A trnI_CAU Triplication Event in the Complete Chloroplast Genome of Paris verticillata M.Bieb. (Melanthiaceae, Liliales)

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Abstract

The chloroplast is an essential plant organelle responsible for photosynthesis. Gene duplication, relocation, and loss in the chloroplast genome (cpDNA) are useful for exploring the evolution and phylogeny of plant species. In this study, the complete chloroplast genome of Paris verticillata was sequenced using the 454 sequencing system and Sanger sequencing method to trace the evolutionary pattern in the tribe Parideae of the family Melanthiaceae (Liliales). The circular double-stranded cpDNA of P. verticillata (157,379 bp) consists of two inverted repeat regions each of 28,373 bp, a large single copy of 82,726 bp, and a small single copy of 17,907 bp. Gene content and order are generally similar to the previously reported cpDNA sequences within the order Liliales. However, we found that trnI_CAU was triplicated in P. verticillata. In addition, cemA is suspected to be a pseudogene due to the presence of internal stop codons created by poly(A) insertion and single small CA repeats. Such changes were not found in previously examined cpDNAs of the Melanthiaceae or other families of the Liliales, suggesting that such features are unique to the tribe Parideae of Melanthiaceae. The characteristics of P. verticillata cpDNA will provide useful information for uncovering the evolution within Paris and for further research of plastid genome evolution and phylogenetic studies in Liliales.

Key words: trnI_CAU triplication, cemA pseudogenization, chloroplast genome, Paris verticillata, Melanthiaceae, Liliales.

Introduction

The chloroplast of plants is believed to have evolved through an endosymbiotic event in which a eukaryotic heterotrophic organism became host to a cyanobacterium, with an interaction between them resulting in chloroplast formation (Douglas 1998; McFadden 1999). It contains a circular double-stranded DNA molecule ranging in length from approximately 100 kb to over 160 kb (Sugiyura 1992). The chloroplast genome (cpDNA) has a quadripartite structure, which includes a large single copy (LSC), a small single copy (SSC), and two inverted repeat (IR) regions. The chloroplast genome contains genes that are responsible for photosynthesis (Sugiyura 1992) and is inherited maternally, paternally, and even biparentally (Corriveau and Coleman 1988; Dong et al. 1992; Birky 1995; Yang et al. 2000; Zhang et al. 2003; Hansen et al. 2007; McCauley et al. 2007). Generally, the gene content and order are highly conserved in cpDNA across plant species. Therefore, cpDNA protein-coding sequences are believed to be useful for exploring phylogenetic relationships among plant species. For example, Jansen et al. (2007) resolved the relationships among major clades of the angiosperms by analyzing 81 genes from the plastid genomes of 64 species. However, gene loss, relocation, and transformation to pseudogenes have also occurred. In parasitic plants such as those belonging to the genus Cuscuta, genes that encode photosynthesis proteins (e.g., ndh genes) are lost and have become pseudogenes due to the lifestyle of dependency on a host plant (McNeal et al. 2007). Lee et al. (2007) reported gene relocation in cpDNA of Jasminum and Menodora (Oleaceae), which was the result of multiple and overlapping inversions. In addition to these mutations, gene duplication events are another characteristic type of phylogenetically informative change in cpDNA. For example, trnF sequences were investigated and found to be duplicated in cruciferous plants, although only part of the gene was duplicated (Schmickl et al. 2007). For these reasons, the number of chloroplast genomes of green plants uploaded to NCBI has risen to 471, of which 372 are from angiosperms (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=2759&opt=plastid#pageTop, last accessed May 13, 2014).
Such increasing resources will allow exploration of evolution among plants. *Paris verticillata* M.Bieb (fig. 1) is a member of the tribe Parideae of the family Melanthiaceae (Angiosperm Phylogeny Group 2009). The species is widespread in East Asia and has been used as folk medicine for asthma and chronic bronchitis (Ahn 1998). Recently, a new phenolic amide and pyrrolizidine alkaloids extracted from the roots of *P. verticillata* were found to have potential cytotoxicity against human tumor cell lines (Lee et al. 2008; Kim et al. 2010). Although the medicinal features of *P. verticillata* have been studied, no genomic work on the plant has yet been conducted. We therefore analyzed the complete chloroplast genome sequence of *P. verticillata* and compared the cpDNA features with previously reported cpDNA in Liliales (Liu et al. 2012; Bodin et al. 2013; Do et al. 2013; Kim JS and Kim J-H 2013). Furthermore, we examined whether the specific changes in cpDNA of *P. verticillata* are found in other species in Liliales to investigate the evolutionary pattern and to provide useful molecular information for further research on this potential medicinal plant.

**Results and Discussion**

**Genome Assembly, Features, and Comparisons with Other Liliales cpDNAs**

The 96,169 reads (range: 40–680 bp) of *P. verticillata* cpDNA generated by the 454 system were assembled against the reference sequence of *Chionographis japonica* (Bodin et al. 2013). Out of them, 327 reads (0.34%) were assembled to *C. japonica*, with an average length of 365 bp and covering 35% of the reference sequences with 97% pairwise identity. Because of the low covering percentage of reads to reference sequence (35%) and low assembly coverage (1.1 x), PCR and Sanger sequencing methods were conducted to complete the cpDNA sequence and to confirm the regions assembled from 454 system reads (supplementary fig. S1, Supplementary Material online).

The cpDNA sequence of *P. verticillata* (accession number: KF433485) was complete and of length 157,379 bp, in which 82,726 bp encompass the LSC region, 17,907 bp the SSC region, and 28,373 bp the length of each IR region (fig. 2). The AT and GC contents are 62.4% and 37.6%, respectively (table 1). The cpDNA consists of 115 unique genes, which are composed of 81 protein-coding genes, 30 tRNAs, and 4 rRNAs. Among the protein-coding genes, nine genes contain one intron and three genes possess two introns (*ycf3*, *clpP*, and *rps12*) of which *rps12* was trans-spliced. In addition, 25 coding regions are duplicated in the IR region. However, only part of *ycf1* was duplicated in the junction between the IRB and SSC regions. The AT and GC contents of Melanthiaceae species were almost the same (62.3% and 37.7%, respectively), whereas these features are different in other families of Liliales (table 1).

The basic features within Liliales were compared among reported cpDNA sequences (table 1). The results indicated that the length of *P. verticillata* (157,379 bp) is similar to that of *Smilax china* (157,878 bp) and longer than those of other species (range: 152,793–155,510 bp). The AT and GC contents of Melanthiaceae species were almost the same (62.3% and 37.7%, respectively), whereas these features are different in other families of Liliales (table 1). The gene content and order are similar among the species examined, but *rps16* was deleted completely in *C. japonica* and partially in *Veratrum patulum*. In addition, *infA* was lost in *S. china*. The IR borders were expanded varyingly not only in Melanthiaceae but also in other taxa (table 1). The IR/SSC boundary was identified by the incomplete duplication of *ycf1* in all species examined, whereas the IRLSC junction was expanded to full *trnH_GUG* (*V. patulum*), part of *rps19* (*Alstroemeria aurea* and

![Fig. 1](https://example.com/f1.png) — Photographs of *Paris verticillata*. (A) Young plant, (B) plant with flower, and (C) close up of flower.
Lilium longiflorum), part of rpl22 (S. china), and part of rps3 (C. japonica and P. verticillata). In general, the cpDNA structure of P. verticillata is similar to those of other Liliales species, such as V. patulum (Do et al. 2013), C. japonica (Bodin et al. 2013), A. aurea, L. longiflorum (Kim JS and Kim J-H 2013), and S. china (Liu et al. 2012) (table 1). The gene content and order are also similar among the species examined. However, the loss of infA and rps16 as well as partial deletion of rps16, which is uncommon in the Liliales, could be unique evolutionary events in such species.
The expansion and contraction of the IR region are highly variable among species, within not only Melanthiaceae but also the order Liliales, in that the junctions were differentiated from trnH_GUG to rps3 (table 1). The IR junction of P. verticillata is in agreement with a previous report (Wang et al. 2008), which suggested that the junction of the order Liliales IR/LSC region included the trnH-rps19 cluster. However, to date, the IR regions have only been examined in four of ten families of Liliales (Bodin et al. 2013; Do et al. 2013). Therefore, further studies covering the junctions of IR and LSC as well as the SSC region in all families of Liliales are required.

**trnI_CAU Triplication and cemA Pseudogenization**

The length of the intergenic spacer (IGS) between rpl23 and ycf2, which contains trnI_CAU, varies among P. verticillata and other Liliales species (303–591 bp; table 1). Paris verticillata possesses the longest IGS, containing three copies of trnI_CAU (fig. 3A). Such a pattern was not detected in other complete cpDNAs from Liliales (Liu et al. 2012; Bodin et al. 2013; Do et al. 2013). Therefore, further studies covering the junctions of IR and LSC as well as the SSC region in all families of Liliales are required.

**Table 1**

Characteristics of Chloroplast Genomes among Liliales

| Species (Family) | Accession number | Protein-coding genes | tRNAs | rRNAs | Length (bp) | AT content (%) | GC content (%) | IRB–SSC junction | IRA–LSC junction | Length of IGS between rpl23 and ycf2 (bp) |
|------------------|------------------|----------------------|--------|--------|-------------|----------------|----------------|----------------|----------------|----------------------------------|
| *Paris verticillata* (Melanthiaceae) | KJ433485 | 81 | 30 | 4 | 157,379 | 62.4 | 37.6 | ycf1 (pseudogene) | rps3 (pseudogene) | 591 bp |
| *Chionographis japonica* (Melanthiaceae) | KF951065 | 80 | 30 | 4 | 154,646 | 62.3 | 37.7 | ycf1 (pseudogene) | rps3 (pseudogene) | 303 bp |
| *Veratrum patulum* (Melanthiaceae) | KF437397 | 81 | 30 | 4 | 153,699 | 62.3 | 37.7 | ycf1 (pseudogene) | rps3 (pseudogene) | 305 bp |
| *Lilium longiflorum* (Liliaceae) | KC968977 | 81 | 30 | 4 | 152,793 | 62.98 | 37.02 | rps19 (pseudogene) | trnH_GUG | 308 bp |
| *Smilax china* (Smilacaceae) | HMS6959 | 80 | 30 | 4 | 157,878 | 62.75 | 37.25 | rps19 (pseudogene) | trnH_GUG | 308 bp |
| *Alstroemeria aurea* (Alstroemeriaceae) | KC968976 | 81 | 30 | 4 | 155,510 | 62.74 | 37.26 | rps19 (pseudogene) | trnH_GUG | 308 bp |

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![Diagram](https://example.com/diagram.png)

**Fig. 3.**—Illustration of trnI_CAU composition in Paris verticillata. (A) Positions of the trnI_CAU copies. (B) The nucleotide sequence of the rpl23-ycf2 IGS of *P. verticillata*, in which three tandem repeat units are highlighted in different colors. The bold italic characters indicate the sequences of *trnI_CAU*.
found in legume chloroplast genomes were generated by homology-facilitated illegitimate recombination. We suggest that the trnl_CAU duplication events may be attributable to homology-facilitated illegitimate recombination, which has occurred twice to generate three tandem repeats in P. verticillata.

The cemA-coding sequence of P. verticillata contained a poly(A) sequence (9 bp) and a small single repeat (SSR) CA unit (fig. 4A). The other species of Liliales have poly(A) sequences of variable lengths (8–12 bp) following the start codon, and C. japonica has the longest such sequence (12 bases). However, the CA SSR was not detected in these taxa. Although the lengths of poly(A) sequences are variable, the amino acid sequences are very similar (fig. 4B). In contrast, the poly(A) sequence and the SSR unit caused a frameshift mutation in cemA of P. verticillata. Consequently, the cemA amino acid sequence of P. verticillata differs from those of other species, except for the first four amino acids (fig. 4B).

In addition to the pseudogenization of hypotetical open-reading frames (ycf15, ycf68) in C. japonica, V. patulum, A. aurea, L. longiflorum, and S. china, dysfunctional protein-coding genes have also been reported in many land plants. Lin et al. (2012) reported the pseudogenization of rpl23 in G. biloba caused by the truncated 5′-region. The loss of functional genes has also been found in parasitic plants. For example, many genes such as atpB, rbcL, ndhF, and rpoC2 were found to have been lost or to be nonfunctional in Cistanche deserticola due to its parasitic lifestyle with its host Haloxylon ammodendron (Li et al. 2013). The cemA gene, assumed to encode a b-type heme protein of unknown function (Willey and Gray 1990), was reported to have been lost in C. deserticola, Epifagus virginiana, Rhizanthella gardneri, and Neotettix nidus-avis (Wolfe et al. 1992; Delannoy et al. 2011; Logacheva et al. 2011; Li et al. 2013). Different lengths of poly(A) sequence have also been observed in other species (Yang et al. 2010). Although transcriptome data have been analyzed, Yang et al. (2010) concluded that whether cemA can be translated into protein remains unclear. The cemA sequenced in this study is suspected to be pseudogene because of the presence of several stop codons caused by the poly(A) sequence and SSR of CA at the beginning of the coding region in P. verticillata (tribe Melanthieae of the Melanthiaceae; fig. 4B). However, it is thought to be functional in other species such as V. patulum (tribe Melanthieae of Melanthiaceae), C. japonica (tribe Chionographideae of Melanthiaceae), A. aurea (Alstroemeriaceae), L. longiflorum (Liliaceae), and S. china (Smilacaceae) because of the absence of internal stop codons. Consequently, this mutation may occur only in the tribe Parideae of Melanthiaceae and could be useful for further research in not only Melanthiaceae but also Liliales species. The loss of cemA in parasitic plants could be explained by the dependence on the host plants. However, P. verticillata is autotrophic. Therefore, further research is required to clarify the impact of cemA pseudogenization in this species.

**Phylogenetic Analysis**

Phylogenetic relationships between species in Liliales and other monocots, and dicots, were explored (fig. 5). The results showed that Liliales was a monophyletic group; the bootstrap value was high (BP 100). Liliales is a sister group of other monocots, and dicots, were explored (fig. 5). The results showed that Liliales was a monophyletic group; the bootstrap value was high (BP 100).
**Fig. 5.**—Phylogenetic tree inferred by RAxML using nucleotide sequences of 76 protein-encoding regions from 40 species. Bootstrap values (>50) are shown above the branches. The light green color box shows the eudicots group whereas the light gray color box indicates the monocots species. The names in the right side of phylogenetic tree represent the classification of species at order level.
and Lolium perenne), Zingiberales (Musa acuminata subsp. malaccensis), and Arecales (Cocos nucifera and Phoenix dactylifera) (BP 91). Within Liliales, Melanthiaceae (including P. verticillata, V. patulum, and C. japonica) is sister to the Smilacaceae (S. china) and Lilieae (L. longiflorum). Also, the Alstroemeriaceae (A. aurea) is sister to the Colchicaceae (Colchicum autumnale and Gloriosa superba). The familial relationships defined in this study were identical to those delineated in a previous work (Kim et al. 2013), in which relationships among all families in the Liliales were investigated.

Conclusions

Here, we report the first data of trnI_CAU triplication and cemA pseudogenization in Melanthiaceae inferred from the complete cpDNA sequence of P. verticillata. Notably, these features were not noted in the previous studies on cpDNA of either the Melanthiaceae or the Liliales. Therefore, these patterns will be useful for understanding the phylogeny and evolution of these species. However, the detailed mechanisms and evolutionary impacts of these findings remain unclear, and further investigations are therefore required.

Materials and Methods

Taxon Sampling, cpDNA Extraction, Sequencing, and Assembly

Paris verticillata was collected in South Korea, and a voucher specimen was deposited in the herbarium of Gachon University (voucher number: GCU02222). The plant materials used in this study were obtained from the Korean National Research Resource Center (Medicinal Plants Resources Bank NRF-2010-0005790) supported by the Korea Research Foundation with resources provided by the Ministry of Education, Science, and Technology in 2013. Fresh leaves (50 g) of P. verticillata were used for chloroplast isolation employing the Percoll gradient buffer method (Kim JS and Kim J-H 2013). A DNeasy Plant Mini Kit (Qiagen, Seoul, South Korea) was used to extract cpDNA from purified chloroplasts. The 454 sequencing system (Roche Applied Science, Penzberg, Germany) was employed to sequence cpDNA of P. verticillata. The raw data were uploaded to Geneious and adjusted manually. The tRNAscan-SE (Schattner et al. 2005) was used to confirm tRNAs. Ambiguous bases in coding regions were checked using data in NCBI (http://blast.ncbi.nlm.nih.gov/, last accessed May 13, 2014) and confirmed by Sanger sequencing. In particular, specific primer pairs were used to verify triplication of trnI_CAU and pseudogenization of cemA, ycf15, and ycf68. The cpDNA map of P. verticillata was constructed using GenomeVx (Connant and Wolfe 2008). The sequences of cemA were aligned using MUSCLE (Edgar 2004), which is included in the Geneious program, and manual adjustments were made when necessary. Sequencher version 5.0 (Gene Codes Co., Ann Arbor, MI) was used to assemble complete sequences of trnI_CAU and cemA. The REPuter program (Kurtz et al. 2001) was used to detect repeat units in the rpl23–ycf2 regions.

Phylogenetic Analysis

Phylogenetic analysis was performed using data on 76 protein-encoding genes from cpDNA sequences of 40 species (supplementary table S1, Supplementary Material online). The nucleotide sequences were aligned using CLUSTALW (Hall 1999), which is included in the Geneious program. Phylogenetic trees were reconstructed using RAxML (Stamatakis et al. 2008), which is available online (http://embnet.vital-it.ch/raml-bb/index.php, last accessed May 13, 2014). Substitution of GTR+G was modeled for the entire data matrix. Maximum-likelihood bootstrap analysis was calculated using 100 replications employing the rapid bootstrapping approach implemented in RAxML. The phylogenetic tree was drawn using FigTree v1.3 program.

Supplementary Material

Supplementary figure S1 and table S1 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org).
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