The taxonomic status of *Angelica purpuraeifolia* and its allies in Korea : Inferences based on ITS molecular phylogenetic analyses

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ABSTRACT: The taxonomy of the umbelliferous species *Angelica amurensis* and its allies was reviewed on the basis of molecular phylogenies derived from sequences of nuclear ribosomal DNA internal transcribed spacer (ITS) regions. Strict consensus of six minimal length 119-step trees derived from equally weighted maximum parsimony analysis of combined nuclear rDNA ITS1 and ITS2 sequences from 29 accessions of *Angelica* and outgroups indicated that *Angelica purpuraeifolia*, known to be endemic to Korea, is the same species as *A. amurensis*. Comparisons of sequence pairs across both spacer regions revealed identity or 1-2 bp differences between *A. purpuraeifolia* and *A. amurensis*. These results indicated that the two taxa are not distinguished taxonomically. Also, nuclear rDNA ITS regions are discussed as potential barcoding loci for identifying Korean *Angelica*.

Keywords: Apiaceae, *Angelica purpuraeifolia*, *Angelica amurensis*, DNA barcode

The Korean endemic *Angelica purpuraeifolia* Chung grows in mountainous areas in Gangwondo, Gyeongsangbugdo, and Chungcheongbugdo (Lee, W. 1996; Park et al., 1997). *A. purpuraeifolia* is characterized by three to four times ternate leaves, obsolete calyx teeth, non-ciliated margins of bracts and bracteoles, and densely hispidulous rachis, peduncles, rays, and pedicels. The species can be also distinguished from other congeners by the presence of purple colors at the base of leaf petioles. *Angelica* species, including *A. purpuraeifolia*, have attracted considerable attention as they have been used as traditional medicine in northeastern Asian countries, including Korea, Japan, and China. Roots of *A. purpuraeifolia* are used in Oriental medicine to relieve pain, fever, perspiration, and anemia (Choi and Park, 1995; Pan and Watson, 2005). Chemical analyses of *Angelica* components have revealed diverse compounds such as coumarines, sesquiterpenes, and polyacetylenes (An et al., 2005; reviewed in Min, 2006). Chi (1967) isolated the coumarine derivative *trans*-khellactone from *A. purpuraeifolia* roots, and more recently, Min (2006) isolated four coumarines (isoscopoletin, oxypeucedanin hydrate, arnottinin, and isokhellactone) and one polyacetylene through repeated column chromatography from *A. purpuraeifolia* roots. Also, *A. purpuraeifolia* derivatives and compounds containing anticancer

bioactivities have been protected by Korean patent law (Lee et al., 2005). However, the taxonomic identity and classification of *A. purpuraeifolia* are unclear because its taxonomic nomenclature is nomen nudum. *A. purpuraeifolia* was first reported by Chung (1956) without a Latin description and type specimens. Following Chung's (1956) treatment of the species, several floras and studies have used this nomenclature to describe *A. purpuraeifolia* (Tou, 1970, 1971; Choi and Park, 1995; Lee, Y. 1996; Park et al., 1997; Feng et al., 2009). In this paper, to taxonomically identify *A. purpuraeifolia*, we present results of a molecular systematic study of *A. purpuraeifolia* and its allies based on nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) sequences. Our goal was to ascertain the taxonomic status and position of the species and to evaluate the utility of the ITS sequences as DNA barcodes for the identification of several *Angelica* species in Korea.

Materials and Methods

Plant materials

Samples of *Angelica* and *Peucedanum* species were obtained through field investigations conducted by the staff of the Korean National Institute of Biological Resources (NIBR) from August 2009 to October 2010. Samples of *Angelica polymorpha* showing diverse morphological variation of leaf shapes and sheaths

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were collected from five different localities. Five accessions of *A. purpureaefolia* were sampled from the Inje and Pyeongchang areas of Gangwon-do and Jirisan of Gyeongsangnam-do province, Korea, respectively. Other species of *Angelica* were collected nationwide in two or three accessions. Herbarium voucher specimens examined in this study were deposited at the herbarium of the NIBR. To compare and align sequences from the *Angelica* species investigated in this study, nrDNA ITS sequences of two *Angelica* accessions, *A. genuflexa* and *A. amurensis*, were obtained from GenBank. In total, 29 accessions were used for molecular phylogenetic analyses including three accessions of the genus *Peucedanum* as outgroups (Table 1).

Experimental strategies

Total genomic DNA was extracted from fresh leaves and herbarium preserved tissues using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Double-stranded DNA of the complete ITS regions in each genomic DNA were polymerase chain reaction (PCR)-amplified using primers "ITS 5" and "ITS 4" (White et al., 1990). Details of the amplification reactions, purification, and alignment are as described in Lee (1998). Pairwise nucleotide differences of unambiguously aligned positions were determined from the distance matrix option in Phylogenetic Analysis Using Parsimony (PAUP*; Swofford, 2000). Phylogenetic analyses of the ITS sequence data were carried out using the heuristic search strategies

Table 1. The 29 accessions of the genus *Angelica* and outgroups examined for nuclear ribosomal DNA internal transcribed spacer sequence variation. Accession numbers of newly investigated sequences start with the letter J. Herbarium acronyms: KB = Korean National Institute of Biological Resources, Incheon.

Taxon	Source or voucher information	GenBank Acc. No.
<i>Angelica polymorpha</i> Maxim.	Korea, Daeam-san, <i>BYLee 090919-16</i> (KB)	JN603222
<i>A. polymorpha</i> Maxim.	Korea, Chiag-san, <i>BYLee 090909-7</i> (KB)	JN603221
<i>A. polymorpha</i> Maxim.	Korea, Gwangdeog-san, <i>BYLee 090823-13</i> (KB)	JN603223
<i>A. polymorpha</i> Maxim.	Korea, Pohang, <i>GYChung 90828001</i> (KB)	JN603224
<i>A. polymorpha</i> Maxim.	Korea, Danyang, <i>CGJang 138</i> (KB)	JN603222
<i>A. polymorpha</i> Maxim.	Korea, Pyeongchang, <i>HK1090155</i> (KB)	JN603224
<i>A. genuflexa</i> Nutt. ex Torr. & A. Gray	Japan, Hokkaido, <i>Xue et al.</i> (2007)	DQ263566
<i>A. dahurica</i> (Fisch. ex Hoffm.) Benth. & Hook.	Korea, Gwangdeog-san, <i>BYLee 090823-11</i> (KB)	JN603213
<i>A. dahurica</i> (Fisch. ex Hoffm.) Benth. & Hook.	Korea, Yonggol-san, <i>HKim 3370</i> (KB)	JN603214
<i>A. dahurica</i> (Fisch. ex Hoffm.) Benth. & Hook.	Korea, Bonghwa, <i>SGKwon & JSMoon 040829</i> (KB)	JN603212
<i>A. purpureaefolia</i> Chung	Korea, Daeam-san, <i>BYLee 090919-1</i> (KB)	JN603226
<i>A. purpureaefolia</i> Chung	Korea, Daeam-san, <i>BYLee 090919-2</i> (KB)	JN603227
<i>A. purpureaefolia</i> Chung	Korea, Inje, <i>YDKim & SHCho s.n.</i> (KB)	JN603228
<i>A. purpureaefolia</i> Chung	Korea, Jiri-san, <i>BYLee 101008-1</i> (KB)	JN603229
<i>A. purpureaefolia</i> Chung	Korea, Pyeongchang, <i>WTLee et al. 1557</i> (KB)	JN603230
<i>A. amurensis</i> Shish.	China, Jirin, <i>Xue et al.</i> (2007)	DQ263581
<i>A. czernaevia</i> (Fisch. & C.A. Mey.) Kitag.	Korea, Daeam-san, <i>BYLee 100619-1</i> (KB)	JN603210
<i>A. czernaevia</i> (Fisch. & C.A. Mey.) Kitag.	Korea, Pyeongchang, <i>SHY 1817</i> (KB)	JN603211
<i>A. cartilaginomarginata</i> (Makino ex Y. Yabe) Nakai	Korea, Goesan, <i>SCKo 411</i> (KB)	JN603207
<i>A. cartilaginomarginata</i> (Makino ex Y. Yabe) Nakai	Korea, Pocheon, <i>WKPaik 1092</i> (KB)	JN603208
<i>A. cartilaginomarginata</i> (Makino ex Y. Yabe) Nakai	Korea, Gaya-san, <i>BYLee 100829-1</i> (KB)	JN603209
<i>A. decursiva</i> (Miq.) Franch. & Sav.	Korea, Modo, <i>JEHan s.n.</i> (KB)	JN603217
<i>A. decursiva</i> (Miq.) Franch. & Sav.	Korea, Chilbo-san, <i>BYLee 101016-1</i> (KB)	JN603215
<i>A. decursiva</i> (Miq.) Franch. & Sav.	Korea, Jugyeob-san, <i>WKPaik 1088</i> (KB)	JN603216
<i>A. gigas</i> Nakai	Korea, Daegwanryeong, <i>BYLee 090909-8</i> (KB)	JN603218
<i>A. japonica</i> A. Gray	Korea, Seogwipo, <i>SKim 1191</i> (KB)	JN603219
<i>Peucedanum hakuunense</i> Nakai	Korea, Bongrim-san, <i>SGKwon & WHKim 1085</i> (KB)	JN603233
<i>P. terebinthaceum</i> (Fisch. ex Trevir.) Fisch. ex Turcz.	Korea, Daegwanryeong, <i>BYLee 090908-2</i> (KB)	JN603232
<i>P. japonica</i> Thunb.	Korea, Deogjeogdo, <i>BYLee 090901-1</i> (KB)	JN603231

of PAUP*. All searches were conducted with 100 random-addition replicates using tree bisection-reconnection (TBR) branch swapping. To identify weakly supported nodes, decay analyses (Bremer, 1988) were conducted until tree storage memory was exhausted. A bootstrap analysis was performed using 100 resampled data sets. All trees were rooted with three accessions of the genus *Peucedanum*, the most closely related taxa to the genus *Angelica*.

Results

ITS sequence analysis

Alignment of all 29 complete ITS 1 and ITS 2 sequences of *Angelica* and its outgroups resulted in a matrix of 441 characters. On average, the ITS 1 region was shorter than ITS 2. The length of ITS 1 in all of the *Angelica* taxa surveyed was 216 bp, except in *A. czernaevia* collected at Daeam-san (215 bp). The length of the ITS 2 region ranged from 219 bp in *A. czernaevia* collected at Daeam-san to 222 bp in *A. japonica* (mean : 220.9 bp). Overall length variation of both spacer regions across all 26 accessions of *Angelica* ranged from 434 bp to 438 bp. These sizes are comparable to values reported for other Apiaceae (Downie et al., 1998; Lee and Downie, 1999; Lee et al., 2010). Of the 441 initial alignment positions, 70 (15.9%) were potentially parsimony-informative, 351 (79.6%) were constant, and 20 (4.5%) were autapomorphic. Both spacers contributed comparable numbers of informative nucleotide substitutions to the phylogenetic analysis. The ITS sequence divergence values among *Angelica* species ranged from 0.2% (between *A. purpureaefolia* at Daeam-san and Jiri-san and *A. amurensis*) to 7.2% (between *A. japonica* and *A. dahurica*, and *A. decursiva* and *A. dahurica*). Intraspecific nucleotide differences were also detected in several species of *Angelica*. ITS sequence divergence among accessions of *A. amurensis* ranged from identity to 0.2% between materials collected in the Ussuri area and three other sites in Korea. Other differences of 0.2% were detected among accessions of *A. cartilaginomarginata*. The 5.8S subunits of 23 *Angelica* taxa were constant in their length (162 bp), but those of three accessions of *A. cartilaginomarginata* were 163 bp in length. Sequence differences of the 5.8S subunits among *Angelica* taxa ranged from identity to 3 bp. Intraspecific nucleotide differences of 1 bp were found among *A. amurensis* accessions (0.6%), while interspecific differences of the 5.8S subunit were not observed among *A. polymorpha*, *A. decursiva*, *A. gigas*, *A. czernaevia*, *A. japonica*, and *A. cartilaginomarginata*.

Phylogenetic analyses and resolution

Results of the analyses of the combined ITS 1 and ITS 2 nucleotide data sets are presented in Fig. 1; separate analyses

of each spacer region alone were not conducted. Parsimony analysis of the 29 combined ITS sequences using equally weighted character states resulted in six maximally parsimonious trees, and the consensus of these six trees with accompanying bootstrap and decay values is presented in Fig. 1. The consensus tree had a length of 119 steps, consistency index (CI) of 0.85 and 0.82, with and without uninformative characters, respectively, and a retention index (RI) of 0.93. In each of the six parsimonious trees, three major groups of taxa were discernable, each without polytomy. The first group included *A. polymorpha*, *A. genuflexa*, *A. dahurica*, *A. amurensis*, *A. czernaevia*, and *A. cartilaginomarginata*. The second group included *A. decursiva* and *A. gigas*, and the third group comprising only *A. japonica* was a sister taxon to the clade including the first and second groups.

Discussion

Taxonomic treatment of *Angelica purpureaefolia* based on ITS sequence-based phylogeny

The major objective of this study was to ascertain the taxonomic status and position of *A. purpureaefolia*, which is endemic to Korea. *A. purpureaefolia* was first recognized as an independent taxon based on the presence of purple colors at the base of leaf petioles, and can be identified by the foul smell of the root (Chung, 1956). However, the species name should be treated as a nomen nudum because no written description in Latin and no designation of type specimens exist. Furthermore, *A. purpureaefolia* has often been confused with *A. anomala*. However, *A. anomala* is clearly distinguished from other Korean species of *Angelica* by the presence of a pubescent or spinulose leaf sheath (Pan and Watson, 2005). On the basis of the ITS-derived phylogeny, *A. purpureaefolia* was not distinguished from *A. amurensis*. As shown in Fig. 1, the phylogeny did not support the monophyly of *A. purpureaefolia*, revealing no synapomorphic characters. None of the six parsimonious trees supported separation of *A. purpureaefolia* from *A. amurensis*. This result was consistent with Feng et al. (2009) molecular systematics investigation of *Angelica* and allied genera from the Hengduan Mountains of China based on nrDNA ITS sequences. Their strict consensus tree derived from maximum parsimony analyses showed polytomous relationships among *A. amurensis*, *A. cincta*, *A. sachalinensis*, and *A. purpureaefolia* (Feng et al., 2009). Comparisons of sequence pairs across both spacer regions in the present study resulted in divergence values and revealed identity between *A. amurensis* and *A. purpureaefolia* at Mt. Daeamsan and Mt. Jirisan. Although some variation of sequence divergence between *A. amurensis* and the other two accessions of *A. purpureaefolia* was shown, from 0.23% to 0.46%, the

Fig. 1. The strict consensus of six minimal length 119-step trees derived from equally weighted maximum parsimony analysis of 29 combined nuclear rDNA ITS1 and ITS2 sequences from the genus *Angelica* and outgroups (CIs with and without uninformative characters = 0.85 and 0.82, respectively; RI = 0.93). Numbers above nodes indicate the number of times a monophyletic group occurred in 100 bootstrap replicates; decay values are presented below.

sequence differences between these two species were quite low compared to species level divergence of other taxa belonging to Apiaceae. Among species of *Agrocharis* and *Lisaea*, pairwise nucleotide divergences ranged from 0.3% to 0.5% and 0.8% to 1.0%, respectively (Lee and Downie, 1999). However, species' delimitations in these two genera are based on fruit secondary

spines that show a high degree of morphological variation. Thus, these characters are only of limited value for delimiting species' boundaries within these genera. The larger and more variable shapes of the secondary spines are considered to have evolved independently in these spiny-fruited umbelliferous plants as an adaptation to locally changing environmental conditions for fruit

Fig. 2. Densely distributed hispidulous hairs on a ray (left) and pedicel (right) that were characters in identifying *A. amurensis* (a sample collected at Daeamsan). Lines are 1 mm long. Photo taken by J.-H. An.

dispersal (Lee, 2002). With the exception of the above genera, pairwise nucleotide divergence of infrageneric species within the umbel family varied in 2.3~5.9% of *Orlaya*, 0.8~6.9% of *Torilis*, and 2.3~6.9% of *Sium* (Lee and Downie, 1999; Lee, 2002; Lee et al., 2010). Genetic divergence among *Angelica* species in Korea, with the exception of *A. purpuraeifolia*, ranged from 2.7% to 7.3%. Therefore, *A. purpuraeifolia*, described as a new species by Chung (1956), might be identical to *A. amurensis* because the sequence divergence between their ITS regions ranged from 0 to 0.46%. Morphological characters also indicated that these two taxa were identical. Densely distributed hispidulous hairs on both rays and pedicels that were characters in identifying *A. amurensis* (Shishkin, 1951) were also found in *A. purpuraeifolia* (Fig. 2). From these molecular and morphological characters, *A. purpuraeifolia* Chung should be treated as a synonym of *A. amurensis* Shishkin.

Nuclear rDNA ITS regions as potential barcoding loci for identifying Korean *Angelica*

Another objective of this study was to assess the potential for using ITS sequences as DNA barcodes for identifying species levels of Korean *Angelica*. Barcode sequence regions should meet three criteria: universality, sequence quality, and discriminatory power (CBOL Plant Working Group, 2009). Therefore, barcode sequences should be relatively easy to amplify using one pair of universal primers. The DNA barcode should also be good at species delimitation. Thus, barcode regions should possess higher interspecific than intraspecific divergence. Recently, the CBOL Plant Working Group recommended the combined sequences of both *rbcL* and *matK* as standard barcodes for vascular plants. However, previous studies have suggested that nuclear rDNA ITS regions exhibit higher interspecific divergence than chloroplast DNA regions in many groups of vascular plants (Chase et al., 2005; Kress et al., 2005; Sass et al., 2007; Chen

et al., 2010). These studies suggested that ITS regions were excellent for species identification, but might be rejected as universal barcodes due to their low efficiency of PCR amplification. Although universal primers for ITS have not been successfully identified for broad taxonomic groups covering all vascular plants, algae, and fungi, ITS regions should be one of the best candidates for barcodes in identifying species within vascular plant families. Previous studies have suggested the value of ITS regions in identifying species of the medicinal plant groups of the family Apiaceae (Downie et al., 1998; Lee and Downie, 1999; Lee, 2002; Spalik and Downie, 2006; Feng et al., 2009), and the present study has also demonstrated the utility of ITS sequences as DNA barcodes for distinguishing among species of the genus *Angelica*. To assess the utility of ITS regions in separating Korean species of *Angelica*, we analyzed multiple accessions of *A. polymorpha*, *A. dahurica*, *A. purpuraeifolia*, *A. czernaevia*, *A. cartilaginomarginata*, and *A. decursiva* collected in diverse areas. Lower sequence divergence or identity among multiple accessions of the same species of the Korean *Angelica* and phylogenetic reconstruction (Fig. 1) demonstrated that the ITS regions were suitable markers for taxonomic discrimination among the species. This suggests that *Angelica* species in Korea may be easily identified by informative variation of sequences in their ITS regions. The results of these analyses, in conjunction with those obtained from a concurrent DNA barcoding study (Lee et al., unpubl. data) using chloroplast DNA regions, will be used to revise the taxonomy of the Korean *Angelica* and its allies.

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