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Complete plastome sequences of *Lonicera* L. species: implications for phylogeny and comparative analysis

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Abstract. *Lonicera* L. is one of the largest and economically significant genera in the family Caprifoliaceae Juss., with a controversial taxonomy. To contribute to its molecular taxonomy, we sequenced the plastomes of *Lonicera* species: *Lonicera caerulea* (two subspecies), *L. tatarica*, and *L. micrantha* – using next-generation sequencing technology and conducted a comparative analysis. Plastome sizes ranged from 153,985 bp in *L. micrantha* to 164,000 bp in *L. caerulea* subsp. *pallasii*, each containing 130 genes, including 85 protein-coding, 37 tRNA, and 8 rRNA genes. Five protein-coding (*rps7*, *rps12*, *ndhB*, *ycf2*, and *ycf15*), 7 tRNA (*trnA-UGC*, *trnI-CAU*, *trnI-GAU*, *trnL-CAA*, *trnN-GUU*, *trnR-ACG*, and *trnV-GAC*), and 4 rRNA (*rrn4.5*, *rrn5*, *rrn16*, and *rrn23*) genes were duplicated. Comparative analysis of *Lonicera* plastome boundaries revealed structural variations in *L. caerulea* subsp. *altaica* and *L. caerulea* subsp. *pallasii*, particularly in *ndhA* gene distribution. Three highly variable, two intergenic (*ycf1-trnN-GUU* and *trnN-GUU-ndhF*) and one genic (*accD*) region were identified. A total of 641 simple sequence repeats were detected in four plastomes. Phylogenetic analyses grouped *Lonicera* samples into two clades corresponding to subgenera *Periclymenum* and *Chamaecerasus*. In this study, the plastid genomes of two subspecies of *L. caerulea* and species *L. micrantha* were sequenced for the first time. The maximum likelihood tree derived from complete plastid genome sequences proved to be the most informative, showing a topology consistent with previous studies. The nucleotide sequences of variable regions (*accD-ycf1-ndhF-trnN-GUU*) demonstrate high potential for use in DNA barcoding and may serve as valuable molecular markers for species phylogenetic studies within the genus *Lonicera*.

Key words: *Lonicera*; Kazakhstan; next-generation sequencing; variable regions; DNA-barcoding markers; simple sequence repeats

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Полные последовательности пластомов видов *Lonicera* L.: значение для филогении и сравнительный анализ

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Аннотация. Род *Lonicera* L. – один из крупнейших и экономически значимых в семействе Caprifoliaceae Juss., таксономия которого остается спорной. С целью внесения вклада в молекулярную таксономию данного рода мы секвенировали пластомы видов *Lonicera* – *L. caerulea* (два подвида), *L. tatarica* и *L. micrantha* – с использованием технологии секвенирования нового поколения и провели сравнительный анализ. Размеры пластомов варьировали от 153 985 п. н. у *L. micrantha* до 164 000 п. н. у *L. caerulea* subsp. *pallasii*; каждый пластом содержал 130 генов, включая 85 генов, кодирующих белок, 37 тРНК и 8 рРНК генов. Пять белок-кодирующих (*rps7*, *rps12*, *ndhB*, *ycf2* и *ycf15*), 7 тРНК (*trnA-UGC*, *trnI-CAU*, *trnI-GAU*, *trnL-CAA*, *trnN-GUU*, *trnR-ACG* и *trnV-GAC*) и 4 рРНК (*rrn4.5*, *rrn5*, *rrn16* и *rrn23*) гена были дуплицированы. Сравнительный анализ границ пластомов у видов *Lonicera* выявил структурные вариации у *L. caerulea* subsp. *altaica* и *L. caerulea* subsp. *pallasii*, особенно в распределении гена *ndhA*. Были идентифицированы три высоковариабельные области: две межгенные (*ycf1-trnN-GUU* и *trnN-GUU-ndhF*) и одна генная (*accD*). В четырех пластомах обнаружено всего 641 простая повторяющаяся последовательность (SSR). Филогенетический анализ сгруппировал образцы *Lonicera* в два клада, соответствующих подродам *Periclymenum* и *Chamaecerasus*. В настоящем исследовании впервые секвенированы пластидные геномы двух

подвидов *L. caerulea* и вида *L. micrantha*. Древо, построенное методом maximum likelihood на основе полных последовательностей пластомов, оказалось наиболее информативным и демонстрировало топологию, согласующуюся с предыдущими исследованиями. Нуклеотидные последовательности вариабельных регионов (*accD-ycf1-ndhF-trnN-GUU*) обладают высоким потенциалом для использования в ДНК-баркодировании и могут служить ценными молекулярными маркерами для филогенетических исследований видов внутри рода *Lonicera*.

Ключевые слова: *Lonicera*; Казахстан; секвенирование нового поколения; вариабельные регионы; маркеры ДНК-баркодирования; простые повторяющиеся последовательности

Introduction

Lonicera L. is the largest genus in the family Caprifoliaceae Juss., comprising approximately 140 species (Wang G.Q. et al., 2024), which are widely distributed across North America, Europe, Asia, and Africa (Donoghue et al., 2001; Wen, 2001). *Lonicera* species exhibit diverse constituents, including saponins, flavonoids, iridoids, phenolic acids, alkaloidal glycosides, etc. (Lin et al., 2008; Ali et al., 2013; Yang Q.R. et al., 2016; Ni, 2017). Moreover, it exhibits a range of biological activities, including antioxidant, anti-inflammatory, antiviral, anti-hepatoma, and hepatoprotective effects (Yoo et al., 2008; Park et al., 2012; Kong et al., 2017; Ge et al., 2018; Liu M. et al., 2020). Besides their biological activities, species of the *Lonicera* genus also hold significant ornamental value and are widely used in landscaping (Hayes, Peterson, 2020; Varlashchenko et al., 2021).

In Kazakhstan, the genus is represented by 22 species (Abdulina, 1999), two of which are listed in the Red Book of Kazakhstan (Baitulin, Sitpayeva, 2014). The species *Lonicera caerulea* subsp. *altaica* (Pall.) Gladkova, *L. caerulea* subsp. *pallasii* (Ledeb.) Browicz, *L. tatarica* L., and *L. micrantha* Trautv. ex Regel are widely distributed across Kazakhstan. According to the Plants of the World Online (https://powo.science.kew.org/) *L. caerulea* subsp. *altaica* is native to a vast range extending from Eastern Europe to Siberia and Mongolia, while *L. caerulea* subsp. *pallasii* is found in Northern and Eastern Europe, Siberia, and Central Asia. *L. tatarica* occurs naturally from Eastern Europe to Central Siberia and northeastern China; in contrast, *L. micrantha* is native to Kazakhstan. These species play a crucial role in the region's floral biodiversity and are of particular ecological and conservation significance. Additionally, they possess medicinal properties and have been traditionally used in folk medicine for various therapeutic applications (Golubev et al., 2022; Boyarskikh, Kostikova, 2023; Taldybay et al., 2024). In Kazakhstan, *Lonicera* species have been studied using botanical (Ametov et al., 2016; Vdovina et al., 2024), phytochemical (Kushnarenko et al., 2016), and biochemical (Vdovina, 2019) assessments.

The phylogenetic relationships within *Lonicera* remain incompletely resolved, presenting ongoing systematic challenges and requiring revisions to its classification (Wang G.Q. et al., 2024). Over time, various classification systems for *Lonicera* have been proposed (Maximowicz, 1877; Rehder, 1903; Nakai, 1938; Hara, 1983). According to A. Rehder (1903), *Lonicera* is divided into two subgenera, *Chamaecerasus* (L.) Rehder and *Periclymenum* (Mill.) Rehder; within the subgenus *Chamaecerasus*, it is further classified into four sections: *Isoxylosteum* Rehder, *Isika* DC., *Coeloxylosteum* Rehder, and *Nintooa* DC. H. Hara (1983) proposed a classification, which was based on the study by C.J. Maximowicz (1877), dividing *Lonicera* into subgenera *Lonicera* and *Caprifolium* (Mill.) Dippel, with further subdivision of subgenus *Lonicera* into four sections (*Isika* (Anderson) Rehder, *Caeruleae* (Rehder) Nakai, *Lonicera* and *Nintooa* (Sweet) Maxim) and five subsections (*Purpurascentes*, *Monanthae*, *Isika*, *Bracteatae*, and *Rhodanthae*). Later, P.S. Hsu et al. (1988) classified *Lonicera* into subgenera *Chamaecerasus* and *Lonicera*; further, subgenus *Chamaecerasus* was divided into four sections (*Coeloxylosteum*, *Isika*, *Isoxylosteum*, and *Nintooa*).

With advancements in molecular genetic technologies, numerous studies have focused on the phylogenetics of *Lonicera*. For instance, M. Nakaji et al. (2015) investigated the phylogenetic relationships among 23 Japanese *Lonicera* species using nucleotide sequences of five plastid non-coding regions (*rpoB-trnC*, *atpB-rbcL*, *trnS-trnG*, *petN-psbM*, and *psbM-trnD*). The findings support the fundamental validity of the classification by H. Hara (1983) of higher taxonomic groups for Japanese *Lonicera* species. M. Srivastav et al. (2023) conducted a phylogenetic analysis using restriction site-associated DNA sequencing (RADSeq). The RADSeq-based phylogenetic tree revealed that the *Coeloxylosteum*, *Isoxylosteum*, and *Nintooa* sections within subgenus *Chamaecerasus* were monophyletic, whereas the *Isika* section was found to be paraphyletic. Using nuclear ribosomal DNA cistron and plastid genome data, X.L. Yang et al. (2024) confirmed the paraphyly of section *Isika* and the monophyly of sections *Coeloxylosteum*, *Isoxylosteum*, and *Nintooa* within subgenus *Chamaecerasus*, aligning with the findings of M. Srivastav et al. (2023). All of the above-mentioned studies have contributed to the classification of the genus *Lonicera*. However, due to widespread hybridization and introgression, the precise taxonomy of *Lonicera* remains unresolved (Wang H.X. et al., 2020).

The plastid is a vital organelle for photosynthesis in plants and possesses its own genome (Howe et al., 2003). The plastome is uniparentally inherited and highly conserved in gene content and organization (Howe et al., 2003). It ranges in size from approximately 120 to 160 kb and exhibits a quadripartite structure consisting of two identical inverted repeats (IR) and two single-copy regions: a large single-copy (LSC) region and a small single-copy (SSC) region (Palmer et al., 1988; Ruhlman, Jansen, 2014).

Advancements in high-throughput sequencing technologies have greatly facilitated plastid genome research, making it more accessible and enabling comprehensive genomic analyses. To date, only a few studies have been conducted on the comparative analysis of *Lonicera* plastid genomes. For example, seven plastid genomes (*L. ferdinandi*, *L. hispida*, *L. nervosa*, *L. fragrantissima* var. *lancifolia*, *L. stephanocarpa*, *L. tragophylla*, and *L. japonica*) (Liu M.L. et al., 2018) and three plastid genomes (*L. japonica*, *L. similis*, and *L. acumi-*

nata) (Yang C. et al., 2023) have been comparatively analyzed. Recent studies have demonstrated that nucleotide sequences of the plastid genome can provide valuable insights for phylogenomic analysis (Luo et al., 2021; Zhao et al., 2023) taxonomic classification (Li Q., 2022; Oyuntsetseg et al., 2024), and species identification, utilizing plastome sequences as a super barcode (Chen X. et al., 2018; Zhang Z. et al., 2019). Moreover, plastid genome nucleotide sequences serve as a valuable resource for identifying species-specific genetic markers, such as DNA barcoding (Hong et al., 2022; Tang et al., 2022; Almerekova et al., 2024), microsatellite (Zhu M. et al., 2021; Yermagambetova et al., 2023), and single nucleotide polymorphism (SNP) markers (Dong et al., 2021). Therefore, we believe that sequencing and comparative analysis of *Lonicera* plastomes can contribute insights into the taxonomic classification and phylogenetic relationships of the genus.

In this study, we sequenced the plastid genomes of *Lonicera* species (*L. caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha*) using Illumina Next Generation Sequencing technology. Among them, *L. caerulea* subsp. *altaica*, *L.caerulea* subsp. *pallasii*, and *L. micrantha* have been sequenced for the first time to date. We conducted a plastome-based analysis to characterize the plastomes of the selected *Lonicera* species. Our analysis included comparative plastome assessments with previously sequenced *Lonicera* species from GenBank, identification of potential molecular markers valuable for DNA barcoding and population genetics, and evaluation of the taxonomic positions of the studied *Lonicera* species.

Materials and methods

Plant leaf material collection and DNA extraction. Fresh leaf samples were collected from the eastern and central parts of Kazakhstan. Detailed information on the collection sites is provided in Table 1. The leaves were dried in silica gel and subsequently used for DNA extraction. Genomic DNA was extracted from the dried *Lonicera* leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle J.J., Doyle J.L., 1987). The quality and concentration of the extracted DNA were assessed using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Plastid genome sequencing, assembly, and annotation. DNA samples that passed Quality Control (QC) analysis were used for subsequent library preparation. The libraries were constructed using the TruSeq Nano DNA Kit (Illumina Inc., San Diego, CA, USA). Plastid genome sequencing was performed on the Illumina NovaSeq 6000 (Illumina Inc.) platform at Macrogen Inc. (Seoul, Republic of Korea). The quality of raw sequencing data was assessed using FastQC 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc, accessed on 02 December 2024). The adapter sequences were removed from the raw reads using Trimmomatic 0.38 (Bolger et al., 2014). *De novo* assembly was conducted using NOVOplasty (Dierckxsens et al., 2017), and plastome annotation was performed using the Plastid Genome Annotator (PGA) (Qu et al., 2019). Gene maps of the annotated plastid genomes of *L. caerulea* subsp. *altaica*, *L.caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha* were drawn using the OrganellarGenomeDRAW tool 1.3.1 (OGDRAW) (Lohse et al., 2007). The newly sequenced plastomes of these species have been deposited in GenBank under the accession numbers PV026015-PV026018.

Table 1. Information on the collection sites of studied *Lonicera* species in Kazakhstan

Species	Collection sites
L. caerulea subsp. *altaica*	Kazakhstan, East Kazakhstan region, Western Altai, foothills of the Ivanovsky ridge
L. caerulea subsp. *pallasii*	Kazakhstan, Karaganda region, Karkaraly district, Karkaraly Mountains
L. tatarica	Kazakhstan, Karaganda region, Bukhar-Zhyrau district
L. micrantha	Kazakhstan, Karaganda region, Karkaraly district, Karkaraly Mountains

Comparative plastome analysis. Comparative plastome analysis of the studied *Lonicera* species was conducted using mVISTA (Frazer et al., 2004) in Shuffle-LAGAN mode, with the plastid genomes of *L. caerulea* (OQ784224) and *L. tatarica* (OQ784187) serving as references. Additionally, the junction sites of the four *Lonicera* plastomes were examined using the IRscope online tool (Amiryousefi et al., 2018), utilizing the same reference genomes, *L. caerulea* (OQ784224) and *L. tatarica* (OQ784187).

Nucleotide variability analysis. The complete plastid genome sequences of the *Lonicera* species were aligned using Geneious Prime® 2025.0.3 (https://www.geneious.com, accessed on 10 February 2025). The aligned sequences were then analyzed for nucleotide variability (*Pi*) using a sliding window approach in DnaSP v6 (Rozas et al., 2003). The sliding window analysis was performed with a window length of 600 bp and a step size of 200 bp.

Simple sequence repeats analysis and comparative genome analysis. Simple sequence repeats (SSRs) in the nucleotide sequences of the four studied *Lonicera* plastomes were identified using MISA software (Beier et al., 2017). The detection thresholds were set as follows: eight repeats for mononucleotide SSRs, four repeats for di- and trinucleotide SSRs, and three repeats for tetra-, penta-, and hexanucleotide SSRs.

Phylogenetic analysis. Phylogenetic analysis was conducted using alignments of complete plastid genome sequences, protein-coding gene sequences, and variable region gene sequences from *L. caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha*, along with GenBank samples, including outgroup species (*Heptacodium miconioides* and *Triosteum himalayanum*). A total of 24 complete plastid genomes were selected to construct phylogenetic trees in order to determine the phylogenetic placement of the studied species within the genus *Lonicera*. The sequence alignment of the complete plastid genomes was conducted using Geneious Prime® 2025.0.3 (https://www.geneious.com, accessed on 12 February 2025). Phylogenetic relationships were inferred using the maximum likelihood (ML) and Bayesian inference (BI) methods. Maximum likelihood trees were generated using IQ-TREE 2.2.2.6 (Nguyen et al., 2015). The software was also used to determine the optimal tree-building model,

identified as GTR+F+I+R2 for complete plastid genome and variable region genes data, and as TVM+F+I+R3 for protein-coding genes data, which were then applied to reconstruct the ML phylogenetic tree. BI phylogenetic trees were reconstructed using MrBayes 3.2.7 (Ronquist et al., 2012). The resulting phylogenetic trees were visualized using FigTree (Rambaut, 2009). The network analysis was performed in SplitsTree4 (Huson, Bryant, 2006) with the Neighbor-Net algorithm.

Results

General features of the four *Lonicera* plastomes

Illumina sequencing generated paired-end reads with an average length of 150 bp for the four *Lonicera* plastomes. The lengths of the plastid genomes of *L. caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha* were 163,889; 164,000; 154,587, and 153,985 bp, respectively (Fig. 1).

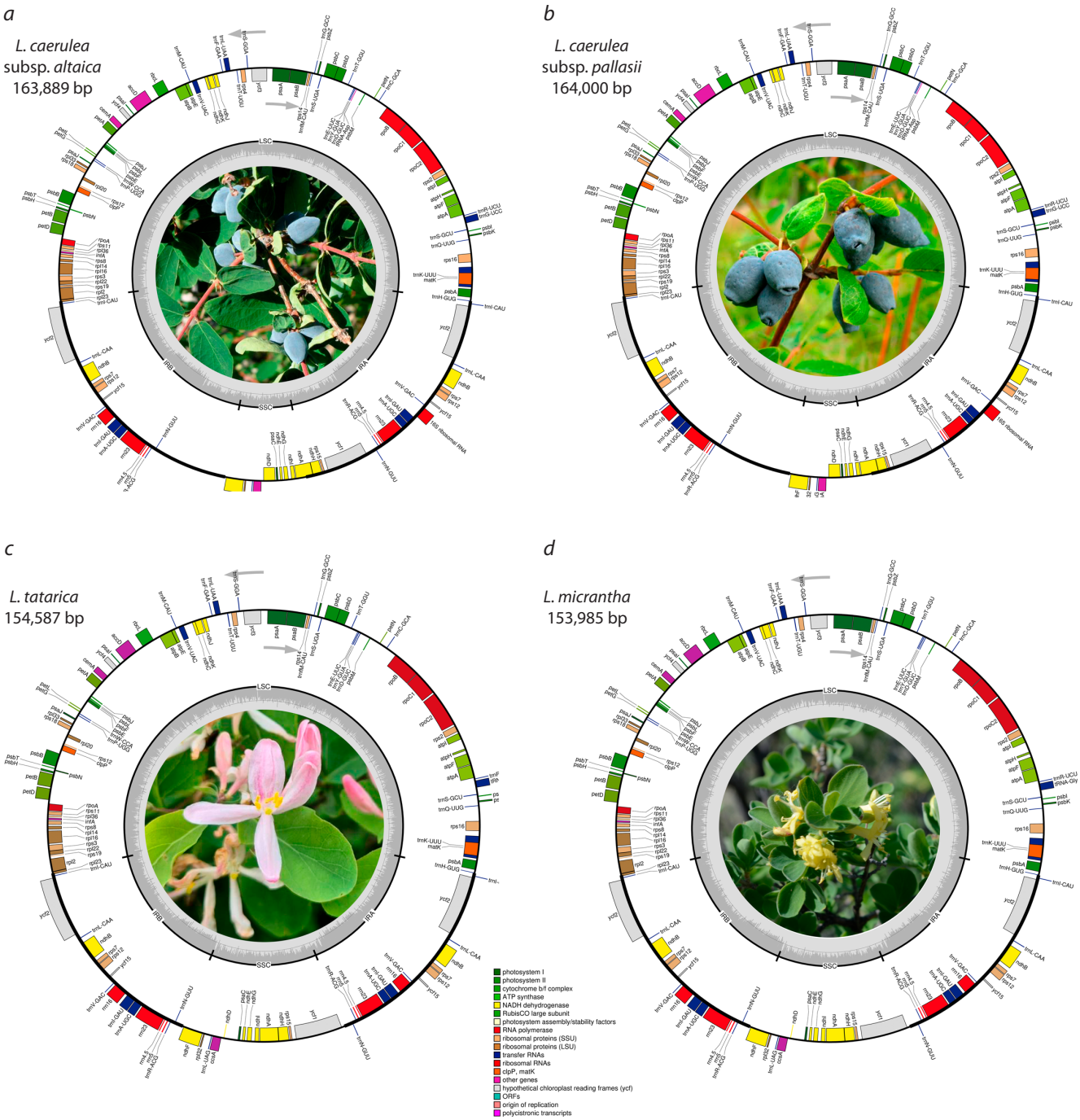

Fig. 1. Plastid genome maps of *L. caerulea* subsp. *altaica* (*a*), *L. caerulea* subsp. *pallasii* (*b*), *L. tatarica* (*c*) and *L. micrantha* (*d*) species.

Genes positioned outside the outer circle are transcribed in a counterclockwise direction, while those inside the circle are transcribed in a clockwise direction. The inner circle represents GC and AT content, with darker gray indicating GC content and lighter gray representing AT content. Genes are color-coded according to their functional categories. The plastid genome map displays a large single-copy (LSC) region, small single-copy (SSC) region, and inverted repeat regions (IRA and IRB).

Table 2. General characteristics of the plastid genomes of the studied *Lonicera* species

Genome features	*L. caerulea* subsp. *altaica*	*L. caerulea* subsp. *pallasii*	*L. tatarica*	*L. micrantha*
GenBank numbers	PV026015	PV026016	PV026017	PV026018
Genome size (bp)	163,889	164,000	154,587	153,985
LSC	88,119	88,813	88,185	88,040
SSC	10,172	10,169	18,750	18,589
IRA	32,799	32,509	23,826	23,678
IRB	32,799	32,509	23,826	23,678
Number of total genes (unique)	130 (115)	130 (115)	130 (115)	130 (115)
Protein genes	85 (80)	85 (80)	85 (80)	85 (80)
tRNA genes	37 (30)	37 (30)	37 (30)	37 (30)
rRNA genes	8 (4)	8 (4)	8 (4)	8 (4)
GC content (%)	38.05	38.12	38.42	38.40
in LSC	36.95	36.92	36.88	36.83
in SSC	32.90	32.84	32.94	33.06
in IRA	40.34	40.59	43.44	43.43
in IRB	40.34	40.59	43.44	43.43

The plastid genome structure consisted of a large single-copy (LSC) region, ranging from 88,040 bp in *L. micrantha* to 88,813 bp in *L. caerulea* subsp. *pallasii*, a small single-copy (SSC) region varying from 10,172 bp in *L. caerulea* subsp. *altaica* to 18,750 bp in *L. tatarica*, and an inverted repeat (IR) region, spanning from 47,356 bp in *L. micrantha* to 65,598 bp in *L. caerulea* subsp. *altaica*. The two inverted repeat regions were designated as IRA and IRB. The total GC content of the four *Lonicera* plastid genomes was relatively consistent, ranging from 38.05 % in *L. caerulea* subsp. *altaica* to 38.42 % in *L. tatarica* plastome. The IR regions exhibited higher GC content (40.34–43.44 %) compared to the single-copy regions, with the LSC region ranging from 36.83 to 36.95 % and the SSC region from 32.84 to 33.06 % (Table 2).

The four assembled plastid genomes of *Lonicera* exhibited identical gene content, intron numbers, and gene order. The plastid genomes of the studied *Lonicera* species comprised 130 genes, including 85 protein-coding genes, 37 tRNA genes, and eight rRNA genes. Among them, five protein-coding genes (*rps7*, *rps12*, *ndhB*, *ycf2*, and *ycf15*), seven tRNA genes (*trnA-UGC*, *trnI-CAU*, *trnI-GAU*, *trnL-CAA*, *trnN-GUU*, *trnR-ACG*, and *trnV-GAC*), and four rRNA genes (*rrn4.5*, *rrn5*, *rrn16*, and *rrn23*) were duplicated within the IR regions of the four *Lonicera* plastid genomes. A total of 17 genes contained introns, of which 16 genes (*rps12*, *rps16*, *rpl2*, *rpl16*, *rpoC1*, *atpF*, *ndhA*, *ndhB*, *petB*, *petD*, *trnA-UGC*, *trnG-UCC*, *trnI-GAU*, *trnK-UUU*, *trnL-UAA*, and *trnV-UAC*) had a single intron, while *ycf3* was the only gene containing two introns. The *rps12* gene exhibited trans-splicing, with its 5′ end located in the LSC region, while its 3′ end was positioned in the IR regions (Fig. 1, Table 3).

Comparative analysis of the four *Lonicera* plastomes

A comparative analysis of the complete plastid genomes of six *Lonicera* species was conducted using mVISTA, with *L. caerulea* (OQ784224) and *L. tatarica* (OQ784187) as reference genomes. The alignment revealed high sequence conservation across the plastomes, with most variations occurring in non-coding regions. Among the coding regions, *accD* exhibited the highest level of divergence. The IR regions were the most conserved, while the LSC and SSC regions displayed higher levels of sequence divergence (Fig. 2).

Inverted repeat expansion and contraction

A comparative analysis of the LSC/IRB/SSC/IRA boundary regions was conducted in the plastomes of *Lonicera* species (*L. caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha*), using *L. caerulea* (OQ784224) and *L. tatarica* (OQ784187) from GenBank as reference sequences. There were structural differences in LSC/IRB/SSC/IRA boundaries of *Lonicera* plastomes. The length of the IR regions ranged from 23,678 to 32,799 bp in four studied *Lonicera* plastomes with some expansion. A notable difference was found in *L. caerulea* subsp. *altaica* and *L. caerulea* subsp. *pallasii* plastomes, where the gene *ndhA*, which crossed over the IRA/SSC boundaries, was similar to those in GenBank (*L. caerulea*). The *ycf1* gene's distance from the IRA region was 246 and 268 bp in *L. tatarica*, and *L. micrantha*, respectively. At the IRB/SSC border, the *ndhF* gene was fully present within the SSC region in all *Lonicera* plastomes, extending into the IRB region with lengths ranging from 41 to 84 bp (Fig. 3).

Nucleotide diversity analysis

To assess nucleotide diversity values, the four *Lonicera* complete plastid genomes in this study were aligned. The aligned nucleotide sequences were then analyzed to calculate the nucleotide diversity of the plastid genome using DnaSP. The results revealed that the *Pi* values in the four *Lonicera* plastomes ranged from 0 to 0.15222. Three highly variable regions were identified: two intergenic regions (*ycf1-trnN-GUU* and *trnN-GUU-ndhF*) and one genic region (*accD*). Among these, the *accD* gene region exhibited the highest *Pi* value (0.15222), followed by the *ycf1-trnN-GUU* region

Table 3. Gene composition and functional categorization of the *L. caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha* plastid genomes

Category	Group of genes	Names of genes
Self-replication	Ribosomal RNA	*rrn4.5* (2), *rrn5* (2), *rrn16* (2), *rrn23* (2)
	Transfer RNA	*trnA-UGC** (2), *trnC-GCA*, *trnD-GUC*, *trnE-UUC*, *trnF-GAA*, *trnfM-CAU*, *trnG-GCC*, *trnG-UCC**, *trnH-GUG*, *trnI-CAU* (2), *trnI-GAU** (2), *trnK-UUU**, *trnL-CAA* (2), *trnL-UAA**, *trnL-UAG*, *trnM-CAU*, *trnN-GUU* (2), *trnP-UGG*, *trnQ-UUG*, *trnR-ACG* (2), *trnR-UCU*, *trnS-GCU*, *trnS-GGA*, *trnS-UGA*, *trnT-GGU*, *trnT-UGU*, *trnV-GAC* (2), *trnV-UAC**, *trnW-CCA*, *trnY-GUA*
	Small subunit of ribosome	*rps2*, *rps3*, *rps4*, *rps7* (2), *rps8*, *rps11*, *rps12** (2), *rps14*, *rps15*, *rps16**, *rps18*, *rps19*
	Large subunit of ribosome	*rpl2**, *rpl14*, *rpl16**, *rpl20*, *rpl22*, *rpl23*, *rpl32*, *rpl33*, *rpl36*
	RNA polymerase	*rpoA*, *rpoB*, *rpoC1**, *rpoC2*
	Translation initiation factor	*infA*
Photosynthesis	ATP synthase	*atpA*, *atpB*, *atpE*, *atpF**, *atpH*, *atpI*
	NADH dehydrogenase	*ndhA**, *ndhB** (2), *ndhC*, *ndhD*, *ndhE*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *ndhJ*, *ndhK*
	Subunits of cytochrome	*petA*, *petB**, *petD**, *petG*, *petL*, *petN*
	Photosystem I	*psaA*, *psaB*, *psaC*, *psaI*, *psaJ*
	Photosystem II	*psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*
	Rubisco	*rbcL*
Other genes	Maturase	*matK*
	Protease	*clpP*
	Envelope membrane protein	*cemA*
	Subunit of acetyl-CoA-carboxylase	*accD*
	C-type cytochrome synthesis gene	*ccsA*
Genes of unknown function	Conserved hypothetical chloroplast ORF	*ycf1*, *ycf2* (2), *ycf3***, *ycf4*, *ycf15* (2)

* Genes containing a single intron, genes containing two introns; (2) – duplicated genes.

(0.10250) and *trnN-GUU-ndhF* (0.09722). Notably, the *accD* region with the highest nucleotide diversity was concentrated in the LSC region of the plastid genome (Fig. 4).

Repeat sequence analysis

This study identified 163, 163, 158, and 157 SSRs in the plastid genomes of *Lonicera caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha*, respectively, resulting in a total of 641 SSRs. Five types of SSRs were identified, including mono-, di-, tri-, tetra-, and hexa-nucleotide repeats. Most of the identified SSR markers were located within the intergenic regions of the plastid genome's LSC region. Detailed information is provided in Supplementary Table S1[1]. Mononucleotide repeats were the most abundant SSR motifs, comprising approximately 72.70 % of the total SSRs, followed by dinucleotide repeats (18.72 %) and tetranucleotide repeats (5.93 %). The most abundant SSR motifs were mononucleotide repeats, which accounted for approximately 72.70 % of the total SSRs, followed by dinucleotide (18.72 %) and tetranucleotide (5.93 %) repeats. Most of the mononucleotide repeats consisted of A/T (451) rather than C/G (15), while the majority of dinucleotide repeats were composed of AT/AT (75) rather than AG/CT (45). Trinucleotide (1.25 %) and hexanucleotide (1.40 %) repeats were rare across the studied plastid genomes but were present in all four plastomes. Pentanucleotide repeats were not found in any of the studied plastomes (Table 4).

Phylogenetic analysis

The ML method was used to reconstruct the phylogenetic trees based on nucleotide sequences of complete plastid genomes (Fig. 5*a*), protein-coding genes (Fig. 5*b*), and variable region genes (Fig. 5*c*). *H. miconioides* and *T. himalayanum* were used as outgroups, while 22 *Lonicera* samples were included as ingroups. The ML trees showed that the analyzed *Lonicera* species were grouped into two clades: the *Periclymenum* subgenus clade and the *Chamaecerasus* subgenus clade. There were five subclades in the ML phylogenetic trees (Fig. 5*a–c*), which represented the sections *Eucarpifolia* and *Phenianthi* (Subclade I) within subgenus *Periclymenum*, section *Isika* (Subclades II and III), sections *Isika* and *Coeloxylosteum* (Subclades IV), and section *Nintooa* (Subclade V) within subgenus *Chamaecerasus*. The phylogenetic tree reveals that *L. tatarica* forms a subclade (IV) consisting of species from the *Coeloxylosteum* section, clustering with the *L. tatarica* (MK970584) sequence from GenBank and indicating a close relationship with *L. maackii* (MN256451) from GenBank. Furthermore, *L. caerulea* subsp. *altaica* and *L. caerulea*

[1] Supplementary Table S1 and Figs S1–S3 are available at: https://vavilovj-icg.ru/download/pict-2025-29/appx29.xlsx

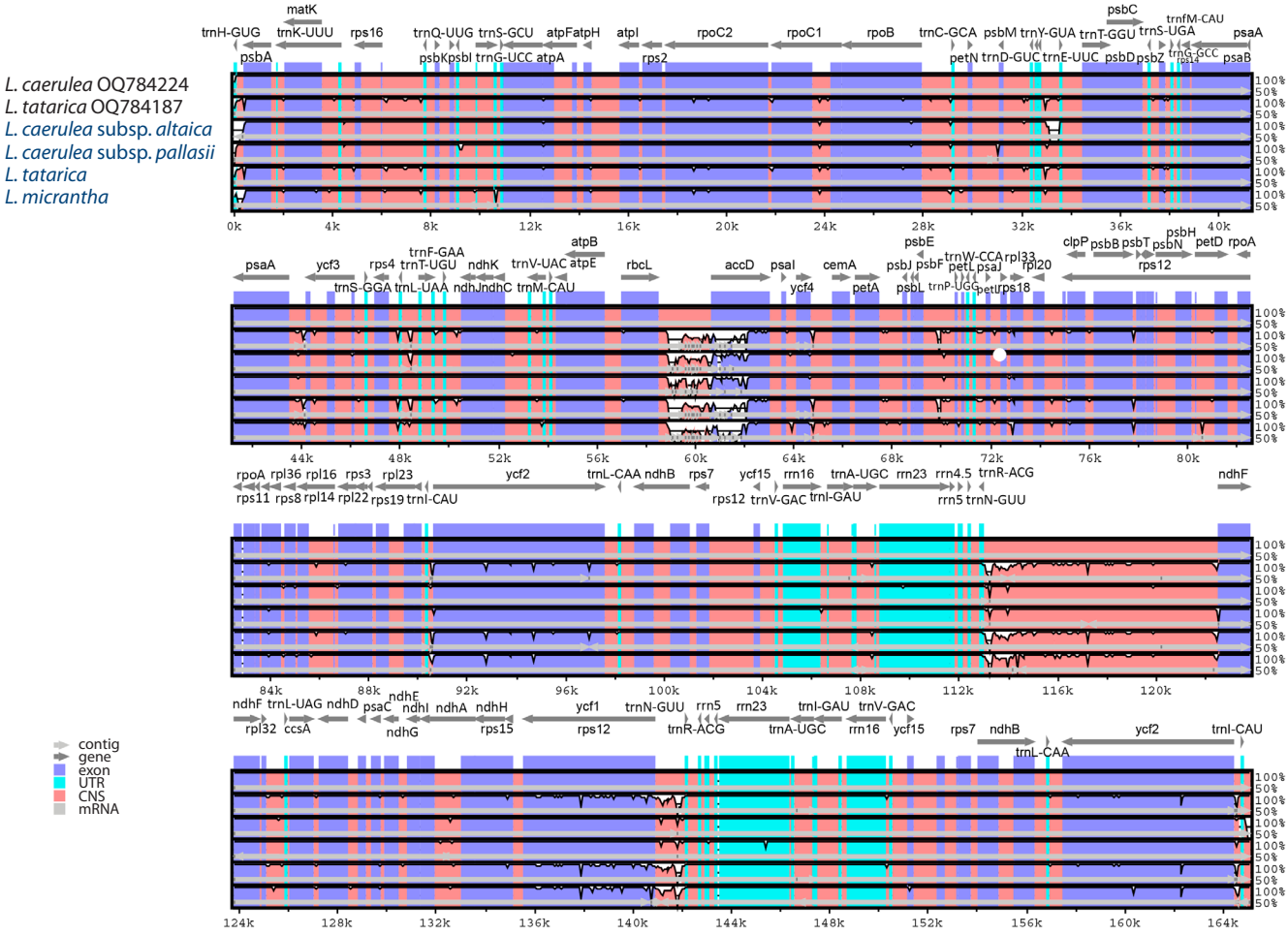

Fig. 2. Comparison of complete plastid genomes of six *Lonicera* samples using mVISTA, with *L. caerulea* (OQ784224) and *L. tatarica* (OQ784187) as reference genomes.

Gray arrows above the alignment indicate gene locations, while different colors distinguish coding and non-coding regions. The horizontal axis represents plastome coordinates, and the vertical scale depicts sequence identity percentages, ranging from 50 to 100 %.

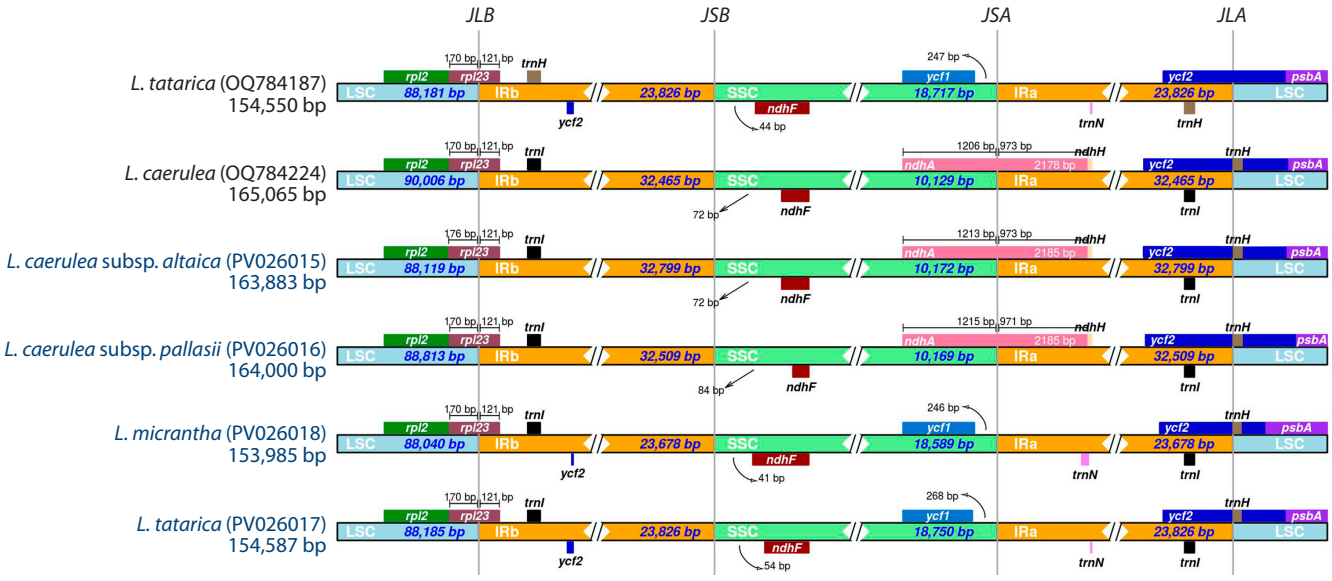

Fig. 3. Comparison of the junctions between the LSC, IR, and SSC regions in *Lonicera* plastomes. Species highlighted in blue were analyzed in this study.

JLB represents the junction between the LSC and IRB regions, JSB marks the boundary between the IRB and SSC regions; JSA indicates the junction between the SSC and IRA regions, and JLA denotes the boundary between the IRA and LSC regions.

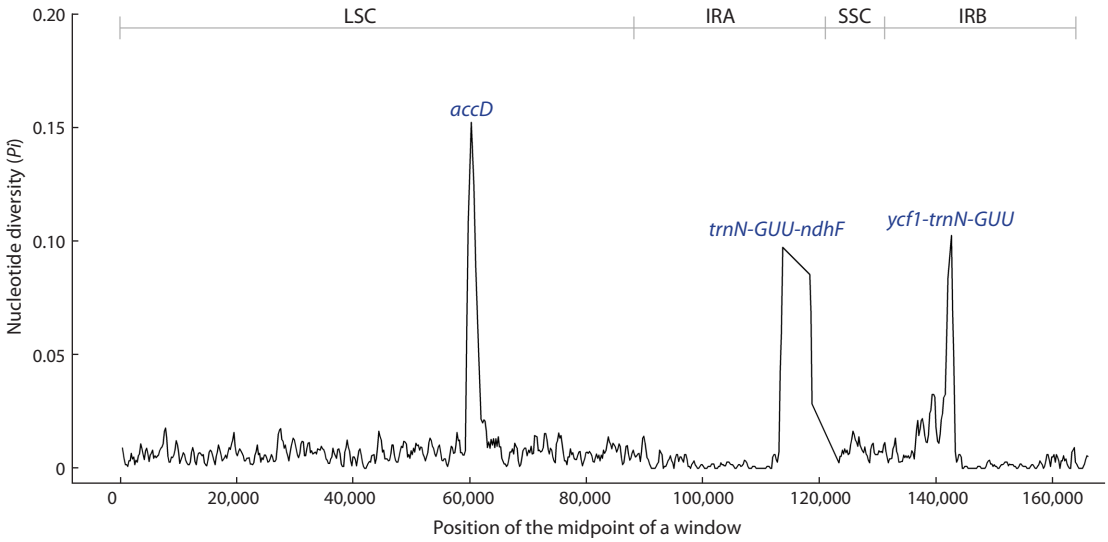

Fig. 4. Nucleotide diversity of the four *Lonicera* plastid genomes using sliding window analysis (window length – 600 bp, step size – 200 bp).

The *X*-axis represents the midpoint position of each window, while the *Y*-axis denotes the nucleotide diversity (*Pi*) value for each window.

Table 4. The number and types of identified simple sequence repeats in the four *Lonicera* plastid genomes

Type	Repeat unit	*L. caerulea* subsp. *altaica*	*L. caerulea* subsp. *pallasii*	*L. tatarica*	*L. micrantha*	Total	%
Mono-	A/T	118	116	109	108	451	72.70
	C/G	4	4	5	2	15	
Di-	AG/CT	10	10	14	11	45	18.72
	AT/AT	19	20	17	19	75	
Tri-	AAC/GTT	1	1	0	1	3	1.25
	AAG/CTT	1	1	1	1	4	
	AAT/ATT	0	0	1	0	1	
Tetra-	AAAG/CTTT	2	2	1	2	7	5.93
	AAAT/ATTT	2	3	3	5	13	
	AATC/ATTG	1	1	1	1	4	
	AATT/AATT	1	1	1	1	4	
	AGAT/ATCT	2	2	3	3	10	
Hexa-	AAAATG/ATTTTC	2	2	0	2	6	1.40
	AATGAT/ATCATT	0	0	0	1	1	
	AAACAT/ATGTTT	0	0	1	0	1	
	AAGGGT/ACCCTT	0	0	1	0	1	
Total		163	163	158	157	641	100

subsp. *pallasii* samples analyzed in this study clustered in one subclade (III) with the *L. caerulea* (OQ784224) and *L. caerulea* subsp. *edulis* (OP345475) sequences from GenBank. Also, *L. micrantha* is positioned within the *Chamaecerasus* subgenus in subclade II and clusters closely with species from the *Isika* section, particularly *L. tangutica* (MZ962399) and *L. microphylla* (OP936076) from GenBank. Most of the described subclades exhibit strong bootstrap support (100 %) at the corresponding nodes, except for subclade II in the ML phylogenetic tree based on complete plastid genome data (Fig. 5*a*), which has moderate support (53 %), indicating high confidence in their overall phylogenetic relationships.

The BI phylogenetic trees were constructed using the same set of samples based on nucleotide sequences from complete plastid genomes (Fig. S1), protein-coding genes (Fig. S2), and variable region genes (Fig. S3). The resulting trees consistently divided the analyzed *Lonicera* species into two distinct clades corresponding to the subgenera *Periclymenum* and *Chamaecerasus*. The topologies of the BI phylogenetic trees were largely congruent with those obtained using ML methods.

To further investigate the relationships and potential reticulation within *Lonicera* species, we constructed a SplitsTree phylogenetic network (Fig. 5*d*) based on complete plastid genome sequences from 22 *Lonicera* and two outgroup samples.

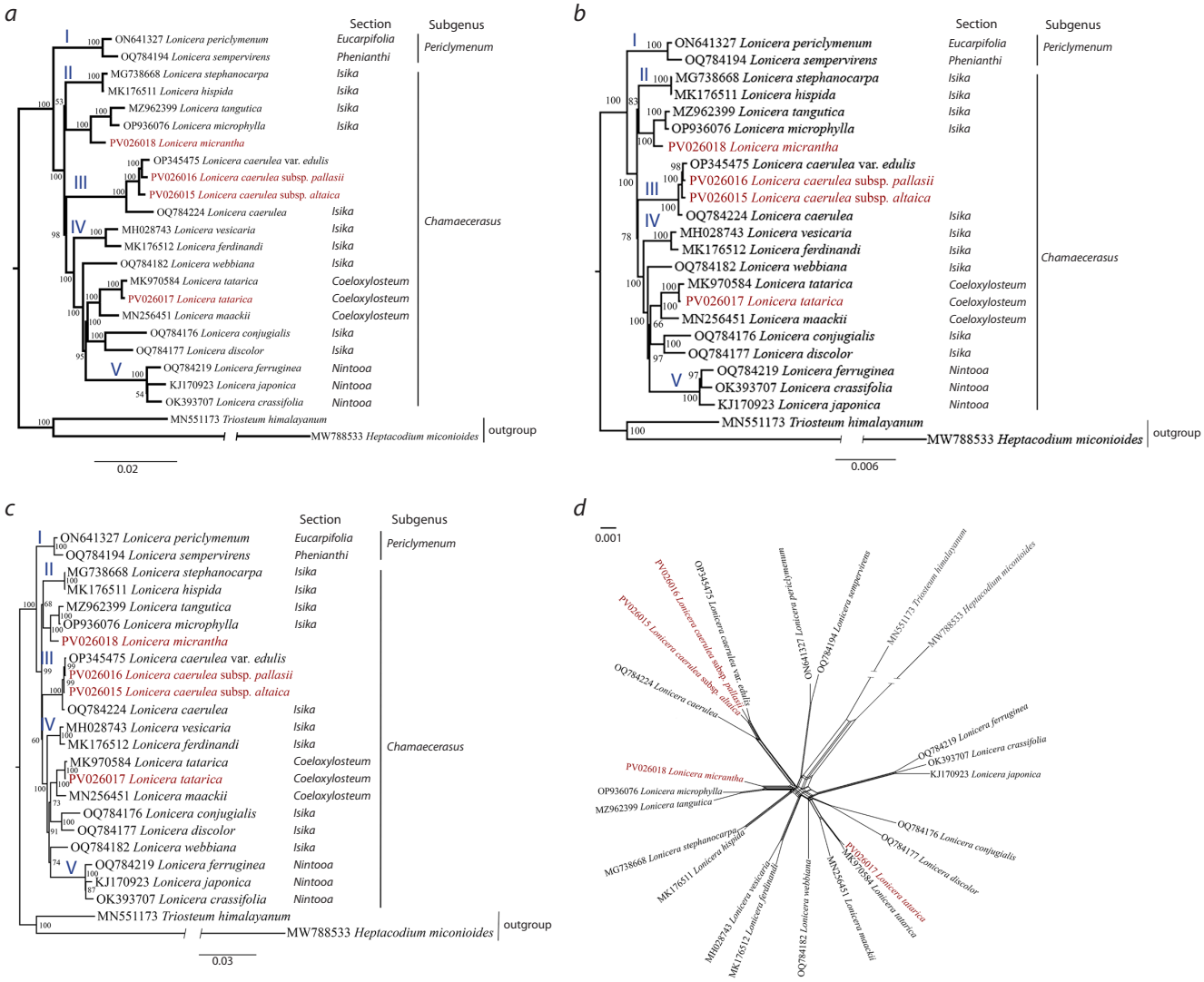

Fig. 5. Maximum likelihood phylogenetic tree of the genus *Lonicera* inferred from nucleotide sequences of the complete plastid genome (*a*), protein-coding genes (*b*), and variable region genes (*c*). Numbers at the nodes of the phylogenetic trees represent bootstrap support values.

Roman numerals (I–V) at the nodes of the phylogenetic trees denote subclade numbers. Splitstree neighbour-net network of 22 *Lonicera* and 2 outgroup plastid genomes (*d*). Species highlighted in red were sequenced in this study.

In this analysis, relationships were generally congruent with those in ML and BI phylogenetic trees. The results of the phylogenetic network coincided with the ML dendrogram (Fig. 5*a–c*). The network indicates that *L. micrantha* has evolved significantly earlier than *L. microphylla* and *L. tangutica.* Also, three subspecies of *L. caerulea* (*altaica*, *pallasii*, and *edulis*) seem to be hybrid forms of the species (Fig. 5*d*).

Discussion

In the present study, the complete plastid genomes of the *L. caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha* were sequenced using next-generation sequencing technology. These genomes were then compared with those of other *Lonicera* species to enhance our understanding of the molecular taxonomy of the genus.

The plastid genomes of the studied *Lonicera* exhibited the typical circular structure found in angiosperms (Palmer et al., 1988; Ruhlman, Jansen, 2014), consisting of an LSC region, an SSC region, and two IR regions (Fig. 1). Our annotation identified a total of 130 genes, including 115 unique genes, consisting of 85 (80 unique) protein-coding genes, 37 (30 unique) tRNA genes, and eight (4 unique) rRNA genes (Table 3). Previous studies have reported slightly different numbers of annotated protein-coding genes, with 82 and 83 genes identified in earlier analyses (He et al., 2017; Liu M.L. et al., 2018; Yang C. et al., 2023). The discrepancies in gene annotation primarily arise from differences in the *ycf15* gene, which are lost in *L. japonica*, *L. ferdinandi*, *L. hispida*, *L. nervosa*, *L. fragrantissima* var. *lancifolia*, *L. stephanocarpa*, *L. tragophylla*, *L. acuminata*, and *L. similis* plastomes. Additionally, we identified the *trnM-CAU* gene, which was not annotated in previous *Lonicera* plastome studies (Frazer et al., 2004). These variations underscore the importance of annotation accuracy, indicating that further comparative analyses are necessary to refine gene identification within the genus.

The genome sizes varied among species, ranging from 153,985 bp in *L. micrantha* to 164,000 bp in *L. caerulea* subsp. *pallasii*. Notably, the plastid genome sizes of *L. cae-*

rulea subsp. *altaica* (163,889 bp) and *L. caerulea* subsp. *pallasii* (164,000 bp) were larger than those of the other two (*L. tatarica*, and *L. micrantha*) studied species (Table 2). The variations in the total length of plastid genomes are typically associated with the expansion and contraction of IR regions (Zhang X.F. et al., 2021). In this study, the IR regions of *L. caerulea* subsp. *altaica* (32,799 bp) and *L. caerulea* subsp. *pallasii* (32,509 bp) were found to be longer than the SSC and LSC regions, contributing to their relatively larger plastome sizes (Fig. 3). These results align with the previously reported plastid genome size of *L. caerulea* (165,065 bp) (Yang X.L. et al., 2024), suggesting that the specific plastid genome lengths observed in the studied *L. caerulea* subspecies may be a common evolutionary characteristic with the *L. caerulea* species.

Molecular markers are essential tools in modern biological research, playing a crucial role in unraveling genetic diversity, phylogenetic relationships, and population dynamics (Wang X.R., Szmidt, 2001; Al-Hadeithi, Jasim, 2021). Among them, DNA barcoding markers offer an efficient approach to species identification by targeting short, conserved regions of the genome (Chac, Thinh, 2023). These markers have made one of the most significant contributions to advancing our understanding of evolutionary processes, establishing DNA barcoding as a core methodology in plant taxonomy (Purty, Chatterjee, 2016; Zhu S. et al., 2022). The highly variable regions in nucleotide sequences of the plastid genome can be used as potential specific DNA barcoding markers for specific plant genera. Using mVISTA (Fig. 2) and sliding window analysis (Fig. 4), we identified three highly variable regions in this study: two intergenic regions (*ycf1-trnN-GUU* and *trnN-GUU-ndhF*) and one genic region (*accD*). These regions show promise as DNA barcoding markers for the phylogenetic analysis of *Lonicera* species. Notably, the *trnN-GUU-ndhF* region has been reported as particularly useful for developing molecular markers in *Lonicera* species (Liu M.L. et al., 2018; Yang C. et al., 2023).

Our study found that the *accD* gene region is the most variable, a finding consistent with previous studies in Asteraceae (Kim et al., 2020) and Fabaceae (Zhang T. et al., 2024). Two intergenic regions (*ycf1-trnN-GUU* and *trnN-GUU-ndhF*) identified in this study were also reported in other plant species. For example, *ycf1-trnN-GUU* was a highly variable region in the plastid genomes of *Parasenecio* (Liu X. et al., 2023) and *Medicago* (Jiao et al., 2023) species. Z. Cao et al. (2023) and W. Xing et al. (2024) reported that the *trnN-GUU-ndhF* intergenic region is hypervariable in the plastid genomes of *Neocinnamomum* taxa and *Pinellia ternata*, respectively. This study identified two highly variable intergenic regions and one genic region as promising candidates for DNA barcoding markers in future research. Nonetheless, further studies are needed to assess the effectiveness of these divergent markers in the phylogenetic analysis of *Lonicera* species.

Another important class of molecular markers is the simple sequence repeat (SSR) markers, which are widely recognized for their value in plant population genetics in assessing genetic diversity, population structure, and evolutionary relationships (Chen F. et al., 2015; Yermagambetova et al., 2024). In our study, we identified a total of 641 SSR markers across the plastid genomes analyzed, with individual counts ranging from 158 in *L. tatarica* to 163 in both *L. caerulea* subsp. *altaica* and *L. caerulea* subsp. *pallasii* plastid genomes (Table 4). Notably, the majority of these SSRs were located in the intergenic regions of the LSC region, a distribution pattern that aligns well with previous findings on angiosperm plastomes (Xia C. et al., 2022; Nyamgerel et al., 2024).

Our results reveal that mononucleotide repeats are the most prevalent SSR motifs across the four *Lonicera* plastomes analyzed, which are common for Caprifoliaceae representatives (Liu H. et al., 2022; Wang L. et al., 2024). Notably, the majority of the mononucleotide repeats were composed of A/T (451) rather than C/G (15), while dinucleotide repeats were predominantly composed of AT/AT (75) as opposed to AG/CT (45). This distribution is consistent with patterns observed in plastid genomes of many other plant species (Li X.Q. et al., 2019; Souza et al., 2019). Numerous studies have demonstrated that SSR markers derived from plastid genome sequences are effective for assessing genetic diversity in plant populations (Jo et al., 2022; Lācis et al., 2022; Guo et al., 2025). The SSR markers identified in our study hold potential for population genetic analyses within the genus *Lonicera*. However, further validation is required to confirm their efficacy and reliability in elucidating the genetic structure of *Lonicera* species populations.

Plastid genome nucleotide sequences have become a powerful tool in phylogenetic studies of different plant genera (Wu et al., 2021; Xia Q. et al., 2023). Their conserved structure, uniparental inheritance, and relatively slow mutation rate make them ideal for resolving evolutionary relationships across diverse plant lineages (Chen J. et al., 2022; Feng et al., 2024). With the development of next-generation sequencing technologies, the rapid and cost-effective sequencing of entire plastid genomes has become increasingly accessible, enhancing their utility in plant taxonomy by providing greater phylogenetic resolution and a deeper understanding of plant evolutionary history. This study utilized complete plastome sequences, protein-coding gene sequences, and variable region gene sequences for the phylogenetic analysis of the studied *Lonicera* species (*L. caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha*), along with publicly available sequences from GenBank, to contribute to a better understanding of phylogenetic relationships within the genus. The ML trees based on the sequences of the complete plastid genome (Fig. 5*a*), protein-coding genes (Fig. 5*b*), and variable region genes (Fig. 5*c*) of the 22 *Lonicera* samples and two outrgoup samples (*H. miconioides* and *T. himalayanum*) was reconstructed.

The phylogenetic analyses in this study revealed that the *Lonicera* species were grouped into two major clades, corresponding to the subgenera *Periclymenum* and *Chamaecerasus*, which is consistent with previous phylogenetic studies (Srivastav et al., 2023; Yang X.L. et al., 2024). Furthermore, the larger clade representing subgenus *Chamaecerasus* was further divided into four distinct subclades corresponding to sections *Isika* (Subclades II and III), sections *Isika* and *Coeloxylosteum* (Subclades IV), and section *Nintooa* (Subclade V). Within subgenus *Chamaecerasus*, species are subclustering into four subclades and align with recognized sectional classifications (Srivastav et al., 2023).

The placement of *L. micrantha*, *L. caerulea* subsp. *altaica* and *L. caerulea* subsp. *pallasii*, which had not been previously

assigned to a section, suggests their belonging to the *Isika* section based on their close clustering with other members of this section. Additionally, the finding supports the assumption that *L. caerulea* subsp. *altaica* and *L. caerulea* subsp. *pallasii* share a common evolutionary history with *L. caerulea*, which is also supported by the plastid genome structure of these species. Furthermore, *L. tatarica* forms a subclade (IV) with *L. tatarica* (MK970584) and *L. maackii* (MN256451) from GenBank, grouping within the *Coeloxylosteum* section. Notably, these samples are positioned between the species of section *Isika*, suggesting a possible evolutionary relationship between these two sections.

The phylogenetic analysis aimed to clarify the phylogenetic positions of the studied *Lonicera* species from Kazakhstan using plastid genome nucleotide sequences, including three newly sequenced ones (*L. caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, and *L. micrantha*) in this study. The genomic data obtained in this study provide valuable resources for future phylogenetic research, contributing to an understanding of evolutionary relationships within the genus *Lonicera* and supporting further taxonomic revisions. Based on the comparison of phylogenetic trees reconstructed using different datasets and methods, we conclude that the maximum likelihood tree derived from complete plastid genome sequences was the most informative, and its topology is consistent with those reported in previous studies (Srivastav et al., 2023; Yang X.L. et al., 2024). Furthermore, the nucleotide sequences of variable regions such as *accD-ycf1-ndhF-trnN-GUU* also demonstrate high potential for use in DNA barcoding, and may serve as valuable molecular markers for species phylogenetic studies within the genus *Lonicera*.

Conclusion

The complete plastid genomes of *L. caerulea* subsp. *altaica*, *L. caerulea* subsp. *pallasii*, *L. tatarica*, and *L. micrantha* exhibited the typical circular structure with four distinct regions. Structural variations were observed in the plastomes of *L. caerulea* subsp. *altaica* and *L. caerulea* subsp. *pallasii*, particularly in genome sizes, which were larger than in the other two species (*L. tatarica* and *L. micrantha*) due to an extended IR region. This finding aligns with previous studies on *L. caerulea* plastomes, further supporting their shared evolutionary history. The nucleotide sequences of variable regions such as *accD-ycf1-ndhF-trnN-GUU* demonstrate high potential for use in DNA barcoding, and may serve as valuable molecular markers for species phylogenetic studies within the genus *Lonicera.* Further studies are required to assess the effectiveness of the identified simple sequence repeats.

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