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Taxonomic status of three taxa of *Elsholtzia* (*E. hallasanensis, E. springia,* and *E. splendens* var. *fasciflora*) (Lamiaceae) based on molecular data

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ABSTRACT: *Elsholtzia hallasanensis, E. springia,* and *E. splendens* var. *fasciflora* (Lamiaceae) were reported recently as new species or new varieties of *E. splendens* according to their morphological characteristics. To reappraise the taxonomic status of these additional taxa and to determine the relationships between all Korean *Elsholtzia* taxa except *E. saxatilis*, which is distributed in North Korea, molecular studies based on the nrDNA (ITS) and cpDNA (*rpl16,* and *trnH-psbA*) sequences of seven taxa of *Elsholtzia* and one outgroup were carried out. The molecular data support that *E. angustifolia* and *E. minima* are distinct species from *E. splendens* and *E. ciliata*, respectively, because they have several private marker genes and show monophyly. The molecular data also support that *E. splendens* has a very close taxonomic relationship with both *E. hallasanensis* and *E. springia.* We found that *E. splendens* var. *fasciflora*, with multiple inflorescence, was based on several private marker genes and on the monophyly of its trees, suggesting that it can be considered as a variety. *Elsholtzia springia*, with the same sequences and the same morphological characteristics with *E. hallasanensis* after transplanting, should be treated as a synonym of *E. hallasanensis*. Moreover, we consider the taxonomic status of *E. hallasanensis* as *E. splendens* var. *hallasanensis* (Y. Lee) N.S. Lee & C.S. Lee, stat. nov.

Keywords: *E. hallasanensis, E. springia, E. splendens* var. *fasciflora,* Lamiaceae, ITS, cpDNA (*rpl16* and *trnH-psbA*)

The genus *Elsholtzia* Wild. (Lamiaceae, tribe Elsholtzieae) consists of approximately forty species that are mainly distributed in temperate regions of the Northern Hemisphere and especially in China, also including Europe and Russia and North Amica (Li and Hedge, 1994). The generic plants are valuable for medicinal purposes and are characterized by opposite leaves, verticillastrate inflorescences, often compact, sometimes in panicles, linear to ovate bracts, campanulate or cylindrical calyx, and nutlet fruit (Li and Hedge, 1994; Jang et al., 2010). The characters used to delimit taxa within *Elsholtzia* are primarily those of a bract shape, pubescent on the abaxial surface, corolla in color, and spike shaped (Jeon and Hong, 2006; Lee et al., 2010).

However, *Elsholtzia* taxa distributed in Korea are controversial due to their taxonomic status and scientific name. In addition, several intraspecific taxa have been described. *Elsholtzia angustifolia* and *E. minima* has been considered as synonyms of *E. splendens* and *E. ciliata,* respectively (Li and Hedge, 1994). *Elsholtzia splendens* f. *alba* and *E. splendens* f. *rosea* were described by Lee (2000). The two formae have characteristics identical to that of *E. splendens* except for only the flower color, which can be white or pink. Several *E. splendens*, collected in Manchuria, Mt. Kumgang and Andong, have been reported as *Elsholtzia splendens* var. *miyasiroana* Kitag (Kitagawa, 1959). These are distinguished from *E. splendens* var. *splendens* by the only leaf characteristics, with narrow to wide lanceolate leaves, an elongate-acuminate leaf apex, and a leaf width of 15 mm. Later, the taxonomic status of *E. splendens* var. *miyasiroana* was moved down to a forma, *E. splendens* f. *miyasiroana* (Kitag.) Y. C. Zhu (Zhu, 1989). Recently, *E. hallasanensis* was described as a new species due to its morphological characteristics, such as its ovate leaf and numerous branches from base of stem, as well as its undivided lower lip (Lee, 2000). However, Jeon and Hong (2006) treated *E. hallasanensis* as a synonym of *E.*

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splendens; it is distributed in sunny areas of Korea and Manchuria. *Elsholtzia springia* Y. Lee, which is similar to *E. hallasanensis*, was also described as a new species according to its morphological characteristics, including one straight and very tiny stem and due to the fact that its flowers bloom very early in the spring (Lee, 2007). Another new variety of *E. splendens* having fascicled inflorescence was also described as *E. splendens* var. *fasciflora* based only on the morphological characteristics (Lee et al., 2010).

Nuclear nrDNA ITS sequences have been used in the analysis of angiosperms (Baldwin et al., 1995) and in the analysis of the evolutionary rate for lower level (genus level and below) phylogeny as well as in advanced phylogenetic studies (Huang et al., 2005; Lee and Downie, 2006). Ribosomal internal transcribed spacer (ITS) sequences (including ITS1, ITS2 and 5.8S rRNA sequences) have conserved lengths and a high degree of variability. They are well suited for generic classification in Lamiaceae studies (Steane et al., 1999; Jamzad et al., 2003).

The documentation of intraspecific variation in cpDNA has become increasingly common (Wagner et al., 1987; Soltis et al., 1991, 1992). It is important to consider both nuclear and chloroplast data as well as data from markers because any significant discordance in the relationships between data sets may serve to identify past hybridization or introgression events (Doyle 1992; Rieseberg and Brunsfeld 1992; Soltis and Kuzoff 1995).

Therefore, the objective of this study is to reappraise the taxonomic status of these additional new taxa based nuclear nrDNA (ITS) and cpDNA (*rpl16* and *trnH-psbA*) sequences analyses.

Materials and Methods

The sources of plant materials for the DNA analysis and the GenBank accession numbers are listed in Table 1. All voucher specimens were deposited into the Ewha Womans University Herbarium (EWH). Leaf materials for DNA extraction were obtained from natural populations in Korea. Thirty four accessions from the *Elsholtzia* taxa with an outgroup, *Collinsonia canadensis* (Wagstaff et al., 1995), were analyzed for the ITS of nrDNA, and *trnH-psbA* and *rpl16* of cpDNA.

Total genomic DNA was extracted using the DNeasy plant mini kit (Qiagen Inc., Valencia, California). Primers used for amplification and sequencing were ITS4, ITS5, ITS2 and ITS3 for the ITS region (White et al. 1990); trnHR and psbA for the *trnH-psbA* intergenic spacer (Sang et al. 1997); and rpl16F71 and rpl16R1516 for the *rpl16* (UAA) intron region (Small et al.

Table 1. Materials of *Elsholtzia* included in the molecular analyses. Voucher specimens were deposited in the herbarium of Ewha Womans University (EWH) and sequences were submitted to the GenBank sequence.

Taxa (Abbreviation)	DNA accessions	Voucher specimens	GenBank accession number
			ITS / *rpl16* / *trnH - psbA*
E. angustifolia	6, 101-2	GB, Munkyeong, Joryeong, *Y. Kim 0410021-3*	JN578052-4 / JN578018-20 / JN577984-6
	59-60	GN, Tongyeong, Mireuksan, *C. Lee 0911030-1*	JN578055-6 / JN578021-22 / JN577987-8
E. ciliata	1, 9	GW, Taebaeksan, *Y. Kim 0410031-2*	JN578057, 59 / JN578023, 25 / JN577989, 91
	2	GW, Samcheok, Odujae, *Y. Kim 0410025*	JN578058 / JN578024 / JN577990
	76	GW, Chuncheon, Obongsan, *K. Hwang 0410011*	JN578062 / JN578028 / JN577994
	112, 115	GG, Gwangju, *Y. Kim 0712001-2*	JN578060-1 / JN578026-7 / JN577992-3
E. hallasanensis	103-4	JJ, Hallasan, *K. Lee & Y. Kim 0410038-9*	JN578063-4 / JN578029-30 / JN577995-6
E. minima	5, 11, 48-9	JJ, Hallasan, *N. Lee et al. 0410021-4*	JN578069-72 / JN578035-8 / JN578001-4
E. splendens var. *splendens*	8	GW, Samcheok, Baekbyeongsan, *Y. Kim 0410011*	JN578073 / JN578039 / JN578005
	24	GB, Munkyeong, Joryeong, *Y. Kim 0410029*	JN578074 / JN578040 / JN578006
	19, 26, 39	CB, Sobaeksan, *N. Lee et al. 0909026-8*	JN578075-7/ JN578041-3 / JN578007-9
	62-3	JJ, Seogwipo-si, Jungmundong, *C. Lee 091002-3*	JN578078, 80 / JN578044-5 / JN578010-1
	77, 79	GG, Seoul, Dobongsan, *Y. Kim 041042-3*	JN578079, 81 / JN578046-7 / JN578012-3
E. splendens var. *fasciflora*	66-7	JJ, Seogwipo-si, Daepodong, *M. Chung & Y. Chung 1011010-1*	JN578065-6 / JN578031-2 / JN577997-8
	74, 75	JJ, Seogwipo-si, Jungmundong, *C. Lee 101004-5*	JN578067-8 / JN578033-4 / JN577999-8000
E. springia	64	JJ, Seogwipo-si, Doneorioreum, *M. Chung 1004017*	JN578084 / JN578050 / JN578016
	65	JJ, Seogwipo-si, Doneorioreum, *N. Lee et al. 1005011*	JN578083 / JN578049 / JN578015
	106	JJ, Seogwipo-si, Doneorioreum, *K. Lee & Y. Kim 0807017*	JN578082 / JN578048 / JN578014
Collinsonia canadensis		USA, West Virginia, 10 Oct. 2004, *M. Roh*	JN578085 / JN578051 / JN578017

1998). Details of the amplification reaction, purification, and alignment methods are identical to those described in Lee et al. (2011).

Two data matrices (the ITS data set and the cpDNA data set; a total 34 accessions representing 7 taxa of *Elsholtzia* and the outgroup), were initially analyzed using an equally weighted, unordered maximum parsimony (MP) approach (Fitch, 1971) implemented in PAUP version 4.0 (Swofford, 2002). Heuristic MP searches were replicated with 1000 random stepwise additions of taxa, TBR branch swapping, and while saving multiple trees. Bootstrap values (Felsenstein, 1985) were calculated from 100 replicate analyses using TBR branch swapping and the random stepwise addition of taxa. The ITS data were analyzed as separate ITS-1, 5.8S and ITS-2 partitions; they were also combined. To examine the extent of conflict between the ITS and cpDNA matrices for a comparable set of taxa, the incongruence length difference (ILD) test of Farris et al. (1995) was implemented using the partition-homogeneity test of PAUP by a heuristic search with 100 replicates and 10 simple additions. All regions were combined with no clear conflict between the nuclear and the plastid regions as examined by the ILD test (P value = 0.41).

Bayesian inference values of the ITS and cpDNA data sets were implemented using MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Likelihood parameters for the Bayesian analysis were calculated using MrModeltest 2.3 (Nylander 2004). The "GTR" model was chosen for ITS, and the "GTR+I" model was selected for cpDNA and ITS + cpDNA under the Hierarchical Likelihood Ratio Test. The Bayesian Markov chain Monte Carlo (MCMC) algorithm was run for 1,000,000 generations with four simultaneous chains (one "cold" and tree "heated"), starting from random trees while sampling every 100 generations. We discarded as burn-in the first 25% of the total number of generations. We generated a 50% majority-rule consensus tree from the remaining trees, in which the percentage of nodes recovered represented their posterior probability (PP). Because the recovered tree topologies resulting from the Bayesian analysis were essentially the same as those resolved using MP, only the MP tree (strict consensus) with nodal support, indicated by both BS and PP values, are presented here.

We also conducted a maximum likelihood (ML) analysis, and the best-fit model was selected based on the likelihood ratio test (Goldman 1993; Whelan and Goldman 2001) implemented in ModelTest 3.7 (Posada and Crandall 1998). Model parameters were imported into PUAP*, and a heuristic search (with the asis addition sequence, TBR branch swapping, and MULPARS option on) was executed.

Results

Nucleotide ITS analysis

Alignment of all 34 complete ITS 1, 5.8S and ITS 2 sequences, representing all possible accessions of the genus *Elsholtzia* and the outgroup, resulted in a matrix of 667 characters. The length of the ITS1 region is 220-222 bp, the length of 5.8S is 172 bps, and the length of the ITS-2 region is 254 to 273 bps. Of the 667 initial alignment positions, 537 sites (80.5%) were identical, 54 sites (8.1%) were parsimony uninformative, and 76 sites (11.4%) were phylogenetically informative (Table 2). Six gaps (indels), ranging from 1 to 8 bps in size, were required to facilitate alignment of all 34 sequences. Pairwise sequence divergence values across the entire ITS region ranged from identical to 11.2% of nucleotides for *Elsholtzia* only. Maximum intraspecific sequence divergence values for *E. angustifolia* (5 accessions), *E. hallasanensis* (2 accessions), *E. minima* (4 accessions), *E. splendens* var. *splendens* (9 accessions), *E. splendens* var. *fasciflora* (4 accessions), and *E. springia* (3 accessions) were all 0% except the 0.4% reading for *E. ciliata* (6 accessions). *Elsholtzia splendens* var. *splendens*, *E. hallasanensis*, *E. splendens* var. *fasciflora*, and *E. springia* had the same ribotype, and the maximum

Table 2. Sequence characteristics of the nuclear rDNA internal transcribed spacers and chloroplast DNA (*trnH-psbA* and *rpl16*), separately and combined, for *Elsholtzia* taxa in Korea and one outgroup.

Characteristic	ITS	*trnH-psbA*	rpl16	Combined cp DNA (contained revision gaps)
Length variation (bp)	651-667	417-440	822-838	1,243-1,257
No. of aligned positions	667	452	844	1,296
No. of constant positions	537 (80.5%)	406	773	1,191
No. of informative positions	76 (11.4%)	26	31	56
No. of variable positions	54 (8.1%)	20	40	49
No. of indels	6	15	16	31
Maximum pairwise sequence divergence within *Elsholtzia*	11.21	5.81	3.16	4.13

divergence values between *E. splendens* var *splendens* and *E. hallasanensis, E. splendens* var. *fasciflora* and *E. springia* were all 0%. Maximum divergence values between these three taxa and *E. angustifolia*, *E. ciliata*, and *E. minima* were 1.69%, 10.59%, and 6.14%, respectively, while the maximum divergence value between *E. minima* and *E. ciliata* was 9.83%.

A maximum parsimony (MP) analysis of all ITS sequences found one equally most parsimonious tree with a tree length (TL) of 143, a consistency index (CI) of 0.9650 (0.9438 excluding uninformative characters), and a retention index (RI) of 0.9878. Bootstrap values for nodes resolved in the strict consensus tree ranged from 58% to 100% (average 84.6%).

The phylogenies estimated using MP (Fig. 1), ML, and Bayesian methods were consistent with one another. All trees strongly supported a monophyly of *Elsholtzia* comprising four major clades: *E. hallasanensis* - *E. splendens* var *splendens* - *E. splendens* var. *fasciflora* - *E. springia* clade, *E. angustifolia* clade, *E. ciliata* clade and *E. minima* clade, with high bootstrap support (BS) and posterior probability (PP). The ModelTest selected "GTR+I" as the best-fit model for the ITS data set, and a subsequent ML analysis found one tree (–lnL = 1566.4115) which was very similar to the strict consensus tree of the MP and Bayesian trees.

Three noncoding Chloroplast DNA analyses and a combined ITS and cpDNA analysis

The region bounded by chloroplast genes examined ranged from 1,243 bp in *E. ciliata* to 2,157 bp in *E. minima.* Of these chloroplast genes, the *trnH-psbA* intergenic spacer was phylogenetically the most informative, at 26 sites (5.7%). Of these chloroplast genes, the *rpl16* intron was phylogenetically the most informative, at 31 sites (3.7%). The multiple alignment of these sequences resulted in a matrix of 1,296 positions. Of the aligned positions, 1,191 (91.8%) were constant, 49 (3.8%) were parsimony uninformative, and 56 (4.3%) were phylogenetically informative. Twenty three (17 in *Elsholtzia*) of 31 (25 in *Elsholtzia*)

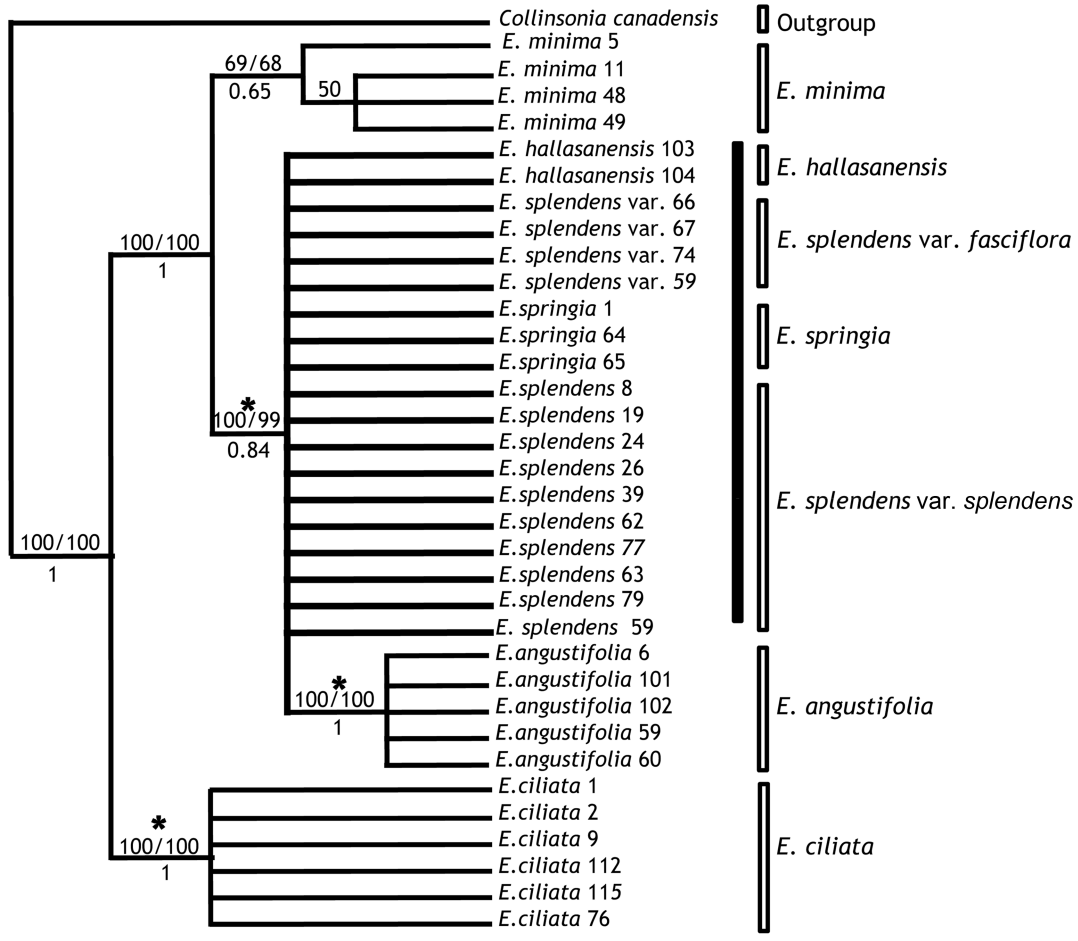

Fig. 1. Strict consensus tree of equally most parsimonious trees based on ITS sequences (TL = 143; CI = 0.9650; RI = 0.9878), bootstrap values above 50% are shown above the branches, and the numbers below branches represent the number of substitutions.

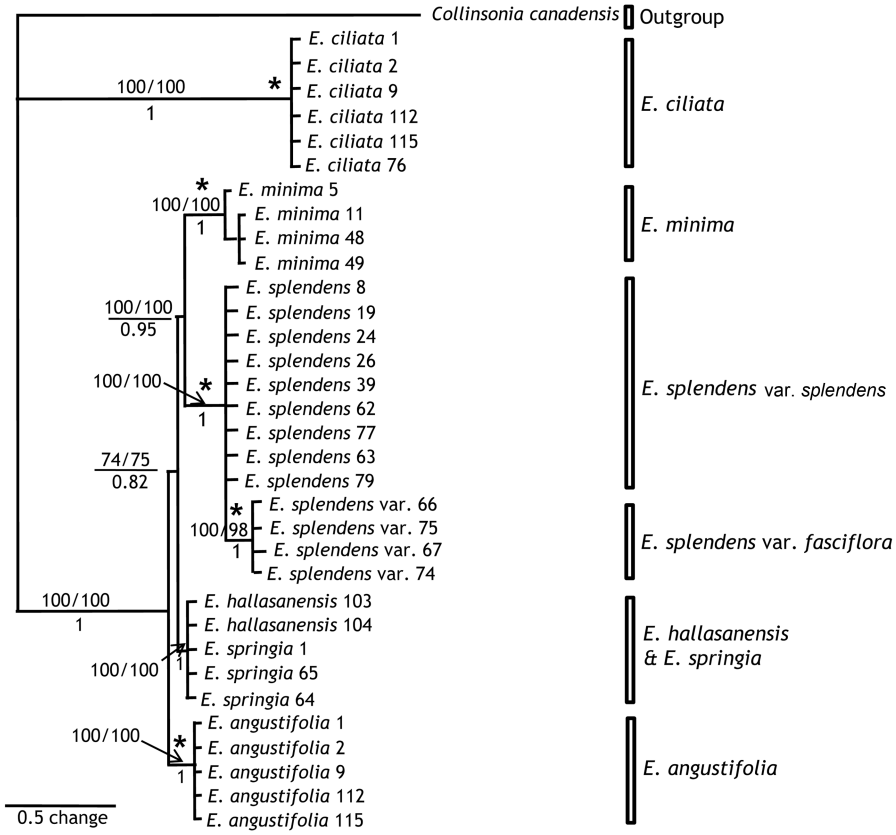

Fig. 2. Strict consensus tree derived from a MrBayesian analysis of Korean *Elsholtzia* species ITS and three noncoding cpDNA sequences (−lnL = 4212.3296).

gaps were potentially informative (Table 2). These 31 informative gaps ranged from 1 to 8 bps in size, with insertions outnumbering deletions by 9 : 17.

Eleven gaps were unique to *Elsholtzia*, and nine additional gaps were restricted to the outgroup, *Collinsonia canadensis*. The largest gap of 7 bps represented a deletion in the *rpl16* intron region in all accessions of *Elsholtzia* examined.

Elsholtzia hallasanensis and *E. springia* had the same haplotypes

Table 3. Important Informative nucleotide sites in ITS and cpDNA (*rpl16*: 09-730; *trnH-psbA*: 888-1,259) to infer the taxonomic status of *Elsholtzia* taxa in Korea.

	ITS	cpDNA
	00000000000000000000	00000000000000000000000111111111
Taxa/positions	00000000011122445 6	00111123344555567889901111122
	22355555891480106 21	06246740179014543892810456855
	09915199806040815 7	94226091357171160812751982009
E. ciliata	**GAT**GG**GCCCGT**CG**CACTC**	**AATC**AAA**−TT−−A−AATCCGGAA**CAA**ATA**
E. minima	ACCGGTT**T**TAACGT-TAT	G**GAT**G**A**GTC−−−G−GAGTCCTGGCAA−−G
E. hallasanensis	ACCGGTTCTAACGT-TAT	GAATA**G**GTC−−−G−GAGTCCTGGCAA−−**G**
E. springa	ACCGGTTCTAACGT-TAT	GAATA**G**GTC−−−G−GAGTCCTGGCAA−−**G**
E. splendens	ACCGGTTCTAACGT-TAT	GAATAAGTC−−−G−GCGTCCTGG**TTT**−−**G**
E. splendens* var. *fasciflora	ACCGGTTCTAACGT-TAT	GAATAAGTC−−−G**T**GCGT**G**CTGG**TTT**−−**G**
E. angustifolia	ACC**AA**TTCTAA**TA**T-TAT	GAATAAGTC−**AT**G−GAGTCCTGGCAA−−**G**

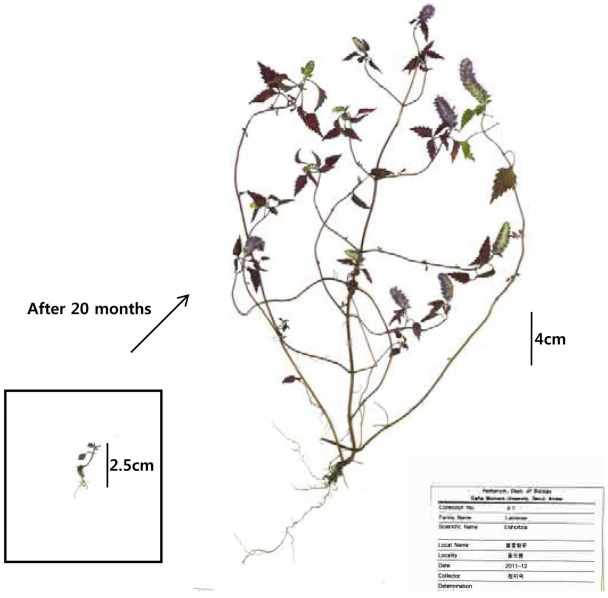

Fig. 3. Growing up shape of *Elsholtzia springia* after 20 months transplanted from Doneori-oreum in Jeju Island to a garden in the lowlands.

and shared one identical specific character state with *E. springia* at position 170 (G). *Elsholtzia splendens* var. *splendens* and *E. splendens* var. *fasciflora* shared seven specific character states at positions 646 (C), 1149 (T) and 1158-1162 (TCTAT). Moreover, *E. splendens* var. *fasciflora* possesses five specific character states within 8 accessions at positions 541 (T) and 891 (G) (Table 3). Pairwise sequence divergence values across the entire cpDNA region ranged from identical to 4.13% of nucleotides for *Elsholtzia* only. The maximum intraspecific sequence divergence values for *E. angustifolia* (5 accessions), *E. ciliata* (25 accessions), *E. hallasanensis* (8 accessions), *E. minima* (6 accessions), and *E. springia* (3 accessions) were 0%, except for *E. splendens* var. *splendens* (9 accessions) and *E. splendens* var. *fasciflora* (4 accessions) which showed 0.24% and 0.23%, respectively. The maximum divergence values between *E. splendens* var. *splendens,* and *E. angustifolia, E. ciliata, E. hallasanensis*, *E. minima, E. splendens* var. *fasciflora,* and *E. springia* were 1.28%, 3.97%, 1.12%, 1.36%, 0.56, and 1.12%, respectively.

The MP analysis found 34,000 equally most parsimonious trees with a TL value of 115, a CI value of 0.9565 (0.9242 excluding uninformative characters), and a RI value of 0.9868. The MP trees were well resolved and the branch was collapsed in the strict consensus tree. For the ML analysis, the ModelTest selected "HKY + G" as the best-fit model for the combined cpDNA sequences. A subsequent analysis found one tree (–lnL = 2371.9375). This ML tree was similar to the MP tree.

A strict consensus Bayesian tree of the combined ITS and three noncoding cpDNA sequences (–lnL = 4212.3296) was found to be similar to the strict consensus tree of cpDNA. *Elsholtzia hallasanensis* and *E. springia* were homoplastic and synapomorphic, as they were branched from a single clade with a prior probability value (PP) of 1.0, whereas *E. splendens* var. *fasciflora* and *E. hallasanensis* were monophyletic, coming from the *E. splendens* var. *splendens* clade with a PP of 1.00 (Fig. 2). On the other hand, *E. splendens* var. *fasciflora* was monophyletic, coming from the *E. splendens* var. *splendens* clade with a PP of 1.00 (Fig. 2). *E. hallasanensis* with *E. springia* was grouped with *E. splendens* and *E. minima* as the same clade with a PP of 1.00 (Fig. 2), although *E. minima* was grouped as another clade separately in the ITS tree with a PP of 1.00 (Fig. 1). Thus, they were grouped with *E. angustifolia*, and *E. ciliata* was formed as a sister group of this group with the same result of the ITS trees (Fig. 1, 2). This Bayesian tree was very similar to the MP and ML trees.

Discussion

The objective of this study was to reappraise the taxonomic status of these additional taxa and to ascertain the relationships between all Korean *Elsholtzia* taxa. We carried out this task based on nrDNA ITS and cpDNA sequences. Our comprehensive analysis of the ITS and cpDNA sequences in this study allowed us to meet our objectives effectively.

Elsholtzia angustifolia and *E. minima* were treated as synonyms of *E. splendens* and *E. ciliata,* respectively (Li and Hedge, 1994). The ITS and cpDNA analysis, however, suggests that *E. angustifolia* and *E. minima* are distinct species from *E. splendens* and *E. ciliata*, respectively, as they have several private marker genes, specifically 51 (A), 55 (A), 146 (T) and 180(A) for ITS along with 490 (A) and 501 (T) for cpDNA in *E. angustifolia*, and 89(T) for ITS along with 64 (G) and 166 (G) for cpDNA in *E. minima* (Table 3). Additionally, *E. angustifolia* and *E. minima* showed monophyly with a PP of 1.0 in the combined ITS and cpDNA phylogenetic trees (Fig. 2). They also showed monophyly.

Elsholtzia hallasanensis and *E. springia* have the same ITS ribotypes and cpDNA haplotype. This molecular data is consistent with the morphological feature in which *E. springia* has the same morphological characteristics as *E. hallasanensis* 20 months after it was transplanted from the Doneorioreum on Jeju Island (Fig. 3), These results support that *E. springia* Y. Lee, which was described as a new species by only its morphological characteristics (Lee, 2007), should also be treated as a synonym of *E. hallasanensis*.

Elsholtzia hallasanensis reported as a new species due to its

morphological characteristics (Lee, 2000), but Jeon and Hong (2006) treated *E. hallasanensis* as a synonym of *E. splendens.* Our molecular data support that *E. hallasanensis* has very close taxonomic relationships with *E. splendens* as well as *E. springia*, because it has the same sequence and forms the same clade in the ITS. It, however, has one specific identical character state at position 170 (G). It is also a sister of the *E. splendens* clade in terms of the combined cpDNA. This molecular result supports that the taxonomic status of *E. hallasanensis* should be positioned down to a variety of *E. splendens*. In addition, *E. splendens* var. *fasciflora* was noted as a new variety by distinguishing it from the typical form of *E. splendens* based on the leaf length, the first leaf number beneath the inflorescence, the inflorescence number and shape, and the involucres shape (Lee et al., 2010). This supports the contention that *E. splendens* var. *fasciflora* can be considered as a variety of *E. splendens* based on several private marker genes and on the monophyly of the trees.

Taxonomic Treatment

We treated the taxonomic status of *E. splendens* var. *hallasanensis* (Y. Lee) N.S. Lee & C.S. Lee, stat. nov. (= synonym *Elsholtzia springia* Y. Lee).

Elsholtzia splendens var. *hallasanensis* (Y. Lee) N.S. Lee & C.S. Lee, stat. nov.

It differs from *E. splendens* var. *splendens* according to the morphological characteristics of the ovate leaf, round involucres, smaller plant height and the numerous branches from the base of the stem.

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