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Phylogeny of *Dystaenia* in subfamily Apioideae (Family Apiaceae) based on ITS sequences

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Abstract

Phylogenetic study of *Dystaenia* which consisted of two species, *D. takesimana* from Korea and *D. ibukiensis* from Japan, was hampered because of its isolated distribution and relatively recent description. In this study, the phylogenetic position of two species of *Dystaenia* in the tribe Apieae of subfamily Apioideae (family Apiaceae) was examined using ITS sequence of nuclear rDNA. The ITS sequences data indicate that *Dystaenia* is a monophyletic group which is most closely related to *Endressia* and *Seseli*. However phylogenetic affinity of *Dystaenia* with two taxonomically related genera, *Angelica* and *Ligusticum*, was not observed. Relatively high sequence divergence value between *D. ibukiensis* and *D. takesimana* implies that they are all distinct species.

Key words: *Dystaenia, Angelica, Ligusticum,* ITS sequence, phylogeny.

Dystaenia Kitagawa was described as a separate taxon by Kitagawa (1937), because of the presence of thick fruit wings and distinct calyx-teeth (Ohwi, 1984). It consists of two species; *D. takesimana* (Nakai) Kitagawa from Ullung Island, Korea and *D. ibukiensis* (Yabe) Kitagawa from north-western part of Honshu, Japan. Originally, *D. takesimana* was described as a new species of

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genus *Angelica* L. by Nakai (1918), and *D. ibukiensis* was descirbed as a member of genus *Ligusticum* L. by Yabe (1902). *Dystaenia* has been left out in the monographs (Drude, 1898; Melchoir, 1964) or in the phylogenetic study (Downie and Katz-Downie, 1996; Plunkett *et al.*, 1996) of Apiaceae, because of its isolated distribution and relatively recent description (Kitagawa, 1937). Consequently, the systematic position of the genus within the Apiaceae is in question.

Recently, *Dystaenia* was considered as congeneric with genus *Ligusticum* based on morphological characters (Hiroe, 1979). Kim *et al.* (1994) reported that *Angelica s.s.* might have close phylogenetic relationship with *Dystaenia* based on the analyses of ITS sequence. The molecular study, however, focused on the systematic position of *Angelica* with limited taxon sampling. In the most recent taxonomic treatment of the Apiaceae (Pimenov and Leonov, 1993), *Angelica* was positioned in the tribe Angeliceae, whereas *Dystaenia* was placed in the tribe Apieae with *Ligusticum*, with the following comment: "The biggest tribe, Apieae, is so large that the relationships among its genera remain unclear".

ITS regions are known to have phylogenetic utility at the interspecific (Baldwin, 1993; Kim and Jansen, 1994) or intergeneric (Baldwin, 1992; Suh *et al.*, 1993) levels, because of its relatively fast evolutionary rate (Baldwin *et al.*, 1995). In this study, by analyzing the ITS sequences, we reexamined the phylogenetic affinity of genus *Dystaenia* with other genera of subfamily Apioideae (Apiaceae), especially with two taxonomically related genera, *Ligusticum* and *Angelica*.

Materials and Methods

Plant samples, PCR amplification and sequencing. The collection and voucher information of the three species from which ITS region was newly sequenced are shown in Table 1. Living materials of *Dystaenia ibukiensis* (Yabe) Kitagawa and *D. takesimana* (Nakai) Kitagawa were collected from the greenhouse of Chonbuk National University, Chonju. *Ligusticum acutilobum* Sieb. *et* Zucc. was collected from the herb garden of Rural Development Administration in Suwon, Korea.

Total genomic DNAs of the three species were isolated from fresh leaf materials using the modified methods of CTAB procedures (Doyle and Doyle, 1987; Choi *et al.*, 1996). The EtOH precipitated DNAs were further purified by ultracentriguation in a CsCl-EtBr gradients (Sambrook *et al.*, 1989).

The nucleotide sequence of ITS (internal transcribed spacer) region of nuclear

rDNA was determined directly from the PCR products using the snap-chill method (Winship, 1989). PCR amplication was carried out with 100μl PCR mixture containing about 100ng of genomic template DNA, 2.5 units of *Taq* (Promega Co.), 200μM dNTPs, 4.0mM $MgCl_2$, 50 *p*mole of primers (ITS1a and ITS4) desinged by White *et al.* (1990). The thermal cycler was programmed to perform an initial 3 minutes denaturation at 95℃, 1 minute annealing at 50℃, 1 minute extention at 72℃. This was followed by 30 amplification cycles with 1 minute denaturation at 95℃, 1 minute annealing at 50℃, and 1 minute extention at 72℃. Finally, the product was terminated with final extention for 10 minutes at 72℃ and soaking at 15℃. To remove unused amplifying primers and dNTPs, the PCR product was purified by Geneclean kit(Bio 101) according to the manufacturer's instruction. The purified double stranded DNA was sequenced using sequenase(ver 2.0, USB), ^{35}S and 4 primers(Table 2).

Sequence analyses and phylogenetic analyses. The sequence boundaries of ITS1, the 5.8S coding region, and ITS2 were determined by comparison to known sequences (Baldwin, 1992; Suh *et al.*, 1993; Kim and Jansen, 1994). The ITS sequences of *Dystaenia* and *Ligusticum* were combined with 20 prealigned ITS sequences

Table 1. Species used for ITS sequence analyses of *Dystaenia* and *Ligusticum*. The voucher specimens were deposited at the herbariums of JUN and AJOU.

Species	Voucher specimens	Collection site
Dystaenia ibukiensis	T. Kawakito(JNU)	Japan, Saruyama, Ishigawa, without collection date
Dystaenia takesimana	B.-Y.Sun(JNU)	Korea, Dodong, Ulneung Isl. 14 Oct. 1993
Ligusticum acutilobum	Choi & Kim(AJOU 13825)	Korea, Suwon(cultivated) 21 Sept. 1997

Table 2. The sequences of primers used in this study

Primers		Sequences
Forward primers	ITS1a	5′-GGAAGGGAAGTCGTAACAAGG-3′
	ITS3	5′-GCATCGATGAAGAACGCAGC-3′
Reverse primers	ITS2	5′-GCTGCGTTCTTCATCGATGC-3′
	ITS4	5′-GCTGCGTTCTTCATCGATGC-3′

from other Apiaceous species which correspond to *Myrrhidendron-Aethusa* clade *sensu* Downie and Katz-Downie (1996). The G+C contents and the degrees of sequence divergence were calculated using Seqspeak (ver. 1.0) and MEGA (ver. 1.0, Kumar *et al.*, 1993), respectively. The phylogenetic trees were reconstructed by the maximum parsimony method implemented in PAUP (ver. 3.1, Swofford, 1993) using the heuristic searches with MULPARS option, 100 random entries of the taxa, and TBR branch swapping. Bootstrap analyses (Felsenstein, 1985) were performed to evaluate the degree of support for given clades.

a. The alignemnt of ITS 1 sequences

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Ligusticum ac   TCGAATCCTG CAATAGCAGA ATGACCCGCT AACACGTCAA CATTTTGGGC GAGCGTCGGG  [ 60]
Dystaenia tak   .......... .......... .......... ....T..... ..A.....A. A..T..T...  [ 60]
Dystaenia ibu   .......... .......... .......... ....T..... ..A.....A. A..T......  [ 60]

Ligusticum ac   GGGCCTCGGT CTCCTGTCTG CGAATCCCTG GTAGGTGGCC ACTCCCGGGT GGCCACTGGC  [120]
Dystaenia tak   .......... .......A.. .......... .......... .......... ..........  [120]
Dystaenia ibu   ..A....... .......A.. .......... .......... .......... ..........  [120]

Ligusticum ac   CTGCAAAATC ATTCGGGCGC GGAATGCGCC AAGGACCTTA AAACTGAATT GTACGTCCGT  [180]
Dystaenia tak   ..C....... ...T...... ......T... .......... .......... .....T...  [180]
Dystaenia ibu   ..C....... ...T....A. ......T... .......... ...T...... ..........  [180]

Ligusticum ac   ATCC-GTTAG CGGGCACCGG CGTCATTCCA AAACAC  [215]
Dystaenia tak   ....C..... ......G... .......... ......  [215]
Dystaenia ibu   ....C..... ......G... .......... ......  [216]
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b. The alignment of ITS 2 sequences

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Ligusticum ac   ATCGTCTTGC CCACAAACCA CTCACACCTG AGAAGTTGTG CCGGTTTGGG GCGGAAACTG  [ 60]
Dystaenia tak   .......... ..C....... .....G.... .......... .......... .....T..  [ 60]
Dystaenia ibu   .......... ..C....... ..G..G.... .......... .......... .....T..  [ 60]

Ligusticum ac   GCCTCCCGTA CCTTGTCGTG CGGTTGGCGG AAAAACGAGT CTCCGGCGAC GGACGTCGCG  [120]
Dystaenia tak   .......... .......... .......... .......... .....T.... ..........  [120]
Dystaenia ibu   .......... .......... .......... .......... .....T.... ..........  [120]

Ligusticum ac   ACATCGGTGG TTGTAAAAGA CCCTCTTGTC TTGTCGTGCG AATCCTCGTC ATCTTAGCGA  [180]
Dystaenia tak   .......... .......... .......... .......... .......... ..........  [180]
Dystaenia ibu   .......... .......... .......... .......... .......... ..........  [180]

Ligusticum ac   GCTCCAGGAC CCTTAGGCAG CACACACTCT GTGCGCTTCG A   [221]
Dystaenia tak   ...T...... ...G...... .......... .......... .   [221]
Dystaenia ibu   ...T...... .......... .......... .......... .   [221]
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Fig. 1. Complete and aligned sequences of ITS1(a) and ITS2(b) for the three species (*Ligusticum acutilobum*, *Dystaenia takesimana*, and *D. ibukiensis*) which were newly sequenced from this study. Dots indicate the same nucleotide as above sequences. Bar means a gap between the sequences. Nucleotide substitutions are shown as A, T, G, C.

Results

Sequence analyses. ITS regions of three species (*Dystaenia takesimana, D. ibukiensis,* and *Ligusticum acutilobum*) were newly sequenced from this study(Fig. 1). Complete and aligned sequences of ITS1 and ITS2 for the three species are provided in Figure 1, and their characterstics are summarized in Table 3.

The nucleotide sequences reported in this paper will appear in the DDBJ/EMBL/GenBank databases with the accession ncembers AB013873, AB013857, and AB013856 respectively. The registered numbers of each sequences in Gene-In of BRIC are 10014, 10018, and 10021, respectively.

Total size of ITS was 437 bp in *Dystaenia ibukiensis* and was 436 bp in *D. takesimana* and *Ligusticum acutilobum*. The size difference between *D. ibukiensis* and the other two species was due to deletions in ITS1 of the latter two species. ITS2 was longer than ITS1 by 5-6 bp in all three species. Proper alignment with previously published sequences introduced 12-13 gaps for the three species, and resulted in a matrix of 449 bp(the data matrix not shown). The G+C contents ranged from 52.3% (ITS1 of *D. ibukiensis*) to 57.7% (ITS1 of *L. acutilobum*). The G+C contents of ITS1 and ITS2 in both species of *Dystaenia* were very similar (Table 2).

Two species of *Dystaenia* exhibited eight nucleotides difference and sequence divergence value of 0.0195 which was calculated by the Kimura's 2 parameter method in MEGA (Kumar *et al.*, 1995). The sequence divergence values of complete data set ranged from 0.0170 to 0.0245 (data not shown). The lowest divergence value was observed between *Ligusticum* and *Angelica*.

Phylogenetic analyses. The integration of the three new sequences with the published and prealigned sequences from 20 Apiaceous genera representing the *Myrrhidendron-Aethusa* clade *sensu* Downie and Katz-Downie (1996) resulted in a

Table 3. Size and G+C contents of ITS1 and ITS2 of nuclear ribosomal DNAs in *Dystaenia* and *Ligusticum*.

Species	ITS1		ITS2	
	Size	G+C(%)	Size	G+C(%)
Dystaenia ibukiensis	216	52.3	221	57.0
Dystaenia takesimana	215	53.5	221	57.5
Ligusticum acutilobum	215	57.7	221	57.5

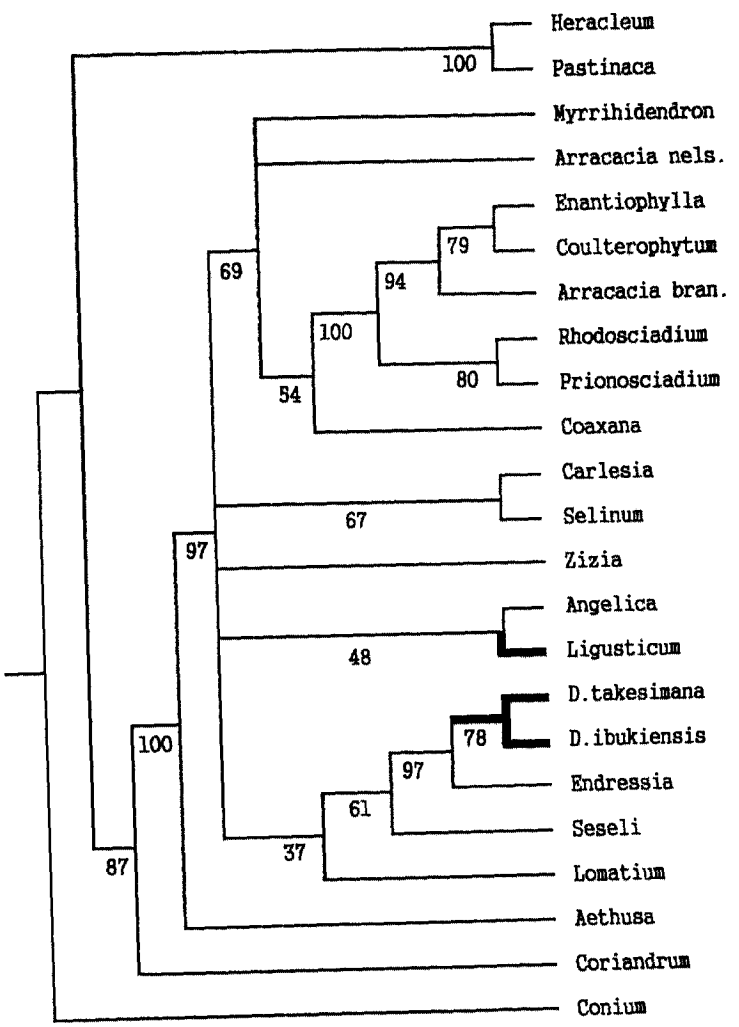

Fig. 2. Strict consensus tree of the 50 maximally parsimonious 315-step trees derived from equally weighted parsimony analysis of combined ITS 1 and ITS 2 sequences(CI excluding uniformative characters=0.618, RI=0.710). Numbers above the node mean the number of times a monophyletic group occurred in one hundred bootstrap replicates. The generic name of taxa are followed Downie and Katz-Downie(1993). The clade of *Dystaenia ibukiensis, D. takesimana,* and *Ligusticum acutilobum* which are analysized in this study are provided with bold lines.

total of 449 sites aligned (data not shown). Among the 449 sites, 182 sites were variable, of which 99 sites were phylogenetically informative. Maximum Fitch parsimony analyses generated 26 equally parsimonious trees, whose strict consensus tree with accompanying bootstrap values is shown in Figure 2. The tree has a length of 315 steps with CI (excluding uninformative sites) of 0.618 and RI of 0.710.

The strict consensus tree produced many unresolved or poorly supported generic relationships, especially for the three genera; i.e. *Ligusticum*, *Angelica* and *Dystaenia* in which we are interested. Two *Dystaenia* species formed a monophyletic group with bootstrap value of 78. The genus grouped with *Endressia* Gay with relatively strong support (bootstrap value of 97). *Seseli* L. positioned as sister clade to the *Dystaenia* and *Endressia* clade. *Ligusticum* and *Angelica* formed a weakly supported clade (bootstrap value of 48), which exhibited no direct affinity with the *Dystaenia* clade.

Discussions

The major intention of this study was to evaluate the relationships among *Dystaenia*, *Ligusticum* and *Angelica* within tribe Apieae of subfamily Apioideae (family Apiaceae). The results, however, did not support the phylogenetic affinity of genus *Dystaenia* with *Angelica* which has been suggested by Kim *et al.* (1994) based on ITS sequence analysis. The close relationship between *Dystaenia* and *Ligusticum* which has been suggested by Hiroe (1979) based on morphology is not observed, either. Instead, the *Dystaenia*, which was confirmed as monophyletic genus in this study, was grouped with *Endressia* and this clade was further grouped with *Seseli*. Therefore, Kitagawa's taxonomic treatment, the separation of genus *Dystaenia* from *Angelica* and *Ligusticum*, was strongly supported by the molecular data(Fig. 2).

The molecular data also suggest the possibility that two phylogenetically close but geographically disjunctly distributed genera, *Dystaenia* in Korea and Japan, and *Endressia* in the Pyrenees only (Tutin, 1974), might have been diverged from ancestral *Seseli s. l.*(including *Libanotis*), which distributes widely from Medditerenean Sea, via the Middle East and China to Northeastern Asia (Hiroe, 1979). These interpretation is supported by two considerations; one is that nearly 80% of extant apioids are native to Eurasia (Mathias, 1971) and the other is that the apioids migrated northward through Africa (via the Middle

East and the Caucasus) into Eurasia, whereupon they experienced their greatest radiation (Plunkett *et al.*, 1996). However, more rigorous taxon sampling (especially from *Seseli*) and solid morphological evidence are needed to evaluate the systematic position of genus *Dystaenia* with high confidency.

Dystaenia takesimana was morphologically similar to its allopatric *D. ibukiensis*, and Hiroe(1979) considered that both species were conspecific. However, molecular data revealed that two species were phylogenetically closely related, but differed in ITS sequence by eight bases, with the nucleotide divergence value of 1.95%(Fig. 1). This value was somewhat similar compared to previous report; 1.3% between *Caulophyllum thalictroides* (L.) Michx. and *C. robustum* Maxim., and 1.6% between *Penthorum sedoides* L. and *P. chinense* Pursh (Lee *et al.*, 1994). The height of *D. takesimana* was 1.5-2 m tall, compared with those of *D. ibukiensis* 0.3-0.7 m (Kitagawa, 1937). Sun *et al.* (1997) indicated that the two species were well distinguished from each other based on the morphology of cell wall of abaxial leaf surface. The morphological evidence and relatively high sequence divergence value in the ITS imply that the two taxa are all distinct species.

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ITS 염기서열에 의한 섬바디속(미나리아과)의 계통분석

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요 약

섬바디속(미나리아과)은 동아시아 고유속으로서 울릉도의 섬바디[*Dystaenia takesimana* (Nakai) Kitagawa]와 일본의 *D. ibukiensis* (Yabe) Kitagawa 두 종으로 구성되어 있다. 본 연구에서는 섬바디속과 왜당귀속(*Ligusticum*)의 rDNA에서 ITS 염기서열을 분석하여 미나리아과 내에서 섬바디속의 계통을 분석하였다. 그 결과 섬바디속은 단계통군이며 *Endressia*와 *Seseli* 두 속과 가장 근연속인 것으로 나타났으며, 분류학적으로 가까운 속으로 알려진 참당귀속(*Angelica*)과 왜당귀속과는 다른 계통인 것으로 분석되었다. 한편 섬바디속내의 *Dystaenia ibukiensis*와 *D. takesimana* 사이의 분기도는 매우 높아서 뚜렷이 독립된 종인 것으로 확인되었다.

주요어 : 섬바디속, 당귀속, 왜당귀속, ITS 서열

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