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Wulfenia **17** (2010): 99–115

Wulfenia
Mitteilungen des
Kärntner Botanikzentrums
Klagenfurt

Local differentiation and hybridization in wild rose populations in Western Ukraine

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Summary: Three species of wild roses, viz. *Rosa spinosissima*, *R. gallica*, and *R. canina* s.l., sympatrically co-occur in Western Ukraine. *R. canina* is the morphologically most diverse of them; its different morphotypes (or morphological species in narrow sense) are characterized by a considerable variability in both vegetative and generative characters. This variability, however, does not correlate with ISSR markers diversity. The latter shows an uneven geographical distribution indicating to a probable differentiation of local populations caused by restricted gene flow. On the contrary, *R. gallica* shows no sign of such a geographical differentiation. However, both species can rarely hybridize, *R. gallica* being always the pollen parent. Hybrids are morphologically diverse, but not strictly intermediate between the parental plants, usually deviating towards one or the other of them in their characters. Some of the hybrids or putative backcrosses are indistinguishable from *R. gallica* itself, what may indicate a probable introgression between the two species. *R. spinosissima* is a rare species in this area, and it is uniform in its characters and seems to be not involved into any hybridization with other species.

Keywords: Rosaceae, *Rosa canina*, *Rosa gallica*, *Rosa spinosissima*, dog-roses, trnV-ndhC, ISSR, hybridization, population

Wild roses are common plants in the south of the Russian Plain in general and in Western Ukraine in particular. They grow in different habitats, though most of them prefer remnants of steppe vegetation and forest margins, often on slopes of hills or eroded gullies. They usually form multispecies stands, mostly composed of dog-roses belonging to the section Caninae (DC.) Ser., though members of other sections like *Rosa majalis* Herrm. of Cinnamomeae (DC.) Ser., or *R. gallica* L. of Gallicanae (DC.) Ser. sections may be present in such stands as well, growing side by side with the dog-roses. The latter are notoriously difficult to be taxonomically identified, what is usually attributed to their inherent hybrid nature and the ability to hybridize with each other and members of other sections (Wissemann & Ritz 2007). The species of dog-roses are commonly delimited on the basis of a set of correlated morphological characters. Their composition is assumed to be more or less established for Central and Western Europe, but opinions about Eastern European species are much more controversial (Klášterský 1968; Henker 2003; Buzunova 2001), up to a point of view that next to nothing is known about them (Wissemann 2003). Western Ukraine is a species-rich Eastern European area, where from many species of dog-roses were described by W. Besser in the XIXth century, and by V. Chrshanovsky in the XXth century (see 'The International Plant Names Index' http://www.ipni.org/ipni/plantnamesearchpage.do).

Both in herbaria and in the wild, dog-rose plants occur which are morphologically intermediate between the described species, commonly regarded as hybrids. However, hybridization experiments with dog-roses (Gustaffson 1944; Wissemann & Hellwig 1997; Werlemark & Nybom 2001; Ritz & Wissemann 2003) reveal that the F1 progeny from interspecific crosses, as a

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rule, is not morphologically intermediate between the parents. Often these hybrid plants are indistinguishable from the maternal plant, or may display hip characters similar to those of the pollen parent, or even possess some novel characters depending on the character combinations of the parental plants. Mostly matroclinal inheritance in dog-roses is due to a very special breeding system called 'balanced heterogamy' (Fagerlind 1951; Wissemann & Ritz 2007). All the dog-roses are allopolyploids with 2n=4x, 5x or 6x, n=7. However, only two genomes of 4, 5 or 6 in a polyploid dog-rose nucleus are pairing and forming bivalents during meiosis. These genomes are transferred both via haploid pollen and polyploid egg cells. The 2, 3 or 4 unpairing genomes form univalents during meiosis and are transferred exclusively via egg cells, being lost during meiosis in pollen mother cells. Such an unequal meiosis results in a highly skewed, mostly matroclinal character inheritance in dog-roses at the morphological (Ritz & Wissemann 2003) as well as the molecular level (Werlemark et al.1999; Werlemark & Nybom 2001).

Studies of wild populations of dog-roses with the use of AFLP markers reveal that marker polymorphism is more correlated with geographic origin of specimens than with their taxonomic identity based on morphology (DeCock et al. 2008). This fact may be due to introgression or to hybridization resulting in the presence of certain hybrid morphotypes arising de novo in geographically distinct localities where two or more rose species meet and hybridize. Similar patterns are found in sympatric populations of wild roses in Russia and Ukraine (Schanzer & Vagina 2007; Schanzer & Kutlunina 2010).

Hence, the aim of the present study is to test if morphological species found in this area can be confirmed genetically, and if any of them may spontaneously hybridize with the others.

Materials and methods

Population sampling: Samples were collected in June 2010 from five localities in Lviv, Ternopil and Ivano-Frankivsk administrative regions of Ukraine in the geographical province of the Podolian elevation. The names and geographical coordinates of the localities, together with the names of species and specimen field numbers of each locality are listed in Appendix 1. The localities are situated at different distances from each other (LG–LO ~ 90 km; LG–LB ~ 21 km; LG–LL ~ 40 km; LV–LO ~ 100 km; LB–LO ~ 100 km; LB–LL ~ 65 km; LL–LV ~ 18 km), so that the most proximate can be grouped together as follows: LG–LB, LL–LV, LO. Their relative geographical positions are shown in Fig. 6.

A total of 76 individuals were sampled for our study. The voucher specimens are deposited in the Herbarium of the Main Botanical Garden in Moscow (MHA). We determined the sampled plants with the key in the 'Flora Europae Orientalis' (Buzunova 2001) according to the taxonomic treatment of the genus suggested there: *Rosa gallica* (16 specimens), *R. canina* L. (26), *R. subcanina* Christ. (13), *R. tomentosa* Sm. (3), *R. caryophyllacea* Bess. (2), *R. spinosissima* L. (3), *R. glauca* Pourr. (1), *R. parviuscula* Chrshan. (3), *R. porrectidens* Chrshan. (2), *R. corymbifera* Borkh. (2), *R. podolica* Chrshan. (1). One specimen morphologically deviated from a typical *R. gallica* and was marked as a putative hybrid. Two specimens of dog-roses did not correspond in their characters to any species in the key and remained undetermined. All the specimens were examined for 20 morphological characters listed in Tab. 1. The choice of the characters for this study depended largely on the characters traditionally used as diagnostic for the species in our sample by other authors, particularly by Buzunova (2001).

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Table 1. Morphological characters used in the study.

Nr.	Character	Unit or states of qualitative characters
1	Bush height	cm
2	Leaflet length	mm
3	Leaflet width	mm
4	Leaflet shape	1 – narrow elliptic, 2 – round, 3 – round with acute tip, 4 – elliptic, 5 – elliptic with acute tip
5	Leaf texture	1 – soft, 2 – medium density, 3 – leathery
6	Leaflet hairyness with simple hairs (above)	1 – glabrous, 2 – sparse, 3 – dense
7	Leaflet hairyness with simple hairs (underneath)	1 – glabrous, 2 – sparse along nerves, 3 – sparse on surface, 4 – dense on surface
8	Leaflet hairyness with glandulous hairs (above)	1 – glabrous, 2 – sparse, 3 – dense
9	Leaflet hairyness with glandulous hairs (underneath)	1 – glabrous, 2 – sparse along nerves, 3 – sparse on surface, 4 – dense on surface
10	Petal colour	1 – white, 2 – pale pink, 3 – pink, 4 – bright pink to magenta
11	Glandulous hairs on pedicel	1 – glabrous, 2 – sparse, 3 – dense
12	Glandulous hairs on sepals	1 – glabrous, 2 – sparse, 3 – dense
13	Hypanthium length	mm
14	Glandulous hairs on hypanthium	1 – glabrous, 2 – sparse, 3 – dense
15	Pedicel length	mm
16	Prickle shape	1 – absent, 2 – hooked, 3 – sickle-shaped, 4 – acicles
17	Sepal shape	1 – entire, 2 – slightly dissected, 3 – pinnate
18	Style head shape	1 – loose, 2 – dense
19	Leaf margin dentations	1 – simple, 2 – double to complex, glandulous
20	Leaf margin teeth	1 – without glands, 2 – with one to few glands, 3 – with many glands

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Table 2. ISSR primers used for PCR.

Primer	Sequence
UBC 840	GAG AGA GAG AGA GAG AAY T
17899a	CAC ACA CAC ACA AG
M7	CAG CAG CAG CAG CAG
M12	CAC ACA CAC ACA (A/G) (C/T)
M9	GAC ACG ACA CGA CAC GAC AC
UBC855	ACA CAC ACA CAC ACA CCY T

DNA extraction: Young leaves were collected from the same plants as the corresponding herbarium specimens and dried in silica gel. DNA was extracted with the NucleoSpin Plant II DNA extraction kit (Macherey-Nagel, Germany) following the manufacturer's instructions.

Marker selection: We used ISSR (Inter Simple Sequence Repeat) markers to study DNA polymorphisms within and between species and to detect putative interspecific hybrids, since they proved to be adequate and useful for these purposes in our previous studies of wild roses (Schanzer & Vagina 2007; Schanzer & Voilokova 2008; Schanzer & Kutlunina 2010). Primers used for PCR were synthesized and purified in PAAG by Syntol Ltd. (Moscow, Russia). They are listed in Tab. 2.

For this study we used the trnV-ndhC intergenic spacer of chloroplast DNA, which was shown to be one of the most variable regions of cp DNA in different groups of flowering plants (Shaw et al. 2007). Though this region was not previously sequenced from any member of the genus *Rosa* by other authors, it proved to be informative in one of our recent studies in wild rose populations (Schanzer et al. 2011). The primer formulas were taken from Shaw et al. (2007) and synthesized by Syntol Ltd. (Moscow, Russia).

ISSR PCR conditions: For amplification of ISSR markers polymerase chain reactions (PCR) were conducted in 20 µl aliquots containing 4 µl of Ready-to-Use PCR MaGMix (200 µM of each dNTP, 1.5 mM $MgCl_2$, 1.5 U 'hot-start' SmarTaqDNA Polymerase and reaction buffer; Dialat Ltd., Moscow, Russia), 14 µl deionized water, 20 pM primer and 10–20 ng of template DNA in a MJ Research PTC-220 DNA Engine Dyad Thermal Cycler (BioRad Laboratories, USA) under the following conditions: 95°C – 3 min (pretreatment); 94°C – 30 s, annealing temperature – 30 s, 72°C – 40 s + 2 s for each cycle (35 cycles), with a final extension step for 4 min at 72°C. The annealing temperature for all ISSR primers used was 50°C. A control, containing all components except genomic DNA, was included in each set of reactions to prove that no contamination occurred.

ISSR PCR reactions were characterized on 1.7% agarose gels in 0.5 × TBE. Gels were stained with ethidium bromide and documented digitally using a GelDoc-It Imaging System (UVP, USA).

Chloroplast DNA PCR conditions and sequencing: The PCR protocol for trnV-ndhC region amplification slightly differed from that for ISSR markers: 94°C – 1 min (pretreatment); 94°C – 30 s, 56°C – 40 s, 62°C – 20 s (30 cycles), with final 2 cycles: 56°C for 40 s, 62°C for

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1 min 20 s. The lower elongation temperature was used because of the high AT content in the target sequence. This improved the work of the polymerase and strongly increased the yield of the PCR product. Double-stranded PCR products were then purified using centrifugation with a solution of ammonium acetate in ethanol. Purified PCR products were cycle sequenced using the ABI PRISM© BigDye™ Terminator v. 3.1 kit (Applied Biosystems) and further analyzed on an ABI PRISM 3730 automated sequencer (Applied Biosystems) at the facilities of Syntol Ltd. (Moscow, Russia).

Analyses of morphological characters: To analyze morphological features we performed Principal Coordinates Analysis (PCoA) as implemented in the PAST v. 2.0 program (Hammer et al. 2001). The same data were analyzed also by cluster analysis using Unweighted Pair Group Method based on Arithmetic mean (UPGMA). Since both qualitative and quantitative characters were analyzed together, Gower similarity measure was used.

Analyses of molecular data: The digital image files of ISSR marker electrophoresis results were analyzed using the Cross Checker 2.91 software (Buntjer 2000). Each fragment that was amplified using ISSR primers and visualized as a band in an electrophoretic gel, was treated as a unit character and scored in terms of a binary code (1/0 = +/–). The resulting matrix was analyzed using PCoA and cluster (UPGMA) analyses as implemented in the PAST v. 2.0 program (Hammer et al. 2001). Jaccard coefficient was used as the measure of genetic similarity.

DNA sequences were aligned manually using BioEdit 7.0.5.3. (Hall 1999) and manually edited afterwards. The alignment was collapsed into haplotypes using TCS 1.21 software (Clement et al. 2000).

Population structure and probability of hybrid origin of particular specimens was analyzed using Bayesian inference with the programs Structure 2.2 (Pritchard et al. 2000; Falush et al. 2007) and NewHybrids 1.1 (Anderson & Thompson 2002). The program Structure assesses probability of subdivision of a sample into K populations basing on calculation of allele frequencies in each of these hypothetical populations using Markov chain Monte Carlo method. We used the admixture model with correlated allele frequencies and the no admixture model with independent allele frequencies for the analyses. The first model implies genetic relatedness of the populations compared, Hardy-Weinberg equilibrium and linkage equilibrium for the markers being analyzed. The second model implies low genetic relatedness of the populations compared. The analyses using both models were applied to the whole sample and to dog-roses separately using the admixture model. The numbers of K=1–7 were tested with 2 replicates per K and 1 million Markov chain Monte Carlo repetitions.

The NewHybrids program uses a similar algorithm of analysis but implies a different model, trying to assess probability of subdividing the sample into a priori classes of genotypes. We used the default model of hybridization between two diploid species implying six possible genotype classes (sp0 – first pure species, sp1 – second pure species, F1 – first generation hybrids, F2 – second generation hybrids, Bx0 and Bx1 – backcrosses). The model implies the following distribution of allele frequencies between the genotype classes: sp0 – 100% homozygous of the first parent species-diagnostic markers; sp1 – 100% homozygous of the second parent species-diagnostic markers; F1 – 100% heterozygous; F2 – 50% heterozygous, 25+25% homozygous of both parent species-diagnostic markers; Bx0 – 50% heterozygous, 50% homozygous of the first parent

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species-diagnostic markers; Bx1 – 50% heterozygous, 50% homozygous of the second parent species-diagnostic markers. Like the model used by the Structure program, this model also implies Hardy-Weinberg equilibrium and linkage equilibrium for the markers being analyzed. The analysis was run for 50 000 repetitions in several replicates to assess the stability of the results.

Results

Principal coordinate analysis (PCoA) gives a fairly well resolved picture of groups corresponding to all the species determined with the key in 'Flora Europae Orientalis' (Fig. 1). The first principal coordinate explains 62.6% of distances, the second one explains 6.7%. As anticipated, the most distant groups correspond to *R. spinosissima*, *R. gallica*, and *R. canina*, while all the other groups gradually fill the gap between *R. canina* and *R. gallica*. The specimen of *R. porrectidens* falls within the cloud of specimens of *R. canina*. Specimens determined as *R. subcanina* form a cloud

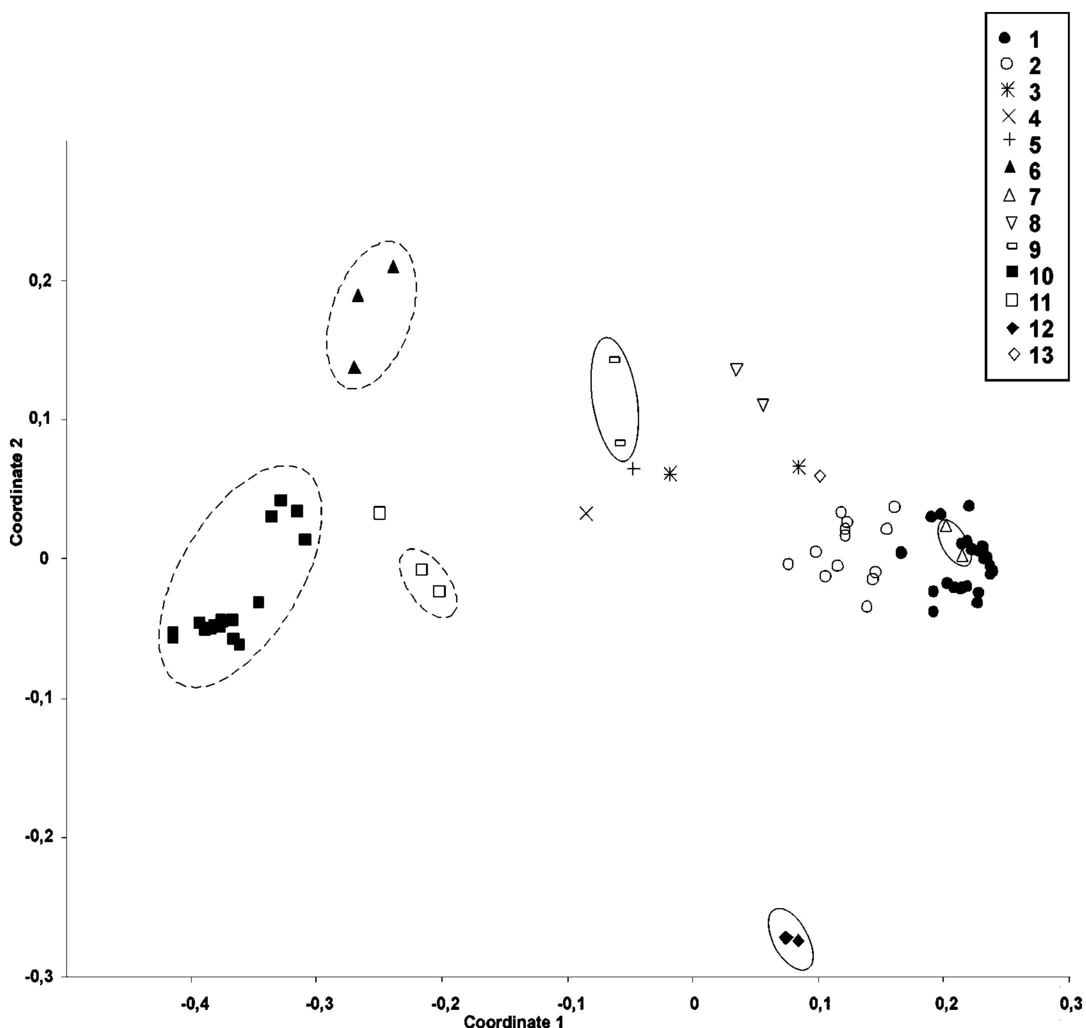

Figure 1. Results of Principal Coordinates Analysis of 20 morphological characters for 76 specimens, Gower similarity measure. 1 – *R. canina*; 2 – *R. subcanina*; 3 – *R.* sp. indet.; 4 – *R. glauca*; 5 – *R. jundzillii*; 6 – *R. tomentosa*; 7 – *R. porrectidens*; 8 – *R. corymbifera*; 9 – *R. caryophyllacea*; 10 – *R. gallica*; 11 – *R. parviuscula*; 12 – *R. spinosissima*; 13 – *R. podolica*.

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Table 3. trnV-ndhC haplotypes. Only variable positions are shown.

haplotype	
G	a**a**--agaaac**t**aaaattctatttct--------tatttctataccattagactatacaatt**gg**-----a
T	a**a**--agaaac**g**aaaattctatttct--------tatttctataccattagactatacaatt**tg**-----a
C	a**c**--agaaac**t**aaaattctatttcttatttctatatttctataccattagactatacaatt**gg**-----a
L	-**c**--agaaac**t**aaaattctatttcttatttctatatttctataccattagactatacaatt**gg**-----a
S	a**a**ta---**tt**ggtgc-

just next to that of *R. canina*. Specimens of *R. parviuscula* and *R. tomentosa* are placed closer to *R. gallica* than to *R. canina*. All the other specimens are placed more or less between *R. gallica* and *R. canina*, somewhat closer to the latter. Of the two undetermined specimens, one is grouped with a specimen of *R. podolica*, the other one is grouped with *R. jundzillii*.

The results of cluster analysis (UPGMA) of morphological data (not shown) are similar to those of PCoA. All the morphological species are clearly distinguished by the set of selected characters and form separate more or less distanced clusters, some with medium to high bootstrap support (1 000 replicates). In Fig. 1, solid line circles surround groups receiving high (90–100) bootstrap support

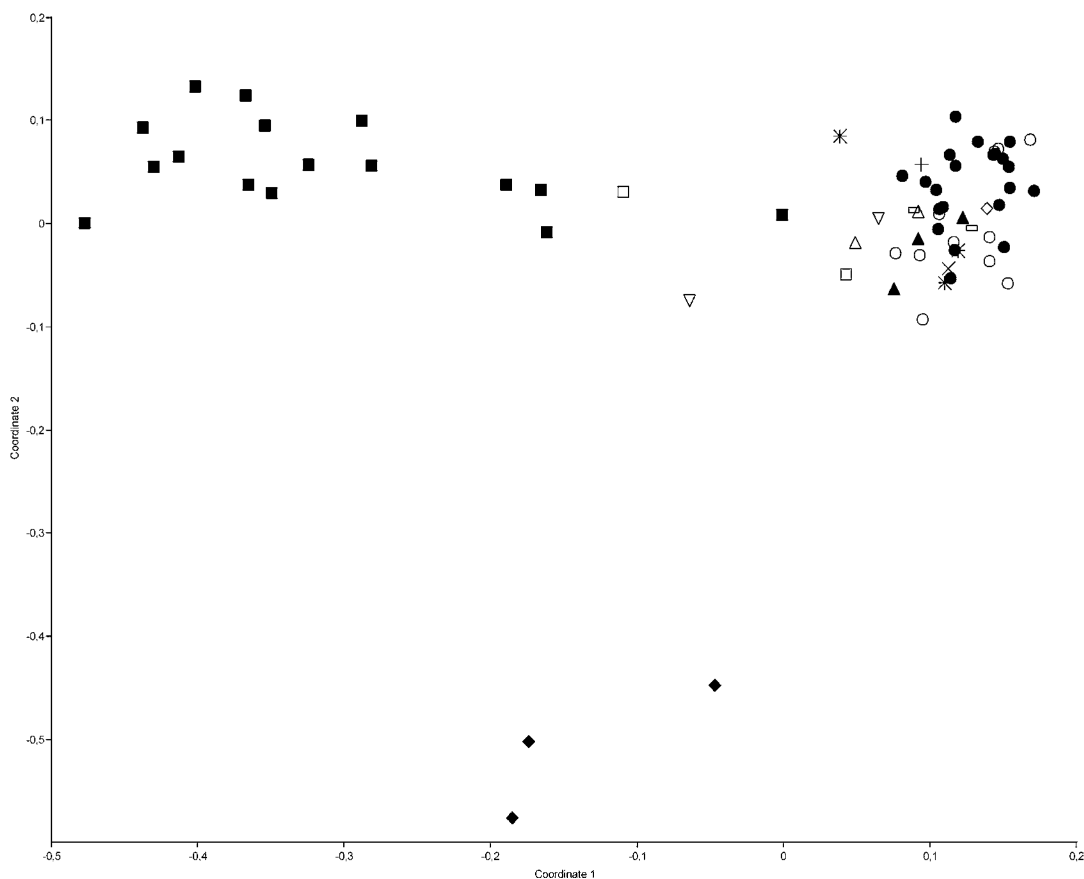

Figure 2. Results of Principal Coordinates Analysis of 82 ISSR markers for 69 specimens, Jaccard similarity measure. Species designation is the same as in Fig. 1.

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in cluster analysis. They are *R. spinosissima* (100), *R. caryophyllacea* (94), and *R. porrectidens* (90). Clusters of *R. tomentosa* (80) and *R. gallica* (71) receive medium support shown by dashed line circles. Several terminal small clusters uniting couples of specimens from the same locality are rather highly supported, too (not shown). All the other clusters receive low to no bootstrap support, what is not surprising given the small number of characters in the matrix. Nevertheless, all the clusters resolve the same groups of specimens as they were determined with the key.

Chloroplast intergene spacer trnV-ndhC was partially sequenced from 58 specimens of total 76 in the sample. The length of the sequence varied between 391 to 442 bp. GenBank accession numbers are given in parentheses after specimen numbers in Appendix 1. The sequences were manually aligned, and the alignment length after editing and introducing gaps was 449. The alignment was converted into haplotypes using TCS software (Clement et al. 2000), gaps were treated as the 5th state. Five haplotypes are recognized, their differences are shown in Tab. 3, and their distribution among the specimens in Figs 3 and 4. Most of the specimens possess haplotype C. The specimen of *R. glauca* bears haplotype L, which differs from C by the only deletion in the 12th position of the alignment. Most specimens of *R. gallica* possess haplotype G,

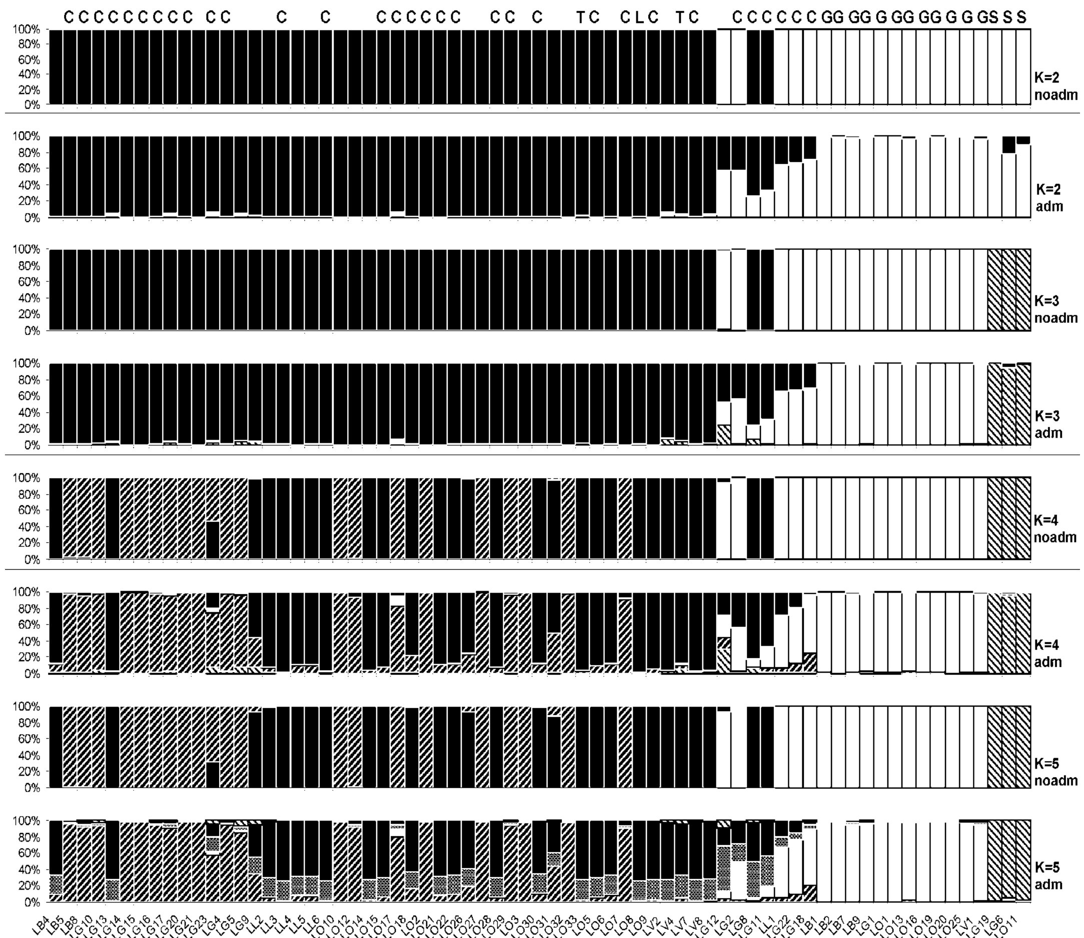

Figure 3. Results of Bayesian analysis in Structure 2.2 program: posterior probabilities of clusterization of 69 *Rosa* specimens into K groups by ISSR marker composition. Specimen numbers are shown below the diagram. trnV-ndhC chloroplast haplotypes are designated above the diagram.

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Table 4. The results of the ISSR data analyses in Structure 2.2: LnP(D) values for different K.

Model	K	LnP(D)
Admixture, allele frequencies correlated	2	-3205.6
	3	-3087.3
	4	**-3020.8**
	5	-3094.5
	6	-3221.5
No admixture, allele frequencies independent	2	-3211.7
	3	-3125.5
	4	**-3030.0**
	5	-3046.6
	6	-3044.4

which differs from the C haplotype by a C–A transition in 27th position of the alignment, and two indels in positions 77–78 and 159–166. Haplotype T is characteristic of the two specimens of *R. tomentosa* and is the closest to the haplotype G, differing from it in two transitions T–G in 144th position, and G–T in 218th position. Haplotype S is the most distanced from them and characteristic of the three specimens of *R. spinosissima*. From the closest haplotype T it differs by a T–G transition in the 244th position, and by four indels, one of which is quite large (positions 138–194).

PCR reactions with six ISSR primers resulted in total 82 reproducible bands. In 7 specimens (LG3, LG7, LV3, LV9, LB10, LO4, LO23) PCR failed with at least one primer, so they were excluded from further analyses. All the bands appeared to be informative, i.e., no one was present in all the specimens or in a single specimen. PCoA analysis separates specimens of *R. spinosissima*, *R. gallica*, and *R. canina* (Fig. 2). The first principal coordinate explains 26.6% of distances; the second one explains 10.1%. However, specimens determined from their morphology as other species of sect. Caninae appear to be either not separable from *R. canina* itself, or are placed in the scatterplot between *R. canina* and *R. gallica*. Several *R. gallica* specimens are deviating toward *R. canina*, too.

Bayesian analyses of the total sample with the Structure software reveal that the highest LnP value is always achieved for K=4, both under admixture and no admixture models (Tab. 4). The diagram in Fig. 3 shows posterior probabilities of assigning particular specimens to one of the groups (K) for K=2–5. For both models used, the program divides the sample into two similar groups under K=2. The first group consists of specimens of *R. spinosissima* (LG19–LO11) and *R. gallica* (LL1–LV1), the second group includes all the specimens initially assigned to *R. canina* and other species of the Caninae section. Seven specimens of *R. corymbifera* (LG12), *R. parviuscula* (LG2, LG8), and *R. gallica* (LG11, LL1, LG22, LG18) show admixed nature as well as two specimens of *R. spinosissima* (LG6, LO11) under the admixture model. Under the no admixture model all the specimens are assigned to one of the two groups with 100% posterior probability. For the number of groups K=3, the program gives a picture nearly identical to K=2

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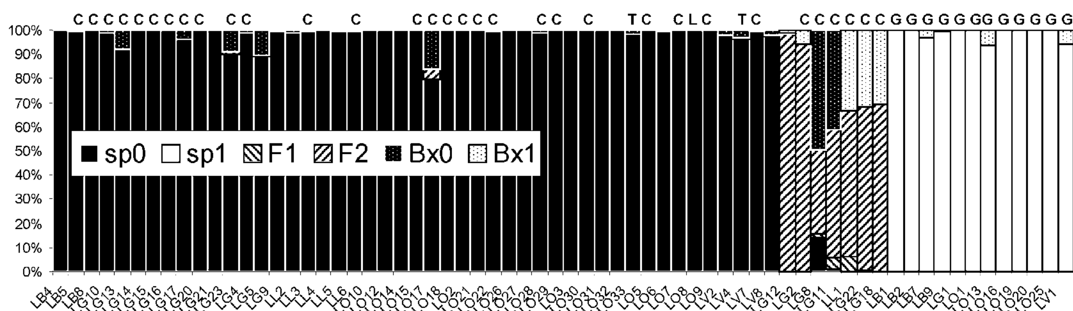

Figure 4. Results of Bayesian analysis in NewHybrids program: posterior probabilities of clusterization of 66 *Rosa* specimens into genotype classes by ISSR marker composition. Specimen numbers are shown below the diagram. trnV-ndhC chloroplast haplotypes are designated above the diagram. sp0 – first parental species; sp1 – second parental species; F1 – first generation hybrids; F2 – second generation hybrids; Bx0, Bx1 – backcrosses.

with only the three specimens of *R. spinosissima* recognized as a separate group. The analyses for K=4 return the same pattern with the major exception that the group representing the sect. Caninae species disentangles into two parts, however, not corresponding to any morphological species. The distribution of trnV-ndhC chloroplast haplotypes among the specimens is shown at the top of the diagram in Fig. 3. Under K=4, the specimens of the first group (*R. spinosissima*) bear haplotype S, those of the second group (most of *R. gallica*) bear haplotype G, the specimens of groups 3 and 4, including the admixed ones, mostly bear haplotype C. Two specimens of *R. tomentosa* (LV4 and LO33) bearing haplotype T appear to belong to different groups, 3 and 4 respectively. The specimen of *R. glauca* with its L haplotype belongs to the third group, together with other haplotype C bearing specimens. The analyses for K=5 and further do not change this pattern, though an intermediate group never represented by any specimen belonging to it with high probability appears under the admixture model.

Since Bayesian analyses in the program Structure revealed some admixed specimens, combining markers from *R. gallica* group and *Rosa* sect. Caninae group, we further analyzed the sample using another model implemented in the NewHybrids program. The specimens of *R. spinosissima* (LG6, LG19, LO11) were excluded from these analyses. The results of the analysis are shown in Fig. 4, with the chloroplast haplotypes indicated for each specimen at the top of the diagram. The program assigns most of the specimens to two parental species corresponding to *R. gallica* and *Rosa* sect. Caninae, respectively. Only seven specimens determined as *R. corymbifera*, *R. parviuscula* and *R. gallica* corresponding to the admixed specimens in the Structure analyses appear to be F2 hybrids, or backcrosses with high posterior probability. Also several specimens of the section Caninae group have small posterior probability of being backcrosses. All the plants of the second parental species (*R. gallica*) bear chloroplast haplotype G, while all the putative hybrids bear haplotype C, as most of the first parental species specimens. The plants bearing haplotypes T and L do not otherwise differ from the rest of the first parental species specimens.

Discussion

At first glance, all morphologically determined species with a few exceptions are more or less clearly distinct from each other on the basis of a set of morphological characters. However, molecular data are in contradiction with the morphology. Both chloroplast and ISSR markers clearly discriminate between species belonging to different sections of the genus, i.e., between

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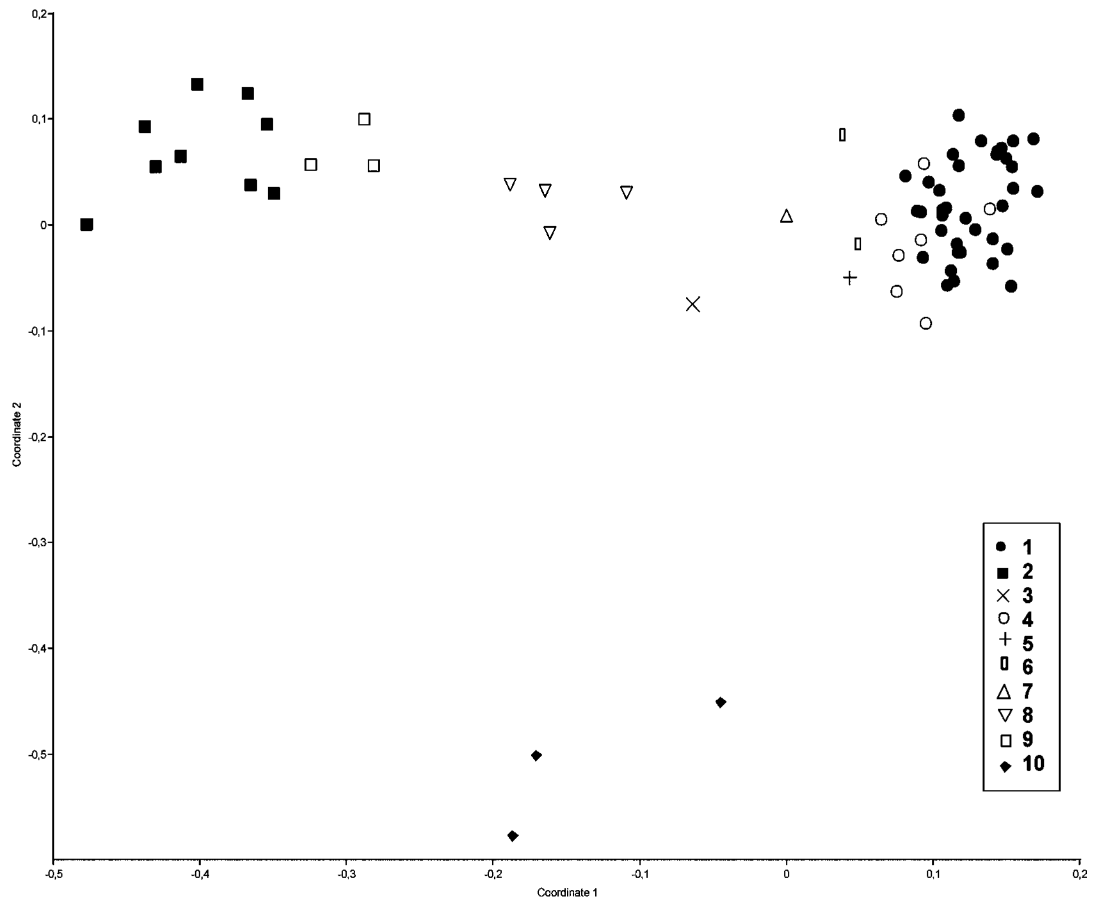

Figure 5. Ordination of the NewHybrids analysis results with PCoA analysis by 82 ISSR markers, Jaccard similarity measure. 1 – sp0 – the first parental species; 2 – sp1 – the second parental species; 3 – F2 – second generation hybrid; 4 – sp0 to less than 10% probability backcross Bx0 to the first parental species; 5 – Bx0, F2 or sp0 with comparable probabilities; 6 – sp0 or Bx0 with less than 10% probability of being F2; 7 – F2 or Bx0 with nearly equal probabilities; 8 – F2 or Bx1 backcross to the second parental species; 9 – sp1 to less than 10% probability backcross Bx1 to the second parental species; 10 – *R. spinosissima*.

R. spinosissima, *R. gallica*, and dog-roses of the section Caninae. However, they fail to discriminate between morphological species of the dog-roses. Chloroplast markers discriminate *R. tomentosa* from the rest of the Caninae section, but ISSR markers do not. All the three specimens of *R. tomentosa* are assessed with the NewNybrids program as pure members of the same parental species as *R. canina* with posterior probability 0.96–0.99. The same relates to the single sampled clone of *R. glauca* included in our study. Its chloroplast trnV-ndhC haplotype (L) differs from the rest of the Caninae haplotypes by a single mononucleotide indel, however, ISSR markers analyzed with the NewHybrids program bring this specimen to the same parental species as the rest of the dog-roses with posterior probability of 0.99. No analyses conducted using the Structure program discriminate *R. tomentosa* and *R. glauca* from the rest of the dog-roses. The other specimens of dog-roses, determined as *R. subcanina*, *R. caryophyllacea*, *R. podolica*, *R. jundzillii*, and one of the two specimens of *R. porrectidens*, share the same chloroplast haplotype (C), and they are assigned to the same parental species by the NewHybrids with posterior probabilities higher than 0.95. PCoA ordination of the NewHybrids analysis results shows a clear pattern of distribution of

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genotype classes (Fig. 5). Specimens of *R. spinosissima* not involved in hybridization are equally distanced from both putative parental species *R. gallica* and *R. canina* in a broad sense. Hybrids F2 and F2-Bx0 (either F2 or less probably backcrosses to *R. gallica*) are placed in the middle between the parental species, while putative backcrosses are strongly shifted to their corresponding parental species.

It is worth mentioning that all putative hybrids and backcrosses to *R. canina* (Bx0) possess the same chloroplast haplotype C as *R. canina*, while all *R. gallica*, including backcrosses Bx1, possess haplotype G. This may be interpreted as *R. gallica* being exclusively the pollen parent in hybridizations with the exception of the three specimens having 2–6% posterior probability of being backcrosses to *R. gallica* and sharing its G haplotype. The specimen LG12 assigned F2 with 0.985 posterior probability is morphologically determined as *R. corymbifera*. This corresponds well to mostly matroclinal inheritance of morphological characters in the section Caninae. However, the specimens assigned as probable F2 hybrids or backcrosses (F2-Bx1) are morphologically either dwarf shrublets (*R. parviuscula*; specimens LG2 and LG8) or rather a typical *R. gallica* (specimens LG18, LG22, LL1). The specimen LG11, initially determined as an atypical and probably hybrid *R. gallica*, falls into this category as well. A possible interpretation for this observation is that morphological type of *R. gallica* may reappear through segregation from hybrid progeny.

The results achieved via Bayesian analyses in Structure and NewHybrids we should, however, treat with major caution. In both cases the underlying models assume populations of diploid outcrossing species. We have not studied chromosome numbers of plants in our sample, but basing on published data from adjacent areas we can reasonably assume that *R. gallica* and *R. spinosissima* in our study are tetraploids with normal meiosis, while members of the Caninae section may be tetra- to hexaploids with heterogamous meiosis (Klášterská 1969; Klášterská & Natarajan 1974; Małecka & Popek 1982, 1984). However, the results of the analyses under all the above mentioned models look quite reasonable, with the majority of the specimens being assigned to separate groups with high posterior probabilities. Moreover, at least for *R. spinosissima* and *R. gallica* these results are strongly correlated with the morphological data. We suppose that deviations of the actual data from the models are not that considerable to render these results as completely erroneous.

The contradiction between morphological and based on ISSR markers subdivisions of the dog-rose group in our analyses may rise a suspicion that the result achieved is artefactual in its nature, due to lack of statistical power in the molecular (ISSR) data set to discriminate between the dog-rose species. The one thing that may lead to such a suspicion is that the *R. canina* s.l. group is subdivided by the Structure program into two parts, which neither correspond to morphological species, nor coincide with the distribution of chloroplast haplotypes L and T, characteristic of *R. glauca* and *R. tomentosa* respectively. However, extrapolation of localities of these two groups onto a geographical map of the area (Fig. 6) shows that they are not arbitrary. The first group marked black in the pie diagrams is concentrated in N and W parts of the area, while the specimens of the second group (grey) are mostly concentrated in the E. The differences in group membership are roughly proportional to the distance between the localities. This observation may serve as an argument in favor of interpretation of morphological variability of dog-roses in the area under consideration as mostly intraspecific, while the variability in ISSR markers reflects the restricted gene flow between the geographically distanced localities. The complex and yet unclear

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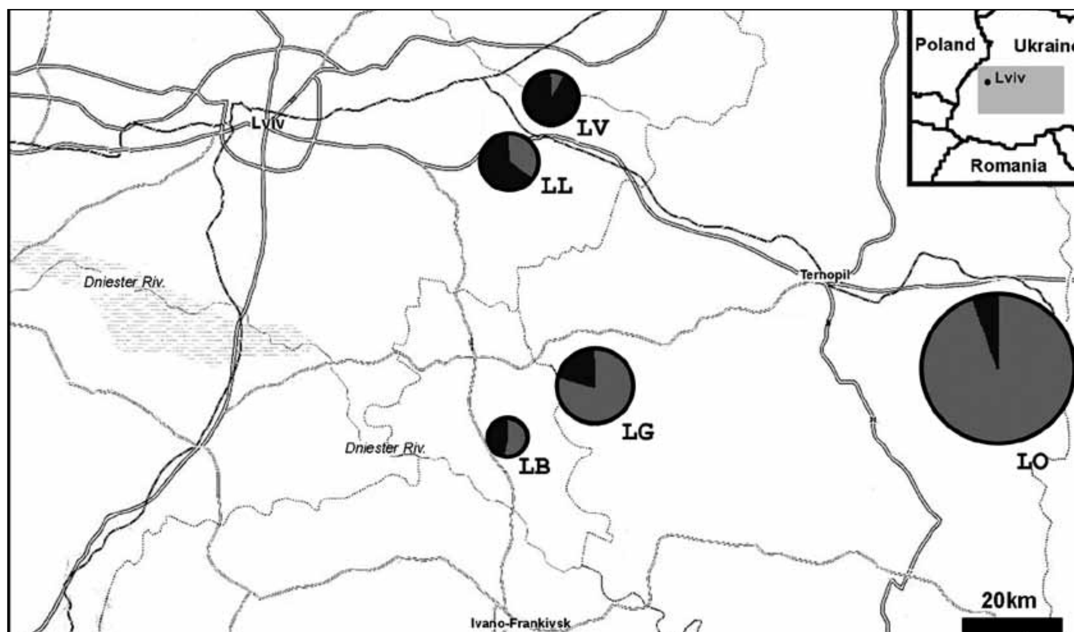

Figure 6. Geographical distribution of dog-rose specimens assigned to groups 3 and 4 in the Structure analysis (K=4). The size of circles approximately reflects the number of specimens sampled from each locality: black sectors – group 3; grey sectors – group 4.

nature of species in *Rosa* section Caninae makes impossible to draw any final conclusion from our data. Nevertheless, at the adopted level of approximation all the dog-rose plants behave as a single species. This, partly, may explain the fact, why some morphotypes of the dog-roses are common like *R. canina*, while others are rare like *R. caryophyllacea* or *R. podolica*, despite they grow together in the same habitat. Absolutely the same pattern is observed in diploid outcrossing populations of *R. majalis* (Schanzer & Voilokova 2008), where morphotypes with glabrous (*R. glabrifolia* C.A. Mey.) or glandulous (*R. gorinkensis* Willd.) leaves may occur in different proportions in populations of otherwise morphologically typical *R. majalis*.

Conclusions

1) *Rosa canina* is the morphologically most diverse species in the area under consideration. Its different morphotypes are characterized by variously pubescent and glandulous leaves, peduncles, hypanthia, and loose to dense heads of styles as well as the presence of two rare chloroplast trnV-ndhC haplotypes, characteristic of *R. tomentosa* and *R. glauca.* These species, however, show no clear differentiation from the rest of the dog-rose specimens studied regarding their ISSR marker compositions; Bayesian analyses include them in *R. canina* with high posterior probabilities. Thus circumscribed, *R. canina* s.l. shows clear geographical differentiation between its eastern and western local populations distanced ca. 100 km from each other, probably due to restricted gene flow.

2) *Rosa gallica* shows no sign of geographical differentiation in this area regarding its morphology or ISSR marker composition. However, it rarely hybridizes with dog-roses, being always the pollen parent. The presence of putative backcrosses indicates to a probable introgression between *R. gallica* and the dog-roses.

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3) *Rosa spinosissima* is the rarest species in this area, which is quite uniform in its characters. It does not seem to be involved into any hybridization with other species.

Acknowledgements

The study was financially supported by RFBR grant no. 10-04-00240a for the first two authors.

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Appendix 1. Sampled localities and specimens composition. GenBank accession numbers of trnV-ndhC sequences are given in parentheses after specimen numbers.

Locality	Label	Geographical coordinates	Sample composition
Lviv region, Zolochiv district, near the Zhulychi village. Nature reserve 'Hora Vysoka', pine wood margin on a slope	LV	N 49º 52' 18" E 24º 53' 03"	*R. gallica* LV1 (HQ404939) *R. podolica* LV8 *R. subcanina* LV2 LV3 LV7 LV9 *R. tomentosa* LV4 (HQ404991)
Lviv region, Zolochiv district, near the Chervone village. Nature reserve 'Hora Lysa and Hora Sypukha', mixed wood margin on a steppe slope	LL	N 49º 48' 06" E 24º 42' 54"	*R. canina* LL5 (HQ404962) *R. gallica* LL1 (HQ404955) *R. subcanina* LL2 LL3 (HQ404980) *R. tomentosa* LL4 *R.* sp. indet. LL6
Ternopil region, Berezhany district, near the Hutysko village. Nature reserve 'Hora Holytsya', steppe slopes	LG	N 49º 24' 22" E 24º 49' 07"	*R. canina* LG3 LG9 LG10 (HQ404978) LG14 (HQ404964) LG15 (HQ404965) LG16 (HQ404966) LG20 (HQ404967) LG21 *R. corymbifera* LG12 LG23 (HQ404986) *R. gallica* LG1 (HQ404940) LG18 (HQ404953) LG22 (HQ404954) *R. gallica* hybr. LG11 (HQ404952) *R. jundzillii* LG17 (HQ404960) *R. parviuscula* LG2 (HQ404959) LG7 (HQ404957) LG8 (HQ404958) *R. porrectidens* LG4 (HQ404956) LG5 *R. subcanina* LG13 (HQ404985) *R. spinosissima* LG6 (HQ404992) LG19 (HQ404993)

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Locality	Label	Geographical coordinates	Sample composition
Ternopil region, Pidvolochysk district, near the Ostapie village. Nature reserve 'Medobory', stony steppe with limestone outcrops	LO	N 49º 23' 10–40" E 26º 04' 5–56"	*R. gallica* LO1 (HQ404945) LO13 (HQ404946) LO16 (HQ404947) LO19 (HQ404948) LO20 (HQ404949) LO23 (HQ404950) LO25 (HQ404951) *R. subcanina* LO2 (HQ404981) LO7 (HQ404982) LO9 (HQ404983) LO10 LO26 LO30 (HQ404984) *R. spinosissima* LO11 (HQ404994) *R. canina* LO3 LO4 (HQ404961) LO6 LO12 LO15 (HQ404963) LO18 (HQ404968) LO21 (HQ404969) LO22 (HQ404970) LO27 (HQ404971) LO29 (HQ404979) LO31 (HQ404972) LO32 (HQ404973) *R. caryophyllacea* LO14 LO28 (HQ404989) *R.* sp. indet. LO5 (HQ404987) LO17 (HQ404988) *R. tomentosa* LO33 (HQ404990) *R. glauca* LO8 (HQ404995) *R. porrectidens* LO9
Ivano-Frankivsk region, Halych district, N of Bovshev village, left bank of Burshtyn reservoir at Hnyla Lypa Riv., Kasova Hora hill. National Nature Park 'Halyts'kyy'	LB	N 49º 13' 30" E 24º 41' 40"	*R. canina* LB4 (HQ404974) LB5 (HQ404975) LB8 (HQ404976) LB10 (HQ404977) *R. gallica* LB1 (HQ404941) LB2 (HQ404942) LB7 (HQ404943) LB9 (HQ404944)

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Digitale Literatur/Digital Literature

Zeitschrift/Journal: Wulfenia

Jahr/Year: 2010

Band/Volume: 17

Autor(en)/Author(s): Fedorova Alina V., Schanzer Ivan A., Kagalo Alexander A.

Artikel/Article: Local differentiation and hybridization in wild rose populations in Western Ukraine 99-115