Lineage-Specific Variation in IR Boundary Shift Events, Inversions, and Substitution Rates among Caprifoliaceae s.l. (Dipsacales) Plastomes

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Abstract: Caprifoliaceae s.l. plastid genomes (plastomes) show that one inversion and two inverted repeat boundary shifts occurred in the common ancestor of this family, after which the plastomes are generally conserved. This study reports plastome sequences of five additional species, Fedia cornucopiae, Valeriana fauriei, and Valerianella locusta from the subfamily Valerianoideae, as well as Dipsacus japonicus and Scabiosa comosa from the subfamily Dipsacoideae. Combined with the published plastomes, these plastomes provide new insights into the structural evolution of plastomes within the family. Moreover, the three plastomes from the subfamily Valerianoideae exhibited accelerated nucleotide substitution rates, particularly at synonymous sites, across the family. The patterns of accD sequence divergence in the family are dynamic with structural changes, including interruption of the conserved domain and increases in nonsynonymous substitution rates. In particular, the Valeriana accD gene harbors a large insertion of amino acid repeat (AAR) motifs, and intraspecific polymorphism with a variable number of AARs in the Valeria accD gene was detected. We found a correlation between intron losses and increased ratios of nonsynonymous to synonymous substitution rates in the clpP gene with intensified positive selection. In addition, two Dipsacoideae plastomes revealed the loss of the plastid-encoded rps15, and a potential functional gene transfer to the nucleus was confirmed.

Keywords: accD; amino acid repeat motifs; clpP; intron loss; positive selection; intracellular gene transfer

1. Introduction

The plastid genome (plastome) of angiosperms is generally conserved and has a quadripartite structure with a pair of inverted repeats (IR) separated by large and small single-copy (LSC and SSC) regions [1]. Plastomes generally range from 120 to 160 kb in length and contain 113 unique genes, 79 protein-coding genes, 30 tRNAs, and 4 rRNAs. However, accumulating data on complete plastome sequences exhibit variations in gene and intron content [2]. Plastid gene loss requires functional transfer to the nucleus before the loss in its plastome [3]. Extensive genome rearrangements, including IR boundary shifts, have also been reported for several lineages such as Campanulaceae [4], Caryophyllaceae [5], Fabaceae [6], Geraniaceae [7], Oleaceae [8], and Papaveraceae [9,10].

Lineage-specific variation in the rate of plastome sequence evolution has been documented in angiosperms [5,11–13]. Higher substitution rates are associated with structural rearrangements [5,14]. Accelerated rates of nucleotide substitution are affected by the whole genome as well as a subset of protein-coding genes. Compared with other plastid-encoded genes, acetyl-CoA carboxylase subunit β (accD), ATP-dependent Clp protease proteolytic subunit (clpP), DNA-directed RNA polymerase subunit α (rpoA), some subunits of ribosomal proteins, and the chloroplast factors ycf1, ycf2, and ycf4, show dynamic acceleration [5,9,11,15–18]. Multiple mechanisms, including dysfunction of DNA replication,
repair, and recombination (DNA-RRR) machinery, localized hypermutation, mutagenic retroprocessing, and pseudogenization, have been hypothesized to explain this acceleration [11,14,16,19].

The most extreme accelerations are found in the plastid-encoded accD and clpP, which have undergone a history of insertions and deletions (indels) of amino acid sequences or intron losses in multiple independent lineages, respectively [9,15]. Several angiosperm lineages have experienced losses in the accD or clpP genes [10,20,21]. The plastid-encoded accD has been functionally replaced via gene transfer to the nucleus or gene substitution in eukaryotic ACCase [15,16,20–26]. However, clear evidence of plastid clpP transfer to the nucleus in angiosperms is lacking. Highly divergent accD and clpP genes are likely caused by compensatory mutations in the interactions between nuclear-encoded plastid-targeted subunits [27,28]. This is because the function of the two genes depends on nuclear-encoded proteins that assemble plastid-localized subunits. For example, the plastid-encoded accD plays an essential role in the fatty acid biosynthesis pathway [29], which is a subunit of the prokaryotic acetyl–CoA carboxylase (ACC) complex [30]. The prokaryotic ACC complex consists of four subunits, three of which are nuclear-encoded proteins: acetyl–CoA carboxylase subunit α (ACCA), biotin carboxyl carrier protein subunit (ACCB), and biotin carboxylase subunit (ACCC). Plastid-encoded clpP is a subunit of the caseinolytic protease (CLP) complex [31] and is involved in multiple processes of chloroplast development [32]. Multiple subunits (CLPP2 to CLPP6) of the CLP complex are encoded in the nucleus [31]. Thus, the nuclear-encoded subunits interact with plastid-encoded subunits.

The honeysuckle family (Caprifoliaceae sensu lato [s.l.]) comprises approximately 825 species in 28–42 genera with widespread cosmopolitan distribution [33]. Caprifoliaceae s.l. has been classified into seven major groups (six subfamilies and one genus): Diervilloideae, Caprifolioideae, Linnaeioideae, Morinoideae, Dipsacoideae, Valerianoideae, and Zabelia (Rehder) Makino [34]. A recent phylogenomic study based on nuclear loci and plastome sequences suggested that Zabelia is recognized as a new subfamily of Zabelioideae [35]. To date, complete plastomes of 22 genera have been sequenced (National Center for Biotechnology Information; NCBI, accessed on 18 August 2021). The sequenced Caprifoliaceae s.l. plastomes range in size from 151.3 to 161.6 kb with a quadripartite organization. Variations in inverted repeat (IR) boundary shifts and nucleotide substitution rates have been documented in the Caprifoliaceae s.l. plastomes [34,36]. Despite broader sampling and sequencing across the family, very little is known about the gene evolution, including structural changes and rate variation in the plastid-encoded accD and clpP genes.

In this study, we generated the complete plastome sequences of three species from the subfamily Valerianoideae and two species from the subfamily Dipsacoideae. Genome organization and nucleotide substitution rates were estimated and compared to the published Caprifoliaceae s.l. plastomes. In particular, the correlation between structural evolution and nucleotide substitution rates in the plastid-encoded accD and clpP genes across this family was examined. In addition, we examined intra- and infraspecific length variations in the accD coding region of Valeriana fauriei Briq. and V. sambucifolia f. dageletiana (Nakai ex F.Maek.) Hara.

2. Results
2.1. Plastome Organization

We sequenced and assembled the complete plastomes of three Valerianoideae (Fedia cornucopiae (L.) Gaertn., V. fauriei, and Valerianella locusta (L.) Later.) and two Dipsacoideae (Dipsacus japonicus Miq. and Scabiosa conosa Fisch. ex Roem. & Schult.) species (Figure S1, see Supplementary Materials). Among the five species, the plastome size ranged from 149,809 bp (V. locusta) to 160,243 bp (D. japonicus) (Table 1). Dipsacus japonicus had the largest LSC (87,066 bp), whereas F. cornucopiae had the smallest SSC (15,862 bp). The GC content of the D. japonicus (38.8%) was higher than that of the other species (Table 1). The three Valerianoideae plastomes encoded 79 protein-coding genes, 30 tRNA genes, and 4 rRNA genes (Table 1). However, the ribosomal protein subunit S15 (rps15) appears to
be a pseudogene in two Dipsacoideae plastomes (Table 1). Functional replacement by gene transfer of *rps15* from plastid to the nucleus was detected in *Dipsacus* transcriptome data (Figure S2). The plastome of *D. japonicus* was missing *trnT-GGU* and contained two *trnE-UUC* with 90.4% nucleotide identity (Figure S3). The duplicated gene content in the IR region varied as a result of IR expansion and contraction. The intron content also varied owing to the loss of intron in the *clpP* gene. The plastome of *F. cornucopiae* was missing the first intron of the *clpP* gene. The plastome of *V. locusta* was missing both introns in the *clpP* gene (Figure S1).
Figure 1. Plastome rearrangement in the analyzed Caprifoliaceae s.l. Schematic diagrams (gray open boxes) of the genomic regions surrounding the inverted repeat (IR) region. Genes drawn below the horizontal line indicate sequences found in the common ancestor of Fedia, Valerianella, and Valeriana are illustrated (orange boxes).

2.2. Elevated Substitution Rates in the Plastomes of the Subfamily Valerianoideae

The 24 Caprifoliaceae s.l. genera and five Adoxaceae genera shared 72 plastid-encoded genes. We excluded seven protein genes from the concatenated data set and substitution rate analysis because they appeared to be pseudogenes or losses (rpl32, ndhF, ycf2, and ycf3) among some species within the family. To examine rate variation in the selected plastid genes among the 24 genera, nonsynonymous (dN) and synonymous (dS) substitution rates were estimated using the phylogenetic tree as a constraint tree (Figure 2). The dN and dS values in pairwise comparisons between Viburnum and Caprifoliaceae s.l. showed that Fedia, Valerianella, and Valeriana from the subfamily Valerianoideae had significantly higher dS rates than the other analyzed species (Wilcoxon rank-sum test, p < 0.001, after Bonferroni correction; Table S1), except for the comparison...
between Valeriana and Pterocephalus. Narodostachys and Patrinia had only significantly higher $d_N$ rates than Weigela (Wilcoxon rank-sum test, $p < 0.05$, after Bonferroni correction; Table S2). In the case of $d_S$ rates, only 11 comparisons between Fedia and six species (Kolkwitzia, Symphoricarpos, Triosteum, Triplostegia, Weigela, and Zabelia), between Valerianella and four species (Symphoricarpos, Triplostegia, Weigela, and Zabelia), and between Valeriana and Weigela showed that Fedia, Valerianella, and Valeriana had significantly higher rates than the other analyzed species (Wilcoxon rank-sum test, $p < 0.05$, after Bonferroni correction; Table S2).

In addition, multiple genes exhibit $d_N / d_S$ ratios that are greater than one, but likelihood ratio tests (LRTs) with Bonferroni correction indicated that $d_N / d_S$ for infA in Acanthocalyx, ndhD in Morinoideae/Linnaeoidae/Valerianoideae/Dipsacoideae/Zabelioi-dae, psbM in Vesalea, rbcL in Weigela, rpoC1 in Valeriana, rpl22 in Adoxa/Tetradoxa/Sinadoxa, rpl32 in Heptacodium, rps2 in Acanthocalyx, rps4 in Scabiosa, rps14 in Pterocephalus, rps16 in Dipsacus/Scabiosa/Pterocephalus, rps18 in Abelia, and rps19 in Acanthocalyx were significantly different (Table S3).

Figure 2. Plastid phylograms of nonsynonymous ($d_N$) and synonymous ($d_S$) substitution rates based on 72 plastid genes. Boxplots of the values of $d_N$ and $d_S$ for individual genes. The box represents values between quartiles, solid lines extend to the minimum and maximum values, outliers are shown as circles and vertical lines in boxes show median values.
2.3. Structural Evolution of Plastid-Encoded accD Gene in Caprifoliaceae s.l. Plastomes

The length of the acetyl-CoA carboxylase beta subunit D (accD) open reading frame (ORF) varied remarkably among the examined Caprifoliaceae s.l. plastomes, ranging from 711 bp in Heptacodium to 2517 bp in Lonicera (Figure 3). The comparison revealed two histories of insertion events within the N- and C-terminal regions, resulting in the expansion or truncation of the accD ORF. The most parsimonious interpretation is that the accD genes were interrupted by the insertion of amino acids in the common ancestor of Caprifoliaceae s.l. (Figure 3). The second insertion of amino acids occurred in the common ancestor of Zabelia, Morinoideae, Linnaeoideae, Valerianoideae, and Dipsacoideae (except Pterocephalus and Triplostegia), which was split into two portions of the conserved domain (Figures 3 and S4).

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Figure 3. Rapid structural evolution of the accD gene. Phylograms show nonsynonymous ($d_N$) and synonymous ($d_S$) substitution rates for the accD among the analyzed Caprifoliaceae s.l. with outgroups. Scale bar indicates the number of substitutions per site. Gray boxes indicate the predicted open reading frames (ORFs). Red rectangles indicate the conserved domain in the ORFs. Asterisks indicate that the ORF has an alternative start codon. Branch lengths are drawn to the same scale based on $d_N$ and $d_S$ substitutions per site. Branches with significantly higher $d_N$/$d_S$ ratios, as determined by likelihood ratio test are marked with asterisks (*, $p < 0.05$; ***, $p < 0.001$ after Bonferroni correction).
In particular, the accD ORFs of Morina, Linnaea, and Zabelia contain a part of the conserved domain, but the catalytic sites are included (Figure S2). Conserved domain (CD) searches identified a portion of Apolipoprotein, MSCRAMM_ClfB, MSCRAMM_SdrC, GAT1, PPK08581, PPK05901, SMC_N, pneumo_PspA, and rplD subfamilies surrounding the conserved domain of accD in the predicted ORF (Table S4). The presence of MSCRAMM_ClfB in Symphoricarpos, Linnaeaeoaideae, and Adoxaceae indicated that this event occurred in the most recent common ancestor of each clade (Table S4). Protein sequence alignment of all inserted regions from the accD was highly divergent with low amino acid identities of 2.9–96.7% (Figure S4). However, two lineages—Dipsacus/Scabiosa and Linnaeoeaideae—had high amino acid identities of 94.3% and 83.1–96.7%, respectively.

The $d_N$ and $d_S$ were calculated for the selected Caprifoliaceae s.l. and outgroup using only the conserved domain sequences to test the effect of the insertion on nucleotide substitution rates of the accD gene. The $d_N$ values for accD in the selected Caprifoliaceae s.l. were significantly higher than in the outgroup (Wilcoxon rank-sum test, $p < 0.05$; Figure S3). Seven branches with $d_N/d_S$ ratios > 1 were detected, butLTRs showed that three branches, Fedia/Valerianella, Triplostegia, and Zabelia, were significantly different ($p < 0.00001$ after Bonferroni correction, Figure 3). The RELAX analysis indicated that the accD experienced significantly intensified selection in the Zabelia, Morinoideae, Linnaeaeoaideae, Valerianoideae, and Dipsacoideae clade ($k = 1.96, p = 0.020, \text{likelihood ratio (LR)} = 5.38$), and the Caprifoliaceae s.l. clade ($k = 2.25, p = 0.032, \text{LR} = 4.60$).

2.4. Length Variation in the Valeriana accD Gene

Valeriana fauriei plastome contains an expanded accD gene, which is interrupted by amino acid repeats (AARs) surrounding the conserved domains. Compared with the other available Valeriana plastomes, V. officinalis contains a truncated accD gene in its genome, whereas V. sambucifolia f. dageletiana contains an expanded accD like V. fauriei accD (Figure 4A). To evaluate the variability of the AAR motifs in the accD gene of V. fauriei and V. sambucifolia f. dageletiana, we designed a PCR primer that targets two hotspot regions of accD (Figure 4A). The amplicon sizes of the region ranged from 966 bp to 1239 bp (Table S5). Alignment of the two region sequences of the 50 individuals with two accD sequences from two plastomes revealed intra-and infraspecific variation of the accD in Valeriana (Figure 4). Two hotspot regions show length variation, consisting of (1) 3 to 10 repeats of “ESTTTTESFAQR” and (2) 5 to 14 repeats of “SDSEEDLIKPD”, although there are one or three different amino acid sequences (Figure 4).

2.5. Correlation between Structural Change and Substitution Rates in the Plastid-Encoded clpP Gene

The phylogenetic distribution of clpP content in the selected Caprifoliaceae plastomes showed that this gene had been pseudogenized multiple times in the family (Figure 5A). BlastN searches using the plastid-encoded clpP from Viburnum identified partial exons or introns of clpP in Pteroccephalus, Triplostegia, Heptacodium, Weigela, Linnaeaeoaideae, Morinoideae, and Zabeliaoidae, which lacked a conserved domain (Figure 5). Only Dipsacus, Scabiosa, Fedia, Leycesteria, Nardostachys, Symphoricarpos, Valerianella, Valeriana, Lonicera, and Triosteum plastomes contain clpP genes that differ in intron content (Figure 5A,B). Similar to the Valerianella clpP gene, the clpP gene of Leycesteria, Lonicera, Symphoricarpos, and Triosteum are missing both introns. The phylogenetic distribution indicated that the loss of the second intron occurred in the Fedia/Valerianella clade (Figure 5).
To examine the correlation between structural changes and substitution rates, $d_S$ and $d_S$ were calculated using the 16 clpP gene sequences available (Figure 5C). Seven branches with $d_N/d_S$ values >1 were detected. However, LRTs revealed that only four branches, Fedia/Valerianella/Valeriana/Nardostachys, Valerianoideae, Lonicera, and Caprifolioideae, were significantly under positive selection ($p < 0.00001$ after Bonferroni correction). The RELAX analysis indicated that clpP experienced significantly intensified selection in the analyzed Caprifoliaceae s.l. ($k = 2.62$, $p = 0.001$, LR = 10.96). Additional analyses indicated that the intronless clpP gene experienced significantly intensified selection in the subfamily Caprifolioideae ($k = 3.18$, $p = 0.000$, LR = 16.06). In Fedia and Valerianella, the clpP gene showed intensified selection, but this was not significant ($k = 1.33$, $p = 0.443$, LR = 0.59).
Figure 5. Structural evolution of the clpP gene: (A) phylogenetic distribution of pseudogene or intron loss among the selected Caprifoliaceae s.l. and five outgroups. Arrowheads indicate the positions of the first (green) and second (blue) intron; (B) schematic diagram of the structure of clpP. Pink boxes indicate the conserved domain of caseinolytic protease; (C) phylogenograms showing nonsynonymous (dN) and synonymous (dS) substitution rates for the clpP genes among 16 species that have intact gene sequences. Branch lengths are drawn to the same scale based on dN and dS substitutions per site. Branches with significantly higher dN/dS ratios determined by likelihood ratio test are marked with asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.001 after Bonferroni correction).

3. Discussion

Sequencing of the F. cornucopiae, V. fauriei, V. locusta, D. japonicus, and S. comosa plastomes revealed that they are distinct from the published plastomes of Caprifoliaceae s.l. These plastomes exhibit dynamic changes in structure, gene and intron content, and lineage-specific rate acceleration. Our results also showed a correlation between mutation rates and structural variation in the accD and clpP genes across Caprifoliaceae s.l. and interruption of the accD gene in the genus Valeriana. The loss of plastid-encoded rps15 was observed in D. japonicus and S. comosa plastomes. Comparative analysis of the gene content among Caprifoliaceae s.l. plastomes suggests the rps15 is lost in the common ancestor of Dipsacus, Scabiosa, and Pterocephalus. Functional replacement of the rps15 from plastid to the nucleus occurs in these lineages, although we found evidence for a plastid-to-nucleus gene transfer in the Dipsacus transcriptome. Additional nuclear transcriptome data for Scabiosa and Pterocephalus are needed for further investigation.

With the five plastomes, we selected the published plastomes of 19 additional genera from Caprifoliaceae s.l. and five genera from Adoxaceae to reconstruct the ancestral plastome in Caprifoliaceae s.l. and rearrangement events in each genus. Our results revealed three synapomorphic events (one inversion and two contractions of IR) in the Caprifoliaceae s.l. (Figure 1). A previous study showed that the inversion associated
with the *ndhF* gene occurred in Adoxaceae [34]. However, compared with angiosperm plastomes, the ancestral Caprifoliaceae *s.l.* plastome has an inversion between *ndhF*. The comparison of the LCBs and IR boundary shift models indicates that independent events occurred in a lineage- or species-specific manner (Figure 1). The model suggests that the IR expansion and contraction were the main mechanisms for changes in gene order in *F. cornucopiae*, *V. fauriei*, and *V. locusta* plastomes. A double-strand break, followed by strand invasion, expansion, and recombination in IR [37], is a potential mechanism for IR expansion in the family. After ancestral inversion, one additional inversion event associated with *ccsA-trnL-rpl32-ndhF* was identified, unique to *Scabiosa*. Dispersed repeats can cause inversions and there is a correlation between the number of repeats and plastome rearrangements [6,38]. However, *Scabiosa* has a small number of repeats in the family, although it has a relatively large number of repeats compared to the outgroups (Table S1). Moreover, dispersed repeat sequences were not found surrounding the inversion block in the *Scabiosa* plastome, which suggests that a different mechanism of inversion may be involved. Several ebb-and-flow expansions and contractions were also observed in the analyzed Caprifoliaceae *s.l.* plastomes.

Lineage-specific variation occurred in the Caprifoliaceae *s.l.* plastomes, showing that *Fedia*, *Valeriana*, and *Valerianella* had significantly accelerated *d*$_S$ in comparison (Figure 2). Structural rearrangements have contributed to higher substitution rates [5,14]. Compared with the analyzed Caprifoliaceae *s.l.* plastomes, the three plastomes showed increased levels of structural divergence (Figure 1). However, IR boundary shifts are the main mechanisms responsible for the genomic changes in this lineage. This variation appears to result from genome-wide acceleration, arguing against localized hypermutation, mutagenic retro-processing, and pseudogenization [16]. Mutated and changed DNA-RRR machinery could be a potential mechanism to explain these phenomena. In the subfamily Valerianoideae, expanded plastome sequencing and examination of organellar-targeted DNA-RRR genes would be needed to explain the causes and consequences of fast-evolving plastomes in this lineage.

Our analysis showed that interrupted *accD* evolution has occurred repeatedly across Caprifoliaceae *s.l.* (Figure 3). Many plastomes show that the *accD* is unrecognized and unannotated because of its extreme divergence [34,36]. In these cases, the *accD* appears to have been lost and possibly transferred to the nucleus, as intracellular gene transfer (IGT) is an ongoing process in angiosperms [3]. Evidence supports the functional replacement of the *accD* gene by gene transfer to the nucleus or gene substitution of nuclear homologs, including coexistence of the nuclear-encoded, plastid-targeted eukaryotic ACCase, prokaryotic ACCase, and the plastid-encoded *accD* [15,16,20,22–26]. However, previous studies have provided some evidence that highly divergent or truncated *accD* genes may be functional in plastids. For example, the divergent copy of the plastid-encoded *accD* in *Lamprocapnos spectabilis* (L.) Fukuhara (Papaveraceae) is transcribed [9]. A functional replacement of truncated *accD* to the nucleus was found in *Trachelium caeruleum* L. (Campanulaceae) [20], *Hypseocharis bilobata* Killip, and *Monsonia emarginata* (L.f.) L’Hér. (Geraniaceae) [15]. In the selected Caprifoliaceae *s.l.* plastomes, many *accD* reading frames remain intact, and several have truncated with the catalytic sites, which suggests that it probably encodes a functional protein. The intensity of both purifying (most branches are *d*$_N$/ *d*$_S$ < 1) and positive selection (three branches, *Fedia/Valerianella*, *Triplostegia*, and *Zabelia* with *d*$_N$/ *d*$_S$ > 1) also indicated that the *accD* is under selective constraint or adaptive changes. To fully understand the evolution of ACCase among Caprifoliaceae *s.l.* genomes, searching the assembled nuclear transcriptomes are required. In addition, we examined the variability of the AAR motifs in the *accD* gene and found evidence of intraspecific length variation in the *Valeriana*. One possible mechanism for length polymorphism is replication slippage and recombination [39]. Similar patterns of the *accD* gene have been previously shown to have repetitive amino acid sequence motifs in *L. spectabilis* [9] and *Medicago truncatula* Gaertn. [40]. Gurdon and Maliga [40] suggested that repetitive amino acid motifs within the *accD* could be recombinationally driven.
Previous studies have shown that multiple lineages experience independent loss [7,14,41–43]. Some cases of losses are because the clpP gene is unrecognized and unannotated owing to high divergence including structural changes. For example, the clpP gene was annotated as a loss in the five Actinidiaceae plastomes [42,43], but the plastomes contain clpP-like ORFs that are missing the two introns with a completely conserved domain. The lack of internal stop codons or frameshifts suggests the functionality of the plastid-encoded clpP. The Geranium and Monsonia clpP genes were annotated as pseudogenes or losses [7,14]; however, extremely divergent and intronless clpP ORFs are found in the lineages [15]. ClpP is involved in important chloroplast processes [32]. If the divergent ORFs are pseudogenes, functional replacement by gene transfer or gene substitution must occur. However, the evolutionary fate of the plastid-encoded clpP loss has not been reported in angiosperms. We identified at least six potential pseudogenizations of clpP and independent losses of one or both introns in the analyzed Caprifoliaceae s.l. (Figure 5). Using the conserved domain sequence of clpP as a query, we did not find any evidence of divergent clpP-like ORFs that were intact. Only two lineages contained the clpP gene, showing dynamics in intron content. The phylogenetic distribution showed the evolutionary history of intron loss events in the subfamily Valerianoideae, in which the loss of the second intron in the common ancestor of Fedia and Valerianella, followed by loss of the first intron independently in Valerianella (Figure 5). The subfamily Caprifolioideae plastome contains intronless clpP, but the history of loss events is unclear from the present data. Direct genomic deletion, exonization of introns, retroprocessing, and gene conversion with foreign copies are possible mechanisms of intron loss [44]. Intensified positive or negative selection in the clpP gene among Caprifoliaceae s.l. suggests that positive selection may act on the nuclear-encoded plastid-targeted genes. Cytonuclear coevolution between plastid- and nuclear-encoded subunits in Caprifoliaceae s.l. should be explored to test this hypothesis.

4. Materials and Methods
4.1. Genome Sequencing, Assembly, and Annotation

Total genomic DNA (gDNA) from V. fauriei and V. locusta from the subfamily Vaerianoideae, as well as D. japonicus, and S. comosa from the subfamily Dipsacoideae (Table S5), were isolated from fresh leaf tissues of a single individual using the Exgene Plant SV Mini Kit (GeneAll, Seoul, South Korea) following the manufacturer’s protocol. The gDNA of F. cornucopiae was provided by the Royal Botanic Gardens Kew DNA and Tissue Collection (Table S5). The gDNAs were sequenced using an Illumina Hiseq2500 sequencing platform (Illumina, San Diego, CA, USA), generating 6 Gb of 150 bp paired-end (PE) reads from a 550 bp insert library.

The PE reads were assembled de novo using Velvet v1.2.10 [45] using multiple k-mers (99 to 141). For each plastome, the longest contigs that reflected a complete plastome with only one copy of the IR were aligned manually, and the consensus was taken as the final genome sequence. Finished plastomes were annotated using a BLAST-like algorithm in Geneious Prime 2021.1.1 (www.geneious.com, accessed on 18 August 2021) with the genes of Nicotiana tabacum L. plastome (NC_001879) as the reference, and the open reading frames (ORFs) were confirmed using the “Find ORFs” option. Circular plastome maps were drawn using OrganellarGenomeDRAW (OGDRAW) v1.3.1 (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html, accessed on 18 August 2021) [46]. The plastomes were deposited in GenBank (accession numbers MZ934745-MZ934749).

4.2. Comparative Analyses

Repetitive DNA sequences in each plastome were identified by performing “blastn” searches using BLAST + v2.6.0 [47] against itself, with a word size of 11, an e-value of $1 \times 10^{-6}$. The newly sequenced plastomes and the 19 published Caprifoliaceae s.l. plastomes were aligned with the outgroup Viburnum betulifolium Batalin from Adoxaceae using the “progressiveMauve” algorithm in Mauve v2.3.1 [48] in Geneious Prime. The National
Center for Biotechnology Information (NCBI) Conserved Domain Database (CDD) v3.19 was used for functional domain annotation (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 18 August 2021) [49]. Transcriptome from Dipsacus asperoides C.Y.Cheng & T.M.Ai was assembled de novo with Trinity [50] using the Sequence Read Archive (SRA) (SRR2043985). The potential nuclear-encoded transcript was identified in the transcriptome by using “blastn” (e-value cutoff of $1 \times 10^{-10}$) with the plastid-encoded rps15 gene sequence from Triplostegia glandulifera Wall. ex DC. as a query. Chloroplast transit peptide (cTP) was predicted by TargetP v1.1 [51].

4.3. Estimation of Substitution Rates

In total, 72 plastid protein-coding genes shared by all selected 29 taxa from newly sequenced plastomes and from the published plastomes were sampled (Table S1). Individual genes were aligned using the back-translation method with MAFFT [52] in Geneious Prime. The constraint tree was generated using the maximum likelihood method in IQ-TREE v2.1.2 [53] with concatenated sequence alignment. To estimate the rates of nucleotide substitution, all genes and the concatenated sequences were analyzed individually. The nonsynonymous ($d_N$) and synonymous ($d_S$) substitution rates for datasets were calculated in PAML v4.8 [54] with the constraint tree. Codon frequencies were estimated using the F3 $\times$ 4 model. LRTs were performed in Hyphy v2.5.23(MP) [55] to test $d_N/d_S$ changes using the MG94xREV codon model. To test for potential relaxed selection, the RELAX [56] implemented in HyPhy was used on the Datamonkey Adaptive Evolution Server (https://www.datamonkey.org/, accessed on 18 August 2021) [57].

4.4. Survey of Variability in the Plastid-Encoded accD Gene

To examine length variation in the accD gene at the inter- and intraspecific levels, 38 V. fauriei individuals and 12 V. sambucifolia f. dagetiana individuals were sampled (Table S5). The gDNAs were extracted from the fresh leaves or herbarium specimens using the GeneAll Kit, or the methods described by Allen et al. [58]. Variable regions in the accD gene were amplified by PCR using specific primers designed with Primer3 in Geneious Prime (64F: 5′-AACCTTTATGATTCGGTTTCTCGT-3′ and 1328R: 5′-ATACCGGTGGTGAATAGCCTCAGTT-3′). Each reaction was 50 µL in volume, including 38.75 µL of distilled water, 5 µL of 10 × Taq Reaction Buffer, 1 µL of dNTPs (10 mM), 0.25 µL of DiaStar™ Taq polymerase (5 units/µL, Solgent Co., Daejeon, Korea), 1 µL of each primer (10 pmole/µL), and 1 µL of total gDNA (20 ng). All reactions consisted of included an initial denaturation step (95 °C for 2 min), 35 cycles of denaturation (95 °C for 20 s), annealing (60 °C for 40 s), and extension (72 °C for 1 min 30 s), followed by a final extension (72 °C for 5 min). The PCR products were purified using a PCR purification kit (MGmed, Korea) according to the manufacturer’s protocol. Sequencing of PCR products was carried out using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at Solgent Co. The nucleotide sequences of the plastid accD copies were aligned using MUSCLE [59] in Geneious Prime.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms221910485/s1, Figure S1: Maps for the newly sequenced plastomes, Figure S2: Nucleotide and amino acid sequences of the nuclear-encoded RPS15 gene from Dipsacus, Figure S3: Duplication of the trnE-UUC gene in Dipsacus japonicus plastome, Figure S4: Structural alignments of Caprifoliaceae s.l. plastomes using Mauve. Figure S5: Amino acid sequence alignments of the plastid-encoded accD of Caprifoliaceae s.l. plastomes using MUSCLE. Figure S6: Boxplots of the values of nonsynonymous and synonymous substitution rates of the plastid-encoded accD for Caprifoliaceae s.l. and outgroups, Table S1: GenBank accession numbers for taxa used in this study, Table S2: Pairwise Wilcoxon rank-sum tests of $d_N$ and $d_S$ values among plastid genes within Caprifoliaceae s.l., Table S3: Positive selection on Caprifoliaceae s.l. plastid genes, Table S4: CD-search results of the plastid-encoded accD gene from Caprifoliaceae s.l., Table S5: Material information and GenBank accession numbers for length variation in the plastid-encoded accD gene.
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