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RAPD based study of genetic variation and relationships among *Lonicera* germplasm accessions

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Blue-berried honeysuckle (*Lonicera caerulea* L.) belongs to the *Caprifoliaceae* Juss. family section *Isika* Rehd., subsection *Caeruleae* Rehd. It produces extra-early ripening berries that are an excellent source of valuable phytochemicals and nutrients. Genetic variation and relationships among 39 *L. caerulea* accessions representing four subspecies, three cultivars, six genetic lines and one native *L. xylosteum* L. accession were characterized by the random amplified polymorphic DNA (RAPD) method. All the accessions were distributed into three groups according to their morphological and phenological characteristics. A total of 105 DNA fragments were scored after amplification of all DNA samples with 11 selected random primers; 83.9% of scored bands were polymorphic. Pair-wise genetic distance (GD) among *L. caerulea* accessions ranged from 0.0 to 0.366. Two accessions were identified as clones of the same genotype. All the accessions were grouped into three clusters. All accessions of *L. caerulea* were distinctly separated from *L. xylosteum* accessions. Our approach based on RAPD analysis was able to reveal genetic specificity only in the subspecies *L. caerulea* L. subsp. *pallasii* Ledeb. The study demonstrated that RAPD analysis is efficient for genotyping blue-berried honeysuckle accessions, and that DNA polymorphism significantly exceeds the morphological diversity of the samples studied.

Key words: *Lonicera caerulea* L., RAPD, genetic relationship, genotyping, subspecies

INTRODUCTION

The *Caprifoliaceae* Juss. family contains about 40 genera and 400 species that are spread over the cool temperate Northern Hemisphere. It is a biologically diverse family consisting of varied life forms ranging from perennial herbs, lianas, shrubs to small trees [1]. There are over 200 species in the genus *Lonicera* L., one of which, *L. xylosteum* L., occurs naturally in Lithuania; other species are introduced and widely cultivated in ornamental planting. Blue-berried honeysuckle (*L. caerulea* L.) belongs to the section *Isika* Rehd., subsection *Caeruleae* Rehd. The volume of the subsection has been a many-year object of investigations and discussions. Different authors single out one to 10–11 species within the subsection because of the differences in the understanding of a species and different research and definition methods. Nowadays there is an appreciable tendency to integrate *altaica*, *caerulea*, *emphyllocalyx*, *kamtschatica*, *pallasii*, *stenantha*, *venulosa* with status subspecies in the *Lonicera caerulea* species.

Blue-berried honeysuckle is a perennial deciduous shrub growing to 2 m. The hermaphrodite flowers of this plant are pollinated by insects. Blue-berried honeysuckle produces early ripening berries which are dark navy to purple in color. Fruits of *L. caerulea* are an excellent source of dietary phytochemicals (anthocyanins, polyphenolics and ascorbic acid) and can be used as natural antioxidants and natural colorants [2, 3]. One more positive feature of this species is extra-early ripening and an outstanding frost resistance of plants and flowers [2]. Blue-berried honeysuckle (*L. caerulea*) is one of commercially promising species of this genus. Fruits of blue-berried honeysuckle are widely harvested in Russia, China and Japan [3].

In Lithuania, blue-berried honeysuckle grows only in private plots and botanical gardens. The collection of blue-berried honeysuckle (*L. caerulea*) of Vilnius University Botanical Garden contains four subspecies, 28 cultivars and 35 genetic lines.

Little is known about blue-berried honeysuckle phylogeny, genetics and population genetic structure. For the study of such anonymous genomes RAPD analysis is widely used. This method is suitable for genotyping, phylogenetic analysis and molecular selection [4–6]. RAPD among other molecular marker methods has considerable advantages because it is fast, not expensive and the development of RAPD markers does not require a prior knowledge of the genome sequence. These markers have been widely used in the phylogenetic analysis of many organisms and a general concordance was demonstrated among the results derived from RAPD and other techniques [7].

In this study, we tested the ability of RAPD markers to genotype *L. caerulea* accessions from the germplasm collection of Vilnius University Botanical Garden and to detect intraspecific genetic variation at the DNA level for understanding subspecies relationships within *L. caerulea* species.

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MATERIALS AND METHODS

Plant material

Thirty-nine accessions of *L. caerulea* and one accession of *L. xylosteum* from the germplasm collection of Vilnius University Botanical Garden were analysed (Table 1). DNA from fresh young plant leaves was isolated using the Genomic DNA purification kit (MBI Fermentas).

RAPD amplification

RAPD-PCRs were carried out in volumes of 25 µl, containing 50 ng of DNA, 2.5 µl 10× *Taq* reaction buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTP, 1 µM primer, 1 U *Taq* DNA polymerase (MBI Fermentas). The thermal cycler (Eppendorf Mastercycler personal) was programmed for one cycle of 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C, and finally by one cycle of 5 min at 72 °C. Amplification products were

Table 1. **Description of *Lonicera* L. material used in the study**

N.	Sample name and characterization	Shrub characterization	Forthcoming
	Group I. Section *Coeloxylosteum* Rehd., subsection *Ochranthae* Zab.		
1	*Lonicera* ***xylosteum*** L. Europe, Russia: West Siberia	Shrub about 3 m, red uneatable berries in late summer	LTU, spontaneous plant
	Section *Isaka* Rehd., subsection *Caerulea* Rehd.		
	Group II. Bushes about 2 m, very early bloom and ripen large blue berries, short rest period of generative buds. Northern regions of Eurasia and North America		
2	*Lonicera* ***caerulea*** L. Small leaves, flowers and berries	Shrub about 1.5 m, spindle-shaped berries 22 × 9 mm, 0.6 g, early ripening	RUS, Meshcherskoe St Exper, 1997, Pom3342
3	*Lonicera caerulea* L. subsp. ***altaica*** Pall. East Europe, Russia: West Siberia; Mongolia, China, in mountain, drought and frost resistance	Shrub about 1.5 m, ovate berries 16 × 10 mm, 0.8 g, extra early ripening	RUS, St. Petersburg, VIR**, 1997, Pom3326
4, 5, 6	*Lonicera caerulea* L. subsp. *Lonicera caerulea* L. subsp. ***pallasii*** Ledeb. Northeast Europe, Russia: Siberia, in taiga, wide leas, frost resistance	Shrub about 1.1 m, oval berries 15 × 9 mm, 0.7 g, early ripening /a, b, c/*	RUS, St. Petersburg, VIR, 1997, Pom3320
7, 8, 9	*Lonicera caerulea* L. subsp. ***stenantha*** Pojark. Russia: West Siberia; Central Asia, Iran, India, in mountain, small leaves, flowers and berries	Shrub about 2.0 m, oval berries 20 × 10 mm, 0.9 g, early ripening /a, b, c/	RUS, St. Petersburg, VIR, 1997, Pom3319
10, 11, 12	*Lonicera caerulea* **'Baktcharskaja'** Russia, VIR, seedling of elite form 16/63	Shrub about 1.5 m, drop-shaped berries 20 × 12 mm, 0.8 g, medium early ripening /a, b, c/	RUS, St. Petersburg, VIR, 1996, Pom3161
13	*Lonicera caerulea* **'Morena'** Russia, VIR, results of far hybridization	Shrub about 1.5 m, jug-shaped berries 30 × 13 mm, 1.4 g, medium early ripening	RUS, St. Petersburg, VIR, 1997, Pom3249
14	*Lonicera caerulea* **'Viola'** Russia, VIR, results of hybridization subsp. *altaica* and *kamtschatika*	Shrub about 1.5 m, cylindrical berries 23 × 10 mm, 1.7 g, medium early ripening /a/	RUS, St. Petersburg, VIR, 1997, Pom3258
15, 16, 17	*Lonicera caerulea* **'Viola'** Russia, VIR, results of hybridization subsp. *altaica* and *kamtschatika*	Shrub about 1.5 m, oval berries 23 × 11 mm, 1.0 g, medium early ripening /b, c, d/	RUS, St. Petersburg, VIR, 1996, Pom3159
18, 19	*Lonicera caerulea* **'96-3'** Lithuania, HBU, seedling	Shrub about 1.0 m, jug-shaped berries 27 × 11 mm, 1.1 g, early ripening /a, b/	LTU, Vilnius, HBU, 1996, Pom3421
20, 21, 22, 23	*Lonicera caerulea* **'96-4'** Lithuania, HBU, seedling	Shrub about 1.0 m, jug-shaped berries 22 × 12 mm, 1.0 g, early ripening /a, b, c, d/	LTU, Vilnius, HBU, 1996, Pom3422
24, 25, 26	*Lonicera caerulea* **'2R'** Lithuania, HBU, seedling	Shrub about 1.5 m, jug-shaped berries 17 × 11 mm, 1.2 g, early ripening /a, b, c/	LTU, Vilnius, HBU, 1996, Pom3028
27, 28	*Lonicera caerulea* **'3U'** Lithuania, HBU, seedling	Shrub about 1.5 m, jug-shaped berries 30 × 11 mm, 1.6 g, early ripening /a, b/	LTU, Vilnius, HBU, 1996, Pom3031

Table 1 (continued)

N.	Sample name and characterization	Shrub characterization	Forthcoming
Group III. Short bushes (about 0.8 m), large leaves, flowers and berries, late bloom and late ripening of round blue berries, frost and drought resistance, long rest period of generative buds. Russia: Arctic, East Siberia, Far East, Kamtschatka, Kurile Islands, Sakhalin, in taiga, Japan			
29	*Lonicera caerulea* L. subsp. ***kamtschatika*** (Sevast.) Pojark.	Shrub about 0.9 m, ovate berries 23 × 12 mm, 1.0 g, late ripening /c/	LVA, Salaspils, HBA, 1991, Pom2635
30, 31, 32	*Lonicera caerulea* L. subsp. ***kamtschatika*** (Sevast.) Pojark.	Shrub about 0.6 m, oval berries 17 × 12 mm, 1.4 g, late ripening /a, d, e/	CZE, Pruhonice, HBA, 1997, Pom3189
33, 34, 35	*Lonicera caerulea* L. subsp. ***kamtschatika*** (Sevast.) Pojark.	Shrub about 1.2 m, oval berries 16 × 10 mm, 0.8 g, late ripening /b, f, g/	LVA, Kalsnava, Arb, 1997, Pom3192
36, 37, 38	*Lonicera caerulea* **'L69-3'** Russia, VIR	Shrub about 0.6 m, ovate berries 18 × 13 mm, 1.1 g, late ripening /a, b, c/	RUS, St. Petersburg, VIR, 1997, Pom3251
39, 40	*Lonicera caerulea* **'639-8'** Russia, VIR, Kurile Islands, seedling	Shrub about 0.5 m, rounded berries 15 × 12 mm, 1.0 g, late ripening /a, b/	RUS, St. Petersburg, VIR, 199, Pom3259

* Letters /a, b, c, etc/ indicate plant accessions with the same name in the dendrogram.

** VIR – N.I. Vavilov Research Institute of Plant Industry (St. Petersburg, Russia).

Table 2. **Oligodeoxynucleotide primers used for RAPD analysis of *Lonicera* L. accessions, number of identified RAPD bands, their size intervals and number of RAPD patterns**

RAPD primer (Roth)	Sequence 5'→3'	Total bands[1]	Polymorphic bands	Size range of DNA fragments [bp]	Number of RAPD patterns[2]
170-03	ACG GTG CCT G	11	11	480–1350	18
170-05	GAG ATC CGC G	11	6	450–1500	13
170-08	CTG TAC CCC C	8	6	480–2300	16
170-10	CAG ACA CGG C	10	10	550–1500	24
380-01	ACG CGC CAG G	11	9	590–2000	22
380-02	ACT CGG CCC C	12	11	700–2550	14
380-03	GGC CCC ATC G	9	6	650–2500	8
380-06	CCC GAC TGC C	10	7	700–2000	18
380-07	GGC AAG CGG G	6	6	890–2400	11
380-08	CGC ACC GCA C	9	9	680–2100	29
380-09	ACG GCG GCT C	8	7	870–1950	6
Total:		105	88	450–2550	177

[1] Total number of RAPD band detected, which are reproducible and useful as molecular markers.

[2] Number of banding patterns that can be distinguished within the group of forty accessions with different RAPD primers.

separated by electrophoresis in 1.5% agarose gels with a Tris-borate-EDTA buffer system. Gels were stained with ethidium bromide, visualized by UV-light and photographed using the BioDocAnalyse (Biometra) system. Marker GeneRuler™ DNA Ladder Mix (MBI Fermentas) was used to determine the size of the DNA fragments. Forty plants including *Lonicera xylosteum* were analysed using 11 primers of arbitrary sequence with the total content of G + C 70% (Roth 170) and 80% (Roth 380) (Table 2). DNA fragments detected not in all accessions profiles were considered as polymorphic.

Data analysis

Matrix of Nei and Li (1979) [8] genetic distance for each pair of accessions was generated for RAPD marker presence or absence data. It was assumed that similarity of fragment size was an indicator of homology. UPGMA (Unweighted Pair Group Method of arithmetic Averages) analysis was performed in TREECON for Windows v.1.3b [9]. Cluster analysis of the 40 accessions, based on the genetic distance matrix, was carried out with the UPGMA [10] using the TREECON v.1.3b [9]. Bootstrap analysis was performed with 1000 replications.

RESULTS

Of the 20 primers tested for their capacity to differentiate among 40 honeysuckle accessions, the best 11 primers showed a polymorphism between accessions and gave reproducible banding patterns. 105 DNA fragments were taken for data analysis in total. The number of RAPD bands scored per primer varied from six (primer 380-07) to 12 (primer 380-02). An average of 9.5 bands was obtained per primer and their size ranged from 450 to 2550 bp. Examples of typical RAPD banding patterns produced

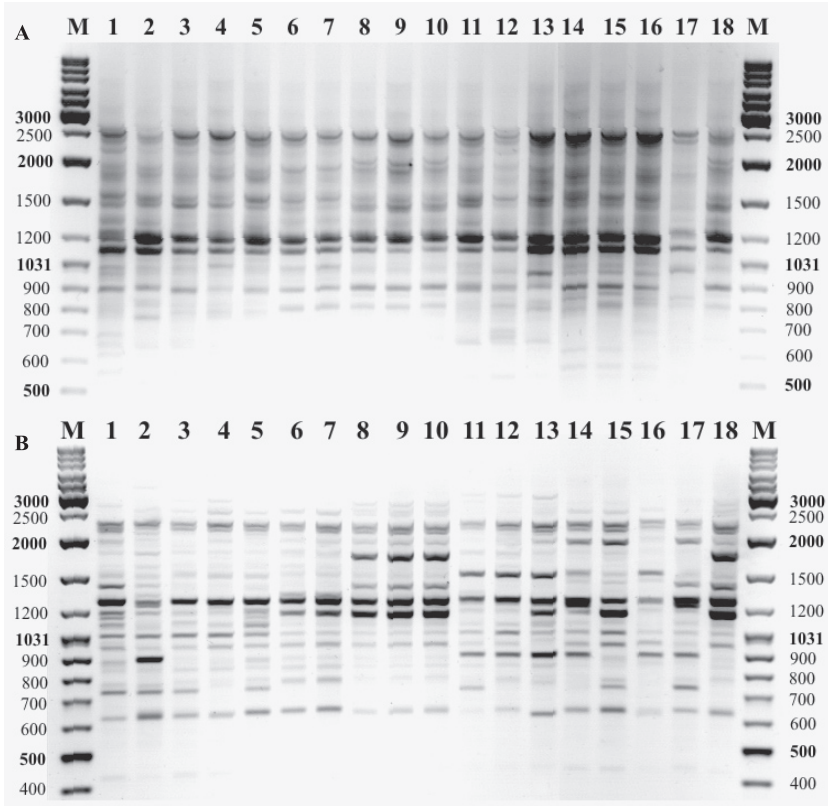

Fig. 1. RAPD analysis with primers 170-03 (A) and 380-08 (B) of *Lonicera caerulea*. M – standard of DNA fragment size GeneRuler™ DNA Ladder Mix (100–10000 bp). Arabic numerals on the top of picture (1, 2, 3... etc.) indicate the code number of the individuals

by primers Roth 170-03 and Roth 380-08 are shown in Fig. 1. The level of DNA polymorphism established in the group of *Lonicera* plants was 83.9%; 88 polymorphic RAPD bands can be considered as molecular markers. The number of RAPD markers identified per primer ranged from six to eleven (Table 2). The highest number of accessions that can be genotyped using 1, 2, and 3 most informative primers were 23, 36 and 36, respectively.

The genetic distance matrix, made on the basis of *L. caerulea* RAPD profiles, revealed values ranging from 0.0 to 0.366. We were able to differentiate all accessions except two examples of *L. caerulea* '639-8' obtained from VIR in 1997 that showed an identical RAPD banding pattern (Fig. 2, Table 1). The other accessions were genetically different and possessed specific RAPD banding patterns. On the basis of these results we can conclude that those two accessions of *L. caerulea* '639-8' are possibly clones of the same genotype.

The generated dendrogram of all 40 accessions (including *L. xylosteum*) showed at least three clusters (Fig. 2). The dendrogram clearly demonstrates the genetic specificity of the species *L. caerulea*. A single sample of *L. xylosteum* was clustered separately from the *L. caerulea* group with a high probability (bootstrap value 99.4%). *L. xylosteum* belongs to section *Coeloxylosteum* Rehd., subsection *Ochranthae* Zab. and morphologically differs from species of the section *Isaka* Rehd., subsection *Caerulea* Rehd. and phenotypicaly was arranged into a separate group (Table 1). The first largest cluster includes 36 accessions and consists of at least 5 subclusters. The second small cluster consists of three samples that present the genetic line 'L69-3'. On the basis of important morphological characters (shrub shape, form of berries, and ripening characteristics) this line was categorized into the third group together with the genetic line 'L69-8' and some other samples of L. *caerulea* (Table 1).

DISCUSSION

We analyzed 39 accessions of the *Lonicera* genetic stock collections obtained from botanical gardens of different countries (Table 1) and currently maintained at the Botanical Garden of Vilnius University. The plants were taxonomically and morphologically different and represented four subspecies, three cultivars and six genetic lines. All the plants studied were accessed according to descriptors and were arranged into three groups according to the most important morphological and phonological characters:

1) bushes about 3 m, red uneatable berries in late summer – *L. xylosteum*;

2) bushes about 2 m, very early blooming and ripening large blue berries, a short rest period of generative buds – *L. caerulea, L. caerulea* subsp. *altaica, pallasii, stenantha,* 'Baktcharskaja', 'Morena', 'Viola', '96-3', '96-4', '2R', '3U';

3) short bushes (about 0.8 m), large leafs, flowers and berries, late bloom and late ripening of round blue berries, frost and dry resistant, a long rest period of generative buds – *L. caerulea* subsp. *kamtschatika*, 'L69-3', '639-8'.

RAPD analysis revealed a considerably higher diversity of the samples. 83.9% of the identified RAPD traits were polymorphic (Table 2). Genetic relationships among studied genotypes in part of cases were confirmed on the basis of these RAPD data (Fig. 2). For example, three accessions of cultivar 'Baktcharskaja' and four accessions of cultivar 'Viola' form distinct groups. Because of the lack of pedigree data it is difficult to judge about the genetic relatedness of those two cultivars, but the genetic relatedness of the individuals of each cultivar is evident. A similar clustering pattern was demonstrated by other groups of related accession (*L. caerulea* '693-3'; *L. caerulea* L. subsp. *pallasii* Lebed.; *L. caerulea* L. subsp. *stenatha* Bojark.).

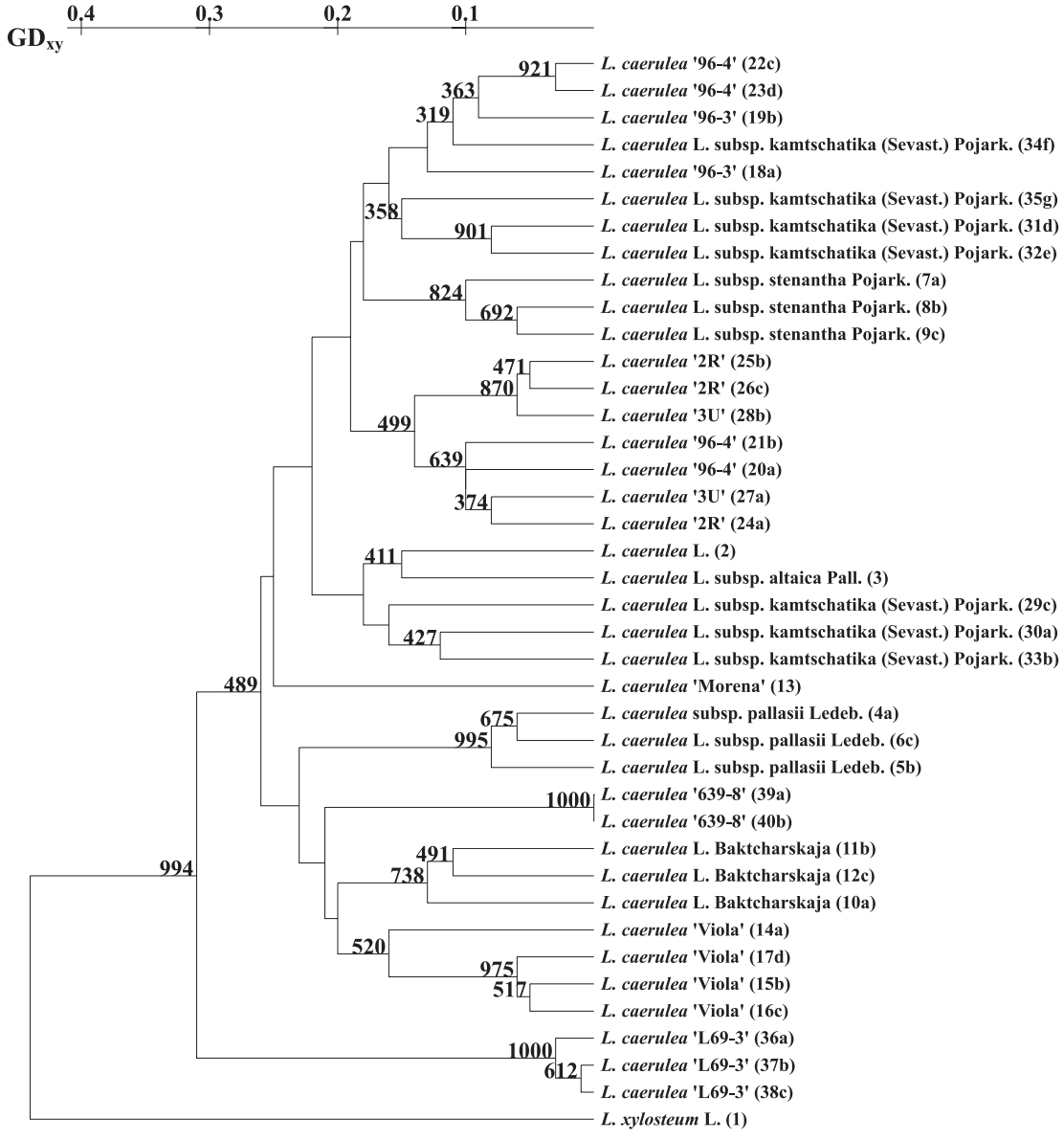

Fig. 2. Genetic relationships among 40 accessions of *Lonicera* based on the binary matrix RAPD traits using the UPGMA algorithm and the Nei and Li genetic distance GD_{xy} [8]. Numbers above branches indicate bootstrap values calculated using 1000 replications

The results obtained in our study demonstrate that the RAPD method can be effectively used to genotype *Lonicera* accessions. All the plants studied, except two possible clones of L. *caerulea* '639-8'; were characterized by a unique RAPD pattern. The genotyping of the accessions can be performed using 5–6 most informative primers. Nevertheless we applied more primers to develop a larger number of RAPD markers, because this gives a possibility to reveal more reliable genetic relationships among the genotypes and taxonomic groups. The use of new molecular methods in the taxonomy of blue-berried honeysuckle is really indispensable. The taxonomy of *L. caerulea* is rather problematic, in spite of the use of classical (based on morphology and anatomy), biochemical and mathematical approaches [11, 12]. A vital necessity, according to some authors, is the development of interspecies taxonomy of *L. caerulea* [12]. In our study, we included individuals of four subspecies of *L. caerulea* (subsp. *kamtschatika*; subsp. *pallasii*; subsp. *stenantha*; subsp. *altaica*) (Table 1). The UPGMA dendrogram based on the genetic distances among the individuals of these subspecies demonstrated the genetic specificity of genotypes of the same taxonomic group (Fig. 3). All accessions of the subspecies *pallasii* and *stenantha* are clustered according to their taxonomic belonging. On the other hand, there are some discrepancies between molecular and taxonomic data. It concerns the group of *L. caerulea* L. subsp. *kamtchatika* (Sevast.) Pojark. The accessions of this subspecies maintained at Vilnius University Botanical Garden collection are grouped in at least two distinct subclusters (Figs. 2, 3). The results presented in the dendrogram (Fig. 3) could be explained by the assumption that our approach, based on RAPD analysis, was able to reveal the genetic specificity of only the subspecies *L. caerulea* L. subsp. *pallasii* Ledeb.

Our study demonstrates that the use of RAPD analysis is efficient for blue-berried honeysuckle germplasm management. This method allows characterizing accessions and establishing genetic relationships between them. Although phylogenetic studies based on RAPD are less informative as compared with

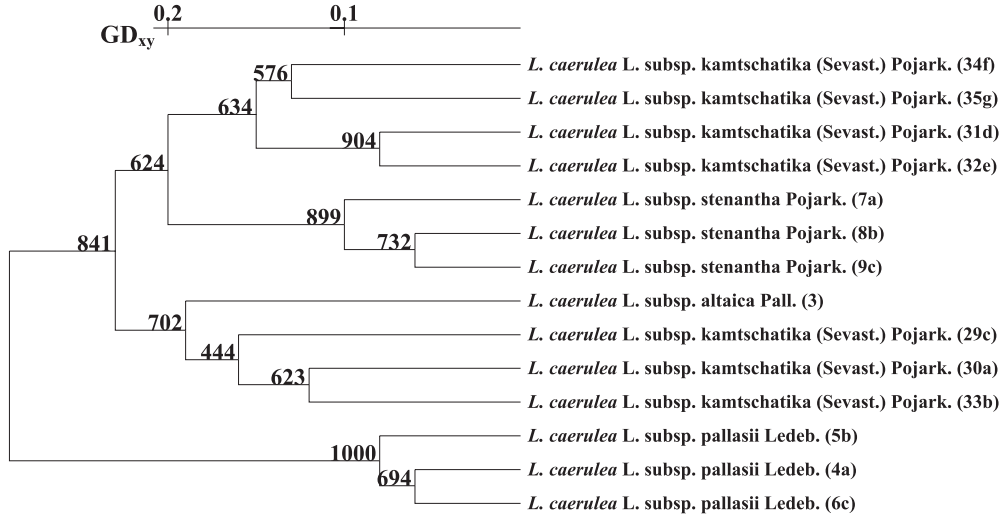

Fig. 3. Dendrogram showing the genetic relationships among individuals of *Lonicera caerulea* from four different subspecies

sequencing [7, 13], this method provided information about the genetic specificity of some *Lonicera caerulea* L. subspecies.

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LONICERA KOLEKCINIŲ PAVYZDŽIŲ GENETINĖS ĮVAIROVĖS IR GIMININGUMO TYRIMAS RAPD METODU

Santrauka

Melsvauogis sausmedis (*Lonicera caerulea* L.) priklauso sausmedinių (*Caprifoliaceae* Juss.) šeimos *Isika* Rehd. sekcijai, *Caeruleae* Rehd. posekcijai. Ši augalų rūšis vertinama dėl labai anksti subrėstančių vertingos cheminės sudėties uogų. Dėl uogų sausmedis gana plačiai auginamas Rusijoje, Kinijoje ir Japonijoje. Vilniaus universiteto Botanikos sodo kolekcijoje šiuo metu yra saugomi keturių melsvauogio sausmedžio porūšių, 28 veislių ir 35 genetinių linijų pavyzdžiai. Siekdami efektyviau panaudoti šią genetinę medžiagą selekciniame ir tiriamajame darbe, mes atlikome 39 melsvauogio sausmedžio kolekcijų pavyzdžių ir vieno paprastojo sausmedžio (*L. xylosteum* L.) pavyzdžio genotipavimą RAPD metodu. Visi tirti pavyzdžiai buvo aprašyti pagal požymių aprašą ir pagal pagrindinius morfologinius bei fenologinius bruožus (krūmo formą, uogų išvaizdą, derėjimo laiką) suskirstyti į tris grupes. RAPD analizės metu buvo atrinkti 105 DNR fragmentai, iš kurių 83,9% buvo polimorfiški. Panaudojus nustatytus molekulinius žymenis buvo genotipuoti visi tirti sausmedžio pavyzdžiai, išskyrus du. Pastarieji greičiausiai yra to paties genotipo klonai. Nustatytas genetinis atstumas tarp tirtų *L. caerulea* pavyzdžių svyravo nuo 0,0 iki 0,366. UPGMA dendrogramoje visi sausmedžių pavyzdžiai buvo suskirstyti į tris grupes. Paprastojo sausmedžio pavyzdys dendrogramoje akivaizdžiai skyrėsi nuo melsvauogio sausmedžio pavyzdžių. Mūsų nustatyti RAPD žymenys gana gerai atspindėjo tirtų pavyzdžių genetinį savitumą, tačiau tarp porūšių toks savitumas buvo nustatytas tik *L. caerulea* L. subsp. *pallasii* Ledeb. porūšiui. Gauti rezultatai rodo, kad RAPD metodas gali efektyviai genotipuoti melsvauogio sausmedžio kolekcinius pavyzdžius, nes DNR polimorfizmas juose gerokai viršija morfologinę įvairovę.