Molecular phylogenetics of Dipsacaceae reveals parallel trends in seed dispersal syndromes

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Abstract. Phylogenetic relationships among 17 taxa of Dipsacaceae were inferred from nucleotide sequence variation in both the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA and the chloroplast $trnL^{(UAA)}$ intron sequences. The combined phylogenetic analysis, carried out by using two taxa from Valerianaceae as an outgroup yielded a single most parsimonious tree, in which Dipsacaceae are divided into two major clades: one including *Lomelosia* and *Pycnocomon*, both in a sister group relationship with a clade containing *Pterocephalus*, *Scabiosa* and *Sixalix*; the other including *Pseudoscabiosa*, *Succisa* and *Succisella* is sister group to *Knautia*, *Pterocephalidium*, *Dipsacus* and *Cephalaria*. The results obtained here greatly differ from previous ones based on classical morphology, but are congruent with recent findings on epicalyx differentiation and with pollen characters. In particular, our results would confirm on molecular grounds the recently restricted circumscription for Scabioseae proposed by other authors. Our phylogenetic hypothesis indicates that adaptations to seed dispersal have been a very strong driving force in Dipsacaceae evolution, with similar selective pressures causing the onset of similar epicalyx shapes and dispersal modes in a parallel fashion in various taxa. For this reason, the gross morphology of the involucel is deceptive in inferring relationships.

Dipsacaceae Juss. (Dipsacales Lindley) is a dicotyledonous family including 12–13 genera and 250–350 species, depending on circumscription, distributed in Eurasia and Africa, with the great majority of the taxa mainly centred around the Mediterranean basin. The family constitutes a monophyletic group (Ehrendorfer 1964a; Verláque 1977a, 1984a,b; Caputo and Cozzolino 1994), whose characteristics are represented by an epicalyx, which encases the fruit and whose shape, symmetry and ornamentations are taxonomically relevant, and by an involucrate head. The family is a derived member of the order Dipsacales, where it is sister group to Valerianaceae (Backlund and Bremer 1997).

Delimitation of taxa within the family has always been subject to controversy; consequently, circumscription of genera and tribes has repeatedly changed over time, because of the overall morphological similarity among the taxa in the family and of their diversity in structural detail. In particular, the classical concepts of *Scabiosa* L. s.l. and *Pterocephalus* (Vaill.) Adans. s.l. included species which are

presently attributed to eight separate genera, most of which with entirely independent phylogenetic histories.

In fact, genus *Scabiosa* was traditionally divided into five sections. After the studies by Verláque (1984a, 1985a, 1986a, b) and Devesa (1984a, b), which demonstrated independent evolutionary histories for its sections, Greuter and Raus (1985) and Greuter et al. (1986) designated *Lomelosia* Rafin. for the species traditionally belonging to sect. *Trochocephalus* Mertens et Koch, *Scabiosa* s.str. for the species belonging to sect. *Sclerostemma* Mertens et Koch, *Sixalix* Rafin. for those formerly belonging to sect. *Cyrtostemma* Mertens et Koch, *Pseudoscabiosa* Devesa for several archaic species formerly isolated in sect. *Asterothrix* Font Quer; in the same period the name *Pycnocomon* Hoffmanns. et Link was resumed in order to refer to the atypical species *Scabiosa rutifolia* Vahl, formerly placed in *Scabiosa* sect. *Pycnocomon*.

Similarly, the genus *Pterocephalus* (Vaill.) Adans., has included in the past various atypical species which have been recently segregated in *Pycnocomon* [*Pycnocomon intermedium* (Lag.) Greuter et Burdet = *Pterocephalus intermedius* (Lag.) Coutinho], *Pterocephalidium* G. López [*Pterocephalidium diandrum* (Lag.) G. López = *Pseudoscabiosa diandra* Devesa = *Pterocephalus diandrus* (Lag.) Lag.] and *Pterocephalodes* V. Mayer et Ehrend. (name adopted for three South-Eastern Asian taxa). As in the case of some of the taxa formerly lumped in a loose concept of *Scabiosa*, the taxa segregated from *Pterocephalus* are not close to the former genus from a phylogenetic point of view.

Correspondingly, the hypotheses of tribal relationships within the family have been greatly modified during time. The main differences among the various phylogenetic hypotheses produced in the recent past (Ehrendorfer 1964a,b, 1965; Verláque 1977a,b, 1984a,b, 1985a,b, 1986a,b; Caputo and Cozzolino 1994, 1995), consisted in the positions of the archaic genera *Succisa* Necker, *Succisella* Beck and *Knautia* (L.) Coult. as compared to the rest of the family and in the relative positions of the above mentioned segregates of *Scabiosa* s.l. However, all the authors listed above concurred towards the idea that tribe Scabioseae, as traditionally circumscribed (i.e. *Lomelosia*, *Pseudoscabiosa*, *Pterocephalidium*, *Pterocephalus*, *Pycnocomon*, *Scabiosa*, *Sixalix*, *Succisa*, and *Succisella*) was monophyletic.

The recent papers by Mayer and Ehrendorfer (1999, 2000) showed that a monophyletic circumscription of Scabioseae should include less taxa than previously suggested. The latter authors also indicated that the homology of parts of the epicalyx, with special reference to a differentiation of its distal region (the corona, which is a membranous expansion in various taxa), has been often misinterpreted, as a consequence of widespread modifications in response to adaptive pressures to fruit dispersal which have fostered rampant homoplasy in both the corona and the calyx. Therefore, Mayer and Ehrendorfer (1999) suggested that a monophyletic circumscription of Scabioseae should include only *Lomelosia*, *Pterocephalus* s.str., *Pycnocomon*, *Scabiosa* s.str. (*Scabiosa* sect. *Scabiosa* in their study), and *Sixalix* (*Scabiosa* sect. *Cyrtostemma* in their study). This more restricted circumscription of Scabioseae would exclude *Pseudoscabiosa*, *Pterocephalidium*, *Succisa*, *Succisella*, as well as *Pterocephalodes*.

This paper aims at a molecular verification of the pattern of descent among dipsacaceous genera, in the light of the aforementioned uncertainties, also in order to test to which extent epicalyx and calyx modifications (which are functional towards seed dispersal) developed homoplasiously. To this purpose, Internal Transcribed Spacer (ITS) regions of nuclear ribosomal DNA and chloroplast *trn*L[(UAA)] introns were sequenced for representative taxa within the family. These sequences were chosen as they are regarded to have a mutation rate which makes them suitable for studies of relationships below the family level (Baldwin et al. 1995, Gielly and Taberlet 1996).

Material and methods

The taxa employed in this study are reported in Table 1. The selection includes representatives from all recognized genera of Dipsacaceae with the exception of *Pterocephalodes*, *Scabiosiopsis* Rech. f., and *Tremastelma* Rafin. (the latter two clearly being, however, derived members of genus *Lomelosia*). All specimens were either field collected by the authors or planted from seeds and cultivated at the Botanical Garden of Naples, Italy. 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 in Caputo et al. (1991) or that of Doyle and Doyle (1990).

ITS 1 and 2 were amplified by using the primers described in Aceto et al. (1999). The chloroplast *trn*L[(UAA)] intron was amplified using the two primers reported by Taberlet et al. (1991). PCR conditions were as described in Aceto et al. (1999). PCR amplification products were purified by using Microcon 100 microconcentrators (Amicon, Danvers, MA, U.S.A.) and double-strand sequenced in both directions by 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, U.S.).

Sequences were then reduced to the appropriate length by aligning them with various sequences available in the literature. Six fictitious unknowns (N's) were added at the 3' terminus of ITS1 in all taxa to prevent terminal misalignments.

Alignments were carried out by using Clustal W ver. 1.81 (Thompson et al. 1994) with default settings, except for the parameter TRANSWEIGHT (which controls the transition/transversion ratio), which was set to one and MAXDIV (which controls the delay of the alignment for the most divergent sequences), which was set to 80%.

Aligned sequences were then visually inspected to correct gap distributions devoid of biological meaning, aiming to reduce the number of gaps. The complete alignment used for all further analyses is available upon request to the senior author.

Cladistic analyses were carried out on the ITS and *trn*L intron data separately, as well as on a combined matrix. Three different sets of analyses were conducted in relation to indel treatment: with indels included in the analyses (scoring gaps as missing data, dataset A), with equivocal indels (i.e. indels susceptible of different reconstructions) excluded (dataset B), and with unequivocal indels given unit weight (nested indels were scored as different states of a multistate character, dataset C).

In order to verify to which extent ambiguous gap positions may influence topology, the elision approach, described by Wheeler et al. (1995), was used. This technique consists in "eliding" various individual alignments into a single combined alignment on which a phylogenetic analysis is carried out.

To this aim, six alignments were carried out for ITS data and for *trn*L intron data separately. These alignments were carried out by using Clustal W ver. 1.81 with the same parameters as above, but with variable gap opening and extension costs at wide intervals in each alignment (PWGAPOPEN = GAPOPEN from 10 to 20 and PWGAPEXT = GAPEXT from 4 to 8). The six alignments obtained plus the original alignment were combined, for each dataset, in a single matrix. These elided ITS and *trn*L intron matrices were analyzed both separately and in combination, in this case always scoring gaps as missing data.

All the manipulations of the matrices, as well as the cladistic analyses, were carried out by using using the cladistic software environment Winclada (Nixon 1999), running Nona (Goloboff 1999) as a daughter process, with the following parameters: hold 100000; hold/100; mult*100; max. The resulting cladograms were investigated with Winclada, which was also used to evaluate congruence between the ITS and *trn*L intron matrices according to the ILD test of Farris et al. (1994), to calculate bootstrap percentages (out of 1000 replicas) and branch support (up to trees 10 steps longer), as well as to plot morphological characters onto the tree shown in Fig. 1.

Comparison of sequence substitution rates between clades was carried out by using the method of Robinson et al. (1998), as implemented in the RRTree software (Robinson-Rechavi and Huchon 2000). The following tests were carried out separately for the ITS and *trn*L intron matrix: the two major clades resulting from the previous analysis, the four major clades, all the genera, four groups of taxa homogeneous in terms of seed dispersal strategies. Significance threshold was calculated as 0.05/numbers of groups in each test.

Table 1. Species, length (bp) and Genbank accession nos. of the sequences used in this study. In the columns labelled "Genbank accession no.", the three values correspond to ITS 1, ITS2 and *trn*L intron, respectively

Species	ITS1 length (bp)	ITS2 length (bp)	*trn*L intron length (bp)	Genbank accession nos.
Cephalaria leucantha (L.) Roem. & Schult.	249	248	513	AJ426523 AJ426524 AJ427376
Cephalaria syriaca (L.) Roem. & Schult.	235	213	512	AJ426525 AJ426526 AJ427377
Dipsacus sylvestris Huds.	223	213	513	AJ426527 AJ426528 AJ427378
Knautia arvensis (L.) Coult.	290	224	526	AJ426529 AJ426530 AJ427379
Lomelosia argentea (L.) Greuter et Burdet	222	228	516	AJ426531 AJ426532 AJ427380
Lomelosia caucasica (MB.) Greuter et Burdet	222	227	519	AJ426533 AJ426534 AJ427381
Pseudoscabiosa limonifolia (Vahl) Devesa	238	230	510	AJ426535 AJ426536 AJ427383
Pterocephalidium diandrum (Lag.) G. López	240	229	523	AJ426537 AJ426538 AJ427382
Pterocephalus perennis Coult.	223	227	547	AJ426539 AJ426540 AJ427384
Pycnocomon rutifolium (Vahl) Hoffmanns. & Link	227	233	537	AJ426541 AJ426542 AJ427385
Scabiosa africana L.	222	226	537	AJ426543 AJ426544 AJ427386
Scabiosa japonica Miq.	225	227	537	AJ426545 AJ426546 AJ427387
Scabiosa uniseta Savi	223	225	537	AJ426547 AJ426548 AJ427388
Sixalix atropurpurea (L.) Greuter et Burdet subsp. *maritima* (L.) Greuter et Burdet	224	226	541	AJ426549 AJ426550 AJ427389
Sixalix farinosa (Cosson) Greuter et Burdet	223	229	541	AJ426551

Table 1 (continued)

Species	ITS1 length (bp)	ITS2 length (bp)	*trn*L intron length (bp)	Genbank accession nos.
Succisa pratensis Moench	243	226	512	AJ426552 AJ427390 AJ426553 AJ426554 AJ427391
Succisella inflexa (Kluk) G. Beck	239	220	513	AJ426555 AJ426556 AJ427392
Patrinia intermedia L.	220	224	514	AJ426557 AJ426558 AJ427393
Valeriana officinalis L.	226	237	497	AJ426559 AJ426560 AJ427394

Results

The lengths of the ITS regions and of the *trn*L introns for all taxa in study, as well as their Genbank accession numbers, are reported in Table 1. ITS1 length ranged from 222 to 290 bp; ITS2 length ranged from 213 to 247 bp; *trn*L intron length ranged from 497 to 541 bp.

The combined matrix including all indels (consensus length 1154 bp, dataset A) produced one most parsimonious cladogram (length = 775, CI = 0.69, RI = 0.60; by excluding uninformative characters, length = 551, CI = 0.57, RI = 0.60).

The combined matrix obtained excluding equivocal indels (consensus length 1042 bp, dataset B) produced 2 equally parsimonious cladograms (length = 609, CI = 0.69, RI = 0.62; by excluding uninformative characters, length = 442, CI = 0.57, RI = 0.62).

The combined matrix obtained excluding equivocal indels and scoring unequivocal indels as multistate characters (consensus length 1042 bp, dataset C) produced 2 equally parsimonious cladograms (length = 656, CI = 0.70, RI = 0.62; by excluding uninformative characters, length = 470, CI = 0.59, RI = 0.62).
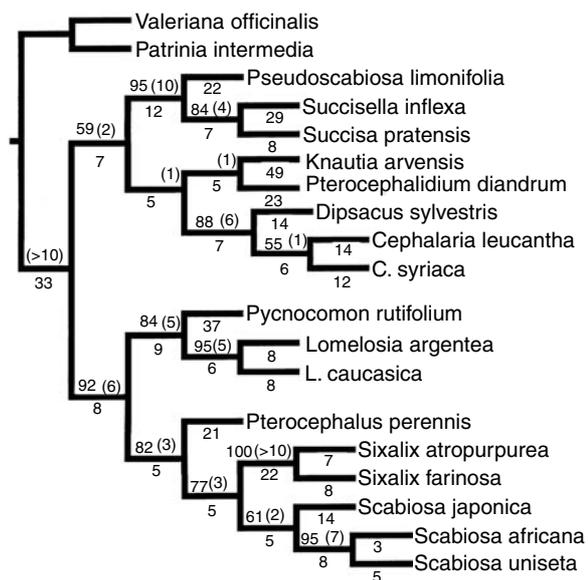

Fig. 1. Single maximum parsimony cladogram obtained for the combined data set including all indels (dataset A). Length = 775, CI = 0.69, RI = 0.60 (by excluding uninformative characters, length = 551, CI = 0.57, RI = 0.60). Numbers below branches represent synapomorphies; numbers above branches indicate bootstrap percentages above 50% (out of 1000 replicas, rounded to the unit); numbers in parentheses above branches indicate branch support (up to 10 steps longer). Support data for the outgroup are not shown

Table 2. Consensus length (bp), number of most parsimonious trees, with length, CI's and RI's for the data sets used in this paper. The second row in the datasets contains the values after removal of uninformative characters. Dataset A = alignment after visual inspection, indels interpreted as missing values; dataset B = all equivocal indels excluded, unequivocal indels interpreted as missing values; dataset C = same as B, unequivocal indels given unit weight

	DATA SET A					DATASET B					DATASET C				
	CL (bp)	n. MP trees	L	Cl	RI	CL (bp)	n. MP trees	L	CI	RI	CL (bp)	n. MP trees	L	CI	RI
ITS	562	21	671	0.66	0.55	472	3	512	0.65	0.57	472	3	547	0.67	0.58
	157		503	0.55	0.55	126		393	0.55	0.57	131		417	0.57	0.58
TrnL	592	2	103	0.90	0.87	570	2	95	0.89	0.87	571	2	107	0.90	0.88
	30		47	0.78	0.87	29		47	0.78	0.87	34		51	0.80	0.88
Combined	1154	1	775	0.69	0.60	1042	2	609	0.69	0.62	1042	2	656	0.70	0.62
	187		551	0.57	0.60	156		442	0.57	0.62	165		470	0.59	0.62

All the results of the ITS, *trn*L intron, and combined cladistic analyses are reported in Table 2.

In all three datasets, the test for incongruence (Farris et al. 1994), run for 20 replicas, showed that the ITS and *trn*L intron matrices were not significantly incongruent. The combined analyses for the three datasets provided cladograms entirely congruent with each other, and the single most parsimonious cladogram obtained for dataset A (Fig. 1) is one of the two equally parsimonious cladograms obtained for dataset B and dataset C.

The elision approach generated a 3919 character matrix (of which 1182 informative) for ITS data, and a 4144 character matrix (of which 246 informative) for the *trn*L intron data. The combined elided dataset (8063 characters, of which 1428 informative) yielded a single cladogram whose topology is very similar to those obtained from the combined datasets A, B, C.

The single most parsimonious cladogram obtained for dataset A (Fig. 1) shows that Dipsacaceae are divided into two major clades. The first clade includes *Pseudoscabiosa*, *Succisa* and *Succisella*, in a sister group relationship with *Knautia*, *Pterocephalidium*, *Dipsacus* and *Cephalaria*; in this latter clade, *Knautia* and *Pterocephalidium* behave as sister groups,

and *Dipsacus* is sister to *Cephalaria*. The other major clade includes *Lomelosia* in a sister group relationship with *Pycnocomon*; this clade is in turn sister to a clade containing *Pterocephalus*, sister group to *Scabiosa* and *Sixalix*.

Bootstrap support out of 1000 replicas (in Fig. 1 only the percentages > 50% are shown) is high for the majority of the clades; the only notable exceptions are the clade including *Knautia* and *Pterocephalidium* and the one immediately below (< 50%). Branch support has a distribution which is, not surprisingly, similar to bootstrap values, and the weakest areas of the cladogram are the same ones characterized by low bootstrap percentages (Fig. 1).

Comparisons of sequence substitution rates (Robinson et al. 1998, Robinson-Rechavi and Huchon 2000) in the ITS and *trn*L intron datasets showed that no one of the tested lineage evolved significantly faster than any other.

The trees from the different analyses of the combined datasets (Table 2) are very similar in their basic structure. In fact, the extra tree obtained for datasets B and C has the relative positions of the *Pseudoscabiosa* clade (clades will be indicated by using the name of their topmost taxon as shown in Fig. 1) and of the *Knautia* clade reversed (data not shown); more-

over, the elided cladogram from the combined dataset has a topology which differs from that shown in Fig. 1 only for the position of *Pterocephalidium*, which is in this case sister group to the *Pseudoscabiosa* clade. From this, we may infer that the positions of the gaps, or the different ways in which they are scored, are not crucial for these datasets. For this reason, we choose the topology corresponding to dataset A and shown in Fig. 1 as a working hypothesis.

The trees obtained for the separate ITS and *trn*L intron analyses (Table 2), albeit statistically congruent, are different. Notably, the ITS datasets (excluding the ITS dataset A, for which the consensus tree is greatly unresolved), show the *Pycnocomon* clade as sister group to *Scabiosa*, in a more nested position than in the cladogram of Fig. 1, as well as inversions of the relative positions of the *Pseudoscabiosa* and *Knautia* clades (data not shown).

The *trn*L datasets alternately show the *Pycnocomon* clade either in the position of Fig. 1 or as sister group to the other major clade in the analysis. In all the *trn*L analyses, extensive collapses occur among the *Pseudoscabiosa*, *Knautia*, and *Dipsacus* clades as a consequence of lack of resolution in the data (data not shown).

The elided ITS and *trn*L intron datasets obviously show similar discrepancies. In addition, the two cladograms from the elided *trn*L intron dataset show *Knautia* as the most basal taxon in the family and *Pseudoscabiosa* sister group to the clade including Scabioseae *sensu* Mayer and Ehrendorfer (1999).

Discussion

The results shown here address various controversial issues in Dipsacaceae relationships, in particular for what attains to the circumscription of tribes and to the monophyly of genera.

Our proposed pattern of descent (Fig. 1) greatly differs from previous ones based on classical morphology, and, in particular, is different from the hypotheses formulated by Ehrendorfer (1964a,b, 1965), Verláque (1977b,

1984a, 1985a) and Caputo and Cozzolino (1994). The hypothesis presented here, however, is entirely congruent (to the extent of the taxa present) with recent findings on epicalyx differentiation (Mayer and Ehrendorfer 1999). In particular, our results confirm on molecular grounds the restricted circumscription for Scabioseae recently proposed by these just mentioned authors. In fact, the clade including *Lomelosia*, *Pterocephalus*, *Pycnocomon*, *Scabiosa*, and *Sixalix* (i.e. Scabioseae *sensu* Mayer and Ehrendorfer) is one of the most supported (Fig. 1, 92% bootstrap value, 6 BS value). Similarly, the sister group relationship between *Pycnocomon* and *Lomelosia*, which has been postulated by Mayer and Ehrendorfer (1999), but entirely excluded by previous authors, is well supported (84% bootstrap, 5 BS).

Other well supported clades are those including *Sixalix* and *Scabiosa* (77% bootstrap, 3 BS) and the two just mentioned genera plus *Pterocephalus* (82% bootstrap, 3BS). Outside Scabioseae, the *Pseudoscabiosa* clade (95% bootstrap, 10 BS) and the *Dipsacus* clade (88% bootstrap, 6 BS) may also be mentioned in this regard.

The least supported clades (bootstrap values < 50%, collapsing in any one-step longer tree) are the clade including *Knautia* and *Pterocephalidium* and that immediately above. The position of *Knautia* is the one of the most widely varying in the present study. The branch leading to *Knautia* is by far the longest in the cladogram (49 autapomorphies, 26 of which non homoplasious), in spite of the fact that we included a species belonging to the most basal subgenus *Trichera* Schrad. (Caputo and Cozzolino 1994). Moreover, removal of *Knautia* from the combined dataset A causes an increase of 100% (from 2 to 4) in the branch support in the clade including *Pseudoscabiosa*, *Succisa*, *Succisella*, *Pterocephalidium*, *Dipsacus*, and *Cephalaria*. We suspect that the position of *Knautia* in the cladogram of Fig. 1 (and, to a lower extent, that of *Pterocephalidium*) may be influenced by low taxonomic sampling in that area of the family. Future studies including representatives of the Eastern sect. *Sphaero-*

dipsacus Lange of genus *Dipsacus*, of genus *Pterocephalodes*, as well as some, putatively more archaic, Eastern European species of *Knautia* (Ehrendorfer 1981) and the aberrant species *Pterocephalus centennii* M. J. Cannon, will possibly contribute to a slightly different placement of *Knautia* in the cladogram. Despite the fact that we regard the place of *Knautia* in the cladogram as a possible artifact and, therefore, we restrain from extensive comments, still, it is worth mentioning that the position of *Knautia* and *Pterocephalidum*, as well as their sister group relationship, is a novel interpretation emerging from our analysis.

All four genera for which at least two species were included appear to be monophyletic (Fig. 1). Given the small number of representatives per genus, testing monophyly at genus level was not a goal of this investigation; however, the species selected for this investigation were chosen in order to maximise intrageneric diversity and/or differences in distribution ranges. For this reason the perennial *Cephalaria leucantha*, belonging to the archaic, Western Mediterranean subg. *Fimbriatocarpus* Szabó, was chosen together with the annual *C. syriaca*, belonging to the more widespread subg. *Cephalaria*. Similarly, the chamaephyte *Sixalix farinosa*, a narrow Tunisian endemic appearing as quite archaic in the genus according to Verláque (1986a), was selected together with the biennial *S. atropurpurea*, a widespread, more derived species. The perennial Japanese endemic *Scabiosa japonica* (regarded as a member of sect. *Prismakena* Bobrov, a group including the most archaic, Asian members of the genus) was selected, along with the chamaephytic South African endemic *S. africana* and with the perennial *S. uniseta*, an Italian representative of the widespread *S. columbaria* species group.

Interestingly enough, genus *Scabiosa* as circumscribed for the past, even in its narrowest delimitation (i.e. including *Lomelosia*, *Sixalix* and *Scabiosa* s.str.) is not monophyletic, as *Pterocephalus* and *Pycnocomon* would be nested in it. On the contrary, the nomenclatural proposal by Mayer and Ehrendorfer (1999), i.e.

to keep genus level for *Lomelosia*, and to use sectional rank within genus *Scabiosa* for *Sixalix* and *Scabiosa* would correctly represent the pattern of descent hypothesized here (Fig. 1).

Our phylogenetic hypothesis suggests that adaptations to seed dispersal have been one of the strongest driving force in Dipsacaceae evolution, with similar selective pressures towards a particular dispersal mode causing the onset of similar epicalyx shapes, and correspondingly, of similar dispersal syndromes, in a parallel fashion in various taxa of the family (Fig. 2A).

Among the taxa used in this analysis, the great majority is anemochorous. *Lomelosia* and *Scabiosa* have evolved the distal part of the epicalyx, above the epicalyx tube (the corona) into a membranous, multinerved, expanded rim, which helps the diaspore to be carried by the wind (pterochorous dispersal, i.e. wind dispersal depending on the presence of a wing). Furthermore, various species of *Lomelosia* (as well as some derived species of *Sixalix*) have a calyx which protrudes from the corona and is covered with short hairs, so exploiting the opportunity of epizoochorous dispersal.

Pycnocomon and *Sixalix* are psammophylous (with the exclusion of the widespread *S. atrorpurpurea* subsp. *maritima*) and disperse by rolling on sand (Verláque 1986b). Both genera show a very reduced membranous corona, which is substituted by a more solid expansion of the distal part of the epicalyx tube, funnel-shaped in *Sixalix* and almost straight in *Pycnocomon*. However, this diminutive corona is homologous to the expanded corona of *Lomelosia* and *Scabiosa* (Mayer and Ehrendorfer 1999). The reduction of the corona in *Pycnocomon* and *Sixalix* occurred homoplasiously, under any character transformation model.
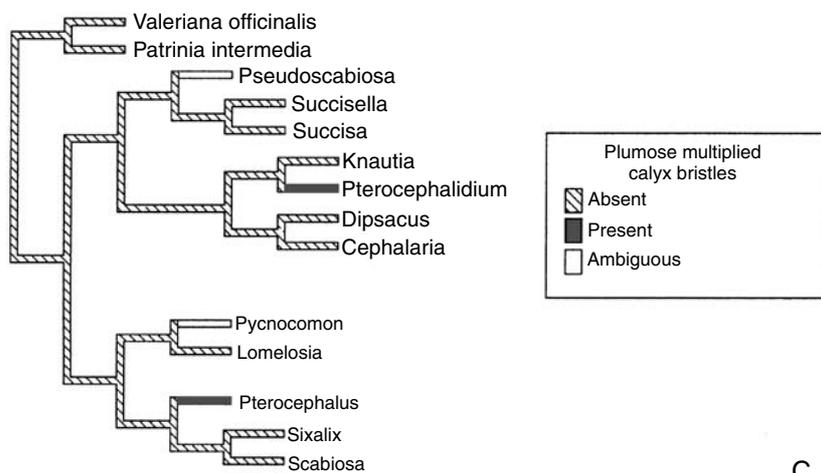
Also *Pseudoscabiosa limonifolia* has a conspicuous membranous corona; however, as Mayer and Ehrendorfer (1999) point out, the distal part of the epicalyx is variable in this genus, with *P. grosii* (Font Quer) Devesa lacking a corona. By plotting presence or absence of the corona onto the cladogram
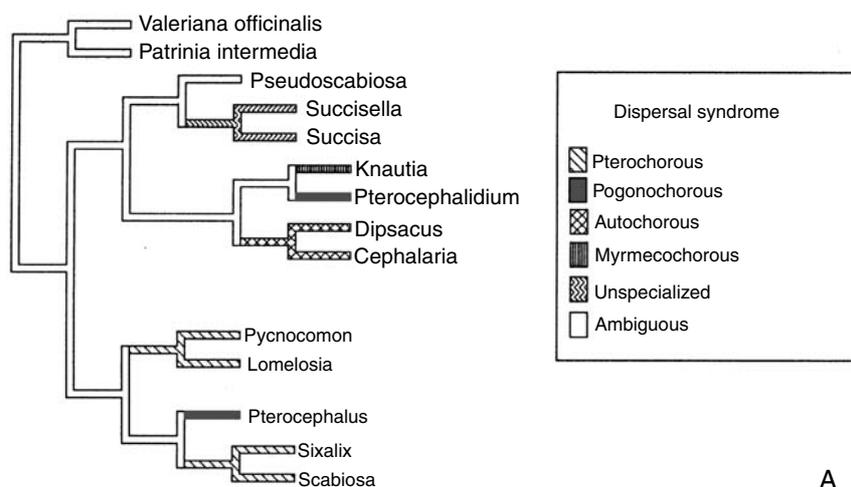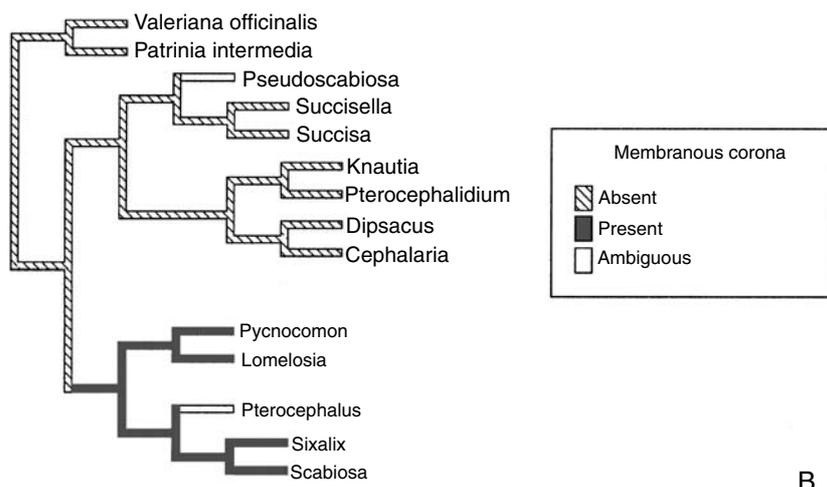
Fig. 2. Morphological characters and ecological adaptations in the studied taxa plotted onto a simplified version of the cladogram in Fig. 1 (unambiguous transformations only). A dispersal syndromes (only those which, based on external evidence, are clearly plesiomorphic in the single terminals are shown). B membranous corona (a very small number of species in *Dipsacus* and *Cephalaria* have a membranous corona, and are not shown here). C plumose multiple calyx bristles. For simplicity's sake, characters which are inapplicable in the outgroup (e.g. the characters related to the epicalyx) have been reconstructed as absent

(Fig. 2B), under all character transformation models, the wide, membranous corona is homoplasious in *P. limonifolia*, as compared to *Lomelosia*, *Pycnocomon*, *Scabiosa*, and *Sixalix*.

Other Dipsacaceae have developed a membranous corona, whether expanded and evidently nervate or minute and without apparent nerves (Mayer and Ehrendorfer 1999, 2000): among them, a genus absent in our analysis, *Pterocephalodes* p.p. (most conspicuous in *P. bretschneideri* (Batal.) V. Mayer et Ehrend.) and *Pterocephalus* p.p. (e.g. *P. pyrethrifolius* Boiss. et Hohen.). In the latter genus, the epicalyces show a great diversity in structural detail, in spite of their general similarity (Mayer and Ehrendorfer 2000). Such variability, especially in the presence and shape of the corona, may be related to the fact that the corona does not seem to have an evident adaptive value towards dispersal and, as a consequence, its presence and shape may not be strictly constrained by selection. The species selected for the present investigation, the Balkanic *P. perennis*, shows a minute membrane joining the teeth present at the top of the epicalyx tube. However, the presence of a corona is regarded as an apomorphic feature in *Pterocephalus*, and the most archaic species of the genus lack it (Mayer and Ehrendorfer 2000, Fig. 25).

Other genera, i.e. *Pterocephalidium* and *Pterocephalus* (as well as *Pterocephalodes* and *Pycnocomon intermedium*, both absent from this analysis) have multiple calyx bristles with a feathery indumentum, aiming at pogonochorous dispersal (i.e. wind dispersal depending on the presence of a pappus). Within the limits of the taxa present in this study, also this character developed homoplasiously (see also Mayer and Ehrendorfer 1999, 2000), irrespectively of the character transformation model chosen (Fig. 2C).

The other major clade observed in the cladogram of Fig. 1, i.e. that including *Cephalaria*, *Dipsacus*, *Knautia*, *Pseudoscabiosa*, *Pterocephalidium*, *Succisa*, and *Succisella* does not show such a high elaboration in the epicalyx morphology. Conversely, the clade shows a greater variety of diaspore dispersal syndromes, not always linked to morphological differentiations in the epicalyx or in the calyx. Notable exceptions in this respect are *Pseudoscabiosa*, *Pterocephalidium* and *Knautia*. In *Pseudoscabiosa*, as already said, a membranous corona is either present [in *P. limonifolia* and *P. saxatilis* (Cav.) Devesa], or absent (in *P. grosii*). Interestingly, the lack of corona in the latter species corresponds to an increase in length, number and hairiness of the calyx bristles, as compared to the other two species, indicating a possible incipient transition from pterochory to pogonochory (or vice-versa).

Pterocephalidium, the only genus in the family having two stamens instead of four, is clearly pogonochorous, with a plumose, pappus-like calyx. However, in this case the distal region of the epicalyx tube is elaborated into a single, very long, flexible awn of uncertain function (López Gonzáles 1987).

In *Knautia* the pedicel of the dorsiventrally flattened epicalyx has developed into an elaiosome, and its dispersion is myrmecochorous (Fig. 2A).

In the other genera, the epicalyx seems to be less directly involved in dispersion (Verláque 1985a, 1986b). In fact, the majority of the species of *Dipsacus* and *Cephalaria* rely on a combination of rigid, often acuminate, involucral/receptacular bracts in the capitulum, and of elastic stems, hereby allowing short-distance projection of diaspores. In some cases these diaspores subsequently adhere to animal furs, indicating either an autochorous or an epizoochorous dispersal syndrome (Fig. 2A), or a combination of both (Verláque 1985a, 1986b). For *Succisa* and *Succisella*, no correspondence between any morphological feature and a dispersal syndrome is observed; both genera are for most part confined to humid environments and have perhaps simplified their epicalyx by loosing features which were less valuable in the that environment. These taxa have been interpreted by various authors (e.g. Verláque 1986b, Caputo and

Cozzolino 1994) as basal within Scabioseae *sensu lato*; however, they are clearly external to that tribe both according to Mayer and Ehrendorfer (1999) and to the data presented here, which show the two genera in a sister relation (with very high bootstrap value and decay index) with *Pseudoscabiosa*.

Despite the differences in dispersal strategies, which have apparently contributed to cause adaptive radiation in various taxa and, as a consequence, may have influenced the evolution rate, comparisons of sequence substitution rates (Robinson et al. 1998, Robinson-Rechavi and Huchon 2000) showed that no one of the tested lineages evolved significantly faster than any other; in particular, not even a comparison among pterochorous, pogonochorous, autochorous, and myrmecochorous groups did show significant differences. However, our choice of taxa is unbalanced under this respect (e.g. only a single species of *Knautia*, i.e. a single myrmecochorous terminal), and also annuals (which constitute derived species in various genera and which often have a higher evolution rate) are underrepresented in our data set. This unbalance may have influenced the results of the test.

For all what said above, also our study, after the work by Mayer and Ehrendorfer (1999) would indicate that the gross morphology of the epicalyx is deceptive in inferring relationships, as the whole family is beset by rampant homoplasies originated by selective constraints towards several diaspore dispersal syndromes.

The presence of a diaphragma, i.e. a protrusion of the distal part of the inner side of the epicalyx tube, which, by enclosing the calyx stalk, secures a better encasing of the achene in the epicalyx, is much more promising in assessing relationships (Mayer and Ehrendorfer 1999, 2000). This structure, together with the corresponding diversification of an epidiaphragma (Mayer and Ehrendorfer 1999), is present in virtually all members of *Lomelosia*, *Scabiosa*, *Sixalix*, and in various species of *Pterocephalus* (including *P. perennis*,

which was used for this study), but absent from all other Dipsacaceae. However, the diaphragma, together with the membranous corona, may be regarded as synapomorphic for Scabioseae *sensu* Mayer and Ehrendorfer (1999). This character may be either reverted more than once (loss in the immediate ancestors of *Pterocephalus* and novel development within the genus) or occurrence in *Pterocephalus* is plesiomorphic. The latter hypothesis, however, is excluded by Mayer and Ehrendorfer (2000).

Palynological and karyological evidences (Verláque 1985a, 1986b; Caputo and Cozzolino 1994; Mayer and Ehrendorfer 1999) are much more supportive of our phylogenetic hypothesis. Dipsacaceae pollen (Verláque 1985a, 1986b) is either triporate (in *Knautia*, *Lomelosia*, *Pterocephalidium*, *Pycnocomon*, and in the aberrant annual *Scabiosa parviflora* Desf.) or tricolpate (in *Cephalaria*, *Dipsacus*, *Pseudoscabiosa*, *Pterocephalus*, *Scabiosa*, *Sixalix*, *Succisa*, and *Succisella*). Tricolpate pollen, also present in all Valerianaceae, is unequivocally reconstructed as plesiomorphic in Dipsacaceae (data not shown); triporate pollen has independently evolved more than once, in *Knautia*/*Pterocephalidium*, in *Lomelosia*/*Pycnocomon*, and in *S. parviflora*.

Chromosome numbers in the family are less amenable to an unequivocal reconstruction. In fact, in the majority of the large genera, chromosome numbers show dysploid variation. However, the haploid numbers appearing as plesiomorphic in the genera in which they occur are $n = 8$ in *Pterocephalidium*, *Scabiosa*, *Sixalix* (Verláque 1985b, 1986a,b), $n = 9$ in *Cephalaria*, *Dipsacus*, *Pycnocomon*, *Pterocephalus* and *Lomelosia* (Verláque 1977a, 1985a), $n = 10$ in *Knautia*, *Pseudoscabiosa*, *Succisa* and *Succisella* (Ehrendorfer 1962, 1981; Verláque 1985b, 1986a). By plotting chromosome haploid numbers on the cladogram (data not shown), it appears that $n = 8$ independently originated twice in the family (in *Pterocephalidium*, as well as in *Scabiosa* and *Sixalix*); $n = 9$ is ambiguous in reconstruction; regardless, it is reconstructed

as plesiomorphic in the family under both an accelerated and a delayed character transformation model (data not shown). However, the character state n = 9 is synapomorphic for the two groups in which it appears only under a delayed transformation model. The state n = 10 is also ambiguous: it appears as uniquely derived in both the *Pseudoscabiosa* clade and *Knautia* only under an accelerated transformation model.

In spite of all the homoplasies described above, it may be noted that our present phylogenetic hypothesis requires a smaller number of homoplasious changes in the presence of diaphragma, pollen morphology and chromosome numbers as compared to previous ones (e.g. Verláque 1986b, Caputo and Cozzolino 1994). Furthermore our evolutionary reconstruction is almost entirely congruent with the novel insights on epicalyx morphology shown by Mayer and Ehrendorfer (1999). The few possible problems of our proposed pattern of descent (e.g. the position of *Knautia*) will be probably solved by both the inclusion of some critical taxa (members of *Pterocephalodes*; basal species of *Dipsacus*, representatives of the derived subgenera of *Knautia*) and the extension of the molecular dataset.

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