


Article

Next-Generation Genome Sequencing of *Sedum plumbizincicola* Sheds Light on the Structural Evolution of Plastid rRNA Operon and Phylogenetic Implications within Saxifragales

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Abstract: The genus *Sedum*, with about 470 recognized species, is classified in the family Crassulaceae of the order Saxifragales. Phylogenetic relationships within the Saxifragales are still unresolved and controversial. In this study, the plastome of *S. plumbizincicola* was firstly presented, with a focus on the structural analysis of *rrn* operon and phylogenetic implications within the order Saxifragaceae. The assembled complete plastome of *S. plumbizincicola* is 149,397 bp in size, with a typical circular, double-stranded, and quadripartite structure of angiosperms. It contains 133 genes, including 85 protein-coding genes (PCGs), 36 tRNA genes, 8 rRNA genes, and four pseudogenes (one *ycf1*, one *rps19*, and two *ycf15*). The predicted secondary structure of *S. plumbizincicola* 16S rRNA includes three main domains organized in 74 helices. Further, our results confirm that 4.5S rRNA of higher plants is associated with fragmentation of 23S rRNA progenitor. Notably, we also found the sequence of putative *rrn5* promoter has some evolutionary implications within the order Saxifragales. Moreover, our phylogenetic analyses suggested that *S. plumbizincicola* had a closer relationship with *S. sarmentosum* than *S. oryzifolium*, and supported the taxonomic revision of *Phedimus*. Our findings of the present study will be useful for further investigation of the evolution of plastid rRNA operon and phylogenetic relationships within Saxifragales.

Keywords: *Sedum plumbizincicola*; Crassulaceae; Saxifragales; *rrn* operon; phylogeny

1. Introduction

The genus *Sedum* comprises more than 420 recognized species, which is the most species-rich member of the family Crassulaceae [1,2]. Some species, formerly classified as *Sedum*, are now assigned to the segregate genera *Hylotelephium* and *Rhodiola* [3–6]. The family Crassulaceae, together with 14 other family members, has been classified in the order Saxifragales. Recently, increasing research

efforts have been focused on the evolution of Saxifragales, however, phylogenetic relationships within the order are still unresolved due apparently to a rapid, ancient radiation [7–12].

Extensive genes were transferred from plastids to the nucleus during evolution. In most land plants, the plastid genome (plastome) is a circular biological macromolecule with a typical quadripartite structure [13–15]. In higher plants, compared with hundreds or thousands of tandem repeats in nuclear ribosomal RNA genes [16–18], typical plastid rRNA genes are characterized by a pair of inverted *rrn* operons, which show the gene order of *rrn16*, *rrn23*, *rrn4.5*, and *rrn5* [19]. With the rapid development of next generation genome sequencing, more and more complete plastid genomes (plastomes) have been deposited in a public database. Recently, a total of over 3000 reference sequences of plastomes were available in GenBank. The plastomes have been widely accepted as a popular tool for phylogenetic studies [7,20–26].

Thus far, 43 plastomes have been obtained in Saxifragales, as shown in Table 1. Currently, there is no report of a plastome for *S. plumbizincicola*, a well-known Zn/Cd hyperaccumulator, which was newly discovered from lead and zinc mining areas in Zhejiang province, China [27]. In this paper, we sequenced the plastome of this species using next-generation genome sequencing. Together with the public sequences, we performed a comparative analysis of plastomes within Saxifragales. Consequently, the aims of this research were (1) to investigate general features of the *S. plumbizincicola* plastome, (2) to examine the structural evolution of the plastid ribosomal RNA operon, and (3) to clarify phylogenetic relationships within the order Saxifragales.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

The fresh leaf samples of *S. plumbizincicola* (code AHNU-KPBK001) were collected from Panjiacun (29°35'16" N, 118°35'19" E) in Zhejiang Province, east China. Genomic DNA extraction was conducted using the Plant Genomic DNA kit (Tiangen, Beijing, China), following the manufacturer's instructions. The library was constructed using a TruSeq DNA PCR-Free Library Prep Kit (Illumina, San Diego, CA, USA) and sequenced on the Illumina HiSeq X Ten (Illumina, San Diego, CA, USA) with the strategy of 150 paired-ends and an insert size of 350 bp.

2.2. Genome Assembly, Gene Annotation, and Sequence Analyses

The paired-end reads were first checked with Fastqc [28] and then trimmed for quality using Trimmomatic 0.39 [29]. After that, obtained clean reads were filtered and assembled with GetOrganelle 1.5.2 [30] using the chloroplast genome of *S. sarmentosum* [7] as reference. The chloroplast genome was annotated with GeSeq [31]. The secondary cloverleaf structures of tRNAs were identified using tRNA-scan SE web server [32]. The secondary structures of rRNAs were predicted by comparison with those of other plant species [33].

2.3. Phylogenetic Analysis

To resolve the phylogenetic relationships among Saxifragales species, two phylogenetic approaches were applied: the maximum likelihood (ML) method in RAxML GUI 1.5b2 [34], as well as the Bayesian inference (BI) method in MrBayes 3.2.7a [35]. With exclusion of the termination codons, 79 protein-coding genes (PCGs) and 4 rRNAs of 37 Saxifragales species were used to construct an evolutionary tree. A phylogenomic study by Yang et al. [36] revealed a sister group relationship between Saxifragales and Rosids. We selected therefore two Vitales species within Rosids (*Vitis heyneana*, NC_039796; *V. vinifera*, NC_007957) as outgroups.

Table 1. Species of plastomes examined in this study.

Family	Species	Accession No.	Reference
Altingiaceae	<i>Liquidambar formosana</i>	NC_023092.1	[7]
Cercidiphyllaceae	<i>Cercidiphyllum japonicum</i>	NC_037940.1	[37]
Crassulaceae	<i>Phedimus kantschaticus</i>	NC_037946.1	[38]
Crassulaceae	<i>Phedimus takesimensis</i>	NC_026065.1	Unpublished
Crassulaceae	<i>Rhodiola rosea</i>	NC_041671.1	[39]
Crassulaceae	<i>Sedum oryzifolium</i>	NC_027837.1	Unpublished
Crassulaceae	<i>Sedum plumbizincicola</i>	MN185459.1	This study
Crassulaceae	<i>Sedum sarmentosum</i>	NC_023085.1	[7]
Daphniphyllaceae	<i>Daphniphyllum oldhamii</i>	NC_037883.1	[8]
Grossulariaceae	<i>Ribes fasciculatum</i>	MH191388.1	[8]
Haloragaceae	<i>Myriophyllum spicatum</i>	NC_037885.1	[8]
Hamamelidaceae	<i>Chunia bucklandioides</i>	NC_041163.1	[40]
Hamamelidaceae	<i>Corylopsis coreana</i>	NC_040141.1	[41]
Hamamelidaceae	<i>Fortunearia sinensis</i>	NC_041487.1	[42]
Hamamelidaceae	<i>Hamamelis mollis</i>	NC_037881.1	[8]
Hamamelidaceae	<i>Loropetalum subcordatum</i>	NC_037694.1	[43]
Hamamelidaceae	<i>Parrotia subaequalis</i>	NC_037243.1	Unpublished
Hamamelidaceae	<i>Sinowilsonia henryi</i>	NC_036069.1	Unpublished
Paeoniaceae	<i>Paeonia brownii</i>	NC_037880.1	[8]
Paeoniaceae	<i>Paeonia decomposita</i>	NC_039425.1	[44]
Paeoniaceae	<i>Paeonia delavayi</i>	NC_035718.1	[45]
Paeoniaceae	<i>Paeonia jishanensis</i>	MG991935.1	[46]
Paeoniaceae	<i>Paeonia lactiflora</i>	NC_040983.1	[47]
Paeoniaceae	<i>Paeonia ludlowii</i>	NC_035623.1	[45]
Paeoniaceae	<i>Paeonia obovata</i>	NC_026076.1	Unpublished
Paeoniaceae	<i>Paeonia ostii</i>	NC_036834.1	Unpublished
Paeoniaceae	<i>Paeonia rockii</i>	NC_037772.1	[48]
Paeoniaceae	<i>Paeonia suffruticosa</i>	NC_037879.1	[8]
Paeoniaceae	<i>Paeonia veitchii</i>	NC_032401.1	Unpublished
Penthoraceae	<i>Penthorum chinense</i>	NC_023086.1	[7]
Iteaceae	<i>Itea chinensis</i>	NC_037884.1	[8]
Saxifragaceae	<i>Bergenia scopulosa</i>	NC_036061.1	[49]
Saxifragaceae	<i>Chrysosplenium aureobracteatum</i>	NC_039740.1	[50]
Saxifragaceae	<i>Heuchera parviflora</i>	KR478645.1	[51]
Saxifragaceae	<i>Heuchera richardsonii</i>	NC_042923.1	Unpublished
Saxifragaceae	<i>Heuchera villosa</i>	NC_042924.1	Unpublished
Saxifragaceae	<i>Mitella diphylla</i>	NC_042925.1	Unpublished
Saxifragaceae	<i>Mitella formosana</i>	NC_042926.1	Unpublished
Saxifragaceae	<i>Mukdenia rossii</i>	NC_037495.1	Unpublished
Saxifragaceae	<i>Oresitrophe rupifraga</i>	NC_037514.1	[52]
Saxifragaceae	<i>Saxifraga stolonifera</i>	NC_037882.1	[8]
Saxifragaceae	<i>Tiarella cordifolia</i>	NC_042927.1	Unpublished
Saxifragaceae	<i>Tiarella polyphylla</i>	NC_042928.1	Unpublished
Saxifragaceae	<i>Tiarella trifoliata</i>	NC_042929.1	Unpublished

For ML analyses, we performed analyses with thorough bootstrap for ten runs and 1000 replicates under the GTRCAT model using RAxML GUI. For BI analyses, the best-fit models for 83 genes were first selected based on Bayesian information criterion (BIC) values in ModelGenerator 0.85 [53], then two simultaneous runs with eight independent Markov chains were run for 10,000,000 generations (sampling every 1000 generations).

3. Results and Discussion

3.1. General Features of *S. plumbizincicola* Plastome

Based on Bowtie2 mapping, in total 19,610,999 reads (21.5% of total reads) were mapped to the reference genome (*S. sarmentosum*, NC_023085), with a 1969× mean coverage (min, 1286×, max, 3664×, standard deviation, 71). The assembled complete plastome of *S. plumbizincicola* (accession number: MN185459.1) is 149,397 bp in size, with a typical circular, double-stranded, and quadripartite structure of angiosperms. The plastome has two identical inverted repeats (IRs, 25,565 bp) separated by a small single copy (SSC, 16,669 bp) and a large single copy (LSC, 81,598 bp), as shown in Figure 1. Approximately 52.0%, 4.3%, and 1.83% of the genome encodes for proteins, rRNAs, and tRNAs, respectively. Whereas, the remaining 41.87% are non-coding regions, including introns, intergenic spaces, and pseudogenes.

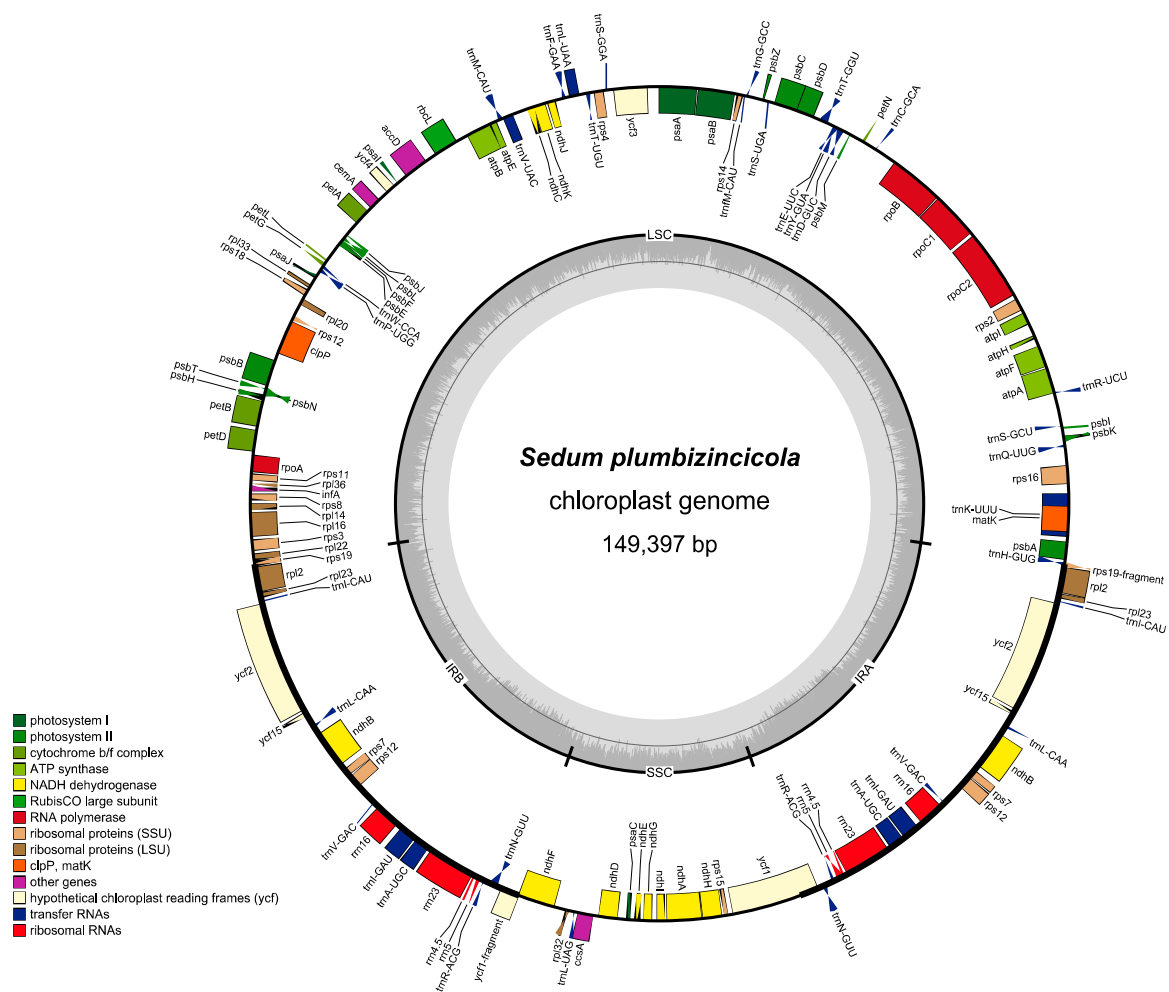


Figure 1. Chloroplast genome annotation map for *Sedum plumbizincicola*. Genes lying outside the circle are transcribed in a clockwise direction, whereas genes inside are transcribed in a counterclockwise direction. Different colors represent different functional groups. The dashed darker and lighter gray in the inner circle denote G + C and A + T contents of chloroplast genome, respectively. LSC, SSC, and IRs mean long single copy, small single copy, and inverted repeat regions, respectively.

Along with new data from this study, we comparatively investigated the structures and properties of plastomes from 44 species, representing 11 families in Saxifragales, as shown in Table 1. The size of plastomes of Saxifragales ranges from 147,048 bp (*Phedimus kamtschaticus*) to 160,410 bp (*Liquidambar*

formosana), as shown in Table S1, and the total of G + C content varies from 36.40% (*Myriophyllum spicatum*) to 38.55% (*Paeonia brownii*).

The plastome of *S. plumbizincicola* contains 133 genes, including 85 protein-coding genes (PCGs), 36 tRNA genes, 8 rRNA genes, and four pseudogenes (one *ycf1*, one *rps19*, and two *ycf15*). Dong et al. [8] reported that *infA* and *rpl32* have been lost from three species of *Paeonia* plastome (*Paeonia brownii*, *P. suffruticosa*, and *P. obovata*). In this study, comparative analysis showed that these two gene loss events occurred in all eleven plastomes of Paeoniaceae. A possible explanation is that the two functional genes have been transferred to the nucleus [8,47,54–60]. Furthermore, Dong et al. [8] observed that the intron of *rpl2* was completely lost in *Saxifraga stolonifera*. There are currently about 640 species in 33 genera recognized within the family Saxifragaceae [61]. Interestingly, in the current study, the intron of *rpl2* was detected in all families in Saxifragales, except for 13 species from the examined 8 genera representing the major lineages of Saxifragaceae, as shown in Table S1, which indicates an early loss of this intron within this lineage. Besides Saxifragaceae, nine other independent losses of *rpl2* intron were reported in dicotyledons [62–75]. The two most probable mechanisms of loss of the *rpl2* intron are homologous recombination and gene conversion [64,76,77].

3.2. Structure Analyses of Plastid Ribosomal RNA Operon

3.2.1. Structure of 16S rRNA

Similar to most other plants, the size of *S. plumbizincicola rrn16* is 1490 bp. In all Saxifragales species examined, the sizes of *rrn16s* are the same as that of *S. plumbizincicola*, except for the family Paeoniaceae, with an insertion (U) between positions 576 and 577 nts. As shown in Table S2, the G + C content of the *rrn16s* of Saxifragales ranges from 56.5% (*Rhodiola rosea*) to 56.9% (*Fortunearia sinensis*, and *Sinowilsonia henryi*). The average G + C content for typical land plants is 56%, whereas this value falls from 52% to 28% for holoparasitic angiosperms, with an increasingly greater number of mutations [78].

We next examined the predicted secondary structure of 16S rRNA in *S. plumbizincicola*. The structure is similar to the models proposed for other plants [78–80], including three main domains organized in 74 helices. In total, 72 mismatched pairs have been detected, and most of them (58/72) are G-U wobble pairs, as shown in Figure 2. Furthermore, we also detected that the position 123 nt of 16S rRNA is cytosine (123-C), whereas other Saxifragales species examined are uracil. To avoid a potential sequencing error, we confirmed the mutation *U123C* by transcriptomic data of *S. plumbizincicola* (accession number: SRR5118122-SRR5118124). For further analysis, the 16S rRNAs from 3125 reference plastomes of land plants deposited in GenBank were investigated. The survey results indicated that only 13 species had the special 123-C, including two hyperaccumulator plants, *Alpinia oxyphylla* and *Curcuma longa* [81,82]. In contrast with non-canonical base pairing (G-U), we particularly observed that the mutation *U123C* of 16S rRNA can form stabilized base pairing (C-G) in helices H120, as shown in Figure 2. However, the underlying biological mechanisms of the mutation *U123C* of 16S rRNA are still unknown.

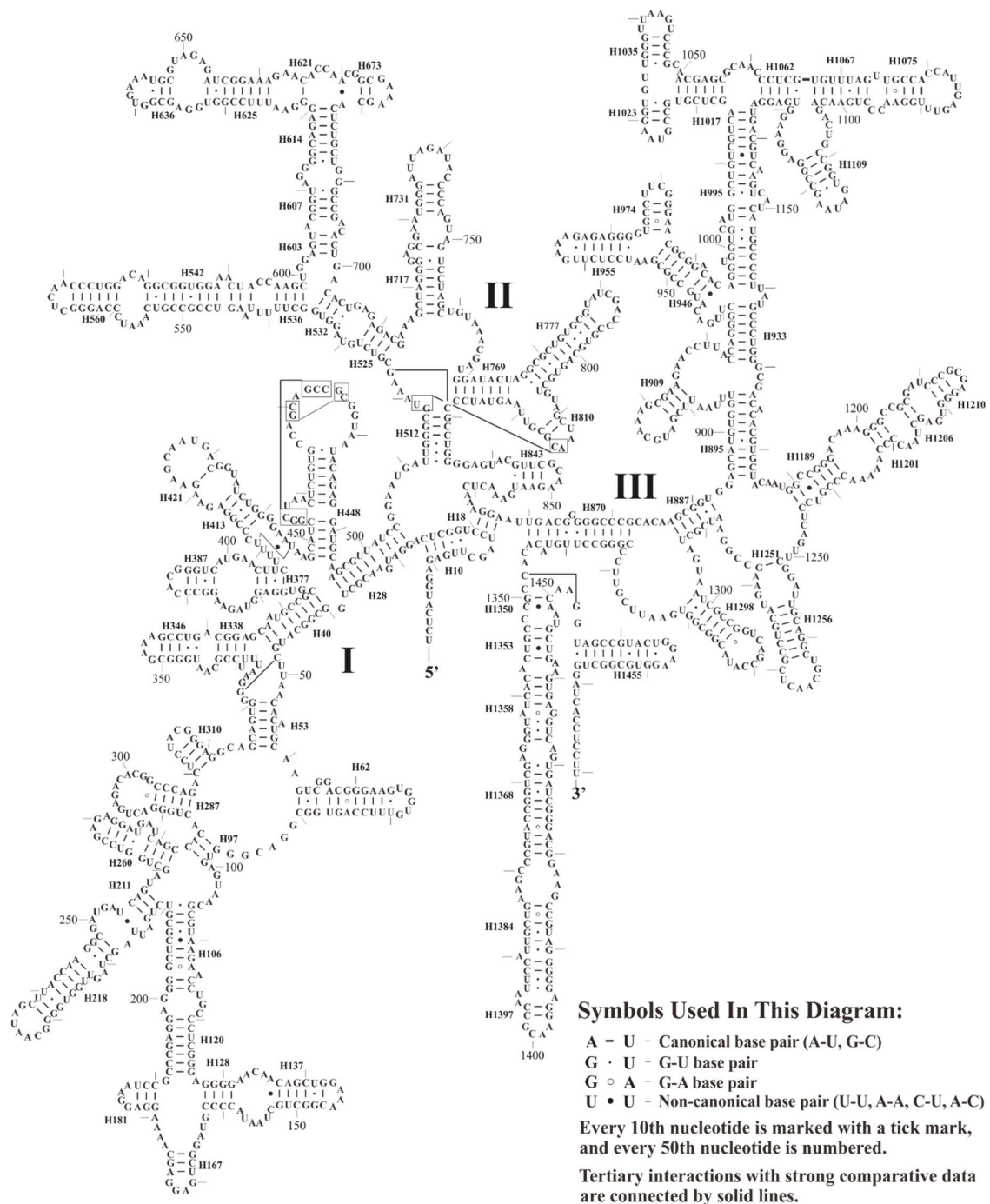


Figure 2. The predicted secondary structure model of 16S rRNA of *S. plumbizincicola*. Roman numbers refer to domain names.

3.2.2. Structure of 23S rRNA and 4.5S rRNA

As can be seen from Table S2, the size of *rnr23* spans from 2089 bp (*Sedum*) to 2857 bp (*Paonia suffruticosa*), and the G + C content ranges from 55.0% (*Corylopsis coreana*, *Loropetalum subcordatum*, and *Chrysosplenium aureobracteatum*) to 55.4% (*M. spicatum*), with an average value of 55.1%. In contrast to *rnr23*, the *rnr4.5* of Saxifragales is remarkably conserved in size (103 bp), with a mean G + C content of 56.7%. The *rnr4.5* and *rnr23* genes are separated by 98–99 bp intergenic spacers (IGd), with G + C content between 57.1% and 60.2%, as shown in Table S2.

The predicted secondary structure of 23S rRNA in *S. plumbizincicola* is similar to the models of Gutell [80,83], containing 149 helices and six domains, as shown in Figure 3. Moreover, a total of 135 mismatched pairs with 101 G-U wobble pairs were found in the structure. We then comparatively analyzed 23S rRNA secondary structures of all investigated taxa in Saxifragales. Remarkably, as shown in Figure 4, the hairpin loops near helix H550 were more divergent than others, including nucleotide substitutions and indels. In particular, these divergent hairpin loops may have potential phylogenetic implications. For instance, all species of Crassulaceae are characterized by six nucleotides (5'-CACUGG-3') in these hairpin loops. In addition, in contrast to *S. plumbizincicola*, *P. suffruticosa* had an extra 46 nts insertion between the helices H1684 and H2037 of 23S rRNA. Our study further shows that the extra insertion may form two additional helices, as shown in Figure 5. Notably, 4.5S rRNA is a unique component of plastid ribosomes from nonvascular (bryophytes) to vascular plants (pteridophytes, gymnosperms, and angiosperms), which is located on the large subunit. Several previous studies of 4.5S rRNA have failed to find known homologues in other types of ribosomes [84–86]. In ongoing follow-up research, 4.5S rRNA has been identified as structurally homologous to the 3' terminus of bacterial, cyanobacterial, and green algal 23S rRNA [19,84,87–90]. Based on sequence identity analysis, 4.5S rRNA of *S. plumbizincicola* and 3' terminus of *Escherichia coli* 23S rRNA (accession number: J01695) share 62.9% nucleotide identity. Interestingly, despite a considerable amount of nucleotide substitutions and indels between these two regions, their secondary structures exhibited similar topology, as shown in Figure 6. This finding confirms once again that 4.5S rRNA of higher plants is associated with fragmentation of 23S rRNA progenitor.

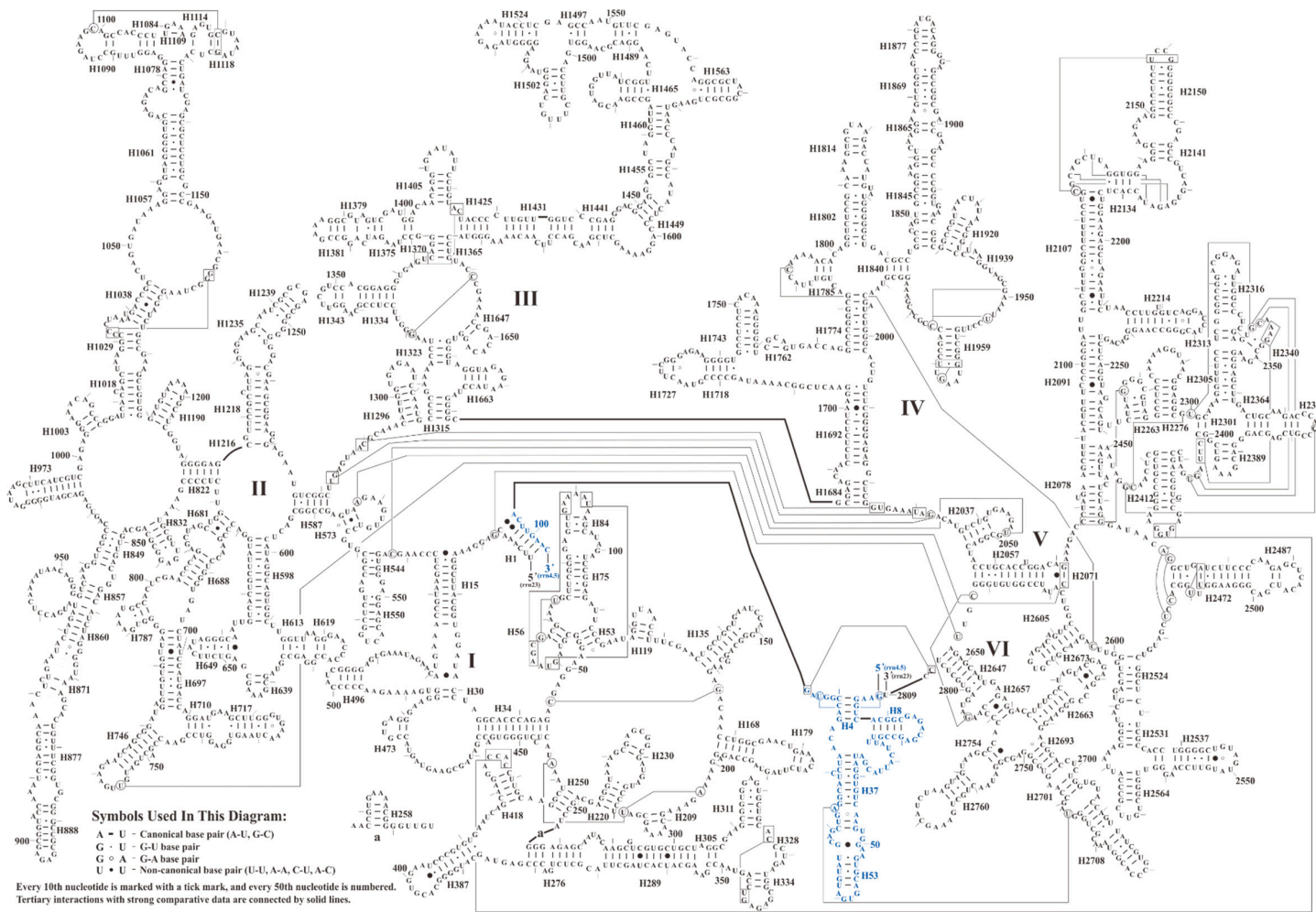


Figure 3. The predicted secondary structure model of 4.5S rRNA and 23S rRNA of *S. plumbizincicola*. Roman numbers refer to domain names. The bases of 4.5S rRNA are presented with blue and the bases of 23S rRNA are presented with black.

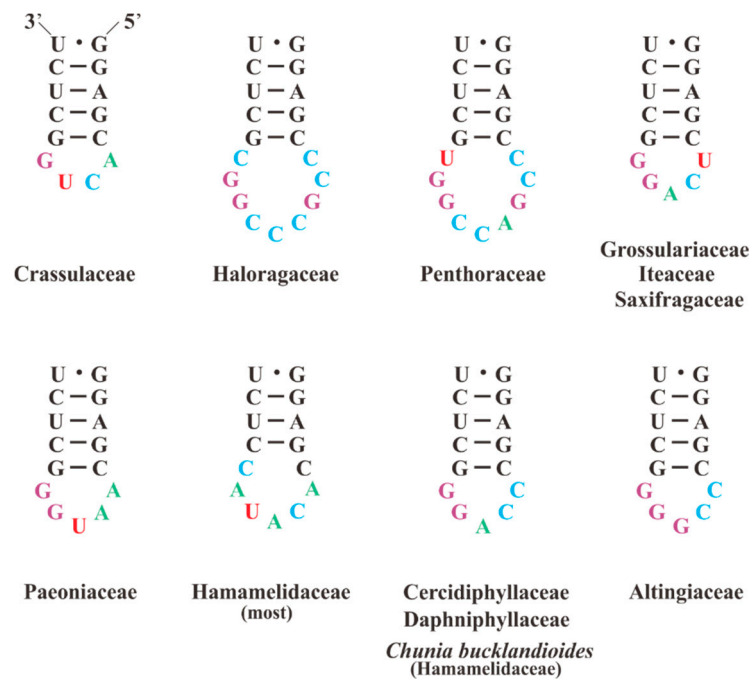


Figure 4. The predicted secondary structure models of H550 of 23S rRNA among Saxifragales species.

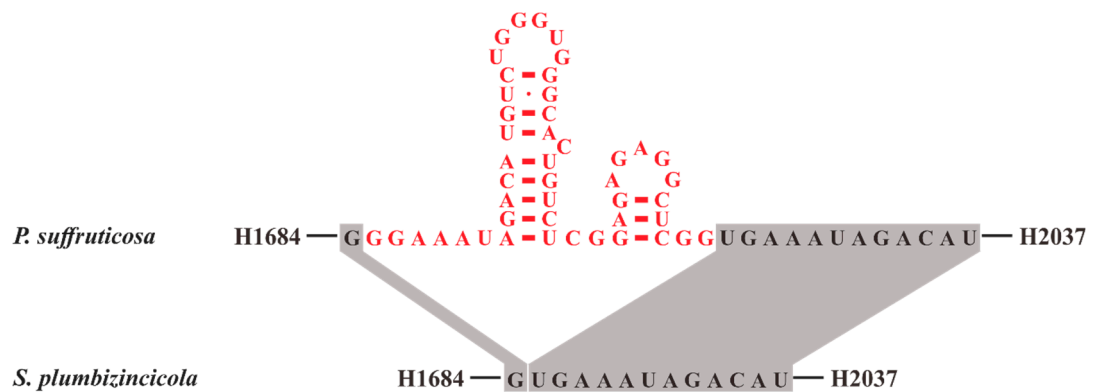


Figure 5. The predicted secondary structure models of the extra insertion between the helices H1684 and H2037 within *Paeonia suffruticosa* 23S rRNA compared with *S. plumbizincicola*. The color red indicates that these bases belong to the extra insertion of *P. suffruticosa*.

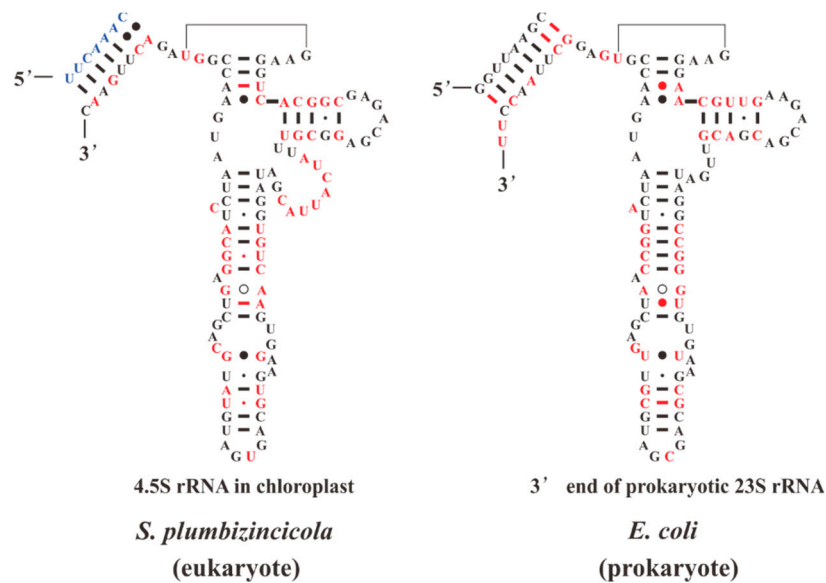


Figure 6. The secondary structure models of the 4.5S rRNA of *S. plumbizincicola* and 3' end of *Escherichia coli* 23S rRNA. The variations between structures are presented with red. The color blue indicates the bases belonging to 23S rRNA of *S. plumbizincicola*.

3.2.3. Structure of 5S rRNA and Evolutionary Implications of Its Putative Promoter

Structurally, 5S rRNA is the smallest RNA component of the large ribosomal subunit in all known organisms [91]. In the *S. plumbizincicola* plastome, *rrn5* and *rrn4.5* are physically linked by the intergenic region (IGe), with the size 219 bp, as shown in Table S2. Besides, the predicted secondary structure of *S. plumbizincicola* 5S rRNA is similar to that of other published studies [92,93], harboring five helices, as shown in Figure 7. Furthermore, our comparative sequence analysis identified a perfectly conserved 121-bp *rrn5* among Saxifragales, with medium G + C content (about 52%), as shown in Table S2. In this study, we also used the 5SRNadb (<http://combio.pl/rrna/>) to survey the G + C content of plastomic *rrn5*. A total of 839 sequences were downloaded and analyzed. The mean G + C content is 50.73%, with the lowest in *Euglena viridis* (32.26%) and the highest in *Staurostrum punctulatum* (59.84%). The survey shows that there is a great variability in G + C content of *rrn5* for photosynthetic euglenoid and green algae.

Based on similarity of nucleotide sequences, Audren et al. [94] found that a prokaryotic type promoter, which is closely related to the bacterial consensus, was located upstream of the *rrn5* and downstream of the stem-loop structure from spinach. However, the putative promoter is inactive both in vivo and in vitro, likely due to the high GC content of the sextama box (TTGGGG) [94,95]. A number of studies have demonstrated that the 5S rRNA gene is transcribed with the other ribosomal genes within the same operon [19,94,96,97]. Notably, the spinach putative promoter was also detected in the similar region from all 44 Saxifragales species. As shown in Figure 8, it contains a sextama box (−35 region, T₁₀₀T₁₀₀G₁₀₀G₁₀₀G₁₀₀G₁₀₀) and a pribnow box (−10 region, C₅₇A₁₀₀A₁₀₀T₁₀₀A₁₀₀T₈₆) separated by 8–29 bp within Saxifragales, as shown in Figure 8. Interestingly, we found the sequence of putative *rrn5* promoters have some evolutionary implications. For example, all spacers between −35 and −10 boxes from 44 investigated species share the 16 common nucleotides (CCTCACAATCACTAGC), except for *Liquidambar formosana* (CCTCTAGC). Due to nucleotide insertion, deletion, and substitution, the ancestral sequence was then further evolved to different apomorphies in diversified lineages within Saxifragales.

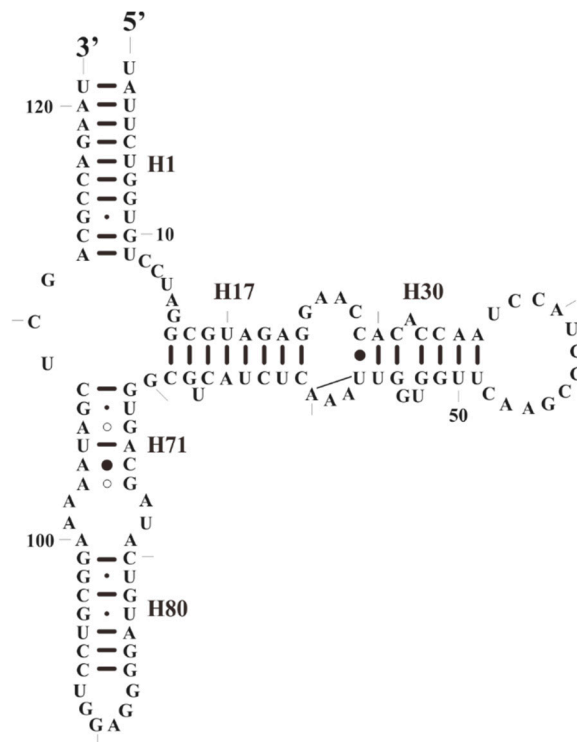


Figure 7. The predicted secondary structure model of 5S rRNA of *S. plumbizincicola*.

		<i>rrn4.5</i>	putative promoter of <i>rrn5</i>		<i>rrn5</i>			
Saxifragales	Crassulaceae	<i>Sedum plumbizincicola</i>	TCAAGAA	TTGGGGCCTCA	---CAAGCACTAGCCAATA---	GAATAT	-----GCTTT	
		<i>Sedum sarmentosum</i>	TCAAGAA	TTGGGGCCTCA	---CAAGCACTAGCCAATA---	GAATAT	-----GCTTT	
		<i>Sedum oryzifolium</i>	TCAAGAA	TTGGGGCCTCA	---CAAGCACTAGCCAATA---	GAATAT	-----GCTTT	
		<i>Phedimus takesimensis</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATA---	GAATAT	-----GCTTT	
	Haloragaceae	<i>Phedimus kantschaticus</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATA---	GAATAT	-----GCTTT	
		<i>Rhodiola rosea</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATA---	GAATAT	-----GCTTT	
		<i>Myriophyllum spicatum</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATA---	GAATAT	-----GCTTT	
		<i>Penthorum chinense</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATA---	GAATAT	-----GCTTT	
	Penthoraceae	<i>Mitella diphylla</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT	
		<i>Mitella formosana</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT	
		<i>Tiarella trifoliata</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT	
		<i>Tiarella polyphylla</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT	
		<i>Tiarella cordifolia</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT	
		<i>Heuchera richardsonii</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT	
		Saxifragaceae	<i>Heuchera villosa</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT
			<i>Heuchera parviflora</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT
			<i>Oreostrophe rupifraga</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT
			<i>Mukdenia rossii</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT
		Grossulariaceae	<i>Bergenia scopulosa</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT
			<i>Chrysosplenium aureobracteatum</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT
	<i>Saxifraga stolonifera</i>		TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT	
	<i>Ribes fasciculatum</i>		TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT	
	Iteaceae		<i>Itea chinensis</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT
			<i>Paeonia jishanensis</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT
			<i>Paeonia decomposita</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT
			<i>Paeonia rockii</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT
	Paeoniaceae		<i>Paeonia suffruticosa</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT
			<i>Paeonia ostii</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT
			<i>Paeonia delavayi</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT
			<i>Paeonia ludowii</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT
		<i>Paeonia brownii</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT	
		<i>Paeonia lactiflora</i>	TCCAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT	
		<i>Paeonia veitchii</i>	TCCAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT	
		<i>Paeonia obovata</i>	TCCAGAA	TTGGGGCCTCA	---CAATCACTAGCCAATAT---	GAATAT	-----GCTTT	
	Hamamelidaceae	<i>Parrotia subaequalis</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACCAGC-----	CAATAG	-----GCTTT	
		<i>Hamamelis mollis</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACCAGC-----	CAATAG	-----GCTTT	
		<i>Sinowilsonia henryi</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACCAGC-----	CAATAG	-----GCTTT	
		<i>Fortunearia sinensis</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACCAGC-----	CAATAG	-----GCTTT	
		<i>Corylopsis coreana</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACCAGC-----	CAATAG	-----GCTTT	
		<i>Loropetalum subcordatum</i>	---AGAA	TTGGGGCCTCA	---CAATCACCAGC-----	CAATAG	-----GCTTT	
Cercidiphyllaceae	<i>Chunia bucklandioides</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT		
	<i>Cercidiphyllum japonicum</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT		
Daphniphyllaceae	<i>Daphniphyllum oldhamii</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT		
	<i>Liquidambar formosana</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT		
Altingiaceae	<i>Spinaclia oleracea</i>	TTAAGAA	TTGGGGCTTCA	---CAATCACTAGC-----	TAATAT	-----CCCTTT		
	<i>Nicotiana tabacum</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	-----GCTTT		
	<i>Zea mays</i>	TTAAGAA	TTGGGACTTCA	---CAATCACTAGC-----	CAATAT	-----TTCT		
	<i>Spirodela oligorhiza</i>	TCAAGAA	TTGGGGCCTCA	---CAATCACTAGC-----	CAATAT	GAATATGCTTT		
	<i>Escherichia coli</i> consensus	TTGACA	TATAAT			
		-35, Sextama box	17 bp	-10, Pribnow box				

Figure 8. The putative promoters of *rrn5* among Saxifragales species.

3.3. Phylogenetic Implications

To investigate the evolutionary relationships among the order Saxifragales, we performed phylogenetic analyses using 83 plastid genes of 44 species. Two species of Vitaceae (*V. heyneana* and

V. vinifera) were employed as outgroups. After alignment, the concatenated sequences are 74,751 bp long. The trees derived from ML and BI analyses display the same topology, as shown in Figure 9. According to the Angiosperm Phylogeny Group (APG) system IV [98], the order Saxifragales comprises 15 families, 11 of which were chosen for the phylogenetic analyses. The order Saxifragales can be generally divided into two clades: core Saxifragales clade (maximum likelihood bootstrap [BS] = 100 and bayesian posterior probability [PP] = 1.0) and Paeoniaceae plus the woody clade ([BS] = 89 and [PP] = 1.0). The former clade is subdivided into two subclades: one containing Crassulaceae, Haloragaceae, and Penthoraceae, and the other comprising three families of Saxifragaceae alliance (Grossulariaceae, Saxifragaceae, and Iteaceae). The latter clade includes Paeoniaceae, Altingiaceae, Cercidiphyllaceae, Daphniphyllaceae, and Hamamelidaceae. In general, the framework of relationships within Saxifragales generated from this study agrees with those reported by Jian et al. [11], Moore et al. [99], and Soltis et al. [12].

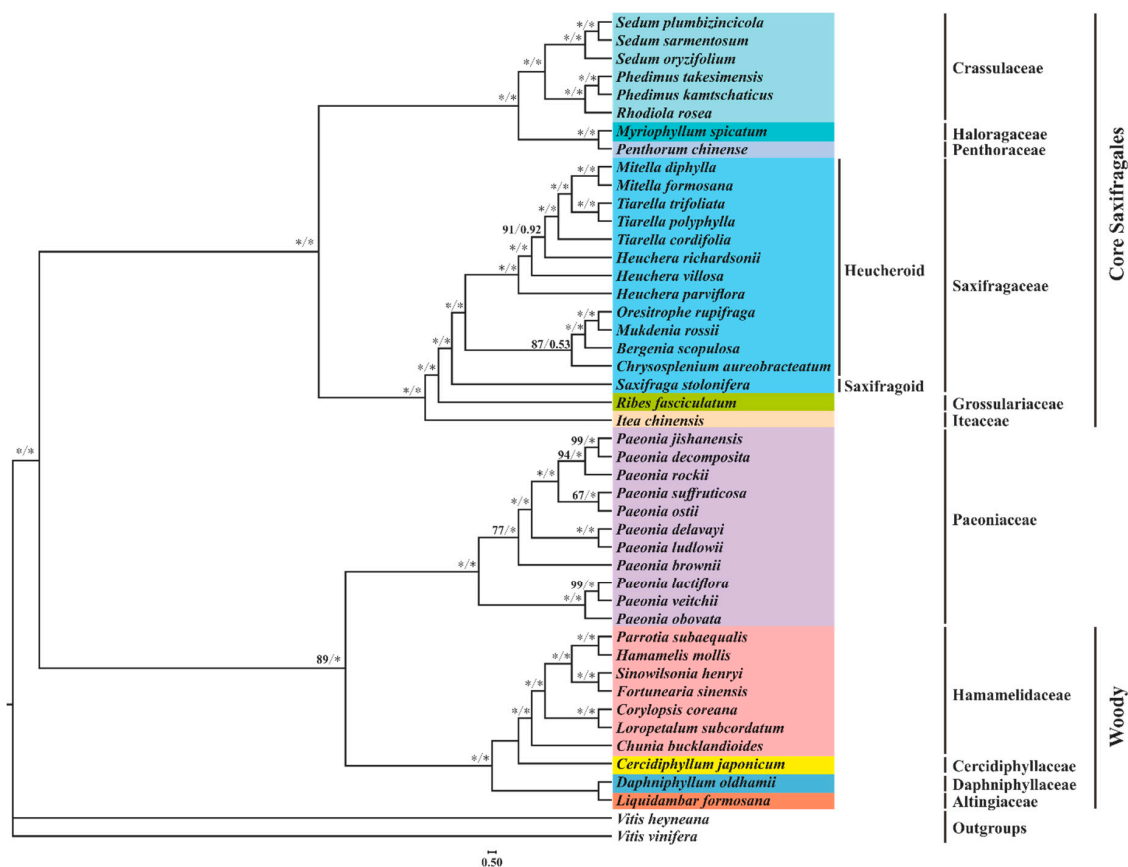


Figure 9. Nucleotide-based phylogenetic tree of 44 Saxifragales species. This analysis is based on 79 protein-coding genes (PCGs) and 4 rRNAs. The maximum likelihood bootstrap (BS) and bayesian posterior probability (PP) values for each node are indicated; * indicates 100% bootstrap or 1.00 PP. The bootstrap percentages < 50% and posterior probabilities < 0.5 were omitted.

In the present study, we found that *S. plumbizincicola* had a closer relationship with *S. sarmentosum* than *S. oryzifolium*. Furthermore, *Sedum* is sister to (*Phedimus* + *Rhodiola*). Species of *Phedimus*, previously treated as members of *Sedum*, have been classified as a separate genus [100,101]. Our data support this taxonomic revision of *Phedimus*.

Within Saxifragaceae alliance, Iteaceae is sister to (Grossulariaceae + Saxifragaceae), with strongly supported nodes ([BS] = 100 and [PP] = 1.0). Furtherly, Saxifragaceae can be divided into two subclades: heucheroid and saxifragoid [61,101]. Within the heucheroid, two genera, *Heuchera* and *Tiarella*, have been suggested as polyphyletic by several chloroplast markers [102,103]. Our present study based on nearly whole plastome sequence data supported this view. However, both morphology and nuclear internal

transcribed spacers (ITS) data have indicated that the two genera are monophyletic [102,104,105]. This incongruence between chloroplast and nuclear gene trees may be due to chloroplast capture [105–110].

Our results also accepted the monophyly of the woody clade, which is sister to the family Paeoniaceae. It is noteworthy that deep-level relationships within Hamamelidaceae are strongly supported. Nevertheless, the closest relatives of this family and relationships among these woody families are still unresolved in our analysis. This might partially be attributed to an ancient, rapid radiation [11]. Therefore, further detailed analyses need be conducted to evaluate the relationships within the woody clade.

4. Conclusions

In the present study, we first sequenced and analyzed the plastome of *S. plumbizincicola*. The genome structure and gene order were revealed, including 85 PCGs, 36 tRNA genes, 8 rRNA genes, and four pseudogenes. Next, we focused on the analyses of the primary and secondary structures of plastid rRNA genes. Notably, we found the sequence of putative *rrn5* promoter has some evolutionary implications within the order Saxifragales. Based on the 83 plastid genes from 44 species, phylogenetic analyses demonstrated that *S. plumbizincicola* had a closer relationship with *S. sarmentosum* than *S. oryzifolium*. Our findings reported here shed light on the structural evolution of plastid rRNA operon and phylogenetic relationships within Saxifragales.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2223-7747/8/10/386/s1>, Table S1: Genomic characteristics of 44 complete Saxifragales plastomes, Table S2: A comparison of sizes, G + C contents in the different regions of ribosomal RNA operon from Saxifragales plastomes.

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