MOLECULAR PHYLOGENETICS

Phylogenetic relationships, taxonomy, and morphological evolution in Dipsacaceae (Dipsacales) inferred by DNA sequence data

Sara E. Carlson[1], Veronika Mayer[2] & Michael J. Donoghue[3]

[1] *Department of Ecology & Evolutionary Biology, Yale University, P.O. Box 208106, New Haven, Connecticut 06520-8106, U.S.A. sara.carlson@yale.edu (author for correspondence)*
[2] *Department of Palynology & Structural Botany, Faculty Center of Botany and Botanical Garden, University of Vienna, Rennweg 14, 1030, Vienna, Austria*
[3] *Department of Ecology & Evolutionary Biology and the Peabody Museum of Natural History, Yale University, P.O. Box 208106, New Haven, Connecticut 06520-8106, U.S.A.*

A phylogenetic study of Dipsacaceae, a Mediterranean-centered clade in the Dipsacales, was conducted using nuclear ITS and three chloroplast markers (*atpB-rbcL*, *trnL-trnF*, *trnS*^{UGA}-*trnG*^{GCC}). This is the first molecular study to assess Dipsacaceae phylogeny in detail, and includes representatives from all major subclades. Maximum parsimony, maximum likelihood, and Bayesian analyses were carried out on the ITS and plastid datasets separately and in combination. For the most part, the ITS and plastid datasets resulted in similar topologies, and the combined data yielded a well-resolved estimate of Dipsacaceae phylogeny. A small Asian clade composed of *Bassecoia* and *Pterocephalodes hookeri* is resolved as sister to the rest of Dipsacaceae. Scabioseae s.str. (*Scabiosa*, *Sixalix*, *Pterocephalus* s.str., *Lomelosia*, *Pycnocomon*) and a "Dipknautid" clade (*Dipsacus*, *Cephalaria*, *Knautia*, *Pterocephalidum*, *Succisa*, *Succisella*, *Pseudoscabiosa*) form the two major lineages within Dipsacaceae. Most of the previously recognized genera are recovered as monophyletic, with the exception of *Pycnocomon*, which is nested within *Lomelosia*. We discuss the taxonomic implications of these results and their significance for understanding character evolution, particularly of the epicalyx in relation to seed dispersal.

KEYWORDS: cpDNA, Dipsacaceae, epicalyx, ITS, Mediterranean, phylogeny

INTRODUCTION

Dipsacaceae (Dipsacales) contains ca. 300 species of perennial and annual herbs and shrubs that occur primarily in the Mediterranean Basin, with about 20% distributed in Asia and Africa. Members of Dipsacaceae occur in open and relatively dry habitats from sea level to high-alpine altitudes, with the exception of a few species that occupy moist meadow habitats. Like other Dipsacales, members of Dipsacaceae have opposite leaves, inferior ovaries, and sympetalous corollas. With members of the Linnina clade they share four stamens (with the exception of *Pterocephalidium diandrum*, which has two) and nuts or achene fruits (Donoghue & al., 2003). Dipsacaceae are distinguished by capitate inflorescences, and additional features include a calyx that is either cup-shaped or divided into four to numerous bristles. In many groups, the radial flowers of the capitula are enlarged, showy, and strongly zygomorphic.

However, the most distinctive feature of Dipsacaceae is an epicalyx that functions in ovary protection, germination, and seed dispersal (Ehrendorfer, 1965a, b; Verlaque, 1984a; Mayer, 1995; Donoghue & al., 2003; Caputo & al., 2004). The epicalyx subtends the calyx and later develops into a tubular organ that closely encloses the ovary (Fig. 1). While other morphological features in Dipsacaeae are fairly conserved, the epicalyx is tremendously diverse in shape and ornamentation, suggesting that selection for different dispersal and/or protection strategies might be driving evolution in this group. To deepen knowledge of the evolution of this feature, a robust hypothesis about the phylogenetic relationships within Dipsacaeae is needed. A phylogeny would also clarify taxonomic delimitations in Dipsacaeae.

Some groups in Dipsacaceae are marked by additional epicalyx structures that are often associated with elaborate wing-like appendages (Fig. 1). The "diaphragma" is formed by a secondary meristem on the inside of the epicalyx (Hilger & Hoppe, 1984), and encloses the stalk of the calyx like a collar. The "epi-diaphragma" is a small attachment located between the diaphragma and the "corona"—a membranous wing-like structure that forms the apical end of the epicalyx. The size and elaboration of the corona is often correlated with the epi-diaphragma, such that epicalyces with horizontal epi-diaphragmata

This paper is dedicated to Fritz Ehrendorfer on the occasion of his 80th birthday.

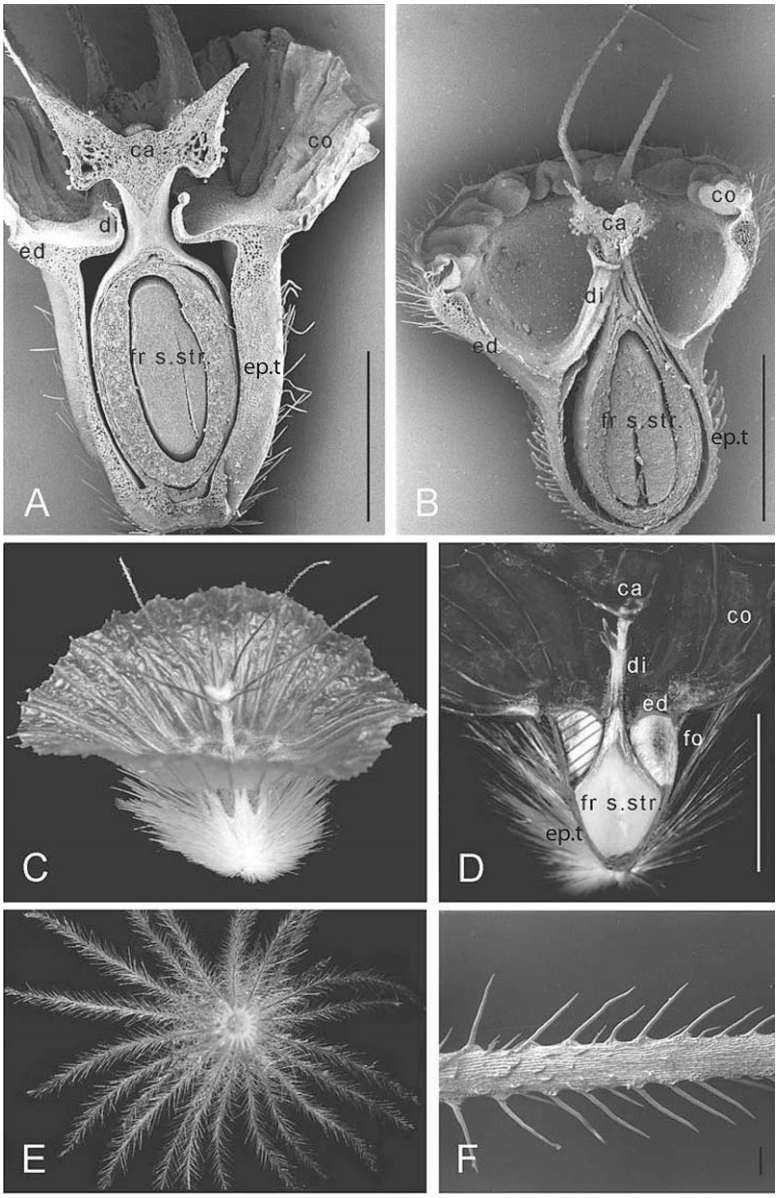


Fig. 1. Morphological variation of structural elements of the diaspore. A, *Scabiosa lucida* with short diaphragma (di), horizontal epi-diaphragma (ed), and expanded corona (co); B, *Sixalix eremophila* with long diaphragma (di), elongated, vertical, lignified epi-diaphragma (ed), and very short corona (co); C & D, *Lomelosia rotata,* with elongated, horizontal epi-diaphragma (ed), covering the pits (= foveoles, fo), and expanded corona (co); E, top view of the plumose calyx bristles of *Pterocephalus frutescens*; F, a single "fringed" calyx bristle of *Lomelosia brachiata*. A and C/D represent "wing-like" coronas, B is "funnel-shaped," and E is "pappus-like." fr s.str. = fruit sensu stricto (= pericarp and seed with embryo), ca = calyx, ep.t = epicalyx tube. Bars: A, B 3 mm; D, 4 mm; F, 100 µm.

tend to have large coronas, and those with long, vertical epi-diaphragmata tend to have small coronas (Mayer & Ehrendorfer, 1999). The epicalyx and the calyx together form the diaspore, and different phenotypes are associated with the many dispersal syndromes found in the group (Ehrendorfer 1965a, b; Verlaque, 1986b; Caputo & al., 2004). Examples of dispersal modifications include the production of a lipid-rich elaiosome at the base of the epicalyx that attracts ants (in *Knautia*), and the formation of winged coronas and elongated spiny calyx bristles that may function in wind dispersal or attachment to animals. In some species, often with reduced or absent coronas, the number of calyx bristles increases to form a pappus-like structure that is associated with long-distance wind dispersal (Niklas, 1992). These observations suggested to previous workers that the epicalyx is a "key innovation" that spurred the adaptive radiation of Dipsacaceae in the Mediterranean Basin (Szabó, 1930; Ehrendorfer, 1964a, 1965a, b; Verlaque, 1977a, 1984a).

The phylogenetic position of Dipsacaceae within the Dipsacales has been studied extensively. Dipsacaceae is included in the herbaceous Valerina clade (Donoghue & al., 2001) of the Caprifoliaceae clade, along with *Triplostegia*, Valerianaceae, and Morinaceae. *Triplostegia* was traditionally part of Valerianaceae in the tribe Triplostegieae (Graebner, 1906; Weberling, 1970; Cronquist, 1988; Backlund, 1996; Brummitt, 1992). However, studies using morphological (Backlund, 1996; Judd & al., 1994; but see Caputo & Cozzolino, 1994), and molecular data (Donoghue & al., 1992; Downie & Palmer, 1992; Pyck &

al., 1999, 2002; Bell & al., 2001; Zhang & al., 2003; Bell, 2004; Bell & Donoghue, 2005) have mostly found *Triplostegia* to be more closely related to Dipsacaceae, with Dipsacaceae + *Triplostegia* sister to Valerianaceae, and this clade in turn sister to Morinaceae.

Taxonomic treatments of Dipsacaceae have differed widely, particularly with regard to the circumscription of tribes and genera. Most authors (Van Tieghem, 1909; Ehrendorfer, 1964a, b; Verlaque, 1977a, b, 1984a, b, 1985a, b, 1986a, b; Caputo & Cozzolino, 1994) have subdivided Dipsacaceae into three tribes: (1) Knautieae (*Knautia*), (2) Dipsaceae (*Dipsacus*, *Cephalaria*), and (3) Scabioseae (*Succisa*, *Succisella*, *Scabiosa* s.l., *Pterocephalus* s.l.). Major revisions to Scabioseae divided *Scabiosa* s.l. into six genera (Devesa, 1984a, b; Greuter & Burdet, 1985; López González, 1987): *Lomelosia* (*Scabiosa* sect. *Trochocephalus* Mert. & Koch), *Scabiosa* s.str. (*Scabiosa* sect. *Scabiosa* = *Scabiosa* L. s.str.), *Pseudoscabiosa* (*Scabiosa* sect. *Asterothrix* Font Quer), *Pterocephalidium* (Lag.) G. Lopez, *Pycnocomon* Hoffmanns. & Link, and *Sixalix* (*Scabiosa* sect. *Cyrtostemma* Mert. & Koch). Recently, Mayer & Ehrendorfer (1999) excluded *Pseudoscabiosa* from Scabioseae, and *Pterocephalus* s.l. was also revised to exclude all three Asian *Pterocephalus* species, which were placed in *Pterocephalodes* (Mayer & Ehrendorfer, 2000). Burtt (1999) had provided the name *Bassecoia* for two species (*B. siamensis*, *B. bretschneideri*) that had been placed in *Pterocephalodes* by Mayer & Ehrendorfer (2000); unfortunately, Burtt's paper was unavailable at the time *Pterocephalodes* was described. Currently, 14 genera are recognized (Devesa, 1984a, b; Greuter & Burdet, 1985; López González, 1987, Burtt, 1999; Mayer & Ehrendorfer, 1999, 2000; Table 1).

Relatively little phylogenetic work has focused on Dipsacaceae. Caputo & Cozzolino (1994) conducted the first phylogenetic study of Dipsacaceae, and their analysis, based on morphology, broadly agreed with traditional interpretations (Van Tieghem, 1909; Ehrendorfer, 1964a, b, 1965a, b; Verlaque, 1977a, b, 1984a, b, 1985 a, b, 1986 a, b). Caputo & al. (2004) inferred a molecular phylogeny of 17 taxa based on the internal transcribed spacer (ITS) and the chloroplast *trnL* intron, which contradicted the previous morphological study and supported the revisions to tribes and genera outlined above. However, limited taxon sampling precluded conclusions regarding the monophyly of the proposed genera. Caputo & al. (2004) found two major clades in Dipsacaceae: Scabioseae s.str. and a clade containing Dipsaceae, Knautieae, and the groups excluded from Scabioseae s.l. (*Pseudoscabiosa*, *Pterocephalidium*, *Succisa*, *Succisella*). The topology within Scabioseae s.str. consisted of: (*Pycnocomon*, *Lomelosia*) ((*Sixalix*, *Scabiosa*) *Pterocephalus*). In the other major clade, they found: (*Pseudoscabiosa* (*Succisa*, *Succisella*) ((*Cephalaria*, *Dipsacus*)(*Knautia*, *Pterocephalidium*))). *Pterocephalodes hookeri* and the two *Bassecoia* species were not included in the analysis of Caputo & al. (2004).

Table 1. Currently recognized genera of Dipsacaceae (arranged alphabetically within the tribes recognized by van Tieghem (1909) showing geographic distribution, number of accepted species, and number of species sampled in this study.

Tribe Genus	Distribution	Species total	Species sampled
Scabioseae			
Bassecoia	Himalayas, SW China, Thailand	2	2
Lomelosia	Circum-Mediterranean, especially E Mediterranean, Caucasus to C Asia	ca. 50	8
Pseudoscabiosa	Iberian Peninsula to Northern Morocco, Sicily	3	3
Pterocephalidium	Iberian Peninsula	1	1
Pterocephalodes	Himalayas, SW China	1	1
Pterocephalus	Irano-Oriental region, extending to E Mediterranean, Macaronesia, E Africa, C Asia	ca. 30	10
Pycnocomon	Iberian Peninsula, circum-Mediterranean	2	2
Scabiosa	E Asia, S and E Africa, circum-Mediterranean	ca. 30	6
Sixalix	Circum-Mediterranean, especially NW Africa	8	2
Succisa	Iberian Peninsula, circum-European, Cameroon	3	1
Succisella	Iberian Peninsula, E Europe	5	1
Dipsaceae			
Cephalaria	S and E Africa, circum-Mediterranean, Caucasus to C Asia	ca. 60	9
Dipsacus	E to C Asia, India, Sri Lanka, circum-European, E Africa, Cameroon	20	3
Knautieae			
Knautia	Circum-European, especially N Mediterranean, Caucasus to C Asia, NW Africa	40–60	8

The objective of the present study was to generate a phylogenetic hypothesis for Dipsacaceae using cpDNA and nrDNA sequences. In this paper, we expand sampling to include 57 species of Dipsacaceae and 3 outgroup species, representing all major subclades, which allows us to test the monophyly of the previously recognized tribes and genera. We have included the two *Bassecoia* species and *Pterocephalodes hookeri*, all from Asia, to clarify their position within Dipsacaceae. In addition, we have more thoroughly sampled the larger and more diverse groups (e.g., *Lomelosia*, *Pterocephalus*, *Cephalaria*). The inferred phylogeny provides a framework for taxonomic rearrangements and for a preliminary discussion of character evolution.

MATERIALS AND METHODS

Plant material. — Sequence data from 60 taxa (57 ingroup and 3 outgroup species), representing the major subclades and geographical regions, were collected from herbarium specimens, silica-preserved material, and GenBank (Appendix). *Scabiosa japonica* was not sequenced for *atpB-rbcL* and *trnS*^{UGA}-*trnG*^{GCG} but *trnL-trnF* and ITS sequences available in GenBank were included in the combined analyses. *Patrinia* (Valerianaceae), *Morina* (Morinaceae), and *Triplostegia* were used as outgroups based on previous molecular work (Pyck & al., 1999; Pyck & Smets, 2000; Bell & al., 2001; Donoghue & al., 2001, 2003).

DNA extraction, amplification, and sequencing. — Total genomic DNA was isolated using the Qiagen DNEasy methods (Qiagen, Valencia, California), or a modified version using beta-Mercaptoethanol and proteinase-K for herbarium specimens (Wurdack & al., 2004.) Four gene regions were amplified and sequenced using standard primers found in the literature (*trnL-trnF* region: Taberlet & al., 1991; *atpB-rbcL* region reverse primer: Manen & al., 1994; *trnS*^{UGA}-*trnG*^{GCC}: Shaw & al., 2005; ITS: White & al., 1990; Urbatsch & al., 2000) with the exception of *atpB-rbcL*, for which a new forward primer was designed (5′ TTTCAAGCGTGGAAACCCCAG 3′). All regions were amplified using standard PCR in 25-µL volume reactions (2 µL genomic DNA, 0.125 µL Qiagen *Taq* polymerase [Qiagen, Valenica, California], 0.5 µL 2.5 mmol dNTPs, 2.5 µL 10× buffer, 2.5 µL Q solution, 0.5 µL 10 mmol $MgCl_2$, 1.5 µL 10 mmol of each primer, 13.875 µL ddH_2O). PCR cycling times were as follows for the different gene regions: *trnL-trnF*: 98°C for 3 min, 35 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, final extension of 72°C for 10 min; *atpB-rbcL*: 94°C for 3 min, 35 cycles of 95°C for 1 min 30 s, 57°C for 2 min, 72°C for 3 min, final extension of 72°C for 10 min; *trnS*^{UGA}-*trnG*^{GCC}: 94°C for 2 min, 35 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 30 s, final extension of 72°C for 10 min; ITS: 94°C for 3 min, 39 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, final extension of 72°C for 10 min. PCR reactions were cleaned using polyethylene glycol (PEG) precipitation (20% PEG 8000 and 2.5 M NaCl) and directly sequenced. Sequences were generated using dye terminator cycle sequencing using ABI PRISM "Big Dye" Primer Cycle Sequencing Ready Reaction kits (Perkin-Elmer, Foster City, California), and visualized using an ABI3730 (Applied Biosystems DNA Analyzer).

Sequence alignment and phylogenetic analysis. — Contiguous sequences were assembled using Sequencher (Gene Codes Corp., Ann Arbor, Michigan). Aligned datasets were generated using Muscle (Edgar, 2004), and adjusted manually in MacClade version 4.06 (Maddison & Maddison, 2000). The aligned matrix is available in TreeBase (www.treebase.org) or on request from the first author.

The data were organized into three main partitions: ITS, chloroplast (cpDNA: *trnL-trnF*, *atpB-rbcL*, *trnS*^{UGA}-*trnG*^{GCC}), and combined ITS and cpDNA datasets. The partition homogeneity test (the ILD test of Farris & al., 1994), as implemented in PAUP*4.0b10 (Swofford, 2002), was used to identify conflict among the different markers. However, tests of incongruence have been shown to be unreliable under certain conditions (Yoder & al., 2001; Hipp & al., 2004), so all partitions were analyzed together regardless of the outcomes of the ILD. Datasets with nodes that were incongruent were also combined, as combining conflicting datasets can sometimes result in a more robust phylogeny (Wiens, 1998). In such cases, the ILD test was used on datasets with the conflicting taxa removed, and the analyses were re-run to ensure that other clades in the tree were not affected. In the combined analyses, nodes sharing conflict between datasets were regarded as questionable. Maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses were performed on all three datasets. MP analyses were conducted using PAUP*4.0b10, ML analyses were conducted using RAxML version 7.0.4 (Stamatakis, 2006; Stamatakis & al., 2008), and BI analyses used MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003).

MP searches were conducted using heuristic search methods with tree bisection reconnection (TBR) branch swapping, collapse of zero-length branches, gaps treated as missing characters, all characters weighted equally, and the MULTREES option in effect. The analyses were repeated 1,000 times with the random addition option. A strict consensus tree summarized sets of equally parsimonious trees. To assess confidence in clades, bootstrap (BS) tests were performed using 1,000 bootstrap replicates with 10 random addition replicates per bootstrap.

Models of molecular evolution were chosen for each marker using AIC (Akaike Information Criteria) scores in Modeltest version 3.7 (Posada & Crandall, 1998). The

cpDNA data were arranged into two separate modeling schemes: (1) all three markers and parameters linked; (2) all three markers unlinked. A similar partitioning strategy was employed for the combined dataset: (1) ITS and the three cpDNA markers linked; (2) ITS unlinked from the three cpDNA markers. In the analyses with unlinked markers, the mutation rate, gamma, and state frequencies were allowed to vary. To select among the different partitioning schemes, likelihood ratio tests (LRTs) were used for the ML analyses to test for statistically significant differences in model fit (Goldman, 1993; Huelsenbeck & Crandall, 1997). For the BI analyses, Bayes factors were used to compare the harmonic means of the marginal log likelihoods that were estimated for the candidate mixed models (Kass & Raftery, 1995).

For the ML analyses, tree searches were executed starting from a random stepwise-addition MP tree and employed the GTRGAMMA (general time-reversible with rate heterogeneity accommodated by a gamma distribution) nucleotide substitution model. ML analyses were run ten times per dataset, starting from ten different starting trees. All free model parameters were estimated with RAxML, with GAMMA model parameters estimated up to an accuracy of 0.1 log likelihood units. Nonparametric bootstrapping under ML was also carried out with RAxML using 1,000 bootstrap replicates. In cases where previously recognized genera were found not to be monophyletic, competing topological hypotheses were statistically contrasted using the Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa, 1999). RAxML bootstrap analyses were carried out using the CIPRES (Cyberinfrastructure for Phylogenetic Research) portal (http://www.phylo.org), while the SH test was computed on a locally compiled binary.

BI analyses were performed assuming default prior parameter distributions set in MrBayes version 3.1.2. Two simultaneous runs were initiated starting from random trees. To ensure that the two runs converged onto a stationary distribution, analyses were run until the average standard deviation of split frequencies was 0.01. Posterior probabilities of trees were approximated using the Metropolis-coupled Markov chain Monte Carlo (MC^3) algorithm with four incrementally heated chains ($T = 0.2$) for 5,000,000 generations and sampling trees every 500 generations. Convergence and sampling intensity were evaluated using the potential scale reduction factor (PSRF) and estimated sample size (ESS). To estimate burn-ins, posterior parameter distributions were viewed using Tracer version 1.4 (Rambaut & Drummond, 2007). The resources of the Computational Biology Service Unit at Cornell University were used in the analyses of the partitioned datasets.

RESULTS

Summary statistics for the MP, ML, and BI analyses of separate and combined datasets are summarized in Table 2. Our combined dataset includes 60 taxa (57 Dipsacaceae species and 3 outgroups). ITS was the most variable marker, with 34% of the characters being parsimony-informative. Of the chloroplast markers, *atpB-rbcL* was most variable (14%), followed by *trnL-trnF* (10%) and *trnS*^{UGA}-*trnG*^{GCC} (7%). The topologies of the trees generated for each partition were generally congruent, although support values were relatively low for the individual cpDNA markers (data not shown). However, while the ITS and cpDNA trees were largely congruent, there were some conflicting nodes (Fig. 2; discussed below), and the ILD test rejected the null hypothesis that the ITS and cpDNA data partitions were derived from the same data pool ($P = 0.01$). When conflicting taxa were removed from the datasets, the ILD test was repeated and still yielded significant incongruence between the datasets. However, removal of conflicting taxa did not affect the topology in other parts of the tree, suggesting that there is a strong congruent phylogenetic signal elsewhere. Combining the cpDNA and ITS gene regions into one dataset resulted in

Table 2. Statistics for the aligned data matrices.

	atpB-rbcL	*trnL-trnF*	*trnS*^{UGA}-*trnG*^{GCC}	ITS	cpDNA	Combined
No. of taxa	59	60	59	60	60	60
Aligned sequence length (nt)	1,024	888	808	631	2,720	3,351
No. of variable sites	292	172	129	325	593	918
No. of informative sites	147	86	56	212	289	501
Length of MP trees	404	221	165	1,048	797	1,862
CI (all characters)	0.83	0.86	0.84	0.48	0.83	0.65
CI (excluding constant sites)	0.71	0.75	0.70	0.45	0.71	0.54
RI	0.92	0.93	0.91	0.72	0.92	0.80
Model of sequence evolution	TVM+G	K81uf+G	TVM+G	GTR+G	GTR+G	GTR+G

CI, consistency index; RI, retention index.

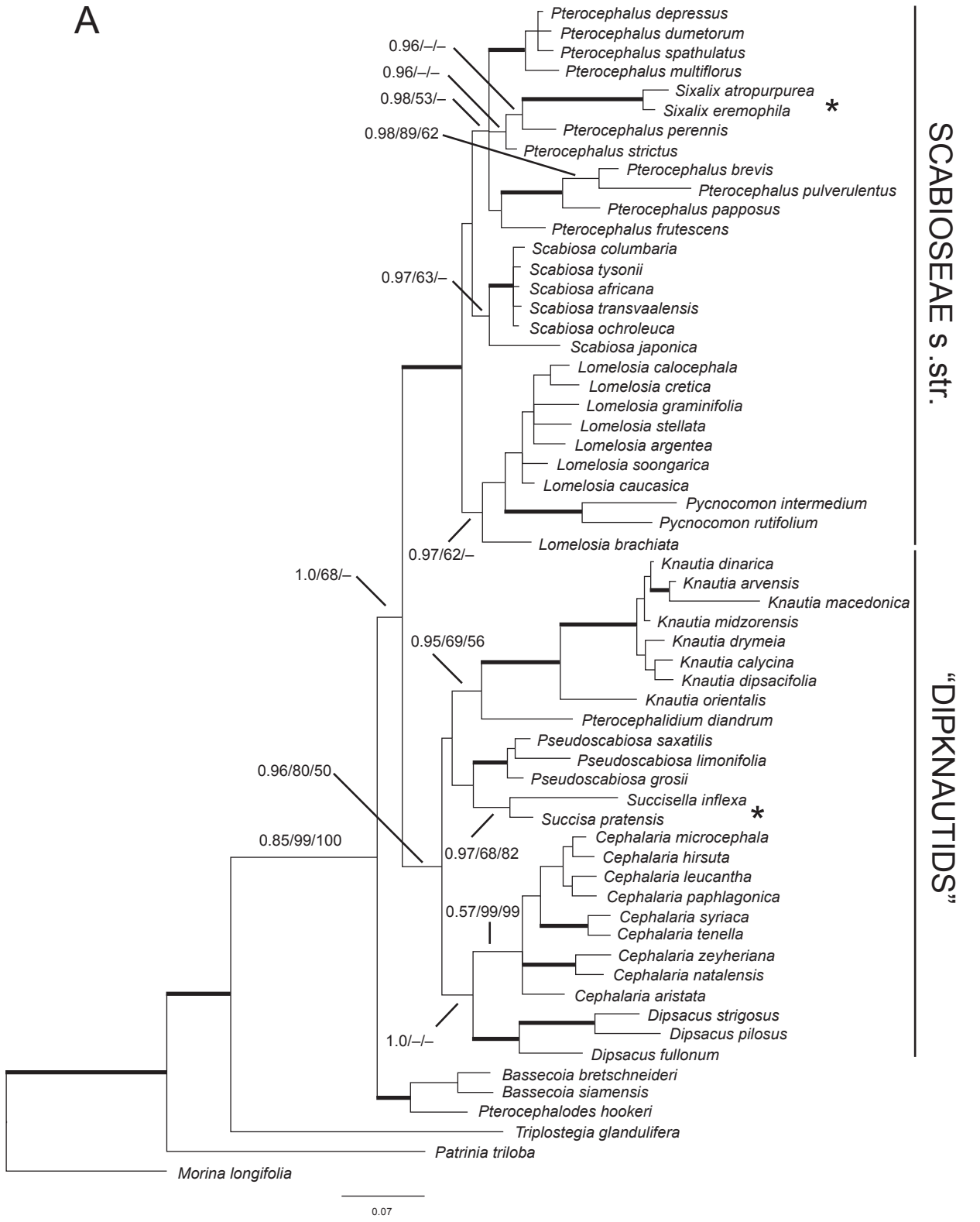
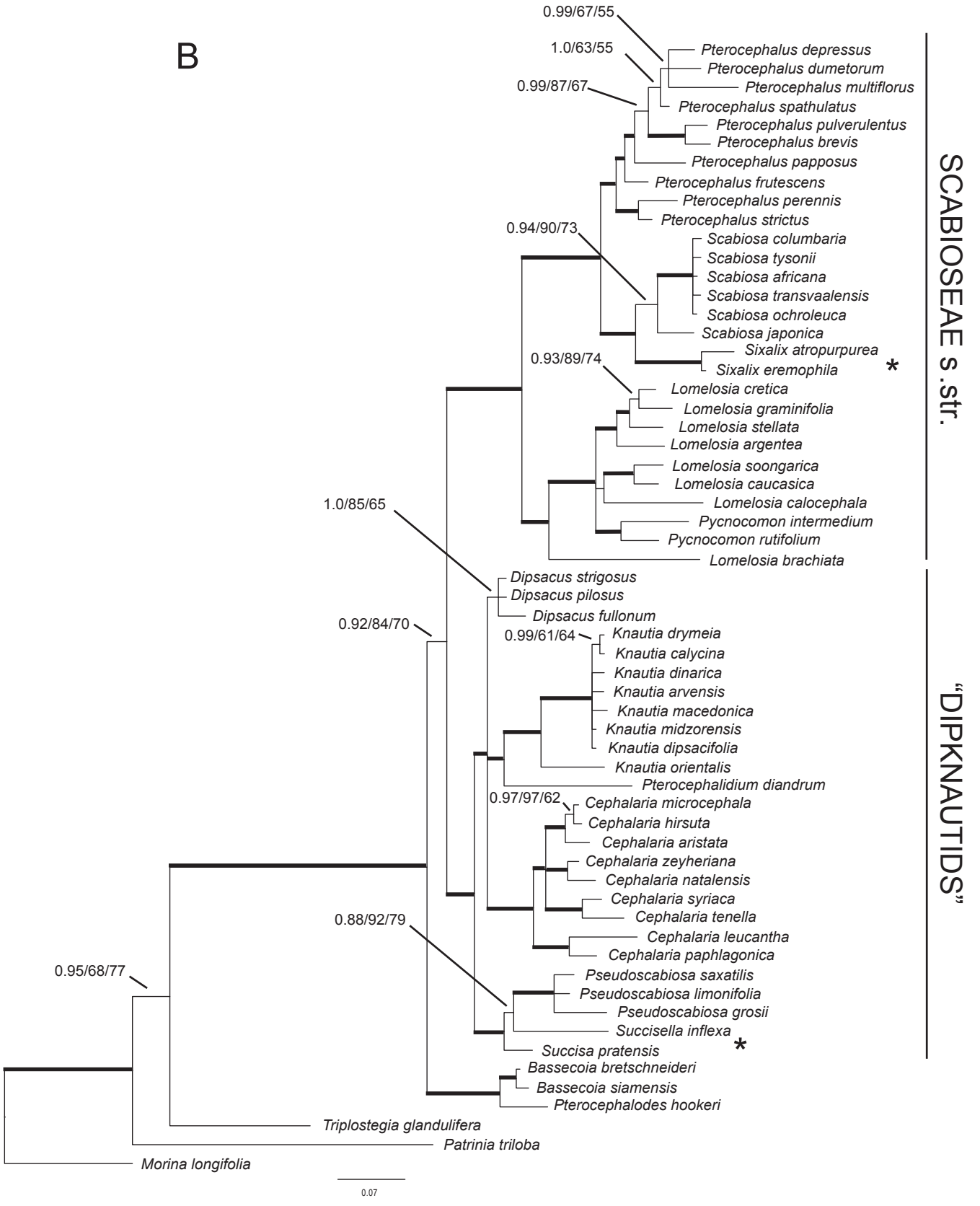


Fig. 2. Bayesian trees of (A) ITS and (B) unpartitioned cpDNA datasets. Thickened branches represent nodes with high bootstrap (BS) and posterior probability (PP) support: maximum likelihood (ML) and maximum parsimony (MP) BS ≥ 70%, PP ≥ 0.95). Support values for major clades that are supported in either the Bayesian, ML, or MP analyses are indicated (PP/ML-BS/MP-BS; "–" indicates BS ≤ 50%). Nodes with no support values indicated have PP ≤ 0.95 and ML-BS and MP-BS ≤ 50%. Incongruent taxa between the two trees are marked with an asterix.


B
0.99/67/55
1.0/63/55
0.99/87/67
Pterocephalus depressus
Pterocephalus dumetorum
Pterocephalus multiflorus
Pterocephalus spathulatus
Pterocephalus pulverulentus
Pterocephalus brevis
Pterocephalus papposus
Pterocephalus frutescens
Pterocephalus perennis
Pterocephalus strictus
0.94/90/73
Scabiosa columbaria
Scabiosa tysonii
Scabiosa africana
Scabiosa transvaalensis
Scabiosa ochroleuca
Scabiosa japonica
Sixalix atropurpurea
Sixalix eremophila
*
0.93/89/74
Lomelosia cretica
Lomelosia graminifolia
Lomelosia stellata
Lomelosia argentea
Lomelosia soongarica
Lomelosia caucasica
Lomelosia calocephala
Pycnocomon intermedium
Pycnocomon rutifolium
Lomelosia brachiata
1.0/85/65
Dipsacus strigosus
Dipsacus pilosus
Dipsacus fullonum
0.92/84/70
0.99/61/64
Knautia drymeia
Knautia calycina
Knautia dinarica
Knautia arvensis
Knautia macedonica
Knautia midzorensis
Knautia dipsacifolia
Knautia orientalis
Pterocephalidium diandrum
0.97/97/62
Cephalaria microcephala
Cephalaria hirsuta
Cephalaria aristata
Cephalaria zeyheriana
Cephalaria natalensis
Cephalaria syriaca
Cephalaria tenella
Cephalaria leucantha
Cephalaria paphlagonica
0.88/92/79
0.95/68/77
Pseudoscabiosa saxatilis
Pseudoscabiosa limonifolia
Pseudoscabiosa grosii
Succisella inflexa
*
Succisa pratensis
Bassecoia bretschneideri
Bassecoia siamensis
Pterocephalodes hookeri
Triplostegia glandulifera
Patrinia triloba
Morina longifolia
0.07
SCABIOSEAE s .str.
"DIPKNAUTIDS"

a well-supported hypothesis of Dipsacaceae phylogeny (Fig. 3); however, areas that showed conflict between the ITS and cpDNA analyses are regarded as questionable.

Phylogenetic data and analyses. — The ITS data matrix consisted of 631 aligned nucleotides, of which 325 were variable and 212 were parsimony informative. The 1,000 MP heuristic searches resulted in a tree length of 1,048 and consistency index (CI) = 0.48 (CI excluding invariant characters = 0.45). MP analysis of the combined cpDNA data matrix (*atpB-rbcL* + *trnL-trnF* + *trnS*^{UGA}-*trnG*^{GCC}) consisted of 2,720 aligned nucleotides, containing 593 variable nucleotides, of which 289 were parsimony informative. The length of the trees was 797 with CI = 0.83 (CI excluding invariant characters = 0.71). The combined ITS and cpDNA data matrix consisted of 3,351 aligned nucleotides, of which 918 were variable and 501 parsimony informative. The heuristic searches resulted in trees with length 1,862 and CI = 0.65 (CI excluding invariant characters = 0.54).

In the ML analyses, the ten independent RAxML runs converged to a similar likelihood score for each dataset (differing up to 0.001). The partitioned datasets significantly fit the data better as determined by LRTs (ln L = –9,914 for the unpartitioned cpDNA dataset, –9,160 for the partitioned cpDNA dataset; ln L = –15,423 for the unpartitioned combined dataset, and –15,672 for the partitioned combined dataset). However, all analyses resulted in the same topology.

Posterior probability distributions of 10,000 sampled trees were obtained for the BI analysis, and samples drawn from the first 1,000 generations (10%) were discarded as burn-in. The average standard deviation of split frequencies approached 0.01 in all analyses, indicating that each MC^3 chain converged to the target distribution. PSRF estimates for all parameters approached 1.0 and ESS values were greater than 10^3, indicating that sampling was adequate. Majority rule consensus trees were generated from the combined post–burn-in samples from each run. The marginal log likelihood values increased with decreasing model complexity. The cpDNA dataset with all markers linked had a marginal log likelihood value of –9,302.79, while the dataset with the three cpDNA markers unlinked was –9,544.34. Similarly, for the BI analyses of the combined data, the dataset with ITS and the three cpDNA markers linked had a marginal log likelihood score of –15,528.23 and the dataset with ITS and the cpDNA markers partitioned was –15,805.42. Evaluation of the different modeling schemes with Bayes factors indicated that the cpDNA and combined datasets with all markers linked were the most robust estimates of Dipsacaceae phylogeny (data not shown). However, all partitioning schemes recovered identical topologies.

Major relationships within Dipsacaceae. — Consistent with previous studies, *Triplostegia* is resolved as sister to Dipsacaceae in all analyses when the tree is rooted along the *Morina* branch (although this relationship is not supported in the BI analysis of the ITS dataset). The analyses revealed three main clades: (1) *Pterocephalodes hookeri* and the two *Bassecoia* species; (2) the tribes Dipsaceae (*Dipsacus*, *Cephalaria*) and Knautieae (*Knautia*), plus additional genera (*Pterocephalidium*, *Pseudoscabiosa*, *Succisa*, *Succisella*), hereafter referred to as the "Dipknautids"; and (3) Scabioseae s.str., consisting of *Pterocephalus* s.str., *Sixalix*, *Scabiosa*, *Lomelosia*, and *Pycnocomon*. The clade consisting of the two *Bassecoia* species and *Pterocephalodes hookeri* was consistently resolved as sister to the rest of the Dipsacaceae. For the purpose of this study, we refrain from providing formal phylogenetic definitions for taxonomic names until we have achieved a better understanding of species level relationships.

Relationships within the "Dipknautids". — In the "Dipknautid" clade, there are two main subclades: (1) a relatively small group containing *Pseudoscabiosa*, *Succisa*, and *Succisella*; and (2) *Dipsacus*, *Cephalaria*, *Knautia*, and the monotypic *Pterocephalidium*. The placement of the former subclade within the "Dipknautids" is unresolved in the ITS tree but supported as sister to the latter clade in the cpDNA tree and the BI analysis of the combined dataset. *Pseudoscabiosa*, a group of three Western Mediterranean endemics with disjunct "relict" distributions, is supported as monophyletic in all analyses. *Succisa* and *Succisella*, also small groups of three and five species, respectively, are sister taxa in the ITS and combined analyses, but in the cpDNA analysis, *Succisella* is sister to *Pseudoscabiosa* (although supported only in the ML and MP analyses).

The monophyly of the Dipsaceae (*Dipsacus*, *Cephalaria*) is supported in the BI analyses of the ITS tree and the BI and ML analyses of the combined dataset, but is unresolved in the cpDNA tree. *Dipsacus* and *Cephalaria* are each monophyletic in all three analyses (although *Cephalaria* is not supported in the BI analyses of the ITS dataset and *Dipsacus* is not supported in the MP analysis of the cpDNA dataset). Within *Dipsacus*, *D. strigosus* and *D. pilosus* (both in *D.* sect. *Sphaerodipsacus*) are sister taxa in the ITS and combined datasets and subtended by *D. fullonum* (*D.* sect. *Dipsacus*). Within *Cephalaria*, the following clades are supported: *C. natalensis* + *C. zeyheriana*, *C. tenella* + *C. syriaca*, *C. leucantha* + *C. paphlagonica*, and *C. hirsuta* + *C. microcephala* (although the latter two clades are not supported in the ITS tree and *C. hirsuta* + *C. microcephala* is not supported by the MP analysis of the cpDNA dataset). In the cpDNA analysis, there is support for *C. leucantha* + *C. paphlagonica* as sister to the rest of *Cephalaria* and for *C. aristata* as being sister to *C. microcephala* + *C. hirsuta*.

Consistent with previous molecular work, *Pterocephalidium diandrum*, an aberrant Spanish endemic

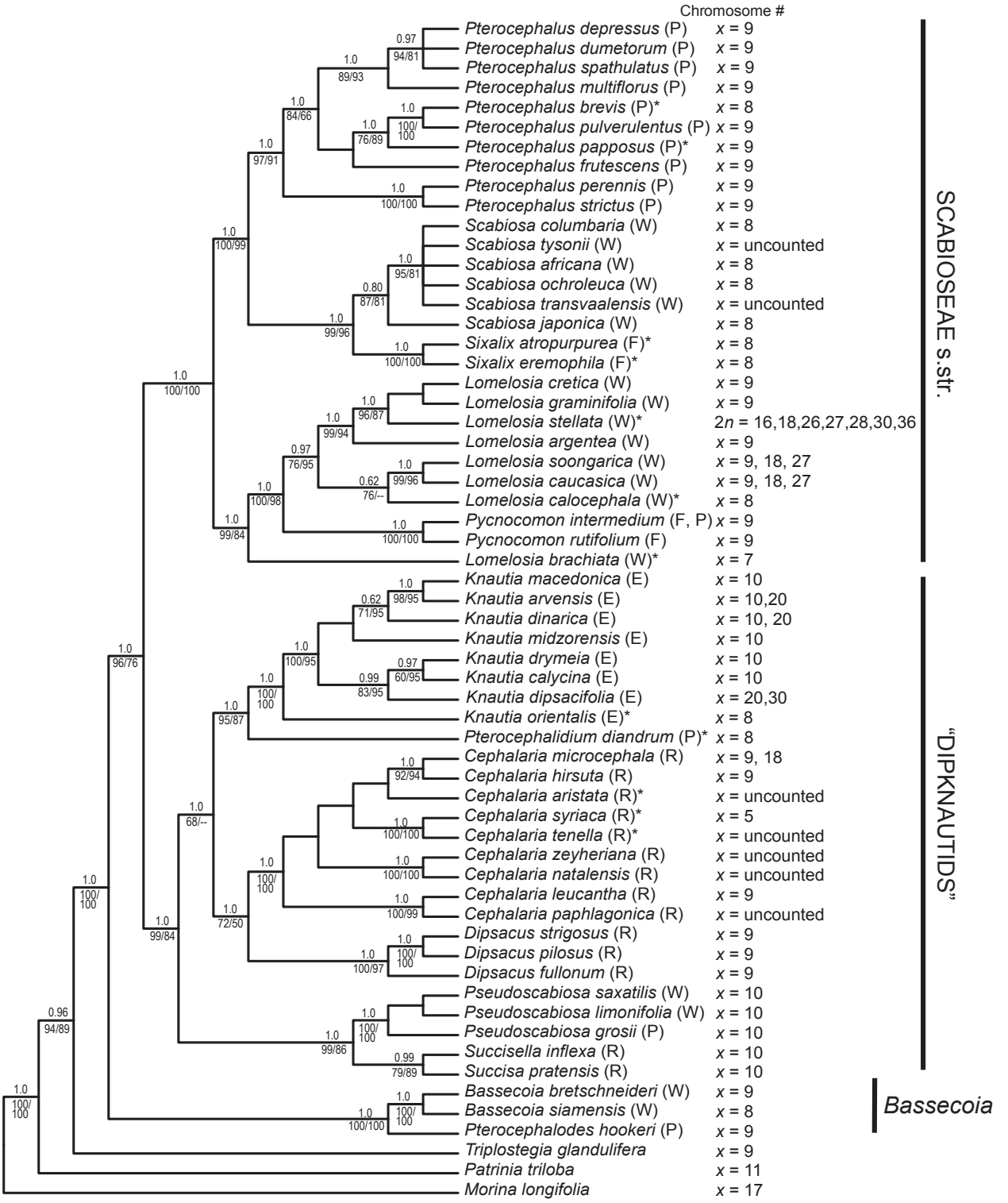


Fig. 3. Bayesian tree of the combined unpartitioned dataset. Numbers above branches represent Bayesian posterior probabilities (PP), and numbers below represent maximum likelihood (left) and maximum parsimony (right) bootstrap (BS) values ("–" indicates BS ≤ 50%). Unmarked nodes have PP ≤ 0.95, and ML-BS and MP-BS ≤ 50%. Chromosome number (based on Verlaque 1985b, 1986a, b), life history (annuals marked with an asterix, all others perennial), and diaspore structure [pappus (P) = calyx bristles multiple and plumose, corona reduced; wing (W) = expanded corona, calyx bristles not plumose; funnel (F) = elongated epi-diaphragma, reduced corona; elaiosome (E); reduced (R) = epicalyx and calyx reduced] are indicated.

originally placed in *Pterocephalus*, is sister to *Knautia*. In the ITS and combined analyses, *K. arvensis* and *K. macedonica* are sister taxa, and in the combined tree, *K. dinarica* subtends this clade in the MP and ML analyses. In the combined analysis, *K. drymeja* and *K. calycina* are sister taxa in the MP and BI analyses, and *K. dipsacifolia* is in turn sister to this clade. *K. orientalis*, an annual species and the only representative of *K.* subg. *Knautia* (Verlaque, 1985b), is supported as sister to the rest of *Knautia*.

Relationships within Scabioseae s.str. — The cpDNA and combined analyses reveal two major clades in Scabioseae s.str.: (1) *Pterocephalus*, *Sixalix*, and *Scabiosa*; and (2) *Lomelosia* and *Pycnocomon*. Relationships in Scabioseae s.str. are not resolved with ITS alone. The placement of *Sixalix* is the major area of conflict between the ITS and cpDNA analyses of the Scabioseae s.str. In the ITS tree, both *Sixalix* species are supported in the BI analysis as being nested within *Pterocephalus* in a clade with *P. perennis* and *P. strictus*, with *P. perennis* appearing as sister to *Sixalix* (this relationship is not supported in the ML of MP analyses). In the cpDNA tree, however, *Sixalix* is sister to *Scabiosa*. In the combined analyses, *Sixalix* forms a clade with *Scabiosa* as in the cpDNA tree.

In *Pterocephalus*, four species with similar epicalyx morphology form a clade in the combined tree: (*P. multiflorus* (*P. spathulatus*, *P. depressus*, *P. dumetorum*)). *Pterocephalus perennis* and *P. strictus* form a clade, which is resolved as sister to the rest of *Pterocephalus* in the cpDNA tree and in the BI and ML analyses of the combined dataset. *Pterocephalus pulverulentus* is sister to *P. brevis*, and this clade is subtended by *P. papposus* in the ITS and combined trees. In the BI analysis of the ITS dataset, the cpDNA tree, and the MP and ML analyses of the combined dataset, *Scabiosa* is monophyletic. Support for species-level relationships in *Scabiosa* is poor, although *S. japonica*, a member of the East Asian *S.* sect. *Prismakena* (Bobrov, 1957), is resolved as sister to the rest of *Scabiosa* in all analyses.

In the other major clade of Scabioseae s.str., *Pycnocomon* is monophyletic and nested within *Lomelosia* (although this relationship is not supported in the MP and ML analyses of the ITS dataset). This finding differs from the study of Caputo & al. (2004) that placed *Pycnocomon* sister to *Lomelosia*. *Lomelosia brachiata* (previously *Tremastelma palaestinum* (L.) Janchen), an Eastern Mediterranean annual, is sister to the remaining species in this clade in the cpDNA and combined trees. In the combined tree, *Pycnocomon* is sister to the remaining *Lomelosia* species except *L. brachiata*. The results of the SH test indicated that when all possible alternative phylogenetic hypotheses for the placement of *Pycnocomon* within Scabioseae s.str. are tested, no topology is significantly better than when *Pycnocomon* is nested within *Lomelosia*. Within *Lomelosia*, the cpDNA and combined analyses support a clade consisting of the widespread *L. argentea* sister to (*L. stellata*, *L. cretica*, *L. graminifolia*). The Eurasian *L. soongarica* and *L. caucasica*, both placed in the "Caucasica" species group by Verlaque (1986b), are also supported as monophyletic in the cpDNA and combined trees.

DISCUSSION

Conflict between cpDNA and nrDNA analyses. — The overall topologies of the ITS, cpDNA, and combined trees are similar. However, this study also reveals some areas of conflict between the cpDNA and ITS datasets. The placement of *Succisella inflexa* within the clade that contains *Pseudoscabiosa* and *Succisa* differs between the two datasets. The conflict between the position of *Sixalix* as either sister to *Scabiosa* or nested within *Pterocephalus* is also striking. Hybridization is thought to be common within some groups of Dipsacaceae (e.g., *Knautia* and *Scabiosa*) and even to have occurred between members of different genera (*Succisoknautia szaboi* Baksay; Baksay, 1952). It is therefore conceivable that the *Sixalix* lineage evolved via hybridization between a species of *Scabiosa* and a species of *Pterocephalus*. Phylogenetic analysis with another nrDNA marker will be needed to address this possibility.

Relationships among the major clades of Dipsacaceae. — The analysis reported here includes the largest sample of Dipsacaceae to date and the combined evidence supports the following phylogenetic conclusions: (1) the small Asian clade that contains *Bassecoia* + *Pterocephalodes hookeri* is sister to a clade consisting of the "Dipknautids" and Scabioseae s.str.; (2) within the "Dipknautids", *Pseudoscabiosa* is monophyletic and forms a clade with *Succisa* and *Succisella* that is sister to the remaining groups; (3) *Cephalaria* and *Dipsacus* are each monophyletic and together form a clade; (4) *Pterocephalidium* and *Knautia* form a clade; (5) within Scabioseae s.str., *Pycnocomon* is monophyletic and nested within *Lomelosia*; and (6) *Pterocephalus*, *Sixalix*, and *Scabiosa* form a clade that is sister to *Lomelosia* + *Pycnocomon*.

Early researchers (Van Tieghem, 1909; Ehrendorfer, 1964a, b; Verlaque, 1977a, b, 1984a, b, 1985a, b, 1986a, b) divided Dipsacaceae into three tribes (Knautieae, Dipsaceae, Scabioseae). Knautieae was distinguished from Dipsaceae and Scabioseae based on the presence of four ribs in the tube of the epicalyx and by an elaiosome. Dipsaceae and Scabioseae have eight-ribbed epicalyces and no elaiosome. Scabioseae was differentiated from Dipsaceae and Knautieae based on a persistent calyx (often modified into bristles) that is elevated on a stalk, while the calyx in Dipsaceae and Knautieae is sessile, deciduous, and cup-shaped. Palynological differences were also thought to differentiate the three tribes, with Knautieae having

triporate pollen with simple apertures and grains that are nearly equal in polar and equatorial dimensions (P/E ≈ 1), Dipsaceae characterized by tricolpate pollen with complex apertures and P ≈ E, and the pollen of Scabioseae being either tricolpate or triporate with simple apertures, and a high P/E ratio (Verlaque, 1984a).

Similar to previous molecular and anatomical studies, our analyses suggest that the major clades in Dipsacaceae do not correspond to the traditionally recognized tribes. *Bassecoia siamensis*, *B. bretschneideri*, and *Pterocephalodes hookeri* were previously placed in *Pterocephalus* based on a persistent calyx modified into 12–24 bristles. Burtt (1999) separated *P. siamensis* and *P. bretschneideri* as the genus *Bassecoia*, but left *P. hookeri* in *Pterocephalus* because it lacks a corona and has plumose calyx bristles, which are characteristic of *Pterocephalus*. Mayer & Ehrendorfer (2000) excluded all three species from *Pterocephalus* because they have floral bracts, pits in the epicalyx, and different epicalyx and pollen morphology. Our study confirms the relationship between *B. siamensis*, *B. bretschneideri*, and *P. hookeri*, and resolves this clade as sister to the remaining Dipsacaceae. This has interesting implications for character evolution. All three species in this clade have traits that were considered derived in Dipsacaceae (Verlaque, 1986a; Caputo & Cozzolino, 1994)—plumose calyx bristles in *P. hookeri* and coronas in *B. siamensis* and *B. bretschneideri*. Our study suggests that the evolution of these traits, presumably in response to selection for wind dispersal, occurred separately in the *Bassecoia* + *P. hookeri* lineage and again elsewhere in the core Dipsacaceae.

The monotypic *Pterocephalidium diandrum* was also previously placed in *Pterocephalus* based on multiple and persistent calyx bristles; however, it shares no other features with *Pterocephalus*. Verlaque (1986a) and Greuter & Burdet (1985) thought that *P. diandrum* was a derived member of Scabioseae and closely aligned with *Pseudoscabiosa* based on the presence of cavities at the pollen poles (a very rare feature in angiosperms; Skvarla & Larson, 1965; Clarke & Jones, 1981). However, *P. diandrum* forms a clade with *Knautia* in our analyses and also in the study of Caputo & al. (2004). *Knautia* was recovered as monophyletic in the ITS and combined analyses, so in a sense "Knautieae" is supported by our analysis, but because of the placement of *P. diandrum*, "Knautieae" should perhaps be revised to include this species. *Pterocephalidium diandrum*, unlike all species of *Knautia*, has an eight-ribbed epicalyx and no elaiosome, and is distinguished from all other Dipsacaceae by an elongated seta (an awn-like appendage) that protrudes from one side of the epicalyx and is of unknown function (perhaps epizoochory). Synapomorphies that unite these two seemingly disparate groups are not immediately obvious; however, *Knautia* and *P. diandrum* share triporate pollen (all other "Dipknautids" have tricolpate pollen), and *P. diandrum* is similar to *K. orientalis* (which is resolved as sister to the remaining *Knautia*) in that they are both annuals with a chromosome number of $x = 8$. *Dipsacus* and *Cephalaria* form a clade in the ITS and combined trees, so there is molecular support for the proposed clade Dipsaceae. Lastly, our study supports the revised circumscription of Scabioseae s.str. proposed by Mayer & Ehrendorfer (1999) and confirmed by Caputo & al. (2004) to include only *Pterocephalus* s.str., *Sixalix*, *Scabiosa*, *Lomelosia*, and *Pycnocomon*.

Based on the current phylogenetic evidence, the major clades of Dipsacaceae, and the characters traditionally used to distinguish them, should be re-evaluated. While there are not yet obvious diagnostic characters for the three major clades found in this study, there are several morphological trends. *Bassecoia* + *Pterocephalodes hookeri* and the "Dipknautids" tend to have mostly spherical capitula composed of 4-merous, ± actinomorphic corollas (*Knautia* is an exception, with 4-merous but zygomorphic flowers), while members of Scabioseae tend to have 5-merous and zygomorphic corollas (however, some species of *Scabiosa* and *Pterocephalus* can also have 4-merous corollas). While some members of both the "Dipknautids" and Scabioseae have diaphragmata (they are absent in *Bassecoia* + *P. hookeri*), they are lignified and thick in the "Dipknautids", and thin and hyaline in the Scabioseae s.str. Pollen and calyx morphology are characters that do not appear to have strong phylogenetic/taxonomic utility.

While the implications of the phylogenetic relationships outlined in this study should be considered at this stage, we would like to see our results confirmed with additional taxon sampling before embarking on a new nomenclature for Dipsacaceae. However, *Pterocephalodes hookeri* and the two *Bassecoia* species have been considered part of the same evolutionary lineage, and our study lends molecular phylogenetic evidence to support this clade. Therefore, we propose that *Pterocephalodes hookeri* be converted to *Bassecoia hookeri*, and we use *Bassecoia*, henceforth, for this Asian clade.

Relationships in the "Dipknautid" clade. — The "Dipknautid" clade contains between 120–150 species, depending on the circumscription of *Knautia*. Most species in this group are perennial, however *Knautia* and *Cephalaria* also contain annual species and *Dipsacus* species are often biennial. There have been several hypotheses regarding relationships in this clade. "Knautieae" has been placed as either sister to the rest of Dipsacaceae (Verlaque, 1984a), sister to Scabioseae s.l. (Caputo & Cozzolino, 1994), or aligned with *Succisa* at the base of Dipsacaceae (Ehrendorfer, 1964a, b, 1965a, b). Our study and that of Caputo & al. (2004) suggest that "Knautieae" is more closely related to *Dipsacus* and *Cephalaria*.

The small clade containing *Pseudoscabiosa*, *Succisella*, and *Succisa* was resolved as sister to the remainder

of the "Dipknautids" in our study (although its position is unresolved in the ITS tree and not supported by the MP analysis of the combined dataset) and in the analyses of Caputo & al. (2004). All species in this clade are perennial with $x = 10$ chromosomes. Our study included all three species of *Pseudoscabiosa* and provides molecular evidence for the monophyly of this group. *Pseudoscabiosa* is characterized by branched star-shaped hairs on the stem and leaves, and tricolpate pollen with cavae (Mayer & Ehrendorfer, 1999). Interestingly, trends in epicalyx morphology follow the same pattern in *Pseudoscabiosa* as they do in the *Bassecoia* clade. Two species in *Pseudoscabiosa* have coronas (*P. limonifolia*, *P. saxatilis*), while the third species (*P. grosii*) lacks a corona and has plumose calyx bristles. The resolution of this clade as potentially sister to the rest of the "Dipknautids" provides further evidence that these structures evolved more than once, even within small clades.

Morphological and molecular evidence supports the close relationship of *Dipsacus* and *Cephalaria*. Characters that unite the two groups include a deciduous, cup-shaped calyx and a chromosome number of $x = 9$ (with some exceptions in *Cephalaria*; e.g., *C. syriaca*). The length of the floral bracts is used to distinguish between the two groups, with *Cephalaria* having longer and tougher bracts than *Dipsacus*. Prickles on the stems and leaves also distinguish many *Dipsacus* species from *Cephalaria*. *Cephalaria* is the larger of the two clades, with ca. 60 species (*Dipsacus* has ca. 20 species) that Szabó (1940) divided into four subgenera—*Lobatocarpus*, *Fimbriatocarpus*, *Phalacrocarpus*, and *Cephalaria* ("Denticarpus" Szabó)—that are distinguished by the number of epicalyx grooves and differences in the reduced corona. *Cephalaria* subg. *Lobatocarpus* (ca. 14 spp.) is composed of South African perennials and thought to be ancestral in *Cephalaria* and most closely related to species assigned to *C.* subg. *Fimbriatocarpus* (3 spp.; Verlaque, 1985b). *Cephalaria* subg. *Phalacrocarpus* contains four annuals and is associated with *C.* subg. *Cephalaria*, the largest subgenus with 43 species divided into seven sections (five for the perennial species and two for the annuals). Several small clades are supported in our study. *Cephalaria natalensis* and *C. zeyheriana*, both of *C.* subg. *Lobatocarpus*, are monophyletic. *Cephalaria tenella* and *C. syriaca*, two Middle Eastern annuals in *C.* subg. *Cephalaria* sect. *Echinocephalae* are also monophyletic. *Cephalaria microcephala* and *C. hirsuta* are both in *C.* subg. *Cephalaria* sect. *Atrocephalae* and form a clade in the cpDNA and combined trees. Two species assigned to different subgenera also form a clade in the cpDNA and combined trees: *C. leucantha* (*C.* subg. *Fimbriatocarpus*) and *C. paphlagonica* (*Cephalaria* sect. *Leucocephalae*).

Knautia (ca. 40–60 spp.) is the most difficult group in Dipsacaceae to resolve taxonomically. Hybridization and polyploidy are frequent and often result in intermediate populations that obscure taxonomic boundaries (Ehrendorfer, 1963, 1965a, b). The number of species in *Knautia* has ranged widely as a result (Szabó, 1905, 1911; Ehrendorfer, 1963; Verlaque, 1985b). *Knautia* is divided into three subgenera: *Trichera*, *Knautia*, and *Tricheranthes* (Verlaque, 1985). All but four *Knautia* species are perennial and assigned to *K.* subg. *Trichera*. *Knautia* subg. *Knautia* and subg. *Tricheranthes* contain few species (one and three, respectively; Verlaque, 1985b) and differ from species assigned to *K.* subg. *Trichera* in being annuals and having fewer flowers in the capitula that are zygomorphic. *Knautia orientalis* (*K.* subg. *Knautia*), in particular, has very reduced capitula with only 5–10 flowers. *Knautia orientalis* is also unusual in that it has $x = 8$ chromosomes while most other *Knautia* species are $x = 10$. Because of these unique features, Verlaque (1985b) and Ehrendorfer (1963, 1965a, b) considered *K.* subg. *Trichera* to be ancestral within *Knautia*, and *K.* subg. *Knautia* to be the most derived lineage. The *Knautia* species sampled in our study are all members of *K.* subg. *Trichera* except for *K. orientalis*. Because *K. orientalis* is resolved as sister to the rest of *Knautia*, our results suggest that this species may not be derived. Additionally, *Pterocephalidium diandrum*, another annual with $x = 8$ chromosomes, is resolved as sister to *Knautia*.

Relationships in Scabioseae s.str. — Scabioseae s.str. contains ca. 120 species, and most of the diversity in epicalyx morphology occurs in this clade where different adaptations for wind dispersal are thought to be driving evolution. Most species in Scabioseae s.str. have additional epicalyx structures (diaphragmata, epi-diaphragmata, and coronas), and tend to modify the apical part of the diaspore in one of three ways: (1) *Lomelosia* and *Scabiosa* have "wing-like" coronas (the size and shape of the corona is especially diverse in *Lomelosia*); (2) *Pycnocomon* and *Sixalix* have a "funnel-shaped" epi-diaphragma that is similar in shape to the corona (it was considered a "lignified corona" by some researchers; Verlaque, 1986a; Caputo & Cozzolino, 1994), although it is not membranous and the corona is reduced; and (3) the corona in *Pterocephalus* is less prominent or absent, and instead the calyx bristles are multiplied and plumose, giving rise to a "pappus-like" structure. The character that distinguishes the two major clades of Scabioseae s.str. is a second ring of sclerenchyma in the tube of the epicalyx that is found only in *Lomelosia* and *Pycnocomon* (Mayer & Ehrendorfer, 1999). The extra sclerenchyma may contribute to the successful establishment of these plants in arid and semi-arid habitats (Mayer, 1995).

The position of *Pterocephalus* within Scabioseae has been inconsistent in the literature—it was either placed as sister to the rest of Scabioseae (Caputo & Cozzolino, 1994; Mayer & Ehrendorfer, 1999) or aligned with *Scabiosa*

(Verlaque, 1984a; Mayer & Ehrendorfer, 2000). Our study and that of Caputo & al. (2004) place *Pterocephalus* in a clade with *Scabiosa* and *Sixalix*. The relative position of *Sixalix* within Scabioseae has also been controversial. Originally, *Pycnocomon* and *Sixalix* were thought to be sister groups because they both contain heterocarpic species and have a "funnel-shaped" epicalyx (Jasiewicz, 1976; Devesa, 1984a; Verlaque, 1986a; Caputo & Cozzolino, 1994). However, Mayer & Ehrendorfer (1999), based on morphological data, and Caputo & al. (2004), based on molecular data, placed *Sixalix* in a clade with *Scabiosa*. Our study cannot confirm this result because of the incongruence between the ITS and cpDNA trees regarding the placement of *Sixalix*.

Pterocephalus is composed of ca. 30 species that are mostly perennial and woody, with very diverse growth forms. The most striking feature of *Pterocephalus* is the pappus-like calyx; however, this character is also found in other groups (e.g., *Bassecoia*, *Pterocephalidium*, *Pycnocomon intermedium*). Unlike other members of Scabioseae s.str., all species currently assigned to *Pterocephalus* lack floral bracts. Our finding in the ITS analysis that *Sixalix* may be nested within *Pterocephalus* suggests a complicated history for this group. While most *Pterocephalus* species are perennial and have $x = 9$ chromosomes, some species are annual (*P. brevis*, *P. papposus*) with $x = 8$ (*P. brevis*), characters that are shared by representatives of *Sixalix*. Also, some *Pterocephalus* species are similar to *Sixalix* in that they have an elongated epi-diaphragma (although not funnel-shaped). If *Sixalix* is nested within *Pterocephalus*, this has implications for the lack of floral bracts as a synapomorphy for *Pterocephalus* and implies that floral bracts were either retained from the ancestor of *Pterocephalus* or re-gained in the lineage leading to *Sixalix*. On the other hand, several synapomorphies support the relationship between *Sixalix* and *Scabiosa*. *Scabiosa* also has a chromosome number of $x = 8$, and *Scabiosa* and *Sixalix* share similar karyotypes, tricolpate pollen, and epicalyx anatomy (Mayer & Ehrendorfer, 1999).

Mayer & Ehrendorfer (1999) proposed a phylogeny for *Pterocephalus* based on different epicalyx "types," with a trend toward increasing complexity of the epicalyx. This phylogenetic hypothesis was based on the observation that some species of *Pterocephalus* completely lack diaphragmata, epi-diaphragmata, and coronas, other species have some structures but not others (e.g., a diaphragma, but not an epi-diaphragma or corona, etc.) and some species have all three structures. Mayer & Ehrendorfer (1999) considered species with all three epicalyx structures to be the most derived in *Pterocephalus*. The results of our study suggest an alternative hypothesis, however. The clade that contains (*P. multiflorus* (*P. spathulatus*, *P. depressus*, *P. dumetorum*)) lacks all three structures ("type 1"; Mayer & Ehrendorfer, 1999), and is nested within *Pterocephalus* in the cpDNA and combined trees. The clade containing *P. perennis* and *P. strictus*, species that have diaphragmata (*P. perennis*) and coronas (both species), is sister to the rest of *Pterocephalus* in the cpDNA and combined trees. It is, therefore, possible that the trend in *Pterocephalus* is toward *decreasing* complexity of the epicalyx, with the diaphragma, epi-diaphragma, and corona becoming reduced in groups that are nested within *Pterocephalus*. Perhaps in the evolution of *Pterocephalus*, the dispersal function has shifted from a "wing" to a "pappus", which does not require the additional epicalyx structures. More complete sampling from *Pterocephalus* (particularly more "type 1" species) is needed to test this alternative hypothesis.

Scabiosa contains ca. 30 species of mostly diploid perennials and is monophyletic in all studies. Species in *Scabiosa* have relatively uniform epicalyces with expanded coronas, and the group is characterized by an interesting biogeographical disjunction between East Asia, Southern and East Africa, and Eurasia. *Scabiosa* sect. *Prismakena* (Bobrov, 1957) was designated for the East Asian representatives of *Scabiosa* that have a quadrilateral epicalyx lacking sclerenchyma and deep grooves in the epicalyx tube. *Scabiosa* sect. *Prismakena* is thought to be ancestral in *Scabiosa* (Verlaque, 1986a), with a pattern of increasing sclerification as *Scabiosa* supposedly moved into Eurasia and Africa. The placement of *S. japonica* (*S.* sect. *Prismakena*) as sister to the remaining *Scabiosa* may be consistent with this view, but more species will need to be included for a critical test. In our study, species-level resolution within *Scabiosa* is particularly poor, and phylogenetic analysis with an additional marker, such as a low-copy nuclear gene, will be needed to further evaluate evolutionary hypotheses.

Past anatomical (Mayer & Ehrendorfer, 1999) and molecular (Caputo & al., 2004) studies placed *Pycnocomon* as sister to *Lomelosia*, but our analyses resolve *Pycnocomon* as nested within *Lomelosia*. Both groups have the same chromosome number ($x = 9$, although a few *Lomelosia* species are $x = 8$ or $x = 7$), triporate pollen, and deep pits and a second ring of sclerenchyma in the epicalyx tube. Species assigned to *Lomelosia* tend to have large, elaborate coronas and horizontal epi-diaphragmata, while the corona in *Pycnocomon* is reduced and the epi-diaphragma is long and vertical, similar to *Sixalix*.

Lomelosia is the largest and most diverse clade in Scabioseae s.str. (ca. 50 species) and has the highest proportion of annuals in Dipsacaceae. Previous researchers (Ehrendorfer, 1965a; Verlaque, 1986b) considered the annual species to be derived within *Lomelosia* and hypothesized that this was responsible for the successful colonization of the (mostly) Eastern Mediterranean by this group. The annual *L. brachiata* is resolved as sister to the rest of *Lomelosia* + *Pycnocomon* in our study. This species was previously placed in a separate genus, *Tremastelma*, because it has

8–10 fringed calyx bristles (instead of five spiny bristles; Fig. 1) and a chromosome number of $x = 7$. Similar to our results with *Knautia orientalis*, the placement of *L. brachiata* in the cpDNA and combined trees does not support the hypothesis that the annual strategy and chromosome loss occur in species that are always nested within *Lomelosia*. *Lomelosia stellata*, another annual, is nested in a clade that contains *L. argentea*, *L. cretica*, and *L. graminifolia* (all perennials), so it does not appear that all annual *Lomelosia* are part of the same evolutionary lineage within *Lomelosia*. Inclusion of more annual species is needed to understand the evolution of this life history in *Lomelosia*.

Implications for character evolution. — The evolution of the epicalyx in Dipsacaceae appears to have facilitated new and diverse ways to protect and disperse seeds. The epicalyx and calyx function in concert to produce different diaspore structures that are presumably associated with different strategies. Within Dipsacaceae, there appears to be a correlation between the size of the corona and the type of calyx bristles: reduced or absent coronas often occur with multiple, pappus-like calyx bristles, and large winged coronas often occur with shorter calyx bristles that are covered in small teeth. The epicalyx tube of species in the *Bassecoia* clade is relatively simple; however, winged coronas and pappus-like calyx bristles are present even among the three species in this clade. The placement of *Bassecoia* as sister to the rest of Dipsacaceae implies that these structures evolved more than once in the diversification of Dipsacaceae, which is counter to earlier interpretations. The placement of (*Pseudoscabiosa* (*Succisa*, *Succisella*)) as potentially sister to the rest of the "Dipknautid" clade further supports this idea, as this small clade also contains species with both wing-like and pappus-like diaspores. Evolutionary shifts between the wing and pappus diaspore strategies appear to be a common theme in the evolution of Dipsacaceae.

The elongated, lignified, funnel-shaped epi-diaphragmata and reduced coronas found in *Pycnocomon* and *Sixalix* represent another type of diaspore structure. The fact that *Pycnocomon* and *Sixalix* are resolved in separated clades suggests that this syndrome also evolved more than once. In the case of *Pycnocomon*, a funnel-shaped epi-diaphragma and reduced corona evolved within a clade of winged *Lomelosia* species. Depending on the position of *Sixalix*, this diaspore type evolved either from within a clade of pappus-like *Pterocephalus* species, or in a clade that is sister to a group of winged *Scabiosa*.

While some calyx and epicalyx characters appear to have evolved multiple times in Dipsacaceae, other characters appear to have evolved just once. The second ring of sclerenchyma in the epicalyx tubes of *Lomelosia* and *Pycnocomon* is one example. The additional ring consists of horizontal lignified fibers that provide protection for the seed and delay germination by preventing water uptake (Mayer, 1995). *Lomelosia* and *Pycnocomon* are found in arid and semi-arid regions that experience a short period of rainfall each year, and the additional ring of sclerenchyma possibly prevents germination until the amount of water available is sufficient to supply the emerging seedling with water. The elaiosome in *Knautia* is another example of a uniquely derived epicalyx character in Dipsacaceae. The elaiosome is formed by a meristem at the base of the epicalyx and is composed mostly of lipids that attract ants (Mayer & Svoma, 1998; Fischer & al., 2008).

Chromosome evolution and life history strategy may also have played an important role in the evolution of Dipsacaceae. While most Dipsacaceae species are perennials, several clades also contain annual species (particularly *Lomelosia*). Early researchers (Ehrendorfer, 1965a, b; Verlaque, 1984a) found that the annual species in Dipsacaceae often have fewer chromosomes, and hypothesized that these are young lineages that diversified in arid habitats. Possible advantages in reducing the number of chromosomes include faster cell division (Bennett, 1972), or bringing together favorable combinations of alleles through chromosome fusion, particularly in harsh environments (Stebbins, 1950). In *Cephalaria*, the base chromosome number is $x = 9$; chromosome counts exist for one annual, *C. syriaca*, which is $x = 5$. In *Pterocephalus*, the annual *P. brevis* is $x = 8$ instead of $x = 9$, and if *Sixalix* is nested within *Pterocephalus*, as suggested by our ITS tree, *S. atropurpurea* and *S. eremophila*, both annuals, also show a reduction to 8 chromosomes. In both cases, the annuals are nested within their respective clades, which provides evidence for the correlated change to the annual habit and reduction in chromosome number. In the *Knautia* + *Pterocephalidium* clade, *K. orientalis* and *P. diandrum* are annuals with $x = 8$ instead of the $x = 10$ found in all other *Knautia* species (except *K. degenii*, a species not included in this study, which is also $x = 8$). Given their phylogenetic relationships, $x = 8$ and the annual habit may have evolved independently in these two lines, though it is also possible that these conditions are ancestral in this clade, which is counter to the hypothesis that annual lineages are derived. Similarly, in the *Lomelosia* + *Pycnocomon* clade, most species are $x = 9$, except for the annual species *L. brachiata* ($x = 7$) and *L. calocephala* ($x = 8$). These two may provide further evidence of multiple correlated changes—but, here too, it could be equally parsimonious to suppose that annuals with low chromosome numbers are ancestral to perennials with higher numbers. Finally, *L. stellata*, another annual species, is highly polymorphic ($2n = 16$, 18, 26, 27, 28, 30, and 36; Verlaque, 1986b). This species is nested within a clade of perennials, providing evidence that the annual habit and chromosome loss may have evolved multiple times in *Lomelosia*.

Our phylogenetic results set the stage for more detailed analyses of character evolution in Dipsacaceae. The possible correlated evolution of functionally interacting structural components of the diaspore (e.g., corona size and plumose vs. non-plumose calyx bristles, etc.) suggest that particular configurations of the epicalyx and calyx are under selection and may form "evolutionary stable configurations" (Wagner & Schwenk, 2000). Further analysis of the co-evolution of such interacting characters will require careful measurements of the constituent morphological elements of the diaspore. In the meantime, the new information presented here on the multiple origins of pappus, wings, and funnels facilitates further exploration of their association with particular ecological conditions. Additionally, our study highlights the need for a more detailed analysis of the possible evolutionary correlation between life history and chromosome numbers. Including more annual species, particularly from clades with large proportions of annuals (e.g., *Lomelosia* and *Cephalaria*), will be necessary to understand how shifts in life history and chromosome number have shaped Dipsacaceae evolution.

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Appendix. Samples of Dipsacaceae and outgroups used in this study.

Taxon, voucher, *atpB-rbcL*, *trnL-trnF*, *trnS*^{UGA}-*trnG*^{GCC}, ITS (sequences of ITS for which two GenBank numbers are present represent ITS1 and ITS2).

Outgroup taxa; *Patrinia* *triloba* Miq., *Eriksson 807* (SBT), AF446951[a], AF447011[a], FJ640693, AY236191[a]; ***Morina*** *longifolia* Wallich ex DC., *Eriksson s.n.* (SBT), AF446945[a], AF447005[a], FJ640692, AY236185[a]; ***Triplostegia*** *glandulifera* Wallich ex DC., *Boufford & al. 28440* (A), AF446949[a], AF447009[a], FJ640694, AY236189[a].

Ingroup taxa; *Bassecoia* *bretschneideri* (Batalin) B.L. Burtt, *Boufford & al. 35478* (A), FJ640591, FJ640646, FJ640696, FJ640751; *Bassecoia siamensis* (Craib) B.L. Burtt, *Smithiand 620049* (E), FJ640592, FJ640647, FJ640697, FJ640750; ***Pterocephalodes*** *hookeri* (Batalin) V. Mayer & Ehrendorfer, *Boufford & al. 28691* (A), AF446946[a], AF447006[a], FJ640695, AY236186[a]; **"Dipknautids"; *Cephalaria*** *aristata* C. Koch, *Holtz & al. 01.102* (E), FJ640605, FJ640656, FJ640711, FJ640757; *Cephalaria hirsuta* Stapf., *Holtz & al. 779* (E); FJ640606, FJ640658, FJ640713, FJ640760/FJ640790; *Cephalaria leucantha* (L.) Roem. & Schult., *Carlson 182* (YU), FJ640598, AJ427376[a], FJ640706, AJ426523[a]/AJ426524[a]; *Cephalaria microcephala* Boiss., *Lamond 5004* (E); FJ640601, FJ640657, FJ640710, FJ640759; *Cephalaria natalensis* Kuntze, *Hilliard & Burdtt 10123* (E), FJ640600, FJ640655, FJ640708, FJ640754; *Cephalaria paphlagonica* Bobrov, *Davis & Coode D38935* (E), FJ640599, FJ640659, FJ640707, FJ640758; *Cephalaria syriaca* (L.) Roem. & Schult., *Assadi & Shirdelpur 13146* (E), FJ640603, AJ427377[a], NS[b], AJ426525[a]/AJ426526[a]; *Cephalaria tenella* Payne, *Von 96072* (WU), FJ640604, FJ640653, FJ640712, FJ640756; *Cephalaria zeyheriana* Szabó, *Turner 346* (WU), FJ640602, FJ640654, FJ640709, FJ640755; ***Dipsacus*** *pilosus* L., no voucher, FJ640608, AY290005[a], FJ640705, AY290016[a]; *Dipsacus fullonum* L., *Cellinese 6001* (YU), FJ640607, FJ640651, FJ640703, NS[b]; *Dipsacus strigosus* Willd. ex Roem. & Schult., *Hewer H.4023* (E); FJ640609, FJ640652, FJ640704, FJ640762; ***Knautia*** *arvensis* (L.) Coult., *Carlson 181* (YU), FJ640617, FJ640666, FJ640722, AJ426529[a]/AJ426530[a]; *Knautia calycina* Guss., *Cellinese 6000* (YU), FJ640618, FJ640663, FJ640721, FJ640765; *Knautia dipsacifolia* Heuff., *Pistarino 2552* (MRSN), FJ640613, FJ640664, FJ640716, FJ640764; *Knautia dinarica* Maly, *Carlson 188* (YU), FJ640615, FJ640662, FJ640718, FJ640767; *Knautia drymeia* Heuff., *Abba' 24-8-1990* (MRSN), FJ640610, FJ640661, FJ640717, NS[b]; *Knautia macedonica* Griseb., *Billiet V786* (BR), FJ640614, AY360115[a], FJ640719, AY360095[a]; *Knautia midzorensis* Form., *Andreev n.s.* (E), FJ640616, FJ640665, FJ640720, FJ640766; *Knautia orientalis* L., *Holtz & Hänel 366* (E), FJ640611, FJ640660, FJ640715, FJ640763; ***Pseudoscabiosa*** *grosii* (Font Quer) Devesa, *Cordoba S-/2.684-8* 1989 (WU), FJ640595, FJ640648, FJ640700, FJ640753; *Pseudoscabiosa limonifolia* (Vahl) Devesa, *Schimmenti & Scafidi s.n.* (MRSN), FJ640597, FJ640649, FJ640701, AJ426535[a]/AJ426536[a]; *Pseudoscabiosa saxatilis* (Cav.) Devesa, *Gardner & Gardner 1058* (E), FJ640596, FJ640650, FJ640702, FJ640752; ***Pterocephalidium*** *diandrum* (Lag.) G. López, *Nydegger 36007* (G), FJ640612, AJ427382[a], FJ640714, AJ426537[a] /AJ426538[a]; ***Succisa*** *pratensis* Moench, *Abba'8-9-1996* (MRSN), FJ640593, AY290007[a], FJ640698, AY290018[a]; ***Succisella*** *inflexa* (Kluk) G. Beck, *No voucher*, FJ640594, AJ427392[a], FJ640699, AJ426555[a]/AJ426556[a]; **Scabioseae s.str.; *Lomelosia*** *argentea* (L.) Greuter & Burdet, *Carlson 189* (YU), FJ640629, AJ427380[a], FJ640744, AJ426531[a] /AJ426532[a]; *Lomelosia brachiata* (Sm.) Greuter & Burdet, *Mayer W-88-07* (WU), FJ640635, FJ640684, FJ640741, FJ640781; *Lomelosia calocephala* (Boiss.) Greuter & Burdet, *Foroughi & al. 12489* (E), FJ640634, FJ640685, FJ640740, FJ640786; *Lomelosia caucasica* (MB.) Greuter & Burdet, *No voucher*, FJ640630, FJ640690, FJ640742, FJ640787; *Lomelosia cretica* (L.) Greuter & Burdet, *Cellinese 6002* (YU), FJ640628, FJ640689, FJ640746, FJ640788; *Lomelosia graminifolia* (L.) Greuter & Burdet, *Abba' 23-7-92* (MRSN), FJ640633, FJ640688, FJ640745, FJ640789/FJ640791; *Lomelosia soongarica* (Schrenk ex Fischer & Meyer) Soják, *Wilson & al. 1331* (E), FJ640631, FJ640691, FJ640747, FJ640785; *Lomelosia stellata* Raf., *Mayer 95037* (WU), FJ640632, FJ640687, FJ640743, FJ640784/FJ640792; ***Scabiosa*** *africana* L., *Rouke 17-3-1980* (E); FJ640621, AJ427386[a], FJ640727, AJ426543[a] /AJ426544[a]; *Scabiosa columbaria* L., *Bell 199* (YU), FJ640620, FJ640669, FJ640724, AY236188[a]; *Scabiosa japonica* Miq., no voucher, NS[b], AJ427387[a], NS[b], AJ426545[a] /AJ426546[a]; *Scabiosa ochroleuca* L., *Gardner & Gardner 3037* (E), FJ640619, FJ640673, FJ640726, FJ640768; *Scabiosa transvaalensis* S. Moore, *Hilliard & Burtt 5960* (E), FJ640624, FJ640672, FJ640723, FJ640769; *Scabiosa tysonii* L. Bolus, *Edwards & al. 3225* (E), FJ640625, FJ640671, FJ640725, FJ640770; ***Sixalix*** *atropurpurea* (L.) Greuter & Burdet, *Carlson 137* (YU), FJ640623, FJ640667, FJ640728, FJ640771; *Sixalix eremophila* (Boiss.) Greuter & Burdet, *Mayer & Jenny s.n.* (BSB), FJ640622, FJ640668, FJ640729, FJ640772; ***Pterocephalus*** *brevis* Coult., *Gutterman s.n.* (E), FJ640642, FJ640677, FJ640739, FJ640776; *Pterocephalus depressus* Coss. & Bal., *Davis 675552* (E), FJ640638, FJ640676, FJ640736, FJ640780; *Pterocephalus dumetorum* Coult., *No voucher*, FJ640639, FJ640674, FJ640735, AM296471[a]/AM296472[a]; *Pterocephalus frutescens* Hochst., *Wood 3095* (E), FJ640640, FJ640680, FJ640730, FJ640774; *Pterocephalus multiflorus* Poech., *Rechinger 62047*(E), FJ640636, FJ640683, FJ640737, FJ640778; *Pterocephalus papposus* Coult., *Edmondson & al. 2330* (E), FJ640643, FJ640681, FJ640681, FJ640731; *Pterocephalus perennis* Coult., *Strid & al. 27695* (E), FJ640641, FJ640675, FJ640732, AJ426539[a]/AJ426540[a]; *Pterocephalus pulverulentus* Boiss. & Balansa ex Boiss., *Collenette 8224* (E), FJ640644, FJ640678, FJ640738, FJ640777; *Pterocephalus spathulatus* (Lag.) Coult., *Gardner & Gardner 1295* (E), FJ640637, FJ640682, FJ640734, FJ640779; *Pterocephalus strictus* Boiss. & Hohen., *Archibald 8316* (E), FJ640645, FJ640679, FJ640733, FJ640775; ***Pycnocomon*** *intermedium* (Lag.) Greuter & Burdet (E), *Molesworth-Allen 8421B*, FJ640626, AM296007[a], FJ640748, FJ640782; *Pycnocomon rutifolium* (Vahl) Hoffmanns. & Link, *Sales & Hedge 02/16* (E), FJ640627, FJ640686, FJ640749, FJ640783.

[a]Sequence acquired from GenBank.
[b]Taxa for which we were unable to obtain sequence data are indicated by "NS".