

# Phylogenetic Contributions of Partial 26S rDNA Sequences to the Tribe Helleboreae (Ranunculaceae)

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**Monophyly and intergeneric relationships of the tribe Helleboreae, sensu Tamura, and related genera were studied using a 1,100-bp segment at the 5' end of the 26S ribosomal RNA gene. Forty-one OTUs, including eight species of the Helleboreae, were either directly sequenced or obtained from previous publications. Data were analyzed using distance and discrete character methods to infer phylogenetic relationships among the included taxa. The inferred phylogeny did not support monophyly of either Helleboreae or Cimicifugeae whose members were intermixed in our inferred phylogeny. This result is congruent with our previous study, which recommended against finely subdividing, suprageneric higher taxa within the R-chromosome group (subfamily Ranuncululoideae, sensu lato) until more molecular data were accumulated. Our data convincingly suggest the presence of the following three monophyletic groups: the *Cimicifuga* group (the clade of *Actaea*, *Cimicifuga*, *Souliea*, *Eranthis*, *Anemonopsis*, and *Beesia*), the *Trollius* group (the clade of *Trollius*, *Megaleranthis*, *Adonis*), and a clade including *Anemonopsis* and *Beesia*. Our data also suggest that *Trollius* and *Megaleranthis* might be congeners and *Eranthis* a paraphyletic group.**

The Ranunculaceae are a large flowering plant family, which include about 2,500 described species and 59 genera. Many species have attractive flowers and substances with strong physiological activity, and are thereby used as ornamental or medicinal plants (Tamura, 1993). This family is also of phylogenetic importance because it is an essentially herbaceous group within an otherwise woody clade of ancient angiosperms (Ro 1996; Ro et al., 1997). Despite their importance, the higher classification of the Ranunculaceae is in an unsatisfactory state (Ro et al., 1997). Based on morphology, different classifications within the family have been proposed by many authors without reaching a satisfactory consensus (Ro, 1996; Ro et al., 1997). This situation may be the combined outcome of the large size of the group and the fact that major morphological characters intergrade among many higher taxa (Ro et al., 1997).

Fortunately, recent developments of molecular data have provided new possibilities for proposing a sound classification of the Ranunculaceae (Johansson and Jansen, 1993; Johansson, 1995; Hoot, 1995; Kosuge et al., 1995; Jensen et al., 1995; Ro et al., 1997; Ro and McPherson, 1997). The results of these molecular

studies are not only highly congruent with each other, but also consistent with the well established portion of the classification based on morphology (Ro et al., 1997). Furthermore, these molecular studies have pinpointed cases of suspicious classification due to morphological homoplasies. For example, Hoot (1995) and Ro et al. (1997) independently found that the genera *Trollius* and *Adonis* were very closely related while previous morphological classification placed them in different subfamilies (see also Fig. 3). They also found that *Xanthorhiza* and *Coptis* were not actually closely related to *Aquilegia* or *Thalictrum*, but were likely to be an ancient lineage basal to most members of the Ranunculaceae (see also Fig. 3). Even though these studies did not include every single genus of the Ranunculaceae yet, some significant improvement of the higher classification of the family had been proposed based on molecular evidence (Jensen et al., 1995; Ro et al., 1997).

In an effort to improve the higher classification of the Ranunculaceae, we selected the tribe Helleboreae for the present molecular analysis. Since Candolle (1817) erected the Helleboreae, Hutchinson (1923) expanded the tribal limit including about half the genera of the Ranunculaceae. Later, Langlet (1932) narrowly defined the tribal limit based on karyotype data. He included subtribes Helleborinae (*Helleborus*, *Eranthis*) and Cimicifuginae (*Anemonopsis*, *Cimicifuga*,

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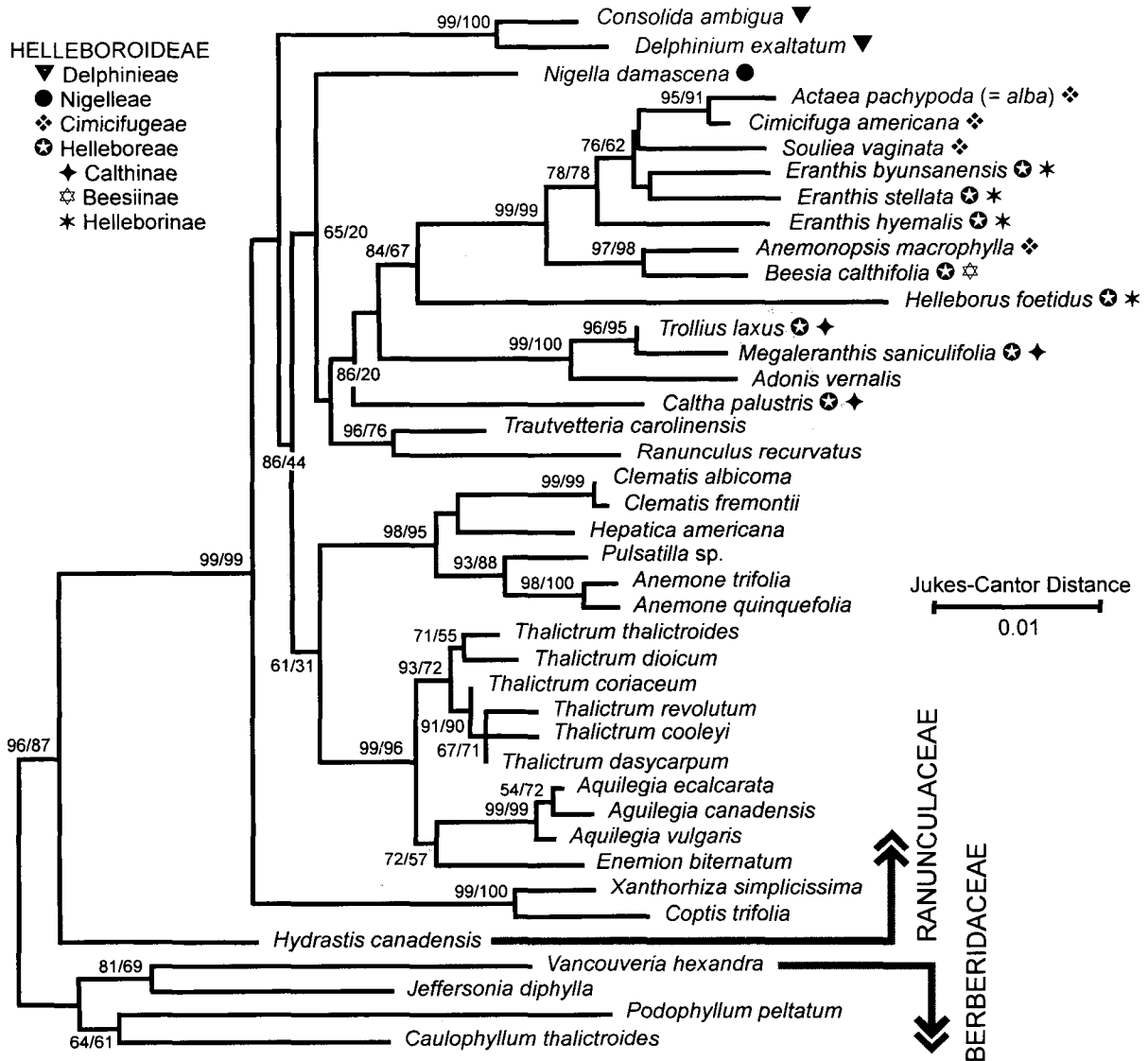
**Table 1.** List of taxa, source of samples, available voucher specimen numbers, and GenBank accession numbers

Species	Collecting source	Collection date	Voucher no. and source	GenBank accession no.
<b>Helleboreae</b>				
<i>Megaleranthis saniculifolia</i> Ohwi	Korea: Kangwon-do, Phyungchang-gun, Mt. Tae-gi	June 14, 1996	YU (K.-E. Ro 355)	AF 131285
<i>Beesia calthifolia</i> (Maxim. ex Oliver) Ulbr.	China: Sechuan Prov., Songapn-xian, Haung-long temple	July 3, 1990		AF 131290
<i>Eranthis byunsanensis</i> Sun	Korea: Chullabuk-do, Boan-gun, Mt. Naebyun	April 26, 1996	YU (K.-E. Ro 378)	AF 131286
<i>Eranthis stellata</i> Maxim.	Korea: Kangwon-do, Dunnae-myun, Whoengsung-gun, Mt. Tae-gi	May 19, 1996	YU (K.-E. Ro 386)	AF 131287
<b>Cimicifugae</b>				
<i>Anemonopsis macrophylla</i> Sieb. & Zucc.	Japan: Honshu Nico-city Jakko-Fall, Hanaish-cho	Sept. 4, 1993		AF 131289
<i>Souliea vaginata</i> (Maxim.) Franch.	China: Sechuan Prov., Songapan-xian, Haung-long temple	July 3, 1990		AF 131288

Species without voucher numbers were provided by K. Kosuge (Kobe University, Japan)

*Actaea*). Based on a comprehensive morphological analysis, Tamura (1995) proposed a revised tribal limit

(as in Fig. 3, right). He also implicated close relationships among the tribes Delphinieae, Nigelleae, Cimici-



**Fig. 1.** Ranunculaceae relationships inferred from a neighbor-joining analysis of Jukes-Cantor distances (940-bp after gaps and sites with missing data were removed). The first number is the Pc value from the standard error test (higher than 50%), and the second number is the Pb from the bootstrap test (2,000 replications).

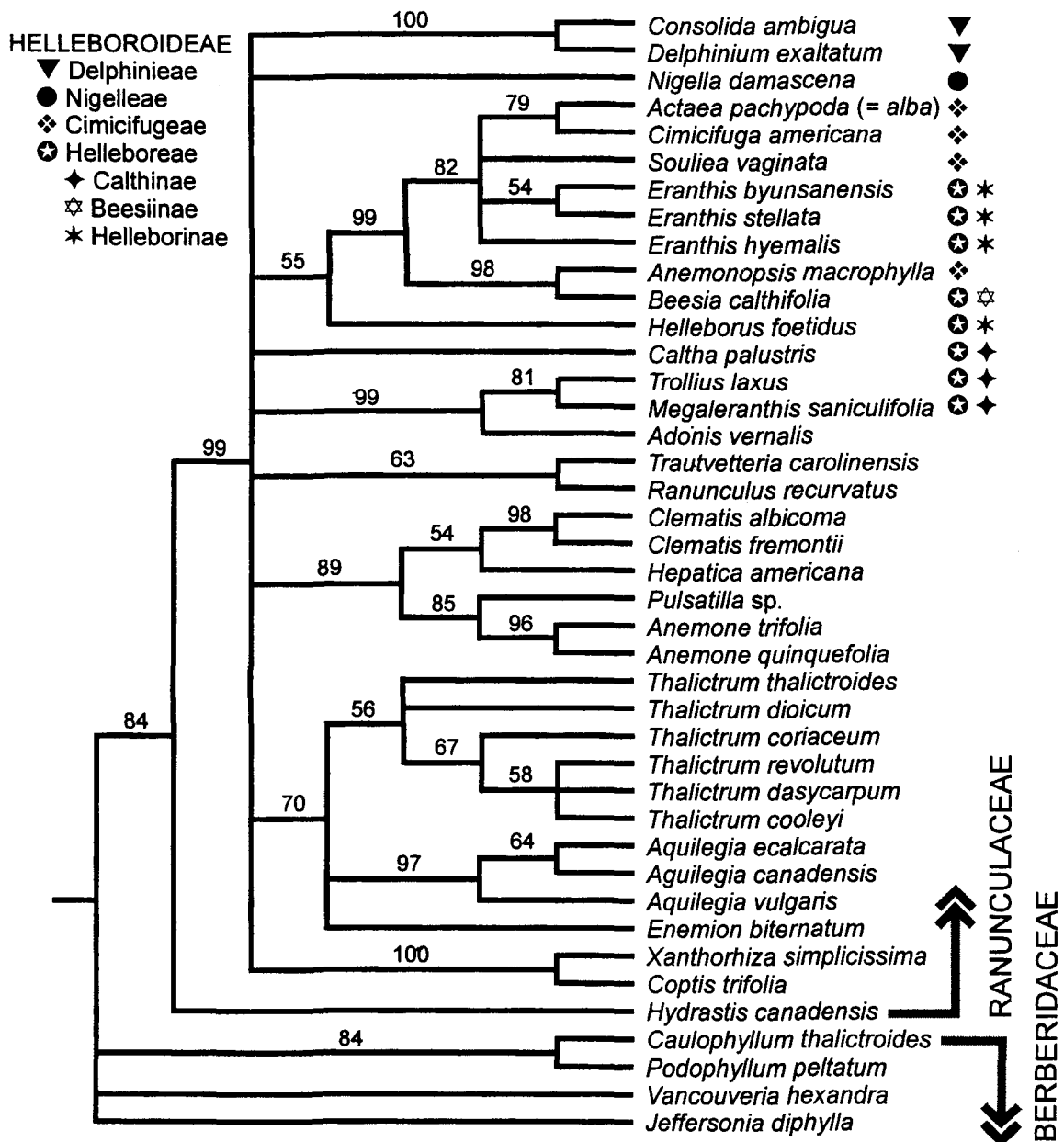


Fig. 2. Ranunculaceae relationships inferred from maximum parsimony analysis. The tree is a 50% majority-rule consensus tree from 500 bootstrap replications with heuristic search using PAUP 4.0b (940-bp after gap and sites with missing data were removed; MaxTrees setting=200). Numbers on branches indicate bootstrap P values higher than 50%.

fugeae and Helleboreae by placing them within the subfamily Helleboroideae. He justified this subfamilial grouping based on their fruit types and karyotypes.

The primary aim of this study was to test the monophyly of the tribe Helleboreae (sensu Tamura, 1995) and to investigate the relationships of the tribe within the subfamily Helleboroideae. We also tried to understand the intergeneric relationships within the tribe Helleboreae. For this study, we sequenced partial 26S rDNA, which was proven to be highly efficient in recovering intergeneric relationships within the Ranun-

culaceae (Ro et al., 1997).

#### Materials and Methods

We newly sequenced a segment approximately 1,100-bp long at the 5' end of the 26S ribosomal RNA gene for six species of Helleboreae and Cimicifugeae sensu Tamura (Table 1). These sequences were combined with the 35 published sequences by Ro et al. (1997). As a result, eight species of the Helleboreae plus seven species representing the other three tribes of

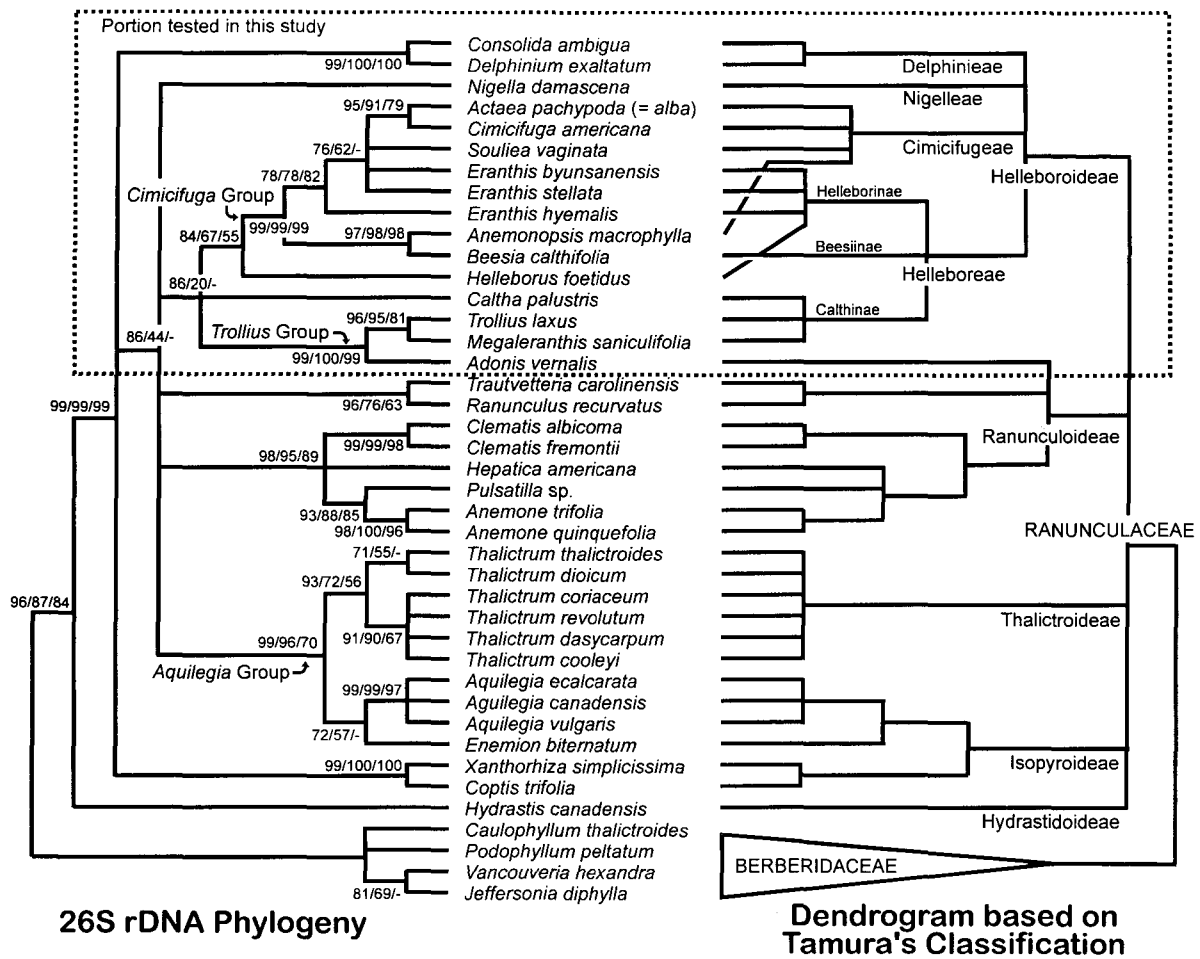


Fig. 3. Comparison between inferred molecular phylogeny and a conventional classification. The tree on the left is a neighbor-joining topology showing branches supported by an interior branch test value (Pc) greater than 70%. The first number is the Pc from the NJ tree, the second is the bootstrap value (Pb) from NJ tree and the third is the Pb from the MP tree. "-" indicates that the clade was not recovered by any particular analysis. The tree on the right is based on the classification by Tamura (1995), and his subfamily, tribe, subtribe names are listed.

the subfamily Helleboroideae, 22 species of the other four subfamilies, and four outgroup taxa (Berberidaceae) were analyzed (Fig. 3). DNA was extracted from freshly frozen or dried herbarium specimens using the method of Doyle (1991) with some modification. The technical details for PCR (Polymerase Chain Reaction) and sequencing reactions were described in Ro et al. (1997).

Alignment of the sequences was conducted using CLUSTAL W software (version 1.7, Thompson et al., 1997). Sites with missing data or gaps were excluded from all analyses. Neighbor-joining (NJ) analysis was performed using the MEGA software (Kumar et al., 1993). The Jukes-Cantor model of nucleotide substitution (Jukes and Cantor, 1969) was selected for the analysis based on the guideline of Nei (1991) for choosing the most appropriate distance measure. For maximum parsimony (MP) analysis, PAUP software (version 4.0b, Swofford, 1998) was used. The reliability of clustering pattern in trees was tested by bootstrapping in the cases of both NJ and MP trees and

by the standard error test for the internal branches of NJ trees (Rzhetsky and Nei, 1992); 2,000 bootstrap replications were used for the NJ tree and 500 bootstrap replications were used for the MP tree.

Voucher specimens are deposited in the Herbarium of the Department of Life Science, Yonsei University, Korea. Sequence data are deposited with GenBank (Table 1). GenBank Accession Numbers for the previously published sequences are listed in Ro et al. (1997).

### Results and Discussion

For each of the 41 species studied, we obtained the 1,020-bp aligned sequence, but the sequence length varied between 980 to 1,010 bp. After excluding sites with missing data or gaps, 940-bp were used for analysis. Among these 940 sites, 192 sites were variable and 124 were informative for parsimony analysis. The average proportion of A:T:C:G is 21.9:18.5:25.6:34.0

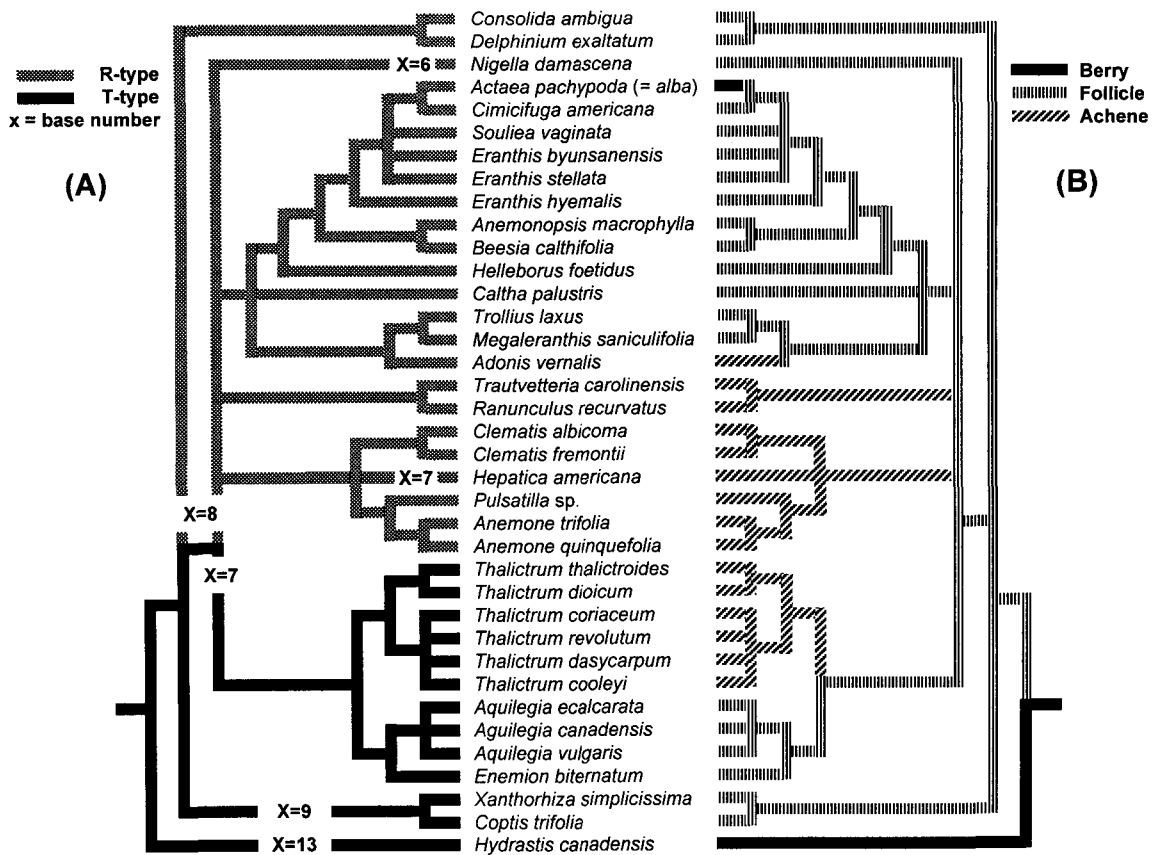


Fig. 4. Chromosome types and base number (A) and fruit types (B) mapped on the neighbor-joining topology supported by Pc values greater than 70% from the standard error test.

with a narrow standard error around the mean, but the nucleotide composition varied substantially in different portions within the sequences. Ro et al. (1997) discussed the pattern of nucleotide variability in conjunction with the G-C content, transition/transversion ratios, and evolutionary rate heterogeneity among lineages.

The inferred phylogenetic trees based on NJ (Fig. 1) and MP methods (Fig. 2) are highly congruent each other, especially for the branches with higher statistical supports. This result is almost identical with the previous molecular study based on the same gene region (Ro et al., 1997), except that additional six species brought about interesting insights into phylogenetic relationships of the tribe Helleboreae (Fig. 3). In the NJ tree, interior branch P values (Pc) from the standard error test tend to be higher than bootstrap P values (Pb). As discussed in Ro et al. (1997), we interpret Pc values greater than or equal to 95% as statistically significant and Pb values greater than 70% as generally informative. In the following discussion, we compared our molecular topology with the classification suggested by Tamura (1995) (Fig. 3).

Our data did not indicate monophyly of the subfamily Helleboreae, *sensu* Tamura (1995). This subfamily

was defined based on the combination of their follicle type fruits and R-type chromosomes. Instead, the mapping of these characters to our molecular topology indicates that these character states are likely to be plesiomorphic (Fig. 4). The R-type chromosome at least defines all the so-called R-chromosome group taxa (Helleboreae plus Ranunculoideae, *sensu* Tamura) (Fig. 4A). Furthermore, the follicle type fruit appears to be even more ancient than the chromosome type, defining the basal branch leading to all the subgroups of the Ranunculaceae excluding *Hydrastis* (Fig. 4B). Therefore, we need to recognize the more phylogenetically based subfamilial classification proposed by Ro et al. (1997). In this classification, Tamura's Helleboreae and Ranunculoideae were merged into the single subfamily Ranunculoideae.

Our molecular topology does not justify monophyly of the tribe Helleboreae either. Instead, this tribe should be viewed as a grade of taxa, which show similar morphological features indicated by Tamura (1995). The following are some important phylogenetic relationships, which have not been recognized by previous morphological analyses:

- (1) A sister-group relationship between *Anemonopsis*

and *Beesia* is strongly supported at 97-98% of all three statistical values (Fig. 3). These taxa are both defined as monotypic genera showing disjunct distributional ranges: *Beesia calthifolia* from China and northern Myanmar, and *Anemonopsis macrophylla* from Japan. Their close relationship has never been suggested before. Moreover, they are morphologically so distinct that they even have been placed in different tribes. Tamura (1990, 1995) classified *Beesia* under the monotypic subtribe Beesiinae within the tribe Helleboreae for having undivided leaves, racemiform inflorescence and a single follicle. On the other hand, he placed *Anemonopsis* in the tribe Cimicifugeae, for having a vigorous rhizome, compound leaves, and aggregated follicles. On the contrary to Tamura's classification, Yang et al. (1995) suggested that *Beesia* might be more closely related to *Cimicifuga* and its allied genera rather than to *Trollius* and its allied genera based on a karyotype study. This result is consistent with our inferred phylogeny, but they did not specify a close relationship between *Beesia* and *Anemonopsis*. The results from other molecular studies (Hoot 1995, Johansson 1995) are also consistent with our conclusion, but, unfortunately, they did not include enough taxa to show a finer resolution of the relationships. These molecular studies, at least, suggested close relationships among *Anemonopsis*, *Cimicifuga*, and *Actaea*. Our data strongly suggest non-monophyletic nature of the tribes Cimicifugeae and Helleboreae, *sensu* Tamura (1995). All the morphological key characters used to define the tribes are broadly overlapping among the included genera. We do find some morphological similarities between *Anemonopsis* and *Beesia* in having the vigorous rhizome, basal leaves, and loose racemiform inflorescence. These characteristics, however, are also found in other genera of the so-called R-chromosome group. Therefore, morphological evolution involving these key characters within the R-chromosome group (Fig. 4) appears highly homoplastic.

(2) We also recognize a larger monophyletic group including *Actaea*, *Cimicifuga*, *Souliea*, *Eranthis*, *Anemonopsis*, and *Beesia* (the *Cimicifuga* group hereafter). Statistical supports for this group are extremely high (99% for all three, statistical tests), but it has never been recognized as such. Instead, the *Cimicifuga* group includes all the members of the tribe Cimicifugeae plus only two genera of the tribe Helleboreae *sensu* Tamura (1995) (Fig. 3). This result is highly congruent with the previous molecular studies involving a limited number of taxa using chloroplast and nuclear genes (Johansson, 1995; Hoot, 1995; Ro et al., 1997). Because of their morphological divergences, it is very difficult to define the *Cimicifuga* group. They at least share vigorous thick rhizomes, follicles, and perennial habit, all of which appear to be symplesiomorphies. Within the *Cimicifuga* group, a close relationship between *Anemonopsis* and *Beesia* has been already discussed

above (sect. 1). In addition, we also find a close relationship between *Cimicifuga* and *Actaea*, which have different fruit types (Fig. 4). This relationship is also strongly supported by previous molecular studies (Johansson, 1995; Hoot, 1995; Ro et al., 1997; Compton et al., 1998a, b). In the study by Compton et al. (1998b), *Cimicifuga* and *Souliea* were synonymized with *Actaea*, but we believe that this nomenclatural act should be reconsidered because of the uncertain phylogenetic position of *Souliea* and the small number of outgroup taxa (*Eranthis hyemalis* and *Anemonopsis macrophylla* only) to test the monophyly of those three genera. The genus *Eranthis* currently includes nine species of spring ephemerals distributed in Eurasia and North America. Nakai (1937) described a separate genus, *Shibateranthis*, but Tamura (1987) later recognized two sections, *Shibateranthis* and *Eranthis*, within the genus *Eranthis*. Section *Shibateranthis* (*E. stellata* plus five species) is characterized by having swellings at the end of the bilobate petal, and section *Eranthis* (*E. hymalis* and *E. longistipitata*) also have the bilobate petal, but without the swellings. Sun et al. (1993) described *E. byunsanensis* from Korea, but this species could not be placed in either section of *Eranthis*, because it has simple cup shape petals instead of bilobate petals. Our data topologically supports the close relationship of this species to *E. stellata*, thus, suggesting that its simple petal might be a specific autapomorphy. Nevertheless, our data do not support the integrity of the genus *Eranthis*, but suggests the paraphyletic nature of the genus. An additional molecular approach involving more variable genes and more *Eranthis* species is needed to further resolve their relationships.

(3) A monophyletic group including *Trollius*, *Megaleranthis*, and *Adonis* is also strongly supported at 99-100% by all three statistical tests (Fig. 3 - *Trollius* group hereafter). This is surprising, because *Adonis* has been classified even in the different subfamily for having the achene type fruit instead of the follicle, and *Trollius* and *Megaleranthis* are grouped together with *Caltha* in the subtribe Calthinae for sharing some superficial similarities (Tamura, 1995). This again indicates the homoplastic nature of the morphological character evolution in the Ranunculaceae. Close relationship of *Trollius* and *Adonis* has also been strongly supported by previous molecular studies (Johansson, 1995; Hoot, 1995; Ro et al., 1997). *Megaleranthis* is a monotypic genus from Korea. Ohwi (1935) erected this genus, and later (Ohwi, 1937) combined it with *Trollius*. Tamura (1966, 1995), however, treated *Megaleranthis* as a separate genus due to its distinct morphological characteristic (a single cauline leaf close to the flower). In a palynological study involving *Megaleranthis* and 14 *Trollius* species, Lee (1989) did not support the separation of *Megaleranthis* from *Trollius*. Our data also shows a close relationship of *Megaleranthis* and *Trollius* (Fig. 3), but we need to analyze more *Trollius*

species to determine whether *Megaleranthis* should be synonymized with *Trollius* or not.

(4) Even though the genus *Helleborus* is the name-bearing taxon for the subfamily Helleboreoideae, the relationship of this genus to other members of the subfamily is not clear. Topologically, it might be a sister-group of the *Cimicifuga* group, but statistical supports are rather weak (84/67/55 - see Fig. 3). The phylogenetic position of *Caltha* is not resolved either.

Partial sequences from the nuclear 26S provide insights into the evolutionary history of the Helleboreae tribe and related taxa. For example, we recognize herein three robust monophyletic groups within the subfamily Helleboreoideae (sensu Tamura, 1995): the *Cimicifuga* group, the *Trollius* group, and the clade including *Anemonopsis* and *Beesia*. Our data also show some interesting relationships (i.e. positions of *Caltha* and *Helleborus*), which need to be tested in the future. As a result of our molecular study, we recommend not to recognize the tribes Cimicifugeae and Helleboreae as such, because they are non-monophyletic (Fig. 3). Furthermore, we are against finely subdividing suprageneric higher taxa within the R-chromosome Group (subfamily Ranunculaceae, sensu Ro et al., 1997) until more data are obtained. We believe that additional molecular studies involving more genes and taxa will greatly contribute to establish a precise higher classification of the Ranunculaceae.

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