Scientific Research

Phylogenetic Relationships in the Genus *Spiraea* (Rosaceae) Inferred from the Chloroplast DNA Region, *trn*L-*trn*F

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ABSTRACT

The 26 taxa of genus *Spiraea* (Rosaceae) in 14 Korean taxa and four taxa of them doubled in the world itself were collected and investigated phylogenetic relationships within these taxa using the chloroplast *trn*L-*trn*F region. Sequence variation within the genus *Spiraea* was mostly due to insertion/deletion. Total alignment sequence of *trn*L-*trn*F region in genus *Spiraea* was 1004 positions, of which 126 were parsimony-informative, 149 variable, 23 singleton, and 847 constant characters. The base furtherance did not show a significant difference among total taxa. Although many taxa of genus *Spiraea* were well separated each other, many branches were not congruent with the taxon positions with botanical names. In addition, sequences of the chloroplast *trn*L-*trn*F region for same species are different from each other according to authors. The taxonomic identification of genus *Spiraea* are in need of revision.

Keywords: Genus *Spiraea*; Chloroplast *trn*L-*trn*F; Phylogenetic Relationships

1. Introduction

Spiraea (also known as meadowsweet) is a genus with 80 - 100 species of shrubs in the subfamily Spiraeoideae (Rosaceae) [1]. They are native to the temperate Northern Hemisphere, with the greatest diversity in eastern Asia [2].

Among plant systematic and phylogeographic studies the chloroplast genome is widely used and generally accepted as an excellent source for molecular information [3,4]. There are several reasons for this. First, the uniparental inheritance ensures orthology of sequences. Second, even within an individual the possibility of recombination between genomes from individual plastids is extremely low, and there are only a few studies describing the occurrence of multimeric chloroplast DNA (cpDNA) genomes or interchromosomal cpDNA recombination [5,6]. Third, dramatic changes in gene content and structure only occurred after the chloroplast genome entered a eukaryotic cell via primary endocytobiosis [7], whereas land plant plastomes are highly conserved [8].

Chloroplast DNA (cpDNA) sequence variations has been widely used to interspecific relationships in plant systematics and phylogeography [9]. Especially, Taberlet *et al.* [9] introduced universal primers to amplify the region comprising the *trn*T (UGA) gene, the *trn*L (UAA) gene. Potter *et al.* [10] reported the relationships in Rosaceae including *Spiraea* densiflora using matK and *trn*L-

*trn*F sequence data.

Genus *Spiraea* is very diverse with interspecific varieties, discriminated by differences in morphology. *Spiraea* has been various divided by different authors into subgenera, sections, series, and cycles [1]. This study represents a preliminary investigation of the phylogeny of the main genera of the *Spiraea* in Korea through the comparative analysis of cpDNA sequences. The aim of this study was to investigate the potential use of the *trn*L (UAA)-*trn*F (GAA) region to identify some Korean species and was compared them with *Spiraea* taxa which were reported in GenBank. These taxa will represent the foundation of an investigation on the potential to understand intraspecific genetic diversity in the world.

2. Materials and Methods

Many individuals of genus *Spiraea* were collected from large effective populations in Korea (**Table 1**) and identified according to Lee [11] and Kim and Sun [12]. Total genomic DNA was extracted from 0.5 - 1.0 g of fresh leaf material. *Stephanandra incise* (Thunb) Zabel of the closely related genus *Stephanandra* was included in the phylogenetic reconstruction as an outgroup.

DNA was extracted using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, USA) according to the manufacturer's protocol.

The *trn*L-*trn*F gene regions of genus *Spiraea* were am-

Table 1. The collection sites and sections of genus *Spiraea* in Korea.

Scientific name	Localities	Section
S. betulifolia Pallas	Sohol-eup, Pocheon-si, Gyeonggi-do	Chamaedryon Ser.
S. blumei D. Don.	Ibanseong-myeon, Jinju-si, Gyeongsangnam-do	Chamaedryon Ser.
S. cantoniensis D. Don.	Ibanseong-myeon, Jinju-si, Gyeongsangnam-do	Chamaedryon Ser.
S. chamaedryfolia var. *pilsa*	Sohol-eup, Pocheon-si, Gyeonggi-do	Chamaedryon Ser.
S. chartacea Nakai	Hongdo, Jindo-gun, Jeollanam-do	Chamaedryon Ser.
S. chinensis Maxim.	Dongdaemun-gu, Seoul	Chamaedryon Ser.
S. fritschiana Schneid	Mt. Nayeon, Pohang-si, Gyeongsangbuk-do	Calospira K. Koch.
S. japonica L.	Ibanseong-myeon, Jinju-si, Gyeongsangnam-do	Chamaedryon Ser.
S. media Schmidt	Mt. Seokgaejae, Samcheok-si, Gangwon-do	Pentactina
S. microgyna Nakai	Mt. Giri, Samjang-myeon, Sancheong-gun, Gyeongsangnam-do	Calospira K. Koch.
S. miyabei Koidz.	Sucheon-dong, Osan-si, Gyeonggi-do	Calospira K. Koch.
S. pseudo-crenata Nakai	Mt. Chiak, Panbu-myeon, Wonju-si, Gangwon-do	Chamaedryon Ser.
S. prunifolia for. simpliciflora Nakai	Ibanseong-myeon, Jinju-si, Gyeongsangnam-do	Chamaedryon Ser.
S. pubescens Turcz.	Mt. Gaya, Hapcheon-gun, Gyeongsangnam-do	Chamaedryon Ser.
S. salicifolia L.	Sohol-eup, Pocheon-si, Gyeonggi-do	Section Spiraria
S. thunbergii Sieb.	Sohol-eup, Pocheon-si, Gyeonggi-do	Chamaedryon Ser.
S. trichocarpa Nakai	Sucheon-dong, Osan-si, Gyeonggi-do	Chamaedryon Ser.

plified using the primer pair to acquire the respective entire region. The final PCR cocktail of 50 μl contained 38 μl water, 5 μl 10% buffer, 3 μl Mg^{+2}, 1 μl dNTPs, 0.25 μl Taq polymerase, and 0.5 μl of each primer. The amplifying reactions were run for 28 cycles of denaturing for 30 s at 95°C, primer annealing for 50 s at 57°C, and elongation for 2 min at 72°C.

PCR products were separated on 1.5% agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Chatsworth, CA) following the manufacture's protocols. The amplified fragments were cloned into a bluescript vector and sequenced using ABI Prism 377 Sequencer (Applied Biosystems Inc., Foster City, CA). At least ten individuals' clones of each taxon were analyzed. The sequencing data were analyzed and edited using the Sequencher software program (Gene Codes Corporation, Ann Arbor, MI, USA).

A pairwise alignment was calculated using the Clustal X program. Phylogenetic relationship were estimated by MEGA5 version 5.0 [13] treating all alignment gaps as missing. The quality of these alignments was assessed with homoplasy indicators [consistency index (CI) [14, 15], retention index (RI) [16], and rescaled consistency index (RC) [16]. The alignments were then checked, and apparent alignment errors were corrected by hand to minimize homoplasy.

Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood

value (lnL), and the number of parameters (including branch lengths) are also presented. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (R) are shown for each model, as well. Codon-based tests of neutrality for analysis between *trn*L-*trn*F sequences of genus *Spiraea* were conducted using the Nei-Gojobori method.

The DNA sequence data of the *trn*L (UAA)-*trn*F (GAA) region were obtained from two different sources. A first source is focusing on this study. All these sequences had been already deposited in GenBank. The second source is GenBank. The sequences of 27 accessions have been selected from previously published studies on *Spiraea* systematics (**Table 2**).

A maximum parsimony tree (MP) was inferred using heuristic search, branch-swapping options and tree bisection-reconnection using MEGA5 [13]. Confidence values for individual branches were determined by a bootstrap analysis with 100 repeated sampling of the data.

3. Results

Sequencings of *trn*L-*trn*F region for seventeen taxa of *Spiraea* in Korea were successful in all of the species. Aligned nucleotides of *trn*L-*trn*F were varied within *Spiraea* varying from 963 bp in *S. cantoniensis* to 975 bp

Table 2. Accession numbers and *trn*L-*trn*F sequences of genus *Spiraea* from GenBank.

Taxa	Distribution	Authors	No. of GenBank
S. alba var. *latifolia* (Aiton) Dippel	USA	Potter *et al.* (2007)	DQ897588
S. betulifolia Pall.	Korea, Japan, Russia	Richardson *et al.* (2000)	AJ390368
S. blumei G. Don.	China, Japan, Korea	Potter *et al.* (2007)	DQ897576
S. canescens D. Don.	Himalaya	Potter *et al.* (2007)	DQ897577
S. cantoniensis Lour.	China, Japan, Korea	Potter *et al.* (2007)	DQ897578
S. chamaedryfolia L.	China, Korea	Oh *et al.* (2010)	GU217793
S. crenata L.	Europe, Asia	Potter *et al.* (2007)	DQ897579
S. decumbens W. Koch.	Europe	Potter *et al.* (2007)	DQ897580
S. densiflora Nutt.	USA	Potter *et al.* (2007)	AF348571
S. douglasii Hook.	British Columbia, Canada	Potter *et al.* (2007)	DQ897581
S. formosana Hayata	Taiwan	Potter *et al.* (2007)	DQ897582
S. fritschiana Schneid.	China, Korea	Potter *et al.* (2007)	DQ897583
S. hypericifolia L.	Europe, Asia	Potter *et al.* (2007)	DQ897586
S. japonica L. f. 1	Japan	Potter *et al.* (2007)	DQ897585, DQ897616
S. lasiocarpa Kar. & Kir.	Russia	Potter *et al.* (2007)	DQ897587
S. miyabei Koidz.	China	Potter *et al.* (2007)	DQ897590
S. prunifolia Sieb. & Zucc.	China, Taiwan, Korea	Potter *et al.* (2007)	DQ897592, GQ428518
S. pubescens Turcz.	China, Korea	Potter *et al.* (2007)	DQ897593
S. salicifolia L.	Europe, Asia	Potter *et al.* (2007)	DQ897594
S. thunbergii Sieb.	Japan, Korea	Potter *et al.* (2007)	DQ897595
S. trichocarpa Nakai	Korea	Potter *et al.* (2007)	DQ897596
S. trilobata L.	Asia	Potter *et al.* (2007)	DQ897597
S. virginiana Brit.	USA	Potter *et al.* (2007)	DQ897599, DQ897600, DQ897601

Sequencings of *trn*L-*trn*F region for seventeen taxa of *Spiraea* in Korea were successful in all of the species. Aligned nucleotides of *trn*L-*trn*F were varied within *Spiraea* varying from 963 bp in *S. cantoniensis* to 975 bp in *S. chinensis*, *S. japonica*, and *S. miyabei* (**Table 3**). The mean nucleotide frequencies for seventeen taxa of genus *Spiraea* in Korea are A = 36.1%, C = 16.2%, G = 16.4%, and T = 31.3%. Total alignment length of genus *Spiraea* in Korea is 1004 positions, of which 126 are parsimony-informative, 149 variable, 26 singleton, and 847 constant characters. Number of segregating sites of genus *Spiraea* in Korea was 143 and nucleotide diversity (π) was 0.034. Under the neutral mutation hypothesis, the probability that D is negative (–1.030) is less than 0.5 (**Table 4**). The negative value of D was observed in the case of deletion/insertion. Thus, deletion/insertion increased the genetic variation. All *trn*L-*trn*F trees generated in Korea exhibited well solved topology with high bootstrap support irrespective of the methods (parsimony) and the setting used. *S. pubescens* and *S. media* were very distinct in MP tree (**Figure 1**). *S. fritschiana* was also very distinct. *S. microgyna* was also very distinct. This result confirmed monophyletic group for all species.

In 27 world accessions, 14 overlapped in Korean taxa and four taxa had doubled in the world itself. Thus, 26 taxa were true. Total alignment length in 27 world accessions is 1004 positions, of which 126 are parsimony-informative, 149 variable, 23 singleton, and 847 constant characters. The base composition did not showed a significant difference among total taxa. The estimated value of the shape parameter for the discrete Gamma Distribution is 1.7129. Substitution pattern and rates were estimated under the Tamura-Nei' model (+G). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+G]). Mean evolutionary rates in these categories were 0.22, 0.51, 0.82, 1.23, 2.22 substitutions per site. The nucleotide frequencies are A = 36.3%, T = 31.3%, C = 16.2%, and G = 16.2%. For estimating ML values, a user-specified topology was used. The maximum Log likelihood for this computation was –2063.296. The analysis involved 42 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 871 positions in the final dataset.

The seven parsimonious trees were recovered from the

Table 3. Base frequencies and total length across fourteen taxa of genus *Spiraea* in Korea using *trn*L-*trn*F analysis.

Taxa	Base (%)				Total (bp)
	T	C	A	G	
Korean taxa					
S. betulifolia	31.3	16.1	36.4	16.1	973.0
S. blumei	31.3	16.1	36.5	16.1	970.0
S. cantoniensis	31.3	16.2	36.2	16.3	963.0
S. chamaedryfolia var. *pilsa*	31.0	16.3	36.1	16.6	971.0
S. chartacea	31.3	16.1	36.5	16.2	971.0
S. chinensis	31.5	15.9	36.3	16.3	975.0
S. fritschiana	31.4	15.9	36.3	16.4	972.0
S. japonica	31.8	15.7	36.2	16.3	975.0
S. media	30.9	17.1	34.7	17.4	972.0
S. microgyna	31.3	16.0	36.3	16.3	974.0
S. miyabei	31.7	15.8	36.3	16.2	975.0
S. pseudo-crenata	31.2	16.0	36.6	16.1	973.0
S. prunifolia for. simpliciflora	31.4	15.9	36.7	16.0	968.0
S. pubescens	30.7	17.4	34.4	17.5	967.0
S. salicifolia	31.3	16.3	36.1	16.4	972.0
S. thunbergii	31.3	16.3	36.3	16.2	972.0
S. trichocarpa	31.3	16.0	36.3	16.3	973.0
Mean	31.3	16.2	36.1	16.4	971.5
World taxa					
S. alba var. *latifolia*	31.5	16.0	36.3	16.2	975.0
S. blumei	31.2	16.2	36.4	16.1	973.0
S. betulifolia	31.1	16.1	36.6	16.3	971.0
S. canescens	31.1	16.2	36.3	16.3	974.0
S. cantoniensis	31.3	16.3	36.1	16.3	968.0
S. chamaedryfolia	31.1	16.2	36.3	16.3	974.0
S. crenata	31.2	16.2	36.4	16.1	973.0
S. decumbens	31.3	16.1	36.5	16.1	968.0
S. densiflora	31.3	16.0	36.4	16.3	975.0
S. douglasii	31.4	16.1	36.2	16.2	974.0
S. formosana	31.5	16.0	36.2	16.2	971.0
S. fritschiana	31.5	16.0	36.2	16.3	975.0
S. hypericifolia	31.3	16.3	36.3	16.2	972.0
S. japonica (1)	31.7	15.9	36.2	16.3	976.0
S. japonica (2)	31.5	16.0	36.2	16.3	975.0
S. lasiocarpa	31.2	16.2	36.5	16.0	973.0
S. miyabei	31.6	16.0	36.1	16.3	975.0
S. prunifolia (1)	31.4	16.3	36.2	16.2	972.0
S. prunifolia (2)	31.2	16.4	36.2	16.3	965.0
S. pubescens	31.3	16.3	36.3	16.2	971.0
S. salicifolia	31.5	16.0	36.2	16.3	975.0
S. thunbergii	31.4	16.2	36.4	16.1	971.0
S. trichocarpa	31.3	16.1	36.5	16.2	971.0
S. trilobata	31.3	16.3	36.4	16.1	971.0
S. virginiana (1)	31.3	16.0	36.4	16.3	972.0
S. virginiana (2)	31.2	16.1	36.6	16.1	968.0
S. virginiana (3)	31.2	16.1	36.5	16.3	971.0
Mean	31.3	16.2	36.3	16.2	972.3

Table 4. Results from Tajima's neutrality test for *trn*L-*trn*F sequences of genus *Spiraea*.

Group	M	S	ps	Θ	Π	D
Korea taxa	17	143	0.153	0.045	0.034	−1.030
Foreign taxa	27	28	0.030	0.008	0.006	−0.635
Total	44	152	0.169	0.039	0.017	−2.049

M = number of sites, S = Number of segregating sites, ps = S/M, Θ = ps/a1, and π = nucleotide diversity. D is the Tajima test statistic.

parsimony analysis with a CI of 0.897, a RI of 0.908, and a RC of 0.814. The most parsimonious tree was recovered from the parsimony analysis with a length of 358 steps and all taxa were most fully resolved (**Figure 2**). All species (including varieties) formed three or four distinct clades with moderate bootstrap support (for convenience, only bootstrap values under parsimony criterion are presented here). All species including varieties in the Korean did not formed one clade. Only *S. japonica* formed one clade. Three accessions (two worlds and one Korean) of *S. japonica* and three *S. vergiana* were divided into different clades. Outgroup was omitted in phylogenetic tree because of the very long branch between genus *Spiraea* and outgroup.

4. Discussion

The internal structure of the genus *Spiraea* is by and large in discordances with other recent and more densely sampled DNA phylogenies of the Spiraeea, such as those of Porter *et al.* [1]. The sparse sampling of *Spiraea* taxa in the present study, together with a relatively high DNA sequence mutation rate, made alignment of *Spiraea* sequences a difficult task, and because of this difficulty, an insertion/deletion complex unique to the one *Spiraea* taxa had to be removed from the data matrix before analysis.

Although all taxa in Korea exhibited well solved topology with high bootstrap, phylogenetic reconstructions revealed notable differences in worldwide taxa (**Figure 2**). This discordance in trees for *Spiraea* may be explained with several possible explanations. First, it is a possibility of species-misidentification. An accurate identification of *Spiraea* species has always been problematic even for expert botanists. This is because of the contradictory classification systems proposed by various researchers, primarily based on morphological characters that could be highly variable depending on the environmental conditions. For example, *S. chamaedryfolia* var. *pilsa* has hairs in flowers. *S. chamaedryfolia* var. *pilsa* is separated from *S. chamaedryfolia* by this character. However ther are very similar to each other. *S. chamaedryfolia* var. *pilsa* are the varieties of *S. chamaedryfolia*. However, they are distinct from each other (**Figure 2**). Second, the evidence that hybridization events most
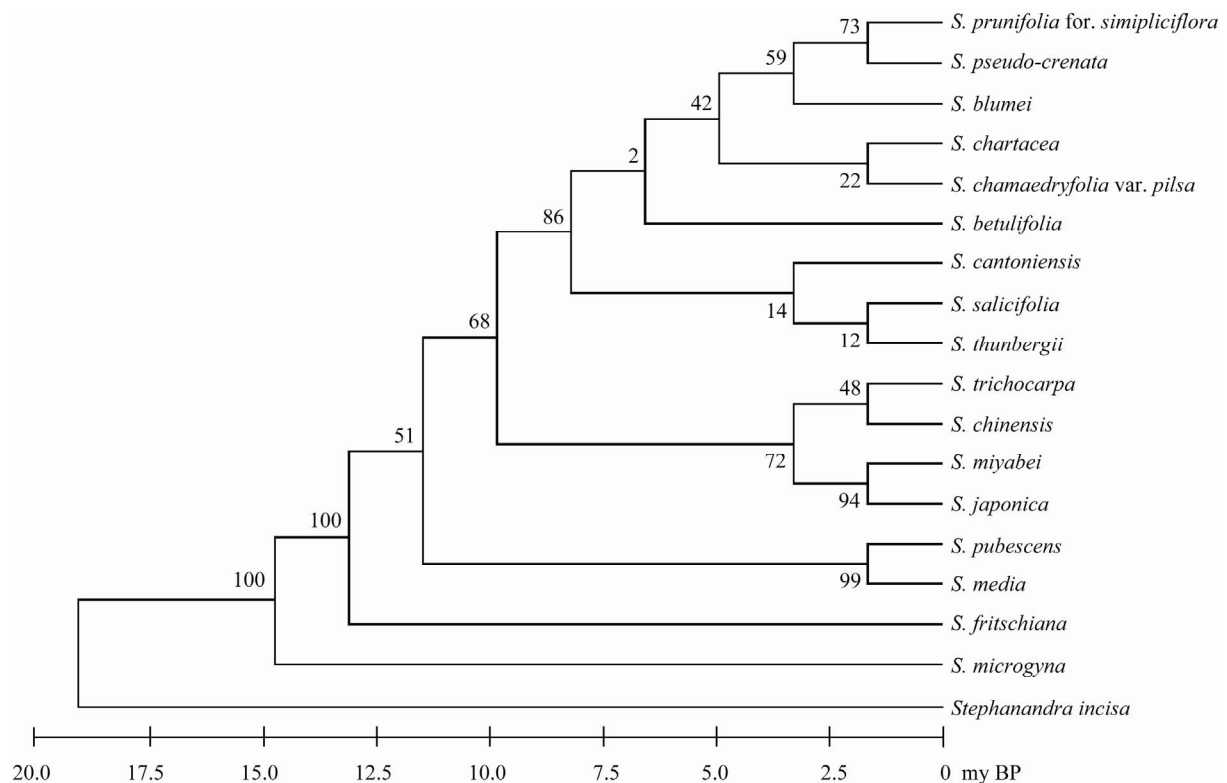

Figure 1. The maximum parsimonious tree for Korean taxa of genus *Spiraea* based on *trn*L-*trn*F using MEGA5. The values of bootstrap were shown in side of vertical lines.
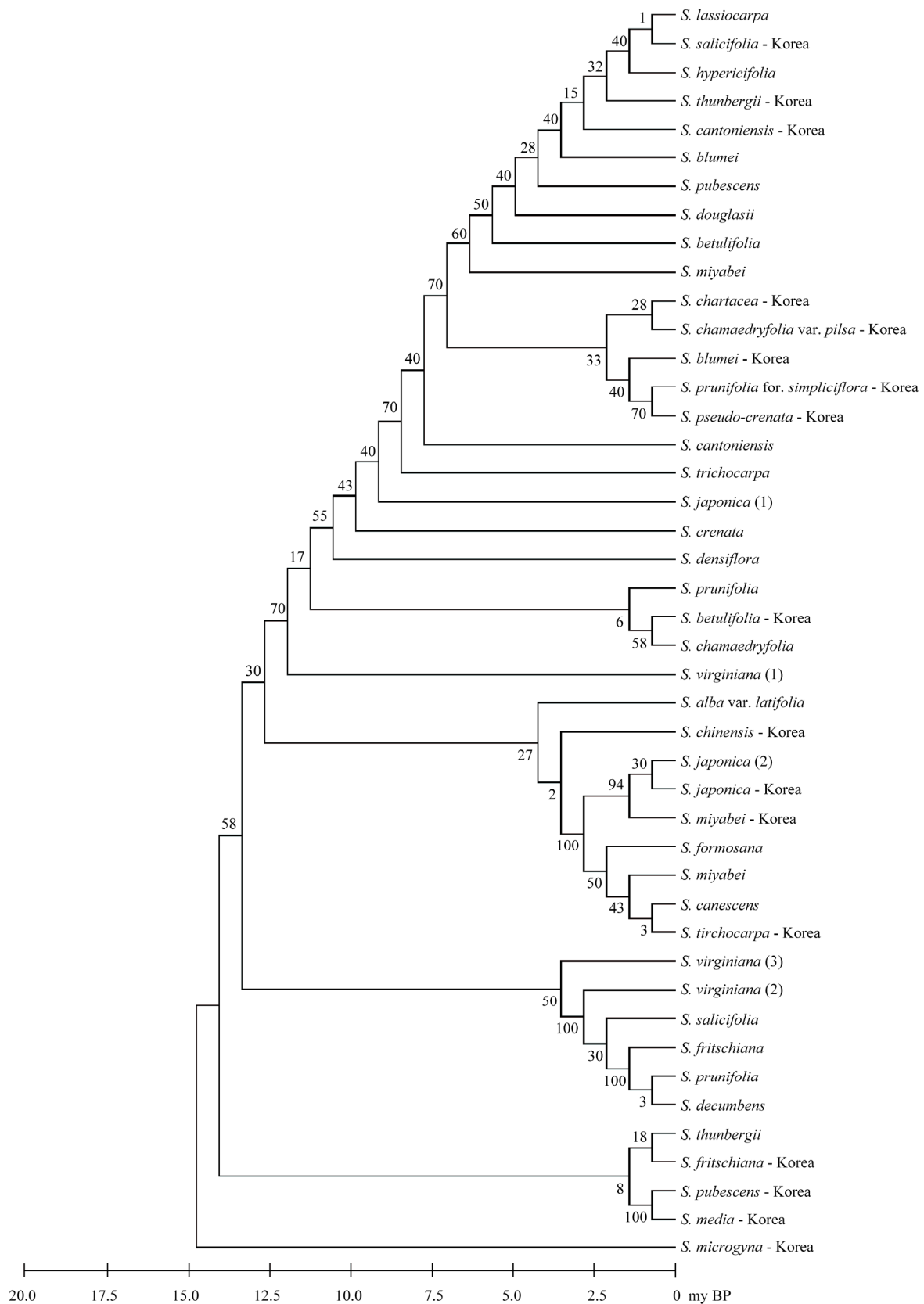
Figure 2. The maximum parsimonious tree for genus *Spiraea* in the worldwide based on *trn*L-*trn*F using MEGA5. The values of bootstrap were shown in side of vertical lines.

likely occurred in several places was provided by the presence in the introduced meadowsweet populations [17]. Although all varieties of *S. japonica* are not hybridized species, hybridization cannot be ruled out. Many varieties of *S. japonica* (var. *acuta*, var. *acuminata*, var. *fortune*, var. *glabra*, var. *japonica*, var. *incise*, var. *ovalifolia,* and var. *stellaris*) are occurred in same regions. Third, the methods and primers of *trn*L-*trn*F may be different ways according to authors. For example, the sequences of combined primer set (forward and reverse primers) are slightly different for each person or species. Some species were well amplified, but other species needed some modified primers for amplification. For one reason or another, fourteen overlapped taxa between Korea and other countries did not same clades for same species (**Figure 2**). In addition, several sequencies of *S. japonica* in GenBank were not accordance with each other.

Inflorescence type, the basis for the recognition of three sections within *Spiraea* [18] does not appear to be a reliable indicator of relationship within the genus (**Figure 2**), but some correlations were nonetheless observed. While compound corymbs, characteristic of section Calospira, are found in *S. fritschiana* var. *obtusifolia*, *S. fritschiana*, and *S. miyabei*. In this study, Calospira was sistered to section Chamaedryon (strongly supported by Bayesian analysis) in some of the most parsimonious trees.

The plants of genus *Spiraea* grow in forest-steppe, steppe, semi-dessert zones, and in subalpine belt of the mountains of the North Hemisphere [20]. This environmental heterogeneity could have resulted in more geographical barriers, higher selection pressures, greater isolation, and less gene flow between populations [18]. All these factors might explain the higher divergence and diversification among *S. japonica* varieties. It is a general principle that the higher the genetic divergence among phylogenetic lines, the earlier the many taxa diverged. This has been widely applied in the interpretation of biogeographical history using sequence data [19]. A possible explanation might be that different areas with different environments will vary in mutation accumulation or divergence maintenance and consequently differ in detectable evolutionary rate.

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