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UNDERSTANDING EVOLUTIONARY RELATIONSHIPS IN THE ANGIOSPERM ORDER APIALES BASED ON ANALYSES OF ORGANELLAR DNA SEQUENCES AND NUCLEAR GENE DUPLICATIONS

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Abstract

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

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I studied evolutionary history in the angiosperm order Apiales, with a special emphasis on interactions between form, time, and space. Four broad categories of problems were addressed: interfamilial relationships in Apiales, the assignment of genera traditionally assigned to the Apiaceae subfamily Hydrocotyloideae, the estimation of divergence times of the major clades, and the reconstruction of the biogeographic history of Apiales. We used molecular markers with different evolutionary properties and rates

derived from the plastid (*trn*D-*trn*T and *rpl*16), nuclear (RPB2), and mitochondrial (*nad1* intron 2) genomes, from more than 250 species representing all major clades in the order. The nuclear RPB2 region exhibited evidence of at least six duplication events in Apiales and provided a rich source of information for understanding the origins of polyploid lineages, especially in Araliaceae. Sequence comparisons among the copies show that exon regions are highly conserved. All copies appear to be functional but may have undergone subfunctionalization. Phylogenetic analyses of the three genomes suggest that Hydrocotyloideae should be divided into as many as six evolutionary lineages, but that most taxa should be included in subfamilies Azorelloideae and Mackinlayoideae. Relationships among and within the major clades of Azorelloideae need further analyses since many genera appeared non-monophyletic (e.g., *Azorella*, *Schizeilema*, and *Eremocharis*). Mackinlayoideae appeared as the earliest diverging lineage of Apiaceae, but the plastid and nuclear trees were incongruent in the placement of the *Platysace* clade relative to Mackinlayoideae and the rest of Apiaceae. Among the remaining clades of suborder Apiineae, Myodocapaceae appeared sister to Apiaceae in both plastid and nuclear trees, preceded by the divergence of Araliaceae and then Pittosporaceae. At the base of the gene trees in Apiales, Griseliniaceae and Torricelliaceae formed successive sisters to Apiineae. The placement of Pennantiaceae as sister to the rest of Apiales was confirmed by plastid data, but was not found in the nuclear trees. The order appears to have originated in the Cretaceous, with Apiineae having an age of c. 100 Mya. Australasia appears to be the most likely center of origin for Apiineae and most of its major clades, except Azorelloideae (South America) and Apioideae-Saniculoideae (sub-Saharan Africa).

CHAPTER 1

The Demise of Subfamily Hydrocotyloideae and the Re-alignment of its Genera across the whole Order Apiales

A. N. Nicolas & G. M. Plunkett

Abstract

As circumscribed by Drude, the umbellifer subfamily Hydrocotyloideae posed a major hindrance to resolving the phylogeny of order Apiales. Previous studies have suggested its polyphyly, but have not had sufficient sampling to address the issue fully. To put an end to the out-dated concept of Hydrocotyloideae, we investigated the placement of 40 of the 42 genera once placed in the subfamily, using extensive taxon sampling across the entire order. Molecular phylogenies were constructed using plastid sequences of the *rpl*16 intron and the *trn*D-*trn*T regions and revealed at least six hydrocotyloid lineages dispersed across both families Apiaceae and Araliaceae. The most speciose of these clades corresponds to the recently erected subfamily Azorelloideae. Another lineage includes genera grouped in Mackinlayoideae, where relationships are well-resolved. *Platysace* appears paraphyletic with respect to *Homalosciadium*, and their placement is well supported as a basal lineage in Apiaceae. The type genus, *Hydrocotyle*, belongs to a supported clade in Araliaceae. The placement of *Hermas* as sister to a clade consisting of Apiaceae subfamilies, Apioideae and Saniculoideae, and *Choritaenia* as sister to *Lichtensteinia* in a clade with affinities to both Apioideae and Saniculoideae, questions the

circumscriptions of the two subfamilies. Finally, plastid data suggest that many former hydrocotyloid genera are are non-monophyletic (e.g., *Azorella*, *Schizeilema*, and *Eremocharis*) and are in dire need of additional phylogenetic and taxonomic studies.

1. Introduction

For over a century, Apiaceae subfamily Hydrocotyloideae has posed a major obstacle to understanding evolutionary relationships throughout the order Apiales, especially between its two biggest families, Apiaceae (= Umbelliferae) and Araliaceae. Drude (1898) placed Hydrocotyloideae in Apiaceae as one of three subfamilies, the others being Apioideae (the "typical umbellifers") and Saniculoideae (including, for example, *Eryngium* and *Sanicula*). In Pimenov and Leonov's (1993) adaptation of Drude's (1898) three-subfamily system, Hydrocotyloideae comprises 42 genera (and c. 470 species) of mostly herbaceous and suffrutescent plants. The group has a worldwide distribution, but its greatest generic diversity is in the Southern Hemisphere, with particularly high levels of endemism in southern South America, Australia, and New Zealand. Hydrocotyloideae include medicinals (e.g., *Centella*, *Mulinum*, *Azorella*), edible plants (e.g., *Centella*, *Diposis*), plants used as ornamentals (e.g., *Trachymene*, *Actinotus, Azorella*, and the aquatic pennywort *Hydrocotyle*), and plants of ethnobotanic importance, some of which have been over-harvested to levels of endangerment (e.g., *Azorella compacta* and *Laretia acaulis*; Hodge, 1960; Wickens, 1995). In differentiating Hydrocotyloideae from the rest of Apiaceae, Drude (1898) emphasized three fruit characters. Unlike apioid and saniculoid fruits, the fruits of Hydrocotyloideae were described as having woody endocarps and

lacking both free carpophores and vittae (oil tubes in the furrows between the main ribs, although rib oil ducts may be present). Drude further divided Hydrocotyloideae into two tribes based on the direction of fruit compression: Hydrocotyleae (with two laterally compressed mericarps) and Mulineae (with dorsally compressed mericarps).

Drude's (1898) classification of Apiaceae was widely followed through the next century, despite alternative delimitations (e.g., Koso-Poljansky, 1916; Cerceau-Larrival, 1962). The traditional view of a close relationship between Apioideae and Saniculoideae has been supported by many studies (see Downie *et al*., 2001), with persisting difficulties in the precise placement of some early-diverging taxa (e.g., *Lichtensteinia*, *Steganotaenia*, and *Polemanniopsis*; Van Wyk, 2001; Liu *et al*., 2003; Calviño *et al*., 2006; Calviño and Downie, 2007). Hydrocotyloideae, on the other hand, were often viewed as intermediates between Araliaceae and the rest of Apiaceae (see Tseng, 1967; Pickering and Fairbrothers, 1970; Rodríguez, 1957, 1971), which led some workers to recognize the hydrocotyloids as a distinct family (Hylander, 1945), or to merge Apiaceae and Araliaceae into a single family (Thorne, 1973). With the advent of phylogenetic studies, particularly those based on molecular data, it has become clear that Hydrocotyloideae are polyphyletic and include genera belonging to several major groups in both families (Plunkett *et al*., 1996, 1997; Downie *et al*., 1998, 2001; Plunkett and Lowry, 2001; Chandler and Plunkett, 2004; Andersson *et al*., 2006). In a revised classification for Apiales, Plunkett *et al*. (2004) divided the hydrocotyloids among three groups: Araliaceae (to which *Trachymene* and the subfamilial-type *Hydrocotyle* were transferred) and two new subfamilies in Apiaceae, Azorelloideae and Mackinlayoideae. That study, however, placed fewer than half of the

genera of Hydrocotyloideae among the major clades of Apiales, and thus a full resolution of relationships among the hydrocotyloids has not yet been achieved. Also, the polyphyly of Drude's tribes and subtribes was addressed through studies based on morphology, fruit anatomy, and molecular data (Henwood and Hart, 2001; Liu, 2004; Chandler and Plunkett, 2004; Andersson *et al*., 2006), but many questions remain unanswered regarding both tribal and generic circumscriptions.

Drude's classification of Hydrocotyloideae suffers from many limitations that contribute to its artificiality. In erecting his system, Drude relied heavily on superficial characters of fruit morphology, which has been proved faulty in many umbellifer subgroups (see Downie *et al*., 2001). His classification also lacked sufficient representation from the Southern Hemisphere, where hydrocotyloids exhibit their greatest diversity (see Tseng, 1967). Subsequent to Drude's system, questions have been raised, though rarely addressed, regarding the distinctiveness of some hydrocotyloid genera (e.g., between *Eremocharis* and *Domeykoa*, or among *Schizeilema*, *Huanaca* and *Diplaspis*; Mathias and Constance, 1962a; Dawson, 1971), which may imply problems with monophyly at the generic level. Despite the progress made by several recent studies, sampling from Hydrocotyloideae has remained limited due largely to a focus on problems at other phylogenetic levels (e.g., subfamily Apioideae in Downie *et al*., 1998, 2000; Plunkett and Downie, 1999; or the order Apiales in Plunkett *et al*. 1996, Plunkett and Lowry 2001, Chandler and Plunkett 2004), or to questions dedicated to a single genus (e.g., *Azorella*; Andersson *et al*. 2006) or a single geographic region (e.g., Australia in Henwood and Hart 2001).

In an effort to resolve the placement of all hydrocotyloid genera, and through this to gain a better understanding of relationships throughout Apiales, the present study includes a wider sampling of taxa than any previous study of the order. This goal follows the conclusions of many studies that emphasize the importance of increased sampling of taxa (Graybeal, 1998; Pollock *et al*., 2002; Zwickl and Hillis, 2002; DeBry 2005), in addition to choosing the appropriate characters. The availability of the necessary taxa has a great impact on phylogeny reconstruction, including an increased likelihood of obtaining a more fully resolved molecular phylogeny. Thus, we have produced over 500 new sequences from two plastid markers, using an extensive generic sampling from Apiales (139 genera) to determine the correct placement for all but two of the hydrocotyloid genera. We also achieved substantial sampling within many of these genera to cover all major geographic regions and to test their monophyly. As a result, we have been able to recover a phylogeny that resolves relationships across Apiales, allowing the placement of hydrocotyloid genera not represented in previous molecular analyses, and to support (or in some cases refute) the placements and relationships suggested by prior studies. In addition, we provide an assessment of the taxonomic value of characters traditionally used to group Hydrocotyloideae and its tribes and subtribes (e.g., the carpophore, woody endocarp, vittae, winged fruits, and petaloid sepals) by noting where structural data available from previous studies are consistent (or inconsistent) with monophyletic groups estimated in our molecular phylogeny.

2. Materials and Methods

2.1. Taxon Sampling

The goal for the taxon sampling (Table 1) was to include an extensive representation of genera from throughout Apiales, with a particular emphasis on representing all major phytogeographic regions where hydrocotyloids are represented. This sampling will provide an extensive framework for the placement of hydrocotyloid genera while improving the phylogenetic inference. Pimenov and Leonov (1993) list 42 genera in subfamily Hydrocotyloideae. Two of these genera, *Turczaninowiella* Koso-Pol. and *Neoturczaninowia* Koso-Pol. appear to be *nomina nuda* for which no specimens or descriptions are known, thus decreasing the number of genera to 40. Mitchell *et al*. (1999) showed a close relationship between the megaherb *Stilbocarpa* (previously placed in Araliaceae) and the hydrocotyloid genera *Schizeilema* and *Azorella*, which suggested the transfer of *Stilbocarpa* to Hydrocotyloideae. The recently erected Australian *Brachyscias*, a monotypic genus, was also added to the list of hydrocotyloid genera based on its morphological affinities with *Chlaenosciadium* (Hart and Henwood, 1999). This brings the total number of extant hydrocotyloid genera back to 42, of which we were able to sample 40 genera. We were unable to sample the monotypic *Asciadium*, a Cuban taxon, known only from the type (dating from 1865), and the critically endangered *Brachyscias verecundus*, known only from two populations in SW Western Australia (Hart and Henwood, 1999). Where available, we also sampled multiple species within genera, especially from the more speciose ones, with emphasis on representation of the type species of each genus and a broad geographic representation of widespread genera. Due to

the high number of genera in Apiaceae subfamily Apioideae and the support for its monophyly by various studies (see Downie *et al*., 2000 and 2001; Sun *et al*., 2004), a representative sample of thirty-five genera was included from Apioideae, with particular emphasis on the early-diverging lineages. We also included all eight genera of subfamily Saniculoideae sensu stricto. We sampled all but two genera from Araliaceae (excluding the two monotypic genera *Anakasia* and *Woodburnia*, the latter only known from the type), both genera of Myodocarpaceae, and all nine genera of Pittosporaceae. The outgroup included samples from the five genera that represent the three earliest diverging families of Apiales: the monogeneric Pennatiaceae and Griseliniaceae, and all three genera of Torricelliaceae (Plunkett, 2001; Kårehed, 2003; APG II, 2003; Chandler and Plunkett, 2004; Plunkett *et al*., 2004).

2.2. Character Sampling

Two sequence regions from the plastid genome were used as sources for molecular markers. These regions contain sequences (tRNAs, spacers, and an intron) which have different evolutionary properties that, theoretically, can yield greater phylogenetic accuracy (Delsuc *et al*., 2002). In the chloroplast genome, the *trn*D-*trn*T spacer region (which also spans *trn*Y and *trn*E) and the *rpl*16 intron are among the most useful markers examined by Shaw *et al*. (2005 and 2007), representing his Tier I and Tier II sequences (respectively) based on the average number of potentially informative characters (PIC). Of these, the *trn*D-*trn*T spacer exhibits one of the highest PIC values of all chloroplast markers tested. Previous studies have successfully used these markers in phylogenetic

studies at various taxonomic levels and across many families, genera, and species, including in Apiales (e.g., Downie, 2000; Ackerfield and Wen, 2003).

2.3. DNA Extraction, PCR, and Sequencing

Most leaf tissue samples were either field-collected and dried using silica gel or harvested from herbarium specimens (Table 1 provides source and voucher information). All sequences used in this study were newly derived, including 268 sequences from the *trn*D-*trn*T region and 272 sequences from the *rpl*16 intron. The two datasets shared in common sequences from 268 samples, representing 263 species across 139 genera of Apiales. Harvesting reliable, high purity, total DNA from fresh, silica-gel dried leaf tissue, or dried herbarium specimens was achieved using the CTAB method of Doyle and Doyle (1987), the DNeasy Plant extraction kit (QIAGEN Inc.), a modified Puregene DNA extraction protocol (Gentra Systems), or following the protocol of Alexander *et al*. (2007) with minor modifications. External and internal primer sets were designed to amplify and sequence regions of the chloroplast genome that included the entire *rpl*16 intron and the *trn*D-*trn*T spacer region (Fig. 1; Table 2). PCR amplifications were produced by thermally cycling a mix of 1 µL of unquantified DNA, 5 µL Sigma JumpStart™ REDTaq® ReadyMix™ Reaction Mix or Promega GoTaq® Green Master Mix, 0.5 µL of each forward and reverse primers (at concentrations of 5 µ*M*), 0.5 µ*M* spremidine (4 m*M*), and 2.5 µL ultrapure water for a total volume of 10 µL. The PCR thermal profile included a 2 min denaturing step at 94ºC, followed by 35 to 40 cycles of denaturation (30 sec at 94ºC), primer-annealing (30 sec at 54ºC), and DNA extension (30 to 90 sec at 70ºC). This was

followed by an extra extension step for 5 min at 72ºC. For many samples, successful amplification could be achieved in a single reaction using the external primers, but for most herbarium samples, amplification of the entire *trn*D-*trn*T region and *rpl*16 intron was achieved in two overlapping fragments sized between 300 and 600 bp, using a combination of external and internal primers (see Table 2).

PCR amplicons were cleaned using ExoSAP-IT (USB Corp.), according to the manufacturer's recommendations, before serving as template for the sequencing reaction. Cycle sequencing reactions were performed by mixing 1 µL of the DYEnamic™ ET Terminator Cycle Sequencing mix (GE Healthcare), 1.5 µL of purified double-stranded PCR product, 0.5 µL primer (5µ*M*), and 3 µL ultrapure water, for a total volume of 6 µL. The amplification program consisted of 40 cycles of 3 steps: 30 sec at 94ºC, 15 sec at 55ºC, and 60 sec at 60ºC. Sequencing products were purified using MontageSeq plates (Millipore Corp.) and then separated electrophoretically on a 96-capillary MegaBACE™ 1000 automated sequencer. The resulting sequences were edited using MegaBACE™ Sequence Analyzer. Complementary (forward and reverse) fragments were assembled and edited using the Sequencher™ 3.0 DNA sequence analysis package (Gene Codes Corporation, Ann Arbor, MI) or by pairwise BLAST (www.ncbi.nlm.nih.gov/BLAST/).

2.4. Sequence Alignment and Phylogenetic Analyses

Sequences were aligned in ClustalX using the default settings (Higgins and Sharpe, 1988), followed by manual adjustments made in BioEdit version 7.0.5 (Hall, 2005). An incongruence length difference (ILD) test (Farris *et al*., 1994) was conducted to assess

congruence among the individual datasets before combining data. This was achieved by running 100 iterations of the partition homogeneity test in PAUP. Maximum parsimony (MP) was employed in the phylogenetic analysis of the separate datasets and of the combined dataset, using PAUP* (Swofford, 2001), with heuristic searches, equally weighted characters, stepwise addition, random addition of sequences, and accelerated-transformation character-state optimization (ACCTRAN). One-hundred replicates were run under the TBR branch-swapping algorithm, saving no more than 1000 trees per replicate. Clade support was estimated using PAUP* to calculate bootstrap values (Felsenstein, 1985) based on 100 pseudo-replicates of full heuristic searches, and these values were compared to estimates from 10,000 pseudo-replicates generated using fast heuristic searches.

For the combined dataset, additional phylogenetic trees were generated using two model-based approaches, maximum likelihood (ML) and Bayesian inference (BI). MODELTEST 3.06 (Posada and Crandall, 1998) was used with PAUP to determine the most appropriate model of sequence evolution to produce the most reliable tree and to reduce the computation time (Posada and Buckley, 2004). Subsequently, the best fitting evolutionary model, GTR + I + Γ, was implemented in the ML and BI analyses on the combined dataset, allowing the programs to estimate the parameters directly from the data. ML analyses were run in GARLI (version 0.95; Zwickl, 2006) using an Apple SuperComputing Cluster under three alternative conditions: (1) parameters were consistent with the default settings, with termination conditions reached after the log likelihood value decreases by no more than 0.01 for 10,000 generations; (2) settings allowed for termination

after the log likelihood values decreases by no more than 0.001 for 100,000 generations; (3) analyses were run for 5,000,000 generations without any premature termination. In total, GARLI was run once under condition 1, ten times under condition 2, and once under condition 3. Two non-parametric bootstrap analyses were performed, the first for 250 iterations under condition 1 and the second for 180 iterations under condition 2.

Bayesian inference analyses were conducted using MrBayes 3.1 under a Metropolis-coupled Markov-chain Monte Carlo (MCMC) sampling for the estimation of the likelihood scores (Huelsenbeck and Ronquist, 2003). Two simultaneous runs were conducted, each for 1,000,000 generations and four incrementally heated chains. Trees generated before the likelihood value stabilized were discarded (the "burn-in") and the remaining trees were used to calculate the posterior probabilities by constructing a majority rule consensus tree (Huelsenbeck and Ronquist, 2001a, 2001b).

3. Results

Complete new sequences were retrieved for the *rpl*16 intron and the *trn*D-*trn*T region from all samples included in this study with the exception of < 100-bp fragment from the *trn*D-*trn*T sequence of *Notiosciadium pampicola*. Comparisons of sequence characteristics and tree descriptions of the two regions are listed in Table 3. Length variation for the *rpl*16 intron ranged from 830 bp in *Azorella trifoliolata* to 1068 in *Pozoa volcanica*, but >85% of the ingroup sequences ranged between 900 and 1000 bp. Within the *trn*D-*trn*T matrix, unaligned sequence lengths varied from 535 bp in *Choritaenia capensis* to 1416 bp in *Gymnophyton isatidicarpum*. However, much of this variation is

due to a large deletion in the *trn*E-*trn*T spacer unique to *Choritaenia capensis*, and a large repeat found in the sequence of the *trn*Y-*trn*E spacer of *Gymnophyton isatidicarpum* and a related species (*Asteriscium closii*). Most ingroup sequences for the *trn*D-*trn*T region (>80%) ranged in size between 850 bp and 1200 bp.

We tested for bias due to subjectivity in sequence alignment by comparing consensus trees produced under MP with bootstrap (10,000 replicates with fast-stepwise addition) based on two versions of the combined dataset. In the first, the entire alignment was used without removing any potentially informative data, and in the second all potentially ambiguous regions were removed from the alignment. The topologies of the two trees were visually congruent and all the clades identical, with only slight differences in bootstrap values, suggesting that the potentially ambiguous regions do not affect phylogenetic inferences. To reduce the amount of excluded data, we realigned all problematic regions after manual pairwise comparisons of all sequences to verify the positions of gaps relative to aligned characters across different groupings of sequences. Through this process, we excluded ambiguous regions totalling 388 aligned characters from the *trn*D-*trn*T partition of the combined dataset. Thus, the final analyses were carried out with an aligned matrix of 5432 characters from the 268 samples common to both datasets (3277 aligned characters from *trn*D-*trn*T region and 2155 characters from the *rpl*16 intron). The individual dataset for the *rpl*16 intron included four more terminals (totaling 272 sequences) compared to the *trn*D-*trn*T and combined datasets. The additional samples necessitated two extra characters in the *rpl*16 intron dataset, and thus the total number of characters of the two individual datasets was 5434.

In general, the *trn*D-*trn*T spacer provided better resolution of relationships within and among major clades, especially within genera of the different clades of family Apiaceae. The strict consensus of trees retrieved for the individual datasets (64,000 trees for *trn*D-*trn*T and 70,000 for *rpl*16) were mostly congruent. Of the few nodes that were different between the two trees, most had <65% bootstrap support (e.g., the placement of *Diposis*, *Klotzschia*, and *Actinotus*; the relationship between the *Asteriscium* clade, the *Bowlesia* clade, and the *Azorella* clade), and thus do not have a major influence on our conclusions regarding phylogenetic relationships (Fig. 2). However, a single difference was well supported in both trees, the placement of *Chlaenosciadium* as sister to *Xanthosia* plus the *Centella* clade in *trnD-trnT* phylogeny but sister to *Xanthosia* alone in the *rpl*16 intron phylogeny (Fig. 2).

The ILD test indicated a lack of significant incongruence between the two data partitions ($p = 0.26$), suggesting their combinability. The combined dataset included 1601 parsimony informative characters (29.47%) and parsimony analysis resulted in the preset upper limit of 40,000 most parsimonious trees with 7321 steps (CI = 0.4790 and RI = 0.8776). The strict consensus of these trees, showing only the major clades with their bootstrap percentages is provided in Fig. 3. The 12 runs of ML analyses in GARLI yielded likelihood scores ranging from -49116.9240 to -49115.1376, with identical tree topology among runs. The bootstrap values retrieved under the two conditions (see Methods) were very similar (with < 5% difference), hence the support values shown on the ML phylogeny represent the average percentages of the two bootsrap runs. The ML tree also showed no topological differences from the two trees retrieved from the BI analyses (see Fig. 4a-d for

topology with ML bootstraps and BI posterior probabilities). Although the placement of *Harmsiopanax* (in Araliaceae) varies between the two topologies (as sister to the *Hydrocotyle-Trachymene* clade in the ML/BI tree, but sister to the rest of Araliaceae in the MP tree), either placement is poorly supported (48% in the MP tree and 69% in the ML tree). In addition to the placement of *Harmsiopanax*, there were evident differences between the two trees in the bootstrap support values of some clades (e.g., *Hermas*, *Lichtensteinia* and *Choritaenia*, *Actinotus*, and Myodocapaceae).

4. Discussion

4.1. Evolutionary Positions of Hydrocotyloideae in Apiales

Drude's Hydrocotyloideae forms a polyphyletic assemblage of at least six main lineages dispersed across both Apiaceae and Araliaceae (Figs. 2-4), and none of Drude's tribes and sub-tribes are monophyletic. One of these six lineages is placed in Araliaceae and includes four genera of tribe Hydrocotyleae (subtribe Hydrocotylinae). Araliaceae appear sister to a clade consisting of Myodocarpaceae and Apiaceae (BS < 65%), whereas Myodocarpaceae appears sister to Apiaceae with significant difference in bootstrap support between the MP and ML trees (55% and 81% respectively). Infra- and inter-familial relationships in Apiales will be addressed in more details in a separate paper through comparisons of plastid, nuclear, and mitochondrial gene phylogenies. Apart from the *Hydrocotyle-Trachymene* lineage, the other five lineages of Hydrocotyloideae are scattered across Apiaceae. The most diverse of these lineages includes genera from the three subtribes of tribe Mulineae. This clade corresponds most closely to the current concept of

subfamily Azorelloideae proposed by Plunkett *et al*. (2004). The second largest clade (seven hydrocotyloid genera) forms part of subfamily Mackinlayoideae, together with two former araliads, *Mackinlaya* and *Apiopetalum*. Members from both subtribes of tribe Hydrocotyleae are included in this clade. Four other hydrocotyloid genera fall within subfamilies Apioideae (*Naufraga* and *Notiosciadium*) or Saniculoideae (*Arctopus* and *Choritaenia*) (Fig. 4b). The *Platysace* clade includes two genera (*Platysace* and *Homalosciadium*) of tribe Hydrocotyleae (subtribe Hydrocotylinae) and is separated from all other species from that tribe. The placement of *Klotzschia* and *Hermas* remains problematic, but they may be considered–with caution–as two distinct lineages in Apiaceae. *Klotzschia* may be arguably treated as part of Azorelloideae and *Hermas* appears to be an early-diverging lineage in Apioideae. A comparison of Drude's classification of Hydrocotyloideae (with the morphological characters used to make this system) with their placement in this study is provided in Table 4.

4.2. Subfamily Azorelloideae

The Azorelloideae form a well supported monophyletic group in Apiaceae that includes 21 of the 42 genera formerly placed in Hydrocotyloideae. *Klotzschia* is sister to the Azorelloideae in the *trnD-trnT* and combined trees (Figs. 2a, 3) but with low bootstrap support (BS = 66%, PP = .98), and this relationship is not found in the tree based on the *rpl*16 data (Fig. 2b). The ambiguous placement of *Klotzschia* prevents us from drawing strong conclusions regarding its relationship to Azorelloideae or Apioideae-Saniculoideae. Azorelloideae are divided into four well supported clades (Fig. 4a) but the relationships

among these four clades remain unresolved. With the exception of *Stilbocarpa* and *Dickinsia*, the genera of Azorelloideae were all included in Drude's tribe Mulineae, but our results do not support his subtribal system (discussed below). All taxa in Azorelloideae conform to Drude's grouping of Hydrocotyloideae based on the presence of a woody endocarp, the lack of vittae, and (with the exception of *Diposis*) distinct rib oil ducts. However, the lack of free carpophores is not synapomorphic among and within the clades of Azorelloideae. The clades are not geographically structured, but instead a series of Australian-South American connections are obvious in each of the three largest clades.

4.2.1. The Azorella *Clade*

Most of the genera in the *Azorella* clade (Fig. 4a) were grouped in Drude's Mulineae subtribe Azorellinae based on the presence of unwinged and non-hollowed fruits, with the exception of *Mulinum* and *Laretia*, which were placed in subtribe Asteriscinae because they possess winged fruits, *Dickinsia*, which was placed in tribe Hydrocotyleae (although it lacked the laterally compressed fruits characterizing the Hydrocotyleae), and *Stilbocarpa*, which was formerly a member of Araliaceae. The *Azorella* clade (Fig. 4a) has two well supported basal branches, the monotypic Mesoamerican *Spananthe* and a clade uniting the Asian *Dickinsia* and the Australian *Diplaspis*. The genera in these two clades are among a minority of taxa in the *Azorella* clade that have free carpophores (Tseng, 1967; Liu, 2004). The core group of the *Azorella* clade includes two sister subclades, the *Schizeilema* subclade and the *Mulinum* subclade. The *Schizeilema* subclade includes two species of *Azorella*, one of which is the type (*A. filamentosa*), in addition to all

representatives from *Schizeilema*, *Huanaca*, and *Stilbocarpa*. The *Mulinum* subclade includes *Mulinum*, *Laretia*, and the remaining species of *Azorella*. Chandler and Plunkett (2004), with more limited sampling, were the first to suggest the lack of monophyly in *Azorella* based on molecular markers, and this finding was later echoed by Andersson *et al*. (2006) using additional sampling of *Azorella* but no samples of *Mulinum*. Martinez (1993a, 1993b) divided *Azorella* into four sections based on phenetic relationships. Our study shows that members of Martinez's section *Azorella* are more closely aligned with *Huanaca*, *Stilbocarpa*, and *Schizeilema* than to other sections of *Azorella*, but relationships among these four genera are not well resolved. *Schizeilema* appears to be polyphyletic, with the New Zealand and Australian species forming one clade (with the Australian species, *S. fragoseum*, nested within the New Zealand species) and the only South American species, *S. ranunculus*, forming a second unrelated clade. The sister-group relationship between *Schizeilema* and *Stilbocarpa* found by Mitchell *et al*. (1999) is not supported by our study. Our results also offer some insight into questions regarding the generic distinctions between *Schizeilema*, *Huanaca*, and *Diplaspis* (see Mathias and Constance, 1971; Van den Borre and Henwood, 1998; Henwood and Hart, 2001) by helping to place the monophyletic *Diplaspis* with sister *Dickinsia*, as sister to the *Schizeilema* and *Mulinum* subclades. Although the two species of *Huanaca* sampled here are monophyletic, and their placement with respect to the polyphyletic *Schizeilema* remains poorly supported, but data from the unsampled species of both genera may provide better insight into the relationship between the two genera. *Schizeilema ranunculus* is the only species of *Schizeilema* reported to have a carpophore, a character shared with all

species of *Stilbocarpa* and *Huanaca* (Grushvitzky *et al*., 1969; Dawson 1971; Mathias and Constance, 1971). The carpophore is reduced to fused ventral bundles in the closely related *Azorella filamentosa* (Liu, 2004), and these bundles are absent altogether in the Australian and New Zealand species of *Schizeilema* (Allan, 1961; Tseng, 1967). Hence the presence of a carpophore is not synapomorphic in the *Schizeilema* subclade.

The *Mulinum* subclade includes species from *Azorella*, *Laretia*, and *Mulinum*. The topology of this subclade suggests that members of Martinez's (1993b) *Azorella* sections Ciliatae and Cirrhosae are non-monophyletic. The South American representative from Cirrhose, *A. lycopodioides*, is sister to the remainder of the *Mulinum* subclade, while the only other members of Cirrhosae, the subantarctic *A. selago* and its segregate *A. macquariensis* (Orchard, 1989), form a sister group to three members of the section Ciliatae (*A. multifida*, *A. pulvinata*, and *A. trifurcata*), which range from Colombia to Argentina. The rest of the Ciliatae (*A. crenata*, *A. biloba*, *A. monantha*, *A. caespitosa*, *A. trifoliolata*, and *A. compacta*) are grouped with *Laretia*, which together forms the sister group to *Mulinum*. Like its sister subclade, the presence or absence of a carpophore is not synapomorphic in the *Mulinum* subclade. Although some members of the genus *Mulinum* have been reported to lack a carpophore, this character is present in all species sampled for this study (Tseng, 1967; Zech, 1992) and these species form a well supported monophyletic group. *Azorella lycopodioides* is one of a few species of *Azorella* reported to have a carpophore, but this structure does not become free at maturity (Tseng, 1967). Two other members of this clade, *Laretia acaulis* and *Azorella compacta* have fused ventral

bundles in place of a carpophore (Tseng, 1967; Liu, 2004) and form a well supported sister relationship.

4.2.2. The Asteriscium *Clade*

The *Asteriscium* clade is a well supported group in Azorelloideae (BS = 100%, PP = 1, Fig. 4a). The clade includes the South American genera *Asteriscium*, *Gymnophyton*, *Pozoa*, *Eremocharis*, and *Domeykoa*, and the Australian genus *Oschatzia*. Two sister subclades emerge, the *Gymnophyton* subclade (*Asteriscium, Gymnophyton, Pozoa,* and *Oschatzia*) and the *Eremocharis*-*Domeykoa* subclade, a grouping that largely agrees with Mathias and Constance's (1962a) conclusions based on mature fruit morphology. The placement of *Oschatzia*, which was not treated by Mathias and Constance (1962a), is noteworthy because it is the the only genus in the *Asteriscium* clade that is not South American, and (together with *Pozoa*) was placed by Drude in a different subtribe of Mulineae (subtribe Azorellinae) due to the lack of winged fruits. The four remining members of this clade were grouped in subtribe Asteriscinae due to the presence of winged fruit without hollows, and this again emphasizes the artificiality of Drude's subtribes. As in the *Azorella* clade, fruit hollows or wings has not proved to be informative at the subtribal level of phylogeny, and without additional information, these two characters are not sufficient to define monophyletic groups.

Both subclades of the *Asteriscium* clade include genera that are non-monophyletic. In the *Gymnophyton* subclade, *Asteriscium* is paraphyletic with respect to *Gymnophyton* (Fig. 4a), due to the placement of *A. closii* (sister to *G. isatidicarpum*). *Asteriscium closii*

had formerly been recognized as the monotypic genus *Bustillosia* (as *B. chilensis* Clos), but Mathias and Constance (1962a) preferred to treat it under *Asteriscium* because of morphological similarities with various species of *Asteriscium*. *Pozoa* is sister to *Asteriscium*-*Gymnophyton* (Fig. 4a) and is the only genus in this subclade to lack a well developed free carpophore (Mathias and Constance, 1962a; Tseng, 1967; Liu, 2004). The sister group to the *Gymnophyton* subclade includes *Domeykoa* and *Eremocharis*, both of which appear non-monophyletic. Both genera lack free carpophores or ventral bundles, and thus the lack of a carpophore is synapomorphic to this subclade but not the whole *Asteriscium* clade. Despite the taxonomic discrepency, the branching pattern in this subclade is consistent with the geographic distributions of the species sampled. One group includes the Peruvian species of *Domeykoa* (*D. amplexicaulis*) and *Eremocharis* (*E. longiraneae*, *E. trpartita*, and *E. triradiata*), whereas the other includes the Chilean species *E. fruticosa* and *D. oppositifolia*. This pattern confirms Mathias and Constance's dilemma as to whether they should unite these two genera and emphasizes the need for more intensive study of generic delimitations in this clade.

4.2.3. The Bowlesia *Clade*

The *Bowlesia* clade consists of four major subclades, one uniting the South American *Bolax* and the Australian *Dichosciadium* (BS > 95%, PP = 1, Fig. 4a), and the other three each with a single genus, the South American *Homalocarpus* (BS = 100%, PP = 1), the American *Bowlesia* (BS = 100%, PP = 1), and the Somalia-Canary Islands monotypic genus *Drusa*. The three latter genera comprised Drude's Mulineae subtribe

Bowlesiinae, which are characterized by unwinged but hollowed fruits, whereas *Bolax* and *Dichosciadium* were included in his subtribe Azorellineae because they lack hollowed fruits. This clade provides another example in Azorelloideae where the lack of a free carpophore is not synapomorphic, since *Drusa* and *Homalocarpus* have free carpophores, *Bolax* and *Bowlesia* have fused ventral bundles, and *Dichosciadium* appears to lack both (Mathias and Constance, 1965; Tseng, 1967; Liu, 2004 and pers. comm. regarding *Bowlesia*). Support for each subclade is strong but the relationships among the four groups need further analysis.

4.2.4. The Diposis *Clade*

The *Diposis* clade includes only the rarely studied genus *Diposis*, which includes three species with edible tubers, each limited to relatively restricted areas of South America (Patagonia, the Pamapas, and Chile, respectively). Drude placed *Diposis* in subtribe Astersicinae based on the presence of fruits with winged lateral ribs. Our data support the placement of *Diposis* in Azorelloideae, but its relation to the other three clades of Azorelloideae is only poorly supported. Species of this genus have a free carpophore but differ from the other genera of Azorelloideae in lacking distinct rib oil ducts (Tseng, 1967; Liu, 2004).

4.2.5. Klotzschia

The three species that comprise the Brazilian endemic genus *Klotzschia* form a monophyletic group (Fig. 4a), sister to the rest of Azorelloideae, but this result is not

strongly supported (BS: MP = 61%; ML = 66%; PP = .98). The genus was traditionally grouped in subtribe Azorellinae due to the lack of wings on the fruit, and has even been suggested to form a link to Araliaceae on the basis of pollen evidence (see Shoup and Tseng, 1977). Our results show strong support for the inclusion of the genus in Apiaceae, but the two separate datasets (Figs. 2a and 2b) do not agree on the placement of this genus within the family. Like some members of Azorelloideae, the fruits of *Klotzschia* have a single fused ventral bundle in place of a carpophore, but differ from fruits from the rest of the Azorelloideae (except *Diposis*) in lacking distinct rib oil ducts (Liu, 2004).

4.3. Subfamily Mackinlayoideae

Subfamily Mackinlyoideae constitutes a well supported clade (Fig. 4c; BS= 100%; PP = 1) sister to the rest of Apiaceae (ML: BS = 86%; MP: BS = 77%; PP = 1). This clade includes two genera formerly placed in Araliaceae, *Apiopetalum* and *Mackinlaya*, and seven hydrocotyloid genera. Of these hydrocotyloids, two well supported sister subclades were retrieved: the *Centella* clade (which includes the mostly South African *Centella*, the Mesoamerican *Micropleura*, and the Australian *Pentapeltis* and *Schoenolaena*) and the *Xanthosia* clade (with the Australian *Xanthosia* and *Chlaenosciadium*). Together, the two subclades are sister to *Mackinlaya*. All of the genera in these two subclades were included in tribe Hydrocotyleae (Pimenov and Leonov, 1993) but our topology does not conform to their assignment in the two subtribes (Hydrocotylinae and Xanthosinae). Hydrocotyleae share the character of laterally compressed fruit, a feature common to all genera of subfamily Mackinlayoideae, with the exception of *Apiopetalum*. The genera sampled here

also share fruits with woody endocarps and no vittae, but vary in regard to the presence of distinct rib oil ducts (see below). Also, all six genera that comprise the *Centella* and *Xanthosia* clades are reported to lack free carpophores; instead, they have ventral bundles (single or paired) fused to the mericarps (Tseng, 1967; Theobald, 1967a; Hart 1998; Liu, 2004).

The sister-group relationship between *Centella* and *Micropleura* has already been reported (Plunkett *et al*., 1996, 1997, Plunkett and Lowry, 2001, Chandler and Plunkett, 2004), and is fully supported in this study. Together, *Micropleura* and *Centella* are sister to the monotypic *Schoenolaena*, and these three genera form a sister group to the ditypic *Pentapeltis*. The Australian genera *Pentapeltis* and *Schoenolaena* have not been previously included in molecular phylogenetic studies and their placement in Mackinlyoideae, as well as the relationships between them is fully supported by our combined analyses (Fig. 4c; BS=100%; PP =1). *Centella* and *Micropleura* were included in subtribe Hydrocotylinae based on the lack of sepals, whereas *Pentapeltis* and *Schoenolaena* were affiliated with tribe Xanthosiinae because they have petaloid sepals. Such incongruence between our gene tree and the traditional subtribal division is also evident in the *Xanthosia* clade, with *Xanthosia* and *Chlaenosciadium* belonging to subtribes Xanthosiinae and Hydrocotylinae, respectively. The placement of the monotypic *Chlaenosciadium* as sister to *Xanthosia* (BS = 81%; PP = 1; Fig. 4c) has not been reported based on molecular data because the former had not been included in any previous molecular-phylogenetic analysis.

As with the divisions of Mulineae, the characters used to divide tribe Hydrocotylineae into subtribes appear to be homoplasious and do not provide a reliable

basis for defining monophyletic groups. Also, the presence of distinct rib oil ducts is not synapomorphic for either the *Centella* or *Xanthosia* subclades. In the *Centella* clade, rib oil ducts are present in *Micropleura*, may be present or indistinct in *Centella*, and are indistinct in *Pentapeltis* and *Schoenolaena* (Tseng, 1967, Theobald, 1967a, Liu, 2004). In the *Xanthosia* clade, such ducts are present in *Xanthosia* but absent in *Chlaenosciadium* (Theobald, 1967).

The Australasian genus *Actinotus* is well supported as a member of subfamily Mackinlyoideae but it is not found among the other former hydrocotyloids. The genus was placed in subtribe Xanthosiinae, with which it shares fruit characters such as a woody endocarp and lack of vittae, but differs from members of this group in having distinct rib oil ducts. Within the subfamily, our results indicate a closer relationship between *Actinotus* and the New Caledonian endemic genus *Apiopetalum* than any of the genera of Drude's subtribe Xanthosiinae. The sister relationship between *Actinotus* and *Apiopetalum* was first reported by Plunkett and Lowry (2001) based on maximum parsimony analysis of plastid *matK* sequences (BS = 71%). Although this sister relationship is not well supported in our MP tree (BS = 55%; Fig. 3), the clade received high support in the ML (BS = 96%) and BI (PP = 1%) analyses (Fig. 4c). The fruits of *Actinotus* are reduced to a single functional carpel and, like *Apiopetalum*, lack carpophores altogether. Further investigation of the morphological and anatomical similarities between the two genera is necessary to test their taxonomic affinities as well as the exact placement of *Actinotus* within Mackinlyoideae.

4.4. Hydrocotyloids transferred to Subfamily Apioideae

Two monotypic genera with very limited distributions are grouped with subfamily Apioideae, *Naufraga* (*N. balearica*) and *Notiosciadium* (*N. pampicola*). Pimenov and Leonov (1993) placed both genera as *incertae sedis* in Hydrocotyloideae. *Naufraga* is a rare species endemic to the Balearic Islands and possibly Corsica, where it is suspected to have gone extinct two years after its initial collection there (Friedlender and Boisselier–Dubayle, 2000). The species was placed in Hydrocotyloideae based on the absence of a free carpophore and the likely presence of a woody endocarp (Constance and Cannon, 1967). Using plastid sequences, however, Downie *et al*. (2000) placed *Naufraga* in the *Apium* clade of Apioideae, sister to *Apium* and our data agree with these results with 100% support (Fig. 4b).

Notiosciadium pampicola is known only from a few collections and has not been included in any previous phylogenetic analysis. The species was reported by Delucchi (2006) to be a critically endangered endemic in Argentina. A report by the Institut Royal des Sciences Naturelles des Belgique (1996) listed it as a constituent of the vegetation of the southern Pampa regions of Uruguay and Argentina. The genus shares characters with many apioid taxa, such as laterally compressed fruits, compound umbels, and entire carpophores. Mathias and Constance (1962b) also noted that it coexists with *Apium uruguayense* in south-central Uruguay and that the two species bear great superficial resemblance to one another. Such resemblance is not surprising in light of the supported placement of *Notiosciadium pampicola* in the apioid superclade, sister to the clade that

includes the terminals from *Arracacia* to *Petroselinum* (Fig. 4b.). However, this sister relationship received low support (BS < 60%; PP = 0.75).

4.5. Hydrocotyloids transferred to Subfamily Saniculoideae and related lineages

The genus *Arctopus* was originally placed in subfamily Saniculoideae (Wolff 1913), but later transferred to Hydrocotyloideae (*incertae sedis*), based on similarities of its inflorescences and flowers (Magin, 1980). However, recent studies have re-established the placement of *Arctopus* in subfamily Saniculoideae (e.g., Plunkett and Lowry, 2001; Liu *et al*., 2003; Chandler and Plunkett, 2004; Calviño and Downie, 2007). *Arctopus* includes three species endemic to the Cape region of South Africa. The plants have simple leaves with marginal setae, characters shared with many Saniculoideae. The three species of *Arctopus* form a monophyletic group within Saniculoideae, sister to another African-endemic genus *Alepidea* (Fig. 4b; BS = 100%; PP = 1). These sister genera share the synapomorphic presence of a carpophore, which is lacking in all other members of Saniculoideae s. str. (Liu *et al*., 2003). In addition, *Arctopus* and *Alepidea* share characters such as sessile female flowers and the presence of kaurenoic acids (Liu *et al*., 2003; Magee, 2008). Together, *Arctopus* and *Alepedia* form a well supported clade sister to the rest of Saniculoideae.

Like *Arctopus*, the monotypic genus *Choritaenia*, is endemic to the Cape region of South Africa. *Choritaenia capensis* was included in tribe Mulineae by Pimenov and Leonov based on the dorsal compression of its fruit, and in subtribe Asteriscinae due to its winged, non-hollowed fruits. Liu *et al*. (2007) described some morphological differences

between *Choritaenia* and the rest of the Mulinoideae, including the distinctively short, bipartite carpophore and the presence of oil vesicles instead of vittae. MP analysis based on DNA sequences of the nuclear ITS region (Calviño *et al*., 2006) placed *Choritaenia* as sister to the *Annesorhiza* clade and *Astydamia* + *Molopospermum* (BS < 50%). Based on the combined analysis of the plastid sequences used herein, *Choritaenia* is not closely related to any of the hydrocotyloid groups, but is rather fully supported as sister to *Lichtensteinia* (Fig. 4b). Together, *Choritaenia* and *Lichtensteinia* form a weakly supported sister group to the rest of Saniculoideae s. str. with *Polemanniopsis* and *Steganotaenia*.

Hermas is a third South African endemic with affinities to the Saniculoideae and early-diverging lineages of Apioideae. First classified as a member of subtribe Asteriscinae based on the presence of winged fruits, molecular data have shown that the genus is quite distinct from other Asteriscinae (Calviño *et al*., 2006). Also, a recently described species, *H. proterantha* (de Villiers and Van Wyk, 2008) lacks the lateral wings that are common to all other species of this genus. Species grouped in *Hermas* share characters with Apioideae (e.g., free carpophores), Saniculoideae (e.g., congested umbels), and Hydrocotyloideae (e.g., woody endocarps, although a newly discovered species, *H. ciliata*, lacks a woody endocarp; De Villiers, Van Wyk and Tilney, pers. comm.). This lends validity to its placement in our trees as sister to the entire Apioideae plus Saniculoideae clade (Fig. 4a), although support for this placement is not very high (BS = 70%; PP = 1).

4.6. Platysace *and* Homalosciadium

The Australian endemics *Platysace* and *Homalosciadium* are well-supported as sister to the rest of Apiaceae, excluding Mackinlayoideae (Fig. 4b; ML: BS = 93%; MP: BS = 87%; PP = 1). The two genera were previously included in tribe Hydrocotyleae based on the presence of laterally compressed mericarps, and in subtribe Hydrocotylinae based on their lack of sepals. Prior to this study, the monotypic *Homalosciadium* (*H. homalocarpum*) had not been sampled for molecular phylogenetic analyses and no comprehensive analyses, molecular or morphological, are available for *Platysace*. Our results call into question the monophyly of *Platysace* due to the placement of the *H. homalocarpum* among the three species of *Platysace* sampled in our study. Fruits of species of both genera share the presence of carpophores and woody endocarps, but lack vittae and (in most cases) distinct rib oil ducts (Tseng, 1967; Hart, 1998; Liu, 2004). The two genera form a well supported, early-diverging lineage within Apiaceae, sister to the rest of Apiaceae (excluding Mackinlayoideae).

4.7. Hyrocotyloids transferred to Araliaceae

Hydrocotyle and *Trachymene*, as well as their respective satellite genera, *Neosciadium* and *Uldinia*, constitute a lineage sister to the rest of Araliaceae. Within this lineage, *Hydrocotyle* and *Neosciadium* form a well-supported clade (BS =100%; PP = 1; Fig. 4d) sister to *Trachymene* and *Uldinia* (also well supported; BS = 100%; PP =1). All four genera were included in Hydocotyleae subtribe Hydrocotylinae based on the presence of laterally compressed fruits and the absence of sepals. The placement of these genera in a

separate lineage from the rest of the Hydrocotyleae demonstrates the ineffectiveness of the morphological characters used by Drude for their classification in providing reliable phylogenetic signal. All four genera have fruits with woody endocarps and without vittae, but only *Hydrocotyle* and *Neosciadium* lack carpophores. The relationship of *Hydrocotyle* and *Trachymene* to Araliaceae, and some of the morphological similarities that link them to members of that family (e.g., sclerified endocarps and bicarpellate gynoecia) were detailed by Chandler and Plunkett (2004). The placement of *Harmsiopanax* as sister to the *Hydrocotyle-Trachymene* clade in the ML and BI trees is not well supported, but is intriguing given the similarities of their morphologies. Like *Hydrocotyle* and *Trachymene*, for example, *Harmsiopanax* has schizocarpic fruits (rare in Araliaceae) and was long thought to show ties to both Araliaceae and Apiaceae (see Frodin and Govaerts 2003). This relationship should be followed up with future investigation into the morphological affinities among these genera.

Hydrocotyle is a widely distributed genus of more than 130 species with remarkable morphological variations both within and among species. In the trees presented here, *Hydrocotyle* appears sister to *Neosciadium*, a monotypic Australian endemic. However, in an earlier version of the datasets, the inclusion of an undetermined species of *Hydrocotyle*, originally misidentified as *Homalosciadium homalocarpum* (*Eichler 22047*), rendered *Hydroctyle* paraphyletic with respect to *Neosciadium* (data not shown). The misidentified species was excluded from our study until it can be properly identified, and questions regarding the monophyly of *Hydrocotyle* must await availability of wider

sampling across the entire morphological and geographic range of the genus (neither of which are represented sufficiently here).

Trachymene is another large genus of more than 55 species, 38 of which are endemic to Australia, while the remaining species are found across Oceania and SE Asia. Previous studies (e.g., Theobald, 1967b; Henwood and Hart, 2001) represented *Trachymene* as a close relative to the sole species of *Uldinia* (*U. ceratocarpa*) which is endemic to Australia. In a recent revision of *Trachymene*, Hart and Henwood (2007) formally transferred *Uldinia* to *Trachymene*, thus reducing to three the number of Hydrocotyloideae genera associated with Araliaceae. In the present study, *Uldinia* appears sister to the three species sampled from *Trachymene*. Theobald (1967) listed the absence of carpophores as a character that separates *Uldinia* from *Trachymene*, but Liu (2004) demonstrated the presence of carpophores in *Uldinia* similar to those found in some *Trachymene* species.

5. Conclusion

Our molecular phylogeny demonstrates that neither Drude's subfamily Hydrocotyloideae, nor his two tribes or five sub-tribes are monophyletic. Although the presence of a woody endocarp and absence of vittae is common to most genera of Hydrocotyloideae (and rare in Apioideae and Saniculoideae), these two characters are homoplasious when considering the entire order Apiales and the placement of the different hydrocotyloid lineages across the phylogeny of the order. The presence or absence of carpophores is not useful at the subfamilial, tribal, and subtribal levels, but this character

may be of much greater value at lower taxonomic levels, especially in the *Azorella* clade, where it exhibits a wide range of variation.

The placement of the former hydrocotyloids is of major importance in understanding evolutionary trends in Apiales. What remains as "subfamily Hydrocotyloideae" includes only the type, *Hydrocotyle*, and the related genera *Neosciadium* and *Trachymene* (with *Uldinia*), which forms a distinct lineage within Araliaceae. The remaining genera of Drude's Hydrocotyloideae are separated into four main lineages in Apiaceae, most of which belong to subfamilies Azorelloideae (including *Klotzschia*) and Mackinlayoideae. The relationships within subfamily Mackinlayoideae are well resolved, with the exception of lingering doubts regarding the placement of *Actinotus*. More work, however, is required to understand relationships within subfamily Azorelloideae. Our study provided great improvements towards understanding this subfamily, but more data are needed to address the many evolutionary questions that remain. These issues include the resolution of the relationships among the major clades and the circumscriptions of many genera within them, especially those that appear to be non-monophyletic (e.g., *Azorella*, *Schizeilema*, *Asteriscium*, *Gymnophyton*, *Eremocharis*, *Domeykoa*). Of the other lineages, *Platysace* (with *Homalosciadium*) appears to be an independent lineage in Apiaceae and it may merit its recognition as its own subfamily. Finally, the placement of *Hermas* and *Choritaenia*, and the sister relationship of Azorelloideae to the *Hermas*+Saniculoideae+Apioideae clade, support the concept that the circumscription of taxa at the level of subfamilies and tribes among the early diverging lineages of Apiaceae are in need of additional revision (Van Wyk, pers. comm.).

TABLES

Table 1. List of species, voucher information, and sources.

Taxa	Voucher	Locality
Family Apiaceae		
Subfamily Hydrocotyloideae		
Actinotus helianthii Labill.	*Donaldson 584*	NSW, Australia
Arctopus dregei Sond.	*Goldblatt 11880*	South Africa
Arctopus echinatus L.	*Bond 1149*	South Africa
Arctopus monacanthus Carmich. ex Harv. & Sond.	*Goldblatt 11676*	South Africa
Asteriscum chilense Cham. & Schlecht.	*Plunkett 2056*	cult., France (Mulhouse)
Asteriscum closii (Kuntze) Math. and Const.	*Muilgara 123*	Chile
Asteriscium glaucum Hieron. & Wolff	*Teillier 972*	Argentina
Azorella biloba (Schlecht.) Wedd.	*Solomon 11669*	Bolivia
Azorella caespitosa Hook.f. (non Cav. 1799)	*Chandler 1124*	Argentina
Azorella compacta Phil.	*Chandler 1093*	Argentina
Azorella crenata (R. & P.) Pers.	*Smith 11882*	Peru
Azorella filamentosa Lam.	*Chandler 1123*	Tierra del Fuego, Argentina
Azorella fuegiana Speg.	*Chandler 1127*	Tierra del Fuego, Argentina

Azorella lycopodioides Gaud.	*Chandler 1119*	Tierra del Fuego, Argentina
Azorella macqueriensis A.E. Orchard	*Jackson 69*	Macquarie Island
Azorella monantha Clos ex Gay	*Chandler 1102*	Mendoza, Argentina
Azorella multifida (R. & P.) Pers.	*Ceron 19429*	Ecuador
Azorella pulvinata Wedd.	*Solomon 16611*	Bolivia
Azorella selago Hook. f.	*Donaldson 234*	Heard Island
Azorella trifoliolata Clos ex Gay	*Chandler 1115*	Argentina
Azorella trifurcata (Gaertn.) Pers.	*RBG Kew 379-81.04150*	Kew
Bolax caespitosa Hombre. & Jacq. ex Decaisne	*Chandler 1122*	Tierra del Fuego, Argentina
Bolax gummifera (Lam.) Spreng.	*Chandler 1126*	Tierra del Fuego, Argentina
Bowlesia flabilis J.F. Macbr.	*Solomon 11625*	Bolivia
Bowlesia lobata Ruiz. & Pav.	*Stein 2003*	Peru
Bowlesia platantifolia Wolff	*Pedersen 13975*	Argentina
Bowlesia tropaeolifolia Gill. & Hook.	*Chandler 1091*	Jujuy, Argentina
Bowlesia uncinata Colla	*Landrum 7581*	Chile
Centella asiatica (L.) Urb.	*Plunkett 1494*	Queensland, Austarlia
Centella linifolia (L.f.) Drude	*Phillipson 5253*	South Africa
Chlaenosciadium gardneri C. Norman	*Keighery 448*	WA, Australia

Choritaenia capensis (Sond.) Burtt Davy	*NMB 5106*	South Africa
Dichosciadium ranunculaceum (F. Muell.) Domin	*Plunkett 1556*	NSW, Australia
Dickinsia hydrocotyloides Franch.	*Wen 5003*	Sichuan, China
Diplaspis cordifolia (Hook.) Hook.f.	*Ratkowsky 195*	Tasmania, Australia
Diplaspis hydrocotyle Hook.f.	*CANB 9501185*	Victoria, Australia
Diposis bulbocastanum DC.	*Claude-Joseph 1360*	Chile
Domeykoa oppositifolia Phil.	*Teillier 547*	Chile
Domeykoa saniculifolia Math. & Const.	*Dillon 8839*	Peru
Drusa glandulosa (Poir.) Bornm.	*Bally 15777*	Somalia
Eremocharis fruticosa Phil.	*Constance, C-2382*	cult., Univ. Calif. Bot. Gard.
Eremocharis longiramea (Wolff) Johnst.	*Quiroz 2543*	Peru
Eremocharis tripartita (Wolff) Math. & Const.	*Sagástegui 14854*	Peru
Eremocharis triradiata (Wolff) Johnst.	*Hutchison 6214*	Peru
Gymnophyton isatidicarpum (Presl ex DC.) Math. & Const.	*Landrum 8218*	Chile
Gymnophyton polycephalum (Gill. & Hook.) Clos	*Chandler 1108*	Mendoza, Argentina
Gymnophyton robustum Clos.	*Zöllner 10279*	Chile
Gymnophyton spinosissimum Phil.	*Zöllner 10383*	Chile

Hermas capitata L.f.	*van Wyk 4103*	South Africa
Hermas villosa (L.) Thunb.	*van Wyk 4100*	South Africa
Homalocarpus dichotomous (Poepp. ex DC.) Math. & Const.	*Taylor 1991*	Chile
Homalocarpus digitatus (Phil.) Math. & Const.	*Teillier 890*	Chile
Homalocarpus integerrimus (Turcz.) Math. & Const.	*Muñoz 2892*	Chile
Homalocarpus nigripetalus (Clos ex Gay) Math. & Const.	*Zöllner 11424*	Chile
Homalosciadium homalocarpum (F. Muell.) H. Eichler	*Lepschi 3646*	WA, Australia
Huanaca acaulis Cav.	*Chandler 1125*	Tierra del Fuego, Argentina
Huanaca andina (Phil.) Phil	*Zöllner 5389*	Chile
Hydrocotyle bonariensis Lam.	*Ware s.n.*	Virginia, USA
Hydrocotyle cf. callicephala Urb.	*Fiaschi 3159*	Brazil
Hydrocotyle javanica Thunb.	*Plunkett 1551*	Fiji
Hydrocotyle cf. javanica	*Plunkett 1999*	Yunan, China
Hydrocotyle modesta Cham. & Schltdl.	*Chandler 1098*	Jujuy, Argentina
Hydrocotyle novae-zealandiae DC.	*Croft 10446*	Tasmania, Australia
Hydrocotyle sibthorpioides Lam.	*Ware 10052*	Virginia, USA
Klotzschia brasiliensis Cham.	*Conceição 752*	Brazil
Klotzschia glaziovii Urb.	*Paula-Souza 6666*	Brazil

Klotzschia rhizophylla Urb.	*Irwim 20245*	Brazil
Laretia acaulis Gill. & Hook.	*Teillier 2504*	Chile
Laretia acaulis Gill. & Hook.	*Zöllner 11719*	Chile
Micropleura renifolia Lag.	*Plunkett 1273*	Oxaca, Mexico
Mulinum albovaginatum Gill. & Hook.	*Chandler 1105*	Mendoza, Argentina
Mulinum chillanense Phil.	*Chandler 1103*	Mendoza, Argentina
Mulinum spinosum (Cav.) Persoon	*Chandler 1099*	Mendoza, Argentina
Mulinum ulicinum Gill. & Hook.	*Chandler 1092*	Jujuy, Argentina
Naufraga balearica Constance & Cannon	*Parc Zoologique et Botanique de la Ville de Mulhouse, 20141*	cult., France
Neosciadium glochidiatum (Benth.) Domin	*Short 2185*	NSW, Australia
Notiosciadium pampicola Speg.	*Gallinal PE-5292*	Uruguay
Oschatzia cuneifolia (F. Muell.) Drude	*Strid 22126*	NSW, Australia
Oschatzia saxifraga (Hook. f.) Walp.	*Ratkowsky 199*	Tasmania, Australia
Pentapeltis peltigera (Hook.) Bunge	*Taylor 2045*	WA, Australia
Pentapeltis silvatica (Diels) Domin	*Keighery 6524*	WA, Australia
Platysace lanceolata (Labill.) Druce	*Davies 7304*	NSW, Australia

Platysace stephensonii (Turcz.) Norman	*Constable 7304*	NSW, Australia
Platysace valida (F. Muell.) F. Muell.	*Plunkett 1547*	Queensland, Australia
Pozoa coriaceae Lag.	*Kiesling 8098*	Argentina
Pozoa volcanica Math. & Constance	*Pedersen 14208*	Chile
Schoenolaena juncae Bunge	*Keighery 8006*	WA, Australia
Schizeilema fragoseum (F. Muell.) Domin	*Gray 4790*	NSW, Australia
Schizeilema haasti Domin	*Wardle 94/170*	South Island, New Zealand
Schizeilema hydrocotyloides Domin	*Bulloch s.n. (WAIK 590)*	South Island, New Zealand
Schizeilema ranunculus Domin	*Pisano 2445*	Tierra del Fuego, Argentina
Schizeilema ranunculus Domin	*Goodall 719*	Tierra del Fuego, Argentina
Schizeilema trifoliolatum Domin	*DeLange s.n. (WAIK 5480)*	North Island, New Zealand
Spananthe paniculata Jacq.	*Barrie 1496*	Guererro, Mexico
Spananthe paniculata Jacq.	*Fiaschi 3167*	Brazil
Stilbocarpa polaris (Hombr. & Jacq.) A. Gray	*Croft 10437*	Tasmania, Australia
Trachymene coerulae Graham	*Crisp 6099*	ACT, Australia
Trachymene glaucifolia (F. Muell.) Benth.	*Letouzey AUS99*	NT, Australia
Trachymene hookeri (Domin) A.E. Holland	*Plunkett 1548*	Queensland, Australia
Trachymene incisa Rudge	*CANB 9613231*	NSW, Australia

Uldinia certocarpa (W.Fitzg.) N.T.Burb	*Symon 12272*	SA, Australia
Xanthosia atkinsoniana F.Muell.	*Adams 1629*	WA, Australia
Xanthosia pilosa Rudge	*Coveny 11135*	NSW, Australia
Xanthosia pusilla Bunge	*Symon 13400*	SA, Australia
Xanthosia rotundifolia DC.	*Bayer WA-94106*	NSW, Australia
Xanthosia tridentata DC.	*Melville 2918*	Victoria, Australia
Subfamily Saniculoideae s. str.		
Actinolema eryngioides Fenzl	*Samuelsson 5540*	Syria
Alepidea capensis R.A. Dyer	*Phillipson 5235*	South Africa
Alepidea peduncularis Steud. ex A. Rich.	*Marshall WK383*	Tanzania
		cult., New York Bot.
Astrantia x rosensimfonie	*Plunkett 1327*	Garden
Astrantia maxima Pall.	*Atha 2458*	Republic of Georgia
Eryngium scaposum Turcz.	*Plunkett 1278*	Mexico
Eryngium yuccifolium Michx.	*Plunkett 1370*	Kansas, USA
Hacquetia epipactus DC.	*Patzak s.n.*	
Petagnaea saniculifolia Guss.	*Hufford 1993-960*	Kew

Sanicula gregari Bickn.	*Ware 9898*	Virginia, USA

Subfamily Apioideae

Aciphylla aurea W.R.B. Oliv.	*RBG Kew, s.n.*	cult., London
Aciphylla glacialis F. Muell. ex Benth.	*Plunkett 1555*	NSW, Australia
Aciphylla simplicifolia F. Muell. ex Benth.	*Plunkett 1557*	NSW, Australia
Aegopodium podograria L.	*Plunkett 1332*	Washington, USA
Andriana tsaratanensis (Humbert) B.-E. van Wyk	*Lowry 5363*	Madagascar
Angelica lucida L.	*Plunkett, s.n.*	Washington, USA
Anginon difforme (L.) B.L. Burtt	*Goldblatt 11173*	South Africa
Anginon paniculatum (Thunb.) B.L. Burtt	*Pimenov s.n.*	South Africa
Anginon ragosum Thunb.	*Constance, C-2399*	cult., Univ. Calif. Bot. Gard.
Anistome aromatica Hook. f.	*Plunkett 2183*	New Zealand
Anisotome haastii Ckn. & Laing	*Bayer 1007*	New Zealand
Anisotome pilifera Ckn. & Laing	*Bayer 1006*	New Zealand
Annesorhiza altiscapa Schltr. ex H. Wolff	*Goldblatt 11111*	South Africa
Apiopetalum glabratum Baill.	*Lowry 4798*	New Caledonia

Apiopetalum velutinum Bail.	*Lowry 4700*	New Caledonia
Apium graveolans L.	*Plunkett 1334*	Washington, USA
Arracacia quadrifida Constance & Affolter	*Plunkett 1268*	Mexico
Astydamia latifolia Baill.	*Mort s.n.*	Canary Islands
Bupleurum salicifolium R. Brown	*Mort s.n.*	Canary Islands
Capnophyllum africanum (L.) Gaertn.	*Goldblatt 11667*	South Africa
Chamarea? sp.	*Goldblatt 11072*	South Africa
Corriandrum sativum L.	*Plunkett 1337*	Washington, USA
Daucus montanus Humb. & Bonpl. ex Spreng.	*Plunkett 1289*	Mexico
Donnellsmithia cordata (Coult. & Rose) Math. & Const.	*Plunkett 1270*	Mexico
Endressia castellana Coincy	*Constance, C-2184*	cult., Univ. Calif. Bot. Gard.
Gingidia montana (J.R. Forst. & G. Forst.) Dawson	*CHR 489446*	New Zealand
Heteromorpha trifoliata (Wendl.) Eckl. & Zeyner	*Plunkett 1345*	cult., Univ. Calif. Bot. Garden
Heteromorpha sp.	*Phillipson 5402*	South Africa
Itasina filiformus? filifolia (Thunb.) Raf.	*Goldblatt 11138*	South Africa
Lagoecia cuminoides L.	*Plunkett 1389*	Washington, USA

Lichtensteinia lacerata	*van Wyk 4098*	South Africa
Lichtensteinia sp. nov.	*van Wyk 4104*	South Africa
Lichtensteinia trifida Cham. & Schltdl.	*Goldblatt 11174*	South Africa
Lignocarpa diversifolia Dawson	*Glenny (CHR 471842)*	Canterbury, New Zealand
Mackinlaya confusa Hemsl.	*Plunkett 1512*	Queensland, Australia
		cult., Huntington Bot.
Mackinlaya macrosciadia (F.Muell.) F.Muell.	*Plunkett 1365*	Garden
Mackinlaya schlechteri (Meisn.) Philipson	*Lowry 5290*	cult., Bogor Bot. Garden
Neogoezia minor Hemsl.	*Plunkett 1272*	Mexico
Oreomyrrhis eriopoda (DC.) Hook.f.	*Plunkett 1558*	NSW, Australia
Petroselinum crispum (Mill.) A.W. Hill	*Plunkett 1478*	Virginia, USA
Peucedanum sp.	*Phillipson 5733*	South Africa
		cult., New York Bot.
Pimpinella saxifraga L.	*Plunkett 1324*	Garden
Polemanniopsis marlothii (H. Wolff) B.L. Brutt	*vanWyk 4105*	South Africa
Pseudocarum laxiflorum (Baker) B.-E. van Wyk	*Lowry 5342*	Madagascar
Scandia geniculata Dawson	*Webb, s.n. (CHR 512012)*	New Zealand
Steganotaenia araliaceae Hochst.	*Plunkett 1832*	South Africa

Stoibrax capense B.L. Burtt	*Goldblatt 11146*	South Africa
Tinguarra montana (Webb ex H. Christ) Hansen & Kunkel	*Mort 1536*	Canary Islands
Xyloselinum leonidii Pimenov & Kljuykov	*Plunkett 2009*	Vietnam

Family Araliaceae

Aralia spinosa L.	*Plunkett 1371*	Washington, USA
Arthrophyllum mackeei	*Lowry 5310*	New Caledonia
Astrotricha latifolia Benth.	*Mackinson 452093*	NSW, Australia
Astrotricha pterocarpa Benth.	*Plunkett 1527*	Queensland, Australia
Astrotricha sp. nov. Isabella	*Plunkett 1551*	Queensland, Australia
Brassaiopsis glomerulata Regel	*Wen 8458*	Yunan, China
Cephalaralia cephalobotrys Harms	*Plunkett 1519*	Queensland, Australia
Cheirodendron bastardianum Frodin	*Price 205*	French Polynesia
Cheirodendron fauriei Hochr.	*Harder 4070*	Hawaii
Cheirodendron platyphyllum (Hook. & Arn.) Seem.	*Harder 4072*	Hawaii
Cheirodendron trigynum (Gaud.) A. Heller	*Johnson 92-110*	Hawaii
Chengiopanax sciadophylloides (Franch. & Sav.) C.B. Shang & J.Y. Huang	*Tsugaru 20947*	Japan

Cuphocarpus aculeatus Decne. & Planch.	*Lowry 5125*	Madagascar
Cussonia spicata Thunb.	*Goldblatt 10490*	South Africa
Dendropanax arboreus (L.) Decne. & Planch.	*Plunkett 1352*	UC BG
Dendropanax hoi C.B. Shang	*Lowry 4903*	Vietnam
Elutherococcus trifoliatus (L.) S.Y. Hu	*Lowry 4972*	Taiwan
		cult., New York Bot.
Fatsia japonica (Thunb.) Decne & Planch.	*Plunkett 1320*	Garden
Fatsia polycarpa Hayata	*Lowry 4968*	Taiwan
Gamblea pseudoevodiifolia (Franch.) C.-B. Shang, Lowry & Frodin	*Wen 8447*	Yunan, China
Gastonia crassa F. Friedmann	*Lowry 6008*	Seychelles
Gastonia duplicata Thouars ex Baill.	*Aridy 299*	Madagascar
Gastonia rodriguesiana Marais	*Lorence 7765*	Nat'l Trop. Bot. Garden
Harmsiopanax ingens Philipson	*Lowry 5266*	Irian Jaya
Hedera helix L.	*Plunkett 1476*	Virginia, USA
Heteropanax fragrans (Roxb.) Seem.	*Plunkett 2032*	Vietnam
		cult., New York Bot.
Kalopanax septemlobus (Thunb.) Koidz.	*Plunkett 1328*	Garden
Macropanax dispermus (Bl.) Ktze.	*Lowry 4940*	Vietnam

Merrilliopanax chinensis Li	*Wen 5068*	Yunan, China
Meryta sinclairii (Hook.f.) Seem.	*Plunkett 1367*	Huntington BG
Metapanax davidii (Franch.) Frodin ex J. Wen & Frodin	*Wen 5005*	Sichuan, China
Motherwellia haplosciadea F. Muell.	*Plunkett 1515*	Queensland, Australia
Munroidendron racemosum (C. N. Forbes) Sherff	*Lorence 7628*	Nat'l Trop. Bot. Garden
Neopanax arboreus (L.f.) Allan	*Plunkett 1353*	UC BG
Neopanax colensoi (Hook.f.) Allan	*Bayer 1002*	New Zealand
Oplopanax elatus Nakai	*Wen 3120*	
Oreopanax capitatus (Jacq.) Decne. & Planch.	*Miller 38*	Chenci, Costa Rica
Osmoxylon boerlegei (Warb.) Philipson	*Takeuchi 15499*	Papua New Guinea
Osmoxylon geelvinkianum Becc.	*Takeuchi 15500*	Papua New Guinea
Osmoxylon insidiator Becc.	*Lowry 5240*	Irian Jaya
Osmoxylon orientale (Guillaumin) B.C. Stone	*Plunkett 1858*	Vanuatu
Panax quinquefolius L.	*Ware 10046*	Virginia, USA
Polyscias guilfoylei (W. Bull) L.H. Bailey	*Lowry 5525*	Irian Jaya
Polyscias multijuga (A. Gray) Harms	*Lowry 5219*	Fiji
Polyscias murrayi (F. Muell.) Harms	*Plunkett 1829*	Australia
Polyscias schmidii Lowry	*Plunkett 2166*	Vanuatu

Pseudopanax ferox Kirk	*Bayer 1003*	New Zealand
Pseudopanax lessonii (DC.) K. Koch	*Plunkett 2193*	New Zealand
Raukaua anomalus (Hook.) A.D. Mitch.	*Mitchell (CHR 5279)*	New Zealand
Raukaua edgerleyi (Kirk) Seem.	*Plunkett 2191*	New Zealand
Raukaua simplex (G. Forst.) A.D.Mitch.	*Plunkett 2184*	New Zealand
Reynoldsia sandwicensis A. Gray	*Plunkett 1359*	cult., Honolulu Bot. Garden
Schefflera arboricola (Hayata) Merr.	*Plunkett 1958*	cult., Florida, USA
Schefflera candelabra Baill.	*Lowry 4762*	New Caledonia
Schefflera costata A.C. Sm.	*Plunkett 1954*	Fiji
Schefflera digitata J. R. Forst. & G. Forst.	*Plunkett 2192*	New Zealand
Schefflera gabriellae Baill.	*Lowry 4792*	New Caledonia
Schefflera cf. lasiogyne	*Neill 11935*	Ecuador
Schefflera macrophylla (Dunn) R. Vig.	*Lowry 4852*	Vietnam
Schefflera morototoni (Aubl.) Maguire, Steyerm. & Frodin	*Hammel 22506*	Costa Rica
Schefflera myriantha Drake	*Mwangulango 501*	Tanzania
Schefflera myriantha Drake	*Lowry 5808*	Madagascar
Schefflera pickeringii (A. Gray) Frodin	*Plunkett 1840*	Fiji
Schefflera rainaliana Bernardi	*Lowry 4994*	Madagascar

Schefflera reginae (Linden ex W. Richards) Frodin	*Lowry 4652*	New Caledonia
Seemannarlia gerrardii (Seem.) R. Vig.	*Phillipson 5470*	South Africa
Sinopanax formosanus (Hayata) Li	*Lowry 4967*	Taiwan
Tetrapanax papyriferus (Hook.) K. Koch	*Plunkett 1344*	cult., Missouri Bot. Garden
Tetraplasandra hawaiiensis A. Gray	*Plunkett 1378*	cult., Honolulu Bot. Garden
Tetraplasandra oahuensis Harms	*Johnson 92-0220*	cult., Honolulu Bot. Garden
Trevesia palmata Vis.	*Plunkett 1329*	cult., New York Bot. Garden

Family Myodocarpaceae

Delarbrea balansae (Baill.) Lowry & G. Plunkett	*Lowry 4714*	New Caledonia
Delarbrea collina Vieill.	*Lowry 4789*	New Caledonia
Delarbrea harmsii R. Vig.	*Lowry 4732*	New Caledonia
Delarbrea michieana (F. Muell.) F. Muell.	*Plunkett 1502*	Queensland, Australia
Myodocarpus crassifolius Dubard & R. Vig.	*Plunkett 5537*	New Caledonia
Myodocarpus fraxinifolius Brongn. & Gris	*Lowry 5308*	New Caledonia
Myodocarpus involucratus Dubard & R. Vig.	*Lowry 5555*	New Caledonia
Myodocarpus pinnatus Brongn. & Gris	*Lowry 1850*	New Caledonia

Family Pittosporaceae

Auranticarpa edentata L.W. Cayzer , Crisp & I. Telford	*Plunkett 1532*	Queensland, Australia
Bentleya spinescens E. M. Bennett	*CANB 9203655*	ACT, Australia
Billardiera cymosa F. Muell.	*Hadlow 522 (CANB 8603985)*	SA, Australia
Billardiera heterophylla (Lindl.) L.W. Cayzer & Crisp	*Plunkett 1361*	cult., Huntington Bot. Garden
Bursaria incana Lindl.	*Plunkett 1530*	Queensland, Australia
Bursaria spinosa Cav.	*Plunkett 1524*	Queensland, Australia
Cheiranthera linearis A. Cunn. ex Lindl.	*Mallinson 359 (CANB 9409910)*	NSW, Australia
Hymenosporum flavum F. Muell.	*Plunkett 1364*	cult., Huntington Bot. Garden
Marianthus ringens F. Muell.	*Cayzer 200*	WA, Australia
Pittosporum brackenridgei A. Gray	*Keppel, s.n.*	Fiji
Pittosporum koghiense Guillaumin	*Lowry 6305*	New Caledonia
Pittosporum rubiginosum (F. Muell.) R.C. Cooper	*Gray 5928*	Queensland, Australia

Pittosporum sp.	*Lowry 6284*	Madagascar
Pittosporum spinescens (F. Muell.) L.W. Cayzer , Crisp & I. Telford	*Plunkett 1534*	Queensland, Australia
Pittosporum tobira (Thunb.) W.T. Aiton	*Plunkett 1388*	Washington, USA
Pittosporum undulatum Vent.	*Plunkett 1831*	Australia
Rhytidosporum alpinum McGill.	*Crisp 8186*	ACT, Australia

Family Torricelliaceae

Aralidium pinnatifidum (Jungh. & deVriese) Miq.	*Soejarto 5981*	Thailand
Melanophylla alnifolia Baker	*Schatz 3552*	Madagascar
Melanophylla aucubifolia Baker	*Schatz 3745*	Madagascar
Melanoplylla modestei G.E. Schatz, Lowry & A.-E. Wolf	*Schatz 3319*	Madagascar
Torricellia tilifolia Harms ex Diels	*Tu, s.n.*	Guizhou, China

Family Griseliniaceae

Griselinia lucida G.Forst.	*Cameron, s.n.*	New Zealand
Griselinia littoralis (Raoul) Raoul	*Bayer 1001*	New Zealand
Griselinia ruscifolia (Clos) Taub.	*Fiaschi 3073*	São Paulo, Brazil

Family Pennantiaceae

Pennantia corymbosa J.R.Forst. & G.Forst.	*Gemmil, s.n*.	New Zealand
Pennantia cunninghamii Miers	*CANB 8203606*	NSW, Australia

Table 2. List of primers used to amplify the *rpl*16 and *trn*D-*trn*T regions in Apiales.

Primer Name	Region	Designation (Fig.1)	Sequence (5´-3´)	Source
rpl16_EX1F	*rpl*16 Exon1	R1	GCTATGCTTAGTGTGYGACTCGTTG	this study
rpl16_EX2BR	*rpl*16 Exon2	R2	CTATGTTGTTTACGGAATCTGGTTC	this study
rpl16_IN400F	*rpl*16 intron	R3	TAAGAAGYGATGGGAACGATGGA	this study
rpl16_IN450R	*rpl*16 intron	R4	GTTYCGCCATCCCGATCAATG	this study
rpl16_EX1Falt	*rpl*16 intron	R5	TAATRACCAACTCATCACTTC	this study
rpl16_MRalt	*rpl*16 intron	R6	TACATATTGGATGGAWTTNTATATC	this study
rpl16_EX2R_Seq	*rpl*16 Exon2	R7	CTTCTCATCCAGCTCCTCGCGAAT	this study
trnD_F	*trn*D	D1	CGGTGCTCTGACCAATTGAACTA	this study
trnT_R	*trn*T	D2	CCGATGACTTACGCCTTACCAT	this study
trnDT_MF	*trn*E-*trn*T spacer	D3	GTGGTTGGTCCGTCAGAAAA	this study
trnDT_MR	*trn*E-*trn*T spacer	D4	TTTTCTGACGGACCAACCAC	this study
trnDT_HydroMF	*trn*E-*trn*T spacer	D5	TCCGGGRRATCTTTCCGTTTTTCATC	this study
trnDT_ HydroMR	*trn*E-*trn*T spacer	D6	GATGAAAAACGGAAAGATYYCCCGGAT	this study
trnE	*trn*E	D7	AGGACATCTCTCTTTCAAGGAG	Shaw *et al.*, 2005
trnE_F	*trn*E	D8	CTCCTTGAAAGAGAGATGTCCT	modified from Shaw *et al.*, 2005

Table 3. Comparison of sequence characteristics and tree matrices of the *rpl*16 intron, *trn*D-*trn*T, and the combined dataset.

	***rpl*16 intron**	***trnD-trnT***	**Combined**
Sequence length	830-1068	535-1416	1507-2419
Aligned sequence length	2157	3277	5432
No. of constant characters	1186	2035	3219
No. of parsimony uninformative characters	284	328	612
No. of parsimony informative characters	687	914	1601
Tree length	3168	4127	7321
Consistency index (CI)	0.4773	0.4853	0.479
Retention index (RI)	0.8816	0.8778	0.8776

Table 4. Traditional division of subfamily Hydrocotyloideae compared to the placement of genera in this study.

Divisions based on Drude (1898) and Pimenov and Leonov (1993)	**Placement in Apiales (this study)**
I. Tribe Hydrocotyleae (Mericarps are laterally compressed)	
a. Subtribe Hydrocotylinae (No sepals)	
Centella	Mackinlayoideae (*Centella* Clade)
Brachyscias	Mackinlayoideae* (*Xanthosia* Clade)
Chlaenosciadium	Mackinlayoideae (*Xanthosia* Clade)
Homalosciadium	*Platysace* clade
Hydrocotyle	Araliaceae
Micropleura	Mackinlayoideae (*Centella* Clade)
Neosciadium	Araliaceae
Platysace	*Platysace* clade
Trachymene	Araliaceae
Uldinia	Araliaceae
b. Subtribe Xanthosiinae (petaloid sepals present)	
Actinotus	Mackinlayoideae
Pentapeltis	Mackinlayoideae (*Centella* Clade)
Shoenolaena	Mackinlayoideae (*Centella* Clade)

Xanthosia	Mackinlayoideae (*Xanthosia* Clade)

II. Tribe Mulineae (Mericarps are dorsally compressed)

a. Subtribe Asteriscinae (fruits are winged and non-hollowed)

Asteriscium	Azorelloideae (*Asteriscium* Clade)
Choritaenia	Apioideae/Saniculoideae
Diposis	Azorelloideae (*Diposis* Clade)
Domeykoa	Azorelloideae (*Asteriscium* Clade)
Eremocharis	Azorelloideae (*Asteriscium* Clade)
Gymnophyton	Azorelloideae (*Asteriscium* Clade)
Hermas	Apioideae/Saniculoideae
Laretia	Azorelloideae (*Azorella* Clade)
Mulinum	Azorelloideae (*Azorella* Clade)

b. Subtribe Azorellinae (fruits are non-winged and non-hollowed)

Azorella	Azorelloideae (*Azorella* Clade)
Bolax	Azorelloideae (*Bowlesia* Clade)
Dichosciadium	Azorelloideae (*Bowlesia* Clade)
Dickinsia	Azorelloideae (*Azorella* Clade)

Diplaspis	Azorelloideae (*Azorella* Clade)
Huanaca	Azorelloideae (*Azorella* Clade)
Klotzschia	Azorelloideae (*Azorella* Clade)
Oschatzia	Azorelloideae (*Asteriscium* Clade)
Pozoa	Azorelloideae (*Asteriscium* Clade)
Schizeilema	Azorelloideae (*Azorella* Clade)
Spananthe	Azorelloideae (*Azorella* Clade)

c. Subtribe Bowlesiinae (fruits are non-winged and hollowed)

Bowlesia	Azorelloideae (*Bowlesia* Clade)
Drusa	Azorelloideae (*Bowlesia* Clade)
Homalocarpus	Azorelloideae (*Bowlesia* Clade)

III. Incertae sedis

Arctopus	Saniculoideae
Asciadium	Unknown
Naufraga	Apioideae
Notiosciadium	Apioideae

*Placement for *Brachyscias* is based on Henwood and Hart, 2001.

FIGURE LEGENDS

Figure 1. Illustration of the plastid regions *rpl*16 and *trn*D-*trn*T, with the approximate location of primers used for PCR and sequencing across Apiales. The size of *rpl16* intron is c. 800 to 1100 bp. In most species, the sizes of *trn*D, *trn*Y, *trn*E, and *trn*T is between 70 and 90 bp, the size of each spacer in the *trn*D-*trn*Y and *trn*Y-*trn*E regions is c. 100 bp and the size of the *trn*E-*trn*T spacer is c. 500 to 800 bp.

Figure 2. Comparison of major clades retrieved with maximum parsimony for the individual data sets, *trn*D-*trn*T (strict consensus of 40,000 trees) and *rpl*16 intron (1b; strict consensus of 70,000 trees). Estimates of branch support based on 100 bootstrap replicates are shown above branches. See Table 3 for tree statistics.

Figure 3. Strict consensus tree showing major clades of the maximum parsimony analysis of the combined dataset. Bootstrap support values are indicated above branches.

Figure 4. Tree retrieved by maximum likelihood and Bayesian inference analyses on the combined dataset. Bootstrap support values (BS) and posterior probabilities (PP) are indicated above or below branches (BS, PP). Designations on some deeper branches: * when BS > 95%; + when BS is from 80% to 94%; x when BS is from 50% to 79%. Values less than 50% are not shown unless the PP = 1.

FIGURES

Figure 1. Illustrations of *rpl*16 and *trn*D-*trn*T with the locations of primers.

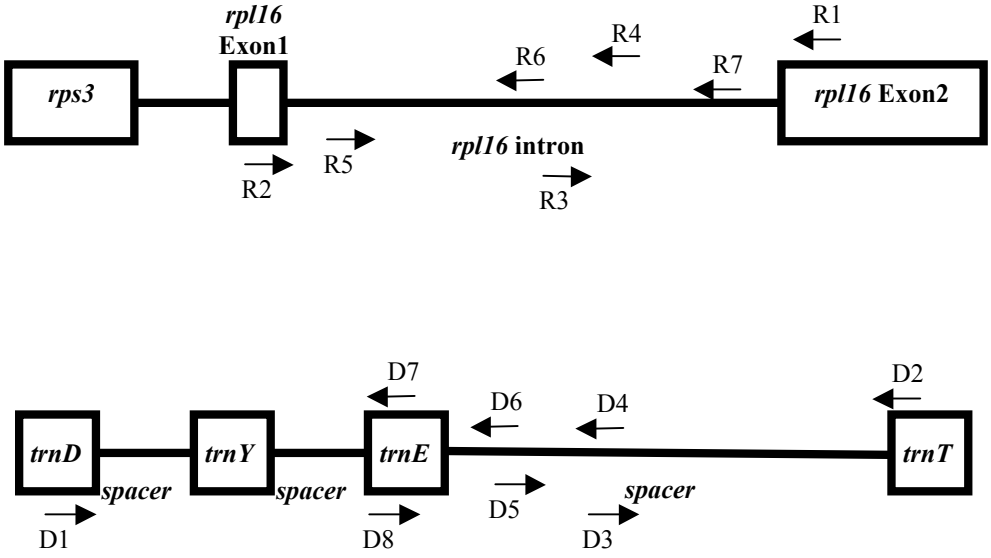


Figure 2. Comaprison of major clades from the maximum likelihood trees of *trn*D-*trn*T and *rpl*16 intron regions.

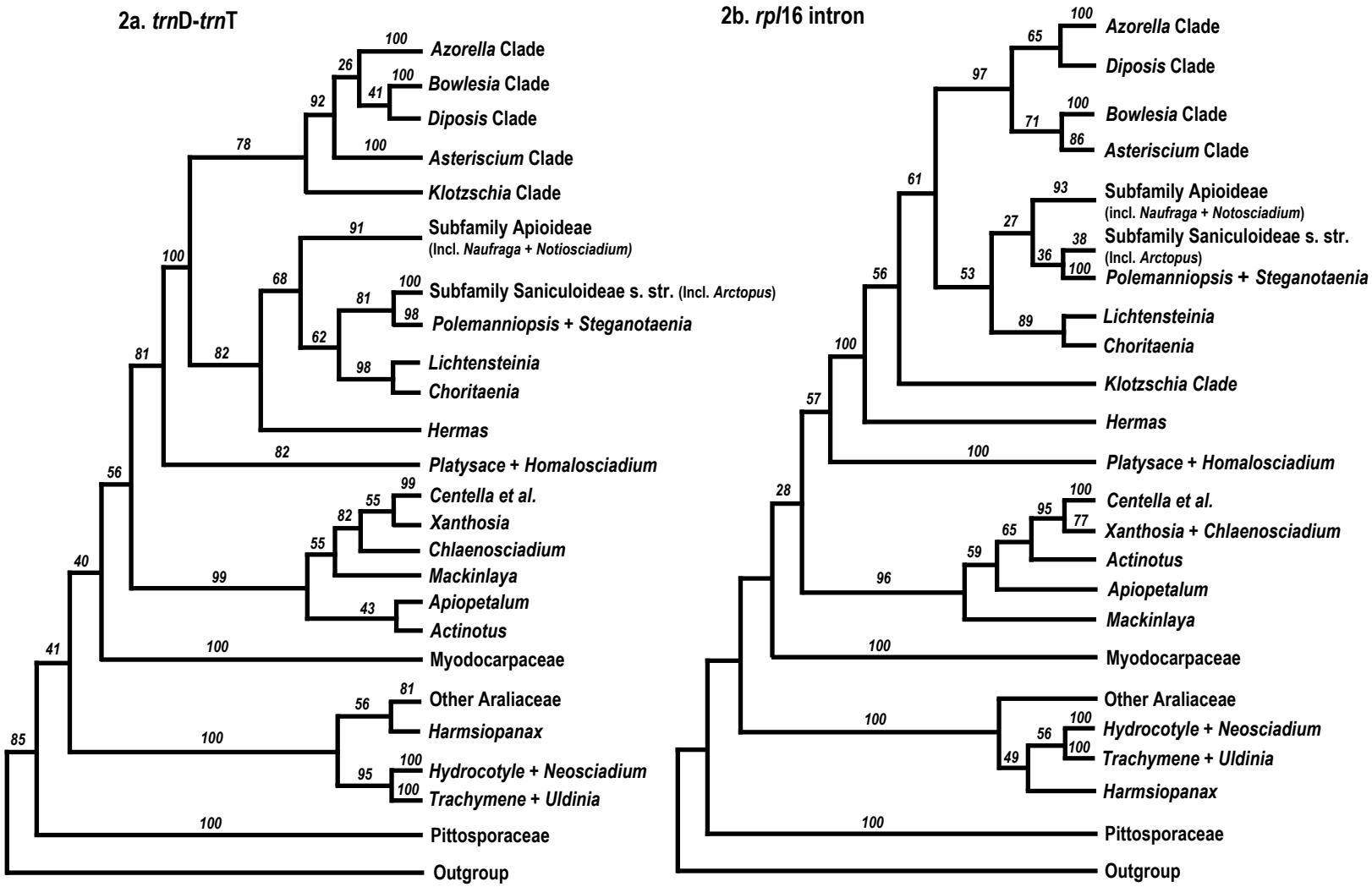


Figure 3. Combined *trn*D-*trn*T and *rpl*16 intron.

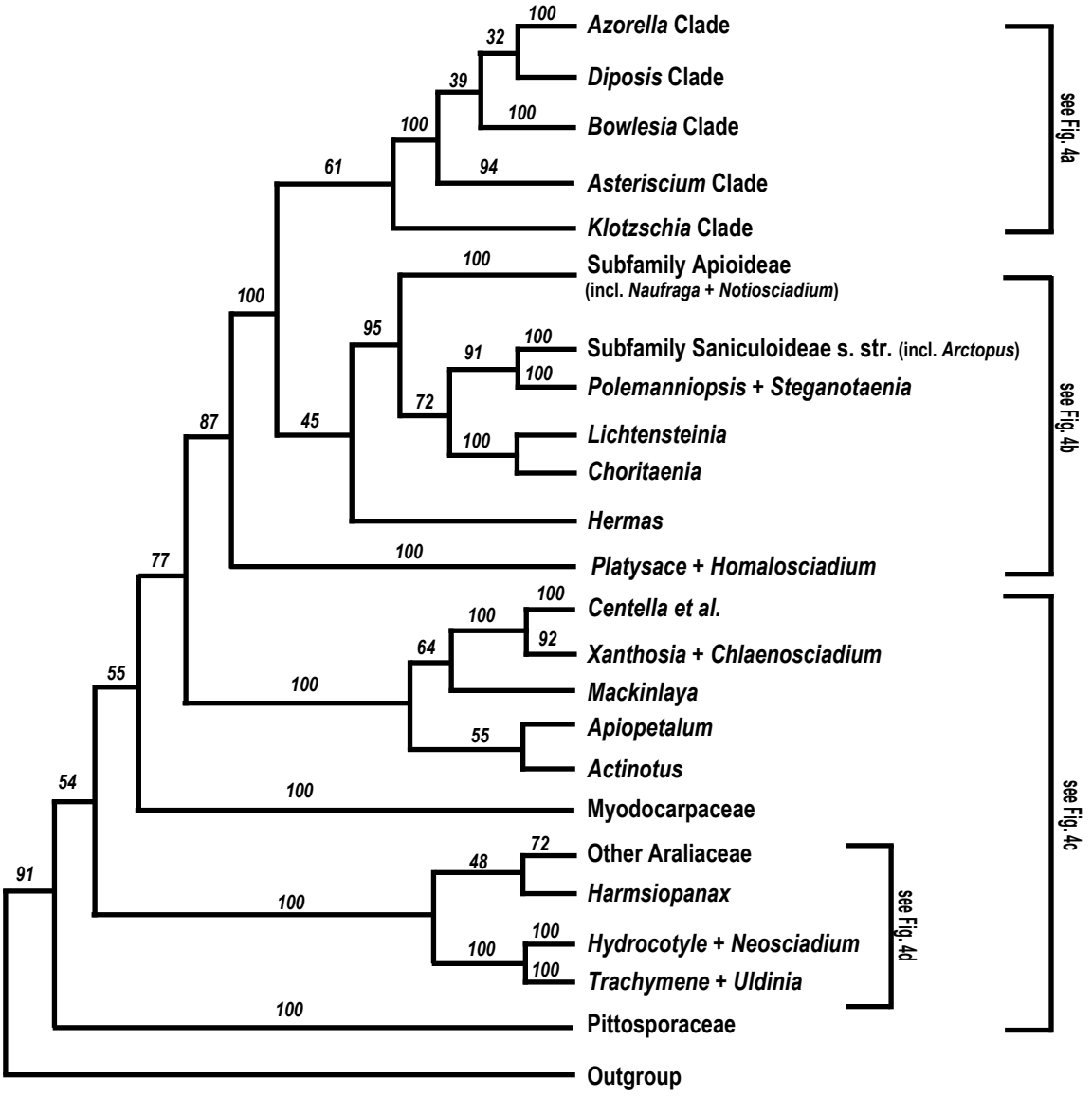


Figure 4. Maximum likelihood phylogeny based on the combined plastid dataset.

Fig. 4a. Azorelloideae.

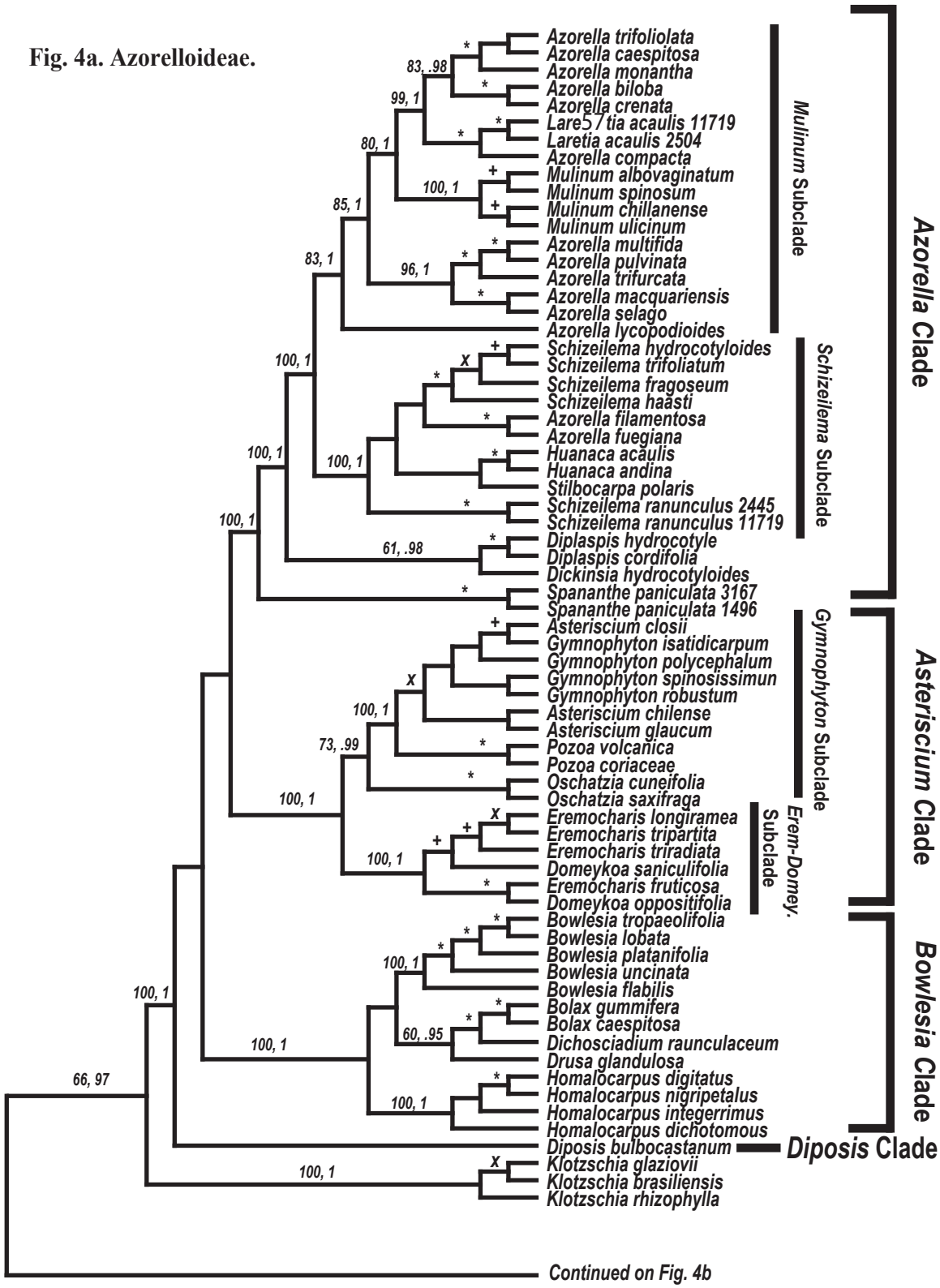


Fig. 4b. Apioideae, Saniculoideae, and *Platysace.*

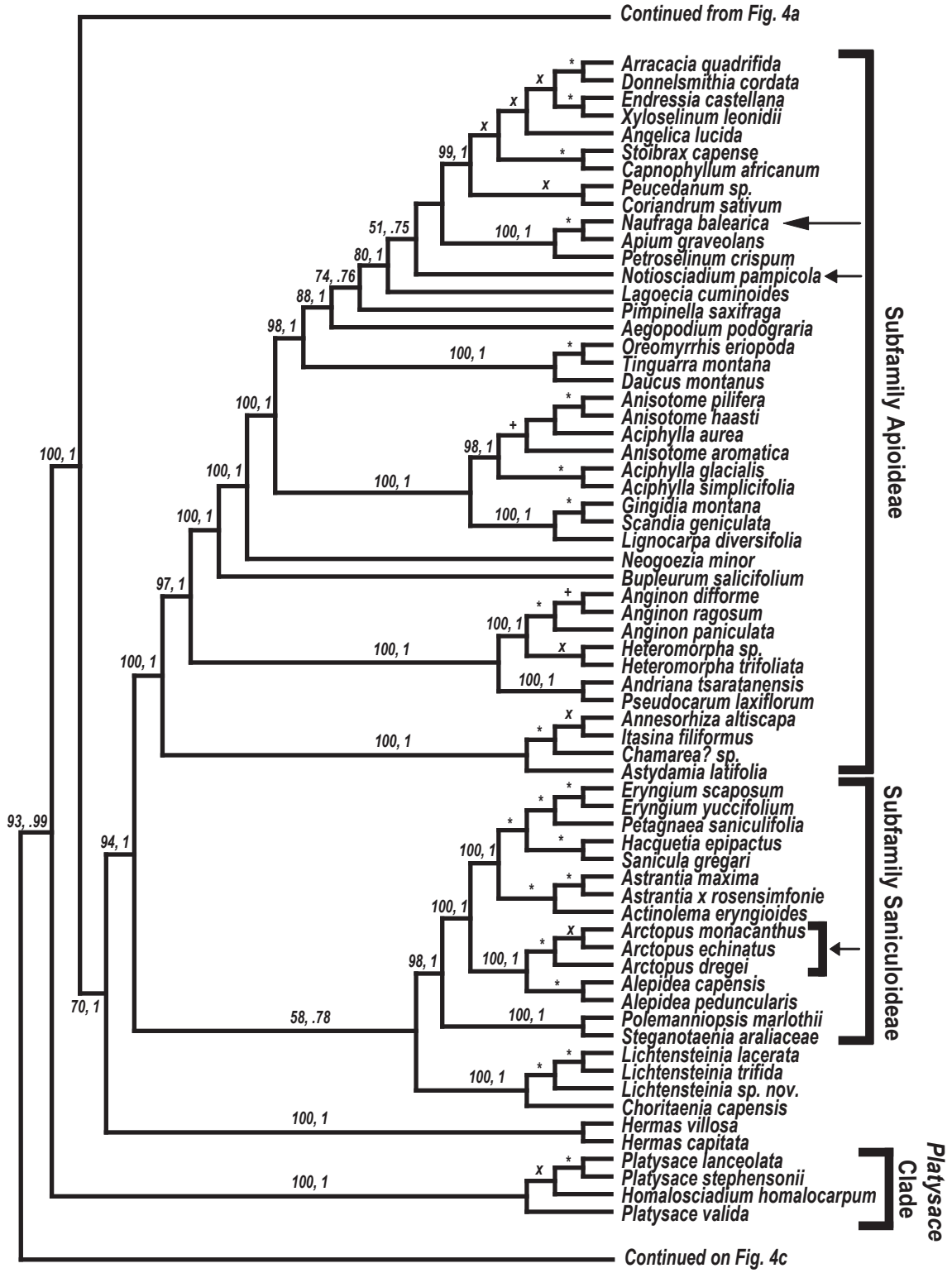


Fig. 4c. Mackinlayoideae, Myodocarpaceae, Pittosporaceae, and outgroups.

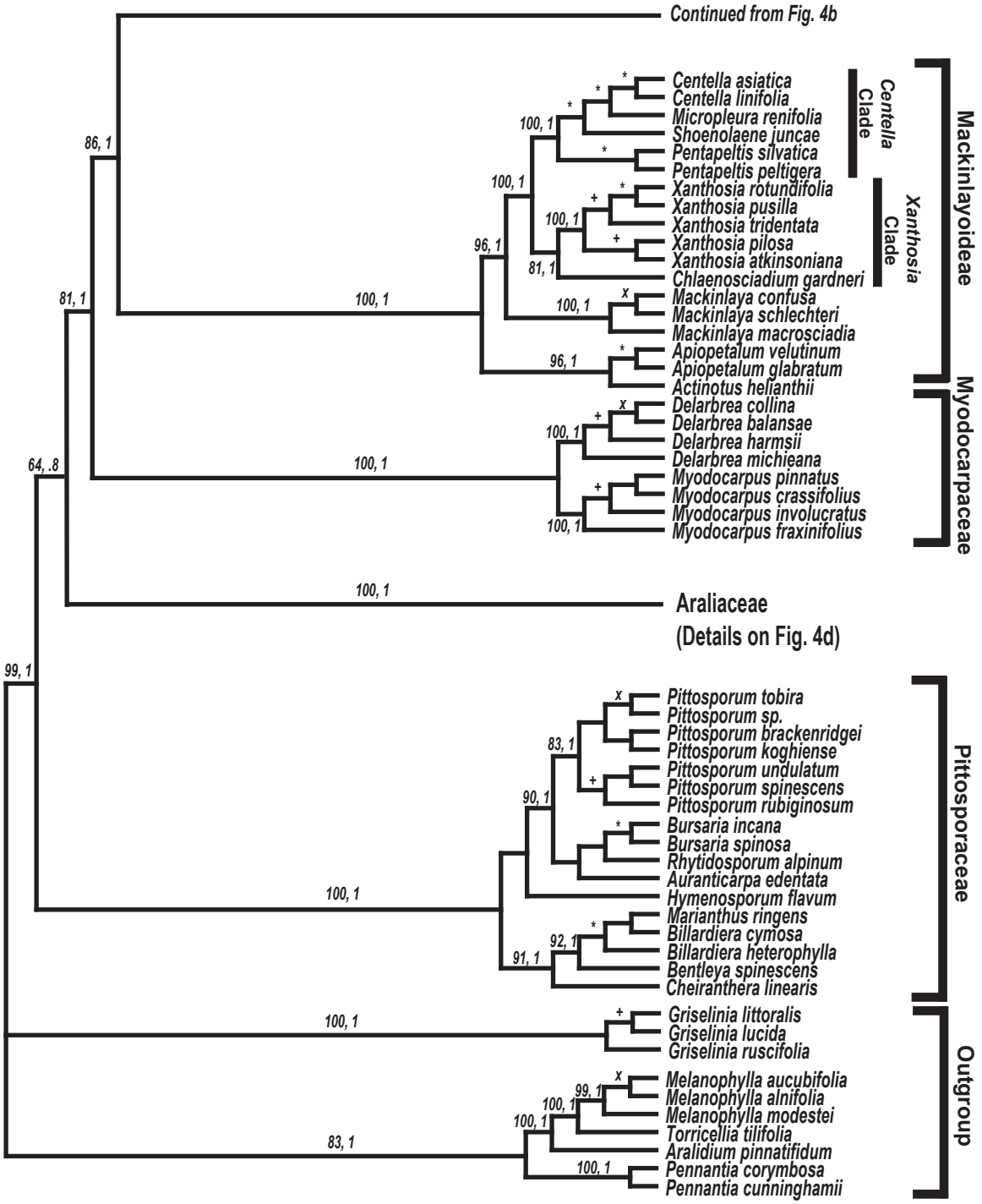
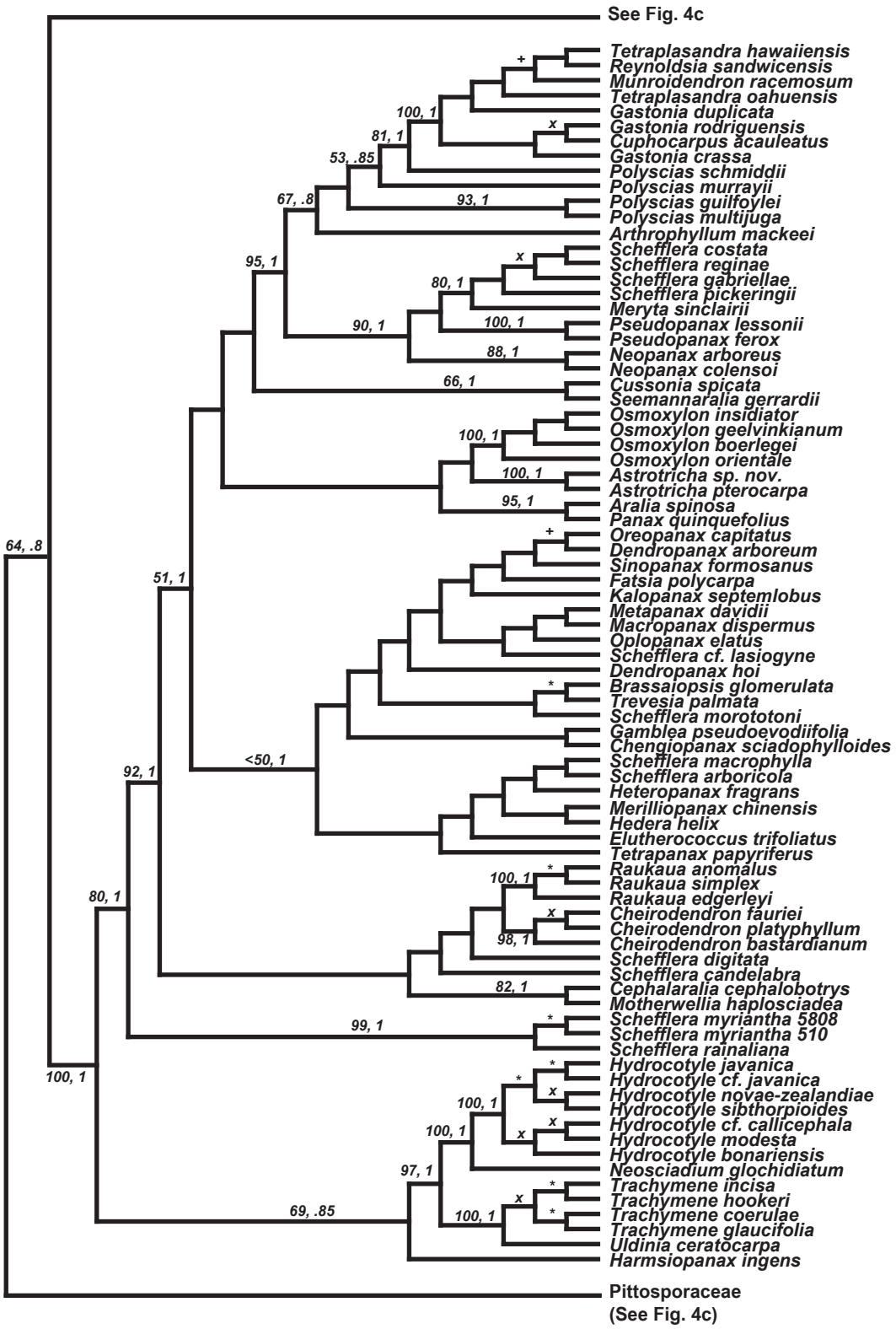


Fig. 4d. Araliaceae.



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CHAPTER 2

Duplication of RPB2 in Apiales: Characteristics of orthologs and paralogs and their implications on the evolution of Apiales

A. N. Nicolas & G. M. Plunkett

Abstract

The second largest subunit of the DNA-dependant RNA polymerase II (RPB2) gene was studied to estimate phylogenetic relationships in the order Apiales. A region from exon 18 to exon 23 (including introns) was sequenced from more than 260 species. This taxon sampling represented all major clades of Apiales, and nearly every genus within these clades (with the exception of Apioideae, where a more representative sampling strategy was applied). Based on interpretations from maximum parsimony and maximum likelihood analyses, at least two copies of RPB2 could be identified in most lineages, representing at least five independent duplication events in the order. The oldest of these duplications can be mapped to the early history of Apiales (c. 100 mya) and appears to precede the divergence of the families of suborder Apiineae (Apiaceae, Araliaceae, Myodocarpaceae, and Pittosporaceae). Both copies place Pittosporaceae as the earliest diverging lineage in the suborder, followed by Araliaceae, Myodocarpaceae, and Apiaceae. Within Araliaceae, several independent duplication events are specific to clades having many known polyploidy species, suggesting that these duplications may have resulted from ancient hybridizations. Following the divergence of subfamily Mackinlayoideae, two

duplication events occurred in Apiaceae. One is shared between subfamilies Apioideae and Saniculoideae, after the divergence of the South African genus *Hermas*. The other is limited to the *Azorella* clade of subfamily Azorelloideae and represents the most recent duplication in Apiineae (c. 55 mya). Both copies demonstrate the polyphyly of *Azorella*, the largest genus in Azorelloideae, and the role of rapid radiation and reticulation in shaping the history of the subfamily. Beyond Apiineae, duplications of RPB2 were detected in the early-diverging families Griseliniaceae and Pennantiaceae, but these appear to be more recent than the earliest duplication found in Apiineae.

1. Introduction

Our understanding of evolutionary relationships in the order Apiales has been hampered by a series of rapid radiations, differences in evolutionary rates among lineages, and the accumulation of homoplasious characters that complicates the task of identifying unique synapomorphies for the major clades of the order. Although molecular data have offered new insights into the evolution of Apiales and its placement among the dicots, many problems remain in understanding the relationships among and within the families of the order. These include the precise relationship among the seven families of Apiales, the proper placement of families Pittosporaceae and Myodocarpaceae, as well as subfamily Mackinlyoideae, the delimitation of subfamilies Apioideae and Saniculoideae in Apiaceae, and the resolution of relationships among the major clades of Azorelloideae. In order to resolve these problems, it is important to sample from sources of data that are as yet untested, with the hope that they will provide additional markers to resolve relationships in

Apiales. In a recent paper aimed at placing 40 of 42 genera once placed within subfamily Hydrocotyloideae (Chapter 1), we retrieved a phylogeny based on new sequences from the plastid *rpl*16 intron and the *trn*D-*trn*T spacer region. Results from that study showed some disagreement with relationships based on nuclear 26S data and two other plastid markers (*mat*K and *rbcl*) presented by Chandler and Plunkett (2004), the last paper on relationships within Apiales that included a thorough sampling across all major clades. To define the nature of these incongruences, we sought a source of data from the nuclear genome that was unlinked to ones used in prior studies to help improve our understanding of the remaining perplexing relationships. Based on recommendations suggested by Denton *et al*. (1998) and especially the work of Oxelman and Bremer (2000) and Oxelman *et al*. (2004), we focused on the RPB2 region of the nuclear genome as a potential candidate to reconstruct intra- and inter-familial relationships in Apiales.

The use of nuclear markers to resolve phylogenetic issues in Apiales has been limited to mostly to rDNA spacers and genes (but see also Mitchell & Wen, 2004). ITS and ETS rDNA sequences have been used mostly at the familial and intergeneric levels (e.g., Downie *et al*., 2000; Wen *et al*., 2001; Valiejo-Roman *et al*., 2002; Plunkett *et al*., 2004*b*, 2004*c*; Tronchet *et al*., 2005) and a single study used sequences from the 26S coding region at the ordinal level (Chandler and Plunkett, 2004). Due to the differences in substitution rates among and within the main lineages of Apiales, it is virtually impossible provide reliable alignments of ITS and ETS sequences across lineages even within Apiaceae (empirical observation), let alone across the order. On the other hand, the 26S gene is highly conserved and did not provide sufficient information to reconstruct a well

resolved phylogeny in Apiales (Chandler and Plunkett, 2004), leaving many questions unanswered. Due to the complexity of the nuclear genome, the reconstruction of nuclear gene trees is more complicated and their use to infer species trees can be less reliable compared to markers from the plastid genome. One cause of this complexity is gene (or even whole genome) duplication, a process that is especially common in plants (Soltis *et al*., 2004). This process often leads to a confusing array of paralogs and orthologs, rendering it difficult to identify homologous copies of the same gene. Nonetheless, when sufficient information is collected about the history of the gene, paralogs and orthologs may be separated with confidence, and the evolution of the different copies may provide valuable insights into the evolution of the taxa.

Recently, to expand the options of informative molecular markers in phylogenetics at different taxonomic levels, much work has been published on the utility of low-copy nuclear genes (reviewed by Sang, 2002; Small *et al*., 2004). Of these genes, DNA-dependent RNA polymerase (RNAP) genes are emerging as potential markers for addressing phylogenetic relationships at different levels. RNAP II is one of three RNAPs identified in eukaryotes that have amino acid sequence homology among fungi, plants, and animals, as well as to the core subunits of the single RNAP found in prokaryotes (Sweester *et al*., 1987; Pati and Weismann, 1990; Kawagishi *et al*., 1993). RNAP II is responsible for the transcription of mRNA and consists of 10 or more subunits in most eukaryotes. The RNAP II genes encoding for the two largest subunits, RPB1 and RPB2, have proved to be very useful in the reconstruction of phylogenies in different groups of fungi (e.g., Liu *et al*., 1999; Zhong and Pfister, 2004; Frøslev *et al*., 2005; Matheny *et al*., 2007; Hofstetter *et*

al., 2007). A single copy of RPB2 has been identified in prokaryotes, fungi, and animals, and it was first suggested that the gene was single copy in plants as well (James *et al*., 1991; Thuriaux and Sentenac, 1992; Archambault and Friesen, 1993; Denton *et al*., 1998). However, two RPB2 paralogs were identified in the asterid Gentianales, Lamiales, Ericales, Solanales, Aquifoliales, and *Escallonia* (Oxelman and Bremer, 2000; Oxelman *et al*., 2004) and the rosid family Malvaceae (Pfeil *et al*., 2004). The two asterid copies, named paralogs *D* and *I*, are both functional and resulted from a duplication event that preceded the divergence of the core eudicot lineages (Oxelman *et al*., 2004; Luo *et al*., 2007). The two copies exhibit size variations in some groups due to the loss of introns 18-23 in paralog *D* (Oxelman *et al*., 2004). In the same study, the *I* paralog was reportedly lost in many asterid groups, including Apiales, which was represented by *Hedera helix* from Araliaceae (including data from exon 11 to exon 24) and *Anthriscus silvestris* from Apiaceae (data from exon 11 to exon 20). Since Denton *et al*. (1998) addressed the utility of RPB2 amino acid, exon, and intron sequences to resolve phylogenies at different levels, the phylogenetic utility of the RPB2 gene has been tested by several studies of angiosperm groups (e.g., Popp and Oxelman, 2001; Oxelman *et al*., 2004; Pfeil *et al*., 2004; Goetsch *et al*., 2005; Thomas *et al*., 2006; Loo *et al*., 2006; Luo *et al*., 2007; Sun *et al*., 2008), but not in Apiales. Because the introns of RPB2 are relatively short, the proximity of exon/intron boundaries makes it easier to delimit sequence regions that would be otherwise difficult to align. This helps to reduce the number of ambiguously aligned regions in the introns, thus producing more potentially-informative characters for phylogenetic reconstructions.

Gene duplication studies and the phylogenetic information they provide have been very limited in Apiales (e.g., Mitchell and Wen, 2004), and none have been targeted at the level of sampling we present in this study. As such, we used extensive sampling of the genera and species within the order to address the following issues: (1) to assess duplication events of the RBP2 gene and identify paralogous and orthologous copies; (2) to test for positive selection and variations in substitution rates within and among copies; and (3) to estimate a phylogeny of Apiales based on the history of the RPB2 gene and address the efficacy of RPB2 intron and exon regions in addressing relationships at different levels within the order.

2. Materials and Methods

2.1. Taxon Sampling

The taxon sampling included an extensive representation of genera from throughout Apiales, with representation of all major clades and, when possible, all major phytogeographic regions. We used a sample nearly identical to that of Chapter 1, which included 139 genera and 263 species (see Table 1, therein) drawn from nearly every genus in the order except Apioideae (where the sampling was more representative, and focused especially on the early diverging lineages). Additional sequences were produced for *Diposis patagonica* Skottsb. (*Puntieri s.n.,* BCRU), *Gymnophyton flexuosum* Clos (*Zöllner 14986*, MO), *Homalosciadium homalocarpum* F. Muell. (*Lepschi 3646*, CANB), and *Hydrocotyle* sp. (*Eichler 22047*, CANB). The outgroup included newly-derived sequences from *Ilex opaca* [Soland.] (*Plunkett 2262*, NY) and *Helwingia japonica* (Thunb.) F. Dietr.

(*Xiang 04C62*, NCSC) from Aquifoliales, and *Lonicera japonica* Thunb. (*Plunkett 2255*, NY) from Dipsacales. We also added previously published sequences of *Valeriana officinalis* L. (GenBank accession number AJ565860) and *Senecio vulgaris* L. (GenBank accession number AJ557132) from Asterales, and *Lonicera* sp. (GenBank accession number AJ565933) from Dipsacales, all included in the study of Oxelman *et al*. (2004). The choice of outgroup taxa was based on previous studies that placed Aquifoliales as sister to a trichotomy comprising Apiales, Asterales and Dipsacales (Plunkett *et al.* 1996; APG II 2003; Judd and Olmstead 2004; Soltis and Soltis 2004).

2.2. DNA extraction, amplification, and sequencing

Most leaf tissue samples were either field-collected and dried using silica gel or harvested from herbarium specimens. Harvesting reliable, high purity, total DNA from fresh, silica-gel dried leaf tissue, or dried herbarium specimens was achieved using the CTAB method of Doyle and Doyle (1987), the DNeasy Plant extraction kit (QIAGEN Inc.), a modified Puregene DNA extraction protocol (Gentra Systems), or following the protocol of Alexander *et al*. (2007) with minor modifications. We targeted a region of the RPB2 gene ranging from the 3′ portion of exon 18 to the 5′ portion of exon 23. For a subset of species (representing different clades across Apiales), we extended this fragment to the 5′ end of exon 24. Primers (Table 1) were designed in exon regions by comparing previously published RPB2 sequences available on GenBank. PCR amplification reactions contains a mixture of 1 μL of unquantified DNA, 5 μL Sigma JumpStart™ REDTaq® ReadyMix™ Reaction Mix or Promega GoTaq® Green Master Mix, 0.5 μL of each

forward and reverse primers (at concentrations of 5 μ*M*), 0.5 μ*M* spermidine (4 m*M*), and 2.5 μL ultrapure water, for a total volume of 10 μL. The PCR thermal profile included a 2 min denaturing step at 94ºC, followed by 35 to 40 cycles of denaturation (30 sec at 94ºC), primer-annealing (30 sec at 54ºC), and DNA extension (30 to 90 sec at 70ºC). This was followed by an extra extension step for 5 min at 72ºC. PCR amplicons were cleaned using ExoSAP-IT (USB Corp.), according to the manufacturer's recommendations, before serving as template for the sequencing reaction.

Although some sequences were produced directly from PCR products (especially within Apiaceae), most were produced from cloned PCR products inserted into plasmid vectors using the StrataClone™ PCR cloning kit (Stratagene) or the Promega pGEM cloning systems (Promega, Madison, Wis.). Four to twelve clones were screened using PCR amplification with M13-20 and M13-27 primers. From these amplicons, one to ten inserts were sequenced. In addition to the M13 primers, inserts were sequenced using the internal RPB2-specific primers (RPB2_EX21R and RPB2_EX20F) in order to attain complete sequences from both complementary strands. Cycle sequencing reactions were performed by mixing 1 μL of the DYEnamic™ ET Terminator Cycle Sequencing mix (GE Healthcare), 1.5 μL of purified double-stranded PCR product, 0.5 μL primer (5μ*M*), and 3 μL ultrapure water, for a total volume of 6 μL. The amplification program consisted of 40 cycles of 3 steps: 30 sec at 94ºC, 15 sec at 55ºC, and 60 sec at 60ºC. Sequencing products were purified using Montage SEQ_{384} plates (Millipore Corp.) and then separated electrophoretically on a 96-capillary MegaBACE™ 1000 automated sequencer. For some

samples derived from herbarium specimens, amplification was performed in two separate pieces with sufficient overlap to ensure that the same copy is being amplified.

2.3. Sequence editing and alignment

Sequences were manually edited using MegaBACE Sequence Analyzer. Complementary strands from individual cloned inserts were compared using BLAST (bl2seq; http://www.ncbi.nlm.nih.gov), as were different clones derived from the same sample. For copies from the same species exhibiting allelic variation less than 1%, a consensus sequence was constructed; when this variation was greater than 1%, both alleles were included in the dataset. Chimeric sequences, detected manually by comparing the sequence to each of the two different copies, were removed from the final data matrix. Sequences were aligned in ClustalX using the default settings (Higgins and Sharpe, 1988), followed by manual adjustments. Long insertions unique to a single sample, as well as certain regions of intron 22 found only in some outgroup taxa were removed from the final alignment. Sequences were compared to annotated sequences in GenBank to determine the exact positions of exons. Subsequently, introns were excised to construct an exon-only data matrix (for comparison to results based on both exons plus introns).

2.4. Data analyses

To compare the phylogenetic placement of our duplicate copies relative to paralogs *D* and *I* described by Oxelman *et al*. (2000 and 2004), a maximum likelihood tree was estimated in GARLI (Zwickl, 2006) using the aligned data matrix of the exon sequences.

For this analysis alone, *Platanus orientalis* (GenBank accession number AY566618) was added to the outgroup because it diverged before the duplication of RPB2 into copies *D* and *I*. We also added the *D* and *I* paralogs from *Escallonia* sp. (GenBank accession numbers AJ565858 and AJ557265), *Solanum lycopersicon* (GenBank accession numbers AJ565934 and AJ565936), and the *I* paralog for *Ilex* × *meserveae* (GenBank accession number AJ557241).

For the data set including aligned regions of both intron and exon sequences, the parsimony ratchet (Nixon, 1999) was implemented in PAUPRat (Sikes and Lewis, 2001) and was run for 10,000 replicates. The 10,000 trees produced from the ratchet analysis were used as starting trees for a heuristic search using TBR swapping in PAUP* version 4.0b10 (Swofford, 2001), with an upper limit of 50,000 trees. Consistency and retention indices (CI and RI) were estimated in PAUP. Support values for nodes were estimated using 200 bootstrap replicates. MODELTEST 3.06 (Posada and Crandall, 1998) was run with PAUP* to estimate the best model of sequence evolution. The recommended model GTR+Γ+I was used for the maximum likelihood analyses in GARLI v. 0.96 Beta (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006). Since GARLI uses a stochastic approach to estimate phylogenies, we followed the recommendation of its authors and performed separate runs estimating the starting tree for the first run by stepwise addition. We used the best scoring tree from each run as the starting tree for the succeeding run. In this approach, the consistency of tree topologies from different runs and similarity in log likelihood values of the resulting trees will reduce the possibility of error. We also set GARLI to perform 100 bootstrap replicates.

To test for significant differences in rates of evolution between exon regions of duplicate copies, we used Tajima's relative rates test as implemented in MEGA 4.0 (Tamura *et al*., 2007), using *Ilex opaca* as the outgroup. Divergence rates within copies of major clades were also compared under the composite likelihood model using Gamma distribution and the transition to transversion ratio (Ts:Tv) calculated in MEGA 4.0. We tested for equivalence in the rates of synonymous (d_S) vs. non-synonymous (d_N) substitutions with the codon-based Z-test (with 1000 bootstrap replicates) as implemented in MEGA 4.0. The codon based Z-test evaluates the deviation from the null hypothesis of neutral evolution where the ratio d_N/d_S is 1. Two alternative hypotheses were tested to check whether positive selection (H_A: $d_N > d_S$) or purifying selection (H_A: $d_N < d_S$) drives the evolution of exon sequences. The significance values were estimated by the codon-based Z-test under the modified Nei-Gojobori method with Jukes-Cantor correction model, which accounts for the bias in Ts:Tv and multiple substitutions at the same site to estimate d_S and d_N.

Exon sequences were translated to amino acid sequences in BioEdit version 7.0.5 (Hall, 2005). Redundant and partial sequences were removed from the matrix and a neighbor-joining (NJ) tree based on the amino acid sequences was constructed in Clustal. Tests for functional divergence between the amino acid sequences of paralogs were carried out in DIVERGE by estimating the coefficient of Type I functional divergence (θ), where $\theta = 0$ under a null hypothesis of no functional divergence between clusters of amino acid sequences in the NJ tree (Gu and Velden, 2002). In Type I functional divergence, the functional constraints vary between the two copies after duplication, leading to different

protein functions. Type II functional divergence tracks significant changes in individual amino acid properties between the two copies, which is beyond the scope of this paper. Likelihood ratio tests (LRT) were conducted in DIVERGE to evaluate the significance value of each θ.

We used BEAST v1.4.8 (Drummod and Rambaut, 2007) to estimate the timing of the duplication events that produced each copy in the different groups. XML input files for BEAST were prepared in BEAUti v1.4.8 (available in the BEAST software package) from a nexus file of aligned sequences. Starting with molecular sequences and a model of evolutionary relationships among these sequences, BEAST uses Bayesian MCMC to estimate posterior distribution of phylogenies and lineages within them. It also uses calibration points at nodes to infer a time-scale for relationships. Hence the result is a Bayesian tree with a posterior probability for each node and an age estimate for each divergence event. We conducted a likelihood ratio test (LRT) to test whether the sequences evolve according to a molecular clock. This was achieved by comparing –ln likelihood values of the best fit model estimated by Modeltest (GTR+Γ+I) with a clock and without a clock. The outgroup was removed and the sample set was trimmed to 169 taxa, representing available copies from all major clades of Apiales, rooted with *Pennantia*. We set calibration points with fossils related to Torricelliaceae (*Toricellia bonesii* (Manchester) Manchester *comb*. *nov*.; Manchester, 1999) and the Asian Palmate clade of Araliaceae (*Dendropanax eocenensis* Dilcher & Dolph; Dilcher and Dolph, 1970). The calibration points were placed at the point of common ancestry for Torricelliace based on fruit fossils collected from lower Eocene deposits. The minimum age was set to 52.2 MYA

and the 95% confidence interval between 48.6 and 55.8 MYA, which spans the lower Eocene epoch. We followed the same concept for the *Dendropanax* leaf fossil and calibrated its clade with a minimum age of 42.9 MYA and 95% confidence interval spanning the middle Eocene from 37.2 to 48.6 MYA. Since estimates for the age of Apiales have not been consistent across previous studies, we did not set a conservative age to the ingroup. Instead we considered prior estimates, calibrations, and sampling limitations to set the age of the ingroup to 100 MYA and relaxed the estimate with 2.5% confidence intervals between 90 and 110 MYA. We used the GTR+Γ+I as the model of evolution and a relaxed clock with uncorrelated lognormal. As recommended by the authors of BEAST, the tree prior was set according to the Yule speciation process, which assumes a constant speciation rate per lineage. The chain was run for 10 million generations with sampling of trees every 1000 generations. The tree file was transferred to TreeAnnotator v1.4.8 (in the BEAST package) to estimate the tree with the maximum sum of clade posterior probabilities (maximum clade credibility tree).

3. Results

The data matrix included 339 new seqeuences of RPB2 that span a region from exon 18 to exon 23. Three previously published sequences were used as additional outgroups, thus bringing the total to 342 sequences. After comparisons of preliminary sequences of intron 23, it proved to be the largest of the introns (> 400 bp) and showed a high degree of variability, rendering it difficult to align among distant groups, so we did not include it in the analyses. However, this intron contained sufficient variability to

provide information within families and genera, and may prove useful at these levels in future phylogenetic studies. The aligned sequence matrix comprised 2610 characters, of which 438 aligned characters represented exon regions. The exon alignment included only two indel regions (of 3 bp and 6 bp) that were potentially synapomorphic to some groups, plus six that were autapomorphic. Most length variations were restricted to the introns. Sequence lengths (excluding outgroups) varied from 819 bp in copy 1 of *Aegopodium podograria* (due to deletion of ~200 bp spanning intron 19, exon 20, and intron 20) to 1393 in *Bowlesia tropaeolifolia* (due to an insertion of ~300 bp in intron 22). Most sequences ranged between 900 and 1050 bp. Sequences from one copy of Pittosporaceae (*RPB2-Pi1*) lack intron 19, but the lengths of these sequences are made up with an insertion in intron 20 that is also unique to *RPB2-Pi1*.

The maximum likelihood tree based on the data from both *D* and *I* copies (analysis not shown) indicates that all samples from Apiales fall within the *D* clade, which makes them homologous to the *D* copy of other asterids (and unrelated to the *I* copy). This justifies the use of the *D* copy of Asterales, Dipsacales, and Aquifoliales as outgroups. One interesting placement retrieved by this analysis was that of *Pennantia* outside of Apiales, and instead as sister to *Ilex opaca* and *Helwingia japonica* (Aquifoliales). Both ML and MP trees showed the presence of several paralogs of the *D* copy, distributed among all the families of Apiales (and within subfamilies of Apiaceae) except Torricelliaceae, for which only one copy was retrieved. Two paralogs were present in Pennantiaceae (*Pen1* and *Pen2*) and in Griseliniaceae (*Gr1* and *Gr2*) (Figs. 1 and 3e). Two copies were identified in Pittosporaceae (*Pi1* and *Pi2*), Araliaceae (*Ar1* and *Ar2*), Myodocarpaceae (*My1* and *My2*),

Mackinlayoideae (*Mk1* and *Mk2*), the *Azorella* clade (*Az1* and *Az2*), and Apioideae + Saniculoideae (*Ap-Sa1* and *Ap-Sa2*) (Figs. 1, 3, and 4). Within Araliaceae copy *Ar1*, two paralogs were identified for the *Pseudopanax* group (*Px1* and *Px2*) and the Asian Palmate clade (*AP1* and *AP2*) (Fig. 3d).

The MP trees for the *D* copy and its paralogs (summary of tree in Fig. 1) was based on 1057 parsimony-informative characters and was 11,548 steps long. The consistency index (CI) was 0.2488 and the retention index (RI) was 0.8081 (Table 2). Most relationships, especially among families and paralogs of *RPB2-D* were well resolved. The ML tree had a -ln likelihood score of -61115.8937 and was fully resolved (summary of tree in Fig. 1, details in Fig. 3). Most nodes representing phylogenetic relationships among and within families had strong bootstrap support in both the MP and ML trees. Both analyses also demonstrated the same placement of *RPB2* paralogs in Apiales (Fig. 1). Major disagreements between the MP and ML trees included the placement of the *Trachymene* clade in copy 2 of Araliaceae (*Ar2*), the placement of the *Hydrocotyle* clade in copy 1 of Araliaceae (*Ar1*), the relationship between Mackinlayoideae and Myodocarpaceae, and the placement and relationship between *Lichtensteinia* and *Choritaenia* (Fig. 1). Although all these placements were resolved, they were not well supported.

The length of the shortest MP tree based on exon data alone was 2124 steps long with 255 parsimony-informative characters (Table 2). The ML tree had a -ln likelihood score of -12362.8375 (Fig. 2). The trees were not as well resolved as those based on the data matrix with both exons and introns, but only a few taxa differed in their placements. For example, Azorelloideae (excluding *Klotzschia*) appears monophyletic in Fig. 2 but not

in Fig.1, and *Harmsiopanax* appears as sister to *Hydrocotyle* in Fig. 2 but not Fig. 1, but the sister relationship between *Harmsiopanax* and *Hydrocotyle* was not well supported.

Tajima's relative rates tests showed no significant differences in the evolutionary rates between the two copies in most clades. However, a significant difference was observed in some Araliaceae, and in all samples of the *Azorella* clade for which both copies were represented ($P < 0.05$ for the null hypothesis of equal rates). The codon-based Z-test of neutrality showed that neither of the two copies is under positive selection ($P = 1$) but are more likely to be under purifying selection due to a significant increase ($P < 0.05$) in the number of synonymous mutations compared to non-synonymous ones for most groups (Table 3). The only exception was *Az2* of *Azorella* ($P = 0.0887$ to reject purifying selection). The LRT on all pairwise comparisons of amino acid clusters representing duplicates showed that Type I functional divergence parameter θ is not significantly higher than 0 ($LRT < 2.5$; $P > 0.1$). This indicates that there is no heterogeneity in function between the two duplicates.

The LRT of rate constancy led to the rejection of a molecular clock ($P \sim 0$), validating the use of a relaxed clock with uncorrelated lognormal distribution of priors. Such a clock considers variation of rates among lineages without assuming *a priori* correlation between a lineage and its ancestor, and usually performs better than other models (Ho *et al*. 2005; Drummond *et al*. 2006). The earliest duplication event dates back to more than 103 Mya, prior to the divergence of Pittosporaceae and after the divergence of Torricelliaceae and Griseliniaceae (Table 4). The most recent duplication within Apiales *s. str.* occurred in the *Azorella* clade of Azorelloideae, dating back to over 55 Mya (Table 4).

The maximum clade credibility tree (Fig. 4) confirms most relationships already established by the ML tree. Two key differences are the placement of *Lichtensteinia* as sister to the remaining copy1 sequences of Apioideae and Saniculoideae (PP = 0.84) and the placement of Torricelliaceae as sister to the two copies of Griseliniaceae (PP = 0.95).

4. Discussion

4.1. Duplication of RPB2

Through this study we demonstrate multiple, independent duplications of RPB2 in Apiales, events not previously reported in this order. One of these copies is homologous to the *D* copy isolated from *Hedera helix* by Olmstead *et al.* (2004). However, the paralogs we retrieved do not correspond to copy *I*, discovered in some groups of angiosperms in that study. Rather, they are a result of duplications of the *D* paralog long after the event leading to the divergence of *RPB2-D* and *RPB2-I*. The duplication of *RPB2-D* occurred at least five times throughout the history of Apiales: within Pennantiaceae, before the divergence of Griseliniaceae and Torriceliaceae, before the divergence of families Pittosporaceae, Araliaceae, Myodocarpaceae and Apiaceae subfamily Mackinlayoideae, and at least twice within family Apiaceae (once within the *Azorella* clade of subfamily Azorelloideae, and once before the divergence of subfamilies Apioideae and Saniculoideae). The oldest of these events dates to the late Cretaceous (103 Mya) and occurred before the divergence of the four core families of Apiales (Apiaceae, Myodocarpaceae, Araliaceae, and Pittosporaceae). Another duplication occurred within core Apiales, dating back to c. 70 Mya, and is shared between Apioideae and

Saniculoideae. A duplication (also c. 70 Mya) occurred outside of core Apiales, giving rise to two copies of RPB2 in Griseliniaceae, perhaps originating in the common ancestor of this family and Torricelliaceae (see below). The duplication event giving rise to the two copies found in Pennantiaceae, however, appears to be unique to that family. This suggests that, despite their membership in Apiales, the nuclear genomes of Griseliniaceae, Torricelliaceae, and Pennantiaceae may be quite divergent, a finding that is also reflected in the morphological characters of these families.

Gene duplication can result from whole genome duplication, duplication of an entire chromosome, duplication within a chromosome by unequal crossing-over, or retroposition through the insertion of cDNA into the genome (reviewed in Zhang, 2003). In many cases, one of the duplicates or paralogs may lose function (pseudogenization) and thus either become highly divergent in its sequence or it may be lost altogether. In contrast to pseudogenization is concerted evolution, where duplicates maintain the same protein and function, with minimal changes in DNA sequences. Two of the most important evolutionary fates of a duplicated gene are subfunctionalization and neofunctionalization. Subfunctionalization is most beneficial in genes with a high functional load, so duplicates can take over subsets of the function of the gene (Nowak *et al*., 1997; Force *et al*., 1999). In these cases, genes accumulate variations in their sequences and diverge without losing function, but the expression of each copy becomes differential to certain tissues (Lynch and Force, 2000). Neofunctionalization implies that sufficient divergence in sequence and function led to the emergence of a new function by one of the duplicates. The survival of distinct gene copies depends on evolutionary pressures and the cellular dynamics that

control neutralism and selectionism (whether positive or purifying selection) in order to decide the fate of synonymous and non-synonymous mutations. Our results show that the number of synonymous substitutions is significantly higher than that of non-synonymous substitutions in both copies, which indicates that the gene is not under positive selection in any of the clades (H_A: $d_N > d_S$ P = 1). Also, the estimates of the parameters of the type I functional divergence (θ) indicate that there is no divergence of functions between the two copies. This means that neofunctionalization can be eliminated as the mechanism driving the divergence of the paralogs. Pseudogenization may also be eliminated because, in addition to the similarity in the ratio of non-synonymous to synonymous mutations in both copies (Table 3), the exon and amino acid matrices did not show frame disruptions and significant length variations between the two copies of any duplication event. Rather, in most clades both copies are under purifying selection (H_A: $d_N < d_S$; $P < 0.05$), a process indicating either concerted evolution or subfunctionalization. Since the variation between the two copies is evident through the comparison of DNA sequences of introns and exons, as well as amino acid sequences of proteins, concerted evolution appears to be less likely, which leaves subfunctionalization as the best explanation for the fate of the two copies. The RPB2 subunit is part of the active center of RNAP II (Hahn, 2004) and the RNAP II enzyme is frequently used in the cell because of its great importance for the transcription of protein-coding genes into mRNA (Young, 1991; Shilatifard *et al*., 2003). Such high demand supports the process of subfunctionalization as the likely reason for the survival of two functional copies. Luo *et al*. (2007) were able to show that both *I* and *D* paralogs of RPB2 are expressed in various plant tissues, with a preferential expression of copy *D* in

vegetative tissues and copy *I* in floral tissues. Since copy *I* was not found in Apiales, it is possible the two copies of *D* isolated here may follow a similar pattern to that described in Luo *et al*. (2007).

Some authors suggested that subfunctionalization and neofunctionalization may both be part of the history of the same gene, and that the former may be a "stepping-stone" to the latter (e.g., Lynch and Force, 2000; He and Zhang, 2005). Subfunctionalization may be a short term process, and after the paralogs lose their ancestral functions in lieu of subfunctions, they may accumulate advantageous mutations and gain new functions. The *Azorella* clade may provide one possible example of the early stage of such a model because the duplication event is more recent (c. 55 Mya) than in other clades. The functional divergence test did not show functional divergence between the two copies of the *Azorella* clade but the relative rates test showed a significant difference in the rates of substitution in exons and almost twice the difference in the mean genetic distance between the two copies. In addition, *Az2* had the highest ratio of non-synonymous mutations compared synonymous ones (0.58) and a low Ts:Tv (1.061), almost three times less than that of copy 1 (Table 3). In addition, copy 2 was not shown to be under either positive or negative selection, but rather under neutral selection (H_A: $d_N \neq d_S$; P = 0.16), although P = 0.0887 for purifying selection may be significant if the cutoff significance level for P was 0.1 (Table 3). This may indicate a progression in copy 2 from subfunctionalization to neofunctionalization, or even pseudogenization.

4.2. Identifying Paralogs and Orthologs – Some Pitfalls to Avoid

Following the duplication of a genomic region, the task of separating orthologs from paralogs without ambiguity may prove very challenging. In a best-case scenario, phylogenetic analysis of the duplicated sequences will produce two well supported clades (one duplicate per clade), each exhibiting a similar or identical topology among the terminals. Our results approached this situation, but not fully. Paralogy and orthology was difficult to discern in some cases because duplications occurred independently in different clades, followed by apparent loss in some clades. The occurrence of duplication events in clades exhibiting significantly different rates of sequence divergence further complicated the task of identifying orthologs. In Apiales, such shifts in evolutionary rates are known to be correlated to life-history differences among species, resulting in branch-length differences of greater than a factor of three in herbs compared to woody trees or shrubs (Smith and Donaghue, 2008). This applies particularly to comparisons of entire clades that are mostly woody (e.g., Araliaceae, Myodocarpaceae, and Pittosporaceae) to those that are mostly herbaceous (e.g., Apiaceae), but also within clades that include both woody and herbaceous taxa (e.g., Mackinlayoideae). Differences in diversification rates may make duplication events appear older in more rapidly evolving lineages (e.g., *Azorella* and Apioideae-Saniculoideae clades) and blur the phylogenetic estimations in rapidly evolving clades that exhibit ambiguity in their delimitations (e.g., the early-diverging clades of Apioideae-Saniculoideae). In this study, we used an advance model of sequence evolution (GTR+Γ+I) incorporated in maximum likelihood analyses that consider rate variations among and within lineages and produce reliable estimates for the placement of duplicates

in the phylogeny. We also used Bayesian approaches (incorporated in BEAST), which account for rate heterogeneity and the lack of correlation between rates along different branches in order to provide greater reliability in the estimation of times of divergence. In addition, we were able to confirm the orthology among duplicate copies in each of the main lineages by comparing introns, exons and amino acid sequences. Exons of orthologous copies are expected to accumulate relatively few variations since they are under purifying selection, regardless of the plant's habit or life history. Amino acid comparisons provide an additional line of evidence in the event that the nucleotide substitutions in the exons were synonymous (and hence not selected). The broad sampling we employed here, including representatives from both the deep and shallow branches across Apiales, increased the chances of retrieving both copies of a duplication where possible, which provides for a more reliable placement of each copy in the overall phylogenetic tree.

Within individual copies of RPB2, we also found evidence of different sequences from the same species. These differences may be ascribed to allelic variation, neopolyploidy, or paleopolyploidy. In some cases, such differences in sequences showed a very small degree of divergence (< 2%), mostly limited to transitions or indels in introns, and these we attributed to allelic variation. In all cases, these alleles comprised a monophyletic clade. Neopolyploids and paleopolyploids were hypothesized based on current knowledge of cytology (albeit rather limited), as well as the monophyly of the sequences and the placement of the sequences compared to other published phylogenies. The distinction between allelic variation and gene duplication becomes especially difficult

in clades with long branches where the monophyly of each copy is not violated even by very close relatives. In these cases, allelic variants, neopolyploids, and even paleopolyploids may all appear monophyletic with low rates of divergence between sequences.

Identification of orthologs may be further complicated by recombination between the two copies, yielding chimeric sequences. This is known to occur *in vivo* through crossing over during meiosis, and *in vitro* as an artifact of molecular techniques, such as PCR (Bradley and Hillis, 1997; Judo *et al*., 1998; Zhou and Hickford, 2000; Jelesko *et al.*, 2004). Our thorough sampling of taxa and the recovery of copies across all major clades facilitated the identification of such recombinants. These recombinants were then excluded from our analyses because they represent two independent evolutionary histories. If left in the data matrix, chimeric sequences may have a significant effect on the placement of other taxa and bias the conclusions of a phylogenetic analysis. In Apiales, we identified at least twelve sequences retrieved from clones as chimeric sequences, each with evident crossing-over points that always occurred in exons, but not always in the same exon (all exons between 18 and 23 served as cross-over points among our recombinants). In the absence of detailed cytological work to detect recombinants and the proximity of the two duplicates on the chromosomes, it is not possible to ascertain whether chimeras arose *in vivo* or were *in vitro* PCR artifacts. However, proximity is certainly brought about during the PCR process, facilitating rearrangements at regions with very high similarity (usually exons). Also, while rare, we did find a very few colonies that contained only fragments of the target PCR product. Such fragmentation in PCR may lead to chimeric sequences through

the joining of fragments from different copies early in the amplification process (Pääbo *et al*., 1990; Bradley and Hillis, 1997). The presence of chimeras with various recombination points along different exons and the retrieval of partial sequences in a few cloning reactions makes us suspect that these sequences are PCR artifacts.

The amplification of the entire target DNA region as two or more separate but overlapping PCR products is sometimes necessary, but can represent another pitfall related to molecular methodology because different primer pairs may favor different paralogs. This was especially important for older samples collected from herbaria, where amplification is typically successful only in small sections of c. 500 bp. In Apiales, for example, amplification with primers RPB2_EX18F and RPB2_EX21R favored one copy in some species of Apioideae (e.g., *Petroselinum crispum* and *Peucedanum* sp.) and Mackinlayoideae (e.g., *Mackinlaya*, *Pentapeltis silvatica*, and *Chlaenosciadium gardneri*), while primers RPB2_EX20F and RPB2_EX23R retrieved another copy for the same species. In such cases, it is important that the overlapping region contains sufficient variation (e.g., intron 20 in our study) to be able to identify which paralog has been amplified. Another issue potentially related to the PCR process is the failure to retrieve one of the two duplicates; this may be difficult to distinguish from the evolutionary loss of one copy. In Apiales, this was evidenced in the *Hydrocotyle* and *Trachymene* clades of Araliaceae, in Torricelliaceae, and in the *Asteriscium* and *Bowlesia* clades of Azorelloideae. We attempted to circumvent this problem by using different combinations of primers and lowering the annealing temperatures, but the same copy was retrieved in all samples of these clades. The differential loss of one copy is a common process that may

follow gene duplication, and this may lead to conflicting signals between phylogenies from different genes. It may be a result of dysploidy, rearrangements within chromosomes, or high divergence in one copy of the sequence following loss of function. Further cytological evidence is imperative to prove that the lack of PCR amplification for one copy indicates the loss of this copy.

4.3. Implications on the Phylogeny of Apiales

The relationships among the families of Apiales retrieved through the phylogenetic analysis of RBP2 data are largely congruent with most relationships estimated by the combined analyses of data from the plastid *rpl16* intron and the *trnD-trnT* spacer (Chapter 1). In the plastid study, *Pennantia* was chosen as outgroup based on the results of Kårehed *et al*. (2001), Kårehed (2003), and Chandler and Plunkett (2004). However, both exon + intron and exon-only trees place *Pennantia* in one of the outgroup clades, sister to the *Ilex-Helwingia* clade of Aquifoliales (Figs. 1-3). We retrieved only a single copy of RPB2 from all three genera that comprise Torricelliaceae. That family appears to be the sister of Griseliniaceae (BS = 83%), but with ambiguity in its placement relative to the two copies of Griseliniaceae (Figs. 1, 3, and 4). Relationships among families Pittosporaceae, Araliaceae, and Myodocarpaceae are the same as those demonstrated by our plastid data (Chapter 1) and by the plastid data (*mat*K and *rbc*L) used by Chandler and Plunkett (2004). All three studies show Pittosporaceae as the earliest diverging lineage of the group, followed by Araliaceae, Myodocarpaceae and Apiaceae (Figs. 1, 3, and 4). The long-held view of *Myodocarpus* and *Delarbrea* (of Myodocarpaceae) as intermediates between

Araliaceae and Apiaceae (Baumann, 1946; Frodin and Govaerts, 2003) is supported with this placement (Figs. 1, 3, and 4). None of our analyses support a sister-group relationship between Pittosporaceae and Myodocarpaceae, a relationship suggested by the nuclear 26S phylogeny estimated in Chandler and Plunkett (2004). Genera in Mackinlayoideae have also been viewed as intermediates between Araliaceae and Apiaceae (see Plunkett and Lowry, 2001; Plunkett *et al*. 2004*a*), and the placement of the subfamily as the earliest diverging lineage of Apiaceae supports this view (Figs. 1, 3, and 4). In the RPB2 phylogeny, the *Platysace* clade (*Platysace* & *Homalosciadium*) appears sister to the rest of Mackinlayoideae and not as successive sister groups to the rest of Apiaceae (Fig1, 3, and 4) as in the plastid phylogeny (Chapter 1). Within Apiaceae, the relationships among the four subfamilies are compatible with our plastid data (Chapter 1), where Mackinlayoideae are sister to the other three subfamilies (Azorelloideae, Saniculoideae, and Apioideae) (Fig. 1). Of the lineages from Azorelloideae, the *Asteriscium* clade alone appears sister to Apioideae-Saniculoideae, but with low support in the exon + intron tree (BS = 57%; Fig. 3b). However, in the exon phylogeny, the *Asteriscium* clade groups with the rest of Azorelloideae (except *Klotzschia*) to form a monophyletic sister group to Apioideae + Saniculoideae, but again support is low (BS < 50%; Fig. 2). Compared to the plastid trees, the RPB2 phylogeny shows a better resolution of relationships among the major groups of Azorelloideae, supporting a sister relationship between the *Azorella* and *Bowlesia* clades and a well supported placement of *Diposis* as sister to the *Asteriscium* clade (Figs. 1 and 3b). *Hermas* is well supported as sister to the Azorelloideae-Saniculoideae clade (BS = 83%; Fig. 3a). Problems remain regarding the placement of *Klotzschia* and the

establishment of a clear relationship between *Lichtensteinia* and *Choritaenia*, and between these taxa and the rest of the Apioideae + Saniculoideae.

4.4. Characteristics and phylogenetic utility of RPB2 duplicates

4.4.1. Early diverging families

Two variants of RPB2 were retrieved from *Pennantia* (*Pen1* and *Pen2,* both forming a clade sister to the outgroup species from Aquifoliales), but we were unable to determine whether these represent duplicated copies or merely alleles of the same locus. If the two variants of *Pennantia* represent a duplication event, it would be the most recent of the duplications detected in this study (mean age of 32.29 Mya). This is also reflected in the remarkably high similarity of the exons (> 95%) and even the introns (> 85%) of the two variants. The only sizeable indel is an insertion of 32 bp at the 3′ end of intron 22 in *Pen2*. All chromosome counts reported for the species of *Pennantia* show a diploid number of $2n = 50$ and no polyploidy has been reported (Murray and De Lange 1995). The high level of intron similarity and the lack of evidence of polyploidy provide reason to suspect that the two variants may represent two alleles of the same locus, perhaps resulting from an old hybridization event within the genus, especially since hybrids between *Pennantia* species have been documented (Gardner and De Lange 2002). It is also worth noting that he placement of *Pennantia* in our phylogeny (among the outgroup taxa), coupled with the lack of morphological links to Apiales, casts some doubts as to its inclusion in the order.

The two RPB2 sequences detected in Griseliniaceae are clearly two distinct copies. Copy 2 (*Gr2*) was much longer than copy 1 (*Gr1*) due to a large insertion (~ 177 bp), but when this long insertion was excluded from the alignment, sequence similarity between the two copies was ~ 85% (> 95% in exons). The placement of the two copies of Griseliniaceae relative to Torricelliaceae was not well supported and varied between the ML and Bayesian trees. However, the two groups always constituted one clade, sister to the rest of Apiales, in trees based on exons + introns (Figs. 1, 3e, and 4b). The placement of *Gr2* as sister to a clade including both *Gr1* and Torricelliaceae in both the MP and ML trees (Fig. 1) may suggest a shared ancestry of the duplication of RPB2 in the two families. We also identified a third variant or RPB2 identified from *Griselinia lucida*, but this was a chimeric sequence, with evidence of recombination at exon 21.

4.4.2. Pittosporaceae

Based on the data matrix that includes both introns and exons, the two copies of RPB2 found in Pittosporaceae (*Pi1* and *Pi2*) are orthologous to the two copies found in Araliaceae, Myodocarpaceae, and Mackinlayoideae (Fig. 1). The placement of these copies is resolved and well supported (*Pi1* BS = 86%; *Pi2* BS = 92%; Figs. 3c and 3e) and agrees with the placement based on chloroplast data (Chapter 1). Our primers did not produce homogeneous PCR products ready for direct sequencing without cloning, but they heavily favored the amplification of *Pi1*. Consequently, *Pi1* was retrieved from clones of all the species sampled from Pittosporaceae, whereas only three of the species had clones with inserts of *Pi2*. As mentioned above (see Results), *Pi1* is distinct from all other RPB2

sequences from Apiales included in this study due the loss of intron 19 (~88 bp) and because of a large insertion at the beginning of intron 20 (~250 bp). The insert did not match any region of any sequence from Apiales or from any other sequence of RPB2 on GenBank, which led us to suspect that it may have resulted from a rearrangement that was old enough to accumulate substitutions and indels over time. Distinctive features in *Pi2* include three large synapomorphic insertions in introns 18 (~30 bp) and 22 (~ 45 bp and 12 bp). No indels were observed in the exons. Excluding indels, sequence identity between the two copies was higher than 77% in the overall sequence and higher than 86% in exons.

Most chromosome counts available for Pittosporaceae have a diploid chromosome number of $2n = 24$ (IPCN; Ito *et al*., 1997; Kiehn, 2005). Although three counts on unidentified species of *Pittosporum* reported 32-36 somatic chromosomes (Ito *et al*., 1997), polyploidy has not been confirmed in the family, nor did we find variation between RPB2 sequences from the same species that were high enough to indicate polyploidy. The most significant variation among clones within the same copy was found in copy *Pi1* of *Pittosporum undulatum*, which showed a difference of nine substitutions (0.87%), only one of which was a transversion. This difference is best interpreted as allelic heterozygosity, which is not surprising when we consider the wide distribution, fast growth, and invasiveness of this species.

Phylogenetic relationships within Pittosporaceae may be inferred from copy *Pi1*, which includes all genera of the family in a fully resolved, well supported lineage (Fig. 3c). Relationships among the nine genera are well resolved and in most cases well supported. Two major clades emerge within this lineage: the first groups *Pittosporum*,

Bursaria, *Rhytidisporum*, and *Auranticarpa* (BS= 59%), while the second groups *Billardiera*, *Marianthus*, *Cheirenthera*, *Bentleya*, and *Hymenosporum* (BS= 80%) (Fig. 3c). The placement of *Hymenosporum* is of greatest interest because prior studies showed it either in a polytomy relative to the other two clades, or in one of the two main clades of Pittosporaceae but with poor support (Chandler *et al.*, 2007; Chapter 1). In the *Pi1* lineage, *Hymemosporum* is sister to the clade comprising *Billardiera*, *Marianthus*, *Cheirenthera*, and *Bentleya*, with considerable bootstrap support in the ML (80%) and MP (67%) trees.

4.4.3. Araliaceae

The taxa sampled from Araliaceae were well represented by both copies of RPB2. Copy 1 (*Ar1*) sequences ranged in length from 996 to 1070 bp, with the exception of *Chengiopanax*, in which intron 19 (~120 bp) was deleted in its entirety. Sequences of copy 2 (*Ar2*) were all slightly smaller than 1000 bp, mostly ranging between 950 and 992 bp. The size variation between the two copies was too small to detect on agarose gels, making it difficult to screen *Ar1* and *Ar2* inserts. The pairwise comparisons between the two copies in Araliaceae (excluding *Hydrocotyle* and *Trachymene*) showed a sequence similarity higher than 70% across the entire region sequenced, and higher than 90% across the exons. Two synapomorphic indels were found in intron 22 of *Ar1*, both insertions of ~15 bp. The PCR process favored the amplification of *Ar1* over *Ar2* in a ratio of about 4:1. A few chimeric sequences were retrieved, exhibiting recombination between the homologous copies at exons 20 (e.g., *Hedera helix*), exon 21 (e.g., *Hedera helix*, *Merilliopanax chinensis*, and *Neopanax colensoi*), and exon 22 (e.g., *Raukaua anomalus*). Both copies

showed evidence of allelic variations within species, most of which had an identity of ~99%. Examples include *Panax quinquifolius* (97.87% identity), *Schefflera morototoni* (99.12%), and *Trevesia palmata* (98.64% identity) of *Ar1*, and *Gastonia rodriguesiana* (98.6 %) of *Ar2*.

In the Araliaceae clade based on copy *Ar1* (Fig. 3d), three main groups provide the greatest information related to the evolution of RPB2. These are the Asian Palmate clade, the *Polyscias-Pseudopanax* clade, and the *Raukaua-Schefflera s. str.* clade, each of which has its own complicated pattern of evolution. Genera of the Asian Palmate clade were divided into two subclades (BS = 69%), with sequence variants (derived from clones of the same samples) identified in *Hedera helix*, *Dendropanax arboreus*, *Chengiopanax sciadophylloides* (ML phylogeny only), and *Trevesia palmata* appearing in both clades (Fig. 3d). The placement of allelic variants from the same samples in different subclades could be explained by a polyploid event in the most recent common ancestor of the two subclades, where hybridization (rather than coalescence) produced the two alleles (*AP1* and *AP2*; Fig. 3d). The inference of polyploidy is further supported by the findings Yi *et al*. (2004), who deduced that a polyploidy event occurred very early in the history of the Asian Palmates, and that the few diploids in this group resulted from later events of ploidal reduction. This is not surprising since the Asian Palmate clade includes a high proportion of polyploids, especially tetraploids ($2n = 48$), and is suspected to have experienced both a rapid radiation and subsequent reticulation events that hindered the resolution of relationships within the group (Mitchell and Wen, 2004; Plunkett *et al*., 2004*b*; Yi *et al*., 2004). Associated with the Asian Palmate genera are the Asian-Pacific genus *Osmoxylon*,

which appears sister to the *AP2* clade of the Asian Palmates (BS = 55%), and the African genera *Cussonia* (BS = 89%) and *Seemannaralia* (BS = 63%), which form successive sisters to the Asian Palmates + *Osmoxylon* clade (Fig. 3d).

The *Polyscias*-*Pseudopanax* clade (Fig. 3d) includes genera associated with *Polyscias sensu lato* (including *Gastonia*, *Reynoldsia*, *Tetraplasandra*, *Munroidendron*, *Cuphocarpus*, and *Arthrophyllum*) and *Pseudopanax* (*Neopanax*, *Meryta*, and Melanesian *Schefflera*). Like other Araliaceae, the taxa in this clade share a base chromosome number $x = 12$, with tetraploidy reported from species of both subclades, but more commonly in the *Pseudopanax* clade. Most species of *Polyscias sensu lato* with reported chromosomal counts are diploid ($2n = 24$), with the exception of *Munroidendron* and *Tetraplasandra* (IPCN, 1979-2007; Yi et al. 2004). The tetraploidy of *Tetraplasandra* may explain the placement of two variants as polyphyletic with respect to the other genera. Similarly, sequences from *Gastonia crassa* appear polyphyletic in *Ar1*. Although chromosome counts have not been reported from any *Gastonia* species, this placement makes us suspect polyploidy in this genus, which warrants further investigation into the origin of known polyploid species in *Polyscias s. lat.* group and whether they originated from ancient hybridization events. Polyploidy in *Polyscias s. lat.* is more likely a relatively recent occurrence, predated by the divergence of the *Polyscias* and *Pseudopanax* subclades.

Of the chromosome counts available from the species of the *Pseudopanax* subclade (*Pseudopanax*, *Neopanax*, Melanesian *Schefflera*, and *Meryta*) (IPCN 1979-2007; Yi *et al*., 2004), all are $2n = 48$, suggesting that tetraploidy may be an ancestral state in the subclade. There is additional evidence of this conclusion from the number of variable sequences of

inserts retrieved for many of its species. The process of sequence editing and comparison in the *Pseudopanax* group proved to be the most complex and confusing in Apiales. Through the ML and MP analyses, it became clear that sequences derived from the same species of this group formed two unrelated clades, one sister to the *Polyscias s. lat.* group (*Px1*; Fig. 3d) as expected based on prior studies, and another (*Px2*; Fig. 3d) associated with the *Raukaua*-*Schefflera s. str.* clade. Within Araliaceae RPB2 copy *Ar1*, different sequences derived from the same species (e.g., *Schefflera pickeringii*, *Schefflera costata*, *Neopanax arborus*, *N. colensoi*, and *Pseudopanax lessonii*) did not appear monophyletic but were instead separated into two distantly related clades, either copy *Px1* or *Px2*. The same pattern is evident in *Ar2*, where one sequence from *Neopanax arboreus* is placed sister to *Raukaua*, whereas the remaining *Ar2* sequences from the *Psuedopanax* group are placed in a clade with *Polyscias s. lat* (Fig. 3e). The most probable explanation for this finding is that an allopolyploid event occurred very early in the evolution of Araliaceae. Hybridization between the ancestors of the *Polyscias* group and the *Raukaua*-*Schefflera s. str.* group, (the extant species of which are mostly diploid) might have led to the emergence of the tetraploid *Pseudopanax* group.

In addition to sequences of the *Pseudopanax* group, the *Raukaua*-*Schefflera s. str.* clade includes many genera with Pacific distribution whose placements were usually unresolved or poorly supported in prior studies (Chandler and Plunkett, 2004; Plunkett *et al*., 2004*b*, 2005). These include the Australian *Cephalaralia*, *Motherwellia*, and *Astrotricha*, the New Zealand *Raukaua*, the mostly Hawaiian *Cheirodendron*, and the Pacific *Schefflera s. str.* (Fig. 3d). Bootstrap support for the clade that includes these taxa

plus *Pseudopanax et al*. is 66%, but the relationships among the genera in this clade are not supported. *Harmsiopanax* is also part of this clade, but in the phylogeny based on exon sequences (BS = 55%; Fig. 2) it appears sister to the *Hydrocotyle* clade. The sister-group relationship between *Harmsiopanax* and the *Hydrocotyle* clade is similar to the relationship estimated by the ML phylogeny based on plastid *trnD-trnT* and *rpl16* data (Chapter 1).

Most previous phylogenetic studies of Apiales have shown that *Hydrocotyle* and *Trachymene* form a clade sister to the rest of Araliaceae (Chandler and Plunkett, 2004; Chapter 1). In the MP phylogeny (Fig. 1), *Hydrocotyle* is sister to copy *Ar1* (BS < 50%) and *Trachymene* sister to *Ar2* (BS = 100%), which is not the same in the ML phylogeny, where *Ar1* is paraphyletic with respect to *Hydrocotyle* (Figs. 1 and 3d) and *Ar2* is paraphyletic with respect to *Trachymene* (Figs. 1 and 3e). Both *Hydrocotyle* and *Trachymene* were sequenced directly from PCR reactions. When cloned, they showed the presence of only one copy, despite relaxation of PCR conditions. This is surprising, considering the high level of polyploidy reported in the two genera, especially *Hydrocotyle* where counts as high as $n = 48$ have been reported for species represented in our study (IPCN, 1979-2007; Pimenov *et al*., 2003). Pseudogenization or differential gene loss may be a factor that hindered the estimation of the organismal relationship between the *Hydrocotyle* and *Trachymene*.

4.4.4. Myodocarpaceae

We retrieved the two copies of RPB2 from both *Myodocarpus* and *Delarbrea*, the only two genera of Myodocarpaceae (copies *My1* and *My2*; Figs. 1, 3, and 4). Size variation was significant between the two copies due to large indels in intron 22, resulting in total length increase of ~150 bp in *My1*, facilitating the identification of each copy on agarose gels. No indels were observed in exons. The two copies showed sequence similarity of ~65% across the entire region (but > 75% when excluding the indels in intron 22) and ~91% in exons. The PCR process favored copy 1 in *Myodocarpus* and copy 2 in *Delarbrea*. Two cloned sequences derived from *Myodocarpus crassifolius* were chimeric, showing a recombination point around the middle of exon 22. Chromosome counts reported for Myodocarpaceae show a gametophyte number $n = 12$. No evidence of polyploidy has been identified in this family and no RPB2-sequence variation within species was great enough to be attributed to anything other than allelic heterozygosity.

In the ML phylogeny, Myodocarpaceae appears as a lineage which diverged after Araliaceae and before Apiaceae. This placement is in agreement with the phylogeny estimated from plastid markers (Chapter 1) but disagrees with the suggestion of a "tentative sister group-relationship" with Pittosporaceae suggested by Chandler and Plunkett (2004). Our placement also agrees with the long-held view of *Myodocarpus* and *Delarbrea* as "morphological intermediates" between Araliaceae and Apiaceae (see Plunkett and Lowry, 2001). In the MP tree, Myodocarpaceae appeared sister to the *Platysace* clade, but with low support (< 50%).

4.4.5. Apiaceae

The subfamilies of Apiaceae are addressed below in three main sections, following the current subfamilial classification of Mackinlayoideae, Azorelloideae, and subfamilies Apioideae and Saniculoideae together. Unlike the other families of the order, most of the genera in Apiaceae are herbaceous and hence exhibit higher rates of substitution than the mostly woody taxa of the rest of Apiales. This is obvious in the pairwise comparisons of homologous sequences and the mean genetic distances of exons where Mackinlayoideae and Apioideae + Saniculoideae have the highest genetic distances (Table 3).

a) Mackinlayoideae (including Platysace *and* Homalosciadium*)*

The placement of Mackinlayoideae relative to Araliaceae and Myodocarpaceae was similar in both copies of RPB2 (*Mk1* and *Mk2*; Figs. 1, 3, and 4). The subfamily shares the orthologous copy 2 with Pittosporaceae (*Pi2*), Araliaceae (*Ar2*), and Myodocarpaceae (*My2*), but this copy is not found in the rest of Apiaceae, as might be expected. Sequence comparison between *Mk1* and *Mk2* was difficult due to the presence of many small indels across the introns, especially intron 22. We were not able to identify long indels that could be used to distinguish one copy from the other. This was exacerbated by the high variation between the main lineages of Mackinlayoideae and the failure to retrieve copy 1 from some key genera (e.g., *Apiopetalum*). Sequence variations between the two copies of the same species in *Xanthosia, Actinotus*, and *Mackinlaya* were < 70%. Polyploids have been reported in *Centella asiatica* and in some species of *Xanthosia* that were not sampled here (Keighery 1982; Hart, 2000; Pimenov *et al*., 2003). We did not encounter any differences

in the same copy within species that could indicate ancient polyploidy, but significant allelic variations due to heterozygosity were observed in *Actinotus helianthii* (98.1% identity) and *Centella linifolia* (98.5% identity, excluding an 82-bp insertion in intron 21 of one of the alleles). No size differences were observed in the exons.

Our PCR process favored *Mk2*, for which we were able to derive sequences from all genera of Mackinlayoideae. Eventually, the *Mk2* clade provided better information regarding phylogenetic relationships in the subfamily. We were able to amplify *Mk1* from *Actinotus*, *Mackinlaya*, *Xanthosia*, *Centella*, and *Pentapeltis*. We also retrieved a copy orthologous to *Mk1* from *Platysace* and *Homalosciadium*, two Australian genera usually associated with Mackinlayoideae. Consequently, orthologs of copy 1 provided a better estimation of relationships relative to other main lineages due to overall better representation of copy 1 across the main lineages of Apiales, and specifically Apiaceae. Relationships within Mackinlayoideae *Mk2* were very well resolved, and in most cases supported (Fig. 3e). In the ML phylogeny of copy 1, *Platysace* and *Homalosciadium* appeared sister to the rest of Mackinlayoideae (BS = 70%). However, in the MP tree, Myodocarpaceae, Mackinlayoideae, and the *Platysace* clade formed a clade that was sister to the rest of Apiaceae, but support for this clade and its internal relationships was poor (BS < 50%; Fig. 1). It is evident that the clade has undergone dysploid or aneuploid reduction from $x = 12$ (in *Apiopetalum*; Yi *et al*., 2004) to $x = 10$ (in *Actinotus*, *Xanthosia*, *Chlaenosciadium*, and *Micopleura*) to $x = 9$ (in *Centella*), and even as low as $x = 5$ (in *Pentapeltis* and *Shoenolaena*) (IPCN, 1979-2007; Keighery, 1983; Pimenov *et al*., 2003), but both copies have been preserved, even in *Pentapeltis*, with only 5 chromosomes.

b) Azorelloideae

Azorelloideae have their highest taxonomic diversity in South America, but there is also a clear connection to Oceania. The subfamily includes four major clades: the *Asteriscium*, *Bowlesia*, *Azorella*, and *Diposis* clades (Chapter 1). The Brazilian genus *Klotzschia* is sometimes placed as sister to the rest of Azorelloideae, but support for this placement has not been very strong. The relationships among the five clades of Azorelloideae are resolved in some trees, but have usually received low support. Unlike results from other studies, RPB2 data do show a well supported sister-group relationship between the *Azorella* and *Bowlesia* clades (BS = 100%; Fig. 3b), but *Klotzschia*, *Diposis*, and the *Asteriscium* clade form a poorly supported group (BS < 50%) sister to Apioideae + Saniculoideae, rather than to *Azorella* + *Bowlesia*. The *Azorella* and *Bowlesia* clades share the same basic chromosome number of $x = 8$, whereas that of the *Asteriscium* clade is $x = 10$ (IPCN, 1979-2007; Constance *et al*., 1976; Pimenov *et al*., 2003). This may indicate a closer relationship in the evolution of the nuclear genome in these two clades.

The *Azorella* clade was the only lineage of Azorelloideae in which two copies of RPB2 were found (*Az1* and *Az2*; Fig. 3b). The duplication event can be mapped to the root of the *Azorella* clade and, apart from the variants detected in *Pennantia* (which may or may not represent duplicate copies, see above), it is the most recent of the duplications reported herein. Sequence identities between *Az1* and *Az2* sequences from those species having both copies ranged between 73% and 77% across the whole region, and between 85% and 90% in the exons alone. Hence, the two copies were easy to align and, when considered separately from the rest of the taxa, resulted in an aligned data matrix of 1143 bp, where

the longest unaligned length was 1052 bp (*Az2* of *Azorella fuegiana*). A few indels (unique to one or the other of the copies) were scattered across the introns, all of which were smaller than 10 bp except for an insertion of 14 bp in intron 19 that was synapomorphic to *Az2*. We also observed one insertion of two codons in exon 22 of *Az1*. On average, *Az2* (slightly more than 1000 bp) was longer than *Az1* (slightly smaller than 1000 bp). One chimeric sequence was retrieved from *Mulinum chilenense*, with a cross-over point at intron 19.

Both copies show that the genus *Azorella* is polyphyletic, a finding consistent with results from plastid data (Chapter 1). Copy *Az1* indicates that *Schizeilema* is paraphyletic, with *S. ranunculus*, the only South American species of *Schizeilema*, forming a lineage apart from the rest of the genus (Fig. 3b). Both copies yielded clades that were mostly resolved and well supported. The relationship among *Schizeilema*, *Huanaca*, *Stilbocarpa*, *Azorella filamentosa*, and *A. fuegiana* (the *Schizeilema et al*. clade) remains unresolved. We suspect that reticulation may have been a major factor in the evolution of this clade. We found three variable sequences in *Stilbocarpa*, with a sequence variation as high as 7%. Two of these sequences were very similar exhibiting ~99% identity, and thus we used only one of them in the data matrix. *Stilbocarpa* is tetraploid ($2n = 48$), so we suspect that that the high allelic variation may be the result of polyploidization. Polyploidy is also very common in *Schizeilema*, where chromosome counts up to $2n = 80$ have been reported. *Schizeilema ranunculus* is the only diploid member of *Schizeilema* ($2n = 16$). *Azorella filamentosa* and *A. fuegiana,* which are affiliated with the other species of *Schizeilema,* are both diploid. *Huanaca acaulis* was reported to have a diploid count of $2n = 18$, but this is

very likely a result of dysploidy or aneuploidy since all reported numbers in the *Azorella* clade suggest a basic chromosome number $x = 8$. The prevalence of polyploidy and the retrieval of clones with high variation in *Stilbocarpa* may be an indication of ancient hybridization events between diploid ancestors ($2n = 16$), and this may account for the lack of resolution among these taxa in the trees resulting from our phylogenetic analyses. Outside of the *Schizeilema et al*. clade, tetraploids have been reported in other *Azorella* species and in *Mulinum*. The placement of one of these tetraploids, *A*. *trifoliolata*, at the base of the *Schizeilema et al*. clade (BS = 100%), differed from that of the plastid phylogeny, where it appears sister to *A*. *monantha* (BS = 100%) in the *Azorella* clade, far from *Schizeilema et al*.. *Azorella monantha* and *A*. *trifoliolata* are not morphologically similar and the latter may represent an allopolyploid whose initial hybridization event may be the source of the incongruent histories recovered from the plastid and nuclear markers.

c) Apioideae-Saniculoideae

Subfamilies Apioideae and Saniculoideae share a duplication event that post-dates the divergence of *Hermas* (BS = 100%; Figs. 1 and 3a). The placement of *Hermas* relative to the rest of Apiaceae has been problematic, but both our plastid phylogeny (Chapter 1) and the RPB2 phylogeny place it as sister to the Apioideae + Saniculoideae lineage (BS = 83%; Fig. 3a). Most species of Apiaceae gave homogeneous PCR products (apart from a few nucleotide polymorphisms), which allowed direct sequencing without cloning. However, these sequences were placed in two different clades and represented two distinct copies. After cloning, we were able to sequence both copies for some genera in Apioideae

(*Petroselinum*, *Peucedanum*, *Notiosciadium*, *Aegopodium*, *Angelica*, and *Astydamia*) and Saniculoideae (*Eryngium* and *Petagnaea*). However, some sequences from genera of Saniculoideae or the early diverging lineages of Apioideae, were placed either with copy *Ap-Sa1* (e.g., *Astrantia* and *Andriana*) or *Ap-Sa2* (e.g., *Heteromorpha*, *Anginon*, *Sanicula*, and *Arctopus*) but not both, despite repeated efforts to adjust PCR conditions and to collect data from additional clones. The lack of both copies from all species did not interfere with the main conclusions of phylogenetic relationships because the clade representing copy *Ap-Sa1* included species from all major clades.

Apiaceae exhibited the greatest sequence variation among species within the individual copies, among alleles of the same species, and between paralogs of RPB2 of the same species when compared to the other main lineages of Apiales (see mean distances; Table 3). This finding may be explained, at least in part, by the great species diversity among the apioids, and by their largely herbaceous habit. Alleles with high variation were recovered in *Ap-Sa1* of *Daucus* (96.31% identity), *Petagnaea* (98.09%), and *Astydamia* (95.17%) (Fig. 3a). The introns included many autapomorphic indels, and the only synapomorphic length variation of considerable size was a ~15 bp indel in intron 22. RPB2 appeared to have great potential to resolve relationships within and among tribes of Apioideae. The species of this clade exhibit a high frequency of dysploidy and polyploidy compared to species from other families and subfamilies of Apiales, suggesting that the history of the nuclear genome in this group is more complicated. More detailed work using multi-copy genes such as RPB2 may uncover patterns of evolution that would not be detected with the use of single-copy nuclear markers or organellar genomes alone. An

example of the complexity of the history of the nuclear genome is evident in the Saniculoideae and some of the early-diverging lineages of Apioideae for which variations in chromosome numbers have been reported. Counts include $n = 12$ (e.g., *Steganotaenia*), which is the same as that of Araliaceae and the other woody families, as well as $n = 11$ (e.g., *Heteromorpha*, *Lichtensteinia*), $n = 9$ (Arctopus), $n = 8$ (e.g., *Alepidea*, *Sanicula*), and $n = 7$ (e.g., *Hermas*, *Astrantia*) (IPCN, 1979-2007; Constance and Chuang, 1982; Pimenov *et al*., 2003). The assignment of the early diverging lineages, mostly from southern and sub-Saharan Africa, to either Apioideae or Saniculoideae remains a major problem in the classification of Apiaceae. Similar problems persist in Azorelloideae (e.g., the placement of *Klotzschia*) and Mackinlayoideae (e.g., *Platysace*). This lack of resolution along the "spine" of the Apiaceae tree creates an obstacle to understanding the duplication of RPB2 in this clade in relation to their cytotaxonomy.

4.5. Putative Model of RPB2 duplication in Apiales

Raven (1975) "cautiously" postulated a base chromosome number $x = 6$ for Umbellales (= Apiales). Yi *et al*. (2004) suggested the same for a more broadly defined Apiales after considering many additional chromosome counts, and the taxonomic transfer of *Hydrocotyle* and *Trachymene* to Araliaceae. The RPB2 phylogeny is largely consistent with these suggestions. The oldest duplication of RPB2 in Apiales appears to be almost as old as the order itself (c. 100 Mya) and probably resulted from a paleopolyploid event that led to the doubling of chromosome numbers to $n = 12$. That number was maintained in Pittosporaceae, Araliaceae, and Myodocarpaceae. In Araliaceae, a more recent polyploid

event may have been brought about through hybridization (or allopolyplodization) between an ancestor of the *Polyscias* group and an ancestor of the *Raukaua-Schefflera s. str.* group, giving rise to the tetraploid *Pseudopanax* group (with $n = 24$). The *Pseudopanax* group maintained copies related to both the *Polyscias* and *Raukaua- Schefflera s. str.* groups. A similar but more recent event may have occurred in the Asian Palmate clade, giving rise to additional tetraploids in Araliaceae. *Hydrocotyle* ($x = 6$ or 12) and *Trachymene* ($x = 11$) have a shorter generation time than the rest of Araliaceae and may have been subject to reductional dysploidy or to polyploidy (in some cases extensively), possibly leading to the loss of one of the RPB2 copies.

Chromosome counts of $n = 12$ were maintained in *Apiopetalum* (Mackinlayoideae) and *Steganotaenia* (Apioideae-Saniculoideae). Within Apiaceae, these woody genera are considered two of the most ancient lineages of their groups. This indicates that the common ancestors to Apiaceae may have shared a count of $n = 12$, as is still found in Pittosporaceae, Araliaceae, and Myodocarpaceae. Due to the herbaceous nature of most Apiaceae, a shorter generation time may have allowed for a higher probability for fixation of chromosomal rearrangements than in the rest of Apiales. Thus, our results are consistent with Raven's (1975) conclusions that the rest of Apiaceae has probably undergone many chromosomal changes, including both descending dysploidy and polyploidy. This would explain the more recent duplication events in Apioideae-Saniculoideae and the *Azorella* clade, and the possible loss of an RPB2 copy in some Apiodeae-Saniculoideae and other lineages characterized by apparent reduction in chromosome numbers (e.g., the *Bowlesia*, *Asteriscium*, and *Hermas* clades). However, this was not the case in Mackinlayoideae,

where both copies of RPB2 persist in both woody and herbaceous species, despite reductions in chromosome numbers.

Based on the phylogeny and age of the RPB2 duplication, we suspect that Griseliniaceae, Torricelliaceae, and Pennantiaceae diverged before the duplication event that led to chromosomal doubling from $x = 6$ to $x = 12$ in the rest of Apiales. These three families may have been subject to polyploidy (multiple times in some cases) and dysploidy events, independent of the one shared by suborder Apiineae (i.e., the "core" families of Apiales). These events would result in an increase from the hypothesized $x = 6$ to the current counts of $n = 18$ in *Griselinia*, $n = 12$ in *Torricellia*, $n = 20$ in *Aralidium*, and $n = 25$ in *Pennantia* (IPCN, 1979-2007; Murray and Delange, 1995). If the duplication of RPB2 resulted from a paleopolyploid event that resulted in $x = 12$ in Apiales, then it will not be possible for Pittosporaceae, Araliaceae, Myodocarpaceae, and Apiaceae to share a prior paleopolyploid event with Griseliniaceae, Torricelliaceae, and Pennantiaceae. Otherwise, the base chromosome counts for the four families would be $x = 24$, which seems very improbable given known counts from extant species.

5. Conclusion

Differences in habits and the ages of clades have contributed to the complexity of resolving phylogenetic relationships in Apiales, and tackling these problems with the use of additional markers remains necessary. Nuclear markers have been underutilized in phylogenetic studies compared to the size and importance of that genome. While plastid markers have provided a broad framework for understanding relationships in plants in

general and Apiales in particular, the unipaternal inheritance and lack of variation render them insufficient (especially for woody groups) when comparing relations at the lower levels. Nuclear markers are much more complex and must be studied carefully because various forms of chromosomal rearrangements may prove difficult to track and may require detailed cytological work to shed better light on sequence evolution. Such events include gene duplication, which is especially widespread in plants. The RPB2 duplicates isolated here provide a small (but very good) example of the degree of information that can be extracted from low-copy number nuclear genes for exploring the history of Apiales. This information can be taken from both copies resulting from the duplication event, or from either one of the markers studied separately. This study provided data from many new sequences, but also yielded information about some structural variation reflected in those sequences, which may help to develop each copy individually for further, more focused phylogenetic studies (e.g., within the various tribes of Apiaceae). Additional nuclear genes should be studied to compare their histories and extrapolate hypotheses on the history of Apiales. Duplication, polyploidization, ancient hybridization, and deep coalescent events provide evidence that can be useful in revising classification systems.

TABLES

Table 1. List of primers developed during this study for the amplification and sequencing of RPB2 regions from exon 18 to 24 in Apiales.

Primer Name	**Region**	**Sequence** (5' to 3')	**Direction**
rpb2EX18_F	Exon18	ATGGAGCATTTGCACTTTAGGCA	Forward
rpb2_EX20F	Exon 20	GGATGAGGAGAAGAAGATGGGAA	Forward
Az_rpb2EX20F	Exon 20	AGGGAYGAGGAGAARAARATG	Forward
Az_rpb2_EX20R	Exon 20	TCCTCNTTAACAAGTGTYCC	Reverse
rpb2EX21_F	Exon 21	TCATATGATAAATTGGACGATGATG	Forward
rpb2_EX21R	Exon 21	TGCAAGACCATCATCGTCCAA	Reverse
rpb2_EX22F	Exon 22	AAGYTTACGCCACAGYGAAAC	Forward
rpb2_EX22R	Exon 22	ACCTGATCCACCATCCCWGT	Reverse
rpb2EX23_F	Exon 23	CAAATGCTGATGGGCTGAGATTTGT	Forward
rpb2_EX23R	Exon 23	TCACCCTTACTTTCACAAATCTCA	Reverse
rpb2_EX23R_alt	Exon 23	ACAGTTCCTTTCTGACCATGCCTA	Reverse
rpb2EX24_R	Exon 24	GCCAAGGAATATCATAGCTGTAAG	Reverse

Table 2. Comparison of data set and tree statistics based on exon and exon+intron data matrices and trees.

	exons	exons-introns
Aligned length	438	2612
MP tree length	2124	11548
Parsimony informative characters	255	1057
Constant characters	104	1225
Consistency index (CI)	0.28	2.25
Retention index (RI)	0.72	0.8081
ML -ln Likelihood	-12363	-61115.8937

Table 3. Comparative exon statistics of the two copies of RPB2, generated in MEGA 4.0. Mean distances within groups were calculated using the composite likelihood model. Mean rates of synonymous (d_S) and non-synonymous (d_N) substitution rates within groups, as well as Z-tests for positive selection and purifying selection, were calculated using the modified Nei-Gojobori model with Jukes Cantor model of evolution and incorporating the transition to transversion ratio (Ts:Tv).

Clade	# seqs.	Mean distance ± S.E.	Ts:Tv bias
Apioideae-Saniculoideae C1	46	0.0625 ± 0.0070	1.936
Apioideae-Saniculoideae C2	18	0.0813 ± 0.0137	1.386
Azorella C1	19	0.0207 ± 0.0033	2.909
Azorella C2	16	0.0408 ± 0.0053	1.061
Mackinlayoideae C1	5	0.0570 ± 0.0134	1.892
Mackinlayoideae C2	14	0.0683 ± 0.0078	2.065
Myodocarpaceae C1	4	0.0118 ± 0.0042	1.32
Myodocarpaceae C2	5	0.0366 ± 0.0065	1.541
Araliaceae C1	89	0.0368 ± 0.0040	1.591
Araliaceae C2	26	0.0471 ± 0.0058	2.512
Pittosporaceae C1	18	0.0196 ± 0.0034	1.746
Pittosporaceae C2	3	0.0214 ± 0.0059	3.276

Clade	d_S ± S.E.	d_N ± S.E.
Apioideae-Saniculoideae C1	0.2206 ± 0.0231	0.0092 ± 0.0037
Apioideae-Saniculoideae C2	0.1941 ± 0.0247	0.0436 ± 0.0067
Azorella C1	0.0570 ± 0.0100	0.0059 ± 0.0020
Azorella C2	0.0526 ± 0.0115	0.0305± 0.0062
Mackinlayoideae C1	0.1993 ± 0.0347	0.0093 ± 0.0038
Mackinlayoideae C2	0.2071 ± 0.0262	0.0186 ± 0.0046
Myodocarpaceae C1	0.0315 ± 0.0115	0.0049 ± 0.0028
Myodocarpaceae C2	0.0705 ± 0.0187	0.0225 ± 0.0071
Araliaceae C1	0.1031 ± 0.0145	0.0134 ± 0.0024
Araliaceae C2	0.1372 ± 0.0180	0.0128 ± 0.0034
Pittosporaceae C1	0.0380 ± 0.0085	0.0128 ± 0.0037
Pittosporaceae C2	0.0663± 0.0186	0.0034 ± 0.0034

Table 3. Continued.

	Positive selection HA: ($d_N>d_S$)		Purifying selection HA: ($d_N<d_S$)	
Clade	Z-statistic	P-value	Z-statistic	P-value
Apioideae-Saniculoideae C1	-8.448	1	8.6275	0
Apioideae-Saniculoideae C2	-5.5334	1	5.7912	0
Azorella C1	-5.173	1	5.1933	0
Azorella C2	-1.365	1	1.3567	0.0887
Mackinlayoideae C1	-5.558	1	5.6382	0
Mackinlayoideae C2	-7.1391	1	7.1028	0
Myodocarpaceae C1	-2.1723	1	2.1949	0.015
Myodocarpaceae C2	-2.4112	1	2.4653	0.0076
Araliaceae C1	-6.248	1	6.5507	0
Araliaceae C2	-6.8049	1	6.7796	0
Pittosporaceae C1	-2.588	1	2.575	0.0056
Pittosporaceae C2	-3.28	1	3.4188	0.0004

Table 4. BEAST estimates of mean root heights (divergence dates) at the nodes of the RPB2 duplications and the 95% high posterior density (HPD) for each date. Estimates were calculated using the GTR+Γ+I evolutionary model, the Yule model of speciation, and a relaxed clock with uncorrelated lognormal.

Node of Duplication	Divergence Date (MYA)	95% HPD (MYA)
Mackinlayoideae-Myodocarpaceae-Araliaceae-Pittosporaceae	103.94	[93.94,113.21]
Griseliniaceae	71.47	[36.05,103.67]
Apioideae-Saniculoideae	69.36	[58.95,80.80]
Azorella Clade	55.78	[43.46,67.91]
Pennantiaceae	32.29	[10.74,87.48]

FIGURE LEGENDS

Figure 1. Comparative summaries of the maximum parsimony (MP; 1a) and maximum likelihood (ML; 1b) phylogenies. The MP tree is the strict consensus of 50,000 most-parsimonious trees generated using heuristic searches and TBR swapping in PAUP*. The ML tree was estimated in Garli using the model GTR +Γ+I. Bootstrap values are shown above branches.

Figure 2. Summary of the tree estimated in GARLI using exon sequences only. Bootstrap values above 50% are shown above branches.

Figure 3 (a-e). Detailed maximum likelihood tree estimated in GARLI from exon and intron sequences with GTR+Γ+I model of evolution. Percentages of 100 bootstrap replicates above 50% are printed above branches.

Figure 4 (a-b). Maximum clade credibility chronogram estimated from trees generated in BEAST after 10 million MCMC generations. Estimates were calculated using the GTR+Γ+I evolutionary model, the Yule model of speciation, and a relaxed clock with uncorrelated lognormal. Calibration points are represented by black diamonds. Grey-block tracks represent branches to nodes of duplication. Posterior probabilities > 85% are shown above the branches.

Fig. 1. Comparison of major clades in the maximum parsimony (MP; Fig. 1a) and maximun likelihood (ML; Fig. 1b) RPB2 phylogenies.

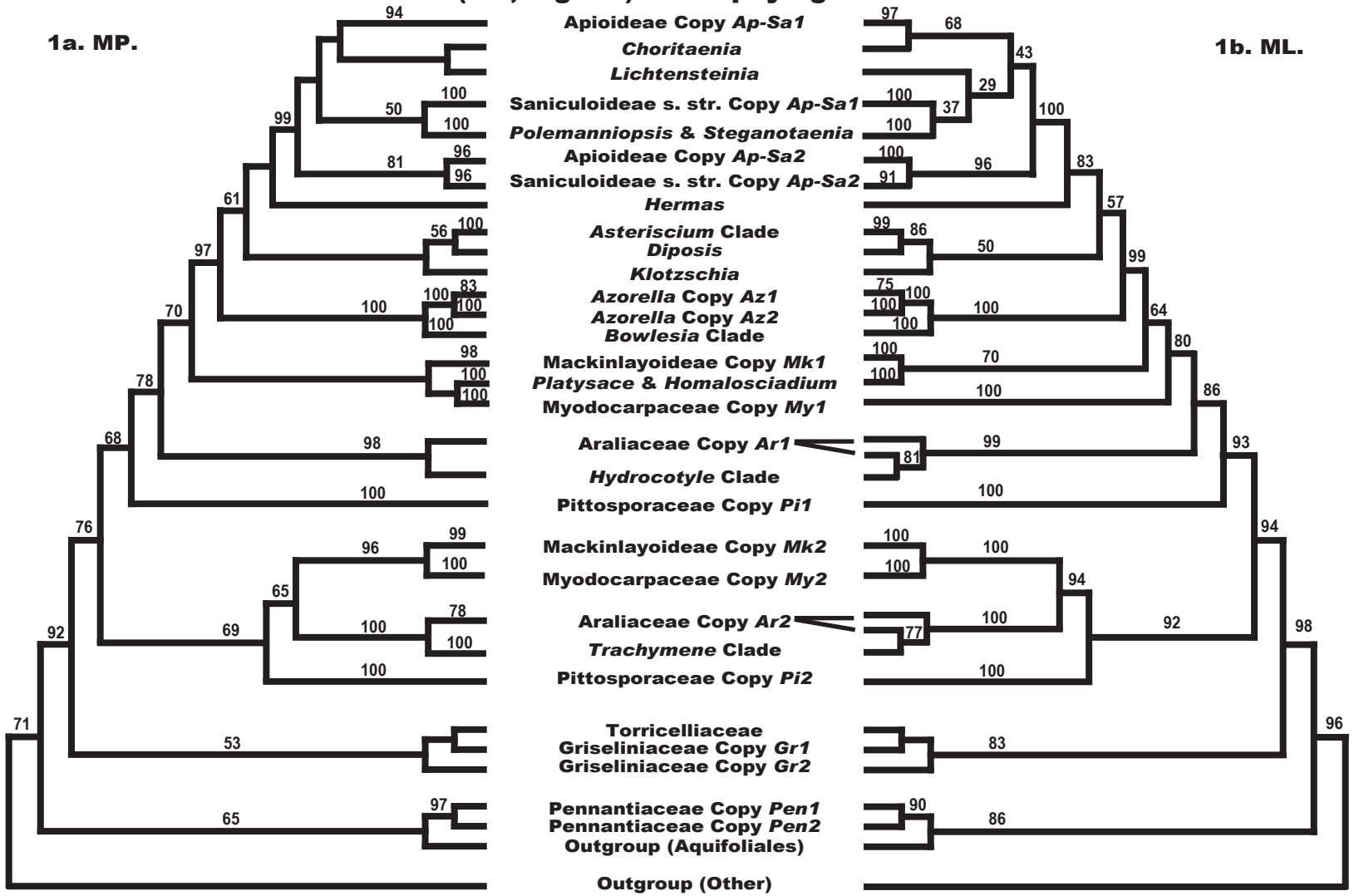


Fig. 2. Summary of the maximum likelihood phylogeny estimated from RPB2 exon sequences.

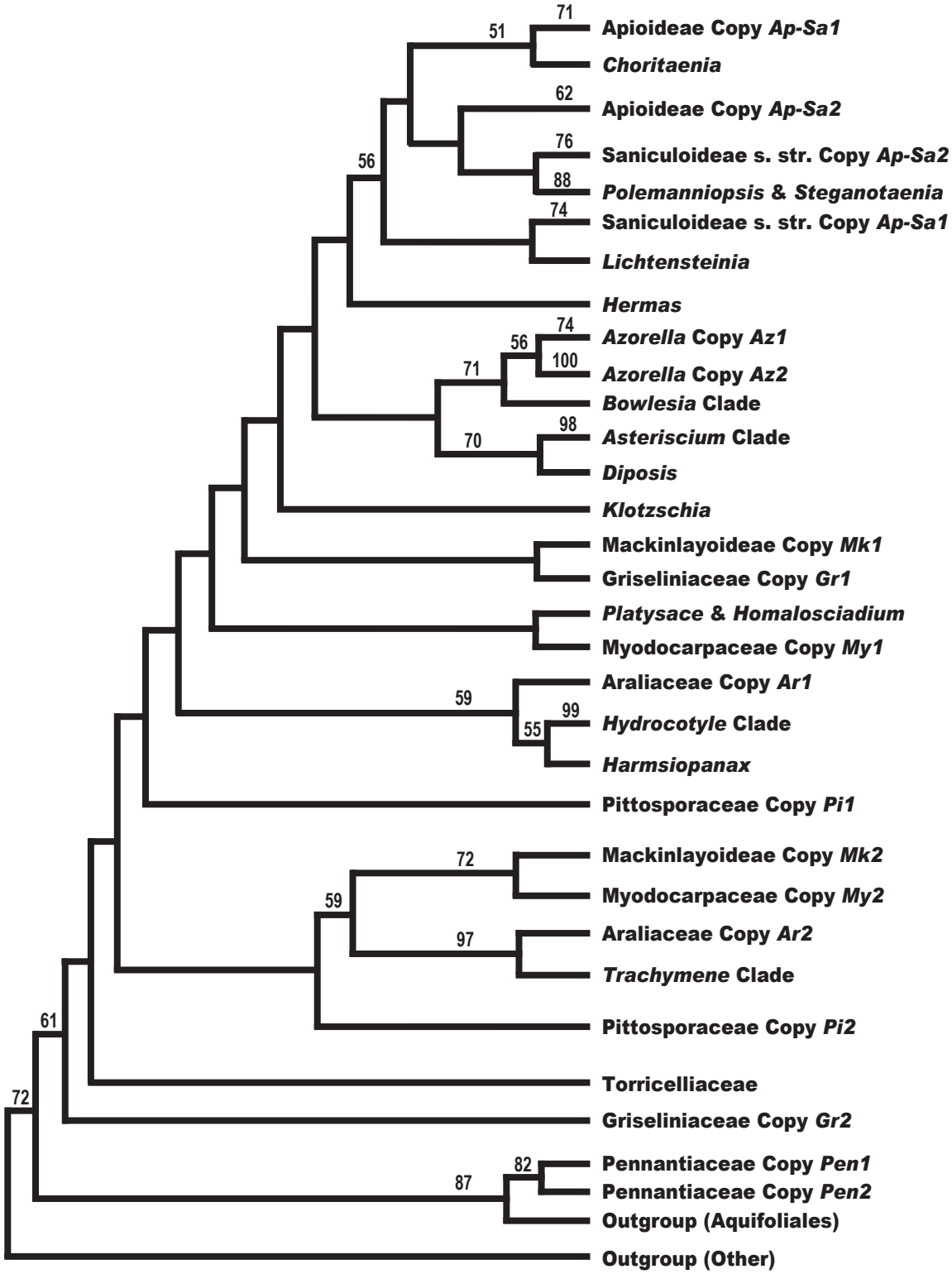


Fig. 3. Maximum likelihood phylogeny of RPB2

3a. Apioideae-Saniculoideae

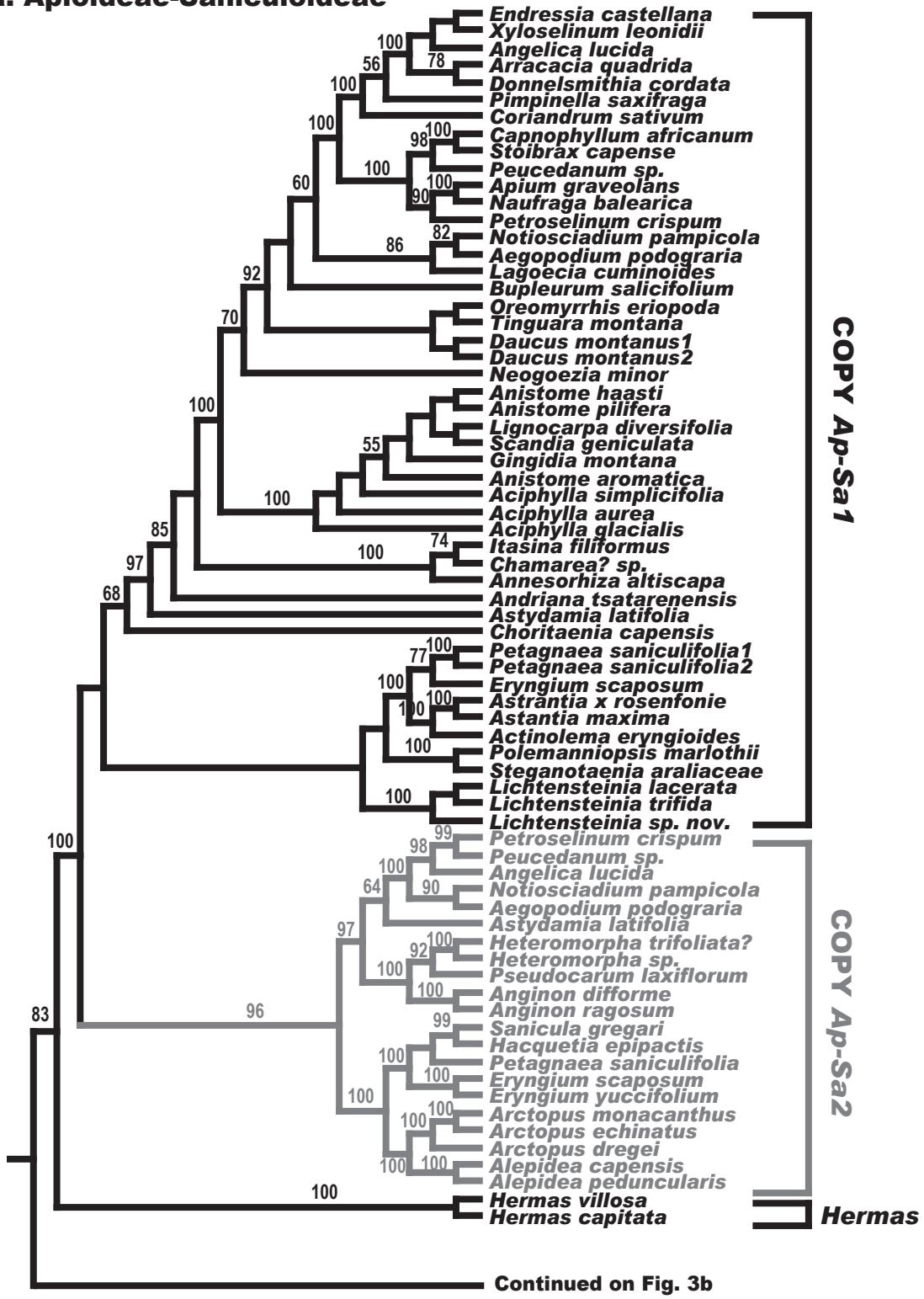


Fig. 3b. Azorelloideae

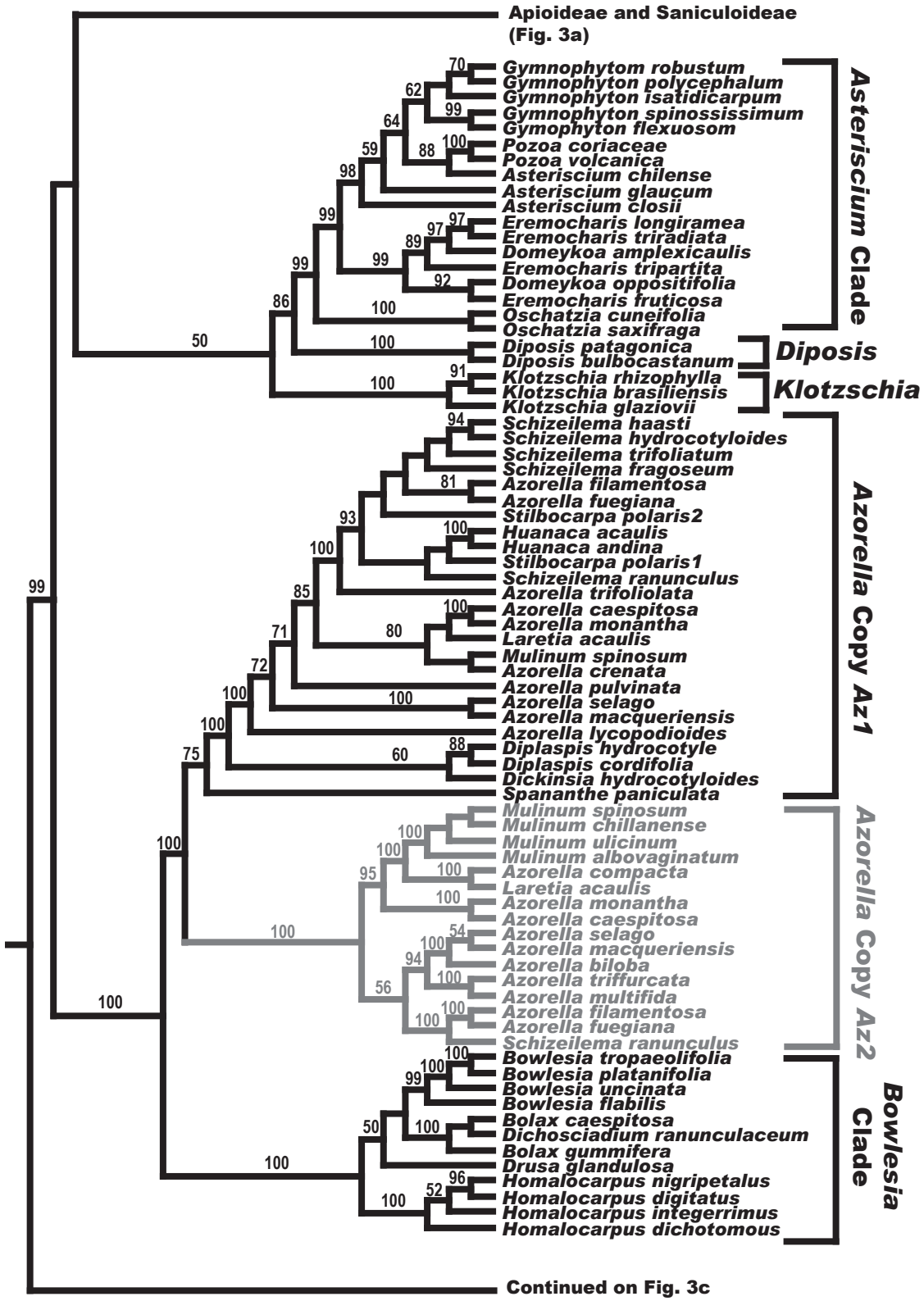


Fig. 3c. Copy 1 Clade of Mackinlayoideae, Myodocarpaceae, and Pittosporaceae

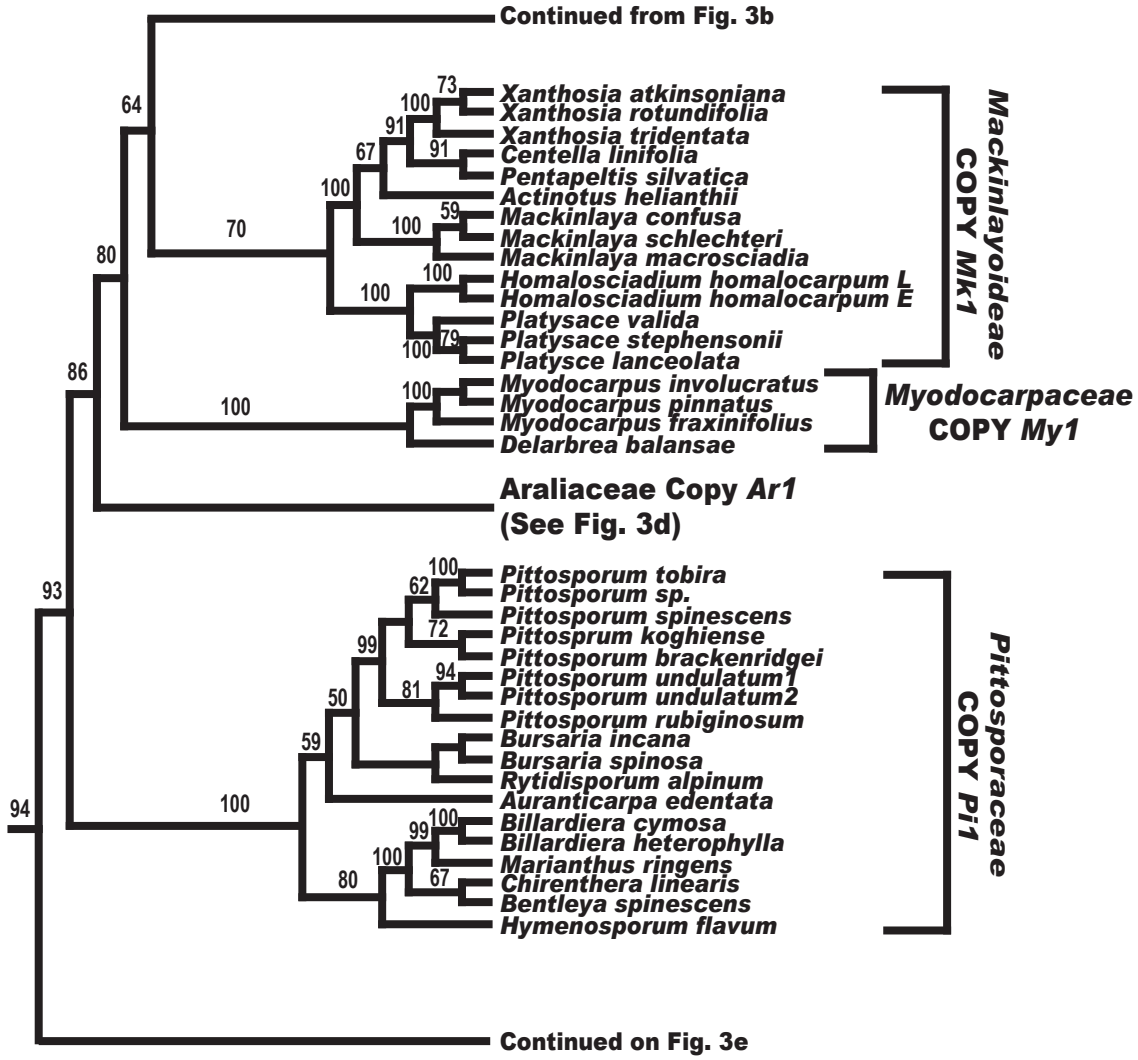


Fig. 3d. Araliaceae Copy *Ar1.*

Fig. 3e. Clade of Copy 2 of Mackinlayoideae, Myodocarpaceae, Araliaceae, and Pittosporaceae, plus clades representing the early diverging families and outgroups.

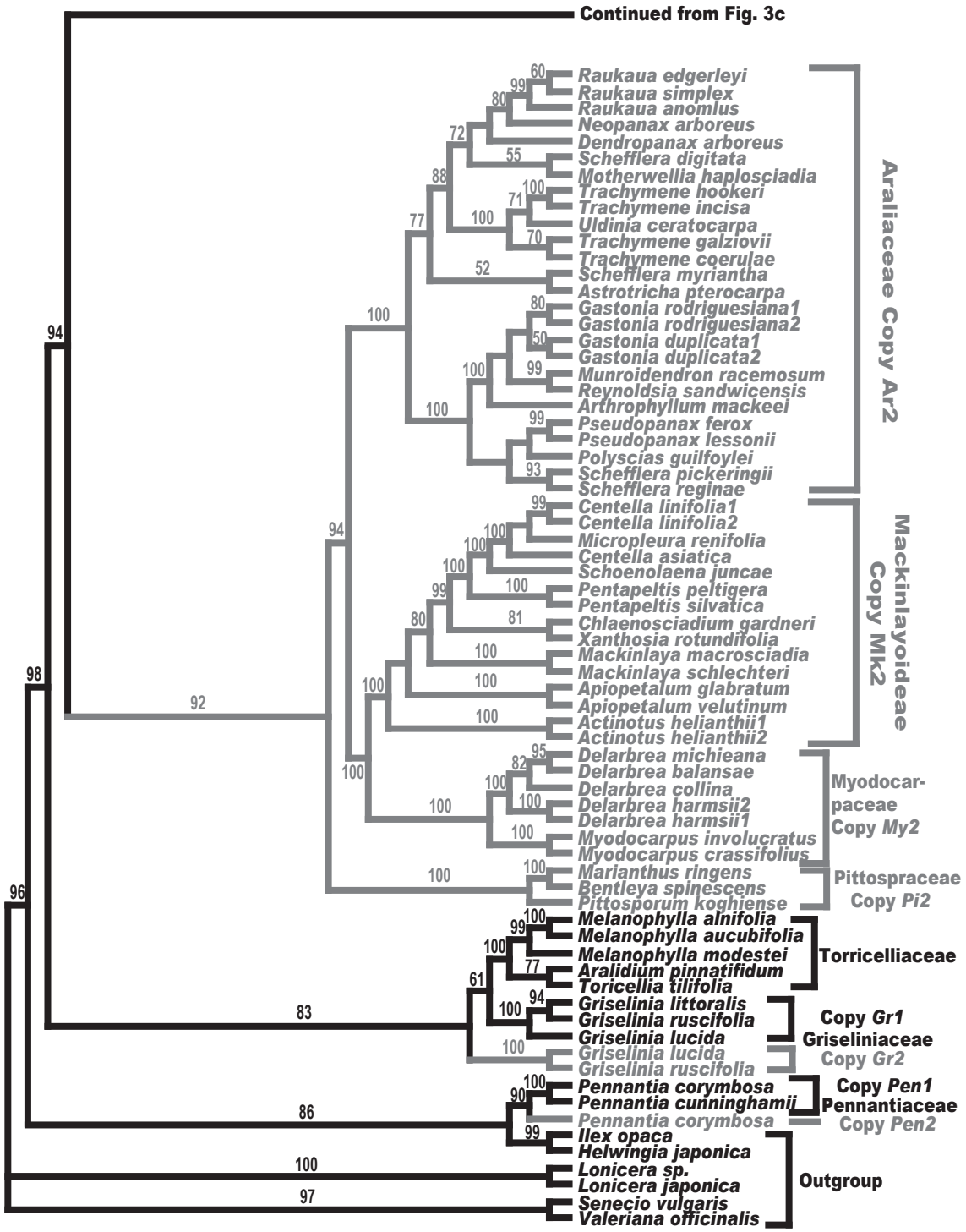


Fig. 4. Bayesian Tree generated in BEAST.

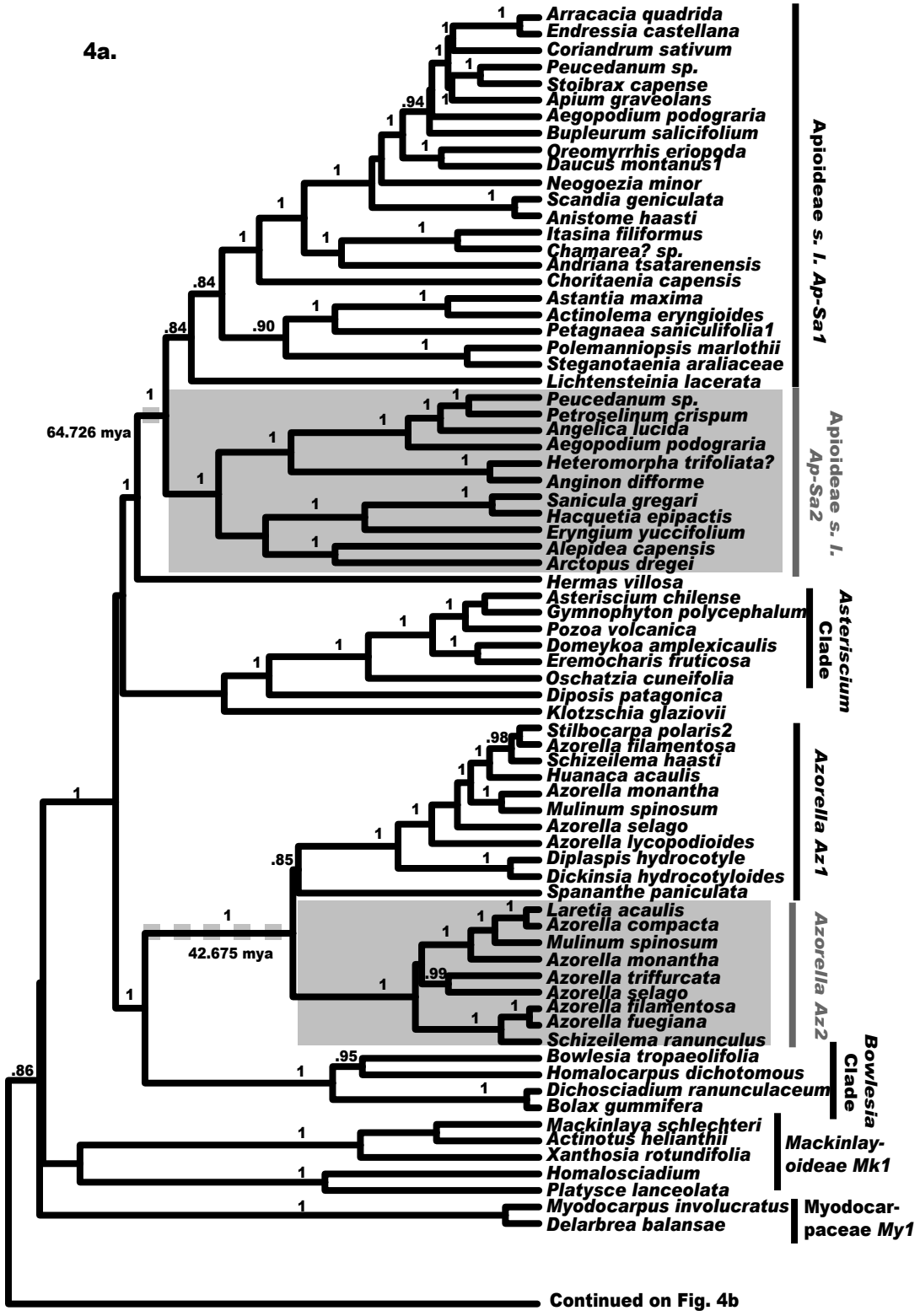
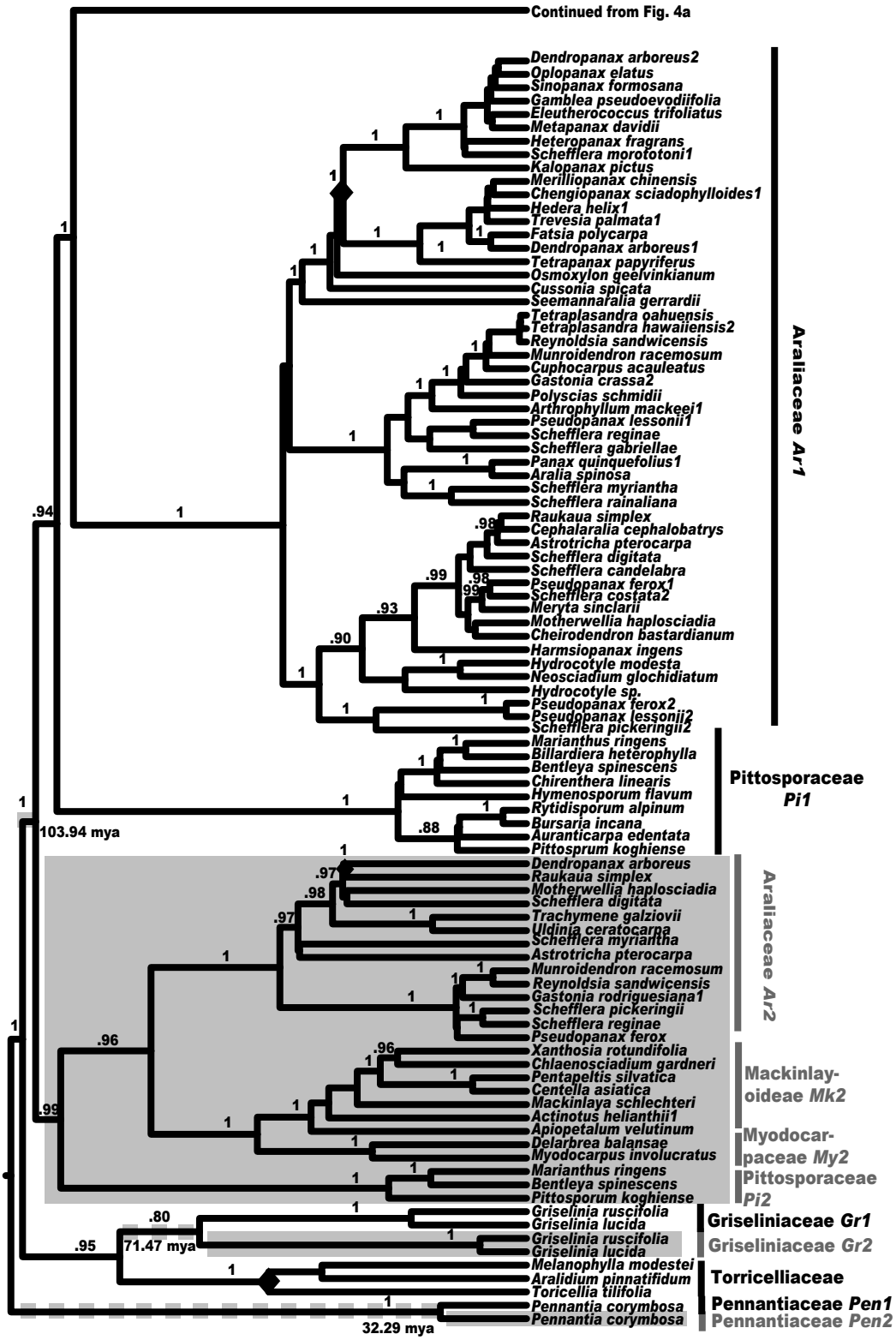


Fig. 4b. Bayesian Tree continued.



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CHAPTER 3

Evolution of Apiales in form, time, and space: Information from the chloroplast, mitochondrial, and nuclear genomes

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Abstract

We generated a dataset of sequences from the *nad1* intron 2 region of the mitochondrion from a representative sample of all major clades across the order Apiales. Phylogenetic analyses were carried out through maximum likelihood (ML) and maximum parsimony (MP) methods. Resulting trees were compared to trees based on plastid *trn*D-*trn*T and *rpl16* sequences (Chapter 1) and nuclear RPB2 exons 18-23 sequences (Chapter 2) to evaluate evidence from three unlinked sources of the genome. The *nad1* intron 2 tree was most useful in resolving well supported relationships within families, but failed to produce many supported ones among families. Trees based on both plastid and nuclear markers were congruent in the placement of Pittosporaceae as the earliest diverging lineage in suborder Apiineae, followed by Araliaceae, Myodocarpaceae, and Apiaceae. Within Apiaceae, Mackinlayoideae appears as the earliest diverging subfamily of Apiaceae, but the placement of the *Platysace* clade was not congruent in the plastid and nuclear trees. In both trees, Azorelloideae diverged after Mackinlayoideae and Platysace, followed by *Hermas*, and then Apioideae + Saniculoideae. Divergence estimates based on the plastid dataset using models with uncorrelated lognormal and uncorrelated

exponential distributions in BEAST suggested a Cretaceous age for both Apiales (> 100 Mya) and suborder Apiineae (c. 100 Mya). This implies that Apiineae, Torricelliaceae, and Griseliniaceae may be of Gondwanan origin. DIVA inferences for the biogeographic history of Apiineae showed an Australasian origin for the order and for each of its four families, including Apiaceae, in which Mackinlayoideae (and the *Platyscae* clade) were also inferred to be of Australasian origin. By contrast, DIVA suggests that Azorelloideae (including *Klotzschia*) had a South American origin, while *Hermas* and Apioideae + Saniculoideae originated in sub-Saharan Africa.

1. Introduction

Form: Apiales comprises more than 500 genera and roughly 5,000 species, with an overall cosmopolitan geographic distribution. The order was traditionally grouped with the dicot subclass Rosidae (e.g., Cronquist, 1988), but its transfer to the asterids is now supported by many molecular studies in which Apiales is placed in a polytomy of the "euasterid II" clade together with Asterales and Dipsacales (Plunkett *et al.*, 1996a; APG II, 2003; Judd and Olmstead, 2004; Soltis and Soltis, 2004). Traditionally, the order included only two families: the largely herbaceous Apiaceae (Umbelliferae), which is mostly temperate in distribution, and the woody Araliaceae, with a predominantly tropical distribution. The circumscriptions and relationship between the two families have long been subject to debate due to difficulties in the circumscription of Apiaceae subfamily Hydrocotyloideae and the placement of genera currently included in subfamilies Mackinlayoideae and Apioideae and family Myodocarpaceae. Adanson

(1763) combined the umbellifers and araliads in a single family (Umbellatae), but this system was not accepted by contemporary authors such as Linnaeus (1764) and de Jussieu (1789), who, among many others, recognized Araliaceae and Apiaceae as separate, but closely related families. The maintenance of Apiaceae and Araliaceae as related but distinct families was adopted by many 20th century botanists, including Takhtajan (1959), Cronquist (1968), and Rodríguez (1971), but a few authors have challenged this classification. Hutchinson (1969), for example, separated the two families between his two major divisions of dicots (Herbaceae and Lignosae) in his controversial system. Conversely, Thorne (1968, 1973) preferred merging the two groups into a single family. Within the past two decades, molecular studies based on Hennig's (1966) cladistic methodology have provided consistent support for grouping the two families within the same order as distinct clades, but with some adjustment in the circumscriptions of each family. Moreover, the order was expanded to include six additional families (Pittosporaceae, Aralidiaceae, Melanophyllaceae, Torricelliaceae, Griseliniaceae, and Pennantiaceae) plus the araliad segregate family Myodocarpaceae (Plunkett *et al.*, 1996*a*, 1997, 2004*a*; Plunkett, 2001; Kårehed, 2003; APG II, 2003). The monogeneric Aralidiaceae, Melanophyllaceae, and Toricelliaceae were shown to constitute a single lineage and were thus merged into Torricelliaceae (Chandler and Plunkett, 2004). However, the exact delimitation of Apiales and the precise relationships among its seven families remain active areas of investigation.

Apiaceae (Umbelliferae) has a very ancient history in botanical research, stretching back to the ancient Greeks (Rodríguez, 1957; Constance, 1971). With more

than 450 genera and 4,000 species, Apiaceae are the largest family of the order Apiales (Pimenov and Leonov, 1993; Judd *et al.*, 2002). The term 'De Umbelliferis Herbis' was first coined by Dodoëns (1583) and the family was first grouped together under Umbelliferae by Tournefort (1694) (see Constance, 1971). Drude (1898) proposed a system in which the family was divided into three subfamilies: Apioideae, Hydrocotyloideae, and Saniculoideae. The relationship between Apioideae and Saniculoideae has been addressed by many studies, with problems arising from the ambiguity of placement of early diverging lineages of Apioideae that exhibit certain affinities to Saniculoideae (e.g. *Lichtensteinia*, *Polemanniopsis*). Recently, the former apioids *Polemanniopsis* and *Steganotaenia* were grouped in Saniculoideae as tribe Steganotaeneae, while the rest of that subfamily comprise tribe Saniculeae (Calviño and Downie, 2007). The status of Hydrocotyloideae has always been more difficult to assess due to the presence of morphological similarities to each of the two other subfamilies of Apiaceae, as well as to Araliaceae. Several studies have demonstrated the polyphyly of this subfamily (Plunkett *et al*., 1996, 1997; Chandler and Plunkett 2004) and we recently placed 40 of the 42 genera previously assigned to hydrocotyloideae in at least six different lineages spread across both Apiaceae and Araliaceae (Nicolas and Plunkett, Chapter 1). Of these, 36 genera remain in Apiaceae, but are distributed among all four currently recognized subfamilies, namely Azorelloideae (with 22 former hydrocotyloid genera), Mackinlayoideae (with 7 genera), Apioideae and Saniculoideae (4 genera). In addition, there are distinct lineages in Apiaceae for the *Platysace* group (2 genera) and *Hermas* (a single genus exhibiting morphological similarities to Azorelloideae,

Apioideae, and Saniculoideae). The four remaining genera (*Hydrocotyle*, *Neosciadium*, *Trachymene*, and *Uldinia*) form a distinct lineage in Araliaceae.

Araliaceae currently comprise 41 genera and more than 1,500 species. This family was traditionally regarded as more "primitive" or less specialized than Apiaceae (Baumann, 1946; Mathias, 1965). The taxa of Araliaceae are more consistent in some features, such as basic chromosome number (usually $x = 12$), but they are more variable than Apiaceae in many anatomical and morphological characters (Darlington and Wylie, 1955; Rodríguez, 1971; Plunkett *et al.*, 2004*c*; Yi *et al.*, 2004). In addition to the transfer of *Hydrocotyle* and *Trachymene* to Araliaceae (see above), the revised classification of Apiales by Plunkett *et al.* (2004*a*) reflected the transfer of three genera (*Stilbocarpa*, *Mackinlaya*, and *Apiopetalum*) from Araliaceae to Apiaceae (see also Mitchell *et al.*, 1999; Wen *et al.*, 2001; Lowry *et al.*, 2004), and of two other genera (*Myodocarpus* and *Delarbrea*) to a new family, Myodocarpaceae (see Plunkett and Lowry, 2001). Within Araliaceae, Plunkett *et al.* (2005) demonstrated the polyphyly of *Schefflera*, the family's largest genus (900+ species). The final infrafamilial revision of Araliaceae has been hindered by the polyphyly of both Hydrocotyloideae (across Apiales) and *Schefflera* (within Araliaceae), the scarcity of diagnostic morphological synapomorphies consistent with molecular data, and poor resolution of relationships among and within the genera of Araliaceae (and the order Apiales in general).

The classification of families Pittososporaceae and Myodocarpaceae in Apiales followed rather opposite tracks. Pittosporaceae (9 genera and ~200 species) had previously been included in subclass Rosidae, but a placement in Apiales was suggested

by molecular data (Chase *et al.*, 1993; Plunkett *et al.*, 1996*a*, 1997) and then formally adopted in the first APG system (1998) on the basis of those studies. However, its precise placement in the order remains unresolved (Plunkett *et al.*, 1996*a*; Downie *et al.*, 2000; Plunkett and Lowry, 2001; Kårehed, 2003; Plunkett *et al.*, 2004*a*; Chandler and Plunkett, 2004). Despite its traditional association with Rosidae, there were much earlier indications of an affinity of Pittosporaceae to Apiales based on chemical data (Hegnauer, 1971; Bohlman, 1971), anatomical characters (Jurica, 1922; Rodriguez, 1971), and cytology (Darlington and Wylie, 1955; Jay, 1969). Its association with Rosidae was influenced most heavily by floral and foliar characters, which differ from most taxa in Apiales (Brewbaker, 1967; Plunkett *et al.*, 1996*a*), but phylogenetic studies suggest that many of the features thought to be derived in Apiales (e.g., low carpel number and simple leaves) are ancestral in Apiales, and many of these features also characterize Pittosporaceae (see Plunkett, 2001). By contrast, Myodocarpaceae are a small family whose taxa have always been included in Apiales, but have recently been segregated from Araliaceae (APG II, 2003; Plunkett and Lowry, 2001; Plunkett *et al.*, 2004*a*, 2004*c*). The three genera originally assigned to Myodocarpaceae (*Myodocarpus*, *Delarbrea*, and *Pseudosciadium*) were long considered araliads because of similarities in habit, inflorescence structure, fruit, and geographic distribution, but they also share many characters with Apiaceae (Oskolski *et al.*, 1997; Plunkett and Lowry, 2001; Lowry *et al.*, 2001). After the family's erection phylogenetic analysis demonstrated that the monotypic *Pseudosciadium* should be included within *Delarbrea* (see Plunkett and Lowry, 2001;

Sprenkle, 2001; Raquet, 2004; Plunkett *et al.*, 2004). Consequently, the family has been reduced to two genera with 17 species (Lowry *et al.*, 2004a).

Among the three remaining families, Torricelliaceae and Griseliniaceae were formerly placed near Cornaceae, but both have their own troubled taxonomic histories (see Philipson *et al.*, 1980; Dillon and Muñoz-Schick, 1993; Schatz *et al.*, 1998; Trifonova, 1998). Genera within these families were artificially grouped in or near Cornaceae, or sometimes between Cornaceae and Apiales (see Rodríguez, 1957, 1971; Murrell, 1993; Plunkett *et al.*, 1997; Plunkett and Lowry, 2001). Over the last decade, there has been a convergence in recognizing an affinity of these genera to Apiales. For example, Philipson (1967) noted the close similarity in wood anatomy between *Griselinia* and Araliaceae, and both Raven (1975) and Goldblatt (1978) suggested an alternative inclusion of *Torricellia* in Araliaceae based on chromosome number. The best support, however, came from molecular phylogenetic studies, which led to their inclusion as separate families in Apiales (Plunkett *et al.*, 1996*a*, 1996*b*, 1997; APG I, 1998; Albach 2001; APG II, 2003; Chandler and Plunkett, 2004; Plunkett *et al.* 2004*a*). A third family, Pennantiaceae, was most recently added to Apiales. This family was erected after the removal of the genus *Pennantia* from Icacinaceae (Kårehed, 2001, 2003) on the basis of both molecular and morphological evidence, a result confirmed (in part) by Chandler and Plunkett (2004). However, relationships of all three of these smaller families to the remaining lineages of Apiales remains poorly resolved and additional studies are needed (APG, II; Judd and Olmstead, 2004; Chandler and Plunkett, 2004).

Two recent studies have provided additional insights into the phylogeny of Apiales. One study aimed at assessing the placement of all genera once placed in subfamily Hydrocotyloideae using the plastid *rpl16* and *trnD-trnT* regions (Chapter 1). The second sought to study patterns of duplications in the nuclear RPB2 genes throughout the order (Chapter 2). Both studies provided extensive sampling (from over 260 species) representing every major lineage in Apiales, and yielded phylogenies that exhibited both agreements and disagreement regarding relationships in Apiales. In the present study, we examine these markers together with a new data set derived from the mitochondrial genome, which to date has not been used in phylogenetic studies of Apiales. Although the mitochondrial genome is the preferred source of molecular markers for phylogenetic studies in many animal groups, it remains underutilized in plant phylogenetics largely because of its overall low rate of nucleotide substitutions (which reduces the number of potentially informative characters) and the high rate of structural rearrangements (which may lead to incorrect inferences of phylogeny). Nevertheless, the genome has provided valuable information about the evolutionary history in seed plants (e.g., Qiu *et al*., 1999; Chaw *et al*., 2000; Barkman *et al*., 2004; Bergthorsson *et al*., 2004). The mitochondrial genome has its own unique history which provides a different source of information about the evolution of the plants, independent of the chloroplast and nuclear genomes. To maximize the chances of detecting nucleotide substitutions, we targeted non-coding regions of the mitochondrial genome. On the basis of literature searches and comparisons of sequences available on GenBank, we selected a region of ~1200 bp that included the second intron of the *nad1* gene. This gene includes five exons

(a-e) that code for subunit 1 of NADH dehydrogenase. Intron 2 is located between exons b and c with a length of less than 1500 bp in most angiosperms and has been used in phylogenetic studies at the interspecific (e.g., *Spiranthes* spp.; Chen and Sun, 1998), intergeneric (e.g., *Pelargonium*; Bakker *et al*., 2000; *Actinidia*; Chat *et al*., 2004), and interfamilial (e.g., Polemoniaceae; Porter and Johnson, 1998; Orchidaceae; Freudenstein and Chase, 2001; Burmanniacaeae; Merckx *et al*., 2006) levels.

Time: The earliest known angiosperm fossils date from Valanginian-Hauterivian deposits, demonstrating the presence of angiosperms in the early Cretaceous (141-132 Mya) (Brenner, 1996). The angiosperm fossil record suggests that these plants underwent a rapid diversification from the Barremian of the early Cretaceous (c. 115 Mya) through the late Cretaceous (c. 90 Mya) (Friis *et al.*, 1999; Herendeen *et al.*, 1999, Magallón-Puebla *et al.*, 1999; Bell *et al.*, 2005). However, there have been several different estimates of the origin of angiosperms, some of which are quite disparate, due largely to differences in methodology or statistical approach (Sanderson and Doyle, 2001). Molecular clocks calibrated with fossil data produced less conservative estimates for an origin of the angiosperms, as early as the Triassic or Jurassic (> 200 Mya) (Sanderson, 1997; Chaw *et al.*, 2004; Bell *et al.*, 2005). The core eudicot lineage was estimated to have diverged from other angiosperms c. 100 – 147 Mya (Bell *et al.*, 2005; Chaw *et al.*, 2004; Bremer, 2000; Wikström *et al.*, 2001). For Apiales, early work by Bessey (1897) reported that fossil "Umbellales" (reflecting on out-dated circumscription that included families Umbelliferae, Araliaceae, and Cornaceae) stretched back to the Cretaceous.

Bessey also estimated that the number of extant species relative to the total number of angiosperms has decreased since the late Cretaceous and Eocene. Much more recently, Magallón and Sanderson (2001) estimated an increase in the diversification rate for Apiales since its divergence. However, this rate may be attributed to recent radiations in the more speciose Apiaceae (~75% of the 4,898 species counted in the study) compared to the other families, which appear to be older but have fewer extant species (e.g., Myodocarpaceae and Pittosporaceae). Also, the divergence time used for Apiales (45.15 Mya) represents an underestimate of that inferred from fossil records (> 60 Mya; Farabee 1993). Within suborder Apiineae, more than 60 fossil taxa have been retrieved for Araliaceae, dating back to the Cretaceous (Europe and North America) and Tertiary (Siberia, Australasia), whereas most fossils referable to Apiaceae were scarce in the Oligocene and Miocene and increased in the Pleistocene (Berry, 1903; Axelrod, 1952; Mathias, 1965). Considering recent advancements in fossil dating and the phylogenetically based classifications, the oldest apialean fossils were collected in Germany and belong to the Maastrichtian flora of the Cretaceous Period (c. 70 Mya) and are placed in the araliad genera *Acanthopanax* (= *Eleutherococcus*) (*A. friedrichii, A. gigantocarpus, A. mansfeldensis,* and *A. obliquocostatus*) and *Aralia* (*A. antiqua*) (Knobloch and Mai, 1986; Bremer *et al.*, 2004). The age of these fossils was among six reference fossil dates used by Bremer *et al.* (2004) to calibrate their molecular clock, resulting in a stem age of 113 Mya for the order Apiales and a crown age of 84 Mya. Schneider *et al.* (2004) estimated a fossil age of 37 Mya and molecular age of 50 to 80 Mya, whereas Wikström (2001) used a fixed fossil age of 69 Mya and estimated the age

of Apiales at 85-90 Mya, with the Araliaceae clade originating 41-45 Mya. Farabee (1993) relied on pollen fossils to date the history of Araliaceae back to the Paleocene (55-65 Mya), but few other studies have provided estimates for the timing of diversification among families and genera in Apiales. Using several alternative estimation models, the present study will make use of paleobotanical evidence and gene phylogenies in an effort to estimate divergence times for the order and its families and genera.

***Space*:** Apiales exhibits an interesting geographic distribution, where Apiaceae are largely north-temperate and Araliaceae mostly tropical. Most of the smaller families are geographically restricted (e.g., Pittosporaceae is largely Australian and Myodocarpaceae is restricted almost exclusively to New Caledonia). Information from fossils and extant species distributions provides evidence that species within Apiales have occupied all major phytogeographic regions and kingdoms, yet very little is known about the historical biogeography of the major apialean clades. Traditional theories of its evolution include an origin of Araliaceae in the Paleotropics during the Creatceous or earlier, and the derivation of the more temperate Apiaceae from proto-araliaceous stock due to climatic changes during the late Cretaceous or Tertiary (Mathias, 1965; Rodríguez, 1971). This Paleotropic-origin theory parallels that of the angiosperms in general (Axelrod, 1952; Shields, 1991) and is supported by the high diversity of "ancient" apialean taxa in tropical regions, specifically Australasia. This region has been of major interest in biogeographic studies at least since the times of Alfred Russel Wallace due to its geology and high levels of endemism for both plants and invertebrates. Different

theories have been proposed to explain both the distribution of taxa within this region and the links of these Australasian taxa to those in other regions of the world. These include vicariance resulting from geological events affecting Gondwana (e.g., Brundin, 1966; Raven and Axelrod, 1972; Nelson, 1975; Linder and Crisp, 1996), and dispersal of surviving taxa across narrow ocean basins or by means of "island stepping stones" (e.g., Carlquist, 1974, 1981; Diamond, 1984; Takhtajan, 1986; Pole, 1994). Explanations supporting dispersal include the submergence of many island and land masses during the Oligocene, implying a post-Gondwanan colonization and diversification (Pole, 1994, 2001; McPhail, 1997). This suggests the affinity between taxa of post-Gandwanan origin on isolated areas may be best explained by long distance dispersal. However, many studies have dated endemic lineages in different Australasian regions to ancient Gondwana-related ancestors, separated by vicariance events (e.g., McLoughlin, 2001; Swenson *et al.*, 2001; Stöckler *et al.*, 2002; Ladiges *et al.*, 2003). Most of these studies attribute this to the presence of refugia, in which some Gondwanan species persisted during periods of submergence and climatic fluctuations. The current distribution of Apiales most likely resulted from both vicariance and long-distance dispersal. Such events can be tested using different biogeographic-reconstruction and estimation tools based on gene phylogenies, geologic histories, current distributions, and patterns of endemism.

The goals of this study are to address the following issues regarding phylogenetic relationships in Apiales: (1) to estimate interfamilial relationships within suborder Apiineae (i.e., among Apiaceae, Araliaceae, Myodocarpaceae,and Pittosporaceae); (2) to

reconstruct relationships among the smaller families of the order (Torricelliaceae, Griseliniaceae, and Pennantiaceae), and to assess the relationships between these groups and suborder Apiineae; (3) to evaluate the utility of the mitochondrial genome in resolving relationships within Apiales, especially through comparisons to phylogenies retrieved from nuclear and chloroplast genomes; (4) to determine divergence estimates for the major clades in Apiales; and (5) to examine the historical biogeography of Apiales. These objectives are addressed by building on the plastid data analyzed in Nicolas and Plunkett (Chapter 1, in review) and orthologs and paralogs of the RPB2 gene region between exons 18 and 23, assembled from Nicolas and Plunkett (Chapter 2). The wide taxonomic sampling in those papers (more than 260 species) will be supplemented by additional character sampling in this study, using data from the second intron from *nad1* of the mitochondrial genome. Many recent studies have demonstrated the importance of increasing both taxon sampling *and* character sampling to reduce phylogenetic error (Swofford *et al.*, 1996; Graybeal, 1998; Mitchell *et al.*, 2000; Rosenberg and Kumar, 2001; Zwickl and Hillis, 2002; Debry, 2005). Extensive representation of taxa yields better estimates of relationships and alleviates problem with long branches (Hillis, 1996), but use of markers from unlinked regions or genomes provides truly independent characters for evolutionary inference. Sampling from such data can provide corroboration of the phylogenetic hypotheses if results are congruent, or provide some insights into past reticulation events in cases of incongruence. As such, our choice of character sampling provides a solid framework to test the phylogenetic

hypotheses in the main lineages of Apiales and a reliable assessment of the utility of the mitochondrial *nad1* intron 2 to resolve relationships at different phylogenetic levels.

2. Materials and Methods

2.1. Taxon and Molecular Marker Sampling

The final sampling for the *nad1* intron 2 phylogeny was chosen from the taxa listed in Table 1 of Chapter 1. We maintained the sampling strategy of representing all available genera (except in cases where our attempts to produce reliable sequences failed), but with a limit to the sampling within each genus to only one or two species. The final sampling included 126 species from more than 100 genera of Apiales. We also added three outgroup taxa, *Sonchus asper* from Asterales (*Plunkett 2257*, NY), *Lonicera japonica* from Dipsacales (*Plunkett 2255*, NY), and *Helwingia japonica* from Aquifoliales (*Xiang 04C62*, NCSC). Most leaf tissue samples were field-collected and dried using silica gel, but a few were harvested from herbarium specimens. All 129 *nad1* intron 2 sequences were generated specifically for this study. Total DNA was extracted using the CTAB method of Doyle and Doyle (1987), the DNeasy Plant extraction kit (QIAGEN Inc.), a modified Puregene DNA extraction protocol (Gentra Systems), or following the protocol of Alexander *et al*. (2007) with minor modifications. External and internal primer sets were designed to amplify a region of c. 1200 bp of *nad1* intron 2 from Apiales and outgroups (Table 1) by comparing sequences of the same region from euasterid II taxa which are available in GenBank. PCR reactions included 1 μL of unquantified DNA, 5 μL Sigma JumpStart™ REDTaq® ReadyMix™ Reaction Mix or

Promega GoTaq® Green Master Mix, 0.5 µL of each forward and reverse primers (at concentrations of 5 µ*M*), 0.5 µ*M* spremidine (4 m*M*), and 2.5 µL ultrapure water for a total volume of 10 µL. The PCR thermal profile included a 2 min denaturing step at 94ºC, followed by 35 to 40 cycles of denaturation (30 sec at 94ºC), primer-annealing (30 sec at 57ºC), and DNA extension (90 sec at 70ºC). This was followed by an extra extension step for 5 min at 72ºC. Some products were amplified in two separate reactions using a combination of external and internal primers to produce two overlapping fragments.

PCR amplicons were cleaned using ExoSAP-IT (USB Corp.), according to the manufacturer's recommendations, before serving as template for the sequencing reaction. Cycle sequencing reactions were prepared by mixing 1 µL of the DYEnamic™ ET Terminator Cycle Sequencing mix (GE Healthcare), 1.5 µL of purified double-stranded PCR product, 0.5 µL primer (5µ*M*), and 3 µL ultrapure water, for a total volume of 6 µL. The amplification program consisted of 40 cycles of 3 steps: 30 sec at 94ºC, 15 sec at 55ºC, and 60 sec at 60ºC. Sequencing products were purified using Montage SEQ_{384} plates (Millipore Corp.) and then separated by electrophoresis on a 96-capillary MegaBACE™ 1000 automated sequencer.

2.2. Sequence Alignment and Data Analyses

Sequences were edited using MegaBACE™ Sequence Analyzer. Complementary (forward and reverse) fragments were compared by pairwise BLAST (www.ncbi.nlm.nih.gov/BLAST/). Sequences used in the final analyses were aligned in ClustalX using the default settings (Higgins and Sharpe, 1988), followed by manual adjustments. An unweighted maximum parsimony analysis was implemented in PAUP*

4.0b10 (Swofford, 2001). We set PAUP* to run 100 replicates of heuristic searches using random addition of sequences and tree-bisection-reconnection (TBR) as the branch-swapping algorithm. A maximum of 1000 optimal trees was saved for each replicate, setting a total upper limit of 50,000 trees for the overall search. Support values for nodes were estimated using 100 bootstrap replicates in PAUP*. MODELTEST (Posada and Crandall, 1998) was run (with PAUP*) to estimate the best model of sequence evolution. The model GTR+Γ+I was used for three separate maximum likelihood analyses in GARLI (Zwickl, 2006). After visually assessing the congruence among tree topologies and similarities in log likelihood values from trees resulting from different runs, we selected the tree with the best likelihood score.

2.3. Inferences of Historical Biogeography and Times of Divergenece

Cladistic biogeography has emerged as the most commonly employed approach to examining historical biogeographical relationships (Ronquist, 1997). This approach uses phylogenetic trees of taxa in an attempt to discover patterns of species distributions that reflect vicariance explanations, using extinction and dispersal where necessary to explain certain distributions (Crisci *et al.*, 2003). An event-based method of reconstruction of areas was implemented in the DIVA software package (Ronquist 1996 and 1997), where vicariance was set as the default cause of speciation (no cost) while accounting for dispersal and extinction events using *a priori* cost assignment (one per event). Due to the large size of our data set and limitations in the number of terminals and characters accepted in DIVA, we constructed three separate DIVA matrices, each with 61 samples and five area characters. Each of the input trees was pruned from the fully resolved ML

plastid phylogeny. The first data set included 61 taxa from Apiaceae and Myodocarpaceae, the second included 61 taxa from Pittosporaceae, Araliaceae, and Griseliniaceae, and the third included 61 taxa representing all major clades across the order. The five geographically-defined characters were Australia + Pacific (area A), the Neotropics + temperate South America (Area B), temperate North America (Area C), sub-Saharan Africa (Area D), and Asia + Europe + North Africa (Area E).

Bayesian estimation of rate variation was assessed using the software BEAST v1.4.8 (Drummond and Rambaut, 2007). The model is optimized through Bayesian MCMC without requiring rate autocorrelation or a starting phylogram, and thus provides a better account for phylogenetic uncertainty (Drummond *et al.*, 2006; Rutschmann, 2006). Both fossils and rates of molecular evolution were used to estimate the time of divergence at different nodes in Apiales. Hence estimates of divergence time were determined by a data matrix of aligned DNA sequences and reliable fossils in order to calibrate branches across various lineages. The data matrix was generated after trimming taxa in the plastid dataset to 161 taxa, which represented all major clades and subclades of the order. We conducted a likelihood ratio test (LRT) to test whether the sequences evolve according to a molecular clock. Fossils used as calibration points included *Toricellia bonesii* (Manchester, 1999) from the lower Eocene and *Dendropanax eocenensis* (Dilcher and Dolph, 1970) from the middle Eocene (detailed in Chapter 2), as well as pollen fossils related to *Steganotaenia* and *Bupleurum* from the lower Eocene, and *Heteromorpha* from the upper Eocene (Gruas-Cavagnetto and Cerceau-Larrival, 1984). The calibration points were placed at the most appropriate points of common

ancestry for each fossil (Fig. 10). The minimum ages for *Torricellia*, *Steganotaenia*, and *Bupleurum* fossils was set at 52.2 Mya and the 95% confidence interval between 48.6 and 55.8 Mya, which spans the entire lower Eocene. We followed the same concept for the fossil of *Dendropanax* and calibrated the clade with a minimum age of 42.9 Mya and 95% confidence interval spanning the middle Eocene from 37.2 to 48.6 Mya, and the fossil of *Heteromorpha,* which was set to a minimum age of 35.55 Mya and 95% confidence interval spanning the middle Eocene from 33.9 to 37.2 Mya. The age of the ingroup was dated to 100 Mya and we relaxed the estimate with 2.5% confidence intervals between 90 and 110 Mya. We used the GTR+Γ+I as the model of evolution and set the tree prior to the Yule speciation process, which assumes a constant speciation rate per lineage. We performed two separate runs with different relaxed clock models, uncorrelated lognormal distribution (UCLN) and uncorrelated exponential distribution (UCED). Both models account for rate variations across branches and do not assume *a priori* correlation between a lineage and its ancestor, although exponential models have a higher variance (Drummond and Rambaut, 2007). For each of the two runs, the chain was run for 20 million generations with sampling of trees every 1000 generations. The tree file was transferred to TreeAnnotator v1.4.8 (in the BEAST package), the burn-in was set to 2000 (10%), and a maximum clade credibility tree was estimated.

3. Results and Discussion

3.1. Characteristics and phylogeny of nad1 *intron 2*

The final *nad1* intron 2 data matrix included 129 sequences with an aligned length of 1725 bp. Individual sequence lengths ranged from 642 bp in *Centella* and *Micropleura*

of Mackinlayoideae to 1227 in *Pittosporum undulatum* of Pittosporaceae. However, most sequences in Apiales had a much narrower range, between 1100 bp and 1200 bp. The shorter lengths found in the herbaceous members of Mackinlayoideae (except *Actinotus*) were due to various deletions spread across the length of the sequence region, the longest one of which was ~500 bp. The *Azorella* clade of Azorelloideae was another group of taxa that exhibited a variety of structural rearrangements, including one major indel greater than 250 bp. The *Trachymene* clade exhibited high variability among species, including an insertion of up to 100 bp. Based on our previous experience with hypervariability in the plastid and nuclear markers, we were prepared for challenges in aligning mitochondrial sequences from the early diverging lineages of the Apioideae + Saniculoideae group and the herbaceous members of Mackinlayoideae. This proved true for the latter group, but not the former. In Apioideae + Saniculoideae, *nad1* intron 2 showed a surprisingly conserved structure, save for a few small indels. We detected an inversion of 20 bp (5′ – AGCCTTTTTCTAAAGGCTCT – 3′) in many taxa belonging to different clades; this ultimately proved to be homoplasious and was thus excluded from the final analyses.

The maximum parsimony (MP) analysis of *nad1* intron 2 resulted in 30,000 trees with a length of 621 steps and a consistency index of 0.8116 (see Table 2 for tree characteristics) and offered greater resolution within the major clades than among them. The best scoring maximum likelihood (ML) tree had a –ln likelihood score of -6632.6566 and showed greater resolution and higher support values than the MP strict consensus tree. In general, we will refer to the ML phylogeny (Fig. 1) to interpret relationships, but

will note important incongruences where the topologies resulting from the two methods disagree.

The early diverging families (Pennantiaceae, Torricelliaceae, and Griseliniaceae) appear in three resolved clades at the base of Apiales in the ML phylogeny only. Support for the placements of Pennantiaceae and Torricelliaceae was < 55% (Fig.1b). Griseliniaceae are sister to the clade uniting the four families that constitute suborder Apiineae (Pittosporaceae, Araliaceae, Myodocarpaceae, and Apiaceae) with 70% bootstrap support. Two major lineages are evident in suborder Apiineae. The first unites family Apiaceae (but excluding Mackinlayoideae) to *Platysace* + *Homalosciadium* (Fig. 1a), but this placement of the *Platysace* clade is not well supported, nor was it retrieved in the MP phylogeny. Both MP and ML phylogenies show five main clades in one of the two lineages of Apiaceae. These are *Klotzschia*, *Hermas*, the *Asteriscium* + *Bowlesia* clade (of Azorelloideae), Saniculoideae + related genera, and Apioideae (but also including members of the *Azorella* group) (Fig. 1a). The relationships among these five subclades were unresolved in the MP tree and resolved with low support in the ML tree (BS < 50%). *Klotzschia* and *Hermas* formed two early diverging clades in Apiaceae but with low support. The rest of this lineage includes three main subclades. Clade 1 includes mostly Apioideae but also members of the *Azorella* group, clade 2 includes *Asteriscium*, *Diposis*, and *Bowlesia* clades of Azorelloideae and is sister to clade 1, and clade 3 includes Saniculoideae and other early lineages of Apiaceae (*Steganotaenia*, *Polemanniopsis*, *Lichtensteinia*, and *Choritaenia*) (Fig. 1a). The placement of the *Azorella* group in Apioideae rather than among the other members of Azorelloideae was

surprising, but appears to represent an artifact due to the large deletion in a region of *nad1* that exhibited the greatest number of synapomorphies uniting the rest of Azorelloideae. Two other results worth noting are the placement of *Lichtensteinia* and *Choritaenia* as sister to Saniculoideae (BS = 64%) and the placement of *Diposis* as sister to the *Asteriscium* clade (BS = 90%).

The second clade of Apiineae includes Mackinlayoideae and the three remaining families of Apiineae (Fig. 1b). The relationships among the clades were unresolved in the MP tree and resolved with low support in the ML tree (Fig.1b). The sister-group relationship between Mackinlayoideae and Pittosporaceae was novel but only weakly supported (BS = 56%), and it did not appear in the MP phylogeny. Although the resolution of relationships among many major groups was poor (especially in the MP tree), the mitochondrial marker resolved relationships in many of the clades, especially at the intergeneric level (e.g., in Pittosporaceae and the major clades of Apiaceae).

3.2. Relationships among the families of Apiales

In our discussion of phylogenetic relationships in Apiales, we draw inferences from all three molecular makers, including data from the mitochondrial (*nad1* intron 2), plastid (*trnD-trnT + rpl16*) and nuclear (RPB2) genomes. The traditional notion of Araliaceae and Apiaceae as a "family pair" is not supported by the results of the markers from the three genomes (Fig. 2). The principle exception is the placement of family Myodocarpaceae relative to Apiaceae and Araliaceae, and in some trees, the placement of subfamily Mackinlayoideae relative to Myodocarpaceae. Although support for these

placements is not very high (< 60%; Fig. 2), this picture of relationships fits with the pre-cladistic view that the taxa now placed in Myodocarpaceae (*Myodocarpus* and *Delarbrea*) represent "bridging groups" between Apiaceae and Araliaceae. These plants share the woody habit of most Araliaceae, and *Delarbrea* also has the drupaceous fruits characteristic of most Araliaceae but with thin endocarps. By contrast, *Myodocarpus* has dry, schizocarpic fruits with free carpophores, reminiscent of many Apioideae. Basic chromosome numbers ($x = 12$) also provide a link between Myodocarpaceae and Araliaceae. However, wood anatomical characters, such as non-septate fibers and thin intervessel pits, suggest a connection to *Apiopetalum* and *Mackinlaya* of Mackinlayoideae, the earliest diverging lineage of Apiaceae (Fig. 2; Plunkett *et al*. 1996a, 1996b, 1997; Oskolski and Lowry, 2000). Evidence from wood anatomy shows some affinities between Araliaceae and woody Apiaceae (excluding Mackinlayoideae) that are not shared with Myodocarpaceae, prompting Oskolski (2001) to conclude that Myodocarpaceae is a distinct lineage of Apiales rather than an intermediate between Apiaceae and Araliaceae (see also Rodríguez, 1957).

Unlike the results inferred from mitochondrial data, Apiaceae, Myodocarpaceae, and Araliaceae form a resolved clade in trees based on plastid (BS = 64%) and nuclear (BS = 86%) data (Fig. 2). The affinity of Pittosporaceae to these three families is evident in the phylogenies estimated from all three genomes. The sister-group relationship of Pittosporaceae to the rest of Apiineae is well supported in the plastid (BS = 99%) and the nuclear (BS = 93%) phylogenies (Fig. 2), but Pittosporaceae is nested well within Apiineae in the mitochondrial tree (sister to Mackinlayoideae), albeit with low support. A

distinguishing morphological feature of Pittosporaceae is the presence of superior ovaries with parietal or axile placentation, whereas the other three families of Apiineae have inferior ovaries with axile (apical) placentation.

The early-diverging families, Griseliniaceae, Torricelliaceae, and Pennantiaceae form successive sister groups to Apiineae. In the mitochondrial and plastid trees, Torricelliaceae appears to have diverged earlier than Griseliniaceae, but in the nuclear topology, the two groups form a clade (Figs. 1 & 2). The three families differ from Apiineae in several features, including the lack of schizogenous secretory canals (see Plunkett 2001) and their pollen morphology, which is usually reticulate in most Apiineae but varies in the three remaining families (Kårehed, 2003). The morphological evidence supporting the inclusion of these families in Apiales is scant. Several features common in Apiineae are also found in most of these groups, such ovary-roof nectaries (Erbar and Leins, *submitted*), a single functional ovule (either per locule or per ovary), drupaceous fruits, and sheathing petiole bases, but there are significant exceptions to each. By contrast, molecular evidence has consistently suggested that these families belong to Apiales as early-diverging lineages. The link between Pennantiaceae and the rest of Apiales is especially tenuous, where only ovary position and low carpel number are shared. In fact, results from two nuclear studies of Apiales have placed it among the outgroups (Dipsaclaes and Aquifoliales) rather than as sister to the rest of Apiales (Chandler and Plunkett, 2004; Nicolas and Plunkett, Chapter 2). Considering the troubled history of Icacinaceae (where *Pennantia* had formerly been placed), we suggest that the affinity between *Pennantia* and the rest of Apiales requires further testing with more

extensive sampling of genera and species of Icacinaceae, its relatives and the other families of Dipsacales and Aquifoliales, as well as Apiales.

3.3. Relationships in Apiaceae

The greatest difficulties in resolving subfamilial relationships in Apiaceae were the placement of taxa formerly grouped in the now obsolete subfamily Hydrocotyloideae and some early-diverging lineages that blurred the circumscriptions of Apioideae and Saniculoideae. The problem of Hydrocotyloideae has largely been put to rest with the re-alignment of its genera across the different subfamilies of Apiaceae and in Araliaceae. In its current circumscription, Apiaceae includes four subfamilies: Azorelloideae, Mackinlayoideae, Apioideae, and Saniculoideae (Plunkett *et al*., 2004*a*). Mackinlayoideae appear to be the earliest-diverging lineage of Apiaceae, followed by Azorelloideae then Apiodeae + Saniculoideae (Fig. 2). The exact circumscription of the latter two subfamilies remains an open subject for debate (see van Wyk, 2001; Calviño *et al*., 2006).

Mackinlayoideae: This subfamily includes two woody genera formerly placed in Araliaceae (*Apiopetalum* and *Mackinlaya*), plus eight mostly herbaceous, former hydrocotyloid genera (*Actinotus*, *Xanthosia*, *Chlaenosciadium* and its segregate *Brachyscias*, *Pentapeltis*, *Shoenolaena*, *Centella*, and *Micropleura*), all of which had been assigned to tribe Hydrocotyleae (Pimenov and Leonov, 1993) based on the presence of laterally compressed fruit. This fruit character is also found in *Mackinlaya*, but it is

absent in *Apiopetaulm*. Members of Mackinlayoideae share several other characters, such as sheathing petiole bases and valvate petals with clawed bases and inflexed tips (detailed in Plunkett and Lowry, 2001). These characters are also shared with many Apiaceae and thus do not represent unique synapomorphies of Mackinlayoideae. *Mackinlaya* and *Apiopetalum* also have features that link them to Araliaceae, such as shrubby to arborescent habits and fleshy fruits, but wood anatomical characters suggest that, collectively, these two genera are distantly related to woody Araliaceae (detailed in Oskolski and Lowry, 2000).

The monophyly of Mackinlayoideae and the resolution of most of its intergeneric relationships is clearly evidenced in all three of our molecular studies (summarized in Fig. 3). The studies with the most extensive sampling are those based on plastid *trn*D-*trn*T + *rpl16* (Chapter 1) and nuclear RPB2 (copy 2) (Chapter 2). Based on DNA sequence data, Mackinlayoideae appear to consist of five main groups: *Apiopetalum*, *Mackinlaya*, *Actinotus*, *Chlaenosciadium-Xanthosia* (the *Xanthosia* group), and *Centella-Micropleura-Shoenolaena-Pentapeltis* (the *Centella* group) (Fig. 3). High rates of sequence variation are apparent among these five groups, especially among the herbaceous taxa. The woody genera, *Apiopetalum* and *Mackinlaya*, appear as distinct lineages that are not sisters within Mackinlayoideae. The separation of these two genera is also reflected in their geographies, with *Apiopetalum* restricted to New Caledonia and *Mackinlaya* ranging from Malesia to Australia. The relationship of these two lineages relative to the mostly Australian *Actinotus* is not clear, and the *nad1* intron 2 phylogeny did not offer a well supported relationship among the three genera. In trees where all

genera of Mackinlayoideae were represented, *Actinotus* appeared either as sister to *Apiopetalum*, as in the combined plastid analysis (ML BS = 96%; Fig. 3a), or as a lineage that diverged earliest in Mackinlayoideae, as in the tree based on RPB2 copy 2 (ML BS < 50%; Fig. 3b). In the same trees, the placement of *Mackinlaya* is well supported as sister to the *Xanthosia* and *Centella* groups (plastid ML BS = 96%; RPB2 ML BS = 80%) (Fig. 3). The main problem remains the relative placements of *Apiopetalum* and *Actinotus* at the base of Mackinlayoideae.

The morphological affinities between (and within) the *Xanthosia* and *Centella* groups were addressed in Chapter 1. The molecular evidence from plastid and nuclear genomes strongly supports the sister relationship between the two groups and suggests a geographic progression from Australia (*Xanthosia*, *Chlaenosciadium*, *Shoenolaena*, and *Pentapeltis*), to Mesoamerica (*Micropleura*) and Africa (*Centella*). A remarkable feature of the *Centella* group is the high number of synapomorphic indels in the sequences of mitochondrial *nad1* intron 2 that are unique to these groups. These characters provide evidence of the association of the Australian *Pentapeltis* and *Shoenolaena* with *Centella* and *Micropleura*, and not with the sympatric *Xanthosia*, in which some authors had once placed them (e.g., Burbidge, 1963). Despite the overwhelming molecular evidence for grouping *Chlaenosciadium*-*Xanthosia* and *Centella*-*Micropleura*-*Shoenolaena*-*Pentapeltis*, we were unable to identify unique morphological characters that could be used as diagnostic features of these subgroups of Mackinlayoideae.

The *Platysace* clade includes both *Platyscae* and *Homalosciadium*, the close relationship between which was first demonstrated by Nicolas and Plunkett (Chapter 1).

Platysace had previously been affiliated with *Trachymene,* whereas *Homalosciadium* was considered a "satellite genus" of *Hydrocotyle* (Henwood and Hart, 2001; Hart and Henwood, 2006). Both genera were grouped in Hydrocotyloideae subtribe Hydrocotylinae, together with *Trachymene* and *Hydrocotyle*, based on the presence of laterally compressed mericarps and petaloid sepals. Hydrocotylinae also included *Centella*, *Micropleura*, and *Chlaenosciadium*, now placed in Mackinlayoideae. Molecular data indicate that *Platysace* and *Homalosciadium* are not closely related to *Hydrocotyle* and *Trachymene*, but rather to Mackinlayoideae (Fig. 2). The relationship of *Platyscae* to *Homalosciadium* has strong support in all three of our phylogenies, but their exact placement in Apiaceae remains questionable. The plastid phylogeny indicates that the *Platysace* clade diverged after Mackinlayoideae and is sister to the rest of Apiaceae (BS = 93%). The nuclear tree shows the *Platysace* clade as sister to Mackinlayoideae (BS = 70%), whereas the mitochondrial tree places it as sister to most Apiaceae (excluding Mackinlayoideae) but with very low support (Fig. 1 & 2). Thus, the placement and taxonomic status of *Platysace* and *Homalosciadium* remain uncertain; one possible solution is to recognize these two genera as a fifth subfamily of Apiaceae, but more detailed studies are necessary.

Azorelloideae: Our analyses of chloroplast markers (Chapter 1) provided strong evidence that many of the former hydrocotyloid genera belong to subfamily Azorelloideae. The same study showed four main clades in the well supported Azorelloideae clade (the *Asteriscium*, *Diposis*, *Azorella*, and *Bowlesia* clades) plus

Klotzschia, tentatively included in Azorelloideae *incertae sedis* (Fig. 2). Interrelationships among the four clades were not strongly supported, but the nuclear (BS = 81%) and mitochondrial (BS = 90%) phylogenies corroborate a sister-group relationship between the *Diposis* and the *Asteriscium* clades (Figs. 1a & 2b). The taxa of these two clades all have winged fruits with lateral ribs, but this character is not unique in Azorelloideae. On the other hand, *Diposis* differs from other Azorelloideae in lacking distinct rib oil ducts in their fruits (Liu, 2004). In the nuclear phylogeny, the *Diposis-Asteriscium* group did not form a monophyletic group with the rest of Azorelloideae, but rather was placed (with low support) as sister to Apioideae + Saniculoideae (Fig. 2b).

The *Asteriscium* clade includes several genera that are shown to be non-monophyletic in both the plastid and nuclear trees (viz., *Asteriscium*, *Eremocharis*, and *Domeykoa*) (Fig. 4). Three main lineages are apparent in this clade, one with *Asteriscium*, *Gymnophyton*, and *Pozoa*, a second with the interdigitated species of *Eremocharis* and *Domeykoa*, and a third with *Oschatzia*, the only Australian genus in the group. The placements of *Pozoa* and *Oschatzia* varied in the three phylogenies. These are the only two genera in the clade to lack winged fruits. In the plastid and mitochondrial phylogenies, *Oschatzia* diverged after the *Eremocharis-Domeykoa* clade, but the situation is reversed in the nuclear topology (Fig. 4). *Pozoa* is sister to *Gymnophyton-Asteriscium* in the plastid and mitochondrial phylogenies, but sister to *Asteriscium chilense* alone in the nuclear tree (BS = 88%).

A sister-group relationship between the *Azorella* and *Bowlesia* clades was supported in the nuclear RPB2 phylogeny (BS = 100%; Fig. 2), but not the plastid or

mitochondrial trees. Within the *Bowlesia* clade, the only well supported relationship among the five genera was found between the South American *Bolax* and the Australian *Dichosciadium* (Fig. 5). *Bowlesia*, *Homalocarpus*, and *Drusa* had traditioanlly been grouped together in subtribe Bowlesiinae due to their hollow fruits, whereas *Bolax* and *Dichosciadium* had been placed in subtribe Azorellinae because they lack this character. *Bolax* also exhibits suffrutescent and mat-forming habits that are very similar to the habits of many species of *Azorella*.

The genera of the *Azorella* clade (*Azorella, Mulinum, Laretia, Schizeilema, Huanaca, Diplaspis, Dickinsia, Stilbocarpa*, and *Spananthe*) exhibit a complex pattern of relationships in the plastid phylogeny (detailed in Chapter 1). The placement of *Spananthe* as the earliest diverging lineage, followed by *Dickinsia* + *Diplaspis*, is congruent between the plastid and nuclear phylogenies (Fig. 6). The remaining problems in the clade include the polyphyly of *Azorella* and *Schizeilema*, and the placement of *Laretia* and *Mulinum*. Character states traditionally used in the classification of these taxa (e.g., the presence or absence of carpophores and wings, and low herbs versus mat- or cushion-forming habits), appear to be poor indicators of relationships and do not provide a solid basis for classification in this clade. Both of the major subclades of the *Azorella* clade included species of *Azorella*. One included *Mulinum* and *Laretia* (the *Mulinum* subclade), while the other included *Huanaca*, *Schizeilema*, and *Stilbocarpa* (the *Schizeilema* subclade) (Fig. 6). The nuclear tree showed a more complicated pattern than the plastid tree. While the relationships in the *Schizeilema* subclade were very similar, the relationships in the *Mulinum* subclade differed between the plastid and nuclear

phylogenies (Fig 6). In one copy of RPB2 (copy *Az1*), *A. trifoliolata* alone was placed as sister to the *Schizeilema* subclade, followed successively by four different subclades that, in the plastid phylogeny, belonged to the *Mulinum* subclade (Fig. 6b). In copy Az2 of RPB2, the *A. trifurcata* and *A. selago* subclades were sister to the *Schizeilema* subclade and did not group in the *Mulinum* subclade (Fig. 6b), but this relationship was not well supported (BS = 56%). Other data (not shown here) indicate that neither *Mulinum* nor *Huanaca* are monophyletic, thus further exacerbating the taxonomic problems, and indicating that the group is in dire need of more study.

One of the main phylogenetic problems remaining in Azorelloideae is the placement of *Klotzschia*. This genus was placed as sister to the rest of Azorelloideae in the plastid study (BS = 66%), sister to the *Diposis* + *Asteriscium* clades in the nuclear study (BS = 50%), and sister to the rest of Azorelloideae + Apioideae + Saniculoideae in the mitochondrial study (BS < 50%) (Figs.1a & 2). The genus was traditionally grouped in subtribe Azorellinae due to the presence of dorsally compressed fruits that lack wings. Within Azorelloideae, *Klotzschia* shares a fruit synapomorphy with *Diposis*, the lack of distinct intrajugal oil ducts (Liu, 2004). *Diposis* may in fact represent the closest relative to *Klotzschia*, and is placed in a clade that diverged just after the divergence of *Klotzschia* in both the plastid and nuclear trees (Fig. 2).

Apioideae-Saniculoideae: The placement of *Hermas* sister to Apioideae + Saniculoideae is congruent in the plastid and nuclear phylogenies (BS > 80%; Fig. 2). The mitochondrial tree did not provide a well supported placement for *Hermas* or a

sister-group relationship between Apioideae and Saniculoideae (Fig. 1a). *Polemanniopsis* + *Steganotaenia* formed a clade that is sister to tribe Saniculeae (*Eryngium*, *Petagnaea*, *Sanicula*, *Hacquetia*, *Astrantia*, *Actinolema*, *Alepideae*, and *Arctopus*) and is supported in both the plastid and mitochondria phylogenies (> 70%), but is not in the nuclear phylogeny (BS < 50%) (Fig. 7). *Choritaenia* + *Lichtensteinia* are sister to Saniculeae + *Polemanniopsis* + *Steganotaenia* in the plastid and mitochondrial phylogenies (BS > 50%), but *Lichtensteinia* alone had that placement in the nuclear phylogeny (BS < 50%), whereas *Choritaenia* was placed as sister to the rest of Apioideae (BS = 68%). Within Saniculeae, different copies of RPB2 were derived from different genera. Despite this, the same overall pattern of relationships can be deduced from all three phylogenies: ((*Arctopus* + *Alepideae*), ((*Astrantia* + *Actinolema*),((*Sanicula* + *Hacquetia*), (*Eryngium* + *Petagnaea*)))) (Fig. 7).

Within the early diverging lineages of Apioideae, the placement of *Astydamia* varies among the three phylogenies. It appears as sister to the *Annesorhiza* clade (*Annesorhiza* + *Itasina* + *Chamarea*) in the plastid tree (BS = 100%), the next diverging lineage after *Choritanea* in the nuclear tree (BS = 97%), and after the divergence of the *Annesorhiza* and *Heteromorpha* (*Heteromorpha* + *Anginon* + *Pseudocarum* + *Andriana*) clades in the mitochondrial tree (BS = 61%) (Figs. 1a & 7). The *Annesorhiza* clade diverged before the *Heteromorpha* clade in the plastid tree (BS = 100%), but the converse relationship was shown in the mitochondrial and nuclear phylogenies, but with low support (BS < 50%) (Figs. 1a & 7). These variations in placement from different markers highlight the persisting problems in resolving relations among the early lineages

of Apioideae + Saniculoideae (van Wyk, 2001; Calviño *et al*., 2006; Calviño and Downie, 2007; Nicolas and Plunkett, Chapters 1 & 2).

Apart from the early lineages described above, *Bupleurum* diverges next in the plastid and mitochondrial trees (BS > 96%). In the plastid phylogeny, *Bupleurum* is succeeded by the *Neogoezia* clade (BS = 100%), the *Anisotome* clade (BS = 100%), and the *Daucus* clade (*Daucus* + *Tinguara* + *Oreomyrrhis*; BS = 98%) (Figs. 1a & 7). The nuclear phylogeny shows the *Anisotome* clade diverging first (BS = 100%), followed by the *Neogoezia* clade (BS = 70%), the *Daucus* clade (BS = 92%), and the *Bupleurum* clade (BS < 50%) (Fig. 7). Since the later diverging lineages of Apioideae were not heavily sampled in our study (due to the large number of genera), we limit our discussion to the placement of *Notiosciadium* since it has only been sampled in two previous molecular studies (Nicolas and Plunkett, Chapters 1 & 2). In the RPB2 phylogeny, *Notiosciadium* and *Aegopodium* are supported sisters (BS = 82%) that together appear as the sister to *Lagoecia* (BS = 86%) (Fig. 7). These three genera appeared as distinct clades in the plastid phylogeny (Fig. 7).

3.4. Relationships in Myodocarpaceae

The sister-group relationship between *Delarbrea* and *Myodocarpus*, as well as the monophyly of each genus, is well supported in all of our phylogenies, just as it has been in many other studies (e.g. Chandler and Plunkett, 2004; Plunkett *et al*. 2004*a*). These two genera are almost exclusively restricted to New Caledonia and have similar wood anatomies, especially in their shared apotrachial axial parenchyma (Oskolski *et al*., 1997,

Lowry *et al*., 2001). The most conspicuous distinguishing feature between the two genera is their fruit types, either dry and schizocarpic in *Myodocarpus* or fleshy and drupaceous in *Delarbrea*. Despite this, the fruits of both genera bear several similarities, notably bicarpelly and large secretory oil vesicles (Lowry, 1986*a* and 1986*b*). The variation in fruit type likely reflects adaptations to different dispersal strategies (Lowry, 1986*a* and 1986*b*).

3.5. Relationships in Araliaceae

Plunkett *et al*. (2004*c*) assigned informal names to three main lineages of Araliaceae: the Asian Palmate group, the *Polyscias-Pseudopanax* group, and the *Aralia* group. The relationships among and within these clades, as well as among many of clades of Araliaceae, remain poorly understood. Based on our results, some affinities appear between the Pacific genera *Raukaua*, *Cheirodendron*, and *Schefflera s. str.*, and sometimes extending further to the Australian genera *Cephalaralia* and *Motherwellia* (Fig.1b & 8). The resolution of these genera in a single clade has neither been consistent nor well supported. Many other clades (e.g., *Astrotricha*, *Harmsiopanax*, *Osmoxylon*, *Cussonia* and *Seemannaralia*, and African members of *Schefflera*) form polytomies within Araliaceae or are retrieved in poorly supported and variable placements in different phylogenic trees. In addition, the *Hydrocotyle-Trachymene* group includes former hydrocotyloids (*Hydrocotyle*, *Neosciadium*, *Trachymene*, and *Uldinia*) that were once placed in Apiaceae, but are more appropriately placed in Araliaceae. Several genera in Araliaceae are poly- or paraphyletic (e.g., *Polyscias*, *Gastonia*, *Cuphocarpus*), but the

polyphyly of Schefflera has the greatest impact on the taxonomy of the entire family. *Schefflera is* currently the largest genus in Araliaceae, but its species are divided into five separate clades, largely reflecting geographic distributions, with clades centered in Asia, the Neotropics, Africa and Madagascar, and the Pacific. The species of Pacific, however, fall in two unrelated clades, one centered in Melansia, and a second smaller (but more broadly distributed) clade representing *Schefflera s. str.* (Plunkett *et al*., 2005).

The Asian Palmate Group (plus *Osmoxylon*, *Cussonia*, and *Seemannaralia*): This clade includes 20 genera with a predominantly Asian distribution, with several notable exceptions. *Dendropanax* is disjunct between Asia and the Neotropics, *Oplopanax* is disjunct between Asia and North America, and *Oreopanax* is restricted to the Neotropics. In addition, two (of the five) clades of the polyphyletic *Schefflera* are found in this group, including both the Asian and Neotropical clades. The plastid phylogeny provides good resolution of the Asian Palmate group, but with very low support (BS < 50%). The nuclear phylogeny offers better support for the clade (BS = 75%), but only with the inclusion of *Osmoxylon* as an early-diverging lineage in one of the subclades (Fig. 8). There is also some evidence that ancient allopolyploidy may have shaped the early history of the tetraploid species in the group (see Chapter 2; Mitchell and Wen, 2004; Yi *et al*., 2004). Judging from our findings and the conclusions of prior studies of the group (e.g., Wen *et al*., 2001; Mitchell and Wen 2004; Plunkett *et al*., 2004*c*), the lack of resolution and support, despite the application of various sources of molecular and morphological evidence, may reflect an early rapid radiation and

subsequent reticulation events. The mitochondrial tree did not provide strong support for any relationships in this clade.

In the nuclear RPB2 data from the Asian Palmate group, we found evidence of another duplication event within the *Ar1* copy of the gene, yielding an additional level of paralogy (copies *AP1* and *AP2*) found only among the Asian Palmates. This may be an indication of ancient allopolyploidy among the ancestors of the species in the clade (Chapter 2). In the resulting cladogram, *Osmoxylon* falls within the Asian Palmate group at the base of the *AP2* subclades (BS = 55%; Fig. 8b). We found no evidence of this placement in the plastid phylogeny, where *Osmoxylon* forms a clade with *Astrotricha* (but with low bootstrap support, BS < 50%; Fig. 8a). In the nuclear cladogram, the African genera *Cussonia* and *Seemannaralia* are successive sister lineages to the Asian Palmate group (including *Osmoxylon*), with strong support (90% and 73%, respectively; Fig. 8b). In the plastid tree, these two genera had a sister-group relationship (BS = 66%) and, in turn, were sister to the *Polyscias-Pseudopanax* group (BS < 50%) in the broader clade that included both the *Polyscias-Pseudopanax* group and the Asian Palmate group (Fig. 8a). Given the palmate structure of the leaf venation, lobing, or division in *Osmoxylon, Cussonia,* and *Seemannaralia*, their association with (or possibly their inclusion in) the Asian Palmate group warrants more detailed study.

The *Polyscias-Pseudopanax* Group: This group unites *Polyscias* and its segregates in one subclade, and Melanesian *Schefflera*, *Meryta*, *Pseudopanax* and *Neopanax* in a second subclade, and is strongly support in the plastid tree (BS = 95%,

Fig. 8a), confirming results from prior studies (e.g., Wen *et al*. 2001; Plunkett *et al*. 2004*c*). The group is not found in the mitochondrial tree (Fig. 1b). The RPB2 gene tree provides an additional perspective into the evolution of this group, due to the detection of two unrelated copies of the gene. Given the predominance of tetraploidy in the taxa of the *Pseudopanax* subclade, it appears that the duplicated copies of the gene track two different evolutionary histories, which is reflected in the two *Pseudopanax* subclades found in the RPB2 tree. One copy reflects the *Polyscias-Pseudopanax* group as we see it in the plastid phylogeny, albeit with lower support (BS = 56%). The second copy, however, shows a relationship to a distant clade that includes *Raukaua et al*. (Fig. 8b). This may be an indication of ancient hybridization events between these groups (see details in Chapter 2). Within the *Pseudopanax* subclade there are four genera of exclusively Pacific distribution (*Pseudopanax*, *Neopanax*, *Meryta*, and Melanesian *Schefflera*). The genera were not placed together in a single supported clade in the mitochondrial phylogeny, but were well-supported in both the plastid phylogeny (BS = 90%) and the *Pseudopanax1* subclade of the nuclear tree (Figs. 1b & 8).

In the plastid and nuclear trees, the *Polyscias* subclade is consistently one of the best supported clades in Araliaceae (plastid BS = 67%; nuclear BS = 99%; Fig. 8). And while the broader *Pseudpanax-Polyscias* group is not resolved in the mitochondrial tree, the smaller *Polyscias* clade does receive support (BS = 69%; Fig. 1b). In this clade, we find additional evidence for the paraphyly of the genus *Polyscias* relative to six other genera, confirming several prior studies (Plunkett *et al*., 2001, 2004*b*; Plunkett and Lowry, *submitted*). This paraphyly ultimately led to the taxonomic inclusion of

Arthrophyllum, *Cuphocarpus*, *Gastonia*, *Munroidendron*, *Reynoldsia*, *Tetraplasandra* under a much broader *Polyscias* to restore monophyly (Lowry and Plunkett, *submitted*).

***Raukaua et al.*:** This group is recognized primarily on the basis of plastid data (Fig. 8a), where it includes *Raukaua*, *Schefflera s. str.*, *Cheirodendron*, *Motherwellia*, and *Cephalaralia*. In the nuclear phylogeny, these genera are also allied, but form a phylogenetic grade at the base of the *Hydrocotyle* clade (Fig. 8b). Neither of the two data sets provided high support for the clade. A similar pattern was shown in Plunkett *et al*. (2004*c*; BS = 53%) in an MP tree based on plastid *trn*L-*trn*F sequences (with the exception of *Motherwellia*, which was placed in the Asian Palmate group), and in their Bayesian analysis of combined *trn*L-*trn*F + ITS data, which united all five genera (PP = 51%). In the mitochondrial phylogeny, these genera (except the unsampled *Cephalaralia*) grouped together with the *Polyscias* clade and *Astroticha*, with 80% bootstrap support (Fig. 1b). In the plastid tree, a sister relationship between *Motherwellia* and *Cephalaralia* is supported (BS = 82%; Fig. 8a), and this relationship may be reflected in their overlapping geographies (in NE Australia), and in their climbing habits and imbricate petal aestivation (which is uncommon in Araliaceae; Plunkett *et al*., *submitted*).

***Aralia* Group:** Apart from the former hydrocotyloids, *Aralia* and *Panax* are the only genera of Araliaceae that include at least some herbaceous species. *Aralia* and *Panax* form a well supported clade in both the plastid (BS = 95%; Fig. 8a) and nuclear cladograms (BS = 100%; Fig. 8b), a result similar to other studies that included better

sampling from these two genera (e.g., Wen *et al*. 2001; Mitchell and Wen, 2004; Plunkett *et al*. 2004*c*). In the nuclear tree, the *Aralia* group is placed in a larger clade that also includes the *Polyscias-Pseudopanax* group and African *Schefflera*. Bootstrap support for this group is 75%, but relationships among its three clades are not well supported (Fig. 8b). An affinity between the *Aralia* group and the *Polyscias-Pseudopanax* group was reported by prior studies (e.g., Wen *et al*., 2001; and Plunkett *et al*., 2004*c*).

African *Schefflera*: The nuclear tree places African *Schefflera* with the Aralia group (see above), but stronger support for its placement as sister to the rest of Araliaceae (excluding the *Hydocotyle-Trachymene* group) came from the plastid phylogeny (BS = 80%; Fig. 8a). Relationships in this group, however, remain tentative, and given the limited sampling used in this study (only two species), it is not prudent to make too many additional inferences.

***Astrotricha* and *Harmsiopanax*:** These two genera do not form a clade, but neither resembles any other genus in Araliaceae, and some authors viewed them as potential links between Araliaceae and Apiaceae (see Frodin and Govaerts, 2003). The affinity to Araliaceae comes from their tropical distributions, woody habits, and paniculate inflorescences. On the other hand, they have bicarpellate, schizocarpic fruits, which are characteristic of most Apiaceae. Molecular studies provide overwhelming support for the inclusion of both genera in Araliaceae, where they are currently classified, but their placement in this family remains unstable. The only well supported placement

for *Astrotricha* was in the mitochondrial *nad1* phylogeny (Fig. 1b), where it is sister to the *Raukaua et al*. group in a larger clade that also includes the *Polyscias* subclade and *Motherwellia*. There is moderate support for this entire clade (BS = 80%), but not for many of the internal relationships. A somewhat similar placement was evident in the RPB2 phylogeny but with low support (BS < 50%; Fig. 8b). In the cladogram resulting from RPB2 copy 2 for Araliaceae (RPB2 *Ar2*), *Astrotricha* is sister to African *Schefflera* with 52% bootstrap support (see Chapter 2). We were not able to find congruence for the placement of *Astrotricha* in our studies (or the studies cited herein), and thus the placement of *Astrotricha* in Araliaceae remains elusive.

Harmsiopanax presents a similar case. Its placement in Araliaceae has been problematic and the genus appears either unresolved or unsupported relative to other genera. The plastid ML phylogeny provides the best supported placement, where it was sister to the *Hydrocotyle-Trachymene* group (BS = 69%; Fig. 8a). The same placement was retrieved in the ML tree based on RPB2 exon sequences (BS = 55%; Chapter 2), but not in the tree based on both exons and introns. In the tree based on copy *Ar1* of the duplicated RPB2 gene (Fig. 8b), *Hydrocotyle* and *Harmsiopanax* are placed in the same clade. This may have been due to sampling difference between the two copies, since copy *Ar1* was not found in *Trachymene,* and copy *Ar2* was not found in either *Hydrocotyle* or *Harmsiopanax*.

***Hydrocotyle-Trachymene* Group:** *Hydrocotyle*, *Neosciadium*, *Trachymene*, and *Uldinia* constitute a lineage that appeared sister to the rest of Araliaceae in the plastid

phylogeny (BS = 100%; Fig. 8a). All four genera had been included in Apiaceae, based on their herbaceous habit and presence of schizocarpic fruits, and further in Hydrocotyloideae subtribe Hydrocotylinae, based on the presence of mericarps with lateral compression, woody endocarps, lack of vittae, and absence of sepals. As discussed in Chapter 1, these characters do not provide reliable phylogenetic signal and do not represent synapomorphies when mapped out on the plastid phylogeny. The suggestion for moving *Hydrocotyle* from Apiaceae to Araliaceae dates back at least to Seemann (1854) who concluded that the valvate corolla aestivation of *Hydrocotyle* warranted this transfer. Other morphological similarities also link the *Hydrocotyle-Trachymene* group to members of Araliaceae, including sclerified endocarps and laterally compressed, bicarpellate fruits, but these characters are also present in some Apiaceae. In addition to the plastid phylogeny, the *Hydrocotyle-Trachymene* group was placed in Araliaceae on the basis of *nad1* intron 2 and RPB2 data, in both cases with high support (BS > 95%; Figs. 1b & 8b). In the RPB2 tree, two paralogous copies were retrieved from this group. In *Hydrocotyle* and *Neosciadium*, we retrieved copy *Ar1* (but not *Ar2*), but in *Trachymene* and *Uldinia*, we found the converse (*Ar2* but not *Ar1*) (Fig. 8b). Within Araliaceae, the *Hydrocotyle-Trachymene* group is most closely related to *Harmsiopanax*, which also has schizocarpic fruits (see previous section). With the exception of the plastid MP phylogeny (Chapter 1), the whole *Hydrocotyle-Trachymene* group did not appear sister to the rest of Araliaceae. In the nuclear MP phylogeny (Chapter 2), the *Hydrocotyle* clade alone appears as sister to the rest of Araliaceae in the *Ar1 clade*, and this same relationship is reflected in the *Ar2* clade, but with *Trachymene* as sister to the

rest of Araliaceae. In the other trees, these genera appeared nested within one of the smaller clades of Araliaceae (*nad1* intron 2 and RPB2 ML phylogenies; Figs 1b & 8b), or sister to *Harmsiopanax* (ML plastid phylogeny; Fig. 8a). Despite this instability among data sets, molecular evidence uniformly places members of the *Hydrocotyle-Trachymene* group in Araliaceae, leaving little doubt as to their status as members of this family (see also Plunkett *et al*., 1997, 2004*a*, 2004*c*; Chandler and Plunkett, 2004).

Within the *Hydrocotyle-Trachymene* group, the *Hydrocotyle* subclade is sister to the *Trachymene* subclade in both the plastid phylogeny (BS = 100%; Fig. 8a) and the mitochondrial phylogeny (BS < 50%; Fig. 1b), a result similar to that of Chandler and Plunkett (2004) based on nuclear 26S rDNA (BS = 72%), plastid *matK* and *rbcL* (BS = 88%) sequences, and the combination of these datasets (99%). One character that distinguishes the two groups is the carpophore, which is lacking in the *Hydrocotyle* subclade but present in the *Trachymene* subclade (Tseng 1967; Henwood and Hart 2001; Liu 2004). In the *Hydrocotyle* subclade, *Hydroctyle* is paraphyletic with respect to the monotypic *Neosciadium* due to the early divergence of a single species of *Hydrocotyle* (*Eichler 22047*). This result is supported in both the *nad1* intron 2 (BS = 99%; Fig. 1b) and RPB2 (BS =100%; Fig. 8b) cladograms, as well as preliminary analysis based on plastid *trn*D-*trn*T and *rpl16* sequences (results not shown). In the *Trachymene* subclade, the monotypic *Uldinia* is either the sister to *Trachymene* (plastid data; Fig. 8a) or nested within a paraphyletic *Trachymene* (mitochondrial data; Fig 1b). Theobold (1967) emphasized several characters that separate *Uldinia* from *Trachymene* (e.g., wing development, floral venation, and the orientation of fibers in the endocarp). Keighery and

Rye (1999), however, considered these differences to be insufficient justification for recognizing a distinct genus, especially given the tremendous variation in fruit features found among other species of *Trachymene*. Thus, they treated the single species of *Uldinia* as *T. certocarpa* (see also Rye, 1999).

3.6. Relationships in Pittosporaceae

The placement of Pittosporaceae as the earliest diverging lineage in suborder Apiineae is well supported in the plastid phylogeny (Fig. 2a) and both copies in the RPB2 phylogeny (Fig. 2b). The family is also placed in suborder Apiineae in the *nad1* intron 2 tree, but without high support for a sister-group relationship (Fig. 1). The same placement (but also without strong support) was reported in the plastid phylogeny of Chandler and Plunkett (2004), the only other study of Apiales that included significant sampling across all families of the order. By contrast, the BI analysis of their combined plastid and nuclear data sets suggested a novel and strongly supported relationship (PP = 98%) between Pittosporaceae and Myodocarpaceae in a clade appearing between the divergence of Araliaceae and Apiaceae.

All phylogenies estimated herein show two defined groups within Pittosporaceae, the first includes *Pittosporum* + *Auraunticarpa* + *Bursaria* + *Rhytidisporum* (the *Pittosporum* group), and the second includes *Bentleya* + *Billardiera* + *Cheirenthera* + *Marianthus* (the *Billardiera* clade) (Fig. 9). The one remaining genus, *Hymenosporum*, was placed as sister to the *Pittosporum* group in the plastid phylogeny (but with weak support; BS < 50%), and sister to the *Billardiera* group in the RPB2 tree (with moderate

support; BS = 80%). The monophyly of the *Pittosporum* group was supported in the plastid (BS = 90%), nuclear (59%), and mitochondrial (85%) phylogenies (Fig. 9). The group included three subclades, *Pittosporum*, the *Bursaria* + *Rhytidisporum* clade, and *Auraunticarpa* (Fig. 9). The species of *Pittosporum* form a well supported clade in all three cladograms (BS > 80%), but the relationship between *Bursaria* and *Rhytidisporum* is only well supported in the RPB2 tree (100%). *Auranticarpa* is placed as sister to *Bursaria* in the mitochondria ML tree (BS = 55%), sister to *Bursaria* + *Rhytidisporum* in the plastid tree (BS < 50%), and sister to the *Pittosporum* and *Bursaria* + *Rhytidisporum* clades in the RPB2 cladograms (BS = 56%). The nuclear tree also shows *Pittosporum* as sister to the *Bursaria* + *Rhytidisporum* subclade (BS = 50%; Fig. 9b).

The monophyly of the *Billardiera* clade was supported in the plastid (BS = 91%), nuclear (100%), and mitochondrial (75%) cladograms (Fig. 9). The relationships between the four genera of this clade were fully resolved in each tree, but were not identical in topology. A sister-group relationship between *Marianthus* and *Billardiera* is well supported in the RPB2 phylogeny (BS = 99%) and weakly supported in the *nad1* intron 2 phylogeny (BS = 51%). The two genera constitute a clade in the plastid phylogeny (BS > 95%), but *Billardiera* appears paraphyletic with respect to *Marianthus* (BS < 50%). This paraphyly was due to the grouping of *Marianthus* (recently re-segregated from *Billardiera*; see Cayzer and Crisp, 2004) with *B*. *cymosa*. These two taxa formed a sister group to *B*. *heterophylla*, which had formerly been treated in the genus *Sollya* (see Cayzer *et al*. 2004). Chandler *et al*. (2007) also showed a paraphyletic *Marianthus* with affinities to *Cheiranthera*, but a sister-group relationship between *Cheiranthera* and

Marianthus was not found in any of our analyses. In the RPB2 phylogeny, *Chiranthera* was sister to *Bentleya* (BS = 67%) and in turn, *Cheiranthera* + *Bentleya* were sister to *Billardiera* + *Marianthus* (BS = 100%) (Fig. 9b). The plastid phylogeny showed *Bentleya* as sister to *Billardiera* + *Marianthus* (BS = 92%) and, in turn, *Bentleya* + *Billardiera* + *Marianthus* were sister to *Cheiranthera* (BS = 91%) (Fig. 9a). *Bentleya* was not sampled in the *nad1* intron 2 analysis, and *Cheiranthera* was sister to *Billardiera* + *Marianthus* in that phylogeny (BS = 75%) (Fig. 9c). Although our sampling was limited in this group, results from our study, coupled with those of Chandler *et al*. (2007), indicate that many questions persist regarding the relationships and generic circumscriptions in the *Billardiera* clade, and that further phylogenetic analyses with much expanded sampling is necessary.

3.7. Divergence and Biogeography

Most approaches for reconstructing biogeographic history involve the use of phylogenetic trees and present-day distributions to understand past events of vicariance, dispersal, and extinction. Although robust methodologies for exploring gene trees provide good inferences of relationships among extant species, the complexities of geological history and unsupported (and sometimes conflicting) evidence regarding area relationships hinder our ability to test hypotheses of vicariance and expose the stochasticity of hypotheses of dispersal. The lack of reliable fossil records also thwarts our ability to ascertain extinction events. All of these issues apply to Apiales, and our objective to understand the biogeography of the order was limited by many of these

complications. As discussed above (see Materials and Methods), DIVA did not accommodate the sample size, and hence we pruned some branches from the plastid tree and analyzed groups separately. Since we discuss divergence and biogeography together, we have superimposed results of the area reconstructions generated by separate runs of DIVA on the chronogram estimated in BEAST, which was also based on the plastid data set (Fig. 10a-c). Times of divergence for select clades in the chronogram are summarized in Table 3 and we also compare some of these estimates to the divergence estimates derived from the nuclear RPB2 data, presented in Chapter 2. Thus, in the following section, we infer biogeographic patterns in Apiales using information drawn from a combination of sources, including the DIVA reconstructions (based on the plastid phylogeny), estimated times of divergence (based on plastid and nuclear estimates), published accounts of geological history, patterns of phylogenetic relationships (based on this and prior studies), and our educated speculations. Through this we hope to provide a framework that attempts to explain the biogeographic relationships of the backbone of Apiales. This initial attempt will produce hypotheses that we anticipate to be the subject of detailed studies in the future.

Starting at the deepest branches in Apiales, the divergence of the Pennantiaceae lineage from the rest of the order occurred in the early Cretaceous, between 120 to 130 Mya. Torricelliaceae diverged later, c. 104 Mya (exponential estimate, or UCED) or 113 Mya (lognormal estimate, UCLN), followed by Griseliniaceae, which diverged from the rest of Apiales c. 100 Mya (UCED) or 110 Mya (UCLN) (Fig. 10b; Table 3). The nuclear phylogeny differed in the placement of Griselineaceae + Torricelliaceae (as sister to order

Apiineae), and in the nuclear chronogram this clade diverged c. 111 Mya, followed by the divergence of Torricelliaceae from Griseliniaceae c. 92 Mya (Chapter 2). Our plastid divergence-date estimates give suborder Apiineae (= Pittosporaceae, Araliaceae, Myodocarpaceae, and Apiaceae) a mid- to late-Cretaceous origin, with a crown age of c. 100 Mya (c. 95 Mya with UCED, c. 103 Mya with UCLN; Table 3). This agrees with the UCLN estimates from the RPB2 phylogeny (Chapter 2), where the crown age is c. 102 Mya. In all cases, the nodes had a posterior probability (PP) higher than 90%.

DIVA provided four likely explanations for the biogeographic history of Apiales, areas A, AD, ABD, or ABDE (Fig. 10). In light of this lack of consensus, we present those scenarios from DIVA that we consider most likely, while keeping in mind the alternative explanations. DIVA reconstructions demonstrate that suborder Apiineae originated in the Australia + Pacific region (area A, Fig. 10b). This indicates that the several alternatives for the origin of the entire order are due to the complex pattern of distributions that characterize these early-diverging families. Considering the ages of Pennantiaceae, Torricelliaceae, Griseliniaceae, and Apiineae, these four clades represent ancient lineages, possibly with Gondwanan origins. Their ancestors may have been isolated in Madagascar, South America, and Australasia during the Cretaceous. Dispersal among the Gondwanan elements remained possible from Australia to South America (through Antarctica) until the Eocene and to Madagascar (through India) later than the Miocene (Gentry, 1982; Schatz, 1996; Sanmartín and Ronquist, 2004). The lack of ancient relatives of these lineages in India, Antarctica, and Africa is due most likely to extinctions.

Griseliniaceae exhibits a South American-New Zealand disjunction with a greater number of species in South America (5 spp.) than New Zealand (2 spp.). Mildenhall (1980) suggested that *Griselinia* appeared in New Zealand in the Miocene, but in our study, the age of this lineage is estimated to be much older (mid-Cretaceous). In general, flowering plants are thought to have arrived in New Zealand during the Eocene as a result of dispersal from Australia and oceanic Pacific Islands or from southern South America through Antarctica (Winkworth *et al*., 2002; Sanmartín and Ronquist, 2004; Wagstaff *et al*., 2006). Given these patterns, it seems that *Griselinia* most likely arrived in New Zealand through long-distance dispersal from South America. Torricelliaceae includes three genera with quite distinct distributions, *Melanophylla* in Madagascar, *Torricellia* from the East Himalayas to western China, and *Aralidium* in western Malesia. The overall distribution in Torricelliaceae may be explained as dispersal events from Madagascar to Malesia and the rest of Asia through India, followed by extinction in India (Schatz, 1996). The tropical flora of South America was largely isolated during most of the Cretaceous (Gentry, 1982), which may explain the divergence of Griseliniacae and its isolation from Torricelliaceae. Interpreting the geographic connection of Pennantiaceae to Torricelliaceae, Griseliniaceae, and Apiineae is more difficult given its placement in the phylogeny and its estimated time of divergence. One likely explanation is that Pennantiaceae is the only extant ancestor of a relative of Apiales that originated during a period of high angiosperm diversification centered in Australasia during the early Cretaceous.

Within Apiineae, radiations within a period of less than 15 Mya led to the origination of Pittosporaceae, Araliaceae, Myodocarpaceae, Mackinlayoideae, and the *Platysace* group (Fig. 10; Table 3). Pittosporaceae diverged during the Cretaceous with a plastid sequence stem age of c. 100 Mya (c. 95 Mya with UCED, c. 103 Mya with UCLN; Table 3). The estimates based on RPB2 copies 1 and 2 were comparable at c. 101 Mya and c. 96 Mya, respectively (Chapter 2). The family has its greatest diversity in Australia, where nearly all of the nine genera are endemic, but the largest genus, *Pittosporum*, has secondary centers of diversity in New Caledonia and New Zealand, and also extends to other islands of the Pacific, into Asia, and across the Indian Ocean to Madagascar and eastern Africa. DIVA reconstructions showed an Australian-Pacific origin for the group (Area A; Fig. 10c). All estimates of the age of the Pittosporaceae crown group were less than 40 Mya, which indicates that diversification within the family was due to post-Gandwanan dispersal from Australasia to the Pacific, Africa, and Asia.

The stem age for Araliaceae ranged from c. 93 Mya (UCED) to c. 100 Mya (UNLG) based on plastid data. Estimates based on the nuclear RPB2 copies *Ar1* and *Ar2* fall between 86 Mya and 96 Mya (Chapter 2). The stem age for the group was greater than 65 Mya in the plastid phylogeny but less than 55 Mya in both copies of RPB2. As discussed in Chapter 2, relations in the nuclear phylogeny were complicated by three factors: (1) the presence of two copies, (2) independent polyploidy events in different clades, and (3) the inability to identify copy 1 from one of the clades (*Trachymene*). Thus, we highlight estimates for well supported clades (having high PP values) from the plastid chronogram, summarized in Fig. 10 and Table 3. One of these supported clades is

the *Hydrocotyle–Trachymene* group (PP = 100%), which appears to have diverged from the rest of Araliaceae more than 65 Mya; the *Hydrocotyle* and *Trachymene* subclades subsequently diverged from one another c. 52 Mya. The clade has an Australian-Pacific origin due largely to the geographic ranges of *Trachymene* and *Neosciadium* and the early diverging sample of *Hydrocotyle* (Fig. 10). *Hydocotyle* and *Trachymene* mark a shift from other Araliaceae to herbaceousness and a preference to temperate habitats, especially in Western Australia, which is their likely center of origin. In the plastid topology, the earliest diverging clade among other Araliaceae is the African *Schefflera* clade (PP = 99.99%), which dates back to the early Eocene with a stem age of c. 54 Mya (UCED) to 61 Mya (UNLG). The age of this divergence is too recent to be considered Gondwanan. Instead, the presence of this clade in Africa and Madagascar is more likely due to long-distance dispersal across the western Indian Ocean Basin (IOB). Dispersal events across the IOB have been reported in many plant groups at different ages (including in other groups of Araliaceae, such as *Polyscias s. lat.*; see Plunkett *et al*., 2004*b*), and may have led to secondary dispersals into Africa across Madagascar (Schatz, 1996; Sanmartín and Ronquist, 2004). The *Raukaua* group diverged from the rest of Araliaceae more than 40 Mya, but support for the group is low. The pattern within the *Raukaua* group shows a Pacific origin and dispersal across the Pacific. The *Polyscias* and *Pseudopanax* subclades diverged 20-25 Mya and both show high species diversity in the Pacific islands. While the *Pseudopanax* subclade is restricted to the Pacific region, the *Polyscias* subclade shows a pattern of dispersal throughout the Paleotropics, that may have originated in Australasia and dispersed multiple times both eastward into the Pacific

and westward into the IOB (see Plunkett *et al*., 2001, 2004*b*; Plunkett and Lowry, *submitted*). A third dispersal to Africa from Australasia appears to explain the distribution of *Seemannaralia* and *Cussonia*. However, it is difficult to make accurate predictions on the routes of these dispersals due to the lack of support and incongruence of the placement of the two genera in different phylogenies. Our estimate for the divergence of the Asian Palmate clade from the rest of Araliaceae is 36-40 Mya, as a result of dispersal from Australasia, and the clade appears to have diversified rapidly in Asia (Fig. 10; Table 3). Resolution in the Asian Palmate group was not sufficient to make further conclusions on diversifications into the Americas, but some authors have suggested a boreotropical origin for the Asian-Neotropical disjunctions in this clade (e.g., Plunkett *et al*., 2004*c*).

A thorough understanding of the biogeography of Araliaceae has been hindered by the many unresolved or unsupported relationships remaining in this family, but like the order as a whole, Araliaceae appears to have had an Australasian origin (Area A; Fig. 10c). New Caledonia, in particular, exhibits extraordinary diversity and endemism in the family. This South Pacific island separated from the Australian continent in the Upper Cretaceous (~65 Mya), but maintained links to Australia through the Miocene (Barlow, 1981). New Caledonia has been devoid of major climatic and volcanic events since the Oligocene (Thorne, 1969; Morat, 1993; Murienne *et al*., 2005), but between the Cretaceous and the Oligocene, major geological events helped to shape the distribution and diversification of the flora on the island (see Lowry, 1998; Murienne *et al*., 2005). The exceptionally high levels of species diversity and endemism in New Caledonia may

be explained by the persistence of refugia that helped to preserve "relictual lineages" during the aridification of Australia and the submergence of other island archipelagoes (during the Oligocene) (Chazeau, 1993; Jaffré, 1993; Lowry, 1998). The island's flora is most similar to that of Australia (Morat, 1993; Morat *et al.*, 2001), but has undergone less alteration due to fewer climatic changes (Stevenson and Hope, 2005). Inhabitation by humans was also delayed in New Caledonia relative to other landmasses (~ 3,000 years BP, compared to Australia, which was inhabited by humans since ~ 50,000 years BP), saving the island's natural habitats from their detrimental anthropogenic effects (the "blitzkrieg" hypothesis; Martin, 1963) and fires that annihilated many forests elsewhere (Miller *et al.*, 2005; Trueman *et al.*, 2005).

Based on plastid data, Myodocapaceae represent an ancient lineage that diverged from the rest of Apiineae c. 89 Mya (UCED) to c. 96 Mya (UNLG); nuclear estimates were c. 93 Mya for copy 1 and c. 73 Mya for copy 2 (Chapter 2). The only two genera in this family, *Delarbrea* and *Myodocarpus*, diverged 20 to 30 Mya (Table 3). The family may constitute lineages that persisted in New Caledonia but went extinct in Australia and other nearby regions during Eocene climatic changes. Most of the 17 species in the family are restricted to New Caledonia, excepting only two species of *Delarbrea* (*D. michieana*, which is endemic to Queensland, and *D. paradoxa*, which ranges from New Caledonia to other nearby Pacific islands). The fruits of these species are dispersed by medium- to large-size birds (Lowry, 1986*b*) and thus may have been dispersed by birds through connections maintained during the Miocene between New Caledonia and Australia through the Sunda arc (Barlow, 1981). One key factor for the diversification of

Myodocarpus may be the adaptability to serpentine soils. Eight of the ten species of *Myodocarpus* grow exclusively on serpentine-like ultramafic soils (Lowry, 1986*a*), an adaptation that may have contributed to species endemism and increased competitive success (Fiedler, 1985; Baker, 1987; Lowry, 1991; Mayer and Soltis, 1994; Morat, 1993; de Kok, 2002).

Mackinlayoideae are another ancient lineage in Apiales, dating to beyond 90 Mya on basis of plastid data (Fig. 10; Table 3). In estimates based on nuclear data (Chapter 2), one of the two paralogous copies of RPB2 diverged from Mypdocarpaceae more than 73 Mya, whereas the other diverged from *Platysace* more than 78 Mya. The divergence of *Actinotus* and *Apiopetalum* occurred more than 55 Mya, followed by *Mackinlaya* (c. 50 Mya). The mostly herbaceous taxa in the clade appear to be older than 40 Mya in the plastid chronogram and c. 34 Mya in the nuclear chronogram. The DIVA reconstruction suggested an Australian + Pacific origin for the clade (Fig. 10). Dispersal within Australasia explains the distribution of *Mackinlaya* from Australia to Malesia. The mostly-herbaceous mackinlayoids (*Xanthosia*, *Chlaenosciadium*, *Pentapeltis*, and *Schoenolaena*) diversified entirely within Australia, but the node ages for these clades do not indicate rapid radiation events. Dispersal to Mesoamerica (*Micropleura*) and Africa (*Centella*) must be invoked to explain the distribution of these taxa, which appear to have diverged after *Schoenolaena*, more than 14 Mya (Table 3).

The *Platysace* clade (*Platysace* + *Homalosciadium*) appears sister to Mackinlayoideae in the RPB2 chronogram (but with low support; PP = 66.3%), and these two clades diverged more than 78 Mya. The plastid chronogram offered ~ 99% support

for its placement as sister to the rest of Apiaceae (i.e., Azorelloideae + Saniculoideae + Apioideae), and an age for the *Platysace* clade of 81-87 Mya. This clade is restricted to Australia and represents the youngest of the major Pacific lineages of Apiales (Fig 10; Table 3). The remaining lineages represent the relatively younger Azorelloideae (South American origin) and Apioideae-Saniculoideae (African origin). A shift from woody habits and tropical climates appears to start with the herbaceous genera of Mackinlayoideae and the *Platysace* clade, both of which show high levels of endemism in temperate southwestern Western Australia.

The split between Azorelloideae and Apioideae-Saniculoideae dates back to 73-79 Mya. The first clade to diverge in Azorelloideae was the *Klotzschia* clade (67-73 Mya), followed by radiation of the four additional clades 59-65 Mya (Fig. 10; Table 3). With the exception of the *Diposis* and *Spananthe* clades, which both diverged more than 45 Mya, the crown ages of the South American *Asteriscium*, *Bowlesia*, and *Azorella* clades ranged between 23 and 44 Mya. These times represent periods of gradual uplifts and other geological changes in the Andes, especially the central and southern Andes (Gentry, 1982; Mégard, 1984). Azorelloideae show a South American origin, and the earliest diverging genus, *Klotzschia*, is the only species of the subfamily in Brazil. The remaining clades exhibit high diversification in the Andes. The diversification of these groups may reflect adaptations either to open areas at high elevations (e.g., *Azorella*), shady, humid areas of high elevations (e.g., *Bowlesia*), or deserts (e.g., *Eremocharis*). Many azorelloids are also characterized by a suffrutescent habit, which may be an adaptation to harsh weather conditions such as severe cold (e.g., *Azorella*) or aridity (e.g.,

Eremocharis). The *Asteriscium*, *Bowlesia*, and *Azorella* clades all include independent dispersal events from South America to the Old World, either to the sub-Antarctic Islands (*Stilbocarpa* and *Azorella*), New Zealand (*Schizeilema*), or Australia (*Diplaspis* in the *Azorella* clade, *Oschatzia* in the *Asteriscium* clade, and *Dichosciadium* in the *Bowlesia* clade).

The earliest diverging lineage in the Apioideae-Saniculoideae group, *Hermas*, split 69-74 Mya (Fig. 10; Table 3). This was followed by the separation of the Saniculoideae and Apioideae clades c. 65 Mya. Based on the DIVA reconstruction, Apioideae-Saniculoideae has an African center of origin. *Hermas* is endemic to southern Africa, as are the early diverging genera in the two clades representing Apioideae and Saniculoideae (e.g., *Polemanniopsis*, *Steganotaenia*, *Heteromorpha*, *Anginon*, *Lichtensteinia*, *Choritaenia*). In Saniculoideae, the earliest diversification out of Africa is estimated to be c. 30 Mya for the clade that unites *Eryngium* + *Sanicula* + *Astrantia* + related genera. In Apioideae, the earliest clade not represented in Africa is the Central American *Neogoezia* (c. 41 Mya) followed by the New Zealand and Australian *Anisotome* group (c. 38 Mya), and then other areas (c. 35 Mya) (Fig. 10a; Table 3), but sampling among the genera of Apioideae is not comprehensive. The apioids experienced a massive diversification in the temperate regions, as evidenced by the large number of its species in Africa (both northern Africa and the Cape region), the Mediterranean, and throughout Eurasia. More detailed sampling and statistical analyses are necessary to explain the complex diversification routes of the very speciose Apioideae-Saniculoideae groups out of (and sometimes back to) Africa.

3.8. Summary and Hypotheses of Biogeographic History

Suborder Apiineae appears to have originated in the Paleotropics, with Australasia as the likely center of origin. Araliaceae, Myodocarpaceae, Mackinlayoideae, and Pittosporaceae are collectively represented by more than 133 endemic species in New Caledonia alone (Morat 1993), and many of these represent early-diverging lineages in their respective clades (i.e., paleo-endemics). Australia likewise has more than 200 endemic species from the same four groups. Thus, either (or possibly both) New Caledonia and/or Australia may have been a center of origin for Apiineae; alternatively, they may merely have served as refugia where the few ancient relicts survived, followed by relatively recent diversifications (leading to a proliferation of neo-endemic species). Dispersals to Madagascar (through the Indian Ocean), to Asia (through Malesia), and into the Americas (either through Antarctica or Asia) provide possible explanations for the geographic histories of Pittosporaceae and Araliaceae to these continents. An Australasian (especially New Caledonian and Australian) distribution is retained in the three clades diverging next in the history of Apiineae (i.e., Myodocarpaceae, Mackinlayoideae, and the *Platysace* clade). Most taxa of Pittosporaceae, Araliaceae, and Myodocarpaceae (plus some Mackinlayoideae) have retained affinities to tropical climates and woody habits. DIVA provides three possible explanations at the node for the *Platysace* clade: Australia/South America, Australia/Africa, or Australia/South America/Africa. The earliest lineages in the South American and African clades are *Klotzschia* and *Hermas*, respectively. The ages of these clades are very similar and the divergence of *Klotzschia* and *Hermas* relative to their respective sister groups shows an

almost identical pattern. Africa and South America started separating more than 120 Mya, but contact was possible until c. 80 Mya (Raven and Axelrod, 1974). Plant dispersals between Africa and South America have been recorded as late as the Eocene (Sanmartín and Ronquist, 2004), later than the age of divergence of the Azorelloideae and Apioideae-Saniculoideae clades. One likely explanation is dispersal from Australia to South America and then to Africa, followed by isolation and diversifications occurring in parallel in Australia, South America, and the Cape region of southern Africa. Radiations in the temperate region of Western Australia were correlated with the development of herbaceousness in Mackinlayoideae. Diversification in South America was followed by rapid diversification throughout the Andes, and dispersal to the sub-Antarctic Islands, New Zealand and Australia. The Cape region of southern Africa was the likely center for the third radiation, in Apiaceae, from where subsequent diversifications occurred throughout Africa and then to the North temperate regions.

4. Conclusion

The *nad1* intron 2 region of the plant mitochondrial genome provides a useful source of data for reconstructing relationships in Apiales, especially within families, where resolution and support were especially strong. The development of additional markers sampled from the mitochondrial genome will probably provide even greater resolution and support for many clades in Apiales. The present study provides a marked improvement in our understanding of phylogenetic relationships in Apiales, with evidence from all three plant genomes, but it also leaves many questions that require

further studies, especially for relationships within some of the major clades. We have also presented for the first time (and with a nearly comprehensive sampling of genera from throughout the order) a detailed estimate of divergence times for all the major clades of Apiales. Nevertheless, it will be necessary to test these estimates using additional markers. Interpreting biogeographic patterns at this level of detail and depth is very complicated, but our study provides an historical-biogeographic framework for all the main lineages of Apiales that can be tested in future studies based on detailed work with additional markers and a focus within individual lineages of the order.

TABLES

Table 1. Primers developed during this study for the amplification and sequencing of the *nad1* intron 2 region in Apiales.

Primer name	Sequence (5' to 3')	Direction	Position
mt_nad1b_IF	GCGTCTCGTCGCAAGGCTCATT	Forward	External
nad1_MR	CCGTCTCATCTTGATTTGGCTA	Reverse	Internal
nad1_MF	CATGGCTGGCTACATACAAGTA	Forward	Internal
nad1c_IR2	CATGTGGCTCGTCCGTGCTT	Reverse	External
nad1INT2_N08F	GAGGTGACTGCAATGAGCAGA	Forward	External
nad1INT2_N08R	AGCGCCTACCAAGCAAAGCT	Reverse	External

Table 2. Sequence characteristics of the mitochondrial *nad1* intron 2 region, and tree statistics based on phylogenetic analyses of this marker.

Sequence length	642-1227
Number of aligned characters	1725
Parsimony-informative characters	240
Number of trees	40000
Number of steps	621
Consistency index (CI)	0.8116
Retention index (RI)	0.9242
Maximum likelihood -ln	-6632.6566

Table 3. Estimates of divergence dates based on BEAST analyses (mean root height; MRH) at selected nodes in the plastid chronogram (Fig. 10), their 95% high posterior density (HPD) intervals, and posterior probabilities (PP). Date estimates are given in millions of years, based on two runs of 20 million generations each, using two relaxed clock models, uncorrelated lognormal distribution (UCLN) and uncorrelated exponential distribution (UNED).

Node	UCLN PP	Plastid UCLN MRH with 95% HPD	UCED PP	Plastid UCED MRH with 95% HPD
Pennantiaceae	1.00	125.27; [103.4,151.94]	1.00	120.80; [96.83,150.50]
Torricelliaceae	1.00	113.74; [99.67,127.71]	1.00	104.49; [95.48,113.44]
Griseliniaceae	0.96	109.54; [95.76,123.73]	0.88	100.85; [91.33,110.61]
Pittosporaceae	1.00	103.21; [90.90,116.26]	1.00	95.73; [85.45,105.78]
Araliaceae	1.00	100.06; [88.45,113.20]	1.00	92.82; [82.74,103.15]
Hydrocotyle and *Trachmene*	1.00	67.64; [48.78,87.53]	1.00	71.30; [55.14,88.06]
African Schefflera	1.00	54.2; [40.95,69.20]	1.00	60.73; [46.22,77.08]
Asian Palmate	0.93	39.11; [36.20,42.80]	0.93	40.52; [32.92,48.23]
Polyscias-Pseudopanax	1.00	28.29; [15.79,38.43]	1.00	25.83; [12.94,41.42]
Raukaua et al.	0.44	43.09; [36.21,50.61]	0.53	51.80; [40.21,66.15]
Myodocarpaceae	1.00	95.64; [84.33,108.18]	1.00	89.16; [79.02,99.35]
Myodocarpus from *Delarbrea*	1.00	23.9; [2.40,52.74]	1.00	21.50; [4.51,68.29]
Mackinlayoideae	1.00	90.55; [80.02,102.79]	0.99	84.94; [75.03,94.56]
Apiopetalum & Actinotus	1.00	66.72; [48.48; 88.68]	1.00	62.65 [41.99,81.42]
Mackinlaya	1.00	57.15; [39.96,76.18]	1.00	51.22; [34.98,69.79]
Xanthosia & Chlaenosciadium	1.00	47.98; [32.69; 65.33]	1.00	41.28; [26.66; 59.06]
Centella & Micropleura	1.00	19.57; [8.54; 32.43]	1.00	14.03; [5.14; 24.72]
Platysace clade	0.97	85.68; [75.37; 96.16]	0.98	81.04; [71.55; 91.11]
Azorelloideae from Apioideae-Saniculoideae	1.00	78.84; [66.99,90.40]	1.00	73.31; [65.26,81.89]

Azorelloideae				
Klotzschia	0.99	73.22; [61.14,84.94]	0.97	67.20; [55.55,78.04]
Azorella (Spananthe)	1.00	52.13; [39.47,63.68]	1.00	47.29; [34.57,60.95]
Azorella (Dickinsia-Diplaspis)	1.00	38.22; [26.11,51.16]	1.00	34.41; [23.28,48.56]
Azorella (rest of Azorella)	1.00	30.03; [20.40,41.20]	1.00	27.45; [17.14,39.72]
Bowlesia	1.00	43.55; [32.30,54.73]	1.00	40.84; [29.84,55.80]
Asteriscium	1.00	38.92; [20.32,55.49]	1.00	32.66; [15.52,50.25]
Diposis	1.00	65.01; [61.14,84.94]	0.54	59.50; [46.58,71.30]
Apioideae-Saniculoideae				
Hermas	0.98	73.25; [63.82,83.58]	0.95	69.60; [61.61,77.61]
Apioideae from Saniculoideae	1.00	65.99; [57.36,74.55]	1.00	64.93; [57.74,72.22]
Lichtensteinia & *Choritaenia* clade	0.96	59.18; [49.91,68.36]	0.94	59.06; [51.83,67.24]
Polemanniopsis & *Steganotaenia* clade	1.00	48.65; [47.69,49.65]	1.00	52.18; [48.01,56.38]
Annesorhiza clade	1.00	58.15; [52.06,65.34]	1.00	58.85; [52.77,65.37]
Heteromorpha clade	1.00	54.42; [49.69,60.20]	1.00	56.10; [50.44,61.68]
Bupleurum clade	1.00	48.64; [47.66,49.60]	1.00	51.45; [47.46,55.43]
Neogoezia clade	1.00	41.66; [37.13,45.68]	1.00	41.97; [34.87,48.16]
Anisotome clade	1.00	38.36; [32.91,43.11]	1.00	37.69; [30.39,44.67]
Rest of Apioideae	0.95	35.96; [30.28,41.06]	0.92	35.05; [27.20,42.01]

FIGURE LEGENDS

Figure 1. Tree retrieved from maximum likelihood (ML) analyses of the mitochondrial *nad1* intron 2 dataset. The tree was generated in GARLI under the model GTR+Γ+I. ML bootstrap support values from 100 replicates are indicated above branches. See Table 1 for tree statistics.

Figure 2. Comparison of major clades retrieved with maximum likelihood (ML) analyses of (a.) the plastid combined dataset (*trn*D-*trn*T + *rpl*16 intron) to that of (b.) the nuclear dataset (RPB2). Both analyses were completed using GARLI and the model GTR+Γ+I. Estimates of branch support (based on 100 ML bootstrap replicates) are shown above branches.

Figure 3. Comparison of phylogenetic relationships in Mackinlayoideae (Apiaceae) based on maximum likelihood (ML) trees for the three data sets: (a.) plastid (*trn*D-*trn*T + *rpl*16 intron), (b.) nuclear (RPB2), and (c.) mitochondrial (*nad1* intron 2). Trees were generated in GARLI under the model GTR+Γ+I. Estimates of branch support based on 100 ML bootstrap replicates are shown above branches.

Figure 4. Comparison of phylogenetic relationships in the *Asteriscium* clade of Azorelloideae (Apiaceae) based on maximum likelihood (ML) trees for the (a.) plastid (*trn*D-*trn*T + *rpl*16 intron), (b.) nuclear (RPB2), and (c.) mitochondrial (*nad1* intron 2)

datasets. Trees were generated using GARLI and the model GTR+Γ+I. Estimates of branch support (based on 100 ML bootstrap replicates) are shown above branches.

Figure 5. Comparison of phylogenetic relationships in the *Bowlesia* clade of Azorelloideae (Apiaceae) based on maximum likelihood (ML) phylogenies for the (a) plastid (*trn*D-*trn*T + *rpl*16 intron), (b) nuclear (RPB2), and (c) mitochondrial (*nad1* intron 2) datasets. Trees were generated using GARLI under the model GTR+Γ+I. Estimates of branch support based on 100 ML bootstrap replicates are shown above branches.

Figure 6. Comparison of phylogenetic relationships in the *Azorella* clade of Azorelloideae (Apiaceae) based on maximum likelihood (ML) phylogenies for the (a) plastid (*trn*D-*trn*T + *rpl*16 intron) and (b) nuclear (RPB2) datasets. Trees were generated using GARLI under the model GTR+Γ+I. Estimates of branch support based on 100 ML bootstrap replicates are shown above branches.

Figure 7. Comparison of phylogenetic relationships in the Apioideae-Saniculoideae (Apiaceae) based on maximum likelihood (ML) phylogenies for the (a) plastid (*trn*D-*trn*T + *rpl*16 intron), (b) nuclear (RPB2), and (c) mitochondrial (*nad1* intron 2) datasets. Trees were generated using GARLI under the model GTR+Γ+I. Estimates of branch support based on 100 ML bootstrap replicates are shown above branches.

Figure 8. Comparison of phylogenetic relationships in Araliaceae based on maximum likelihood (ML) phylogenies for the (a) plastid (*trn*D-*trn*T + *rpl*16 intron) and (b) nuclear (RPB2 copy *Ar1*) datasets. Trees were generated using GARLI under the model GTR+Γ+I. Estimates of branch support based on 100 ML bootstrap replicates are shown above branches.

Figure 9. Comparison of phylogenetic relationships in Pittosporaceae based on maximum likelihood (ML) phylogenies for the (a) plastid (*trn*D-*trn*T + *rpl*16 intron), (b) nuclear (RPB2), and (c) mitochondrial (*nad1* intron 2) datasets. Trees were generated using GARLI under the model GTR+Γ+I. Estimates of branch support based on 100 ML bootstrap replicates are shown above branches.

Figure 10. Maximum clade credibility chronogram estimated from trees based on plastid (*trn*D-*trn*T + *rpl*16 intron) data and generated in BEAST after 20 million MCMC generations. Estimates were calculated using the GTR+Γ+I evolutionary model, the Yule model of speciation, and a relaxed clock with uncorrelated lognormal. Calibration points are represented by black diamonds. Relevant historical biogeography area relationships were estimated in DIVA and superimposed on the chronogram with letter designations for the five areas used.

Figure 1. *NAD1* INTRON 2 Maximum Likelihood Phylogeny.

Fig. 1a. Apiaceae.

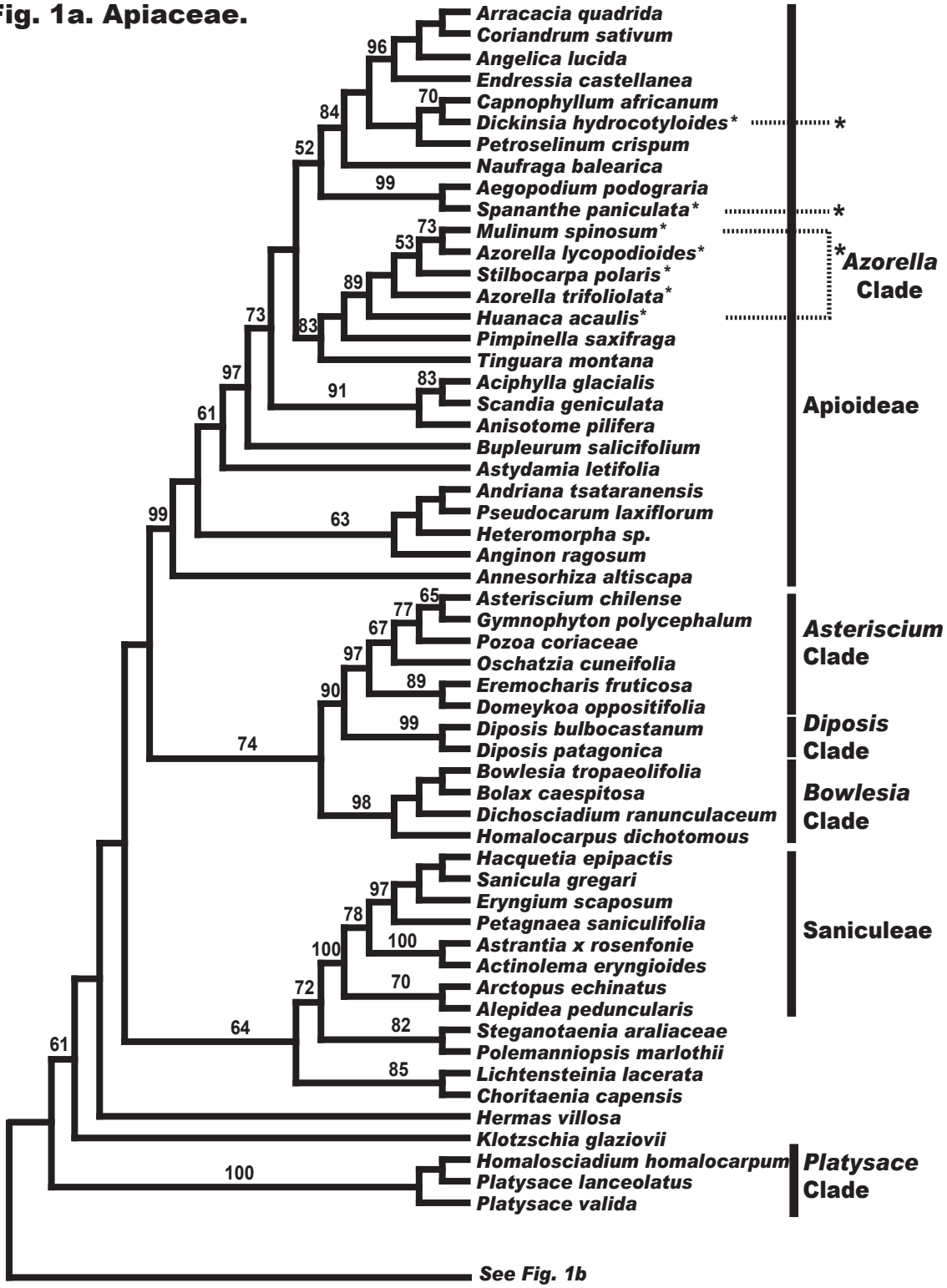


Fig. 1b.

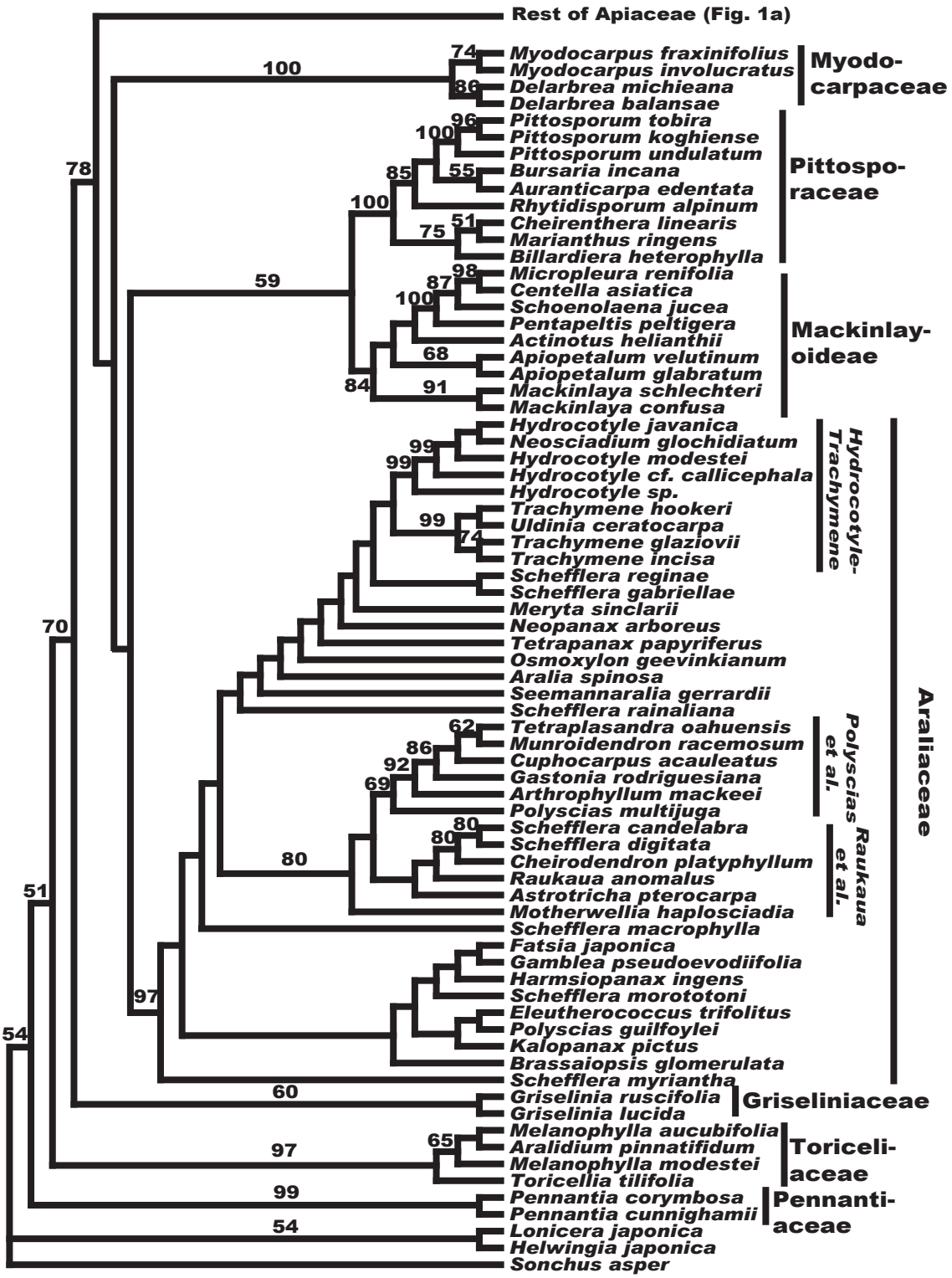

Rest of Apiaceae (Fig. 1a)
Myodocarpus fraxinifolius
Myodocarpus involucratus
Delarbrea michieana
Delarbrea balansae
Myodo-carpaceae
Pittosporum tobira
Pittosporum koghiense
Pittosporum undulatum
Bursaria incana
Auranticarpa edentata
Rhytidisporum alpinum
Cheirenthera linearis
Marianthus ringens
Billardiera heterophylla
Pittospo-raceae
Micropleura renifolia
Centella asiatica
Schoenolaena jucea
Pentapeltis peltigera
Actinotus helianthii
Apiopetalum velutinum
Apiopetalum glabratum
Mackinlaya schlechteri
Mackinlaya confusa
Mackinlay-oideae
Hydrocotyle javanica
Neosciadium glochidiatum
Hydrocotyle modestei
Hydrocotyle cf. callicephala
Hydrocotyle sp.
Trachymene hookeri
Uldinia ceratocarpa
Trachymene glaziovii
Trachymene incisa
Hydrocotyle-Trachymene
Schefflera reginae
Schefflera gabriellae
Meryta sinclarii
Neopanax arboreus
Tetrapanax papyriferus
Osmoxylon geevinkianum
Aralia spinosa
Seemannaralia gerrardii
Schefflera rainaliana
Tetraplasandra oahuensis
Munroidendron racemosum
Cuphocarpus acauleatus
Gastonia rodriguesiana
Arthrophyllum mackeei
Polyscias multijuga
Polyscias et al.
Schefflera candelabra
Schefflera digitata
Cheirodendron platyphyllum
Raukaua anomalus
Astrotricha pterocarpa
Motherwellia haplosciadia
Raukaua et al.
Schefflera macrophylla
Fatsia japonica
Gamblea pseudoevodiifolia
Harmsiopanax ingens
Schefflera morototoni
Eleutherococcus trifolitus
Polyscias guilfoylei
Kalopanax pictus
Brassaiopsis glomerulata
Schefflera myriantha
Araliaceae
Griselinia ruscifolia
Griselinia lucida
Griseliniaceae
Melanophylla aucubifolia
Aralidium pinnatifidum
Melanophylla modestei
Toricellia tilifolia
Toriceli-aceae
Pennantia corymbosa
Pennantia cunnighamii
Pennanti-aceae
Lonicera japonica
Helwingia japonica
Sonchus asper

Figure 2. Summaries of the maximum likelihood phylogenies of the plastid *trn*D-*trn*T and *rpl*16 intron (2a) and nuclear RPB2 (2b).

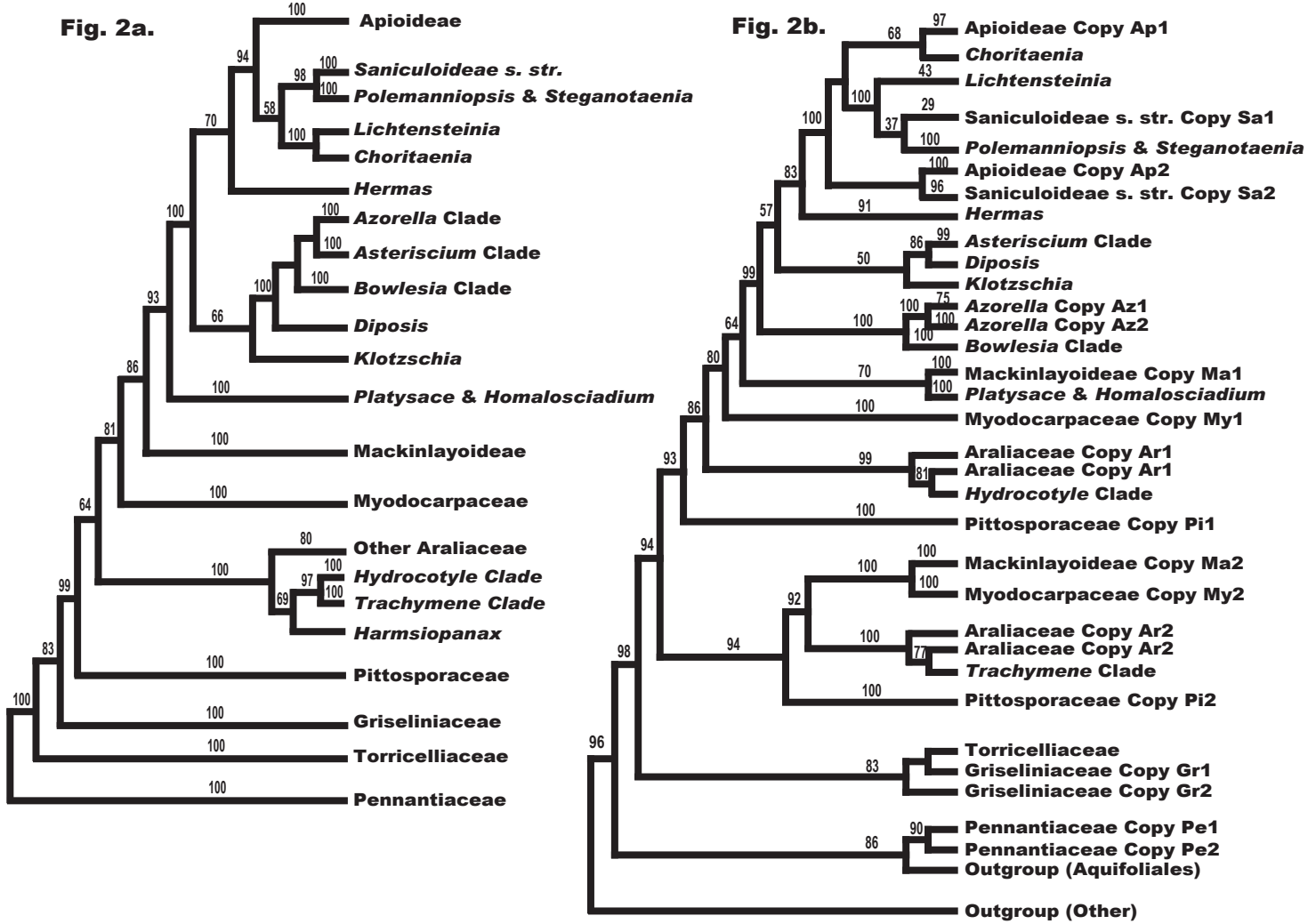
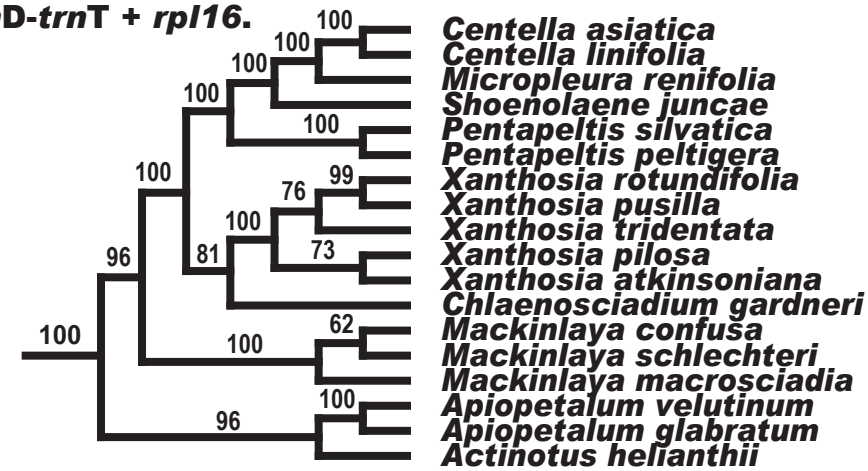
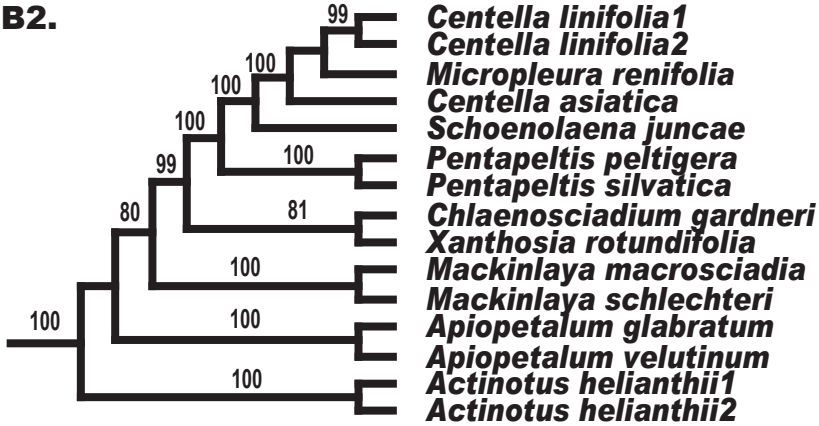


Figure 3. Mackinlayoideae.

a. Plastid *trn*D-*trn*T + *rpl16*.



b. Nuclear RPB2.



c. Mitochondrial *nad1* intron2.

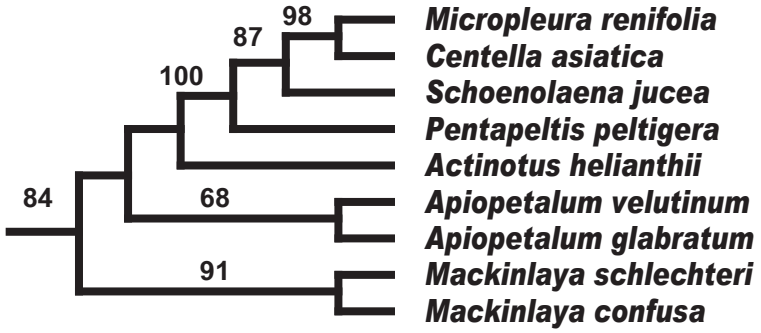
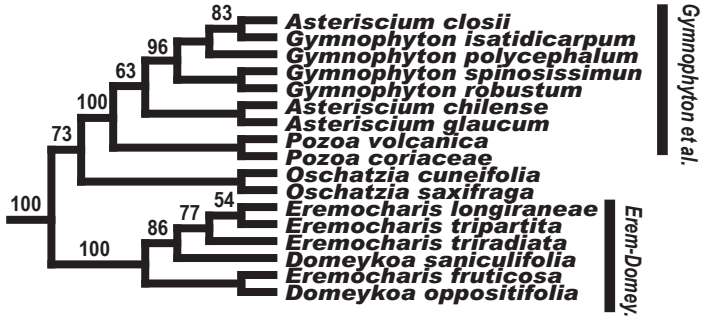
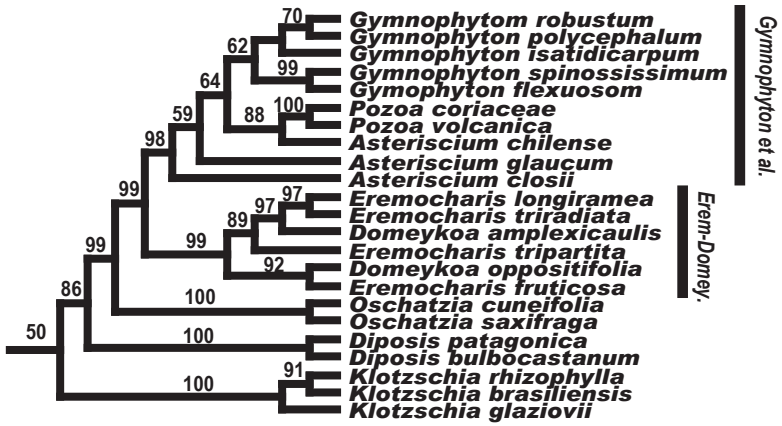


Figure 4. Azorelloideae; *Asteriscium* Clade.

a. Plastid *trn*D-*trn*T + *rpl16*.



b. Nuclear RPB2.



c. Mitochondrial nad1 intron2.

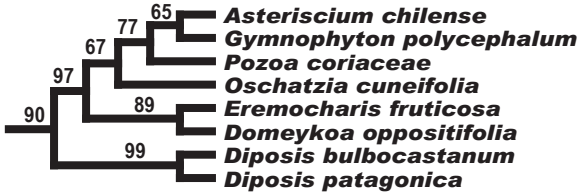
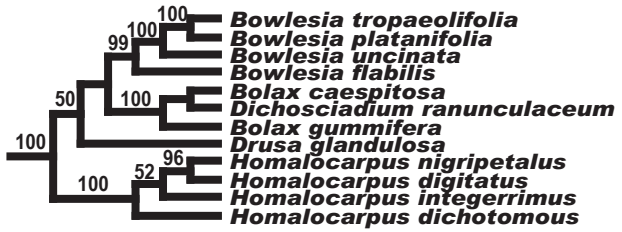


Figure 5. Azorelloideae; *Bowlesia* Clade.

a. Plastid *trn*D-*trn*T + *rpl16*.

b. Nuclear RPB2.



c. Mitochondrial nad1 intron2.

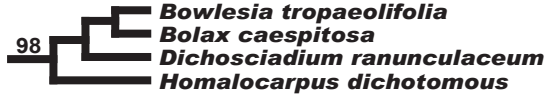
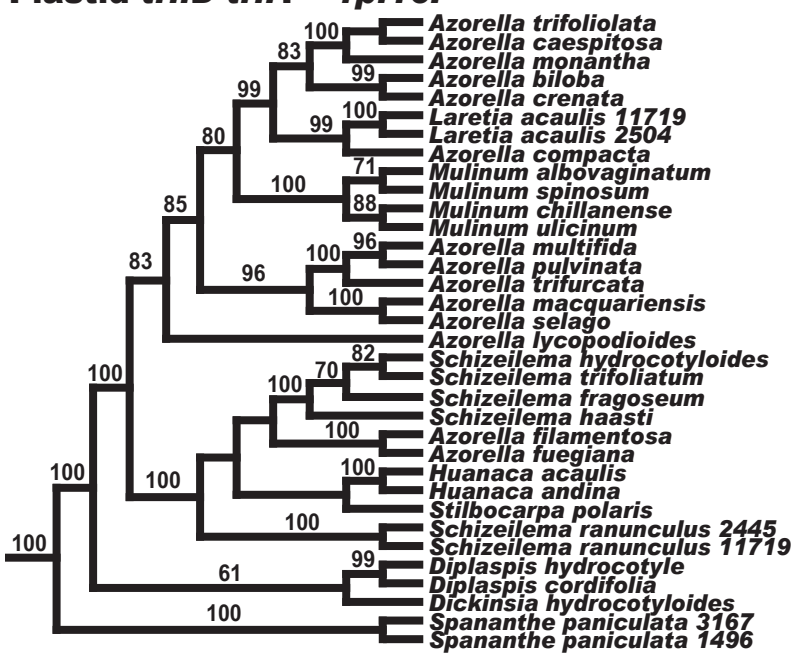


Figure 6. Azorelloideae; *Azorella* Clade.

a. Plastid *trn*D-*trn*T + *rpl16*.



b. Nuclear RPB2.

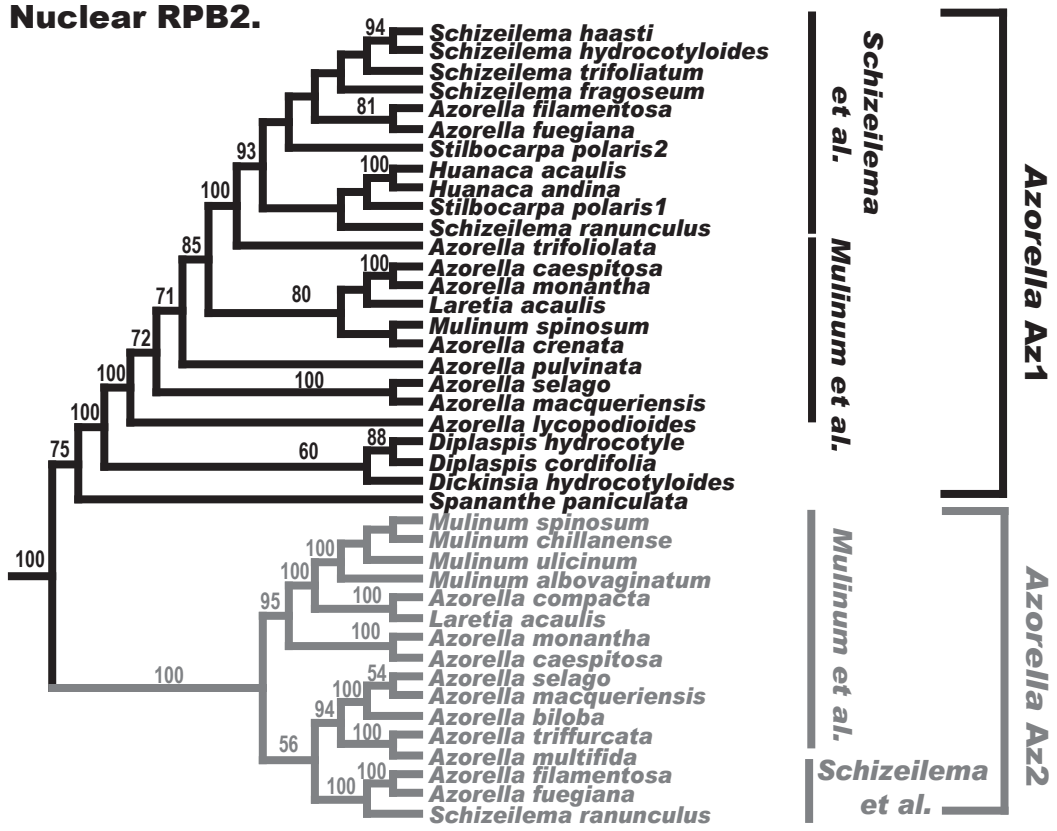


Figure 7. Apioideae-Saniculoideae.

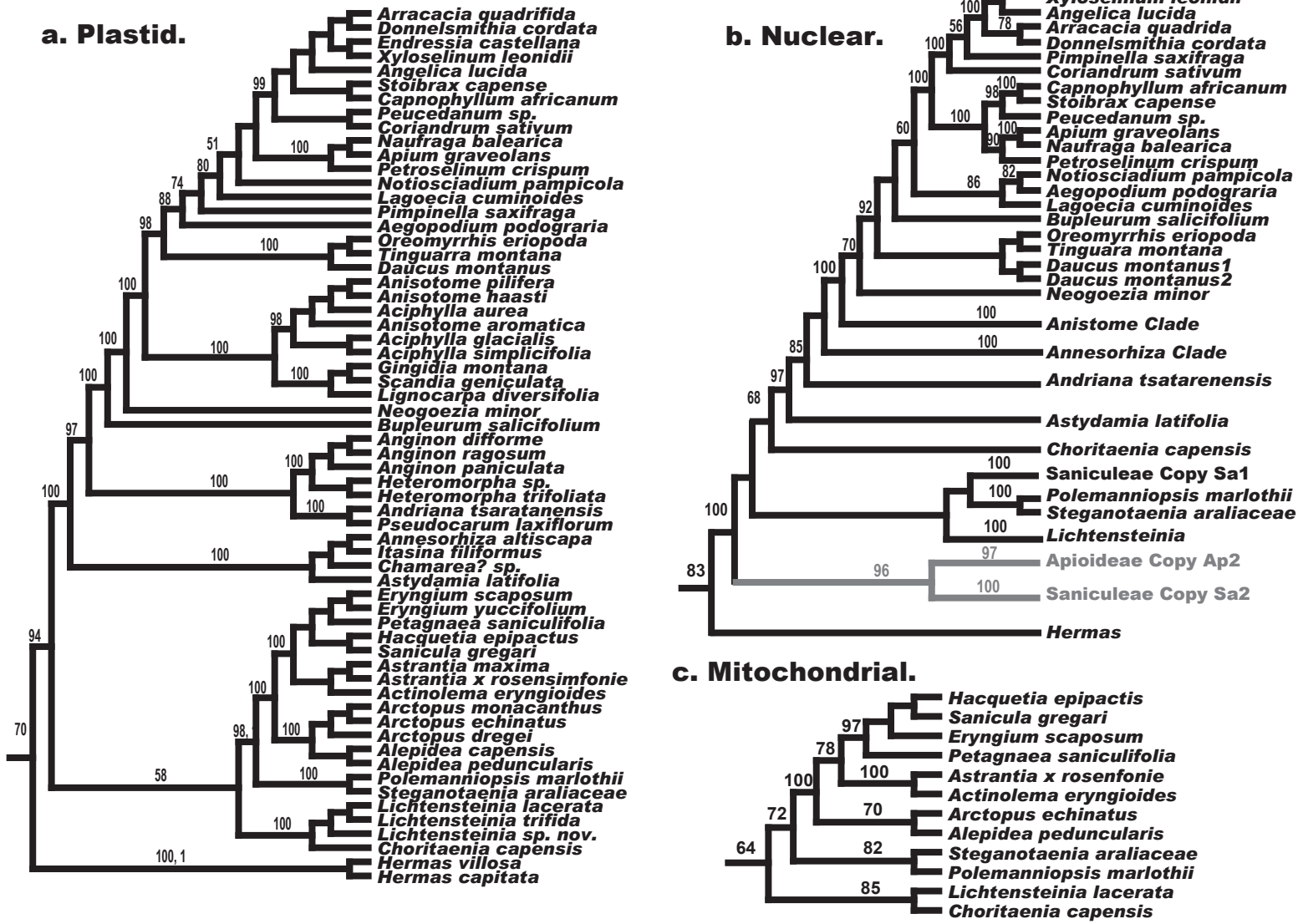


Figure 8. Araliaceae plastid and nuclear maximum likelihood phylogenies.

Fig. 8a. Plastid *trn*D-*trn*T + *rpl16*.

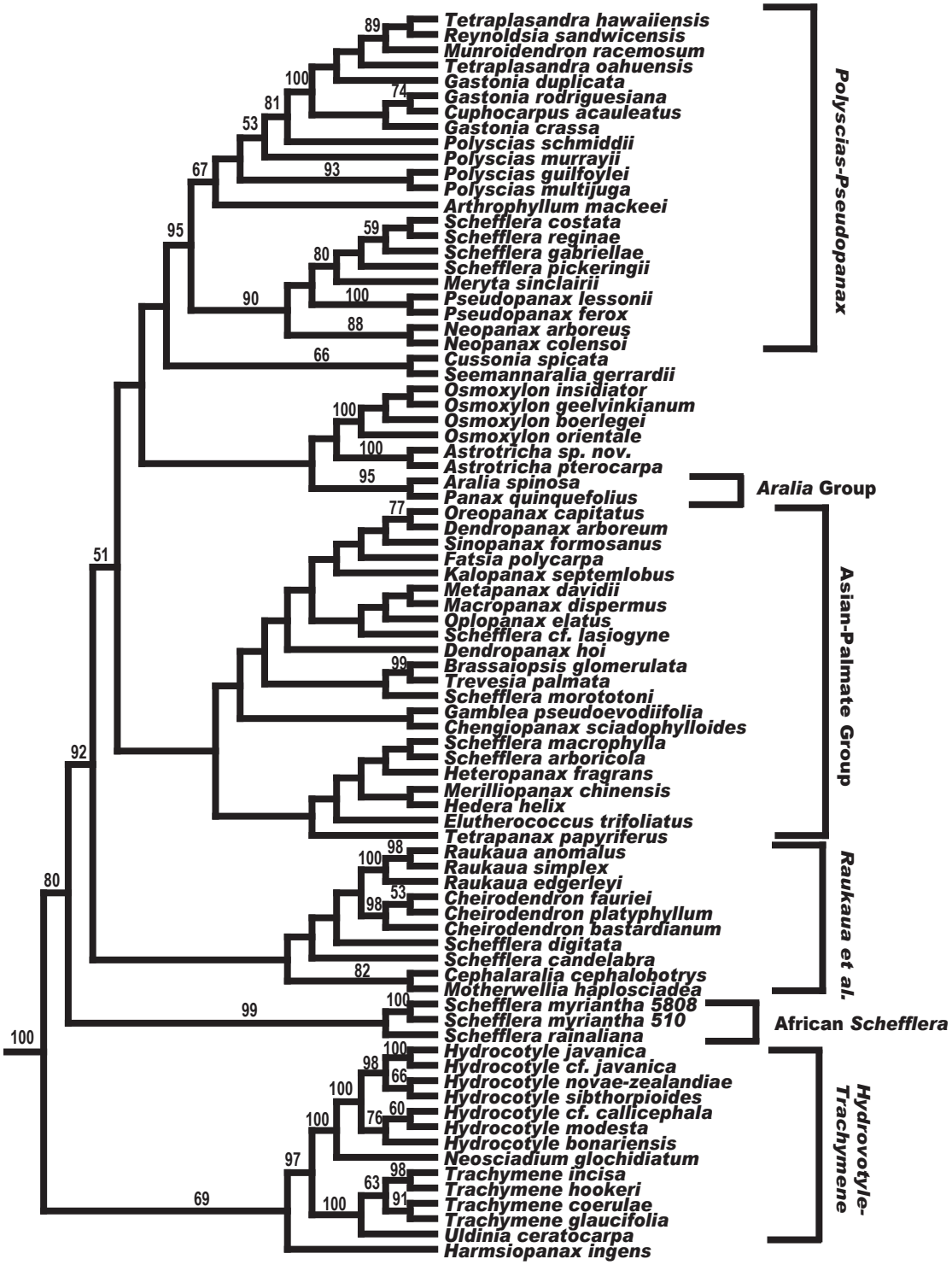


Fig. 8b. Araliaceae RPB2 Ar1.

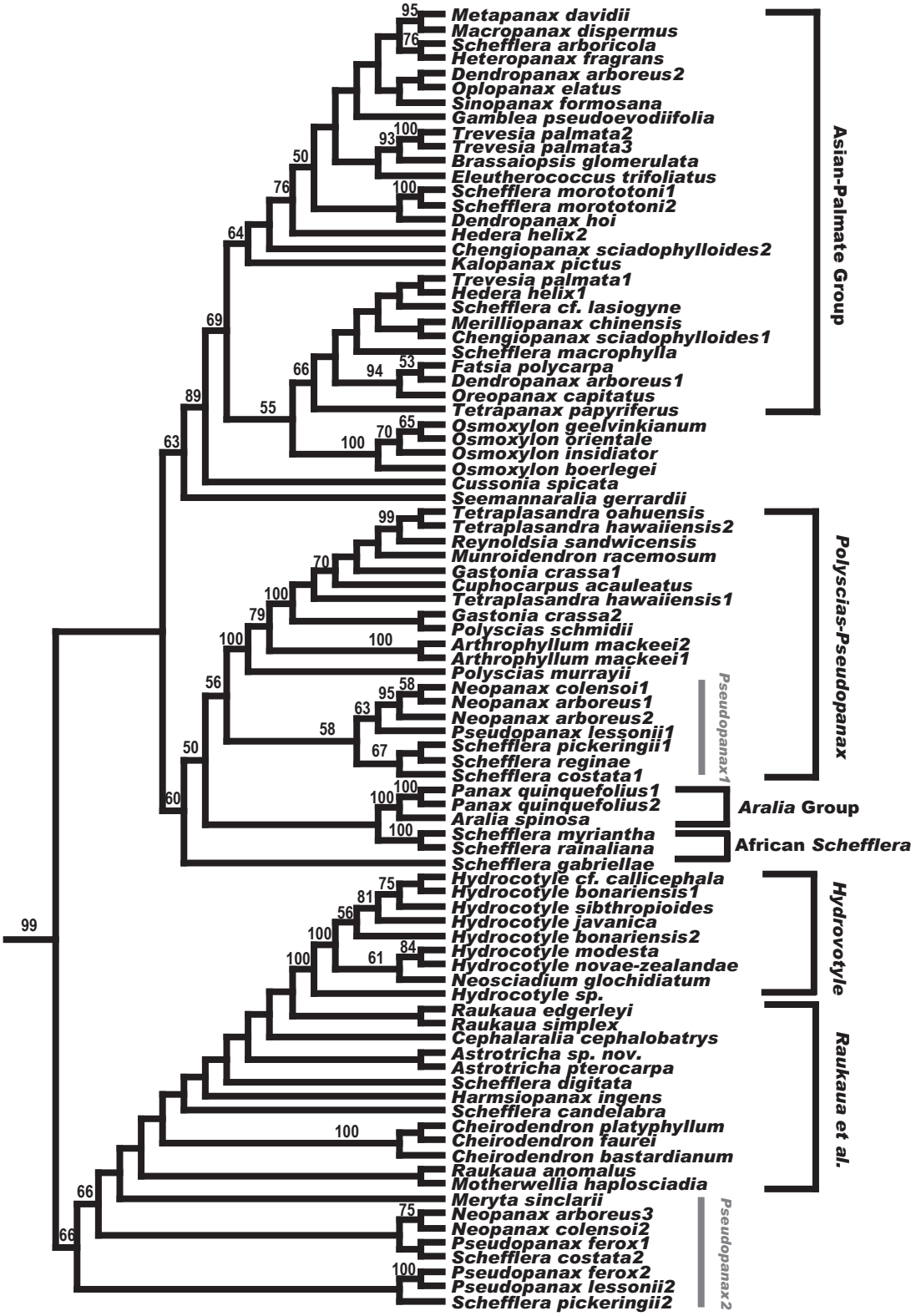
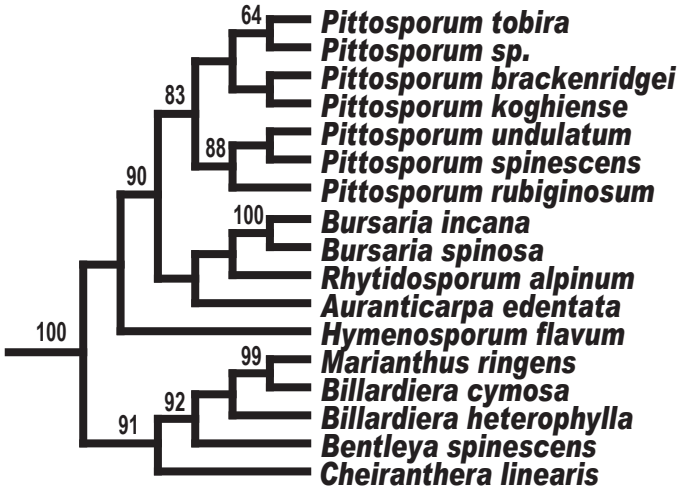
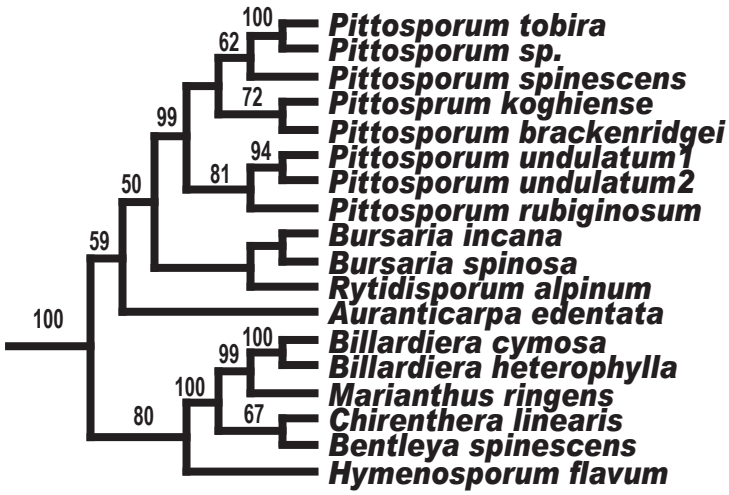


Figure 9. Pittosporaceae.

a. Plastid *trn*D-*trn*T + *rpl16*.



b. Nuclear RPB2.



c. Mitochondrial *nad1* intron 2.

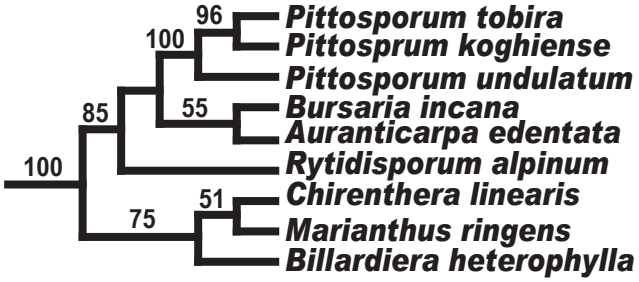


Figure 10. Bayesian Chronogram generated in Beast with origin of some clades as estimated in DIVA.

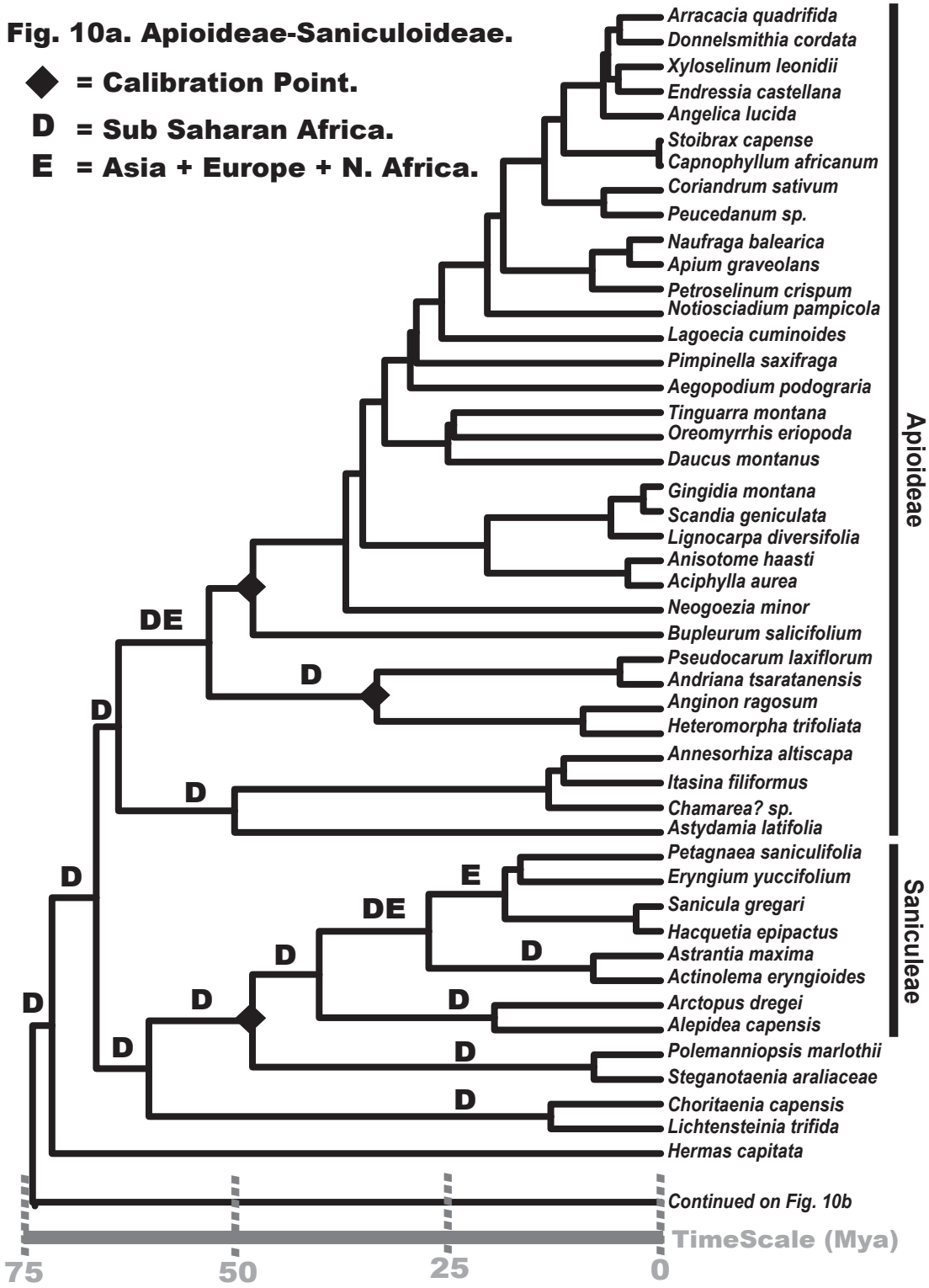


Fig. 10b. Azorelloideae, *Platysace*, Mackinlayoideae, and Myodocarpaceae.

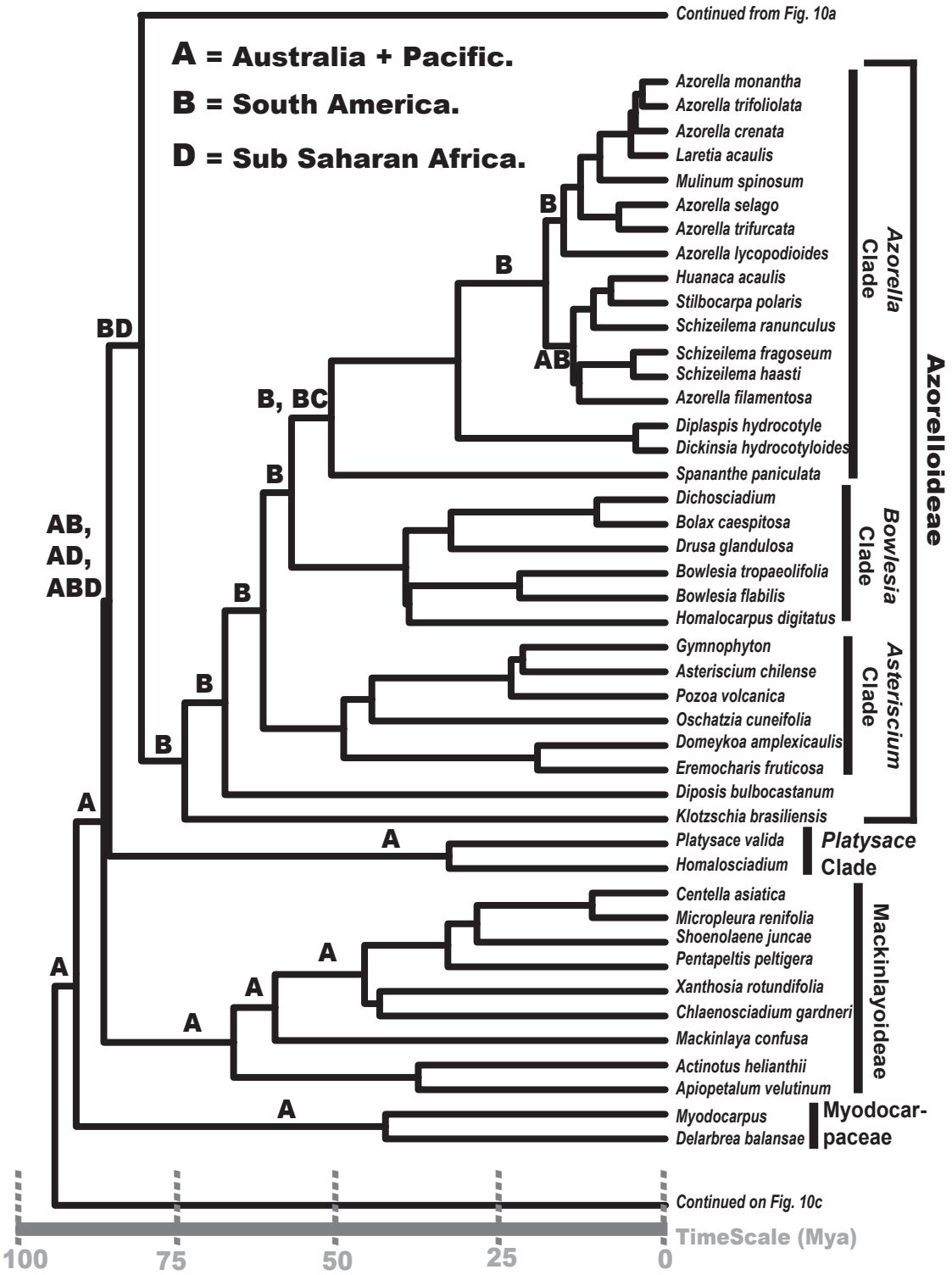
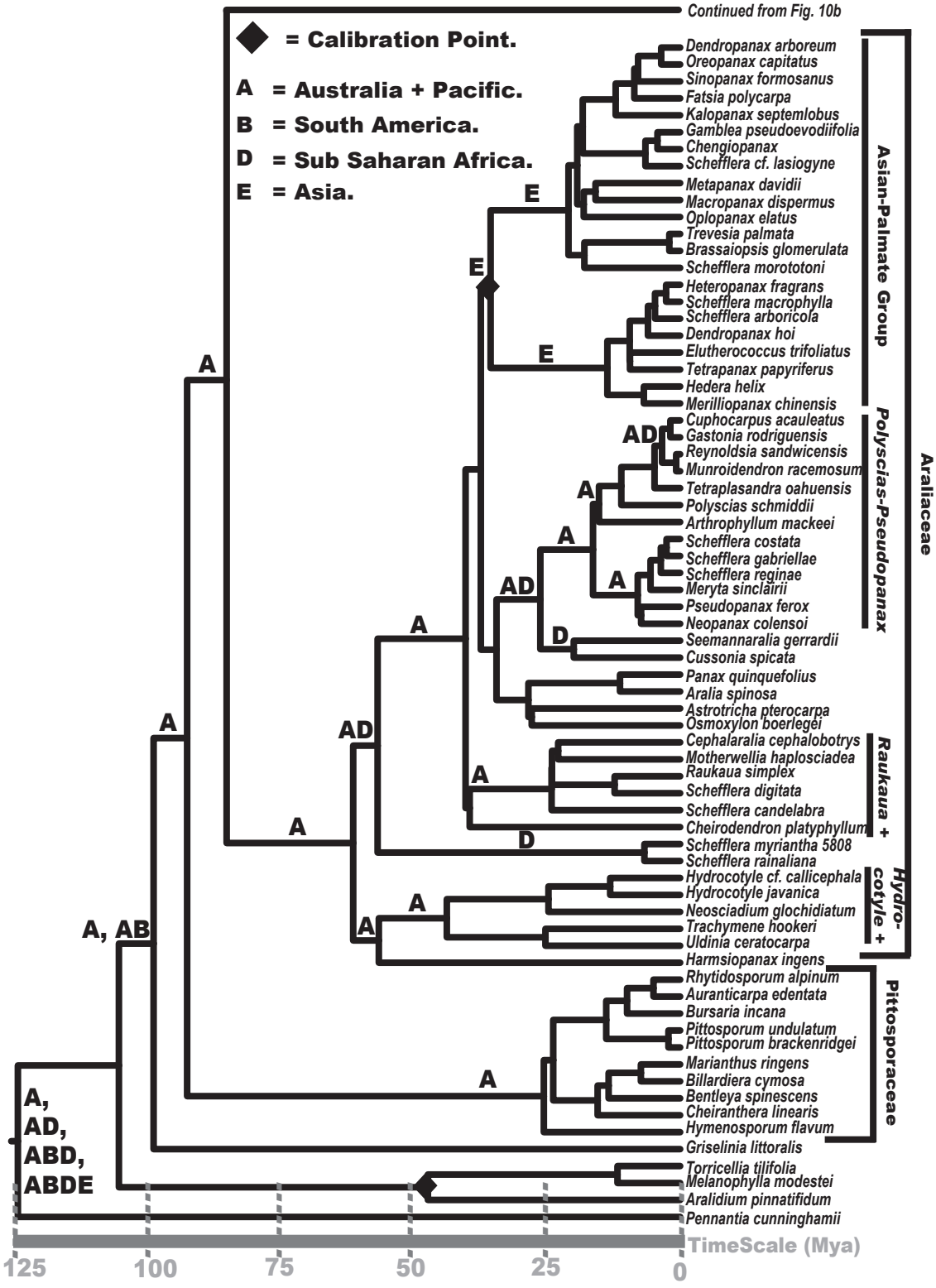


Fig. 10c. Araliaceae, Pittosporaceae, and the early-diverging families.



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