

Towards a molecular phylogeny of *Apiaceae* subfamily *Apioideae*: additional information from nuclear ribosomal DNA ITS sequences

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Abstract: Evolutionary relationships among 116 representatives (80 genera) of *Apiaceae* (*Umbelliferae*) subfam. *Apioideae* were investigated by comparative sequencing of the two internal transcribed spacers of the 18S–26S nuclear ribosomal DNA repeat. The resultant phylogenies, inferred using maximum parsimony and neighbor-joining methods, clarified the relationships of several genera whose phylogenetic placements have heretofore been problematic. Comparisons between the phylogenies inferred and the distribution of several phytochemical (coumarins, flavonoids, and phenylpropenes) and morphological (stomates, pollen, and cotyledonary shape) characters were also made, revealing that many of these characters (like those morphological and anatomical characters of the fruit) are highly homoplastic. It is not surprising then that systems of classification of *Apioideae* based on these characters, particularly with regard to tribal and subtribal designations and relationships, are unsatisfactory. The results of recent serological investigations of the subfamily support several relationships proposed herein using molecular data.

Evolutionary relationships among those plants belonging to *Apiaceae* (*Umbelliferae*) subfam. *Apioideae* have been particularly difficult to resolve. This lack of knowledge stands in stark contrast to the large amount of attention this group has received over the past 30–40 years. Comparative data are available for a wide selection of characters (reviewed in HEYWOOD 1971a, and CAUWET-MARC & CARBONNIER 1982), paralleling the major systematic approaches or techniques developed over this course of time. The system of classification proposed by DRUDE (1897–1898) in ENGLER & PRANTL's 'Die natürlichen Pflanzenfamilien' is the most widely used today. More recent systems are available, such as those of KOSOPOLJANSKY (1916) and CERCEAU-LARRIVAL (1962, 1979), but because these are either limited in scope or more controversial than DRUDE's system, they have not been widely adopted. In his treatment of *Apioideae*, the largest of the three subfamilies

of *Apiaceae*, DRUDE followed KOCH (1824), DE CANDOLLE (1830), and ROMPEL (1895) in the emphasis of several characters, such as the presence or absence of calcium oxalate crystals in the cells of the fruit wall, the degree and orientation of fruit compression, the number, character, and distribution of mericarp ribs and dorsal vittae, the shape of the mericarp commissural face, and the nature of the endosperm. Serious doubts have been cast on the validity of using such characters to diagnose evolutionary relationships (HEYWOOD 1971b, 1982). Indeed, recent cladistic analyses of molecular data (DOWNIE & KATZ-DOWNIE 1996; DOWNIE & al. 1996, 1998; KONDO & al. 1996; PLUNKETT & al. 1996b; VALIEJO-ROMAN & al. 1998) support the earlier observations of many (e.g. THEOBALD 1971, DAVIS 1972, CRONQUIST 1982, HEDGE & al. 1987, SHNEYER & al. 1992), showing that DRUDE's system, particularly his tribal and subtribal designations, are unnatural. DRUDE's subfamily *Apioideae*, however, is evidently monophyletic (DOWNIE & al. 1996, 1998; PLUNKETT & al. 1996a, 1997; VALIEJO-ROMAN & al. 1998).

A curious feature about the *Apiaceae* (and that of many other early described temperate families containing an assortment of edible, medicinal, or poisonous plant; WALTERS 1961) is that many apioid tribes and subtribes are characterized by a small number of large genera (e.g. *Angelica*, *Ferula*, *Peucedanum*, *Pimpinella*, and *Seseli*), which comprise the greater part of the group in terms of species number, plus a large number of small genera, many of which are mono- or bitypic (HEYWOOD 1971b). Whether this pattern represents phylogeny accurately or is simply a reflection of taxonomic practice in subfam. *Apioideae* is not altogether clear. The affinities of these smaller genera, many of which have been described from the Old World, are largely unknown. HEYWOOD (1971b) supposed that although some of these small genera are of dubious value, many seem distinct and taxonomically isolated. To confound matters, some of these larger genera are likely artificial, held together by a few esoteric characters (HEYWOOD 1971b, LAVROVA & al. 1983, VASIL'eva & PIMENOV 1991, SHNEYER & al. 1995).

As part of our continuing investigations into the higher level relationships of *Apiaceae* subfam. *Apioideae*, we use the results of cladistic analysis of the two internal transcribed spacers of the 18S–26S nuclear rDNA repeat (i.e. ITS 1 and ITS 2) to address the following objectives: (1) To ascertain the historical relationships among available Old World representatives of the subfamily, including such genera as *Anethum*, *Exoacantha*, *Hansenia*, *Karatavia*, *Komarovia*, *Laser*, *Lecokia*, *Oedibasis*, *Parasilaus*, *Pyramidoptera*, and *Tommasinia* whose phylogenetic placements have heretofore been refractory or controversial. (2) To interpret patterns in the evolution of selected phytochemical (i.e. coumarins, flavonoids, and phenylpropenes), palynological, anatomical, and morphological characters. Although these characters have been surveyed widely in the subfamily, trends in their evolution and their reliability in demarcating taxonomic groups have rarely been considered outside of the framework of DRUDE's (1897–1898) classificatory system. Because this system is regarded by many as being highly unnatural, particularly with regard to its tribal/subtribal categories, we wanted to reevaluate these characters in a more rigorous phylogenetic context. (3) To compare our results to those inferred for the subfamily using serological techniques (SHNEYER & al. 1992, 1995). Many of the species examined by SHNEYER & al. were included in our study, thus the utility of

systematic serology, a tool which has seen relatively little use in plant systematic studies, in establishing relationships within *Apioideae* can be assessed.

Materials and methods

Plant accessions. One hundred and sixteen representatives of *Apiaceae* subfam. *Apioideae* were examined for nuclear rDNA ITS sequence variation. Complete ITS sequences for 49 accessions are reported here for the first time (Table 1); these were combined with 67 previously published ITS sequences (DOWNIE & al. 1998). Of the 80 genera examined, 27 (and possibly as many as 29) are monotypic, and 8 are bitypic (PIMENOV & LEONOV 1993). Although the selection of taxa was based primarily upon the availability of living material and, to a lesser extent, the taxonomic interests of our respective laboratories, many of the accessions chosen were included because they represented precisely the same species that were used in two serological investigations (SHNEYER & al. 1992, 1995), enabling a comparison between their results and ours.

Experimental strategy. Details of the DNA extractions, the PCR (polymerase chain reaction) amplifications (including primer locations and characteristics), and the DNA purification and sequencing strategies used are provided elsewhere (DOWNIE & KATZ-DOWNIE 1996). In summary, the sequence data were obtained through direct sequencing of double-stranded templates derived from the PCR procedure. Both spacer regions were sequenced in their entirety on both strands.

Data collection and analysis. The DNA sequences were aligned using the program CLUSTAL V (HIGGINS & al. 1992), and the resulting alignments were manually adjusted as necessary. Only the two spacers were included in the analysis; sequence data from the intervening 5.8S subunit were incomplete for many taxa, and those data that were available were not sufficiently variable to warrant additional sequencing. Several ITS regions were difficult to read or align unambiguously because of compressions or numerous base insertion/deletion (indel) events. These regions of ambiguity (ranging in size from 2–26 positions) were excluded from the distance calculations and phylogenetic analyses. Pairwise nucleotide differences of unambiguously aligned positions were determined using the distance matrix option in PAUP (SWOFFORD 1993); positions with gaps in any one taxon were treated as missing for all taxa. Transition/transversion (Ts/Tv) rate ratios over a subset of the maximally parsimonious trees, and the distribution of the number of inferred changes per character over a single shortest tree, were calculated using MacClade version 3.01 (MADDISON & MADDISON 1992). The nucleotide sequence data reported in this study have been deposited with GenBank. Accession numbers for the 49 ITS 1 and ITS 2 sequences obtained as part of this investigation are provided in Table 1; accession numbers for the remaining 67 ITS sequences are presented in DOWNIE & al. (1998). The complete aligned matrix is available from SRD upon request.

Phylogenetic analyses were implemented using maximum parsimony (PAUP version 3.1.1; SWOFFORD 1993) and distance (PHYLIP's version 3.5 NEIGHBOR program; FELSENSTEIN 1993) methods. For the parsimony analysis, the length of the shortest trees was determined by initiating 500 heuristic searches each using random addition starting trees, with tree bisection-reconnection (TBR) branch swapping and MULPARS selected, but saving no more than five of the shortest trees from each search. These trees were then used as starting trees for TBR branch swapping (with MULPARS and steepest descent selected). Because the number of equally most parsimonious trees could not be ascertained, the maximum number of trees saved was set at 5000 and these trees were permitted to swap to completion. The strict consensus tree obtained from these 5000 trees was then saved as a topological constraint (CATALÁN & al. 1997). Once more, 500 random-order-entry replicate

Table 1. Accessions of *Apiaceae* subfam. *Apioideae* examined for nuclear ribosomal DNA ITS sequence variation. These ITS data have been deposited with GenBank as separate ITS 1 and ITS 2 sequences (accession numbers in brackets). Information for those taxa included in the phylogenetic analyses but not listed below is presented in DOWNIE & al. (1998). Herbarium acronyms according to HOLMGREN & al. (1990). Unless otherwise indicated, cultivated specimens are vouchered at their respective institutions

Taxon	Source and/or voucher, and GenBank accession number
<i>Aciphylla subflabellata</i> W. R. B. OLIV.	cult. Royal Botanic Garden, Edinburgh, U.K. (no. 19693044) [ITS 1: AF008646; ITS 2: AF009125]
<i>Angelica cincta</i> BOISS.	cult. Moscow State University Botanical Garden, Russia (MW) [ITS 1: AF008601; ITS 2: AF009080]
<i>A. decurrens</i> (LEDEB.) B. FEDTSCH.	Russia, Siberia, Irkutsk Region; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008599; ITS 2: AF009078]
<i>A. purpurascens</i> (AVE-LALL.) GILLI	Georgia, Caucasus, Adjara, Beshumi; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 1463 (MW) [ITS 1: AF008611; ITS 2: AF009090]
<i>A. sylvestris</i> L.	Russia, Moscow Region, Oka Valley; cult. Moscow State University Botanical Garden, Russia, T. A. OSTROUMOVA s.n. (MW) [ITS 1: U78414; ITS 2: U78474]
<i>A. tatarica</i> BORDZ.	Georgia, Caucasus, Borjomi, Tabatskhuri Lake; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 851 (MW) [ITS 1: AF008610; ITS 2: AF009089]
<i>Aphanopleura trachysperma</i> BOISS.	Armenia, Ararat Region, Vedi, V. MANAKIAN s.n. (MO) [ITS 1: AF008629; ITS 2: AF009108]
<i>Aulacospermum anomalum</i> (LEDEB.) LEDEB.	cult. Royal Botanic Garden, Edinburgh, U.K. (no. 19932275) [ITS 1: AF008641; ITS 2: AF009120]
<i>A. simplex</i> RUPR.	Kirghizia; cult. Moscow State University Botanical Garden, Russia (MW) [ITS 1: AF008640; ITS 2: AF009119]
<i>Azilia eryngioides</i> HEDGE & LAMOND	cult. Royal Botanic Garden, Edinburgh, U. K. (no. 19840179) [ITS 1: AF008620; ITS 2: AF009099]
<i>Cnidiocarpa alaiica</i> PIMENOV	Tadjikistan, Kichik-Karamyk; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 1332 (MW) [ITS 1: AF008615; ITS 2: AF009094]
<i>Cnidium silaefolium</i> (JACQ.) SIMONKAI	cult. Moscow State University Botanical Garden, Russia (MW) [ITS 1: AF008614; ITS 2: AF009093]
<i>Conioselinum scopulorum</i> (A. GRAY) COULT. & ROSE	USA, Colorado, Garfield Co., Flat Tops Wilderness, J. P. VANDERHORST 3883 (RM) [ITS 1: AF008634; ITS 2: AF009113]
<i>C. tataricum</i> HOFFM.	Kirghizia, Ottuk, Baidula Gorge; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008623; ITS 2: AF009102]
<i>Cortia depressa</i> (D. DON) LEUTE	cult. Royal Botanic Garden, Edinburgh, U.K. (no. 19892739) [ITS 1: AF008607; ITS 2: AF009086]
<i>Eleutherospermum cicutarium</i> (BIEB.) BOISS.	Russia, N Caucasus, Chechen Republic, Harami Pass; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 166 (MW) [ITS 1: AF008637; ITS 2: AF009116]
<i>Erigenia bulbosa</i> (MICHX.) NUTT.	USA, Illinois, Alexander Co., Shawnee National Forest, L. R. PHILLIPPE 23573 (ILLS) [ITS 1: AF008636; ITS 2: AF009115]

Table 1 (continued)

Taxon	Source and/or voucher, and GenBank accession number
<i>Exoacantha heterophylla</i> LABILL.	Israel, Golan, S of Mevo-Hamma, A. LISTON 535/1 (UCB) [ITS 1: AF008617; ITS 2: AF009096]
<i>Fuernrohria setifolia</i> C. KOCH	Armenia, Caucasus, Sachlu; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008633; ITS 2: AF009112]
<i>Hansenia mongholica</i> TURCZ.	Russia, Altai, Karakol Lakes; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 18 (MW) [ITS 1: AF008643; ITS 2: AF009122]
<i>Heracleum aconitifolium</i> WORONOW	Russia, Krasnodarsky Region, N Caucasus, Kawkazsky Reserve, Abago; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008625; ITS 2: AF009104]
<i>Karatavia kultiassovii</i> (KOROVIN) PIMENOV & LAVROVA	Kazakhstan, Syrdariinsky Karatau Gorge, Mynzhilke; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 164 (MW) [ITS 1: AF008612; ITS 2: AF009091]
<i>Laser trilobum</i> (L.) BORKH.	Azerbaijan, Caucasus, Vel-Veli-Chai; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008644; ITS 2: AF009123]
<i>Ligusticum canadense</i> (L.) BRITTON	USA, North Carolina, Jackson Co., Bull Pen Road, S. R. HILL 25934 (ILLS) [ITS 1: AF008635; ITS 2: AF009114]
<i>L. physospermifolium</i> ALBOV	Russia, Stavropol Region, Teberdinsky Reserve; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008616; ITS 2: AF009095]
<i>Malabaila secacul</i> (MILL.) BOISS.	Jordan, Irbid, Jordan University of Science and Technology, J. LAHHAM 26 [ITS 1: AF008627; ITS 2: AF009106]
<i>Oedibasis platycarpa</i> (LIPSKY) KOSO-POL.	Kazakhstan, Syrdariinsky Karatau Gorge, Boroldai; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008632; ITS 2: AF009111]
<i>Opopanax hispidus</i> (FRIV.) GRISEB.	Turkey, Manissa, Sipulus Mt.; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 56 (MW) [ITS 1: AF008624; ITS 2: AF009103]
<i>Parasilau asiaticus</i> (KOROVIN) PIMENOV	Tadjikistan, Nikolayevsky Spusk; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008642; ITS 2: AF009121]
<i>Pastinaca armena</i> FISCH. & C. A. MEY.	Azerbaijan, Caucasus, Karabakh, Lachin; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 568 (MW) [ITS 1: AF008626; ITS 2: AF009105]
<i>Peucedanum caucasicum</i> (BIEB.) K. KOCH	Russia, Stavropol Region, Popovka; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008618; ITS 2: AF009097]
<i>P. cervaria</i> (L.) LAPEYR.	Ukraine, Carpathian Mts., Holmez, near Uzhgorod; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008608; ITS 2: AF009087]

(contd.)

Table 1 (continued)

Taxon	Source and/or voucher, and GenBank accession number
<i>P. pschavicum</i> BOISS.	Russia, S Ossetia, Bad; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 112 (MW) [ITS 1: AF008619; ITS 2: AF009098]
<i>Phlojodicarpus popovii</i> SIPLIV.	cult. Royal Botanic Garden, Edinburgh, U. K. (no. 19932315) [ITS 1: AF008604; ITS 2: AF009083]
<i>Pleurospermum foetens</i> FRANCH.	cult. Royal Botanic Garden, Edinburgh, U. K. (no. 19910914) [ITS 1: AF008639; ITS 2: AF009118]
<i>P. uralense</i> HOFFM.	Russia, Altai Mts., Charyshskoya; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008638; ITS 2: AF009117]
<i>Polylophium panjutinii</i> MANDEN. & SCHISCHK.	Georgia, Caucasus, Migaria Mt., YU. V. DAUSHKEVICH. s.n. [ITS 1: AF008645; ITS 2: AF009124]
<i>Psammogeton canescens</i> (DC.) VATKE	Afghanistan, Fariah Prov., between Farahrood and Shindand, I. HEDGE & al. W7684 (E) [ITS 1: AF008630; ITS 2: AF009109]
<i>Pyramidoptera cabulica</i> BOISS.	Afghanistan, Bamian Prov., near Tachan; cult. Moscow State University Botanical Garden, Russia, I. GUBANOV & al. 738 (MW) [ITS 1: AF008631; ITS 2: AF009110]
<i>Seseli gracile</i> WALDST. & KIT.	cult. Moscow State University Botanical Garden, Russia (MW) [ITS 1: AF008605; ITS 2: AF009084]
<i>S. libanotis</i> (L.) W. D. J. KOCH	Russia, Moscow Region, Oka Valley; cult. Moscow State University Botanical Garden, Russia, T. A. OSTROUMOVA s.n. (MW) [ITS 1: AF008603; ITS 2: AF009082]
<i>S. mucronatum</i> (SCHISCHK.) PIMENOV	Kirghizia, Talas Gorge, Kara-Bura; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008606; ITS 2: AF009085]
<i>S. peucedanoides</i> (BIEB.) KOSO-POL.	Russia, N Caucasus, Kabardino-Balkar Republic, Baksan River; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 79 (MW) [ITS 1: AF008613; ITS 2: AF009092]
<i>Spermolepis inermis</i> (NUTT. ex DC.) MATHIAS & CONSTANCE	USA, Illinois, Rock Island Co., Cordova, R. A. EVERS 80062 (ILLS) [ITS 1: AF008602; ITS 2: AF009081]
<i>Sphaenolobium tianschanicum</i> (KOROVIN) PIMENOV	Kazakhstan, Talas Gorge, Badam; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. 19 (MW) [ITS 1: AF008622; ITS 2: AF009101]
<i>Sphenosciadium capitellatum</i> A. GRAY	USA, Oregon, G. MASON 7531 (ILL) [ITS 1: AF008600; ITS 2: AF009079]
<i>Thyselium palustre</i> (L.) HOFFM.	Russia, Moscow Region, Peski; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV s.n. (MW) [ITS 1: AF008621; ITS 2: AF009100]
<i>Tommasinia verticillaris</i> (L.) BERTOL.	cult. Moscow State University Botanical Garden, Russia (MW) [ITS 1: AF008609; ITS 2: AF009088]
<i>Zosima orientalis</i> HOFFM.	Turkey, Tortum-Erzurum; cult. Moscow State University Botanical Garden, Russia, M. G. PIMENOV & al. s.n. (MW) [ITS 1: AF008628; ITS 2: AF009107]

searches were initiated as above, saving five trees from each search. However, in this analysis, only those trees that do not fit the constraint tree were saved. No additional minimal-length trees were found, suggesting that the strict consensus tree adequately summarizes the available data, even though the exact number of shortest trees is not known. Bootstrap values (FELSENSTEIN 1985) were calculated from 100 replicate analyses using a heuristic search strategy, simple addition sequence of the taxa, and TBR swapping. Owing to the large size of the data set and the many resultant minimal length trees, a MAXTREE limit of 100 trees per replicate was set. Each indel was mapped a posteriori onto one of the resulting minimal-length cladograms in the most parsimonious way possible in an effort to ascertain their congruence with a phylogeny generated using nucleotide substitutions only.

For the neighbor-joining analysis, distance matrices were calculated using the DNADIST program of PHYLIP and the numbers of nucleotide substitutions were estimated using KIMURA'S (1980) two-parameter method. Three Ts/Tv rate ratios (i.e. 1.0, 1.5, and 2.0) were used, but all resulted in the same topology. A bootstrap analysis of these data was done using 100 resampled data sets generated with the SEQBOOT program prior to calculating the distance matrices and neighbor-joining trees. PHYLIP'S CONSENSE program was then used to construct a strict consensus tree.

All trees computed were rooted with *Heteromorpha arborescens*. Phylogenetic analyses of plastid *rbcL* (PLUNKETT & al. 1996a) and *rpoCl* intron (DOWNIE & al. 1996, 1998) sequences reveal that this taxon may represent the earliest diverging lineage within *Apiaceae* subfam. *Apioideae*. *Bupleurum* and *Anginon*, two other basal apioids, also were considered but high nucleotide divergence in the former and the difficulty in amplifying the ITS 1 region of the latter precluded them from being used (DOWNIE & al. 1998). Similarly, partial ITS sequences obtained for representatives of *Apiaceae* subfamilies *Hydrocotyloideae* and *Saniculoideae* could not be readily aligned owing to high sequence divergence (DOWNIE & KATZ-DOWNIE 1996). In the absence of additional molecular data, it is indeed probable that *Heteromorpha* may not represent the most basal element within subfam. *Apioideae*. However, until these data are obtained, rooting the tree with *Heteromorpha* seems reasonable.

Assessment of character evolution. To determine patterns of evolution of several selected phytochemical, anatomical, and morphological characters, their occurrences were tabulated next to the strict consensus tree resulting from parsimony analysis of the ITS sequence data. The characters we have chosen to examine have been taken from the literature, and primarily from those studies where a broad array of *Apioideae* species was surveyed. Moreover, many of these studies used these data explicitly to infer higher level relationships in the family (e.g. CERCEAU-LARRIVAL 1962, GUYOT 1971, HARBORNE 1971). We emphasize that our survey of the non-English literature was not comprehensive. While it is likely that character data exist for several species where we have found none, we are more interested in the broad trends seen in the evolution of these characters than in documenting their every occurrence. We have also chosen, at this time, not to include a detailed analysis of the characters of the fruit. This topic will be the subject of a manuscript currently in preparation.

Results

ITS sequence analysis. Alignment of all 116 ITS 1 and ITS 2 DNA sequences resulted in a matrix of 490 positions. Ten positions from ITS 1 and 38 positions from ITS 2 (for a total of 48 positions) were deleted because of alignment ambiguity. Characteristics of these aligned sequences, as separate and combined

Table 2. Sequence characteristics of the two internal transcribed spacer (ITS) regions, separately and combined, in 116 accessions of *Apiaceae* subfamily *Apiioideae* (*nt* nucleotides)

ITS region	Length range (nt)	Aligned length (nt)	No. of excluded sites	% sequence divergence (range)	No. of unambiguous alignment gaps	No. of informative alignment gaps
ITS 1	204–221	241	10	0–35.5	32	17
ITS 2	211–227	249	38	0–34.3	32	12
ITS 1 & ITS 2	427–444	490	48	0–34.6	64	29

ITS region	No. of constant sites (and %)	No. of informative sites (and %)	No. of autapomorphic sites (and %)	No. of variable sites (and %)	% G & C content range (and mean)
ITS 1	52 (22.5%)	161 (69.7%)	18 (7.8%)	179 (77.5%)	50.0–59.7 (55.0)
ITS 2	45 (21.3%)	147 (69.7%)	19 (9.0%)	166 (78.7%)	50.0–62.1 (56.4)
ITS 1 & ITS 2	97 (21.9%)	308 (69.7%)	37 (8.4%)	345 (78.1%)	51.5–59.8 (55.7)

spacer regions, are presented in Table 2. Although ITS 1 was, on average, slightly shorter than ITS 2, it provided more parsimony-informative characters. The ratio of terminal taxa (116) to phylogenetically informative sites for both spacers (308) was 1:2.7. In direct pairwise comparisons of all unambiguous positions across all accessions, sequence divergence values ranged from identity to 34.6% of nucleotides. Despite the high values of some pairwise comparisons, the interspersions of conserved and variable sites through both spacers promoted sequence alignability. Among congeners, sequence divergence values ranged from identity to 20.5% (Table 3). Sixty-four gaps, ranging between 1 and 14 bp in size, were introduced to facilitate alignment. For each spacer region, the number of gaps in each size category and the number of gaps autapomorphic vs. informative for parsimony analysis are presented in Fig. 1. The vast majority of these gaps were a single bp in size. The placement of gaps in most indel regions was unambiguous because of flanking conserved sites. No evidence of obvious ITS length variants, representative of multiple rRNA repeat types, was observed.

ITS phylogenetic analyses. Parsimony analysis of both spacers for 116 accessions resulted in many thousands of maximally parsimonious topologies. The strict consensus of 5000 of these trees, with accompanying bootstrap values, is presented in Fig. 2. These trees have a length of 2236 steps, consistency indices (CI's) of 0.313 and 0.298, with and without uninformative characters, respectively, and a retention index (RI) of 0.682. Alongside the strict consensus tree (Fig. 2) are the tribal designations of PIMENOV & LEONOV (1993), modified slightly from those of DRUDE (1897–1898), and those *Apiioideae* groups (numbered 1–10) recognized in an earlier phylogenetic study based on plastid *rpoC1* intron and ITS sequences

Table 3. Range in pairwise percent sequence divergence for those genera where two or more species were examined. Asterisks denote genera that are not monophyletic based on the results of this study

Genus	No. of species examined	% sequence divergence
<i>Aciphylla</i>	4	0.5–2.4
<i>Angelica</i> *	12	0.5–7.0
<i>Aulacospermum</i>	2	0.5
<i>Cnidium</i> *	3	0–11.6
<i>Conioselinum</i> *	3	1.7–14.6
<i>Heracleum</i> *	3	1.4–8.2
<i>Laserpitium</i> *	2	13.8
<i>Ligusticum</i> *	4	2.7–20.5
<i>Pastinaca</i>	2	2.2
<i>Peucedanum</i> *	4	3.1–9.7
<i>Pleurospermum</i>	2	3.6
<i>Seseli</i> *	7	3.1–9.2

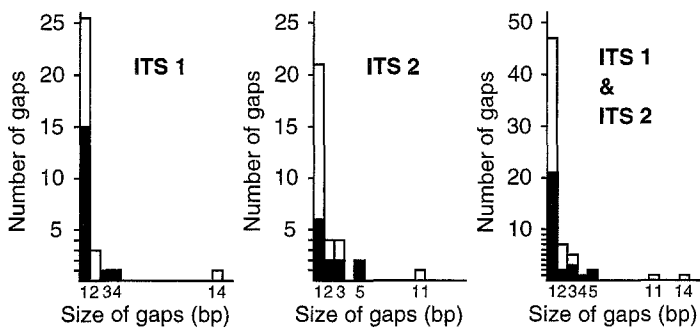
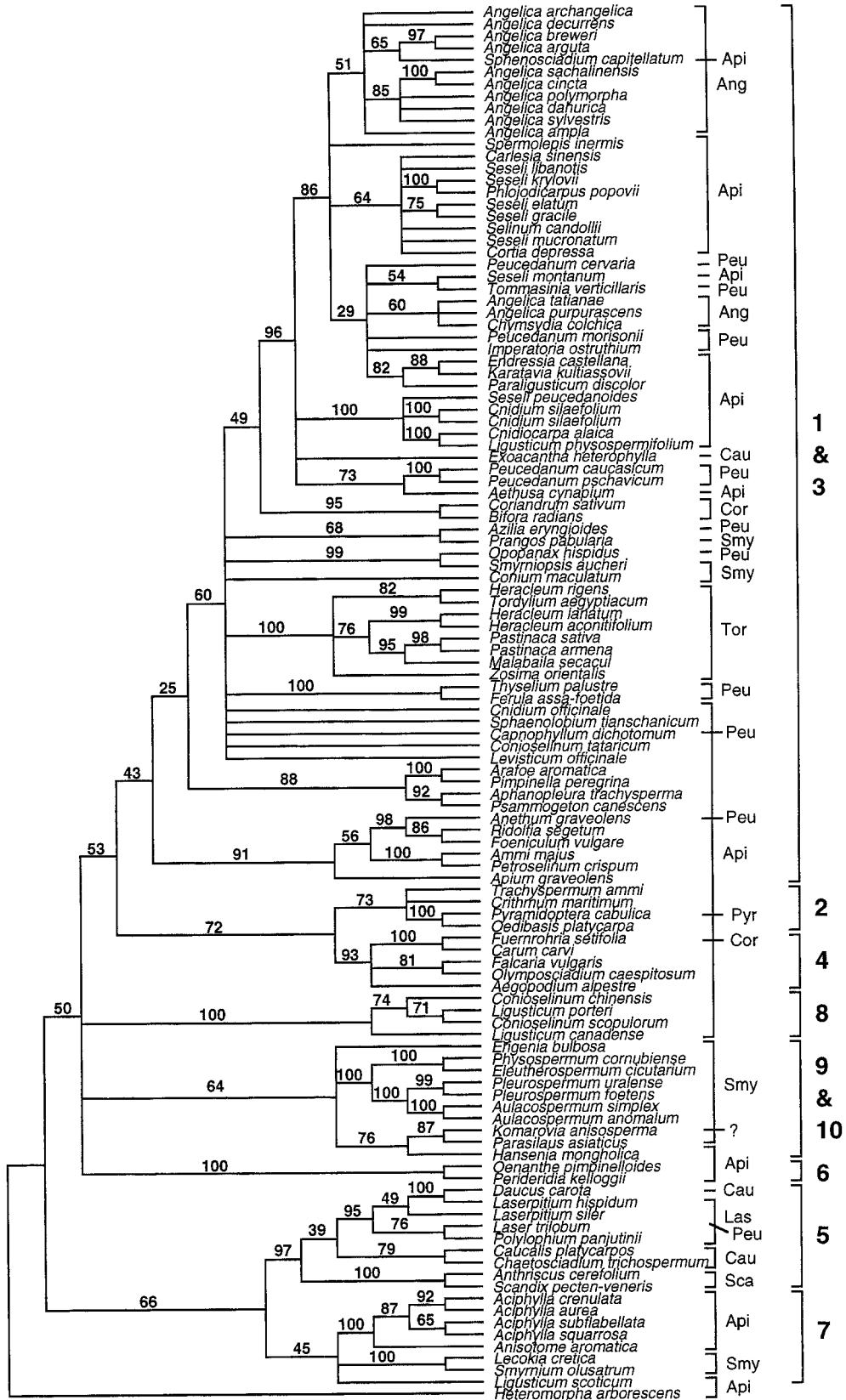


Fig. 1. Characteristics of the gaps inferred in the alignment of 116 ITS 1 and ITS 2 sequences from *Apiaceae* subfam. *Apioidae*. For each spacer region and combined, the number of gaps in each size category and the distribution of autapomorphic (open bar) or informative (shaded bar) gaps are provided

(DOWNIE & al. 1998). Because the relationships between apioid groups 1 and 3 and between groups 9 and 10 varied depending upon the type of sequence analyzed (i.e. ITS or *rpoC1* intron) and the method of tree construction used (i.e. maximum parsimony, maximum likelihood, or neighbor-joining), each of these two pairs of groups have been combined (as group 1 & 3, and group 9 & 10). To facilitate comparisons between the results of an earlier study (DOWNIE & al. 1998) and those obtained herein, these groups are indicated in all subsequent tree figures. The average Ts/Tv ratio among all ITS sequences across 200 trees chosen randomly from the 5000 maximally parsimonious trees was 1.5. One of these 5000 trees was arbitrarily selected (Fig. 3) to show branch lengths and the distribution of the 29



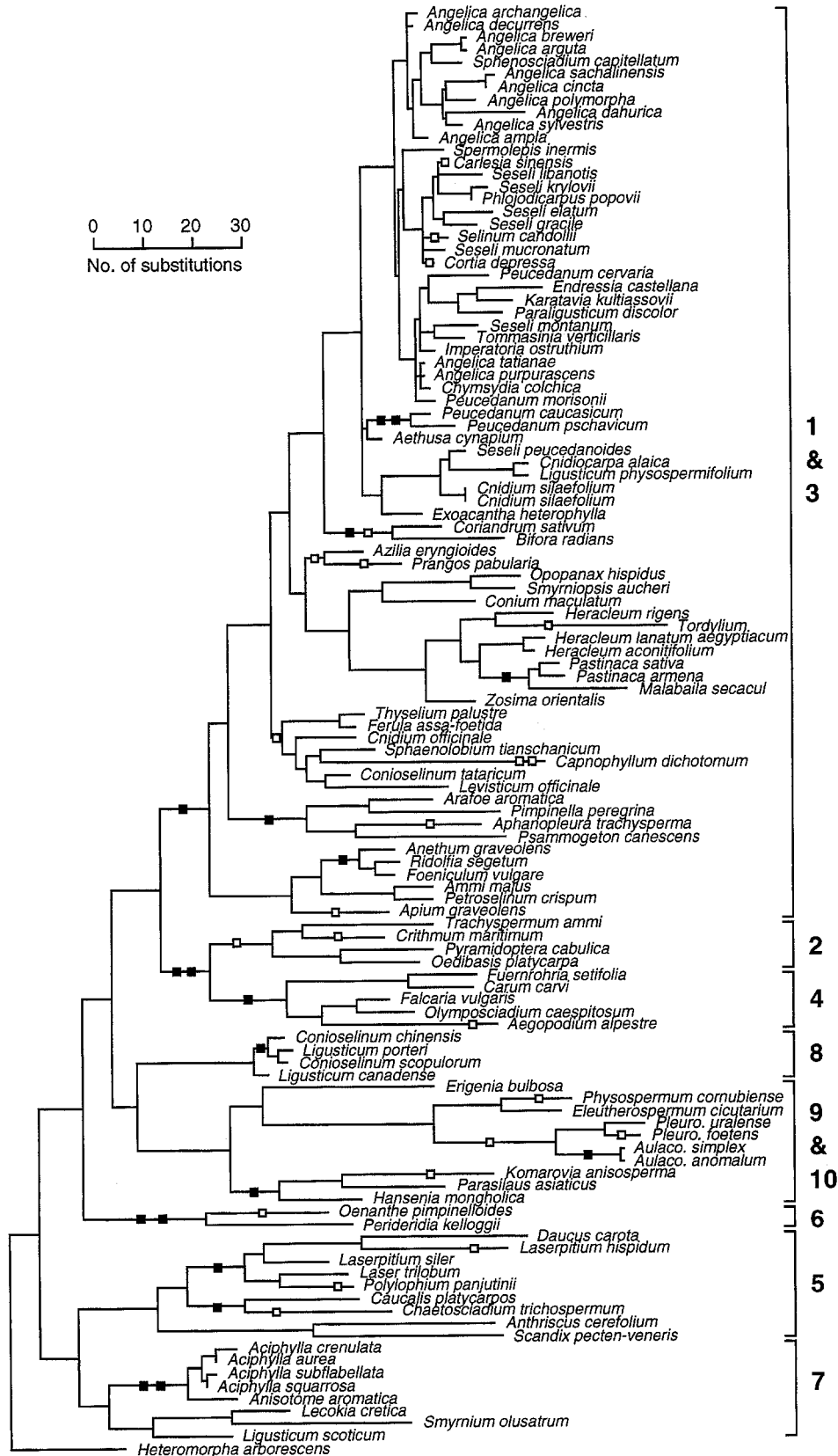
informative alignment gaps. Of these 29 gaps, 19 are unique; the remaining 10 alignment gaps required 23 indel events, because each was inferred to occur 2–3 times on the resultant phylogeny. Although some indels map without homoplasy onto this tree, and provide support for some otherwise weakly supported clades, over half are homoplastic. Similarly, the distribution of the number of inferred changes per character on this single tree reveals that many characters change multiple times. Of the 442 unambiguously aligned sites in the multiple alignment, 27 of these required 14–23 evolutionary changes over this tree, with the average number of steps per character being 5.06. The neighbor-joining tree, calculated with a Ts/Tv rate ratio of 1.5 based on the inferred frequencies in the minimal length trees derived from the parsimony analysis, is presented in Fig. 4. Trees of identical topology were obtained with Ts/Tv rate ratios of 1.0 and 2.0 (not shown).

ITS phylogenetic resolutions. With the exception of apioid groups 2 and 4, which arise within group 1 & 3 in the neighbor-joining tree (Fig. 4), both maximum parsimony and neighbor-joining methods yield the same major clades. These clades are the same as those recognized previously (DOWNIE & al. 1998), although their composition varies slightly owing to the different species sampled. In both analyses presented herein, groups 2 and 4 (the “*Crithmum*” and “*Aegopodium*” clades, respectively, of DOWNIE & al. 1998) comprise sister taxa, as do groups 5 and 7 (the “*Daucus*” and “*Aciphylla*” clades, respectively), yet the relationships of each of these pairs of groups to the other major clades are not clear. Group 1 & 3 is the largest clade, comprising the “*Angelica*” and “*Apium*” clades (and the “*Crithmum*” and “*Aegopodium*” clades in the neighbor-joining tree) of DOWNIE & al. (1998). The remaining major clades have been designated as follows: Group 6 – the “*Oenanthe*” clade; Group 8 – the “*Conioselinum*” clade; and Group 9 & 10 – the “*Komarovia*” and “*Physospermum*” clades, respectively. Within group 1 & 3, several smaller clades (with varying levels of bootstrap support) are discernible.

The discrepancies observed between the phylogenies presented in Figs. 2 and 4 are largely attributable to poorly supported nodes, resulting from too few and/or too many conflicting characters. As stated above, the ratio of terminal taxa to



Fig. 2. Strict consensus of 5000 maximally parsimonious 2236-step trees derived from equally-weighted parsimony analysis of 116 nuclear rDNA ITS 1 and ITS 2 sequences from *Apiaceae* subfam. *Apioideae* (CI excluding uninformative characters = 0.298, RI = 0.682). Numbers at the nodes indicate the number of times that group occurred in 100 bootstrap replicates. Tribal classification presented is that of PIMENOV & LEONOV (1993), modified from that of DRUDE (1897–1898); abbreviations indicate tribes *Scandiceae* (Sca), *Caucalideae* (Cau), *Coriandreae* (Cor), *Smyrnieae* (Smy), *Pyramidoptereae* (Pyr), *Apiaceae* (Api), *Angeliceae* (Ang), *Peucedaneae* (Peu), *Tordylieae* (Tor), and *Laserpitieae* (Las). ? indicates tribal placement is “*incertae sedis*”. Numbers to the right indicate those groups of *Apioideae* recognized in an earlier phylogenetic study using plastid *rpoCl* intron and ITS sequences (DOWNIE & al. 1998), and are as follows: Group 1 & 3 (“*Angelica*” and “*Apium*” clades), Group 2 (“*Crithmum*” clade), Group 4 (“*Aegopodium*” clade), Group 5 (“*Daucus*” clade), Group 6 (“*Oenanthe*” clade), Group 7 (“*Aciphylla*” clade), Group 8 (“*Conioselinum*” clade), and Group 9 & 10 (“*Komarovia*” and “*Physospermum*” clades)



parsimony-informative characters (1:2.7) is low, and the data are decidedly homoplastic. Thus, it is not surprising that the bootstrap values supporting the basal (and many other) nodes in the ITS trees are also correspondingly low. When these basal nodes, characterized by bootstrap values $\leq 50\%$, are treated as ambiguous (that is, they are collapsed to yield polytomies), the two trees are highly consistent with respect to the major groups recognized.

As observed in previous molecular systematic studies, the phylogenies inferred herein provide very little support for DRUDE'S (1897–1898) often cited system of classification for the subfamily. Although the umbellifers display a remarkable array of morphological and anatomical modifications of their fruits, the almost exclusive use of these characters to delimit suprageneric groups has confounded understanding of evolutionary relationships. DRUDE'S tribes *Apieae*, *Smyrnieae*, and *Peucedaneae* (tribes *Apieae*, *Smyrnieae*, *Peucedaneae*, *Tordylieae*, and *Angeliceae* sensu PIMENOV & LEONOV 1993), the largest in the subfamily, are clearly not monophyletic with many independent derivations (Fig. 2). The small tribe *Coriandreae* is also not monophyletic, with the clade of *Coriandrum* and *Bifora* separated from the monotypic *Fuernrohrria*. The separation of *Fuernrohrria* from *Coriandreae* was suggested previously (VINOGRADOVA 1995). With the exception of *Exoacantha*, DRUDE'S tribes *Dauceae* and *Laserpitieae*, and *Scandiceae* subtribes *Scandicinae* and *Caucalidinae* (= PIMENOV & LEONOV'S tribes *Laserpitieae*, *Scandiceae*, and *Caucalideae*) comprise a clade (group 5, Fig. 2). The genus *Exoacantha*, placed in *Dauceae* by DRUDE and in *Caucalideae* by BENTHAM (1867) and BOISSIER (1872), is clearly excluded. The close relationship between *Daucus* and those members of DRUDE'S *Scandiceae* subtribe *Caucalidinae* (*Caucalis* and *Chaetosciadium* in our study) supports the classificatory systems of BENTHAM (1867) and BOISSIER (1872), in which the spiny-fruited members of *Apioideae*, with both primary and secondary ridges on their fruit, were united as tribe *Caucalideae*. The inclusion of members of tribe *Laserpitieae* in this clade supports, in part, the earlier work by TAMAMSCHIAN (1947) in suggesting an affinity between *Laserpitium* and *Daucus* based on carpological characters. Tribe *Scandiceae*, represented in this study by *Anthriscus* and *Scandix*, is monophyletic.

Of the 80 genera of *Apioideae* included in our study, 12 were represented by more than one species (Table 3), and of these, eight (*Angelica*, *Cnidium*, *Conioselinum*, *Heracleum*, *Laserpitium*, *Ligusticum*, *Peucedanum*, and *Seseli*) are not monophyletic. Many of these genera are species-rich, with their generic boundaries exceedingly difficult to comprehend (PIMENOV & LEONOV 1993, VALIEJO-ROMAN & al. 1998).

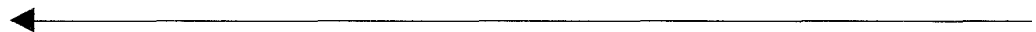
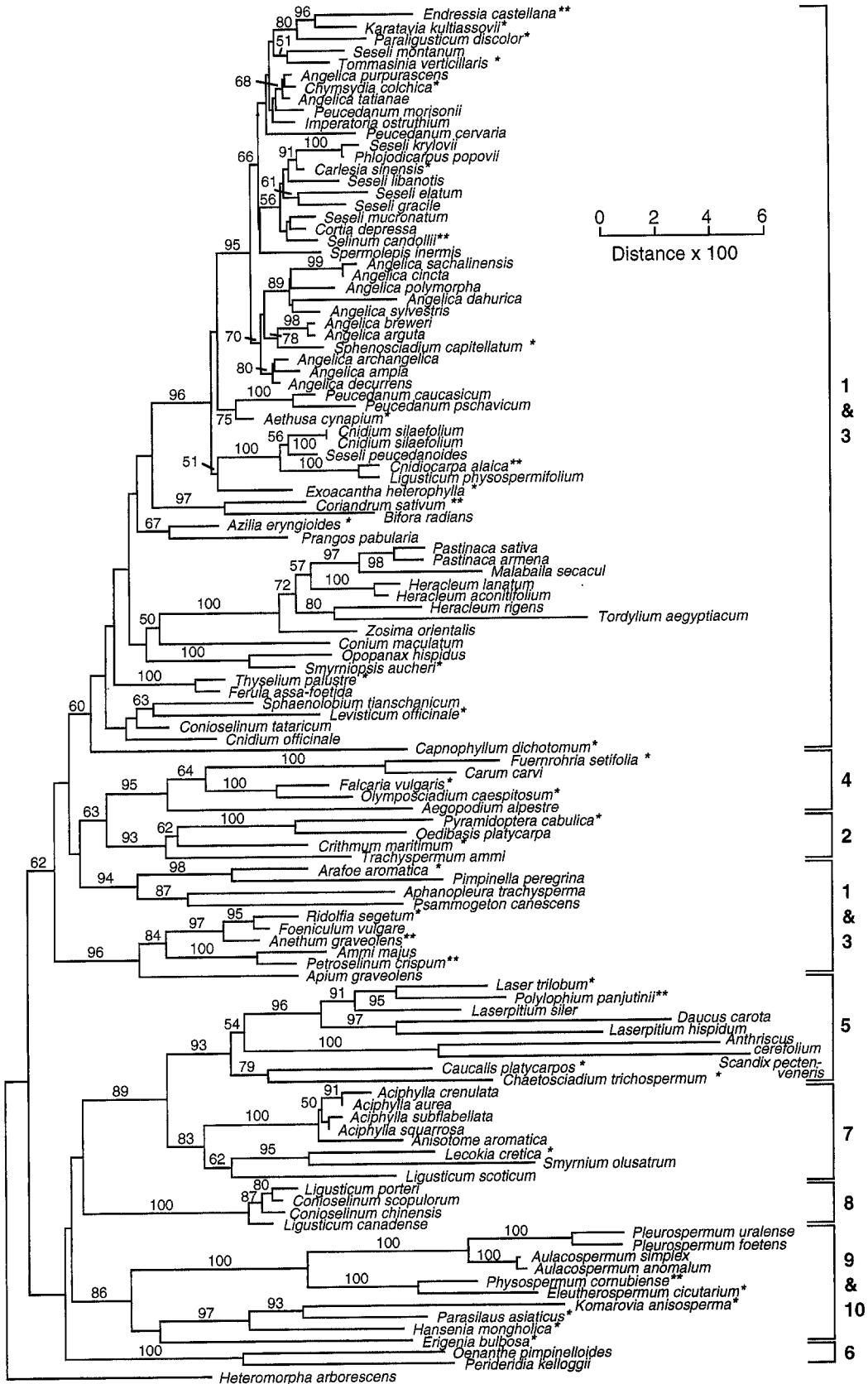


Fig. 3. One of 5000 maximally parsimonious 2236-step trees derived from equally-weighted parsimony analysis of 116 nuclear rDNA ITS 1 and ITS 2 sequences from *Apiaceae* subfam. *Apioideae* (CI excluding uninformative characters = 0.298, RI = 0.682). Lengths of branches are proportional to the number of inferred nucleotide substitutions occurring along them (note scale bar). The distribution of 42 indels inferred from the 29 informative alignment gaps is also indicated, with solid boxes denoting synapomorphies unique to one group and open boxes denoting homoplastic characters. Clade designation, identified as numbers 1–10, is as explained in Fig. 2



Discussion

We recognize the limitations of ITS data in addressing issues of relationship within the subfamily; these have been addressed elsewhere (SOLTIS & KUZOFF 1993, DOWNIE & al. 1998). The homoplastic nature of these data, the high sequence divergence, and the small sizes of the spacers all conspire to reduce the utility of this region in resolving relationships across the entire subfamily. Yet within several of the major clades identified, much resolution and high bootstrap support are achieved. Although the major clades resolved are highly concordant with those inferred using more conserved plastid gene and intron sequences (PLUNKETT & al. 1996b, DOWNIE & al. 1998), the relationships among them are largely ambiguous. The deeper level relations in the subfamily will have to await additional data, particularly from the more conservatively-evolving plastid genome.

The phylogenetic affinities of problematic genera. Despite the poor resolution and bootstrap support among the ancestral nodes of the ITS trees (Figs. 2 and 4), the locations of several Old World taxa which have previously eluded phylogenetic placement are revealed. The monotypic genera *Komarovia*, *Parasilaua*, and *Hansenia* (along with the North American *Erigenia* in the neighbor-joining tree; Fig. 4) comprise a clade, sister group to *Physospermum*, *Eleutherospermum*, *Aulacospermum*, and *Pleurospermum* (group 9 & 10). All of these genera, save *Hansenia* which has been treated in tribe *Apiaceae* and *Komarovia* whose placement has not been previously known, have been placed in tribe *Smyrniaceae* (PIMENOV & LEONOV 1993). The genus *Laser*, which traditionally has been treated in tribe *Laserpitieae*, was placed alongside *Peucedanum* (tribe *Peucedaneae*) by SHISHKIN (1951) and maintained there by PIMENOV & LEONOV (1993). Its affinities are clearly with other *Laserpitieae* representatives, such as *Laserpitium* and *Polylophium* (group 5). *Laserpitieae* arises from within a paraphyletic *Caucalideae*. The genus *Lecokia* is allied with *Smyrnum*, comprising a clade well away from the other *Smyrniaceae* representatives with which they have been long associated (group 7). Serological investigations of SHNEYER & al. (1992, and described below) suggested that *Smyrnum* may be isolated within the family, forming a monotypic tribe or subtribe (*Lecokia* was not examined). Similarly, HEDGE & al. (1987) treated *Smyrniaceae* in a narrow sense, including only *Smyrnum* and *Smyrniopsis* in the tribe (*Lecokia* was treated in tribe *Apiaceae*). The close affinities among *Smyrnum*, *Lecokia*, *Ligusticum scoticum*, and the Australasian *Aciphylla* and *Anisotome* (group 7) are interesting indeed, as is the proposed sister group relationship between this clade and the “*Daucus*” clade (group 5). *Phlojodicarpus* and the monotypic *Tommasinia*, *Karatavia*, and *Exoacantha*,



Fig. 4. Neighbor-joining tree inferred from analysis of 116 nuclear rDNA ITS 1 and ITS 2 sequences from *Apiaceae* subfam. *Apioideae* using a transition/transversion rate ratio of 1.5. Branch lengths are proportional to distances estimated from the two parameter method of KIMURA (scale distance is given as 100 times this value). Numbers at the nodes indicate bootstrap estimates for 100 replicate analyses; values <50% are not indicated. Clade designation is as explained in Fig. 2. Monotypic genera are designated with a single asterisk; bitypic genera are designated with two asterisks (PIMENOV & LEONOV 1993)

placed in tribes *Peucedaneae*, *Apiaceae*, and *Caucalideae*, respectively (PIMENOV & LEONOV 1993), all fall within the large apioid group 1 & 3. *Phlojodicarpus* and *Tommasinia* each have a close relationship with *Seseli*, but the latter is clearly not monophyletic. *Karatavia*, a recent segregate of *Selinum* (LAVROVA & al. 1987), is sister to *Endressia*. *Pyramidoptera* and *Oedibasis* form a strongly supported clade in group 2, allied with *Crithmum* and *Trachyspermum*. The genus *Anethum*, submerged by BENTHAM (1867) into *Peucedanum* and referred to tribe *Peucedaneae* by DE CANDOLLE (1830), BOISSIER (1872), SHISHKIN (1951), and PIMENOV & LEONOV (1993), clearly shows an affinity with genera *Foeniculum*, *Ammi*, *Petroselinum*, *Ridolfia*, and *Apium*, all traditionally regarded as belonging to tribe *Apiaceae* (group 1 & 3).

Among the few New World exemplars included in our study, a close relationship is seen among *Sphenosciadium capitellatum*, *Angelica breweri* GRAY, and *A. arguta* NUTT. In the *matK*-derived phylogeny of PLUNKETT & al. (1996b), *Angelica lucida* L. and *Sphenosciadium* are sister taxa. *Ligusticum canadense*, *L. porteri*, *Conioselinum scopulorum*, and *C. chinensis* comprise a clade (group 8); neither *Ligusticum* or *Conioselinum* are monophyletic. The closest relative of *Spermolepis* is not evident in our cladograms and, as stated above, *Erigenia* appears to be allied with several Old World representatives of tribe *Smyrnieae*.

HEYWOOD (1971b) has stated that many small genera in the subfamily appear to be taxonomically isolated and, of these, some may be relictual. Of the 80 genera included in our study, 27 are monotypic (with an additional two – *Chymsydia* and *Sphenosciadium* – being either monotypic or bitypic) and eight are bitypic (PIMENOV & LEONOV 1993). The distribution of these monotypic and bitypic genera are indicated by single and double asterisks, respectively, in Fig. 4. These genera are widely distributed throughout the tree. While an argument can be made suggesting that *Sphenosciadium* and, perhaps, *Chymsydia* be merged with *Angelica*, our sampling of the larger genera is just too sparse to ascertain the validity of HEYWOOD's statement. It is of interest to note, however, that there are indeed some clades that include predominantly monotypic genera. One of these clades contains such genera as *Komarovia*, *Parasilaus*, *Hansenia*, and *Erigenia*, which, according to the distance tree (Fig. 4), may constitute long-evolving separate lineages. The same situation may occur within apioid groups 2 and 4.

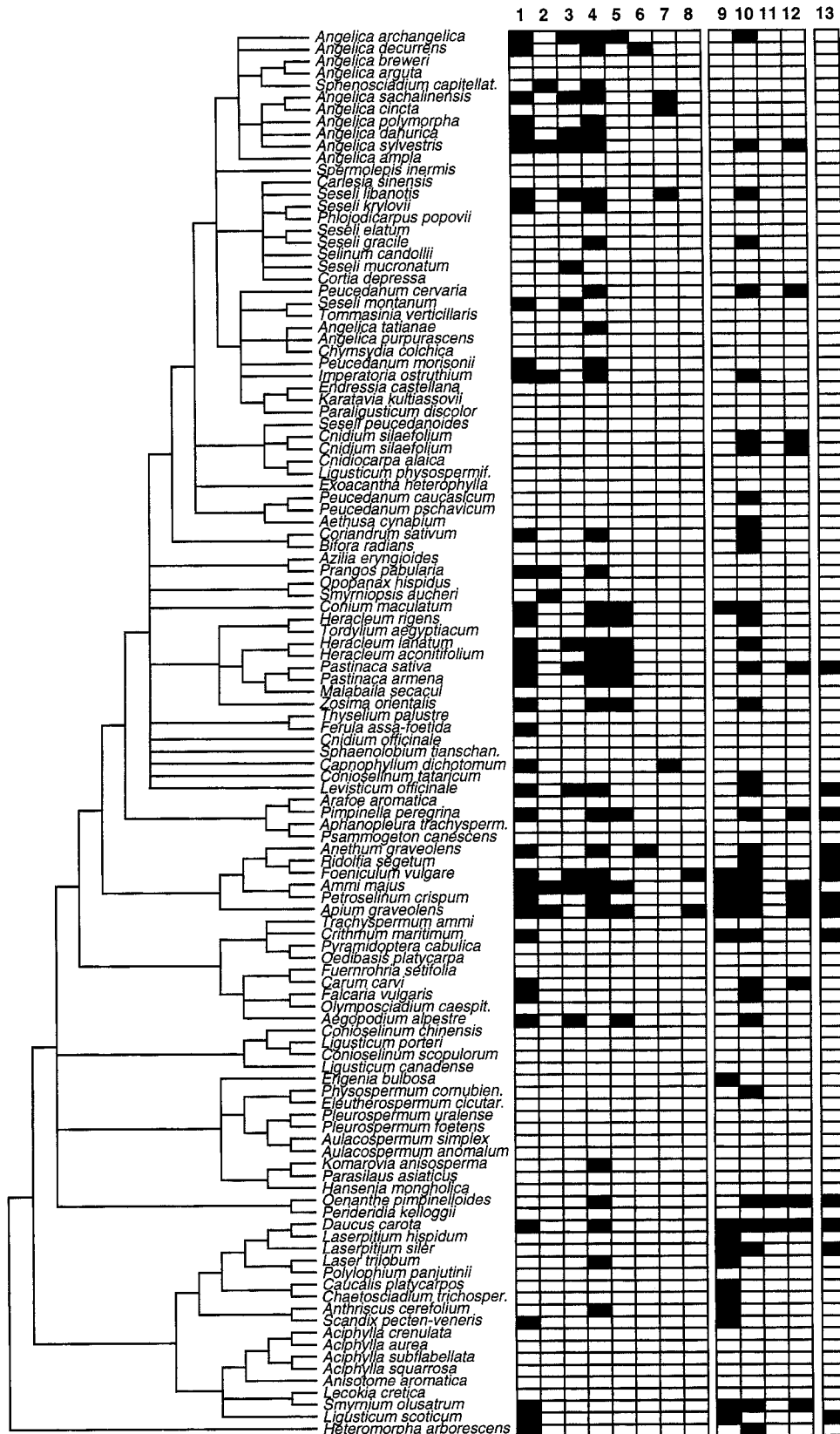
Character evolution. Within *Apiaceae* subfam. *Apioideae*, a considerable amount of phytochemical, anatomical, cytological, morphological, and palynological data are available (reviewed in HEYWOOD 1971a, and CAUWET-MARC & CARBONNIER 1982). However, patterns in the evolution of many of these characters, and their reliability in demarcating taxonomic groups, have yet to be considered outside of the framework of DRUDE's (1897–1898) classificatory system (for an exception see PLUNKETT & al. 1996b). Here we reconsider the evolution of several selected characters in the independent context of an ITS-derived phylogeny, in spite of the problems inherent in using these ITS data to infer relationships at such deep levels. It is reassuring, however, that many of the major clades inferred in our trees are also apparent, for the most part, in those phylogenies constructed using several plastid gene and intron sequences (KONDO & al. 1996, PLUNKETT & al. 1996b, DOWNIE & al. 1998) and in a phylogeny based on the comparative analysis of restriction sites obtained from throughout the entire chloroplast genome

(PLUNKETT & DOWNIE, unpubl. data). Thus, we believe it is reasonable to assume that the phylogenies inferred using these molecular data reflect the species phylogeny.

Members of subfam. *Apioideae* are abundantly supplied with secondary metabolites and, as a result, much work has been done on identifying these products and in assessing their utility as taxonomic markers. Several chemical constituents are widespread, if not universal, in the subfamily. These include the polyacetylenes, mono- and triterpenes, and the rare oligosaccharides apiose and umbelliferose (CROWDEN & al. 1969, BOHLMANN 1971, HEGNAUER 1971). Chemical compounds having a restricted distribution within the subfamily are numerous, and include the sesquiterpene lactones, alkaloids, naphthalide-type lignans, 2-methylchromones, coumarins, flavonoids, and phenylpropenes (HARBORNE & al. 1969; HARBORNE 1971; HEGNAUER 1971, 1982; NIELSEN 1971; HARBORNE & WILLIAMS 1972; CARBONNIER & al. 1982; GONZALEZ & GALINDO 1982; PLOUVIER 1982; HOLUB & BUDESINSKY 1986; HOLUB & al. 1987). Of these, only the last three compounds show sufficient variation and were surveyed widely enough to be potentially useful phylogenetically. The patterns of character evolution for each of these three types of data, in the context of the relationships inferred in the strict consensus tree, are discussed below. We realize that the simple presence or absence of any one particular compound is not a definitive indicator of relationship. Moreover, problems are sometimes encountered in scoring these chemical data, and often only positive results are reported.

Coumarins. A comprehensive survey of plant coumarins (from their discovery in 1820 through 1989) has been presented by MURRAY & al. (1982) and MURRAY (1991). These studies and others (i.e. NIELSEN 1971) show that members of *Apioideae* are especially rich in substituted coumarins, both in abundance and in structural diversity. Several distinct structural classes of coumarins exist, including i) simple coumarins, ii) furanocoumarins, iii) dihydrofuranocoumarins, iv) pyranocoumarins, and v) dihydropyranocoumarins. Both furanocoumarins and pyranocoumarins, and their dihydroderivatives, occur in two structural forms: linear and angular. In *Apioideae*, linear pyranocoumarins occur only in their dihydro form (NIELSEN 1971, MURRAY & al. 1982).

The pattern of coumarin evolution in *Apioideae*, considered in the context of the major structural classes outlined above and the 49 species for which data are available, is presented in Fig. 5. We have not considered the relative numbers of coumarins in each structural class, as done by NIELSEN (1971), but simply show the distribution of these compounds. General trends in the evolution of these compounds are as follows: 1) The rarity of taxa possessing only simple coumarins (character 1). These taxa belong to several widely separate clades, and include *Ferula*, *Crithmum*, *Carum*, *Falcaria*, *Scandix*, *Smyrniium*, *Ligusticum scoticum*, and *Heteromorpha*. 2) The widespread and scattered occurrences of the two forms of dihydrofuranocoumarins (characters 2 and 3) in apioid group 1 & 3. With the exception of the one angular dihydrofuranocoumarin detected in *Aegopodium*, these compounds are lacking in all other umbellifer groups. 3) The almost ubiquitous occurrence of linear furanocoumarins (character 4), with the restriction of angular furanocoumarins (character 5) to *Angelica archangelica*, *Conium*, *Heracleum*, *Pastinaca*, *Zosima*, *Pimpinella*, *Ammi*, *Apium*, and *Aegopodium*. Of



these nine genera, angular furanocoumarins are most numerous and structurally diverse in *Heracleum*, *Pastinaca*, and *Zosima*. Although coumarin data were not available for *Malabaila secacul*, they are available for *M. dasycarpa* and *M. graveolens* (MURRAY & al. 1982). These species, like the closely allied *Heracleum*, *Pastinaca*, and *Zosima*, are rich in angular furanocoumarins. 4) The sporadic occurrences of both linear (character 6) and angular (character 7) dihydrofuranocoumarins in three species of *Angelica*, and in *Seseli libanotis*, *Capnophyllum*, and *Anethum*. 5) The presence of angular pyranocoumarins (character 8) only in the closely related *Foeniculum* and *Apium*.

Angular furanocoumarins are formed by a biosynthetic pathway distinct from that which leads to the linear furanocoumarins, although both groups share the same simple coumarin precursor, umbelliferone (STECK & BROWN 1970, MURRAY & al. 1982). Plants containing angular furanocoumarins are found in at least six derived clades (character 5, Fig. 5), indicating that the biosynthesis of these compounds evolved more recently than their widespread linear counterparts (character 4). Linear furanocoumarins, such as xanthotoxin, are toxic to generalist larval herbivores (BERENBAUM 1978), and it has been suggested that the biosynthetic pathway leading to the synthesis of angular furanocoumarins may have been an evolutionary response by the plants to selective pressures exerted by specialist herbivores that had adapted to feeding on linear furanocoumarins (BERENBAUM & FEENY 1981). If this is indeed the case, then this response occurred at least six times independently during the evolution of *Apioideae*.

Flavonoids. It has been suggested that flavones are derived from flavonols (HARBORNE 1967), thus the absence of flavones and their glycosides in subfamilies *Hydrocotyloideae* and *Saniculoideae* and their widespread occurrence in subfam. *Apioideae* indicate that the latter subfamily is evolutionarily specialized (HARBORNE 1971). Even within *Apioideae*, variation in flavonoid type is apparent. While flavonol aglycones and their glycosides (character 10, Fig. 5) are ubiquitous among the taxa surveyed, flavones (character 9) are restricted to several isolated lineages. Flavones are present in the “*Daucus*” clade, and in the clade containing *Apium*,

◀
 Fig. 5. Distribution of selected phytochemical character states on the strict consensus tree derived from parsimony analysis of 116 nuclear rDNA ITS 1 and ITS 2 sequences from *Apiaceae* subfam. *Apioideae*. Shaded boxes represent the presence of a particular chemical compound. The characters are as follows: 1) simple coumarins; 2) linear dihydrofuranocoumarins; 3) angular dihydrofuranocoumarins; 4) linear furanocoumarins; 5) angular furanocoumarins; 6) linear dihydrofuranocoumarins; 7) angular dihydrofuranocoumarins; 8) angular pyranocoumarins; 9) flavone aglycones and glycosides, 10) flavonol aglycones and glycosides; 11) flavonoid sulphates; 12) methylated flavonoids; and 13) phenylpropenes. Character information was obtained from CROWDEN & al. (1969), HARBORNE & al. (1969), HARBORNE (1971), NIELSEN (1971), HARBORNE & WILLIAMS (1972), HARBORNE & KING (1976), CARBONNIER & al. (1982), MURRAY & al. (1982), SALEH & al. (1983), MURRAY (1991), and HEATH-PAGLIUSO & al. (1992). The data presented for *Aegopodium alpestre*, *Pimpinella peregrina*, and *Oenanthe pimpinelloides* were taken from *A. podagraria*, *P. saxifraga*, and *O. aquatica*, respectively. Each of these congeners is monophyletic (DOWNIE & al. 1998, and unpubl. data)

Petroselinum, *Ammi*, and *Foeniculum*. Flavones have also been reported from *Conium*, *Crithmum*, *Erigenia*, *Smyrniium*, and *Ligusticum scoticum*. The unusual sulphated flavonoids (character 11) are restricted to *Oenanthe* and *Daucus*, whereas the structurally complex *O*-methylated flavonoids (character 12) have evolved multiple times. Both of these latter two flavonoid classes do not appear to be of any systematic significance.

Because members of tribes *Laserpitieae*, *Caucalideae*, and *Scandiceae* (group 5) possess a similar yet diverse and structurally complex flavonoid chemistry (largely of the flavone-type), it was viewed that these taxa are closely related and probably represent an advanced group (or groups) within the subfamily (CROWDEN & al. 1969, HARBORNE 1971, HARBORNE & WILLIAMS 1972). While the ITS trees do show a close relationship among these taxa, they also indicate that this group is positioned relatively basal in the cladograms despite their specialized chemistry and characteristic fruits.

Phenylpropenes. A considerable number of phenylpropenes occur in subfam. *Apiioideae* (character 13, Fig. 5), with myristicin occurring relatively widely (HARBORNE & al. 1969, HARBORNE 1971). Because these compounds are reported to occur in many economically important umbellifers (e.g. *Pastinaca*, *Levisticum*, *Anethum*, *Foeniculum*, *Petroselinum*, *Apium*, *Daucus*, and *Ligusticum scoticum*), their distribution may actually reflect a bias in sampling. Phenylpropenes are widely distributed within the subfamily, and do not appear to be useful taxonomically.

Stomata. By studying the development and appearance of mature stomata in leaf epidermal peels of *Apiaceae*, GUYOT (1971) characterized, and proposed a relationship among, seven major stomatal types. Five of these stomatal types occur within subfam. *Apiioideae*, ranging from mesoperigenous anomocytic (most simplest developmentally) to either paracytic, diacytic or mesogenous anisocytic (most complex developmentally). In some plants, both anomocytic and anisocytic (and occasionally bicytic) stomatal types were observed, with stomatal development being partly of the mesoperigenous type; GUYOT (1971) considered these stomata as having intermediary characteristics. In addition, different types of stomata were found to exist simultaneously on the same leaf. The distribution of these different stomatal types, based on the results of GUYOT (1971) and GUYOT & al. (1980), is illustrated in Fig. 6 (characters 1–5).

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Fig. 6. Distribution of selected stomatal, cotyledonary, and palynological character states on the strict consensus tree derived from parsimony analysis of 116 nuclear rDNA ITS 1 and ITS 2 sequences from *Apiaceae* subfam. *Apiioideae*. Shaded boxes represent the occurrences of that particular character state. The characters are as follows: 1) mesoperigenous anomocytic stoma; 2) mesoperigenous anisocytic stoma; 3) bicytic paracytic stoma; 4) bicytic diacytic stoma; 5) mesogenous anisocytic stoma; 6) long “L-type” cotyledons; 7) round “R-type” cotyledons; 8) subrhomboidal pollen; 9) oval pollen; 10) subrectangular pollen; and 11) equatorially-constricted pollen. Character information was obtained from CERCEAU-LARRIVAL (1962, 1963, 1965), GUYOT (1971), and GUYOT & al. (1980). In several instances, the distribution of character states is representative for a genus and not necessarily the species indicated

While mesoperigenous anomocytic stomata (character 1, Fig. 6) were considered by GUYOT (1971) as being the least complex developmentally (and possibly plesiomorphic), and its occurrence in *Heteromorpha* is consistent with this hypothesis, these stomata are also detected in *Smyrnum*, *Caucalis*, and *Psammogeton*, and in several species not included in our study (e.g. *Heracleum sphondylium* L., *H. mantegazzianum* SOM. & LEV., and *Thapsia villosa* L.). Those taxa bearing only the specialized stomatal types (i.e. characters 3–5, Fig. 6), such as *Seseli elatum*, *S. montanum*, *Cnidium silaefolium*, *Anethum*, *Ridolfia*, *Crithmum*, *Falcaria*, *Aegopodium*, *Oenanthe*, and *Daucus*, are also distributed throughout the tree. GUYOT (1971) cautioned that those tribes (sensu CERCEAU-LARRIVAL 1962) characterized by his “primitive” or “highly evolved” stomatal types may not necessarily be linked, and our results certainly confirm this. OSTROUMOVA (1987, 1990) carried out a study comparable to that of GUYOT (1971), recognizing six major stomatal types within *Apiaceae*. Again, we were unable to discern any trend in the evolution of these stomatal types when these characters were mapped onto the ITS strict consensus tree (not shown).

Pollen and seedling morphology. CERCEAU-LARRIVAL (1962, 1971), from her studies on the correlations between pollen morphology and the presence or absence of either round (“lignée cotylédonaire R”) or long (“lignée cotylédonaire L”) cotyledons, supported by evidence from the inflorescence, fruit, and adult vegetative (especially leaf) morphology, proposed an original division of *Apiaceae* into five subfamilies (*Bupleuroideae*, *Endressioideae*, *Azorelloideae*, *Eryngioideae*, and *Apioideae*) and 38 tribes. Subsequent palynological investigations (CERCEAU-LARRIVAL 1963, 1965) resulted in five additional tribes being recognized. CERCEAU-LARRIVAL’s detailed study of nearly 1500 species of *Apiaceae* revealed five distinct pollen types based on the internal contour of the endexine. These pollen types, ranked from “very primitive” to “highly evolved”, were classified as subrhomboidal, subcircular, ovoid, subrectangular, and equatorially-constricted. Those tribes of DRUDE (1897–1898) possessing taxa with more than one of these pollen types were declared invalid and split into palynologically homogeneous units, thus bringing the total number of tribes recognized in subfam. *Apioideae* from eight (sensu DRUDE) to 34. CERCEAU-LARRIVAL (1962) established an evolutionary scenario, where small perennial plants having seedlings with small cotyledons and adults with simple, entire, linear leaves, small glabrous fruits, and small subrhomboidal pollen (e.g. *Bupleurum*) were regarded as primitive, and tall annual plants with large cotyledons, dissected leaves, large and spiny fruits, and large equatorially-constricted pollen (e.g. *Caucalis*, *Orlaya*, and *Turgenia*), were regarded as highly advanced.

Figure 6 illustrates the distribution of CERCEAU-LARRIVAL’s cotyledon shape (L vs. R; characters 6 and 7, respectively) and pollen (subrhomboidal, ovoid, subrectangular, equatorially-constricted; characters 8–11, respectively) characters with respect to the inferred phylogeny. Her interpretation of cotyledonary shape and pollen evolution finds little support in the ITS-derived phylogeny. These characters occur throughout the tree, and trends in their evolution are difficult to discern. The same conclusion was reached by PLUNKETT & al. (1996b) when the evolution of these characters was assessed against a similar phylogeny generated using cpDNA *matK* sequence data.

While pollen and cotyledon characters do little to highlight the higher-level relationships within the subfamily, it is of interest that when other characters are used (such as those from the fruit, inflorescence, adult vegetative morphology, and leaf ontogeny), some trends are established that are supported by cladistic analyses of molecular data. For example, CERCEAU-LARRIVAL (1962, 1967) reported that *Bupleurum* represents a species characterized by many plesiomorphic features. Indeed, in the cladograms presented by PLUNKETT & al. 1996b and DOWNIE & al. (1996, 1998), *Bupleurum* occupies a basal position. Some of the most specialized taxa, according to CERCEAU-LARRIVAL, are those members belonging to tribe *Caucalideae* (e.g., *Turgenia*, *Caucalis*, *Orlaya*, *Torilis*, *Lisaea*, and *Daucus*). These taxa comprise a strongly supported monophyletic group (DOWNIE & al. 1998) that are well-differentiated morphologically from other umbellifers. The ITS trees, however, do not shed any light on the relative advancement of these taxa.

Systematic serology. Relationships among members of *Apiaceae* have been elucidated using serological data (PICKERING & FAIRBROTHERS 1970, 1971; SHNEYER & al. 1991, 1992, 1995). Although these studies included only a small number of taxa, the serological groups identified are consistent with many of those clades inferred using ITS and other DNA sequences. The earliest of these studies supported DRUDE's (1897–1898) division of the family into three subfamilies, and indicated that subfam. *Apioideae* is more similar serologically to subfam. *Saniculoideae* than to subfam. *Hydrocotyloideae* (PICKERING & FAIRBROTHERS 1970). The sister group relationship between *Apioideae* and *Saniculoideae* has since been substantiated by cladistic analyses of plastid *rpoC1* intron and *rbcL* sequences (DOWNIE & al. 1996, 1998; PLUNKETT & al. 1996a). Subsequent analyses by PICKERING & FAIRBROTHERS (1971) revealed five serological groupings within *Apioideae*, corresponding to DRUDE's tribes *Scandiceae* (three genera examined; *Myrrhis*, *Osmorhiza*, and *Torilis*), *Coriandreae* (*Coriandrum*), *Apieae* (*Carum* and *Foeniculum*), *Peucedaneae* (*Angelica*, *Levisticum*, *Peucedanum*, *Pastinaca*, and *Heracleum*), and *Dauceae* (*Daucus*). Representatives of tribes *Coriandreae*, *Apieae*, and *Peucedaneae* (groups 1–4 in our study) had the highest protein similarity, whereas those members of tribes *Scandiceae* and *Dauceae* (both in group 5 in our study) were each serologically distinct.

SHNEYER & al. (1992, 1995) used serological data to assess relationships among representatives of DRUDE's tribes *Smyrnieae* and *Peucedaneae* and several outgroup genera. In all, seed proteins were extracted from 50 taxa, and antisera were produced for 11 species. The genera included in these studies, as well as a summary of their results, are presented in Fig. 7. The 11 genera examined of tribe *Smyrnieae* (SHNEYER & al. 1992) fell into five separate and unrelated taxonomic groups (Fig. 7A); moreover, some of these groups included non-*Smyrnieae* representatives. Their results suggested that tribe *Smyrnieae* sensu DRUDE is not monophyletic. Other results similar to ours included the isolated position of *Smyrnum*, and the serological similarities between *Physospermum*, *Aulacospermum*, and *Pleurospermum*, and between *Parasilaus* and *Komarovia*. In our analyses, *Physospermum*, *Aulacospermum*, and *Pleurospermum* comprise a group sister to a clade containing *Parasilaus* and *Komarovia* (group 9 & 10). Similarly, *Smyrnum* (plus *Lecokia*) comprises a clade far removed from any other member of *Smyrnieae* examined.

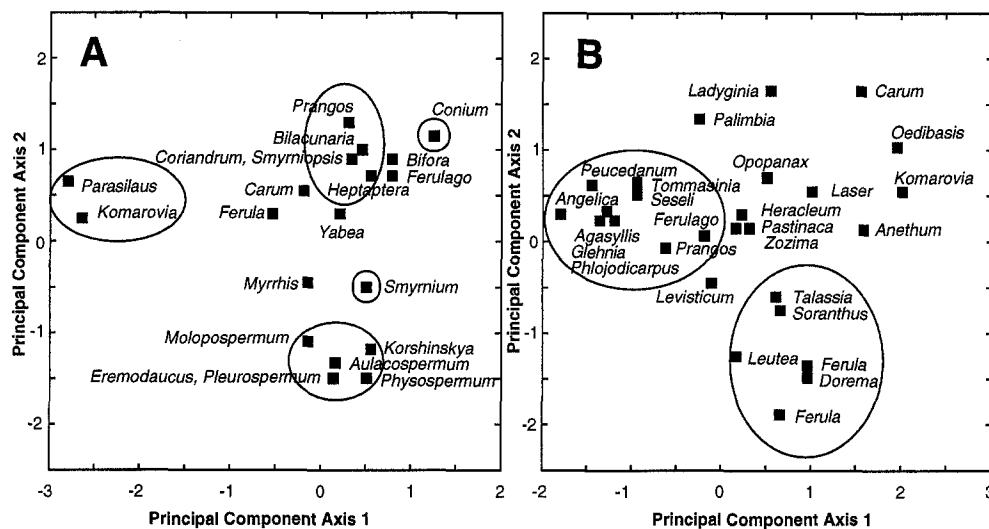


Fig. 7. Ordinations of representative genera of *Apiaceae* subfam. *Apioideae* generated by plotting the first two principal components using mean serological similarity values (redrawn from SHNEYER & al. 1992, 1995). A Based on these results and those of cluster analyses, five serological groups are distinguished in tribe *Smyrnieae* (outlined). B In tribe *Peucedaneae*, two groups (outlined) of serologically similar taxa are distinguished. In most major groups identified in both ordinations, non-*Smyrnieae* and non-*Peucedaneae* taxa are present, indicating the heterogeneity of these tribes

A subsequent study by SHNEYER & al. (1995) examined the serological affinities of 27 species of tribe *Peucedaneae* (sensu DRUDE) and nine representatives from other *Apiaceae* tribes. Two major complexes of genera were identified, and are presented in Fig. 7B. The first complex includes such genera as *Peucedanum*, *Angelica*, *Seseli*, *Phlojodicarpus*, *Tommasinia*, and *Prangos*. *Peucedanum* and *Angelica* are serologically very similar, as are *Seseli* and *Tommasinia*. The six genera comprising this first complex, in addition to the serologically similar *Heracleum*, *Pastinaca*, and *Zosima*, all fall within group 1 & 3 in the ITS cladograms. Here the latter three genera, along with *Tordylium* and *Malabaila*, comprise a clade, as do *Seseli montanum* and *Tommasinia*. A close relationship is also apparent between several *Peucedanum* and *Angelica* exemplars included in our study. The second complex of genera distinguished contains six species, of which only *Ferula* was included in our study.

Despite the technically complex procedures involved in measuring the serological reaction, the various factors that may affect this reaction, and the problems encountered in interpreting these data for systematic purposes (such as the assumption of homologous proteins and the measurement of serological similarity), investigations employing serology have been shown to have much systematic value (CRAWFORD 1990). In *Apiaceae*, the serological results obtained by PICKERING & FAIRBROTHERS (1970, 1971) and SHNEYER & al. (1992, 1995) are consistent, in part, to those results obtained from phylogenetic analyses of plastid and nuclear sequence data. When protein similarity is high between two species, it

is very likely that they are closely related evolutionarily. Thus, a case can be made for the supposed close relationship between *Pleurospermum* and *Eremodaucus* (and, perhaps, *Molopospermum*), *Prangos* and *Bilacunaria*, *Phlojodicarpus*, *Glehnia*, and *Agasyllis*, and *Ferula* and *Dorema*, even though the latter two or three species of each set have yet to be sequenced.

Conclusions

The phylogenies inferred herein for *Apiaceae* subfam. *Apioideae* using nuclear rDNA ITS sequence variation, with regard to the major clades identified, are similar to those estimated using plastid *matK* and *rpoC1* intron sequences (PLUNKETT & al. 1996b, DOWNIE & al. 1998). These molecular data provide very little support for DRUDE's (1897–1898) system of classification of the *Apioideae* or for alternative systems based largely on morphological and anatomical characters of the fruit, or pollen, stomata, and seedling morphology (KOSO-POLJANSKY 1916; CERCEAU-LARRIVAL 1962, 1979). The homoplastic nature of many of these nonmolecular characters, inferred by mapping these data (or the tribal categories derived from these data) onto the ITS-derived cladograms, has led to much confusion and differences in taxonomic treatment.

Taxonomic relationships among Old World *Apioideae* are particularly complex, with both species-rich genera whose generic delimitations are poorly known and many monotypic and bitypic genera. In this study, we have attempted to ascertain the phylogenetic relationships of several problematic genera. However, given the approximately 400 genera recognized in the subfamily and the observation that many of the larger genera are likely not monophyletic (PIMENOV & LEONOV 1993), our sampling of 80 genera is indeed small. Nevertheless, relationships are proposed that can be tested as other data and more material become available for analysis.

These molecular systematic studies of *Apioideae* supply a new set of data with which to assess evolutionary relationships within the subfamily, and the results to date look promising indeed. Complete resolution of relationships, however, will require the continued sampling of Old World representatives and, likely, the simultaneous analysis of all available molecular evidence. A revised classification of *Apioideae* is our goal for the future. In order to achieve this goal, much work needs to be done in integrating the expanding body of evidence from molecular studies with conventional morphological, anatomical, palynological, and phytochemical data. Critical analyses of these nonmolecular data, of which this paper is but a start, are a prerequisite to obtaining the best classification possible.

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