

PHYLOGENY OF *GALIUM* L. (RUBIACEAE) FROM KOREA AND JAPAN BASED ON CHLOROPLAST DNA SEQUENCE

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Keywords: Chloroplast DNA; Korean-Japan *Galium*; Molecular data; Phylogeny.

Abstract

The present paper deals with the phylogeny and inter- and intragenic relationships using four chloroplast DNA sequences within 19 *Galium* L. species from Korea and Japan. Maximum parsimony and Bayesian analyses were conducted to clarify the relationships among the sections and species. The strict consensus tree had three main clades. Clade I comprises of the only individuals of *G. paradoxum* Maximowicz (sect. *Cymogalia*), which is distinguished by opposite leaves in the genus, supported by the 100% bootstrap value (PP: 0.98); Clade II consists of members of eight sections (sect. *Galium*, sect. *Hylaea*, sect. *Kolgyda*, sect. *Trachygalium*, sect. *Leptogalium*, sect. *Orientalgalium*, sect. *Aparine*, and sect. *Leiogalium*); Clade III comprises members of eight sections (sect. *Baccogalium*, sect. *Lophogalium*, sect. *Platygalium*, sect. *Relbunium*, sect. *Depauperata*, sect. *Aparinoides*, sect. *Leiogalium* and *Trachygalium*). The sect. *Leptogalium* which includes two taxa namely *G. tokyoense* Makino and *G. dahuricum* var. *lasiocarpum* (Makino) Nakai is paraphyletic. Four taxa of *Trachygalium* group (*G. trachyspermum* A. Gray, *G. gracilens* (A. Gray) Makino, *G. pogonanthum* Franch. & Sav., *G. koreanum* Nakai) were placed from sect. *Cymogalia* to sect. *Platygalium* based on molecular and morphological data.

Introduction

Galium L., the largest genus of the tribe Rubieae in the family Rubiaceae (Robbrecht and Manen, 2006), is taxonomically diverse and comprises over 650 species (Govaerts, 2006). *Galium* is divided into 16 sections based on characters of leaf and fruit by Ehrendorfer *et al.* (2005). The species of *Galium* are distributed centrally in temperate regions and are mostly annual and perennial herbaceous plants. The genus is characterized by more than two leaf-like whorls, number of divided petal, rudimentary calyx and a two locular ovary.

Phylogenetic relationships among species of tribe Rubieae including eleven genera have been studied by many researchers (Ehrendorfer *et al.*, 1994, 2014; Manen *et al.*, 1994; Manen and Natali, 1995; Natali *et al.*, 1995, 1996; Soza and Olmstead, 2010). Molecular phylogenetic studies using chloroplast DNA *atpB-rbcL* intergenic region have shown monophyly of the tribe Rubieae with seven major clades, and confirmed that genera *Asperula* and *Galium* is not a monophyletic group (Manen *et al.*, 1994; Natali *et al.*, 1995, 1996). Soza and Olmstead (2010) conducted more clearly molecular phylogenetic analysis of tribe Rubieae using three chloroplast DNA makers and their results indicated that *Galium* is polyphyletic, and species of *Galium* occur in three major clades (Clades III, V, VII). Recently, phylogenetic relationships study of tribe Rubieae including

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some *Galium* species by Ehrendorfer *et al.* (2014) has evaluated that genus *Galium* is paraphyletic. Although there have been several phylogenetic study to investigate relationships of tribe Rubieae, very little is known about phylogenetic relationships among Korean species of *Galium*. Soza and Olmstead (2010) determined the phylogenetic relationships among Rubieae including members of *Galium* but this study included only three common species distributed in Korea and Japan. In Korea, twenty taxa of seven sections are currently recognized (Lee, 1995; Lee, 1979; Lee, 2004). *G. koreanum* Nakai, *G. verum* var. *asiaticum* for. *pusillum* (Nakai) M. Park are endemic to Korea and latter species is restrictedly distributed in Mt. Halla of Jeju Island. *G. kikumugura* Ohwi is broadly expanded to Japan. Jeong and Pak (2009, 2012) conducted morphological and somatic chromosome number counts of Korean *Galium*. These studies however, provided very little phylogenetic relationships among the species. Therefore, further studies are needed to understand their phylogenetic relationships among Korean *Galium* species and taxonomic position of Korean and Japan taxa within the *Galium* spp. occurring worldwide. This study aims to clarify inter-and intragenic relationships within Korean and 10 Japanese *Galium* species, and to determine the taxonomic position of Korean endemic taxa within the closely related *Galium* spp. using the chloroplast DNA sequences.

Materials and Methods

Plant materials

Total 19 species of *Galium* distributed in Korea and Japan were collected (Table 1). We selected two outgroup taxa [*Didymaea alsinoides* (Cham. and Schltld.) Standl., and *Rubia cordifolia* L.] based on the results of the analyses of Soza and Olmstead (2010). The sequences of *Galium* and outgroups obtained from National Center for Biotechnology Information (NCBI) database with the exception of sequences of sample from Korea-Japan. All sources and voucher specimens of materials were deposited at the Herbarium of Kyungpook National University (KNU).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from fresh leaf tissues and field-collected silica-gel dries tissue using the 2 % hexa decyltrimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987). We amplified the *rpoB-trnC* region and *trnC-ycf6* region with primers designed by Demesure *et al.* (1995). The *trnL-trnF-ndhJ* region was amplified using primers published in Taberlet *et al.* (1991) and Shaw *et al.* (2007) (Table 2). Polymerase chain reaction (PCR) conditions were an initial denaturation of 94°C for 5 min, 35 cycles of 94°C denaturation for 30 s, 48°C-57°C annealing for 30 s extension for 1m, and final extension at 72°C for 10 min. PCR products were purified using the QIAquick PCR purification kit following the instructions of the manufacturer. Sequencing reactions were carried out for the purified PCR products using Big Dye Terminator Cycle Sequencing reagents (Applied Biosystem, Foster city, CA, USA). For sequencing, we used the same primers as those used for PCR. All sequences have been deposited in GenBank (Table 1).

Data analysis

The DNA sequences were aligned with Clustal X (Thompson *et al.*, 1997). All chloroplast regions were combined and analyzed using Maximum Parsimony (MP) and the Bayesian analyses. Gaps introduced from the alignment were treated as missing characters in subsequent analyses. MP analyses were conducted in a PAUP* (version 4.0b 10; Swofford, 2003) using a heuristic searches with TBR branch swapping and MULTREES option. Relative support of various monophyletic groups revealed in the most parsimonious trees was examined with the bootstrap

Table 1. Sampling sites of plant materials used for phylogenetic analyses.

Taxon	Locality	Voucher	GenBank acc. No.			
			<i>trnC-ycf6</i>	<i>trnF-ndhJ</i>	<i>TrnL</i>	<i>rpoB-trnC</i>
Sect. <i>Aparine</i>						
<i>Galium spurium</i> var. <i>echinospermon</i>	Chilgok-gun, Korea	J20050310	KC339150	KC339020	KC339085	LC062539
Sect. <i>Aparinoides</i>						
<i>G. trifidum</i>	Jeju-si, Korea	J20060807	KC339148	KC339018	KC339083	LC062537
	Tokyo metro, Japan	M20100501	KC339149	KC339019	KC339084	LC062538
Sect. <i>Cymogalia</i>						
<i>G. paradoxum</i>	Pyeongchang-gun, Korea	J20090814	KC339164	KC339034	KC339099	LC062552
	Jeongseon-gun, Korea	J20050618	KC339163	KC339033	KC339098	LC062551
	Muju-gun, Korea	J20100844	KC339162	KC339032	KC339097	LC062550
Sect. <i>Hylaea</i>						
<i>G. trifloriforme</i>	Ulleung-gun, Nari, Korea	J20080621	KC339204	KC339074	KC339139	LC062581
	Ulleung-gun, Korea	J20080635	KC339203	KC339073	KC339138	LC062580
	Ulleung-gun, Taehwa, Korea	J20080603	KC339205	KC339075	KC339140	LC062582
	Miyagi, Japan	J20100748	KC339206	KC339076	KC339141	LC062583
<i>G. japonicum</i>	Ulleung-gun, Nari, Korea	J20080611	KC339207	KC339077	KC339142	LC062584
	Ulleung-gun, Nari, Korea	J20080612	KC339151	KC339021	KC339086	LC062540
	Jeongeup-si, Korea	J20100845	KC339209	KC339079	KC339144	LC062585
	Jeju-si, Korea	J20070901	KC339210	KC339080	KC339145	LC062586
	Miyagi, Japan	J20100758	KC339211	KC339081	KC339146	LC062587
	Yamagata, Japan	J20100759	KC339212	KC339082	KC339147	LC062588
Sect. <i>Leptogalium</i>						
<i>G. dahuricum</i> var. <i>lasiocarpum</i>	Namyangju-si, Korea	J20100897	KC339189	KC339059	KC339124	LC062569
	Pyeongchang-gun, Korea	J20090807	KC339192	KC339062	KC339127	LC062571
	Yeongwol-gun, Korea	J20080926	KC339155	KC339025	KC339090	LC062543
	Seongju-si, Korea	J20100658	KC339188	KC339058	KC339123	LC062568
	Jecheon-si, Korea	J20091021	KC339190	KC339060	KC339125	LC062570
	Yamagata, Japan	J20100708	KC339194	KC339064	KC339129	LC062573
<i>G. kikumugura</i>	Mt. Zao, Japan	J20100765	KC339200	KC339070	KC339135	LC062577
<i>G. pseudoasprellum</i>	Miyagi, Japan	J20100789	KC339202	KC339072	KC339137	LC062579
<i>G. tokyoense</i>	Pocheon-si, Korea	J20070938	KC339195	KC339065	KC339130	LC062572
	Pocheon-si, Korea	J20090808	KC339193	KC339063	KC339128	LC062574
	Tokyo metro, Japan.	M20090503	KC339197	KC339067	KC339132	LC062575
Sect. <i>Platygalium</i>						
<i>G. boreale</i>	Yeongwol-gun, Korea	J20050625	KC339152	KC339022	KC339087	LC062541
	Mongolia	L20090830	KC339153	KC339023	KC339088	LC062542
<i>G. gracilens</i>	Sunchen-si, Korea	J20090801	KC339181	KC339051	KC339116	LC062566
	Hwasun-gun, Korea	J20090830	KC339180	KC339050	KC339115	LC062565
<i>G. kamtschaticum</i> var. <i>yakusimense</i>	Jeju-si, Korea	J20070907	KC339166	KC339036	KC339101	LC062553
<i>G. koreanum</i>	Sancheong-gun, Korea	J20100808	KC339186	KC339056	KC339121	LC062567
<i>G. kinuta</i>	Yeongwol-gun, Korea	J20050626	KC339167	KC339037	KC339102	LC062554
<i>G. pogonanthum</i>	Hamyang-gun, Korea	J20090504	KC339172	KC339042	KC339107	LC062559
	Jeju-si, Korea	J20050706	KC339171	KC339041	KC339106	LC062558
<i>G. trachyspermum</i>	Inje-gun, Korea	J20080906	KC339170	KC339040	KC339105	LC062546
	Andong-si, Korea	J20070751	KC339157	KC339027	KC339092	LC062545
	Gyeongju-si, Korea	J20100913	KC339159	KC339029	KC339094	LC062547
	Geoje-si, Korea	J20090327	KC339156	KC339026	KC339091	LC062544
	Miyagi, Japan	J20100723	KC339160	KC339030	KC339095	LC062548
	Yamagata, Japan	J20100747	KC339161	KC339031	KC339096	LC062549
Sect. <i>Galium</i>						
<i>G. verum</i> var. <i>asiaticum</i>	Geoje-si, Korea	J20100524	KC339173	KC339043	KC339108	LC062563
	Jeju-si, Korea	J20090685	KC339174	KC339044	KC339109	LC062562
	Fukui, Japan	M20100503	KC339176	KC339046	KC339111	LC062561
<i>G. verum</i> var. <i>trachycarpum</i> f. <i>nikkoense</i>	Ulsan metro., Korea	J20050830	KC339198	KC339068	KC339133	LC062576
	Tokushima, Japan	J20100732	KC339177	KC339047	KC339112	LC062564
<i>G. verum</i> var. <i>asiaticum</i> f. <i>pusillum</i>	Jeju-si, Korea	J20050807	KC339175	KC339045	KC339110	LC062560

method (Felsenstein, 1985). Bootstrap values were calculated from 1,000 replicates with the random addition and heuristic search options. The Bayesian phylogenetic analyses were conducted with MrBayesver 3.1.2 (Ronquist and Huelsenbeck, 2003). The suitable model was determined to be GTR+I+G for combined sequence data by MrModeltest 2.3 (Nylander, 2004). Each Morkov chain was started from a random tree and run for 1,000,000 generations, sampling a tree every 100 generations. Burn-in time was estimated from the plot of likelihoods generated using the 'sump' command in MrBayes. Posterior probabilities (pp) were based on analysis of post-burn-in tree. Nodes were considered highly supported when pp values were higher than 0.95 (Felsenstein, 1985).

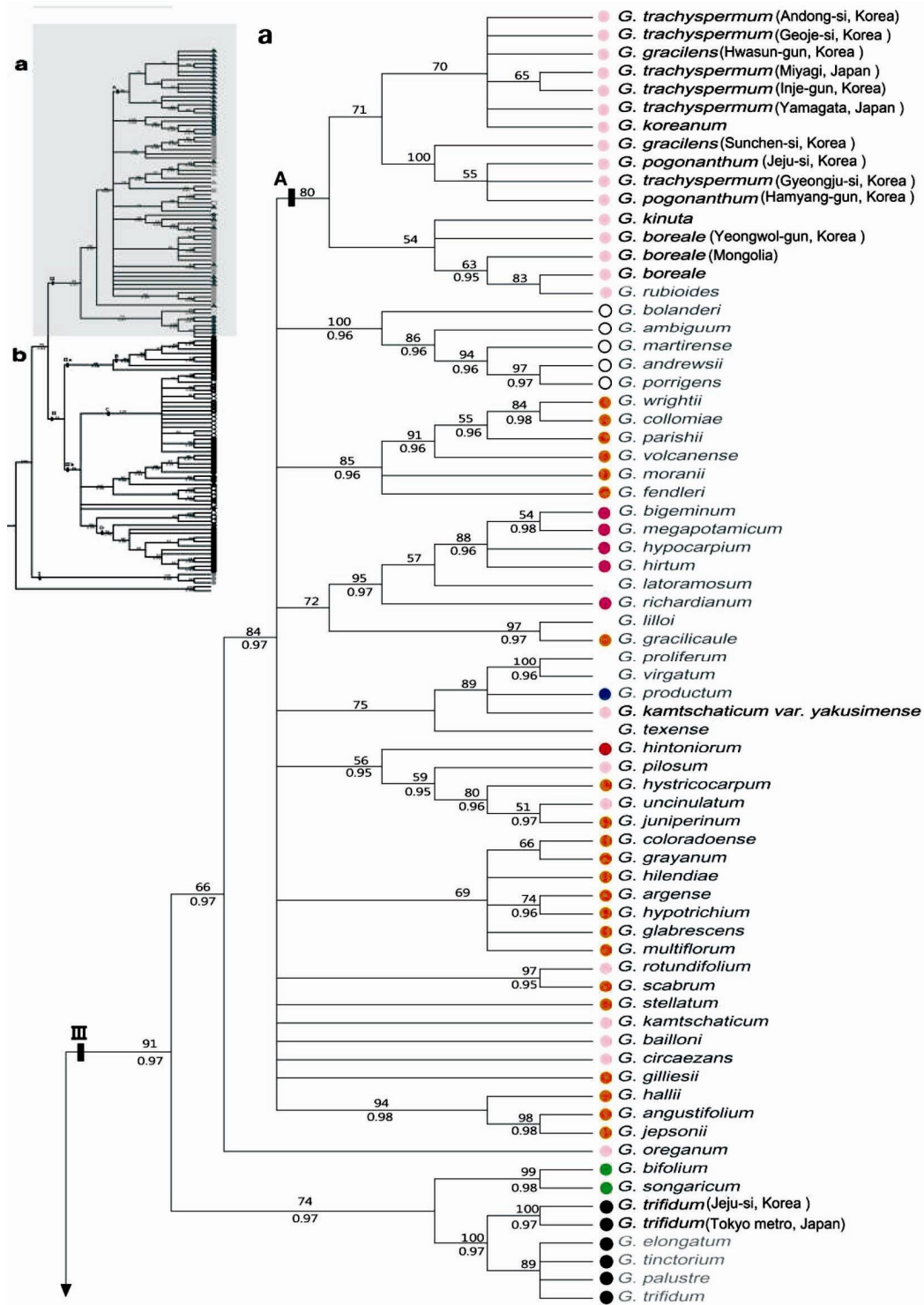
Results and Discussion

Sequence characteristics

The total of 4,341 lengths of the aligned sequences was used for phylogenetic analysis. Of a total of investigated character sites, 2,793 characters were constant and 824 characters were parsimony informative including out groups. The parsimony analyses generated 10,620 most parsimonious trees with a total length of 2,970 steps, a consistency index of 0.65 and a retention index of 0.88. The MP tree with bootstrap values (BP) and PP are shown in Fig. 1.

Phylogenetic analyses

The strict consensus tree had three main clades (clade I, clade II and clade III). Clade IV is highly supported by the 100% bootstrap value (PP: 0.98) and was sister to the rest of the species, which were grouped in two other clades. This clade was only composed of the individuals of *G. paradoxum* Maxim. Clade IIa is supported 99% bootstrap value (PP<0.95). Clade IIb consists of two highly supported subclades (subclade IIa and IIb). Subclade IIa included three taxa: *G. dahuricum* var. *lasiocarpum* (Makino) Nakai., *G. pseudoasprellum* Makino and *G. triflorum* Michx. comprising of Group B. *G. triflorum* (sect. *Trachygalium*) was sister to *G. dahuricum* var. *lasiocarpum* from Korea-Japan and *G. pseudoasprellum* from Japan (99% bootstrap value). Subclade IIb is supported by 91% bootstrap value (PP<0.95). This subclade contained 8 taxa from Korea-Japan. It was further divided into Group C and D. Group C contained members of three sections (sect. *Galium*, sect. *Leiogalium* and sect. *Leptogalium*) which are identified by Soza and Olmstead (2010), *G. tokyoense* Makino, *G. kikumugura*, and three species belonging to sect. *Galium* from Korea-Japan. But the *G. verum* group from Korean and Japanese were not well resolved. In the Group D, *G. japonicum* (Maxim.) Makino & Nakai from Korea and Japan is monophyletic, although the individuals of *G. trifloriforme* Kom. did not form monophyletic group. These two taxa share its most recent common ancestor with *G. spurium* var. *echinospermum* (Wallr.) Hayek and *G. odoratum* (L.) Scop (61% bootstrap value (PP: 0.96)). Clade III is supported by 91% bootstrap value (PP: 0.97), comprising eight sections; sect. *Baccogalium*, sect. *Lophogalium*, sect. *Platygalium*, sect. *Leiogalium*, sect. *Trachygalium*, sect. *Relbunium*, sect. *Depauperata*, sect. *Aparinoides*. The members of sect. *Depauperata*, and sect. *Aparinoides* are sister to the rest of the species within this Clade. *G. trifidum* L. is paraphyletic and unresolved within the clade. Group A in Clade III included four taxa from *G. trachygalium* group (*G. gracilens* (A. Gray) Makino, *G. koreanum*, *G. pogonanthum* Franch. & Sav. and *G. trachyspermum* A. Gray) and members of sect. *Platygalium* (BS: 80%, PP<0.95). The previous classification based on morphological study of the four taxa of the *G. trachygalium* group was not resolved (Jeong and Pak, 2009). The individuals from the same taxa did not even form the monophyletic. *G. kinuta* Nakai & Hara belonging to sect. *Platygalium* with *G. boreale* L. was resolved as paraphyletic.



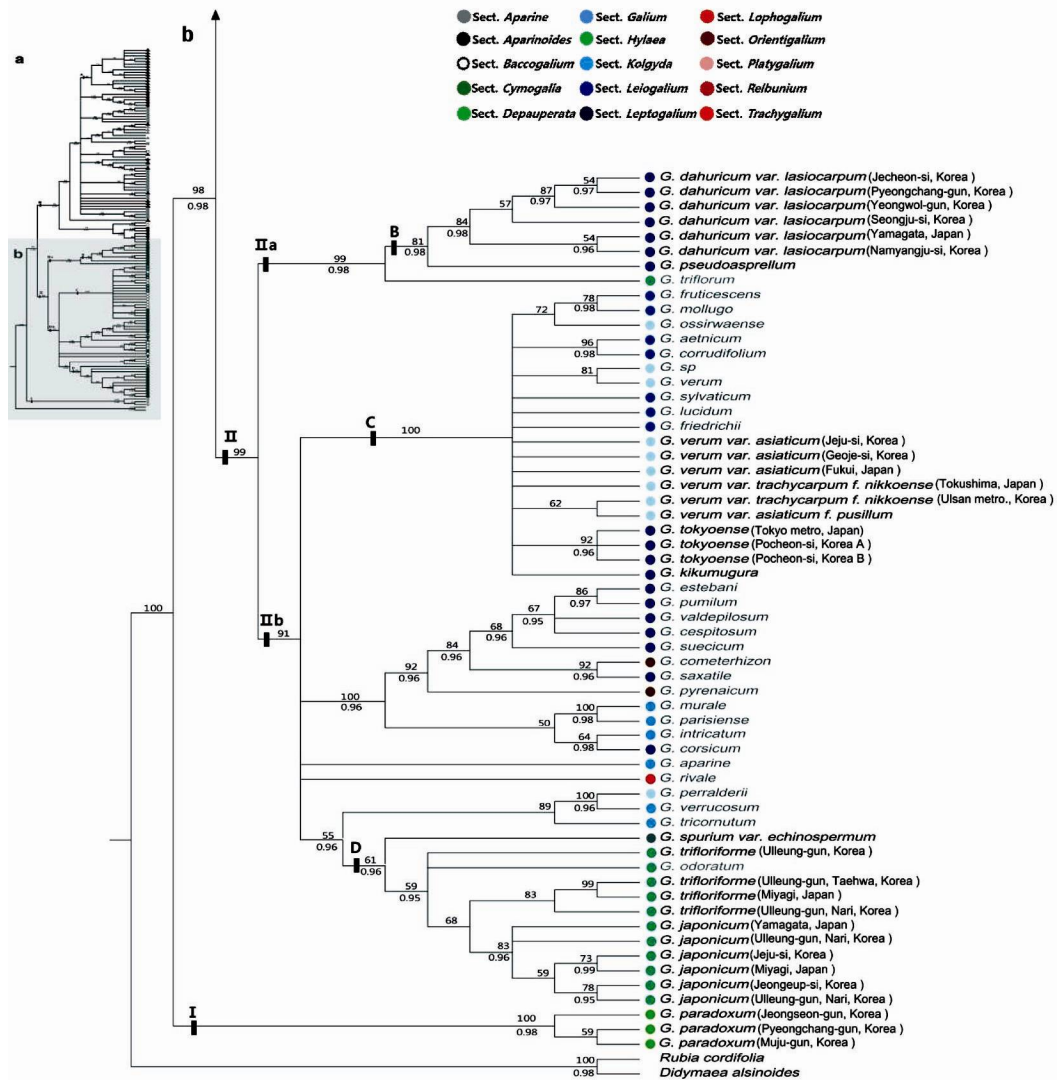


Fig. 1. Strict consensus tree of genus *Galium* based on Chloroplast DNA data, Bootstrap values and posterior probabilities are shown above and below branches, respectively. Different shapes were used for sectional treatments (taxon without shape “represents not classified”). Species in black represent the taxa sampled in this study.

Phylogenetic relationships of Korean-Japanese *Galium*

The phylogenetic relationships among Korean *Galium* and some of Japanese *Galium* were, for the first time, assessed in this study. We confirmed that the cpDNA phylogeny has significantly higher resolution and better support than previous study in Korean-Japanese *Galium* using morphological and chromosome number data by Jeong and Pak (2009, 2012). In some of taxa, our data were incongruent with previous classifications of Korean-Japanese *Galium* based on morphological data.

G. paradoxum was sister to the group consisting of the rest of the *Galium* species (Fig 1). It also support the study of Ehrendorfer *et al.* (2014) using the plastid DNA sequences. The species is a perennial herb with opposite leaves, a pair of scale-like small stipules, one vein, white petiole and corolla, and rotate flowers. *G. paradoxum* was placed into a sect. *Cymogalia* based on the characters of inflorescence and hairs of a fruit (Pobedimova *et al.*, 2000; Ehrendorfer *et al.*, 2005). Its main distributions is in eastern Asia (Ehrendorfer *et al.*, 2014), and mainly occurs in moist high elevations in mountain forests.

The taxa in the clade II have whorls of six or eight leaf-like organs. The five taxa from Korea-Japan are contained in Group C. The taxa of *G. verum* group (sect. *Galium*; *G. verum* var. *asiaticum* Nakai, *G. verum* f. *nikkoense* var. *trachycarpum* (Nakai) Ohwi and *G. verum* var. *asiaticum* f. *pusillum*) showed polytomies in the MPtree with weak PP. *G. verum* var. *asiaticum* is widely distributed throughout Korea and Japan. In our study, *G. verum* var. *asiaticum* have five chloroplast types from five individuals. But we cannot find morphological variation among the individuals. The three taxa are erect and have whorls of six or more than leaf-like organs, inflorescences of branched panicles with white or yellow flowers, and glabrous fruits. These three taxa don't exhibit significant morphological differences. But the plant and leaves size of *G. verum* var. *asiaticum* f. *pusillum* are smaller than those of other two taxa, and Korean endemic species in Mt. Halla on Jeju Island (Lee, 2004). It formed a clade with *G. verum* f. *nikkoense* var. *trachycarpum* from Ullsan-si (eastern part of Korea) with weak BS. It could provide crucial information for origin of Korean endemic, *G. verum* var. *asiaticum* f. *pusillum*. It needs additional study to investigate the origin and in these evolutionary relationship among these taxa. The four taxa of *G. dahuricum* group from Korea-Japan; *G. dahuricum* var. *lasiocarpum*, *G. kikumugura*, *G. tokyoense*, and *G. pseudoasprellum*, are have been included into sect. *Trachygalium* (Ehrendorfer *et al.*, 2005). There is no study of phylogenetic using molecular makers before. The four taxa of *G. dahuricum* group occur in East Asia, and have serious identification problems and taxon delimitation due to severe variations in the morphology of leaves, seed hairs and flower and inflorescences (Chen and Ehrendorfer, 2011). We confirmed the phylogenetic relationship among these taxa, for the first time. *G. kikumugura* and *G. tokyoense* were included in Group C. *G. kikumugura* having whorls of four leaf-like organs and fruit with generally hooked hairs were closely related to *G. tokyoense*, morphologically (Yamazaki, 1993). Lee (1995) reported the distribution of *G. kikumugura* in Korea but we could not find the distribution during the this study although the species is widely distributed in Japan. We also could not confirm *G. kikumugura* specimens collected from Korea at Korean and Japan herbria. Therefore we assumed that the distribution report of this taxa by Lee (1995) was based on misclassification. *G. pseudoasprellum* was treated as synonyms of *G. dahuricum* by Ehrendorfer *et al.* (2005), but in our results did not support his opinion. *G. pseudoasprellum* is similar to *G. dahuricum* var. *lasiocarpum*, morphologically but it can be distinguished from *G. dahuricum* based on leaf shapes, which whorl of 6 elliptic or lanceolate leaves. *G. tokyoense* has glabrous fruit and white flower compare with *G. dahuricum* var. *lasiocarpum*. Previous studies based on morphology (Yamazaki, 1993; Pobedimova *et al.*, 2000; Chen and Ehrendorfer, 2011) were argument for classification of *G. tokyoense*. We confirmed that the *G. tokyoense* and *G. dahuricum* var. *lasiocarpum* were polyphyletic. Also our result is supported that previous classification that *G. tokyoense* be regarded as a species. *G. kamchaticum* Steller *ex* Schultes & J. H. Schultes and *G. kamchaticum* var. *yakusimense* (Masamune) Yamazaki were place to clade with polytomy at MP tree with weak PP value. *G. kamchaticum* is distributed in an alpine meadow of worldwide with centers of the diversity in eastern Asia and eastern North America (Ehrendorfer *et al.*, 2005). *G. kamchaticum* var. *yakusimense* is smaller leaf and tall than *G. kamchaticum*. This species is

erect, with round leaves, one vein, whorls of four leaf-like organs, 4-parted white, and a fruit with generally hooked hairs.

We confirmed that *G. kinuta* is closer to *G. boreale*. Two taxa usually occur in northern part of Korean peninsula, especially in the mountain forests in lower elevation. The somatic chromosome number of *G. kinuta* and *G. boreale* were 4X ($2n=44$) and/or 2X ($2n=11$), respectively (Jeong and Pak, 2009). *G. kinuta* is erect, four leaf-like organs, three veins, branched panicles of inflorescences, and white flowers. *G. kinuta* and *G. boreale* are generally very similar in morphology and can be distinguished by the characters of leaf-shape.

Table 2. Primers used for amplification of cpDNA regions in this study.

Region	Primer	Sequence (5'-3')	Annealing temperature (°C)	References
<i>trnC-ycf6</i>	trnC ^{GCA} F	CCAGTTCRAATCYGGGTG	52	Demesure <i>et al.</i> (1995)
	ycf6R	GCCCAAGCRAGACTTACTATATCCAT		Demesure <i>et al.</i> (1995)
<i>trnF-ndhJ</i>	ndhJ	ATGCCYGAAGTTGGATAGG	57	Shaw <i>et al.</i> (2007)
	TabE	GGTTCAAGTCCCTCTATCCC		Taberlet <i>et al.</i> (1991)
<i>TrnL intron</i>	c	CGAAATCGGTAGACGCTACG	55	Taberlet <i>et al.</i> (1991)
	d	GGGGATAGAGGGACTTGAAC		Taberlet <i>et al.</i> (1991)
<i>rpoB-trnC</i>	rpoBb	CGGATATTAATAKMTACATACG	55	Soza and Olmstead (2010)
	rpoBd	GTTGGGGTTTACATATACT		Soza and Olmstead (2010)

The *G. trachygalium* group consisted of four species; *G. trachygalium*, *G. pogonanthum*, *G. gracilens*, which occur in both Korea and Japan, and *G. koreanum* endemic to Korea. Although, the four taxa placed into Group A, our data did not provide insights into the specific phylogenetic relationships among *G. trachygalium* group species. These taxa are characterized by whorls of four leaf-like organs, cymose inflorescences with several terminal flowers, 4-parted rotate flowers and tuberculate fruit. The identification and delimitation of these species are usually difficult because they are very similar in morphology. The four species are distinguished by the differences in leaf size, shape, and fruit hairs (Jeong and Pak, 2012). These taxa usually occur in the near or same population, and share a common habitat. The somatic chromosome number of these species are 2X ($2n=22$) and/or 4X ($2n=44$) (Jeong and Pak, 2009). This inconsistencies phylogeny can be explained the speciation processes of the *G. trachygalium* group. But it is yet to be determined whether incomplete lineage sorting of ancestral polymorphisms in the population, or chloroplast capture by hybridization and introgression. It needs additional study to understand origin and clear relationship among these taxa. *G. trachyspermum*, *G. pogonanthum* and *G. gracilens* previously been placed into a sect. *Cymogalia* by Yamazaki (1993) but our data showed that these four taxa including *G. koreanum*, are more closely related to members of sect. *Platygalium* (Table 1). We suggest that the four taxa have to be transferred to sect. *Platygalium* based on molecular and morphological data.

Acknowledgement

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1A6A1A05011910).

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(Manuscript received on 2 October 2015; revised on 1 November 2016)