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Brief Report

Estimation of the Genome Size and Complete Chloroplast Genome in *Adenophora remotiflora*: Genome Structures, Comparative Genomics, and Phylogenetic Diversity

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Abstract: *Adenophora remotiflora* is a wild perennial plant used as oriental medicine and ornamental flowers in East Asia. The haploid genome size of *A. remotiflora* was estimated at 3.9 Gb with a 2.42% heterozygosity ratio. The chloroplast genome of 174,455 base pairs (bp) shows a circular map structure, and has four conserved regions consisting of a large single-copy region of 108,423 bp, a small single-copy region of 10,444 bp, and a pair of inverted repeats (each 27,794 bp). A total of 108 unique genes were annotated, comprising 74 protein-coding genes, 4 ribosomal RNA genes, and 30 transfer RNA genes. A total of 155 repeat sequences were identified, and comparative genome structures were characterized among the *Adenophora* species. Phylogenetic diversity showed that *A. remotiflora* is in a close position within the *Adenophora* genus, and *Adenophora erecta* is in the closest evolutionary position.

Keywords: *Adenophora remotiflora*; *Adenophora* species; chloroplast genome; genome size

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1. Introduction

The scattered-flower ladybell (*Adenophora remotiflora* Miq.) of the Campanulaceae family is a perennial herbaceous plant that inhabits East Asia. *A. remotiflora* is a traditional oriental medicine, and its flowers are cultivated for ornamental purposes. Its extract has been reported to have health promotion effects, including antioxidant functions for improving skin conditions, managing chills, and clearing coughs [1]. The Campanulaceae family consists of more than 1000 species [2], and *Adenophora* is a large genus with a complex taxonomic history. The *Adenophora* species are important medicinal and horticultural plants, and there is an increasing demand for various cultivars with high medicinal herb content and disease resistance [3].

Chloroplasts play a major role in the photosynthesis metabolic pathway, pathway signaling, and the production of multiple compounds, such as flavonoids [4,5]. The plants' chloroplast genome has a circular map structure with four conserved regions: a large single-copy region (LSC), a small single-copy region (SSC), and two separated inverted repeats (IRs) [6]. Chloroplast genome sequences are widely used in genome evolution studies and phylogenetic classification [7,8].

The *Adenophora* genus species have very different genomic structures due to many sequence inversions [9]. Although previous studies focused on the *Adenophora* species, their taxonomic and evolutionary positions are unclear, because of the recombination of many genes [10]. Therefore, chloroplast genome assembling of *A. remotiflora* is useful for clarifying the phylogenetic diversity and evolutionary tendencies.

Here, we estimate the genome size in *A. remotiflora* for the first time, and the chloroplast genome was assembled using de novo sequencing (NCBI accession no. OP920648). Comparative genome structure analyses with phylogenetic diversity were performed for the *Adenophora* genus clade.

2. Materials and Methods

2.1. Plant Material, Sequencing, and Genome Size Estimation

A. remotiflora was collected from Yeongyang County, Gyeongsangbukdo, Republic of Korea (1085 m, N 36°48′23.87″, E 129°05′22.78″). Genomic DNA was isolated from fresh leaves. DNA integrity, purity, and concentration were confirmed using a NanoDrop 2000 (Thermo, Waltham, MA, USA) with 1% agarose gel. Illumina paired-end (PE) libraries with two 125 bp read lengths were constructed, and sequenced on an Illumina HiSeq X platform (Illumina, San Diego, CA, USA) according to the Illumina manual protocol. Among the sequenced raw reads, poor-quality reads (PHRED score < 20) were filtered via the trimming check of the CLC Assembly Cell (CLC, Aarhus, Denmark). The estimation of genome size was performed using two genome estimation tools (Jellyfish, GenomeScope) [11,12]. The optimal *k*-mer value was calculated by changing the *k*-mer distribution (17, 19, 21, 23, 25, 27, 29, and 31). According to the optimal *k*-mer value, the genome size was estimated using two genome estimation tools.

2.2. Chloroplast Genome Assembly, Gene Annotation, and Characteristic Visualization

Using quality-filtered Illumina PE reads, de novo assembly was performed using the CLC genomic assembler (CLC, Aarhus, Denmark) after read preprocessing. The primary assembled contigs were aligned to the chloroplast sequences of *Adenophora erecta* (NC_036222) downloaded from Organelle Genome Resources of the NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 20 July 2023). The primary aligned contigs were manually curated (i.e., base correction, circularization, and gap-filling) using the reference function of the CLC Assembler (CLC, Aarhus, Denmark). Filtered sequences were annotated using the GeSeq [13], and predicted genes were manually curated and confirmed using Artemis [14]. The multilayer structure of the chloroplast map was visualized using CPGView [15].

2.3. Repeat Sequences of A. remotiflora

Three repeat types of the chloroplast sequences were analyzed as simple sequence repeats (SSRs): dispersed and tandem repeats. For the short-repeat sequences (repeat units < 7 bp), SSRs and short tandem repeats were identified using the MIcroSAtellite (MISA) identification tool [16]. The unit size/minimum repeat parameters were set as 10/mono-nucleotides, 6/di-, 5/tri-, and 4/multi-. For the long-repeat sequences (repeat units $\geq$ 7 bp), dispersed repetitive sequences were distinguished using VMATCH [17] based on the following setting: repeat distance (3); the repeat length (30) was specified. The long tandem repeats were detected using Tandem Repeats Finder [18] with the following settings: matching ratio (80%), indel ratio (10%), matches (2), mismatches (7), size (500), and score (50).

2.4. Chloroplast Sequence Comparison among the Adenophora Species

Eight chloroplast sequences of the *Adenophora* species were downloaded from Organelle Genome Resources of the NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 20 July 2023) as reference sequences: *A. remotiflora* (KP889213), *A. remotiflora* (OP920648), *A. erecta* (NC_036222), *A. divaricate* (NC_036221), *A. kayasanensis* (MZ365443), *A. racemose* (MT012303), *A. stricta* (NC_036223), and *A. triphylla* (MT649408). To compare the structural differences, multiple layer alignments were performed to detect the deletion, inversion, and re-arrangement events using Mauve [19]. Gene content similarities were displayed using mVISTA [20], and junction genes of boundary regions were evaluated using IRScope [21].

2.5. Phylogenetic Diversity among the Campanulaceae Family

To clarify the phylogenetic diversity, a total of 15 genome sequences were downloaded from Organelle Genome Resources of the NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 20 July 2023). These sequences were eight *Adenophora* species, namely *Hanabusaya asiatica* (NC_024732), *Trachelium caeruleum* (NC_010442), *Codonopsis lanceolate* (MH251613), *Platycodon grandifloras* (NC_035624), *Viburnum carlesii* (MN985820), *Sambucus nigra* (NC_045061), and *Helianthus annuus* (NC_007977), with the last three species used as the outgroup. The 62 protein-coding genes commonly shared among 15 chloroplast sequences were subjected to evolutionary position and phylogeny relationship analysis. The commonly shared genes were multi-aligned using MAFFT [22], and phylogenetic diversity was shown using MEGA11 with the employment of a maximum likelihood (ML) method [23]. The ML trees were reconstructed using the GTR-GAMMA model, as it is the best substitution model, and bootstrap iteration was set to 1000.

3. Results and Discussion

3.1. Genome Size Estimation of A. remotiflora

A. remotiflora was sequenced using an Illumina HiSeq X platform (Illumina, San Diego, CA, USA), resulting in 1680 million reads and 253.7 Gb raw sequence length. After filtering and correction, trimmed 188.2 Gb sequences were derived with a 139 bp average read length and 41.5% GC content. For whole-genome size estimation, two genome estimation tools (GenomeScope and Jellyfish) were used based on the trimmed sequences. Because the estimated efficiency of the genome size is affected by changing *k*-mer values, we evaluated the optimal genome size via varying the *k*-mer frequency values in both methods. Using the GenomeScope method, the genome size was predicted to be between 3.7 Gb and 3.9 Gb. The optimal *k*-mer value for the highest predicted model fit was 23; then, haploid genome size was estimated to be 3.9 Gb (Figure 1a). Using the Jellyfish method, the genome size was predicted to be between 3.7 Gb and 3.8 Gb. The genome size using the optimal *k*-mer value (k = 23) was 3.8 Gb (Figure 1b). The haploid genome size was estimated to be 3.8–3.9 Gb with a 2.42% heterozygosity ratio, and only 541.9 Mb unique genome sequences (13.9%) were detected. It is assumed that *A. remotiflora* (2n = 2x = 36) has many heterogeneous parts between the haplotypes in the parent genome.

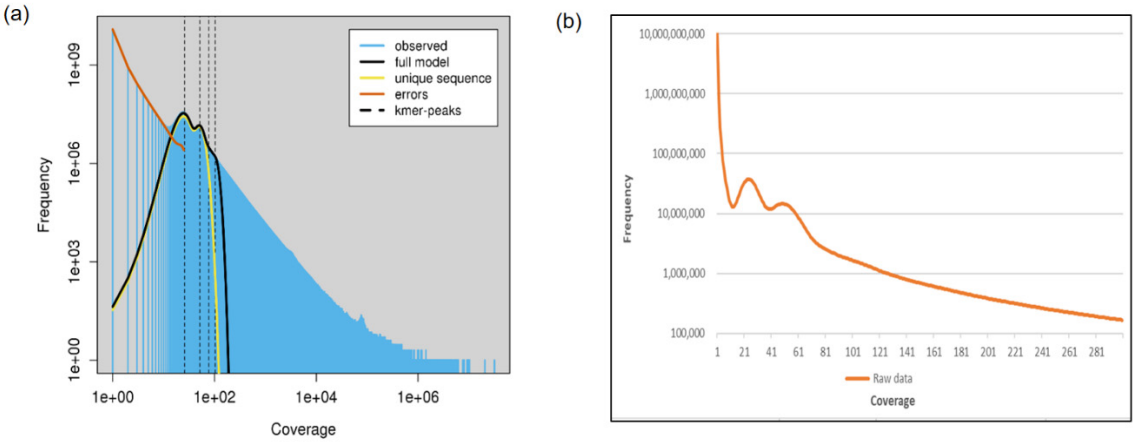

Figure 1. Genome size estimation of *A. remotiflora* (**a**) Whole-genome size with optimal *k*-mer frequency values were measured using GenomeScope profile plots. (**b**) Whole-genome sizes were measured using Jellyfish.

3.2. Chloroplast Genome Assembly of A. remotiflora

For the assembly construction of the chloroplast genome, 1.5 Gb sequences were re-extracted from the trimmed 188.2 Gb sequence dataset. The assembled genome presented a circular-shaped structure with 174,455 bp. The circular structure had four conserved regions consisting of LSC (108,423 bp), SSC (10,444 bp), and a pair of IRs (IRa and IRb; each

27,794 bp). A total of 131 genes were functionally predicted including 23 multicopy genes (Table 1). The 23 duplicated genes were excluded from the 131 gene dataset, resulting in 108 unique genes being annotated (Table 1, Figure 2).

Table 1. Chloroplast genome features and identified genes in *A. remotiflora*.

Category	Group	Name of Genes
Photosynthesis	Photosystem I	*psaA*, *psaB*, *psaC*, *psaI*, *psaJ*
	Photosystem II	*psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*, *ycf3*, *psbJ* *
	Cytochrome [1]	*petA*, *petB*, *petD*, *petL*, *petN*, *petG* *
	ATP synthase	*atpA*, *atpB*, *atpE*, *atpF*, *atpH*, *atpI*
	NADH [2]	*ndhC*, *ndhD*, *ndhE*, *ndhF*, *ndhJ*, *ndhK*, *ndhA* *, *ndhG* *, *ndhH* *, *ndhI* *
	Rubisco [3]	*rbcL*
Self-replication	SSU ribosome [4]	*rps2*, *rps3*, *rps4*, *rps7*, *rps8*, *rps11*, *rps14*, *rps16*, *rps18*, *rps19*, *rps12* *, *rps15* *
	LSU ribosome [5]	*rpl2*, *rpl14*, *rpl16*, *rpl20*, *rpl22*, *rpl32*, *rpl33*, *rpl36*
	Polymerase [6]	*rpoA*, *rpoB*, *rpoC1*, *rpoC2*
	rRNA genes	*rrn16* *, *rrn23* *, *rrn4.5* *, *rrn5* *
	tRNA genes	*trnC-GCA*, *trnD-GUC*, *trnE-UUC*, *trnF-GAA*, *trnfM-CAU*, *trnG-GCC*, *trnG-UCC*, *trnH-GUG*, *trnK-UUU*, *trnL-UAA*, *trnL-UAG*, *trnM-CAU*, *trnQ-UUG*, *trnR-UCU*, *trnS-GCU*, *trnS-GGA*, *trnS-UGA*, *trnT-GGU*, *trnT-UGU*, *trnV-UAC*, *trnY-GUA*, *trnA-UGC* *, *trnR-AGC* *, *trnI-GAU* *, *trnL-CAA* *, *trnN-GUU* *, *trnP-UGG* *, *trnV-GAC* *, *trnW-CCA* *, *trnI-CAU* **
Other genes	Cytochrome [7]	*ccsA*,
	Envelope [8]	*cemA*,
	Maturase	*matK*
	ORFs [9]	*ycf2*, *ycf4*, *ycf1* *

[1] chloroplast cytochrome b_6f complex, [2] NADH dehydrogenase (quinone), [3] subunit of rubisco, [4] small subunits, [5] large subunits, [6] RNA polymerase, [7] C-type cytochromes genes, [8] envelope membrane proteins, [9] open reading frames, multicopy genes (* two, ** three copies).

This genome has 19 intron-containing genes consisting of 11 protein-coding genes and 8 transfer RNA genes (*trnG-UCC*, *trnK-UUU*, *trnL-UAA*, *trnV-UAC*, *trnA-UGC* (×2), and *trnI-GAU* (×2)). Among these genes, three genes (*rps12* (×2) and *ycf3*) contain two introns, and sixteen genes have one intron. In particular, three genes (*trnA-UGC*, *trnI-GAU* and *rps12*) have two copies. In the RNA splicing, we identified nine splicing genes consisting of eight cis-splicing and one trans-splicing (*rps12*) gene (Figure S1). The *ycf3* cis-splicing gene has two introns, which is important in photosynthesis [9].

Chloroplast genome genes were categorized into three functional groups: photosynthesis, self-replication, and "other genes" [24]. The annotated 108 genes of *A. remotiflora* were categorized into 44 genes in the photosynthesis metabolism, 58 expression genes in the self-replication, and 6 genes in the "other genes" category. Among the classified genes, the photosynthesis category was functionally divided into six groups (Table 1). This category had two dominant gene families, such as five genes of the photosystem I group and sixteen genes of the photosystem II group. The self-replication category comprised five groups, including 34 encoded genes from ribosomal and transfer RNAs. The "other genes" category was divided into four groups, and three genes (*ycf1*, *ycf2*, and *ycf4*) as open reading frames, whose gene functions are unclear, were identified.

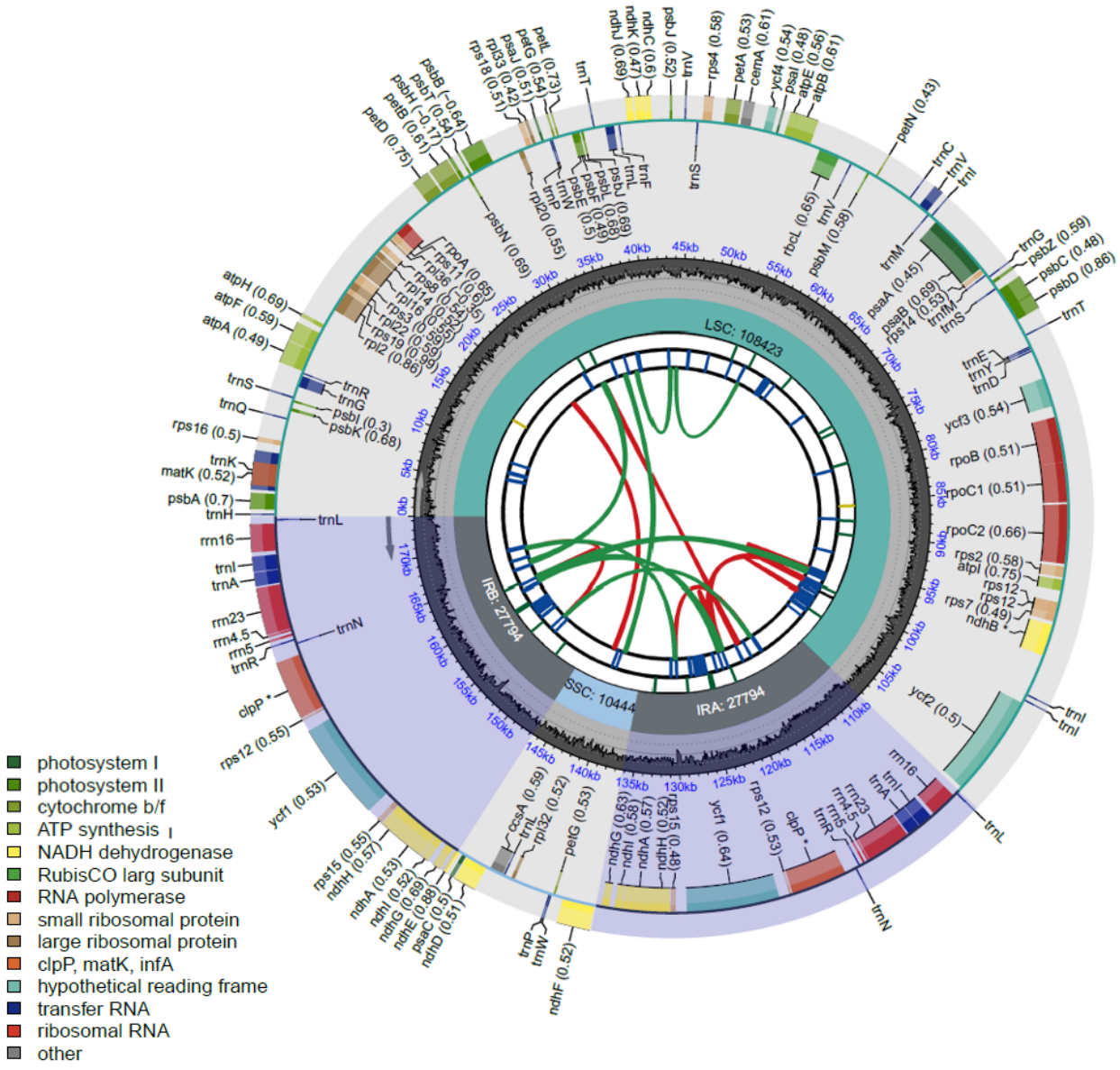

Figure 2. Chloroplast genome diagram of *A. remotiflora*. Schematic map has six circular tracks. The first, second and third circles represent patterns of various repeat sequences, the fourth circle shows the four conserved regions, and the fifth and sixth circles show the GC content and gene names.

3.3. Identification of Repeat Sequences in A. remotiflora

The genome size and structural changes are mainly affected by length differences in repetitive sequences. The repeat sequences are a major cause of gene recombination, sequence divergence, and genomic rearrangement [25]. Three types of repetitive sequences were identified to reveal the structural changes. In total, 155 repeat sequences were detected, covering 25 SSRs, 49 dispersed repeats, and 81 tandem repeats.

In the short-repeat sequences (repeat units < 7 bp), detected SSRs were composed of 22 mono-nucleotides, two di-nucleotides, and one tri-nucleotide (Table S1). All SSRs were 10–15 bp long, and the A/T repeat units of mono-nucleotides showed a 90.1% ratio. Generally, SSRs are used to evaluate molecular markers, genetic diversity, and evolutionary position [26,27].

Regarding the long-repeat sequences (repeat units $\geq$ 7 bp), we detected 49 dispersed repeats and 81 tandem repeats. The dispersed repeats comprised two types: 22 palindromic matches and 27 direct-forward matches (Table S2). The tandem repeats had a period size (repeat unit size) ranging from 9 to 114 bp per copy, and repeat copy numbers ranged

from 1.9 to 25.0 copies (Table S3). Dispersed and tandem repeats are the main cause of sequence variation, recombination, and rearrangement [28,29]. Therefore, 155 detected repeat sequences could be used to distinguish genetic diversity and identify potential molecular markers for the *Adenophora* species.

3.4. Comparison of Chloroplast Genome Sequences

Multiple genome alignments are widely used to analyze complex gene structures and genome collinearity [30]. We aligned local collinear blocks (LCBs) to characterize the genome structure of *A. remotiflora*. The nine LCBs were identified among eight *Adenophora* species, and these LCBs had small structural rearrangements except *A. triphylla* (Figure 3). In particular, *A. triphylla* differed from that of the other *Adenophora* species. This chloroplast genome is presumed to be highly variable, because it has extensive morphological variation, and a large chloroplast genome size [31].

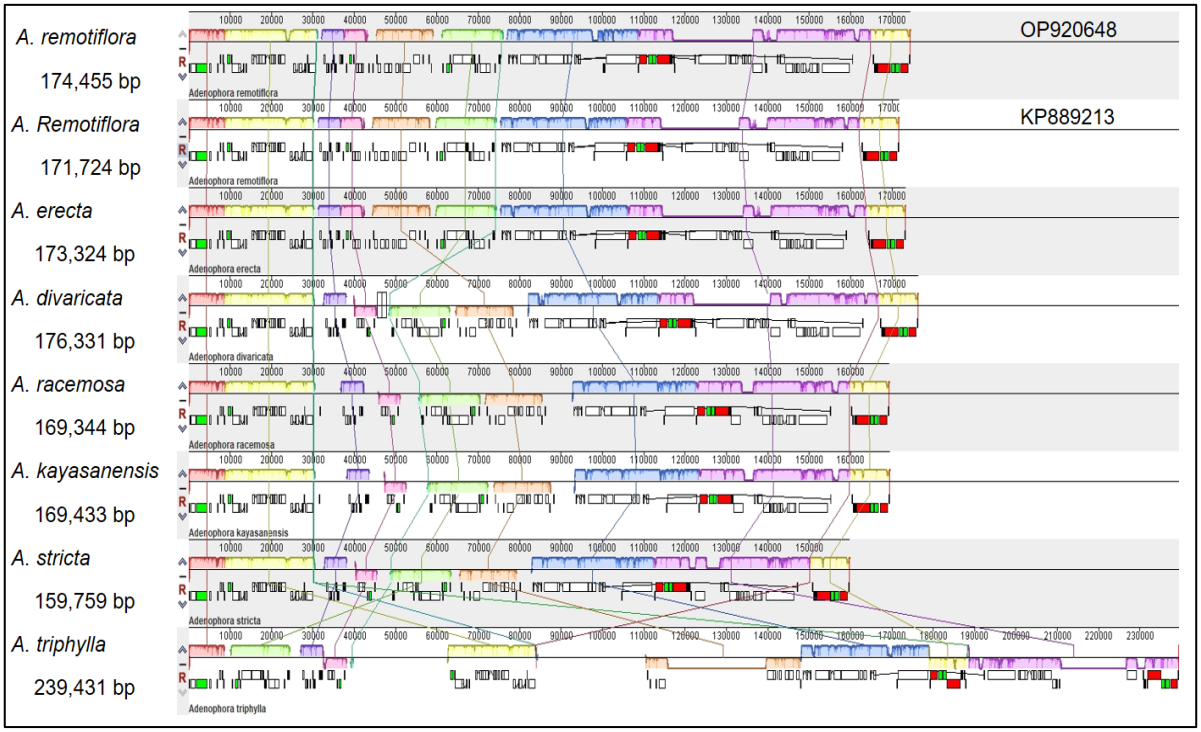

Figure 3. Features of locally collinear blocks (LCBs) using Mauve alignment. The colored LCB boxes indicate the most conserved collinear regions, and block histograms show the sequence similarity.

In the chloroplast genome, rearrangement/recombination of sequences is a cause of adaptive evolution and advantageous mutation [32]. The multilayer alignment was performed in the eight *Adenophora* species using mVISTA. The sequence alignment results were unclear because of the many layers of genes. To clarify, we compared the alignment results using only two *A. remotiflora*, i.e., *A. remotiflora* from this study (NCBI accession No. OP920648), and another from a previous report (NCBI accession no. KP889213). These alignment results showed that the chloroplast genome size differed by 2731 bp, and both *A. remotiflora* have many highly conserved sequences, except the *petG* gene (Figure 4). We assumed that the *petG* gene, known as components of the cytochrome complex subunit, was the gene variation point between the two *A. remotiflora* specimens.

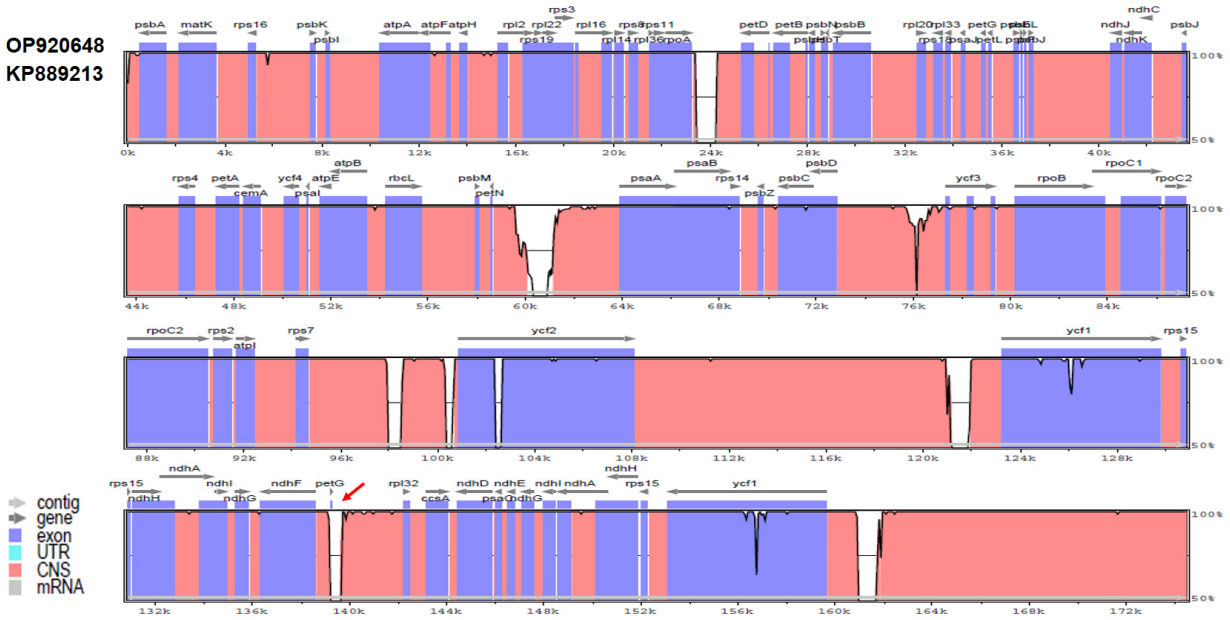

Figure 4. Chloroplast nucleotide sequence alignments between *A. remotiflora* (this study, NCBI accession no. OP920648) and another *A. remotiflora* sequence (NCBI accession no. KP889213) using the mVISTA program. Red arrow indicates the location of the *petG* gene, and gray arrows show the direction and position of each gene. The white areas indicate the dissimilar regions, and gene name is shown at the top.

3.5. Comparison of Inverted Repeats Region

Inverted repeats (IR) contraction and expansion events are a major source of chloroplast sequence variation [33]. IR boundary comparison was performed between two *A. remotiflora* (OP920648 and KP889213). Both IR region sizes ranged from 27,437 to 27,794 base pairs (bp). In the LSC/IR border region, five genes (*trnL*, *trnH*, *psbA*, *rrn16*, and *ycf2*) were found, and four genes (*psaC*, *ndhF*, *ndhE*, and *ndhG*) were found in the SSC/IR border (Figure 5). Among the 10 *ndh* family genes identified in *A. remotiflora* (Table 1), only the *ndhE* gene (303 bp length) was found in the SSC/IRa (JSA). In particular, a *ndhE* pseudogene fragment of 157 bp size was found in the SSC/IRb (JSB) of *A. remotiflora* (KP889213). This pseudogene moved by one base pair from the SSC to the IRb. Therefore, we assumed that the two *A. remotiflora* had sequences for plant species distinction in the JSB border regions.

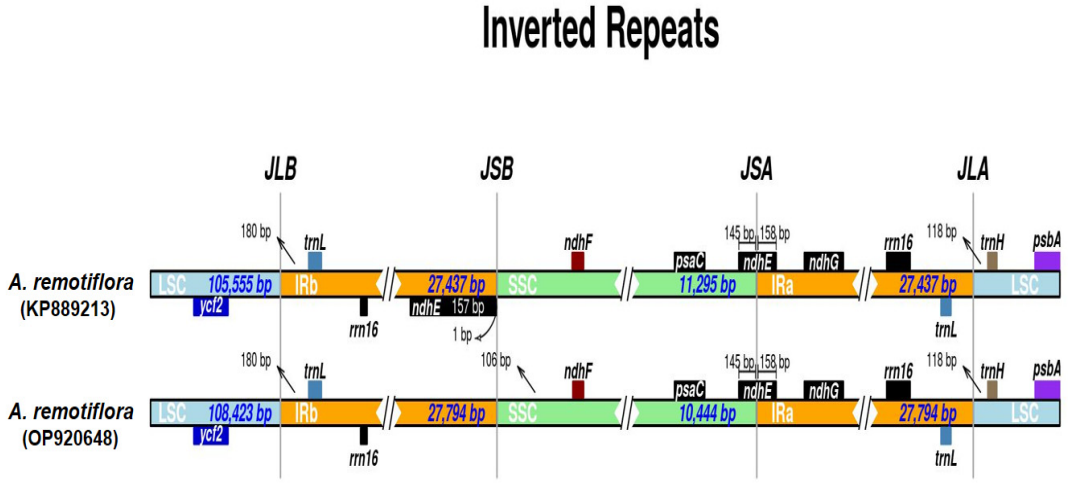

Figure 5. Comparative analyses of junction positions in the boundary regions (LSC, SSC, Ira, and IRb) between two *A. remotiflora* (OP920648 and KP889213). The numbers represent the distances or gene length, and gene names are indicated around boxes.

3.6. Phylogenetic Diversity in the Campanulaceae Family

In the plant chloroplast genome, phylogenetic trees were used in the determination of evolutionary positions, and phylogeny construction was employed at different taxonomic levels [34]. To better evaluate the Phylogenetic diversity among the Campanulaceae family, a ML tree was reconstructed using 62 commonly shared genes, with the closely related three species of *Viburnum carlesii*, *Sambucus nigra*, and *Helianthus annuus* as an outgroup. All phylogenetic relationships were consistent with traditional taxonomy based on morphology-based classification in the Campanulaceae family [35]. Eight *Adenophora* species were clustered in the closest position, and most species showed high bootstrap support values. The phylogeny results indicated that *A. remotiflora* was within the *Adenophora* clade and was the closest relative of *A. erecta* (Figure 6).

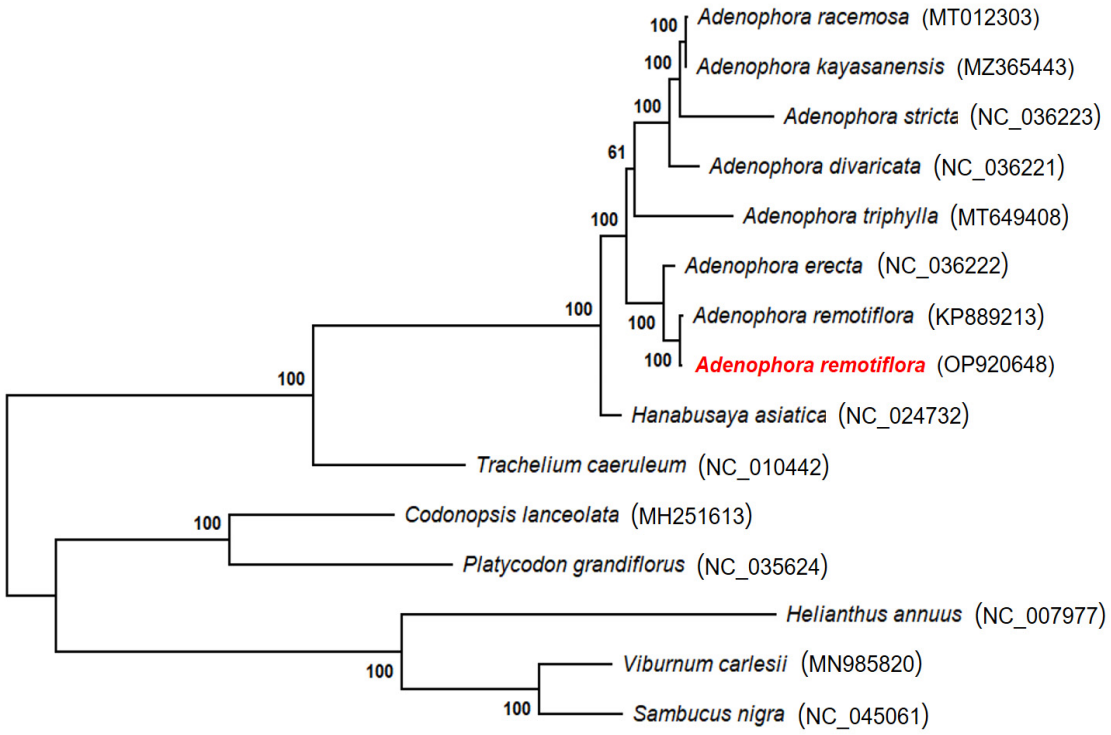

Figure 6. Phylogenetic diversity of 62 protein-coding genes commonly shared among 15 chloroplast genome sequences. The *Viburnum carlesii*, *Sambucus nigra*, and *Helianthus annuus* are outgroup. Numbers associated with branch nodes show the ML bootstrap support values. Red text indicates *A. remotiflora* used in this study.

4. Conclusions

Genome size and chloroplast genome were estimated in *A. remotiflora.* The complete chloroplast genome features were compared among eight *Adenophora* species. The chloroplast genome was characterized based on multilayered alignments, repeat sequences, comparative divergence, and boundary regions. The genome structure is well conserved throughout the *Adenophora* genus, except for *A. triphylla.* These data will increase the available sequences for the Campanulaceae family, and 155 identified repeats have the potential to be used as molecular markers for the *Adenophora* species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14010275/s1, Figure S1. Schematic representation of the eight *cis*-splicing genes in *A. remotiflora*. Table S1. Characteristics of simple sequence repeats in *A. remotiflora*, Table S2. Characteristics of the dispersed repeats in *A. remotiflora*. Table S3. Characteristics of the tandem repeats in *A. remotiflora.*

Author Contributions: Supervision, and Data validation: W.-S.Y. and Y.-K.K.; Data collection, Information analysis, Writing, and Editing: W.-S.Y., C.-K.K., and Y.-K.K. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The sequences of the *Adenophora remotiflora* Miq. chloroplast genome were deposited in the NCBI/GenBank with number PRJNA905878, including SAMN31887054 BioSample, and SRP410123 rad archive sequences.

Conflicts of Interest: The authors declare no conflicts of interest.

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