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Phylogenetic analysis of the polymorphic 4× species complex *Lonicera caerulea* (Caprifoliaceae) using RAPD markers and noncoding chloroplast DNA sequences

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Abstract: The blue-berried honeysuckle (*Lonicera caerulea* L.) is one of the most representative species of the genus *Lonicera* L. in horticulture. This article presents the results of research on the taxonomy of blue-fruited honeysuckles, which is quite complicated due to the phenotypic plasticity, ability to hybridize and distribution across different ecological zones. We used the random amplified polymorphic DNA (RAPD) markers and sequencing of seven chloroplast DNA (cpDNA) regions (*trnH-psbA*, *rpS12-rpL20*, *trnL-trnF*, *trnS-trnG*, *trnG*, *rpS16* and *trnS-psbZ*) to assess the phylogenetic relationships among the taxa within the polymorphic 4× species complex *L. caerulea* and to determine the position of *Lonicera boczkarnikowae* Plekh. and *Lonicera venulosa* Maxim. within this complex. *Lonicera chrysantha* Turcz. ex Ledeb., *L. orientalis* Lam. and *L. xylosteum* L. were used as the outgroup species. The RAPD and cpDNA analyses both indicated that all of the studied taxa of the blue-fruited honeysuckle form a single cluster consisting of two subclusters. A second cluster includes the outgroup species. According to the cpDNA analysis, *L. boczkarnikowae* and *L. venulosa* belong to the subcluster that includes the taxa of the polymorphic tetraploid complex *L. caerulea.* A separate subcluster within the cluster of blue-fruited honeysuckles contains *L. altaica* and *L. edulis.*

Key words: *Lonicera* L.; blue-fruited honeysuckle; noncoding cpDNA regions; molecular markers; intraspecific phylogeny

Introduction

Many plant species that earlier were not greatly valued or cultivated are now being intensively investigated with a view to using them more widely for human needs (Badenes et al. 2004). Such plants are frequently distinguished by unique biological and agronomic features (e.g., valuable nutritional properties, resistance to biotic and abiotic stress). The blue-berried honeysuckle (*Lonicera caerulea* L.) is one of the species of the genus *Lonicera* L. most commonly utilized in horticulture. Due to its fruit quality and biological properties (early ripening, frost hardiness and pest resistance), this species is becoming popular in countries with a temperate climate.

L. caerulea is a medium-sized perennial shrub distributed throughout the boreal forests of Eurasia and North America that produces edible blue berries. The berries of the blue-berried honeysuckle contain high levels of phenols, flavonoids and anthocyanins that account for their antibacterial, antioxidant and anti-inflammatory properties (Chaovanalikit et al. 2004; Svarcova et al. 2007). *L. caerulea* is a relatively new commercial species that was first cultivated in the 19th century in Russia (Plekhanova 2000). Scientific breeding of the species began in the 20th century in the former Soviet Union. Breeding was primarily conducted using the germplasm of *L. caerulea* subsp. *altaica, L. caerulea* subsp. *edulis*, *L. caerulea* subsp. *kamtschatica* and *L. boczkarnikowae* (Thompson 2008).

L. caerulea is assigned to the section *Isika* Rehd., subsection *Caeruleae* Rehd. This section is characterized by solid branches and accessory buds with zygomorphic and nearly actinomorphic corollas with 1-3 nectaries (Theis et al. 2008). The composition of the *Caeruleae* Rehd. subsection has long been a topic of discussion. Different authors discern one to 19 species within this subsection of *Caeruleae* (Plekhanova & Rostova 1994; Sheiko 2007). Many scientists still dispute whether it is one species *L. caerulea* composed of several subspecies and/or varieties or a group of different species (Plekhanova & Rostova 1994; Streltsina et al. 2006; Thompson 2008). There are two main taxonomic evaluations of blue-fruited honeysuckles. In the first taxonomic classification of *L. caerulea* ever performed, A. Rehder (1903) grouped all of the ecogeographical races into one polymorphic species divided into eight subspecies and eight forms. This phylogenetic classifica-

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tion of *L. caerulea* is accepted by many scientists (Nakai 1938; Hultén 1971; Browicz 1974; Thompson 2008). The second point of view is represented by Pojarkova (1958) and many other Russian botanists (P.S. Pallas, N.S. Turczaninow, N.V. Riabova, V.N Voroshilov). In a monographic assessment of the genus *Lonicera* for The Flora of the USSR, Pojarkova assigned 10 species to subsection *Caeruleae*. Some other Russian scientists assigned a smaller number of species to subsection *Caeruleae*. For example, Riabova recognized 4 species (Riabova 1980), and Voroshilov recognized 5 species (Voroshilov 1992; Plekhanova & Rostova 1994). On the basis of variations in the morphological, anatomical and biochemical characteristics of the *Lonicera* in subsection *Caeruleae,* Plekhanova and Rostova (1994) suggested that *L. altaica*, *L. caerulea*, *L. emphyllocalyx, L. kamtschatica*, *L. pallasii*, *L. stenantha*, *L. turczaninowii*, *L. villosa* and the tetraploid race of *L. edulis* belong to the tetraploid circumholarctic polymorphic species *L. caerulea* (according Thompson (2008), polymorphic 4× complex *L. caerulea*). The relationship of two more taxa of blue-fruited honeysuckle *L. boczkarnikowae* Plekh. and *L. venulosa* Maxim. with the 4× polymorphic complex is unclear. These taxa are considered by different authors as separate species (Plekhanova 2000; Boyarskih & Chernyak 2012). The existence of multiple taxonomic evaluations also means that several names are used for the same taxa. For example, *L. kamtschatica* is considered a separate species, a subspecies or a variety. Phylogenetic investigation of cultivated plants and adjustment of their taxonomy are important for management, using the genetic resources of species and forecasting the success of gene introgression from related species (Handa et al. 2006).

Molecular assays have not been used to study the taxonomic problem of the blue-fruited honeysuckles in spite of the wide use of DNA markers in phylogenetic studies and plant barcoding (Harris 1999; Shaw et al. 2005; 2007; Hollingsworth et al. 2011). RAPD (Williams et al. 1990) analysis is one of simplest molecular methods used in plant phylogenetic studies. Earlier studies of *L. caerulea* using DNA markers either did not consider the problem of the inter- and intraspecific taxonomy of this species (Miyashita & Hoshino 2010; Smolik et al. 2010; Naugžemys et al. 2011) or were based on only a small number of RAPD markers (Naugžemys et al. 2007). The dominant nature of RAPD markers, their low reproducibility and the lack of homology among some bands of similar size reduced the potential utility of these markers for molecular taxonomy (Harris 1999). In spite of these drawbacks, RAPD assays using them as additional molecular markers are valuable because they provide a more detailed understanding of genetic relationships, especially if these markers are located in different genomes within the cells (Makarevitch et al. 2003). For this reason, our analysis of variation in the group of honeysuckle taxa is mainly based on chloroplast DNA (cpDNA) markers and RAPD markers were used only as supplemental data. The application of RAPD assays and cpDNA sequencing (Shaw et al. 2005) in *L. caerulea* taxonomic studies should provide additional information and more precisely describe the status of the blue-berried honeysuckle as a polymorphic species, eliminating some of the contradictions regarding the taxonomy of this species.

The aims of the study were to resolve the relationships within the polymorphic 4× complex *L. caerulea* using molecular marker methods, to clarify the relationships of *L. venulosa* Maxim. and *L. boczkarnikowae* Plekh. with the *L. caerulea* and compare the results of this analysis with the previous data from the taxonomic treatments of *L. caerulea* by other authors.

Material and methods

Plant materials

This investigation concerned the taxa of the blue-fruited honeysuckle *L. caerulea* L., *L. altaica* (syn. *L. caerulea* L. subsp. *altaica* (Pall.) Gladkova), *L. edulis* Turcz. ex Freyn (syn. *L. caerulea* var. *edulis* Turcz. ex Herder), *L. emphyllocalyx* (Maxim.) Nakai (syn. *L. caerulea* subsp. *emphyllocalyx*), *L. kamtschatica* (Sevast.) Pojerkova (syn. *L. caerulea* L. subsp. *kamtschatica* (Sevast.) Gladkova, *L. caerulea* var. *kamtschatica* Sevast.), *L. pallasii* Ledeb. (syn. *L. caerulea* L. subsp. *pallasii* Ledeb.), and *L. stenantha* (syn. *L. caerulea* L. subsp. *stenantha*), *L. venulosa* Maxim. (syn. *L. caerulea* subsp. *venulosa* (Maxim.) Worosh.), and *L. boczkarnikowae* Plekh. Three species of *Lonicera* (*L. chrysantha* Turcz. ex Ledeb., *L. orientalis* Lam., *L. xylosteum* L.) that are morphologically and genetically divergent from the blue-fruited honeysuckles (Theis et al. 2008) were included in our study as the outgroup species. All of the accessions of the different taxa used in this study were obtained from different sources and are stored in the collection of the Vilnius University Botanical Garden (Table 1).

DNA extraction and RAPD-PCR

Total DNA was extracted from fresh young leaves using the genomic DNA purification kit (Thermo Fisher Scientific Baltics), following the manufacturer's instructions. The DNA quantity and quality were determined spectrophotometrically and electrophoretically. DNA amplifications were conducted in 25 μL reaction mixtures containing the following components: 1× PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM primers, 1 U of *Taq* DNA polymerase (Thermo Fisher Scientific Baltics) and 50 ng of total DNA. The 50 decameric primers used in this study were purchased from Karl Roth GmbH & Co. KG (Germany). All of the amplifications were conducted using an Eppendorf Mastercycler® gradient (Eppendorf AG, Germany). The PCR conditions were those previously optimized (Naugžemys et al. 2011): 4 min at 94℃ as the initial DNA denaturation step, followed by 35 cycles of 60 s at 94℃, 60 s at 35℃ and 120 s at 72℃. The last cycle was followed by a final extension step of 72℃ for 5 min. The reaction mixture without DNA was used as a negative control to detect contamination. Reproducibility was assessed by comparing at least two independent reactions. Approximately 94% of the amplified DNA fragments were reproducible. Only the reproducible DNA bands were analyzed.

RAPD–PCR data analysis

The presence or absence of an individual DNA fragment in the PCR products of the plants of genus *Lonicera* L. was scored as "1" or "0", respectively. Non-reproducible, unclear

Table 1. The taxa of *Lonicera* used in the study, their origin and the GenBank® accession numbers for their *trnH-psbA*, *rpS12-rpL20*, *trnL-trnF*, *trnS-trnG*, *trnG*, *rpS16*, and *trnS-psbZ* regions.

	Lonicera L. taxa	N[1]	Provenance	GenBank® accession number for seven cpDNA regions						
				trnH-psbA	*rpS12-rpL20*	*trnL-trnF*	*trnS-trnG*	*trnG*	*rpS16*	*trnS-psbZ*
1	*L. caerulea*	3	Meshcherskoje, St Expert[2], 1997	KC429681	KC429693	KC429705	KC429717	KC429729	KC429741	KC429753
2	*L. kamtschatica*	6	Tartu[3], 1991	KC429682	KC429694	KC429706	KC429718	KC429730	KC429742	KC429754
3	*L. stenantha*	4	VIR[4], 1997	KC429683	KC429695	KC429707	KC429719	KC429731	KC429743	KC429755
4	*L. pallasii*	3	VIR[4], 1997	KC429684	KC429696	KC429708	KC429720	KC429732	KC429744	KC429756
5	*L. altaica*	1	VIR[4], 1997	KC429685	KC429697	KC429709	KC429721	KC429733	KC429745	KC429757
6	*L. edulis*	3	Reykjavik[5], 1995	KC429686	KC429698	KC429710	KC429722	KC429734	KC429746	KC429758
7	*L. boczkarnikowae*	1	VIR[4], 1997	KC429687	KC429699	KC429711	KC429723	KC429735	KC429747	KC429759
8	*L. emphyllocalyx*	1	VIR[4], 1997	KC429688	KC429700	KC429712	KC429724	KC429736	KC429748	KC429760
9	*L. orientalis*	1	VIR[4], 1992	KC429689	KC429701	KC429713	KC429725	KC429737	KC429749	KC429761
10	*L. venulosa*	1	VIR[4], 1997	KC429690	KC429702	KC429714	KC429726	KC429738	KC429750	KC429762
11	*L. xylosteum*	1	VU Botanical garden[5]	KC429691	KC429703	KC429715	KC429727	KC429739	KC429751	KC429763
12	*L. chrysantha*	1	Turku[7], 1993	KC429692	KC429704	KC429716	KC429728	KC429740	KC429752	KC429764

[1]N – number of studied samples; [2]Plant Experimental Station, Meshcherskoje, Russia; [3]Botanical Garden of Tartu University, Estonia; [4]VIR – N.I. Vavilov Research Institute of Plant Industry, Saint Petersburg, Russia; [5]Reykjavik Botanical Garden, Iceland; [6] Botanical Garden of Vilnius University, Lithuania; [7]Botanical Garden of Turku University, Finland

or poorly resolvable DNA fragments (bands) were not registered. The genetic distance between individuals (GD_{xy}) was calculated according to the method of Nei & Li (1979). The relationship between the samples based on genetic distances was assessed by the neighbor-joining (NJ) and unweighted-pair group method with arithmetic means (UPGMA) grouping methods using the TREECON program v.1.32 for Windows (Van De Peer & De Wachter 1994). The bootstrap analysis was conducted with 1000 iterations.

Amplification of specific cpDNA regions, cloning and sequencing

To explore the seven regions of cpDNA (*trnH-psbA*, *rpS12-rpL20*, *trnL-trnF*, *trnS-trnG*, *trnG*, *rpS16*, *trnS-psbZ*) PCR amplification was performed as described in Shaw et al. (2005). The PCR products were electrophoresed in 0.8% agarose gels, then excised and purified using a GeneJET Gel Extraction Kit, following the manufacturer's protocol (Thermo Fisher Scientific Baltics). The isolated DNA fragments were cloned into the pTZ57R/T vector using the InsTAclone PCR Cloning Kit (Thermo Fisher Scientific Baltics). Recombinant clones were selected using the *lacZ* system on FastMedia™ LB Agar Amp IPTG/X-Gal (Thermo Fisher Scientific Baltics). Plasmid DNA was isolated using a 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 a 3130xl Genetic Analyzer (Applied Biosystems, USA) using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Each DNA region was sequenced using the primers designed by Shaw et al. (2005). Two or three clones per sample from each taxon were sequenced. The sequences of the studied regions of all of the taxa were deposited in GenBank® (Table 1).

Sequence analysis

The sequencing results were evaluated using the ChromasPro v.1.7.5 (Technelysium Pty Ltd, Australia) and MEGA 5.2 (Tamura et al. 2011) software. The forward and reverse sequences for each clone were edited and assembled using the Sequencher 5.2 software (Gene Codes Corporation, USA). Homology searches were performed using the BLAST algorithm (Altschul et al. 1990) in the NCBI database. The phylogenetic analysis was conducted and the dendrograms were constructed using the maximum likelihood (ML) analysis method and the Tamura-Nei model in the PAUP 4.0b10 program (Swofford 2003). Indels were not included in the analysis. The bootstrap analysis was conducted with 1000 iterations. The topology of the dendrogram constructed by other methods (neighbor-joining and maximum parsimony) was consistent. The ML method was chosen because it is the method most frequently used to analyze sequences of cpDNA regions (Bell et al. 2001; Bell & Donoghue 2005; Jacobs et al. 2010; Bell 2010).

Results

RAPD analysis of the genomes of the investigated samples using 12 primers revealed 132 polymorphic loci (Table 2). The size of the DNA fragments obtained ranged from 270 bp to 2500 bp. The average level of DNA polymorphism was 78.1% ± 13.5%. The maximum number of polymorphic loci (100%) was detected with primer 380–02 and the minimum (43.8) with primer A–03. The average number of polymorphic loci determined using one primer was 11. None of the samples showed an identical pattern of amplified DNA fragments. The estimation of the genetic distance between the taxa indicated that the closest relationship (GD_{xy} = 0.188) was between *L. boczkarnikowae* and *L. kamtschatica*. The largest genetic distance (GD_{xy} = 0.610) was between *L. caerulea* and *L. chrysantha* (data not shown).

The UPGMA dendrogram generated on the basis of genetic distance values reflects the genetic relatedness among the *Lonicera* taxa (Fig. 1). The dendro-

Table 2. The RAPD primers used to study the *Lonicera* species and the characteristics of the RAPD-PCR products generated with these primers and scored for the phylogenetic analysis.

RAPD primer (ROTH)	Sequence 5'→3'	Total DNA fragments	Monomorphic fragments	Polymorphic loci	Polymorphic loci %	Size range of DNA fragments (bp)
170–08	CTGTACCCCC	19	5	14	73.7	490–2100
170–10	CAGACACGGC	15	3	12	80	470–1700
380–01	ACGCGCCAGG	14	3	11	78.6	490–1700
380–02	ACTCGGCCCC	10	–	10	100	510–1800
380–07	GGCAAGCGGG	16	3	13	81.3	560–1960
A–01	CAGGCCCTTC	12	2	10	83.3	700–2300
A–02	TGCCGAGCTG	12	3	9	75	580–2500
A–03	AGTCAGCCAC	16	9	7	43.8	680–1700
A–04	AATCGGGCTG	12	2	10	83.3	560–1900
A–05	AGGGGTCTTG	13	2	11	84.6	580–2100
A–09	GGGTAACGCC	18	6	12	66.7	270–1180
A–11	CAATCGCCGT	15	2	13	86.7	370–1400
Total / average		172	40	132	78.1 ±13.5	270–2500

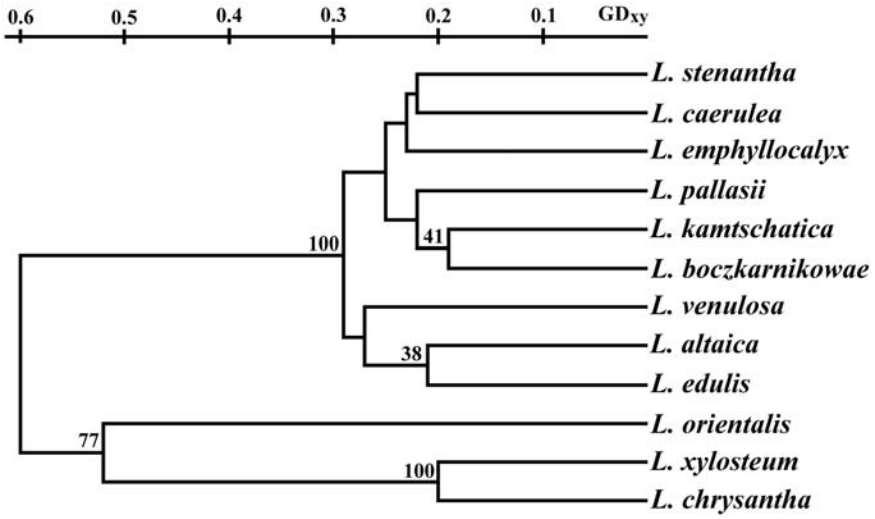

Fig. 1. UPGMA dendrogram of the *Lonicera* species based on their RAPD markers, constructed according to the genetic distance protocol of Nei & Li (1979). The axis at the top of the figure shows the genetic distance coefficient values. The bootstrap values (%) were obtained by 1000 iterations.

gram consists of two clusters that are significantly different (with strong bootstrap support). The first cluster includes all of the taxa of blue-fruited honeysuckles belonging to section *Isika* subsection *Caeruleae,* together with the taxa of the polymorphic 4× complex of *L. caerulea*; the second cluster includes *L. orientalis* (section Isika subsection *Rhodanthae*) and two representatives of section *Coeloxylosteum* subsection *Ochranthae*–*L. chrysantha* and *L. xylosteum*. A similar grouping of the taxa was generated using the NJ method (data not shown).

The relationships among the twelve taxa were determined using the sequences of seven noncoding regions (*trnH-psbA*, *rpS12-rpL20*, *trnL-trnF*, *trnS-trnG*, *trnG*, *rpS16*, and *trnS-psbZ*) of the chloroplast genome (Table 3). The individual cpDNA fragments obtained using samples of different taxa vary in length. The length of the *trnH-psbA* intergenic spacer ranges from 455 to 481 bp. The longest regions (481 nt) were found in seven taxa (*L. altaica, L. boczkarnikowae*, *L. chrysantha*, *L. emphyllocalyx*, *L. kamtschatica*, *L. stenantha* and *L. venulosa*), and the shortest (455 bp) was in *L. xylosteum*. The length of the *rpS12-rpL20* spacer ranges from 887 to 907 bp; the longest is in representatives of eight taxa, and the shortest is in *L. edulis.* The length of the *trnL-trnF* spacer ranges from 1001 to 1016 bp; the longest is in four taxa (*L. kamtschatica*, *L. pallasii*, *L. stenantha*, and *L. venulosa*), and the shortest is in *L. emphyllocalyx*. The length of the *trnS-psbZ* spacer ranges from 983 to 994 bp; the longest is in two taxa (*L. caerulea* and *L. venulosa*), and the shortest is in *L. xylosteum*. The length of the *rps16* intron ranged from 890 to 913 bp; the longest is in *L. caerulea, L. kamtschatica* and the shortest is in *L. emphyllocalyx*. The length of the *trnS-trnG* region ranged from 850 bp (*L. xylosteum*) to 880 bp (*L. emphyllocalyx*). After sequencing and alignment, the cpDNA sequences were used in a phylogenetic analysis. The combined length of the obtained aligned sequence was 6015 nucleotides (Table 4).

Table 3. The lengths of the seven cpDNA regions (bp) in the samples of twelve different taxa of the genus *Lonicera*.

Taxon No.*	*trnH-psbA*	*rpS12-rpL20*	*trnL-trnF*	*trnS-trnG*	*trnG*	*rpS16*	*trnS-psbZ*	Total cpDNA bp
1	457	888	1004	855	783	913	994	5894
2	481	907	1016	878	783	913	993	5971
3	481	907	1016	879	783	892	987	5945
4	476	907	1016	851	784	910	990	5934
5	481	907	1015	851	783	893	989	5919
6	457	887	1002	855	783	912	993	5889
7	481	907	1005	879	783	894	990	5939
8	481	907	1001	880	784	890	993	5936
9	457	901	1003	850	783	908	991	5893
10	481	907	1016	879	783	911	994	5971
11	455	907	1011	850	786	911	983	5903
12	481	906	1009	878	783	911	985	5953
Total	5669	10838	12114	10385	9401	10858	11882	71147
Average	472.42	903.17	1009.50	865.42	783.42	904.83	990.17	5928.92

* 1, *L. caerulea*; 2, *L. kamtschatica*; 3, *L. stenantha*; 4, *L. pallasii*; 5, *L. altaica*; 6, *L. edulis*; 7, *L. boczkarnikowae*; 8, *L. emphyllocalyx*; 9, *L. orientalis*; 10, *L. venulosa*; 11, *L. xylosteum*; 12, *L. chrysantha*

Table 4. A comparison of the seven cpDNA non-coding regions.

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

* PI, Parsimony informative character

Comparing the sequences from the 12 *Lonicera* species revealed that 235 of the 6015 aligned positions were variable, 56 of which were parsimony informative. A total of 179 single nucleotide polymorphisms (SNP) were found. The highest percentage of variable position (5.7%) was detected in *trnH-psbA* region. In the *trnG* region were identified only 2.7% of variable positions. The highest number of parsimony-informative positions (16) was identified in the *trnS-psbZ* region. Only two of the parsimony-informative positions were detected in the *rpS12-rpL20* spacer. However, 45 SNPs in this region were established (Table 4).

A total of 31 indels larger than 1 nt were identified (Fig. 2); some of them are unique to particular taxa. For example, a 5-bp deletion in the *trnH-psbA* region is unique to *L. stenantha*; a 3-bp deletion in the *trnS-trnG* region is unique to *L. chrysantha*; a 5-bp deletion in the *trnL-trnF* region is unique to *L. orientalis* and an 11-bp deletion in the *trnS-trnG* region is unique to *L. venulosa*. The highest number (10) of indels (2 bp or larger) was identified in the *trnL-trnF* region. The largest gap (26 bp) in the aligned sequences was discovered in the *trnH-psbA* regions of *L. altaica*, *L. chrysantha*, *L. orientalis* and *L. xylosteum*. The longest average genetic distance (0.013) among the samples studied was also found in the *trnH-psbA* region.

The ML dendrogram was generated from composite sequence-based analysis (Fig. 3). Two distinct clusters of taxa were revealed by this analysis. The first cluster consists of two subclusters that are composed of the taxa of blue-fruited honeysuckles. The first subcluster of this cluster is represented by *L. caerulea*, *L. emphyllocalyx*, *L. kamtschatica*, *L. pallasii* and *L. stenantha* as members of the polymorphic tetraploid complex and two species (*L. boczkarnikowae, L. venulosa*) that were not included in the study of Plekhanova and Rostova (1994). Two taxa of the polymorphic tetraploid complex (*L. altaica* and *L. edulis*) diverge from the first subcluster to form a second, separate subgroup. The second cluster, as in the RAPD analysis, consists of *L. chrysantha*, *L. orientalis and L. xylosteum*, which formed the outgroup in our study. The comparison of genetic divergence of taxa in different clusters showed closer genetic relationships in the blue-fruited honeysuckle cluster. The average genetic distance among taxa in this cluster was 0.0077, while the average genetic distance in the cluster of outgroup species was 0.0081. The average genetic distance among all taxa was – 0.0088. Close genetic relationships were iden-

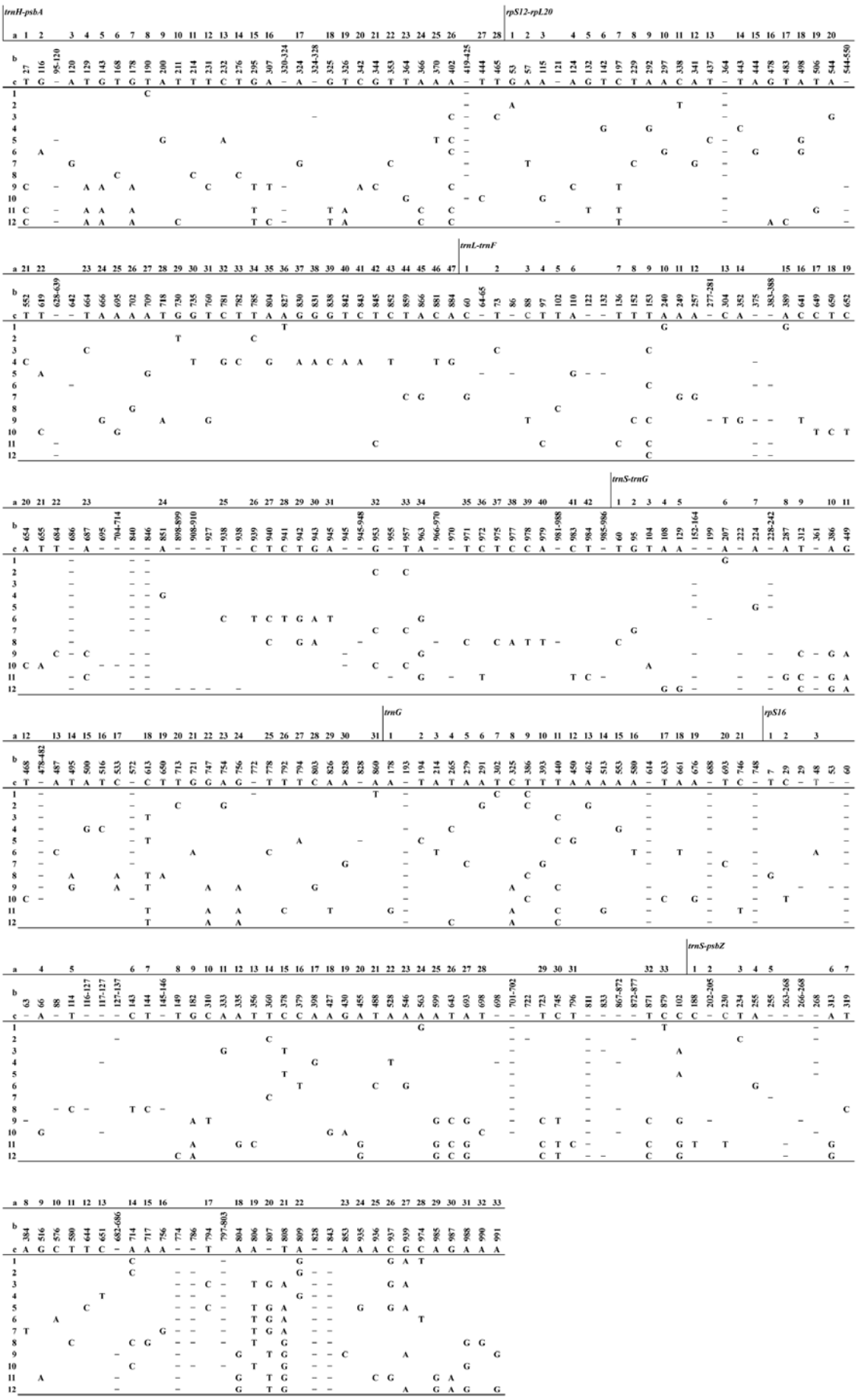

Fig. 2. Variable nucleotide positions in the seven regions of chloroplast DNA of the twelve taxa of *Lonicera* in the study.

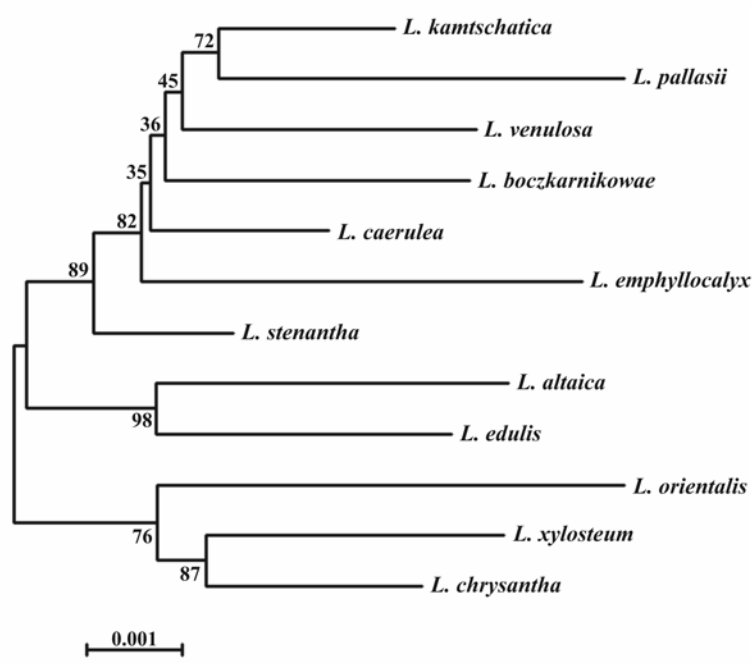

Fig. 3. Phylogenetic analysis of the twelve taxa of genus *Lonicera* based on the sequences of seven non-coding cpDNA regions. The dendrogram was constructed by applying the ML method and the algorithm of the Tamura-Nei model. The bootstrap values (%) were obtained by 1000 iterations.

tified between *L. caerulea* and *L. venulosa* (0.0049). A bit higher genetic distance (0.0061) was established between *L. caerulea* and *L. boczkarnikowae* (data not shown; Fig. 3).

Discussion

To assess the phylogenetic relationships among the taxa within 4× species complex *L. caerulea* and some other representatives of subsection *Caeruleae*, we applied the RAPD assay and sequencing of seven cpDNA non-coding regions. In addition to the taxa of the blue-fruited honeysuckle, we included several taxa of ornamental and medicinal honeysuckles that were used as the outgroup species in our study. In most cases, the results of previous phylogenetic investigations obtained using the RAPD markers are consistent with the results obtained using other methods (Kochieva et al. 2002; Katsiotis et al. 2003; Makarevitch et al. 2003; Yang et al. 2008). In our study the phylogenetic trees generated using the RAPD data or the cpDNA data had some similarities and some discrepancies.

The separation of the studied taxa into two clusters is evident and statistically significant in both of the dendrograms. The dendrograms obtained using the nuclear or the chloroplast DNA markers clearly indicate all of the taxa belonging to polymorphic 4× complex of *L. caerulea* group into one cluster. The structure of the second cluster, consisting of *L. chrysantha*, *L. orientalis* and *L. xylosteum,* is identical in both dendrograms and clearly identifies the close genetic relationship between *L. chrysantha* and *L. xylosteum.* Taxonomically, they are assigned to section *Coeloxylosteum* subsection *Ochranthae*, whereas *L. orientalis* belongs to section *Isika* Rehd., subsection *Rhodanthae.* An undoubted relationship among these three species was confirmed by the high bootstrap values and the results of investigations by others (Theis et al. 2008; Smith 2009).The structure of the first cluster, which includes the taxa of blue-fruited honeysuckles, appears more divergent when the dendrograms generated using the different molecular data are compared (Figs 1, 3). Both of the dendrograms demonstrate the monophyletic origin of the blue-fruited honeysuckle; however, this cluster appears to contain two subclusters. All of the taxa included in our study that belong to subsection *Caeruleae* Rehd. and are related to the polymorphic tetraploid species complex *L. caerulea* fall within the first cluster. The polymorphic 4× complex *L. caerulea*, settled in the vast territory, is in the process of formation of new taxa (Riabova 1980). The formation occurs in two ways (Sheiko 2007). The first way – changes in the number of chromosomes. Consequently, polyploid species may show exploitation of a new niche and reproductive isolation (Miyashita et al. 2011). In our study all assessed representatives of different taxa were tetraploids (Žilinskaite, unpublished data). Although diploid and tetraploid populations are found near each other, no triploid forms have been detected in the wild until now, indicating the difficulty of seed production in the crosses between diploid and tetraploid forms of blue-fruited honeysuckles and reduced viability of hybrids (Miyashita et al. 2011). The second way of new taxa forming inside the *L. caerulea* includes separation of morphologically and ecologically divergent populations (Sheiko 2007). Such view of speciation defines species as segments of population-level evolutionary lineages (Leaché et al. 2009). In Siberia and the Far East,

the distribution areas of some tetraploid taxa overlap, and interspecific hybridization occurs (Plekhanova & Rostova 1994). This process complicates the intraspecific taxonomic evaluation of the polymorphic 4× complex of *L. caerulea*. Regarding this group of tetraploid races, Plekhanova & Rostova (1994) noted some biological peculiarities of *L. altaica*, *L. kamtschatica* and *L. stenantha* and emphasized the need to determine the intraspecific taxonomy of *L. caerulea*. In the present study we also reveal some signs of the formation of new taxa inside polymorphic tetraploid species *L. caerulea*. Both RAPD and cpDNA dendrograms show the split of cluster of blue-fruited honeysuckle in two subclusters, which indicates intraspecific divergence. The molecular analysis performed in our study indicates that *L. altaica* and *L. edulis* are in a subcluster separate from the group containing the other blue-fruited honeysuckles (Figs 1, 3). The ranges of *L. altaica* and *L. edulis* are situated close by each other with partial overlap in some locations. Moreover, our results indicate a close phylogenetic relatedness between *L. emphyllocalyx*, *L. kamtschatica* and *L. pallasii,* which is in agreement with the results obtained by Plekhanova and Rostova (1994).

The most recent and comprehensive analysis of the phylogenetic relationships within subsection *Caeruleae* Rehd. was conducted by Plekhanova and Rostova (1994). These authors applied the method of principal component analysis to assess the variations in the morphological, anatomical and biochemical characteristics of representatives of this subsection. Their analysis indicated that the elements of similarity of the tetraploid races of *L. altaica*, *L. caerulea*, *L. edulis*, *L. emphyllocalyx, L. kamtschatica*, *L. pallasii*, *L. stenantha, L. turczaninowii* and *L. villosa* dominated the differences, and the authors suggested grouping them into the single tetraploid polymorphic species *L. caerulea*. This conclusion is consistent with results of our investigation. Both the RAPD- and cpDNA sequence-based dendrograms demonstrate an obvious grouping of *L. caerulea* and the other taxa of the polymorphic tetraploid complex into one cluster. Therefore, we propose that the status of *L. venulosa* and *L. boczkarnikowae,* which are often regarded as a separate species, should be the same as that of the other members of the first cluster and that they should be considered intraspecific taxa of *L. caerulea*.

In spite of the naturally occurring hybridization between races of the same ploidy (Plekhanova & Rostova 1994), artificial interspecific hybridization has not been applied to the breeding of the blue-fruited honeysuckle; instead, the development of new cultivars of *L. caerulea* has been based on the open pollination and selecting wild plants with exclusive characteristics or hybridizing the existing superior cultivars (Kuklina 2007; Miyashita & Hoshino 2010). We believe that the intraspecific grouping of the taxa of the blue-fruited honeysuckle established in our study could be used to plan which parental plants from different taxa to breed by artificial hybridization to expand the genetic basis of the modern cultivars.

In conclusion, our DNA-based taxonomic study showed some signs of the divergence inside the tetraploid circumholarctic polymorphic species *L. caerulea*. On the other hand, sequencing of cpDNA regions revealed close genetic relationships among *L. caerulea* and two taxa (*L. venulosa* and *L. boczkarnikowae*) which indicates that these taxa should not be considered separate species.

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