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МОРФОЛОГИЧЕСКАЯ И МОЛЕКУЛЯРНАЯ ОЦЕНКА ГЕНЕТИЧЕСКИХ РЕСУРСОВ *LONICERA CAERULEA* L. НА ДАЛЬНЕМ ВОСТОКЕ

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Актуальность. Жимолость съедобная, *Lonicera caerulea* L. s.l., – перспективная ягодная культура, чрезвычайно важная для здорового питания. Значительная пищевая и лекарственная ценность плодов, очень высокая органолептическая их оценка могут позволить жимолости занять достойное место на рынке здоровой пищи. **Цель.** Неясный таксономический статус дальневосточных форм и высокий уровень варьирования морфологических особенностей различных органов, особенно – плодов, послужили основанием для реализации двустороннего российско–чешского проекта по изучению жимолости. **Материалы и методы.** Основные полевые исследования были проведены в Северо–восточной части распространения жимолости на Камчатке и Сахалине. Все обследованные ценопопуляции были охарактеризованы экологически, геоботанически, фитосоциологически. Кроме того, были оценены факторы угрозы ценопопуляциям, эти данные явились предпосылкой для рекомендаций по сохранению жимолости *in situ*. Девяносто один образец был собран для гербария, *in vitro* сохранения и молекулярного исследования. Кроме того, плоды 20 российских культурных сортов жимолости были включены в сравнительную молекулярную оценку с природными экотипами. **Результаты и обсуждение.** В результате проведенного AFLP анализа весь исследованный материал разделился на три группы. Две группы сформированы *L. caerulea,* собранной на Камчатке, и большинством культурных форм жимолости. Одна группа объединяет только генотипы сахалинских растений. При этом различить образцы, собранные на Сахалине и Камчатке, по морфологическим признакам очень трудно, что отражается в существующих таксономических системах.

Ключевые слова: жимолость, *Lonicera caerulea* L., географическое распространение, Камчатка, Сахалин, молекулярно–генетический анализ, *in situ* сохранение.

MORPHOLOGICAL AND MOLECULAR EVALUATION OF THE FAR EAST FRUIT GENETIC RESOURCES OF *LONICERA CAERULEA* L.

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Background. Honeysuckle, *Lonicera caerulea* L. s.l. is a very promising fruit important for healthy nutrition. High nutritional and medicinal value of its fruits and excellent results of organoleptic evaluation may enable honeysuckle to fill the gaps in the healthy food market. **Objective.** Unclear taxonomic status and high variation in morphology of the sweet–fruit Far East forms initiated a joint project. **Materials and methods.** Based on the Czech–Russian bilateral project, a research programme was undertaken in the north–eastern part of the area of honeysuckle distribution in Kamchatka and Sakhalin. The identified localities were characterized ecologically and in the context of vegetation, phytosociological features were noted, and threat factors were assessed as a prerequisite for *in situ* conservation planning. Ninety–one samples were collected for the herbarium, propagation *in vitro* and molecular characterization. Additional 20 available Russian fruit cultivars were evaluated and compared with ecotypes. Wild ecotypes and cultivars were included in molecular evaluation. **Results and discussion.** Cluster analysis based on AFLP data shows three clusters. Two clusters are formed by *L. caerulea* collected from Kamchatka and by most of honeysuckle varieties. One cluster unites only genotypes from Sakhalin localities. Morphologically it is very difficult to distinguish between genotypes from Sakhalin and Kamchatka, and it supports the taxonomic concept on the subspecific level.

Key words: honeysuckle, *Lonicera caerulea* L. s.l., geographical distribution, Kamchatka, Sakhalin, molecular characterization, *in situ* conservation.

Introduction

Modern large–scale horticulture is based on a small range of high yielding cultivars grown under intensive conditions. Market demands are, or can be, much wider however. Minor fruits represent a great potential for diversification of cultivated species spectrum. Honeysuckle is a promising fruit crop for a combination of highly positive advantages: stable annual fruiting, earliness and high biochemical parameters of fruit. J. Heinrich (Heinrich et al., 2008) mentioned that the fruits of *Lonicera caerulea* L. s.l. are a promising source of health beneficial substances that exhibit antiadherent, antioxidant and chemoprotective properties. Their regular consumption is a good preventative

for serious chronic diseases of civilization, such as cancer, hypertension, atherosclerosis, diabetes and cardio–vascular diseases.

The genus *Lonicera* L. (blue honeysuckle) is widespread over the territory of Eurasia and North America. Blue honeysuckle, as an independent taxon, was identified by botanists as early as in the pre–Linnaean period. Clusius in 1583 provided a description and an image of honeysuckle with fruit. Linnaeus (1753) described blue honeysuckle as the species *Lonicera caerulea*. In 1903, blue honeysuckles were classified by A. Rehder (1903) into the subsection *Caeruleae* represented by only one Linnean species, *Lonicera caerulea*. This subsection is a subject of continuing discussions among the experts concerning its volume, structure and the status of the incorporated taxa. On the contrary A. Poyarkova recognized 10 species within the USSR territory out of the 16–17 known *Lonicera* species: *L. altaica* Pall., *L. baltica* Pojark., *L. buschiorum* Pojark., *L. caerulea* L., *L. edulis* Turcz. ex Freyn, *L. iliensis* Pojark., *L. kamtschatica* (Sevast.) Pojark., *L. pallasii* Ledeb., *L. stenantha* Pojark. and *L. turczaninovii* Pojark. Plekhanova assessed results of the complex studies and identified seven subspecies in the polymorphic tetraploid species *L. caerulea* (Plekhanova, 2007): subsp. *altaica* (Pall.) Plekhanova; subsp. *emphellocallyx* (Maxim.) Plekhanova; subsp. *kamtschatica* (Pojark.) Plekhanova; subsp. *pallassii* (Ledeb.) Browich; subsp. *stenantha* (Pojark.) Hult. ex Skvortsov and subsp. *venulosa* (Maxim.) Worosh.

The authors studied collected samples as genetical resources of *Lonicera caerulea* from Kamchatka and Sakhalin (Holubec et al, 2007; Holubec, Smekalova 2012; 2013) and compared them with cultivated material.

Material and methods

The Far East distribution of *Lonicera coerulea* was visited during the RUS–CZE bilateral research project in 2011–2013 (Sakhalin 2011, 2012; Kamchatka, 2013) and additional information was used from previous author´s visits in 1993, 1995, 2005 and 2007 in Kamchatka (Table 1; Fig. 1, 2).

Localities of honeysuckle were found on the basis of herbarium (LE) and literature data and from local people. Herbarium specimens were evaluated. Visited sites were localised and characterised botanically and ecologically, phytosociological relevés were noted on square plots 4 × 4 m.

Morphological and especially fruit characters of selected plants were evaluated according to adapted descriptor list (Plekhanova, Korneichuk, 1988). Vegetative samples were collected for herbarium, for propagation (vegetative and in vitro) and for molecular analysis. Fruit samples were collected and

evaluated on size, weight and organoleptically on taste sugars, acids, bitterness (Table 2).

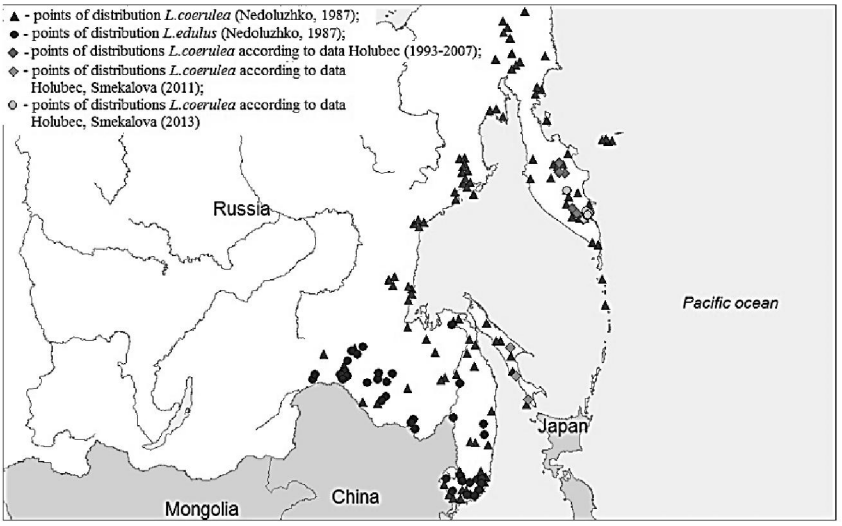

Рис. 1. Распространение голубой жимолости на Дальнем Востоке (авторы карты: Т. Н. Смекалова, Л. В. Багмет)
Fig. 1. Distribution of blue honeysuckle in the Far East (authors of the map: T. Smekalova and L. Bagmet)

Collected vegetative samples were propagated by cuttings, grafting or *in vitro*. Plants were grown in the experimental fields in the Czech Republic. New collected material was compared with current mainly Russian cultivars from the available honeysuckle collection in the Mendel University Brno, Czech Republic. In total, 91 genotypes were evaluated (Table 1).

Таблица 1. Число исследованных образцов *Lonicera caerulea*
Table 1. The number of *Lonicera caerulea* accessions used in evaluation

Region/ Year of collection	1993	2007	2011	2012	2013	Total
Kamchatka	10	12	–	–	26	48
Sakhalin	–	–	11	12	–	23
Cultivated varieties	–	–	–	–	–	20
Total	–	–	–	–	–	91

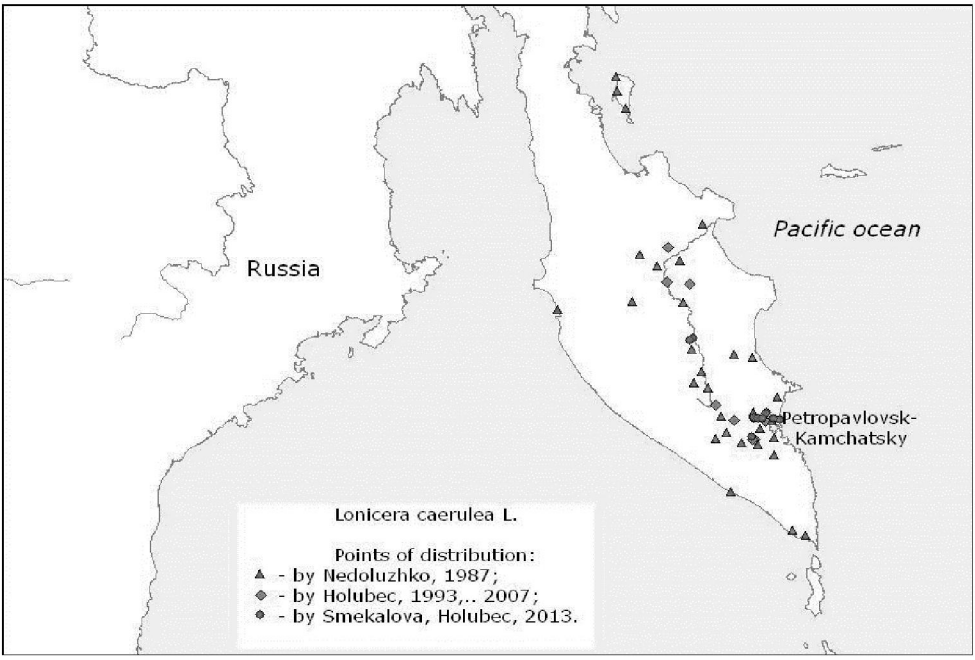

Рис. 2. Распространение голубой жимолости на Камчатке (авторы карты: Т. Н. Смекалова, Л. В. Багмет)
Fig. 2. Distribution of blue honeysuckle in Kamchatka (authors of the map: T. Smekalova and L. Bagmet)

DNA was extracted from at least three leaves per sample using CTAB detergent according to the optimised protocol. Leaves were ground in liquid nitrogen using mortar and pestle and immediately moved into 700 µl of 2% CTAB. Sometime during incubation for 30 minutes at 60°C 10 mg of polyvinylpirrolidon was added. Then, a shake–out in chlorophorm – isoamylalcool mixture (54:1) was done twice. DNA was precipitated in acid medium by 1x volume of absolute ethanol. DNA pelet was washed twice by incubation in 70% ethanol at 4°C each for 1 hour. Finally, DNA was diluted in an appropriate volume of TE buffer. DNA was run in 0,8% agarose gels to verify the quality and the concentration. λ HindIII (Fermentas, Vilnius, Lithuania) was used to determine the size and the concentration of DNA.

Таблица 2. Морфологических особенности *Lonicera coerulea* природных ценопопуляций Камчатки (исследовано 26 растений) и Сахалина (исследовано 32 растения, вес плодов растений Сахалинской популяции не был определен из–за перезрелого состояния)

Table 2. Morphologic evaluation of *Lonicera coerulea* from wild populations in Kamchatka (selected 26 plants) and Sakhalin (selected 32 plants, fruit weight in Sakhalin populations was not measured because of overripeness)

		Leaf		Fruit				
		Mean (mm)	St. Dev.	Mean (mm)	St. Dev.	Max (mm)	Weight (g)	Weight max (g)
Sakha–lin	length	51,44086	14,28782	17	2,685942	23	–	–
	width	22,99462	6,168934	10,125	1,778175	12	–	–
	l/w	2,24825	0,315643	1,737444	0,440153		–	–
Kam–chatka	length	49,02174	9,812451	19,084	2,344982	27	0,6912	1,03
	width	22,01449	4,855557	10,076	0,943283	12	–	–
	l/w	2,259189	0,3512	1,908378	0,281209	–	–	–

Amplified fragment length polymorphism markers were generated with the Applied Biosystems kit for plants (Applied Biosystems, Foster City, CA, USA). DNA digestion was carried out using the restriction enzymes *Eco*RI and *Mse*I (Vos et al., 1995). Eighty primer combinations were tested, and eighteen pairs were selected for further analyses. These combinations of primer pairs were chosen because they generated a high number of scorable fragments with a range of sizes (100–500 bp). The selective amplification, with *Mse*I primers and fluorescently marked *Eco*RI primers, was performed as a multiplex PCR in a Labcycler (SensoQuest GmbH, Göttingen, Germany) with a reaction mixture of 10 µl containing the following: 0,2 mM dNTP, 1 µM *Mse*I primer, 3,0 × 0,5 µM *Eco*RI primers, 1 U *Taq* polymerase (Qiagen GmbH, Hilden, Germany), 1x buffer with 10 mM $MgCl_2$ and 1 µl diluted (1:20) preselective amplification reaction. Amplification products were separated by capillary electrophoresis in an ABI PRISM 310 (Applied Biosystems) and analysed using GeneScan and Genotyper software (Applied Biosystems). Based on the presence or absence of AFLP amplification in each of amplicon size category, a binary matrix was built and use for data analysis.

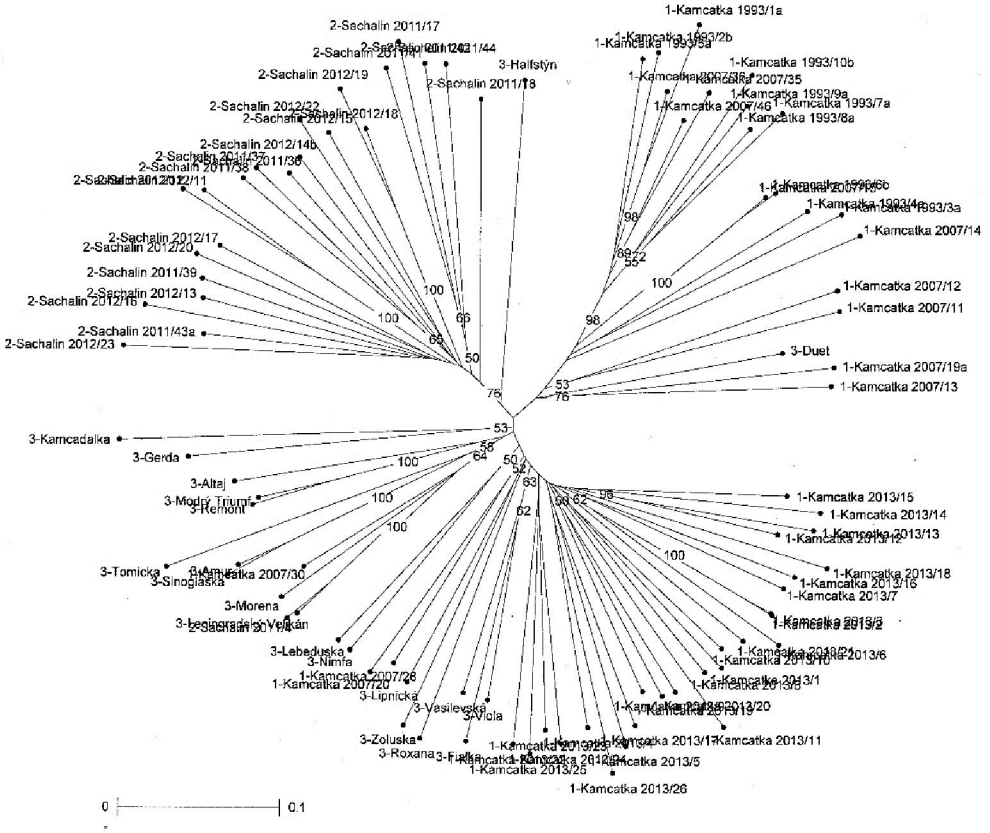

Рис. 3. Дендрограмма, построенная по данным AFLP: 1 – камчатские образцы; 2 – сахалинские образцы; 3 – культурные образцы жимолости

Fig. 3. Dendrogram based on AFLP data: 1–genotypes collected from kamchatka; 2–genotypes collected from sakhalin; 3–cultivated honeysuckle varieties

Cluster analysis was performed to study the relationships among genotypes. On the basis of the presence or absence of an amplification product, binary data matrices were built. A dissimilarity matrix was computed with DARwin software using the Jaccard coefficient (Perrier et al. 2003; Perrier, Jacquemoud–Collet, 2006). A dendrogram was constructed using an

unweighed neighbour joining method. Bootstrap analysis with 2000 replicates was performed to estimate the robustness of a tree.

Results and discussion

Lonicera caerulea was found on 6 localities in central and south Sakhalin island mostly in *Abies sachalinensis* Fr. Schmidt taiga and forest clearings. Samples from 32 plants were collected and evaluated. In Kamchatka 9 localities were visited in 2013, mainly in *Betula ermannii* Cham. or *Alnus fruticosa* Rupr. mixed open forests and clearings. Samples of 26 plants were collected and evaluated. Ecological conditions of visited sites vary very widely in connection to altitude, soil type, water availability and vegetation type. Localities on Sakhalin island were rather humid, water logged forests, while in Kamchatka the sites were on volcanic bedrocks characterised by excessive drainage. Morphological traits were compared in populations of Sakhalin and Kamchatka.

Morphological and especially fruit characters showed a large variation in both regions Sakhalin and Kamchatka. Leaf length width, shape index and hairiness varied considerably and they do not allow finding distinguishing traits. Similar differences were found in the size, shape and hairiness of fruits. The variation in fruit size in Sakhalin was 12–23 × 8–14 mm and in Kamchatka 10–27 × 8–12 mm and the mean fruit weight was 0,60–1,03 g (based on the weight of 20 fruits). Fruit shape was variable in both regions being from near rounded, oval to spindle form, short and long cylindric. Hairiness of leaves was highly variable in both regions. Hairiness of young branches was dense on plants from Kamchatka while sparse or absent (soon disappearing) on plants in Sakhalin and southern Kuril Islands. It can be supported by inspected LE herbarium specimens (Sakhalin: Egorova 2772, 3531; Maximovicz 18.05.1956; Iturup: Pobedinova and Konovalova 1194, 1107, Egorova 5587; Kunashir: Ashretu 359, Berezancev 27.08.1975, Egorova 904; Urup: Egorova 2195; Shikotan: Gordeev 29.06.1954). Hairs on fruit apexes were usually straight and always present in Kamchatka, while sparse, curly or often absent in Sakhalin.

Additional traits connected with plant use as fruit crop were evaluated: taste of fruits as balance of sugars and acids, presence of bitterness, fruit firmness, abscission ease, earliness, etc. While fruits in Kamchatka were all without any bitterness, fruits from the Sakhalin area produced various levels of acceptable bitterness. However, it is not comparable with the heavy bitterness of *Lonicera caerulea* from the main land and other Euro–Asiatic localities. The

fruit firmness was partly affected by ripeness, however long fruits, especially cylindric, were usually firmer.

All collected wild ecotypes, including cultivated material from previous missions, were evaluated by AFLP to reveal genetic diversity level among genotypes from Kamchatka, Sakhalin and cultivated varieties. In total, 586 alleles were detected by the analysis of 91 samples using 18 primer combinations. Cluster analysis based on data showed three main clusters (Fig. 3) but with low bootstrap values. The first cluster is set up by genotypes collected in Sakhalin and by the variety Halfstýn. Genotypes collected in Kamchatka in 1993 and 2007 form the second cluster together with the variety Duet. And in the third cluster there are genotypes collected in Kamchatka in 2007, 2013, one genotype from Lopatino, Sakhalin and most of analysed honeysuckle varieties. The former estimation that the plant in Lopatino looked rather like cultivated variety was confirmed and can be declared as cultivar 'Leningradski Velikan'.

On the basis of measured morphologic data and AFLP analysis of Far East material, it is not possible to distinguish more taxa on specific level within the complex *Lonicera caerulea* in Kamchatka and Sakhalin and it is in agreement with results of A. Skvortsov and A. Kuklina (2002) for the main land. However, the material from Kamchatka differs from those in Sakhalin on the level of DNA. It can be supported by two morphological traits: hairiness of young branches and hairiness of fruit apexes. Based on these data it could be possible to classify the found genotypes into two geographical subspecies: *L. caerulea* subsp. *kamtschatica* (Pojark.) Plekhanova, and another subspecies with distribution in Sakhalin and most likely in Kuril islands, but not in sense of V. Nedoluzhko (1986) [subsp. *edulis* (Turcz. ex Regel) Hultén], which was devoted to diploid materials only.

There is also interesting that while genotypes collected from Sakhalin in two different years form the same cluster, genotypes from Kamchatka are divided in two clusters according sampling in the wild or in cultivation with the exception of three genotypes collected in 2007 (Fig. 3). More analyses should be done to test the hypothesis and it is a subject of our future study.

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