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Circumscription and phylogeny of Apiaceae subfamily Saniculoideae based on chloroplast DNA sequences

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Abstract

An estimate of phylogenetic relationships within Apiaceae subfamily Saniculoideae was inferred using data from the chloroplast DNA *trnQ-trnK* 5′-exon region to clarify the circumscription of the subfamily and to assess the monophyly of its constituent genera. Ninety-one accessions representing 14 genera and 82 species of Apiaceae were examined, including the genera *Steganotaenia*, *Polemanniopsis*, and *Lichtensteinia* which have been traditionally treated in subfamily Apioideae but determined in recent studies to be more closely related to or included within subfamily Saniculoideae. The *trnQ-trnK* 5′-exon region includes two intergenic spacers heretofore underutilized in molecular systematic studies and the *rps16* intron. Analyses of these loci permitted an assessment of the relative utility of these noncoding regions (including the use of indel characters) for phylogenetic study at different hierarchical levels. The use of indels in phylogenetic analyses of both combined and partitioned data sets improves resolution of relationships, increases bootstrap support values, and decreases levels of overall homoplasy. Intergeneric relationships derived from maximum parsimony, Bayesian, and maximum likelihood analyses, as well as from maximum parsimony analysis of indel data alone, are fully resolved and consistent with one another and generally very well supported. We confirm the expansion of subfamily Saniculoideae to include *Steganotaenia* and *Polemanniopsis* (as the new tribe Steganotaenieae C.I. Calviño and S.R. Downie) but not *Lichtensteinia*. Sister group to tribe Steganotaenieae is tribe Saniculeae, redefined to include the genera *Actinolema*, *Alepidea*, *Arctopus*, *Astrantia*, *Eryngium*, *Petagnaea*, and *Sanicula*. With the synonymization of *Hacquetia* into *Sanicula*, all genera are monophyletic. *Eryngium* is divided into "Old World" and "New World" subclades and within *Astrantia* sections *Astrantia* and *Astrantiella* are monophyletic.

Keywords: Apiaceae; Saniculoideae; cpDNA *trnQ-trnK* 5′-exon; Phylogeny; Indels

1. Introduction

Apiaceae subfamily Saniculoideae, as treated by Drude (1898) and Wolff (1913), comprises two tribes (Saniculeae and Lagoecieae), nine genera (*Actinolema* Fenzl, *Alepidea* F. Delaroche, *Arctopus* L., *Astrantia* L., *Eryngium* L., *Hacquetia* Neck. ex DC., *Lagoecia* L., *Petagnaea* Caruel, and *Sanicula* L.), and approximately 330 species. The subfamily has a bipolar distribution, but is better represented in the southern hemisphere than its sister group, subfamily Apioideae (Mathias, 1971; Downie et al., 2001). *Sanicula* and *Eryngium* are each cosmopolitan and are the only genera of Saniculoideae represented in the western hemisphere. They also account for the majority of the species of the subfamily, with 39 and about 250 species, respectively (Pimenov and Leonov, 1993). *Hacquetia*, *Lagoecia*, and *Petagnaea* are each monotypic and occur in Europe and/or Asia, as do *Actinolema* (two species) and *Astrantia* (nine species; Pimenov and Leonov, 1993). *Arctopus* and *Alepidea*, with three and 20 species each, occur in Africa. The subfamily is of some economic and ecologic importance. Several species

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are endangered, some are used for culinary or medicinal purposes, many are ornamentals, and others are noxious weeds. The plants are mostly herbaceous, with often spiny or bristly simple leaves. Their flowers are arranged primarily in simple (rarely compound) umbels or heads that are surrounded by showy bracts. Their fruits comprise an exocarp covered in scales, bristles, or prickles (or rarely are glabrous or tuberculate), a mesocarp with calcium-oxalate crystals scattered throughout, and a parenchymatous endocarp. The most common base chromosome numbers of the subfamily are $x = 7$ and $x = 8$.

Since the treatments of Saniculoideae by Drude (1898) and Wolff (1913), the composition of the subfamily has changed only slightly. *Lagoecia* has been transferred to subfamily Apioideae (Plunkett et al., 1996; Downie et al., 2000a; Valiejo-Roman et al., 2002), its affinity to the apioid umbellifers having been suggested previously (Koso-Poljansky, 1916; Cerceau-Larrival, 1962; Tseng, 1967; Guyot, 1971; Magin, 1980). *Arctopus* was transferred to and maintained within the traditionally circumscribed Apiaceae subfamily Hydrocotyloideae (Froebe, 1964; Magin, 1980; Constance and Chuang, 1982; Pimenov and Leonov, 1993), but later returned to Saniculoideae upon analyses of molecular data (Plunkett and Lowry, 2001; Chandler and Plunkett, 2004). *Oligocladus* Chodat and Wilczek, provisionally included in subfamily Saniculoideae by Pimenov and Leonov (1993), finds affinity among the higher apioid umbellifers (C.I. Calviño and S.R. Downie, unpublished data). The most dramatic change in circumscription of Saniculoideae, however, was the recent addition of the African apioid genera *Steganotaenia* Hochst., *Polemanniopsis* B. L. Burtt, and *Lichtensteinia* Cham. and Schltdl., primarily on the basis of cladistic analysis of fruit anatomical characters (Liu et al., 2003). The sister group relationship between *Steganotaenia*/*Polemanniopsis* and subfamily Saniculoideae was first revealed by Downie and Katz-Downie (1999) using chloroplast DNA (cpDNA) *rps16* intron sequences. Their results suggested that *Steganotaenia* and *Polemanniopsis* be removed from subfamily Apioideae, but they were uncertain whether the circumscription of Saniculoideae should be expanded to include these genera. *Steganotaenia* and *Polemanniopsis* have morphological features similar to subfamily Saniculoideae, such as intrajugal vittae (oil ducts in the ribs of the fruits that are associated with vascular bundles) and the absence of commissural and vallecular vittae (oil ducts in the commissure and furrows of the fruits, respectively), but characters reminiscent of subfamily Apioideae are also apparent, such as large compound umbel inflorescences. A subsequent molecular phylogenetic study of southern African Apiaceae also indicated a sister group relationship between *Steganotaenia*/*Polemanniopsis* and subfamily Saniculoideae (Calviño et al., 2006). The genus *Lichtensteinia*, however, comprised a monogeneric clade sister group to all other members of subfamily Apioideae. Furthermore, Calviño et al. (2006) reported that, in the study of Liu et al. (2003), the fruit characters uniting *Lichtensteinia* and *Steganotaenia*/*Polemanniopsis* with subfamily Saniculoideae were plesiomorphic in the family, thus the inclusion of these three genera within an expanded Saniculoideae was influenced by these symplesiomorphies. The placement of *Steganotaenia* and *Polemanniopsis* into an expanded Saniculoideae was not implemented by Calviño et al. (2006), given that the only evidence clearly justifying the sister group relationship between these genera and Saniculoideae is that of the *rps16* intron and these sequence data supported the relationship only weakly.

While molecular data have been useful to corroborate the monophyly of subfamily Saniculoideae and reveal its sister group relationship to subfamily Apioideae, no molecular systematic study to date has focused explicitly on infrasubfamilial relationships of all of its genera and the phylogenetic placements of *Steganotaenia*, *Polemanniopsis*, and *Lichtensteinia* remain uncertain. Furthermore, a recent study of Apiaceae using nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequences has suggested that *Eryngium* is paraphyletic and that *Hacquetia* should be treated as part of *Sanicula* (Valiejo-Roman et al., 2002). These phylogenetic hypotheses, however, were based exclusively on ITS sequence comparisons, and among distant members of Apiaceae alignment of these sequences is highly problematic. Moreover, several molecular genetic processes impact ITS sequences in ways that may confound phylogenetic inference (Álvarez and Wendel, 2003), such as the divergent paralogous ITS sequences detected in a few members of the early branching *Annesorhiza* clade within subfamily Apioideae (Calviño et al., 2006).

The major objective of this study is to estimate phylogenetic relationships within Apiaceae subfamily Saniculoideae using molecular data. We examine the cpDNA *trnQ-trnK* 5′-exon region (hereafter, called *trnQ-trnK*), a region encompassing primarily three large noncoding loci (i.e., the *trnQ-rps16* intergenic spacer, *rps16* intron, and *rps16-trnK* intergenic spacer; Fig. 1). Over the past decade, the *rps16* intron has been used increasingly in phylogenetic studies of both Apiaceae and other angiosperms (Lidén et al., 1997; Oxelman et al., 1997; Downie and Katz-Downie, 1999; Kelchner, 2002; Shaw et al., 2005), but the spacer regions flanking the *rps16* gene have been rarely considered for such a purpose (Hahn, 2002). A recent study of two apioid genera using these intergenic spacers established their higher rate of molecular evolution (in both nucleotide substitutions and length mutations) over other cpDNA loci used in molecular systematic investigations of Apiaceae (Lee and Downie, 2006). Herein, we examine the efficacy of this region in resolving phylogeny at different taxonomic levels within the family, including the use of indels for phylogeny estimation. Ancillary objectives include clarification of the circumscription of subfamily Saniculoideae (with emphasis on the phylogenetic placements of *Steganotaenia*, *Polemanniopsis*, and *Lichtensteinia*) and an assessment of the monophyly of its constituent genera. We present the first explicit phylogenetic hypothesis for the subfamily and a revised classification that reflects this phylogeny.

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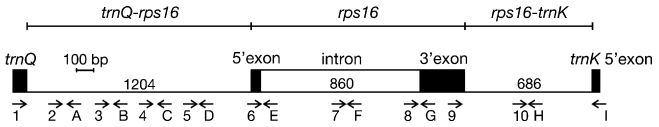

Fig. 1. Map of the 3117-bp locus of tobacco cpDNA (Shinozaki et al., 1986) showing the relative positions of genes *trnQ*, *rps16*, and (in part) *trnK*. The gene *rps16* is interrupted by an intron, and only the 5′-exon of gene *trnK* is shown. The sizes of the two intergenic spacer regions and intron are presented in base pairs (bp). Scale bar is 100 bp unit. The arrows represent the directions and approximate positions of the primers used in PCR amplification and/or DNA sequencing. Forward primers are designated 1–10; reverse primers are designated A–I. These primer sequences, written 5′ to 3′, are as follows: 1, CCC GCT ATT CGG AGG TTC GA (trnQ); 2, TCG CAA TAA GAA AGA ACC TC (Alepidea-trnQ-1F); 3, GAG GAA ATG CTT AGC TTA AG (trnQ-2); 4, CAG AGA CTG TTG TTC AGT GT (Alepidea-trnQ-2F); 5, GCT TAT GAG TTG AAT C (trnQ-3); 6, TTT GAA ACG ATG TGG TAG A (5exon-C); 7, TAA GAA GCA CCG AAG TAA TGT C (rps16-C); 8, TTT CTC GAG CCG TAC GAG GAG (rps16-2); 9, TTC CTT GAA AAG GGC GCT CA (3′exon-1); 10, GCG TCT ATG TAG TGC CAA TC (trnK-1); A, ACG GAA GGG AGA CTC TCT AA (Alepidea-trnQ-R); B, GTC ACT GAA ATA GAA CG (trnQ-R); C, ATC AGA TGA ACG AGT GGG (Alepidea-trnQ-1R); D, CTC AAT AGG AGA TAT TGA CCC (trnQ-1R); E, ATC GTG TCC TTC AAG TCG CA (rps16-1R); F, AAT GGC GTT TCC TTG TTC (rps16-CR); G, ACC CAC GTT GCG AAG AT (3′exon-CR); H, GTT CGA TAC ACT GTT GTC (trnK-1R); I, TAC TCT ACC GTT GAG TTA GC (trnK).

2. Materials and methods

2.1. Accessions examined

Ninety-one accessions representing 14 genera and 82 species of Apiaceae were examined for cpDNA *trnQ-trnK* sequence variation. For a list of the accessions with corresponding DNA accession number and voucher information, please see online Supplementary Appendix A. *Rps16* intron data for 13 of these accessions were obtained previously (Downie and Katz-Downie, 1999; Calviño et al., 2006; online Supplementary Appendix A); intron data for the remaining accessions, and data from the *trnQ-rps16* and *rps16-trnK* intergenic spacer regions for all 91 accessions were obtained specifically for this study. These accessions represent all genera traditionally included within subfamily Saniculoideae (with the exception of *Lagoecia*, which is now placed in tribe Pyramidoptereae of subfamily Apioideae; Downie et al., 2000a), plus the African genera *Steganotaenia*, *Polemanniopsis*, and *Lichtensteinia* which have been traditionally treated in subfamily Apioideae. *Steganotaenia* and *Polemanniopsis* have been determined in recent studies to be more closely related to or included within subfamily Saniculoideae (Downie and Katz-Downie, 1999; Liu et al., 2003; Calviño et al., 2006). The circumscription of Saniculoideae was further expanded by Liu et al. (2003) with the inclusion of *Lichtensteinia*, but this treatment was not adopted by Calviño et al. (2006). For those genera divided into sections (i.e., *Astrantia*, *Eryngium*, *Alepidea*, and *Sanicula*; Grintzesco, 1910; Wolff, 1913; Weimarck, 1949; Shan and Constance, 1951), at least one representative of each section was included. Lack of adequate material for DNA extraction precluded our sampling from *Eryngium* sections *Gigantophylla* and *Pseudojunceae* (consisting of one and two species, respectively), *Sanicula* sect. *Tuberculatae* (three species), and *Alepidea* sect. *Stellata* (two species). In addition, the availability of an ITS phylogeny for *Sanicula* ensured that representatives from each of its major lineages were considered (Vargas et al., 1998, 1999). Monotypic genera were represented by two or more accessions.

All phylogenetic trees were rooted with *Hermas*, as a previous study revealed a sister group relationship between the *Hermas* clade and the clade of Apioideae and Saniculoideae plus *Steganotaenia*/*Polemanniopsis* (Calviño et al., 2006). As additional outgroups, we included one accession each of *Anginon* Raf. (tribe Heteromorpheae) and *Annesorhiza* Cham. and Schltdl. (*Annesorhiza* clade). These genera constitute early branching lineages of subfamily Apioideae and were included to assess the phylogenetic position of *Lichtensteinia* relative to subfamilies Saniculoideae and Apioideae.

2.2. Experimental strategy

Leaf material for DNA extraction was obtained from herbarium specimens, botanic gardens, or the field (online Supplementary Appendix A). For most accessions, total genomic DNA was obtained from about 20 mg of dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA); for several accessions extracted during previous studies, the modified hexadecyltrimethylammonium bromide (CTAB) protocol of Doyle and Doyle (1987) was used instead, as detailed in Downie and Katz-Downie (1996, 1999).

The region bounded by and including chloroplast genes *trnQ* and *trnK* 5′-exon and containing the *rps16* intron is 3117 bp in size in tobacco (Shinozaki et al., 1986). Flanking the gene *rps16* are the *trnQ-rps16* and *rps16-trnK* intergenic spacer regions and in tobacco cpDNA these spacers are 1204 and 686 bp in size, respectively (Fig. 1). The strategies employed to obtain these sequence data are presented elsewhere (Downie and Katz-Downie, 1996, 1999; Calviño et al., 2006), with only slight modifications herein. We first performed a long-PCR on a few accessions of *Eryngium*, using primers anchored in genes *trnQ* and *trnK* 5′-exon that were constructed by comparing published gene sequences from tobacco and rice and choosing regions highly conserved between them (Shinozaki et al., 1986; Hiratsuka et al., 1989). All other primers were subsequently designed based on these *Eryngium* data. In total, 19 primers were used for PCR and/or DNA sequencing of the entire *trnQ-trnK* region (Fig. 1). Four of these primers were constructed specifically for *Alepidea* because primers trnQ-3 (primer 5) and trnQ-1R (primer D) could not be used due to a 238-bp deletion unique to this genus, and primer trnQ-2 (primer 3) did not anneal to *Alepidea* (and other African members of Saniculoideae, and Apioideae) because of a point mutation at the extreme 3′-end of the primer binding site. For PCR

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amplifications of the *trnQ-rps16* intergenic spacer using any of the primers internal to this region, the annealing temperature was decreased from 53 to 48 °C and the $MgCl_2$ concentration was decreased from 2.75 to 1.5 mM. For sequencing of the *rps16-trnK* spacer for those DNAs where homopolymer regions or secondary structure formation obstructed the reaction, 1 μl of dGTP BigDye terminator (ABI Prism® dGTP BigDye™ Terminator v3.0) was added to the standard sequencing cocktails. Simultaneous consideration of both DNA strands across the entire region for most taxa permitted unambiguous base determination. GenBank reference numbers for all sequences are presented in the online Supplementary Appendix A.

2.3. Sequence comparisons and phylogenetic analyses

Sequence chromatograms were edited manually using Se-Al (Rambaut, 2002). DNA sequences were aligned initially using the default pairwise and multiple alignment parameters in the computer program CLUSTAL X (gap opening cost = 15.00, gap extension cost = 6.66, DNA transition weight = 0.50; Jeanmougin et al., 1998) then rechecked and adjusted manually as necessary. Gaps were positioned to minimize nucleotide mismatches. A matrix of binary-coded indels was constructed to incorporate length-mutational information into the phylogenetic analysis. Gaps of equal length in more than one sequence were coded as the same presence or absence character state if they could not be interpreted as different duplication or insertion events. Indels of similar location but with different lengths were coded as multiple binary characters. In several regions, gap coding was problematic because of homopolymers or indirect duplications of adjacent elements in two or more taxa. These gaps were not scored and these ambiguous regions were excluded from subsequent analysis.

Some regions of the alignment were scored as missing. Only data for the *rps16* intron were available for *Polemanniopsis marlothii* (DNA Accession No. 1333; online Supplementary Appendix A) and *Steganotaenia araliacea* (Accession Nos. 1373 and 1385) because these accessions were used in a prior study and their DNAs were no longer available (Downie and Katz-Downie, 1999). Data for the *trnQ-rps16* spacer and about half of the *rps16-trnK* spacer could not be obtained for *Arctopus echinatus* 2559 despite our repeated but unsuccessful attempts to PCR-amplify these regions. Similarly, parts of both spacers in *Sanicula chinensis* could not be PCR-amplified. Portions of the *rps16* 3′-exon were missing data (between primers G and 9; Fig. 1), attributable to the positions of the primers anchored in this exon used to amplify the regions flanking it. However, this exon had little to no variation among all other accessions, hence the absence of these data did not affect the phylogenetic results. Overall, missing data represented 5.5% of the entire matrix.

Boundaries of the genes *trnQ*, *rps16*, and *trnK* 5′-exon were determined by comparison of the DNA sequences to corresponding boundaries in tobacco cpDNA (Shinozaki et al., 1986). The determination of boundary sequences for the six major structural domains of the *rps16* group II intron was based on similar boundary sequences inferred for tobacco, mustard, and other Apiaceae (Michel et al., 1989; Neuhaus et al., 1989; Downie and Katz-Downie, 1999). Characterization of the three cpDNA regions and six *rps16* intron structural domains was facilitated using MacClade version 4.07 (Maddison and Maddison, 2005), BioEdit version 6.0.7 (Hall, 1999), and PAUP version 4.0b10 (Swofford, 2002). Uncorrected pairwise nucleotide distances of unambiguously aligned positions were determined using the distance matrix option of PAUP*. Average sequence divergence estimates were calculated both within and between genera. Relative evolutionary rates were estimated by plotting the pairwise corrected genetic distance (according to the model selected by Modeltest version 3.7; Posada and Crandall, 1998) of one region versus the other. The slope of the linear regression was taken as the relative rate value (Pochon et al., 2006). Because both variables are independent or subject to natural variability, we performed a Model II (geometric mean) linear regression and not the standard Model I. The Model II method (for equations see Barker et al., 1988) effectively minimizes the sum of the squares of the deviations of the observations from the line in both axis by measuring the offsets along a line perpendicular to the regression line.

The data matrices were each analyzed using maximum parsimony (MP) as implemented by PAUP*. For matrices representing either the entire *trnQ-trnK* region, the entire region plus scored indels, or only scored indels, heuristic searches were performed for 100,000 replicates with random addition of taxa and tree-bisection-reconnection (TBR) branch swapping. For matrices of partitioned data (i.e., intron or spacer regions), with and without indels, the heuristic search strategies employed by Calviño et al. (2006) were followed. Bootstrap values were calculated from 100,000 replicate analyses using "fast" stepwise-addition of taxa and only those values compatible with the 50% majority-rule consensus tree were recorded. The number of additional steps required to force particular taxa into a monophyletic group was examined using the constraint option of PAUP*.

The relative utility of the three cpDNA regions in resolving phylogenetic relationships and the effect of incorporating length-mutational events into the phylogenetic analyses were assessed by comparing the results of MP analysis of each data partition, with or without indels included as additional characters, against those major clades inferred from MP analysis of the entire *trnQ-trnK* region plus binary-scored alignment gaps (because analysis of the latter yielded trees of greatest resolution and highest bootstrap support overall). Comparisons were made of the number of major clades recovered in each of these analyses and their corresponding bootstrap support values (Felsenstein, 1985). Additional comparative data included the number of parsimony informative characters, the number and length

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of maximally parsimonious trees, and measures of relative homoplasy. In comparing the consistency and retention indices of each data partition, with or without indels, each group of characters was optimized onto the most parsimonious trees inferred from analysis of the entire *trnQ-trnK* region plus binary-scored alignment gaps.

Bayesian inference of the entire *trnQ-trnK* region (indel characters not included) was conducted using MrBayes version 3.1.1 (Huelsenbeck and Ronquist, 2001). The program was run in parallel on an IBM pSeries 690 system at the National Center for Supercomputing Applications at UIUC. Prior to analysis, Modeltest was used to select an evolutionary model of nucleotide substitution that best fits these data, as selected by the Akaike Information Criterion estimator. The settings appropriate for the best-fit TVM+I+G model were put into a MrBayes block in PAUP* (nst = 6, rates = invgamma). The priors on state frequencies and rates and variation across sites (shape of the gamma distribution) were estimated automatically from the data assuming no prior knowledge about their values (Dirichlet default option). From different random starting trees, four independent Bayesian analyses (nruns = 4) were run for 10 million generations and the trees saved to a file every 100 generations (i.e., a total of 400,000 trees was sampled). Four simultaneous Markov chain Monte Carlo (MCMC) chains were used and branch lengths of the trees were saved. Variation in likelihood scores to determine apparent stationarity was examined graphically for each independent run using the program Tracer version 1.2.1 (A. Rambaut and A. Drummond, University of Oxford, unpublished data). The states of the chain that were sampled before stationarity (i.e., the "burn in" of the chain) were discarded and the posterior probability values for each bipartition of the phylogeny were determined from the remaining trees. To summarize and compare the samples from each analysis, the sump and sumt commands of MrBayes were used. MCMC convergence was also explored by examining the potential scale reduction factor (PSRF) convergence diagnostics for all parameters in the model (provided by the sump and sumt commands) and graphically using the cumulative, compare, and absolute difference options of the program AWTY online (Wilgenbusch et al., 2004).

The data matrix of the entire *trnQ-trnK* region (indel characters not included) was also analyzed using the maximum likelihood method as implemented by PAUP*. The results obtained were congruent to those inferred by the Bayesian analysis; hence they will not be discussed further.

3. Results

3.1. Sequence comparisons

Sequence characteristics of each of the three cpDNA data partitions are presented in Table 1. On average, the size of the *trnQ-rps16* intergenic spacer in Saniculoideae is smaller than that of outgroups Apioideae and *Hermas* as a result of several large deletions. Sizes of the *rps16* intron and *rps16-trnK* intergenic spacer are approximately the same between ingroup and outgroup taxa. Alignment of all partitioned regions for 91 accessions of Apiaceae resulted in a matrix of 4846 positions. Of these, 445 were excluded from the analysis because of alignment ambiguities. The remaining 4401 aligned positions yielded 871 parsimony informative characters. In addition, 322 unambiguous alignment gaps were inferred, of which 189 were parsimony informative. The latter ranged in size from 1 to 1009 bp and their frequency in relation to size for each data partition is shown in Fig. 2. Most indels were 10 bp or shorter in size. The average size of insertion across all three regions was 6 bp, whereas the average size of deletion across all regions ranged from 5 to 56 bp as a result of several large deletions in the *trnQ-rps16* inter-

Table 1
Sequence characteristics of the cpDNA *trnQ-trnK* region, analyzed as three separate data partitions, for 91 accessions of Apiaceae

Sequence characteristic	*trnQ-rps16*	*rps16*	*rps16-trnK*
Length variation (range/average in bp)			
Saniculoideae	780–1613/1370	1066–1133/1086	800–934/875
Apioideae and *Hermas*	1442–1776/1684	1107–1121/1113	746–890/859
No. aligned positions	2386	1247[b]	1213[c]
No. positions eliminated	280	41	124
No. positions not variable	1424	970	720
No. positions autapomorphic	233	65	118
No. positions parsimony informative	449	171	251
No. unambiguous alignment gaps	172	56	94
No. unambiguous alignment gaps parsimony informative	102	36	51
Sequence divergence (range/average in %)	0–14.0/6.4	0–8.2/3.2	0–14.0/6.2
Between Saniculoideae genera	1.6–10.8/6.7	0.7–5.6/3.3	1.8–10.0/6.7
Within Saniculoideae genera	0–3.0/1.1	0.2–0.9/0.5	0.3–3.1/1.3
Total No. parsimony informative characters[a]	551	207	302

Length variation is presented for both Saniculoideae (82 accessions, including *Steganotaenia* and *Polemanniopsis*) and outgroups Apioideae and the *Hermas* clade (9 accessions).

[a] Number of parsimony informative nucleotide substitutions plus number of parsimony informative gaps.

[b] Of the total aligned positions of the *rps16* gene, 40 and 197 bp correspond to the 5′- and 3-′exons, respectively.

[c] Includes 25 bp of the *trnK* 5′-exon.

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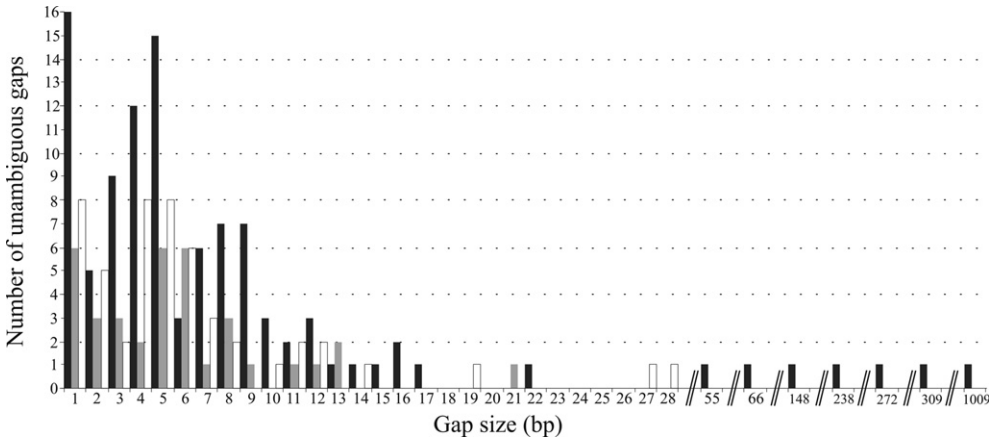

Fig. 2. Frequency of unambiguous gaps in relation to gap size inferred in the alignment of 91 cpDNA *trnQ-trnK* sequences. One-hundred and eighty nine gaps, of 1–1009 bp in size, were potentially informative across all three partitions. Each bar represents the total number of parsimony informative gaps for each partition: *trnQ-rps16* (black bars); *rps16* (gray bars); *rps16-trnK* (white bars).

genic spacer (black bars in Fig. 2). Relative to the outgroup *Hermas*, these 189 informative gaps represent a minimum of 77 deletion and 112 insertion events. For each data partition, the ratio of informative substitutions to informative indels is similar (4.4–4.9:1); when the three regions were considered collectively, this ratio is 4.6:1.

Of the three data partitions, the *trnQ-rps16* region displays the most parsimony informative characters (i.e., nucleotide substitutions plus indels; Table 1). The two intergenic spacers had high levels of sequence divergence at all taxonomic levels considered, with maximum divergence values of 3.1% among saniculoid congeners (*rps16-trnK*), 10.8% between saniculoid genera (*trnQ-rps16*), and 14.0% across subfamilies. The slopes of the linear regressions in pairwise comparisons indicate that the intergenic spacers are evolving about twice as fast as that of the *rps16* intron (*trnQ-rps16* vs. *rps16*, $m = 1.75$, $R^2 = 0.9189$; *trnQ-rps16* vs. *rps16-trnK*, $m = 1.01$, $R^2 = 0.8402$; *rps16-trnK* vs. *rps16*, $m = 1.73$, $R^2 = 0.8191$). No significant differences were observed in the relative evolutionary rates between the two intergenic spacer regions.

For each of the six major structural domains of the cpDNA group II *rps16* intron, characteristics of the aligned sequences are presented in Table 2. Domain I is the largest, ranging between 475 and 503 bp among Saniculoideae, whereas domains V (34 bp) and VI (24–38 bp) are the smallest. Domains V and VI are also the most conserved evolutionarily, with few informative positions, low nucleotide sequence divergence, and very few or no alignment gaps. The small sizes of domains III and VI in Saniculoideae relative to the outgroup taxa are due to two deletions of 21 and 13 bp, respectively. These deletions occur in all non-African saniculoid taxa and in "Old World" *Eryngium*, respectively.

3.2. Phylogenetic analyses

MP analysis of 4401 unambiguously aligned positions representing the entire *trnQ-trnK* region and 189 binary-

Table 2
Sequence characteristics of the six major structural domains of the cpDNA group II *rps16* intron for 91 accessions of Apiaceae

Sequence characteristic	Intron domain					
	I	II	III	IV	V	VI
Length variation (range in bp)						
Saniculoideae	475–503	80–96	48–75	126–143	34	24–38
Apioideae and *Hermas*	485–507	92–101	66–70	136–141	34	37–38
No. aligned positions	567	119	79	159	34	38
No. positions eliminated	33	8	0	0	0	0
No. positions not variable	416	84	64	120	34	33
No. positions autapomorphic	32	5	3	11	0	3
No. positions parsimony informative	86	22	12	28	0	2
No. unambiguous alignment gaps	30	9	5	10	0	2
No. unambiguous alignment gaps parsimony informative	18	5	5	7	0	1
Maximum sequence divergence (%)						
Saniculoideae	5.9	11.3	12.6	9.4	0	5.4
All accessions	8.9	14.4	13.7	14.5	0	8.1

Length variation is presented for both Saniculoideae (82 accessions, including *Steganotaenia* and *Polemanniopsis*) and outgroups Apioideae and the *Hermas* clade (9 accessions).

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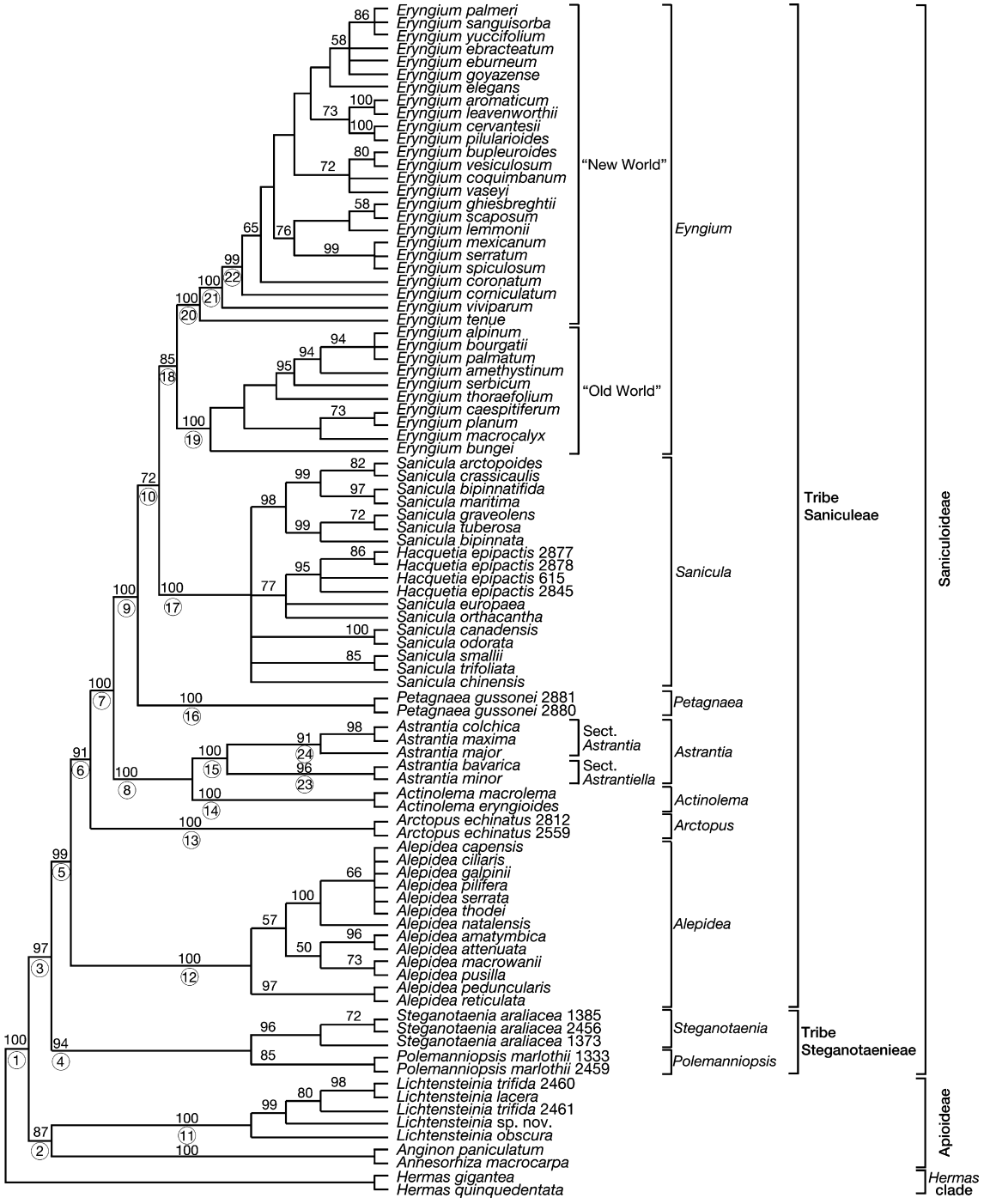

Fig. 3. Strict consensus of 4320 minimal length 2249-step trees derived from equally weighted maximum parsimony analysis of 91 cpDNA *trnQ-trnK* sequences plus 189 binary-scored alignment gaps (CIs = 0.7781 and 0.7215, with and without uninformative characters, respectively; RI = 0.9487). Numbers above the branches are bootstrap estimates for 100,000 replicate analyses using "fast" stepwise addition of taxa; values <50% are not indicated. Circled numbers below the branches correspond to the 24 recognized clades indicated in Table 3 and discussed in the text.

scored informative indels resulted in 4320 minimal length trees, each of 2249 steps (consistency indices, CIs = 0.7781 and 0.7215, with and without uninformative characters, respectively; retention index, RI = 0.9487). The strict consensus of these trees is presented in Fig. 3. No relationship was apparent between size of indel and its level of homoplasy, with the exception that all indels 16 bp or greater in size were not homoplastic when considered across all MP trees (Fig. 4). Indeed, 82% of all indels were not homoplastic when optimized on these trees. The percentage of homoplastic indels from each of the three data partitions was similar, ranging from 11 to 16%. Twenty-four major clades were identified on the strict consensus tree and are described in Table 3. These clades represent a variety of taxonomic levels, such as sections and other infrageneric groupings, genera, subtribes, tribes, and subfamilies. Sup-

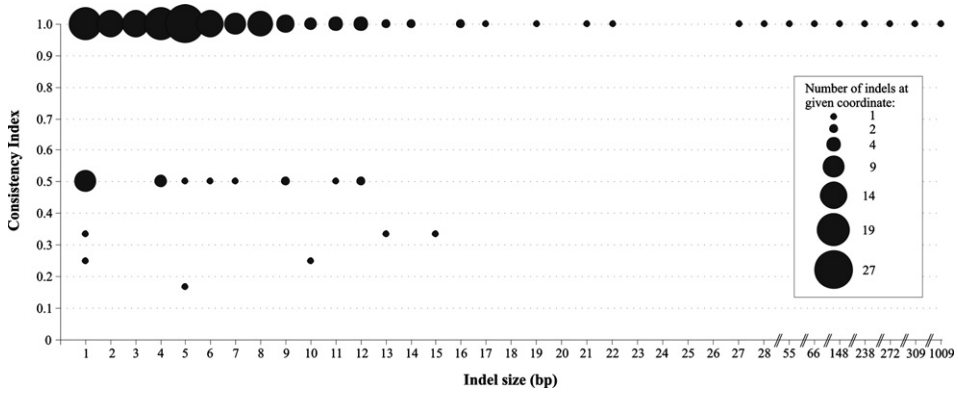

Fig. 4. Levels of homoplasy, as measured by the consistency index, in relation to indel size considered over the MP trees obtained from the analysis of the *trnQ-trnK* + indels matrix. The diameter of each dot corresponds to the number of indels at a given coordinate.

port for each of these clades is generally quite strong, with bootstrap values ranging between 72 and 100% (averaging 96%). The same 24 major clades were recovered when indels were excluded from the analysis; bootstrap values on the resultant strict consensus tree (not shown) ranged between 68 and 100% (averaging 93%; Table 3). For some clades (i.e., Nos. 3, 4, and 18; Table 3), bootstrap support levels decreased considerably upon the exclusion of indel characters. The results of MP analyses of partitioned data, with or without indels as additional characters, and their comparisons to the results of the aforementioned analyses are presented in Table 3. The *trnQ-rps16* data partition plus indels was the only matrix that recovered all major clades inferred by analysis of the entire *trnQ-trnK* region plus indels. The

Table 3
Comparison of bootstrap support values calculated from MP analysis of combined or partitioned data, with and without their corresponding binary-coded indel matrices, for the 24 major clades of Apiaceae identified in Fig. 3 and described here

Relationship	Clade	*trnQ-trnK* + indels	*trnQ-trnK*	*trnQ-rps16* + indels	*trnQ-rps16*	*rps16* + indels	*rps16*	*rps16-trnK* + indels	*rps16-trnK*
Subfamilial	1	100	100	100	100	100	100	100	100
	2	87	79	84	78	77	63	35	32
	3	97	77	95	74	70	59	32	22
Tribal	4	94	82	100	100	80	78	94	92
	5	99	94	96	89	94	86	83	70
Subtribal	6	91	83	57	53	95	90	52	46
	7	100	99	100	100	100	99	83	77
	8	100	100	100	100	100	100	100	100
	9	100	100	98	92	94	94	100	99
	10	72	68	51	47	38	33	55	45
Generic	11	100	100	100	100	92	91	97	97
	12	100	100	100	100	100	100	100	100
	13	100	99	n/a	n/a	97	94	96	89
	14	100	100	100	100	87	87	100	100
	15	100	99	99	88	79	79	95	94
	16	100	100	100	100	100	100	100	100
	17	100	100	99	84	80	83	98	88
	18	85	72	55	54	84	72	46	30
Infrageneric	19	100	100	100	100	100	98	100	100
	20	100	100	100	100	91	90	84	85
	21	100	100	100	100	89	89	100	99
	22	99	96	88	91	78	78	75	5
	23	96	93	63	63	65	64	87	75
	24	91	91	63	63	60	55	26	26
Average		96	93	89	86	85	83	81	74

Clade 1, Apioideae, Saniculoideae; clade 2, Apioideae; clade 3, Saniculoideae; clade 4, Tribe Steganotaenieae; clade 5, Tribe Saniculeae; clade 6, *Arctopus*, *Actinolema*, *Astrantia*, *Petagnaea*, *Sanicula*, *Eryngium*; clade 7, *Actinolema*, *Astrantia*, *Petagnaea*, *Sanicula*, *Eryngium*; clade 8, *Actinolema*, *Astrantia*; clade 9, *Petagnaea*, *Sanicula*, *Eryngium*; clade 10, *Sanicula*, *Eryngium*; clade 11, *Lichtensteinia*; clade 12, *Alepidea*; clade 13, *Arctopus*; clade 14, *Actinolema*; clade 15, *Astrantia*; clade 16, *Petagnaea*; clade 17, *Sanicula*; clade 18, *Eryngium*; clade 19, "Old World" *Eryngium*; clade 20, "New World" *Eryngium*; clade 21, "New World" *Eryngium* except *E. tenue*; clade 22, "New World" *Eryngium* except *E. tenue* and *E. viviparum*; clade 23, *Astrantia* sect. *Astrantiella*; clade 24, *Astrantia* sect. *Astrantia*. Sequence data for the *trnQ-rps16* intergenic spacer region were unobtainable for one of two accessions of *A. echinatus*, thus this clade did not occur in the analysis of this data partition and is marked as not applicable

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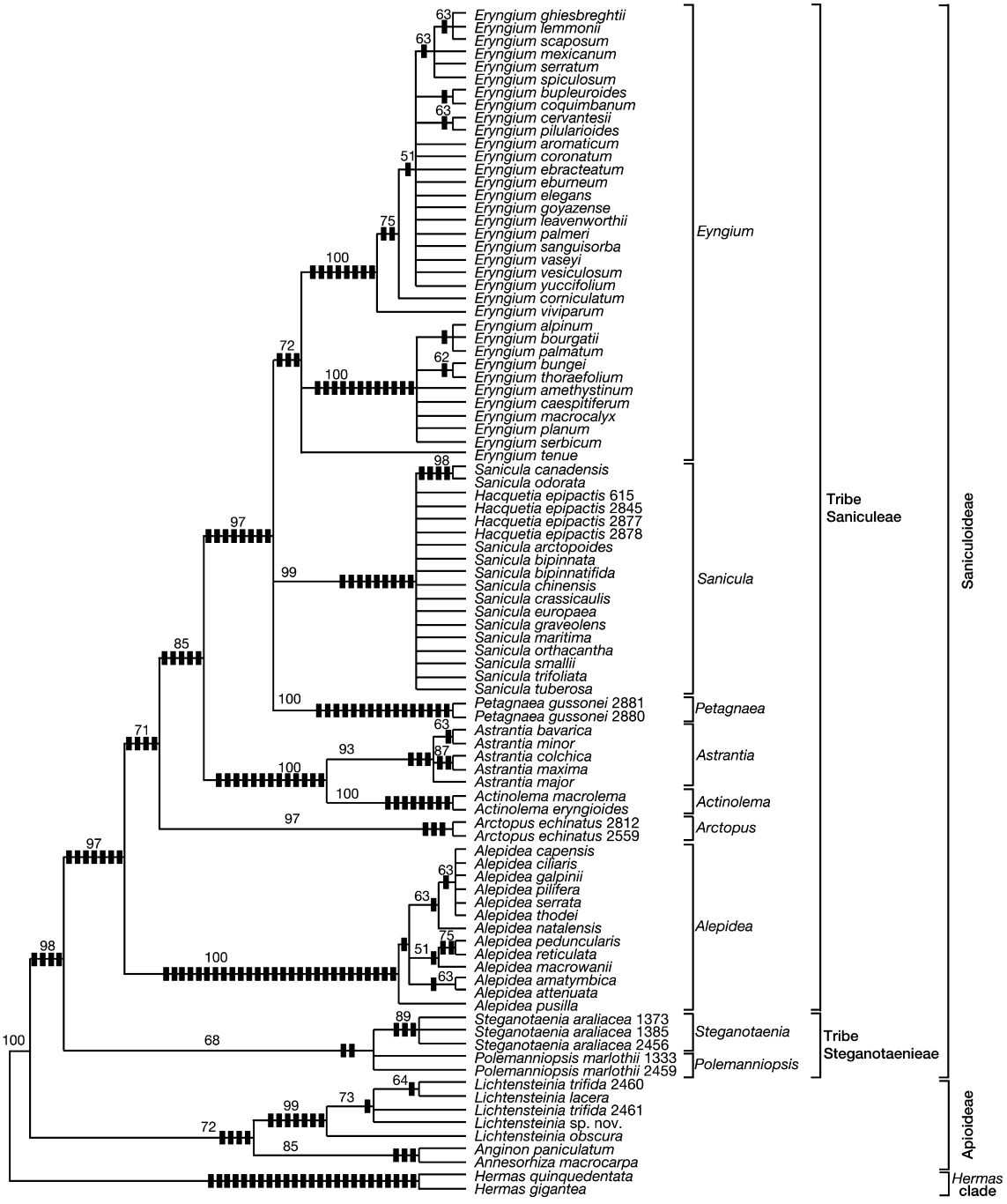

Fig. 5. Strict consensus of 2016 minimal length 217-step trees derived from equally weighted maximum parsimony analysis of 189 binary-scored and parsimony informative alignment gaps (CIs = 0.8710, RI = 0.9842). Numbers above the branches are bootstrap estimates for 100,000 replicate analyses using "fast" stepwise addition of taxa; values <50% are not indicated. Black boxes along branches indicate the relative distributions of indels, as inferred along one arbitrarily selected minimal length tree. The names of the major clades recovered from MP analysis of indel data are marked with brackets.

rps16 plus indels matrix recovered all major clades but one (a subtribe, clade No. 10), whereas the *rps16-trnK* data partition plus indels performed poorest, with several major clades not recovered in the strict consensus tree derived from these data. Considering the three data partitions, bootstrap support values are generally highest for the *trnQ-rps16* plus indels matrix (averaging 89%), but for several major clades these values are higher for one of the other two regions. In general, the incorporation of indels into an analysis resulted in higher bootstrap support values than when they were not included, and the simultaneous analysis of data from all three regions plus binary-scored alignment gaps resulted in trees of greatest resolution and highest bootstrap support values (Table 3). This was also true when compared to results of MP analyses of any combination of two regions (data not shown).

MP analysis of the matrix representing only the 189 binary-scored informative indels from the entire *trnQ-trnK*

region resulted in 2016 minimal length trees, each of 217 steps (CI = 0.8710, RI = 0.9842). The strict consensus of these trees (Fig. 5) is highly consistent with the strict consensus tree inferred using both nucleotide substitutions and indels (Fig. 3), with most major clades recovered. For the former, bootstrap support values ranged from 51 to 100%, averaging 81%. To reveal the distribution of indels throughout the phylogeny, the pattern of indel distribution along one arbitrarily selected minimal length tree was mapped onto the strict consensus tree inferred from indel data. Patterns of indel distribution are important in supporting the sister group relationship between *Steganotaenia*/*Polemanniopsis* and subfamily Saniculoideae, as well as the sister group relationship between *Lichtensteinia* and other members of subfamily Apioideae. Two unique indels (a 6-bp insertion in *trnQ-rps16* and a 12-bp insertion in *rps16* intron domain I) are shared by *Steganotaenia* and *Polemanniopsis*. Four to five synapomorphic indels support the branch leading to *Steganotaenia*, *Polemanniopsis*, and Saniculoideae, depending upon the reconstruction. Four of these (representing 4- and 148-bp deletions, and 4- and 5-bp insertions) occur within the *trnQ-rps16* intergenic spacer region; a single 1-bp deletion occurs within *rps16* intron domain IV. Three of these deletions, however, are nested within larger deletions in other saniculoid taxa. As an example, the 148-bp deletion in *Steganotaenia*, *Polemanniopsis*, *Alepidea*, *Arctopus*, *Actinolema*, and *Astrantia* cpDNAs is nested within a 272-bp deletion in *Sanicula, Hacquetia*, and *Eryngium* and a 1009-bp deletion in *Petagnaea*. Four synapomorphic indels support the clade of *Lichtensteinia*, *Anginon*, and *Annesorhiza* (representing a single 2-bp deletion and 3-, 4-, and 5-bp insertions). Not a single indel supports the union of *Lichtensteinia* with *Steganotaenia* and *Polemanniopsis* or with any member of subfamily Saniculoideae. Each of the genera *Eryngium*, *Petagnaea*, *Astrantia*, *Actinolema*, *Arctopus*, *Alepidea*, and *Steganotaenia* are supported by synapomorphic indels; the genera *Sanicula* and *Hacquetia* unite as a clade, supported by nine synapomorphic indels. No autapomorphic indel was found for the monotypic genus *Polemanniopsis*.

A comparison of the number and length of minimal length trees resulting from MP analysis of combined or separate data partitions, with or without indels, or with just binary-scored indels, is presented in Table 4. Analyses of partitioned data results in the preset maximum tree limit of 20,000 trees, whereas MP analyses of the entire *trnQ-trnK* region, with and without indels, or of just the indels matrix alone, results in a lower and definite number of trees. Comparisons of overall homoplasy, calculated by optimization of each data partition (with or without indels), the entire *trnQ-trnK* region (without indels), or the matrix of indel characters onto the MP trees inferred by analysis of the *trnQ-trnK* plus indels matrix, reveal that the indels matrix had the least level of homoplasy. In general, when indels were incorporated into the phylogenetic analysis, CI and RI values increased. The *trnQ-rps16* plus indels matrix had the lowest level of homoplasy (CI = 0.7514, RI = 0.9547), whereas the *rps16-trnK* matrix plus indels was the most homoplastic (CI = 0.6794, RI = 0.9387).

Table 4
A comparison of the number and length of minimal length trees and overall levels of homoplasy resulting from MP analysis of combined or separate data partitions, with and without corresponding indels, or of just indel data from the entire *trnQ-trnK* region

Data partition	Number of MP trees	Length	CI	RI
trnQ-trnK	7455	2026	0.7036	0.9415
All indels	2016	217	0.8475	0.9809
trnQ-rps16 + indels	>20,000	1141	0.7514	0.9547
trnQ-rps16	>20,000	1016	0.7408	0.9494
rps16 + indels	>20,000	394	0.7152	0.9492
rps16	>20,000	352	0.6920	0.9430
rps16-trnK + indels	>20,000	699	0.6794	0.9387
rps16-trnK	>20,000	642	0.6558	0.9282

Homoplasy indices were calculated by optimizing each data partition over the MP trees obtained from the analysis of the *trnQ-trnK* + indels matrix (No. of MP trees = 4320; length = 2249 steps; CI = 0.7215; RI = 0.9487). CI, consistency index, excluding uninformative characters; RI, retention index.

The four independent Bayesian analyses showed MCMC convergence for all parameters in the best-fit model (PSRF reached 1 for all parameters). Moreover, the absolute difference graphic produced by AWTY online showed no significant variability among independent runs. Pairwise comparisons between tree files of each run showed no difference in the posterior probabilities of all splits for paired MCMC analyses. In all independent runs, the likelihood values reached stationarity by generation 200,000; however, the cumulative graphics produced by the program AWTY online showed that the posterior probabilities of the splits stabilize after 5 million generations, showing that tree topologies are finally being sampled in proportion to their posterior distribution and that the chains actually reached stationarity after 5 million generations. Given these results, the first 50,000 trees of each run were discarded as "burn in" and a 50% majority rule consensus tree that summarizes topology and branch length information was calculated based upon the remaining 200,000 trees (Fig. 6).

The phylogenies estimated using MP, Bayesian, and maximum likelihood analyses of the entire *trnQ-trnK* region are each highly resolved and consistent with one another. The five included accessions of *Lichtensteinia* comprise a clade sister group to the clade of *Anginon* + *Annesorhiza* (87% bootstrap, 100% posterior probability). The genera *Eryngium*, *Sanicula*, *Hacquetia*, *Petagnaea*, *Astrantia*, *Actinolema*, *Arctopus*, and *Alepidea* comprise a strongly supported monophyletic group (99% bootstrap, 100% posterior probability). This clade comprises subfamily Saniculoideae, as traditionally circumscribed (but excluding *Lagoecia*). *Steganotaenia* and *Polemanniopsis* are well supported sister taxa (94% bootstrap, 100% posterior probability) and this clade is sister group to Saniculoideae (97% bootstrap, 97% posterior probability). Constraining *Steganotaenia* + *Polemanniopsis* and the clade comprised of *Anginon*, *Annesorhiza*, and *Lichtensteinia* to monophyly in a subsequent MP analysis

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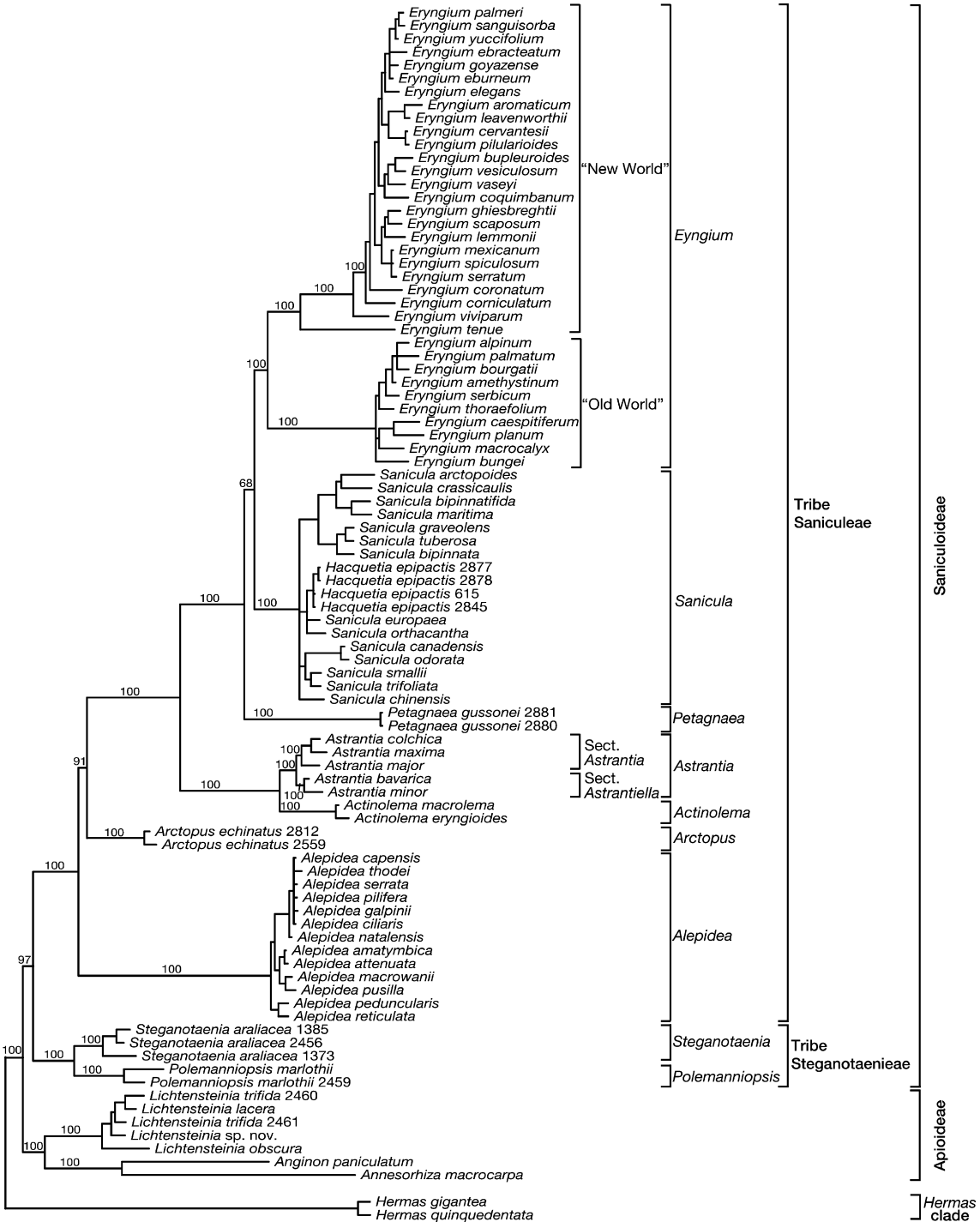

Fig. 6. Fifty-percent majority-rule consensus of 200,000 trees derived from Bayesian analysis of 91 cpDNA *trnQ-trnK* sequences. Numbers at nodes are posterior probabilities values; these values are indicated only for *Steganotaenia*, *Polemanniopsis*, *Anginon* + *Annesorhiza*, and the 24 recognized clades indicated in Table 3 and discussed in the text.

resulted in trees eight steps longer than those without the constraint invoked (length = 2257 steps), indicating that *Steganotaenia* and *Polemanniopsis* are indeed more closely related to subfamily Saniculoideae than they are to subfamily Apioideae (the latter including *Lichtensteinia*). With the exception of *Sanicula*, all genera are monophyletic. The monotypic genus *Hacquetia* arises within a paraphyletic *Sanicula* and comprises a clade along with *Sanicula europaea* and *S. orthacantha*. Constraining *Sanicula* to monophyly results in trees four steps longer (length = 2253 steps) than those without the constraint. Intergeneric relationships within Saniculoideae are fully resolved: (((((*Eryngium*, *Sanicula* + *Hacquetia*), *Petagnaea*), (*Astrantia*, *Actinolema*)), *Arctopus*), *Alepidea*). The genus *Eryngium* is divided into two well-supported subclades according to their geographic distribution, designated herein as "Old World" and "New

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World." Within *Astrantia*, sections *Astrantia* and *Astrantiella* are each resolved as monophyletic.

4. Discussion

4.1. Phylogenetic utility of the cpDNA trnQ-trnK region

The cpDNA *trnQ-trnK* region encompasses three large noncoding loci, of which only the *rps16* intron has been extensively characterized and used in phylogenetic study (summarized in Kelchner, 2002). To obtain greater phylogenetic resolution at the intergeneric and infrageneric levels than that which could be obtained using only the *rps16* intron (Downie and Katz-Downie, 1999; Calviño et al., 2006), additional data from the *trnQ-rps16* and *rps16-trnK* intergenic spacer regions were considered. Such noncoding loci are under less functional constraints than coding or intron regions and, therefore, should provide greater levels of variation for phylogenetic analyses (Learn et al., 1992; Gielly and Taberlet, 1994; Downie et al., 1996; Kelchner, 2000, 2002; Shaw et al., 2005).

The issue of the relative frequencies of nucleotide substitutions vs. indels in noncoding cpDNA sequences has been much discussed. Depending upon the region and taxonomic rank considered, indels may occur more, equally, or less often than nucleotide substitutions (Golenberg et al., 1993; Clegg et al., 1994; Gielly and Taberlet, 1994; Small et al., 1998; Shaw et al., 2005). In our study and across the entire *trnQ-trnK* region, the ratio of informative substitutions to indels was 4.6:1; this value was similar to those ratios calculated for each of the separate regions. Despite their lower frequency than nucleotide substitutions, the inclusion of indels in phylogenetic analyses improves levels of branch support and resolution of relationships. In analyses of combined or partitioned data, the inclusion of indel characters yielded more resolved phylogenies with lower levels of overall homoplasy and with higher bootstrap support values. These results add to the growing body of knowledge that indels provide reliable characters for phylogenetic analysis (Graham et al., 2000; Simmons et al., 2001; Geiger, 2002; Vogt, 2002; Hamilton et al., 2003; Müller and Borsch, 2005). Our observation that indels of 10 bp or shorter are the most common size class is in agreement with previous observations of other noncoding markers (Graham et al., 2000). It has been suggested that there is no apparent trend of decreased homoplasy with increased indel size (Simmons et al., 2001; Müller and Borsch, 2005) and our data support these findings. However, all indels of 16 bp or longer were not homoplastic, and a close examination of the results of Müller and Borsch (2005) reveals the same, suggesting that there might be a size threshold for decreased probability of length mutations.

Sequence comparisons of the three cpDNA loci revealed that the two intergenic spacers are evolving about twice as fast as that of the *rps16* intron and pairwise nucleotide divergence values for the spacers are, on average, two times higher than that for the *rps16* intron at all taxonomic levels considered. Group II introns in the chloroplast genomes of land plants, such as the *rps16* intron, show a strong relationship between the functional importance of its secondary structure and the probability of mutational change, with those domains essential for intron-associated functions most conserved evolutionarily (Michel et al., 1989; Learn et al., 1992; Downie et al., 1996, 1998, 2000b; Kelchner, 2002). While the numerous conserved regions within the *rps16* intron may explain its lower rate of mutational change relative to the intergenic spacers, the functional (and, consequently, structural) constraints within the latter regions and their effects on mutational processes are less understood.

No significant differences were detected in relative evolutionary rates between the two intergenic spacers. However, the *trnQ-rps16* spacer contributes more informative characters to the phylogenetic analysis than do both the *rps16* intron and *rps16-trnK* spacer region combined. MP analysis of separate data sets reveals that the *trnQ-rps16* region yields phylogenies that are most resolved and with greater branch support than the other regions, and resolution of relationships is obtained at diverse hierarchical levels. Also, relative to the *rps16* and *rps16-trnK* regions, the *trnQ-rps16* intergenic spacer region results in trees with higher CI and RI values. A major problem with the *trnQ-rps16* locus, however, is the size of deletions which can occur in this region (such as the 1009 bp deletion in *Petagnaea*). Such large deletions may be disastrous for phylogenetic analysis if they result in a reduction of available informative nucleotide substitutions.

Overall, the greatest resolution of relationships and the highest levels of branch support were achieved by simultaneous analysis of all nucleotide and indel data. Had we restricted this study to a continued sampling of only *rps16* intron sequences, all major clades inferred by MP analysis of combined data plus indels would have been recovered save one, but many of these would have been supported only weakly to moderately. Similarly, resolution of infrageneric relationships would have been minimal. Continued investigations of Apiaceae phylogeny, at a variety of hierarchical levels, would benefit by consideration of the entire *trnQ-trnK* locus.

4.2. Subfamily Saniculoideae

Wolff's (1913) revision of subfamily Saniculoideae is comprehensive and predominant and, until recently, changes to his system have been minimal. Like the classification of Drude (1898), Wolff recognized two tribes (Saniculeae and Lagoecieae) and nine genera (*Actinolema*, *Alepidea*, *Arctopus*, *Astrantia*, *Eryngium*, *Hacquetia*, *Lagoecia*, *Petagnaea*, and *Sanicula*) within the subfamily. Molecular studies have supported the transfer of *Lagoecia* to subfamily Apioideae (Plunkett et al., 1996; Downie et al., 2000a). The Argentinean genus *Oligocladus* has been described as *incertae sedis* within Saniculoideae (Pimenov and Leonov, 1993); our unpublished work, however, places

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this genus in tribe Selineae of subfamily Apioideae (C.I. Calviño and S.R. Downie, unpublished data). Liu et al. (2003), primarily on the basis of cladistic analysis of fruit anatomical characters but also considering the available molecular evidence (Downie and Katz-Downie, 1999), expanded the circumscription of subfamily Saniculoideae to include the African genera *Steganotaenia*, *Polemanniopsis*, and *Lichtensteinia*. Based on our results and those obtained in a concurrent study of southern African Apiaceae (Calviño et al., 2006), we present a revised classification of Saniculoideae that more accurately reflects its evolutionary history. We reject the transfer of the genus *Lichtensteinia* into Saniculoideae and recognize *Steganotaenia* and *Polemanniopsis* as comprising a new, distinct tribe within the subfamily that is a well-supported sister group to tribe Saniculeae. The latter is redefined to include the genera *Actinolema*, *Alepidea*, *Arctopus*, *Astrantia*, *Eryngium*, *Petagnaea*, and *Sanicula*. *Hacquetia epipactis* is included within *Sanicula* and, as such, all genera are monophyletic. Both tribes are clearly delineated morphologically, but only molecular data support their sister group relationship. In addition to nucleotide substitutions, the distribution of several synapomorphic indels supports the inclusion of *Steganotaenia* and *Polemanniopsis* in Saniculoideae. As yet, however, we are unaware of any unique anatomical or morphological character that would support the union of these taxa.

The Namibian genus *Phlyctidocarpa* Cannon and W.L. Theob., also treated in subfamily Apioideae (Cannon and Theobald, 1967; Pimenov and Leonov, 1993), may constitute yet another member of subfamily Saniculoideae based on our preliminary molecular study. Like *Steganotaenia* and *Polemanniopsis*, *Phlyctidocarpa* resembles most apioid umbellifers in habit, but has fruit features similar to those found in Saniculoideae (Theobald and Cannon, 1973). Fruit anatomical and further molecular studies of this unusual monotypic genus are in progress and the results should clarify its phylogenetic placement (B-E. van Wyk et al., University of Johannesburg, unpublished data).

4.3. Lichtensteinia

Liu et al. (2003) included the African apioid genus *Lichtensteinia* in their broadened circumscription of Saniculoideae based primarily on the shared presence of large intrajugal secretory ducts and the absence of both commissural and vallecular vittae. These same characters, however, occur among other early branching members of Apiaceae, thus the inclusion of *Lichtensteinia* alongside *Steganotaenia*, *Polemanniopsis*, and other Saniculoideae is based on shared plesiomorphies rather than synapomorphies (Calviño et al., 2006). We are not aware of any morphological or anatomical character supporting the union of *Lichtensteinia* with Saniculoideae that does not also include members of Apiaceae subfamilies Azorelloideae and Mackinlayoideae. The results of a concurrent molecular phylogenetic study of southern African Apiaceae with expanded sampling of early branching lineages of subfamily Apioideae revealed that *Lichtensteinia* comprises a monogeneric clade that is sister group to all other members of subfamily Apioideae (Calviño et al., 2006). The same results are supported herein upon expanded sampling of Saniculoideae and consideration of additional molecular data. *Lichtensteinia*, therefore, should be maintained within subfamily Apioideae.

4.4. Tribe Steganotaenieae

The African genera *Steganotaenia* and *Polemanniopsis*, arborescent and shrubby plants that were treated previously in subfamily Apioideae, are monophyletic sister taxa and this clade is a well-supported sister group to subfamily Saniculoideae, as traditionally circumscribed. Such relationships were proposed initially using *rps16* intron sequences (Downie and Katz-Downie, 1999) and later corroborated through a greater sampling of early branching lineages of subfamily Apioideae (Calviño et al., 2006), but in each of these studies bootstrap support for the sister group relationship between *Steganotaenia*/*Polemanniopsis* and Saniculoideae was weak. As a result of examination of fruit anatomical characters and in light of the molecular evidence, Liu et al. (2003) transferred *Steganotaenia* and *Polemanniopsis* to subfamily Saniculoideae. Upon consideration of additional cpDNA data from previous studies, and comprehensive sampling of all genera and most infrageneric groups traditionally considered in Saniculoideae, our results corroborate the expansion of the subfamily to include *Steganotaenia* and *Polemanniopsis*, as the entire group is strongly supported as monophyletic. The alternative would be to recognize the clade of *Steganotaenia*/*Polemanniopsis* as a new subfamily but, by doing so, its sister group relationship to Saniculoideae would not be reflected in the classification and it would comprise only two genera and four species.

Steganotaenia and *Polemanniopsis* are woody, occur in southern Africa (*Steganotaenia* is widespread throughout tropical Africa), and have fruits with 2–3 wings and 5 prominent intrajugal secretory ducts per mericarp. At maturity, those intrajugal ducts associated with the wings expand forming enormous cavities (Liu et al., 2003). This combination of characters sets them apart from all other genera of the subfamily. We formally recognize this clade at the tribal level and propose the new name, Steganotaenieae C. I. Calviño and S. R. Downie.

Steganotaenieae C.I. Calviño and S.R. Downie *Trib. Nov. Generalignosa austro-africana alis mericarpiorum 2–3 bene evoluta; vittae intrajugales 5 magnae, alarum permagnis (cavitates)*. Type genus: *Steganotaenia* Hochst. In Flora 27 (1), Besondere Beilage 4 (1844). Also includes *Polemanniopsis* B.L. Burtt.

4.5. Tribe Saniculeae

With the transfer of *Lagoecia* to subfamily Apioideae and taxonomic realignments suggested by the molecular

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phylogenies, further changes to the infrasubfamilial classification of Saniculoideae are necessary. Tribe Saniculeae, as traditionally circumscribed (Drude, 1898; Wolff, 1913), consists of *Actinolema*, *Alepidea*, *Astrantia*, *Hacquetia*, *Eryngium*, and *Sanicula*. The inclusion of both *Petagnaea* and *Arctopus* (genera traditionally treated in tribe Lagoecieae) among these taxa suggests that tribe Saniculeae, to be monophyletic, must be redefined to include these genera. Tribe Saniculeae was characterized by Drude and Wolff as having mature fruits with both mericarps fertile (as is typical of most Apiaceae), whereas members of tribe Lagoecieae have fruits with only one fertile (or developed) mericarp. Our expanded circumscription of Saniculeae includes plants characterized by fruits with an exocarp often covered in scales, bristles, or prickles, a mesocarp with calcium-oxalate crystals scattered throughout, and umbels or heads that are surrounded by showy bracts. This revised definition of tribe Saniculeae corresponds precisely to Drude's conception of subfamily Saniculoideae (upon the exclusion of *Lagoecia*).

Alepidea is well supported as monophyletic (100% bootstrap, 100% posterior probability) and is sister group to all other members of tribe Saniculeae. The genus comprises perennial herbs with rosulate, simple leaves and capitate inflorescences. It is mainly distributed in southern Africa, with a few species extending northward to Zimbabwe, Kenya, and Ethiopia (van Wyk, 2000). Historically, *Alepidea* and *Eryngium* have been considered closely related (Bentham, 1867; Dümmer, 1913; Turmel, 1950). Both genera share similar inflorescence types and leaves, with the main difference between them being the absence of floral bracts in *Alepidea*. It was further thought that *Eryngium* and *Alepidea* had a common Asian origin, whereupon the lineages that diverged into these genera dispersed along two different routes: one northwestward to colonize Europe and then the New World, and the other southward towards eastern Africa (Turmel, 1950). Our results, however, reject the hypothesis of a sister group relationship between *Alepidea* and *Eryngium*. The morphological characters once thought to unite these genera, while similar in appearance, have evolved independently for there are clear structural differences in the capitate inflorescences and leaves between the genera (C. I. Calviño et al., unpublished data). Moreover, the origin of *Alepidea* is likely southern African rather than Asian. The infrageneric taxonomy of *Alepidea* has long been problematic despite several revisionary studies (Sonder, 1862; Dümmer, 1913; Wolff, 1913; Weimarck, 1949; Hilliard and Burtt, 1982; Burtt, 1991). Weimarck (1949) recognized six sections based on fruit surface features and types of marginal cilia on the basal leaves. The sampling of *Alepidea* herein represents all sections save one, and the phylogenetic results obtained indicate that these sections are artificial. Features of the marginal cilia display intergradation of character states among the sections (Hilliard and Burtt, 1982; Burtt, 1991) and we are of agreement with Burtt (1991) in suggesting that these sections be abandoned.

Arctopus consists of three dioecious species endemic to the Cape Floristic Region of South Africa. Its distinctive characters include an acaulescent habit, deciduous leaves, spiny leaf margins, a single developed mericarp, and lack of differentiation between sepals and petals in female flowers. Monophyly of the genus was confirmed using ITS and cpDNA *trnL-F* sequences (A.R. Magee et al., University of Johannesburg, unpublished data). Once treated in the traditionally recognized Apiaceae subfamily Hydrocotyloideae (Froebe, 1979; Magin, 1980; Constance and Chuang, 1982; Pimenov and Leonov, 1993), molecular data clearly support the original placement of this genus within subfamily Saniculoideae. *Arctopus* is sister group to the large clade of *Actinolema* through *Eryngium* (Fig. 3).

Astrantia and *Actinolema* are each monophyletic and collectively comprise a strongly supported clade sister group to the clade of *Petagnaea*, *Sanicula* (including *Hacquetia*), and *Eryngium*. The sister group relationship between *Actinolema* and *Astrantia* is also supported by ITS sequences (Valiejo-Roman et al., 2002) and several shared morphological characters (Grintzesco, 1910; Wolff, 1913). *Actinolema* comprises two annual species distributed in Anatolia and the Caucasus. They have entire basal leaves and sessile umbels with leaf-like involucral bracts. *Astrantia* includes nine perennial species characterized by divided basal leaves and conspicuous, colored involucral bracts. Its distribution is sympatric to *Actinolema* in SW Asia, but its range extends further west to the Balkan Peninsula, Carpathian Mountains, Alps, Apennines, and Pyrenees (Wörz, 1999). Two sections are recognized in *Astrantia*: sect. *Astrantia* (= sect. *Macraster* Grintzesco), including *A. major, A. maxima*, and *A. colchica*; and sect. *Astrantiella*, including *A. bavarica* and *A. minor* (Grintzesco, 1910; Wolff, 1913). Section *Astrantia* displays rigid involucral bracts and acute calyx teeth longer than the petals, whereas section *Astrantiella* has membranous involucral leaves and obtuse calyx teeth longer or as long as the petals (Wolff, 1913; Wörz, 1999). Each of these sections is monophyletic in the molecular phylogenies presented herein.

Petagnaea is endemic to the Nebrody Mountains of NE Sicily, Italy. Its only species, *P. gussonei* (= *Petagnia saniculifolia* Gussone), is categorized as endangered according to the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List (Gianguzzi et al., 2004). *Petagnaea* comprises a monotypic lineage sister to the clade of *Eryngium* + *Sanicula* (including *Hacquetia*). Its isolated phylogenetic position in Saniculoideae corroborates the need for further conservation actions for this species.

The genus *Sanicula*, upon the inclusion of *Hacquetia epipactis*, comprises a well supported monophyletic group. Both genera are morphologically very similar, with *Hacquetia* separated from *Sanicula* primarily by its showy involucral bracts and the absence of prickles on the fruit exocarp. Many species of *Sanicula*, however, also lack fruit prickles. Indeed, the absence of such prickles characterizes three different lineages of *Sanicula* (Vargas et al., 1999).

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Sanicula orthacantha (sect. *Pseudopetagnia*) and *S. hacquetoides* (sect. *Tuberculatae*, not sampled here) also display prominent and leafy involucres similar to those seen in *Hacquetia*. Shan and Constance (1951) suggested an affinity among *Hacquetia* and *Sanicula* sects. *Tuberculatae* and *Pseudopetagnia* and in our study, *Hacquetia epipactis* allies with *S. orthacantha* and *S. europaea*. The results of phylogenetic analyses of ITS sequences also show *Hacquetia* nested within *Sanicula* (Valiejo-Roman et al., 2002). On the bases of morphological similarities, the intergradation of characters previously used to delimit the genera, and results of molecular phylogenetic analyses, *H. epipactis* is best treated within the genus *Sanicula*. The name *Sanicula epipactis* (Scop.) E.H. Krause is already available for use.

Eryngium is the largest genus in the family with about 250 species (Pimenov and Leonov, 1993). It is easily distinguished by its capitate inflorescences and single bract per flower and it has long been recognized as a natural group. The genus, however, is extremely variable morphologically and interspecific relationships are obscure. In the present study, we examined 35 species of *Eryngium*, a 4- to 6-fold increase in sampling over any previous study (Downie and Katz-Downie, 1999; Valiejo-Roman et al., 2002). These species represent 34 of 36 sections of *Eryngium* recognized by Wolff (1913). On the bases of the phylogenetic results presented herein, *Eryngium* is confirmed as monophyletic (85% bootstrap, 100% posterior probability). Furthermore, three synapomorphic indels support the monophyly of *Eryngium* (Fig. 5). These results are in contrast to those inferred by Valiejo-Roman et al. (2002) using ITS sequences in which *Sanicula* occurred within a paraphyletic *Eryngium*. A reanalysis of the DNA sequences used by Valiejo-Roman et al., which contained representatives of all major subfamilies of Apiaceae, could not be aligned unambiguously because of high nucleotide sequence divergence and numerous indels. Tree topologies were not stable to alternative pairwise and multiple alignment parameters assigned by CLUSTAL X and, in contrast to the results of Valiejo-Roman et al., a monophyletic *Eryngium* could result in some of our analyses of their data. At such deep levels of comparison, ITS sequences are just too divergent for phylogenetic analyses (Downie et al., 2001; Hardway et al., 2004). In our analyses, *Eryngium* is sister group to *Sanicula*; this relationship, however, is not strongly supported (72% bootstrap, 68% posterior probability).

The cpDNA phylogenies presented herein show two strongly supported major clades within *Eryngium*: "Old World," representing species from Eurasia and North Africa; and "New World," representing species primarily from the western hemisphere, but also from Australia and the western Mediterranean. Similar groupings of species were recognized by Wolff (1913): "Species gerontogeae" and "Species americanae and australienses," respectively. Wolff (1913) included *Eryngium tenue*, *E. viviparum*, and *E. corniculatum* (distributed in the western Mediterranean) in his "Species gerontogeae" ("Old World") group, but indicated that these species are closely related to those of North America. In the molecular phylogenies, these three species fall as successive sister lineages at the base of the "New World" clade, suggesting that *Eryngium* of the New World may have had their origin from western Mediterranean ancestors. A recent, yet preliminary, classification of *Eryngium* has been presented by Wörz (2004) based on morphology, but it does not reflect the major clades recognized herein using molecular data. Further molecular systematic studies on *Eryngium* are in progress and the results should illuminate the naturalness of the sections described by Wolff (1913) while contributing to a modern classification of this taxonomically complex genus (C.I. Calviño et al., unpublished data).

In summary, we revise the circumscription of Apiaceae subfamily Saniculoideae and present an estimate of phylogenetic relationships within the subfamily using data from the cpDNA *trnQ-trnK* 5′-exon region, which includes two intergenic spacers that have been heretofore previously underutilized in molecular systematic studies. The region presents a large number of phylogenetically informative indels that, when analyzed by themselves, yield trees of great resolution and high levels of overall branch support. With the exception of the monotypic genus *Polemanniopsis*, synapomorphic indels support the monophyly of all recognized genera within the subfamily. When used with nucleotide substitution data, the inclusion of indels in phylogenetic analyses of both combined and partitioned data sets improves resolution of relationships, increases branch support values, and decreases levels of overall homoplasy. The continued acquisition of sequence data from the *trnQ-trnK* 5′-exon region for assessing relationships at a variety of hierarchical levels within Apiaceae seems worthwhile, given the large number of parsimony informative characters obtained, as well as adequate levels of sequence divergence observed even among congeners. We have redefined subfamily Saniculoideae to include the African genera *Steganotaenia* and *Polemanniopsis* (as the new tribe Steganotaenieae), but not *Lichtensteinia*. The latter should be maintained within subfamily Apioideae. Sister group to tribe Steganotaenieae is an expanded tribe Saniculeae, which includes *Actinolema, Alepidea, Arctopus, Astrantia, Eryngium, Petagnaea*, and *Sanicula*. The genus *Hacquetia* is synonymized with *Sanicula* and, as such, all genera are monophyletic. Intergeneric relationships within tribe Saniculeae are fully resolved and, generally, very well supported. Studies are in progress which will use the resultant phylogeny to evaluate patterns of character state evolution within the subfamily and to formulate hypotheses of ancestral character states and biogeography using objective methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.01.002.

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