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MOLECULAR PHYLOGENY AND FLORAL EVOLUTION OF PENAEACEAE, OLINIACEAE, RHYNCHOCALYCACEAE, AND ALZATEACEAE (MYRTALES)[1]

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We derive detailed relationships among and within the African Penaeaceae, Oliniaceae, Rhynchocalycaceae, and Central and South American Alzateaceae based on six chloroplast data sets that include sequences of all genera and most species. This is the first study addressing intrafamilial relationships of Penaeaceae and Oliniaceae based on molecular data. All analyses conducted on the six separate and combined data sets produce similar tree topologies without any major conflicts. The resulting phylogenies suggest that the monospecific New World Alzateaceae is sister to the three African taxa and that the monospecific Rhynchocalycaceae is sister to Oliniaceae/Penaeaceae. Within Penaeaceae, our results partially contradict traditional generic circumscriptions, suggesting, for example, that *Brachysiphon* and *Stylapterus* are paraphyletic. Within the monogeneric Oliniaceae the analyses reveal two well-supported clades. We also performed comparative studies of floral development and morphology of Penaeaceae, Oliniaceae, and Rhynchocalycaceae. We analyze the results of these comparativel studies in the context of the molecular phylogeny to test competing hypotheses of perianth organ homology in Penaeaceae and Oliniaceae. Our analyses show that flowers of both families are most parsimoniously interpreted as having an obhaplostemonous organization. The respective homology of calyx and corolla among the three families is further supported by congruent patterns of floral development and structural similarities including anatomical and histological features.

Key words: Alzateaceae; chloroplast DNA; floral development; molecular phylogeny; Oliniaceae; Penaeaceae; perianth homology; Rhynchocalycaceae.

The Myrtales are a well-delimited order that is characterized by the combined occurrence of vestured pits and bicollateral bundles in the wood (van Vliet and Baas, 1984). Recent molecular studies recognize 14 families within Myrtales: Alzateaceae, Combretaceae, Crypteroniaceae, Heteropyxidaceae, Lythraceae, Melastomataceae, Memecylaceae, Myrtaceae, Oliniaceae, Onagraceae, Penaeaceae, Psiloxylaceae, Rhynchocalycaceae, and Vochysiaceae (e.g., Conti, Litt, and Sytsma, 1996; APG, 1998; Savolainen et al., 2000). Several studies, both nonmolecular (Johnson and Briggs, 1984) and molecular (Conti et al., 1997; Clausing and Renner, 2001), investigated the relationships among myrtalean families. Phylogenetic studies of *rbcL* sequences (Conti, Litt, and Sytsma, 1996; Conti et al., 1997) confirmed a clade, earlier defined from nonmolecular data (Johnson and Briggs, 1984), that comprises four myrtalean taxa with a Western Gondwanan distribution: the South African Penaeaceae and Rhynchocalycaceae, the South and East African Oliniaceae, and the Central and South American Alzateaceae. This Western Gondwanan clade is strongly supported as sister to a fifth taxon, the Southeast Asian Crypteroniaceae (Clausing and Renner, 2001; Conti et al., 2002). These five taxa are characterized by flowers with an ephemeral anther endothecium (Tobe and Raven, 1983a, b, 1984a, b, c, d, 1987a, b; Johnson and Briggs, 1984). The present study focuses on the four Western Gondwanan taxa: Penaeaceae, Oliniaceae, Rhynchocalycaceae, and Alzateaceae.

The Penaeaceae are a clearly circumscribed taxon that comprises 23 species in seven genera endemic to the southern and southwestern parts of the Cape Province of South Africa. They are shrubs and undershrubs confined to the ''fynbos'' vegetation typical of the Cape Floristic Region. In the past, Penaeaceae have either been placed in Myrtales or close to Thymelaeaceae (e.g., Gilg, 1894b; Supprian, 1894), the latter sometimes also included in Myrtales (e.g., Cronquist, 1981). More recent authors agree on the placement of Penaeaceae in Myrtales and the exclusion of Thymelaeaceae from this order (e.g., Dahlgren and Thorne, 1984; Johnson and Briggs, 1984; Thorne, 1992; Takhtajan, 1997). The family is relatively well described through studies on pollen morphology (Patel, Skvarla, and Raven, 1984), vegetative anatomy (Carlquist and Debuhr, 1977; van Vliet and Baas, 1984; Dickie and Gasson, 1999), embryology (Tobe and Raven, 1984c), and a series of mainly taxonomical papers by Dahlgren (Dahlgren, 1967a, b, c, 1968, 1971) and Dahlgren and van Wyk (1988). The flowers of Penaeaceae have only a single whorl of perianth organs that alternate with the stamens. The perianth has variously been interpreted as being apetalous (e.g., Cronquist, 1981; Dahlgren and Thorne, 1984; Johnson and Briggs, 1984; Dahlgren and van Wyk, 1988), i.e., the flowers as being obhaplostemonous (number of stamens equals number of sepals and stamens are in alternisepalous position) or as consisting of petals only (Rao and Dahlgren, 1968), i.e., the flowers as being haplostemonous

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(number of stamens equals number or petals and stamens are in alternipetalous position). Other authors did not specify the nature of the perianth (e.g., Dahlgren, 1967a, b, c, 1968, 1971; Rourke, 1995).

The Oliniaceae comprise about eight species confined to the single genus *Olinia*. They are trees and shrubs found in the montane and coastal forests of eastern and southern Africa, and also on the island of St. Helena, where the genus was most probably introduced from South Africa (Cufodontis, 1960). Most authors of traditional classifications associated Oliniaceae with myrtalean families (Cronquist, 1981; Dahlgren and Thorne, 1984; Thorne, 1992; Takhtajan, 1997). A sound taxonomic treatment of the family is not yet available (Sebola and Balkwill, 1999). The homology of the three perianth whorls of Oliniaceae has been much debated. From the outside of the flower inwards, the following three alternating whorls have been identified: (1) a whorl of truncate, minutely toothlike structures, (2) a whorl of conspicuous, petal-like organs, and (3) a whorl of small, scale-like organs. The stamens are positioned in the same radii as these latter scale-like organs. Two hypotheses have been proposed for the homology of these three whorls: the first interprets the flowers as being haplostemonous, i.e., the outermost organs are interpreted as a reduced calyx, the middle whorl as a corolla, and the innermost organs as either scales of unspecified nature (e.g., Verdcourt, 1975), as staminodes (e.g., Cronquist, 1981), or as stipules of the petals (e.g., Rao and Dahlgren, 1969; Sebola and Balkwill, 1999). The second hypothesis describes the flowers as being obhaplostemonous, i.e., the organs of the outermost whorl are interpreted as ''teeth'' of unspecified nature, the middle whorl as calyx, and the innermost whorl as corolla (e.g., Gilg, 1894a; Dahlgren and van Wyk, 1988).

The Rhynchocalycaceae comprise the single species *Rhynchocalyx lawsonioides*, a rare, evergreen tree endemic to the Eastern Cape and KwaZulu-Natal in South Africa, that grows mainly on forest margins, often near watercourses. The genus was first described in Lythraceae (Oliver, 1894), but later was included in an expanded Crypteroniaceae (van Beusekom-Osinga and van Beusekom, 1975). However, the distinctive embryology of *Rhynchocalyx* prompted its elevation to family rank (Johnson and Briggs, 1984; Tobe and Raven, 1984d). The flowers of *R. lawsonioides* are clearly obhaplostemonous, i.e., the stamens are in alternisepalous position.

The Alzateaceae comprise the single species *Alzatea verticillata*, represented by trees restricted to submontane tropical forests of Bolivia, Peru, Panama, and Costa Rica. *Alzatea verticillata* was earlier referred to Lythraceae (e.g., Lourteig, 1965) and was later included in the broadly circumscribed Crypteroniaceae (van Beusekom-Osinga and van Beusekom, 1975). Based on wood anatomical (van Vliet and Baas, 1984) and embryological features (Tobe and Raven, 1984a), the genus was then raised to family rank (Graham, 1984). The flowers of *A. verticillata* are generally described as apetalous (e.g., Graham, 1984; Johnson and Briggs, 1984; but see van Beusekom-Osinga and van Beusekom, 1975).

To date, no molecular phylogenetic study based on an intensive taxon sampling of this Western Gondwanan clade of Myrtales has been published and neither has the floral morphology been comparatively investigated in a phylogenetic context. In addition, intrafamilial relationships in both Penaeaceae and Oliniaceae have never been addressed using molecular data. Therefore, several unresolved phylogenetic and morphological questions remain to be answered. For example: What are the detailed phylogenetic relationships among and within Penaeaceae, Oliniaceae, Rhynchocalycaceae, and Alzateaceae? Are the traditionally recognized genera of Penaeaceae monophyletic? What are the perianth organs of Oliniaceae and Penaeaceae homologous to? Here we present the results of phylogenetic analyses performed on DNA sequences from six chloroplast markers for most species of this Western Gondwanan clade of Myrtales. In addition, floral development and floral structure in Penaeaceae, Oliniaceae, and Rhynchocalycaceae are described and compared. We analyze the results of our comparative morphological study in the context of the molecular phylogeny to address questions of perianth organ homology in Penaeaceae and Oliniaceae. Further, we attempt to elucidate the evolution of a number of floral features in Penaeaceae, some of which relate to pollination syndromes and others that are important for intrafamilial classification. The resulting paper provides an example of how the integration of molecular phylogenetic and comparative developmental/morphological approaches can contribute to the problem of organ homology assessment.

MATERIALS AND METHODS

Plant material and DNA extractions—We included 26 species representing all ten genera of Penaeaceae, Oliniaceae, Rhynchocalycaceae, and Alzateaceae in our molecular analyses. In addition, we sampled all five subspecies of *Penaea cneorum* described by Dahlgren (1971), for a total of 30 sampled taxa. As outgroup taxon we used *Crypteronia paniculata* from the Crypteroniaceae, which unequivocally have been shown to be sister to the clade comprising the four ingroup families (Conti, Baum, and Sytsma, 1999; Clausing and Renner, 2001; Conti et al., 2002). Taxon names, voucher information, and GenBank accession numbers are archived at http://ajbsupp.botany.org/v90. We extracted total genomic DNA from silica dried leaf material. Leaf tissue was homogenized using glass beads and a Retsch MM 2000 shaker (Retsch, Haan, Germany). For the subsequent DNA extraction, we applied the DNeasy Plant Mini Kit (Qiagen, Basel, Switzerland) to all species except *Brachysiphon rupestris*, *Endonema lateriflora*, and *Stylapterus fruticulosus*, which were extracted with the sodium dodecyl sulphate (SDS) extraction protocol as described by Eichenberger, Gugerli, and Schneller (2000).

Polymerase chain reaction and DNA sequencing—Six chloroplast regions, the *rps16* intron, *rpl16* intron, the *trnS-G* intergenic spacer, the *atpB-rbcL* intergenic spacer, the *psbA-trnH* intergenic spacer, and part of the *matK* exon, were amplified for all taxa. Primers, corresponding annealing temperatures, and literature sources for the primers are presented in Table 1. All polymerase chain reactions (PCR) were performed on a Biometra TGradient thermocycler (Biometra, Göttingen, Germany), applying a thermal cycling program consisting of 34 cycles of 0.5 min at 95°C, 1 min at the primer-specific annealing temperatures given in Table 1, and 1.7 min at 72°C, followed by a terminal extension of 10 min at 72°C. In order to successfully detect amplified DNA target regions and possible contamination, the PCR products were examined on agarose gels with a negative control. Successfully amplified PCR products were purified with the QIAquick PCR Purification Kit (Qiagen).

The same primers (Table 1) were used for the cycle-sequencing reactions, which were carried out using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems, Applera Europe BV, Rotkreuz, Switzerland). For a few taxa, we were initially unable to sequence the entire *rpl16* intron; in these cases two additional internal primers, MF and MR, were used. These primers (see Table 1) were designed with the aid of the software Oligo 6.67 (Molecular Biology Insights, Cascade, Colorado, USA). The sequenced products were cleaned with MicroSpin G-50 columns (Amersham Pharmacia Biotech Europe, Dübendorf, Switzerland) to remove excess dye terminators before loading on an ABI Prism 377 DNA sequencer (Perkin Elmer). Sequencher 3.1.1 (Gene Codes, 1998) was used to edit and assemble complementary strands. Base positions were individually

TABLE 1. Primer information.

cpDNA region	Primers		Annealing temperature	Primer source
rps16 intron	rpsF	5′-GTGGTAGAAAGCAACGTGCGACTT-3′	55°C	(Oxelman, Lidén, and Berglund, 1997)
	rpsR2	5′-TCGGGATCGAACATCAATTGCAAC-3′		(Oxelman, Lidén, and Berglund, 1997)
rpl16 intron	F71	5′-GCTATGCTTAGTGTGTGACTCGTTG-3′	52°C	(Baum, Small, and Wendel, 1998)
	R1516	5′-CCCTTCATTCTTCCTCTATGTTG-3′		(Baum, Small, and Wendel, 1998)
	MF	5′-GAACGACGGAACCTGTGAATAC-3′		This project
	MR	5′-ATCTATTTCAGTTGTCGGGTTAGC-3′		This project
trnS-G intergenic spacer	trnS	5′-GCCGCTTTAGTCCACTCAGC-3′	55°C	(Hamilton, 1999)
	trnG	5′-GAACGAATCACACTTTTACCAC-3′		(Hamilton, 1999)
atpB-rbcL intergenic spacer	Oligo 2	5′-GAAGTAGTAGGATTGATTCTC-3′	52°C	(Manen, Natali, and Ehrendorfer, 1994)
	Oligo 5	5′-TACAGTTGTCCATGTACCAG-3′		(Manen, Natali, and Ehrendorfer, 1994)
	Oligo 7	5′-CCCTACAACTCATGAATTAAG-3′		(Manen, Natali, and Ehrendorfer, 1994)
psbA-trnH intergenic spacer	psbAF	5′-GTTATGCATGAACGTAATGCTC-3′	52°C	(Sang, Crawford, and Stuessy, 1997)
	trnHR	5′-CGCGCATGGTGGATTCACAAATC-3′		(Sang, Crawford, and Stuessy, 1997)
matK exon (part)	matK3F	5′-AAGATGCCTCTTCTTTGCAT-3′	50°C	(Sang, Crawford, and Stuessy, 1997)
	matK3R	5′-GATCCGCTGTGATAATGAGA-3′		(Sang, Crawford, and Stuessy, 1997)

checked for agreement between the complementary strands. We aligned the sequences visually in Sequencher 3.1.1. Variable positions were double-checked to ensure that base calls were consistent at informative positions. To ensure that unexpected results, such as the paraphyly of *Brachysiphon*, were not the product of any mistake during laboratory work, we re-extracted and resequenced all five species of *Brachysiphon*. For *Brachysiphon fucatus* and *Stylapterus micranthus*, second accessions were extracted, sequenced, and compared to the first accessions.

Indel coding and inversions—We coded insertion or deletion events (indels, listed in Table 2) as additional, binary characters applying the ''simple indel coding method'' described by Simmons and Ochoterena (2000). Only potentially informative indels bordered by unambiguously aligned nucleotide stretches were coded. We omitted single-nucleotide indels resulting from the differing lengths of strings of the same nucleotide (e.g., four A's vs. five A's), as this type of indel may be the product of experimental error (Downie et al., 1998; McDade and Moody, 1999) or evolutionary lability (Small et al., 1998).

Within the intergenic spacer between *psbA* and *trnH* we detected a 7-base pair (bp) inversion (starting at position 123 in the aligned matrix) present in eight taxa of Penaeaceae (*Glischrocolla formosa*, *Penaea cneorum* subsp. *cneorum*, *P. cneorum* subsp. *gigantea*, *P. cneorum* subsp. *ruscifolia*, *P. dahlgrenii*, *P. mucronata*, *Sonderothamnus speciosus*, and *Stylapterus ericifolius*). This 7-bp region is flanked by two conserved inverted repeats (each 21 bp long), probably forming the stem region of a stem-loop secondary structure, with the inversion corresponding to the loop. As inversions may show high levels of parallelism and reversal, we removed the region containing this inversion from the analyses in order to eliminate potential scoring of homoplasious synapomorphies (Kelchner, 2000).

Phylogenetic analyses—Cladistic analyses were performed using the Maximum Parsimony (MP) optimization of the software package PAUP* (Phylogenetic Analyses Using Parsimony) 4.0b8 (Swofford, 2000). We included only informative nucleotide and indel characters in the analyses, and all characters and character state transitions were weighted equally (Fitch, 1971). The individual data matrices for each of the six DNA regions plus a combined data matrix of the six regions were analyzed by employing an heuristic search strategy using 1000 replicates with random taxon-addition, tree-bisection-reconnection (TBR) branch swapping, and keeping all most-parsimonious trees. Relative support for different clades was estimated with the bootstrap (Felsenstein, 1985) option implemented in PAUP* employing a full heuristic search with 1000 replicates, simple addition sequence, and TBR branch swapping. To assess congruence among pairwise combinations of the six data sets we carried out 1000 replicates of the partition homogeneity test (Farris et al., 1995) as implemented in PAUP*, employing a full heuristic search with simple taxon-addition sequence, tree-bisection-reconnection (TBR) branch swapping, and saving all most-parsimonious trees. In addition, a homogeneity test with the same settings was conducted to assess congruence between the indel characters and the substitution characters.

Morphological/anatomical/histological methods—Out of the currently eight recognized species of Oliniaceae we studied floral development and morphology of two species (*Olinia emarginata* and *O. ventosa*). For Penaeaceae we collected floral material for all genera except for the monotypic genus *Glischrocolla*. We studied floral development and morphology of several species including *Brachysiphon acutus*, *Endonema retzioides*, *Penaea dahlgrenii*, *Saltera sarcocolla*, and *Stylapterus fruticulosus*. Both in Penaeaceae and Oliniaceae, respectively, our studies revealed that floral development and basic floral organization are very similar within each family. In the results section of the present study floral development and morphology of one representative of Penaeaceae (*Endonema retzioides*) and Oliniaceae (*Olinia emarginata*) are described. However, the conclusions on organ homology drawn from the study of these two representative species are valid for the entire families. Rhynchocalycaceae is represented by its only member *Rhynchocalyx lawsonioides*. For scanning electron microscopy (SEM) and microtome sectioning, living material was fixed in formalin-acetic acid-alcohol (FAA) and subsequently stored in 70% ethanol. For SEM investigations, the material was dehydrated in ethanol and acetone, critical point dried, and sputter coated with gold. For microtome sectioning, specimens were embedded in 2-hydroxyethyl methacrylate (Kulzer's Technovit 7100; Heraeus Kulzer, Wehrheim, Germany), cut using a Microm HM rotary microtome 355 (Microm, Walldorf, Germany) at 5 μm, and stained with toluidine blue and ruthenium red (for detailed description of embedding and staining method, see Igersheim [1993] and Igersheim and Cichocki [1996]). Permanent slides are deposited at the Institute of Systematic Botany of the University of Zurich (Z).

RESULTS

Size and structure of molecular data sets—The aligned length of the combined data set summed up to 5381 positions, of which 267 (5.0%) were potentially parsimony informative (see Table 3 for information on individual and combined data sets). Among the six individual data sets the percentages of informative characters were highest in the *rps16* intron and the intergenic spacer between *trnS* and *trnG* (5.9%) and lowest in the intergenic spacer between *atpB* and *rbcL* (2.3%). All the data sets were complete. The combined data matrix included 47 parsimony-informative indels (Table 2), each between 1 and 226 bp in length. Of these 47 indels, 15 are most parsimoniously reconstructed as insertions and 31 as deletions; for one (C_7; Table 2) parsimony reconstruction is ambiguous, as

TABLE 2. Informative indels coded as additional binary characters.

cpDNA region/ code	Starting position	Length	Type (as compared to outgroup)	Taxa	State
rps16 intron					
A_1	99	1	insertion	*Olinia*	synapomorphic
A_2	114	5	insertion	Penaeaceae	synapomorphic
A_3	165	11	deletion	*Olinia*	synapomorphic
A_4	194	5	insertion	*Olinia*	synapomorphic
A_5	305	5	insertion	*Olinia*	synapomorphic
A_6	386	5	deletion	all except *Alzatea*	synapomorphic
A_7	631	5	deletion	*Brachysiphon rupestris, B. mundii, Endonema, Saltera sarcocolla, Stylapterus ericoides*	homoplasious
A_8	866	8	deletion	all except *Alzatea*	synapomorphic
A_9	948	1	insertion	Penaeaceae	synapomorphic
A_{10}	955	1	deletion	Penaeaceae	synapomorphic
rpl16 intron					
B_1	18	15	deletion	*Olinia*, Penaeaceae	synapomorphic
B_2	161	7	deletion	Penaeaceae	synapomorphic
B_3	176	6	insertion	*Olinia*	synapomorphic
B_4	333	8	deletion	*Brachysiphon fucatus, B. microphyllus, Penaea, Stylapterus ericifolius, S. ericoides, S. fruticulosus*	synapomorphic
B_5	357	1	deletion	*Olinia*	synapomorphic
B_6	562	6	insertion	*Olinia*	synapomorphic
B_7	628	7	insertion	*Sonderothamnus petraeus, Stylapterus micranthus*	homoplasious
B_8	687	32	deletion	*Olinia*	synapomorphic
B_9	705	1	insertion	*Brachysiphon mundii, Saltera sarcocolla, Sonderothamnus*	synapomorphic
trnS-G intergenic spacer					
C_1	116	4	deletion	*Olinia*	synapomorphic
C_2	161	1	insertion	*Olinia*	synapomorphic
C_3	246	7	deletion	Penaeaceae	synapomorphic
C_4	262	2	deletion	Penaeaceae	synapomorphic
C_5	271	2	insertion	Penaeaceae	synapomorphic
C_6	280	2	insertion	Penaeaceae	synapomorphic
C_7	433	4	inapplicable in outgroup	*Olinia emarginata, O. vanguerioides* vs. other *Olinia* species	synapomorphic
C_8	480	4	deletion	*Olinia capensis, O. radiata, O. ventosa*	synapomorphic
C_9	487	18	deletion	*Olinia*	synapomorphic
C_{10}	596	9	deletion	*Olinia*	synapomorphic
C_{11}	640	16	insertion	*Penaea* except *P. dahlgrenii*	synapomorphic
atpB-rbcL intergenic spacer					
D_1	20	6	insertion	*Olinia*	synapomorphic
D_2	118	208	deletion	*Olinia*	synapomorphic
D_3	128	198	deletion	Penaeaceae, *Rhynchocalyx*, inapplicable in *Olinia*	?
D_4	441	6	deletion	*Olinia capensis, O. radiata, O. ventosa*	synapomorphic
D_5	474	8	deletion	*Endonema, Glischrocolla*	homoplasious
D_6	571	2	insertion	*Olinia*	synapomorphic
D_7	596	13	deletion	Penaeaceae	synapomorphic
psbA-trnH intergenic spacer					
E_1	128	5	deletion	Penaeaceae	synapomorphic
E_2	135	3	deletion	Penaeaceae	synapomorphic
E_3	170	9	deletion	*Olinia*	synapomorphic
E_4	208	3	deletion	*Olinia*	synapomorphic
E_5	208	226	deletion	all Penaeaceae except *Brachysiphon rupestris, Endonema, Glischrocolla*	synapomorphic
E_6	440	1	deletion	Penaeaceae	synapomorphic
E_7	451	80	deletion	Penaeaceae	synapomorphic
E_8	501	4	deletion	*Olinia*	synapomorphic
E_9	527	13	deletion	*Olinia emarginata, O. vanguerioides*	synapomorphic
matK exon (part)					
F	44	6	deletion	Penaeaceae	synapomorphic

TABLE 3. Description of cpDNA data sets and resulting trees (excluding uninformative characters).

cpDNA region/matrix	Aligned length	No. of informative nucleotide positions[a]	No. of informative indels	No. of steps	CI	RI	No. of trees
rps16 intron	968	56 (5.8)	10	93	0.78	0.94	20
rpl16 intron	1169	63 (5.4)	9	98	0.82	0.94	36
trnS-G intergenic spacer	882	52 (5.9)	11	102	0.74	0.89	23 544
atpB-rbcL intergenic spacer	1045	24 (2.3)	7	39	0.87	0.96	1356
psbA-trnH intergenic spacer	578	29 (5.0)	9	48	0.94	0.98	819
matK exon (part)	730	41 (5.6)	1	57	0.84	0.96	15
Combined	5372	265 (4.9)	47	446	0.80	0.93	24
Combined without indels	5372	265 (4.9)	0	393	0.78	0.93	144
Combined only indels	0	—	47	52	0.90	0.98	434

it is inapplicable in the outgroup. The highest number of indels (11) was found in the intergenic spacer between *trnS* and *trnG*, whereas only a single informative indel was present in the *matK* exon (Tables 2, 3). Nineteen of the 47 parsimony-informative indel characters were synapomorphic for *Olinia* and 14 for Penaeaceae (see Fig. 1). Two deletions (A_6 and A_8; Table 2) in the *rps16* intron were synapomorphic for a clade containing Penaeaceae, *Olinia*, and *Rhynchocalyx*. A 15-bp deletion (B_1; Table 2) in the *rpl*16 intron was synapomorphic for the clade consisting of *Olinia* and Penaeaceae. Three of the 47 indel characters were resolved as homoplasies on the strict consensus tree of the combined analysis (indels A_7, B_7, D_5; Table 2).

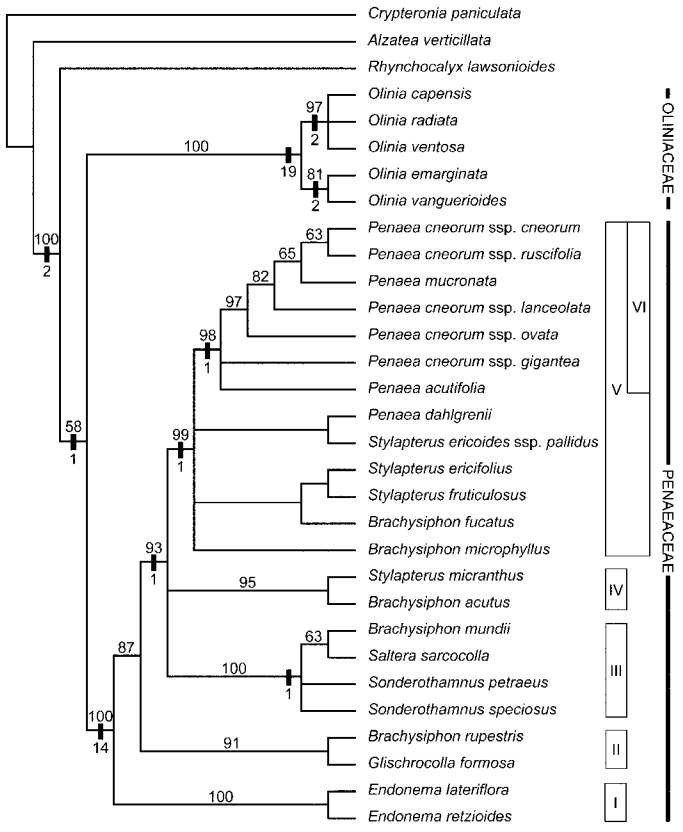


Fig. 1. Strict consensus of 24 shortest trees resulting from maximum parsimony analysis using the combined data from six chloroplast markers and 47 indel characters (265 informative characters; 446 steps; CI = 0.80, RI = 0.93, excluding uninformative characters). Bootstrap values are shown above branches. Bars and numbers below indicate presence and number of indel characters supporting particular branches (see Table 2). Well-supported clades within Penaeaceae are marked I–VI.

Phylogenetic reconstruction—Descriptive values for the trees that resulted from the analyses of the separate and combined data sets are listed in Table 3. Pairwise comparisons among the six data sets with the partition homogeneity test did not reject their homogeneity. The lowest *P* value (0.210) resulted from the comparison of the *rpl16* intron with the *psbA-trnH* intergenic spacer, whereas the highest value ($P = 0.976$) resulted from the comparison of the *trnS-G* intergenic spacer and the *rps16* intron. In addition, visual comparisons among the strict consensus trees found in parsimony analyses of the separate data sets (Fig. 2) showed only minor topological conflict, and only clades with weak branch support were involved in any of the discrepancies. The partition homogeneity test comparing indels vs. substitution characters resulted in a *P* value of 0.846 and a comparison of the strict consensus trees derived from the separate data sets, i.e., combined indel characters vs. combined substitution characters, does not reveal any topological conflicts (Fig. 2).

Maximum parsimony analysis of the combined data set, inclusive of indel characters, resulted in 24 shortest trees (446 steps, consistency index [CI] = 0.80, retention index [RI] = 0.93, excluding uninformative characters). The strict consensus tree (Fig. 1) of the combined analysis supports a clade formed by Penaeaceae, *Olinia*, and *Rhynchocalyx* (bootstrap support [BS] = 100%). This clade is further supported by two indel characters (A_6, A_8; Table 2). The sister-group relationship between *Olinia* and Penaeaceae is only weakly supported (BS = 58%). This weak support is also reflected in the strict consensus trees of the individual data partitions, where only the *rpl16* intron data set lends some support (BS = 59%) to *Olinia* as sister to Penaeaceae, whereas none of the other five data sets resolves relationships among *Rhynchocalyx*, *Olinia*, and Penaeaceae. Support for the sister relationship between *Olinia* and Penaeaceae mainly stems from a 15-bp deletion in the *rpl16* intron (B_1; Table 2). When this indel character is excluded from the analyses, a trichotomy with Penaeaceae, *Olinia*, and *Rhynchocalyx* is formed, both in the strict consensus of the *rpl16* intron data set and in the combined data set.

The monophyly of *Olinia* and Penaeaceae is well supported by 100% BS and 19 and 14 indel characters, respectively. Within *Olinia*, two well-supported clades were found. One consists of *Olinia emarginata* and *O. vanguerioides* (BS = 81%) and the other comprises the three remaining species (BS = 97%; Fig. 1). Each of the clades is supported by two indel characters (C_8, D_4, and C_7, E_9, respectively; Table 2).

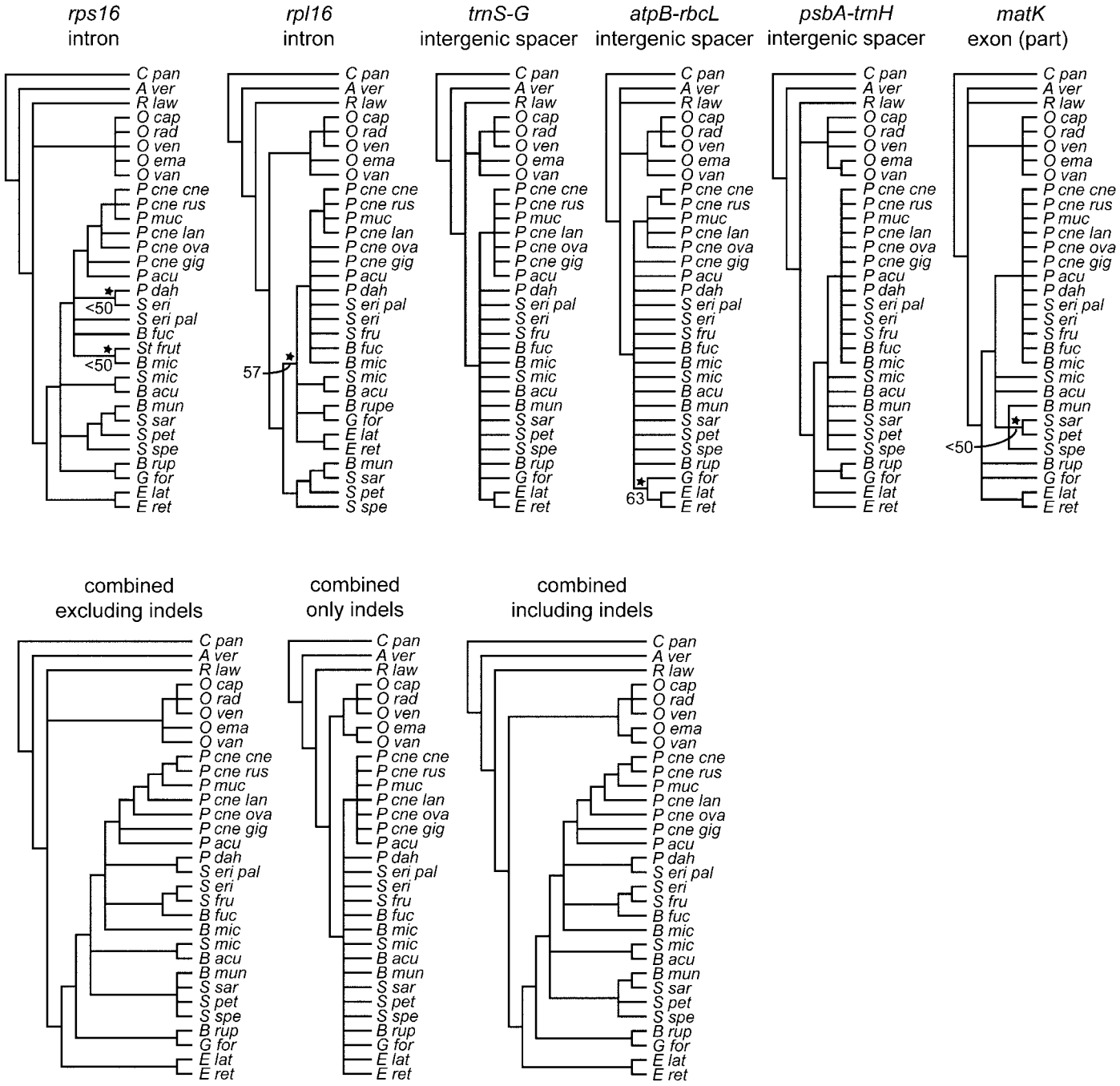


Fig. 2. Comparisons among the most-parsimonious (MP) strict consensus trees of the six separate data sets, the combined data set excluding indel characters, the combined indel characters of the six data sets, and the combined sequence data including indel characters (identical to Fig. 1). DNA sequence and MP tree descriptions are given in Table 3. Stars, with corresponding bootstrap support values, indicate clades not present in the strict consensus tree of the combined data sets (Fig. 1).

Within Penaeaceae, several well-supported clades are defined. The strict consensus tree (Fig. 1) supports the sister-group relationship of *Endonema* with the rest of the family (BS = 87%). The two species of the genus *Endonema* form a clade with 100% BS. In turn, the clade of *Glischrocolla formosa* and *Brachysiphon rupestris* (BS = 91%) is sister to the clade formed by *Penaea*, *Stylapterus*, *Brachysiphon* pro parte (p.p.), *Saltera*, and *Sonderothamnus* (BS = 93%), a relationship further supported by a deletion (E_5; Table 2) of 226 bp in the intergenic spacer between *psbA* and *trnH*. This topology contradicts the strict consensus of the *rpl16* intron trees, supporting (BS = 57%) a sister-group relationship between a clade with *Sonderothamnus*, *Saltera*, and *Brachysiphon mundii* and the rest of Penaeaceae. In the strict consensus tree of the combined analysis, *Sonderothamnus*, *Saltera*, and *Brachysiphon mundii* form a strongly supported clade (BS = 100% and an insertion in the *rpl16* intron), which, in turn, forms part of a strong (BS = 95%) trichotomy with *Stylap-*

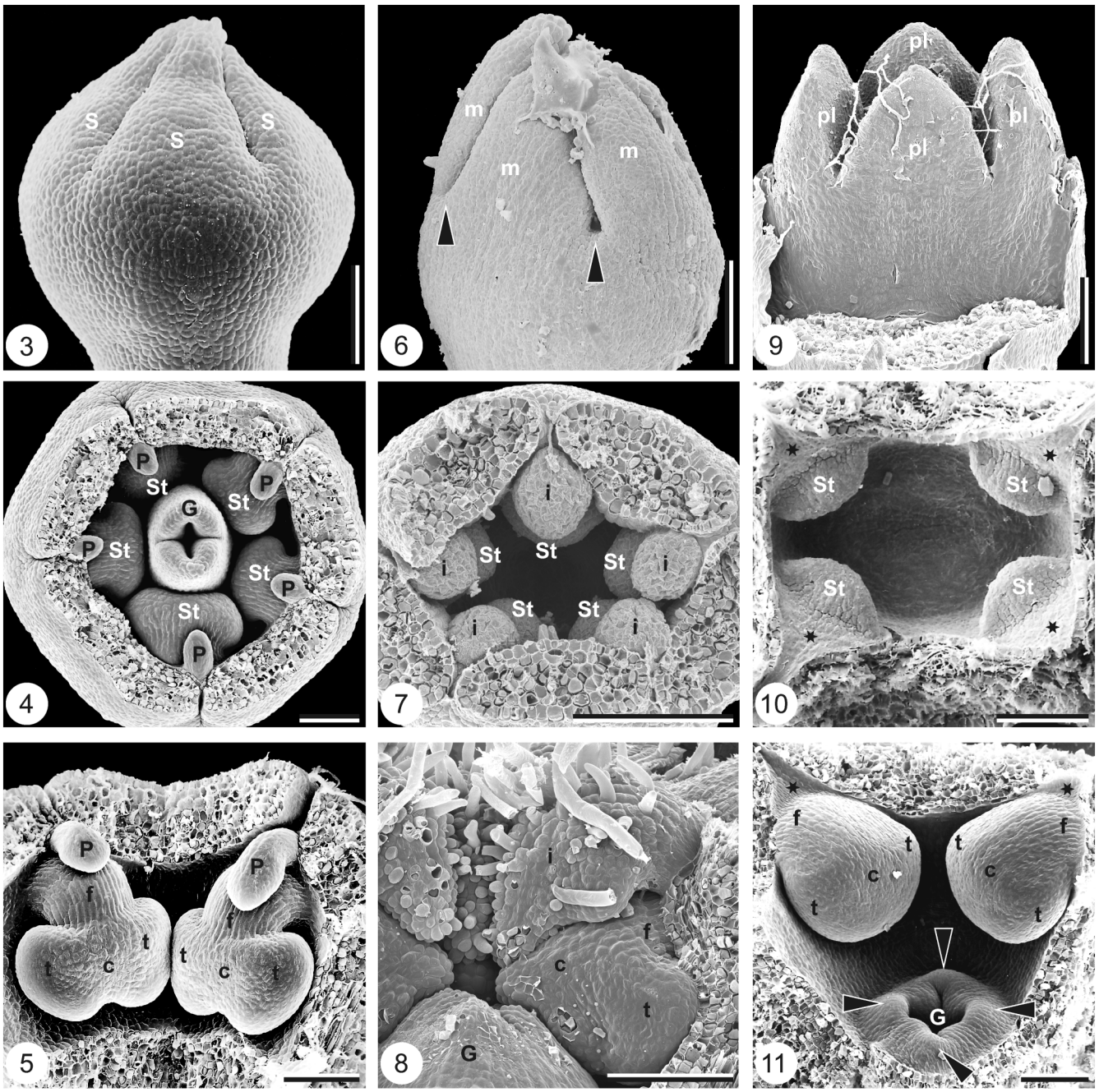
terus micranthus and *Brachysiphon acutus* and a third clade comprising the remaining species of Penaeaceae. This latter clade receives 99% BS and shares an 8-bp deletion in the *rpl16* intron (B_4; Table 2). Other well-supported groups are confined to a clade comprising all *Penaea* species except *Penaea dahlgrenii* (BS = 98% in the combined analysis). The members of this *Penaea* clade also share a 16-bp indel in the *trnS-G* spacer (C_{11}; Table 2). Within the *Penaea* clade, four of the five subspecies of *Penaea cneorum*, together with *Penaea mucronata*, form a solid clade (BS = 97%) that is part of a trichotomy with the fifth subspecies of *Penaea cneorum* and *Penaea acutifolia*.

Floral development and structure—Floral development and structure are described with emphasis on characters that help to clarify floral organization and organ homology among Rhynchocalycaceae, Oliniaceae, and Penaeaceae.

Rhynchocalyx lawsonioides (Rhynchocalycaceae)—Flowers are bisexual; penta- to heptamerous, except for the two-merous gynoecium; obhaplostemonous; and the ovary is superior (Figs. 3, 4). Sepals are inserted on the rim of a short hypanthium (Figs. 3, 4). They are broad-based and triangular, and their aestivation is valvate (Figs. 3, 12, 19). Sepals are 8–11 cell layers thick, and each sepal is served by three vascular bundles (Fig. 18). Petals alternate with the sepals and are inserted on the adaxial side of the hypanthium rim (Figs. 4, 5). Their development is retarded relative to the sepals in young buds (Figs. 4, 5). In early developmental stages, petals are incurved, club-shaped, with a narrow base and a slightly thickened apex (Fig. 5). In older stages, petals have a linear claw and an expanded, circular blade with an irregularly shaped outline (Fig. 13). Petal blades are 3–5 cell layers thick. Each petal is served by a single vascular bundle (Fig. 19). Stamens are produced opposite, i.e., just below the petals on the adaxial side of the hypanthium rim (Figs. 4, 5). In bud, stamens are wrapped by the petal blades (Fig. 13), which at anthesis spread to expose the stamens (not shown). Stamens are strongly incurved in bud, with their pollen sacs directed towards the hypanthium (Figs. 5, 19). The apex of the ovary is tapering into a short style with a terminal, papillate stigma (Fig. 13). Scattered cells with oxalate druses are present in all floral organs except for the petals. In the calyx they are restricted to the basal part of the sepals and are concentrated in the adaxial parenchymatic tissue (Fig. 20).

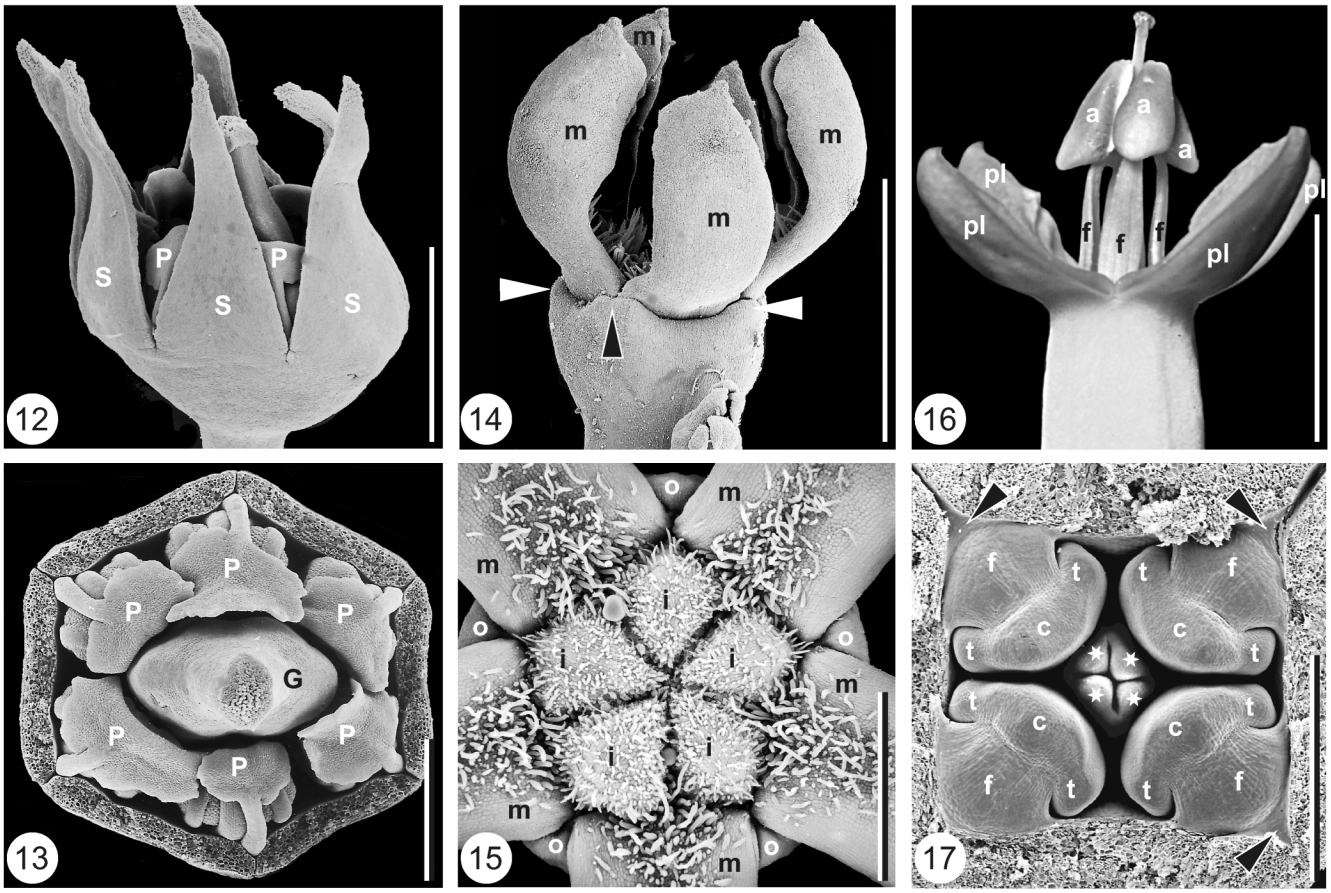
Olinia emarginata (Oliniaceae)—Flowers are bisexual and pentamerous, and the ovary is inferior. In the following description of the perianth, we refer to the three whorls of organs as to outer, middle, and inner whorl, respectively. In early developmental stages only the two inner whorls are present and the transition from the hypanthium to the organs of the middle whorl is continuous and smooth (Figs. 6, 7). The organs of the outer whorl arise only later during floral development and are then alternating with the organs of the middle whorl (Figs. 14, 15, 22). At anthesis, the organs of the outer whorl are small, blunt, and tooth-like and are inserted on top of the somewhat thickened hypanthium rim (Figs. 14, 15). These ''teeth'' are irregular in size and shape, sometimes being slightly two-tipped (black arrow head in Fig. 14). They are not vascularized (Fig. 22). The organs of the middle whorl have a broad base (Figs. 14, 15) and are triangular in shape in early developmental stages (Fig. 6). Due to the expansion of the hypanthium rim, the organs of the middle whorl appear inserted on the adaxial side of the rim in old buds and anthetic flowers (Figs. 14, 15). Aestivation is, at least in early stages, more or less valvate. However, due to their rapid growth and their lingulate shape, the lobes often overlap with each other in an irregular pattern (Fig. 6), and in later stages, aestivation is apert (i.e., without touching of neighboring organs; Fig. 14). The organs of the middle perianth whorl are 9–11 cell layers thick (Fig. 21), and towards their base, the adaxial surface is covered by unicellular trichomes (Figs. 15, 22). Each organ of the middle whorl is supplied by three main vascular bundles, of which the two lateral ones bifurcate close to the base (Fig. 21). The inner whorl of the perianth and the stamens are initiated at the adaxial side of the hypanthium rim (Fig. 7). Organs of the inner whorl alternate with those of the middle whorl, and their development is retarded relative to latter ones (Fig. 7). They are incurved and bend over the stamens in young stages and later close the entrance to the floral tube (Figs. 8, 15). Organs of this inner whorl have a narrow base (Figs. 8, 22). The inner organs are covered by unicellular trichomes, which, in bud stage, fill the space between the individual organs (Fig. 15). At anthesis, the inner organs are vertical; thus, the entrance to the floral tube is open (not shown). The blades of the inner organs are approximately six cell layers thick and each organ is supplied by a single vascular bundle that trifurcates within the blade (Fig. 22). Stamens are produced opposite, i.e., just below the lobes of the inner perianth whorl on the adaxial side of the hypanthium rim (Fig. 7), and they are strongly incurved with their pollen sacs directed towards the hypanthium (Fig. 8). They remain incurved during anthesis, thus leaving only a narrow entrance to the floral tube (not shown). The carpels alternate with the stamens, and the style ends in a capitate stigma with secretory papillae (not shown). Scattered cells with oxalate druses are present in all floral organs except for the style. In the organs of the middle perianth whorl they are relatively rare and restricted to the adaxial, parenchymatic tissue in the basal part of the organs (Fig. 23). Single oxalate druses are present in the parenchymatic tissue of the organs of the inner perianth whorl.

Endonema retzioides (Penaeaceae)—Flowers are bisexual and tetramerous, and the ovary is superior. The perianth consists of a single whorl of organs, which are inserted on the rim of a long, tubular hypanthium (Fig. 16). Perianth organs have a broad base and are triangular in shape in young stages (Fig. 9). Aestivation is apert in early developmental stages (Fig. 9) and valvate in later stages (Fig. 25). The perianth organs become exceedingly thick and fleshy during floral development (Fig. 24), and at anthesis they are numerous cell layers thick. Each perianth lobe is supplied by three main vascular bundles (Fig. 24). In early developmental stages, floral buds show strikingly large, triangular, ''empty'' areas between the adjacent flanks of two neighboring perianth lobes and the corresponding stamen primordium, which is located just below on the adaxial side of the hypanthium rim (Figs. 10, 17). Stamens are strongly incurved in bud with their pollen sacs directed towards the hypanthium (Figs. 11, 17, 25). At anthesis, the stamens are upright and exserted from the floral tube (Fig. 16). The carpels alternate with the stamens (Fig. 11). Already at relatively young developmental stages, the commissural regions (i.e., the regions where neighboring carpels are fused)



Figs. 3–11. Floral development of *Rhynchocalyx lawsonioides* (Figs. 3–5), *Olinia emarginata* (Figs. 6–8), and *Endonema retzioides* (Figs. 9–11). **3.** Young floral bud, viewed from the side; simply-valvate aestivation of calyx. **4.** Floral bud, sepals removed, viewed from above. **5.** Dissected floral bud with strongly incurved stamens and incurved petals. **6.** Young floral bud, viewed from the side; irregular aestivation of middle perianth organs; arrowheads indicate where outer perianth organs will develop in later developmental stages (see Fig. 14). **7.** Floral bud, middle perianth organs removed, viewed from above. **8.** Dissected floral bud with strongly incurved stamens and inner perianth organs. **9.** Young floral bud, viewed from the side; apert aestivation of perianth lobes. **10.** Floral bud, perianth lobes removed, viewed from above; stars indicate ''empty'' areas between adjacent perianth lobes and stamen. **11.** Dissected floral bud with strongly incurved stamens; stars indicate ''empty'' areas between adjacent perianth lobes and stamen; arrowheads indicate carpel tips (alternating with stamens, see Fig. 17). Scale bars = 100 μm.

Figure Abbreviations (applicable to Figs. 3–26): a, anther; c, connective; e, epidermis; f, filament; G, gynoecium; i, inner perianth organ of Oliniaceae; m, middle perianth organ of Oliniaceae; o, outer perianth organ of Oliniaceae; P, petal; pc, petal claw; pl, perianth lobe in Penaeaceae; ps, pollen sac; S, sepal; St, stamen; t, theca.



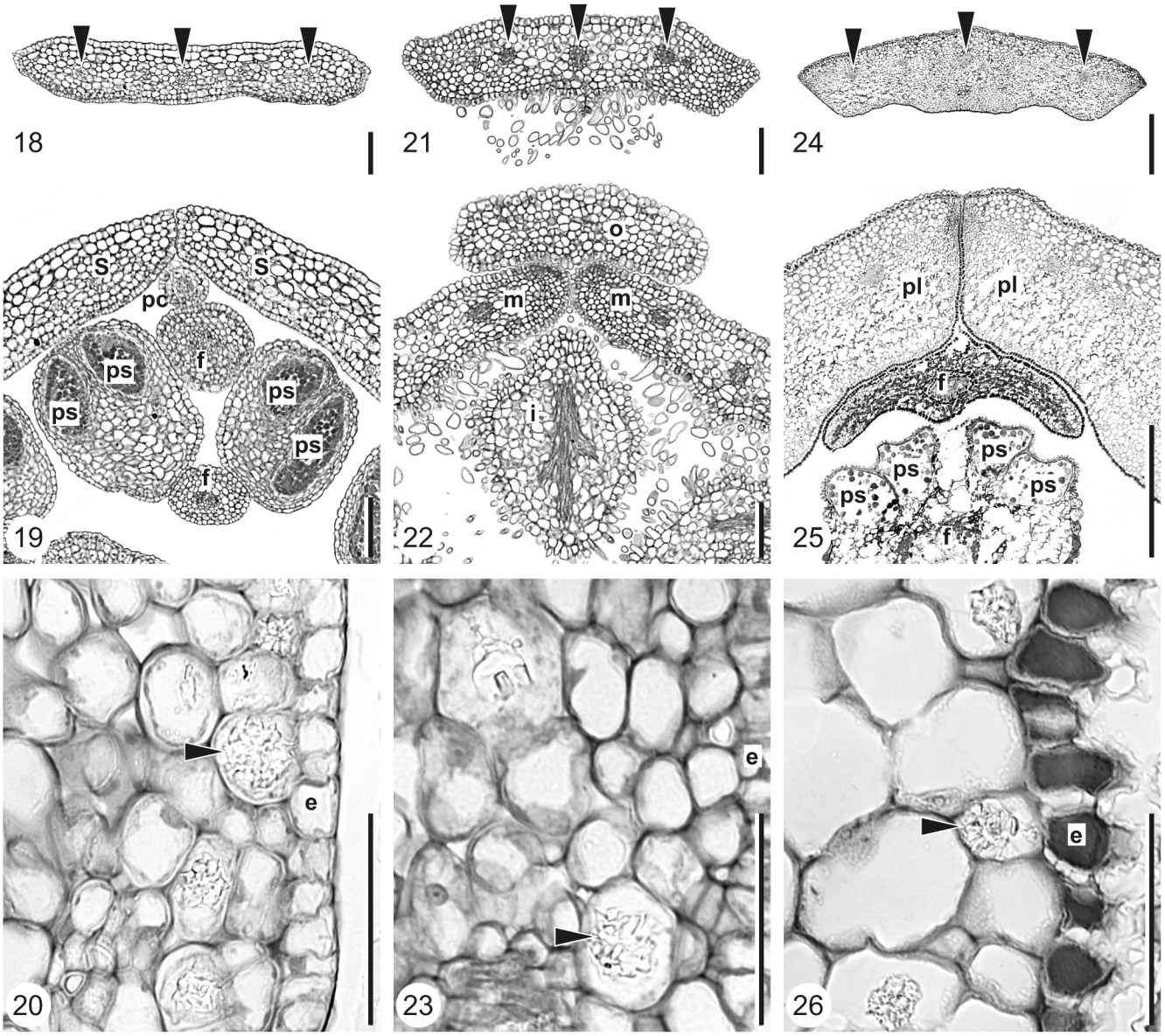
Figs. 12–17. Floral structure of *Rhynchocalyx lawsonioides* (Figs. 12, 13), *Olinia emarginata* (Figs. 14, 15), and *Endonema retzioides* (Figs. 16, 17). **12.** Flower at the beginning of anthesis, viewed from the side. **13.** Floral bud just before anthesis, sepals removed, viewed from above; note clawed petals. **14.** Old floral bud, viewed from the side. White arrowheads indicate thickened hypanthium rim with outer perianth organs alternating with middle perianth organs. Black arrowhead indicates a slightly bifid outer perianth organ. **15.** Floral bud just before anthesis, viewed from above; middle perianth organs spread, inner perianth organs still incurved. **16.** Distal part of anthetic flower; perianth lobes spread, stamens erect. **17.** Floral bud, perianth lobes removed, viewed from above. Arrowheads indicate ''empty'' areas between adjacent perianth lobes and stamen, and stars indicate commissural lobes of gynoecium (opposite stamens, see Fig. 11). Scale bars in Figs. 12, 14, 15 = 1 mm; in Figs. 13, 17 = 0.5 mm; in Fig. 16 = 1 cm.

extend beyond the carpel tips, giving the false impression of the carpels being positioned in the same sectors as the stamens (Fig. 17). At anthesis, the style ends in a quadrangular, capitate stigma, which consists of four commissural lobes. Scattered cells with oxalate druses are present in all floral organs except for the uppermost part of the style. In the perianth organs they are restricted to the basal part and are concentrated in the adaxial parenchymatic tissue (Fig. 26).

DISCUSSION

Phylogenetic relationships among families—Our results based on six cpDNA regions confirm the monophyly of Oliniaceae and Penaeaceae, respectively, and the interfamilial relationships found in previous molecular studies (Clausing and Renner, 2001; Conti et al., 2002), with Alzateaceae sister to the three African taxa and Rhynchocalycaceae sister to the clade of Penaeaceae and Oliniaceae. This African clade was also strongly supported by the analyses of Clausing and Renner (2001) and Conti et al. (1997). The strong molecular support for the clade formed by the three African families, excluding the New World Alzateaceae, contrasts with results based on nonmolecular characters (Johnson and Briggs, 1984), which support sister relationships between Rhynchocalycaceae and Alzateaceae and between Oliniaceae and Penaeaceae, respectively. The data matrix by Johnson and Briggs (1984) did not contain any exclusive synapomorphies for the three African families, but it included two derived features shared by Rhynchocalycaceae and Alzateaceae, i.e., libriform fibers (walls with simple pits) in the wood and a reduced carpel number. However, libriform fibers are also present in Oliniaceae (van Vliet and Baas, 1984), and reduced carpel number (relative to the number of perianth organs and stamens) is also present in some members of *Olinia* (Rao and Dahlgren, 1969). The occurrence of a glandular swelling or mucro (areola) at the leaf tip in Rhynchocalycaceae, Oliniaceae, and some species of Penaeaceae (*Saltera*, *Sonderothamnus*) has been proposed as a possible synapomorphy for the three African families (Dahlgren and van Wyk, 1988).

Our cpDNA results corroborate the sister-group relationship between Oliniaceae and Penaeaceae that was already supported in previous molecular (Conti et al., 1997, 2002; Clausing and Renner, 2001) and nonmolecular studies (Johnson and Briggs, 1984), but only when a 15-bp deletion in the *rpl16* intron (B_1; Table 2) is added to the combined data set (BS =



Figs. 18–26. Transverse sections of old floral buds of *Rhynchocalyx lawsonioides* (Figs. 18–20), *Olinia emarginata* (Figs. 21–23), and *Endonema retzioides* (Figs. 24–26). **18.** Transverse section (TS) of basal region of sepal; arrowheads indicate main vascular bundles. **19.** TS at the level just above petal insertion on hypanthium rim. Filament appears twice due to strongly incurved orientation of stamen; note petal claw with a single vascular bundle. **20.** Close-up of TS from the adaxial subepidermal parenchymatic tissue in basal part of a sepal; arrowhead indicates oxalate druse. **21.** TS of basal region of middle perianth organ. Arrowheads indicate main vascular bundles. **22.** TS at the level just above insertion of inner perianth organs on hypanthium rim. Inner perianth organ appears approximately longitudinal due to its incurved orientation in the bud. **23.** Close-up of TS from the adaxial subepidermal parenchymatic tissue in the basal part of a middle perianth organ; arrowhead indicates oxalate druse. **24.** TS of basal region of perianth lobe. Arrowheads indicate main vascular bundles. **25.** TS at the level just above the hypanthium rim. Filament appears twice due to strongly incurved orientation of stamen. **26.** Close-up of TS from the adaxial subepidermal parenchymatic tissue in the basal part of a perianth lobe; arrowhead indicates oxalate druse. Scale bars in Figs. 18, 20, 21, 22 = 100 μm; in Figs. 24, 25 = 1 mm; in Figs. 20, 23, 26 = 50 μm.

58%; see Fig. 2). Earlier molecular studies also found only low or moderate bootstrap support for the Oliniaceae/Penaeaceae clade (Conti et al., 1997, BS $<$ 50%; Clausing and Renner, 2001, BS = 68%/74%). The only synapomorphy supporting the clade with Penaeaceae and Oliniaceae in the analysis by Johnson and Briggs (1984) was what they called ''reduction of stamen filaments.'' However, filaments, although small in Oliniaceae, are well developed in both families, and in many species of Penaeaceae they are even massive and long (e.g., in *Endonema*, *Glischrocolla*, *Saltera*; see Fig. 16 for *Endonema retzioides*; see also Dahlgren and van Wyk [1988]). Patel, Skvarla, and Raven (1984) noted distinct similarities in pollen structure of Oliniaceae and Penaeaceae, including a psilate surface and a particular exine structure with an exceedingly thick foot layer and tectum, and a thin columella layer with an infratectal granular layer.

The three African families are each very distinctive, both morphologically and molecularly (BS = 100% for both Oliniaceae and Penaeaceae; see Fig. 1), but their detailed relationships are weakly resolved. The long branches subtending

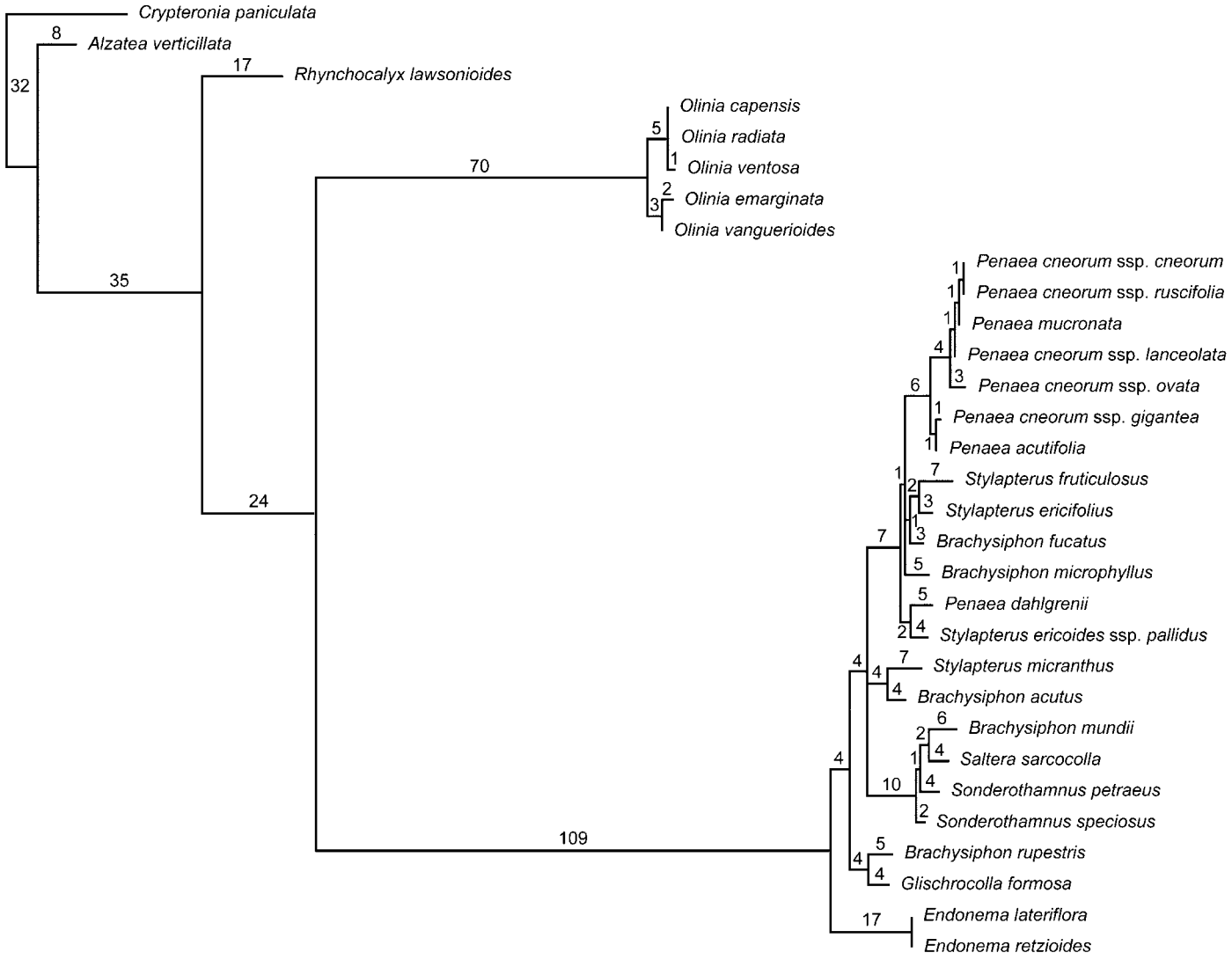


Fig. 27. Phylogram for one of the 24 shortest trees from the combined data set and 47 indel characters. Branch lengths are reported above the branches.

the crown groups of Oliniaceae and Penaeaceae may, in part, explain the apparent contradiction between distinctness and resolution (see Fig. 27, phylogram). A possible explanation for the observed lack of resolution is that the stem lineages of Oliniaceae, and especially Penaeaceae, represent groups of relatively ancient origin that accumulated many apomorphies during their independent evolutionary trajectories. The radiation that gave rise to the ancestors of Rhynchocalycaceae, Oliniaceae, and Penaeaceae has probably been obscured by the changes that have later accumulated over a long period of time, both at the molecular and nonmolecular level, thus making it difficult to establish phylogenetic relationships among the three families. After a relatively long period of independent evolution, Oliniaceae and Penaeaceae appear to have radiated rather rapidly, a process that left its signature in the short branches of the crown groups of these two families. A similar evolutionary scenario has been proposed for the radiation of the South African Cape genus *Phylica* (Rhamnaceae; Richardson et al., 2001). In the future, we plan to use molecular dating approaches to further investigate the implications of the observed pattern for the rate of molecular evolution and time of radiation of the groups studied here.

In summary, molecular evidence strongly supports the monophyly of the three African families Rhynchocalycaceae, Oliniaceae, and Penaeaceae and lends only weak support for the sister group relationship of Oliniaceae and Penaeaceae. At the nonmolecular level, a possible synapomorphy for the African clade is glandular leaf tips, and a specialized pollen structure supports the sister-group relationship of Oliniaceae and Penaeaceae.

Phylogenetic relationships within Oliniaceae—The short branches (Fig. 27) and polytomies (Fig. 1) within Oliniaceae mirror the confusing taxonomy of this family (Sebola and Balkwill, 1999), where a variable number of species, from as few as one (e.g., Sonder, 1862) to seven or eight have been recognized (e.g., Dahlgren and van Wyk, 1988; Sebola and Balkwill, 1999). Geographically, most species are restricted to South Africa, while one species (*Olinia vanguerioides*) occurs in Zimbabwe and Mozambique and one, *O. rochetiana*, in

northeastern South Africa, extending north into tropical East Africa. Verdcourt (1978) states that *O. rochetiana* is closely related to *O. vanguerioides*, differing in little but flower size. Among the five species sampled, our results strongly support a clade with *O. emarginata* and *O. vanguerioides* (BS = 81%) and a clade including *O. capensis*, *O. ventosa*, and *O. radiata* (BS = 97%). In addition, each clade is supported by two indels (Fig. 1, Table 2). Geographically, the latter three species are restricted to coastal forests in the western and/or southern parts of South Africa, whereas *O. emarginata* extends from the southeast to the northeast of South Africa and *O. vanguerioides* occurs even farther north in Zimbabwe and Mozambique (van Wyk and van Wyk, 1997; Sebola and Balkwill, 1999). Morphologically, species delimitation is problematic within *Olinia*, and a careful monographic treatment and morphological/anatomical study of the genus are greatly needed. For this reason it is currently difficult to determine morphological synapomorphies that are congruent with the clades retrieved in our molecular study. Stomata types may represent a potentially useful synapomorphy, as they are reported to be mostly anomocytic on the leaves of *O. emarginata* and *O. vanguerioides* and mostly paracytic in *O. radiata* and *O. ventosa* (Mújica and Cutler, 1974).

Phylogenetic relationships within Penaeaceae—Phylogenetic relationships among species of Penaeaceae have not been investigated from a molecular standpoint or with a rigorous analytical approach. Delimitation and relationships of genera within Penaeaceae have been controversial in the past. Morphologically, the family is rather uniform and differences pertain mainly to the relative size and shape of the vegetative and reproductive organs, whereas distinct, qualitative differences are rare. Already in 1894, Supprian noted that due to the close relationships among all Penaeaceae, and the occurrence of taxa with transitional morphological features, generic delimitation was difficult. These difficulties are also reflected in earlier taxonomic treatments of the family, for example de Candolle (1857) recognized six genera, which Baillon (1877) reduced to only three and Gilg (1894b) extended again to five. The currently accepted generic circumscriptions and systematic relationships among the genera are mainly based on taxonomic revisions by Dahlgren (Dahlgren, 1967a, b, c, 1968, 1971). He recognized seven genera and 21 species, although he also found it challenging to clearly circumscribe genera, especially due to the occurrence of transitional species, for example, *Stylapterus ericifolius* between *Stylapterus* and *Brachysiphon* and *Brachysiphon mundii* between *Brachysiphon* and *Saltera*/*Sonderothamnus* (Dahlgren, 1967a). Since Dahlgren's work, two new species have been described, *Penaea dahlgrenii* (Rourke and McDonald, 1989) and *Brachysiphon microphyllus* (Rourke, 1995). Rourke (1995) stated that these new species are not easily assigned to any particular genus, further emphasizing the lack of clear-cut boundaries between the genera. A detailed comparative study of leaf anatomy of Penaeaceae (Dickie and Gasson, 1999) concluded that, due to variability and inconsistency, leaf anatomical characters are not useful for systematic purposes within the family, a conclusion that corroborates Dahlgren's view (Dahlgren, 1968). Furthermore, leaf anatomical characters, when mapped on our molecular tree, revealed no congruence with molecular clades (not shown). Similarly, wood anatomical features track more closely habitat type (e.g., xeromorphic vs. mesomorphic adaptations) than taxonomic groupings (Carlquist and Debuhr, 1977).

The difficult interpretation of generic circumscription is also reflected in the short branches found within the Penaeaceae crown group (Fig. 27). Despite the relatively low number of informative characters within Penaeaceae, our analysis of the combined data sets strongly supports several clades (marked I–VI in Fig. 1) and suggest that some of Dahlgren's genera, specifically *Brachysiphon* and *Stylapterus* (Dahlgren, 1967a), are not monophyletic (see Fig. 1).

Clade I (BS = 100%, Fig. 1; branch length [BL] = 17, Fig. 27) comprises the two species of the genus *Endonema* and is sister to the rest of the Penaeaceae. This result strongly confirms the proposed monophyly of the genus (Dahlgren, 1967c). *Endonema lateriflora* and *E. retzioides* share large, tubular, and brightly colored flowers typical of a bird-pollination syndrome (Dahlgren, 1967c). Both species have strongly incurved stamens in bud, a feature regarded as a synapomorphy for *Endonema* (Dahlgren, 1967c), but this feature is also present in *Brachysiphon acutus* and *B. mundii* (Fig. 28; J. Schönenberger, personal observation). Strongly incurved stamens also characterize Oliniaceae, Rhynchocalycaceae, and Alzateaceae; hence they appear to represent the ancestral condition in Penaeaceae. Weberling (1988) identified a potential synapomorphy for *Endonema*, i.e., the specialized inflorescence with two or more flowers situated in the leaf axil on a young branch that continues vegetative growth, while inflorescences are terminal in the rest of Penaeaceae. Both species are restricted to a relatively small area in the Riversonderend Mountains in the Cape Province and occur in similar habitats on south-facing slopes, often near a watercourse (Dahlgren, 1967c).

Clade II (BS = 91%, BL = 4), comprising *Glischrocolla formosa* and *Brachysiphon rupestris*, is sister to the remaining members of Penaeaceae. To date, *G. formosa* and *B. rupestris* have never been suggested to be closely related to one another and the former was thought to be distinct from all other members of the family. The bird-pollinated, long, tubular, brightly colored flowers of the monospecific *Glischrocolla*, with two pendant and two ascending ovules in each locule, seemed to imply a close relationship to *Endonema*, which also has the same number and arrangement of ovules (Dahlgren, 1967b). *Glischrocolla* differs from *Endonema* mainly in having upright stamens in bud, five- vs. three-colporate pollen, and terminal vs. lateral inflorescences. *Brachysiphon* sensu Dahlgren (1968) is a taxonomically difficult genus that is not easily circumscribed. Dahlgren (1968) stated that *B. acutus* and *B. fucatus* are in some respects similar to members of the genus *Stylapterus*, while *B. rupestris* and *B. mundii* in some features resemble *Saltera* and *Sonderothamnus*. Using Dahlgren's criteria, *B. microphyllus*, which was recently described (Rourke, 1995), could be placed either in *Brachysiphon* or *Sonderothamnus* and only the occurrence of bracts with entire margins favored its assignment to the former. The occurrence of two erect and two pendant ovules in the middle part (axially) of each locule, a consistent feature of *Endonema* and *Glischrocolla* (Dahlgren, 1967b, c), appears occasionally in *B. rupestris* (Phillips, 1926, 1951) and might provide a morphological link to *G. formosa*. Both *G. formosa* and *B. rupestris* grow on ledges or in crevices on sandstone rock precipices, the former at rather high (1200–1500 m) and the latter at lower altitudes (30–400 m). At present, the two species are only known from a few populations and are narrowly distributed in close, but not overlapping, areas of the Western Cape Province.

Clades III, IV, and V form a strongly supported monophyletic group (BS = 93%, BL = 4, 226-bp deletion in the *psbA*-

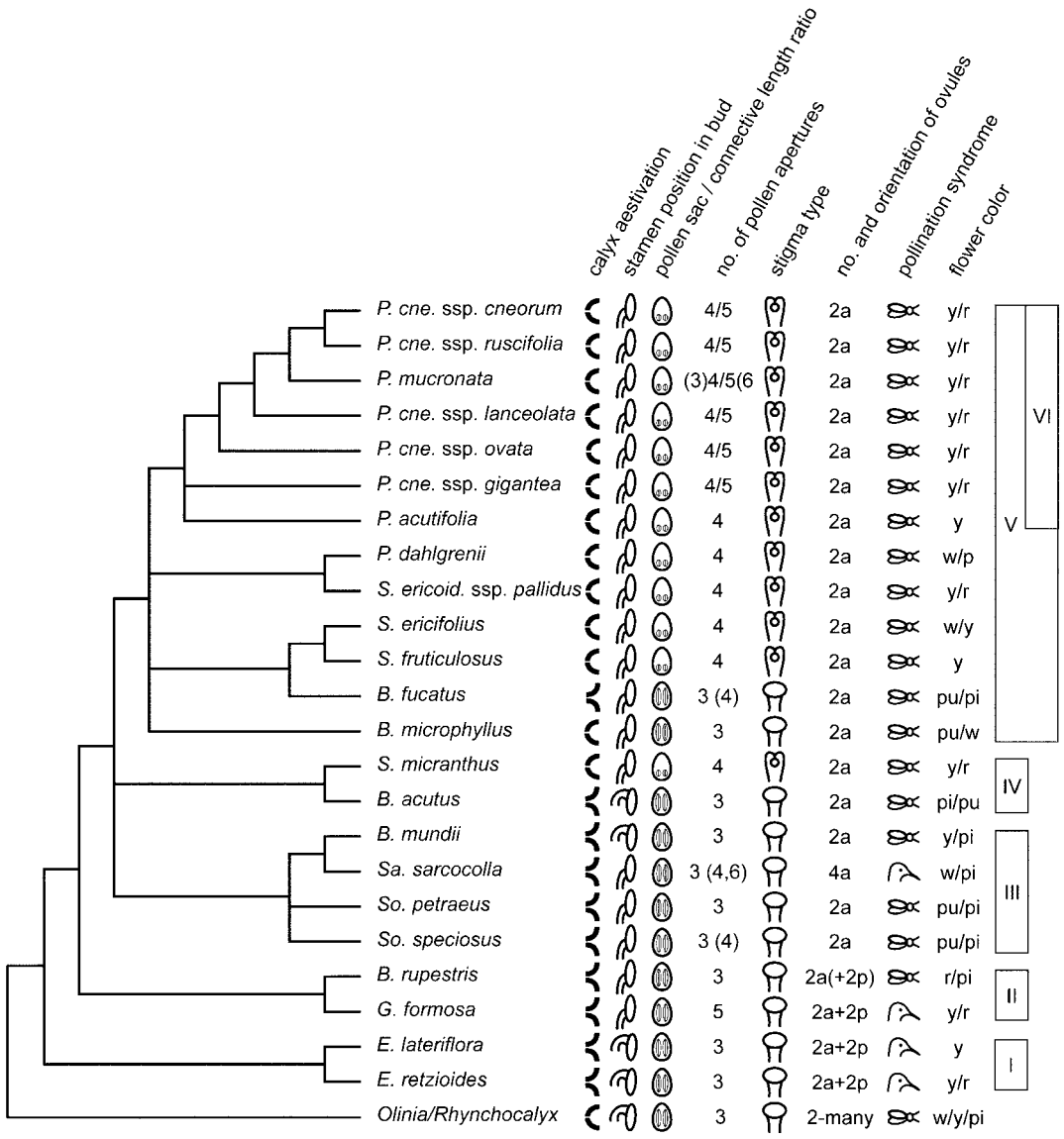


Fig. 28. Distribution of floral characters in Penaeaceae on the strict consensus tree of the combined data sets and 47 indel characters. Calyx aestivation: simply-valvate, reduplicative-valvate. Stamen position in bud: erect, strongly incurved. Pollen sac/connective length ratio: pollen sacs shorter than half of the connective length, pollen sacs longer than half of the connective length. Stigma type: stigmatic area restricted to angles between commissural lobes, stigma terminal. Number and orientation of ovules: a = ascending, p = pendant. Pollination syndrome: insect-pollination syndrome, bird-pollination syndrome. Flower color: pi = pink, pu = purple, r = red, w = white, y = yellow. See Fig. 27 for spelled-out species names. Data from Dahlgren (1967a, b, c, 1968, 1971); Patel, Skvarla, and Raven (1984); Phillips (1926, 1951); and J. Schönenberger, personal observation.

trnH spacer). Further, all members of this group have only erect ovules (two per locule, except for *Saltera sarcocolla*, which has four) inserted basally in the locule (Fig. 28). Relationships among the three clades are not resolved.

Clade III (BS = 100%, BL = 10, insertion in the *rpl16* intron) comprises *Sonderothamnus* (two species), *Saltera* (monospecific), and *Brachysiphon mundii*. Dahlgren (1968) listed a number of similarities between *Sonderothamnus* and *Saltera*, including specialized leaf tips (with areolae), which are always present in *Sonderothamnus* and at least in some individuals of *Saltera*. Denticulate-fimbriate bract and bracteole margins represent another feature shared by both genera. From a morphological point of view, *B. mundii* does not share any obvious synapomorphies with *Saltera* and *Sonderothamnus*, but we have been unable to detect any traits that clearly distinguish it from these genera and would thus contradict the close relationship among the taxa of clade III. Before Dahlgren's treatment of Penaeaceae (Dahlgren, 1968), the two species of *Sonderothamnus* were included in a broadly circumscribed *Brachysiphon* (e.g., Gilg, 1894b; Phillips, 1951; Barker, 1963). Dahlgren knew *B. mundii* only from a single specimen in post-floral stage (the type specimen) and he remarked that the collection was rather incomplete. Therefore, it is possible that a more detailed study of anthetic flowers may reveal characters linking *B. mundii* with *Saltera* and *Sonderothamnus*. The two species of *Sonderothamnus* and *B. mundii* have narrow distributions, while *Saltera sarcocolla* is relatively widespread in the southwestern part of the Cape Province. The distribution of *Saltera* overlaps with that of *Sonderothamnus*, while *B. mundii* has a more eastern distribution and is the only

species within Penaeaceae that is restricted to limestone rocks (Dahlgren, 1968; Goldblatt and Manning, 2000).

Clade IV (BS = 95%, BL = 4) comprises *Stylapterus micranthus* and *Brachysiphon acutus*, a pair of species that had never been suggested to be closely related. To rule out the possibility that this unexpected result may be the product of experimental error, we double-checked our sequencing results (as described in the Materials and Methods section) and confirmed our species identifications (see http://ajbsupp.botany.org/v90/). As the floral structure of these two species does not suggest a sister relationship, it is necessary to propose an alternative evolutionary process that may explain this unexpected pattern. In this regard, it is important to note that our phylogenetic reconstruction is derived exclusively from chloroplast DNA sequences; hence, it represents a maternal phylogeny. Therefore, the possibility cannot be ruled out that the relationship between *B. acutus* and *S. micranthus* may represent a further example of hybridization followed by chloroplast capture (see, e.g., Soltis and Kuzoff, 1995; Wolfe and Elisens, 1995; Sang, Crawford, and Stuessy, 1997; Conti et al., 1999).

Clade V (BS = 99%, BL = 7; deletion in the *rpl16* intron, B_4, Table 2) comprises the remaining species of the genera *Brachysiphon* and *Stylapterus* and all species of *Penaea*. Most species in the clade have white or yellow flowers, simply valvate aestivation of the perianth lobes, relatively short pollen sacs, and stigmatic areas that are restricted to the angles between commissural stigma lobes. Exceptions for either one of the abovementioned characters are found in *B. microphyllus* and *B. fucatus* (see Fig. 28). Clade V is formed by a polytomy with four branches, of which only one supports a strongly monophyletic group (clade VI; BS = 98, BL = 6; insertion C_{11}, Table 2).

Clade VI comprises all *Penaea* species except for *P. dahlgrenii*. The latter species was described by Rourke and McDonald (1989) only after Dahlgren's (1971) monograph of *Penaea*. In the protologue of *P. dahlgrenii*, Rourke and McDonald (1989) remarked that the species is so unlike any other *Penaea* that its placement in the genus might be regarded as questionable. Our results lend some support to this view and confirm the monophyly of *Penaea* sensu Dahlgren (1971). The sister relationship of *P. dahlgrenii* with *Stylapterus ericoides* is weakly supported by molecular data (BS < 50%, BL = 2) and seems unlikely from a biogeographic standpoint, as both species are narrow endemics separated by rather long distances (ca. 150 km; Dahlgren, 1967a; Rourke and McDonald, 1989). In addition, because all species of Penaeaceae have ant-dispersed seeds with elaiosomes, dispersal over long distances seems rather unlikely (Bond and Slingsby, 1983). Considering the weak support for this clade, it is possible that additional DNA sequence data may change the relationships of *Penaea dahlgrenii* and *Stylapterus ericoides*. Within clade VI (*Penaea* sensu Dahlgren) the five subspecies of *P. cneorum* (Dahlgren, 1971) do not form a monophyletic group, but are paraphyletic with *P. mucronata* (Fig. 1). Both *P. mucronata* and *P. cneorum* are relatively widespread and variable in their phenotypic characters (Dahlgren, 1971). To resolve phylogenetic relationships and species delimitation of these two species more informative characters and a much denser sampling at the population level are necessary. Another weakly supported clade within clade V comprises *Stylapterus ericifolius*, *S. fruticulosus*, and *Brachysiphon fucatus*. This clade seems unlikely from a morphological perspective, as *B. fucatus* differs conspicuously from the two *Stylapterus* species in flower color, perianth aestivation, and stigmatic characteristics. Conversely, some morphological characters, including the smaller and more rounded commissural stigma lobes on the style apex, long papillate instead of ciliate dehiscence margins of anthers, and a different ovary shape, suggest that *S. ericifolius* (and *S. dubius*, not included here) may be transitional in their morphology between *Stylapterus* and *Brachysiphon* (Dahlgren, 1967a). Finally, the unresolved relationships of *Brachysiphon microphyllus* within clade V reflect the difficulty of assigning this morphologically distinctive species to any genus within Penaeaceae (Rourke, 1995).

Perianth homology—The question of perianth homology in Penaeaceae, and especially in Oliniaceae, has been extensively debated (e.g., Fernandes and Fernandes, 1962; Rao and Dahlgren, 1968; Verdcourt, 1978; Sebola and Balkwill, 1999). Different interpretations of homology were based on evidence from floral anatomy and morphology (e.g., Rao and Dahlgren, 1969) and floral organization and architecture (e.g., Dahlgren and van Wyk, 1988), but were never integrated in an explicit phylogenetic framework. Here, we interpret the results of our analyses of floral development and structure in the context of the molecular phylogeny.

In Myrtales, the perianth generally consists of calyx and corolla (e.g., Dahlgren and Thorne, 1984; Johnson and Briggs, 1984). Among the closest relatives of Oliniaceae and Penaeaceae, the perianth either is typically biseriate, as in most Crypteroniaceae and in Rhynchocalycaceae, or the petals are absent, as in Crypteroniaceae p.p. (in *Crypteronia*, e.g., van Beusekom-Osinga and van Beusekom, 1975) and in Alzateaceae (e.g., Graham, 1984; but see van Beusekom-Osinga and van Beusekom, 1975; they describe Alzateaceae as having rudimentary petals). As our study and earlier molecular phylogenetic analyses (Clausing and Renner, 2001; Conti et al., 2002) identified Rhynchocalycaceae as sister to the clade formed by Oliniaceae and Penaeaceae, we decided to compare perianth development and structure of these three families.

Although anthetic flowers of the three families appear to be rather different, the study of early developmental stages revealed clear similarities. In all three families young floral buds have a whorl of broadly attached organs (i.e., sepals of Rhynchocalycaceae, middle whorl Oliniaceae, perianth whorl of Penaeaceae) with valvate aestivation that enclose and protect the other floral organs (Figs. 3, 6, 9). Also anatomically and histologically these broadly attached perianth lobes of the three families share several features, i.e., they are relatively thick, they are served by three main vascular bundles, and they contain cells with oxalate druses restricted to the adaxial parenchymatic tissue in their basal part (Figs. 18–26). These shared features indicate that the middle whorl of perianth organs of Oliniaceae and the perianth organs of Penaeaceae are homologous to the sepals of Rhynchocalycaceae. The combination of traits that characterizes these organs, including their large size and protective function in early development, valvate aestivation, broad attachment, and three main vascular bundles, is typical for sepals (Endress, 1994). In Rhynchocalycaceae and Oliniaceae a whorl of narrowly attached organs (i.e., petals of Rhynchocalycaceae, inner whorl of Oliniaceae) alternating with the sepals is initiated on the inner side of the hypanthium rim. These narrowly attached organs emerge in a more or less transversal position from the hypanthium rim and later bend downwards over the stamens (Figs. 4, 5, 7, 8). In both families

these organs are delayed in their development as compared to the sepals and, although they are involved in pollinator attraction (Dahlgren and van Wyk, 1988), they are relatively small at anthesis. At the beginning of anthesis they bend back and outwards and expose the stamens (see also Dahlgren and van Wyk, 1988). Also anatomically and morphologically these innermost perianth organs of Rhynchocalycaceae and Oliniaceae are similar, as they are thin and served by a single vascular bundle (Figs. 18–26). The shared features indicate the homology of the petals of Rhynchocalycaceae and the inner perianth organs of Oliniaceae. The combination of delayed development, relatively thin organs, narrow attachment, supply by a single vascular bundle, and attractiveness to pollinators is characteristic for petals (Endress, 1994).

In Penaeaceae, a conspicuous ''empty'' area between adjacent perianth lobes and the stamen positioned between them marks floral buds in early developmental stages (Figs. 10, 11). Such an ''empty'' area is unusual in a developing flower, where organs are normally densely packed to reduce damage from desiccation, frost, heat, or insects (Endress, 1994). Exceptions to such dense packing are mainly present in groups where organs are aborted during floral development. Examples are found in the Lamiales, where the adaxial stamen is mostly reduced (Endress, 1999), or in certain groups of Fabaceae, where petals and/or stamens are initiated early during floral ontogeny but are later aborted at different stages of development (Tucker, 1988, 2001). The ''empty'' areas in the floral buds of Penaeaceae are probably the remnants of a whorl of petals. Most likely, petals are still initiated on the meristematic flower apex of Penaeaceae, but their development is suppressed immediately after initiation, producing the conspicuous ''empty'' areas, which lack any distinct primordia. Initiation necessarily precedes appearance, and organ primordia may be present without being detectable as three-dimensional structures (Endress, 1992, 1999). Based on their vascular anatomical studies of *Glischrocolla formosa*, Rao and Dahlgren (1968) interpreted the perianth lobes of Penaeaceae as petals, and the flowers, therefore, as being haplostemonous, but in later publications, Dahlgren and collaborators (Dahlgren and Thorne, 1984; Dahlgren and van Wyk, 1988) described the flowers of Penaeaceae as obhaplostemonous. Our own morphological developmental studies support the interpretation of perianth organs of Penaeaceae as sepals; hence, they also support the obhaplostemonous nature of the flowers.

An interesting feature that characterizes both Oliniaceae and Penaeaceae is that the perianth organs display a mixture of ecological functions. The sepals of both families are responsible for the protection of young floral organs. In Oliniaceae the sepals lose that function in later developmental stages; protection of stamens and carpels is then provided by the petals, which close the entrance to the floral tube (Fig. 15). At anthesis, the sepals of Oliniaceae are colored, relatively large and lingulate in shape, and share the function of pollinator attraction with the smaller, but also colored petals (see also Dahlgren and van Wyk, 1988). In Penaeaceae the sepals provide protection to the other floral organs until anthesis. Pollinator attraction is shared between the colored sepals and the colored hypanthium (see also Dahlgren and Thorne, 1984).

The homology of the outer whorl of the Oliniaceae perianth, formed by small tooth-like structures, has long been debated. In a study of floral anatomy of *Olinia*, Rao and Dahlgren (1969) suggested that the ''teeth'' correspond to reduced sepals and the middle whorl to petals, hence the flowers were interpreted as haplostemonous. The innermost whorl was interpreted as being formed by the fusion of pairs of adjacent petal stipules. This view was later adopted by Sebola and Balkwill (1999). However, no structural evidence was found to support this hypothesis, and the main argument was based on organ arrangement, i.e., on the opposite position of stamens and organs of the inner whorl (Rao and Dahlgren, 1969). Our investigations show that the tooth-like structures on the hypanthium rim of Oliniaceae are clearly different from the perianth organs of both Rhynchocalycaceae and Penaeaceae. In addition, the ''teeth'' arise only late during floral development (Fig. 6), a pattern that would be highly unusual for a reduced floral organ whorl. If the ''teeth'' were interpreted as reduced sepals, one would expect them to be initiated in the normal sequence, i.e., as the first organs on the developing floral apex, which are then aborted during later stages of development. This temporal sequence of floral organ initiation and subsequent abortion has been documented for all categories of floral organs and for a broad range of taxa, for example, for the reduced calyces of some Acanthaceae (Schönenberger and Endress, 1998). However, if the tooth-like structures of the Oliniaceae perianth do not represent the calyx, to what do they correspond? Mayr (1969) summarizes possible interpretations of comparable structures (the so-called epicalyx) in Lythraceae, another family of Myrtales. The epicalyx in Lythraceae has been interpreted as consisting of either simple outgrowths in the region of the congenitally united sepals (see also Dahlgren and Thorne, 1984), as a separate whorl of bracteole-derived structures, or as fused calyx stipules. The often bifid structure of the ''teeth'' in Oliniaceae (e.g., Fig. 14) fits best with the interpretation of united stipules. However, the final answer to the question of the nature of the teeth-like structures remains elusive.

Myrtalean flowers generally have a double perianth (e.g., Cronquist, 1981; Takhtajan, 1997) and the ancestral condition of the androecium appears to be diplostemony, i.e., the flowers have twice as many stamens as petals or sepals and the outer whorl of stamens is in episepalous position (Ronse Decraene and Smets, 1991). Flowers of Crypteroniaceae have a double perianth (*Dactylocladus*, *Axinandra*) or are apetalous (*Crypteronia*) and are diplostemonous (*Axinandra*) or obhaplostemonous (*Crypteronia*, *Dactylocladus*) (van Beusekom-Osinga and van Beusekom, 1975; Pereira, 1996). As in *Rhynchocalyx*, the petals in the flowers of *Dactylocladus* and *Axinandra* are enveloping the stamens in bud (Pereira, 1996). Flowers of *Alzatea* are obhaplostemonous (e.g., Lourteig, 1965; Graham, 1984) and mostly described as apetalous (e.g., Dahlgren and Thorne, 1984; Graham, 1984), whereas van Beusekom-Osinga and van Beusekom (1975) described the flowers of *Alzatea* as having rudimentary petals. Thus, there are two obvious evolutionary trends in the flowers of Crypteroniaceae, Alzateaceae, and Rhynchocalycaceae, i.e., (1) the reduction of petals and (2) the reduction or loss of the episepalous whorl of stamens, which leads to obhaplostemonous flowers. Not only do our developmental and structural studies suggest that the flowers of Oliniaceae and Penaeaceae should also be interpreted as obhaplostemonous, but our phylogenetic study also supports this interpretation, as it is most parsimonious to assume a common basic organization of the flowers in the entire clade formed by the four Western Gondwanan families plus Crypteroniaceae. In this phylogenetic framework (Fig. 1), obhaplostemony (i.e., stamens alternating with the sepals, represented by the middle perianth whorl in Oliniaceae and by the

only perianth whorl in Penaeaceae) constitutes a clear synapomorphy for the clade. Conversely, if the floral ''teeth'' of Oliniaceae were interpreted as sepals and the organs of the middle whorl as petals, and/or the perianth organs of Penaeaceae as petals, the flowers would be haplostemonous, a less parsimonious interpretation of character state distribution.

Conclusions—Our cpDNA study confirms earlier results in supporting Alzateaceae as sister to the African families and Rhynchocalycaceae as sister to the clade formed by Oliniaceae and Penaeaceae. Further, our study provides a molecular phylogenetic hypothesis of the detailed relationships within Oliniaceae and Penaeaceae. Our results resolve *Endonema* as sister to all other Penaeaceae and strongly support several groups within the family, but contradict previous generic circumscriptions of Penaeaceae by suggesting that *Brachysiphon* and *Stylapterus* sensu Dahlgren (1967a, 1968) are not monophyletic. Definite taxonomic adjustments within the family will have to await results based on the analysis of additional molecular, preferably nuclear markers and a detailed study of floral morphology.

In addition, our molecular data indicate a relatively long, independent evolution of the stem lineages and a relatively recent origin of the crown groups of Penaeaceae and Oliniaceae, respectively. This phylogenetic pattern raises important questions pertaining to, for example, whether the radiations of the two crown groups occurred simultaneously and also whether these radiations coincided with climatic changes, as suggested for other South African taxa (Richardson et al., 2001). The answers to questions on the tempo of evolution in these African families should be addressed with molecular dating methods.

Our developmental and structural analyses of the flowers of Rhynchocalycaceae, Oliniaceae, and Penaeaceae demonstrate that the flowers of all three families have the same basic organization, i.e., they are obhaplostemonous. The calyx of Rhynchocalycaceae is homologous to the middle perianth whorl in Oliniaceae and to the single perianth whorl of Penaeaceae. The corolla in Rhynchocalycaceae is homologous to the inner whorl of perianth organs in Oliniaceae. The flowers of Penaeaceae are apetalous or, more precisely, the petals are highly reduced. Calyx and corolla homology of the three families is supported by congruent developmental patterns, morphological/anatomical similarities, and phylogenetic reconstruction.

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