Phylogenetic analysis of chloroplast *rps*16 intron sequences reveals relationships within the woody southern African Apiaceae subfamily Apioideae

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Abstract: Evolutionary relationships among 48 genera of Apiaceae (Umbelliferae) were inferred using maximum parsimony, maximum-likelihood, and neighbor-joining analyses of chloroplast DNA *rps*16 intron and adjacent *rps*16 3′ exon sequences. Emphasis was placed on woody members of Apiaceae subfamily Apioideae endemic to southern Africa, a region hypothesized to be the place of origin of this largely herbaceous subfamily. The resultant phylogenies were highly concordant and indicate that the apioid genera *Polemanniopsis* and *Steganotaenia* form a clade sister to Apiaceae subfamily Saniculoideae. The African genera *Anginon*, *Dracosciadium*, *Glia*, *Heteromorpha*, and *Polemannia* also comprise a clade and likely represent the most basal elements within Apioideae. *Heteromorpha*, however, is not monophyletic, with *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. var. *abyssinica* (A. Rich.) H. Wolff and *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. var. *arborescens* arising in separate subclades. Progressing up the trees, *Annesorhiza* then *Bupleurum* fall as successive sister taxa to all remaining Apioideae. The major clades recognized within subfamily Apioideae are largely congruent with those inferred using other types of molecular evidence. Sequence divergence is similar to that of other chloroplast introns, including being generally low among congeners and woody taxa. While the *rps*16 intron has seen very little use in molecular systematic studies to date, this study demonstrates its ability to discern high-level relationships within Apiaceae.

Key words: Apiaceae, Apioideae, chloroplast *rps*16 intron, phylogeny, southern Africa, Umbelliferae.

Résumé : Les auteurs ont déduit les relations évolutives qui existent entre 48 genres d'Apiaceae (Ombelliferae) en appliquant les analyses de parsimonie maximum, de ressemblance probable maximale et de liaison avec le voisin, à l'examen des séquences de l'ADN de l'intron chloroplastique *rps* 16 et de l'exon adjacent *rps* 16 3′. Les auteurs ont mis l'accent sur les entités ligneuses des Apiaceae, sous-famille Apioideae, endémiques du sud de l'afrique, une région hypothétiquement perçue comme lieu d'origine de cette grande sous-famille d'herbacées. Les phylogénies obtenues concordent étroitement et indiquent que les genres apioïdes *Polemanniopsis* et *Steganotaenia* forment un clade frère de la sous-famille Saniculideae au sein des Apiaceae. Les genres africains *Anginon, Dracosciadium, Glia, Heteromorpha,* et *Polemannia* constituent également un clade et représentent vraisemblablement les éléments les plus fondamentaux au sein des Apioideae. Cependant, l'*Heteromorpha* n'est pas monophylétique, l'*Heteromorpha arborens* var. *abyssinica* et l'*Heteromorpha arborescens* var. *arborescens* apparaissant dans des sous-clades distincts. En montant dans les dendrogrammes, les *Annesorhiza* suivis des *Bupleurum* se situent comme des taxons frères pour tous les Apioideae qui restent. Les principaux clades reconnus dans la sous-famille des Apioideae correspondent généralement à ceux déduits à partir de d'autres types de preuves moléculaires. La divergence des séquences est semblable à celle de d'autres introns, incluant le fait d'être généralement faible entre les congénères et les taxons ligneux. Alors que l'intron *rps* 16 n'a connu que peu d'utilisation dans les travaux de systématique moléculaire jusqu'ici, cette étude démontre son aptitude à percevoir les relations de niveau supérieur chez les Apiaceae.

Mots clés : Apiaceae, Apioideae, intron chloroplastique *rps* 16, phylogénie, sud de l'Afrique, Ombelliferes.

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Introduction

The obvious distinctive characters of many umbellifers, such as herbs with hollow or pith-filled stems, pinnately divided leaves with sheathing bases, small unspecialized flowers in compound umbel inflorescences, and specialized fruits consisting of two single-seeded mericarps, make them easily identifiable and, as a consequence, one of the first groups of flowering plants to be widely recognized (Constance 1971). While this homogeneity holds true for the vast majority of species of Apiaceae subfamily Apioideae, particularly those of the North Temperate Zone, genera exist in the southern hemisphere that do not conform to this morphological stereotype. These plants may include small trees, shrubs, or distinctly woody subshrubs, with sometimes quite atypical leaf and fruit morphology. Considering that the family Apiaceae (Umbelliferae) is largely herbaceous and its puta-

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tive sister family Araliaceae not, these woody umbellifers are of immense phylogenetic importance.

It has been suggested that herbaceous Apioideae probably evolved from montane tropical woody ancestors of at least shrub to small tree dimensions, perhaps similar in habit to present-day *Myrrhidendron* of Central America, and *Diplolophium*, *Heteromorpha*, and *Steganotaenia* of Africa (Dawson 1971). Indeed, cladograms inferred using chloroplast DNA (cpDNA) restriction sites (Plunkett and Downie 1999), or *rbc*L (Plunkett et al. 1996a), *rpo*C1 intron (Downie et al. 1996*a*, 1998) or *rpl*16 intron (Downie et al. 2000) sequences have shown that several predominantly woody apioids endemic to subsaharan Africa (specifically, *Anginon rugosum* (Thunb.) Raf., *Glia prolifera* (Burm. f.) B.L. Burtt, and either *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. var. *arborescens* or *Heteromorpha arborescens* var. *abyssinica* (A. Rich) H. Wolff (as *Heteromorpha trifoliata* (Wendl.) Eckl. & Zeyh.) depending upon the study) form a clade sister to all other examined Apioideae. In contrast, phylogenetic analysis of chloroplast *mat*K sequences suggests that the primarily herbaceous apioid genus *Bupleurum* occupies this position, although *Heteromorpha* and *Anginon* fall one node away (Plunkett et al. 1996*b*). Cerceau-Larrival (1962, 1971), from her studies on pollen morphology and cotyledon type, supported by evidence from leaf ontogeny, inflorescences, fruits, and adult vegetative morphology, suggested that the ancestral apioids were likely small-statured perennial species with simple, entire, linear leaves, subrhomboidal-shaped pollen, and unspecialized glabrous fruit and likely very similar to present-day *Bupleurum*. While *Bupleurum* is a primarily herbaceous genus of largely Eurasian and North African distribution, some species are distinctly woody (e.g., *Bupleurum fruticosum* L.) and one, *Bupleurum mundii* Cham. & Schlechtd., is endemic to southern Africa. Moreover, the latter species is unique in having subrhomboidal-shaped pollen, considered the most primitive form of pollen in the genus, if not the entire family (Cerceau-Larrival 1971). Some of the earliest microfossils known for Apiaceae, dated from the early Tertiary (Eocene), are referred to extant *Bupleurum* and *Heteromorpha* (Gruas-Cavagnetto and Cerceau-Larrival 1982).

In addition to *Anginon*, *Glia*, and *Heteromorpha*, three other distinctly woody apioids are endemic to subsaharan Africa: *Polemannia*, *Polemanniopsis*, and *Steganotaenia*. Similarities in wood anatomy and other characters between some of these genera (and *Bupleurum*) and Araliaceae (Rodríguez 1957, 1971; Burtt 1988, 1991), suggest their basal position within Apioideae. The relationships of these taxa to other African endemics (such as *Annesorhiza*, *Diplolophium*, and *Dracosciadium*) and to the more northern, herbaceous elements of the subfamily are not clear. *Steganotaenia*, once submerged in *Peucedanum* (Drude 1898; Engler 1921) but now regarded as a distinct yet closely allied genus (Norman 1934; Cannon 1978; Townsend 1989; Burtt 1991; Thulin 1991), is sister to *Sanicula* (Apiaceae subfamily Saniculoideae) based on *rbc*L sequence comparisons of very few Apiaceae taxa (Backlund and Bremer 1997). *Polemanniopsis* was removed from *Polemannia* by Burtt (1988) with the comment that no close ally could be found. Based on morphological comparisons, affinities between *Heteromorpha* and *Polemannia* and between *Anginon* and *Glia* have been expressed (Winter and van Wyk 1996; van Wyk et al. 1997). Explicit hypotheses of relationships that include a broad representation of these woody African apioid genera are, however, lacking.

Here, we use cladistic analysis of *rps*16 intron sequences to infer the historical relationships among these woody and other endemic African apioids. We also assess their placement in a broader phylogeny of Apiaceae. Compared with the wealth of data now available from rapidly evolving cpDNA loci, relatively few phylogenetic studies have focused explicitly on chloroplast introns. Such studies include those within genes *trn*L (UAA) (Fangan et al. 1994; Gielly and Taberlet 1994), *trn*V (UAC) (Clegg et al. 1986; Learn et al. 1992), *rpl*16 (Dickie 1996; Jordan et al. 1996; Kelchner and Clark 1997; Downie et al. 2000), *rpo*C1 (Downie et al. 1996*a*, 1996*b*, 1998), and *rps*16 (Oxelman et al. 1997). Pairwise comparisons of the 17 chloroplast introns shared between tobacco (*Nicotiana tabacum* L.) and rice (*Oryza sativa* L.) indicate that the *rps*16 intron is one of the most divergent, with 67% sequence similarity (Downie et al. 1996*a*). We have chosen to analyze this intron given its potential for variation, the ease by which this region can be isolated from herbarium material and sequenced using standard methodologies, and the success others have had in using this locus for phylogenetic inference in plant groups at comparable taxonomic levels (Lidén et al. 1997; Oxelman et al. 1997).

The major objectives of this study are (*i*) to evaluate the utility of the chloroplast *rps*16 intron in estimating phylogeny within the family Apiaceae and (*ii*) to ascertain the phylogenetic placement of the predominantly woody southern African apioid genera *Anginon*, *Glia*, *Heteromorpha*, *Polemannia*, *Polemanniopsis*, and *Steganotaenia*. The positions of three other genera (*Annesorhiza*, *Diplolophium*, and *Dracosciadium*), all herbaceous perennials or shrubs endemic to southern or tropical Africa (Burtt 1991), will also be considered. Given that subfamily Apioideae may have originated in southern Africa and that the woody habit is likely plesiomorphic in the family (Dawson 1971; Rodríguez 1971; Plunkett et al. 1996*a*, 1996*b*), phylogenetic study of these African plants is critical to understanding the origin and early diversification of subfamily Apioideae.

Methods

Plant material

Fifty species (36 genera) of Apiaceae subfamily Apioideae, 12 species (5 genera) of Apiaceae subfamily Saniculoideae, 11 species (7 genera) of Apiaceae subfamily Hydrocotyloideae, and 1 species of Araliaceae (*Aralia chinensis* L.) were included in this study (Table 1). Within subfamily Apioideae, two accessions each of *Heteromorpha involucrata* and *Steganotaenia araliacea* were also considered, bringing the total number of accessions examined to 76. Subfamilial placement of these taxa is based on Pimenov and Leonov (1993). Drude's (1898) tribal and subtribal categories have not been used, as their artificiality has been demonstrated or expressed by many (reviewed in Downie et al. 1998).

Of the nine endemic African genera considered in this investigation, *Glia* and *Polemanniopsis* are each monotypic (Burtt 1988, 1991), *Dracosciadium* consists of 2 species (Hilliard and Burtt 1986), *Polemannia* and *Steganotaenia* each comprise 3 species (Hilliard and Burtt 1986; Burtt 1991; Thulin 1991), *Diplolophium* has 5 species (Burtt 1991), *Anginon* has 12 species (Allison and

Table 1. Accessions of Apiaceae subfamily Apioideae and related taxa examined for chloroplast *rps*16 intron DNA sequence variation.

Taxon	Source and voucher information	GenBank accession No.
Apiaceae subfamily Apioideae		
Aegokeras caespitosa (Sibth. & Sm.) Raf.	Cult. RBGE (No. 19100154) from plant obtained from University of Cambridge Botanic Garden, England	AF110541
Aethusa cynapium L.	Cult. UIUC from seeds obtained from Jardin botanique de Caen, France, *Downie 337* (ILL)	AF110539
Anethum graveolens L.	Cult. UIUC from seeds obtained from University of Oldenburg Botanic Garden, Germany, *Downie 157* (ILL)	AF110542
Angelica archangelica L.	Cult. UIUC from seeds obtained from University of Joensuu Botanical Garden, Finland, *Downie 79* (ILL)	AF110536
Anginon rugosum (Thunb.) Raf.	South Africa, Western Cape, *Batten 1018* (UC), cult. UCB, Constance pers. coll. No. C-2399	AF110573
Anginon verticillatum (Sond.) B. L. Burtt	South Africa, summit of the Ploegberg complex, 20 Sept. 1989, *Viviers 2111* (E)	AF110574
Anisotome aromatica Hook. f. var. *pinnatisecta* Allan	New Zealand, South Island, Canterbury, Mt. Hutt, *Corden 29* (E), cult. RBGE (No. 19881687)	AF110550
Annesorhiza altiscapa Schltr. ex H. Wolff	South Africa, Nieuwoudlville, Glenlyon Farm, 15 August 1993, *Batter AB1192* (E)	AF110582
Anthriscus caucalis M. Bieb.	Cult. UIUC from seeds obtained from Jardin botanique de Caen, France, *Lee 44* (ILL)	AF110549
Apium graveolens L.	Cult. UIUC from seeds obtained from Conservatoire et Jardins botaniques de Nancy, France, *Downie 258* (ILL)	AF110545
Aulacospermum anomalum Ledeb.	Russia, Altayskiy Kray, cult. RBGE (No. 19932275) from seeds obtained from Moscow State University Botanical Garden, Russia	AF110558
Aulacospermum simplex Rupr.	Kirghizia, cult. Moscow State University Botanical Garden, Russia	AF110557
Bupleurum americanum J. M. Coult. & Rose	U.S.A., Wyoming, Teton Co., Gros Ventre Area, 7 July 1994, *Hartman 47328* (RM)	AF110563
Bupleurum angulosum L.	Pyrenees, *Younger 2565* (E), cult. RBGE (No. 19861043)	AF110568
Bupleurum chinense DC.	Cult. UIUC from seeds obtained from Shanghai Botanic Garden, China, *Downie 409* (ILL)	AF110565
Bupleurum falcatum L.	Cult. Moscow State University Botanical Garden, Russia, seeds obtained from Wroclaw Botanic Garden, Poland, 1988	AF110566
Bupleurum fruticosum L.	Spain, Jaén, Cazorla, Sierra de Pozo, 6 Dec. 1991, *McBeath 2592* (E), cult. RBGE (No. 19921249)	AF110569
Bupleurum ranunculoides L.	Cult. UIUC from seeds obtained from Hungarian Academy of Sciences Botanical Garden, Vácrátót, Hungary, *Downie 94* (ILL)	AF110564
Bupleurum rotundifolium L.	Cult. UIUC from seeds obtained from Jardin botanique de Caen, France, *Downie 304* (ILL)	AF110567
Conium maculatum L.	Cult. UIUC from seeds obtained from Conservatoire et Jardins botaniques de Nancy, France, *Downie 241* (ILL)	AF110546
Crithmum maritimum L.	Cult. UIUC from seeds obtained from Quail Botanical Gardens, California, *Downie 345* (ILL)	AF110540
Cymopterus montanus Nutt. ex Torr. & A. Gray	U.S.A., Colorado, El Paso Co., Rockrimmon Rd., 18 May 1982, *Hartman 13968* (RM)	AF110534
Daucus carota L.	Cult. UIUC from seeds obtained from Jardin botanique de Montréal, Canada, *Downie 386* (ILL)	AF110547
Diplolophium somaliense Verdc.	Africa, *MT 9176* (E)	AF110562
Dracosciadium italae Hilliard & B. L. Burtt	South Africa, Natal, Ngotshe District, Itala Nature Reserve, Lovwsburg Escarpment, 21 Jan. 1983, *Porter 620* (E)	AF110581
Eleutherospermum cicutarium (M. Bieb.) Boiss.	Russia, N Caucasus, Chechen Republic, Harami Pass, 2 July 1976, *Pimenov et al. 166* (MW), cult. Moscow State University Botanical Garden, Russia	AF110561
Erigenia bulbosa (Michx.) Nutt.	U.S.A., Illinois, Alexander Co., Shawnee National Forest, 13 April 1994, *Phillippe 23573* (ILLS)	AF110554
Foeniculum vulgare Mill.	Cult. UIUC from seeds obtained from National Botanic Gardens, Glasnevin, Ireland, *Downie 187* (ILL)	AF110543
Glia prolifera (Burm. f.) B. L. Burtt	South Africa, Cape Province, Fernkloof Nature Reserve, 4 February 1992, *Barker 96/A* (E), cult. RBGE (No. 19923034)	AF110572
Heracleum lanatum Michx.	U.S.A., California, Marin Co., Muir Woods, *Downie 579* (ILL)	AF110537

Table 1. (*continued*).

Taxon	Source and voucher information	GenBank accession No.
Heteromorpha arborescens (Spreng.) Cham. & Schltdl. var. *arborescens*	Cult. UIUC from seeds obtained from Real Jardín Botánico, Spain, *Downie 42* (ILL)	AF110575
Heteromorpha arborescens (Spreng.) Cham. & Schltdl. var. *abyssinica* (A. Rich.) H. Wolff	Malawi, Mt. Mulanje, Linji (Litchenya) Plateau, 17 Feb. 1986, *Chapman & Chapman 7223* (E)	AF110578
Heteromorpha involucrata Conrath	Tanzania, Mbeya District, Mshewe Rapids, 9 Feb. 1990, *Lovett et al. 4142* (E)	AF110576
Heteromorpha involucrata Conrath	Tanzania, Mbeya District, Punguluma Hills above Mshewe and Muvwa villages, 12 Feb. 1990, *Lovett et al. 4158* (E)	AF110577
Heteromorpha pubescens Burtt Davy	South Africa, Transvaal, Letaba 2 District, Lekgalameetse Nature Reserve, 10 Apr. 1990, *Balkwill et al. 5602* (E)	AF110580
Heteromorpha stenophylla Welw. ex Schinz var. *transvaalensis* (Schltr. & H. Wolff) P. J. D. Winter	South Africa, Transvaal, Barberton District, Songimvelo Game Reserve, 5 Dec. 1991, *Balkwill et al. 6665* (E)	AF110579
Komarovia anisosperma Korovin	Uzebekistan, Zeravschan Mts., Urgut, 30 May 1978, *Pimenov* et al. 178 (MW), cult. Moscow State University Botanical Garden, Russia	AF110555
Oenanthe pimpinelloides L.	Cult. UIUC from seeds obtained from Jardin botanique National de Belgique, Belgium, *Downie 273* (ILL)	AF110553
Pastinaca sativa L.	Cult. UIUC from seeds obtained from Johannes Gutenberg University, Germany, *Downie 70* (ILL)	AF110538
Petroselinum crispum (Mill) A.W. Hill	Cult. UIUC from seeds obtained from Institut für Pflanzengenetik und Kulturpflanzenforschung, Germany, *Downie 34* (ILL)	AF110544
Physospermum cornubiense (L.) DC.	Ukraine, Crimea, Alikat-Bogaz Pass, 4 Apr. 1974, *Pimenov & Tomkovich s.n.* (MW), cult. Moscow State University Botanical Garden, Russia	AF110556
Pleurospermum foetens Franch.	China, Yunnan, 12 October 1990, *Chungtien et al. 1181* (E), cult. RBGE (No. 19910914)	AF110559
Pleurospermum uralense Hoffm.	Russia, Altai Mts., Charyshskoya, 13 Sept. 1989, *Pimenov et al. s.n.* (MW), cult. Moscow State University Botanical Garden, Russia	AF110560
Polemannia montana Schltr. & H. Wolff	South Africa, Natal, Underberg District, 27 Jan. 1975, *Hilliard & Burtt 7751* (E)	AF110570
Polemannia simplicior Hilliard & B. L. Burtt	South Africa, Eastern Cape, Barkly East District, 6 Feb. 1983, *Hilliard & Burtt 16487* (E)	AF110571
Polemanniopsis marlothii (H. Wolff) B. L. Burtt	South Africa, Western Cape, Piekniek Klippe, Pakhuis Pass, N Cedarberg, 13 Oct. 1987, *Taylor 11817* (E)	AF110597
Sium latifolium L.	Cult. UIUC from seeds obtained from Hungarian Academy of Sciences Botanical Garden, Vácrátót, Hungary, *Downie 97* (ILL)	AF110552
Smyrnium olusatrum L.	Cult. UIUC from seeds obtained from University of Oldenburg Botanical Garden, Germany, *Downie 141* (ILL)	AF110551
Steganotaenia araliacea Hochst.	South Africa, Transvaal, Pilgrim's Rest District, SW of Hoedspruit, 10 Oct. 1987, *Balkwill* & *Cadman 3779* (E)	AF110595
Steganotaenia araliacea Hochst.	Tanzania, Arusha Region, Arumeru District, Arusha, *Gereau* & *Mziray 1684* (MO)	AF110596
Torilis arvensis (Huds.) Link	U.S.A., Illinois, Champaign Co., Urbana, *Downie 816* (ILL)	AF110548
Zizia aurea (L.) W. D. J. Koch	Cult. UIUC from seeds obtained from Jardin botanique de Montréal, Canada, *Downie 393* (ILL)	AF110535
Apiaceae subfamily Saniculoideae		
Astrantia major L. ssp. *major*	Switzerland, Sept. 1986, *Schilling 2937* (E), cult. RBGE (No. 19861407)	AF110594
Eryngium alpinum L.	Austria, Wien, Heldenfriedhof, cult. RBGE (No. 19820697) from seeds obtained from Salzburg University, Austria	AF110583
Eryngium alternatum J. M. Coult. & Rose	Mexico, Jalisco, Puerto de las Cruces, *Fuentes 654* (UC), cult. UCB, Constance pers. coll. No. C-2377	AF110590
Eryngium coronatum Hook. & Arn.	Paraguay, Paraguarí, Arroyo Yuquyty, E of Nueva Italia, 14 December 1989, *Zardini & Velazquez 17068* (UC), cult. UCB, Constance pers. coll. No. C-2389	AF110586
Eryngium mexiae Constance	Cult. UCB, Constance pers. coll. No. C-2418	AF110589

Table 1. (*concluded*).

Taxon	Source and voucher information	GenBank accession No.
Eryngium planum L.	Cult. UIUC from seeds obtained from National Botanic Gardens, Glasnevin, Ireland, *Downie 191* (ILL)	AF110584
Eryngium proteaflorum D. Delaroche	Mexico, Mexico, Estada, *Walker 765* (E), cult. RBGE (No. 19930224)	AF110585
Eryngium spiculosum Hemsl.	Mexico, Michoacán, Jiquilpan, *Ruiz 3402* (UC), Cult. UCB (No. 94.0960), Constance pers. coll. No. C-2415	AF110588
Eryngium yuccifolium Michx.	U.S.A., Illinois, Brown Co., *Tyson s.n.* (UC), cult. UCB (No. 86.0104)	AF110587
Hacquetia epipactis (Scop.) DC.	Cult. RBGE (No. 19694625)	AF110591
Petagnaea saniculifolia Guss.	Cult. RBGE (No. 19695641)	AF110593
Sanicula canadensis L.	U.S.A., Illinois, Champaign Co., Urbana, *Downie 737* (ILL)	AF110592
Apiaceae subfamily Hydrocotyloideae		
Azorella trifurcata Pers. "Nana"	Cult. RBGE (No. 19760821)	AF110599
Bolax gummifera (Lam.) Spreng.	Cult. RBGE (No. 19361025)	AF110600
Centella asiatica (L.) Urb.	Cult. UCB, Constance pers. coll. No. C-2198	AF110603
Centella erecta (L. f.) Fern.	U.S.A., Florida, Wakulla Co., St. Marks Wildlife Refuge, 12 Apr. 1971, *Godfrey s.n.* (UC), cult. UCB, Constance pers. coll. No. C-1477	AF110602
Centella hirtella Nannf.	Argentina, Corrientes, Santa Rosa, February 1978, *Eskuche s.n.* (UC), cult. UCB, Constance pers. coll. No. C-2108	AF110605
Centella triflora (R. & P.) Nannf.	Chile, Valdivia, Instituto de Botánica, Universidad Austral, Apr. 1980, *Romero & Klempau s.n.* (UC), cult. UCB, Constance pers. coll. No. C-2140	AF110604
Eremocharis fruticosa Phil.	Chile, Antofagasta, Quebrada Coquimbo, Taltal, *Dillon & Teillier 5082* (UC), cult. UCB, Constance pers. coll. No. C-2382	AF110598
Hydrocotyle pusilla A. Rich.	Equador, *Ornduff 9683* (UC), cult. UCB, Constance pers. coll. No. C-2353	AF110608
Hydrocotyle rotundifolia Wall.	Cult. Missouri Botanical Garden (No. 895612)	AF110607
Klotzschia rhizophylla Urb.	Brazil, Minas Gerais, Serra do Cipo, *Pirani CFSC 12909* (UC), cult. UCB, Constance pers. coll. No. C-2414	AF110601
Xanthosia atkinsoniana F. Muell.	Australia, cult. Moscow State University Botanical Garden, Russia	AF110606
Araliaceae		
Aralia chinensis L.	Cult. UIUC from seeds obtained from Shanghai Botanic Garden, China, *Downie 407* (ILL)	AF110609

Note: These sequence data have been deposited with GenBank under the accession numbers cited (AF110534–AF110609). Herbarium acronyms are according to Holmgren et al. (1990). RBGE, Royal Botanic Garden Edinburgh; UIUC, University of Illinois at Urbana-Champaign; UCB, Botanical Garden of the University of California at Berkeley.

van Wyk 1997), *Annesorhiza* has 12–15 species (Burtt 1991), and *Heteromorpha* comprises 7 species with 7 varieties (Winter and van Wyk 1996). We have followed the recent treatments for *Anginon* and *Heteromorpha* and, in the absence of studies suggesting otherwise, assumed that the remaining taxa are each monophyletic. Our work to date has centered on resolving the suprageneric relationships within subfamily Apioideae and in the phylogenetic placement of genera whose relationships have heretofore been obscure. In this regard, our sampling of herbaceous endemic African genera is admittedly sparse, being largely based on what material was available for analysis, with some non-monotypic genera represented by only one or two species (Table 1). Nevertheless, our results provide the necessary framework and explicit phylogenetic hypotheses from which future revisionary and other systematic studies can proceed.

Genera not restricted to Africa were included in this study with the aim of representing most major lineages of Apioideae, as defined previously (Plunkett et al. 1996*b*; Downie et al. 1998, 2000; Katz-Downie et al. 1999; Plunkett and Downie 1999). Twelve major clades of Apioideae have been identified; here we included representation from 11 of them. The *Conioselinum* clade (group 8) of Downie et al. (1998) and Katz-Downie et al. (1999) was omitted because of lack of material for analysis; this clade includes representatives of two non-monophyletic herbaceous genera of cosmopolitan distribution, *Conioselinum* and *Ligusticum*. The 11 apioid groups represented in this study are described elsewhere in this paper and are illustrated on all tree figures presented herein. Consideration was also given to those taxa included in previous phylogenetic studies based on *rpo*C1 or *rpl*16 intron sequences, so that a comparison among the results of each of these studies can be made. To assess the ability of the *rps*16 intron to resolve low-level relationships within the family, seven species of *Bupleurum* (Apioideae), eight species of *Eryngium* (Saniculoideae), and four species of *Centella* (Hydrocotyloideae) were examined. While each of these genera are presumably monophyletic, they represent particularly troublesome groups whose infrageneric relationships have been difficult to resolve. Moreover, both Old and New World species of *Bupleurum* were included, representing herbaceous and woody members, to assess the phylogenetic position of this genus relative to *Heteromorpha* and other putatively basal apioids.

Experimental strategy

Leaf material for DNA extraction was obtained either directly from the field, from plants cultivated from seed in the greenhouse, from accessioned plants cultivated at several botanic gardens, or for the majority of the southern African species, from herbarium specimens. Total genomic DNAs were isolated from herbarium material using a slightly modified version of Doyle and Doyle's

(1987) CTAB procedure. Upon the addition of 1.0% sodium bisulfite and 1.0% polyvinylpyrrolidone (PVP) to the 2× CTAB isolation buffer, approximately 30 mg of leaf tissue was ground in a mortar with pestle. The homogenate was incubated at 60°C for 30 min prior to treatment with chloroform – isoamyl alcohol. Dried herbarium leaf tissue from samples as old as 25 years yielded DNAs suitable for polymerase chain reaction (PCR) amplification and sequencing. The CTAB procedure was also used to extract total genomic DNA from living material. These DNAs were purified by centrifugation in cesium chloride – ethidium bromide gradients.

For all 76 accessions, a region containing the complete *rps*16 intron and about half of its 3′ exon was amplified using the PCR method and primers *rps*16 5′ exon (AAACGATGTGGNAGNAA RCA) and *rps*16 3′ exon (CCTGTAGGYTGNGCNCCYTT) in an equimolar ratio (primers written 5′ to 3′). These primers were designed by comparing published *rps*16 exon sequences from tobacco, rice, mustard (*Sinapsis alba* L. cv. Albatros), and barley (*Hordeum vulgare* L.) and choosing regions highly conserved among them (Shinozaki et al. 1986; Hiratsuka et al. 1989; Neuhaus et al. 1989; Sexton et al. 1990). In tobacco cpDNA, the *rps*16 intron is 860 base pairs (bp) in size, the 3′ end of primer *rps*16 5′ exon is three positions away from the 5′ exon – intron junction, and the 3′ end of primer *rps*16 3′ exon is 135 positions downstream from the intron – 3′ exon junction (Shinozaki et al. 1986). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa).

Details of the amplification reactions were the same as those presented in Downie and Katz-Downie (1996), with the exception of an increase in the $MgCl_2$ concentration from 1.5 to 3.0 mM. Each PCR reaction proceeded as follows: (*i*) 1 min at 94°C; (*ii*) 1 min at 53°C; and (*iii*) 1 min at 72°C. The first cycle was preceded by an initial denaturation step of 30 s at 94°C. A 10-min 72°C extension period followed completion of the 35 thermal cycles. All taxa examined possessed an intron in chloroplast gene *rps*16. The ensuing PCR fragments were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and sized against *Eco*RI–*Hin*dIII digested lambda DNA standards and (or) a positive control (i.e., a PCR product from tobacco cpDNA). Successful PCR amplifications resulted in a single DNA band of about 1100 bp. After isolation in agarose, the amplification products were purified using the Elu-Quik DNA Purification Kit (Schleicher & Schuell, Keene, N.H.) according to the manufacturer's instructions.

All sequencing was done using an Applied Biosystems, Inc. (Foster City, Calif.) 373A automated DNA sequencer with Stretch upgrade at the Genetic Engineering Facility of University of Illinois at Urbana–Champaign's Biotechnology Center. Cycle sequencing reactions were carried out in a PTC-100 thermocycler (M.J. Research, Inc., Cambridge, Mass.) using the purified PCR products, AmpliTaq DNA polymerase, and fluorescent dye-labeled terminators (Perkin-Elmer Corp, Norwalk, Conn.). The reaction conditions were as specified by the manufacturer, with the addition of 5% dimethylsulfoxide (DMSO). The sequencing products, after purification with Centri-Sep spin columns (Princeton Separations, Adelphia, N.J.), were resolved by electrophoresis in 4% acrylamide gels. Primers *rps*16 5′ exon and *rps*16 3′ exon each generated 600–700 (and occasionally up to 800) bases per reaction with little background and few ambiguities. All automated output was checked visually and edited for correct automated base-calling. Simultaneous consideration of both DNA strands across the entire intron gave sufficient overlap for unambiguous base determination in nearly all cases.

Multiple sequence alignment and gap coding

The DNA sequences were aligned initially using CLUSTAL W version 1.7 (Thompson et al. 1994), copied into the data editor of PAUP* version 4.0.0d64 (D. Swofford, Smithsonian Institution, Washington, D.C.), and realigned manually. Gaps were coded by hand and positioned to minimize nucleotide mismatches. Gaps of equal length in more than one sequence were coded as the same character state if they could not be interpreted as different duplication or insertion events. Similarly located but different-length indels were coded as multiple binary characters. In several regions of the alignment, gap coding was particularly problematic; these regions were excluded from the analysis. In the maximum parsimony analysis, indels and substitutions were given equal weights. Gaps were not treated as separate characters in the maximum-likelihood and neighbor-joining analyses.

Pairwise nucleotide differences of unambiguously aligned positions were determined using the distance matrix option in PAUP*. Alignment gaps in any one sequence were treated as missing data for all taxa. These divergence values were calculated simply as the proportion of divergent sites in each direct pairwise comparison. Transition/transversion (Ts/Tv) rate ratios over a subset of the maximally parsimonious trees were calculated using MacClade version 3.01 (Maddison and Maddison 1992). Polytomies were arbitrarily resolved and ambiguous base calls ignored. The nucleotide sequence data reported in this study have been deposited with the GenBank Data Library (accession numbers are provided in Table 1), and the complete aligned data matrix can be obtained directly from the authors.

Phylogenetic analysis

The resulting alignment and gap codes were analyzed initially using equally weighted maximum parsimony (MP). Two data matrices were considered: the full 76-taxon matrix and a reduced matrix of 60 accessions. For the full matrix, maximally parsimonious trees were sought using PAUP* and the heuristic search strategies described in Downie et al. (1998), based on those presented in Catalán et al. (1997). The length of the shortest trees was determined by initiating 500 random addition replicate searches, 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 a maximum tree limit of 5000. The strict consensus of these 5000 trees was subsequently used as a topological constraint. Once more, 500 random addition replicate searches were initiated as above, saving no more than five trees from each search. However, only those trees that did not fit the constraint tree were saved. As no additional trees were found at the length of the initial 5000 trees, this suggested strongly that the strict consensus tree does adequately summarize the available evidence, even though the exact number of trees at that length 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 branch swapping, with a maximum tree limit of 500 trees per replicate set.

Given the large number of taxa, the inability to ascertain the number of equally most parsimonious trees, and the limitation imposed on the bootstrap analysis, a subsequent MP analysis was carried out upon the removal of 16 taxa. A smaller data matrix was also necessary to decrease the amount of computational time required to complete the maximum likelihood analysis, particularly when global branch swapping is invoked (described below). Excluded were six of the seven species of *Bupleurum*, seven of the eight species of *Eryngium*, and three of the four species of *Centella*. The species within each of these genera had very similar or identical DNA sequences and are monophyletic based on the results of the MP analysis of the full matrix. MP analysis of the reduced (60-taxon) matrix, using 500 random addition replicate searches, TBR branch-swapping, and mulpars selected, resulted in a finite number of minimal length trees. Bootstrap analyses were carried out as above, but without a maximum tree limit.

Table 2. Characteristics of the multiple alignment of 76 (full matrix) or 60 (reduced matrix) cpDNA *rps*16 intron and flanking *rps*16 3′ exon sequences from Apiaceae and the outgroup *Aralia chinensis*.

Characteristic	Full matrix	Reduced matrix
Nucleotide sites		
Length variation (bp)	868–1018[a]	868–1018
Mean length variation (bp)	974[b]	976
No. of total aligned positions	1302	1297
No. of aligned positions excluded (%)	260 (20.0)	260 (20.0)
No. of aligned positions constant (%)	653 (50.2)	662 (51.0)
No. of aligned positions autapomorphic (%)	142 (10.9)	168 (13.0)
No. of aligned positions parsimony informative (%)	247 (19.0)	207 (16.0)
Gaps		
No. of unambiguous alignment gaps	113	104
No. of unambiguous alignment gaps parsimony informative	42	32
Sequence divergence (range in %)		
All accessions	0–12.3	0–12.3
All included Apioideae accessions	0–9.7	0–9.3

[a]Length variation of only the *rps*16 intron is 758–908 bp.

[b]Mean length variation of only the *rps*16 intron is 864 bp.

The reduced (60-taxon) matrix was also analyzed using neighbor-joining (NJ) and maximum-likelihood (ML) methods. Distance trees were obtained from NJ analyses of the reduced matrix (Saitou and Nei 1987), estimated using Kimura's (1980) two-parameter method as implemented in PAUP*. Two Ts/Tv rate ratios were used (1.0 and 2.0), with the former approximating the expected ratio of Ts to Tv as inferred by the MP analysis. A bootstrap analysis was done using 1000 resampled data sets. Using the program fastDNAml (version 1.0.6; Olsen et al. 1994), ML trees were inferred using a Ts/Tv rate ratio of 2.0, randomizing the input order of sequences (jumble), and invoking the global branch swapping search option. The heuristic analysis was repeated until three separate runs, each starting with a different random number seed, produced the same highest (least negative) log likelihood value. Empirical base frequencies were derived from the sequence data and used in the ML calculations. Bootstrapping of the ML data was computationally prohibitive.

All trees computed were rooted with *Aralia chinensis*, the only accession of Araliaceae included in this study. Phylogenetic analyses of molecular data (Plunkett et al. 1996a, 1997) corroborate traditional taxonomic evidence (Dahlgren 1980; Thorne 1992) in suggesting that Araliaceae are closely allied to Apiaceae.

Results

Sequence analysis

Among all 76 representatives of Apiaceae and Araliaceae examined, the *rps*16 intron varied in length from 758 (*Diplolophium somaliense*) to 908 bp (*Annesorhiza altiscapa*) and averaged 864 bp. Percent G+C content ranged from 31.8 to 37.2%, averaging 34.6%. All sequencing reactions culminated in an additional 110 bp of sequence from the adjacent *rps*16 3′ exon region (about half the entire length of the exon), with no length variation. Alignment of all 76 *rps*16 intron and flanking 3′ exon sequences resulted in a matrix of 1302 positions (Table 2). However, because of frequent length mutations of varying sizes within particular regions of the intron confounding interpretation of homology, it was necessary to exclude 21 regions (260 alignment positions) from the analysis. These ambiguous regions ranged in size from 2 to 38 positions (averaging 12 positions), with many of them characterized by tracts of poly-As, -Gs, and -Ts. Of the remaining 1042 unambiguously aligned positions, 653 were unvarying, 142 were variable but uninformative for parsimony analysis, and 247 were informative (Table 2). A total of 113 unambiguous gaps was required for proper alignment of these sequences. These gaps ranged in size from 1 to 117 bp, averaging 7 bp (Fig. 1). Relative to the outgroup *Aralia chinensis*, these gaps represent a minimum of 61 insertion and 48 deletion events; four gaps could not be polarized, as they were unique to *Aralia*. Forty-two of these 113 gaps were informative for parsimony analysis, ranging in size between 1 and 22 bp, and averaging 5 bp (Fig. 1). Measures of pairwise sequence divergence ranged from identity to 12.3% (the latter between *Torilis arvensis* and *Hydrocotyle rotundifolia*). Three species of *Eryngium* (*E. alternatum*, *E. mexiae*, and *E. yuccifolium*), two species of *Bupleurum* (*B. americanum* and *B. chinense*), and *Anginon rugosum* and *Glia prolifera* each yielded identical DNA sequences. Within subfamily Apioideae, the greatest sequence divergence occurred between *Torilis* and *Bupleurum rotundifolium*, with a value of 9.7%. Sequence characteristics of the reduced matrix, including the number of aligned and parsimony informative positions, are provided in Table 2.

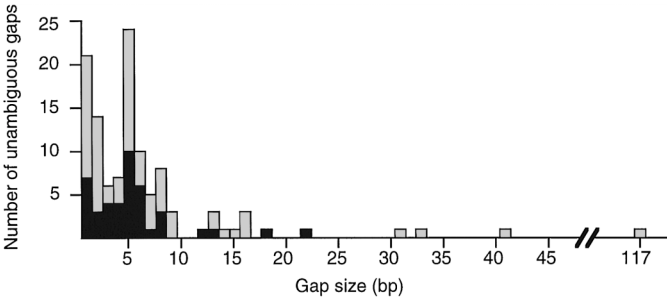
Like other plastid group II introns, the intron in chloroplast gene *rps*16 exhibits considerable conservation of secondary structure and is characterized by six centrally radiating structural components (designated as domains I–VI; Michel and Dujon 1983; Michel et al. 1989). Each of these structural regions includes highly conserved stem portions and, generally, less conserved loop portions. The determination of conserved domain boundary sequences for the *rps*16 intron in Apiaceae was based on similar boundary sequences inferred for tobacco and mustard (Michel et al. 1989; Neuhaus et al. 1989). For each of these six domains and across all 76 intron sequences (the flanking *rps*16 3′ exon portions were excluded), the number of constant, autapomorphic, informative, and excluded aligned positions, the range in overall size, the maximum pairwise sequence divergence, and the number of unambiguous alignment gaps were calculated (Table 3). Domain I is the largest, averaging 485.7 bp in

Table 3. Sequence characteristics of the six major structural domains of the cpDNA group II *rps*16 intron across 76 accessions of Apiaceae and the outgroup *Aralia chinensis* (Araliaceae).

Characteristic	Intron domain					
	I	II	III	IV	V	VI
Length variation (range in bp)	466–517	70–108	48–78	21–150	34–34	21–34
Length average (bp)	485.7	86.8	66.5	134.3	34.0	33.5
No. of total aligned positions	658	161	82	201	34	34
No. of aligned positions excluded	110	80	17	53	0	0
No. of aligned positions constant	335	41	32	77	28	23
No. of aligned positions autapomorphic	78	15	13	20	3	8
No. of aligned positions parsimony informative	135	25	20	51	3	3
No. of unambiguous alignment gaps	54	23	12	21	0	3
No. of unambiguous alignment gaps parsimony informative	22	10	3	5	0	2
Maximum sequence divergence (%)	13.5	30.2	24.1	27.4	11.8	14.7

size, whereas domains V and VI are the smallest, ranging between 21 and 34 bp. Domains V and VI are most conserved evolutionarily, with relatively few informative positions, low sequence divergence, and very few or no alignment gaps. Variation among the remaining four domains was difficult to assess owing to their relative differences in size. In domain II, approximately 50% of the region was excluded because of alignment ambiguity, and pairwise sequence divergence values just slightly exceeded 30% of nucleotides. Domain III had proportionally the most variable nucleotide positions, followed closely by domains IV then I. The small size of the *rps*16 intron in *Diplolophium somaliense* is due to a 117-bp deletion, representing the almost complete removal of domain IV. The 21 nucleotides remaining form a stem structure and represent the extreme 5′ and 3′ termini of this domain. Gaps of 31, 33, and 41 bp (Fig. 1) also represent large deletions and are located in domains II, I, and I, respectively.

Fig. 1. Frequency of unambiguous gaps in relation to gap size inferred in the alignment of 76 cpDNA *rps*16 intron and flanking 3′ exon sequences from Apiaceae and the outgroup *Aralia chinensis* (average gap size 7 bp). Solid bars indicate phylogenetically informative gaps.



Phylogenetic analysis

MP analysis of 1042 unambiguously aligned *rps*16 intron and flanking exon nucleotide positions and 42 parsimony informative alignment gaps resulted in more than 10 000 minimal length trees before termination of analysis. The strict consensus of 5000 of these trees, each of length 869 steps, consistency indices (CIs) of 0.6709 and 0.5972, with and without uninformative characters, respectively, and retention index (RI) of 0.8788, is shown in Fig. 2 with accompanying bootstrap values.

The MP analysis of the reduced matrix, which included 1037 unambiguously aligned nucleotide positions and 32 informative gaps (Table 2), resulted in 288 minimal length trees each of 793 steps (CIs = 0.6797 and 0.5745, with and without uninformative characters, respectively; RI = 0.8452). The strict consensus of these 288 trees, with accompanying bootstrap values, is presented in Fig. 3. Analysis of the data without the 32 scored informative gaps resulted in 216 maximally parsimonious trees each of 755 steps (CIs = 0.6715 and 0.5564, with and without uninformative characters, respectively; RI = 0.8358). The topology of this strict consensus tree was nearly identical to that when the gaps were included, with the exception of the four regions in Fig. 3 highlighted by arrows. The exclusion of gap scoring from the analysis results in (*i*) *Crithmum* and *Aegokeras* forming a clade, (*ii*) the collapse of the branch leading to *Erigenia*, (*iii*) a paraphyletic *Pleurospermum* from which *Aulacospermum* is derived, and (*iv*) *Heteromorpha arborescens* var. *arborescens* sister to the clade of *Anginon, Glia,* and *Polemannia*.

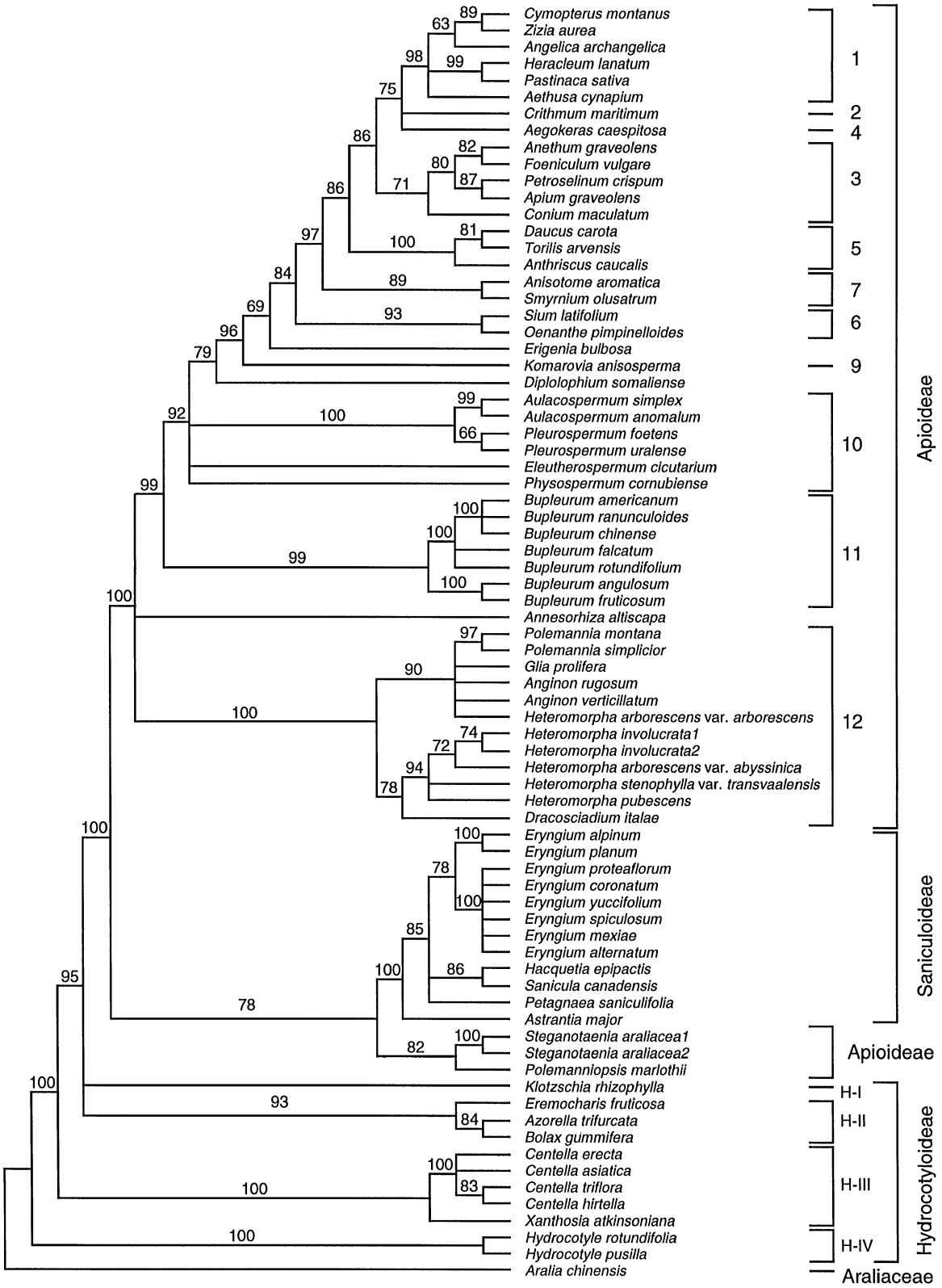
Distance trees obtained from NJ analysis, estimated from the two-parameter method of Kimura (1980) using either a Ts/Tv rate ratio of 1.0 or 2.0 and the reduced data matrix, were topologically identical. The tree constructed with a rate ratio of 2.0 is presented in Fig. 4A. The best ML tree, calculated with a Ts/Tv rate ratio of 2.0, had a log likelihood value of –6276.34774, and appears in Fig. 4B.

Phylogenetic resolutions

Phylogenies estimated using MP, NJ, or ML methods reveal that, in the context of those species examined, Apiaceae subfamily Apioideae is monophyletic if *Steganotaenia* and *Polemanniopsis* are excluded. Apiaceae subfamily Saniculoideae is also monophyletic, and sister to *Steganotaenia* plus *Polemanniopsis*. Subfamily Hydrocotyloideae is not monophyletic, consisting of three (NJ; Fig. 4A) or four (MP and ML; Figs. 2, 3, and 4B) basally branching lineages.

Similar groupings of taxa within subfamily Apioideae are recognized in all trees. These major clades, identified by numbered brackets in Figs. 2–4, coincide with those same groups recognized on the basis of parsimony analysis of *rpo*C1 intron sequences (Downie et al. 1998). Group 1, the

Fig. 2. Strict consensus tree of 5000 minimal length 869-step trees derived from equally weighted maximum parsimony analysis of 76 *rps*16 intron and flanking 3′ exon sequences from Apiaceae and the outgroup *Aralia chinensis* using 1042 unambiguously aligned nucleotide positions and 42 parsimony informative gaps (excluding uninformative characters, CI = 0.5972; RI = 0.8788). Numbers above the nodes are bootstrap estimates for 100 replicate analyses (based on a set maximum tree limit of 500 trees per replicate). Numbered groups of Apiaceae circumscribed on the basis of parsimony analysis of cpDNA *rpo*C1 intron sequences (Downie et al. 1998; see text for descriptions). H-I to H-IV indicate the four major lineages of Apiaceae subfamily Hydrocotyloideae.



Angelica clade, is strongly supported. The genera *Crithmum* and *Aegokeras* (syn. *Olymposciadium*), representing the *Crithmum* (group 2) and *Aegopodium* (group 4) clades (Downie et al. 1998; Katz-Downie et al. 1999), either unite as sister taxa in the ML (Fig. 4B) or MP trees (when gaps are excluded as additional characters; Fig. 3) or fall as successively basal taxa to group 1 in the NJ tree (Fig. 4A). Group 3 (the *Apium* clade) arises as sister to apioid groups 1, 2, and 4 (Figs. 2–4A) except in the ML tree (Fig. 4B), where *Conium* forms a separate branch. Resolution of relationships among the latter three groups is variable depending upon the method of tree construction used. Successively basal to group 1–4 is the *Daucus* clade (group 5), the *Aciphylla* clade (group 7), the *Oenanthe* clade (group 6), *Erigenia bulbosa*, and *Komarovia anisosperma* (the latter representing group 9, the *Komarovia* clade). The North American monotypic genus *Erigenia* has yet to be unambiguously assigned to any specific group of umbellifers. While nuclear rDNA internal transcribed spacer (ITS) data suggest an affinity of this taxon to apioid groups 9 and 10 (Katz-Downie et al. 1999), data from the *rpl*16 intron (Downie et al. 2000), like that of the *rps*16 intron, position this taxon as an isolated lineage between apioid groups 6 and 9.

The tropical African *Diplolophium*, represented by *D. somaliense*, is sister in all trees to apioid groups 1–9. Group 10, the *Physospermum* clade, has been delimited previously as comprising the genera *Physospermum*, *Eleutherospermum*, *Aulacospermum*, and *Pleurospermum* (Katz-Downie et al. 1999; Downie et al. 2000). Other than the NJ tree (Fig. 4A), where these four genera are monophyletic, the relationships among them are not clear. While *Aulacospermum* and *Pleurospermum* form a strongly supported clade in all trees, *Eleutherospermum* and *Physospermum* are variably positioned. The *Bupleurum* clade (group 11) is sister to apioid groups 1–10. A dichotomy exists among the seven examined species of *Bupleurum* (Fig. 2): *B. angulosum* and the shrubby, evergreen *B. fruticosum* comprise one subclade; the remaining five species comprise the other. Pairwise sequence divergence estimates among the seven species of *Bupleurum* are low, ranging between 0.1 and 5.0%.

The last major clade within subfamily Apioideae is group 12, previously designated as the *Heteromorpha* clade (Downie et al. 1998). In addition to *Heteromorpha*, this well-supported group consists of the African endemics *Anginon, Glia, Polemannia*, and *Dracosciadium*. Two subclades are recognized. *Polemannia montana* and *P. simplicior* unite as a strongly supported group alongside *Glia*, *Anginon*, and *Heteromorpha arborescens* var. *arborescens*. However, owing to low sequence divergence (0–0.8%), relationships among the members of this subclade cannot be discerned (*Glia* and the two species of *Anginon* have virtually identical *rps*16 intron sequences). The second subclade consists of *Dracosciadium* and all but one of the *Heteromorpha* accessions. Here, divergence values among pairwise comparisons are also low, ranging between 0.2 and 1.5%. The placement of *H. arborescens* var. *arborescens* away from *H. arborescens* var. *abyssinica* (= *H. trifoliata* (Wendl.) Eckl. & Zeyh.) and all other examined *Heteromorpha* is suggestive that both *H. arborescens* and the genus itself are not monophyletic. In most previous studies, as seen here in the

Fig. 3. Strict consensus tree of 288 minimal length 793-step trees derived from equally weighted maximum parsimony analysis of 60 *rps*16 intron and flanking 3′ exon sequences from Apiaceae and the outgroup *Aralia chinensis* using 1037 unambiguously aligned nucleotide positions and 32 parsimony informative gaps (excluding uninformative characters, CI = 0.5745; RI = 0.8452). Numbers above the nodes are bootstrap estimates for 100 replicate analyses (with no maximum tree limit set per replicate). The four arrows indicate regions that vary (discussed in text) when the 32 informative gaps are excluded and the analysis rerun (resulting in 216 minimal length trees each of 755 steps; excluding uninformative characters, CI = 0.5564; RI = 0.8358). *Het.*, *Heteromorpha*.

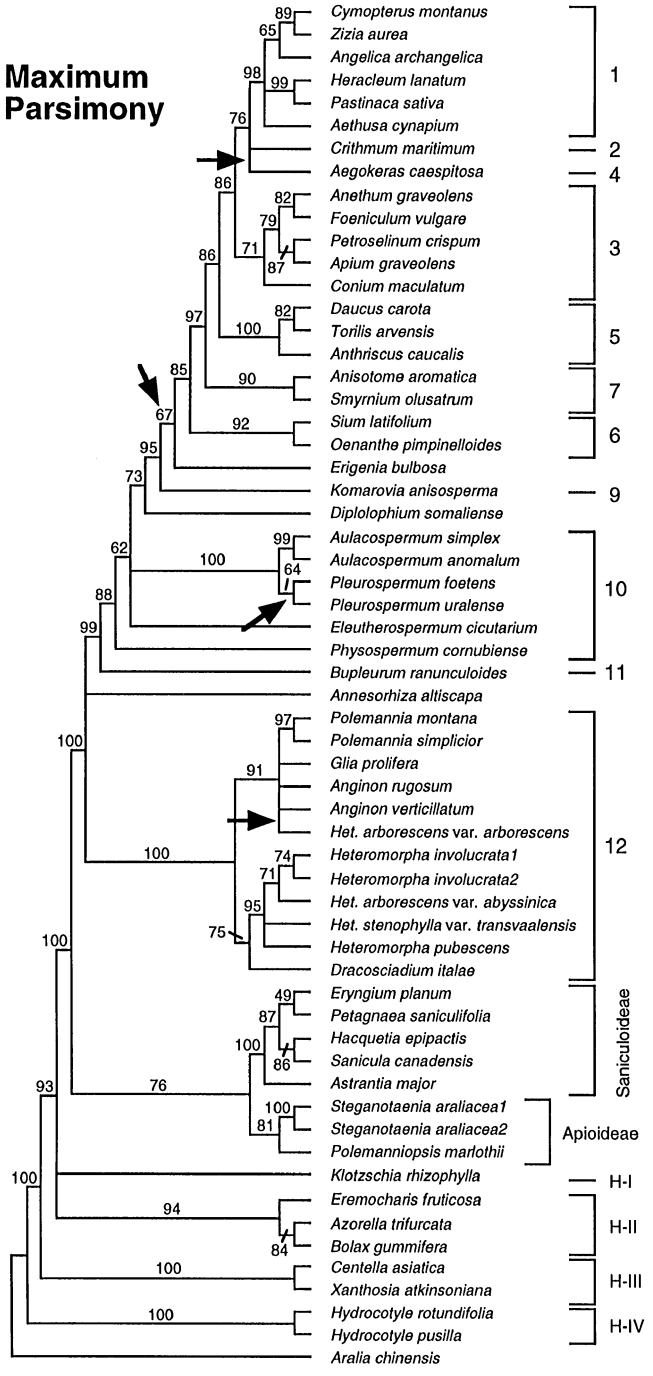
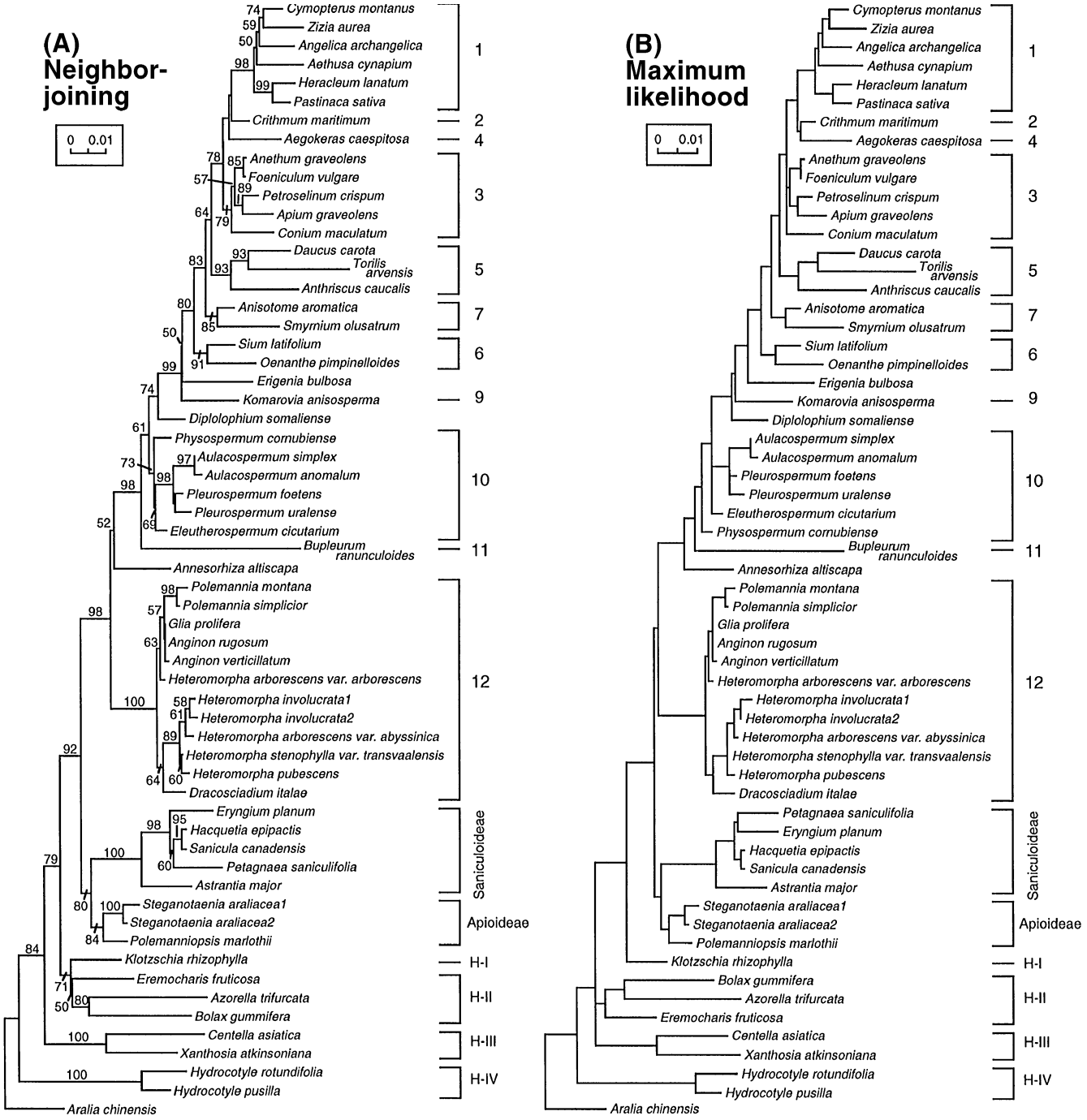


Fig. 4. Neighbor-joining (A) and maximum likelihood (B) trees inferred from 60 unambiguously aligned cpDNA *rps*16 intron and flanking 3′ exon sequences from Apiaceae and the outgroup *Aralia chinensis* using a transition/transversion rate ratio of 2.0. Branch lengths in Fig. 4A are proportional to distances estimated from the two-parameter method of Kimura. Values above the nodes indicate bootstrap estimates for 1000 replicate analyses; values <50% are not indicated. The maximum-likelihood tree in Fig. 4B had a log likelihood value of –6276.34774. Branch lengths are proportional to the number of expected nucleotide substitutions per site. Bootstrap values were not computed.



NJ and ML trees (Fig. 4), the *Heteromorpha* clade is clearly sister to all other examined Apioideae. In contrast, the strict consensus trees inferred using MP (Figs. 2 and 3) reveal a basal trichotomy within Apioideae, with the African *Annesorhiza altiscapa* occurring as a separate lineage alongside the *Heteromorpha* clade.

Steganotaenia and *Polemanniopsis*, treated as members of subfamily Apioideae in all existing taxonomic accounts, occur together as sister group to Apiaceae subfamily Saniculoideae. This assemblage is sister to all other members of subfamily Apioideae. The two accessions of *Steganotaenia araliacea* differ by six intron nucleotide positions (0.6% divergence) and unite with 100% bootstrap support. The five genera examined of Apiaceae subfamily Saniculoideae form a strongly supported clade. *Hacquetia* and *Sanicula* are clearly allied in all trees, but the relation-

ship between them and *Eryngium* and *Petagnaea* is not resolved. A major dichotomy in *Eryngium* is evident, with *E. alpinum* and *E. planum* sister to all other *Eryngium* species. Within *Eryngium*, pairwise sequence divergence ranges from 0 to 2.2% of nucleotides, with *E. alternatum*, *E. mexiae*, and *E. yuccifolium* having identical nucleotides across all intron positions.

Apiaceae subfamily Hydrocotyloideae is not monophyletic, with three or four basally branching lineages inferred in all trees: *Klotzschia* (H-I); *Eremocharis*, *Azorella*, and *Bolax* (H-II); *Centella* and *Xanthosia* (H-III); and *Hydrocotyle* (H-IV). The first two lineages unite in the NJ tree (Fig. 4A). While *Hydrocotyle* consistently occurs as sister to all other Apiaceae, the relationships among the three other clades are equivocal. *Centella* is monophyletic, with only 0.1–0.4% of all intron nucleotide positions varying.

Discussion

Molecular characteristics of the *rps*16 intron

The chloroplast gene *rps*16, encoding ribosomal protein S16 (Neuhaus et al. 1989), is interrupted by a group II intron in many different land plants (Downie and Palmer 1992). For those species where sequence data are available, this intron varies considerably in length, from 707 to 951 bp (Oxelman et al. 1997). In Apiaceae, the length of the *rps*16 intron varies between 758 and 908 bp (averaging about 864 bp). The former occurs in *Diplolophium somaliense* and reflects the almost complete removal of domain IV from the intron, whereas the latter (in *Annesorhiza altiscapa*) reflects a 33-bp duplication of adjacent sequence in domain I. A total of 113 unambiguous gaps was inferred in the alignment of 76 *rps*16 intron sequences; many more existed but were in those regions of the alignment excluded from the analysis. Like other noncoding regions of the chloroplast genome, this locus is clearly evolving rapidly, as evidenced by the accumulation of many length mutations (Curtis and Clegg 1984; Zurawski and Clegg 1987; Clegg and Zurawski 1992).

Group II introns are excised from mRNA transcripts via a series of self-catalyzed reactions (Michel et al. 1989) and show a strong relationship between the functional importance of its structural features and probability of evolutionary change (Learn et al. 1992; Clegg et al. 1994). Intron domains V and VI, and portions of domain I, such as the regions housing the exon binding sites, are required for proper processing of the transcript and, therefore, evolve most slowly (Learn et al. 1992). With regard to the *rps*16 intron, domains V and VI are indeed highly conserved, with relatively few indels and a high degree of sequence conservatism. In contrast, the low sequence conservation of domains II and III suggests that these regions may not be integral to proper functioning of the intron, and the almost complete absence of domain IV in *Diplolophium somaliense* certainly confirms that some regions are indeed dispensable. These results parallel those obtained for several other chloroplast introns, where domains II, III, and IV can be either highly variable, completely unstructured (consisting of runs of As and Ts), or absent (Kohchi et al. 1988; Michel et al. 1989; Learn et al. 1992; Downie et al. 1998, 2000).

Unlike chloroplast genes *rpl*2, *rpl*16, and *rpo*C1, where both intron-containing and intron-absent genes have been reported (Downie et al. 1991; Downie and Palmer 1992; Downie et al. 1996*b*), we are unaware of any plants possessing an intact *rps*16 gene that lacks a complete intron. However, sequencing studies have revealed that the *rps*16 gene is totally absent from the chloroplast genomes of *Marchantia polymorpha* L. (Ohyama et al. 1986), *Pinus thunbergii* Parl. (Tsudzuki et al. 1992), *Pisum sativum* L. (Nagano et al. 1991), and *Epifagus virginiana* (L.) Bart. (Wolfe et al. 1992). Furthermore, filter hybridization studies suggest the gene's absence, either in its entirety or part, from the chloroplast genomes of many additional Fabaceae, as well as from representatives of Connaraceae, Eucommiaceae, Fagaceae, Krameriaceae, Linaceae, Malpighiaceae, Passifloraceae, Polygalaceae, Salicaceae, Turneraceae, and Violaceae (Downie and Palmer 1992; Doyle et al. 1995). Obviously, the absence of chloroplast gene *rps*16 in these taxa precludes this region from being used in comparative analyses.

Phylogenetic utility of the *rps*16 intron

The phylogenetic estimates proposed herein are largely congruent to those inferred using either *rbc*L, *mat*K, *rpo*C1 intron, or *rpl*16 intron sequences (Plunkett et al. 1996*a*, 1996*b*, 1997; Downie et al. 1998, 2000), or cpDNA restriction sites (Plunkett and Downie 1999), and attest to the utility of the *rps*16 intron for inferring phylogeny within Apiaceae. Given the disparity in size and species composition of each of these data sets, a rigorous empirical comparison among them cannot be made. In general, *rps*16 intron sequence divergence is similar to that reported for other chloroplast introns. The *rpo*C1 and *rps*16 introns appear to be evolving most similarly (both exhibiting about 12% divergence across the same breadth of sampling), and the phylogenetic relationships proposed by each are, in many instances, identical and similarly supported, with comparable levels of homoplasy. Clearly, the *rps*16 intron provides a useful addition to other intron and gene regions for discerning high level relationships within Apiaceae. To assess the ability of the *rps*16 intron to resolve infrageneric relationships, multiple accessions of *Bupleurum*, *Centella*, *Eryngium*, and *Heteromorpha* were examined. While some resolution was achieved within each of these genera, sequence divergence was generally low and, again, comparable with that observed for other chloroplast introns (Downie et al. 1998, 2000). Further resolution of relationships at this level will have to come from studies of more rapidly evolving DNA regions.

The utility of the plastid *rps*16 intron in phylogeny estimation in other plant groups is furthered by two other factors. First, the exon-specific primers were constructed to be universal among angiosperms, being based on consensus sequences from tobacco, mustard, barley, and rice. We have used these primers successfully to amplify the entire intron region from a variety of monocot and dicot families, and as long as an intact and functional chloroplast *rps*16 gene exists, these primers should anneal. Second, the entire intron is easily PCR amplified and sequenced using standard methodologies, even from DNAs extracted from herbarium specimens up to 25 years old. In fact, this was one of the prime reasons we chose to analyse this region, as PCR amplifications of other genomic regions were either unsuccessful or, as in the case of the nuclear rDNA ITS region, yielded DNA

sequences for basal Apioideae, Saniculoideae, and Hydrocotyloideae that were too divergent to align unambiguously with most other Apioideae. While the *rps*16 intron has seen very little use in phylogenetic studies to date (e.g., Lidén et al. 1997; Oxelman et al. 1997), we show that it can be a useful addition to the repertoire of the plant molecular systematist.

Relationships within Apiaceae subfamily Apioideae

All phylogenetic analyses of molecular data to date reveal a close relationship among apioid groups 1–4. These groups are collectively called the "apioid superclade," as the relationships among them are not entirely clear (Plunkett and Downie 1999). Variously associated with this superclade are the *Daucus* (group 5), *Oenanthe* (group 6), and *Aciphylla* (group 7) clades. The repeated pattern of poor resolution among these clades increasingly suggests their rapid and simultaneous radiation. More basally branching lineages include the *Komarovia* clade (group 9) and the *Physospermum* clade (group 10); in this study, the latter is not monophyletic. Situated between groups 9 and 10 is tropical African *Diplolophium somaliense*. *Diplolophium* contains both herbaceous perennial and woody members and is considered allied to central African *Physotrichia* (Norman 1923; Townsend 1989). Additional study is necessary to confirm the phylogenetically isolated position of *Diplolophium* (and *Physotrichia*) within Apioideae.

Successively basal within Apioideae, in many but not all previous phylogenetic studies, are the *Bupleurum* (group 11) and *Heteromorpha* (group 12) clades. With the exception of the *mat*K study of Plunkett et al. (1996*b*), where *Bupleurum* arises as sister taxon to all other apioids, and the studies of Cerceau-Larrival (1962, 1971), where it was hypothesized that ancestral Apioideae were likely similar to some present-day *Bupleurum* species, all other studies of plastid DNA sequence and restriction site data indicate that the *Heteromorpha* clade (expanded herein to include *Anginon*, *Dracosciadium*, *Glia*, and *Polemannia*) is sister to all other Apioideae taxa (Plunkett et al. 1996*a*; Downie et al. 1998, 2000; Plunkett and Downie 1999). While our sampling of *Bupleurum* is low relative to the 180–190 species recognized in the genus by Pimenov and Leonov (1993), we have included both Old World and New World taxa, in addition to herbaceous and woody members. It is increasingly evident that *Bupleurum* does not occupy the most basal position within subfamily Apioideae, although the position of the putatively ancestral *B. mundii* (not included here) has yet to be determined (Cerceau-Larrival 1971; Burtt 1991).

It has been suggested that herbaceous Apioideae probably evolved from woody ancestors (Dawson 1971; Plunkett et al. 1996*a*, 1996*b*), and the predominantly woody habit of many members of the *Heteromorpha* clade certainly adds credence to this hypothesis. However, the placement of *Annesorhiza*, a poorly known southern African endemic of some 12–15 herbaceous perennial species (Burtt 1991), as one branch of a trichotomy at the base of the subfamily in Figs. 2 and 3, indicates that a herbaceous ancestry for Apioideae cannot be ruled out completely. *Annesorhiza* is presumably monophyletic, with its characteristic leaves and unique expanded and lignified vascular bundles (van Wyk and Tilney 1994). Additional studies of *Annesorhiza* are especially warranted, given their placement near *Heteromorpha* and other basal, woody apioids.

The woody southern African apioids

The delimitation of species within *Heteromorpha* has been historically complex, with the extremely polymorphic *H. arborescens* and *H. trifoliata* being particularly problematic (Townsend 1985; Burtt 1991). In the most recent revision of the genus, Winter and van Wyk (1996) divided *H. arborescens* into five varieties (with one of these, *H. arborescens* var. *abyssinica*, encompassing the geographically widespread *H. trifoliata*). However, because of the existence of intermediate forms and few diagnostic characters, their boundaries are often blurred, leading Winter and van Wyk (1996) to consider the *H. arborescens* species complex as a paraphyletic assemblage. In our study, *Heteromorpha* is not monophyletic, with *H. arborescens* var. *abyssinica* and *H. arborescens* var. *arborescens* occurring in separate subclades, the latter allied with *Anginon*, *Glia*, and *Polemannia*. *Heteromorpha* is widely regarded as monophyletic, because of its dissimilar (i.e., heteromorphic) winged mericarps derived from the expansion of all five sepaline ribs (Winter et al. 1993; Winter and van Wyk 1996). Therefore, the anomalous placement of *H. arborescens* var. *arborescens* may be due to other factors, such as hybridization, lineage sorting, or simply the failure of the *rps*16 intron to adequately resolve relationships among these woody plants owing to its high sequence conservation.

The close relationship between *Anginon* and *Glia* is corroborated by the shared presence of heavily cutinized outer cell walls of the fruit epidermis, a feature not seen in other southern African apioids (Allison and van Wyk 1997; van Wyk et al. 1997). While morphological and anatomical differences between *Anginon* and *Glia* exist (van Wyk et al. 1997), their *rps*16 intron sequences are quite conserved, with *A. rugosum*, *A. verticillatum*, and *Glia prolifera* differing by only one nucleotide position. *Heteromorpha* and *Polemannia* are similar vegetatively and, in the absence of fruiting material, have occasionally been confused (Hilliard and Burtt 1986). Characters uniting these genera include a woody habit, smooth bark (which peels in horizontal bands in *H. arborescens*), and pedately to pinnately compound leaves with entire margins (Winter and van Wyk 1996). The fruit of *Polemannia*, however, is not heteromorphic, and the leaves of *Heteromorpha* lack the distinctive intramarginal vein of *Polemannia* (Hilliard and Burtt 1986; Winter and van Wyk 1996). The proposed sister group relationship between *Dracosciadium* and all species of *Heteromorpha* (except *H. arborescens* var. *arborescens)* is unexpected. *Dracosciadium* is distinct among these southern African Apioideae because of its palmate or peltate-digitate leaves and herbaceous perennial habit; no obvious synapomorphy between *Heteromorpha* and *Dracosciadium* is apparent.

Steganotaenia *and* Polemanniopsis

Our results suggest that *Steganotaenia* and the monotypic *Polemanniopsis* are sister taxa and that this clade is sister to Apiaceae subfamily Saniculoideae. In *Steganotaenia*, both a stylopodium and vittae (secretory canals) are absent or rudimentary, and the plants commonly flower before the leaves are produced. The combined absence of these characters is

very rare, if not unique, among apioid umbellifers. *Polemanniopsis* also possesses unusual features, such as heteromorphic, strongly winged mericarps with the wings having an internal cavity which, in the young fruit, contains oil droplets. These heteromorphic fruits apparently develop differently from those in *Heteromorpha* (Winter and van Wyk 1996). Other diagnostic characters include a seed not fused to the pericarp, minutely ruminate endosperm and, like *Steganotaenia*, no commissural vittae (Burtt 1988). The shared absence of vittae in *Polemanniopsis* and *Steganotaenia* is synapomorphic. Further comparisons of these two genera should undoubtedly yield additional synapomorphies.

The placement of *Steganotaenia* and *Polemanniopsis* alongside subfamily Saniculoideae cannot easily be reconciled. While similarities to Saniculoideae exist (such as obsolete or reduced stylopodia, and absent or rudimentary vittae), features suggesting subfamily Apioideae are also apparent (such as compound umbel inflorescences). A recent study incorporating *rbc*L sequence data reported a sister group relationship between *Steganotaenia araliacea* and *Sanicula gregari* (Backlund and Bremer 1997). The latter, however, was the sole representative of Saniculoideae and only one of five genera of Apiaceae considered. Similarities to Araliaceae have also been put forth, such as growth habit (the supposed pachycaul ancestry of arborescent *Steganotaenia* is unique in Apiaceae but common among woody Araliaceae; Burtt 1988), and a chromosome number of n = 12 (common in Araliaceae but atypical in Apioideae where n = 11 prevails; Burtt 1991). Similarly, the minutely ruminate endosperm of *Polemanniopsis* is unknown in Apiaceae but fairly common in Araliaceae (Burtt 1988). Based on diverse evidence, *Steganotaenia* and *Polemanniopsis* should be removed from Apioideae. Their transfer to an expanded Saniculoideae, or their placement in some yet to be described suprageneric taxon, will require further study.

Conclusions

Comparative sequence analysis of the chloroplast *rps*16 intron, a region little used in molecular systematic studies to date, has much potential to discern high-level relationships within Apiaceae. To this end, we are now expanding our sampling to include representation from other apioid genera, including those for which only herbarium material is available. Our long-term goals are to produce an explicit phylogenetic hypothesis for subfamily Apioideae using molecular characters and, in combination with detailed investigations of morphology and fruit anatomy, a modern classification. While much more work needs to be done, information on the early diversification of the subfamily, as provided herein using these intron data, is critical to achieving these goals. In this study, we have gained insight into the affinities of nine genera endemic to Africa, including several whose relationships have heretofore been largely unknown. Several relationships, such as the proposed affinity between *Polemanniopsis* and *Steganotaenia* and the putative position of herbaceous *Annesorhiza* among basal Apioideae, and the observation that *Heteromorpha* may not be monophyletic, are surprising indeed and deservant of further study. Burtt (1991) listed 24 genera of umbellifers endemic to subsaharan Africa, of which 21 are treated in subfamily Apioideae. Given the importance of this region in the early evolution of Apioideae, the phylogenetic affinities of each of these remaining endemic African apioid genera must be assessed to attain a clearer understanding of evolutionary relationships within the group.

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