers were successfully used in studies of hybridization in various plant groups (Wolfe et al. 1998, Goldman 2008, Khajudparn et al. 2012). For testing the hypothesis on the occurrence of interspecific hybridization between *L. corniculatus* and *L. stepposus* a study of morphological variability and genetic polymorphism of ISSR markers in nine natural populations of *L. corniculatus*, *L. ucrainicus* and *L. stepposus* was conducted (Kramina et al. 2012). The inference of that work was that *L.* × *ucrainicus* might arise as a result of hybridization between the tetraploid species *L. corniculatus* and the diploid species *L. stepposus* (Kramina et al. 2012). As a whole, data of nrITS DNA of 14 *Lotus* accessions do not contradict the idea about hybrid origin of *L.* × *ucrainicus* (Kramina et al. 2012). However, in Kramina et al. (2012) the number of samples was very low. Both presumed parent taxa are underrepresented in that study, i.e. *L. corniculatus* and *L. stepposus* were presented by two local populations each only. Some of the results obtained by Kramina et al. (2012) do not necessarily confirm a hypothesis of hybrid origin of *L.* × *ucrainicus* and may be explained by other ways. The first results need to be checked on a more representative material, sampled from various parts of distribution areas.

The goals of the present study were:

To study morphological and genetic (ISSR) variation in a wide set of samples of *L. corniculatus*, *L. stepposus* and *L. ucrainicus* and to investigate potential gene flow between the species.

To test a hybrid status of individuals morphologically identified as *L. ucrainicus* on a more representative material.

Materials and methods

Plant sampling

Specimens from 24 local populations were sampled from the European part of Russia and Ukraine in 2007–2012. Description of localities and number of studied specimens for each sample are presented in Table 1. A map showing geographical distribution of localities is presented in Fig. 1. Based on diagnostic morphological characters (Kramina 2000), the material was initially identified in the following way: six populations of *Lotus stepposus* Kramina (Sb, Sl, K, H,Vb and S), five populations of *L. corniculatus* L. var. *corniculatus* (G, Hz, L, Sv, Kr), six populations of *L. corniculatus* L. var. *hirsutus* Koch (Od, B1, B2, U, V, Kv) and seven populations with intermediate morphological characters identified as *Lotus ucrainicus* (Vd, Vi, P, M, R, O and Km). All sampled individuals (n = 119) were collected as herbarium specimens and used for morphological studies. Leaves from each specimen were dried in silica gel and used for DNA extraction and ISSR analysis.

Analysis of morphological characters

We selected nine morphological characters which had demonstrated the widest interspecific variation in the previous study (Kramina et al. 2012), including those of leaf, pubescence, partial inflorescence and flower (see Table 2). All characters were measured in 119 specimens belonging to *L. corniculatus* (n = 26), *L. corniculatus* var. *hirsutus* (n = 30), *L. ucrainicus* (n = 27), and *L. stepposus* (n = 36). Statistical analysis of morphological characters was conducted using STATISTICA version 7.1 software for Windows (StatSoft Inc. 2006). To study variability

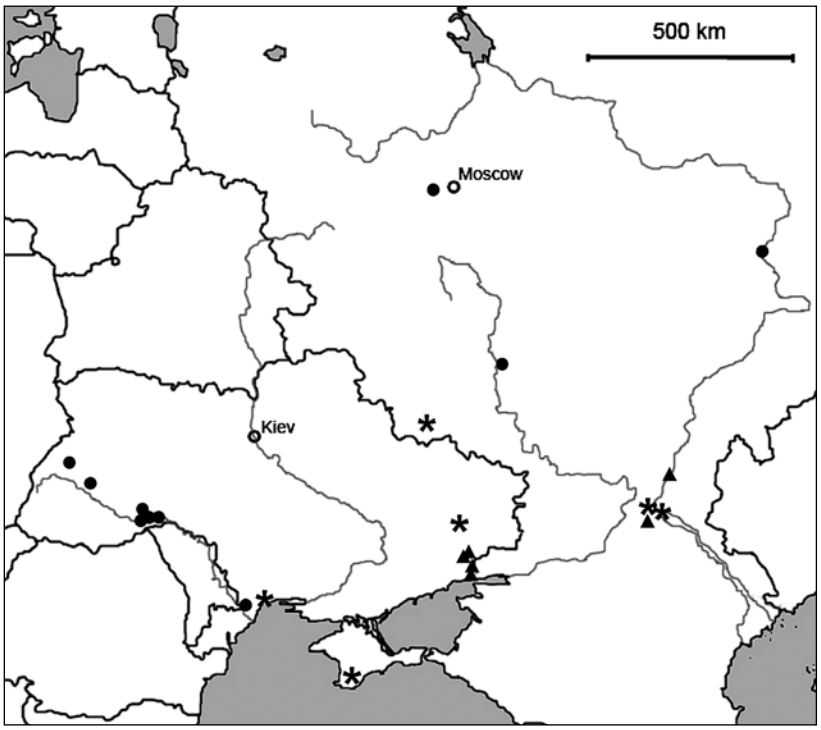

Figure 1. Map of the European part of Russia and Ukraine showing the location of sampled populations of *Lotus corniculatus* (filled circles), *L. stepposus* (filled triangles) and the putative hybrid *L. ucrainicus* (asterisks).

of each morphological character within every studied taxon a nested design analysis of variance (ANOVA) was carried out. 'Population' and 'species' were used as factors and each morphological quantitative character as a dependent variable. Random factor 'population' was nested in fixed factor 'species'. Further, a comparison of means of four studied taxa was conducted using Unequal N honestly significant difference (HSD) criteria (i.e. modified Tukey's test for unequal groups). Characters whose means were significantly different in supposed parental species (i.e. *L. corniculatus* and *L. stepposus*) were revealed, and then their means were compared with corresponding means in a species of supposed hybrid origin (i.e. *L. ucrainicus*).

In consideration of a slight interspecific overlap of character variability ranges, a multivariate Discriminant Analysis (DA) of quantitative characters was conducted. DA requires multivariate normalcy of data in each group, however, several authors demonstrated sufficient robustness of this method which permits some departure from normality in experiments (see for example Lachenbruch & Goldstein 1979). For the initial classification of samples in DA, C and K groups were combined into one common group Co (*L. corniculatus*) because their morphological differences were not very significant. The groups S (*L. stepposus*) and U (*L. ucrainicus*) were used as two other separate groups. DA was conducted using a set of seven morphological characters (FML, FUL, FCA, COAV, FLOB, LHAIR, STYLE), important for species delimitation. As far as some of characters in the set are ratios (FML=LML/WML, FUL=LUL/WUL, FCA=LTUB/LLOB, Table 2), for more correct DA application, original characters (LML, WML, LUL, WUL, LTUB, LLOB) were included in the analysis instead of ratios, together with four remaining important characters (COAV, FLOB, LHAIR, STYLE). So, the more correct DA was conducted using a set of ten continuous characters.

Table 1. Sample localities, geographical coordinates and number of studied individuals for each sample.

Species	Sample index	Sample name	Sample localities	Geographical coordinates	No. of specimens
L. comiculatus	G	Gutisko	Ukraine, Ternopol Region, Berezhany Distr., near Gutisko village, mount Golitza, meadow-steppe vegetation, S slope, N. Sytchak & A. Kagalo, 24.07.2011	49°24'N 24°49'E	4
	Hz	Hometz	Ukraine, Lviv Region, NE outskirts of Lviv city, mount Hometz, meadow-steppe vegetation, E slope, N. Sytchak & M. Kagalo, 17.07.2011	49°53'N 24°03'E	4
	L	Lutzino	Russia, Moscow Region, Odintzovo Distr., 0.5 km W from Lutzino village, meadow by the road, T. E. Kramina, 03.07.2008	55°42'N 36°46'E	6
	Sv	Sviyaga	Russia, Ulyanovsk Region, Ulyanovsk city, flood plain meadow of Sviyaga River, A. V. Maslennikov, 15.07.2008	54°20'N 48°20'E	7
	Kr	Krivoborye	Russia, Voronezh Region, Ramon Distr., near Krivoborye village, meadow on limestone, T. E. Kramina & I. A. Schanzer, 19.06.2008	52°02'N 39°10'E	5
	Od	Odessa-1	Ukraine, Odessa Region, Odessa city, on the beach, M. Chumachenko, 04.07.2011, Belyaevka Distr., Majory Village, steppe, T. Vasilyeva, 24.06.2008	46°32'N 30°17'E	2
	B1	Bakota-1	Ukraine, Khmelnytskyi Region, Kamianets-Podilskyi Distr., near Kashtanivka village, upland meadow, A. A Kagalo, T. E. Kramina & S. V. Polevova, 29.07.2009	48°34'N 27°00'E	5
	B2	Bakota-2	Ukraine, Khmelnytskyi Region, Kamianets-Podilskyi Distr., near Kashtanivka village, Dnestr river bank, A. A. Kagalo, T. E. Kramina & S. V. Polevova, 29.07.2009	48°34'N 27°00'E	5
	U	Ustye	Ukraine, Khmelnytskyi Region, Kamianets-Podilskyi Distr., near Ustye Village, near Smotrich river mouth, A. A. Kagalo, T. E. Kramina & S. V. Polevova, 29.07.2009	48°34'N 26°39'E	6
	V	Vrublivtzy	Ukraine, Khmelnytskyi Region, Kamianets-Podilskyi Distr., near Vrublivtzy village, cliff by Dnestr river, A. A. Kagalo, T. E. Kramina & S. V. Polevova, 30.07.2009	48°36'N 26°46'E	6
	Kv	Kavalery	Ukraine, Khmelnytskyi Region, Kamianets-Podilskyi Distr., 1.5 km W from Verbka village, steppe slope and near the road, A. A. Kagalo, T. E. Kramina & S. V. Polevova, 30.07.2009	48°47'N 26°39'E	6
L. ucrainicus	Vd	Kleban-Byk	Ukraine, Donetzk Region, Kleban-Byk storage reservoir, from upland to water meadow, V. M. Ostapko, T. E. Kramina & I. A. Schanzer, 14.08.2008	48°27'N 37°43'E	6
	Vi	Visloye	Russia, Belgorod Region, Visloye village, in the meadow, N. M. Reshetnikova, 05.08.2008	50°45'N 36°36'E	4
	P	Park	Russia, Volgograd city, park for recreation, by the road, T. E. Kramina, 05.07.2007	48°43'N 44°32'E	5
	M	Mamayev Kurgan	Russia, Volgograd, Mamayev burial mound, in upland meadow, T. E. Kramina, 03.07.2007	48°44'N 44°32'E	5
	R	Rybachy	Russia, Volgograd Region, between Rybachy and Noven'koye villages, in wet meadow, T. E. Kramina, 04.07.2007	48°42'N 44°45'E	4
	O	Odessa-2	Ukraine, Odessa Region, outskirts of Odessa city, Vapnyarka village, M. Adzhalak, 10.07.2004, near Odessa city, Bezkarovaynaya, 15.07.2007	46°35'N 30°53'E	2
	Km	Crimea	Ukraine, The Crimea, Bakhchisaray Distr., near Nauchny settlement, mount Sel-Bukhra, Unknown collector, 27.10.2011	44°43'N 34°00'E	1

L. stepposus	Sb	Starobeshevo	Ukraine, Donetzk Region, near Starobeshevo village, V. M. Ostapko, T. E. Kramina & I. A. Schanzer, 13.08.2008	47°45'N 38°02'E	6
	Sl	Starolaspa	Ukraine, Donetzk Region, near Starolaspa village, V. M. Ostapko, T. E. Kramina & I. A. Schanzer, 13.08.2008	47°33'N 37°59'E	6
	K	Konkovo	Ukraine, Donetzk Region, near Konkovo village, V. M. Ostapko, T. E. Kramina & I. A. Schanzer, 13.08.2008	47°20'N 38°10'E	6
	H	Khomutovka	Ukraine, Donetzk Region, near Samsonovo village, Nature Reserve 'Khomutovskaya step', meadow on the left bank of Gruzskiy Elanchik river, V. M. Ostapko, T. E. Kramina & I. A. Schanzer, 12.08.2008	47°15'N 38°08'E	6
	Vb	Verkhny Balykley	Russia, Volgograd Region, Bykovo Distr., 2 km S of Verkhny Balykley, in steppe gully, T. E. Kramina, 02.07.2007	49°30'N 45°10'E	6
	S	Sarepta	Russia, Volgograd, near railway station Sarepta, by the track, T. E. Kramina, 06.07.2007	48°31'N 44°30'E	6

Analysis of ISSR band patterns

DNA was extracted from dry leaves taken from herbarium specimens (20 mg leaf tissue) with NucleoSpin Plant kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. To study inter-simple sequence repeats (ISSRs) seven oligonucleotide primers complementary to simple sequence repeats (HB12, HB13, M1, M12, UBC840, UBC855 and UBC868) were used (Table 3). Compared to the previous paper (Kramina et al. 2012), less informative primers M2, M3, M4, M7 and M9 were excluded from ISSR analysis, and a new primer UBC868 was added.

Polymerase chain reaction (PCR) mixtures contained 10–20 ng template DNA, 20 pM primer, and ready-to-use MaGMix [200 µM of each dNTP, 1.5 mM $MgCl_2$, 1.5 U Taq-polymerase, and reaction buffer (Dialat Ltd., Russia). The reaction volume was 20 µl. PCR was conducted in a MJ Research PTC-220 DNA Engine Dyad (Bio-Rad Laboratories, USA) thermocycler. PCR conditions were as follows: 95°C for 3 min (preliminary denaturation), 94°C for 30 s, annealing temperature for 30 s and 72°C for 40 s + 2 s for each cycle (for 35 cycles). Annealing temperatures were first calculated as T = 4 (G + C) + 2 (A + T), and finally selected after PCR with a gradient of annealing temperatures conducted on a restricted set of accessions. The final annealing temperature of selected primers is presented in Table 3.

Amplification products were separated by electrophoresis on 1.7% agarose (Amresco Inc., USA) gel in 0.59 Tris-borate-ethylenediaminetetraacetic acid (TBE) with ethidium bromide (0.5 µg/ml) staining at 125 V and photographed with a digital camera. Photographs of gels were analysed using Cross Checker 2.91 (Buntjer 2000). A binary matrix of presence/absence of equal-length fragments was constructed for further analysis.

The reproducibility of ISSR bands was analysed using the approach described by Bonin et al. (2004). Twelve DNA samples (about 10% of all samples) were chosen randomly from the set of already extracted samples. For these samples, second PCR analyses with the same primers and reaction conditions were conducted. Matrices obtained for first and second PCR analyses were then compared to calculate the error rate, which was estimated as the ratio between observed number of phenotypic differences and total number of phenotypic comparisons (Bonin et al.

Table 2. Morphological characters and mean differences among the studied taxa *Lotus corniculatus* (C, n = 26), *L. corniculatus* var. *hirsutus* (K, n = 30), *L. ucrainicus* (U, n = 27) and *L. stepposus* (S, n = 36). Results of nested design ANOVA (p-value for F) and significance of difference among species means tested using Unequal N HSD criteria (modified Tukey's criteria): — not significant; * $p \le 0.05$; ** $p \le 0.01$; *** $p \le 0.001$.

#	Character label	Morphological character	Nested ANOVA p-value among species/among populations	Difference between group means					
				S from C	S from K	S from U	C from K	U from C	U from K
1	FML =LML/WML	Length to width ratio of the terminal leaflet of a middle stem leaf	***/—	***	***	***	—	**	—
2	FUL =LUL/WUL	Length to width ratio of the terminal leaflet of an upeer stem leaf	***/—	***	***	***	—	***	**
2	PBCA	Pubescence density on calyces (grades 1–9)	**/***	*	*	—	—	—	—
4	LHAIR	Trichome length on calyces (mm)	***/**	***	***	***	*	***	—
5	FLOB	Portion of narrow part of calyx teeth (%)	***/—	***	***	*	—	*	***
6	COAV	Flower length (mm)	***/***	***	***	***	**	***	*
7	UMAV	Number of flowers per umbel	*/***	—	—	—	—	—	—
8	FCA =LTUB/FLOB	Calyx tube length to calyx lobe length ratio	***/—	***	***	—	—	***	**
9	STYLE	Style length (mm)	***/*	***	***	***	—	**	—

2004). Degree of incongruence in the presence/absence of fragments in two matrices were counted. Sixty nine occurrences of incongruence were scored in 2328 comparisons. So, the calculated reproducibility index was 69/2328 = 0.02964 (or 2.96%).

At first, the matrix of all samples was analysed using Microsoft Excel. The total number of bands and the number of polymorphic bands were counted. It would be ideal to find species-specific marker bands (i.e. bands that are present in all individuals of one species and in none of the other) (Archibald et al. 2004), however, no such markers were revealed in studied species of *Lotus*. So, less strict methods for defining marker bands were applied in this study.

First, to reveal species-characteristic bands for each studied species, a method described by Záveská et al. (2011) was used. This method has already been applied in the previous paper (Kramina et al. 2012), and proved to be effective in *Lotus* studies. According to this approach, ISSR bands were identified as exclusive for each supposed parental species (i.e. *L. corniculatus* and *L. stepposus*), when they were represented with frequency ≥ 70% in at least one of its population and absent from second presumable parental species. Then, these bands were checked for presence in the species of supposed hybrid origin (i.e. *L. ucrainicus*). Later, each exclusive band of the presumed parental species was categorized as private or shared with *L. ucrainicus*. The presence of private bands in *L. ucrainicus* was also checked.

Second, to reveal patterns of gene flow among studied species, a method used by Archibald et al. (2004) was applied. According to this approach, those bands that occurred in one presumable parental species in at least a 25% higher frequency than the other were considered species-typical bands. Band frequencies in the proposed hybrids were then examined to determine if they were intermediate to those found in the two putative parental species.

Table 3. Characteristics of seven oligonucleotide primers complementary to simple sequence repeats.

Primer Label	Primer 5'-3' sequence	Annealing temperature °C
HB12	$[(CAC)_3GC]$	48.5
HB13	$[(GAG)_3GC]$	48.5
M1	$[(AC)_8CG)]$	50
M12	$[(CA)_6(A/G)(C/T)]$	49.5
UBC840	$[(GA)_8AYT]$	50
UBC855	$[(AC)_8CYT]$	50
UBC868	$[(GAA)_6]$	50

The matrix of the presence/absence of ISSR bands was then analysed using principal coordinate analysis (PCO) implemented in the PAST program (Hammer et al. 2001). Jaccard coefficient was used as the measure of genetic similarity (Jaccard 1912). Then, a neighbor-joining analysis (NJ) (Saitou & Nei 1987) was run in SplitsTree v. 4.13.1 (Huson 1998), and again Jaccard metric was used as a genetic distance.

Population structure and probability of hybrid origin of particular specimens were analysed using Bayesian inference with the program Structure 2.2 (Pritchard et al. 2000; Falush et al. 2007). The program Structure 2.2 assesses probability of subdivision of a sample into K populations based on calculation of allele frequencies in each of these hypothetical populations using the Markov chain Monte Carlo method. The admixture model with correlated allele frequencies was used. The model implies genetic relatedness of compared populations, Hardy-Weinberg equilibrium and linkage equilibrium for the analysed markers. Numbers K = 1–8 were tested with 10 replicates per K value and 500 000 Markov chain Monte Carlo repetitions. For identification of the real K, the maximum value of the estimated logarithm of the posterior probability of data ["Ln P(D)"] returned by Structure is often used. Following Evanno et al. (2005), we refer to Ln P(D) as L(K). However, Evanno et al. (2005) demonstrated that the distribution of L(K) did not show a clear mode for the true K. They used a delta K (DK) approach for estimation of the real K. It is based on the second-order rate of change of the likelihood function with respect to K and demonstrates a clear peak at the true value of K (Evanno et al. 2005). Delta K is calculated by the formula: $DK = m(|L(K+1) - 2L(K) + L(K-1)|)/s[L(K)]$, where m is the mean and s is the standard deviation (Evanno et al. 2005). We compare these two methods (i.e. maximum value of L(K) and DK) for estimation of the real number of clusters in our dataset and revealing of genetically admixed samples.

The genetic differentiation of studied samples and the levels of variation in ISSR patterns were analysed in Arlequin ver. 3.5.1.3 program (Excoffier & Lischer 2010) and by analysis of molecular variance (AMOVA). AMOVA analyses are based on the pairwise squared Euclidean distances among molecular phenotypes (Stewart & Excoffier 1996) and allow to calculate components of molecular variance and their significance levels (based on permutation procedures) for the following hierarchical levels: among species, among populations within species and among individuals within populations.

To determine if genetic distances were correlated with geographic distances, a Mantel test (Mantel 1967) was performed using Arlequin software. Geographic distances were calculated based on geographical coordinates for each population (Table 1). Gene flow between pairs of populations was calculated by the formula: $N_em = 0.25\times(1/Fst-1)$ on the basis of Φ_{ST} value estimates obtained in AMOVA (Schmidt & Jensen 2000).

Results

Morphological variation

Nested design ANOVA

The material was divided into four species groups: C (*L. corniculatus*), K (*L. corniculatus* var. *hirsutus*), U (*L. ucrainicus*) and S (*L. stepposus*). Group means of all studied characters (1–9) were significantly different (Table 2), however, among-species differences of means were less prominent in PBCA and UMAV characters. For the rest of characters (i.e. FML, FUL, LHAIR, FLOB, COAV, FCA, and STYLE) differences were significant at $p \leq 0{,}001$.

A pairwise comparison of means in four groups (C, K, U and S) using Unequal N HSD criteria led to the following results. Means of C (*L. corniculatus*) and K (*L. corniculatus* var. *hirsutus*) groups were very close and not significantly different for the majority of characters, except for LHAIR and COAV, which varied also on among-population level. So, for multivariate morphological analysis it was decided to combine these groups into one large group Co (*L. corniculatus*, $n = 56$). The group S (*L. stepposus*) was the most distinct from C and K (*L. corniculatus*), their means were different at $p \leq 0{,}001$ for all characters, excluding PBCA and UMAV, which are not very important for species discrimination. Character means of the group U (*L. ucrainicus*) were not different from those of S (*L. stepposus*) in PBCA, UMAV and FCA characters and from K (*L. corniculatus* var. *hirsutus*) in PBCA, UMAV, FML, LHAIR and STYLE characters. After exclusion of less significant characters PBCA and UMAV, two different sets of characters were obtained. In the first set, including a character FCA only, character mean was similar in U and S. And in the second set (including FML, LHAIR and STYLE) the means were alike in U and K. The characters PBCA and UMAV were considered less important for discrimination of studied species and excluded from further analyses. Differences between typical *L. corniculatus* (C) and *L. corniculatus* var. *hirsutus* (K) were small and revealed in two characters only (Table 2), so for further analyses these groups were combined into one common group *L. corniculatus* (Co).

Discriminant Analysis

DA was conducted using ten continuous morphological characters and original classification of dataset into three groups (Co – *L. corniculatus* (including both var. *corniculatus* and var. *hirsutus*), U – *L. ucrainicus*, S – *L. stepposus*). The analysis revealed that percentage of correct classification in the whole dataset was 89.1%, and it was maximal for the diploid species *L. stepposus* (97.2% of correct identification, 1 misclassified specimen) and minimal for a species of a presumable hybrid origin *L. ucrainicus* (70.4%, 8 misclassified specimens). Two canonical variables (roots) were obtained, the first of which described 96% of the total between-group variance. Wilks'Lambda ≈ 0.13 suggests rather good separation of the groups. On a scatterplot (Fig. 2), individuals of *L. stepposus* and *L. corniculatus* form non-overlapping clouds of dots which are separated by a lax cloud of specimens of *L. ucrainicus*. Cluster of *L. stepposus* accessions is the most compact and slightly overlaps with a cluster of *L. ucrainicus* (Vd7 is the only specimen of

L. ucrainicus which is situated among dots of *L. stepposus* cluster). Accessions of *L. corniculatus* form a cloud of dots which is wider and more overlapping with the one of *L. ucrainicus*. Species clusters are situated in an order S – U – Co alongside the first root, the second root is not relied on interspecific differences. For the first root, COAV character had the highest absolute value of standardized coefficient (–0.66) and the characters COAV and LHAIR had the highest correlation coefficients with this root, so these features are the most important for species discrimination.

ISSR analysis

ISSR band composition

PCR with seven primers enabled to generate 194 ISSR fragments, of which 192 were polymorphic (Table 4). About 95% of all loci were polymorphic in the tetraploid species *L. corniculatus*. In the putative hybrid species *L. ucrainicus* the portion of polymorphic loci was also high (89%), while in the diploid *L. stepposus* it was lower (about 79%).

Species-exclusive amplicons

Nine exclusive amplicons were counted in *L. corniculatus* (Table 4). None of them was fixed (i.e. present in all accessions). Only one exclusive amplicon was private for *L. corniculatus* and 8 of 9 bands were shared with *L. ucrainicus*. *Lotus stepposus* possessed two exclusive amplicons, both shared with *L. ucrainicus*. *Lotus ucrainicus* had the only its-own private amplicon, absent in the two other studied species.

Species-typical amplicons and their occurrence in putative hybrids

Although no species-specific ISSR loci were found in presumable parental species *L. corniculatus* and *L. stepposus*, 73 loci occurred with at least 25% difference in band frequencies between these two species (Table 5). These loci were used to examine gene flow. For 57 of these loci, presumable

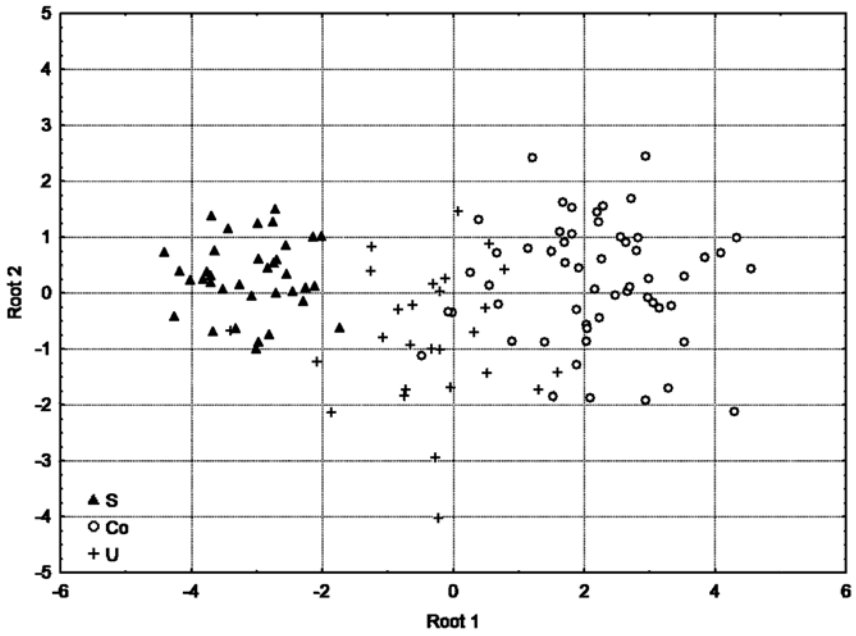

Figure 2. Scatterplot showing the results of Discriminant analysis performed by using 10 morphological characters in 119 individuals. Species symbols: S – *Lotus stepposus*, Co – *L. corniculatus*, U – *L. ucrainicus*.

Table 4. Summary of ISSR amplicon counting for the total matrix and for three studied species (number of polymorphic loci, number of exclusive amplicons).

Total number of amplicons 194							
Number of polymorphic loci 192							
	No. of polymorphic loci (% of all)	No. of exclusive amplicons	No. of fixed exclusive amplicons	No. of private exclusive amplicons	No. of exclusive amplicons shared with:		
Species					Co	U	S
L. corniculatus (Co)	184 (95)	9	—	1	—	8	—
L. × *ucrainicus* (U)	173 (89)	—	—	1	—	—	—
L. stepposus (S)	154 (79)	2	—	—	—	2	—

hybrid individuals of *L. ucrainicus* were characterized by band frequencies intermediate to those which were found in the putative parental species *L. corniculatus* and *L. stepposus*. This result may suggest their real hybrid nature. From these 57 loci, *L. stepposus* had higher frequency for 31 loci and *L. corniculatus* for 26 loci. And for the remaining 16 of 73 loci putative hybrids of *L. ucrainicus* had extreme band frequencies compared to parental species, i.e. they had highest or smallest frequency values among three studied taxa. These 16 loci do not necessarily support a hypothesis about a hybrid origin of *L. ucrainicus*.

Ordination by PCO

Using the data matrix containing 192 polymorphic ISSR amplicons, principal coordinate analysis based on Jaccard distance was conducted. The first three principal coordinates described 14.5%, 4.8% and 4.3% of the total distance matrix, respectively. On a scatterplot of the first two principal coordinates (Fig. 3), three noncontacting clusters of dots corresponding to *L. corniculatus* (C and K), *L.* × *ucrainicus* (U) and *L. stepposus* (S) can be seen. Cluster of *L.* × *stepposus* dots is the most compact, while clusters of two other species are laxer. Individuals of C and K groups are mixed within the cluster of *L. corniculatus* without any order, which supports the idea that genetically they form one common group. Groups C and K are partly separated alongside the coordinate 3 (figure is not presented), but this coordinate describes 4.3% of the total distance only and is less important than two first coordinates.

NJ analysis

NJ analysis of total 194 ISSR loci produced a single, unrooted tree (Fig. 4). Presumed parental species (*L. stepposus* and *L. corniculatus*) form large clades on the opposite sides of the main branch. Several clades of putative hybrid species *L. ucrainicus* are placed between them. They do not form a compact cluster. The only individual of *L. stepposus* from the sample Starobeshevo (i.e. Sb12, marked by asterisk on Fig. 4) is clustered with individuals of *L. ucrainicus*, which is an evidence of its admixed genetic nature. Bootstrap support for the majority of groups within the tree was very low (not shown). As a rule, populations were not resolved as separate groups within the neighbor-joining tree, with the exception for three local populations of *L. stepposus* (H, Vb, and S), which form separate clades. Two of them, Vb and S from Volgograd region, were characterized by high bootstrap support values equal to 82.4 and 81, respectively, while for the rest tree, the support more than 50% was mainly obtained only for small groups of individuals, not corresponding to local populations or species.

Genetic differentiation, gene flow and analysis of molecular variance

Genetic differentiation (Φ_{ST}) between most pairs of populations was rather high. Most values (ca. 70%) varied between 0.21 and 0.49, only ca. 30% pairs of populations demonstrated lower (0.04–0.2) genetic differentiation (table of values it not presented). Variation in the ISSR banding pattern was highly significant among species, among populations and within populations (p = 0.00000; Table 6). The major part of variation was found within populations (66.86%, $\Phi_{ST} = 0.331$, p = 0.00000). Variation among species (18.49%, $\Phi_{CT} = 0.185$, p = 0.00000) was higher than that among populations within species (14.64%, $\Phi_{SC} = 0.180$, p = 0.00000) (Table 6), which shows that gene flow among studied *Lotus* species is more restricted than within each of the species. The average number of individuals exchanged between populations per generation (N_em) was not very high (0.505).

A low positive but highly significant correlation was found between genetic distance (Φ_{ST}) and geographic distance (r = 0.312, p= 0.000000, Mantel test) in the analysis of all 24 local populations. However, if populations of each species were analysed separately, quite different results were obtained. Maximum correlation was found for populations of diploid species *L. stepposus* (r = 0.765, p = 0.009), for populations of more widespread species *L. corniculatus* it was smaller (r = 0.505, p = 0.019), while for hybrid populations of *L. ucrainicus* it had minimum value and was not significant (r = 0.173, p = 0.188).

Analysis by Structure v.2.2

Analysis of allele frequencies was conducted for expected group number K equal 1, 2, 3, 4, 5, 6, 7 and 8. For each K value, the analysis was repeated 10 times. All repeats for each K gave close results for small K values (1, 2 or 3). Starting from K = 4, L(K) was unstable and varied from one repeat to another. The maximum value of L(K) returned by Structure was obtained for K = 8,

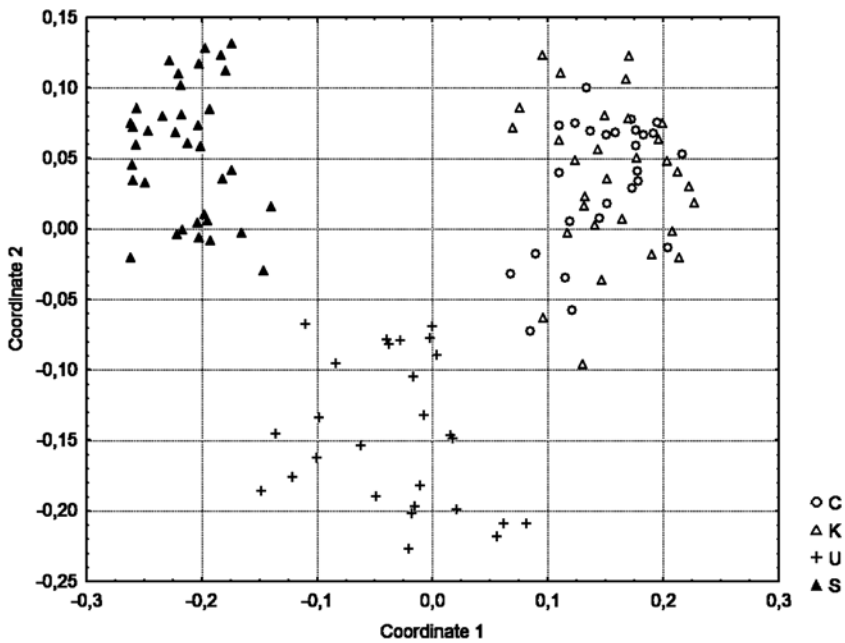

Figure 3. Scatterplot showing the results of Principal coordinate analysis performed by using 192 ISSR markers in 119 individuals. Species symbols: C – *L. corniculatus*, K – *L. corniculatus* var. *hirsutus*, U – *L. ucrainicus*, S – *L. stepposus*.

Table 5. Band frequencies of the 73 species-typical ISSR loci (differ by at least 25% between the putative parental species, *Lotus corniculatus* and *L. stepposus*). *Co, U and S indicate the band frequencies for *Lotus corniculatus*, putative hybrids *L. ucrainicus* and *L. stepposus*, respectively.

Loci with intermediate 'hybrid' band frequencies	ISSR band frequencies in*			Species with higher frequency
	Co	U	S	
HB12-25	0.59	0.04	0	Co
HB12-26	0.64	0.33	0.33	Co
HB13-2	0.59	0.56	0.31	Co
HB13-4	0.64	0.52	0.17	Co
HB13-6	0.79	0.48	0.06	Co
HB13-7	0.39	0.19	0	Co
HB13-13	0.45	0	0	Co
HB13-14	0.29	0.07	0	Co
HB13-16	0.34	0.22	0	Co
HB13-26	0.27	0.15	0	Co
M12-24	0.68	0.56	0.22	Co
M12-27	0.84	0.81	0.17	Co
UBC840-11	0.98	0.89	0.72	Co
UBC855-3	1	0.96	0.61	Co
UBC855-9	0.52	0.37	0.25	Co
UBC855-11	0.54	0.11	0	Co
UBC855-14	0.61	0.26	0.17	Co
UBC855-19	0.3	0.11	0	Co
UBC855-20	0.64	0.22	0	Co
UBC868-8	0.5	0.19	0.17	Co
UBC868-17	0.98	0.78	0.06	Co
UBC868-27	0.61	0.15	0	Co
M1-3	0.96	0.41	0.17	Co
M1-15	0.43	0.22	0.11	Co
M1-17	0.52	0.04	0.03	Co
M1-25	0.41	0.22	0	Co
HB12-2	0.64	0.78	1	S
HB12-8	0.23	0.37	1	S
HB12-9	0.11	0.15	0.53	S
HB12-15	0	0.11	0.55	S
HB13-3	0.64	0.89	0.97	S
M12-3	0.04	0.26	0.56	S
M12-5	0.21	0.44	0.94	S
M12-7	0.31	0.37	0.75	S
M12-11	0.3	0.41	0.81	S

M12-18	0.25	0.63	0.94	S
M12-25	0.04	0.15	0.36	S
M12-28	0.09	0.26	0.47	S
UBC840-6	0.04	0.41	0.67	S
UBC840-9	0.59	0.85	0.92	S
UBC840-18	0.05	0.56	0.81	S
UBC840-20	0.04	0.19	0.69	S
UBC840-24	0.07	0.22	0.42	S
UBC840-28	0.09	0.11	0.58	S
UBC855-1	0.05	0.52	0.64	S
UBC855-10	0.55	0.74	0.89	S
UBC868-1	0.02	0.15	0.31	S
UBC868-5	0.46	0.67	0.72	S
UBC868-7	0.29	0.48	0.69	S
UBC868-10	0.25	0.63	0.75	S
UBC868-25	0.04	0.07	0.33	S
M1-1	0.23	0.81	0.94	S
M1-7	0.46	0.7	1	S
M1-10	0.43	0.44	0.83	S
M1-16	0.43	0.7	0.89	S
M1-18	0.27	0.52	0.75	S
M1-23	0.25	0.37	0.64	S
Loci with extreme 'hybrid' band frequencies	Co	U	S	
HB12-5	0.59	0.26	0.97	
HB12-14	0.39	0.26	0.72	
M12-10	0.09	0	0.86	
M12-21	0.32	0.37	0.03	
M12-22	0.63	0.07	0.14	
UBC855-2	0.18	0.11	0.61	
UBC840-5	0.34	0.3	0.67	
UBC840-7	0.38	0.44	0.11	
UBC855-12	0.3	0	0.64	
UBC855-13	0.96	1	0.42	
UBC855-22	0.46	0.07	0.19	
UBC868-6	0.88	0.22	0.36	
UBC868-13	0.38	0.33	0.67	
UBC868-18	0.34	1	0.92	
M1-5	0.41	0.26	0.78	
M1-19	0.59	0.19	0.28	

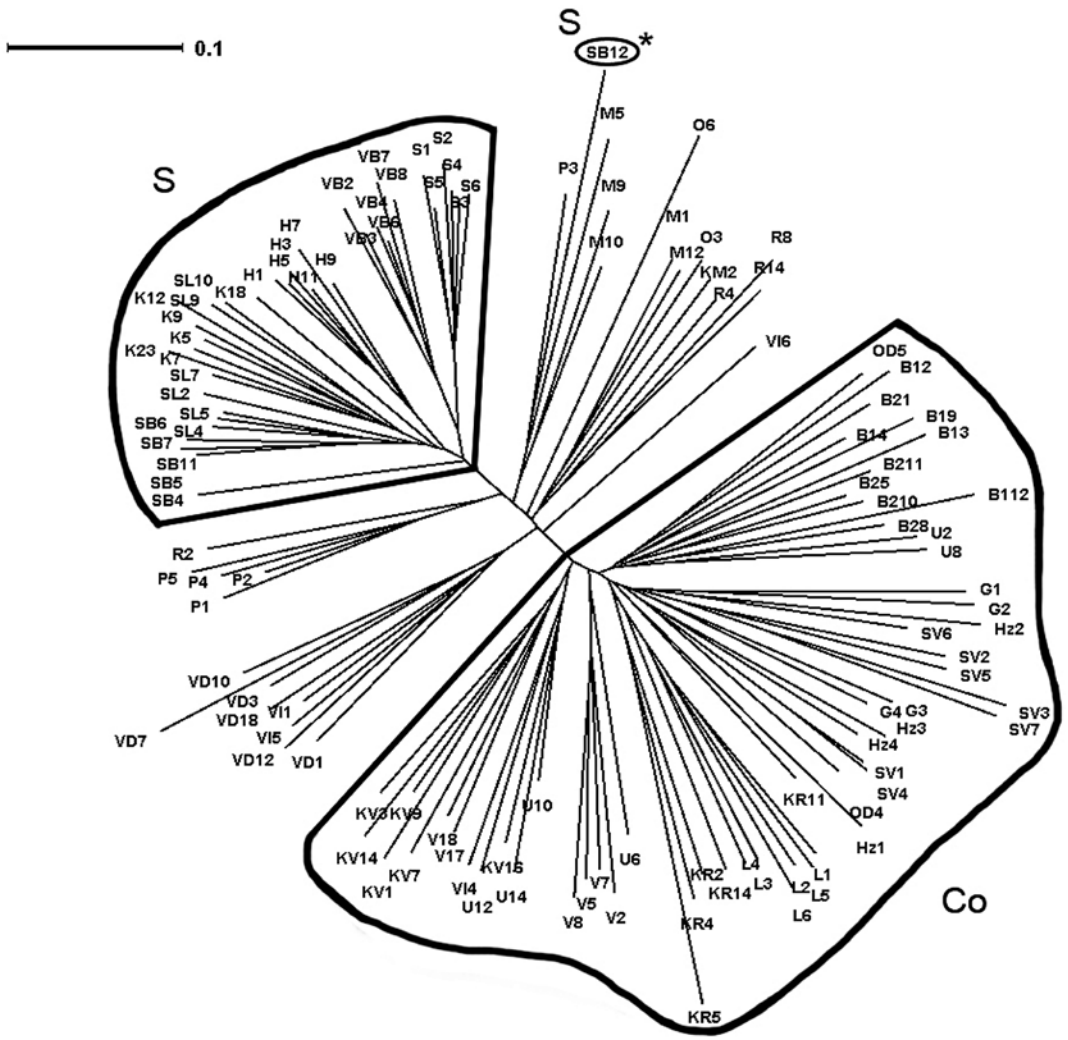

Figure 4. Neighbor-joining dendrogram inferred for 119 individuals with 194 ISSR loci, using Jaccard similarity coefficient (Jaccard 1912). Two main clusters, including *L. corniculatus* (Co) and *L. stepposus* (S) individuals, are outlined with thick lines. The only individual of *L. stepposus* which stands separately from the main species cluster (Sb12) is marked with an asterisk. Individuals of *L. ucrainicus* are not outlined. All individuals are marked with the population symbol (from Table 1) and an individual number.

however, this was an area of instability of L(K); the next highest value of L(K) was obtained for K = 3 (Fig. 5 A).

For K = 5, 6, 7 and 8, L(K) varied greatly, so Structure was not run with K > 8. The plateau of mean values of K was not well pronounced. If the maximum value of L(K) is used as a criterion for estimation of the real K, the conclusion would be that the real K value is 3 (Fig. 5 A).

The peak of the distribution of DK corresponds to K = 2 (DK = 412), but this peak does not stand out sharply against other values of DK (Fig. 5 B). The values of DK for K = 3 is also rather high (DK = 307). So, the statement that the real K value is 2 may not be so categorical. On Fig. 6 we demonstrate probable patterns of allele frequencies corresponding to the presence of two (Fig. 6 A) or three (Fig. 6 B) groups in the studied dataset. The following description of the behaviour of the three studied species in different variants of the analysis can be given:

Table 6. Summary of analysis of molecular variance (AMOVA) of populations of *Lotus corniculatus* (11 populations), *L. ucrainicus* (7 populations) and *L. stepposus* (6 populations). The analysis is based on ISSR data. Levels of significance are based on 1000 iteration steps.

	Variance component			
Level of variation	df	Absolute	%	p
Among species	2	6.15	18.49	0.000
Among populations	21	4.87	14.64	0.000
Within populations	95	22.24	66.86	0.000

In both variants of analysis (i.e. with K = 2 and K = 3), the group Co (*L. corniculatus*) containing subgroups C (*L. corniculatus*, accessions G, Hz, L, Sv and Kr) and K (*L. corniculatus* var. *hirsutus*, accessions Od, B1, B2, U, V and Kv), was presented by almost pure population, i.e. the first parental species. The group S (*L. stepposus*, accessions Sb, Sl, K, H, Vb and S) was another nearly pure population, i.e. the second parental species (Fig. 6A, B).

In the case of K = 2, the group U (*L.* × *ucrainicus*, accessions Vd, Vi, P, M, R, O and Km) was admixed and its specimens possessed genetic material from two parental species (Co and S) (Fig. 6A).

In the case of K = 3, the group U formed the third almost pure population with few slightly admixed specimens (Fig. 6B). According to ISSR spectra, probability is not more than 14% for accessions P3 and R2 to attend to *L. stepposus* and less than 10% for accessions O3 and Km2 to be classified as *L. corniculatus*. This variant of Structure analysis was also characterized by the presence of several admixed accessions in the parental species groups Co and S. Thus, the accession L6 identified as *L. corniculatus* according to morphological data was admixed with U group (the probability to attend to *L.* × *ucrainicus* 0.62). The accession Kr5 (Co group) had the probability ratio to attend to Co:U = 0.19:0.81. The accession U12 of *L.* × *ucrainicus* is characterized by a slite genetic mixture with *L. corniculatus* (the probability 0.11) and an individual Sb12 of *L. stepposus* is admixed with U group (with more than a half probability).

The results obtained for K > 3 can be briefly summarized as follows. The species *L. stepposus* (S) in the majority of analyses was a pure group (except for a specimen Sb12, which was often admixed). In some variants of analysis the group S was separated into two subgroups corresponding to Russian and Ukrainian populations. The second parental species *L. corniculatus* for K > 3 very often was broken down into several subgroups which were not very pure.

Discussion

The results of morphological and molecular analyses conducted on a set of *Lotus* samples from a wide territory of Russia and Ukraine have demonstrated the presence of three distinct groups corresponding to taxonomic concepts of *L. corniculatus*, *L. ucrainicus* and *L. stepposus*.

Morphological studies revealed that individuals of *L. ucrainicus* are characterized by a transitional morphology between the two other studied species, *L. corniculatus* and *L. stepposus*. It is apparent from the results of multivariate morphological analysis as well as from variation patterns of individual characters in the three taxa. *Lotus ucrainicus* usually possesses a combination of characters of presumable parental species: *L. ucrainicus* is close to *L. stepposus* in the calyx tube to teeth ratio and to *L. corniculatus* var. *hirsutus* in style length and index of leaflets of middle stem leaves. As

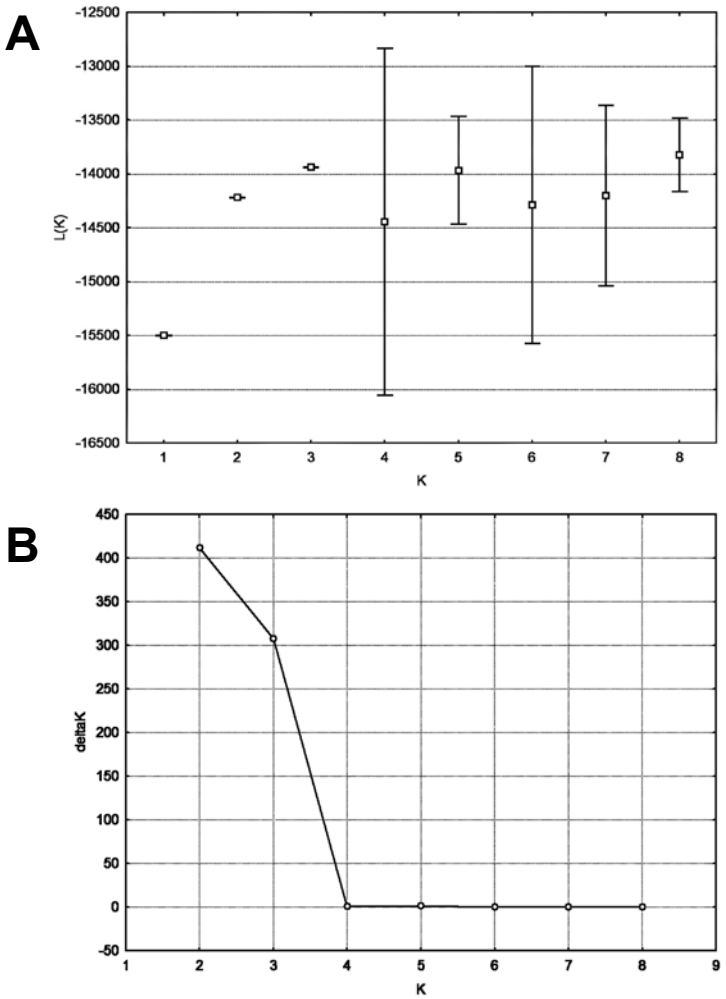

Figure 5. Structure v.2.2 software results. A – Mean L(K) (±standard deviation, SD) over 10 runs for each K value. B – DK calculated as DK = m|L''(K)|/s[L(K)]. The modal value of this distribution may serve as an estimate of true K.

for the morphological features important for delimiting all three studied species (i.e. flower length and index of leaflets of upper stem leaves), their variability ranges in *L. ucrainicus* slightly differ from those in two other species. Such pattern of morphological variation may suggest a hybrid origin of *L. ucrainicus*. This idea has been advanced in earlier publications (Kramina 2000; Kramina et al. 2012), but now it is illustrated and supported by more representative sampling.

The major trends in ISSR marker distribution remained unchanged compared to the previous paper (Kramina et al. 2012). *Lotus ucrainicus* shares all species-exclusive ISSR amplicons from *L. stepposus* and the majority of such markers from *L. corniculatus*. This may suggest that *L. ucrainicus* appeared through hybridization between the two mentioned species. As for species-typical ISSR markers, the picture is more complicate. Seventy three of such markers (i.e. those that occurred in one presumable parental species in at least a 25% higher frequency than in the other) were revealed. For the majority of them, *L. ucrainicus* has an intermediate band frequency which agrees with its presumable hybrid nature. However, for a small part (about 22%) of species-typical markers, *L. ucrainicus* has extreme frequency values, i.e. smaller or larger than in two putative parental species. This fact does not necessarily support the hybrid hypothesis. Several explanations

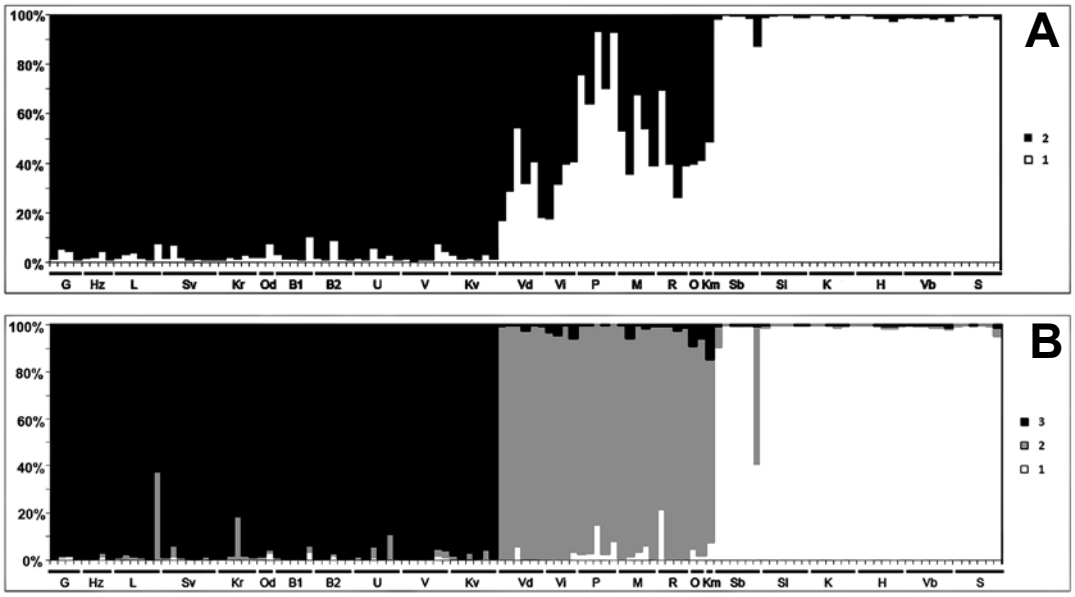

Figure 6. Two barplots obtained as a result of Bayesian analysis in Structure v.2.2. Posterior probabilities of clusterization of 119 *Lotus* individuals into K clusters by ISSR marker composition. Symbols of local populations are the same as in Table 1. Presumed number of clusters in the studied dataset: A – K = 2; B – K = 3.

of similar pattern of genetic variation in *Zaluzianskya* species were proposed by Archibald et al. (2004) and may be applied to *Lotus* species as well. The first explanation is connected to dominant nature of ISSR markers. If both parents are polymorphic for all marker bands, some hybrid individuals may lack a particular marker because they inherited the absent 'allele' from two heterozygous parents. Then, if we analyse band frequencies in studied species, the presumed hybrid taxon may differ significantly from putative parental taxa. However, if population band frequencies are taken into account, it becomes apparent that in hybrid populations the frequencies are indistinguishable from those in at least some of the populations of one or both parental species (Archibald et al. 2004).

The neighbor-joining tree constructed on ISSR data suggests a geographic component of genetic diversity and a gene flow between populations, especially within each studied species. Individuals of local populations of *L. corniculatus* are mixed in clades, the same can be related to *L. ucrainicus*. In Volgograd region the populations of the diploid species *L. stepposus* form distinct clusters, while Ukrainian populations are intermingled in clades and one specimen (Sb12) is even genetically admixed with *L. ucrainicus*. Intermediate position of *L. ucrainicus* individuals between clusters of two other species on NJ tree and PCO scatterplot is congruent with the possibility of their hybrid nature.

The amount of variance among populations (15%) was considerably lower than in comparable studies on *Gentianella germanica* (37%; Fischer & Matthies 1998), *Pedicularis palustris* (44%; Schmidt & Jensen 2000) and *Astragalus cremnophylax* var. *cremnophylax* (63%; Travis et al. 1996). It is comparable to that obtained for *Hedysarum grandiflorum* Pall. (17%; Schanzer & Suprun 2012), but some restrictions of gene flow exist among studied *Lotus* populations. Among-species genetic variability (18.5%) indicates that species *L. corniculatus*, *L. ucrainicus* and *L. stepposus* are differentiated from each other and gene flow between them is more restricted

than within each of them. The fact that the major part of molecular variation is presented by within-population variability, together with rather high fixation indices ($\Phi_{ST} > 0.1$), gives evidence of genetic isolation of populations studied.

According to Mantel test results, geographical component in genetic variance in *L. stepposus* and *L. corniculatus* is of high or middle value, while for *L. ucrainicus* it is not significant. This means that the genetic distances among its populations are not associated with geographical distances, which again may be an indirect evidence of hybrid origin of the latter.

The most probable number of groups in the studied set of populations, in accordance with Structure software results, is two or three. This supports the idea of the presence of three species or two species and a number of genetically admixed individuals, corresponding to *L. ucrainicus* in this taxonomic group.

As a result, patterns of ISSR band frequencies in the studied dataset may be explained by at least two ways. On the one hand, such patterns may be caused by a partially restricted bidirectional gene flow between *L. corniculatus* and *L. stepposus* through introgressive hybridization with *L. ucrainicus*. On the other hand, such a picture of genetic variation may be due to putative recent colonization from a common ancestor, which explains genetic similarity of all three *Lotus* species. The first explanation is preferable due to the following reasons. *Lotus ucrainicus* has the only characteristic ISSR amplicon and there is no significant correlation between genetic variation and geographical distance among its populations. These facts may serve as evidence of later origin of this taxon compared to *L. stepposus* and *L. corniculatus*. So, *L. ucrainicus* looks like a taxon, which appeared recently through hybridization or is being differentiated at present time. *Lotus ucrainicus* is less distinguishable from *L. corniculatus* than from *L. stepposus*. This may be due to the same ploidy level 2n = 4x, revealed in *L. corniculatus* and the majority of studied *L. ucrainicus* individuals by flow cytometry analysis (Kramina et al. 2012). Cases of tetraploid hybrid progeny formation after crossing between tetraploid *Lotus corniculatus* and diploid *L. tenuis* have been described (Negri & Veronesi 1989; Rim & Beuselinck 1992) and discussed previously (Kramina et al. 2012). The formation of unreduced 2n gametes may be supposed in diploid parent species *Lotus stepposus*, as it was demonstrated for the close species *L. tenuis* (Rim & Beuselinck 1992).

Morphological and ISSR analyses allowed more precise identification of the diploid species *L. stepposus*. In the majority of analyses conducted, the limits of the species are determined identically with six populations included (Sb, Sl, K, H, Vb and S). Sb12 is the only accession which demonstrates admixed genetic composition. Ideas about more or less clear limits of the diploid species *L. stepposus* and its distinction from *L. corniculatus* have already been discussed by Kramina et al. (2012), where only two of its populations (from Volgograd region) had been studied. Now these ideas are supported by new results. In the present paper, several Ukrainian samples were added, including a sample H collected in the Nature reserve 'Khomutovskaya step', in the locus classicus of *L. stepposus*. Inclusion of the material from the locus classicus confirmed our general understanding of this species and its particular traits.

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Address of the author:

Dr Tatiana E. Kramina
Department of Higher Plants
Biological Faculty
Lomonosov Moscow State University
1(12), Leninskie Gory
Moscow 119234
Russia
E-mail: tkramina@yandex.ru