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Article

Complete Chloroplast Genome Sequences of *Clematis*: IR Expansion and Relative Rates of Synonymous Substitutions

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Abstract: Genus *Clematis* is one of the largest within Ranunculaceae. Here we report the chloroplast genome of two *Clematis* species, *C. brachyura* and *C. trichotoma* endemic to Korea. The chloroplast genome lengths of *C. brachyura* and *C. trichotoma* are 159,532 bp and 159,170 bp, respectively. Gene contents in the complete chloroplast genomes of these two *Clematis* species are identical to that of most Ranunculaceae and other angiosperms. However, our data results demonstrated that genus *Clematis* has inversion and rearrangement events concerning gene *rps4* gene, *rps16* to *trnH* region, and *trnL* to *ndhC* region, and IR regions expansion. Comparison of IR regions among Ranunculaceae species revealed that *Clematis* species contained six protein coding genes (*infA*, *rps8*, *rpl14*, *rpl16*, *rps3*, and *rpl22*) usually found in the long single copy (LSC) region of other species. Phylogenetic analysis demonstrated that genus *Clematis* is closely related to genus *Ranunculus*. Differences in repeat structure, substitution rates, and IR expansion in genera *Clematis* and *Ranunculus*, explained their relationship. *Clematis* species showed slightly higher tandem repeats content than *Ranunculus* species. The six protein-coding genes showed lower synonymous substitution rates in the IR of *Clematis* species than in the LSC of *Ranunculus* species. Overall, the chloroplast genomes and results presented here provide important information on the evolution of Ranunculaceae.

Keywords: *Clematis*; Chloroplast genome; Rearrangement; Inversion; IR expansion; Synonymous substitution rate

1. Introduction

Family Ranunculaceae comprises 59-62 genera and approximately 2,500 species that have two chromosome types: R (*Ranunculus*) type with large chromosomes and T (*Thalictrum*) type with small chromosomes [1, 2]. Ranunculaceae were classified based on morphological characters until chromosomal characters became the most important criterion for classification [1, 3]. Genus *Clematis*, a member of Ranunculaceae, consists of 250-350 species, including some medicinal plants [4], which are distributed in all continents except Antarctica. *Clematis brachyura* and *C. trichotoma* are particularly valuable plants endemic to Korea [5].

The chloroplast (cp) genome consists of a large single copy (LSC), a small single copy (SSC), and two inverted repeat (IR) regions. In angiosperms, cp genomes of generally range from 120 to 160 kb in length and contain 110 to 130 genes. Gene contents and gene order are highly conserved in most angiosperms [6, 7] although many studies have reported changes in cp genome including gene loss

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[8, 9], inversion or deletions [10, 11], and expansion or contraction of the IR region [12-16]. The IR regions range from 15 to 30 kb in length and contain four ribosomal RNA (rRNA) genes, five transfer RNA (tRNA) genes and four coding genes. In recent studies, the IR regions have been use as important markers given their variability [17, 18], especially in plants, presenting large-scale expansions [14, 19], or loss of IR region [20, 21]. Previous studies suggested that mononucleotide, dispersed (>16 bp), tandem repeats and substitution rates are important contributors to cp genome size and structural evolution [13, 15, 22, 23].

Most recently, sequencing of complete cp genomes in Ranunculaceae revealed the transfer of *rpl32* gene to the nucleus [24], phylogenetic and genomic structure analyses in *Aconitum* spp. [25, 26] and the evolutionary history of Ranunculaceae [27]. Many studies focused on *Aconitum* species because this is a traditional herbal medicine in Asia [26, 28, 29].

The present study reports the complete cp genome of two species (*C. brachyura* and *C. trichotoma*) within genus *Clematis* and its major objectives were to 1) characterize and compare the structure, gene organization, and phylogenetic relationships within Ranunculaceae, 2) analyze the inversions, rearrangements, and IR expansions within *Clematis*.

2. Materials and Methods

2.1. *Chloroplast genome sequencing, assembly and annotation*

Total genomic DNA from *Clematis brachyura* and *C. trichotoma* were isolated from fresh leaf tissues of a single plant using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) and sequenced using the Illumina Miseq platform (Illumina Inc., San Diego, Ca). A total 8,572,072 bp and 7,505,088 bp pair-end sequence read of 300 bp was generated from the sequencing library, respectively. All contigs were de novo assembled into contigs with Velvet v. 1.2.03 [30]. Thereafter, complete chloroplast sequences were assembled with reference genome of *Clematis terniflora* (NC_028000) and assembled into a complete genome by overlaps using Geneious v 10.2.2 [31].

Complete cp genome sequences were annotated using DOGMA [32] and all identified tRNA genes were annotated using tRNAscan-SE [33]. Circular genome maps were drawn in OGDRAW [34]. To compare the structure and genes present in Ranunculaceae cp genomes, sequences from the different plants were aligned using MAFFT [35] and Geneious v. 10.2.2.

2.2. Phylogenetic analysis

A total of 71 coding genes of 15 Ranunculaceae were compiled in to a single file comprising 55,759 bp and aligned with MAFFT. Fourteen Ranunculaceae (including *C. brachyura* and *C. trichotoma*) were selected as in-groups and *Nuphar* (DQ354691) was included as outgroup (**Table 1**). Maximum likelihood (ML) analyses were performed using RAxML v7.4.2 using 1000 bootstrap replicates and the GRT+I+G model [38].

2.3. Repeat structure and substitution rate analysis

Tandem repeats (forward and reverse) were detected using REPuter [36]. The minimum repeats sizes was set at 30 bp and at a sequence indent greater than 90%. The simple sequence repeats (SSRs) were detected using Phobos v. 3.3.12 (http://www.ruhr-uni-bochum.de/ecoevo/cm/com_phobos.htm). The total number of repeats was compared across species considering mononucleotide (>8bp). One copy of the large IR was removed from each genome before repeat analyses.

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Protein-coding genes (*rpl14*, *rpl16*, *rpl2*, *rpl22*, *rps19*, *rps3* and *rps8*) of IR expansion region were extracted from each sequenced genome and previously published genomes *Clematis* (NC_02800 and KM652489), *Ranunculus* (DQ359689, KX557270 and KX639503) and *Aconitum* (NC_029829) genomes, were included as outgroup. Extracted sequences were aligned using MAFFT. Synonymous substitution (Ks) rates were analyzed in DnaSP [37].

3. Results

3.1. *Chloroplast genome and gene contents in genus Clematis* 3.1.1. Subsubsection

3.1. *Chloroplast genome and gene contents in genus Clematis*

Total cp genome size was 159,532 bp in *C. brachyura* (KM104710) and 159,170 bp in *C. trichotoma* (KM104711) and ranged within these values (**Figure 1, Table 1**), which is consistent with that from *C. fusca* var. *coreana* (KM652489) and *C. terniflora* (NC_02800). All of the *Clematis* genomes displayed a typical quadripartite structure, comprising two IRs (17,997-18,105 bp) separated by the LSC (79,339-79,478 bp) and SSC (17,997-18,105 bp) regions. The GC content of the cp genomes obtained here is 38%, which is consistent with all *Clematis* cp genomes. There are 112 unique genes, including 79 protein-coding genes, 29 tRNA genes and four rRNA genes.

Compared with Ranunculaceae, total genome sizes ranged from 146.9 to 160.8 kb. Species within *Clematis* have an expanded IR with approximately 5kb, comprising five or six coding genes (**Table 1**).

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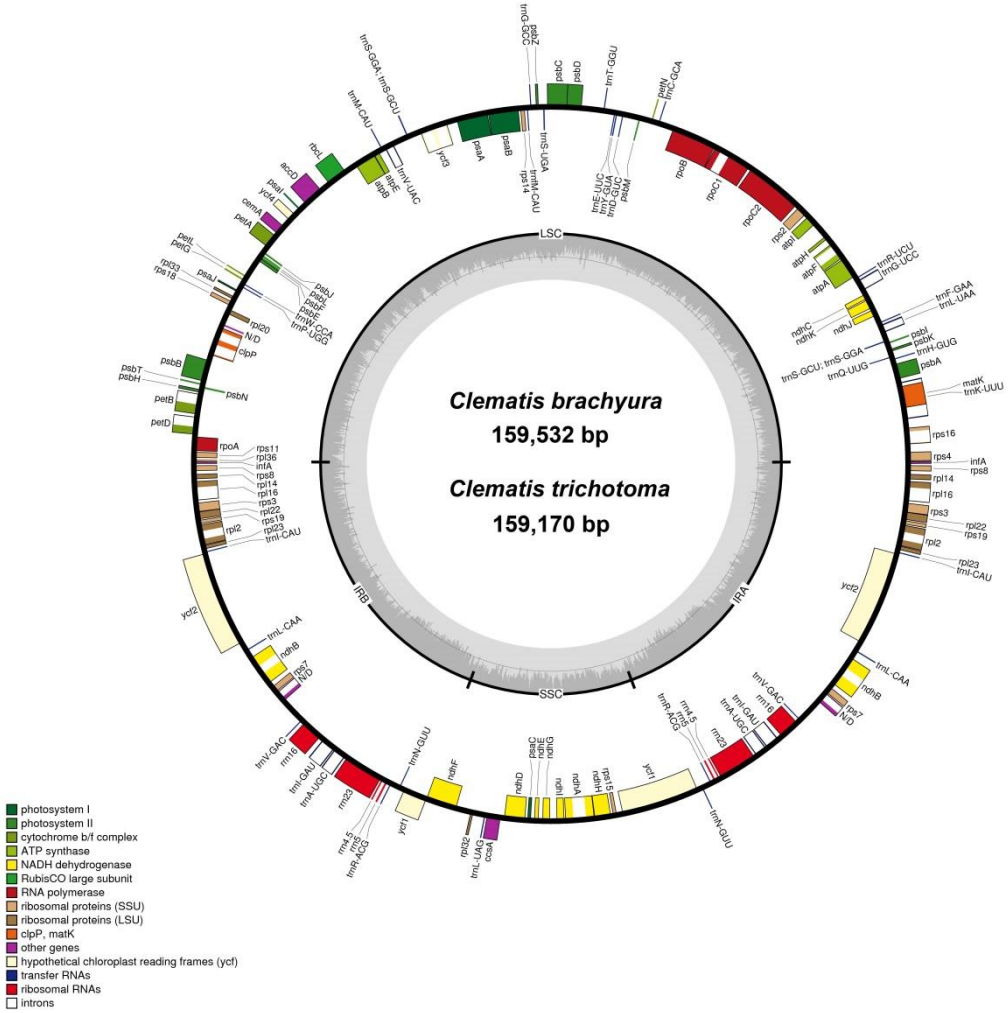

Figure 1. Chloroplast genome of *Clematis brachyura* and *C. trichotoma*. Genes inside the circle are transcribed clockwise, while genes outside the circle are transcribed counter-clockwise. Dark gray and light gray inner circles corresponds to GC and AT contents, respectively.

3.2. Phylogenetic relationships and gene loss in Ranunculaceae

The phylogenic analysis conducted for the 71 protein-coding genes of 15 Ranunculaceae (**Figure 2**) revealed several monophyletic groups with strong support (100% bootstrap values). Genera *Clematis* and *Ranunculus* form two well-supported monophyletic sister groups (100% bootstrap values), which is consistent with previous finding for family Ranunculaceae [2, 39].

Previous studies revealed that gene *infA* was lost or pseudogenation in angiosperm [18, 20, 40]. In the present study, *Ranunculus*, *Thalictrum*, and *Gymnaconitum* show an internal stop codon in *infA*. Park et al. [24] reported that *rpl32* was lost in *Thalictrum*. According to the present study, *Aconitum*, *Gymnaconitum* and *Actaea* also lost *rpl32* (**Figure 2**).

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117 **Table 1.** Ranunculaceae chloroplast genomes compared in this study.
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Species	Total Size (bp)	LSC size (bp)	SSC size (bp)	IR size (bp)	Genes in IR region (coding genes/tRNA/rRNA)	GC content in IR region	Accession number
***Clematis brachyura* (CB)**	159,532	79,341	18,105	31,043	13/7/4	42.1%	KM104710
***Clematis trichotoma* (CT)**	159,170	79,339	17,997	30,917	12/7/4	42.0%	KM104711
Clematis fusca var. *coreana* (CF)	159,609	79,478	18,044	31,048	13/7/4	42.0%	KM652489
Clematis terniflora (CTE)	159,528	79,328	18,092	31,054	13/7/4	42.0%	NC_028000
Ranunculus macranthus (RM)	155,129	84,637	18,910	25,791	6/7/4	43.5%	DQ359689
Ranunclus occidentalis (RO)	154,474	83,532	21,282	24,830	6/7/4	43.6%	KX557270
Ranunclus austro-oreganus (RA)	154,493	83,582	21,249	24,831	6/7/4	43.6%	KX639503
Aconitum chiisanense (AC)	155,934	86,559	17,054	26,161	6/7/4	43.0%	NC_029829
Gymnacotium gymnandrum	157,327	88,107	16,940	26,140	6/7/4	41.1%	NC_033341
Thalictrum coreanum	155,088	84,733	17,549	26,403	7/7/4	43.3%	NC_026103
Megaleranthis saniculifolia	159,924	88,326	18,382	26,608	7/7/4	43.1%	FJ597983
Tollius chinensis	160,191	88,522	18,405	26,632	7/7/4	43.1%	NC_031849
Coptis chinenesis	155,484	84,567	17,393	26,762	5/7/4	43.0%	NC_036485
Actaea racemosae	146,906	92,594	18,674	17,819	5/7/4	45.0%	NC_034704
Nuphar advena	160,866	90,379	18,817	25,835	7/7/4	43.3%	DQ354691

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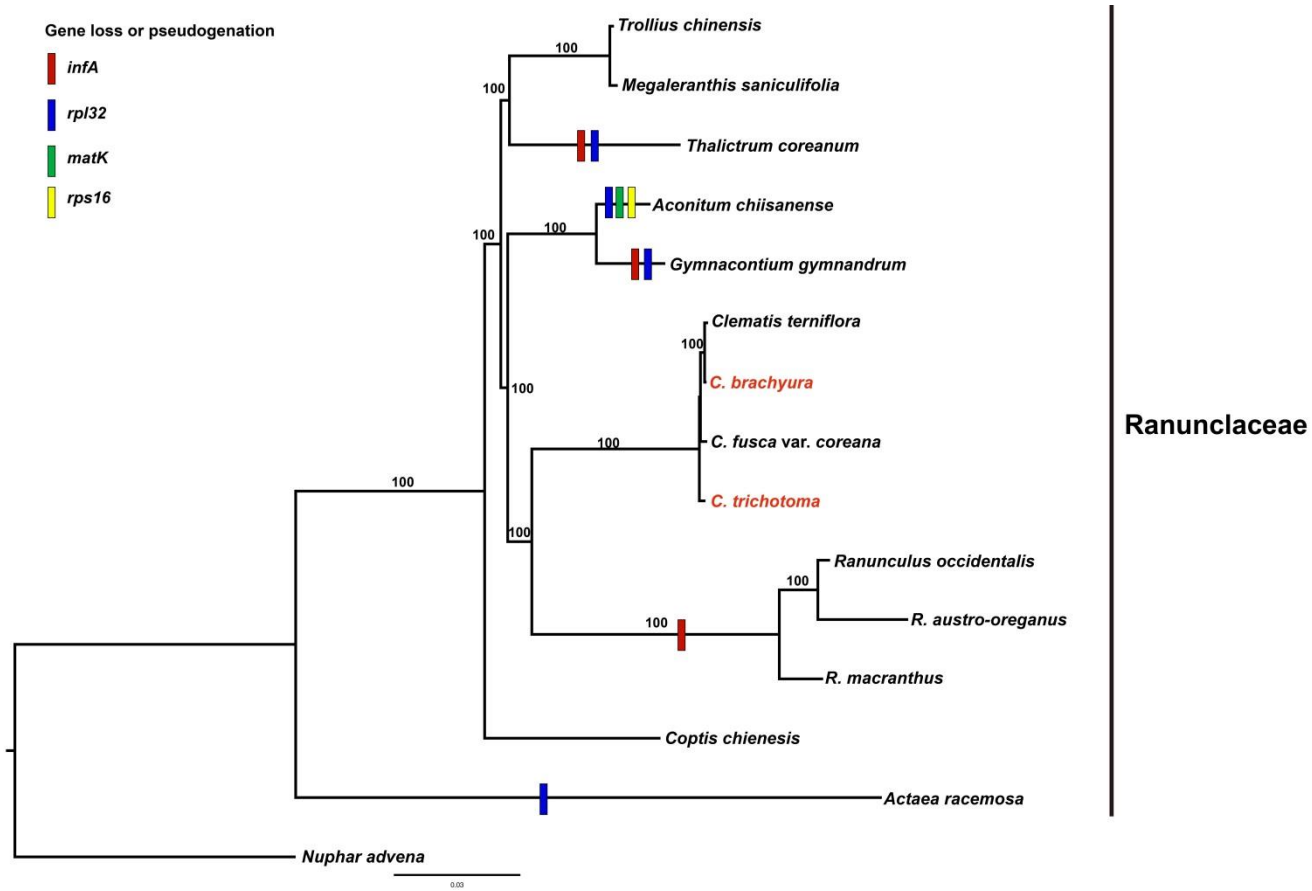

Figure 2. Phylogenetic tree reconstruction of 15 taxa using maximum likelihood, based on concatenated sequences of 71 protein-coding genes and gene loss.

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3.3. *Inversion, rearrangement, and IR expansion in genus Clematis*

Chloroplast genomes of Ranunculaceae are highly conserved. However, rearrangements, inversions and IR expansions have been detected in cp genomes of *Clematis* species. A small inversion (**Figure 3A**) and a large inversion (**Figure 3B**) were detected in the LSC region between genes *rps4* gene and *rps16* and to the *trnH* region, respectively (**Figure 3**). Rearrangements such as shifting *trnL* to *ndhC* region (**Figure 3C**) and shifting *rpl22* to the *infA* region (**Figure 4**) were also detected. Shifting *rpl22* to the *infA* region is considered an IR expansion in genus *Clematis*.

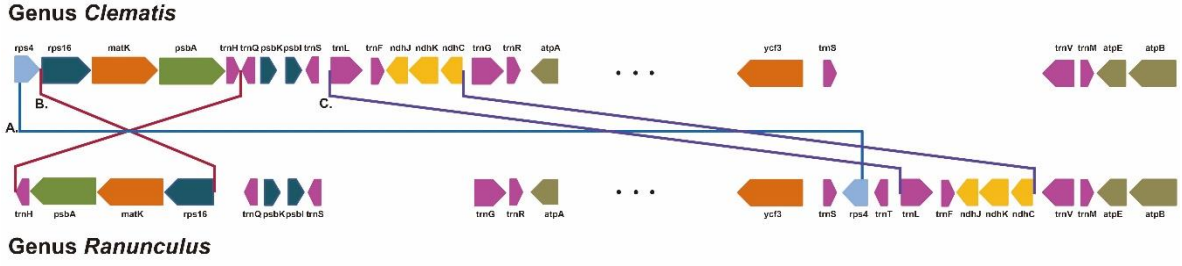

Figure 3. Inversions and rearrangements in genus *Clematis*. A: rearrangement of gene *rps4*. B: inversion of *rps16* to the *trnH* region C: rearrangement of *trnL* to the *ndhC* region.

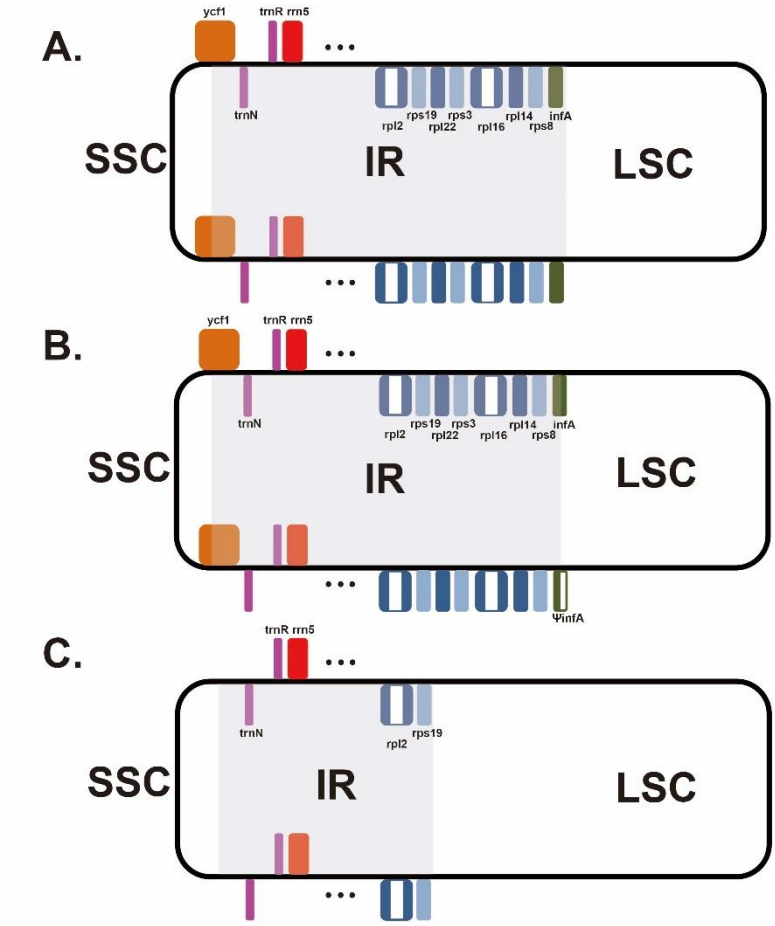

Figure 4. The inverted repeat (IR) region expansion in genus *Clematis*. A: IR region of Clematis brachyuran B: IR region of C. trichotoma, C: IR region of genus Ranunculus species. SSC, small single copy, LSC, long single copy.

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149 *3.4. Repeat content*

150 Tandem repeats and repetitive sequences were compared between *Clematis* and *Ranunculus* species
151 (**Table 2**). Repetitive sequences were longer in *Clematis* species (1,369 to 1,402 bp) than in *Ranunculus*
152 species (1,073 to 1,487 bp) and tandem repeats were also longer in *Clematis* species (385 to 461 bp)
153 than *Ranunculus* species (212 to 265 bp). Among the eight cp genomes compared (including
154 *Aconitum*), four *Clematis* species contained the longest tandem repeats (more than 73 bp).
155

156 **Table 2.** Summary of repetitive sequences and tandem repeats contents *Clematis* and *Ranunculus* species
157 (*outgroup).

Species	Size (bp)	Repetitive sequence (bp)	Tandem repeats (bp)
***Clematis brachyura* (CB)**	128,500	1,381	385
***Clematis trichotoma* (CT)**	128,248	1,369	461
Clematis fusca var. *coreana* (CF)	128,561	1,402	427
Clematis terniflora (CTE)	128,474	1,390	385
Ranunculus macranthus (RM)	129,338	1,487	265
Ranunculus occidentalis (RO)	129,644	1,073	212
Ranunculus austro-oreganus (RA)	129,662	1,136	212
Aconitum chiisanense (AC)*	129,773	1,155	313

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159 *3.5. Synonymous substitution rates*

160 Synonymous substitution rates were compared between the IR and LSC regions of four *Clematis*
161 and three *Ranunculus* species. The values of *Ks* were markedly higher for LSC than for IR genes in
162 both genera (Figure 5A, B). Ranunculaceae have six protein-coding genes (*infA*, *rps8*, *rpl14*, *rpl16*,
163 *rps3*, and *rpl22*) in the LSC region. However, in *Clematis* genomes, these six protein-coding genes
164 shifted from the LSC region to IR regions (**Figure 4, 5**). For five of these genes (except *infA*), *Ks*
165 values were substantially lower in IR than LSC region (**Figure 5C**). Gene *infA* was lost in genus
166 *Ranunculus.* Genes (*rpl14*, *rpl16*, and *rps19*) exhibited extremely lower values in the IR of *Clematis*
167 species than in the LSC of *Ranunculus* species.

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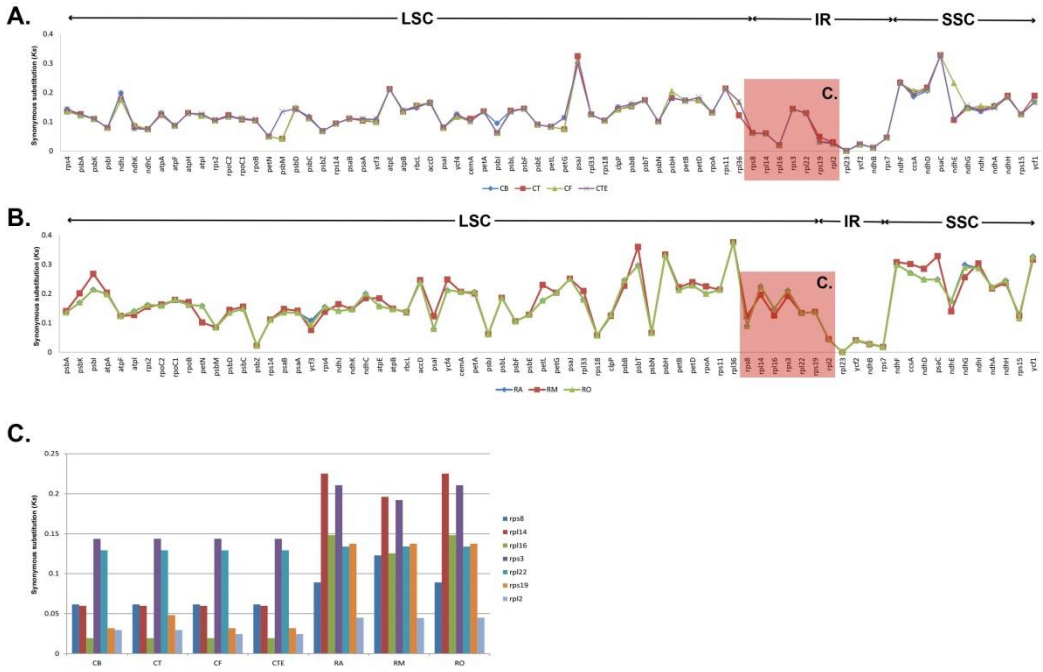

Figure 5. Synonymous substitutions (*Ks*) in genera *Clematis* and *Ranunculus*. A: *Ks* in *Clematis* species. B: *Ks* in *Ranunculus* species. C: *Ks* for genes in the junction of long single copy (LSC) and inverted repeat (IR) regions, including genes relocated into or out of these regions.

4. Discussion

4.1 *Structure of chloroplast genomes and comparative analyses*

Previous studies showed that typical cp genomes of angiosperms have similar size, gene order and gene contents [41, 42]. Chloroplast genome sizes of Ranunculaceae generally range from generally146-155 kb (expect for *Trollius*), but *Clematis* species cp genomes are approximately 159 kb in size. Gene order within LSC region and IR regions also differs between *Clematis* and other Ranunculaceae genera. In particular, the IR regions expanded into the LSC region by shifting six protein-coding genes (*infA*, *rps8*, *rpl14*, *rpl16*, *rps3*, and *rpl22* usually found in the LSC region. Similar patterns have observed in *Tetracentron* and *Trochodendron* within family Trochodendraceae [43], where five protein-coding genes (*rps8*, *rpl14*, *rpl16*, *rps3*, and *rpl22*) are shifted to the IR regions. A previous study suggested poly A regions might play an important role in IR expansion [12]. In *Clematis* species, IR extension ends in the LSC region and the IR region is over 70% AT-rich with numerous possible poly A tracts that might have played a role in its expansion.

The cp genome of *Clematis* species has similar gene contents to that of other Ranunculaceae genera. However, Clematis species present rearrangements and inversions in the LSC region. Hoot and Palmer [44] reported that *Anemone* and related genera (*Clematis*, *Pulsatilla*, *Hepatica* and *Knowltonia*) underwent inversion and rearrangement events throughout cp genome evolution. Several studies suggested that tandem repeats or AT-rich regions occurred rearrangement and plastome size evolution in angiosperm such as *Silene* [23], *Trifolium* [45], and Geraniaceae [13]. For example, repetitive sequence and tandem repeats within the contraction/expansion IR region of some *Sileneae* species is dramatically higher than in other *Sileneae* species [23]. However, the genus *Clematis* species show tandem repeats content slightly higher than closely related taxa or outgroup (**Table 2**).

4.2 *Gene shifts from LSC to IR reduced synonymous substitution rates*

The synonymous substitution rate is lower in the IR than in the single copy (SC) regions of angiosperm cp genomes [46]. Zhu et al. [16] demonstrated that genes moved from the SC into IR regions in land plants demonstrated lower synonymous substitution rates consistent with that observed for original genes in IR regions.

In this study, we examined synonymous substitution rates of five LSC genes (*rps8*, *rpl14*, *rpl16*, *rps3*, and *rpl22*) shifted to IR in *Clematis* species and LSC located genes in *Ranunculus* species (**Figure 5C).** We found that the five shifted genes in *Clematis* species (LSC to IR genes) exhibited lower synonymous substitution rates than genes located in the LSC region of *Ranunculus* species. These results support that the duplicative nature of the IR reduces the synonymous substitution rate within this region, as stated in a previous study [16].

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

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