VILNIUS UNIVERSITY

Donatas Naugžemys

STUDY ON GENETIC DIVERSITY AND PHYLOGENETIC RELATIONSHIPS IN GENUS *LONICERA* L. USING DNA MARKER METHODS

Summary of doctoral dissertation
Biomedical science, biology (01 B)

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Doctoral dissertation will be defended at the public session of the Council of Biological Science at 14.00 p.m. on 04 March, 2011 in the Great auditorium of the Faculty of Natural Sciences, Vilnius University.
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VILNIAUS UNIVERSITETAS

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SAUSMEDŽIO (*LONICERA* L.) GENTIES ATSTOVŲ GENETINĖS ĮVAIROVĖS IR FILOGENETINIAI TYRIMAI DNR ŽYMENŲ METODAIS

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INTRODUCTION

Many plant species that earlier have not been greatly valued or cultivated now are intensively investigated with a view to using them more widely for human needs. Such plants are frequently distinguished by unique biological and agronomic features (e.g. resistance to biotic and abiotic stress). The blue-berried honeysuckle attracted the interest of breeders as late as in the middle of the last century.

The blue-berried honeysuckle (*Lonicera caerulea* L.) is a medium-size perennial shrub ripening blue edible berries, which is distributed in boreal forests of Eurasia and North America. *L. caerulea* as a commercial species is rather new; it started to be cultivated in the 19th century in Russia (Plekhanova, 2000). Scientific breeding of the species started in the 20th century in the Soviet Union (Janick, Paull, 2008). Due to frost hardiness (flowers survive -8°C/-10°C) and early ripening of berries, the blue-berried honeysuckle is a valuable berry shrub from the viewpoint of breeding. The berries of the blue-berried honeysuckle contain a lot of phenols, flavonoids and anthocyanins determining antibacterial, antioxidant and anti-inflammatory features of honeysuckle fruit (Chaovanalikit et al., 2004; Svarcova et al., 2007).

Investigations on the blue-berried honeysuckle in Lithuania started in 1979, after assembling a honeysuckle collection in the Botanical Garden of Vilnius University (Žilinskaitė et al., 2007). Along with classical methods, modern methods which are based on genome DNA analysis and which do not depend on environmental conditions or stage of development of an individual should be applied to enable more efficient management and practical use of the honeysuckle collection of the Botanical Garden of Vilnius University. DNA marker based methods (Semagn et al., 2006), one of the most popular of which is the Random Amplified Polymorphic DNA (RAPD) method, are widely used for investigation and breeding of cultivars all over the world. This method is efficient and considerably cheap and because of single short primers of arbitrary nucleotide sequence used does not require original information on genome sequences of the species under investigation (Williams et al., 1990; Atienzar, Jha, 2006).

Because of polymorphism and wide distribution range, the blue-berried honeysuckle is interesting taxonomically, and scientists are still disputing whether this is one species composed of several subspecies and varieties, or a group of different species (Плеханова, Ростова, 1994; Janick, Paull, 2008). So far, classical methods have been used to solve this problem. To date, nuclear and organelle DNA analysis methods are being introduced in the systematics of plants and living organisms (Harris, 1999; Shaw et al., 2005; 2007). Chloroplast DNA (cpDNA) region sequencing is becoming widely used in plant molecular taxonomy. Application of RAPD method and cpDNA sequencing in *L. caerulea* genome analysis should provide more valuable information and describe more precisely the status of the blue-berried honeysuckle as a polymorphic species, and eliminate some contradictions in the taxonomy of this species. The use of molecular genetic methods in investigating the genome of genus *Lonicera* L. should open new opportunities for solving theoretical and practical issues, handling and using honeysuckle genetic material, and increasing breeding efficiency of new cultivars.

Aim of the study

The aim of the study is to examine genetic diversity of the *Lonicera* L. collection of the Botanical Garden of Vilnius University, carry out intra-specific and inter-specific

phylogenetic analysis through methods of molecular markers, and compare sequencing results with homologous cpDNA sequences registered in databases.

Task of research

1. To genotype samples from the honeysuckle collection of the Botanical Garden of Vilnius University by using the RAPD method.
2. To establish genetic relationship between investigated genotypes and, based on the data obtained, describe more precisely the origin of some samples.
3. To determine the level of genetic differentiation of genetic lines and elite cultivars.
4. To evaluate possibilities of the use of genetic lines in the breeding of the blue-berried honeysuckle and in increasing genetic diversity of new cultivars.
5. To determine species-specific RAPD markers for *L. caerulea* L., examine polymorphism of their DNA sequences, evaluate their molecular origin, and search for similar sequences in databases.
6. Based on the results of RAPD analysis and cpDNA sequencing, to carry out phylogenetic analysis of several species and subspecies of genus *Lonicera* L. by applying modern methods of bioinformatics.
7. To compare genetic similarity trees of the investigated taxa of honeysuckle genus constructed on the basis of RAPD data with the trees constructed on the basis of sequencing data of non-coding cpDNA regions, and compare the data obtained with the results of honeysuckle phylogenetic investigations obtained by other authors.
8. To give recommendations on further handling and breeding of the blue-berried honeysuckle collection.

The statements being defended

1. The RAPD method is suitable for investigation of genetic diversity of the *Lonicera* L. collection and for genotyping *L. caerulea* L. samples.
2. The blue-berried honeysuckle (*L. caerulea* L.) distinguishes by high DNA polymorphism.
3. The dendrograms of genetic relationship of *L. caerulea* L. cultivars constructed on the basis of RAPD markers do not conflict with the data on the origin of cultivars.
4. Some monomorphic RAPD loci of the blue-berried honeysuckle (*L. caerulea* L.) can be used as species-specific markers.
5. Based on the sequencing results of non-coding regions of honeysuckle chloroplast DNA (cpDNA) specific sequences, it is possible to differentiate closely related taxa of genus *Lonicera* L.
6. The results of phylogenetic analysis of genus *Lonicera* L. obtained by RAPD method are similar to the results of cpDNA sequencing.
7. *L. caerulea* L. forms a monophyletic group with other taxa of edible honeysuckles.
8. The honeysuckle genetic lines derived from the Vilnius University Botanical Garden can be used for broadening the genetic base of elite cultivars of *L. caerulea* L.

Scientific novelty of the study

Investigations on genetic diversity and intra-specific taxonomy of the blue-berried honeysuckle (*L. caerulea* L.) for the first time were conducted using DNA marker methods (RAPD and sequencing of cpDNA non-coding regions). *L. caerulea* L. representatives were found to have high level of DNA polymorphism. A species-specific RAPD marker was identified and sequenced for this polymorphic complex species. The

sequencing of six cpDNA non-coding regions of twelve taxa samples of genus *Lonicera* L. (*trnH-psbA*, *5'rpS12-rpL20, trnL-trnF*, *trnS-trnG*, *rpS16, trnS-psbZ*) was done for the first time. Phylogenetic analysis of cpDNA non-coding sequences showed that subspecies of the blue-berried honeysuckle described in scientific literature do not form one claster, but group with other taxa of disputable status and form with them a monophyletic group.

This molecular investigation complies with the position of Rehder (1903), Plekhanova and Rostova (1994) stating that *L. caerulea* L. is a polymorphic species to which, along with subspecies *L.* subsp. *kamtschatica*, *L.* subsp. *stenantha*, *L.* subsp. *pallasii*, *L.* subsp. *altaica,* the species distinguished by some other authors, namely *L. venulosa* (Maxim.) Worosh., *L. edulis* Turcz. ex Freyn, *L. emphyllocalyx* (Maxim.) Nakai, *L. boczkarnikowae* Plekhanova, can be attributed.

Scientific and practical significance

Oligonucleotide primers suitable for investigation of genetic diversity and phylogenetic analysis of the blue-berried honeysuckle (*L. caerulea* L.) have been determined and used in genotyping a hundred of samples from the honeysuckle collection of the Vilnius University Botanical Garden. Comparison of genetic diversity of genetic lines and elite cultivars of the blue-berried honeysuckle collection of the Vilnius University Botanical Garden assessed by RAPD method showed that these genetic lines could be used as an additional source of genetic diversity for the creation of new honeysuckle cultivars. A species-specific RAPD marker established for *L. caerulea* L. can be used in the analysis of hybrids of the blue-berried honeysuckle and other species. The sequences of six cpDNA non-coding regions (*trnH-psbA*, *5'rpS12-rpL20, trnL-trnF*, *trnS-trnG*, *rpS16, trnS-psbZ*) of twelve *Lonicera* L. taxa were established and will be registered in the NCBI database. Phylogenetic investigation of cultured plants and adjustment of their taxonomy are important in management and using species genetic resources and forecasting success of gene introgression from related species.

The presentation and approbation of the results

One scientific paper is accepted for publication in ISI Web of Science Journal List issue. Two scientific papers on the topic of the dissertation were published one in ISI Master Journal List issues and scientific papers in Lithuanian reviewed scientific periodicals. The results of the research were presented at three international conferences and seminars.

The structure and volume of the dissertation

The dissertation contains the following chapters: Short list of acronyms, Introduction, Review of the literature (4 subchapters), Materials and methods (2 subchapters), Results of the study (2 subchapters), Discussion of results (3 subchapters), Conclusions, List of publications and Conference abstracts, Acknowledgements, List of references (305 reference sources). The volume of dissertation is 163 pages (excluding appendix) and Appendix (4 tables and sequences of six cpDNA regions of twelve different taxa of genus *Lonicera* L.). The dissertation is illustrated with 25 figures and 27 tables. The dissertation is written in Lithuanian with the summary in English.

MATERIALS AND METHODS

The object of study is plants of genus *Lonicera* L. from the collection of the Vilnius University Botanical Garden. Most accessions of honeysuckle originated from Russia. The investigation covers 13 species (*L. caerulea* L., *L. alpigena*, *L. vesicaria* Kom., *L. praeflorens* Lam., *Lonicera* x *bella* f. candida Zabel, *L. xylosteum* L., *L. chrysantha* Turcz. ex Ledeb., *L. venulosa* (Maxim.) Worosh., *L. edulis* Turcz. ex Freyn, *L. emphyllocalyx* (Maxim.) Nakai, *L. boczkarnikowae* Plekhanova, *L. orientalis* Lam.), four subspecies (*L. caerulea* L. subsp. *kamtschatica*, *L. caerulea* L. subsp. *pallasii*, *L. caerulea* L. subsp. *altaica*, *L. caerulea* L. subsp. *stenantha*), 28 cultivars (*'Narymskaya', 'Desertnaya', 'Salyut', 'Sinyaya Ptitsa', 'Lazurnaya', 'Altayskaya', 'Rannyaya', 'Start', 'Fialka', 'Roksana', 'Parabelskaya', 'Morena', 'Volshebnica', 'Velikan', 'Chernichka', 'Goluboje Vereteno', 'Obilnaya', 'Valentina', 'Lyulia', 'Kuvshinovidnaya', 'Bugristaya', 'Zolushka', 'Regel-683', 'Bakcharskaya', 'Viola', 'Vasyuganskaya', 'Tomichka', 'Leningradskii Velikan'*) and 32 genetic lines of *Lonicera caerulea* L. (2R, 3-5, 2-1, 3-79, 3R, 3U, 3-80, 10-32, 1L, 1G16, 1E, 2D, 2C63, 1B43, 2K, 2S, 19, 1N73, 22T12, 1U, 1V, 2E, 1T, 1M, 1P, 96-1, 96-2, 96-3, 96-4, 69-3, 10, 32).

DNA extraction and RAPD-PCR

Total DNA was extracted from fresh young leaves, using the Genomic DNA purification kit (*Fermentas*, Lithuania). DNA quantity and quality were measured with a spectrophotometer and electrophoretically. Amplifications were carried out in 25 µl of reaction mixtures containing the following components: 1× PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of the primer, 1.0 u of *Taq* DNA polymerase (*Fermentas*, Lithuania) and 50 ng of total DNA. Fifty decameric primers used in this study were purchased from *Karl Roth GmbH & Co. KG* (Germany). All amplifications were carried out in *Eppendorf Mastercycler*® *ep gradient* (*Eppendorf* AG, Germany) programmed for 35 cycles: 4 min at 94 °C initial DNA denaturation step, followed by 35 cycles of 60 s at 94 °C, 60 s at 35 °C, 120 s at 72 °C. The last cycle was followed by the final extension step for 5 min at 72 °C. The reaction mixture without DNA was used as a negative control to avoid contamination. Amplification products were fractionated in 1.5% agarose gel (1×TBE) and visualized with ethydium bromide. RAPD-PCR reproducibility was assessed by comparing at least two independent reactions.

Amplification of specific cpDNA regions, cloning and sequencing

To explore the six regions of cpDNA (*trnH-psbA*, 5'*rpS12-rpL20*, *trnL-trnF*, *trnS-trnG*, *trnS-psbZ* IGS regions and *rpS*16 intron) the PCR conditions were carried out as described in Shaw et al. (2005) methodological recommendations. The isolated DNA fragments were cloned into the pTZ57R/T vector using the *InsTAclone*™ PCR Cloning Kit (*Fermentas*, Lithuania). Recombinant clones were selected using lacZ' system on the *FastMedia*™ *LB Agar Amp IPTG/X-Gal* (*Fermentas*, Lithuania). Plasmid DNA was isolated using *NucleoSpin*® *Plasmid* kit (*MACHEREY–NAGEL GmbH&Co. KG*, Germany). The cloned inserts were sequenced at the Sequencing Center of the Institute of Biotechnology (Lithuania) with the *3130xl* Genetic Analyzer (*Applied Biosystems*, USA) using *BigDye*® *Terminator v3.1 Cycle Sequencing* Kit (*Applied Biosystems*, USA).

Phylogenetic analysis

Sequences of cloned fragments were evaluated with *ChromasPro v.1.5* (*Technelysium*, Australia) and *MEGA 5* (Tamura et al., 2011). A homology search was carried out with *BLAST* algorithm (Altschul et al. 1990) at the *NCBI* database. Phylogenetic analysis was conducted and dendrograms were constructed using the ML (**M**aximum **L**ikelihood) analysis method and Tamura-Nei model. Homologous sequences were also searched for in EMBL-EBI and DDBJ databases.

Data analysis

The presence or absence of an individual DNA fragment in DNA samples of plants of genus *Lonicera* L. was scored as "1" or "0", respectively. Non-reproductive, unclear or poorly resolvable DNA fragments were not registered. Genetic distance between individuals (GD_{xy}) was calculated according to Nei and Li (1979). Relationship between samples based on genetic distances was assessed by NJ and UPGMA grouping methods. Molecular genetic diversity analysis (AMOVA) was carried out using GenAlEx v6.41 program (Peakall, Smouse, 2006). Bootstrap analysis was conducted with 1000 iterations.

RESULTS

Initially, we used 20 primers and analyzed 40 genotypes and found that RAPD method can be effectively used in genotyping honeysuckle collection samples, because DNA polymorphism considerably exceeds their morphological diversity. 84.3% DNA polymorphism was found in the group of genotypes under investigation (Naugžemys et al., 2007). In the second stage of investigation, 30 new primers were additionally tested. An optimal set of twelve primers was determined for the genotyping of the collection. The primers were used to genotype a hundred honeysuckle collection samples of different taxonomic rank and breeding value, including species, subspecies, cultivars and genetic lines. The highest level of DNA polymorphism (97.8%) was found for the samples representing species.

To estimate the potential of blue-berried honeysuckle genetic lines for increasing genetic diversity of cultivars, 32 genetic lines and 19 elite cultivars were examined. Genomic DNA of the genotypes was investigated using 12 informative RAPD primers from earlier experiments. Having investigated DNA of 51 samples, 149 RAPD bands (loci) were determined, of which 124 (83.2%) were polymorphic (Table 1).

Table 1. DNA polymorphism of *L. caerulea* L. genetic lines and elite cultivars
1 lentelė. *L. caerulea* L. genetinių linijų ir elitinių veislių DNR polimorfizmas

RAPD primer (ROTH)	Sequence 5'→3'	Total bands	Monomorphic bands	Polymorphic bands	Polymorphic bands %	Size range of DNA fragments (bp)
170–08	CTGTACCCCC	14	1	13	92.8	490 – 2100
170–10	CAGACACGGC	14	–	14	100	470 – 1800
380–01	ACGCGCCAGG	12	3	9	75	490 – 1700
380–02	ACTCGGCCCC	10	–	10	100	510 – 1800
380–07	GGCAAGCGGG	14	2	12	85.7	560 – 1960
A–01	CAGGCCCTTC	11	–	11	100	700 – 2300
A–02	TGCCGAGCTG	11	5	6	54.5	580 – 2450
A–03	AGTCAGCCAC	10	4	6	60	680 – 2480
A–04	AATCGGGCTG	12	5	7	58.3	560 – 1900
A–05	AGGGGTCTTG	13	1	12	92.3	490 – 2100
A–09	GGGTAACGCC	14	1	13	92.8	270 – 1180
A–11	CAATCGCCGT	14	3	11	78.5	370 – 1400
Total / average		**149**	**25**	**124**	**83.2 ±17**	**270 – 2480**

Genetic relationship between cultivars and genetic lines was assessed by UPGMA method (Fig. 1). All genotypes were divided into two large groups: group I composed of all cultivars and seven genetic lines (‘*32*’, ‘*L-69-3*’, ‘*10*’, ‘*96-2*’, ‘*96-3*’, ‘*96-1*’, ‘*96-4*’), and group II composed solely of genetic lines. UPGMA analysis results rather closely corresponded to available scanty data on the origin of cultivars.

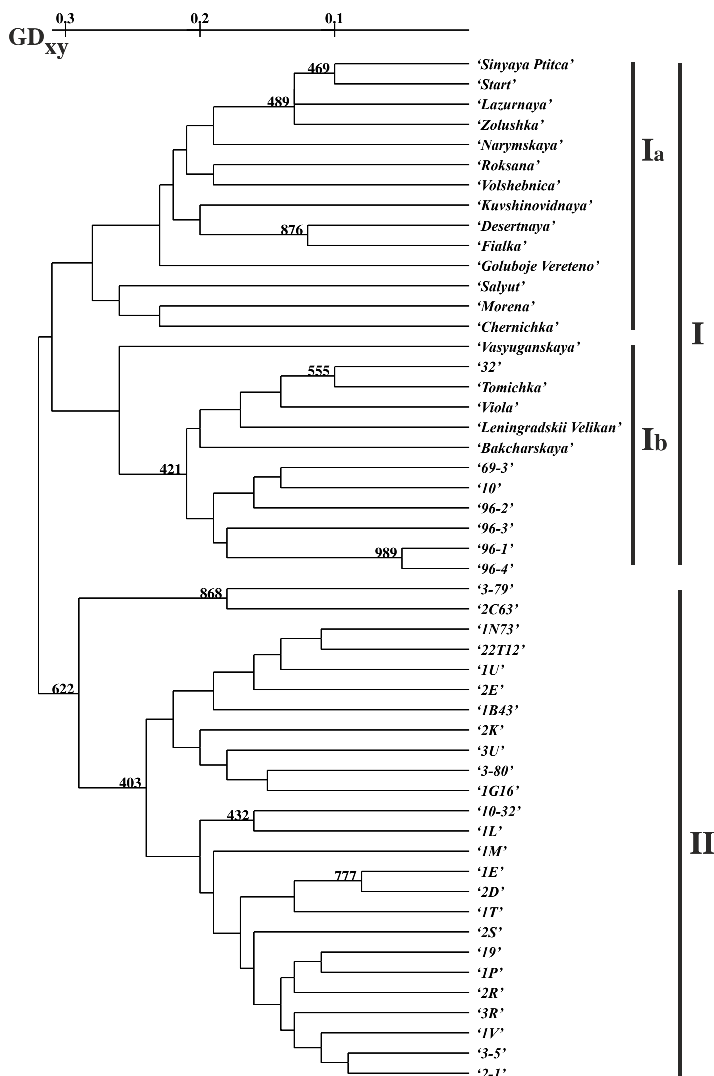

Figure 1. Genetic diversity of the accessions of *L. caerulea* L. cultivars and genetic lines according to RAPD loci. Dendrogram constructed on the basis of UPGMA method using Nei and Li (1979) genetic distance matrix. Bootstrap values obtained after 1000 iterations

1 pav. *L. caerulea* L. veislių ir genetinių linijų pavyzdžių genetinė įvairovė pagal RAPD-PGR lokusus. Dendrograma sudaryta UPGMA metodu, naudojant Nei ir Li genetinių atstumų matricą. Įkėlų vertės gautos atlikus 1000 iteracijų

AMOVA also showed obvious genetic differentiation between the investigated genetic lines and cultivars ($\Phi_{PT} = 0.189$, $P \leq 0.001$). Differences between genetic lines and cultivars are not only due to difference in allele frequencies, but also to group-specific loci.

Out of 124 RAPD loci, eleven (170–$08_{490/570/890}$; 170–$10_{950/1300}$; 380–01_{530}; 380–02_{1500}; A–$09_{680/700/1031}$; A–11_{790}) were specific only to genetic lines, and six loci (380–01_{490}; A–$09_{300/560/790}$; A–$11_{510/600}$) were specific to cultivars. We analysed RAPD spectra of 51

honeysuckle samples and determined 25 monomorphic bands (Table 1). In order to verify whether monomorphic RAPD bands include those characteristic solely of *L. caerulea* species, we supplemented a group of genotypes under investigation with the samples of four *L. caerulea* subspecies (*L. caerulea* L. subsp. *kamtschatica* Sevast., *L. caerulea* L. subsp. *stenantha* Pojark., *L. caerulea* L. subsp. *altaica* (Pall.), *L. caerulea* L. subsp. *pallasii* (Ledeb.) Browicz)) and twelve species (*L. caerulea* L., *L. alpigena*, *L. vesicaria* Kom., *L. praeflorens* Lam., *Lonicera* x *bella* f. candida Zabel, *L. xylosteum* L., *L. chrysantha* Turcz. ex Ledeb., *L. venulosa* (Maxim.) Worosh., *L. edulis* Turcz. ex Freyn, *L. emphyllocalyx* (Maxim.) Nakai, *L. boczkarnikowae* Plekhanova, *L. orientalis* Lam.) and carried out their RAPD analysis with the same primers. Out of 149 RAPD bands analysed, 96.6% were polymorphic. The mean genetic distance in the group of subspecies and species was 0.453 ± 0.148, with the lowest value being 0.188, and the highest 0.708. Having compared RAPD profiles of all samples, we found one RAPD band (ca. 570 bp) characteristic of all samples attributed to L. *caerulea* (Fig. 2).

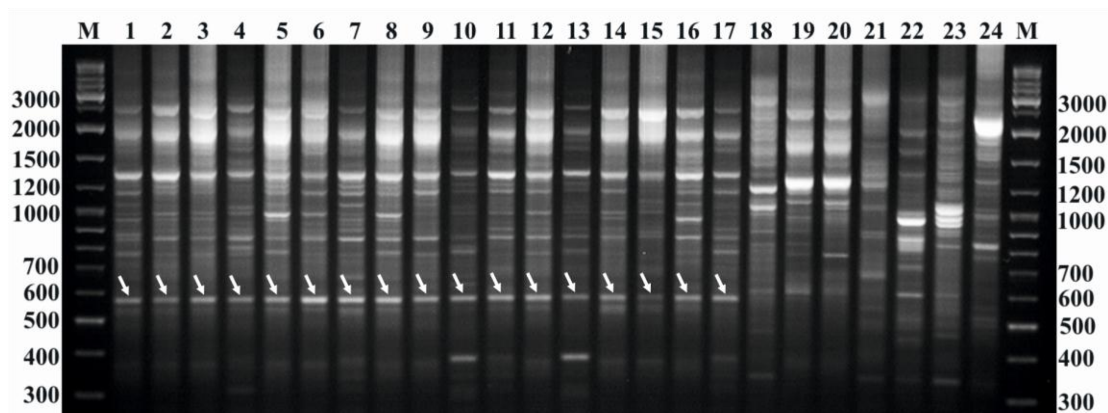

Figure 2. Species-specific RAPD marker (marked by an arrow) of tetraploid polymorphic *Lonicera caerulea* L., determined by Roth A–02 primer. 1 – *Lonicera caerulea* L. subsp. *kamtschatica* (Pojark.), 2 – *Lonicera caerulea* L. subsp. *stenantha* (Pojark.), 3 – *Lonicera caerulea* L. subsp. *pallasii* (Ledeb.), 4 – *Lonicera caerulea* L. subsp. *altaica* (Pall.), 5 – '*Desertnaya*', 6 – '*Salyut*', 7 – '*Sinyaya Ptitsa*', 8 – '*Fialka*', 9 – '*Morena*', 10 – '*Viola*', 11 – '*Vasyuganskaya*', 12 – '*Tomichka*', 13 – '*Leningradskii Velikan*', 14 – *Lonicera caerulea* L., 15 – *Lonicera edulis* Turcz. ex Freyn, 16 – *Lonicera boczkarnikowae* Plekhanova, 17 – *Lonicera emphyllocalyx* (Maxim.) Nakai, 18 – *Lonicera chrysantha* Turcz. ex Ledeb., 19 – *Lonicera xylosteum* L., 20 – *Lonicera orientalis* Lam., 21 – *Lonicera vesicaria* Kom., 22 – *Lonicera alpigena* L., 23 – *Lonicera* x *bella* f. candida Zabel, 24 – *Lonicera praeflorens* Batalin. M – DNR size marker „GeneRuler^{TM} DNA Ladder Mix“ (100–10000 bp)

2 pav. Tetraploidinei polimorfinei *Lonicera caerulea* L. rūšiai savitas RAPD žymuo (pažymėtas rodykle) nustatytas su Roth A–02 pradmeniu.

This species-specific marker was determined by Roth A–02 primer and, therefore, referred to as $A02_{570}$. In order to determine its molecular-genetic origin, we analysed DNA fragments of the same size, corresponding to $A02_{570}$ band in RAPD profiles of three genotypes (*L. caerulea* L., *L. caerulea* L. subsp. *kamtschatica* (Pojark.) and *L. caerulea* L. subsp. *stenantha* (Pojark.)). These DNA fragments were cloned into pTZ57R/T vector. The sequencing of cloned fragments showed that they all are of the same size (568 bp). However, polymorphism (ca. 3.2%) in DNA sequence was also found. No similar sequences were detected when searching for homologous sequences by BLAST in GenBank® database. Using sequencing data of $A02_{570}$ DNA fragment (Fig. 3) two primers complementary to the ends of cloned DNA fragment, i.e. $A02_{570D}$

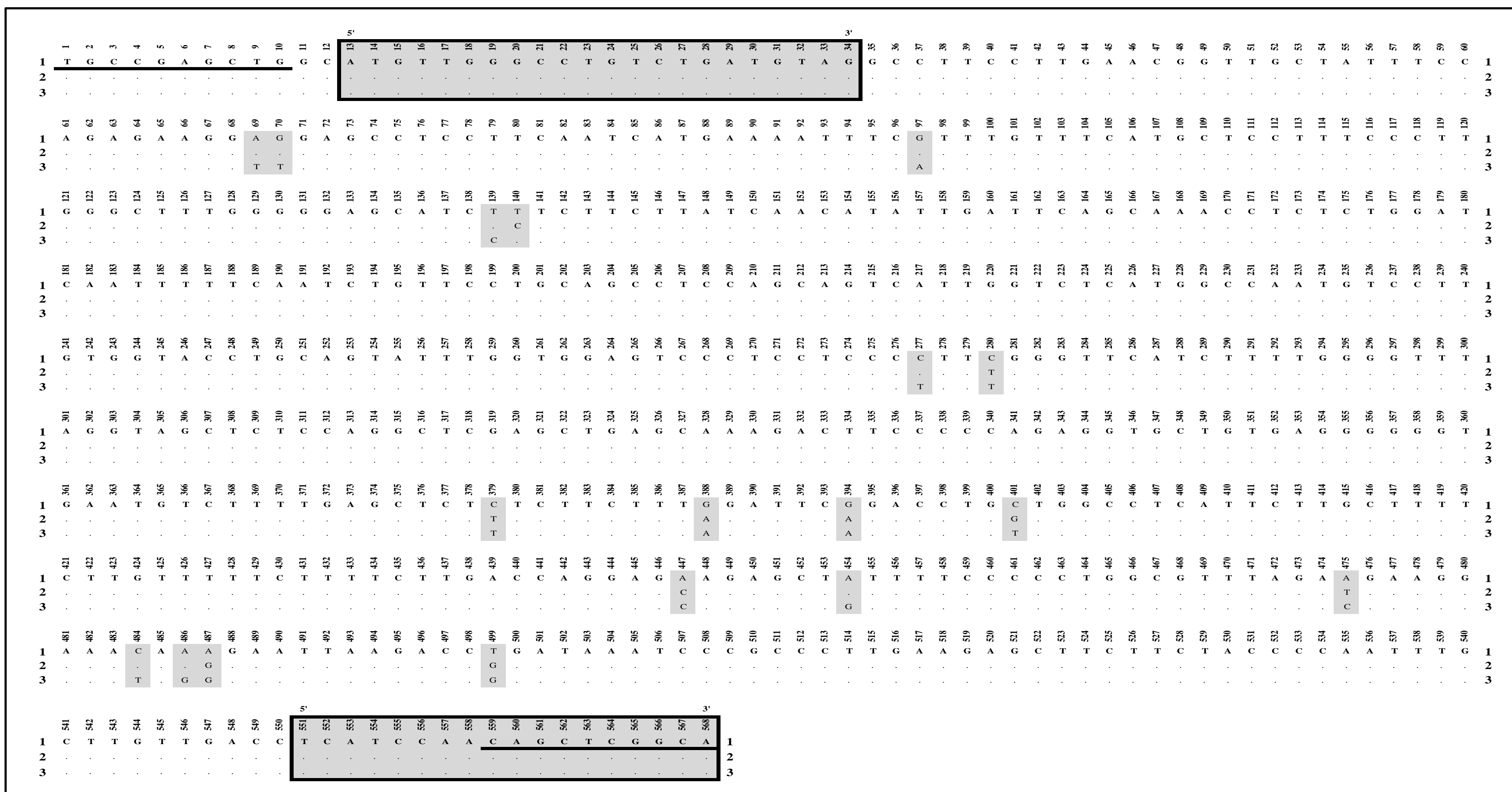

Figure 3. Sequencing results of species-specific samples of cloned DNA fragments of *Lonicera caerulea* L.: 1 – *L. caerulea* L., 2 – *L. caerulea* L. subsp. *stenantha* (Pojark.), 3 – *L. caerulea* L. subsp. *kamtschatica* (Pojark.) Plekhanova. Primer A–02 annealing sites are underlined. SCAR primer annealing sites are marked grey

3 pav. *Lonicera caerulea* L. rūšiai savitų, klonuotų DNR fragmentų pavyzdžių sekoskaitos rezultatai: 1 – *L. caerulea* L., 2 – *L. caerulea* L. subsp. *stenantha* (Pojark.), 3 – *L. caerulea* L. subsp. *kamtschatica* (Pojark.) Plekhanova. Pradmens A–02 prisijungimo vietos pabrauktos. SCAR pradmenų jungimosi vietos pažymėtos pilkai

(5'ATGTTGGGCCTGTCTGATGTAG3') and $A02_{570R}$ (5'TGCCGAGCTGTTGGATGA3'), were developed. Based on the derived primers we wanted to convert RAPD $A02_{570}$ marker into a more reliable and convenient SCAR marker. However, such conversion of RAPD marker into SCAR changed the original DNA polymorphism. 568 bp DNA fragment was observed not only in representatives of *L. caerulea* L., but in all investigated samples.

In parallel, economic traits of the most perspective genetic lines were investigated and compared with respective characteristics of standard cultivars. We analysed three-year data and assessed the size and chemical composition of berries. The results of investigation showed that genetic lines (‘*1L*’, ‘*2C*’, ‘*3U*’, ‘*3-5*’, ‘*96-4*’) distinguish not only by a greater weight of berries, but also by higher resistance to fungal diseases, compared with other lines and cultivars (Žilinskaitė et al., 2007).

In order to establish more objectively phylogenetic relationships of twelve taxa samples of genus *Lonicera* L., we applied RAPD method and the sequencing of six cpDNA non-coding regions. RAPD analysis of genomes of investigated samples revealed 132 polymorphic loci. The size of analysed DNA fragments was from 270 bp to 2500 bp. The greatest number of polymorphic loci (100%) was detected with primer 380–02 and the lowest (43.8%) with A–03. 11 polymorphic loci on average were determined using one primer. DNA polymorphism was 78.1% in the group of investigated taxa.

Based on the calculated genetic distances, UPGMA and NJ dendrograms were constructed, which show possible phylogenetic relationship of investigated taxa (Fig. 4, 5)

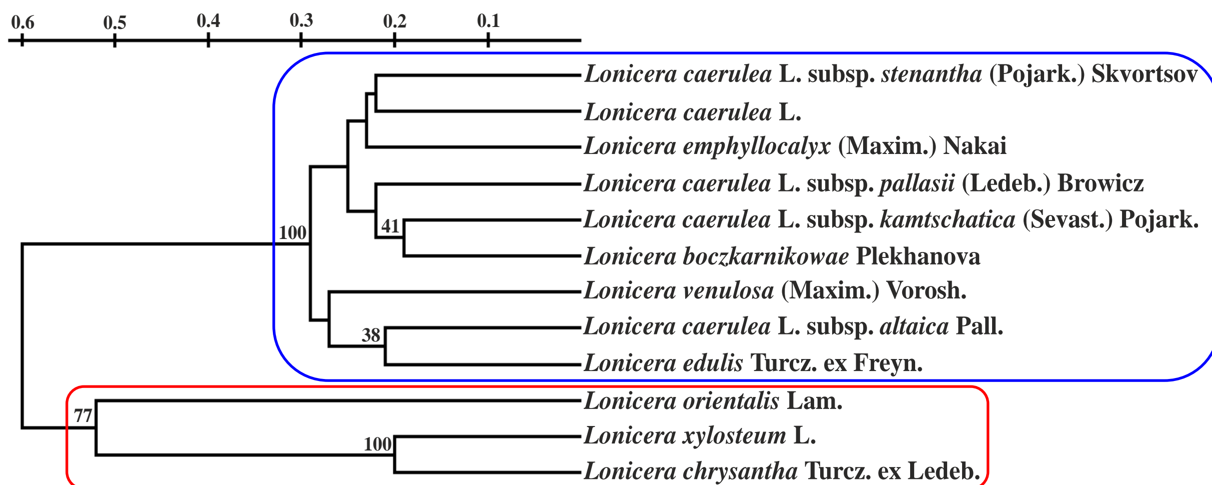

Figure 4. UPGMA dendrogram of *Lonicera* L. species and subspecies, constructed according to genetic distance of Nei and Li (1979). Bootstrap values (%) obtained after 1000 iterations

4 pav. *Lonicera* L. genties rūšių ir porūšių UPGMA dendrograma, sudaryta pagal Nei ir Li (1979) genetinius atstumus. Įkėlų vertės (%), gautos atlikus 1000 iteracijų

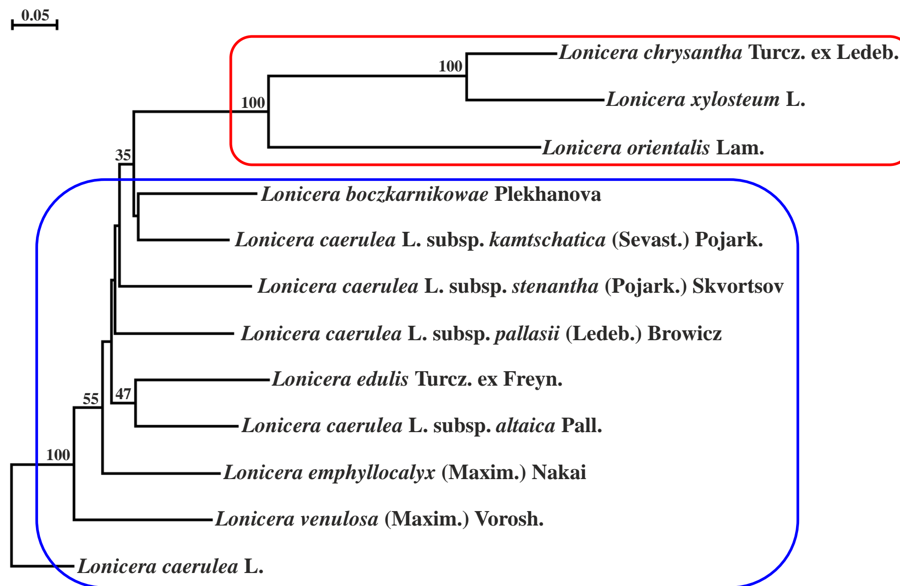

Figure 5. Phylogenetic dendrogram of *Lonicera* L. species and subspecies, constructed by NJ grouping method according to genetic distances of Nei and Li (1979). Bootstrap values (%) obtained after 1000 iterations

5 pav. *Lonicera* L. rūšių ir porūšių filogenetinė dendrograma, sudaryta NJ grupavimo metodu pagal Nei ir Li (1979) genetinius atstumus. Įkėlų vertės (%), gautos atlikus 1000 iteracijų

In order to determine phylogenetic relationships of *Lonicera* L. species and *L. caerulea* L. subspecies more exactly and to estimate suitability of RAPD-PGR method in *Lonicera* L. taxonomy, the sequencing of six non-coding cpDNA regions of twelve taxa of genus *Lonicera* L. was carried out. After sequencing and alignment, cpDNA sequences were used in phylogenetic analysis. Initially, quantitative and qualitative analysis of sequenced DNA fragments was done using programs of bioinformatics processing of sequencing results "Sequencing Analysis 5.2" and "Chromas 2.33". The sequencing of 71146 nucleotides of six cpDNA non-coding regions of twelve taxa of genus *Lonicera* L. was carried out (Table 2).

Table 2. Lengths of six cpDNA regions (bp) estimated in samples of twelve different taxa of genus *Lonicera* L.

2 lentelė. cpDNR šešių regionų ilgiai (bp), nustatyti dvylikoje skirtingų *Lonicera* L. genties taksonų pavyzdžių

Taxon Nr.*	*trnH-psbA*	*5'rpS12-rpL20*	*trnL-trnF*	*trnS-trnG*		*rpS16*	*trnS-psbZ*	Total cpDNR bp
				5'trnG2S	*5'trnG2G*			
1	481	907	1016	878	783	912	993	5945
2	481	907	1016	879	783	892	987	5966
3	476	907	1016	851	784	910	990	5916
4	481	907	1015	851	783	893	989	5931
5	455	907	1011	850	786	911	983	5916
6	481	906	1009	878	783	911	985	5962
7	481	907	1016	879	783	911	994	5950
8	481	907	1001	880	784	890	993	5936
9	457	901	1003	850	783	908	991	5893
10	481	907	1005	879	783	894	990	5960
11	457	888	1004	855	783	913	994	5892
12	457	887	1002	855	783	912	993	5880
Total	**5669**	**10838**	**12114**	**10385**	**9401**	**10857**	**11882**	**71146**
Average	**472.42**	**903.17**	**1009.50**	**865.42**	**783.42**	**904.75**	**990.17**	**5928.92**

* – 1 – *Lonicera caerulea* L. subsp. *kamtschatica* (Sevast.) Pojark.; 2 – *Lonicera caerulea* L. subsp. *stenantha* (Pojark.) Skvortsov; 3 – *Lonicera caerulea* L. subsp. *pallasii* (Ledeb.) Browicz; 4 – *Lonicera caerulea* L. subsp. *altaica* Pall.; 5 – *Lonicera xylosteum* L.; 6 – *Lonicera chrysantha* Turcz. ex Ledeb.; 7 – *Lonicera venulosa* (Maxim.) Vorosh.; 8 – *Lonicera emphyllocalyx* (Maxim.) Nakai; 9 – *Lonicera orientalis* Lam.; 10 – *Lonicera boczkarnikowae* Plekhanova; 11 – *Lonicera caerulea* L.; 12 – *Lonicera edulis* Turcz. ex Freyn.

According to the summary of sequencing results (Table 2), the lengths of individual cpDNA fragments of samples of different taxa are rather variable.

The *trnH-psbA* intergenic spacer was the smallest, with length ranging from 457 bp to 481 bp in the investigated samples. The longest (from 1002 bp to 1016 bp) cpDNA sequences were in *trnL-trnF* intergenic spacer. A comparison of variability of six cpDNA non-coding regions is presented in Table 3.

Table 3. Comparison of variability of six cpDNA non-coding regions
3 lentelė. cpDNR šešių nekoduojančių regionų kintamumo palyginimas

Statistic	Combined	*trnH-psbA*	*5'rpS12-rpL20*	*trnL-trnF*	*trnS-trnG*		*rpS16*	*trnS-psbZ*
					5'trnG2S	*5'trnG2G*		
Range of raw length (bp)		455–481	887–907	1001–1016	850–880	783–785	890–913	983–994
Aligned length (nt)	6015	488	908	1019	885	787	915	1013
No. of conservative sites	5763	460	861	974	854	763	880	971
No. of variable sites	235	28	47	42	31	21	33	33
PI*	56	9	2	8	7	4	10	16
Single nucleotide polymorphism (SNP)	179	19	45	34	24	17	23	17
Range of pairwise distance		0–0.30	0.002–0.024	0.001–0.019	0.002–0.014	0.001–0.010	0–0.016	0.002–0.017
Average		0.013	0.009	0.008	0.008	0.006	0.008	0.008

* – PI – *Parsimony informative character*

We carried out multiple analysis of sequencing data of all six cpDNA regions and obtained the aligned sequence of 6015 nucleotides (Table 3). The length of conservative sequences was 5763 nucleotides, and the length of variable sequences was 235 nt. In total, 56 parsimony informative sites and 179 polymorphisms of single nucleotide were determined. Having evaluated all determined cpDNA sequences, we carried out phylogenetic analysis and drew a dendrogram, using ML method and Tamura-Nei model (Figure 6).

All honeysuckle cpDNA sequences determined during sequencing were compared with nucleotide sequences registered in GenBank® / EMBL / DDBJ and PDB databases of NCBI institute. The search was performed using BLASTN v2.2.24. It revealed that cpDNA *5'rpS12-rpL20* intergenic spacer of genus *Lonicera* L. in family *Caprifoliaceae* had never been studied. We also did not find cpDNA *trnS-psbZ* region sequences of genus *Lonicera* L. in the databases.

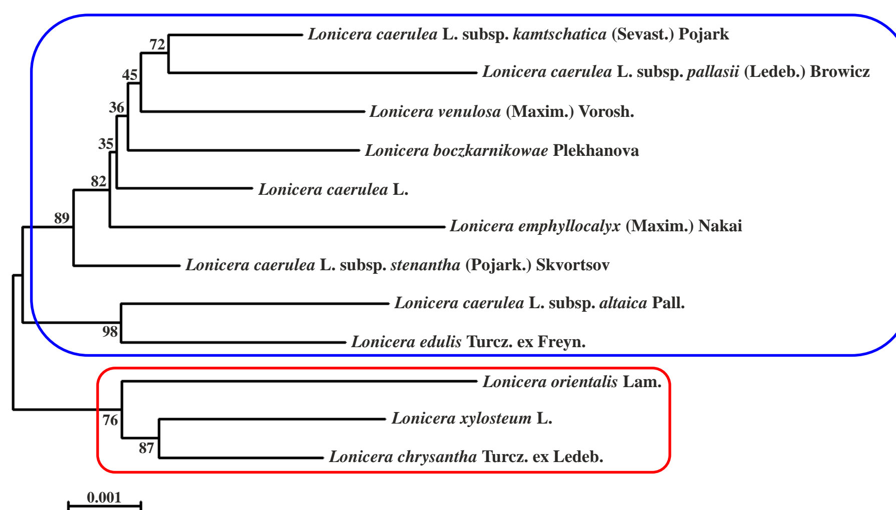

Figure 6. Phylogenetic analysis of twelve taxa of genus *Lonicera* L. based on sequencing of six non-coding cpDNA regions. Dendrogram made by applying ML method and Tamura-Nei model algorithm. Bootstrap values (%) obtained after 1000 iterations

6 pav. *Lonicera* L. genties dvylikos taksonų filogenetinė analizė, atlikta remiantis cpDNR šešių nekoduojamų regionų sekoskaita. Dendrograma sudaryta naudojant ML metodą ir Tamura-Nei modelio algoritmą. Įkėlų vertės (%) gautos atlikus 1000 iteracijų

A search for sequences similar to cpDNA *trnH-psbA* region resulted in six similar sequences of the species of genus *Lonicera* L. and of two species of closely related species of *Caprifoliaceae* family. A dendrogram was drawn based of these data (Fig. 7).

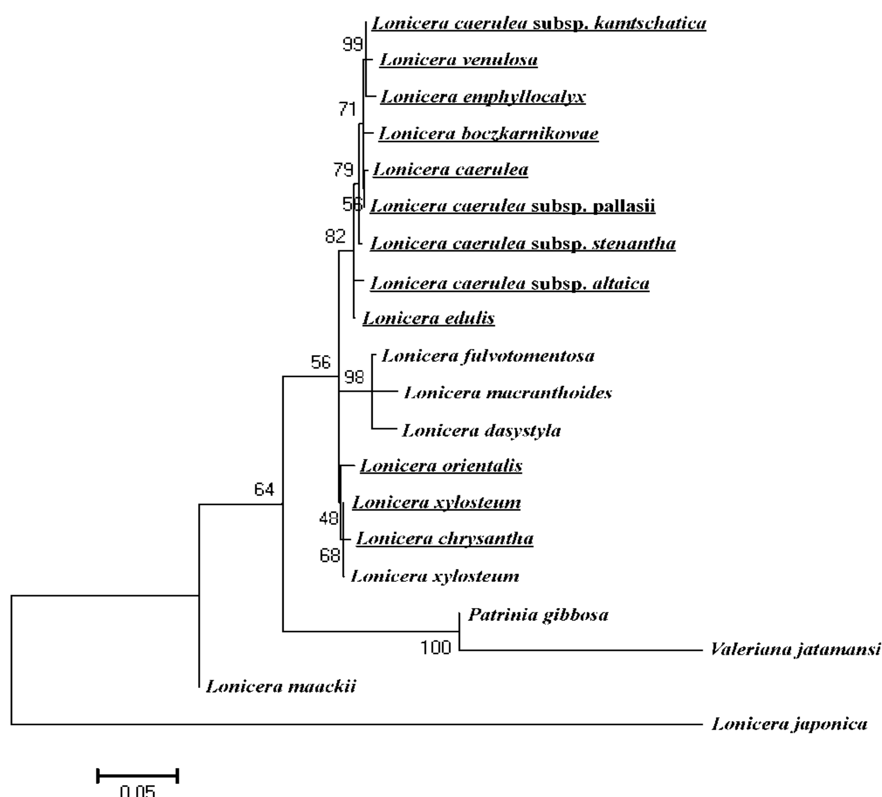

Figure 7. Dendrogram of genetic relationship between taxa of genus *Lonicera* L. and two related *Caprifoliaceae* species (*P. gibbosa*, *V. jatamansi*) based on sequencing data of *trnH-psbA* cpDNA region by using ML method and Tamura-Nei model algorithm. Bootstrap values

(%) obtained after 1000 iterations. The names of taxa of genus *Lonicera* L. studied are underlined

7 pav. *Lonicera* L. genties taksonų ir dviejų gimininingų *Caprifoliaceae* šeimos rūšių *P. gibbosa* ir *V. jatamansi* gimininingumo dendrograma, sudaryta remiantis *trnH-psbA* cpDNR regiono sekoskaitos duomenimis, naudojant ML statistinį analizės metodą ir Tamura-Nei modelio algoritmą. Įkėlų vertės (%) gautos atlikus 1000 iteracijų. Mūsų tirtų *Lonicera* L. genties taksonų pavadinimai pabraukti

It is evident from the dendrogram, as from the dendrograms drawn based on our data (Fig. 4 and 6), that all taxa of honeysuckles bearing edible fruits form one cluster. It reveals monophyletic character of the group. Comparison of cpDNA *trnL-trnF* region sequences with sequences registered in databases shows that the sequences we have determined are similar to twelve registered sequences of the species of genus *Lonicera* L. A search for cpDNA sequences homologous to *rpS16* gene intron sequence revealed two homologous sequences belonging to the species of genus *Lonicera* L. A comparison of cpDNA *trnS-trnG* region *5'trnG2S* part sequences with sequences registered in databases revealed more than a hundred of homologous sequences of species of genus *Lonicera* L. (Theis et al., 2008; Smith, 2009). We compared cpDNA *trnS-trnG* region *5'trnG2G* part sequences with sequences registered in databases and found 12 sequences similar to those we determined. Seven of the latter belong to the species of genus *Lonicera* L. (Bell, 2010).

DISCUSSION

Development of new cultivars largely depends on genetic collections containing genotypes distinguishing by valuable and accessible genetic diversity. In order to use a collection efficiently, estimation of its genetic diversity is necessary, because variability of morphological markers is insufficient for effective management of genetic resources. The best way to investigate genetic features of plants is by using DNA markers (Weising et al., 2005). For many years already DNA markers have been regarded as an important and efficient measure for management of genetic collections and gene banks and use of genetic diversity in breeding programs (Rafalski, Tingey, 1993). Multiple studies show that RAPD method is one of the most suitable for assessment of genetic diversity (Angiolillo et al., 2006; Carelli et al., 2006; Sensoy et al., 2007; Cordeiro et al., 2008). We used this method to assess genetic diversity of the honeysuckle collection of the Vilnius University Botanical Garden, to genotype honeysuckle collection samples and estimate the potential of genetic lines as an additional source of genetic diversity. At the beginning of investigation, we selected RAPD primers, i.e. selected oligonucleotide primers suitable for investigation of *Lonicera* L. and determined reliably reproducible RAPD markers. Selection of primers was conducted in two stages. Initially, we tested 20 primers by using them for investigation of 40 *Lonicera* L. genotypes of different taxonomic status. The investigation showed that DNA polymorphism determined by RAPD method was sufficient for genotyping even very close genetically honeysuckle plants. DNA polymorphism estimated by RAPD method was more informative than morphological diversity. In the second stage, we additionally tested 30 oligonucleotide primers. Upon evaluation of the results of both stages, 12 primers for further investigation were chosen. A similar number of primers for investigation of collections of different plant species were used by other authors (Gimenes et al., 2000; Carelli et al., 2006; Sensoy et al., 2007; Cordeiro et al., 2008). By using 12 primers, we determined

149 RAPD loci with high DNA polymorphism (88.1%) in a hundred of genotypes. The estimation of DNA polymorphism in the groups of genotypes representing species, subspecies, cultivars and genetic lines showed that the greatest polymorphism, as expected, was between species (97.8%). Based on available data we can state that our study of the blue-berried honeysuckle collection by using molecular markers is the first such type published study of this species (Naugžemys et al., 2007). Some oligonucleotide primers selected in our investigation were later used in inter-specific hybrid study of *L. caerulea* L. var. *emphyllocalyx* and *L. gracilipes* var. *glabra* Miquel conducted by other authors (Miyashita & Hoshino, 2010). Recently, there was published an investigation of genetic diversity by ISSR method of fourteen accessions of *L. caerulea* L. from Poland and Russia (Smolik et al., 2010). By using 11 selected ISSR primers, 129 ISSR loci were determined, of which 64% were polymorphic. Whereas RAPD and ISSR loci in genome are different and studied accessions belonged exclusively to *L. caerulea* L. and the number of studied accessions was considerably lower, the lower level of genetic diversity determined does not object to the results obtained in our study.

The broadening of the genetic base of cultivated plants is highly relevant in modern plant breeding, because only a minor part of genetic diversity found in natural populations is used in breeding programs (Baur, 1914; Haskell, 1960; Mekbib, 2008; Wouw et al., 2009). For this reason, cultivated plants are more vulnerable to unfavourable conditions, different pests and diseases. The blue-berried honeysuckle is not an exception. Though it is a new culture from the viewpoint of breeding, the breeding of blue honeysuckle is more and more frequently performed by using already existing cultivars. As the works of different authors show, natural populations of different species are very important source of additional genetic material (Stalker, 1980; Cox et al., 1984; Sharma et al., 2003; Wouw et al., 2009). The genetic base of a particular culture can be broadened by using genetic material of natural populations of the same species and of related species (Cooper et al., 2001; Miyashita, Hoshino, 2010). Whereas morphological markers have lower diversity and greatly depend on environmental conditions, RAPD markers were used in our work to estimate the potential of honeysuckle genetic lines originating from natural populations from the collection of the Botanical Garden for increasing genetic diversity of cultivars. Genetic differentiation of 32 genetic lines and 19 elite cultivars was estimated. DNA polymorphism determined using twelve oligonucleotide primers was sufficient for genotyping the selected samples of elite cultivars and genetic lines (Fig. 1). Genetic relationship determined by UPGMA method corresponded in most cases to available genealogical data of investigated cultivars. Similar results showing that molecular and genealogical data correlate were obtained by other authors (Doldi et al., 1997; Кудрявцев и др., 2003; Raddová et al., 2003). The fact that the results of UPGMA cluster analysis of genetic relationship of investigated cultivars and lines objectively reflect the similarities and differences of these two genotype groups can be confirmed by PCoA and AMOVA. PCoA shows that plants of groups under consideration tend to group separately in the two-dimensional coordinate space (Fig. 8).

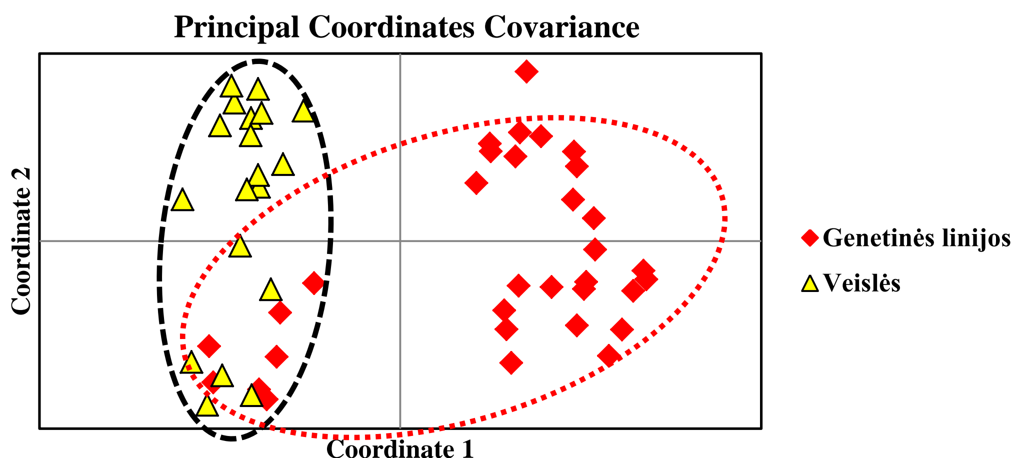

Figure 8. Principal coordinate analysis of blue-berried honeysuckle genetic lines and cultivars

8 pav. Melsvauogio sausmedžio genetinių linijų ir veislių principinė koordinačių analizė

In order to assess the level of genetic variation within and among different (morphologically, genetically, geographically) groups of germplasm accessions some authors use AMOVA method (Jakse et al., 2004, Carelli et al., 2006, Soleimani et al., 2007), which is traditionally applied in population genetics when dominantly inherited DNA markers are used (Nybom, 2004). Results obtained by AMOVA also confirmed that these two genotype groups differ significantly from each other. Though the major part of genetic diversity is characteristic of both genotype groups, a significant part of genetic diversity (18.9%) is group-specific. Several genetic lines of the honeysuckle collection of the Vilnius University Botanical Garden do not submit by their agronomic and biological features to the recognized cultivars. The comparison of some agronomic and biological features of fourteen *L. caerulea* L genetic lines and seven cultivars showed that some genetic lines (‘*1L*’, ‘*2C*’, ‘*3U*’, ‘*3-5*’, ‘*96-4*’) distinguish by great weight of berries and resistance to fungal diseases (Žilinskaitė et al., 2007).

Taxonomic assessment of *L. caerulea* is very confusing so far (Плеханова, Ростова 1994; Janick, Paull, 2008), and there is much unclearness in denominations. Therefore, species-specific markers should be used when handling blue-berried honeysuckle collections and analysing hybrids. In our work we determined $A02_{570}$ marker, which is species-specific for polymorphic *L. caerulea* L. The cloning of this DNA fragment from three taxonomically different samples (*L. caerulea* L., *L. caerulea* L. subsp. *stenantha* Pojark. and *L. caerulea* L. subsp. *kamtschatica* Sevast.) and its sequencing showed low polymorphism (≈ 3.2%). The BLAST search for homologous sequences did not yield any results.

Summarizing the results of investigation of genetic lines and elite cultivars of the blue-berried honeysuckle we can state that genetic lines of the blue-berried honeysuckle collection of the Vilnius University Botanical Garden are valuable from the viewpoint of breeding, because they can be used as a source of additional genetic material in creating new cultivars. The cross of the most perspective genetic lines and particular elite cultivars might lead to development of valuable hybrids.

RAPD markers are rather widely used for investigation of phylogenetic relationships between different taxa. In most cases the results of phylogenetic investigations obtained

by RAPD method coincide with the results obtained by other methods (Kochieva et al., 2002; Katsiotis et al., 2003; Makarevitch et al., 2003; Yang et al., 2008). RAPD bands are often amplified from repetitive palindromic sequences (Williams et al., 1990; Devos, Gale, 1992), which are important in phylogenetic and taxonomic investigations (Nkongolo et al., 2001). As the UPGMA and NJ dendrograms (Fig. 4 and 5) shows, the grouping of twelve taxa of genus *Lonicera* L. according to genetic relationship rather closely corresponded to their morphological traits and biological features. All taxa that are somehow related to polymorphic *L. caerulea* L. fall within the same cluster. It is worth mentioning that among all these taxa samples there are such the fruit whereof can be used for food. In Siberia and Far East, the distribution areas of these species overlap and the process of inter-specific hybridization takes place. Honeysuckle species *L. chrysantha* and *L. xylosteum* ripening red berries are grouped together in the dendrogram. *L. orientalis* also falls within the same cluster though morphologically this species differs more from the two species mentioned above. Undoubted relationship between these three species is confirmed by high bootstrap values and results of investigations by other authors (Theis et al., 2008; Smith, 2009). According to the dendrogram, there is no obvious grouping of *L. caerulea* and its subspecies; therefore, we think that the status of subspecies should be the same as of *L. venulosa*, *L. emphyllocalyx*, *L. edulis* and *L. boczkarnikowae,* which are often regarded as separate species.

Along with RAPD analysis we used the sequencing of non-coding cpDNA regions, as the main method for establishment of phylogenetic relationships between honeysuckle taxa specimens. DNA sequencing is statistically more reliable and allows forming more exact phylogeny trees compared with RAPD method (Blattner et al., 2001; Gehrig et al., 2001; Makarevitch et al., 2003). We investigated six cpDNA regions, the significance of which for phylogenetic investigations is different. According to Shaw et al. (2007), the variability of cpDNA non-coding regions differs markedly. The areas distinguished by high polymorphism of nucleotides are very important in establishing phylogenetic relationships of taxa of the lowest rank (Gielly, Taberlet, 1994; Shaw et al., 2005).

cpDNA *trnH-psbA* region is used for the creation of plant DNA barcode (Kress et al., 2005; Cowan et al., 2006; Kress, Erickson, 2007; Lahaye et, al., 2008; Whitlock et al., 2010). In our study, the greatest number of variable sites (5.7%) was determined in this cpDNA region. Based on our results obtained by different methods and on investigations of other authors, we can infer that cpDNA *trnH-psbA* region of the honeysuckle is suitable for intra-specific phylogenetic analysis of the blue-berried honeysuckle (Bell, Donoghue, 2005; Ferri et al., 2009; Kress et al., 2009; Chen et al., 2010; Yang et al., 2011). We are the first to use the sequencing data of *5'rpS12-rpL20* region in phylogenetic investigation of genus *Lonicera* L. and *Caprifoliaceae* family. The number of polymorphic sites in the sequence of *5'rpS12-rpL20* region constituted 5.2%. This intergenic spacer distinguished by the greatest number of single nucleotide polymorphisms (45 SNP) compared to the investigated sequences of other cpDNA regions (Table 3). Rather contradictory results were obtained when investigating cpDNA *trnL-trnF* region. In this region, we determined a sufficient number of variable sites (5.3%); however, phylogenetic analysis of the sequencing data of this region did not show such clear separation of taxa samples of the group of *L. caerulea* from the group of *L. chrysantha*, *L. xylosteum* and *L. orientalis*. We suppose that cpDNA *trnL-trnF* region is less informative in phylogenetic analysis of honeysuckle species compared with other

cpDNA regions we have studied. This can be corroborated by the results of other authors, which show that cpDNA *trnL-trnF* region often yields poor resolution in some taxonomic groups (Hardig et al., 2000; Klak et al., 2003; Muellner et al., 2003; Samuel et al., 2003). A joint dendrogram was drawn using ML method; other additional methods (NJ, ME, UPGMA, MP) were also used. ML method was chosen as the most frequently used in the analysis of sequences of cpDNA regions (Bell et al. 2001; Bell, Donoghue, 2005; Jacobs et al. 2010; Bell, 2010). Tamura-Nei model was also applied (Tamura, Nei, 1993). The joint dendrogram (Fig. 6) drawn by using the sequencing data of all six cpDNA regions shows that all taxa samples are clearly separating, most tree branches are reliable, because they are supported by sufficiently high bootstrap values. In the dendrogram we can see two clusters, grouping honeysuckle taxa belonging to *L. caerulea* L. complex polymorphic species (first cluster), and honeysuckle species *L. chrysantha*, *L. xylosteum* and *L. orientalis* (second cluster). The dendrogram also shows that the first cluster is composed of two subclusters. The smaller subcluster is composed of *L. edulis* and *L. caerulea* subsp. *altaica* accessions. These two taxa group together in the dendrogram drawn based on RAPD markers, too (Fig. 4 and 5). A *L. venulosa* sample is also grouping in RAPD-UPGMA dendrogram. More distinct differences between RAPD and cpDNA phylogenetic trees are observed in the larger subcluster of the first cluster. In the dendrograms drawn according to cpDNA sequencing and RAPD analysis, *L. chrysantha, L. xylosteum* and *L. orientalis* form one cluster. *L.* chrysantha is more genetically related to *L. xylosteum* than *L. orientalis* is. Genetic relationship of these three species is showed by other authors, too (Theis et al., 2008; Smith 2009).

We applied software package TREECON according to Nei and Li (1979) to calculate pairwise genetic distances between twleve *Lonicera* L. taxa samples, by using RAPD and sequencing data. We applied Spearman's distribution and calculated intercorrelation between these genetic distances. The mean positive correlation obtained $r = 0.59$ ($p < 0.0001$) allows stating that genetic distances obtained according to RAPD and cpDNA data intercorrelate. This indirectly confirms similarity of obtained phylogenetic dendrograms (Fig. 4 and 6).

The results of our study do not conflict with phylogenetic relationships of honeysuckle species determined by other authors (Theis et al., 2008; Smith, 2009). Intra-specific phylogenetic investigations of *L. caerulea* L. by using DNA markers (RAPD and cpDNA sequencing) are new and, therefore, incomparable. Dendrograms constructed both according to sequencing data obtained in our study (Fig. 6) and according to these data supplemented with homologous sequences of related taxa from the databases (Fig. 7) show that taxa of honeysuckles bearing edible fruits form a monophyletic group. However, subspecies of *L. caerulea* L. do not form one clade in a monophyletic group as could be expected if they were treated as taxa of lower rank compared to species offered by some authors.

CONCLUSIONS

1. RAPD method is suitable for genotyping collection samples of genus *Lonicera* L. By using twelve oligonucleotide primers, a hundred of honeysuckle samples of different taxonomic rank were genotyped.
2. The blue-berried honeysuckle (*L. caerulea* L.) distinguishes by high (83.2%) DNA polymorphism.

3. The UPGMA dendrogram of the cultivars of the blue-berried honeysuckle (*L. caerulea* L.) constructed by using RAPD markers is in compliance with the data on the origin of these cultivars.
4. A species-specific RAPD marker for complex polymorphic *L. caerulea* L. species is identified and sequenced.
5. Out of six investigated regions (*trnH-psbA*, *5'rpS12-rpL20, trnL-trnF*, *trnS-trnG*, *rpS16, trnS-psbZ*) two chloroplast DNA regions (*trnH-psbA* and *5'rpS12-rpL20*) are the most suitable for phylogenetic investigations of *Lonicera* L. taxa.
6. The sequencing data of cpDNA *5'rpS12-rpL20* region is used for the first time for phylogenetic study of plants belonging to family *Caprifoliaceae*. This sequence is distinguished by the greatest number of single nucleotide polymorphisms compared with the studied sequences of other cpDNA regions.
7. Phylogenetic dendrograms based on RAPD markers and cpDNA sequencing data are similar.
8. *L.* subsp. *kamtschatica*, *L.* subsp. *stenantha* and *L.* subsp. *pallasii*, *L.* subsp. *altaica* as subspecies of *L. caerulea* L. do not form one cluster in phylogenetic dendrogram and do not differ in their taxonomic status from *L. venulosa* (Maxim.) Worosh., *L. edulis* Turcz. ex Freyn, *L. emphyllocalyx* (Maxim.) Nakai and *L. boczkarnikowae* Plekhanova, which are treated by some authors as separate species.
9. Phylogenetic analysis of the sequencing data of cpDNA *trnH-psbA*, *5'rpS12-rpL20, trnL-trnF*, *trnS-trnG*, *rpS16* and *trnS-psbZ* regions shows that all investigated edible honeysuckle samples of different taxa of genus *Lonicera* L. form a monophyletic group.
10. Genetic lines of the blue-berried honeysuckle derived in the Vilnius University Botanical Garden distinguish by unique RAPD loci and show other genetic differences from elite cultivars; therefore, they can be used in honeysuckle breeding to increase genetic diversity of new cultivars. Some of these lines distinguish by good economic characters.

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LIST OF PUBLICATIONS

List of publications on the dissertation topic

1. **Naugžemys** D., Žilinskaitė S., Kleizaitė V., Skridaila A., Žvingila D. 2011. Assessment of Genetic Variation among Elite and Wild Germplasm of Blue Honeysuckle (*Lonicera caerulea* L.). *Baltic Forestry*, in press.

2. **Naugžemys D**., Žilinskaitė S., Denkovskij J., Patamsytė J., Literskis J., Žvingila D. 2007. RAPD based study of genetic variation and relationships among *Lonicera* germplasm accessions. *Biologija*, 53 (3): 34-39.
3. Žilinskaitė S., **Naugžemys D**., Radaitienė D., Žvingila D. 2007. Investigation of blue-berried honeysuckle lines and cultivars at Vilnius university botanical garden. *Sodininkystė ir daržininkystė*, 26 (3): 47-56.

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1. Žilinskaitė S., **Naugžemys D**., Radaitienė D., Žvingila D. 2007. Investigation of blue-berried honeysuckle lines and cultivars at Vilnius university botanical garden. Breeding of horticultural plants and investigation of cultivars. Present and future. Babtai; 5.
2. **Наугжемис Д**., Жилинскайте С., Жвингила Д. 2008. „Интродукция, изучение голубой жимолости (*Lonicera* L., подсекция *Caeruleae* Rehd.) в Ботаническом саду Вильнюсского университета. Международная научно-методическая конференция.,нтродукция Нетрадиционных, редких растений. Россия, Мичуринск, Том 1: 125-127.
3. **Наугжемис Д**., Жилинскайте С., Жвингила Д. 2009. Изучение генетического родства RAPD – методом между сортами, видами жимолости (*Lonicera* L.) Ботанического сада Вильнюсского университета. (http://lonicera-conference.narod.ru/eng_version.html)

Participation in conferences:

1. VIII Международная научно-методическая конференция "Интродукция нетрадиционных, редких растений". 2008. Россия, г. Мичуринск, 8-12,юня.
2. ‘1st Virtual International Scientific conference on *Lonicera caerulea* L.’ 2009. Russia, 23 March – 23 April.

Publication not directly related to dissertation topic:

1. Meškauskaitė E., **Naugžemys D**., Žvingila D., Naujalis J.R. 2010. Morphological and genetic differentiation of *Saxifraga hirculus* L. (*Saxifragaceae*) populations in Lithuania. *Acta Biologica Universitatis Daugavpiliensis*, *Supplement*, 2: 69-78.
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5. **Naugžemys D.**, Aučina A., Žvingila D., Skridaila A. 2006. Comparison of DNA polymorphism in seedlings of *Pinus sylvestris* L. from different populations by RAPD markers. *Biologija*, 1: 30-35.

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SANTRAUKA

Nemažai augalų rūšių, kurios praeityje nebuvo labai vertinamos ir kultivuojamos, dabar intensyviai tiriamos norint jas plačiau panaudoti žmonių reikmėms. Dažnai tokie augalai pasižymi unikaliomis biologinėmis ir agronominėmis savybėmis (pvz., atsparumu biotiniam ir abiotiniam stresui). Viena iš tokių rūšių, kurios selekcija susidomėta tik praėjusio amžiaus viduryje, yra melsvauogis sausmedis.

Melsvauogis sausmedis (*Lonicera caerulea* L.) – vidutinio dydžio daugiametis krūmas, subrandinantis melsvos spalvos valgomas uogas, paplitęs borealiniuose Eurazijos ir Šiaurės Amerikos miškuose. *L. caerulea* kaip komercinė rūšis yra palyginti nauja, pradėta kultivuoti XIX a. Rusijoje (Plekhanova, 2000). Sausmedžio mokslinė selekcija prasidėjo XX a. viduryje Tarybų Sąjungoje (Janick, Paull, 2008). Dėl didelio atsparumo šalčiui (žiedai ištveria -8°C/-10°C) bei ankstyvo derėjimo melsvauogis sausmedis yra vertinga selekciniu požiūriu uogakrūmių rūšis. Sausmedžio uogose gausu fenolinių junginių, flavonoidų ir antocianinų, kurie nulemia antibakterines, antioksidantines ir priešuždegimines sausmedžio vaisių savybes (Chaovanalikit et al., 2004; Svarcova et al., 2007).

Melsvauogio sausmedžio tyrimai Lietuvoje prasidėjo 1979 m., sukūrus Vilniaus universiteto Botanikos sodo sausmedžių kolekciją (Žilinskaitė et al., 2007). VU Botanikos sodo sausmedžių kolekcijos efektyvesniam tvarkymui ir praktiniam panaudojimui selekcijoje be klasikinių metodų reikalingi ir šiuolaikiniai, kurie pagrįsti genomo DNR analize ir nepriklauso nuo aplinkos poveikio bei individo raidos stadijos. Veislių tyrimams ir selekcijai pasaulyje plačiai naudojami DNR žymenų metodai (Semagn et al., 2006), iš kurių vienas populiariausių yra RAPD (angl. ***R****andom* ***A****mplified* ***P****olymorphic* ***DNA***) metodas, nes yra efektyvus, palyginti pigus ir dėl naudojamų atsitiktinės sekos pradmenų nereikalauja pradinės informacijos apie tiriamos rūšies genomo sekas (Williams et al., 1990; Atienzar, Jha, 2006).

Dėl savo polimorfiškumo ir didelio užimamo arealo melsvauogis sausmedis įdomus ir taksonominiu požiūriu, nes iki šiol ginčijamasi, ar tai viena rūšis, sudaryta iš kelių porūšių ir rasių, ar tai skirtingų rūšių grupė (Плеханова, Ростова, 1994; Janick, Paull, 2008). Iki šiol šios problemos sprendimui buvo naudojami klasikiniai metodai. Pastaruoju metu tiek augalų, tiek kitų gyvų organizmų sistematikai plačiai naudojami branduolio ir organoidų DNR analizės metodai (Harris, 1999; Shaw et al., 2005; 2007). Molekulinėje augalų taksonomijoje vis plačiau naudojama chloroplastų DNR (cpDNR) regionų sekoskaita. Dėl to RAPD metodo ir cpDNR sekoskaitos panaudojimas *L. caerulea* analizei turėtų suteikti naujos vertingos informacijos ir patikslinti melsvauogio sausmedžio, kaip polimorfinės rūšies, statusą, pašalinti kai kuriuos prieštaravimus šios rūšies sistematikoje. Molekulinių genetinių metodų panaudojimas tiriant *Lonicera* L. genties genomą turėtų atverti naujas galimybes sprendžiant daugelį teorinių ir praktinių uždavinių, tvarkant ir naudojant sausmedžių genetinę medžiagą bei padidinti naujų veislių selekcijos efektyvumą.

Darbo tikslas ir uždaviniai:

Ištirti VU Botanikos sodo sausmedžio (*Lonicera* L.) kolekcijos genetinę įvairovę, atlikti vidurūšinę ir tarprūšinę filogenetinę analizę molekulinių žymenų metodais, palyginti sekoskaitos rezultatus su cpDNR sekomis, registruotomis duomenų bazėse.

1. Genotipuoti VU Botanikos sodo melsvauogio sausmedžio kolekcijos pavyzdžius RAPD metodu.
2. Nustatyti tirtų genotipų giminingumo laipsnį ir, remiantis šiais duomenimis, patikslinti kai kurių pavyzdžių kilmę.
3. Nustatyti genetinių linijų kolekcijos pavyzdžių ir elitinių veislių genetinės diferenciacijos lygį.
4. Įvertinti genetinių linijų panaudojimo melsvauogio sausmedžio selekcijai ir naujai kuriamų veislių genetinės įvairovės didinimui galimybes.
5. Nustatyti *L. caerulea* L. rūšiai savitus RAPD žymenis, ištirti jų DNR sekų polimorfizmą, įvertinti jų molekulinę prigimtį, atlikti panašių sekų paiešką duomenų bazėse.
6. Remiantis RAPD analizės ir cpDNR sekoskaitos rezultatais atlikti *Lonicera* L. genties keleto rūšių ir porūšių filogenetinę analizę šiuolaikinės bioinformatikos metodais.
7. Palyginti tirtų sausmedžio genties atstovų genetinio panašumo medžius, nubraižytus RAPD duomenų pagrindu, su medžiais, nubraižytais remiantis cpDNR nekoduojančių regionų sekoskaitos duomenimis. Gautą informaciją palyginti su kitų autorių gautais sausmedžio filogenetinių tyrimų rezultatais.
8. Pateikti rekomendacijas, kaip pagerinti tolesnius melsvauogio sausmedžio kolekcijos tvarkymo ir selekcijos darbus.

Ginamieji teiginiai

1. RAPD metodas yra tinkamas *Lonicera* L. kolekcijos genetinei įvairovei tirti ir *L. caerulea* L. pavyzdžiams genotipuoti.
2. Melsvauogiui sausmedžiui (*L. caerulea* L.) yra būdingas didelis DNR polimorfizmas.
3. *L. caerulea* L. veislių giminingumo dendrogramos, sudarytos RAPD žymenų pagrindu, neprieštarauja veislių kilmės duomenims.
4. Kai kurie melsvauogio sausmedžio (*L. caerulea* L.) monomorfiniai RAPD lokusai gali būti panaudoti kaip rūšiai saviti žymenys.
5. Remiantis sausmedžio chloroplastų DNR (cpDNR) nekoduojančių specifinių regionų sekoskaitos rezultatais, galima diferencijuoti artimai giminingus *Lonicera* L. genties atstovus.
6. *Lonicera* L. genties filogenetinės analizės rezultatai, gauti RAPD metodu ir cpDNR sekoskaitos duomenų pagrindu, yra panašūs.
7. *L. caerulea* L. rūšis sudaro monofiletinę grupę su kitais, valgomus vaisius vedančiais, sausmedžiais.
8. VU Botanikos sode išvestos sausmedžio genetinės linijos gali būti naudojamos *L. caerulea* L. elitinių veislių genetinei bazei praplėsti.

Darbo mokslinis naujumas

Pirmą kartą melsvauogio sausmedžio (*L. caerulea* L.) genetinės įvairovės tyrimams bei vidurūšinės taksonomijos problemoms spręsti buvo panaudoti DNR žymenų metodai (RAPD ir cpDNR nekoduojančių regionų sekoskaita). Nustatyta, kad *L. caerulea* L. atstovams būdingas didelis DNR polimorfizmas. Identifikuotas ir sekvenuotas RAPD žymuo, savitas šiai polimorfinei kompleksinei rūšiai. Pirmąkart atlikta *Lonicera* L. genties dvylikos atstovų cpDNR šešių nekoduojančių regionų (*trnH-psbA*, *5'rpS12-*

rpL20, trnL-trnF, *trnS-trnG*, *rpS16, trnS-psbZ*) sekoskaita. cpDNR nekoduojančių sekų filogenetinė analizė parodė, kad mokslinėje literatūroje aprašyti melsvauogio sausmedžio porūšiai nesudaro vienos klados, o grupuojasi su kitais diskutuotino statuso taksonais ir kartu su jais sudaro monofiletinę grupę.

Šie molekuliniai tyrimai patvirtina A. Rehder'io (1903) ir M. Plekhanovos (1994) nuomonę apie tai, kad *L. caerulea* L. yra polimorfinė rūšis, kuriai be porūšių *L.* subsp. *kamtschatica*, *L.* subsp. *stenantha*, *L.* subsp. *pallasii*, *L.* subsp. *altaica* galima priskirti ir kai kurių autorių išskiriamas rūšis – *L. venulosa* (Maxim.) Worosh., *L. edulis* Turcz. ex Freyn, *L. emphyllocalyx* (Maxim.) Nakai, *L. boczkarnikowae* Plekhanova.

Mokslinė ir praktinė darbo reikšmė

Nustatyti oligonukleotidiniai pradmenys, kurie tinka melsvauogio sausmedžio (*L. caerulea* L.) genetinės įvairovės tyrimams ir filogenetinei analizei. Su jais genotipuota šimtas VU Botanikos sodo sausmedžio kolekcijos pavyzdžių. Palyginus VU Botanikos sodo melsvauogio sausmedžio genetinių linijų ir elitinių veislių genetinę įvairovę, įvertintą RAPD metodu, nustatyta, kad šios genetinės linijos gali būti naudojamos kaip papildomas genetinės įvairovės šaltinis naujoms sausmedžių veislėms kurti. *L. caerulea* L. rūšiai nustatytas savitas RAPD žymuo gali būti naudojamas melsvauogio sausmedžio ir kitų rūšių hibridų analizei. Dvylikos *Lonicera* L. genties atstovų cpDNR šešių nekoduojančių regionų (*trnH-psbA*, *5'rpS12-rpL20, trnL-trnF*, *trnS-trnG*, *rpS16, trnS-psbZ*) sekos bus užregistruotos ir papildys NCBI duomenų bazę. Kultūrinių augalų filogenetiniai tyrimai, jų taksonomijos patikslinimas yra svarbu kaupiant ir naudojant rūšies genetinius išteklius, prognozuojant genų introgresijos iš giminingų rūšių sėkmę.

Išvados

1. RAPD metodas yra tinkamas *Lonicera* L. genties augalų kolekciniams pavyzdžiams genotipuoti. Panaudojus dvylika oligonukleotidinių pradmenų genotipuota šimtas skirtingo taksonominio rango sausmedžio pavyzdžių.
2. Melsvauogis sausmedis *Lonicera caerulea* L. pasižymi dideliu (83,2%) DNR polimorfizmu.
3. Melsvauogio sausmedžio *L. caerulea* L. veislių UPGMA dendrograma, sudaryta naudojant RAPD žymenis, neprieštarauja šių veislių kilmės duomenims.
4. Identifikuotas ir sekvenuotas *L. caerulea* L. polimorfinei kompleksinei rūšiai savitas RAPD žymuo.
5. Du chloroplastų DNR regionai – *trnH-psbA* ir *5'rpS12-rpL20* – iš šešių ištirtų (*trnH-psbA*, *5'rpS12-rpL20, trnL-trnF*, *trnS-trnG*, *rpS16, trnS-psbZ*) yra tinkamiausi *Lonicera* L. genties atstovų filogenetiniams tyrimams.
6. Pirmą kartą *Caprifoliaceae* šeimos augalų filogenetiniams tyrimams panaudoti cpDNR *5'rpS12-rpL20* regiono sekoskaitos duomenys. Ši seka pasižymėjo didžiausiu vieno nukleotido polimorfizmų skaičiumi palyginti su kitų cpDNR regionų tirtomis sekomis.
7. Filogenetinės dendrogramos, sudarytos remiantis RAPD žymenimis ir cpDNR sekoskaitos duomenimis, yra panašios.
8. *L. caerulea* L. porūšiai *L.* subsp. *kamtschatica*, *L.* subsp. *stenantha* ir *L.* subsp. *pallasii*, *L.* subsp. *altaica* nesudaro vienos klados filogenetinėje dendrogramoje ir savo taksonominiu statusu nesiskiria nuo *L. venulosa* (Maxim.) Worosh., *L. edulis*

Turcz. ex Freyn, *L. emphyllocalyx* (Maxim.) Nakai ir *L. boczkarnikowae* Plekhanova, kurias kai kurie autoriai vertina kaip atskiras rūšis.

9. Filogenetinė analizė, atlikta pagal cpDNR *trnH-psbA*, *5'rpS12-rpL20, trnL-trnF*, *trnS-trnG*, *rpS16* ir *trnS-psbZ* regionų sekoskaitos duomenis rodo, kad visi ištirti *Lonicera* L. genties valgomųjų sausmedžių atstovai sudaro monofiletinę grupę.
10. VU Botanikos sodo kolekcijoje saugomos melsvauogio sausmedžio genetinės linijos, pasižyminčios savitais RAPD lokusais, gali būti naudojamos sausmedžio selekcijoje naujų veislių genetinei įvairovei didinti. Kai kurios iš šių linijų pasižymi ir geromis ūkinėmis savybėmis..

CURRICULUM VITAE

Personal information

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Education:

2000 – 2004 Bachelor degree in Ecology and Environmental, Šiauliai University.

2004 – 2006 Master degree in biology, Vilnius University.

2006 – 2010 PhD student, Department of Botany and Genetics, Faculty of Natural sciences, Vilnius University.

The winner of Lithuanian academy of Sciences Prize for the high school students‘ research papers in biology, medicine and geosciences behind the work of „Analysis of genetic diversity in yellow marsh Saxifrage (*Saxifraga hirculus* L.) populations“ (Scientific supervisor – prof. Donatas Žvingila, 2006).

Current position and workplace

From 2007 specialist, Departments – Laboratory of Plant Physiology and Isolated Tissue Cultures, Botanical garden, Vilnius University.

Assistant at the Department of Environmental Research, Faculty of Natural Sciences, Šiauliai University.

Scientific Interest

Populations genetics, plant genetics, molecular phylogenetics, evolution, molecular markers, biotechnology, bioinformatics methods.

CURRICULUM VITAE

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Išsilavinimas:

2000 – 2004 Ekologijos ir aplinkotyros bakalauras, Šiaulių universitetas.

2004 – 2006 Biologijos (genetika) magistras, Vilniaus universitetas.

2006 – 2010 doktorantas, botanikos ir genetikos katedra, Gamtos mokslų fakultetas, Vilniaus universitetas.

2006 m. Lietuvos mokslų akademijos aukštųjų mokyklų studentų mokslinių darbų premijos laureatas, biologijos, medicinos ir geomokslų skyriuje už darbą „Genetinės įvairovės tyrimas pelkinės uolaskėlės (*Saxifraga hirculus* L.) populiacijose“ (darbo vadovas – prof. Donatas Žvingila).

Dabartinės pareigos ir darbovietė

Nuo 2007 m. specialistas, Augalų fiziologinių ir biocheminių tyrimų laboratorija, Botanikos sodas, Vilniaus universitetas.

Asistentas, Aplinkotyros katedra, Gamtos mokslų fakultetas, Šiaulių universitetas.

Moksliniai interesai

Populiacijų genetika, augalų genetika, molekulinė filogenetika, evoliucija, molekuliniai žymenys, biotechnologija, bioinformatikos metodai.