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Molecular Reexamination of Korean Umbelliferae Based on Internal Transcribed Spacer Sequences of rDNA: *Ligusticum tenuissimum* (Nakai) Kitagawa and *Libanotis coreana* (Wolff) Kitagawa

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The taxonomic status of *Ligusticum tenuissimum* (Nakai) Kitagawa and *Libanotis coreana* (Wolff) Kitagawa has been controversial in Korea. We examined their phylogenetic relationships by comparing the internal transcribed spacer (=ITS) sequences of the nuclear ribosomal RNA gene (=rDNA) with another seven species in the Korean Umbelliferae. Our results support the legitimacy of the nomenclatural combination with *L. tenuissimum* Kitagawa rather than with *Angelica tenuissima* Nakai. We also suggest using *L. coreana* (Wolff) Kitagawa instead of *Seseli coreanum* Wolff because *L. coreana* has been completely separated from most taxa of the genus *Seseli*. In addition, we have confirmed at least three phylogenetic lines in the genus *Ligusticum*. One is the core group of *Ligusticum* that comprises *L. tenuissimum* and four other species. This clade includes the *Ligusticum hultenii* line and *Ligusticum scothicum*, known as an anomaly of *Ligusticum*. The second clade is composed of *Ligusticum physospermifolium* with *Seseli monatum*, which indicates the polyphyly of *Ligusticum*. The third line is *Ligusticum acutilobum*, clustered as a sister grade of *Dystaenia*.

Keywords: Internal transcribed region (ITS), *Libanotis coreana*, *Ligusticum tenuissimum*, rDNA, Umbelliferae

The family of Umbelliferae in Korea comprises 27 to 33 genera and 62 to 78 species, depending upon authors (Nakai, 1952; Chung, 1958; Lee, 1979). Umbelliferae has been studied throughout Korea, Japan, and Manchuria, and many new species has been reported in Korea (Nakai, 1919, 1952; Kitagawa,1937, 1941, 1963). Eighteen species (ca. 22% of the total) are endemic there (Nakai, 1952). This is not a high ratio compared with that for Asian Umbelliferae, which is approximately 46% (Heywood, 1971). Most of these endemic taxa in Korea have not been reexamined taxonomically.

Among the Korean endemic Umbelliferae, two genera, *Bupleurum* and *Dystaenia*, have been examined phylogenetically, on the basis of their ITS sequences of rDNA (Choi et al., 1996, 1998). Although the resolution of ITS sequences for the subfamily and tribal level of Umbelliferae is not completely congruent with other data from *rpo*C1 or cpDNA (Downie et al., 1996; Plunkett and Downie, 1999), these sequences are very useful in assessing phylogenetic relationships at lower taxonomic levels, such as among genera or species. Their rates of divergence are relatively high compared with protein coding or transcribed regions of *rbc*L and 18S/26S ribosomal DNA respectively (Baldwin et al., 1995; Downie and Katz-Downie, 1996; Choi and Kim, 1999). The resolution of ITS sequences is also useful for analyzing the identity of the genus *Dystaenia* and the genealogy of the Korean *Bupleurum* (Choi et al., 1996, 1998).

The genus *Ligusticum* L., one of the largest and most complex genera in Umbelliferae, comprises three species in Korea; *Ligusticum hultenii* Fernald, *Ligusticum tenuissimum* (Nakai) Kitagawa, and *Ligusticum acutilobum* Sieb. et Zucc. The species *L. tenuissimum* (Nakai) Kitagawa previously was named *Angelica tenuissima* Nakai from Korea (Nakai, 1919). However, Kitagawa (1941) changed this taxon to the genus *Ligusticum*, based on speciemens from Manchuria. Because of Kitagawa's recombination on *L. tenuissimum* (=*A. tenuissima*), the two scientific names for the same taxon have been confused in Korea (Nakai, 1952; Chung, 1958; Lee, 1979; Lee, 1996).

Libanotis coreana (Wolff) Kitagawa was reported through the taxonomical change of *Seseli coreanum* Wolff as an endemic species from Korea (Kitagawa, 1937, 1963); genus *Libanotis* has been treated as synonymous with the genus *Seseli*. In Korea, the two generic names for one taxon also have long been mixed (Nakai, 1952; Chung, 1958; Lee, 1979; Lee, 1996).

In this study, we used ITS sequences of rDNA to

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reexamine the taxonomic status of *L. tenuissimum* and *L. coreana*. ITS sequences of rDNA from these taxa were compared with other related species and genera in Umbelliferae. In addition, the molecular phylogenetic relationships of *L. tenuissimum* and *L. coreana* were analyzed with other related taxa in that family.

MATERIALS AND METHODS

Plant Collections and ITS Sequencing

Nine species in Umbelliferae, belonging to *Ligusticum, Angelica, Libanotis, Pimpinella*, and *Peucedanum*, were collected from Korea and far eastern Russia (Table 1). Other taxa, which are not endemic to Korea, were surveyed to compare the taxonomic status of *Ligusticum* and *Libanotis* within the family.

Leaves to be used as DNA sources were collected in the field and stored at –70°C. With minor modification (Choi and Kim, 1999), the method described by Doyle and Doyle (1987) was followed in extracting total genomic DNA. DNA was purified by ultracentrifugation in CsCl-ethidium bromide gradients, followed by alcohol precipitation (Sambrook et al., 1989). Automated DNA sequencing of the ITS1, 5.8S, and ITS2 regions was performed with an ABI prime ready reaction deoxyterminator FS cycle sequencing kit (Model 377, ver.2.1.1). PCR primers for ITS sequencing were designed by White et al. (1990). Our nucleotide sequences appear in the DDBJ/EMBL/GenBank databases; accession numbers are included in Table 1.

Analysis of ITS Sequences and Phylogenetic Relationships

The sequence boundaries of ITS1 and ITS2 were determined by comparing them with published sequences from Umbelliferae (Downie and Katz-Downie, 1996). Because we knew from preliminary sequence analysis that the three taxa of *Ligusticum* were not grouped into the same clade, we used additional ITS sequences of Umbelliferae that were deposited in GenBank (Zhang and Madden, 1997; see Table 1). The ITS sequences were combined with 31 prealigned ITS sequences from other species in subfamily Apioideae and 1 species in subfamily Saniculoideae. Sequences were aligned using the ClustalW program (Thomson et al., 1994), and then finally adjusted by eye. The alignment of sequences is available from the corresponding author upon request.

Phylogeny were reconstructed with the maximum parsimony algorithm method, via

PAUP (ver.4.01b; Swofford, 1999). We conducted heuristic searches with equal weighting of character state changes. Pairwise nucleotide differences of unambiguously aligned positions were determined using the DISTANCE MATRIX option in PAUP. Our bootstrap analyses also were performed with PAUP to assess the degree of support for any particular branch on the strict consensus tree. Bootstrap values were calculated from 100 replicate analyses, through heuristic searches. We estimated the amount of phylogenetic information in the parsimony analyses, using consistency and retention indices. In addition, tree distance was calculated using the neighbour-joining method of tree construction, implemented through MEGA (Kumar et al., 1993). Distance matrices were calculated using the MEGA program, and the number of nucleotide substitutions (excluding gaps) were estimated according to Kimura's two-parameter method.

We utilized the fast the fast DNA mL program (ver.1.0.6) to estimate the maximum-likelihood phylogeny, based on the procedures of Felsenstein (1985, 1993). A maximum-likelihood tree was inferred using a transition/transversion ratio of 2.0. Here, we randomly selected the sequence addition order (JUMBLE) and invoked the GLOBAL branch swapping search option. Empirical base frequencies were derived from the sequence data and used in the maximum-likelihood calculations.

RESULTS

TS Sequence Variations in Umbelliferae

The sequence sizes for ITS1 varied from 201 bp (*L. hultenii*) to 221 bp (*Pimpinella* species). The 5.8 S coding region was 162 bp long for all species. Sequence sizes for ITS2 varied from 212 bp in *L. coreana* to 224 bp in *L. hultenii*, and from 217 to 223 bp in tribe Peucedaneae. GC content of ITS1 ranged from 53.7% in *Angelica dahurica* to 56.9% in genus *Peucedanum*. The GC content of ITS2 ranged from 53.2% in *Peucedanum terebinthaceum* to 57.0% in *L. hultenii*; this was the highest value for any species in this study (data not shown).

The range of sequence divergence for ITS1 was 1.7 to 23.1 between species in tribe Apieae, and 0.0 to 8.4 in tribe Peucedaneae. Sequence divergences of ITS2 were slightly higher, ranging from 1.8 to 35.8 in tribe Apieae and from 0.0 to 11.9 in tribe Peucedaneae. The total range of combined sequence divergences varied from 1.7 to 27.8 between species in tribe Apieae, and from 0.0 to 9.8 in tribe Peucedaneae. The lowest combined divergence value (0.0) was observed within the genus *Angelica*, the next lower (0.0 to 4.1) was within the genus *Peucedanum*. The combined value for ITS sequence divergence were highest in the genus *Ligusticum* (2.6 to 27.8).

Phylogenetic Relationships of *L. tenuissimum* and *L. coreana*

Maximum parsimony analyses generated 10 equally parsimonious trees (Fig. 1). The consensus tree was 764 steps long, with a CI value of 0.627 and a RI value of 0.710. Although a strict consensus tree was produced, it poorly supported tribal relationships, especially for the genus *Ligusticum* (Fig. 1).

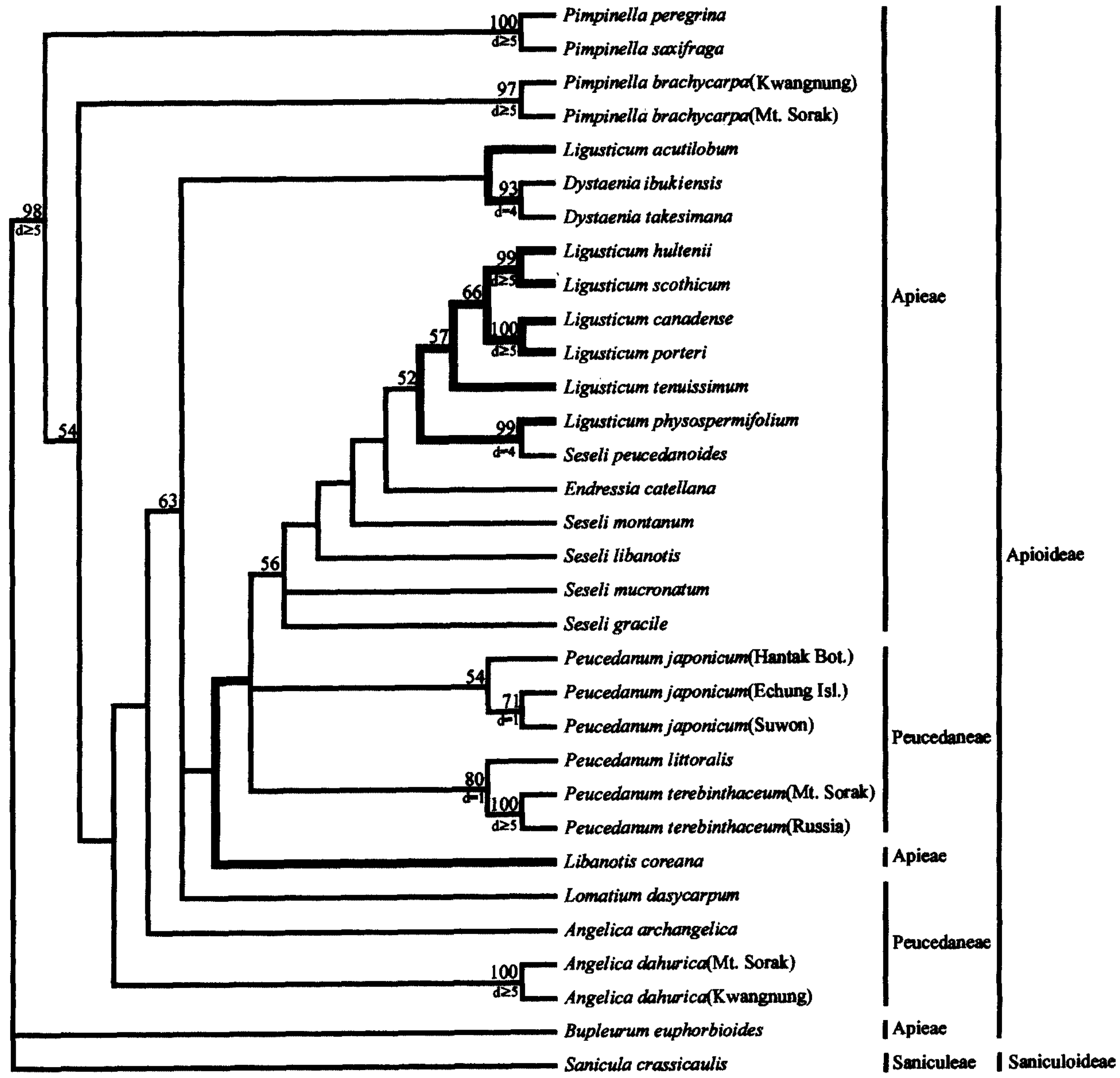

Figure 1. Strict consensus of the 10 maximally parsimonious 764-step trees derived from equally weighted parsimony analysis of combined ITS1 and ITS 2 sequences (CI = 0.627, RI = 0.710). Values above the nodes indicate the number of times a monophyletic group occurred in 100 bootstrap replicates. Decay analyses with tree length 5 steps longer than most parsimonious trees could not be done because of computational constraints.

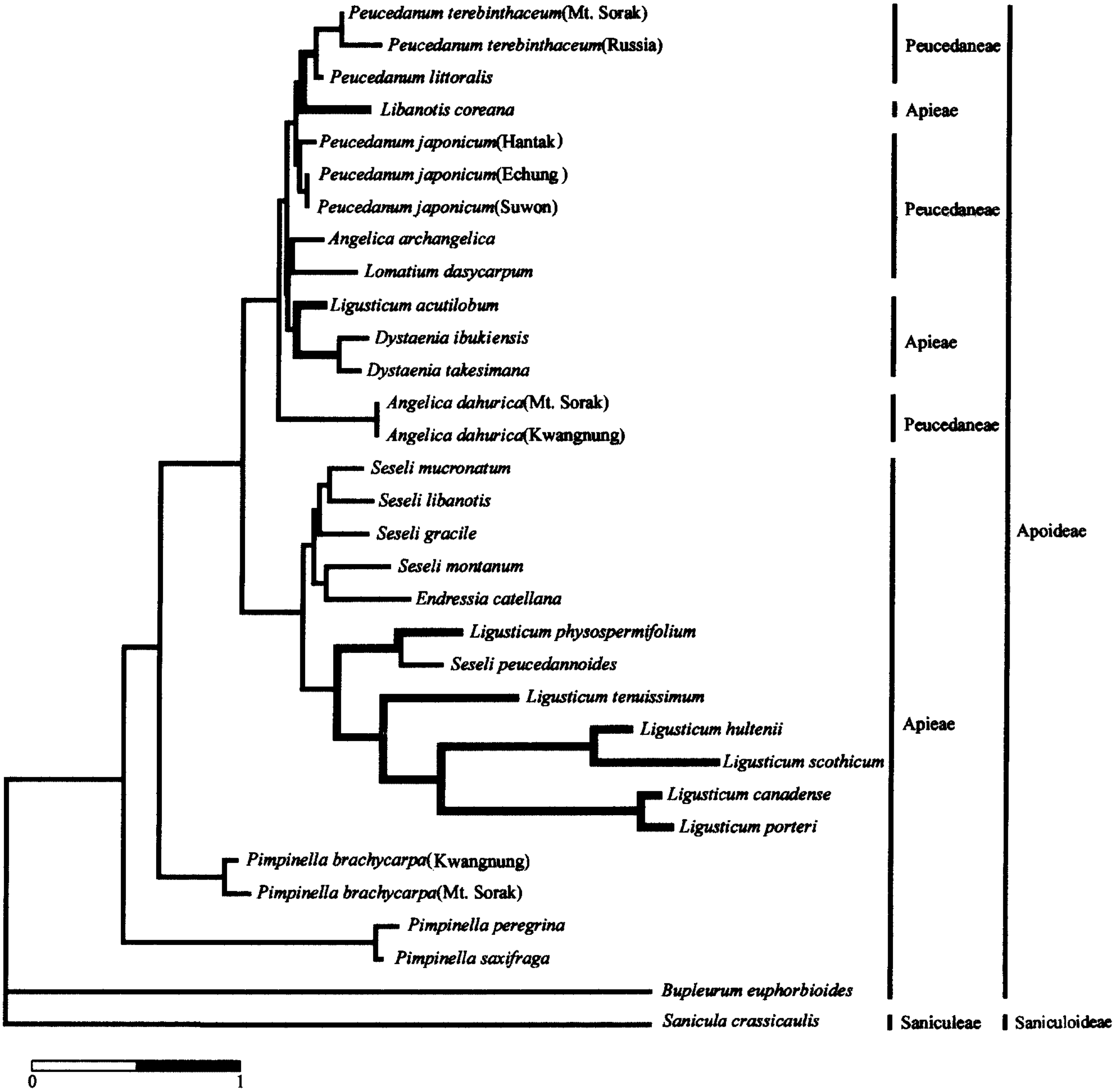

Figure 2. Tree obtained from the neighbour-joining analysis of substitution rates estimated from the two-parameter method of Kimura (1980) for combined ITS1 and TS2 sequences, using a transition/transversion ratio of 2.0.

The maximum parsimony tree from ITS sequence analysis showed that *L. coreana* made a clade separate from *Seseli* (Fig. 1). However, *L. coreana* was nested in the *Peucedanum* clade on the neighbour-joining tree (Fig. 2), and formed a sister group with *Peucedanum* on the maximum-likelihood tree (Fig. 3). In all the trees obtained from analyses of maximum parsimony, neighbour-joining, and the maximum-likelihood, *L. coreana* was apparantly more closely related to *Peucedanum* than to *Seseli*.

In contrast, *L. hultenii* was grouped into the same clade with *Ligusticum scothicum*, which made a sister line of *L. tenuissimum* in all three trees (Figs. 1, 2, and 3). All the trees obtained from the maximum parsimony, neighbour-joining, and maximum-likelihood methods demonstrated that *L. tenuissimum* formed a monophyletic group with *Ligusticum canadense* and *L. hultenii.*

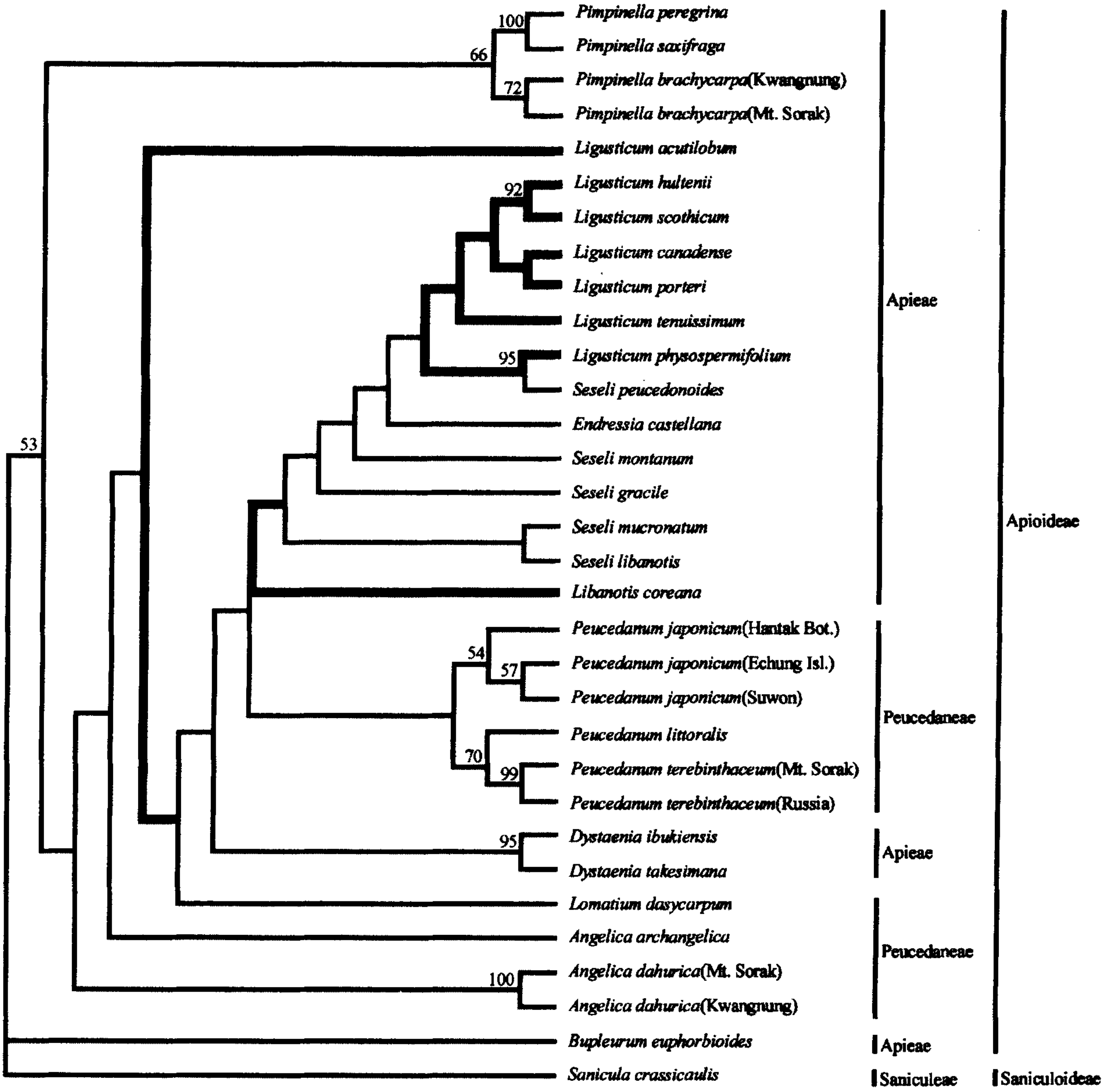

Figure 3. Maximum-likelihood tree constructed from unambiguous ITS sequences, using a transition/transversion ratio of 2.0. Values above the nodes indicate the number of times a monophyletic group occurred in 100 bootstrap replicates. Bootstrap value scored using NNI option PAUP (Swofford, 1999).

DISCUSSION

The relative size of ITS1 and ITS2 varies considerably among groups of angiosperms (Baldwin et al., 1995). The sizes of ITS1 and ITS2 for *L. coreana* and genus *Ligusticum* lay intermediate in the range of that reported previously for other angiosperms (i.e., 187 to 298 bp for ITS1 and 187 to 252 bp for ITS2; reviewed in Baldwin et al., 1995; Downie and Katz-Downie, 1996; Choi et al., 1998). In our group of angiosperms investigated, nucleotide divergence values for ITS1 were generally similar to those for ITS2, with a few exceptions (Baldwin et al., 1995). The nucleotide divergence for *L. coreana* and genus *Ligusticum* nucleotide were greater between sequences of ITS2 than between those of ITS1. This suggests that ITS2 changes faster evolutionarily than does ITS1.

The GC content of ITSs ranges from approximately 30 to 77% in angiosperms (Baldwin et al., 1995; Choi and Kim, 1999). Contents for both ITS sequences in

L. coreana and genus *Ligusticum* fell within the range reported for other angiosperms. In our ITS1 sequences, GC content was slightly higher than in the ITS2 sequences, except for the genus *Angelica*.

One objective of our study was to reexamine the taxonomic status of the endemic species of Korean Umbelliferae, using ITS sequences. According to our results, the taxonomical change from *A. tenuissima* Nakai to *L. tenuissimum* (Nakai) Kitagawa and from *S. coreanum* Wolff to *L. coreana* (Wolff) Kitagawa clearly are well supported by our ITS sequence analyses (Figs. 1, 2, and 3).

Many of the large genera in Apioideae (Umbelliferae) are not monophyletic (Pimenov and Leonov, 1993; Katz-Downie et al., 1999), although many authors agree with Drude's classification system (1898) on the high level occuring in Umbelliferae (Downie et al., 1996; Downie and Katz-Downie, 1996).

The delimitation of the generic concept in *Ligusticum* (*s. l.*) is still controversial (Heywood, 1971; Kondo et al., 1996). This genus was suggested as being one of the polyphyletic groups on the basis of several molecular data sets (Kondo et al., 1996; Plunkett and Downie, 1999). In fact, our study also demonstrated that the genus *Ligusticum* L. is no longer a monophyletic group (Heywood, 1971; Kondo et al., 1996; Downie et al., 1996; Plunkett and Downie, 1999). This is apparant why many authors have not been satisfied with the taxonomical position of *Ligusticum*, especially in Korea.

Ligusticum acutilobum Sieb. et Zucc. may have a closer relationship with *Dystaenia* than with *Angelica*

Table 1. Species collections used for ITS sequence analysis of genera *Pimpinella, Ligusticum, Libanotis, Angelica,* and *Peucedanum* (Umbelliferae) from Korea. Accession numbers are for DDBJ/GenBank databases.

Species	Source and Voucher	Accession NO.
Subfamily Apioideae		
Tribe Apieae (Ammieae)		
Subtribe Apiinae (Carinae)		
Pimpinella brachycarpa (Kom.) Nakai	Korea, KwangNung Arboretum, 26 Aug. 1994 [AJOU 13848]	AF169258/AF169259 (KS102365*/KS102366*)
Pimpinella brachycarpa (Kom.) Nakai	Korea, Mt. Seolack, 23 July 1996 [AJOU 13846]	AF169260/AF169261 (KS102367*/KS102368*)
Subtribe Seselinae		
Ligusticum acutilobum Sieb. et Zucc.	Korea, Suwon (cultivated) 21 Sept. 1997 [AJOU 13825]	AF169262/AF169263 (KS102369*/KS102370*)
L. hultenii Fern.	Russia, 17 July 1997	AF169264/AF169265 (KS102371*/KS102372*)
L. tenuissimum Kitagawa	Korea, Hantack Botanical Garden, 16 July 1999 [AJOU 20271]	AF205404/AF205405 (KS102758*/KS102759*)
Libanotis coreana (Wolff) Kitagawa	Cultivated in Chonbuk Nal't University	AF169266/AF169267 (KS102373*/KS102374*)
Tribe Peucedaneae		
Subtribe Angelicinae		
Angelica dahurica (Fisch.) Benth. & Hook.	Korea, Mt. Seolack, 23 July 1996 [AJOU 13845]	AF169268/AF169269 (KS102375*/KS102376*)
Angelica dahurica (Fisch.) Benth. & Hook.	Korea, KwangNung Arboretum, 5 Aug. 1994 [AJOU 13849]	AF169270/AF169271 (KS102377*/KS102378*)
Subtribe Peucedaneae (Ferulinae)		
Peucedanum japonicum Thunb.	Korea, Hantack Botanical Garden, 13 July 1997 [AJOU 13832]	AF169272/AF169273 (KS102379*/KS102380*)
Peucedanum japonicum Thunb.	Korea, Echung Isl. 21 July 1995 [AJOU 13827]	AF169274/AF169275 (KS102381*/KS102382*)
Peucedanum japonicum Thunb.	Korea, Suwon (cultivated) 21 Sept. 1997 [AJOU 13829]	AF169276/AF169277 (KS102383*/KS102384*)
Peucedanum littoralis	Russia, Marina station 16 July 1997	AF169278/AF169279 (KS102385*/KS102386*)
Peucedanum terebinthaceum Fisch.	Korea, Mt. Seolack, 15 May 1995 [AJOU 13830]	AF169280/AF169281 (KS102387*/KS102388*)
Peucedanum terebinthaceum Fisch.	Russia, 7 July 1997	AF169282/AF169283 (KS102389*/KS102390*)

Accession numbers in GeneNuri of BRIC, Korea (*s.str.*), based on ITS sequence analysis (Figs. 1, 2, and 3). Hiroe(1979) and Kitagawa (1937) described *L. acutilobum* as a a member of *Angelica* in Japan, but our results do not support their description of *Augelica cutiloba*. In both the maximum-parsimony and the neighbour-joining trees, *L. acutilobum* made a sister group with *Dystaenia* rather than with *Angelica* (Figs. 1 and 2).

Likewise, our data always placed *L. hultenii* in the same clade with *L. scothicum*; this was considered an anomaly in the old concept of *Ligusticum* (Plunkett and Downie, 1999). Besides their morphological similarities, two taxa of *Ligusticum* show similar habitat, i.e., *L. hultenii* is distributed along the seacoast of northeastern Korea while *L. scothicum* is a seaside plant in Europe and North America. *L. tenuissimum* was originally classified in the genus *Angelica* by Nakai (1919). Our results from all three trees, however, indicate that *L. tenuissimum* belongs to a monophyletic group with *L. canadense, L. hultenii,* and *Ligusticum porteri,* rather than with genus *Angelica* (Figs. 1, 2, and 3). We strongly believe that the scientific name of *L. tenuissimum* should be placed under *Ligusticum* instead of under *Angelica* (Kitagawa, 1937; Hiroe, 1979).

Maximum-likelihood analysis of ITS sequences of the genus *Pimpinella* and the genus *Peucedanum* were fully resolved to show a monophyletic group for all species examined in those genera (Figs. 1, 2, and 3). In this study, *Angelica* and *Seseli* were not grouped into a single clade. This finding is similar to the *rpo*C1 intron-based phylogeny of Downie and Katz-Downie (1996) and the ITS region phylogeny of Katz-Downie et al.(1999).

Interestingly, *L. coreana* was located in a separate clade in the maximum parsimony tree (Fig. 1). However, this species was nested between *Peucedanum* taxa in the neighbour-joining tree (Fig. 2), but was not resolved from the genus *Peucedanum* in the maximum-likelihood tree (Fig. 3). Because the tribe that contains *Peucedanum was* consistently nested in the tribe Apieae from all three trees, these two tribes must be reexamined on the tribal level.

Two main tribes of Umbelliferae -- Peucedaneae and Apieae -- were identified as either polyphyletic or perhaps requiring revision (Figs. 1, 2, and 3). Our results also show the necessity for analyzing the other endemic taxa of Umbelliferae in Asia and South Korea that have not yet been thoroughly reexamined (Kitagawa, 1937; Nakai, 1952; Heywood, 1971; Hiroe, 1979).

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