RESEARCH ARTICLE

The complete chloroplast genome sequences of three *Adenophora* species and comparative analysis with Campanuloid species (Campanulaceae)

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Abstract

We report the complete chloroplast genomes of three *Adenophora* species, and analyzed these compared them to five published Campanuloid plastomes. The total genome length of *Adenophora divaricata*, *Adenophora erecta*, and *Adenophora stricta* ranged from 159,759 to 176,331 bp. Among the eight Campanuloid species, many inversions were found to be only in the LSC region. IR contraction was also identified in the plastid genome of *Adenophora stricta*. Phylogenetic analyses based on 76 protein coding genes showed that Campanuloids are monophyletic, and are composed of two major groups: *Campanula s. str.* and *Rapunculus*. When we compared each homologous locus among the four *Adenophora* species, ten regions showed high nucleotide divergence value (>0.03). Among these, nine loci, excepting *ycf3-rpoB*, are considered to be useful molecular markers for phylogenetic studies and will be helpful to resolve phylogenetic relationships of *Adenophora*.

OPEN ACCESS

Citation: Cheon K-S, Kim K-A, Yoo K-O (2017) The complete chloroplast genome sequences of three *Adenophora* species and comparative analysis with Campanuloid species (Campanulaceae). PLoS ONE 12(8): e0183652. <https://doi.org/10.1371/journal.pone.0183652>

Editor: Xiu-Qing Li, Agriculture and Agri-Food Canada, CANADA

Received: January 20, 2017

Accepted: August 8, 2017

Published: August 22, 2017

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1D1A3B03931131), and Kangwon National University.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Campanulaceae *s. str.* consists of approximately 1,046 species that are primarily distributed in temperate regions [1–4]. This family was divided into three groups, including Platycodonoids, Wahlenbergioids, and Campanuloids based on the capsule morphology [5], which was strongly supported by molecular phylogenetic studies based on nuclear ribosomal ITS (nrITS) and three chloroplast DNA (cpDNA) markers [4, 6–7].

The genus *Adenophora*, which belong to Campanuloids, is a perennial herbaceous plant with approximately 50–100 species that are distributed in temperate regions in Asia and Europe. This genus was first described by Fischer [8], and then, a classification system was established based on various studies [9–17] according to morphological characteristics, such as the phyllotaxy, presence or absence of petioles, shape of the calyx, and length of the disk. However, there are still many different opinions regarding the sections and subsections within the *Adenophora* classification system because very similar morphological characteristics are found the species. Additionally, many taxonomic studies [4, 6–7, 18–27] have been conducted in

Campanulaceae, however, the phylogenetic relationships among the *Adenophora* remain to be elucidated.

Among the three *Adenophora* species discussed in this study, *A. erecta* is a very important species since it is an endemic species to Ulleung-do Island in Korea. This species was first described as a new species in 1997 [28] and is distinguished from *A. remotiflora* because the leaves are compactly arranged along the upper part of the stem, while the length of the disc is shorter than the width. Furthermore, *A. divaricata* and *A. stricta* are distributed in China, Japan, and Korea. In addition, *A. divaricata* differs from *Adenophora pereskiiifolia* because the cylindrical disk is more than 1.2 times longer than the width, while the presence of trichome in the calyx tube is characteristic of *A. stricta* and distinguishes *A. stricta* from closely allied taxa, such as *Adenophora lamarckii* and *Adenophora polyantha* [29].

The structure of the chloroplast genome is highly conserved among land plants [30]. However, gene order changes due to rearrangements sometimes occur and can be used to obtain very important phylogenetic information [31–33]. Campanulaceae are known to have very different chloroplast genome structures in a genus or species due to many rearrangements [30–31, 34–38]. Therefore, the chloroplast genome structure of Campanulaceae is very useful for identifying their unclear phylogenetic relationships. However, plastid genome research studies in Campanulaceae have not been extensively performed. Only a few species (*Adenophora remotiflora*, *Campanula punctata*, *Campanula takesimana*, *Hanabusaya asiatica*, and *Trachelium caeruleum*) were sequenced for the whole cp genome [30, 35–38].

In this study, we report the chloroplast genome sequences of three *Adenophora* species (*A. divaricata*, *A. erecta*, *A. stricta*), and compared these sequences to five published Campanuloid chloroplast genomes. The specific goals of this study were to (1) confirm the genome features of the three *Adenophora* species, (2) the changing tendency of the chloroplast genome structure of Campanuloids, and (3) identify the divergence hotspot regions to provide information regarding useful molecular markers for future phylogenetic studies in *Adenophora*.

Materials and methods

Ethics statement

Adenophora divaricata, *A. erecta*, and *A. stricta* are not an endangered or protected species. We did not collect plant materials from any privately owned or protected area that required permission. The plant materials of *A. divaricata*, *A. erecta*, and *A. stricta* were collected at Mt. Hanseok (38°03'21"N, 128°16'52"E) in Gangwon-do Province, Ulleung Island (37°32'55"N, 130°54'19"E) in Gyeongsangbuk-do Province, and Sunyu Island (35°48'47"N, 126°24'31"E) in Jeollabuk-do Province in South Korea, respectively.

Voucher specimens

The voucher specimen of three species was deposited at Kangwon National University Herbarium (KWNU). The voucher numbers are KWNU87503 (*A. divaricata*), KWNU80073 (*A. erecta*), and KWNU79662 (*A. stricta*).

DNA extraction, sequencing, assembly and genome mapping

Total DNA was extracted from approximately 100 mg of fresh leaves using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). The total genomic DNA was used for sequencing by an Illumina MiSeq (Illumina Inc., San Diego, CA, USA) platform at LabGenomics (<http://www.labgenomics.com/>). Three *Adenophora* species were sequenced to produce 3,754,935–4,223,586 raw reads with a length of 301 bp. These paired-end reads were aligned of

Adenophora remotiflora cp genome (accession no. KP889213) as a reference. After screening these paired-end reads through alignment with *A. remotiflora* cp genome, 325,221 to 560,114 reads were extracted. A *de novo* assembly was performed using Geneious v.7.1.8 (Biomatters Ltd., Auckland, New Zealand). The chloroplast genome coverage was estimated using the CLC Genomics Workbench v7.0.4 software (CLC-bio, Aarhus, Denmark). The cp genome coverages of the sequencing data of *A. divaricata*, *A. erecta*, and *A. stricta* were 524, 779, and 947×, respectively. In addition, the junction of large single copy (LSC), small single copy (SSC), and inverted repeat (IR) regions, along with the end points of inversion and IR contraction, were reconfirmed by PCR and Sanger sequencing.

The protein coding genes, tRNAs, and rRNAs in the plastid genome were predicted and annotated by Dual Organellar GenoMe Annotator (DOGMA) using the default parameters [39]. Based on this initial annotation, the putative starts and stops and the intron positions were determined by comparisons with homologous genes in other Campanulaceae cp genomes. tRNAs were confirmed using tRNAscan-SE [40]. A circular plastid genome map was drawn using the OGDRAW program [41].

Comparative analysis of the genome structure and phylogenetic analysis

The chloroplast genome sequences of five Campanuloid species, i.e., *Adenophora remotiflora* (accession no. KP889213), *Campanula punctata* (accession no. KU198434), *Campanula takesimana* (accession no. KP006497), *Hanabusaya asiatica* (accession no. KJ477692), and *Trachelium caeruleum* (accession no. EU090187), were obtained from the GenBank. A genome structure comparison of eight Campanuloid species, including the three *Adenophora* species in this study and five downloaded Campanuloid species, was performed using Mauve [42].

In total, 76 protein coding genes (S1 Table) in the eight Campanuloid species and one out-group (*Brighamia insignis*, KT372780) were compiled into a single file and aligned with MAFFT v.7 [43]. In addition, the *rpl23* and *infA* genes were excluded from the phylogenetic analysis data matrix, since most of these gene regions were deleted, and only a few regions existed. Before Maximum likelihood (ML) analysis, a search for the best fitting substitution model was performed using jModeltest v. 2.1.10 [44]. Based on the Akaike Information Criterion (AIC) and Akaike Information Criterion with Correction (AICc), GTR+I was the best model. ML analysis was performed using RAxML v7.4.2 [45] with 1,000 bootstrap replicates and the GTR+I model. Bayesian inference was performed using MrBayes v3.0b3 [46].

Divergence hotspot identification in *Adenophora*

Four chloroplast genomes, including *A. divaricata*, *A. erecta*, *A. remotiflora* and *A. stricta*, were analyzed to identify rapidly evolving molecular markers that can be used in future phylogenetic studies in *Adenophora*. Both coding genes and non-coding region fragments >200 bp were extracted separately from each plastid genome by applying the “Extract” option in Geneious v.7.1.8 (Biomatters Ltd., Auckland, New Zealand). Then, the homologous loci were aligned individually using MAFFT v.7 [43]. To analyze the nucleotide diversity (π), the total number of mutations (Eta), the average number of nucleotide differences (K) and the parsimony informative characters (PICs) were determined using DnaSP v.5.10 [47].

Results

Genome features of the three *Adenophora* species

The chloroplast genomes of *A. divaricata* (accession no. KX462129), *A. erecta* (accession no. KX462130), and *A. stricta* (accession no. KX462131) have been submitted to GenBank of

Table 1. Comparison of chloroplast genome features of three *Adenophora* species.

Feature	<i>Adenophora divaricata</i>	<i>Adenophora erecta</i>	<i>Adenophora stricta</i>
Genome size (bp)	176,331	173,324	159,759
LSC	113,353	105,861	112,321
SSC	8,648	11,267	27,238
IR	27,165	28,098	10,100
Number of unique protein coding genes	78	78	78
Number of tRNAs	30	30	30
Number of rRNA	4	4	4
G+C (%)			
LSC	37.1	37.5	37.1
SSC	33.0	35.0	35.4
IR	42.2	41.8	51.0
Total genome	38.5	38.7	38.5

<https://doi.org/10.1371/journal.pone.0183652.t001>

National Center for Biotechnology Information (NCBI). The plastid genome sizes of three *Adenophora* species ranged from 159,759 to 176,331 (Table 1 and Fig 1). The genome is composed of an LSC region (105,861–113,353 bp), SSC region (8,648–27,238 bp), and two IR copies (10,100–28,098 bp). Their overall GC contents are almost identical (38.5–38.7%). The chloroplast genomes of the three *Adenophora* species each contain 112 unique genes (Table 1). In the three *Adenophora* cp genomes, the following three genes (*rpl23*, *infA*, and *clpP*) are presumably nonfunctional: (1) only 46 bp of the 3' end of *rpl23* exists, (2) only 202 bp in the middle of *infA* remain, and (3) only 38 bp in exon1 of *clpP* exists. Additionally, two tRNAs (*trnI*-CAU and *trnV*-GAC) and one gene (*psbJ*) had an additional one and two copies, respectively. In the chloroplast genomes of two species (*A. divaricata* and *A. erecta*), a portion of three genes (*psbB*, *ycf3*, and *rrn23*) was duplicated in the IRs. In *rps12*, the 3' exon and 5' exon were located in the LSC and IRs, respectively. However, the 5' exon in *rps12*, and portion of *ycf3* and *rrn23* were located in the SSC in *A. stricta* due to the IR contraction (Fig 2).

Comparison of the chloroplast genome structure in eight Campanuloid species

The gene order changes in the plastid genomes of the eight Campanuloid species were confirmed only in the LSC region (except for *A. stricta*). The gene order among the three *Campanula s. str.* species (*C. punctata*, *C. takesimana*, and *T. caeruleum*) and between the two sect. *Remotiflorae* species (*A. erecta* and *A. remotiflora*) of *Adenophora* was exactly the same (S1 Fig).

Among the three *Campanula s. str.* species and *Hanabusaya*, two inversions of large gene blocks (*trnT*-*ndhC* and *psbM*-*trnS*) were found. In addition, two inversions between *Hanabusaya* and sect. *Remotiflorae* and between sect. *Remotiflorae* and the remaining *Adenophora* species (*A. divaricata* and *A. stricta*) were identified (Fig 3).

The IR/LSC and IR/SSC borders in the eight Campanuloid plastid genomes were compared (Fig 4). Two genes (*ycf2* and *trnH*-GUG) in the eight Campanuloid species were all located in the LSC. *Ycf2* was separated from the LSC/IRb border by 191 bp (*C. punctata* and *C. takesimana*) to 341 bp (*T. caeruleum*), and *trnH*-GUG was separated from the IRa/LSC border by 118 bp (in five *Rapunculus* species) to 140 bp (*T. caeruleum*). In addition, *trnL*-CAA was found in the IR (separated from the LSC/IRb and IRa/LSC border by 172–180 bp), and *ndhF* was

Fig 1. Chloroplast genomes of *A. divaricata*, *A. erecta*, and *A. stricta*. Genes inside the circle are transcribed clockwise, and genes outside the circle are transcribed counter-clockwise. The dark-gray inner circle corresponds to the GC content, and the light-gray represents the AT content. (A) *A. divaricata* chloroplast genome, (B) *A. erecta* chloroplast genome, and (C) *A. stricta* chloroplast genome.

<https://doi.org/10.1371/journal.pone.0183652.g001>

<https://doi.org/10.1371/journal.pone.0183652.g002>

<https://doi.org/10.1371/journal.pone.0183652.g003>

Fig 4. Comparison of the LSC, IR, and SSC junction positions in the eight Campanuloid chloroplast genomes.

<https://doi.org/10.1371/journal.pone.0183652.g004>

located in the SSC (separated from the IRb/SSC border by 105–218 bp). The IRb/SSC borders extended into *ndhE* to create a *ndhE* pseudogene in seven species (except for *A. stricta*). The lengths of the *ndhE* pseudogene were 159 bp in two species of *Campanula* and 158 bp in the remaining five species. In the *A. stricta* cp genome, the *psbB* pseudogene was duplicated in the IR near the IRb/SSC and SSC/IRa border, while the *clpP* pseudogene was located in the SSC adjacent to the SSC/IRa border.

Meanwhile, the cp genome of *A. stricta* lost duplication copies of *ndhG*, *ndhI*, *ndhA*, *ndhH*, *rps15*, *ycf1*, 5' exon of *rps12*, and *clpP* due to the IR contraction (Fig 2).

Phylogenetic analysis of eight Campanuloid species using 76 protein coding genes

The ML tree, which used 76 protein coding genes from the eight species, revealed that Campanuloids were monophyletic. The Campanuloids formed the following two clades: The *Campanula s. str.* clade and the *Rapunculus* clade. All nodes in the phylogenetic tree were strongly supported, with 100% bootstrap (BP) values and 1.00 Bayesian posterior probabilities (PP).

In the *Campanula s. str.* clade, *T. caeruleum* is the earliest diverging lineage, which was identified as a sister to the other species in this group. Within the *Rapunculus* clade, *H. asiatica* formed a basal branch and was a sister to all other taxa. Additionally, *A. erecta* and *A. remotiflora* as well as *A. divaricata* and *A. stricta* formed a clade (Fig 5).

Divergence sequence hotspot regions in *Adenophora*

In total, 141 loci (62 coding genes and 79 non-coding regions) were compared across the four *Adenophora* species. The Pi values ranged from 0 (*petB*, *trnI* intron, and *rrn23-rps15*) to

Fig 5. Phylogenetic tree reconstruction based on 76 protein coding genes using the ML. Bootstrap values are shown below the clades, and Bayesian posterior probabilities are shown above the clades.

<https://doi.org/10.1371/journal.pone.0183652.g005>

Fig 6. Comparison of the Pi value in four *Adenophora* species. (A) Coding genes and (B) Non-coding regions.

<https://doi.org/10.1371/journal.pone.0183652.g006>

0.17886 (*ndhF-rpl32*). Ten of these loci, i.e., *rpoA-petD* (0.06819), *psbT-psbB* (0.03145), *psbB-rpl20* (0.10200), Ψ *psbJ-trnD* (0.03801), *trnC-petN* (0.08722), *trnV-ΨpsbJ* (0.04408), *ycf3-rpoB* (0.10918), *ndhB-trnI* (0.07050), *ndhF-rpl32* (0.17886), and *ycf1* (0.03011), showed high values (greater than 0.03). Additionally, eight of these regions were located in the LSC, while *ycf1* and *ndhF-rpl32* were located in the IR and SSC, respectively (Fig 6 and S2 Table).

Discussion

Chloroplast genome organization in Campanuloids

The LSC, SSC, and IR regions in the three *Adenophora* cp genomes varied in length. In particular, the length of the IR regions in *A. stricta* was significantly shorter than that in the other species, which is thought to be due to the IR contraction. The length of the LSC region in *A. erecta* was approximately 7,000 bp shorter than that in the other species, and the lengths of the SSC and IR regions in *A. erecta* were approximately 2,500 bp and 900 bp longer than those in the *A. divaricata* cp genome, respectively. It is believed that the length difference in the LSC region was due to a change in the length of the intergenic spacer (IGS) in *psbJ-ndhC*, *trnT(UGU)-psbJ* and *psbJ-ycf3*, which is the end point of the inversion. Additionally, the length difference in the SSC and IR regions was identified to be caused by the length difference in the IGS regions in portion of *ycf3-rps12(5')*, which is located in the IR regions, and *ndhF-rpl32*, which is located in the SSC region, regardless of the inversion.

The Campanuloid plastomes were identified to have a very different gene order when compared to the cp genome of *B. insignis* belonging to Campanulaceae *s. l.* This is important evidence that many rearrangements in the cp genome occurred among the Campanulaceae species. However, it is very difficult to estimate the exact gene order change process by only the cp genomes analyzed so far, since the plastid genome of species belonging to Wahlenbergioids and Platycodonoids has not been analyzed in any species to date.

The results of this study revealed that the chloroplast genome structure of two species of *Campanula* (*C. punctata* and *C. takesimana*) was almost identical to that of *Trachelium* regarding the gene order and duplication of certain genes. In addition, we confirmed that the genome structures two *Remotiflorae* species (*A. erecta* and *A. remotiflora*) of *Adenophora* were the same. Therefore, we believe that the cpDNAs of members of *Campanula s. str.* and sect. *Remotiflorae* evolved via identical routes. Genome structure modifications in the phylogenetic groups of *Campanula s. str.* and *Rapunculus* were identified only in the LSC region (except for *A. stricta*). Duplicated copies of *psbJ* and *trnV-GAC* were located at the inversion endpoint (Fig 3). This duplication might be attributed to inversions, and these duplicated genes might have caused multiple inversion events within Campanuloids.

Many interspecific and intergeneric relationships of Campanuloids currently remain unclear. Specifically, the phylogenetic relationships among *Adenophora* are currently unresolved. In this study, the genome structures of each section of *Adenophora*, such as sect. *Remotiflorae* (*A. erecta* and *A. remotiflora*), sect. *Platyphyllae* (*A. divaricata*), and sect. *Microdiscus* (*A. stricta*) were different. Therefore, we believe that the chloroplast genome structure can be used as an important characteristic to differentiate the *Adenophora*.

Among the 11 plastid-encoded *ndh* genes, a loss of function of *ndhK* in the chloroplast genome has been reported in *Pinus thunbergii* and *Phalaenopsis aphrodite* [48–49]. Haberle et al. [30] have suggested that *ndhK* might be a pseudogene in *T. caeruleum* because it contains multiple internal stop codons. We compared *ndhK* in *T. caeruleum* to that in the seven Campanuloid species. The results of the comparison revealed that *ndhK* in *T. caeruleum* was not a pseudogene. Its earlier identification as a pseudogene might be due to an error in the annotation process. In conclusion, *ndhK* in the chloroplast genomes of Campanuloid species is a functional gene (S2 Fig).

Phylogenetic implications

Recent phylogenetic studies [4, 6–7] have proposed that Campanuloids can be divided into the following two clades: *Campanula s. str.* clade and *Rapunculus* clade. The results of this study were based on 76 protein coding genes, and these two clades were well supported and clearly distinguished by the gene order in the plastid genome structure (Fig 5).

The phylogenetic tree constructed in this study showed that *A. erecta* and *A. remotiflora* (sect. *Remotiflorae*) formed a sister clade to the *A. divaricata* (sect. *Platyphyllae*) and *A. stricta* (sect. *Microdiscus*) clade (Fig 5). Therefore, it is hypothesized that sect. *Remotiflora* had the earliest divergence among the section of *Adenophora*. Additionally, sect. *Platyphyllae* had an earlier divergence from a common ancestor than sect. *Microdiscus* because *A. stricta* had a unique IR structure among the Campanulaceae due to the IR contraction (Figs 2 and 5).

Meanwhile, *H. asiatica* is a very important plant resource since it is monotypic and one of the six endemic genera in Korea. It was first described as *Symphyandra asiatica* Nakai [50] due to its connate anthers but was segregated into a new genus, *Hanabusaya*, based on its morphological characteristics [51]. However, the phylogenetic position of *Hanabusaya* as an endemic genus remain unclear despite the many studies that have been performed [7, 18, 24–26, 52]. In this study, *Hanabusaya* formed a sister clade to the *Adenophora* clade. Thus, *Hanabusaya* has

evolved through a different evolutionary route than the morphologically similar *Campanula s. str.* species, including *Campanula* and *Symphyanthra*. Additionally, *Hanabusaya* has a unique cp genome structure compared to that in the other species used in this study (Fig 3). Therefore, the phylogenetic position of *Hanabusaya* was well supported in this study as an endemic genus. However, only a few species were included in this study, and thus, we believe that further studies that include various species are needed to clarify the phylogenetic position of *Hanabusaya*.

Useful molecular markers information for the phylogeny of *Adenophora*

Despite the many phylogenetic studies performed [4, 6, 25–26], the phylogenetic relationships among the *Adenophora* species remain unclear. We believe that this is due to nucleotide variations in molecular markers, such as *atpB*, *matK*, *rbcL*, *atpB-rbcL*, *atpF-atpH*, *rpl16*, *rpoC1*, and *trnL-trnF*, which were very low in previous studies (Fig 6 and S2 Table).

The results of this study showed that the nucleotide diversity in ten regions, including one coding gene and nine non-coding regions, had relatively high calculated values (>0.03). However, among these regions, one non-coding region (*ycf3-rpoB*) was not considered suitable as a phylogenetic marker because it had a very small number of PICs compared to the Eta and/or aligned length (S2 Table).

Therefore, nine regions (*rpoA-petD*, *psbT-psbB*, *psbB-rpl20*, Ψ *psbJ-trnD*, *trnC-petN*, *trnV-ΨpsbJ*, *ndhB-trnI*, *ndhF-rpl32*, and *ycf1*) are considered useful markers for evaluating the phylogenetic relationships among the *Adenophora* species.

Conclusions

We provide the first report of the complete plastid genome sequences of *A. divaricata*, *A. erecta*, and *A. stricta* and compared these sequences to those of five Campanuloid species in Campanulaceae. The results of the genome structure comparison confirmed many inversions. The phylogenetic analyses showed that Campanuloids were divided two major groups. The divergence hotspots clarified in this study could be used as molecular markers that will be helpful for elucidating the phylogenetic relationships among the *Adenophora* species.

Supporting information

S1 Fig. The gene order comparison among eight Campanuloid chloroplast genomes using MAUVE program.

(TIF)

S2 Fig. Nucleotide alignment of chloroplast copies of *ndhK* genes for eight Campanuloid species.

(TIF)

S1 Table. The length and aligned length of 76 protein coding genes used for phylogenetic analyses.

(XLSX)

S2 Table. Eta, Pi value, and PICs of 141 homologous loci.

(XLSX)

Author Contributions

Conceptualization: KSC KOY.

Data curation: KSC KAK.

Formal analysis: KSC KAK.

Funding acquisition: KOY.

Investigation: KSC KAK KOY.

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