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Phylogenetic Relationships of the Korean *Trigonotis* Steven (Boraginaceae) Based on Chloroplast DNA (cpDNA) and Nuclear Ribosomal Markers (nrDNA) Region

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Abstract - We performed phylogenetic analyses of a total of 21 acessions covering 5 species in the Korean Trigonotis and one outgroup species using nuclear ribosomal ITS and chloroplast *rbcL, matK, ndhF* sequences. Outgroup were chosen from the closely related genus *Lithospermum zollingeri.* Both parsimony and Bayesian Inference methods were used to reconstruct the evolutionary history of the group. The evidence collected indicated that phylogenetic relationships among Korean *Trigonotis* species are unresolved based on nuclear marker (ITS), as the same as based on separated chloroplast sequences. While the phylogenetic relationships of Korean *Trigonotis* species almost clearly were resolved in combined chloroplast sequences. Thus, the members of *Trigonotis coreana* can be distinguished to the members of *Trigonotis peduncularis* in combined cpDNA sequences and *Trigonotis nakaii* was treated as a synonymed to *Trigonotis radicans* var. *sericea.* In addition, the MP and BI analysis showed *Trigonotis icumae* as sister of the remained Korean *Trigonotis* species based on combined molecular markers (BI: PP = 1).

Key words - nrDNA, cpDNA and *Triogonotis*

Introduction

The genus *Trigonotis,* the small one in family Boraginaceae, consists of about 58 species, distributed in Asia, E. Europe (Wang, 1982; Zhu et al., 1995), China (39 species), with 33 endemic species. Members of the *Trigonotis* genus is perennial, biennial herbs, or rarely annual plants and grown in grassy places and in lowland areas. It has single or several stems and cespitose, erect to diffuse, hispid or pilose, rarely glabrous. Calyx is 5-lobed or 5-parted. Corolla is blue or white with center yellow; throat appendages 4. Cymes are solitary or dichotomously branched, ebracteate or lower pedicels bracteate, rarely all bracteates (flowers extra axillary).

Morphological characteristics of *Trigonotis* genus are arguments and confusing. Maximovicz (1872) collected *Omphalodes sericea* that was transferred to *Trigonotis* by Johnston (1937). One year later, Ohwi (1953) put a taxon into synonymy of what he considered to be *Trigonotis radicans* together with *Omphalodes sericea* Maximovicz and *Omphalodes aquatic* is an additional synonym of *Trigonotis radicans*. According to Popov (1953), he identified Maximowicz' and other author plants under the name of *Trigomotis radicans* as *Trigonotis coreana* Nakai (1917). Furthermore, Lee (1996) mentioned that *Trigonotis icumae* (Maxim.) is synonym to *Omphalodes icumae* Maxim. *Trigonotis peduncularis* (Treviranus) Bentham is synonym *Eritrichium japonicum* Miq., *Eritrichium pedunculare* (Trevir.) A.DC., *Myosotis chinensis.* A.DC., *Myosotis peduncularis* Trevir., and *Trigonotis radicans* var. *sericea* is synonym to *Omphalodes sericea* Maxim., *T. coreana* Nakai, *T. sericea* (Maxim.) I.M. Johnston, *T. nakaii* Hara. Morphological classification is not only time-consuming but also it may not distinctly distinguished closely related species. In the recent years, molecular studies that have provided insights about genes based on rRNA gene sequences are more popular. The existence of unique substitutions plays a key role in distinguishing among closely related species. One of the useful tools for phylogenetic inference at the generic and infrageneric level in plants is the Internal Transcribed Spacer (ITS) region of the 18S-5.8S-26S nuclear ribosomal DNA (nrDNA) that has used well as a phylogenetic

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marker in most groups of flowering plants (Baldwin, 1992). However, there are several problems in the concerted evolution, the presence of pseudogenes as well as the existence of paralogs and orthologs (Bailey *et al*., 2003; Feline and Rosello', 2007; Mayol and Rossello', 2001; Soltis *et al*., 2008). The chloroplast genome has been proved as a useful tool for species identification as well as in ecological and evolutionary studies (Newmaster *et al*., 2006; Kress and Erickson, 2007; Lahaye *et al.,* 2008). The slowly evolving gene *rbc*L, indicating that can be used at the generic level. Thus, in the interordinal or intrafamilial level, the utility of *rbcL* is limited. However, it sometimes has been used for phylogenetic analyses at species levels in angiosperms (Xiang *et* al., 1993; Azuma *et* al., 2000; Jovanovic' and Cvetkovic', 2010). The *mat*K and *ndhF* gene are applied in phylogenetic reconstructions at high taxonomic levels, such as Order or Family, however, sometimes also at low taxonomic categories, such as Genus or Species (Wolfe, 1991; Olmstead *et al*., 1998 Hilu *et al*., 2003; Muller *et al*., 2006; Chase *et al*., 2007;). In the present paper, we focus on sequence data from the nuclear internal transcribed spacer regions (ITS) and chloroplast DNA (cpDNA) to find out genetic diversity and the phylogenetic relationships among Korean *Trigonotis* genus.

Materials and Methods

Taxon sampling

The taxon sample comprised 20 individuals covering 5 species and scored for analyses. Those include five individuals of *Trigonotis nakaii,* five individuals of *Trigonotis radicans* var. *sericea,* two individuals of *Trigonotis icumae,* three individuals of *Trigonotis coreana* and five individuals of *Trigonotis peduncularis*, all from the Herbarium. We used

Table 1. Taxa included in the present study, with voucher information on country of location, herbarium. GN (Gyeongsangnam-do),CB (Chungcheongbuk-do), JN (Jeollanam-do), GW (Gangwon-do), GB (Gyeongsangbuk-do), JB (Jeollabuk-do)

Accession	Locality	Specimen Vouchers
Trigonotis nakaii Hara (T01)	GN, Miryang-si, Pyochungsa	YNUH0406
Trigonotis nakaii Hara (T02)	JB, Gochang, Mt. Seonunsan	YNUH0006
Trigonotis nakaii Hara (T03)	GN, san-cheong-gun, Sam-jang-myeon, Mt. Jirisan	KHB1067120
Trigonotis nakaii Hara (T04)	JB, Gokseong-gun, Mt. Tong-myeong-san	KHB1102046
Trigonotis nakaii Hara (T05)	CB, Boeun-gun, Naesongni-myeon, Mansu-ri	KWNU57636
Trigonotis radicans var. *sericea* (Maxim.) Hara (T06)	JB, Boseong-gun, Mundeok-myeon , Juksan-ri, Daewonsa	KHB1073595
Trigonotis radicans var. *sericea* (Maxim.) Hara (T07)	JB, Gochang-gun, Mt. Seonunsan	KHB1107968
Trigonotis radicans var. *sericea* (Maxim.) Hara (T08)	JN, Boseong-gun, Bongnae-myeon, Mt. Cheonbongsan	KHB1098995
Trigonotis radicans var. *sericea* (Maxim.) Hara (T09)	JN, Boseong-gun, Mundeok-myeon, Juksan-ri, Daewonsa jubyeon	KHB1088453
Trigonotis radicans var. *sericea* (Maxim.) Hara (T10)	JN, Hwasun-gun, Nam-myeon, Unsan-ri, Mt. Malbongsan	KHB1088533
Trigonotis icumae (Maxim.) Makino (T11)	GB, Sangui-ri, Budong-myeon, Mt. Juwangsan	KHB1187802
Trigonotis icumae (Maxim.) Makino (T12)	GW, Dutasan (Mt.), Hajang-myeon, Samcheok-si	KHB1170955
Trigonotis coreana Nakai *(T13)*	GB, Daegu, Mt. apsan	YNUH0044
Trigonotis coreana Nakai (T14)	GB, Chilgok-gun Yeonhodong	YNUH08054
Trigonotis coreana Nakai (T15)	JN, Yeongdae bongwan dwitpyeon	YUNH1690027
Trigonotis peduncularis (Trevir.) Benth. ex Heml (T16)	GB, Cheongdo-gun Unmun-myeon, Mt. Unmunsa	YNUH0505
Trigonotis peduncularis ((Trevir.) Benth. ex Heml (T17)	GN, Namhae-gun Mijo-myeon Nogumaeul Haeanga	KHB1164795
Trigonotis peduncularis (Trevir.) Benth. ex Heml (T18)	JN, Hampyeong-gun Haebo-myeon, Mt. Moaksan	KHB1158275
Trigonotis peduncularis (Trevir.) Benth. ex Heml (T19)	GW, Chuncheon-si, Mt. Bonguisan	KWNU66396
Trigonotis peduncularis (Trevir.) Benth. ex Heml (T20)	Jeju-si, Yongnunioreum	KWNU74892
Lithospermum zollingeri A. DC.	GB, Daegu, Dalseonggun, Mt. hwaryongsan	YNUH00022

Lithospermum zollingeri as an outgroup because of morphological similar characters and *Trigonotis* 's sister group (Table 1).

DNA extraction, amplification and sequencing

Total genomic DNA from dried leaf materials were collected from Herbarium and extracted by a modified CTAB protocol from Doyle and Doyle (1987). For this study, three chloroplast markers, such as *rbc*L, O1F and O1R (Les, 1994); *mat*K, T590F and T1320R (Sang *et al*., 1997); *ndh*F included two primer pairs, 599F-1354R and 1318F-2110R (Sweeney and Price. 2000; Olmstead and Sweere, 1994) were conducted to evaluate the suitability of various markers for the present study and the internal transcribed spacers from the nuclear ribosomal DNA (ITS) (White *et al*., 1990) was used. The *ndh*F gene was amplified in two overlapping fragments, while the others were amplified in one fragment, using the primers listed in the Table 2. Polymerase chain reaction (PCR) conditions for the amplification were as follows : 96℃ for 1min followed by 30 to 35 cycles of 95℃ for 30 sec, 50 to 56℃ for 30 sec, 72℃ for 1 min and a final extension of 72℃ for 10 min. The amount of product was quantified using agarose gel electrophoresis with a low mass DNA ladder using the QIAquick PCR purification Kit (Qiagen, Crawley), following the manufactures' protocol. After that, the PCR production was sent to the Solgent Company for sequencing.

Phylogenetic analyses

All regions were sequenced using both forward and reverse primers. Sequences were assembled in Sequencher 3.0 (Gene Codes, Ann Arbor, Michigan, USA) and aligned manually in MacClade v. 408 (Maddison and Maddison, 2005). A consensus sequence was generated when the sequences from the forward and reverse primer were aligned for each taxon. Indels were coded separately using the simple indel coding method (Simmons and Ochoterena, 2000). Regions with ambiguous alignments were excluded. The phylogenetic analyses were set up in two steps. In the first step, the sequences of the nrDNA and cpDNA were analysed separately. In the second step, a combined analysis of the nrDNA and cpDNA was carried out. The combined matrix is used for incongruence between nuclear and chloroplast markers. Molecular trees based on the unweighted pair group method with arithmetic mean (UPGMA), Neighbor Joining (NJ), Maximum Parsimony (MP) were performed in PAUP* version 4.0b10 (Swofford, 2002). Heuristic bootstrap analysis was performed with 1,000 bootstrap replicates, 100 random addition cycles per bootstrap replicate, TBR swapping and equal weights. The resolution of species was characterized by calculating the percentage of species recovered as monophyletic based on phylogenetic trees. Clades with bootstrap percentages of 50%-74% are described as weakly supported; 75%-89% as moderately supported; 90%-100% as strongly supported.

Table 2. Oligonucleotide primers used in the PCR amplification and sequencing of DNA marker loci

Region	Primer sequemce from the 5' -3'	Pre-melt/ Amplication/ Final extention/ Number of cycles in the amplication	Reference
ITS4 ITS5	TCCTCCGCTTATTGATATGC GGAAGTAAAAGTCGTAACAAGG	95℃ (3 min)/ 95℃ (30sec) + 56℃ (30 sec) + 72℃ (1 min)/ 72℃ (10 min)/ 35	White et al.1990
rbcL O1F O1R	ATGTCACCACAAACAGAGACTAAAGC CTTCTGCTACAAATAAGAATCGATCTCTCCA	95℃ (4 min)/ 95℃ (30 sec) + 52 -54℃ (1 min) + 72℃ (1 min)/ 72℃ (7 min)/ 35-40	Les (1994) Tsukaya et al. (1997)
*mat*K T590F T1320R	AAGACCCCTCTTCTTGCAT GATCCGCTATAATAATGAGA	96℃ (2 min)/ 95℃ (1min) + 52℃ (1 min) + 72℃ (1 min)/ 72℃ (10 min)/ 35	Sang et al (1997)
ndhF 599F 1354R 1318F 2110R	TAGGTCTTTATTGGATAAC AAATGTCCTTCAAAAGTAAG GGATTAAC(CT)GCATTTTATATGTTTCG CCCCCTA(CT)ATATTTGATACCTTCTCC	95℃ (3 min)/ 94℃ (20 sec) + 45℃ (40sec) + 72℃ (1 min)/ 72℃ (5 min)/ 35	Sweeney & Price (2000) Olmstead & Sweerel (1994)

Bayesian analyses were reconstructed with MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). Searches were conducted using two independent runs, each performed with four simultaneous chains. In addition, the statistical test was used: the Incongruence length difference (ILD) test as a suitable first step in detecting incongruences (Cunningham. 1997; Hipp *et al*., 2004). The ILD test, called the partition homogeneity test (PHT) in PAUL* version 4.0b10 (Swofford, 2003), computed 1000 replicates with MAX-TREES option set to 100 and was executed on the combined dataset, excluding coded indels, and after removing constant characters from the matrix. The PHT was conducted on the combined chloroplast dataset as well, to test two chloroplast markers against each other.

Results

Sequence characteristics of the Korean Trigonotis species

Sequences for *Trigonotis* and outgroup taxa were generated for all plastid and nuclear markers selected. The data sets showed different levels of sequence variation and contained various indels (Table 3). The aligned sequences derived from all the cpDNA regions and the ITS revealed differences in the sequences length of 20 accessions covering 5 species in Korean *Trigonotis* and *L. zollingeri* as an outgroup. The tree length of ITS region is the highest, comparing to other primers. The *rbc*L data sets included 83 steps, with a CI of 0.976; a RI of 0.818; 0.62% of all characters were potentially parsimony-informative; 562 characters were constant and 76 characters were parsimony-uninformative. The MP analysis of the *mat*K regions comprised 30 steps, 670 of constant and 29 of parsimony-uninformative. Although the data sets of *mat*K marker were the lowest parsimony-informative (0.14%), the consistency and retention index were the highest ratio (CI=1 and RI=1), similarity with *ndh*F regions. The *ndh*F sequences resulted in an aligned matrix of 1525 characters. The MP analysis for this marker was 77 of tree length, 1450 characters were constant and 22 characters parsimony-uninformative (Table 3). The phylogenetic relationships are unresolved based on the NJ, MP and BI analysis of each chloroplast marker. The topology of *rbc*L provided better resolution at lower levels, with *Trigonotis* species. The topology of *mat*K data sets was similar to the topology of *ndh*F sequences, showing the unresolved relationships within clades. Overall, *mat*K presented the lowest percentage of informative sites, comparing to *ndh*F (0.14% for *mat*K and 3.48% for *ndh*F, respectively).

Phylogenetic analysis of the combined plastid sequences

The aligned chloroplast sequences were 2867 characters; 2,697 characters were constant; 164 characters are parsimony-uninformative while only 6 characters were parsimony-informative. Maximum parsimony analysis resulted in 176 steps, with CI of 0.972 and RI of 0.839, resulting in less homoplasy than ITS data. When analyzing the chloroplast regions, they share the same history due to their linkage, no

Table 3. Sequence characteristics and tree statistic of the cpDNA and ITS regions from the maximum-parsimony (MP) analysis

Characteristics	cpDNA			nrDNA	Combined cpDNA	Combined cpDNA and ITS
	*rbc*L	*mat*K	*ndh*F	ITS		
LAS (bp)	627-645	698-718	1525	685-719	2867	3526
TL	83	30	77	187	176	1623
PICs*	4(0.62%)	1(0.14%)	53(3.48%)	86(13.11%)	6(0.21%)	87(2.47%)
CI	0.976	1.000	1.000	0.930	0.972	0.900
RI	0.818	1.000	1.000	0.957	0.839	0.945
RC	0.798	1.000	1.000	0.890	0.815	0.935
Constant	562	670	1450	498	2697	1939
Parsimony-uninformative	76	29	22	72	164	1500

LAS, length of aligned sequences; TL, tree length; PICs, parsimony-informative characters (number and percent); CI, consistency index, RI, retention index; HI, homoplasy index; RC, rescaling consistency index.

suprising in finding of the same topologies. We cannot see error or misalignment when checking the alignment of the coding spacers of taxa. The coding region has fewer changes and the signal appears to be overhelmed in the combined analyses. In the BI analysis, the AICc selected the GTR+ Γ+I substitution model. In the combined plastid sequences, the NJ, MP and BI analysis were shown the same topology trees. The results provided *Trigonotis radicans* var. *sericeae* species were related to *Trigonotis nakaii* and *Trigonotis icumae*. In the contrast, the phylogenetic relationships were resolved between *Trigonotis peduncularis* and *Trigonotis coreana* with low bootstrap (BS = 57%) and high posterior probabilities (PP = 0.87).

Phylogenetic analysis of ITS data sets

The maximum parsimony tree from the phylogenetic analysis of DNA sequences of the ITS of 20 accessions covering 5 species in Korean *Trigonotis* and *L. zollingeri* as an outgroup. The aligned data set of the ITS of nuclear ribosomal DNA is 656bp in length with sequences varying from 685bp (*Trigonotis peduncularis*) to 719bp (*Trigonotis nakaii*). In the phylogenetic analysis, the tree length of ITS regions is the highest (187 step) one among the other molecular markers, 72 characters were parsimony-uninformative and 498 were constant for ITS regions. The MP analysis, ITS showed the highest ratio of informative sites (13.11%), while parsimony-informative of *mat*K kept the smallest ratio (0.14%) among 5 markers, with the consistency index (CI = 0.930) and retention index (RI = 0.957) for the parsimony trees (Table 3). The broad phylogenetic relationships are similar between the NJ, MP and BI analyses and divided into two main clades: one clade with strongly bootstrap and high posterior probabilities (BS= 100%; PP = 0.92) included *Trigonotis radicans* var. *sericea*, *Trigonotis nakaii* and *Trigonotis icumae*. In this clade, *Trigonotis icumaae* is a sister of the remained species. Within clade E, *Trigonotis radicans* var. *sericea* formed a polyphyletic. Although clade E has morderately bootstrap and high posterior probabilities values (BS = 84%; PP = 0.99), the internal phylogenetic relationships were unresolved. The second lineage, with

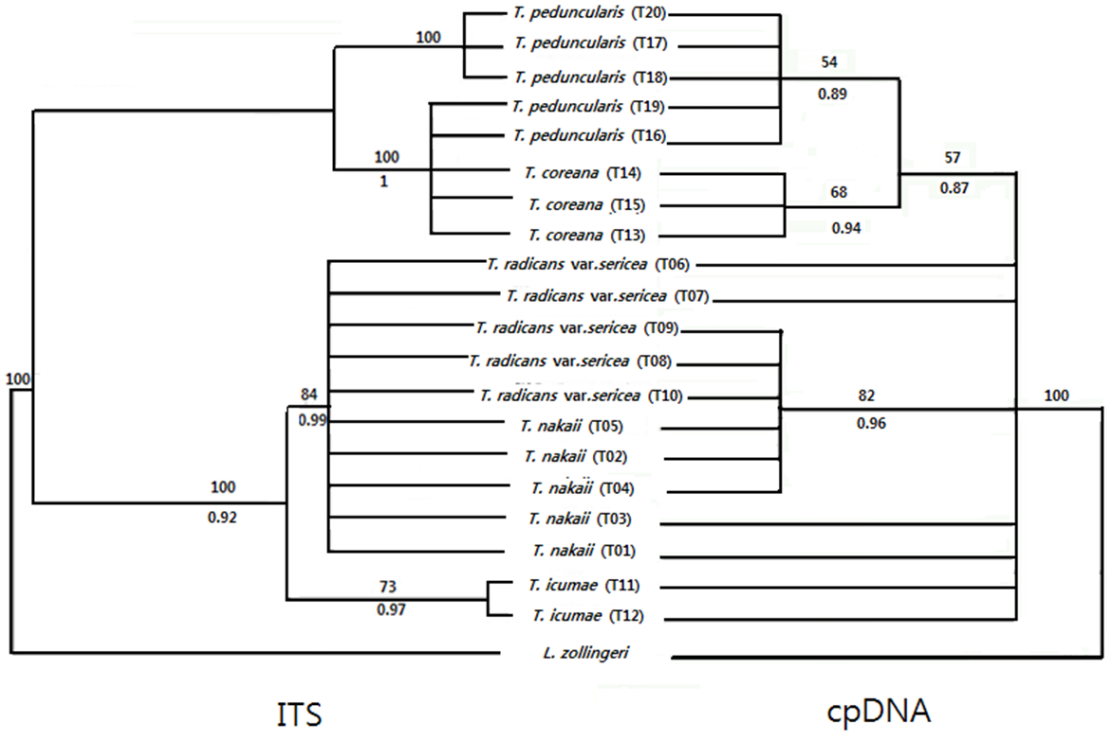

Fig. 1. Maximum parsimony analysis based on the analysis of nrDNA (left) and cpDNA (right) of *Trigonotis* species as a topological constraint and *L.zolligeri* as an outgroup. Bootstrap values are shown above branches and posterior probabilities values are shown under branches.

moderately bootstrap and high posterior probabilities values (BS = 85%; PP = 0.95), *Trigonotis peduncularis* was polyphyletic. Within this clade, although two terminal clades were generated with strongly bootstrap and high posterior probabilities (BS100; PP = 1), the phylogenetic relationships were unresolved (Fig.1).

Phylogenetic analyses based on combined cpDNA and ITS data

The A total of 3526 characters were generated when combined cpDNA and nrDNA (ITS) sequences, 87 characters were parsimony-informative. The heuristic search produced 1623 steps with 1939 of constant and 1500 of parsimony uninformative. Maximum parsimony analysis resulted in 176 steps, with CI of 0.972; RI of 0.839 and RC of 0.935 (Table 3). The ILD test is used to examine of phylogenetic incongruence between nrDNA and cpDNAs, the incongruence between the nrDNA and chloroplast data sets was no significant incongruence (p = 0.01), thus they were analyzed in combination. By the way, the combined data tree topology had the same structure as the cpDNA phylogeny. The topology trees of Korean *Trigonotis* taxa from the NJ, MP and BI analysis provided the similar results. Phylogentic relationships are very similar between the total evidence trees obtained in the BI and MP analyses. Both MP and BI trees provided that phylogenetic relationships of *Trigonotis* taxa were divided into two main clades. Clade A was weakly bootstrap and low posterior probabilities values (BS = 60%, PP = 0.63) and the relationship of *Trigonotis icumea,* receiving strongly bootstrap and high posterior probability, was resolved with the related species (Fig.2). In the clade D, *Trigonotis peduncularis* can be only distinghuished to *Trigonotis coreana* in BI analysis. In the MP tree, *Trigonotis peduncularis* was related to *Trigonotis coreana.*

Discussion

In the recent years, the comparative sequence analysis in systematic is an important tool for inferring phylogenetic relationships because of its fastening and convenience.

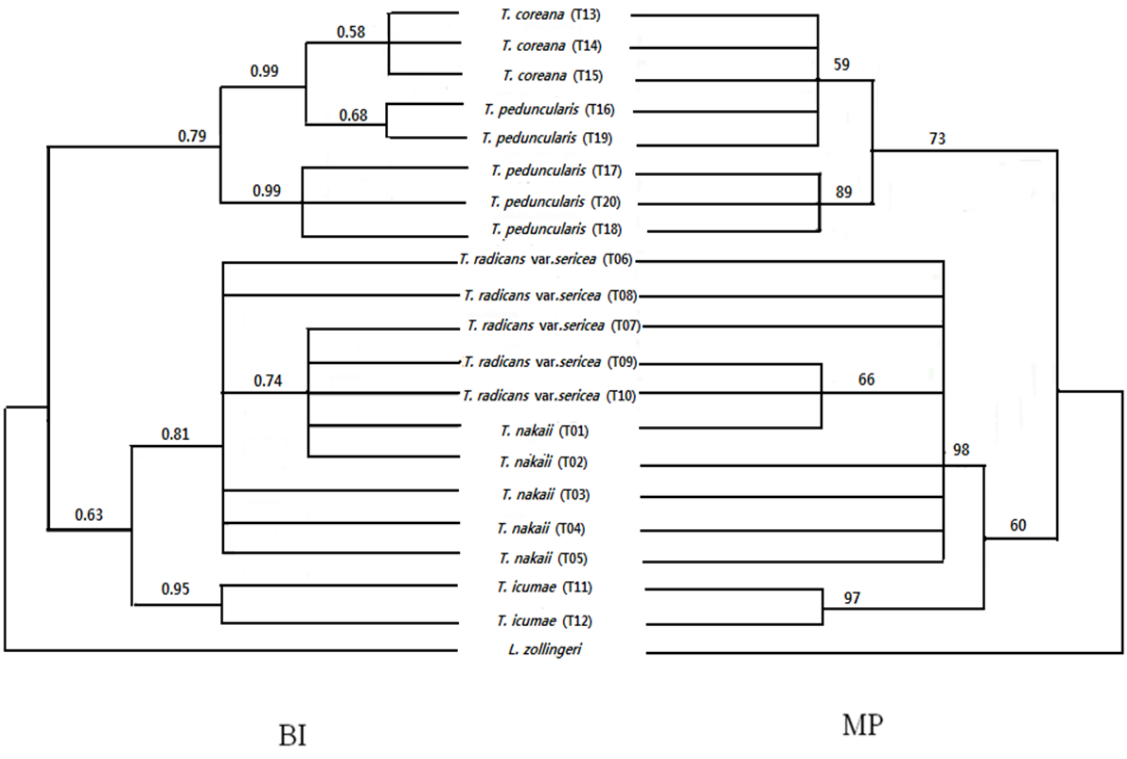

Fig. 2. Phylogenetic tree of 21 *Trigonotis* individuals and *L. zollingeri* as an outgroup from MP analysis and BI analysis based on combined cpDNA and nrDNA. The posterior probabilities and Bootstrap values are shown above branches.

Phylogenetic inference and elucidation of the evolutionary processes that generate biological diversity have been accomplished even at lower taxonomic levels using chloroplast genome and the ITSs of the nrDNA. In the present study, all the three cpDNA primers used successfully amplified the target regions in the *Trigonotis* species. Attempts to amplify the *rpo*C1 region were not successful in all the taxa. In this study, *ndh*F provided the highest number of parsimony-informative characters followed by *rbc*L and *mat*K regions (Table.3). Of the cpDNA regions used in this study, the individual gene analyses have provided such a topology but low or <50% support. (Savolainen *et al.,* 2000a). The intergenic spacers were useful in the inference of phylogenetics at low taxonomic level in genera, the genus Korean *Trigonotis* is a particular example because the phylogenetic relationships between species are clearly unresolved.

Overall, the ITS data set provided a weaker signal than chloroplast data sets. The ITS topology was also less resolved the internal relationship between *Trigonotis* species, comparing to the chloroplast markers results (Fig.1). Tests clearly provided that the major relationships found by the plastid sequences were similar to those based on ITS. However, in general the internal relationships between species within section are unresolved. The findings are supported by Candolle‘s concept (1846) that *Trigonotis* var. *sericea* is synonym to *Trigonotis nakaii.* In addition, the results are shown that the phylogenetic relarionships between *Trigonotis icumae* and *Trigonotis* species are unresolved based on combined chloroplast sequences, comparing to ITS sequence (Fig.1). Furthermore, in the MP tree, *Trigonotis coreana* was within *Trigonotis peduncularis* group based on the ITS sequence but these species formed a separated group based on chloroplast sequences (Fig.1). Similarity, the phylogenetic relationship of *Trigonotis nakaii* and *Trigonotis radicans* var. *sericeae* were resolved in plastid tree, while this relationship cannot be found in ITS marker. In addition, *Trigonotis* taxa formed monophyletic group based on the combined plastid data sets, by contract, polyphyletic groups were reconstructed based on the ITS sequences (Fig.1). According to Small *et al*., (2004), nuclear DNA data sets in phylogenetic studies have traditionally been limited in classification between orthologous and paralogous sequences. However, at the infrageneric level, the ITS marker is a useful tool for resolving phylogenetic relationships at different taxonomic levels. The different combined data set is a contentious issue and numerous suggested methods for approaching the problems (Huelsenbeck *et al*., 1996), despite of the low incongruence or low bootstrap support for the incongruent nodes then data can be deemed to be combinable (e.g. Mason-Gamer and Kellogg 1996). The phylogenetic relationships of closely related taxa are inadequate resolution when using few cpDNA loci related to the low number of parsimony-informative characters (Rokas *et al*., 2003). Thus, the phylogenetic relationships of Korean *Trigonotis* taxa based on combined nuclear ribosomal DNA and chloroplast DNA markers were partly resolved. Both MP and BI trees showed the polyphyly of *Trigonotis* species and reconstructed major clades: the clade I included *Trigonotis coreana* and *Trigonotis peduncularis* and clade II comprised the remained taxa. Based on combined molecular markers, both MP and BI trees, shown *Trigonotis coreana* is related to *Trigonotis peduncularis*. These interesting results are conflict to the previous studies that *Trigonotis radicans* var. *sericea* and *Trigonotis coreana* are synonym species (De Candolle, 1846). The conclusions can be drawn from this study: firstly, the phylogenetic relationships of *Trigonotis* taxa were well unresolved based on separated chloroplast markers (not shown) because these regions do not contain enough information to resolved relationships between closely related genera. Both ITS and cpDNA trees, the DNA sequences resulted from the *Triogonotis peduncularis* is closest to *Trigonotis coreana* and *Trigonotis radicans* var. *sericea* is related to *Trigonotis nakaii*. Secondly, the combination of nuclear ribosomal DNA and chloroplast sequences helps to discriminate between *Triogonotis* species and provides more information for inference of phylogeny. Using additional rapidly evolving genomic regions is desirable to provide insight needed to improve our understanding of plant evolution. Ongoing studies further base on the non-coding chloroplast regions to find out the phylogenetic relationships within *Trigonotis* genus because the non-coding regions of chloroplast DNA are supplied to evolve more rapidly than coding regions.

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