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Molecules and Cells

Complete Chloroplast DNA Sequence from a Korean Endemic Genus, *Megaleranthis saniculifolia*, and Its Evolutionary Implications

Young-Kyu Kim, Chong-wook Park[1], and Ki-Joong Kim*

The chloroplast DNA sequences of *Megaleranthis saniculifolia,* an endemic and monotypic endangered plant species, were completed in this study (GenBank FJ597983). The genome is 159,924 bp in length. It harbors a pair of IR regions consisting of 26,608 bp each. The lengths of the LSC and SSC regions are 88,326 bp and 18,382 bp, respectively. The structural organizations, gene and intron contents, gene orders, AT contents, codon usages, and transcription units of the *Megaleranthis* chloroplast genome are similar to those of typical land plant cp DNAs. However, the detailed features of *Megaleranthis* chloroplast genomes are substantially different from that of *Ranunculus,* which belongs to the same family, the Ranunculaceae. First, the *Megaleranthis* cp DNA was 4,797 bp longer than that of *Ranunculus* due to an expanded IR region into the SSC region and duplicated sequence elements in several spacer regions of the *Megaleranthis* cp genome. Second, the chloroplast genomes of *Megaleranthis* and *Ranunculus* evidence 5.6% sequence divergence in the coding regions, 8.9% sequence divergence in the intron regions, and 18.7% sequence divergence in the intergenic spacer regions, respectively. In both the coding and noncoding regions, average nucleotide substitution rates differed markedly, depending on the genome position. Our data strongly implicate the positional effects of the evolutionary modes of chloroplast genes. The genes evidencing higher levels of base substitutions also have higher incidences of indel mutations and low Ka/Ks ratios. A total of 54 simple sequence repeat loci were identified from the *Megaleranthis* cp genome. The existence of rich cp SSR loci in the *Megaleranthis* cp genome provides a rare opportunity to study the population genetic structures of this endangered species. Our phylogenetic trees based on the two independent markers, the nuclear ITS and chloroplast *matK* sequences, strongly support the inclusion of the *Megaleranthis* to the *Trollius*. Therefore, our molecular trees support Ohwi's original treatment of *Megaleranthis saniculifolia* to *Trollius chosenensis* Ohwi.

INTRODUCTION

The chloroplast (cp) genomes of vascular plants are highly conserved in terms of gene contents and genome structures as compared to other genomes (Palmer, 1991; Raubeson and Jansen, 2005). Two large inverted repeats (IR) ranging from 12-75kb separate the large single copy (LSC) and small single copy (SSC) regions (Palmer, 1990; 1991). The cp genome sizes of land plants ranged from 100 to 200 kb due to differences in the lengths of the IR regions. Expansions and contractions of IR regions both into the SSC and LSC regions are primarily responsible for the differing sizes of the cp genomes. The gene orders and the polycistronic transcription units of cp genome are also conserved among the majority of the vascular plants (Hermann et al., 1991; Kanno and Hirai, 1993; Shinozaki et al., 1988). However, the gene orders of the cp genomes are sometimes rearranged in independent plant groups (Cosner et al., 1997; 2004; Doyle et al., 1992; Hachitel et al., 1991; Hiratsusuka et al., 1989; Hoot and Palmer, 1994; Jansen and Palmer, 1987; Kim et al., 2005; Knox et al., 1993; Lee et al., 2007; Lidholm and Gustafsson, 1991; Milligan et al., 1989; Palmer and Thompson, 1981; Palmer et al., 1987a; 1987b; Sasaki et al., 2005; Strauss et al., 1988) and shows powerful phylogenetic utilities.

Inversion mutations are the most frequent rearrangements in the cp genomes. In most cases, dispersed repeats have been demonstrated to promote inversions via intramolecular recombination (Lee et al., 2007; Ogihara et al., 1988; Palmer, 1991). Gene relocations, such as transposition, gene duplication and insertion, and gene and intron losses are also mediated by the inversion mechanism (Lee et al., 2007). Several evolutionary hot spot regions evidencing high levels of insertion/deletion and base substitutions are concentrated on some intergenic spacers (Palmer, 1991; Raubeson and Jansen, 2005). Several comparative studies have documented the phylogenetic utility of chloroplast genome structures at higher taxonomic levels. However, only a few detailed comparative evolutionary studies have been conducted using the complete cp genome in closely

School of Life Sciences, Korea University, Seoul 136-701, Korea, [1]School of Biological Sciences, Seoul National University, Seoul 152-742, Korea
*Correspondence: kimkj@korea.ac.kr

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related genera or species.

The genus *Megaleranthis saniculifolia* Ohwi is monotypic, and is one of six genera endemic to Korea. The species is quite rare, and evidences disjunctive distribution patterns in the high mountain areas of Jiri, Sobaek, Halla, and Jeombong. It belongs to the family Ranunculaceae, which consists of approximately 59 genera and 2,500 described species. The family is distributed widely throughout temperate and cold temperate regions of the Northern hemisphere (Tamura, 1993). In Korea, this family is comprised of 29 genera and 104 species. The family has long been considered to be important to both horticulture and medicine. Several family members, including *Ranunclulus, Anemone, Clematis,* and *Delphinium* have been developed into ornamental plants due to their attractive and showy flowers. The genus *Megaleranthis* also has the potential to be developed into an ornamental herbaceous plant. Many members of families have also been utilized in both traditional and modern medicine, due to their rich and diverse alkaloid compounds. Several different systematic treatments of the Ranunculaceae have been previously published (Hoot, 1991; Jansen, 1966; 1968; Johansson and Jansen, 1991; Leppik, 1964; Santisuk, 1979; Tamura, 1966).

The genus *Megaleranthis* is classified into a member of the Tribe Trollieae or the Tribe Heleboreae along with *Trollius, Calathodes* and *Caltha,* on the basis of karyotype, pollen exine, and general morphology (Lee, 1989; 1990; Tamura, 1990; Yuan and Yang, 2006). Among them, several researchers proposed a close relationship between *Megaleranthis* and *Trollius* (Kim and Lee, 1987; Lee, 1989; 1990; Owhi, 1937). In addition, close relationships to the *Adonis* also reported in molecular data (Jensen et al., 1995; Ro et al, 1997). *Megaleranthis* however, can be distinguished from *Calathodes* by the presence of petals and the absence of interstitial growth of the receptacles after flowering. *Megaleranthis* can be readily distinguished from the *Trollius* by the presence of involucral bracts and the absence of nectariferous pits in the flowers, despite its karyological and pollen similarities. Therefore, most authors have maintained that *Megaleranthis* is a distinctive Korea endemic genus (Park et al., 2007; Tamura, 1995). There remains substantial disagreement with regard to the phylogenetic relationships between *Megaleranthis saniculifolia* and other related genera.

In this study, we have analyzed the structural organization and evolutionary modes of the cp genome from a monotypic and an endemic Korean plant genus, *Megaleranthis saniculifolia*. The comparative sequence analysis of the whole chloroplast genomes between *Megaleranthis saniculifolia* and related genera will provide us with better information regarding the evolution of the cp genome. In addition, the comparative data will contribute to our better understanding of the origin and evolution of endemic genera in Korea. This data may also provide correct information regarding the phylogenetic position of *Megaleranthis saniculifolia* in the family Ranunculaceae.

MATERIALS AND METHODS

Plant materials and DNA extraction

For the whole cp genome analysis, *Megaleranthis saniculifolia* was collected from the central part of Korea (Mt. Sobaek). Three individual plants forming a clone were collected from wild habitats (A voucher specimen is kept at the Korea University Herbarium, Seoul, Korea, http://pdbk.korea.ac.kr). The taxa utilized for comparative phylogenetic study are listed in Table 1. Total DNA was isolated from the fresh leaves by the CTAB method (Doyle and Doyle, 1987) and DNA was purified via ultracentrifugation in cesium chloride/ethidium bromide gradients (Sambrook et al., 1989).

PCR amplification and sequencing

We utilized long-range PCR amplification strategies for the whole genome amplifications conducted in this study. Approximately 45 overlapping fragments, ranging from 4 kb to 10 kb, were amplified using the self-designed primers. The amplification primers were designed on the basis of comparative cp DNA sequences analysis among *Nicotiana, Panax, Jasminum,* and *Ranunculus* (Kim and Lee, 2004; Lee et al., 2007; Raubeson et al., 2007; Shinonzaki et al., 1986). Each fragment was sequenced using a series of cp DNA primers that covers both directions at a length of 400 bp to 600 bp. For the phylogenetic analysis, the chloroplast *matK* region was amplified and sequenced using a pair of primers (F:AACTAGTCGGATGGAGTAG)/(R:TGGGTTGCTAACTCAATGG). In addition, nuclear ribosomal ITS regions were amplified and sequenced using a pair of primers (*ITS1*:TC CGTAGGTGAACCTGCGG)/(*ITS4*:TCCTCCGCTTATTGATATGC). The PCR products were purified with the MEGAquick-spin kit (iNtRON, Korea) and then the cleaned products were sequenced with an ABI 3730X1 automatic sequencer.

Chloroplast genome analysis

The sequence fragments were assembled using Sequencer 4.5 (Gene Code Corporation, USA). Gene annotations and comparative analysis were conducted using the BLAST (BLASTN, PHI-BLAST, BLASTX), ORF finder program from the National Center for Biotechnology Information (NCBI) and DOGMA (Wyman et al., 2004). Codon usage and A-T contents were analyzed using MEGA4 (version 3.1). Repeating sequences were searched using REPuter (Kurtz et al., 2001). The locations and secondary structure of tRNA, rRNA, intron regions and other parts of the genomes were evaluated using tRNAscan-SE (version 1.21) and mFOLD (version 3.3) software. The DnaSP (version 4.50) and mVISTA programs were utilized for comparisons of similarities among chloroplast genomes.

Phylogenetic analysis

59 ITS region sequences and 20 *matK* sequences were obtained from NCBI (Table 1). All other sequences were generated in this study and deposited in the NCBI database (Genbank accession numbers are provided in Table 1). For the phylogenetic study, all homologous gene sequences were aligned with the CLUSTAL algorithm (Higgins et al., 1996) and edited with the MEGA program (Kumar et al., 2001). Parsimony analysis was conducted using PAUP version 4.0b10 (Swofford, 2002). We reconstructed the maximum parsimony (MP) trees for each gene. Gaps were treated as missing data and all characters were equally weighted. The amount of support for monophyletic groups was evaluated by 1,000 bootstrap replicates (Farris et al., 1996). In addition to MP analysis, a neighbor joining (NJ) tree with 1,000 bootstrap replications was also constructed (Saitou and Nei, 1985).

RESULTS

General features of *Megaleranthis saniculifolia* cp genome

The complete chloroplast sequences of *Megaleranthis saniculifolia* is 159,924 bp in length (GenBank FJ597983). It harbors a pair of inverted repeat regions (IRa and IRb), each consisting of 26,608 bp. The two IR regions divided the genome into the large single copy (LSC) region and the small single copy (SSC) region. The LSC region is 88,326 bp and the SSC region is

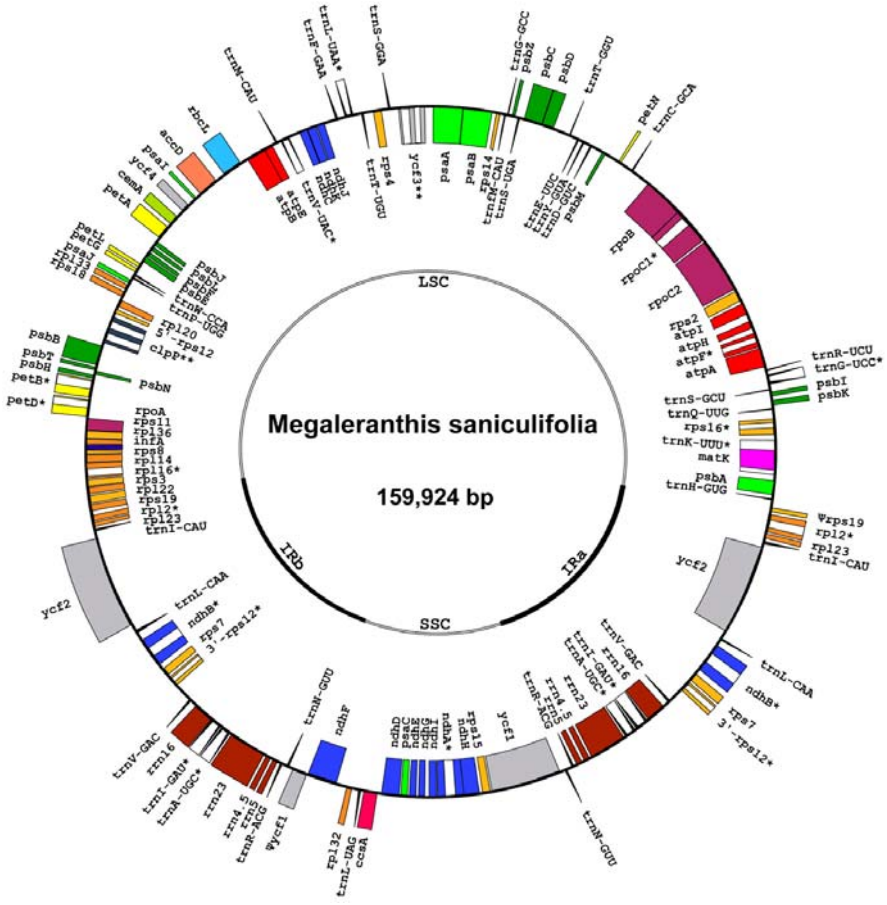

Fig. 1. The gene map of *Megaleranthis saniculifolia* cp genome. A pair of thick lines at the inside circle represent the inverted repeats (IRa and IRb; 18,382 bp each), which separate the large single copy region (LSC; 88,326 bp) from the small single copy region (SSC; 26,608 bp). Genes drawn inside the circle are transcribed clockwise, while the outside genes are transcribed counterclockwise. Intron-containing genes are marked by asterisks.

18,382 bp (Fig. 1). The 113 individual genes in the *Megaleranthis* chloroplast genome are provided in Fig. 1 and Table 2. The 19 genes are located on the IR regions. The 61 protein coding genes and 22 tRNA genes are located in the LSC region, whereas the 12 protein coding genes and one tRNA gene are located in the SSC region. The *Megaleranthis* chloroplast genome is composed of 55% coding regions and 45% non-coding regions. A total of 18 genes include one or two introns (Table 3). Among these, *rps12*, *clpP,* and *ycf3* harbor two introns. The *rps12* gene is the unique divided gene in which the 5′ end exon is located in the LSC region and two 3′ end exons and introns are duplicated and located on the IR regions.

The overall GC and AT contents of the *Megaleranthis* chloroplast genome were 38% and 62%, respectively. The AT contents in the IR regions amount to 57%, whereas, the AT contents in the LSC and SSC regions were 64% and 68%, respectively. The low AT content in the IR regions is attributed to the low AT contents of the four rRNA (*rrn16*, *rrn23*, *rrn4.5*, *rrn5*) genes in the regions. The AT contents of protein coding regions is approximately 62%, with the first position of codon at 55%, second position at 61% and the third position at 69%, respectively. The nucleotide frequencies of *Megaleranthis* cp genomes are provided in Table 4.

The 30 tRNA genes representing 20 amino acid species are identified in the *Megaleranthis* cp genome by similarity search and computer prediction. The codon usages of the *Megaleranthis* cp genome are summarized in Table 5. Six of the 30 tRNA gene species, *trnK-UUU*, *trnG-UCC*, *trnL-UAA*, *trnV-UAC*, *trnI-GAU* and *trnA-UGC*, contained intervening sequences at the anticodon stem/loop or D-stem regions. The high AT content at the third codon position (Table 4) reflects the codon usage bias to A or T. The codon usage frequencies of the stop codons are also biased to A or T at both the second and third codon positions.

Angiosperm chloroplast genomes evidence a wide range of length variations at the inverted repeats and single copy region boundaries regions owing to the frequent expansions and contractions of the IR regions. The detailed comparison of inverted repeats (IR) - single copy (SC) boundaries among *Megaleranthis*, *Nicotiana*, *Nandina*, *Ranunculus* and *Arabidopsis* are shown in Fig. 2. This data is reflective of the various border positions even in closely related genera of the same family, such as *Megaleranthis* and *Ranunculus*. The various pseudogenes, such as *rps19* and *ycf1,* are created at the IR-SC boundaries. The IR of *Megaleranthis* extended into the *rps19* gene and inserted into the *rps19* pseudogene in 104 bp lengths at the IRa/LSC border, whereas the IR of *Ranunculus* extended into the *trnH-GUG* gene and inserted into the *trnH-GUG* pseudogene with 23 bp lengths at the IRb/LSC border.

Distribution of cp SSR

The simple sequence repeats (SSR) in which the same nucleotide sequence unit is repeated more than 10 times were identified from 54 different locations on the *Megaleranthis* cp genome (Table 6). The majority (36) of the 54 SSR loci are homopolymers, whereas 14 loci are diSSR, and two loci are triSSR. Tetra- and pentaSSR also occurred in two locations. All of the 36 homopolymer loci were composed of multiple A's or

Table 1. Taxa used for chloroplast DNA sequencing and phylogenetic analysis

Taxon	DNA number[a]	GenBank accession number	
		ITS	*matK*
Megaleranthis saniculifolia Ohwi	2007-0164	FJ597996*	FJ598002*
Actaea asiatica Hara	2006-0742	FJ597984*	
Adonis amurensis Regel & Radde		AB361620	
Adonis annua L.		AY148280	
Adonis multiflora Nishikawa & Koji Ito		AB361622	
Adonis pseudoamurensis W.T. Wang		AF454935	
Adonis ramosa Franch.		AB361618	
Adonis shikokuensis Nishikawa & Koji Ito		AB361623	
Adonis vernalis L.		AJ347910	AJ414340
Anemone acutiloba Laws.			DQ994677
Anemone americana (DC.) Hara		AY055386	AF542590
Anemone amurensis Kom.		EF139271	
Anemone antucensis Poepp.		AY056049	
Anemone blanda Schott ex Kotschy		AY055402	
Anemone caffra Harv.		AY055399	
Anemone canadensis L.		AY055387	
Anemone caroliniana Walter		AY055403	
Anemone cernua Thunb.			AB110531
Anemone crassifolia Hook.		AY055398	
Anemone demissa Hook. f. ex Thomson		AY055392	
Anemone drummondii S. Watson		AY055404	
Anemone flaccida Fr. Schmidt		AY055391	AB110530
Anemone hupehensis Hort. ex Boynton		AY055397	
Anemone keiskeana T. Ito ex Maxim.		AY055390	
Anemone knowltonia Burtt Davy		AY055401	
Anemone koraiensis Nakai	2006-0360	FJ597985*	
Anemone montana Hoppe ex Sturm		AM267281	
Anemone multifida Standl.		AY055405	
Anemone narcissiflora L.		AY055393	
Anemone nemorosa L.		AM267278	
Anemone obtusiloba Lindl.		AY055394	
Anemone occidentalis S. Watson		AY055400	
Anemone patens L.		AM267280	
Anemone pendulisepala Y.N. Lee		EF139262	
Anemone reflexa Ledeb. & Spreng.	2006-0309	FJ597986*	
Anemone richardsonii Hook.		AY055388	
Anemone rivularis Wall.		AY055396	
Anemone stolonifera Maxim.		EF139243	
Anemone sylvestris L.		AM267276	
Anemone tenuicaulis (Cheeseman) Parkin & Sledge		AY055389	
Anemone transylvanica Heuff.			DQ994670
Anemone trullifolia Hook. f. & Thomson		AY055395	
Caltha appendiculata Pers.		AY365385	
Caltha dionaeifolia Hook.		AY365389	
Caltha introloba F. Muell.		AY365387	

(Continued)

Taxon	DNA number[a]	GenBank accession number	
		ITS	*matK*
Caltha leptosepala subsp. *howellii* (Huth) P.G. Smit		AY365395	
Caltha leptosepala DC. subsp. *leptosepala*		AY365394	
Caltha natans Pall.		AY365398	
Caltha novae-zelandiae Hook. f.		AY365388	
Caltha obtusa Cheeseman		AY365386	
Caltha palustris L.			AB069845
Caltha palustris L. var. *membranacea* Turcz.	2006-0361	FJ597987*	FJ597997*
Caltha sagittata Cav.		AY365391	
Caltha scaposa Hook. f. & Thomson		AY365396	
Cimicifuga acerina (Siebold & Zucc.) Tanaka			AF353578
Cimicifuga foetida L.	2006-1485	FJ597988*	
Cimicifuga heracleifolia Kom.	2006-0341	FJ597989*	
Cimicifuga simplex Wormsk. ex DC.	2006-1511	FJ597990*	FJ597998*
Eranthis hyemalis Salisb.		Z98273	AJ414342
Eranthis pinnatifida Maxim.	2001-0496	FJ597991*	
Eranthis stellata Maxim.	2006-0341	FJ597992*	FJ597999*
Hepatica acutiloba DC.		AM267285	
Hepatica asiatica Nakai	2006-0300	FJ597993*	
Hepatica falconeri (Thomson) Juz.			DQ994675
Hepatica henryi Steward			DQ994674
Hepatica insularis Nakai	2006-0421	FJ597994*	FJ598000*
Hepatica maxima Nakai	2005-0599	FJ597995*	FJ598001*
Hepatica nobilis Schreb.			DQ994668
Hepatica nobilis Mill. var. *asiatica* (Nakai) H. Hara			DQ994672
Hepatica transsilvanica Fuss		AM267283	
Thalictrum aquilegiifolium L.	2006-0391		FJ598003*
Thalictrum dioicum L.		U75663	
Trollius acaulis Lindl.		AY148265	
Trollius altaicus C. A. Mey.			AY515233
Trollius asiaticus L.		AY148267	
Trollius chinensis Bunge		AY148268	AY515235
Trollius europaeus L.		AY148270	AY515236
Trollius hondoensis Nakai	2004-1055		FJ598004*
Trollius japonicus Miq.	2004-1264		FJ598005*
Trollius laxus Salisb.		AY148266	
Trollius ledebourii Rchb.		AY148271	AY515237
Trollius membranostylis Hulten		AY148272	
Trollius pulcher Makino			AY515239
Trollius pumilus D. Don		AY148273	
Trollius ranunculinus (Sm.) Stearn		AY148277	
Trollius riederianus Fisch. & C.A. Mey.		AY148278	AY515241
Trollius yunnanensis Ulbr.			AY515238

[a]DNA numbers are from Plant DNA Bank of Korea (http://pdbk.korea.ac.kr). Asterisks indicate new sequences generated in this study.

T's. 10 of the diSSR loci were composed of multiple AT's or TA's. The SSR loci also contribute to the A-T richness of the cp genome of *Megaleranthis*. The 36 of the total SSR loci occurred in the intergenic spacers and six of the SSR loci occurred in the intron regions, respectively.

Phylogenetic analysis

For phylogenetic analysis using the nuclear internal transcribed spacer (ITS) region, the sequences of 72 taxa were assembled and aligned. 13 sequences were generated in this study (GenBank FJ597984-FJ597996) and the others were obtained from the NCBI database (Table 1). The aligned data matrix consists of 678 characters, and *Thalictrum dioicum* was used to outgroup for the analysis. The strict MP consensus tree was 1,339 steps in length, with a consistency index (CI) of 0.512, a retention index (RI) of 0.863, and a rescaled consistency index

Table 2. Genes contained in *Megaleranthis* chloroplast genome (total 113 genes)

DNA numbers	Group of genes	Name of genes[a]
Self replication	rRNA genes	*rrn16(x2), rrn23(x2), rrn4.5(x2), rrn5(x2)*
	tRNA genes	*trnA-UGC*(x2), trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnfM-CAU, trnG-GCC, trnG-UCC*, trnH-GUG, trnI-CAU(x2), trnI-GAU*(x2), trnK-UUU*, trnL-CAA(x2), trnL-UAA*, trnL-UAG, trnM-CAU, trnN-GUU(x2), trnP-UGG, trnQ-UUG, trnR-ACG(x2), trnR-UCU, trnS-GCU, trnS-GGA, trnS-UGA, trnT-GGU, trnT-UGU, trnV-GAC(x2), trnV-UAC*, trnW-CCA, trnY-GUA*
	Small subunit of ribosome	*rps2, rps3, rps4, rps7(x2), rps8, rps11, rps12*(x2, part), rps14, rps15, rps16*, rps18, rps19(x2, part)*
	Large subunit of ribosome	*rpl2*(x2), rpl14, rpl16*, rpl20, rpl22, rpl23(x2), rpl32, rpl33, rpl36*
	DNA dependent RNA polymerase	*rpoA, rpoB, rpoC1*, rpoC2*
Genes for photosynthesis	Subunits of NADH-dehydrogenase	*ndhA*, ndhB*(x2), ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK*
	Subunits of photosystem I	*psaA, psaB, psaC, psaI, psaJ*
	Subunits of photosystem II	*psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ*
	Subunits of cytochrome b/f complex	*petA, petB*, petD*, petG, petL, petN*
	Subunits of ATP synthase	*atpA, atpB, atpE, atpF*, atpH, atpI*
	Large subunit of rubisco	*rbcL*
Miscellaneous proteins	Translational initiation factor	*infA*
	Maturase	*matK*
	Protease	*clpP***
	Envelop membrane protein	*cemA*
	Subunit of Acetyl-CoA-carboxylase	*accD*
	c-type cytochrom synthesis gene	*ccsA*
Genes of unknown function	Conserved open reading frames (ORF, *ycf*)	*ycf1(x2, part), ycf2(x2), ycf3**, ycf4*

[a]One and two asterisks reflect one- and two-intron containing genes, respectively. Genes located in the IR regions are indicated by the (x 2) symbol after the gene name.

(RC) of 0.441. Figure 3 shows one of the MP tree topologies with bootstrap values in excess of 50%. *Megaleranthis* was nested in the *Trollius* group, whereas *Adonis, Caltha* and *Eranthis* form sister groups.

For the chloroplast DNA tree, 29 *matK* sequences are aligned including (Sequences for 9 taxa are generated in this study, GenBank FJ597997-FJ598005) and subjected to MP analysis. The aligned data matrix consisted of 981 characters, and we employed *Thalictrum aquilegifolium* as the outgroup. The strict consensus MP tree has 489 steps, with a CI of 0.861, a RI of 0.903, and a RC of 0.778. *Megaleranthis* was also nested in *Trollius. Adonis, Caltha*, *Cimicifuga,* and *Eranthis* form a close generic group with *Trollius* (Fig. 4).

DISCUSSION

Comparative analysis of *Megaleranthis* and *Ranunculus* cp genomes

Megaleranthis is one of six endemic Korean vascular plant genera and includes a single species (Park et al., 2007). This small herbaceous plant is rare in the wild and listed as an endangered species of Korea. The 64 cp genomes have been sequenced completely from a variety of seed plants (Jansen et al., 2007) and are available from public databases such as NCBI. However, the majority of these sequences are limited to crop plants or large-sized plants because of the need for a large quantity of materials for the isolation of the cp DNA. We completed the chloroplast genome sequences of *Megaleranthis* from small quantities of leaf materials using combined methods of a series of overlapping long-range PCR and extensive primer designs. The availability of the complete cpDNA genome from rare and endemic genera will provide a rare opportunity to understand not only the evolutionary modes of the chloroplast genome itself, but also the genetic diversities of rare and endangered species.

The complete chloroplast genome of *Megaleranthis* is 159,924 bp with a LSC region of 88,326 bp, an SSC region of 18,382 bp, and two IR regions of 26,608 bp each (Fig. 1). We compare the organization and gene contents of the *Megaleranthis* cp genome to the cp genomes available from the NCBI public database. Overall, the cp genome of *Megaleranthis* displays structures typical of land plants (Ohyama et al., 1986;

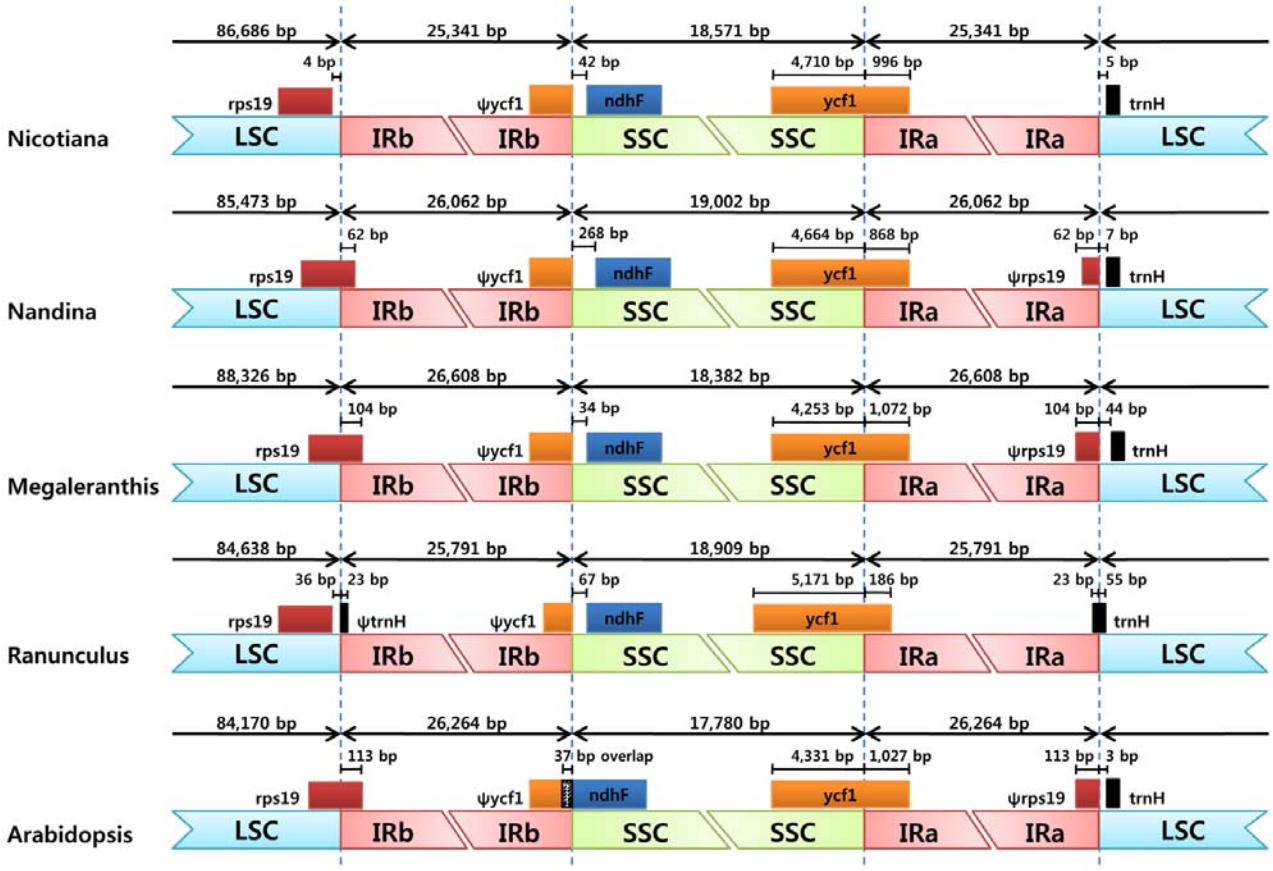

Fig. 2. The comparison of the LSC, IR and SSC border regions among five chloroplast genomes.

Table 3. The lengths of intron and exon for splitting genes in the *Megaleranthis* cp genome

Gene	Exon I	Intron I	Exon II	Intron II	Exon III
trnA-UGC	38	802	35		
trnG-UCC	23	712	48		
trnI-GAU	37	950	35		
trnK-UUU	37	2496	35		
trnL-UAA	35	509	50		
trnV-UAC	39	578	35		
rps12[a]	114	-	232	538	26
rps16	40	889	227		
rpl2	391	661	434		
rpl16	9	974	399		
rpoC1	430	771	1613		
ndhA	553	902	536		
ndhB	777	716	756		
petB	6	844	642		
petD	8	712	496		
atpF	145	740	410		

[a]The *rps12* gene is divided. The 5′-*rps12* is located on the LSC region and the 3′-*rps12* is located on the IR regions.

Palmer, 1991; Shinonzaki et al., 1986). The numbers of genes and introns are similar to the typical cp genomes (Tables 2 and 3). The gene order and AT composition of *Megaleranthis* cp DNA are also similar to the typical cp DNA genome (Table 4). We compared the detailed features of *Megaleranthis* cp genomes with the previously sequenced *Ranunculus* cp genome (Raubeson et al., 2007) because the two genera belong to the same family, Ranunculaceae. The detailed features of the *Megaleranthis* cp genomes differ substantially from that of *Ranunculus* (Fig. 5).

Table 4. The base compositions of *Megaleranthis* chloroplast genome

Regions	T(U)	C	A	G	Sequence no.
LSC	32.4%	18.6%	31.3%	17.6%	88,326 bp
IR	28.3%	20.9%	28.6%	22.2%	26,608 bp
SSC	33.8%	17.0%	34.3%	14.9%	18,382 bp
Total	31.3%	19.4%	30.7%	18.6%	159,924 bp

First, the *Megaleranthis* cp DNA is 4,797 bp longer than that of *Ranunculus* because of expanded IR into SSC region and duplicated sequence elements in the several spacer regions of the *Megaleranthis* cp genome. For example, the 1,072 bp of the 5′ portion of the *ycf1* gene was incorporated into the IR region and generated a pseudogene with a length of 1,072 bp (Fig. 2), whereas the length of the *ycf1* pseudogene is only 186 bp in *Ranunculus*. The 3′ portion of the *rps19* gene in *Megaeranthis* also exists as a pseudogene at the boundary of IR and LSC; however, the gene is located on the LSC in *Ranunculus*.

Table 5. The codon usage of the *Megaleranthis* chloroplast genome

TTT	Phe(F)	823	TCT	Ser(S)	463	TAT	Tyr(Y)	682	TGT	Cys(C)	190
TTC	Phe(F)	438	TCC	Ser(S)	290	TAC	Tyr(Y)	169	TGC	Cys(C)	72
TTA	Leu(L)	732	TCA	Ser(S)	359	TAA	END(*)	38	TGA	END(*)	20
TTG	Leu(L)	501	TCG	Ser(S)	159	TAG	END(*)	21	TGG	Trp(W)	409
CTT	Leu(L)	495	CCT	Pro(P)	360	CAT	His(H)	411	CGT	Arg(R)	313
CTC	Leu(L)	154	CCC	Pro(P)	197	CAC	His(H)	147	CGC	Arg(R)	101
CTA	Leu(L)	310	CCA	Pro(P)	282	CAA	Gln(Q)	607	CGA	Arg(R)	310
CTG	Leu(L)	146	CCG	Pro(P)	128	CAG	Gln(Q)	187	CGG	Arg(R)	102
ATT	Ile(I)	956	ACT	Thr(T)	455	AAT	Asn(N)	829	AGT	Ser(S)	339
ATC	Ile(I)	371	ACC	Thr(T)	222	AAC	Asn(N)	236	AGC	Ser(S)	105
ATA	Ile(I)	621	ACA	Thr(T)	374	AAA	Lys(K)	860	AGA	Arg(R)	413
ATG	Met(M)	557	ACG	Thr(T)	127	AAG	Lys(K)	286	AGG	Arg(R)	152
GTT	Val(V)	472	GCT	Ala(A)	559	GAT	Asp(D)	744	GGT	Gly(G)	544
GTC	Val(V)	149	GCC	Ala(A)	200	GAC	Asp(D)	199	GGC	Gly(G)	153
GTA	Val(V)	491	GCA	Ala(A)	362	GAA	Glu(E)	870	GGA	Gly(G)	648
GTG	Val(V)	172	GCG	Ala(A)	149	GAG	Glu(E)	311	GGG	Gly(G)	256

Asterisks indicate the stop codon.

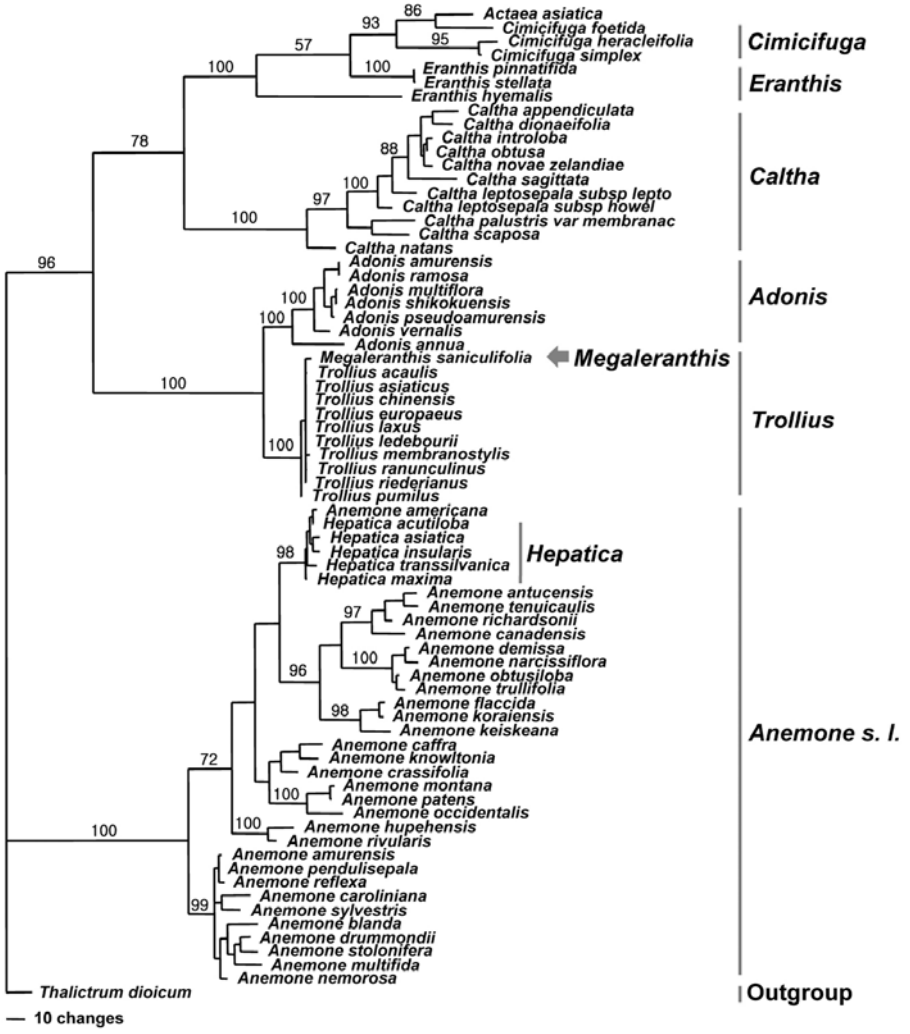

Fig. 3. Maximum parsimony tree resulting from the nuclear ITS region. Bootstrap values over 50% are shown above the branches.

Table 6. Distribution of simple sequence repeats (SSRs) loci in the *Megaleranthis* cp genome.

Base	Length	No. of SSR	Coordinations
A	10	3	300-309, 79512-79521, 159794-159803
	11	4	60999-61009, 75428-75438, 100064-100074, 132198-132208
	12	3	8040-8051, 9636-9647, 112531-112542
T	10	12	5009-5018, 6473-6482, 12867-12876, 13564-13573, 26826-26835, 47499-47508, 55391-55400, 63442-63451, 73906-73915, 88448-88457, 128781-128790, 130405-130414
	11	8	27924-27934, 63814-63824, 84217-84227, 118742-118752, 119028-119038, 125849-125859, 130980-130990, 148177-148187
	12	2	4457-4468, 135709-135720
	14	2	19110-19123, 74637-74650
	15	2	17037-17051, 115722-115736
AT	10	5	1681-1690, 20478-20487, 51895-51904, 118009-118018, 118030-118039
	12	1	9168-9179
	18	1	67667-67684
CA	10	1	32323-32332
TA	10	5	33589-33598, 49040-49049, 54842-54851, 59152-59161, 117981-117990
TC	10	1	127819-127828
ACC	12	1	42824-42835
GAA	12	1	2077-2088
ATAT	16	1	67667-67682
TAAGA	15	1	10570-10584

The coordinations are the nucleotide number positions beginning at the IRa/LSC junction (Fig. 1).

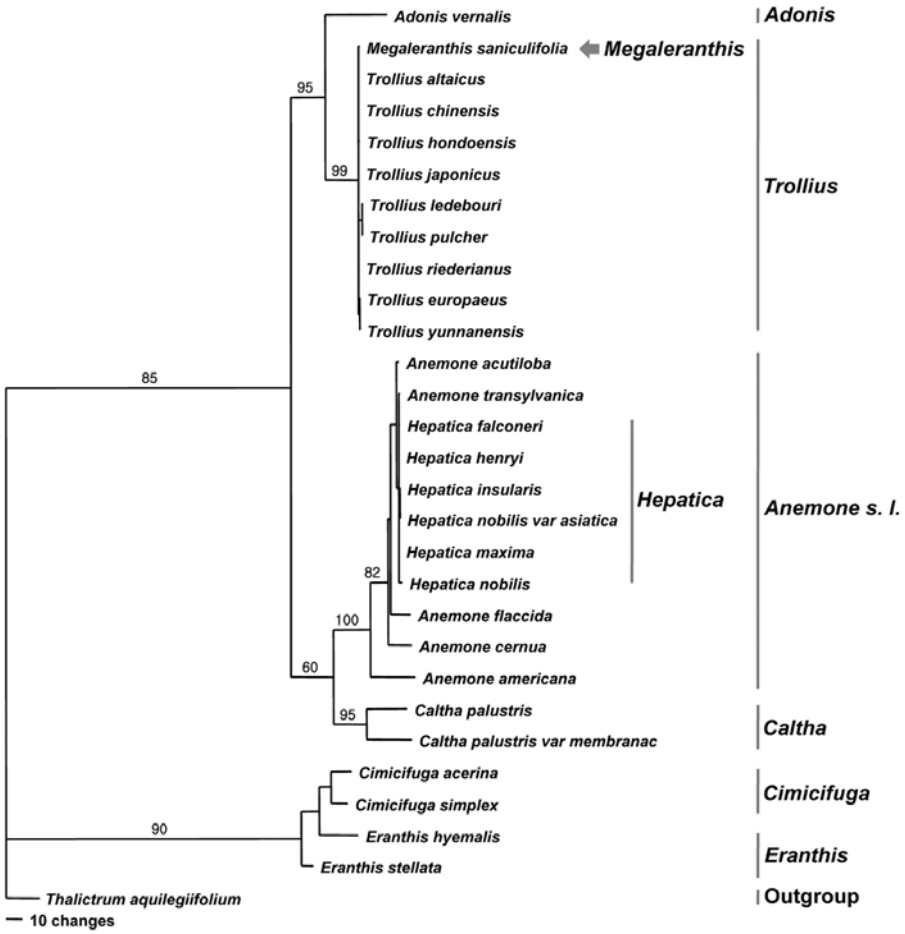

Fig. 4. Maximum Parsimony tree resulting from the chloroplast *matK* region. Bootstrap values in excess of 50% are shown above the branches.

Table 7. The comparisons of protein coding genes and *rrn* genes between *Ranunculus* and *Megaleranthis*

Region	Gene	Ran[a] (bp)	Meg[a] (bp)	Indel (Evt.)	No. of different site	Nucleotide diversity (%)	Ks[b]	Ka[b]	Ka/Ks
LSC & IR	*rps12*[a]	372	372	0 (0)	3	0.806	0.0209	0.0036	0.17
LSC	*psbA*	1062	1062	0 (0)	26	2.448	0.1075	0.0012	0.01
LSC	*matK*	1524	1527	-3 (1)	154	10.105	0.2093	0.0823	0.39
LSC	*rps16*	237	267	-30 (1)	19	8.017	0.2753	0.0315	0.11
LSC	*psbK*	186	186	0 (0)	10	5.376	0.1689	0.0278	0.16
LSC	*psbI*	111	111	0 (0)	5	4.505	0.2158	0.0000	0.00
LSC	*atpA*	1524	1524	0 (0)	88	5.774	0.2218	0.0146	0.07
LSC	*atpF*	552	555	-3 (1)	25	4.529	0.1148	0.0285	0.25
LSC	*atpH*	246	246	0 (0)	3	1.220	0.0459	0.0000	0.00
LSC	*atpI*	744	744	0 (0)	23	3.091	0.1199	0.0053	0.04
LSC	*rps2*	711	711	0 (0)	41	5.767	0.2042	0.0214	0.10
LSC	*rpoC2*	4152	4152	0 (6)	273	6.607	0.1655	0.0419	0.25
LSC	*rpoC1*	2043	2043	0 (0)	97	4.748	0.1673	0.0165	0.10
LSC	*rpoB*	3213	3216	-3 (1)	159	4.949	0.1884	0.0136	0.07
LSC	*petN*	90	90	0 (0)	2	2.222	0.1019	0.0000	0.00
LSC	*psbM*	105	105	0 (0)	3	2.857	0.1308	0.0000	0.00
LSC	*psbD*	1062	1062	0 (0)	33	3.107	0.1394	0.0012	0.01
LSC	*psbC*	1422	1422	0 (0)	54	3.797	0.1593	0.0037	0.02
LSC	*psbZ*	189	189	0 (0)	4	2.116	0.0682	0.0070	0.10
LSC	*rps14*	303	303	0 (0)	13	4.290	0.1281	0.0216	0.17
LSC	*psaB*	2205	2205	0 (0)	67	3.039	0.1405	0.0012	0.01
LSC	*psaA*	2253	2253	0 (0)	76	3.373	0.1461	0.0035	0.02
LSC	*ycf3*	507	513	-6 (1)	12	2.367	0.0706	0.0104	0.15
LSC	*rps4*	606	606	0 (0)	24	3.960	0.1378	0.0121	0.09
LSC	*ndhJ*	477	477	0 (0)	16	3.354	0.1410	0.0055	0.04
LSC	*ndhK*	684	654	30 (2)	33	5.093	0.1548	0.0228	0.15
LSC	*ndhC*	363	363	0 (0)	16	4.408	0.1626	0.0127	0.08
LSC	*atpE*	399	402	-3 (1)	18	4.511	0.1675	0.0132	0.08
LSC	*atpB*	1497	1497	0 (0)	70	4.676	0.1780	0.0098	0.06
LSC	*rbcL*	1428	1428	0 (0)	46	3.221	0.1299	0.0046	0.04
LSC	*accD*	1491	1476	15 (3)	120	8.130	0.2051	0.0565	0.28
LSC	*psaI*	111	111	0 (0)	3	2.703	0.1219	0.0000	0.00
LSC	*ycf4*	555	555	0 (0)	35	6.306	0.2147	0.0255	0.12
LSC	*cemA*	690	690	0 (0)	56	8.116	0.2050	0.0565	0.28
LSC	*petA*	969	969	0 (0)	60	6.192	0.2200	0.0226	0.10
LSC	*psbJ*	123	123	0 (0)	2	1.626	0.0619	0.0000	0.00
LSC	*psbL*	117	117	0 (0)	1	0.855	0.0423	0.0000	0.00
LSC	*psbF*	120	120	0 (0)	2	1.667	0.0686	0.0000	0.00
LSC	*psbE*	252	252	0 (0)	7	2.778	0.1280	0.0000	0.00
LSC	*petL*	96	96	0 (0)	5	5.208	0.2299	0.0000	0.00
LSC	*petG*	114	114	0 (0)	5	4.386	0.1998	0.0000	0.00
LSC	*psaJ*	135	135	0 (0)	6	4.444	0.2070	0.0000	0.00
LSC	*rpl33*	201	201	0 (0)	10	4.975	0.1524	0.0258	0.17
LSC	*rps18*	306	306	0 (0)	7	2.288	0.0725	0.0086	0.12
LSC	*rpl20*	360	354	6 (1)	20	5.650	0.1133	0.0421	0.37
LSC	*clpP*	606	606	0 (0)	31	5.116	0.1624	0.0220	0.14
LSC	*psbB*	1527	1527	0 (0)	79	5.174	0.2191	0.0086	0.04

(Continued)

Region	Gene	Ran[a] (bp)	Meg[a] (bp)	Indel (Evt.)	No. of different site	Nucleotide diversity (%)	Ks[b]	Ka[b]	Ka/Ks
LSC	*psbT*	102	102	0 (0)	6	5.882	0.2965	0.0000	0.00
LSC	*psbN*	132	132	0 (0)	4	3.030	0.1375	0.0000	0.00
LSC	*psbH*	222	222	0 (2)	18	8.145	0.1040	0.0767	0.74
LSC	*petB*	648	648	0 (0)	26	4.012	0.1707	0.0041	0.02
LSC	*petD*	510	504	6 (1)	27	5.357	0.2390	0.0053	0.02
LSC	*rpoA*	1041	987	54 (3)	68	6.890	0.2036	0.0355	0.17
LSC	*rps11*	417	417	0 (0)	23	5.516	0.2133	0.0098	0.05
LSC	*rpl36*	114	114	0 (0)	8	7.018	0.3800	0.0000	0.00
LSC	*infA*	249	234	15 (4)	36	15.385	0.1921	0.1622	0.84
LSC	*rps8*	399	399	0 (0)	21	5.263	0.1344	0.0291	0.22
LSC	*rpl14*	369	369	0 (0)	17	4.607	0.2112	0.0036	0.02
LSC	*rpl16*	408	408	0 (0)	20	4.902	0.1711	0.0133	0.08
LSC	*rps3*	654	657	-3 (1)	47	7.187	0.1901	0.0456	0.24
LSC	*rpl22*	540	534	6 (1)	30	5.618	0.1733	0.0295	0.17
LSC total/average		43845	43764	81 (30)	2213	5.066	0.0812	0.0441	0.54
IR	*rps19*	279	279	0 (0)	11	3.943	0.1364	0.0141	0.10
IR	*rpl2*	825	825	0 (0)	12	1.455	0.0344	0.0081	0.24
IR	*rpl23*	282	282	0 (0)	1	0.355	0.0000	0.0046	0.00
IR	*ycf2*	6885	6867	18 (9)	181	2.646	0.0402	0.0231	0.57
IR	*ndhB*	1533	1533	0 (0)	20	1.305	0.0329	0.0069	0.21
IR	*rps7*	468	468	0 (0)	2	0.427	0.0178	0.0000	0.00
IR	*rrn16*	1491	1491	0 (0)	3	0.201	-	-	-
IR	*rrn23*	2810	2810	0 (0)	12	0.427	-	-	-
IR	*rrn4.5*	103	103	0 (0)	0	0.000	-	-	-
IR	*rrn5*	121	121	0 (0)	0	0.000	-	-	-
IR total/average		14797	14779	18 (9)	242	1.640	0.0388	0.0177	0.46
SSC	*ndhF*	2217	2229	-12 (6)	264	11.957	0.3082	0.0847	0.27
SSC	*rpl32*	162	174	-12 (1)	15	9.259	0.3149	0.0488	0.15
SSC	*ccsA*	951	972	-21 (2)	124	13.039	0.3434	0.0941	0.27
SSC	*ndhD*	1509	1503	6 (1)	109	7.252	0.2507	0.0285	0.11
SSC	*psaC*	246	246	0 (0)	14	5.691	0.3019	0.0000	0.00
SSC	*ndhE*	306	306	0 (0)	12	3.922	0.1057	0.0216	0.20
SSC	*ndhG*	534	534	0 (0)	35	6.554	0.2129	0.0277	0.13
SSC	*ndhI*	543	543	0 (0)	45	8.287	0.2526	0.0444	0.18
SSC	*ndhA*	1092	1089	3 (1)	84	7.713	0.2604	0.0300	0.12
SSC	*ndhH*	1182	1182	0 (0)	64	5.415	0.2221	0.0138	0.06
SSC	*rps15*	273	273	0 (0)	24	8.791	0.1446	0.0795	0.55
SSC	*ycf1*	5367	5325	42 (40)	831	16.637	0.2886	0.1640	0.57
SSC total/average		14382	14376	6 (50)	1621	11.585	0.2705	0.0884	0.33
Total/average		73024	72919	105 (89)	4076	5.628	0.1093	0.0489	0.45

[a]The *rps12* gene is included in the LSC region. Ran and Meg mean *Ranunculus* and *Megaleranthis,* respectively.
[b]Ks and Ka represent the synonymous and nonsynonymous substitutions, respectively.

We aligned all of the sequences of the protein coding genes, introns, and intergenic spacer regions between *Megaleranthis* and *Ranunculus*. The numbers and lengths of indels, the number of polymorphic sites, nucleotide diversity, synonymous (Ks) and nonsynonymous (Ka) substitutions, and Ka/Ks values were calculated in each of the aligned gene regions (Tables 7-9). The total length of the gene coding region and intron region is similar in the two genera. However, the total length of the spacer region is substantially longer in *Megaleranthis* than in *Ranunculus*. This is principally due to the large duplicated insertion elements in the five spacer regions, including *petN-psbM, psbM-trnD, trnT-L, ycf4-cemA*, and *ycf2-trnL* (Table 9).

Second, the chloroplast genomes of *Megaleranthis* and *Ranunculus* evidence a sequence divergence of 5.6% in the coding regions, 8.9% in the intron regions, and 18.7% in the intergenic spacer regions, respectively. This means that the intron region evolves twice as fast as the gene coding region and the intergenic spacer region evolves twice as fast as the

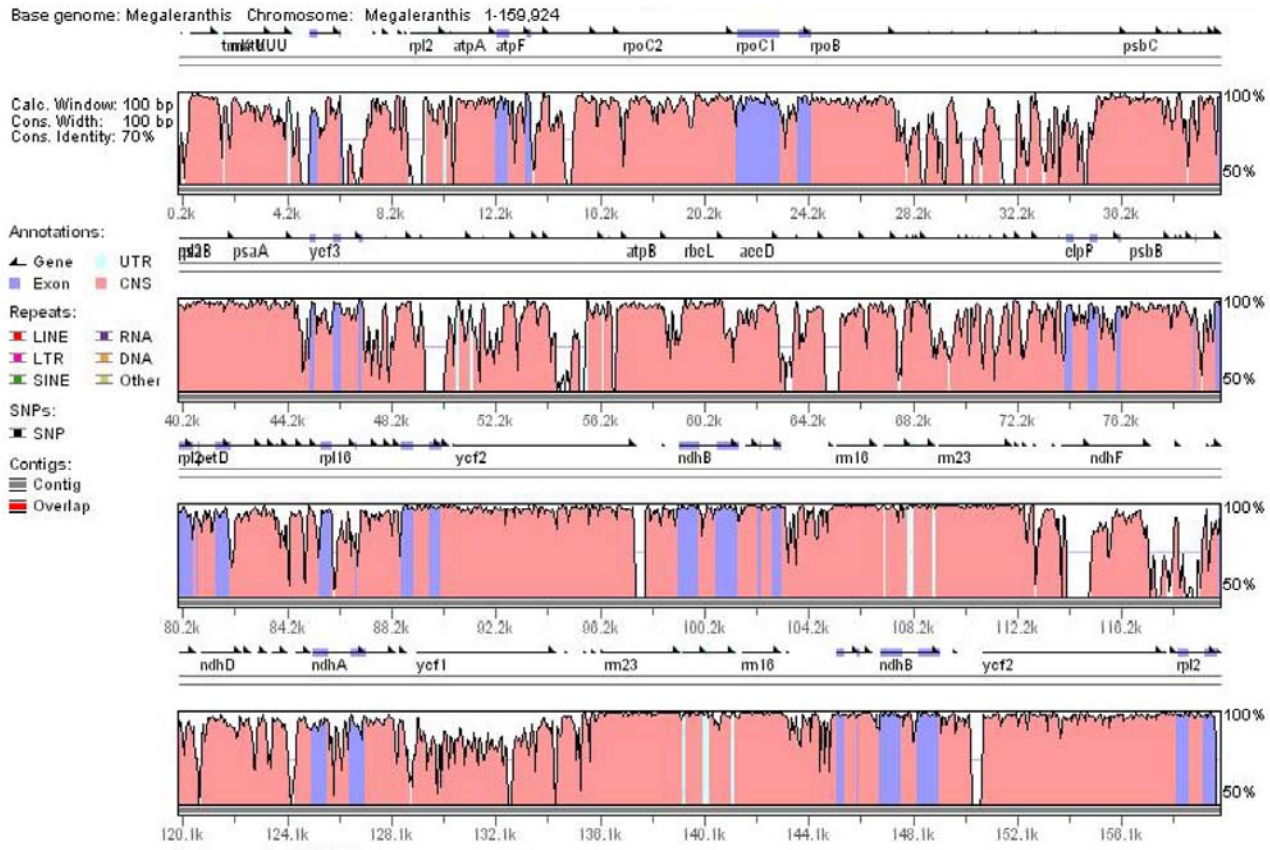

Fig. 5. The comparison of cp genome between *Ranunculus* and *Megaleranthis*. The coordination of base pairs is provided in the Fig. 1.

intron regions. The differential base substitution rates according to the regions are probably attributable to the different selection constraints depending on the region (Palmer, 1991). In the coding region, average nucleotide substitution rates differ markedly depending on the genome position. The genes on the IR region evidence only a 1.6% divergence, whereas the genes on the LSC and SSC evidence divergence rates of 5.1% and 11.6%, respectively. Therefore, the average evolutionary rates among the IR, LSC, and SSC regions are 1:5:11. This is similar to the 1:5 ratios of the previous estimations between the IR region and the single copy region (Wolfe et al., 1989).

Slower nucleotide substitution in the IR region is due principally to the frequent recombination events and subsequent base collection mechanism between two strands of homologous IR sequences (Palmer, 1991; Wolfe et al., 1989). This data strongly supports the notion of positional effects of the evolutionary modes of chloroplast genes. Five genes, including *ycf1* (16.6%), *infA* (15.4%), *ccsA* (13.0%), *ndhF* (11.9%), and *matK* (10.1%) evidence sequence divergences of more than 10%, whereas the *rps12* (0.8%) and *psbL* (0.9%) evidence divergences of less than 1% in the same SC region. The genes with higher levels of base substitutions evidence strong bias toward the Ka/Ks ratios (Table 7), as well. The genes with higher levels of base substitutions also exhibit higher incidences of indel mutations. All indels in the coding genes are multiples of three (Table 7). The correlation between base substitutions, indel mutations, and Ka/Ks ratios have also been reported in a previous study (Jansen et al., 2007; Kim and Lee, 2004). The rapidly evolving genes may prove useful in phylogenetic and DNA barcoding studies at the genus and species levels, whereas the slowly evolving genes may prove useful for phylogenetic studies at higher taxonomic levels.

Third, the *Megaleranthis* and *Ranunculus* cp genomes differ substantially in the noncoding regions. The divergence in the spacer region ranges from 0% to 39%, as compared to 0 to 16% in the coding region. Twelve intergenic spacer regions evidence sequence divergences of more than 30%. Seven of them are located in the LSC and the others are located in the SSC (Table 9). The wide range of evolutionary divergences was observed both in the base substitutions and indel mutations, due to lesser selection constraints in the spacer regions as compared to the coding regions. Therefore, the spacer regions are probably reflective of a more accurate positional effect as compared to the coding regions. For example, the average base substitutions of intergenic spacers among the IR, LSC, and SSC regions are 4.4%, 20.4%, and 29.5%, respectively. This corresponds to an approximate 1:4:6 ratio for the IR, LSC, and SSC regions, respectively.

Abundant indel mutations ranging from 1 bp to 695 bp were noted between the two chloroplast noncoding regions. Most of the small size indels were attributed to the slippage mispairing mechanism of short repeated sequences occurring during DNA replications. Several medium size indels of up to 695 bp are associated with the short direct or indirect repeat sequences in the two chloroplast genomes in Ranunculaceae. This suggests that genetic recombination also contributes to indel mutations in these genomes. Differential positional effects are also apparent in the intron regions. For example, the average base substitutions of intron regions among IR, LSC, and SSC are 1.7%, 10.3%, and 16%, respectively (Table 8). This corresponds to an

Table 8. Comparison of intron regions between *Ranunculus* and *Megaleranthis*

Region		Gene	Ran[a] (bp)	Meg[a] (bp)	Indel	No. of different site	Nucleotide diversity (%)
LSC	Intron	*trnK-UUU*	2542	2494	48	271	10.945
LSC	Intron	*rps16*	849	887	-38	94	11.177
LSC	Intron	*trnG-UCC*	695	710	-15	58	8.406
LSC	Intron	*atpF*	717	738	-21	67	9.599
LSC	Intron	*rpoC1*	794	769	25	69	9.200
LSC	Intron1	*ycf3*	744	752	-8	71	9.834
LSC	Intron2	*ycf3*	715	712	3	59	8.310
LSC	Intron	*trnL-UAA*	491	507	-16	46	9.504
LSC	Intron	*trnV-UAC*	582	576	6	30	5.263
LSC	Intron1	*clpP*	675	657	18	54	8.424
LSC	Intron2	*clpP*	833	809	24	91	11.461
LSC	Intron	*petB*	802	842	-40	129	16.125
LSC	Intron	*petD*	724	710	14	59	8.322
LSC	Intron	*rpl16*	1019	972	47	165	17.497
LSC total/average			12182	12135	47	1263	10.678
IR	Intron	*rpl2*	659	659	0	14	2.124
IR	Intron	*ndhB*	716	714	2	13	1.857
IR	Intron	*3′-rps12*	535	536	-1	10	1.869
IR	Intron	*trnI-GAU*	949	948	1	21	2.232
IR	Intron	*trnA-UGC*	800	800	0	5	0.625
IR total/average			3659	3657	2	63	1.733
SSC	Intron	*ndhA*	1008	900	108	138	16.028
Total/average			16849	16692	157	1464	8.968

[a]Ran and Meg represent *Ranunculus* and *Megaleranthis,* respectively.

approximate 1:5:8 ratio among the IR, LSC, and SSC regions, respectively. High levels of base substitutions are observed in the introns of *rpl16* (17.5%, LSC), *petB* (16.1%, LSC), and *ndhA* (16.0%, SSC). The cp genomes of *Megaleranthis* and *Ranunculus* in the same family evidence a sequence similarity of 91.3% (Fig. 6).

The utility of cp SSR

The extent and utility of chloroplast SSR remains poorly understood. The cp SSR was initially reported in studies of *Pinus radiata* and *Oryza sativa* (Cato and Richardson, 1996; Powell et al*.,* 1995; Provan et al*.,* 1996) and the potential utilities of cp SSR for the population genetics were suggested. Kim and Lee (2004) also reported 18 and 29 SSR loci from *Panax schingseng* and *Nicotiana tobacum* cp genomes, respectively. The majority of these SSR are homopolymers of A or T, and they are located on specific spacer regions. Only 4 of 47 SSR loci from the *Panax* and *Nicotiana* cp genomes are homopolymers of Gs or Cs, and they are located on the gene coding regions. A total of 54 SSR loci were identified from the *Megaleranthis* cp genome (Table 6). This is the largest number of SSR loci ever reported from a single cp genome. Furthermore, 14 loci are dipolymers of AT, TA, CA, and TC. Two loci are tripolymers of ACG and CAA. Furthermore, tetra- and pentapolymers of $(ATAC)_{n=16}$ and $(TAAGA)_{n=15}$ were also discovered from the *Megaleranthis* cp genome. The existence of rich SSR loci in the *Megaleranthis* cp genome provides us with a rare opportunity to study the population genetic structures of this endangered species.

Phylogenetic relationship of *Megaleranthis saniculifolia*

Megaleranthis is a monotypic and endemic genus in Korea. Ohwi (1935) first described the genus and he later transferred the species into *Trollius* (1937). Tamura (1966; 1990; 1995) recognized the separate generic status of *Megaleranthis* and most recent Korean flora treat the genus as a distinctive endemic genus (Lee, 2007; Park et al*.,* 2007). Lee (1989) and Lee and Blackmore (1992) have reported the same pollen types of *Megaleranthis* with the species of *Trollius*. Similar striate pollen exines have also been reported from the *Calathodes* (Lee, 1992). The close relationship among the *Calathodes, Trollius,* and *Megaleranthis* was also supported by the results of chromosome studies (Lee and Yeau, 1985; Yuan and Yang, 2006). In addition to the three genera, the close relationships of the *Adonis* to the group were also bolstered by independent molecular data (Jensen et al*.,* 1995; Ro et al*.,* 1997).

However, the available molecular data is not sufficient to elucidate the specific relationships among the species *Trollius, Megaleranthis, Calathodes,* and *Adonis,* due principally to the limited sampling. Our phylogenetic trees based on the two independent markers, specifically the nuclear *ITS* and chloroplast *matK* sequences, support the inclusion of the *Megaleranthis* in the *Trollius* species (Figs. 3 and 4). The *Trollius-Megaleranthis* clade is strongly supported by 100% bootstrap values both in

Table 9. Comparison of intergenic spacer regions between *Ranunculus* and *Megaleranthis*

Region	Gene spacer		Ran[a] (bp)	Meg[a] (bp)	Indel	No. of different site	Nucleotide diversity (%)
LSC	*trnH-GUG*	*psbA*	304	332	-28	104	31.420
LSC	*psbA*	*trnK-UUU*	279	257	22	59	22.957
LSC	*trnK-UUU*	*rps16*	490	750	-260	141	29.375
LSC	*rps16*	*trnQ-UUG*	1059	1219	-160	354	34.336
LSC	*trnQ-UUG*	*psbK*	343	335	8	27	8.084
LSC	*psbK*	*psbI*	409	417	-8	52	12.903
LSC	*psbI*	*trnS-GCU*	136	83	53	33	39.759
LSC	*trnS-GCU*	*trnG-UCC*	324	741	-417	88	27.586
LSC	*trnG-UCC*	*trnR-UCU*	204	163	41	30	19.481
LSC	*trnR-UCU*	*atpA*	58	141	-83	8	13.793
LSC	*atpA*	*atpF*	85	66	19	7	10.606
LSC	*atpF*	*atpH*	457	473	-16	103	23.303
LSC	*atpH*	*atpI*	805	1040	-235	166	21.475
LSC	*atpI*	*rps2*	217	217	0	25	11.792
LSC	*rps2*	*rpoC2*	245	178	67	38	21.348
LSC	*rpoC2*	*rpoC1*	188	176	12	18	10.405
LSC	*rpoC1*	*rpoB*	26	26	0	5	19.231
LSC	*rpoB*	*trnC-GCA*	1019	1256	-237	162	17.143
LSC	*trnC-GCA*	*petN*	725	851	-126	123	17.723
LSC	*petN*	*psbM*	838	1271	-433	312	38.095
LSC	*psbM*	*trnD-GUC*	581	1114	-533	120	20.870
LSC	*trnD-GUC*	*trnY-GUA*	422	432	-10	133	32.759
LSC	*trnY-GUA*	*trnE-UUC*	69	59	10	3	5.085
LSC	*trnE-UUC*	*trnT-GGU*	735	687	48	207	30.942
LSC	*trnT-GGU*	*psbD*	1484	1594	-110	214	15.598
LSC	*psbC*	*trnS-UGA*	182	188	-6	20	11.111
LSC	*trnS-UGA*	*psbZ*	351	334	17	39	11.782
LSC	*psbZ*	*trnG-GCC*	355	391	-36	64	18.182
LSC	*trnG-GCC*	*trnfM-CAU*	192	183	9	43	24.294
LSC	*trnfM-CAU*	*rps14*	162	162	0	14	8.642
LSC	*rps14*	*psaB*	120	150	-30	12	10.000
LSC	*psaB*	*psaA*	25	25	0	1	4.000
LSC	*psaA*	*ycf3*	710	653	57	96	15.047
LSC	*ycf3*	*trnS-GGA*	925	886	39	178	20.917
LSC	*trnS-GGA*	*rps4*	293	333	-40	64	22.695
LSC	*rps4*	*trnT-UGU*	347	347	0	40	12.422
LSC	*trnT-UGU*	*trnL-UAA*	760	1334	-574	231	31.862
LSC	*trnL-UAA*	*trnF-GAA*	348	409	-61	52	15.160
LSC	*trnF-GAA*	*ndhJ*	695	707	-12	132	20.465
LSC	*ndhJ*	*ndhK*	114	192	-78	19	16.667
LSC	*ndhK*	*ndhC*	43	53	-10	3	6.977
LSC	*ndhC*	*trnV-UAC*	1190	1450	-260	324	28.125
LSC	*trnV-UAC*	*trnM-CAU*	182	182	0	19	10.440
LSC	*trnM-CAU*	*atpE*	147	257	-110	37	25.170
LSC	*atpB*	*rbcL*	744	744	0	113	15.893
LSC	*rbcL*	*accD*	701	691	10	84	12.805
LSC	*accD*	*psaI*	713	664	49	179	28.778
LSC	*psaI*	*ycf4*	424	421	3	61	15.099
LSC	*ycf4*	*cemA*	272	838	-566	35	13.011

(Continued)

Region	Gene spacer		Ran[a] (bp)	Meg[a] (bp)	Indel	No. of different site	Nucleotide diversity (%)
LSC	*cemA*	*petA*	221	225	-4	39	17.808
LSC	*petA*	*psbJ*	423	376	47	96	26.301
LSC	*psbJ*	*psbL*	119	135	-16	6	5.042
LSC	*psbL*	*psbF*	22	22	0	0	0.000
LSC	*psbF*	*psbE*	9	9	0	0	0.000
LSC	*psbE*	*petL*	1195	1237	-42	197	17.220
LSC	*petL*	*petG*	189	178	11	29	16.384
LSC	*petG*	*trnW-CCA*	164	140	24	35	25.000
LSC	*trnW-CCA*	*trnP-UGG*	155	154	1	17	11.111
LSC	*trnP-UGG*	*psaJ*	369	365	4	70	19.663
LSC	*psaJ*	*rpl33*	403	417	-14	80	21.053
LSC	*rpl33*	*rps18*	165	170	-5	38	23.171
LSC	*rps18*	*rpl20*	277	269	8	75	28.736
LSC	*rpl20*	*5'-rps12*	807	787	20	81	10.479
LSC	*5'-rps12*	*clpP*	172	199	-27	33	19.298
LSC	*clpP*	*psbB*	406	415	-9	30	7.407
LSC	*psbB*	*psbT*	180	183	-3	16	9.143
LSC	*psbT*	*psbN*	79	77	2	13	16.883
LSC	*psbN*	*psbH*	102	102	0	3	2.941
LSC	*psbH*	*petB*	126	119	7	16	13.445
LSC	*petB*	*petD*	210	192	18	20	10.417
LSC	*petD*	*rpoA*	185	221	-36	30	18.868
LSC	*rpoA*	*rps11*	65	66	-1	3	4.615
LSC	*rps11*	*rpl36*	112	112	0	13	11.607
LSC	*rpl36*	*infA*	116	122	-6	14	12.727
LSC	*infA*	*rps8*	71	126	-55	20	28.169
LSC	*rps8*	*rpl14*	276	190	86	42	22.340
LSC	*rpl14*	*rpl16*	140	143	-3	32	24.806
LSC	*rpl16*	*rps3*	187	149	38	27	18.121
LSC	*rpl22*	*rps19*	57	71	-14	5	8.772
LSC total/average			28499	32443	-3944	5472	20.361
IR	*rps19*	*rpl2*	112	65	47	12	18.462
IR	*rpl2*	*rpl23*	18	18	0	1	5.556
IR	*rpl23*	*trnI-CAU*	165	165	0	2	1.212
IR	*trnI-CAU*	*ycf2*	83	68	15	0	0.000
IR	*ycf2*	*trnL-CAA*	606	1042	-436	24	3.967
IR	*trnL-CAA*	*ndhB*	564	566	-2	29	5.142
IR	*ndhB*	*rps7*	316	331	-15	5	1.592
IR	*rps7*	*3'-rps12*	53	53	0	1	1.887
IR	*3'-rps12*	*trnV-GAC*	1859	1890	-31	59	3.305
IR	*trnV-GAC*	*rrn16*	228	227	1	2	0.881
IR	*rrn16*	*trnI-GAU*	294	294	0	7	2.381
IR	*trnI-GAU*	*trnA-UGC*	64	64	0	0	0.000
IR	*trnA-UGC*	*rrn23*	152	152	0	4	2.632
IR	*rrn23*	*rrn4.5*	98	98	0	3	3.061
IR	*rrn4.5*	*rrn5*	224	227	-3	4	1.786
IR	*rrn5*	*trnR-ACG*	235	254	-19	7	3.043
IR	*trnR-ACG*	*trnN-GUU*	589	662	-73	40	6.791
IR	*trnN-GUU*	*ψycf1*	1018	323	695	51	15.789
IR total/average			6678	6499	179	251	4.356

(Continued)

Region	Gene spacer		Ran[a] (bp)	Meg[a] (bp)	Indel	No. of different site	Nucleotide diversity (%)
SSC	*ψycf1*	*ndhF*	66	33	33	12	36.364
SSC	*ndhF*	*rpl32*	839	986	-147	291	34.934
SSC	*rpl32*	*trnL-UAG*	769	1021	-252	295	39.021
SSC	*trnL-UAG*	*ccsA*	121	123	-2	41	35.965
SSC	*ccsA*	*ndhD*	211	252	-41	57	30.978
SSC	*ndhD*	*psaC*	124	125	-1	7	5.645
SSC	*psaC*	*ndhE*	267	270	-3	51	19.691
SSC	*ndhE*	*ndhG*	245	248	-3	47	20.435
SSC	*ndhG*	*ndhI*	374	405	-31	95	26.389
SSC	*ndhI*	*ndhA*	78	78	0	11	14.103
SSC	*ndhH*	*rps15*	146	147	-1	30	21.429
SSC	*rps15*	*ycf1*	397	409	-12	92	24.533
SSC total /average			3637	4097	-460	1029	29.518
Total /average			38814	43039	-4225	6752	18.728

[a]Ran and Meg represent *Ranunculus* and *Megaleranthis,* respectively.

the ITS and the *matK* trees. Our data does not provide conclusive species relationships among the *Trollius*. However, the results suggest that *Magaleranthis sanisulifolia* is more closely related to the group of species including *T. asiatica*, *T. altaicus*, and *T. chinensis*. Using multiple genes for phylogenetic analysis may enhance species resolution in *Trollius.* We continued this study using 10 cp gene regions that evidence high levels of sequence diversity (Tables 7-9, K.-J. Kim et al., unpublished data). Our molecular trees support Ohwi's original treatment of *Megaleranthis saniculifolia* to *Trollius chosenensis* Ohwi (1937). This conclusion is also comparable to the palynotaxonomic suggestion (Lee and Blackmore, 1992). Therefore, the distinctive morphological characters of *Megaleranthis* may be considered to be independently derived autapomorphies, rather than synapomorphies with other genera and species.

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