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ORIGINAL ARTICLE

A phylogenetic analysis of Dipsacaceae based on four DNA regions

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Abstract Authors studied the phylogeny of Dipsacaceae using maximum parsimony and Bayesian analyses on sequence data from chloroplast (*trn*L intron, *trn*L–*trn*F intergenic spacer, *psb*B–*psb*H gene complex) and nuclear genomes (ITS1 and ITS2). Both data partitions as well as their combination show that Dipsacaceae is a monophyletic group. Topology in tribe Scabioseae is similar to those of other recent studies, except for the position of *Pycnocomon*, which is nested in *Lomelosia*. *Pycnocomon*, the pollen and epicalyx morphologies of which closely resemble those of *Lomelosia*, is interpreted as a psammophilous morphotype of *Lomelosia*, and its nomenclature has been revised accordingly. Exclusion of *Pseudoscabiosa*, *Pterocephalidium*, *Pterocephalodes* (and probably *Bassecoia)*, *Succisa*, *Succisella* from Scabioseae is confirmed. *Pterocephalodes hookeri* is the sister group to the rest of the family. Its remoteness from *Pterocephalus* has been confirmed on molecular grounds. Lack of evident synapomorphies for various clades is interpreted as a possible consequence of fast adaptative radiation.

Keywords Dipsacaceae · Internal transcribed spacers (ITS) · Phylogeny · *psb*B–*psb*H · *trn*L intron · *trn*L–*trn*F intergenic spacer

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Introduction

Dipsacaceae Juss. (Dipsacales Lindl.), the teasel family, includes 250–350 species (Ehrendorfer 1965; Verláque 1977a) of annual to perennial herbs and shrubs; native mostly to Mediterranean and temperate climates, they are found in north temperate Eurasia and in tropical to southern Africa. Synapomorphies for the family are the epicalyx, which encloses the indehiscent, achene-like fruit and represents the dispersal unit of all species, and a scapous, dense, involucrate head (Caputo and Cozzolino 1994). The family is a derived member of the order Dipsacales. Parsimony analyses based on both morphological and molecular data have indeed repeatedly confirmed the sister group relationship between Valerianaceae Batsch (in particular with *Triplostegia* Wall. ex DC.) and Dipsacaceae (Judd et al. 1994; Backlund and Donoghue 1996; Bell et al. 2001; Donoghue et al. 2001; Bell and Donoghue 2003; Zhang et al. 2003; Bell 2004; Pyck and Smets 2004).

Morphological, palynological and karyological evidence, and investigations of seed dispersal (Ehrendorfer 1964a, b, 1965; Verláque 1977a, b, 1984a, b, 1985a, b, 1986a, b; Caputo and Cozzolino 1994) have not contributed to a consensus about the evolutionary history of the family, and relationships between taxa have been contradictorily reconstructed in previous literature.

Summarizing a quite long account of taxonomic intricacies, the family has been historically regarded as divided into three tribes: Dipsaceae, including *Dipsacus* L. and *Cephalaria* Schrad.; Knautieae, including only *Knautia* L.; and Scabioseae, including *Pterocephalus* (Vaill.) Adans. s.l., *Pycnocomon* Hoffmanns. et Link, *Scabiosa* L. s.l., *Succisa* Necker and *Succisella* Beck. The studies by Verláque (1977a, b, 1984a, b, 1985a, b, 1986a, b) and their nomenclatural consequences (Devesa 1984; Greuter and Burdet 1985; López Gonzáles 1987) caused segregation of various entities, mainly from the genus *Scabiosa*, some of which are phylogenetically distant from the former genus. *Scabiosa* s.l. has been therefore subdivided on the account of differences in epicalyx structure into *Lomelosia* Rafin., *Scabiosa* s.str., *Sixalix* Rafin. and *Pseudoscabiosa* Devesa. Similarly, from the genus *Pterocephalus* (Vaill.) Adans., *Pycnocomon intermedium* (Lag.) Greuter et Burdet (= *Pterocephalus intermedius* (Lag.) Coutinho), and *Pterocephalidium* G. López (*Pterocephalidium diandrum* (Lag.) G. López = *Pterocephalus diandrus* (Lag.) Lag.) were segregated.

More recently, Mayer and Ehrendorfer (1999) critically reviewed the epicalyx structure by employing microscopical techniques and suggested that Scabioseae, in order to be monophyletic, should include fewer genera than previously thought. Moreover, they segregated three Asian *Pterocephalus* species (namely, *Pterocephalus bretschneideri* (Batalin) Pritz., *Pterocephalus hookeri* (C. B. Clarke) Pritz. and *Pterocephalus siamensis* (Craib) Verláque) into genus *Pterocephalodes* Mayer et Ehrendorfer (Mayer and Ehrendorfer 2000) demonstrating its remoteness from *Pterocephalus* s.s. on morphoanatomical grounds. However, Burtt (1999), a few months earlier (and Mayer and Ehrendorfer were not aware of it) had removed *Pterocephalus bretschneideri* and *Pterocephalus siamensis* from the genus *Pterocephalus*, creating a new genus *Bassecoia* Burtt for them. Given the fact that the three species seem to belong to a monophyletic unit (Mayer and Ehrendorfer 2000), that no name is available for *Pterocephalodes hookeri* under *Bassecoia*, and that our paper does not directly deal with the nomenclature of these taxa, from now onwards we will loosely refer to the monophylum including the three species as "*Pterocephalodes/Bassecoia*".

As a consequence of what was mentioned above, Dipsacaceae presently include 14 genera (species number are from Verláque 1986b; Mayer and Ehrendorfer 1999, 2000): *Lomelosia* (approx. 50 species), *Pterocephalus* (approx. 30 species), *Pycnocomon* (two species), *Scabiosa* (approx. 30 species), *Sixalix* (eight species), all included in the narrower circumscription of Scabioseae as redefined by Mayer and Ehrendorfer (1999); *Cephalaria* (approx. 80 species), *Dipsacus* (approx. 40 species), *Pseudoscabiosa* (three species), *Pterocephalidium* (one species), *Pterocephalodes/Bassecoia* (one and two species), *Succisa* (four species), *Succisella* (three species), putatively belonging to Dipsaceae according to Mayer and Ehrendorfer (2000); and *Knautia* (at least 50 species), which the above-mentioned authors did not investigate. We reported the main infrafamilial taxa in which Dipsacaceae are divided in Table 1.

This taxonomic complexity is related to rampant parallelism amongst various genera, especially in dispersal syndromes (Mayer and Ehrendorfer 1999, 2000; Caputo et al. 2004). In anemochorous taxa, indeed, a wide membranous corona (i.e. the distal part of the epicalyx) homoplasiously develops (e.g. *Lomelosia*, *Pseudoscabiosa* p.p., *Scabiosa*), or calyx bristles are multiplied and more or less plumose (e.g. *Pseudoscabiosa grosii*, *Pterocephalidium*, *Pterocephalodes/Bassecoia*, *Pterocephalus*, *Pycnocomon intermedium*). In few species both conditions occur (e.g. *Lomelosia brachiata*, *Pterocephalus pyrethrifolius* Boiss. et Hohen. and *Pterocephalus wendelboi* Rech. f., *Bassecoia bretschneideri* Burtt and *B. siamensis* Burtt).

The only genus-level molecular investigation available for the family (Caputo et al. 2004) is based on a 1,042 character matrix originating from nuclear ribosomal (internal transcribed spacers, ITS) and chloroplast (*trn*L intron) DNA data for 19 taxa. The authors of the last mentioned contribution confirm the findings by Mayer and Ehrendorfer (1999) on Scabioseae. However, the authors themselves felt their taxonomic sampling to be scanty, especially within *Dipsacus* and *Cephalaria*, and since *Pterocephalodes/Bassecoia* was absent from their dataset.

We present here an expanded molecular investigation, which includes 35 taxa and four DNA regions: the ITS region of nuclear ribosomal DNA (nrDNA), which has provided valuable information for resolving phylogenetic relationships across a wide range of taxonomic levels (Baldwin et al. 1995; Suh et al. 1993; Sun et al. 1994; Bayer et al. 1996); the chloroplast DNA *trn*L–*trn*F region (consisting of a non-coding spacer between *trn*L [UAA] and *trn*F [GAA] genes) and the adjacent *trn*L (UAA) intron region, which are employed for phylogenetic investigations in restricted taxonomic circumscriptions for their average mutation rates (Fangan et al. 1994; Gielly and Taberlet 1995a, b, 1996); the chloroplast DNA *psb*B–*psb*H gene complex, that includes parts of the named genes, which encode two subunits of the photosystem II, the non-coding spacers between them as well as the *psb*T and *psb*N genes coding uncertain small photosystem II proteins (Bukharov et al. 1988).

Materials and methods

Taxon sampling

A total of 28 species representing a wide sampling of diversity of Dipsacaceae were included in this work

Table 1 Synopsis of the infrafamilial taxa of Dipsacaceae

Tribe	Genus	Section/subgenus	Representative species
Dipsaceae	*Cephalaria* Schrad.	subg. *Cephalaria*	*Cephalaria joppensis* (Rchb.) Coult.
		subg. *Fimbriatocarpus* Szabó	*Cephalaria leucantha* (L.) Roem. et Schult.
		subg. *Lobatocarpus* Szabó	*Cephalaria natalensis* Kuntze
		subg. *Phalacrocarpus* Szabó	*Cephalaria syriaca* (L.) Roem. et Schult.
	Dipsacus L.	Sect. *Dipsacus*	*Dipsacus laciniatus* L.
			Dipsacus strigosus Roem. et Schult.
			Dipsacus sylvestris Huds.
		Sect. *Sphaerodipsacus* Lange	*Dipsacus japonicus* Miq.
			Dipsacus pilosus L.
	Pseudoscabiosa Devesa		*Pseudoscabiosa grosii* (Font Quer) Devesa
			Pseudoscabiosa limonifolia (Vahl) Devesa
	Pterocephalidium G. López		*Pterocephalidium diandrum* (Lag.) G. López
	Pterocephalodes V. Mayer et Ehrend.		*Pterocephalodes hookeri* (C. B. Clarke) V. Mayer et Ehrend.
	Succisa L.		*Succisa pratensis* Moench
	Succisella Beck		*Succisella inflexa* (Kluk) G. Beck
Knautieae	*Knautia* (L.) Coult.	Sect. *Trichera* (Schrad.) Rouy	*Knautia arvensis* (L.) Coult.
Scabioseae sensu Mayer et Ehrendorfer	*Lomelosia* Rafin.	Sect. *Callistemma* (Mert. et Kock) Mayer et Ehrend.	*Lomelosia brachiata* (Sm.) Greuter et Burdet
		Sect. *Lomelosia*	*Lomelosia cretica* (L.) Greuter et Burdet
			Lomelosia palestina (L.) Rafin.
			Lomelosia simplex (Desf.) Rafin.
	Pterocephalus (Vaill.) Adans.		*Pterocephalus dumetorum* (Brouss. ex Willd.) Coult.
	Pycnocomon Hoffmans. et Link		*Pycnocomon intermedium* (Lag.) Greuter & Burdet.
			Pycnocomon rutifolium (Vahl) Hoffmanns. & Link
	Scabiosa L.		*Scabiosa africana* L.
			Scabiosa japonica Miq.
			Scabiosa uniseta Savi
	Sixalix Rafin.		*Sixalix atropurpurea* (L.) Greuter et Burdet subsp. *maritima* (L.) Greuter et Burdet
			Sixalix farinosa (Cosson) Greuter et Burdet

The last column indicates our chosen representative species

(Tables 1, 2). Seven outgroup taxa (Table 2) were chosen on the basis of previous molecular phylogenetic studies (see above) indicating that Valerianaceae and Morinaceae J. Agardh are the sister groups of Dipsacaceae. Choice of outgroups was aimed at including the most archaic members of the above mentioned families: *Acanthocalyx* (DC.) M. Cannon (Morinaceae), *Patrinia* Juss. and *Nardostachys* DC. (Valerianaceae), and *Triplostegia* (variously regarded as a member of Dipsacaceae, Valerianaceae or of the monogeneric Triplostegiaceae [Hoeck] Airy Shaw). In particular, the latter genus is considered (Zhang et al. 2003; Bell 2004; Pyck and Smets 2004) to be the sister group to Dipsacaceae.

Voucher information and GenBank accessions for all samples are listed in Table 2. Specimens for DNA extraction were either field-collected by the authors or planted from seeds and cultivated at the Botanical Garden of Naples, Italy.

DNA extraction and sequencing

Leaves were collected at flowering time. Voucher specimens of the examined plants are deposited at NAP. Total DNA was extracted following either the procedure described by Doyle and Doyle (1990) or employing the GenElute^{TM} Plant Genomic DNA Miniprep Kit (Sigma Aldrich). ITS1 and ITS2 were amplified using the primers

Table 2 Species, GenBank accession numbers and origins of the samples in study

Species	ITS GenBank accession nos.	*trn*L–*trn*F IGS GenBank accession nos.	*trn*L intron GenBank accession nos.	*psb*B–*psb*H GenBank accession nos.	Origin
Acanthocalyx nepalensis (D.Don) M. J. Cannon	AY290015	AY290004	AY290004	–	Bell and Donoghue (2003)
Centranthus ruber (L.) DC	AY310448	AF446986	AF446986	AM392458	Bell 2004 (ITS1, ITS2, *trn*L–*trn*F, *trn*L); Voucher no. 1, Avino & Caputo, NAP (*psb*B–*psb*H)
Cephalaria joppensis (Rchb.) Coult.	AM296451 AM296452	AM296188	AM295996	AM392459	Voucher no. 2, Avino and Caputo, NAP
Cephalaria leucantha (L.) Roem. et Schult.	AJ426523 AJ426524	AM296189	AJ427376	AM392460	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 3, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Cephalaria natalensis Kuntze	AM296453 AM296454	AM296190	AM295997	AM392461	Voucher no. 4, Avino and Caputo, NAP
Cephalaria syriaca (L.) Roem. et Schult.	AJ426525 AJ426526	AM296191	AJ427377	AM392462	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 5, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Dipsacus japonicus Miq.	AM296455 AM296456	AM296192	AM295998	AM392463	Voucher no. 6, Avino and Caputo, NAP
Dipsacus laciniatus L.	AM296457 AM296458	AM296193	AM295999	AM392464	Voucher no. 7, Avino and Caputo, NAP
Dipsacus pilosus L.	AM296459 AM296460	AM296194	AM296000	AM392465	Voucher no. 8, Avino and Caputo, NAP
Dipsacus strigosus Roem. et Schult.	AM296461 AM296462	AM296195	AM296001	AM392466	Voucher no. 9, Avino and Caputo, NAP
Dipsacus sylvestris Huds.	AJ426527 AJ426528	AM296196	AJ427378	AM392467	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 10, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Knautia arvensis (L.) Coult.	AJ426529 AJ426530	AM296197	AJ427379	AM392468	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 11, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Lomelosia brachiata (Sm.) Greuter et Burdet	AM296463 AM296464	AM296198	AM296002	AM392469	Voucher no. 12, Avino and Caputo, NAP
Lomelosia cretica (L.) Greuter et Burdet	AM296465 AM296466	AM296199	AM296003	AM392470	Voucher no. 13, Avino and Caputo, NAP
Lomelosia palestina (L.) Rafin.	AM296467 AM296468	AM296200	AM296004	AM392471	Voucher no. 14, Avino and Caputo, NAP
Lomelosia simplex (Desf.) Rafin.	AM296469 AM296470	AM296201	AM296005	AM392472	Voucher no. 15, Avino and Caputo, NAP
Morina longifolia Wallich	AY236185	AF446975	AF446975	AM392473	Bell 2004 (ITS1, ITS2, *trn*L, *trn*L–*trn*F); Voucher no. 16, Avino and Caputo, NAP (*psb*B–*psb*H)
Nardostachys jatamansi (Jones) DC.	AY236190	AF446980	AF446980	–	Bell 2004
Patrinia villosa L.	AM296475 AM296476	AM296215	AM296009	AM392474	Voucher no. 17, Avino and Caputo, NAP
Pseudoscabiosa grosii (Font Quer) Devesa	AM296449 AM296450	AM296202	AM296006	AM392457	Voucher no. 18, Avino and Caputo, NAP
Pseudoscabiosa limonifolia (Vahl) Devesa	AJ426535 AJ426536	AM296203	AJ427383	AM392475	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 19, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)

Table 2 continued

Species	ITS GenBank accession nos.	*trn*L–*trn*F IGS GenBank accession nos.	*trn*L intron GenBank accession nos.	*psb*B–*psb*H GenBank accession nos.	Origin
Pterocephalidium diandrum (Lag.) G. López	AJ426537 AJ426538	AM296204	AJ427382	AM392476	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 20, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Pterocephalodes hookeri (C. B. Clarke) V. Mayer et Ehrend.	AY236186	AF446976	AF446976	–	Bell 2004
Pterocephalus dumetorum (Brouss. ex Willd.) Coult.	AM296471 AM296472	AM296205	AM296008	AM392477	Voucher no. 21, Avino and Caputo, NAP
Pycnocomon intermedium (Lag.) Greuter et Burdet.	AM296473 AM296474	AM296206	AM296007	AM392478	Voucher no. 22, Avino and Caputo, NAP
Pycnocomon rutifolium (Vahl) Hoffmanns. et Link	AJ426541 AJ426542	AM296207	AJ427385	AM392479	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 23, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Scabiosa africana L.	AJ426543 AJ426544	AM296208	AJ427386	AM392480	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 24, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Scabiosa japonica Miq.	AJ426545 AJ426546	AM296209	AJ427387	AM392481	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 25, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Scabiosa uniseta Savi	AJ426547 AJ426548	AM296210	AJ427388	AM392482	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 26, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Sixalix atropurpurea (L.) Greuter et Burdet subsp. *maritima* (L.) Greuter et Burdet	AJ426549 AJ426550	AM296211	AJ427389	AM392483	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 27, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Sixalix farinosa (Cosson) Greuter et Burdet	AJ426551 AJ426552	AM296212	AJ427390	AM392484	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 28, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Succisa pratensis Moench	AJ426553 AJ426554	AM296213	AJ427391	AM392485	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 29, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Succisella inflexa (Kluk) G. Beck	AJ426555 AJ426556	AM296214	AJ427392	AM392486	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 30, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)
Triplostegia glandulifera Wall. ex DC.	AY236189	AF446979	AF446979	–	Bell 2004
Valeriana officinalis L.	AJ426559 AJ426560	AM296216	AJ427394	AM392487	Caputo et al. 2004 (ITS1, ITS2, *trn*L); Voucher no. 31, Avino and Caputo, NAP (*trn*L–*trn*F, *psb*B–*psb*H)

A dash indicates that the sequence was not obtained or was not available in previous literature

reported in Aceto et al. (1999), the *trn*L (UAA) intron and the *trn*L–*trn*F intergenic spacer (IGS) region were amplified using the primers indicated in Taberlet et al. (1991), and the *psb*B–*psb*H region was amplified using the primers reported in Bukharov et al. (1988).

PCR reactions (reaction volume 50 µL, primer concentration 50 pm/µL) were carried out for 30 cycles in a Perkin-Elmer Cetus 9600 thermocycler (Perkin-Elmer, Norwalk, CT, USA). Initial conditions were as follows (other details are reported in Aceto et al. 1999): 1 min denaturation at 94°C, 1 min annealing at 55°C (ITS and *trn*L intron) or 50°C (*trn*L–*trn*F IGS and *psb*B–*psb*H region), and 45 s extension at 72°C. Samples were denatured for 5 min at 94°C before the beginning of the first cycle; extension time was increased by 3 s/cycle; and extension was further prolonged for 7 min at the end of the last cycle. PCR amplification products were purified using GFX^{TM} PCR DNA (Amersham Pharmacia Biotech) and/or the Gel Band Purification Kit (Amersham Pharmacia Biotech). PCR fragments were directly sequenced using a modification of the Sanger dideoxy method (Sanger et al. 1977) as implemented in a double strand DNA cycle sequencing system with fluorescent dyes.

Sequence reactions were then loaded into a 373A Applied Biosystems Automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

After removing 5.8S from the literature sequences, an alignment was carried out using ClustalX (Thompson et al. 1997) with default parameters, then visually inspected to correct the regions in which identical sequences were reconstructed by the software as having different gap distribution, aiming at minimizing the number of gaps. The resulting data matrices (available from the corresponding author) were analysed using maximum parsimony (MP), coding gaps as missing data. The combined matrix was investigated both under a MP criterion and by employing Bayesian inference (BI, Huelsenbeck and Ronquist 2001); gaps in the combined matrix which, after visual inspection, were still uncertain in reconstruction were excluded by the further investigations; remaining gaps were either coded as missing data or scored as binary characters in two independent analyses. For this latter purpose, the insertion/deletion (indel) events were coded using simple indel coding (Simmons and Ochoterena 2000) as implemented in the computer program SeqState (Müller 2005). We thus created two different combined matrices (both available from the corresponding author), one with and another without indel characters and both were analysed under MP and BI criteria.

All the manipulations of the matrices, as well as the majority of the MP analyses, were carried out using the cladistic software environment Winclada (Nixon 1999), running Nona (Goloboff 1999a) as a daughter process with the following settings: a maximum storage space of 10,000 trees; a tree storage space per iteration of 100; one hundred iterations of an algorithm that randomizes the addition order of taxa creates a Wagner tree and swaps its branches by tree bisection–reconnection; and further branch swapping (via tree bisection and reconnection) on all the found trees. Several small areas of the alignment, amounting to a total of 165 characters in the four DNA regions (see "Results"), showed equivocal indel patterns and were therefore excluded from the analysis.

Various MP analyses as well as all the bootstrap (1,000 replicas, Felsenstein 1985) and decay analyses (up to ten steps longer trees, Bremer 1994) were carried out using the TNT software (Goloboff et al. 2003), which can handle large datasets in reasonable times. Settings for the MP analyses were identical to those described above. To avoid being trapped in local length minima, the "new technology" search approach was also used (Goloboff 1999b) with default settings (except for what follows). This approach consists in a very aggressive search for minimum length trees (initial level 100, finding minimal length ten times), followed by the production (via branch swapping) of independent hits to that length.

To assess the potential incongruence between the nuclear and the chloroplast matrices, the incongruence length difference (ILD) test of Farris et al. (1994) was carried out (200 replicas, tree space 10,000, ten iterations per replica, ten trees per iteration) as implemented in Winclada.

As any alignment obtained using clustering procedures will be influenced by gap opening and extension parameters, the elision approach, described by Wheeler et al. (1995), was used to evaluate how sensitive topology is to ambiguous gap positions. This technique consists in "eliding" various individual alignments into a single combined alignment on which a phylogenetic analysis is carried out. Six alignments were generated for all the markers separately. These alignments were generated using ClustalX (Thompson et al. 1997) with the same parameters as above, but with variable gap opening and extension costs at wide intervals in each alignment (PWGAPOPEN = GAPOPEN from 5 to 25 and PWGAPEXT = GAPEXT from 2 to 11). The six alignments obtained plus the original alignment was combined, for each dataset, into a single matrix. These elided matrices were analysed both separately and in combination, in this case scoring all gaps as missing characters.

When multiple sequence alignment and tree searches are conducted in two disconnected processes, as is common in phylogenetic studies, the resulting trees are optimal for the multiple alignment but the multiple alignment may not be optimal per se, as no optimality criterion is imposed on the search. For this reason, a direct optimization approach (Wheeler 1996) was attempted. Direct optimization aims at finding all phylogenetically optimal alignments under a specific cost set and does not require any manipulation of the sequences prior to the analysis. In the present study, we used direct optimization as implemented in the program POY ver. 3.0.11 (Wheeler et al. 2003, documentation by De Laet and Wheeler 2003). POY implements direct optimization (Wheeler 1996) that constructs phylogenetic hypotheses directly without the intervening step of multiple sequence alignment. Given the computational difficulties, search parameters allowed for only 100 replicas, without branch swapping, keeping two trees per replica, employing the algorithm by Goloboff (1999b) during the search and outputting the implied alignment only for the first of the obtained trees. All other parameters were set as default.

To infer the phylogeny of our plants, a Bayesian analysis (Rannala and Yang 1996; Mau and Newton 1997; Mau et al. 1999; Huelsenbeck et al. 2001) was also carried out. BI is similar to maximum likelihood (ML) in that the user postulates a model of evolution and the program searches for the best trees that are consistent with both the model and the data. However, it differs from ML in that whilst ML seeks the tree that maximises the probability of observing data given that

tree, Bayesian analysis seeks the tree that maximises the probability of the tree given the data and the model for evolution. Analyses were carried out with the software MrBAYES (v3.1; Ronquist et al. 2005) under the model suggested by the computer program MrAIC (Nylander 2004), which estimates likelihood scores under different models using the program PHYML (Guindon and Gascuel 2003). The general time reversible model (GTR, Lanave et al. 1984) with a gamma distribution of substitution rates for the molecular data was suggested for all the partitions except that of *trn*L where the Hasegawa–Kishino–Yano (Hasegawa et al. 1985) plus gamma distribution was considered. A restriction-site model was applied for the indel data (as suggested by Ronquist et al. 2005). Two Markov chains (the "cold" chains) were run in parallel and each of them was flanked to other three simultaneous "heated" chains (Metropolis coupled Markov chain Monte Carlo; Huelsenbeck and Ronquist 2001) to avoid getting trapped in a local maximum. The chains were run for 4,000,000 generations sampling only once every 100 generations, collecting 40,000 trees at the end. In order to avoid the early pre-convergence trees in our analyses, we discarded the first 25% (burn in) of the trees, calculating the majority rule consensus tree on the remaining trees. For each analysis the datasets were partitioned and the partitions unlinked.

Sequence substitution rates were compared between clades using the method of Robinson et al. (1998), as implemented in the RRTree software (Robinson-Rechavi and Huchon 2000), equally weighing all nucleotides and all sequences with a 0.01 significance threshold for all tests, not taking into account the topology and employing default distance for non-coding regions.

PTP tests (Faith and Cranston 1991) were carried out (both on the matrix in which indels were scored as missing data and in the matrix in which indels were coded as binary characters) using TNT (Goloboff et al. 2003); with the null hypothesis that the length of MP trees does not significantly differ from that of trees obtained from the randomized data (i.e. that data do not express any cladistic structure, apart that produced by chance alone). One hundred permutations were produced.

Ancestral states of morphological characters have been reconstructed using the software Mesquite (Maddison and Maddison 2008), both under a MP and maximum likelihood models (all characters unordered).

Results

The ITS1 and ITS2 data matrix for the 35 accessions consisted of 589 characters, 273 of which are informative. Parsimony analysis (excluding equivocal indels in positions 58–96 and 282–303 of the ITS1 alignment and in pos. 14–62, 95–99 and 215–219 of ITS2) resulted in five equally parsimonious trees (see Table 3 for summary statistics). In the strict consensus tree (Fig. 1a), Dipsacaceae are monophyletic with *Pterocephalodes hookeri* sister to a clade including the rest of the family. This latter group shows a basal polytomy within which various monophyletic units occur: one including *Succisa* and *Succisella*, another including the two species of *Pseudoscabiosa*, and two larger clades. One of them, fully resolved, includes *Knautia* and *Pterocephalidium* in a sister group relationship with the species of *Cephalaria* and *Dipsacus*; the other includes all the other taxa of the family. In this latter clade, genus *Pycnocomon* is nested within *Lomelosia*.

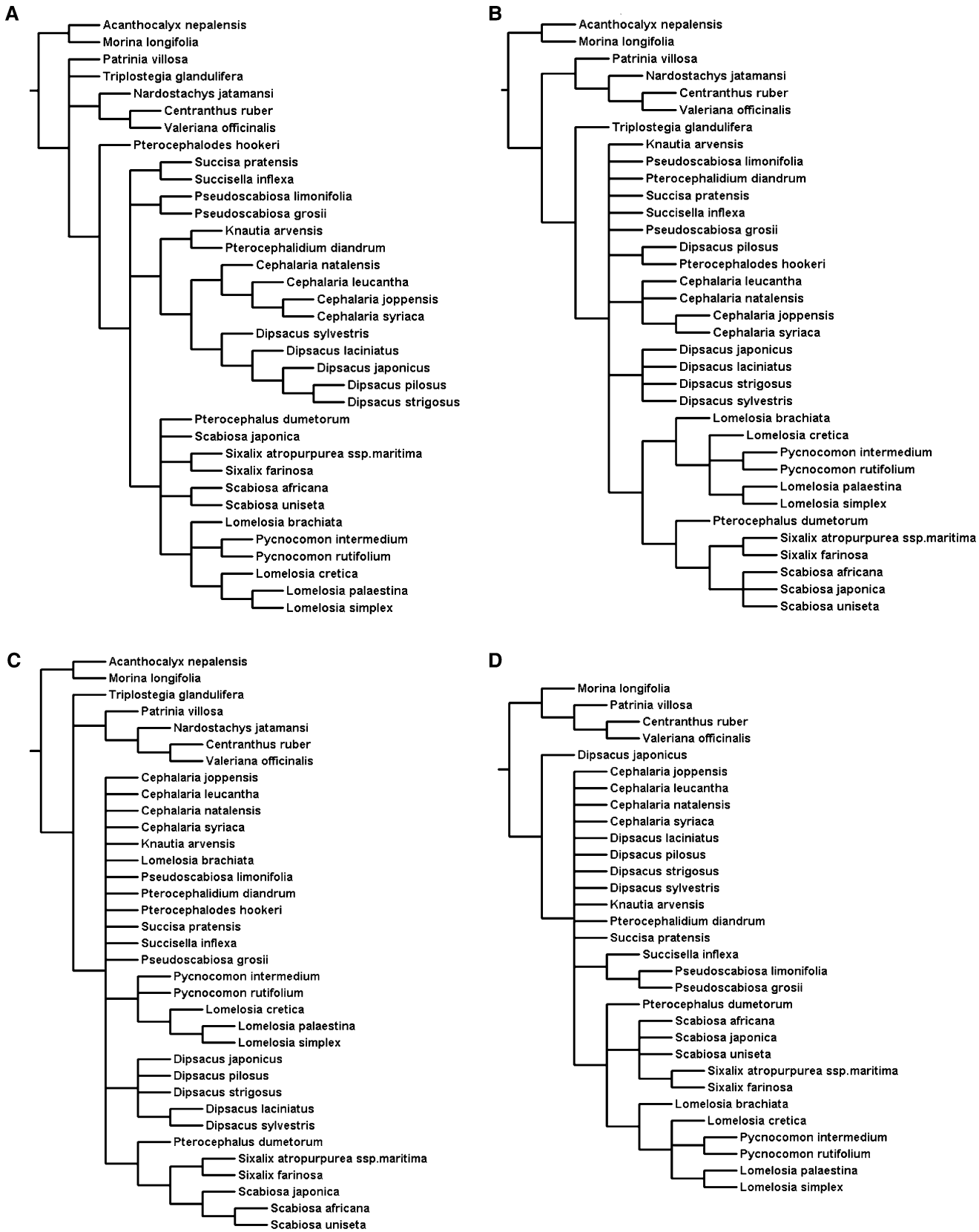
Table 3 Consensus length (CL), number of most parsimonious trees, with length, CI's and RI's for the data sets used in this paper

Matrix	Summary statistics				
	CL (bp)	No. of MP trees	*L*	CI	RI
ITS	589	5	885	0.55	0.58
	273		764	0.48	0.58
*trn*L–F IGS	404	4	157	0.87	0.91
	63		100	0.80	0.91
*trn*L intron	627	273	155	0.89	0.89
	54		81	0.79	0.89
*psb*B–*psb*H	670	1	121	0.91	0.87
	45		57	0.80	0.87
Combined	2,290	1	1,332	0.65	0.68
	436		1,014	0.55	0.68

The second row in the datasets contains the values after removal of uninformative characters

The *trn*L–F IGS data matrix for the 35 accessions consisted of 404 characters (63 of which are informative). Parsimony analysis (excluding equivocal indels in pos. 232–233 of the *trn*L–F alignment) resulted in four equally parsimonious trees (see Table 3 for summary statistics). Also this strict consensus tree (Fig. 1b) shows various collapses, and the few visible clade include one with *Pterocephalodes hookeri* and *Dipsacus pilosus*, another with the remaining species of *Dipsacus*, a group including all investigated species of *Cephalaria*, a clade including *Lomelosia* and *Pycnocomon*, and another with *Pterocephalus*, *Scabiosa* and *Sixalix*.

The *trn*L intron data matrix for the 35 accessions consisted of 627 characters, 54 of which are informative. Parsimony analysis (excluding equivocal indels in positions 149–159 of the *trn*L alignment) resulted in 273 equally parsimonious trees (see Table 3 for summary statistics). The strict consensus tree (Fig. 1c) shows extensive collapses and the ingroup shows only few clades occurring in all the MP cladograms. In particular, a clade including the two species of *Pycnocomon* and three species of


A
Acanthocalyx nepalensis
Morina longifolia
Patrinia villosa
Triplostegia glandulifera
Nardostachys jatamansi
Centranthus ruber
Valeriana officinalis
Pterocephalodes hookeri
Succisa pratensis
Succisella inflexa
Pseudoscabiosa limonifolia
Pseudoscabiosa grosii
Knautia arvensis
Pterocephalidium diandrum
Cephalaria natalensis
Cephalaria leucantha
Cephalaria joppensis
Cephalaria syriaca
Dipsacus sylvestris
Dipsacus laciniatus
Dipsacus japonicus
Dipsacus pilosus
Dipsacus strigosus
Pterocephalus dumetorum
Scabiosa japonica
Sixalix atropurpurea ssp.maritima
Sixalix farinosa
Scabiosa africana
Scabiosa uniseta
Lomelosia brachiata
Pycnocomon intermedium
Pycnocomon rutifolium
Lomelosia cretica
Lomelosia palaestina
Lomelosia simplex
B
Acanthocalyx nepalensis
Morina longifolia
Patrinia villosa
Nardostachys jatamansi
Centranthus ruber
Valeriana officinalis
Triplostegia glandulifera
Knautia arvensis
Pseudoscabiosa limonifolia
Pterocephalidium diandrum
Succisa pratensis
Succisella inflexa
Pseudoscabiosa grosii
Dipsacus pilosus
Pterocephalodes hookeri
Cephalaria leucantha
Cephalaria natalensis
Cephalaria joppensis
Cephalaria syriaca
Dipsacus japonicus
Dipsacus laciniatus
Dipsacus strigosus
Dipsacus sylvestris
Lomelosia brachiata
Lomelosia cretica
Pycnocomon intermedium
Pycnocomon rutifolium
Lomelosia palaestina
Lomelosia simplex
Pterocephalus dumetorum
Sixalix atropurpurea ssp.maritima
Sixalix farinosa
Scabiosa africana
Scabiosa japonica
Scabiosa uniseta
C
Acanthocalyx nepalensis
Morina longifolia
Triplostegia glandulifera
Patrinia villosa
Nardostachys jatamansi
Centranthus ruber
Valeriana officinalis
Cephalaria joppensis
Cephalaria leucantha
Cephalaria natalensis
Cephalaria syriaca
Knautia arvensis
Lomelosia brachiata
Pseudoscabiosa limonifolia
Pterocephalidium diandrum
Pterocephalodes hookeri
Succisa pratensis
Succisella inflexa
Pseudoscabiosa grosii
Pycnocomon intermedium
Pycnocomon rutifolium
Lomelosia cretica
Lomelosia palaestina
Lomelosia simplex
Dipsacus japonicus
Dipsacus pilosus
Dipsacus strigosus
Dipsacus laciniatus
Dipsacus sylvestris
Pterocephalus dumetorum
Sixalix atropurpurea ssp.maritima
Sixalix farinosa
Scabiosa japonica
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Dipsacus pilosus
Dipsacus strigosus
Dipsacus sylvestris
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Pterocephalidium diandrum
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Succisella inflexa
Pseudoscabiosa limonifolia
Pseudoscabiosa grosii
Pterocephalus dumetorum
Scabiosa africana
Scabiosa japonica
Scabiosa uniseta
Sixalix atropurpurea ssp.maritima
Sixalix farinosa
Lomelosia brachiata
Lomelosia cretica
Pycnocomon intermedium
Pycnocomon rutifolium
Lomelosia palaestina
Lomelosia simplex

◀ **Fig. 1** Trees obtained from the four separate maximum parsimony analyses (see text for details). **a** Consensus of five equally parsimonious trees for the ITS1 and ITS2 data matrix (tree length = 885; CI = 0.55; RI = 0.58; excluding autapomorphies: tree length = 764; CI = 0.48; RI = 0.58). **b** Consensus of four equally parsimonious trees for the *trn*L–F IGS data matrix (tree length = 157; CI = 0.87; RI = 0.91; without uninformative characters: tree length = 100; CI = 0.80; RI = 0.91). **c** Consensus of 273 equally parsimonious trees for the *trn*L intron data matrix (tree length = 155; CI = 0.89; RI = 0.89; without uninformative characters: tree length = 81; CI = 0.79; RI = 0.89). **d** Single most parsimonious tree for the *psb*B–*psb*H data matrix (tree length = 121; CI = 0.91; RI = 0.87; without uninformative characters: tree length = 57; CI = 0.80; RI = 0.87). Matrices for trees **a**–**c** include 35 taxa and matrix for tree **d** includes 31 taxa

Lomelosia, another including all species of *Dipsacus* and a third including *Pterocephalus* and the species of *Scabiosa* and *Sixalix*.

The *psb*B–*psb*H data matrix included only 31 accessions (sequences for this marker were missing for *Acanthocalyx*, *Nardostachys*, *Pterocephalodes* and *Triplostegia*) and consisted of 670 characters, 45 of which are informative. Parsimony analysis (excluding equivocal indels in pos. 156–164, 200–218 and 375–378 of the *psb*B–*psb*H alignment) resulted in a single most parsimonious tree (see Table 3 for summary statistics). The MP cladogram exhibits numerous polytomies (Fig. 1d). *Dipsacus japonicus* is sister to the rest of the family, and only a clade including *Succisella* and *Pseudoscabiosa* and another including *Pterocephalus*, *Scabiosa*, *Sixalix*, *Lomelosia* and *Pycnocomon* can be observed.

The ILD test (Farris et al. 1994) indicated that the nuclear and choloroplastic matrices were not significantly incongruent ($P = 0.3980$). As a consequence, the four data sets were combined in a single matrix. No literature sequences were available in the *psb*B–*psb*H region for three outgroups and one ingroup taxa (namely *Acanthocalyx*, *Nardostachys*, *Pterocephalodes* and *Triplostegia*), and plant material was not available; therefore, after exploratory analyses (both scoring with gaps as missing data and coding them as binary characters) which showed that topological distribution of the other OTUs did not change by excluding those four taxa, the mentioned regions of these taxa were substituted with unknown characters. The combined matrix of 35 samples consisted of 2,290 characters (436 of which are informative).

Parsimony analysis (excluding all the equivocal indels listed above, gaps treated as missing data) resulted in one single MP cladogram (see Table 3 for summary statistics). This fully resolved tree (Fig. 2) was rooted with *Morina* and *Acanthocalyx* (Morinaceae). A clade including all investigated Valerianaceae is sister to a clade in which *Triplostegia* is in its turn sister to Dipsacaceae. The family is divided into two major clades, one of which (Clade A) includes *Pterocephalus*, *Sixalix* (two species), *Scabiosa* (three species), *Lomelosia* (four species) and *Pycnocomon* (two species); the other major clade (the reason why we do not call it "Clade B", but prefer to give that name to a monophyletic subset of this clade will be clear below) includes *Pterocephalodes*, *Succisa*, *Succisella*, *Pseudoscabiosa* (two species), *Knautia*, *Pterocephalidium*, *Cephalaria* (four species) and *Dipsacus* (five species).

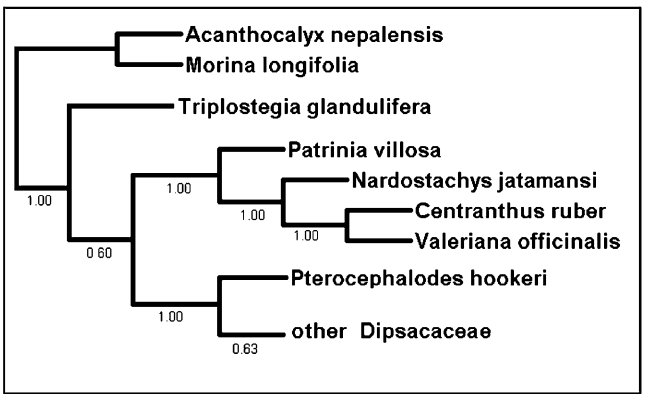
Clade A is in its turn divided into two monophyletic groups; one of them shows *Pterocephalus* at the base, in a sister group relationship with *Sixalix* and *Scabiosa*. This latter genus is represented in this investigation by a ladderized sequence of *Scabiosa japonica*, *Scabiosa africana* and *Scabiosa uniseta*. The other monophyletic group within Clade A includes *Lomelosia* and *Pycnocomon*. *Lomelosia brachiata* is basal to a group including the two species of *Pycnocomon*, which are sister to a ladderized group including *L. cretica*, *L. palaestina* and *L. simplex*.

The other major clade of the tree in Fig. 2 shows *Pterocephalodes* as sister to the remaining taxa. This latter clade (Clade B) is in its turn divided into two monophyletic units. The first includes *Succisa* and *Succisella* in a sister group relationship and both in their turn sister to a clade including *Pseudoscabiosa limonifolia* and *Pseudoscabiosa grosii*. The other clade shows the sister genera *Knautia* and *Pterocephalidium* to be sister to a clade including *Cephalaria* and *Dipsacus*. Within *Cephalaria*, *C. natalensis* is basal, followed by a ladderized group including *C. leucantha*, *C. joppensis* and *C. syriaca*. Genus *Dipsacus* shows *D. sylvestris* sister to the clade composed of *D. japonicus*, *D. pilosus* and *D. strigosus*.

Bootstrap support and clade decay index are rather low, and, however, higher within Clade A than in Clade B plus *Pterocephalodes hookeri* (Fig. 2). In particular, in the latter group, only the clade including *Succisa*, *Succisella* and *Pseudoscabiosa*, the clade of genus *Cephalaria* and that of genus *Dipsacus* show bootstrap percentages above 50%.

The elision approach generated a 15,954 character matrix (3,855 of which are informative) for the combined elided dataset. Analysis yielded a single cladogram (35 accessions; tree length = 15,242; CI = 0.60; RI = 0.65; without uninformative characters: tree length = 12,438; CI = 0.51; RI = 0.65) whose topology (data not shown) differs from that of the MP combined analysis with gaps as missing data (Fig. 2) in that *Triplostegia* is sister to a clade including Valerianaceae and Dipsacaceae; *Pterocephalodes* is sister group to all remaining Dipsacaceae; *Pterocephalidium* and *Knautia* are ladderized at the base of a clade including *Pseudoscabiosa*, *Succisa* and *Succisella*. Therefore, it appears that indel distribution does not influence topology to a great extent (especially in the light of the Bayesian topology discussed below).

BI (gaps treated as missing data) produced a consensus tree whose topology is rather similar to that of the single MP tree described above. The differences (Fig. 2, boxes)



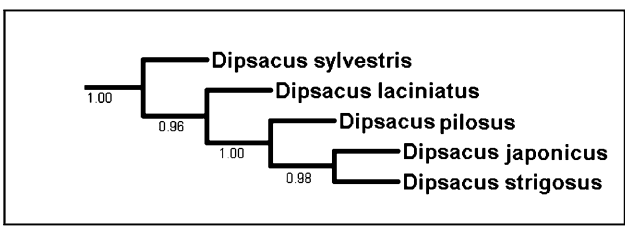


Fig. 2 Single MP cladogram for the combined matrix with indels regarded as missing data (tree length = 1,332; CI = 0.65; RI = 0.68; without uninformative characters: tree length = 1,014; CI = 0.55; RI = 0.68). The combined dataset includes 35 samples and 2,290 characters (436 of which informative). *Numbers* on internodes represent the following: above branch, decay index (in *bold* type) and number of unambiguous changes; below branch posterior probability and bootstrap percentages (*italicized*). Bootstrap values below 50% are not reported. Support for the most inclusive ingroup not shown. The two major clades of Dipsacaceae have been labelled with "*A*" and "*B*". Different resolutions obtained in Bayesian inference analysis (*boxes*) (see text for details)

are the position of *Triplostegia* (sister to a clade including Valerianaceae and Dipsacaceae), the position of *Pterocephalodes hookeri* (sister to the rest of the family) and in the relative positions of two species of *Dipsacus*. Posterior probabilities are quite low (especially for the sister group relationship between Valerianaceae and Dipsacaceae, for the position of *Pterocephalodes hookeri* and for the relationships between *Cephalaria* and *Dipsacus*).

As we mentioned before, another parsimony analysis and BI were conducted on the same combined matrix, but coding gaps as binary.

Ninety-three characters were generated, and therefore the final matrix was 2,483 characters long (507 informative characters). Phylogenetic analysis resulted in two MP cladograms (tree length = 1,610; CI = 0.66; RI = 0.67; without uninformative characters: tree length = 1,170; CI = 0.53; RI = 0.67), one of which is shown in Fig. 3. This tree shows few differences when compared to that of MP in Fig. 2. Amongst them, the position of *Triplostegia*, which is now sister to a clade including Dipsacaceae and representatives of Valerianaceae and the position of *Pterocephalodes hookeri*, which is the basalmost taxon in the family. A minor difference is the position of *D. japonicus*, which is here sister to *D. strigosus*. Interestingly enough, the topology of this tree is identical to that of the tree obtained by the Bayesian analysis of the complete matrix without indels (Fig. 2, boxes). The strict consensus tree (not shown) indicates that the clade including *Succisa*, *Succisella* and *Pseudoscabiosa* collapses in such a way that relationships with the rest of Dipsaceae and with Scabioseae are unclear. BI produced a majority rule consensus tree whose topology is identical to that of the MP tree (Fig. 3) described above (this is the reason why we preferred to show one cladogram instead of the consensus for the MP analysis).

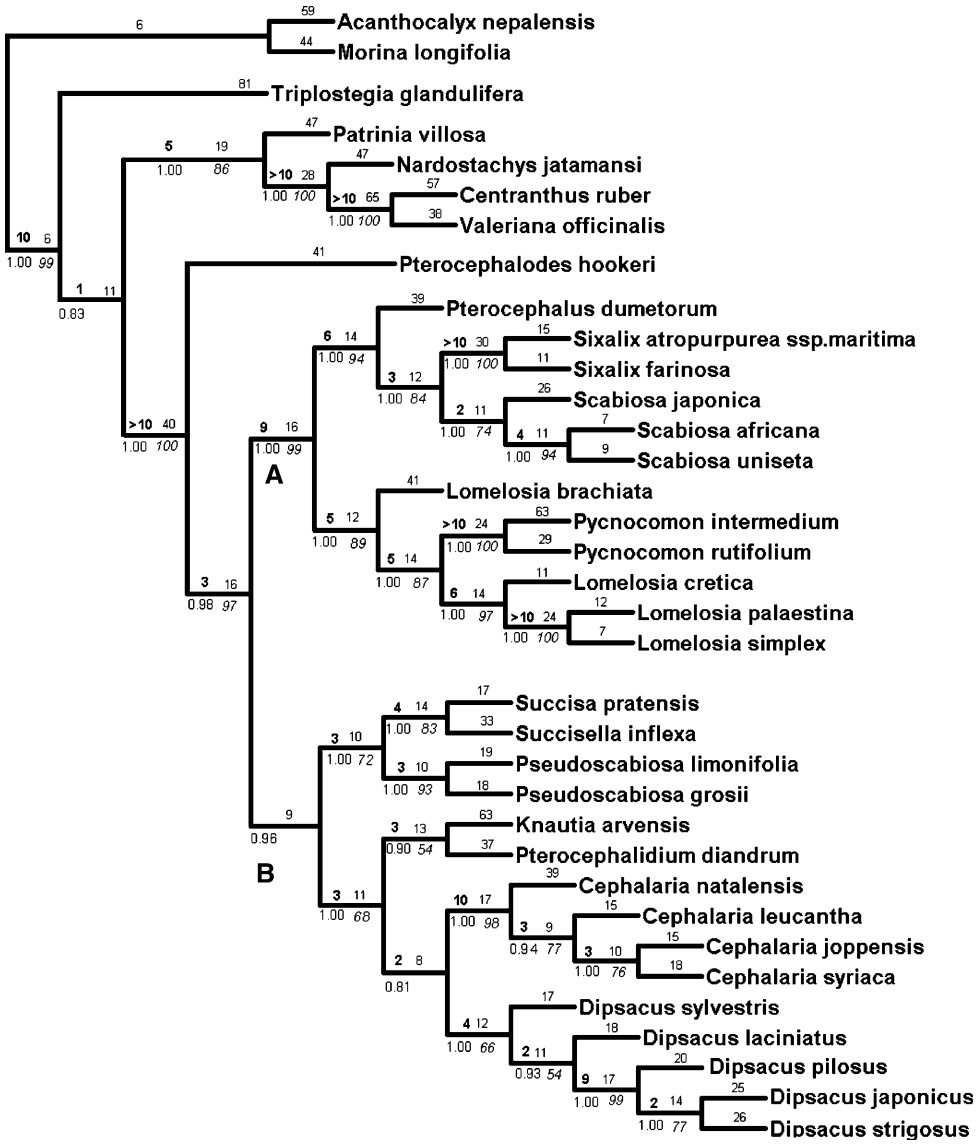


Fig. 3 One of the two MP cladograms for the combined matrix with indels scored as binary characters (tree length = 1,610; CI = 0.66; RI = 0.67; without uninformative characters: tree length = 1,170; CI = 0.53; RI = 0.67), the combined dataset includes 35 samples and 2,483 characters (507 of which informative). *Numbers* on internodes represent the following: above branch, decay index (in *bold* type) and number of unambiguous changes; below branch posterior probability and bootstrap percentages (*italicized*). Bootstrap values below 50% are not reported. Support for the most inclusive ingroup not shown. The clades labelled "*A*" and "*B*" include the same OTUs as in Fig. 2

Bootstrap support, clade decay index and posterior probabilities are (Fig. 3) much higher in this case than in the tree of Fig. 2.

Obviously, the elision approach was not repeated on the combined matrix with indels scored as binary characters.

Direct optimization (Wheeler 1996, 2003) of sequences yielded a 2,534 character matrix (390 of which are informative). Phylogenetic analysis (gaps were regarded as missing data) resulted into two equally parsimonious cladograms (35 accessions, tree length = 1,446; CI = 0.66; RI = 0.72; excluding uninformative characters: tree length = 1,097; CI = 0.56; RI = 0.72). The consensus tree topology (not shown) is more similar to that of Fig. 3 (i.e. that obtained scoring indels as binary characters) than to that in Fig. 2. In particular, the only difference between the directly optimized topology and that of Fig. 3 is that a sister group relationship occurs between the clade including *Knautia* and *Pterocephalidium* and that including *Pseudoscabiosa*, *Succisa* and *Succisella*.

Comparison of sequence substitution rates (Robinson et al. 1998; Robinson-Rechavi and Huchon 2000) amongst tribes, major clades, genera and informal ecological groups (e.g. anemochorous vs. mirmecochorous) of the cladogram in Fig. 2 using both the complete matrix (gaps as missing data) and its partitions showed that no lineage evolved significantly faster than any other (P always < 0.05). This test was not carried out on the matrix including indels coded as binary characters.

The average branch lengths of the internal nodes in the MP trees were 9.8 (Fig. 2) and 16.5 (Fig. 3) and were significantly different ($P < 0.0001$) from the average lengths of terminal branches, which were 19.8 (Fig. 2) and 30.4 (Fig. 3).

As shortness of internal nodes may indicate fast radiation (Shaffer and McKnight 1996), but may also depend upon the lack of phylogenetic signal in the data set, we verified our data for the presence of such signal by carrying out PTP tests (Faith and Cranston 1991) as implemented in TNT (Goloboff et al. 2003), both on the matrix expressing the tree shown in Fig. 2 and on that expressing the tree shown in Fig. 3. These tests allowed in both cases rejection of the the null hypothesis that data do not have cladistic structure ($P \leq 0.01$).

To verify which morphological and karyological characters may be interpreted as synapomorphies for various clades, corolla lobe numbers, pollen types and chromosome haploid numbers have been traced (Fig. 4) on the MP cladogram shown in Fig. 3. A MP model was chosen in spite of its drawbacks in reconstructing ancestral states (Cunningham et al. 1998) after observing that, for all three characters, reconstruction was identical to that employing maximum likelihood (within the significance threshold indicated by the software).

Discussion

Increasing the number of taxa and doubling the number of base pairs over the only previous family level molecular analysis (Caputo et al. 2004) allowed us to investigate various details of Dipsacaceae phylogeny and to better understand the evolution of various characters. The presence, in our investigation, of *Pterocephalodes hookeri* as well as various species of genera *Cephalaria*, *Dipsacus* and *Lomelosia* and two species of *Pycnocomon* and *Pseudoscabiosa* indeed allows insights on the phylogeny of these genera.

First of all, some comments are appropriate on the position of *Pterocephalodes hookeri*. As mentioned earlier, *Pterocephalodes/Bassecoia* has a history of taxonomic intricacies which is even more complicated than usual for the family. Regardless of the name, the three species which are included in the genera clearly seem to be related, even if the presence of tetramerous corolla and floral bracts, and lack of a diaphragma (Mayer and Ehrendorfer 2000), as well as the multiplied calyx bristles are not necessarily synapomorphic (indeed, at least the second and third characters are probably synplesiomorphic). *P. hookeri*, which was employed in our analysis, is regarded as the basalmost taxon in the group, given the fact that *B. bretschneideri* and *B. siamensis* share some presumably derived characters (amongst which a developed corona) in the cladogram by Mayer and Ehrendorfer (2000; Fig. 25, sub *Pterocephalodes bretschneideri* V. Mayer et Ehrend. and *Pterocephalus siamensis* V. Mayer et Ehrend.).

Pterocephalodes hookeri appears as basal to Clade B in the MP tree of Fig. 2; however, in the MP analysis of the nuclear DNA partition of our original data set (Fig. 1a), in the elision approach, in the MP investigation combined matrix in which indels were coded as binary characters (Fig. 3), in both Bayesian analyses (Figs. 2box, 3) and in the direct optimization investigation, it is indeed the sister group to the rest of the family; in addition, forcing topology of the tree in Fig. 2 with *Pterocephalodes* as sister to the rest of the family would only cause a 1-step increase in the length of the MP tree. Given the fact that in the MP cladogram shown in Fig. 3 (which is topologically identical to the Bayesian consensus), a basal position of *Pterocephalodes hookeri* is strongly supported (97% bootstrap value, clade decay index di = 3, posterior probability $P = 0.98$), we provisionally accept its position as indicated by both MP investigation and Bayesian analysis of Fig. 3, even if data from the other two species potentially belonging to the clade that are lacking. Mayer and Ehrendorfer (2000) already indicated that *Pterocephalodes/Bassecoia* was far removed from *Pterocephalus* and they suggested that its position would be within our Clade B, although with a quite different topology than that

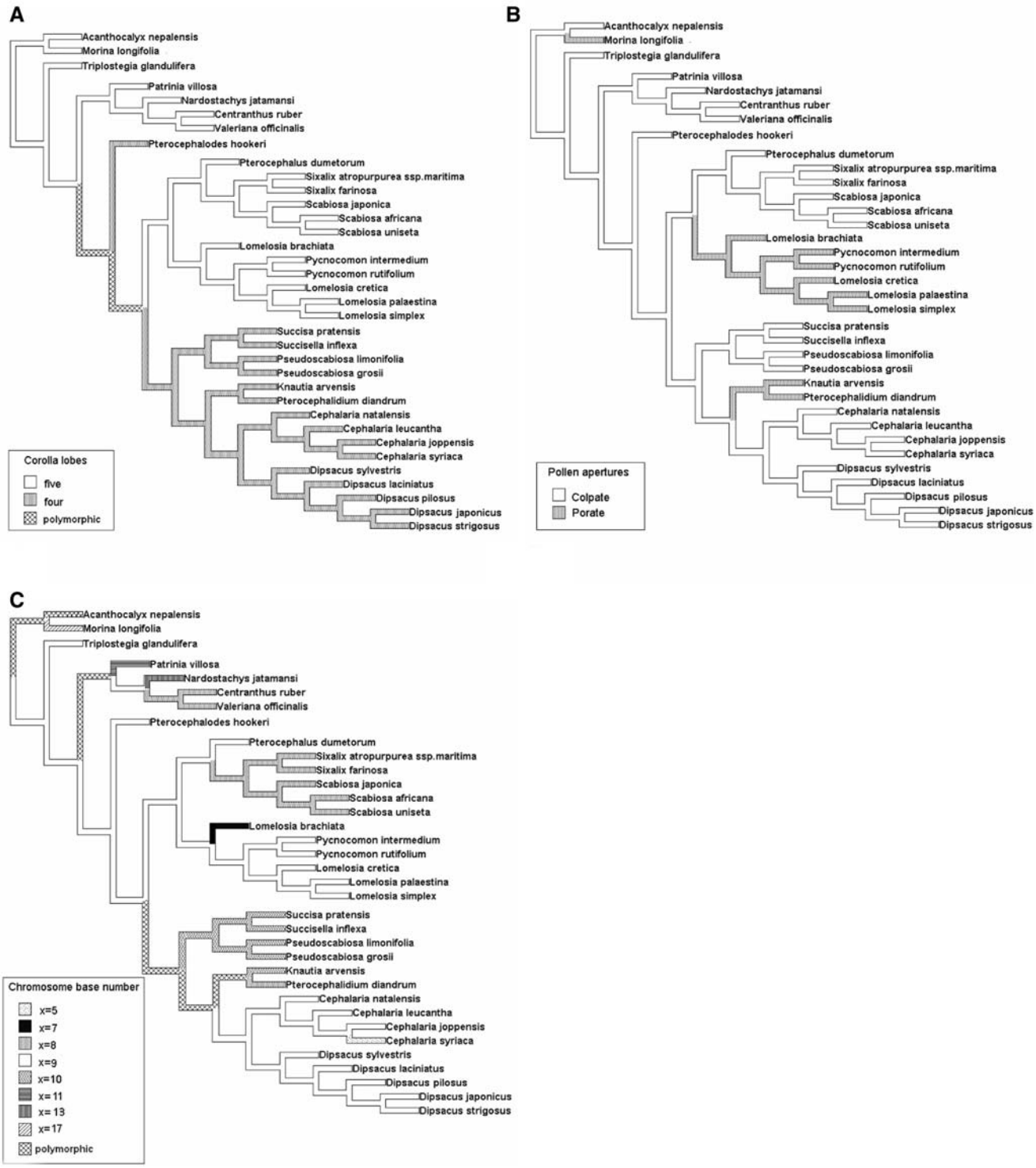


Fig. 4 Unambiguous changes of corolla lobe numbers (**a**), pollen types (**b**) and chromosome base numbers (**c**) plotted on the tree shown in Fig. 3. Characters are unordered and have been reconstructed under a maximum parsimony criterion after observing that state distribution was identical to that obtained using a maximum likelihood criterion (within the significance threshold). In **c** *Valeriana officinalis* was reconstructed as $x = 8$, even if its diploid number is $2n = 14$, as the great majority of basal species in the genus have $2n = 16$ or multiples; for graphical reasons, *Acanthocalyx* was reconstructed as polymorphic, whereas its chromosome number is unknown. Chromosome numbers are from Verláque (1986b) and from Mayer and Ehrendorfer (1999, 2000)

is shown in Fig. 2. Its basal position in the family (Fig. 3) is the first non-morphological indication of its remoteness from *Pterocephalus*. Interestingly enough, *Pterocephalodes hookeri* has a chromosome haploid number $n = 9$ (Mayer and Ehrendorfer 2000), whereas the other two species, according to the above-mentioned authors, have a putatively different chromosome number $n = 8$. Regardless, as *Pterocephalodes hookeri*, as previously said, is presumably basal in the *Pterocephalodes/Bassecoia* group (Mayer and Ehrendorfer 2000), we reconstructed *Pterocephalodes/Bassecoia* as primitively having $n = 9$ (which is the plesiomorphic number for the family, Fig. 4c).

The sister group relationship between *Succisa*/*Succisella* and *Pseudoscabiosa* (Fig. 2: bootstrap value 54%, clade decay index di = 2, posterior probability $P = 1.0$; Fig. 3: bootstrap value 72% clade decay index di = 3, posterior probability $P = 1.0$) is difficult to document under a morphological perspective. In fact, these taxa share various symplesiomorphies, which make them alike superficially (e.g. globose capitula). These taxa share a base chromosome number $x = 10$, which, however, at present may not be regarded as synapomorphic, given the uncertainty in reconstruction (Fig. 4c). *Pseudoscabiosa* is another genus in which, as in *Pterocephalodes/Bassecoia*, the shape of the corona (and possibly its role in dispersal) varies. Interestingly, *Pseudoscabiosa grosii*, the only species of the genus whose corona is made of four lobes (as opposed to a membranous rim) also has up to six long, plumose, calyx bristles, whereas the other two species, which have a membranous corona, have four much shorter more or less glabrous calyx bristles.

Another interesting issue is the relative position of *Knautia* and *Pterocephalidium*. Their sister group relationship was already documented in Caputo et al. (2004), but the authors indicated that their position needed further verification. Our expanded analysis with two further markers and 16 further taxa gave the same evidence (elision topology excluded). Posterior probability for the clade and decay index ($P = 0.98$, di = 3) are comparatively high, but bootstrap percentage is, in this case, very low (<50%). Interestingly enough, *Knautia* and *Pterocephalidium* are the only two members of Clade B sharing triporate pollen (Verláque 1985a, b), a character state which is synapomorphic for the clade, although homoplastic for the family (Fig. 4b). Forcing a topology in which *Knautia* is sister to the clade including *Succisa*, *Succisella* and *Pseudoscabiosa* would cause an increase in three steps on the tree of Fig. 2 and six steps on the tree of Fig. 3 in length when compared to the MP solution; forced topologies with *Knautia* as sister to the rest of Clade B show an increase of seven steps in both trees; forcing the said genus as sister to the rest of the family would cause a 10-step increase for Fig. 2 and 14 steps for Fig. 3.

Knautia, a member of the separate, monogeneric tribe Knautieae in virtually all literature, has been variously considered very close to *Succisa* and *Succisella* (Ehrendorfer 1964b, 1965), mainly as a consequence of a base haploid chromosome number $x = 10$; as sister group to the rest of the family (Verláque 1985a); or as sister to a group that corresponds to Scabioseae sensu lato, *non* Mayer and Ehrendorfer (Caputo and Cozzolino 1994). The morphological autapomorphies of *Knautia* within the family (i.e. zygomorphic epicalyx, epicalyx pedicel transformed into an elaiosome, receptacular bracts substituted by stiff hairs, absence of evident ribs on the epicalyx tube) may be easily justified in the light of myrmechocory (Ehrendorfer 1962), the diaspore dispersal syndrome in *Knautia*, which is unique in the family.

Within *Cephalaria*, the topology of the cladograms is entirely compatible with the indications by Verláque (1985a, 1986b) and Caputo and Cozzolino (1994). In fact, *C. natalensis*, belonging to the South African subg. *Lobatocarpus* Szabó (with a four-lobed corona) is basal within the genus, followed by *C. leucantha*, belonging to the paleoendemic Mediterranean subg. *Fimbriatocarpus* Szabó (with a fimbriated corona), and two representatives of subg. *Cephalaria* (with a eight-toothed corona), distributed from the Mediterranean area to Iran. This topology would call for an early vicariance event between South Africa (stem lineage of subg. *Lobatocarpus*) and the Mediterranean basin (stem lineage of the rest of the genus).

Topology of our trees for the representative species of genus *Dipsacus* does not correspond to the inferences drawn from the morphology. In fact, amongst the five species employed, two (*D. japonicus* and *D. pilosus*) belong to the allegedly archaic (Verláque 1985b) section *Spaerodipsacus* Lange, characterised by globose capitula and absence of prickles (two likely plesiomorphies), whereas the other three belong to sect. *Dipsacus*, with oblong capitula and spiny. However, apparently neither section is monophyletic, as *D. pilosus* and *D. japonicus* are nested together with the other species. This would suggest that spinosity and elongated capitula are plesiomorphic for the genus. However, in this case, we prefer not to expound further, as no central to East Asian species is present in our analysis, and topology of the *trn*L intron consensus tree (Fig. 1c) would give different suggestions. However, by having added literature sequences of *D. mitis* (for which only ITS is available) or *D. asper* or *D. asperoides* (for which only *trn*L intron and *trn*L–F IGS sequences are available) to a subsample matrix including only *Dipsacus* and *Cephalaria* (data not shown) in no case a monophyletic sect. *Sphaerodipsacus* or *Dipsacus* was obtained, as well as in no case a single *Sphaerodipsacus* species was basal.

Mayer and Ehrendorfer (1999), on the basis of an accurate morphological study, suggested an improved

circumscription of tribe Scabioseae, including only *Pterocephalus*, *Lomelosia*, *Pycnocomon*, *Scabiosa* and *Sixalix* (which corresponds to Clade A of Fig. 2). They, however, only hint at a circumscription of Dipsaceae (Mayer and Ehrendorfer 2000) giving no indication on the position of *Knautia*. We restrain from any further comment on the matter, given the fact that, even if the majority of our analyses would suggest that tribe Dipsaceae can be circumscribed as including *Cephalaria*, *Dipsacus*, *Knautia*, *Pseudoscabiosa*, *Pterocephalidium*, *Succisa* and *Succisella* (Clade B of Figs. 2, 3), alternative topologies exist, in which either the *Pseudoscabiosa*/*Succisa*/*Succisella* clade is not included in Clade B or *Pterocephalodes hookeri* is included and belongs to Clade B; moreover, our investigation includes only a single species of *Knautia*, out of that at least 50 known. Finally, no morphological synapomorphy is known, at present, for Clade B. A tetramerous corolla (Fig. 4a) is present in all members of Clade B (Verláque 1985a; Mayer and Ehrendorfer 1999, 2000), but also in *Pterocephalodes*/*Bassecoia*. Pentamery is regarded as a plesiomorphic condition for Dipsacales, and within Scabioseae, tetramery is quite sporadic (Van Steenis 1948; Weberling 1975, 1978; Verláque 1985a; Cannon and Cannon 1984; Donoghue et al. 2003).

Phylogeny of Clade A of our investigation (i.e. Scabioseae sensu Mayer and Ehrendorfer) is identical, for the taxa in common, to that depicted in Caputo et al. (2004) and in Mayer and Ehrendorfer (2000) and therefore will not be discussed here in detail. It shows, however, a difference, when compared to what was indicated by Mayer and Ehrendorfer (1999; Fig. 14), in the position of genus *Pterocephalus*.

Given this discrepancy, we chose *Pterocephalus dumetorum* as representative of the genus *Pterocephalus* in our investigation, as it belongs to the most archaic group of species ("type 1") as far as epicalyx morphology is concerned according to Mayer and Ehrendorfer (2000).

Particularly interesting is the topology shown in the cladogram of Figs. 2 and 3 for the species of genus *Lomelosia*. In fact, its basalmost species is *L. brachiata* (formerly placed in the monospecific genus *Tremastelma* Rafin.), a Mediterranean annual with multiplied calyx bristles (ten instead of five) and a quite robust epicalyx with eight large foveoles. Even if the number of species in our investigation is too low to draw general conclusions, all previous literature (Verláque 1986a, b) indicates that annuals with large foveoles are quite derived in the genus. Moreover, genus *Pycnocomon*, with its prismatic epicalyx, adapted to roll on sand (Verláque 1986b), appears as clearly nested in *Lomelosia* (Figs. 2, 3), and may be interpreted only as a psammophilous morphotype of the latter genus, in spite of its divergent morphological characteristics (connate involucrum, heterocarpy).

Given the evidence shown above, either two species of *Pycnocomon* should be included in *Lomelosia*, or a different generic name should be used for *L. brachiata*. For this latter species, a host of names is available, under at least four genera. A suitable name, i.e. *Tremastelma palaestinum* (L.) Janchen is available, and some authoritative literature sources (Verláque 1977b, 1986b) do recommend to employ it. However, Mayer and Ehrendorfer (1999), recommend to keep *L. brachiata* within *Lomelosia* "because *Tremastelma* shares all important synapomorphies with *Lomelosia* (e.g. elongated horizontal epidiaphragma, deep pits, second sclerenchyma ring). Its specific apomorphies (chromosome number $x = 7$ and the multiplied flattened calyx) do not contradict such an interpretation" (Mayer and Ehrendorfer 1999). However, they recommend to keep it in a section of its own, *Lomelosia* sect. *Callistemma* (Mert. et Koch) V. Mayer et Ehrend.

By the same token, *Pycnocomon* and *Lomelosia* share triporate (Fig. 4b) and operculate pollen, and epicalyx morphology with deep foveoles and with a second sclerenchyma ring in its anatomy structure (Verláque 1986a; Mayer and Ehrendorfer 1999). In particular, epicalyx enclosure in *Pycnocomon* and its very lignified structure are very similar to those of some morphologically archaic *Lomelosia* species like *L. cyprica* not present in our analysis (Verláque 1986a). However, they also share chromosome base number ($x = 9$), which is plesiomorphic for the family.

As a consequence, we regard including the two species of *Pycnocomon* within *Lomelosia* as appropriate, so as to complete the nomenclatural work on Dipsacaceae by Devesa (1984), Greuter and Burdet (1985), López Gonzáles (1987), Burtt (1999) and Mayer and Ehrendorfer (2000). We are conforted in doing this by further unpublished evidence based only on ITS and *trn*L intron on a larger set of species of *Lomelosia*.

Lomelosia rutifolia (Vahl) Avino et Caputo comb. nova

≡*Scabiosa baetica* Boiss., Elench. Pl. Nov. 58–59 (1838)
≡*Pycnocomon rutifolium* (Vahl) Hoffmanns. & Link, Fl. Portug. 2: 94, pl. 88 (1820–1824)
≡*Scabiosa urceolata* Desf., Fl. Atlant. 1: 122 (1798), nom. illeg.
≡*Scabiosa divaricata* Lam., Tabl. Encycl. 1: 250 (1792), nom. illeg., non Jacq.
≡*Scabiosa rutifolia* Vahl, Symb. Bot. 2: 26 (1791)

Lomelosia intermedia (Lag.) Avino et Caputo comb. nova
≡*Pycnocomon intermedium* (Lag.) Greuter & Burdet in Willdenowia 15: 76 (1985)
≡*Pterocephalus intermedius* (Lag.) Cout., Fl. Portugal 594 (1913)

≡*Pterocephalus broussonetii* Coult. in DC., Prodr. 4: 653 (1830)
≡*Pterocephalus lusitanicus* Coult. in DC., Prodr. 4: 653 (1830)
≡*Asterocephalus intermedius* Lag., Elench. Pl. [8] (1816)
≡*Scabiosa gramuntia* sensu Brot., Fl. Lusit. 1: 145 (1804), non L.

In conclusion, Dipsacaceae would consist of two large monophyletic units derived from plants originating from the stem lineage of *Pterocephalodes/Bassecoia*. No morphological synapomorphies for the two major clades can be easily found; this is especially frustrating in Scabioseae, which are morphologically cohesive as presently circumscribed. A more or less developed membranous corona (i.e. the condition of all members of *Lomelosia*, *Pycnocomon*, *Scabiosa* and *Sixalix*) is absent from the majority of members of *Pterocephalus* (and also present in few members of Clade B and *Bassecoia*); by the same token, the diaphragma (a protrusion of the distal part of the inner side of the epicalyx tube, which contributes to a better encasing of the fruit present in *Lomelosia*, *Pterocephalus* p.p., *Pycnocomon*, *Scabiosa* and *Sixalix*) is absent from various members of *Pterocephalus*.

Mayer and Ehrendorfer (1999, 2000) suggested a basal haploid chromosome number $x = 9$ as synapomorphic for Scabioseae but, as shown in Fig. 4c, it is indeed plesiomorphic for the family.

At present, therefore, no morphological synapomorphy may be found for the tribe; as only relevant sequence length mutation, the *trn*L intron sequences of all Scabioseae show a tandem duplication of a CATWGAA motif (starting from pos. 283 in the *Pterocephalus dumetorum* sequence, GenBank acc. no. AM296205); however, the diaphragma may be a potential synapomorphy for Scabioseae as circumscribed here in case we assume that it was secondarily lost in the species of *Pterocephalus* in which it is absent. This, however, would contradict the hypothesis of Mayer and Ehrendorfer (2000), who clearly indicate the diaphragma in *Pterocephalus* as homoplasiously developed as compared to that in *Lomelosia*, *Pycnocomon*, *Scabiosa* and *Sixalix*.

The difference in the length between internal and terminal branches (i.e. the fact that lower internodes are defined by fewer character state changes) may indicate that early evolution in Dipsacaceae was characterised by a period of fast radiation (Shaffer and McKnight 1996). Such difference may also be related to constant diversification followed by recent extinction. However, the absence of true morphological synapomorphies in the major clades of the family (see earlier in the "Discussion"), and their presence in the majority of genera may be better explained by postulating a fast timing in the early cladogeny of the family (causing the quick, parallel onset of separate body plans from a common ancestor). Even if further study is needed in order to make our hypothesis less speculative, we would like to notice that besides the two largest clades of the family (Clades A and B in Fig. 3), also other well-supported monophyla in our investigation (*Pseudoscabiosa/Succisa/Succisella*, *Pterocephalus/Scabiosa/Sixalix*, *Cephalaria/Dipsacus*), in spite of morphological resemblance (which, for what just said, is mainly a consequence of synplesiomorphies), lack clear shared derived morphological traits.

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