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Original Research Article

Phylogenetic Relationships in Korean *Elaeagnus* L. Based on nrDNA ITS Sequences

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Abstract - Molecular phylogenetic analyses of Korean *Elaeagnus* L. were conducted using seven species, one variety, one forma and four outgroups to evaluate their relationships and phylogeny. The sequences of internal transcribed spacer regions in nuclear ribosomal DNA were employed to construct phylogenetic relationships using maximum parsimony (MP) and Bayesian analysis. Molecular phylogenetic analysis revealed that Korean *Elaeagnus* was a polyphyly. *E. umbellata* var. *coreana* formed a subclade with *E. umbellata.* Additionally, the genetic difference between *E. submacrophylla* and *E. macrophylla* was very low. Moreover, *E. submacrophylla* formed a branch from *E. macrophylla,* indicating that *E. submacrophylla* can be regarded as a variety. However, several populations of this species were not clustered as a single clade; therefore, further study should be conducted using other molecular markers. Although *E. glabra* f. *oxyphylla* was distinct in morphological characters of leaf shape with *E. glabra.* But *E. glabra* f. *oxyphylla* was formed one clade by molecular phylogenetic with *E. glabra.* Additionally, this study clearly demonstrated that *E. pungens* occurs in Korea, although it was previously reported near South Korea in Japan and China. According to the results of ITS regions analyses, it showed a resolution and to verify the relationship between interspecies of Korean *Elaeagnus.*

Key words - *Elaeagnus*, Elaeagnaceae, ITS, Molecular phylogeny

Introduction

Elaeagnus L. is a member of the family Elaeagnaceae with about 60 species distributed in Asia, Australia, southern Europe and North America (Heywood *et al*., 2007). The genus *Elaeagnus* is characterized by alternate leaves, most parts with silvery white or brown scales, tubular calyx, 4-lobed stamens equal in number and alternating with the sepals and fruit usually containing eight longitudinal ribs. According to the Angiosperm Phylogeny Group (APG) system, *Elaeagnus* belongs to the order Rosales, Eurosids. The initial taxonomic research on *Elaeagnus* L. was conducted by Linnaeus (1753), who established the genus, and Schlechtendal (1857, 1860) and Servettaz (1908), who conducted taxonomic research on all species of *Elaeagnus* L. in the world. Schlechtendal (1860) recognized 28 species in the genus and Servettaz (1908) reported 44 species through monographs. Servettaz also divided *Elaeagnus* L. into two sections, *Sempervirentes* of evergreen group and *Deciduae* of deciduous group, which were supported by Zofu (1965) and Chang (1983). Britton and Brown (1913) designated *Elaeagnus angustifolia* as the type species of *Elaeagnus* L. Sun *et al*. (2010) eliminated the section name *Deciduae* by Art. 22.1 of the International Code of Nomenclature for algae, fungi and plants (ICN) (Melbourne Code) (McNeill *et al*., 2012) and used the autonym, *Elaeagnus*. To date, 60 species have been reported, including several species along with variants (Cronquist, 1981; Ohwi, 1984; Takhtajan, 1991; Lee, 1996).

Regarding Korean *Elaeagnus* L. was first conducted by Hemsley (1894), who recorded two species, *E. macrophylla* and *E. umbellata*, from the Korean peninsula. Since then, *Elaeagnus L.* has been reported by Palibin (1900), Servettaz (1909), Nakai (1911), Mori (1921), Nakai (1952) and Chang (1983). Meanwhile, Lee (1980) introduced six species and four varieties of Korean native species, and as well as one exotic species, *E. multiflora*, from Japan. Lee (1996) subsequently excluded *E. umbrellata* var. *parvifolia* and *E. umbrellata* var. *longicarpa*, and substituted them with *E. glabra* var.

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oxyphylla to *E. glabra* f. *oxyphylla* by recognizing seven species, one variety and one forma. Later, Lee (2007) changed this to six species and two varieties.

However, there are many problems in hybrid species. Previous studies have shown different results assuming putative parants of hybrids. For *E. submacrophylla*, it is a hybrid of *E. pungens* × *E. macrophylla* by Servettaz (1909), but it has been reported hybrids *E. glabra* × *E. macrophylla* in Korea by Lee (1996). In addition, *E. maritima* is divided in view that Ohwi (1984), Ohba (2003) and Lee (2007) regarded hybrid of species *E. glabra* × *E. macrophylla*, but Lee (1996) was referred to as intermediate type of head or hybrid species. Yoo *et al*. (1994) also investigated the distribution and characteristics of soil underlying the habitat of Korean *Elaeagnus*, while Ki (2004) conducted research on the *Elaeagnus* trichome, Koh (2005) conducted a taxonomic study, and Lee (2006) investigated the external morphology.

In the recent years, many studies have been undertaken on this plant, concentrating on it is agricultural and ornamental values. Fruits and other parts of the plant bodies are consumed for their nutritional values in Asia (Wang *et al*., 2006; Fu and Wang, 2007). Additionally, many species are commonly used in traditional medicine to treat symptoms such as cough, diarrhea, itching and foul sores. Phytochemical studies have shown various medicinal properties of this genus, including antioxidant and anti-tumor (Kim *et al*., 2007; Lee *et al*., 2011; Lee and Seo, 2011).

Despite the abundance of research on *Elaeagnus* L., there is still a need to record species with accuracy owing to the differing opinions among researchers regarding species and variety, and difficulty in identifying species caused by high variation within Korean taxonomic groups. In the present paper, the main objectives were to phylogenetic relationships among Korean *Elaeagnus*, to assess the amount of congruence between the inferred relationships and the existing classification. We used internal transcribed spacer (ITS) region of nuclear DNA because they have been phylogenetically useful in most groups of flowering plants at generic and infrageneric levels (Baldwin, 1992; Yoo and Park, 2012). The main objectives of this study include (1) construction of the phylogenetic relationship of Korean *Elaeagnus* based on ITS sequences; (2) testing the hypothesis of the hybrid origin in *E. submacrophylla* and *E. maritima.* This information should contribute to developing a reasonable classification system and to a better understanding of the evolution of *Elaeagnus L.*

Materials and Methods

Plant materials

All materials were collected from natural populations or specimens in the herbarium of Sung Kyun Kwan University (SKK; Korea) and the herbarium of the University of Texas (TEX; USA). Previous molecular phylogenetic analyses indicated sister clades from *Hippophae* and *Shepherdia* (Sun *et al*., 2002). Therefore, *Hippophae rhamnoides* subsp. *yunnanensis*, *Hippophae rhamnoides* subsp. *wolongensis, Shepherdia argentea,* and *Shepherdia canadensis* were selected as outgroups. All species samples were provided with voucher information and symbols (Table 1). In this study, We contained unrecorded species *E. pungens,* which was discovered in the Tongyeng, Gyeongsangnam-do (Unpublished).

DNA extraction, PCR amplification and DNA sequencing

Plant fresh leaves or herbarium specimens were used for isolation of genomic DNA. Total genomic DNA was extracted using the method described by Loockerman and Jansen (1996). For this study, the internal transcribed spacers form the nuclear ribosomal DAN (ITS) (White *et al*., 1990) was used. Genomic DNA samples were quantified using a UV-vis spectrophotometer (Geldoc-it™ imaging system, Upland, California, USA). PCR cocktail (25 μl) consisted of 250 ng genomic DNA, 1X Diastar™ *Taq* DNA buffer, 0.2 mM of each dNTP, 10 pM of each primer (ITS5, ITS4) and 0.025 U of Diastar™ *Taq* DNA Polymerase (SolGent Co., Korea). The ITS amplification conditions were as follows: initial denaturation at 95℃ for 2 min, followed by 35 cycles of 95℃ for 20 sec, 56℃ for 40 sec, and 72℃ for 50 sec, with a final extension at 72℃ for 5min, after which samples were held at 8℃. Amplification of genomic DNA was conducted in a PTC-200 thermo cycler (Peltier Thermal Cycler, MJ Research, Waltham, MA, USA). PCR products were purified by using a Gel & PCR purification system kit (Solgent Co., Korea) according to the manufacturer's protocols. Purified PCR products were sequenced with specific primers by an ABI (Applied Biosystems,

Table 1. Taxa used in this study

Taxon	Abbreviation	Collector / Voucher	Locality	Accession No.
E. umbellata Thunb. (보리수나무)	EU04	O. Son 2091010 (YNUH)	JejuIsland, Aewoleup	JQ062482
	EU07	O. Son 2100604a (YNUH)	Gyongsangnam-do, Jinhaesi	JQ062483
	EU11	O. Son 2100604b (YNUH)	Gyongsangbuk-do, Mt. Palgong	JQ062484
	EU20	O. Son 2100614a (YNUH)	Gyongsangbuk-do, Jangsugun Janggyemyeon	JQ062485
	EU21	O. Son 2100614b (YNUH)	Gyongsangbuk-do, Jangsugun Gyenammyeon	JQ062486
E. umbellata var. *coreana* (H.Lév.) H.Lév. (왕보리수나무)	EUC01	S. Kim 2070519a (YNUH)	Gyongsangbuk-do, Uljingun Bukmyeon Mt. Eungbong	JQ062479
	EUC02	H. Lee 2070519b (YNUH)	Gyongsangbuk-do, Uljingun Bukmyeon Mt. Eungbong	JQ062480
	EUC03	O. Son 2070519c (YNUH)	Gyongsangbuk-do, Uljingun Bukmyeon Mt. Eungbong	JQ062481
E. multiflora Thunb. (뜰보리수)	EMF01	O. Son 2100604 (YNUH)	Gyongsangnam-do, Jinhaesi Ungdong	JQ062476
	EMF04	O. Son 2100605 (YNUH)	Gyongsangnam-do, Changwonsi Mt. Bulmo	JQ062477
	EMF07	O. Son 2100605a (YNUH)	Gyongsangnam-do, Jinhaesi Dudong	JQ062478
E. macrophylla Thunb. (보리밥나무)	EMP05	O. Son 2091024a (YNUH)	UlleungIsland, Ulleng-gun Ulleng-eup Dokdo	JQ062496
	EMP25	O. Son 2101024c (YNUH)	Gyongsangnam-do, Geojesi	JQ062497
	EMP27	O. Son 2090825 (YNUH)	UlleungIsland, Ulleng-gun Ulleng-eup Seomyeon	JQ062498
E. submacrophylla Serv. (큰보리장나무)	ESM01	O. Son 2091010 (YNUH)	JejuIsland, Donnaeko	JQ062489
	ESM04	O. Son 2091009 (YNUH)	JejuIsland, Sanyangri	JQ062491
	ESM10	O. Son 2100219 (YNUH)	Jeollanam-do, Haenam Mt. Duryun	JQ062493
	ESM12	O. Son 2100220 (YNUH)	Jeollanam-do, Haenam Songgilmyeon Songhwari	JQ062494
E. glabra Thunb. (보리장나무)	EG06	J. Koh 050206 (SKK)	JejuIsland, Donnaeko	JQ062499
	EG07	J. Kim 051031 (SKK)	JejuIsland, Donnaeko	JQ062500
	EG08	O. Son 2100221a (YNUH)	Jeollanam-do, Wandogun	JQ062501
	EG09	O. Son 2110221b (YNUH)	JejuIsland, yeondong	JQ062502
	EG10	O. Son 2110221c (YNUH)	JejuIsland, yeondong	JQ062503
E. glabra f. *oxyphylla* (Servett.) W.T.Lee (좁은잎보리장나무)	EGO01	J. Koh 050205 (SKK)	JejuIsland, Donnaeko	JQ062504
E. maritima Koidz. (녹보리똥나무)	EMT01	O. Son 2100220a (YNUH)	Jeollanam-do, BogilIsland Bojilmyeon	JQ062504
	EMT02	O. Son 2100220b (YNUH)	Jeollanam-do, BogilIsland Bojilmyeon	JQ062505
	EMT03	J. Koh *et al*. 051023 (SKK)	Jeollanam-do, Wandogun Gunoemyeon Youngpungri	JQ062506
	EMT04	H. Koh 050423 (SKK)	JejuIsland, Hangyeongmyeon Gotjawal	JQ062507
E. pungens Thunb.	EP01	O. Son 2101018a (YNUH)	Gyongsangnam-do, Tongyeongsi Mt. Miruk	JQ062487
	EP02	O. Son 2101018b (YNUH)	Gyongsangnam-do, Tohgyungsi Mt. Miruk	JQ062488
Shepherdia canadensis Nutt.	SC	Merrill King and Robert M. Gravey. 13940 (TEX)	Colorado, Larminer contry, USA.	JQ517290
Shepherdia argentea Nutt.	SA	Marian MacLord. 678 (TEX)	Colorado, Moffat contry, USA	JQ517289
Hippophaë rhamnoides subsp. *yunnanensis* Rousi	HRY	Sun *et al*. Y22	Litang, Sichuan	AF440250
Hippophae ramnoides subsp. *wolongensis* Y. S. Lian	HRW	Sun *et al*. W110	Wenchuan, Sichuan	AF440252

California, USA) 3730xl DNA analyzer by Solgent Co. (Daejeon, Korea).

Data assembly and alignment

The sequence fragments were assembled and aligned using Geneious pro v5.5 (Drummond *et al*,. 2011). Alignment gaps were treated as missing characters. PAUP* ver 4.0b10 (Swofford, 2003) was used for parsimony analyses, which were conducted following widely used protocols for bootstrapping (Felsenstein, 1985). For the heuristic analyses, tree searches were performed with 1000 random sequence additions and tree bisection-reconnection (TBR) branch swapping. The

consistency index (CI), retention index (RI) and rescaled consistency index (RC) were obtained by PAUP*. Bayesian inference (BI) analysis was conducted using MrBayse v3.2.1 (Ronquist and Huelsenbeck, 2003) with Markov chain Monte Carlo (MCMC) analyses of 500,000 generations for nuclear ribosomal DNA (nrDNA), and trees were sampled every 500 generations. Convergence of the MCMC was assessed by calculating the effective sample size (ESS) of the combined runs. All estimated parameters showed > 1,000 significant of ESS. The first 25% of the generations were discarded as the burn-in. To estimate the posterior probability (PP) of recovered branches, 50% majority rule consensus trees were created.

Results

Base sequence analysis and variation

Total Genomic DNA was isolated from 34 individuals of 13 taxa and used as templates to amplify ITS base sequences. These amplified products were purified, then sequenced and identified, after which they were deposited in the GenBank database (Table 1). An arrangement of the ITS base sequence of 13 taxonomic groups and 34 individuals, including the outgroup, resulted in a total length of 645-650 bp without any significant difference. The base sequences arranged were 663 bp in total. The length of ITS 1 and 2 was 262-268 bp and 217-220 bp, respectively, and 5.8S showed 16 bp in all taxonomic groups. Overall, *Shepherdia canadensis* showed the shortest length of ITS (644 bp), while two species of the genus *Hippophae* showed the longest length of 650 bp. The lengths of ITS 1 (263 bp) and 2 (217 bp) in the in-group were the same, with the exception of *E. umbellata* EU04. A total of 542 bases among the 663 bp base sequence of arranged ITS showed no variations, while the remaining 136 bases varied. Among these, 97 taxonomically available characters were identified. The average base sequence composition of the whole group was 21.2% A, 23.8% T, 26.6% G, and 28.3% C. The G-C base composition determines the structure, and physical characters of DNA were shown to be 52.92% for ITS 1, 51.81% for 5.8S, and 53.49% for ITS 2. When base sequence variation including the outgroup was considered, two species in the genus *Hippophae*, *E. glabra* and *E. glabra* f. *oxyphylla*, showed 0-15.686%. The base sequence variation in the in-group, *E. multiflora*, *E. glabra* and *E. glabra* f. *oxyphylla* , was 0-2.046%. The in-group base sequence variation in each section showed similar results, with 0-1.094% being observed for the *Sempervirentes* section and 0-1.251% for the *Elaeagnus* section.

Molecular phylogenetic study

The Maximum Parsimony (MP) of Korean *Elaeagnus* L. based on the ITS revealed 14 parsimony trees containing 165 steps, with a consistency index (CI) of 0.909, retention index (RI) of 0.945, and rescaled consistency index (RC) of 0.859. MP tree analysis revealed that the in-group, *Elaeagnus*, showed more similarity to *Shepherdia* than to the other genera in the outgroup. It also formed a polyphylic group with a 100% bootstrap value that was divided into clade of *E. pungens*, section *Sempervirentes* and section *Elaeagnus*. *E. multiflora*, placing it in the deciduous section. *Elaeagnus* formed one distinct clade with a 98% bootstrap value. *E. umbellata* and *E. umbellata* var. *coreana* had a bootstrap value of 71%, while *E. umbellata* var. *coreana*, which has ovate-lanceolate leaves unlike *E. umbellata*, shared synapomorphy and formed a clade with a 62% bootstrap value. *E. pungens* of section *Sempervirentes* formed one clade with an 87% bootstrap value. Another section, *Sempervirentes*, formed a clade with a bootstrap value of less than 50%. Clade of *E. submacrophylla*-*E. macrophylla,* which has silvery white scales on the back of the leaf, and clade of *E. maritima* - *E. glabra* - *E. glabra* f. *oxyphylla* which has brown scales on the back, fall into this category. *E. macrophylla* and *E. submacrophylla* formed clades with less than 50% bootstrap value, but *E. submacrophylla* showed a bootstrap value of 63%. *E. maritima*, *E. glabra*, and *E. glabra* f. *oxyphylla* formed a clade with a bootstrap value of 61%, but *E. maritima* formed a polytomy, rather than a clade. *E. glabra* formed two different clades with bootstrap values of 76% and 83% because of monophyletic collapse and was not separated from *E. glabra* f. *oxyphylla* since it has narrower leaves than *E. glabra* (Fig. 1).

Bayesian inference (BI) analysis revealed that *E. pungens* had a posterior probability (PP) of 0.55, which was lower than the independent MP values and closer to the clade of section *Elaeagnus*, while creating one independent clade. The PP of section *Elaeagnus* was 0.98, while that of section *Semper-*

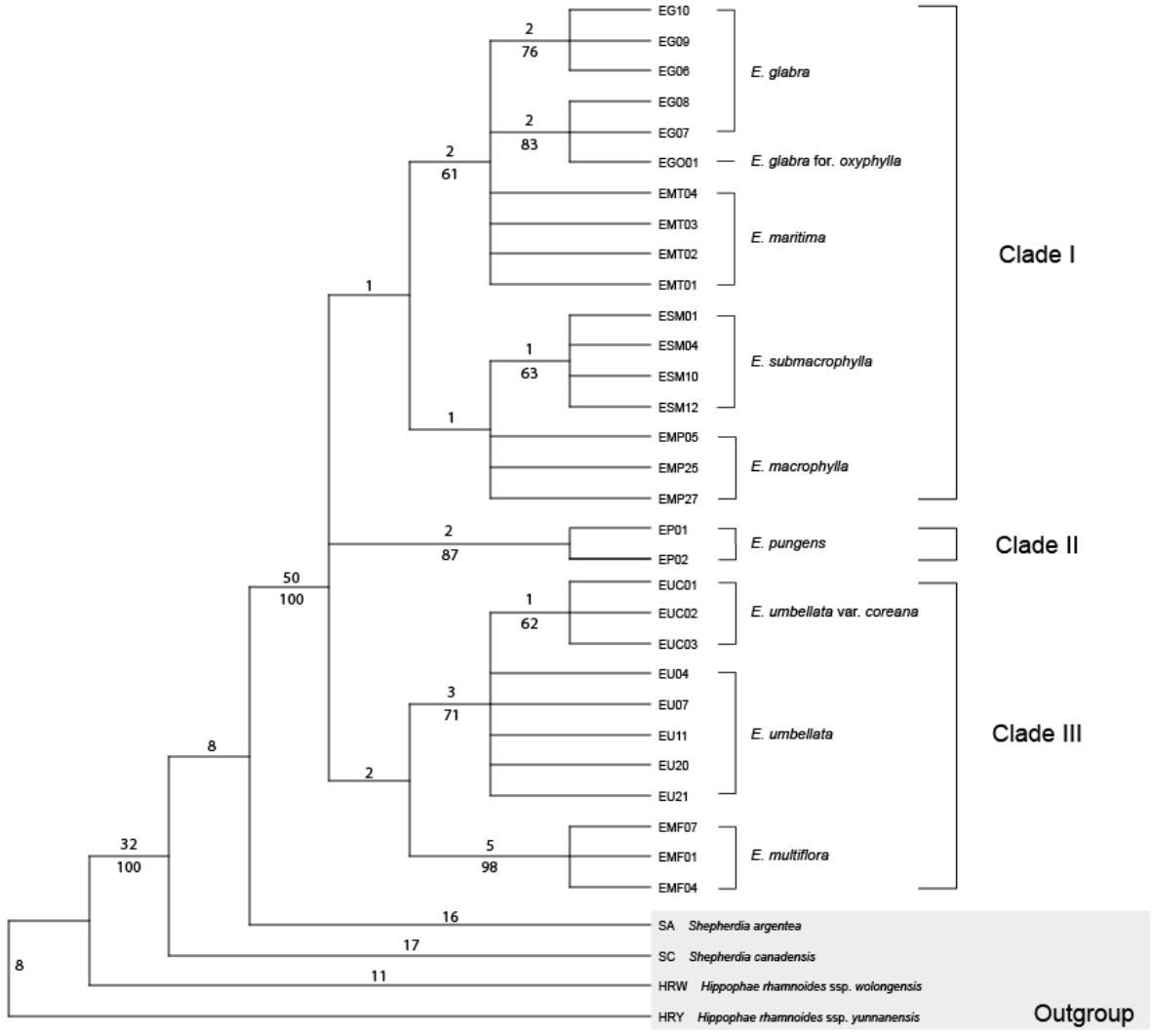

Fig. 1. Strict consensus tree (Length = 117, CI = 0.909, RI = 0.945) based on 61 phylogenetically informative changes in ITS sequences of 34 taxa. The bootstrap values are found below branches and the number of changes is indicated above branches.

virentes was 0.6. *E. multiflora*, which belongs to section *Elaeagnus*, was located at the lowest level, with a PP of only 9.9 along with MP. Species belonging to section *Sempervirentes* showed the same result in MP. The *E. submacrophylla* - *E. macrophylla* clade had a PP of 0.91, while the clade had a PP of 0.81. *E. glabra* was shown to be a polyphyletic taxon (Fig. 2).

Discussion

Phylogenetic inference and elucidation of the evolutionary processes that generate biological diversity have been accomplished even at lower taxonomic levels using ITS of the nrDNA. In this study, a molecular phylogenetic study of Korean *Elaeagnus* L. based on nrDNA ITS sequences was conducted and classification systems and relationships between taxonomic groups were analyzed. Our results indicate that both MP and BI tree showed the polyphyly of *Elaeagnus* species (Fig. 1, Fig. 2). MP tree was constructed tritomy: Clade I included *E. glabra*, *E glabra* f. *oxyphylla*, *E. maritima*, *E. submacrophylla* and *E. macrophylla,* Clade II was involved *E. pungens* and Clade III was comprised the remained taxa. Unlike MP tree, BI tree was divided into two major groups. Clade I consisting of *E. glabra*, *E glabra* f. *oxyphylla*, *E. maritima*, *E. submacrophylla* and *E. macrophylla* have a weak support posterior probabilities of 0.6 as a monophyletic group. Calde II consisting of *E. pungens, E. umbellata, E.*

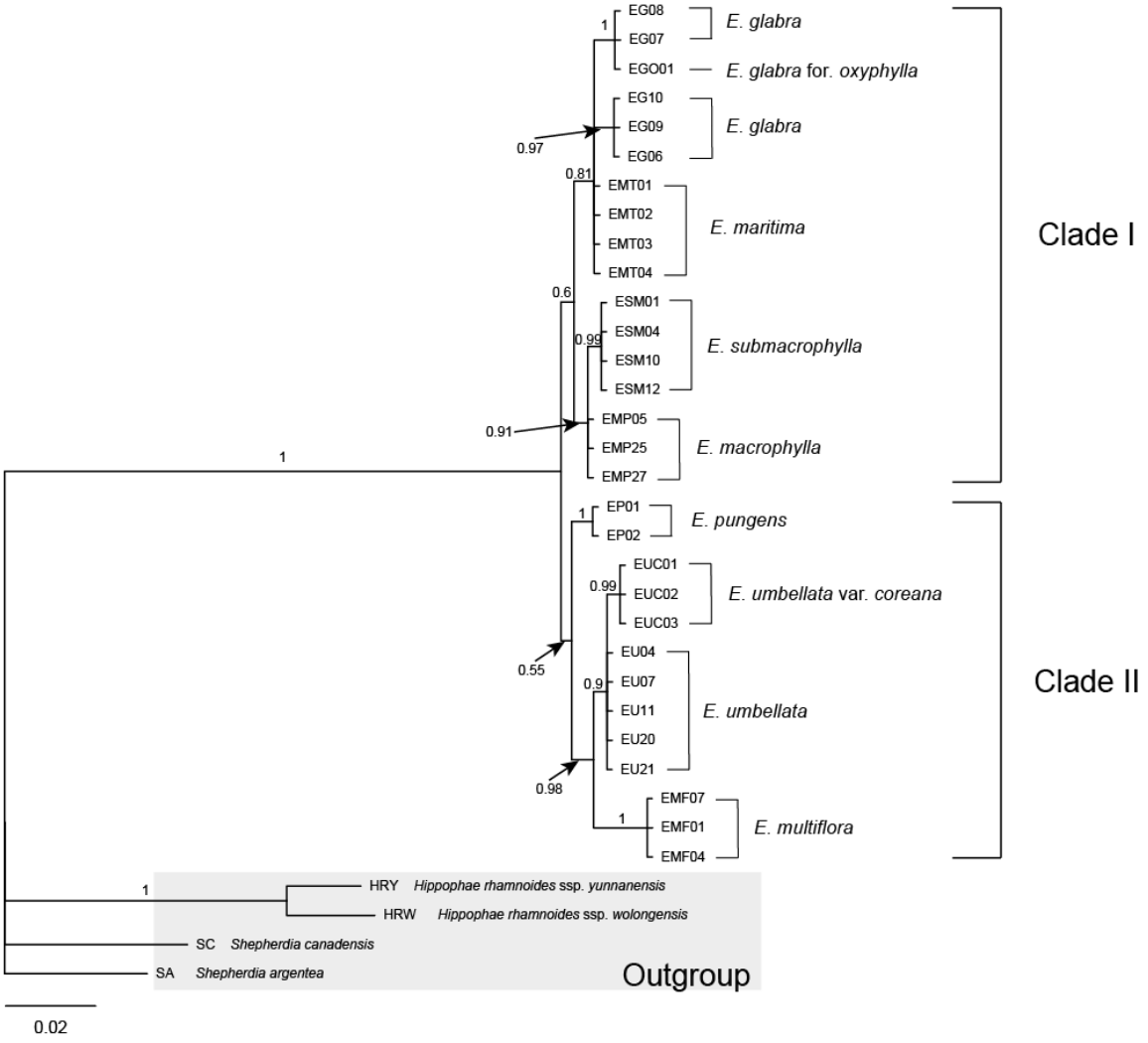

Fig. 2. Bayesian tree of ITS sequences of 34 taxa. Numbers above the branches are posterior probabilities > 0.5.

umbellata var. *coreana,* and *E. multiflora* is monophyletic in phylogenetic analysis. However, results from ITS data are not concordant with morphological data Servettaz (1909). *E. multiflora* was an independent species as it formed a separate a clade with a bootstrap value of 98% and a posterior probabilities of 1, without combining with other groups in the MP and BI tree based on nrDNA ITS sequences. Additionally, *E. multiflora* has a thorn and the earliest blooming time, as well as a fruit stalk that is longer than its flower stalk when the oval fruit ripens. Based on these evidence, *E. multiflora* has been classified as a distinct species. Sequence of *E. umbellata* var. *coreana* formed a subclade with those of *E. umbellata* with strong support posterior probabilities of 0.99, but MP tree was found to have a relatively low bootstrap value (62%). The taxonomic opinion that *E. umbellata* var. *coreana* has been classified as a variety due to morphological differences in the leaves when compared to *E. umbellata* by Lee (1980). In the present study, the findings reported by Lee (1966) are tenable because it formed one independent clade by branching from the *E. umbellata*. Similarity. *E. submacrophylla* was sister to a clade of *E. macrophylla*, and monophyletic group. it is reasonable to consider *E. submacrophylla* a variation of *E. macrophylla* instead of a hybrid, since it reappeared in the clade of *E. macrophylla*, which has leaves that are oval or ovate. *E. maritima* calde showed that it formed a polytomy. Although it did form a clade with a relatively low bootstrap with *E. glabra*, it showed a discontinuity in form and molecular characteristics from *E. macrophylla*. Based on these results, it

can be considered its own species. The most notable pattern in the ITS data is the phylogenetic position of *E. pungens*. *E. pungens* that is not previously been reported in Korea, was discovered from Tongyeong and Southern Gyungsang province of South Korea. In terms of growth period, it is reasonable to include *E. pungens* in section *Sempervirentes*. However, *E. pungens* formed an independent clade with strong support bootstrap value of 87% in the MP tree. By contract, in the BI tree, *E. pungens* was sister to a clade of section *Elaeagnus* (i.e., *E. umbellata*, *E. umbellata* var. *coreana*, *E. multifloa*) as a monophyletic group in clade II. It is necessary to include more maker. *E. glabra*, Both MP and BI trees, formed a clade with relatively high reliability and had polyphyletic group. This also showed different patterns from the external morphology and molecular phylogenetic study. These findings indicate that more methods are needed to evaluate organisms at the population level, and that such methods should consider interspecific hybrids, convergence and parallel evolution. In addition, the results are shown that the phylogenetic relationships between *E. glabra* and *E. glabra* f. *oxyphylla* are unresolved based on ITS sequence (Fig. 1, Fig. 2). *E. glabra* f. *oxyphylla* was recognized by morphological differences in leaves, that is, lanceolate-shaped leaves (Lee, 1996). On the basis of the ITS-derived phylogeny, *E. glabra* f. *oxyphylla* was closely related to *E. glabra* (EG07, EG08). However, the phylogeny did not support monophyly of *E. glabra* f. *oxyphylla*, revealing no synapomorphic characters.

In conclusion, ITS phylogeny gives relatively high statistical support for the group of *E. glabra*-*E. glabra* f. *oxyphylla* clade, *E. submacrophylla*-*E. macrophylla* clade and *E. umbellata*-*E. umbellata* var. *coreana* clade in BI and MP tree. However, phylogenetic relationships among species of *Elaeagnus* revealed by ITS data are not consistent with the traditional inter-section classification system.

In general, molecular approaches have been more effective than morphological or cytogenetic ones in the identification of interspecific hybridization (Kaplan and Fehrer, 2007). DNA sequencing data, internal transcribed spacer (ITS) region of nuclear DNA, is a useful method in detection of hybrid and evolutionary trends of low-level taxa (Baldwin, 1992; Kim *et al*., 1994; Ainouche and Bayer, 1997; Sun *et al*., 2002). In the genus Korea *Elaeagnus,* hybrid origins of two species, *E. submacrophylla* and *E. maritima* have been suggested previously (Servattazd, 1908; Lee, 1980; Lee, 1996; Ohwi, 1984; Ohba, 2003; Lee, 2007). *E. submarophylla* have been classified separately from *E. macrophylla* due to its twig color and the form and texture of its leaves. For this reason, Servattaz (1908) considered it a variation *E. pungens* × *E. macrophylla*. Furthermore, Lee (1996) considered it a variation of *E. glabra* × *E. macrophylla*. ITS analysis have shown that *E. glabra* and *E. pungens* are not related, but *E. macrophylla* has topology of the *E. submacrophylla*. In addition, DNA evidence supported the hybrid species better than variety species. *E. maritima* is similar in appearance to *E. glabra*, but with more oblong and leathery leaves and reddish brown scales on the back of the leaves, which make the two species completely different. Ohwi (1984), Ohba (2003), and Lee (2007) described *E. maritima* as a hybrid of *E. glabra* × *E. macrophylla*, while Lee (1996) described it as a hybrid or intermediate form. However, a phylogenetic tree based on the nrDNA ITS region showed that it formed a polytomy. Although it did form a clade with a relatively low bootstrap with *E. glabra*, it showed a discontinuity in form and molecular characteristics from *E. macrophylla*. Based on ITS results, it can be considered its hybrid species. In order to confirm the putative parents, It need to be conducted additional research using plastid marker. And its implications in phylogeny will be discussed further study.

In the present study, ITS data of two putative hybrids did not cleary prove the relationship of each hybrids and their potential parents. Thus, Ongoing studies further base on the chloroplast genome to find out the potential maternal species of two hybrids because the plastid locus has generally been used to determine evolutionary relationships and genomic origin.

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