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Phylogeny and Biogeography of Isophyllous Species of *Campanula* (Campanulaceae) in the Mediterranean Area

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ABSTRACT. Sequence data from the nuclear internal transcribed spacer (ITS) were used to infer phylogenetic relationships within a morphologically, karyologically, and geographically well-defined group of species of *Campanula* (Campanulaceae), the *Isophylla* group. Although belonging to the same clade within the highly paraphyletic *Campanula*, the *Rapunculus* clade, members of the *Isophylla* group do not form a monophyletic group but fall into three separate clades: (i) *C. elatines* and *C. elatinoides* in the Alps; (ii) *C. fragilis* s.l. and *C. isophylla* with an amphi-Tyrrhenian distribution; and (iii) the *garganica* clade with an amphi-Adriatic distribution, comprised of *C. fenestrellata* s.l., *C. garganica* s.l., *C. portenschlagiana*, *C. poscharskyana*, and *C. reatina*. Taxa currently classified as subspecies of *C. garganica* (*garganica*, *cephallenica*, *acarnanica*) and *C. fenestrellata* subsp. *debarensis* are suggested to be best considered separate species. The molecular dating analysis, although hampered by the lack of fossil evidence, provides age estimates that are consistent with the hypothesis that the diversification within the *garganica* clade was contemporaneous with the climatic oscillations and corresponding sea-level changes during the late Pliocene and Pleistocene. Dispersal-vicariance analysis suggests that the *garganica* clade originated east of the Adriatic Sea, from where it reached the Apennine Peninsula.

KEYWORDS: amphi-adriatic distribution, dispersal-vicariance analysis, molecular dating, taxonomy.

Campanula L. is the largest genus within the Campanulaceae and comprises ca. 300 species distributed mainly in extra-tropical areas of the northern hemisphere (Meusel and Jäger 1992; Shulkina et al. 2003). A major center of species diversity is found in the Mediterranean region, where ca. 250 species occur (Geslot in Greuter et al. 1984). Many of those are edaphically and microclimatically specialized chasmophytes, and as such are often narrowly distributed endemics (Damboldt 1965b; Kovanda 1970a; Pignatti 1982).

The delimitation of *Campanula* is quite unclear, and the genus appears to be residual after the exclusion of morphologically well-characterized, mainly monotypic, groups as separate genera. Recent molecular phylogenetic studies (Eddie et al. 2003) have shown that genera such as *Azorina* Feer, *Campanulastrum* Small, *Edraianthus* DC., and *Phyteuma* L. nest within *Campanula*. *Campanula*, including well-marked segregates, groups in three major clades: (1) the *Campanula* s. str. clade, which additionally includes *Azorina*, *Edraianthus*, *Trachelium* Tourn. ex L. and others; (2) the *Rapunculus* clade, which additionally includes *Adenophora* Fisch., *Asyneuma* Griseb. & Schenk, *Campanulastrum*, *Legousia* Durand, *Phyteuma*, and others; and (3) a small unnamed third clade, which additionally includes *Gadellia* Schulkina and *Musschia* Dum. The relationships among these three clades and to the genus *Jasione* L. are unresolved.

The taxonomic complexity at the generic level also occurs, not only at the infrageneric level within *Campanula* s. str. (e.g., the generic recognition of taxa such as subgenus *Roucela* (Dumort.) Damboldt), but also at the sectional and subsectional level. Many authors during the last two centuries tried to develop a workable classification of this large genus (e.g., de Candolle 1830; Boissier 1875; Nyman 1878–1882; Janchen 1958; Gadella 1964, 1966a,b; Contandriopoulos 1984; Kolakovsky 1994) but none of these systems seems to be successfully predictive of phylogenetic relationships. In addition, taxonomic systems have been erected on the basis of geographically limited floristic studies, such as the Flora SSSR (Fedorov 1957) or Flora of Turkey (Damboldt 1978), and are thus difficult to apply to *Campanula* of other regions (e.g., Witasek 1906; Hruby 1930, 1934; Podlech 1965; Damboldt 1965b; Kovanda 1970b, 1977; Kovanda and Ančev 1989). Such classifica-

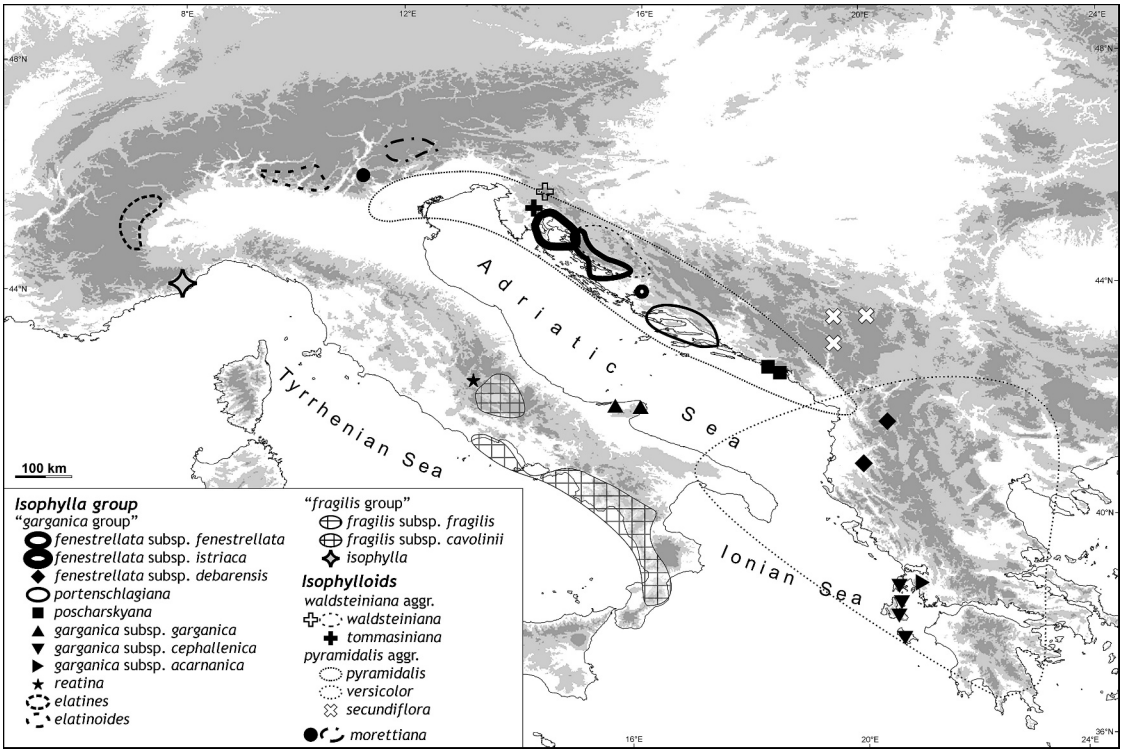

FIG. 1. Distribution areas of isophyllous *Campanula* species in the central Mediterranean area (following Damboldt 1965b; Lovašen-Eberhardt and Trinajstić 1978; Pignatti 1982; Surina in Kovačić and Surina 2005).

tions were based on morphological characters partly refined by karyological data, but the evolution of the morphological and karyological characters and possible problems with homoplasy are poorly understood in the Campanulaceae as a whole. A sound molecular phylogenetic hypothesis would address these questions and could eventually aid the construction of a more predictive taxonomic classification.

Despite these problems, some smaller groups within *Campanula* are well-characterized morphologically, karyologically, and biogeographically, suggesting that they may be monophyletic (e.g., Carlström 1986; Runemark and Phitos 1996; Eddie and Ingrouille 1999; Oganesian 2001; Sáez and Aldasoro 2003). One of those, hereafter called the *Isophylla* group, comprises European species characterized by isophylly, long petiolate basal leaves with kidney-shaped to cordate blades, distinctly petiolate cauline leaves with cordate to ovate blades, calyces without appendages between the lobes, erect capsules with basal pores (rarely with irregular splits), and orbicular to ovate seeds with narrowly ridged testa (Damboldt 1965b). This group has a highly fragmented distribution in the central Mediterranean region, and its species are mostly allopatric and confined to small, often compact areas (Fig. 1).

The *Isophylla* group is further divided by Damboldt (1965b) into a Tyrrhenian ''*fragilis* group'' (i.e., *C. fragilis* aggr. of Geslot in Greuter et al. 1984: *C. fragilis* subsp. *fragilis* and subsp. *cavolinii* [together *C. fragilis* s.l.], *C. isophylla*) and a predominantly peri-Adriatic ''*garganica* group'' (i.e., *C. elatines* aggr. of Geslot in Greuter et al. 1984: *C. fenestrellata* subsp. *fenestrellata*, subsp. *istriaca* and subsp. *debarensis* [together *C. fenestrellata* s.l.], *C. garganica* subsp. *garganica*, subsp. *acarnanica* and subsp. *cephallenica* [together *C. garganica* s.l.], *C. elatines*, *C. elatinoides*, *C. portenschlagiana*, *C. poscharskyana*, and *C. reatina*; Table 1). This split is supported by the presence of two different chromosome numbers, $2n = 32$ in the ''*fragilis* group'' and $2n = 34$ in the ''*garganica* group.'' Trinajstić (in Lovašen-Eberhardt and Trinajstić 1978) recognizes these groups as series *Fragiles* Trin. and *Garganicae* Trin., respectively. Additionally, on the basis of leaf hairiness and pollen size he separates *C. elatines* and *C. elatinoides* from the ''*garganica* group'' (sensu Damboldt 1965b) as separate series *Elatines* Trin. (Table 1), rendering series *Garganicae* exclusively amphi-adriatic in distribution (Fig. 1).

TABLE 1. Classification of the *Isophylla* group sensu Damboldt (1965b). [a]Treated as separate species in the main text of Damboldt (1965b), but reduced to subspecies in a ''note added in proof'' (Damboldt 1965b: 358).

Damboldt (1965b)	Lovašen-Eberhardt and Trinajstić (1978)	This study
***fragilis*-''Gruppe'' (group)**	***Campanula* sect. *Campanula* subsect. *Elatines* (Wohlfarth) Trinajstić ser. *Fragiles* Trinajstić**	***fragilis* clade**
C. fragilis Cyrillo	*C. fragilis* Cyrillo	*C. fragilis* Cyrillo
C. fragilis subsp. *fragilis*		*C. fragilis* subsp. *fragilis*
C. fragilis subsp. *cavolinii* (Tenore) Damboldt		*C. fragilis* subsp. *cavolinii* (Tenore) Damboldt
C. isophylla Moretti	[not treated]	*C. isophylla* Moretti
***garganica*-''Gruppe'' (group)**	–	–
–	***Campanula* sect. *Campanula* subsect. *Elatines* (Wohlfarth) Trinajstić ser. *Elatines***	***elatines* clade**
C. elatines Linné	*C. elatines* Linné	*C. elatines* Linné
C. elatinoides Moretti	[not treated]	*C. elatinoides* Moretti
–	***Campanula* sect. *Campanula* subsect. *Elatines* (Wohlfarth) Trinajstić ser. *Garganicae* Trinajstić**	***garganica* clade**
C. portenschlagiana Roemer & Schultes	*C. portenschlagiana* Roemer & Schultes	*C. portenschlagiana* Roemer & Schultes
C. poscharskyana Degen	*C. poscharskyana* Degen	*C. poscharskyana* Degen
''Sammelart'' (species aggregate) *C. garganica* Tenore		
C. garganica subsp. *acarnanica* (Damboldt) Damboldt[a]	[not treated]	*C. acarnanica* Damboldt
C. garganica subsp. *cephallenica* (Feer) Hayek[a]	[not treated]	*C. cephallenica* Feer
C. garganica subsp. *garganica*	*C. garganica* Tenore	*C. garganica* Tenore
''Sammelart'' (species aggregate) *C. fenestrellata* Feer		
C. fenestrellata subsp. *debarensis* (Rechinger f.) Damboldt[a]	*C. debarensis* Rechinger f.	*C. debarensis* Rechinger f.
C. fenestrellata subsp. *fenestrellata*	*C. fenestrellata* Feer [s. str.]	*C. fenestrellata* subsp. *fenestrellata*
C. fenestrellata subsp. *istriaca* (Feer) Damboldt	*C. istriaca* Feer	*C. fenestrellata* subsp. *istriaca* (Feer) Damboldt
[not known yet]	[not known yet]	*C. reatina* Lucchese

A few other species are considered to be closely related to the *Isophylla* group (reviewed in Damboldt 1965b), and will hereinafter be called the ''isophylloids.'' The delimitation of this group, however, is somewhat problematic, particularly with respect to those species that differ from the *Isophylla* group, and most other isophylloids, by distal pores on the capsule, instead of basal ones. Following the widely accepted intraspecific classification of *Campanula* by Boissier (1875), who distinguished sect. *Rapunculus* with distal pores from sect. *Campanula* with basal pores, Damboldt (1965b) consequently treated isophylloids with distal pores, such as *C. raineri* or *C. adsurgens*, as members of sect. *Rapunculus*, while all other isophyllous species belonged to sect. *Campanula*.

In addition, there are problems concerning those isophylloids that share some morphological characteristics with the otherwise well-separated harebells of subsect. *Heterophylla* (Wit.) Fed. (sensu Kovanda 1970a,b; 1977). In particular, the isophylloid *C. waldsteiniana*-aggr. (*C. waldsteiniana*, *C. tommasiniana*) has been joined with the heterophyllous *C. pulla* and *C. cespitosa* (Nyman 1878–1882). Additionally, overall morphological similarity of the heterophyllous *C. hercegovina* to the *C. waldsteiniana*-aggr. blurs the distinction between the isophylloids and subsect. *Heterophylla*. Of those taxa most often assigned to the isophylloids, the *C. waldsteiniana*-aggr., the *C. pyramidalis*-aggr. (*C. pyramidalis*, *C. versicolor*, *C. secundiflora*), and *C. morettiana* occur in the same geographic regions as members of the *Isophylla* group (Fig. 1). Among those species, *C. morettiana* is anomalous in that it has a campanulaceous corolla and is reported to have indehiscent capsules.

The first aim of this study is to test the monophyly of the *Isophylla* group sensu Damboldt (1965b) and to establish molecular phylogenetic hypotheses for (i) the relationships within the *Isophylla* group; (ii) those of the *Isophylla* group to other groups, in particular, the isophylloids, and (iii) those of the isophylloids to subsect. *Heterophylla*, particularly the *Rotundifolia* group (sensu

Kovanda 1970a,b, 1977). The second aim is to use the phylogenetic hypotheses to interpret the biogeographic history of the peri-Adriatic *Isophylla* group.

Material and Methods

Taxon Sampling. The sampling strategy focused on taxa of the *Isophylla* group and all currently recognized species and subspecies of this group are included. Within the isophylloids, priority was given to taxa with similar distribution areas as members of the *Isophylla* group (Fig. 1), namely the *C. waldsteiniana*-aggr., the *C. pyramidalis*-aggr., and *C. morettiana*. Several other species are mentioned in the context of isophyllous bluebells (Damboldt 1965b). From those, due to the availability of material only *C. arvatica* s.l. (incl. *C. adsurgens*) and *C. raineri* are included.

As outlined above, the distinction between isophylloids and subsect. *Heterophylla* is equivocal in some cases. Of species formerly thought to be closely related to the *C. waldsteiniana*-aggr., *C. pulla* (northeastern Alps), *C. cespitosa* (eastern Alps), and *C. hercegovina* (Hercegovinian mountains) are included in our study; no material was available for *C. excisa* (Penninian Alps). Several species unambiguously assigned to subsect. *Heterophylla*, such as *C. scheuchzeri* or *C. rotundifolia*, are also included.

Due to the lack of a modern generic classification system for *Campanula*, it is difficult to decide a priori which species could be used as outgroups. In a recent molecular phylogenetic study based on nuclear ITS sequences (Eddie et al. 2003), no members of the isophyllous core groups were included. However, preliminary analyses (data not shown) indicated that the species under study belong to a well-supported clade, which includes *C. pyramidalis* and *C. arvatica* (both investigated by Eddie et al. 2003), and accordingly other species of this clade such as *C. carpatica*, *C. hawkinsiana*, *C. herminii*, *C. lusitanica* are included as well. Although further sampling of *Campanula* was primarily guided by the availability of material, it was attempted to cover as wide as possible the taxonomic range of European *Campanula*. As already suggested by the study of Eddie et al. (2003), several smaller genera nest within *Campanula* s.l., and therefore representatives of these groups are included as well. As the ultimate outgroup for this study, the genus *Jasione* was chosen for two reasons. First, this morphologically very distinct group is placed as one of the transitional taxa in the study of Eddie et al. (2003) with unresolved relationships to any of the *Campanula* lineages. Second, ITS sequences of *Jasione* could still be aligned to sequences of *Campanula*, while those of more distant outgroups (e.g., *Canarina canariensis* (L.) Vatke or *Wahlenbergia nutabunda* (Guss.) A. DC.) introduce severe alignment problems, thus increasing potential errors in the resultant phylogeny. A list of taxa included in this study with collecting and voucher information is provided in Appendix 1.

Molecular Methods. Total genomic DNA was extracted from silica-dried or rarely from air-dried leaf material following the CTAB extraction protocol (Doyle and Doyle 1987) with slight modifications (Schönswetter et al. 2002). The ITS1-5.8S-ITS2-region of the nuclear ribosomal DNA was amplified using primers 17SE and 26SE (Sun et al. 1994). The reaction mix for polymerase chain reaction (PCR) of 50 µl contained 1 × PCR buffer (Sigma-Aldrich, Vienna, Austria), 200–500 ng of DNA-template, 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), 0.04 µM of each primer, 0.38 units of Red Taq DNA polymerase (Sigma-Aldrich, Vienna, Austria), and 2.5 mM $MgCl_2$. In cases of poor amplification results, PCR was repeated with 0.02% BSA (Sigma-Aldrich, Vienna, Austria) and/or 5% DMSO (Sigma-Aldrich, Vienna, Austria). PCR was conducted with the following conditions: denaturation for 4 min at 94°C; 35 cycles with 1 min at 95°C, 1 min at 48°C, 1 min at 72°C; final elongation for 10 min at 72°C. PCR products were purified from 1% agarose gels using the GFX PCR DNA & Gel Band Purification Kit (Amersham Biosciences Europe, Vienna, Austria) according to the manufacturer's instructions. Sequencing was done using the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, USA) and analyzed on an ABI PRISM 377 DNA autosequencer (Applied Biosystems).

Sequences of *Campanula hercegovina* and *C. fragilis* subsp. *fragilis* were not readable due to the presence of a high number of ambiguous sites (>1% of the sequence), and therefore ITS sequences of these two species were cloned. The purified fragment was ligated into the pGEM-T easy vector (Promega, Madison, Wisconsin) according to manufacturer's instructions. The plasmids were transformed into *E. coli* JM109 competent cells (Promega, Madison, Wisconsin) and blue/white screening was used to identify transformants, the DNA of which was isolated using a standard mini-prep method (Sambrook et al. 1989). The clones were digested with *Eco*RI (Promega, Madison, Wisconsin) to check the insert length. Positive clones were sequenced using primers 17SE and 26SE and analyzed on an ABI 377 automated sequencer as described above. Of three clones checked, three different sequences were obtained, which had different lengths of ITS1 (±1 bp), thus accounting for the reading problems in the uncloned sequence. Sequences have been deposited in GenBank under accession numbers DQ304566–DQ304630 (Appendix 1).

Sequence Alignment. The boundaries of ITS1 and ITS2 were determined with the sequence of *Senecio vulgaris* (Asteraceae; AF422136). Regions for which no sequence data are available (5.8 S region and parts of the 3′-end of ITS2 for six species) were coded as missing data, resulting in a total of 2.3% of cells with missing data. The alignment was conducted using the multiple alignment mode in ClustalX 1.81 (Thompson et al. 1997) with DNA transition weight of 0.5 and penalties of 15 for gap-opening and 6.66 for gap extension. The alignment was adjusted manually using BioEdit 5.0.9 (Hall 1999) and is available from TreeBASE (study number S1526).

Phylogenetic Analyses. In order to assess the influence of missing data on the phylogenetic analyses, two data sets were analyzed, one including all data and one excluding insertions restricted to an arbitrarily chosen threshold of one fourth or less of taxa (≤18 taxa: 44 characters). All phylogenetic analyses of these two data sets yielded very similar results (data not shown), and only those from the full data set are shown. Maximum parsimony analyses were conducted using PAUP* 4.0b10 (Swofford 2001) with the following settings: random addition sequence with 1000 replicates and no more than 1000 trees saved per replicate, TBR branch swapping, MulTrees on, steepest descent option not in effect, swap on best trees only, and collapse branches if minimum length is zero. Characters were treated as unordered and of equal weight, and gaps were treated as missing data. Clade support was assessed using bootstrap with 1000 replicates (heuristic search options as above, except random addition sequence with 10 replicates saving no more than 500 trees per replicate). As best-fit substitution model the Symmetrical Model with a gamma distribution accounting for heterogeneity among sites and a proportion of invariable sites (SYM+Γ+I) was selected using the Akaike Information Criterion as implemented in MrModeltest 2.2 (Nylander 2004). Maximum likelihood analysis was performed with PAUP* 4.0b10 (Swofford 2001) using the

parameters suggested by MrModeltest (nst = 6 base = equal rmat = (0.7509 2.0867 1.3084 0.2991 4.2157) rates = gamma shape = 1.1084 pinvar = 0.2773). As tree search method we used the likelihood ratchet (Vos 2003), employing 200 iterations and 15% of characters being re-weighted. The instruction file for PAUP to run the ratchet script was generated with the program PAUPRat (Sikes and Lewis 2001). The obtained maximum likelihood trees were identical, and one of those was used as a starting tree in another heuristic search and TBR-swapped until completion. Clade support was again assessed using bootstrap with 100 replicates saving only one tree per replicate, starting trees obtained via neighbor joining and TBR branch swapping. Bayesian analysis was performed with MrBayes 3.1 (Ronquist and Huelsenbeck 2003) using models with six substitution types (nst = 6), a gamma distribution for describing rate heterogeneity across sites and a proportion of invariable sites as suggested by MrModeltest. Values for all parameters, such as the shape of the gamma-distribution, were estimated during the analyses. The settings for the Metropolis-coupled Markov chain Monte Carlo (MC^3) process were: three runs with four chains each (one cold, three heated chains using the default incremental heating scheme) were run simultaneously for 5×10^6 generations each with trees being sampled every 100^{th} generation using the default priors (flat Dirichlet priors for the substitution matrix and state frequencies; uniform prior (0.05, 50) for the shape parameter of the gamma distribution; uniform prior (0, 1) for the proportion of invariable sites; all topologies equally probable; exponential prior (10) for the branch lengths). Convergence of the independent runs was assessed via (i) comparing likelihood scores and means and variances across runs, and via (ii) the variance of split frequencies (mcmcdiagn = yes) calculated every 5000^{th} tree discarding the first 10% of trees (relburnin = yes burninfrac = 0.1). Convergence was considered to have been reached when the variance of split frequencies was < 0.01, which was the case after c. 1.3 mio generations and a corresponding burn-in of c. 1300 trees. The posterior probability (PP) of the phylogeny and its branches was determined from the combined set of trees discarding the first 5000 trees of each run (i.e. the set of trees discarded for the convergence diagnostics when the runs were completed), which comprises 135000 trees.

Alternative phylogenetic hypotheses (described in Results) were tested in a maximum likelihood framework using the Shimodaira-Hasegawa-Test (SH-test, Shimodaira and Hasegawa 1999) as implemented in PAUP 4.0b10 employing 10,000 bootstrap replicates to generate a test distribution by the RELL (re-sampling estimated log-likelihood) method. The strategy for the constrained maximum likelihood searches was the same as for the unconstrained searches described above. Because the SH-test is known to be very conservative (i.e., less likely to reject the null hypothesis of all trees in the set being equally good explanations of the data; Goldman et al. 2000), we also used a Bayesian approach by determining the posterior probabilities of alternative topologies from the combined set of trees after the burn-in period (see above), considering alternative topologies with posterior probabilities of 0.05 or more as not significantly worse (Huelsenbeck et al. 2002; Steele et al. 2005).

Age Estimation and Biogeographic Analysis. We tested for clock-like evolution of substitution rates in the total data set using relative rate tests as implemented in the program K2WuLi (Jermiin 1996) based on pairwise comparisons of K2P sequence differences using *Jasione montana* as outgroup. We used this method because the presence of many closely spaced divergences in the ITS data render search times under the clock constraint as required for likelihood ratio testing unrealistically long (Barker 2004). The relative rate tests detected numerous cases of rate heterogeneity among ITS sequences at a significance level of $P = 0.05$ (z scores > |1.96|) also involving species of the core groups of interest, namely *C. garganica* subsp. *cephallenica* vs. *C. garganica* subsp. *acarnanica* (z score = 2.04). To obtain a conservative estimate of the divergence time for this group (i.e., the crown group age of the *garganica* clade: Figs. 2–4), we used the longest edge within this clade, bl_{max}, which leads from the root of this clade to *C. garganica* subsp. *acarnanica*, and derived the divergence time t according to the formula $t = bl_{max}/r$, where r is the substitution rate. Uncertainties in branch lengths estimates were accounted for by re-calculating the divergence time by using the maximal and minimal branch lengths as derived from adding or subtracting the standard error from the respective edges. The origin of the whole group (i.e., the stem group age of the *garganica* clade) might substantially predate the time of diversification, and therefore we obtained a conservative estimate of the time of origin by dating the node separating the clade of interest from the other species as described above.

In several studies, substitution rates per site and year were established for particular groups (Richardson et al. 2001). The lowest rates are proposed in the woody Winteraceae ($3.2–5.7 \times 10^{-10}$ substitutions per site and year; Suh et al. 1993) and in herbaceous perennial *Begonia*/Begoniaceae (3.2×10^{-10} substitutions per site and year; Plana et al. 2004). If these are applied to the clade of isophyllous *Campanula* species (assuming a uniform molecular clock), the dates of origin of its species are up to more than 50 million years ago (mya). This is far beyond the oldest known Campanulaceae pollen fossils from the Miocene and Pliocene (Mildenhall 1980; Benton 1993) and even beyond the inferred age of 41 mya for the clade including *Campanula* and *Codonopsis* (Wikström et al. 2001), the latter genus being a member of the basal clade within Campanulaceae (Eddie et al. 2003). The majority of reported substitution rates are considerably higher, and we used those established for the herbaceous perennial *Soldanella* (Primulaceae) of 8.34×10^{-9} to 3.89×10^{-8} substitutions per site and year (Zhang et al. 2001), which is the widest range of rates proposed so far.

In a second approach, we used the penalized likelihood approach (PL) of Sanderson (2002a) as implemented in the program r8s ver. 1.70 (Sanderson 2002b) to produce ultrametric trees. In PL a saturated model in which each lineage has its own rate is combined with a roughness penalty, which forces rates to change smoothly from branch to branch. The tradeoff between smoothness and goodness-of-fit of the data to the saturated model is determined by a smoothing parameter. The optimal value for this smoothing parameter is estimated from the data via a cross-validation procedure as described in Sanderson (2002a,b). In practice, several values are tested, and the one resulting in the lowest cross validation-score is chosen for the final run. PL was conducted using a smoothing parameter of 0.01 and the TN (truncated Newton) algorithm. Several runs from different perturbations of different starting points aborted, and finally only 5 perturbations per each of 5 random starting points (num_restarts = 5, num_time_guesses = 5) were employed. We also applied non-parametric rate smoothing (NPRS; Sanderson 1997) using the Powell algorithm as implemented in r8s with 10 perturbations per each of 10 random starting points. NPRS estimates rates and times via a least-squares smoothing criterion that penalizes rapid rate changes from branch to neighboring branch on a tree, which might lead to overfitting the data. The fossil record of Campanulaceae in general is very poor (Muller 1981) and does not provide any calibration points for the groups covered in this study. We therefore arbitrarily set the age of the root node to 1000 to obtain relative ages for the nodes of interest. These were then

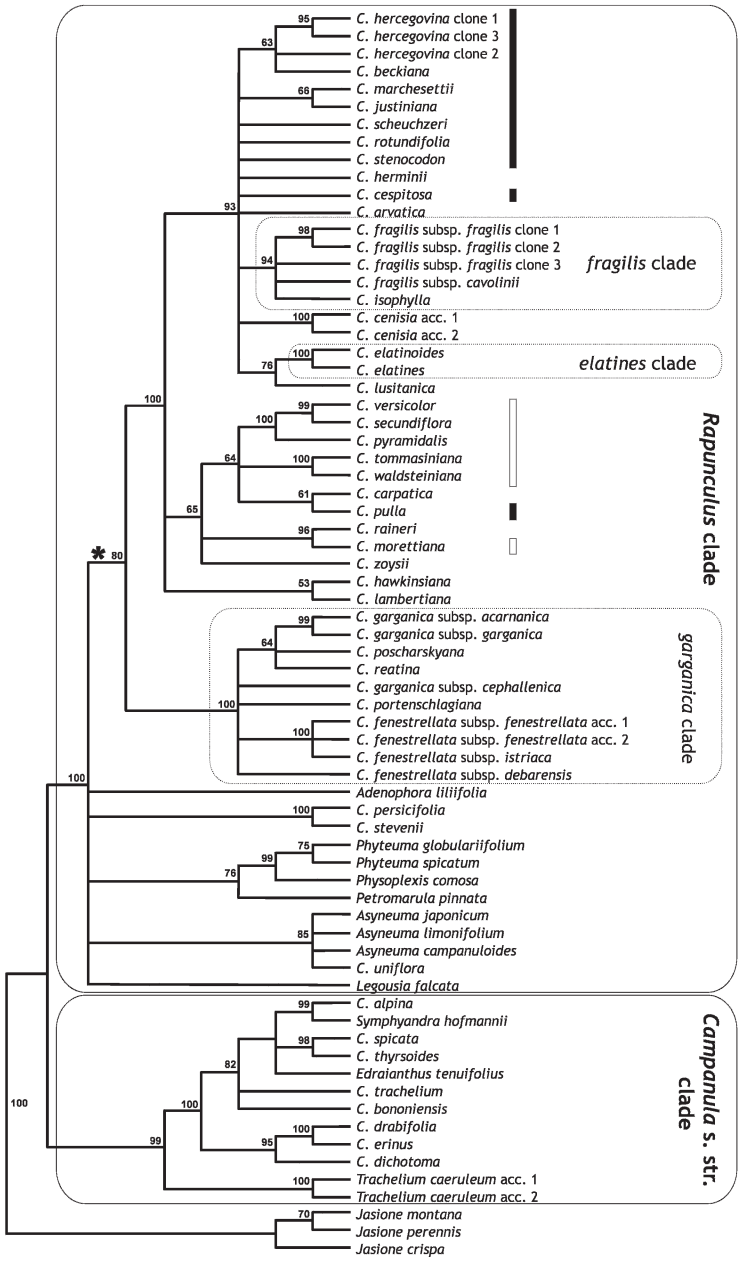

FIG. 2. Phylogenetic relationships of the *Isophylla* group and the isophylloids of *Campanula* inferred from maximum parsimony analysis of the nuclear ITS region: strict consensus tree of 78,913 equally most parsimonious trees. Numbers above branches are bootstrap values ≥50. The tree is rooted at the midpoint between *Jasione* and the other taxa. Circumscriptions of the *Rapunculus* and the *Campanula* s. str. clade as well as the three components of the *Isophylla* group (*elatines*, *fragilis* and *garganica* clade) are indicated; black and white bars mark subsect. *Heterophylla* and the isophylloid *Campanula* species, respectively, and the asterisk marks the core clade (see text for details).

translated into absolute ages by setting the age of the root (i) to 41 mya, the age of the clade including *Campanula* and *Codonopsis* as inferred by Wikström et al. (2001); or (ii) to 23 mya, the approximate beginning of the Miocene, from which period campanulaceous fossils are known (Mildenhall 1980; Benton 1993). Because both are very conservative estimates of the maximum ages of the group of interest, we do not provide confidence intervals for the PL and NPRS estimates.

Ancestral areas of the core group of isophyllous *Campanula* species were inferred using dispersal-vicariance analysis as implemented in the program DIVA 1.2 (Ronquist 1996). Because our focus is on the biogeographic relationships across the Adriatic Sea, only two areas were defined, one west and one east of the Adriatic Sea (areas W and E, respectively). We used the topology from Bayesian analysis (see above), randomly resolving polytomies involving *C. fenestrellata* s.l. (subsp. *fenestrellata* and subsp.

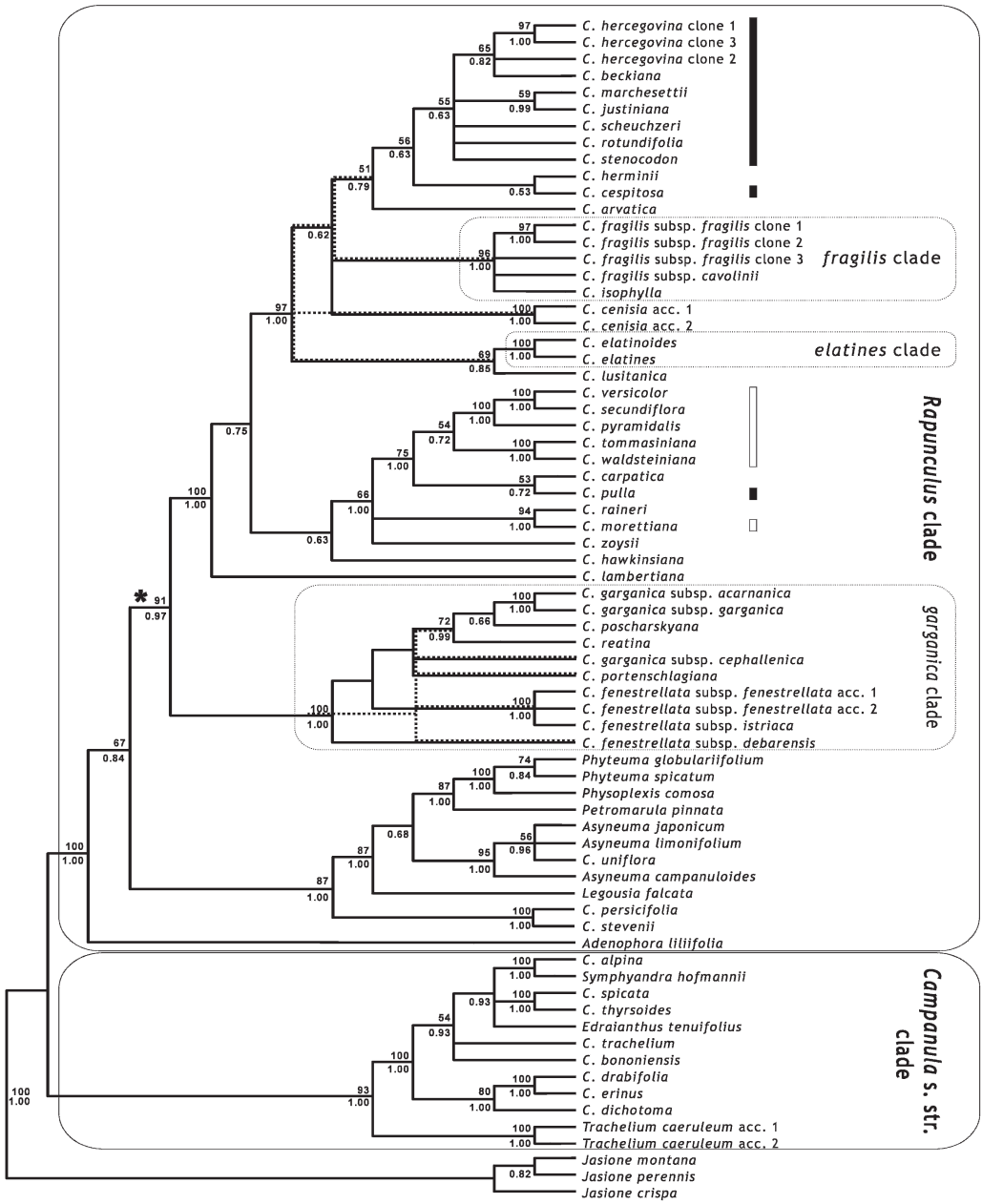

FIG. 3. Phylogenetic relationships of the *Isophylla* group and the isophylloids of *Campanula* inferred from maximum likelihood (strict consensus of three maximum likelihood trees; solid lines) and Bayesian analysis (dotted lines) of the nuclear ITS region. Numbers above branches are bootstrap values ≥50 (maximum likelihood), numbers below branches are posterior probabilities ≥0.5 (Bayesian inference). The tree is rooted at the midpoint between *Jasione* and the other taxa. Circumscriptions of the *Rapunculus* and the *Campanula* s. str. clade as well as the three components of the *Isophylla* group (*elatines*, *fragilis* and *garganica* clade) are indicated; black and white bars mark subsect. *Heterophylla* and the isophylloid *Campanula* species, respectively, and the asterisk marks the core clade (see text for details).

istriaca treated as one terminal), *C. portenschlagiana*, and *C. garganica* subsp. *cephallenica*. Because the taxa involved in those polytomies have identical distributions (all occur in the eastern side of the Adriatic Sea), this does not have any effect on the number of inferred events. While the clade including the remaining taxa, among them the two taxa distributed west of the Adriatic Sea (*C. reatina* and *C. garganica* subsp. *garganica*), is well supported, the relationships among those taxa are ambiguous (see Results). We therefore analyzed all topological variants concerning

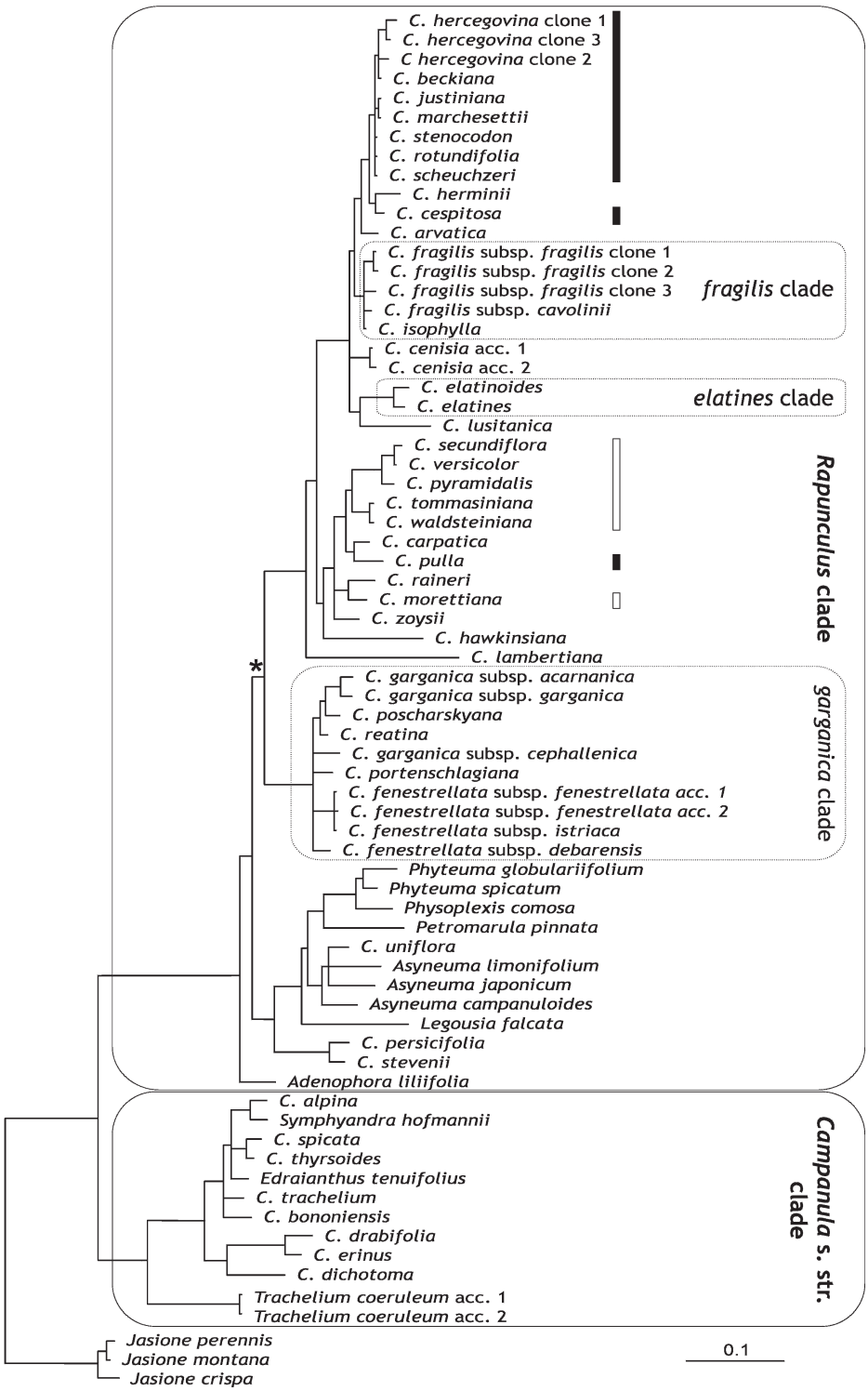

FIG. 4. Phylogenetic relationships of the *Isophylla* group and the isophylloids of *Campanula* inferred from Bayesian analysis of the nuclear ITS region showing branch lengths (calculated as mean values from all trees of the posterior set, where the branch is present). The tree is rooted at the midpoint between *Jasione* and the other taxa. Circumscriptions of the *Rapunculus* and the *Campanula* s. str. clade as well as the three components of the *Isophylla* group (*elatines*, *fragilis* and *garganica* clade) are indicated; black and white bars mark subsect. *Heterophylla* and the isophylloid *Campanula* species, respectively, and the asterisk marks the core clade (see text for details).

this clade, which had posterior probabilities greater than 0.05.

Results

Molecular Parameters. The length of ITS1 ranges from 271 bp in *Symphyandra hofmannii* to 279 bp in several *Campanula* species, e.g., *C. elatines* and *C. stenocodon*, that of 5.8 S is 163 bp (except in *C. alpina* with 164 bp), and that of ITS2 ranges from 248 bp in *C. cenisia*, *C. justiniana* and other *Campanula* species to 294 bp in *Asyneuma limoniifolium*. In the aligned data set, ITS1 has 300 characters, 5.8 S has 164 characters and ITS2 has 294 characters resulting in a total length of 758 characters. The number of variable characters is 388, of which 307 are parsimony-informative.

Phylogenetic Relationships. Maximum parsimony analysis resulted in 78,913 most parsimonious trees (length = 1160, C. I. excluding uninformative characters = 0.4832, R. I. = 0.8345, r. C. I. = 0.4367), and the strict consensus tree is shown in Fig. 2. Maximum likelihood analysis using the likelihood ratchet resulted in 108 topologically identical maximum likelihood trees out of 451 trees sampled. Swapping one of those trees to completion did not result in any changes and gave three maximum likelihood trees (-ln = 7113.82982). These trees are topologically nearly identical to the consensus tree obtained from Bayesian analysis (harmonic mean -ln = 7267.89; Figs. 3, 4), with the differences restricted to nodes with weak statistical support in both methods.

All three analyses infer two major clades (Figs. 2–4), which are called hereinafter the *Campanula* s. str. clade and the *Rapunculus* clade following Eddie et al. (2003). Within the *Campanula* s. str. clade (bootstrap support [BS] 99/93 from maximum parsimony/maximum likelihood analysis, posterior probability [PP] 1.00), the genus *Trachelium*, represented by two accessions of *T. caeruleum* (BS 100/100, PP 1.00), is sister to a clade (BS 100/100, PP 1.00) including several species of *Campanula* as well as the representatives of *Symphyandra* and *Edraianthus* included in our study. Because the species of the *Campanula* s. str. clade are not the main focus of this study, the sampling is less extensive. However, a noteworthy clade in this group is that which includes the annual species *C. dichotoma*, *C. erinus*, and *C. drabifolia* (BS 95/80, PP 1.00), the latter two being sister-species (BS 100/100, PP 1.00).

Similar to the *Campanula* s. str. clade, the *Rapunculus* clade (BS 100/100, PP 1.00) includes, apart from *Campanula* species, taxa traditionally treated as separate genera. Among these, *Petromarula* and the sister taxa *Physoplexis* and *Phyteuma* (BS 99/100, PP 1.00) form one clade (BS 76/87, PP 1.00), which in maximum likelihood and Bayesian analysis is sister to a clade that includes species of *Asyneuma* plus *Campanula uniflora* (BS 85/95, PP 1.00), although this is only weakly supported (BS < 50, PP 0.68). Maximum likelihood and Bayesian analyses (Fig. 3) suggest *Legousia* and a small clade of *C. persicifolia* and *C. stevenii* (BS 100/100, PP 1.00) as consecutive sister groups to the clade including *Phyteuma* and *Asyneuma* (BS 87, PP 1.00 and BS 87, PP 1.00, respectively). A proposed relationship to a clade including only *Campanula* species, among them all isophyllous species investigated (hereinafter called the core clade, indicated by an asterisk in Figs. 2–4), is statistically only weakly supported by maximum likelihood and Bayesian analyses (BS 51, PP 0.82), rendering the phylogenetic relationships between the core clade and the clade including *C. persicifolia* and *Phyteuma* among others and to *Adenophora* equivocal. In maximum parsimony, the relationships between the core-clade, *C. persicifolia* plus *C. stevenii*, *Adenophora*, *Legousia*, *Asyneuma* plus *C. uniflora*, and the clade including *Petromarula*, *Physoplexis* and *Phyteuma* are unresolved (Fig. 2).

Most *Campanula* species of the *Rapunculus* clade belong to the core clade (BS 80/91, PP 0.97), which again is divided into two clades. The smaller of the two (BS 100/100, PP 1.00), the *garganica* clade (Figs. 2–4), comprises the isophyllous species *C. fenestrellata* s.l., *C. garganica* s.l., *C. portenschlagiana*, *C. poscharskyana*, and *C. reatina*. The hypothesis that this group is closely related to the remaining isophyllous species (*C. elatines*, *C. elatinoides*, *C. fragilis* s.l., *C. isophylla*; see below) is rejected (SH-test: -ln = 7188.44288, δ -ln = 74.61307, $p < 0.001$; posterior probability = 0). Within the *garganica* clade, *C. fenestrellata* subsp. *fenestrellata* and subsp. *istriaca* form a monophyletic group (BS 100/100, PP 1.00). Although *C. fenestrellata* subsp. *debarensis* is not inferred as sister to the other two subspecies, but as a distinct lineage with unresolved relationships, a monophyletic *C. fenestrellata* s.l. cannot be rejected based on our data (SH-test: -ln = 7113.88939, δ -ln = 0.05958, $p = 0.9297$; posterior probability = 0.2185). *Campanula garganica* subsp. *garganica* groups with *C. garganica* subsp. *acarnanica* (BS 99/100, PP 1.00), and consecutively with *C. poscharskyana* (BS -/< 50, PP 0.66) and *C. reatina* (BS 64/72, PP 0.99). Although *C. garganica* subsp. *cephallenica* does not group with the other taxa of *C. garganica*, a monophyletic *C. garganica* s.l. is not rejected by the SH-test (-ln = 7119.88099, δ -ln = 6.05118, $p = 0.5721$), but is by the Bayesian test (posterior probability = 0.0032).

The larger clade (BS 100/100, PP 1.00) is more heterogeneous and includes the remaining iso-

phyllous species as well as members from other groups (Figs. 2–4). From the isophyllous species, *Campanula fragilis* s.l. and *C. isophylla* group together (BS 94/96, PP 1.00) as well as *C. elatines* and *C. elatinoides* (BS 100/100, PP 1.00). Although not inferred by any method used, a monophyletic clade of *C. elatines/elatinoides* and *C. fragilis* s.l./*isophylla* is only rejected in the Bayesian approach (posterior probability = 0.0029), but not the maximum likelihood-based SH-test (-ln = 7117.37384, δ -ln = 3.54403, $p = 0.7112$). On the other hand, the hypothesis of a closer relationship between *C. elatines/elatinoides* and *C. fenestrellata* s.l. and others (that is the ''*garganica*-group'' of Damboldt 1965b) is rejected (SH-test: -ln = 7183.22010, δ -ln = 69.39029, $p < 0.001$; posterior probability = 0). Both *C. elatines/elatinoides* and *C. fragilis* s.l./*isophylla* belong to a well supported clade (BS 93/97, PP 1.00) that includes all accessions of heterophyllous species included in this study, e.g., *C. beckiana* and *C. rotundifolia*, as well as *C. arvatica*, *C. cespitosa*, *C. cenisia*, *C. herminii*, and *C. lusitanica* (Figs. 2–4). The relationships among these species or species groups, however, are unclear, and even congruently suggested relationships, such as *C. lusitanica* and *C. elatines/elatinoides*, are only weakly supported (BS 76/69, PP 0.85).

Other *Campanula* species, which formerly have been connected to the isophyllous groups, do not belong to the same clade as *C. fragilis* s.l. or *C. elatines*. Instead, the species aggregates *C. tommasiniana/waldsteiniana* (BS 100/100, PP 1.00) and *C. pyramidalis/secundiflora/versicolor* (BS 100/100, PP 1.00) group together (BS -/54, PP 0.72). Within the latter, *C. secundiflora* is strongly suggested as sister species to *C. versicolor* (BS 99/100, PP 100). A closer relationship to the only weakly-supported species pair *C. pulla/carpatica* (BS 61/53, PP 0.72) is moderately supported (BS 64/75, PP 1.00). Similarly, a relationship to *C. zoysii* and a small clade including *C. morettiana* and *C. raineri* (BS 96/94, PP 1.00) is only weakly supported (BS 65/66, PP 1.00). The phylogenetic relationships of this group to the clade including *C. fragilis* s.l. and *C. elatines* (see above) and to *C. hawkinsiana* and *C. lambertiana* are unclear or, if any are suggested, these are insufficiently supported (Figs. 2–4).

Age Estimation and Biogeographic Analysis. Several substitution rates for ITS sequences have been suggested (Richardson et al. 2001; Plana et al. 2004). The slowest among those would suggest a highly unlikely ancient origin for the clade exclusively consisting of isophyllous species (e.g., *C. fenestrellata*) even older than the inferred origin of the family Campanulaceae (see Materials and Methods). Using the maximum and minimum of a range covered by the majority of published substitution rates in other taxa (8.34×10^{-9} to 3.89×10^{-8} substitutions per site and year: Zhang et al. 2001), more plausible divergence times are estimated. The origin of the *garganica* clade is dated to between (11.17–)8.00(–4.83) and (2.40–)1.72(–1.04) mya, while its diversification started between (5.78–)3.63(–1.48) and (1.24–)0.78(–0.32) mya. The clade involved in the trans-Adriatic disjunction (*C. reatina*, *C. poscharskyana*, *C. garganica* subsp. *garganica*, and *C. garganica* subsp. *acarnanica*) is estimated to have diversified between (3.80–)2.57(–1.34) and (0.81–)0.55(–0.29) mya. Similar estimates are obtained from the penalized likelihood/non-parametric rate smoothening approaches. If the root is taken as 23 mya, the origin of the whole clade is inferred as 9.2/9.2 mya, while its diversification started around 4.7/4.8 mya; the clade involved in the trans-Adriatic disjunction is dated as 2.8/2.8 mya. If 41 mya is assumed at the root, the corresponding age estimates are 16.3/16.5, 8.4/8.5 mya and 5.0/5.0 respectively.

The dispersal-vicariance analysis was conducted on three topologies differing in the relationship between *C. garganica* subsp. *garganica*, *C. garganica* subsp. *acarnanica*, *C. poscharskyana* and *C. reatina* (Fig. 5). All suggested scenarios are equally costly in requiring either two dispersals (Fig. 5a, b [scenario 2], c) or one dispersal and one extinction [Fig. 5b [scenario 1]). The ancestor of *C. garganica* subsp. *garganica* and *C. garganica* subsp. *acarnanica* is suggested to have been distributed in both areas (EW) with a subsequent vicariance event leading to the current distribution. The ancestral distribution of the ancestor of the clade including the two subspecies of *C. garganica* plus *C. reatina* and *C. poscharskyana* is either reconstructed as EW (Fig. 5a, b [scenario 2]) or as E only (Fig. 5b [scenario 1], c). If these reconstructions are weighted by the posterior probabilities of each alternative topology, EW has a probability of 0.779104 (0.654756 + 0.248696 / 2) and E of 0.215007 (0.090659 + 0.248696 / 2), respectively. These probabilities do not sum up to unity, but to 0.994111, which is the posterior probability of the clade of interest.

Discussion

***Phylogenetic Relationships within* Campanula.** Phylogenetic studies in *Campanula* are complicated not only by the large number of species but also by the strong incongruence of previous taxonomic classifications with the phylogenetic relationships both at the inter- and the intrageneric level (Eddie et al. 2003), rendering it difficult to decide a priori

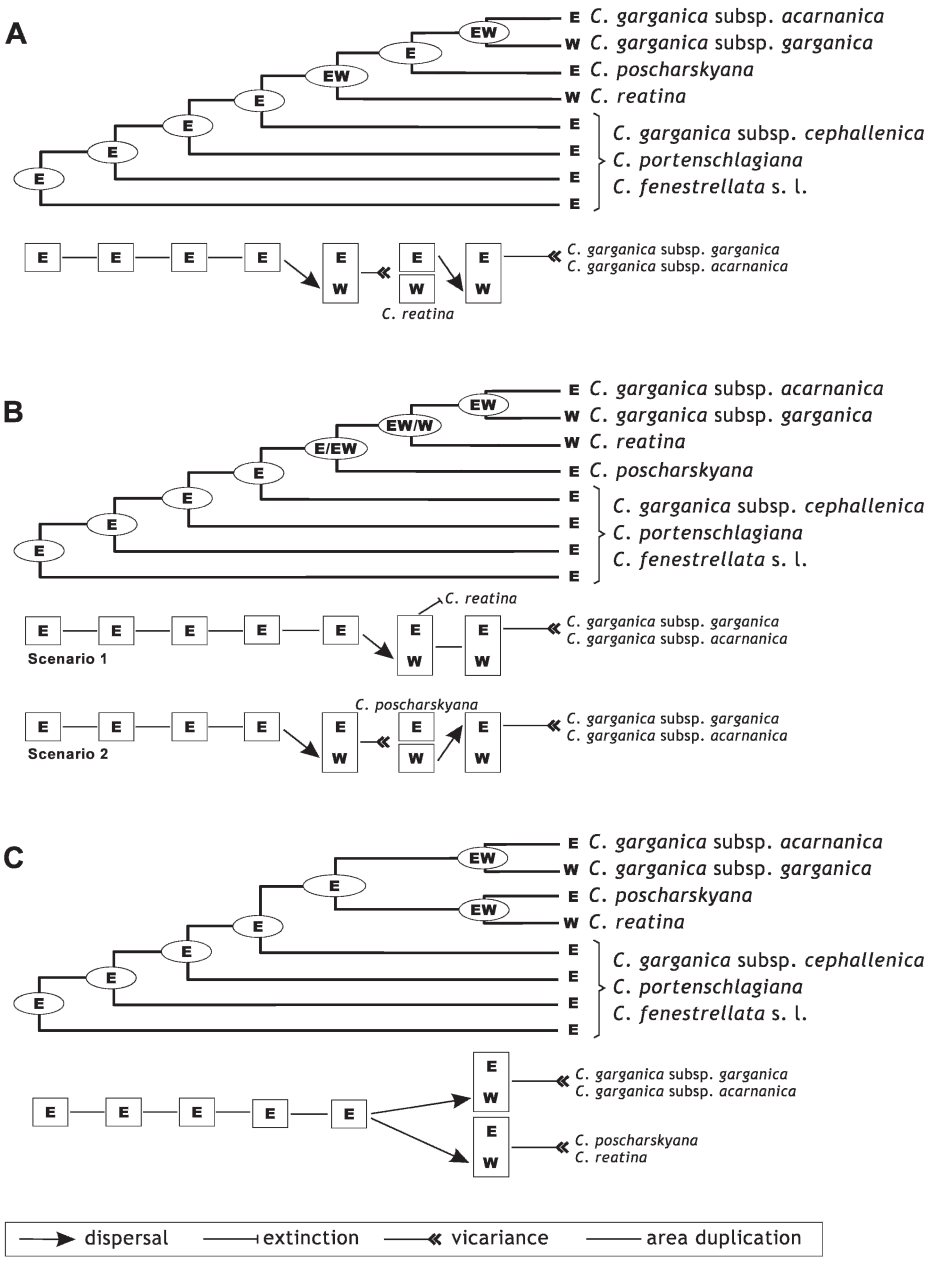

FIG. 5. Dispersal-vicariance-analysis of the species of the *garganica* clade. A–C. Three alternative topologies concerning the relationship of *C. garganica* subsp. *garganica*, *C. garganica* subsp. *acarnanica*, *C. reatina*, and *C. poscharskyana* (see text for details) and the respective reconstructions of ancestral areas. Note that the phylogenetic relationships of the remaining taxa (*C. garganica* subsp. *cephallenica*, *C. portenschlagiana*, and *C. fenestrellata* s.l.) are resolved randomly, but because all have the same distribution (E) it does not affect the analysis.

which taxa should be included in a phylogenetic analysis. This is well exemplified by the results of this study. The *Isophylla* group includes a relatively small number of taxa that are congruent morphologically and biogeographically, and its taxa were therefore considered as a close-knit group (Damboldt 1965b; Lovašen-Eberhardt and Trinajstić 1978). Nevertheless, the *Isophylla* group constitutes three distinct phylogenetic lineages (Figs. 2–4). Due to the lack of predictability of previous taxonomic systems, it cannot be ruled out that some members of these three clades are not included in this study because they have been classified differently. Nevertheless, the circumscription of each of these three groups fits well with its morphology, karyology, habitat require-

ments, and distribution (see below). This together with a dense sampling among sympatric species of other putatively related groups, such as the isophylloids, reduces the possibility that phylogenetically close species were not included in this study.

The restricted sampling of *Campanula* species outside the groups of interest (*Isophylla* group, isophylloids) calls for caution concerning interpretation of inferred phylogenetic relationships, because crucial taxa might not be included. Nevertheless, a few proposed relationships are worth mentioning. Within the *Campanula* s. str. clade, the three annual species *C. dichotoma*, *C. drabifolia*, and *C. erinus* form a monophyletic group. Based on the absence or presence of calyx appendages between the calyx teeth, these species have been assigned to two subgenera: *Megalocalyx* (*C. dichotoma*) and *Roucela* (*C. drabifolia* and *C. erinus*; Damboldt 1976). Both subgenera share a Mediterranean distribution with most species in the east Mediterranean and the Middle East, and both groups exhibit considerable variation in haploid chromosome numbers, being $n = 7, 8, 10, 14, 28$ in subg. *Roucela* (reviewed in Carlström 1986) and $n = 8, 10, 11, 12$ in subg. *Megalocalyx* (reviewed in Sáez and Aldasoro 2003). Further studies including all species of these groups are needed to test the proposed close relationship and to establish a phylogenetic framework for investigating the evolution of chromosome base numbers in this group.

Among those genera included in the *Rapunculus* clade, *Phyteuma* and *Physoplexis* are very closely related, and the distinction of *Physoplexis* from *Phyteuma* is only weakly supported by the molecular data (Eddie et al. 2003; this study). *Physoplexis* is a monotypic genus of the southeastern European Alps and differs from the European endemic *Phyteuma*, by, among other characters, the inflorescence (umbel instead of spikes or capitula), flower color (pink instead of blue, blackish-violet, or yellowish-white), and its chromosome base number ($x = 17$ instead of $x = 10, 11, 12, 14$; Bolkhovskikh et al. 1969). Investigations, including a broader sampling within *Phyteuma*, are currently being undertaken to clarify the taxonomic status of *Physoplexis* in relation to *Phyteuma* (G. M. Schneeweiss, unpubl.). The closest relative of this group is equivocal, and might either be *Asyneuma* (Eddie et al. 2003) or *Petromarula* (this study). Surprisingly, *Campanula uniflora* groups with *Asyneuma*, rendering this genus possibly paraphyletic. *Campanula uniflora* is a circum-arctic species with no known close relationships. In its morphology it displays neotenous floral characteristics and is quite unlike any species of *Asyneuma*. *Asyneuma* is characterized by (sub)sessile flowers with corollas divided nearly to the base, narrow corolla lobes, and capsules dehiscing by pores at or above the middle (Damboldt 1968b, 1969, 1970). However, none of these characters is unique to *Asyneuma*, and the exact circumscription of *Asyneuma* is still controversial (Lakušić and Conti 2004). The sampling in this group is very incomplete, and the inclusion of additional species is required to clarify if this genus is monophyletic and what are its closest relatives among the *Campanula* species.

Phylogeny of the* Isophylla *Group. In the following, we will use the term ''clade'' to describe the phylogenetically defined groups found in this study to terminologically differentiate them from the ''groups'' as defined by Damboldt (1965b). A revised classification of the *Isophylla* group is presented in Table 1. The *Isophylla* group falls into three distinct clades. One, the *fragilis* clade, is congruent with the *fragilis* group of Damboldt (1965b), with sect. *Elatines* ser. *Fragiles* of Trinajstić (Lovašen-Eberhardt and Trinajstić 1978) and with the *C. fragilis* aggregate of Geslot (Greuter et al. 1984). This mainly Tyrrhenian clade (Fig. 1) includes *Campanula isophylla* and *C. fragilis* s.l. and is characterized by a chromosome number of $2n = 32$ (unique within the *Isophylla* group), pseudo-umbellate inflorescences, more or less bowl-shaped corollas with broad lobes, long acuminate hairs at the filament bases, yellow or reddish-brown pollen, and strongly shiny ochre seeds. *Campanula fragilis* subsp. *fragilis* differs from subsp. *cavolinii* by larger dimensions of the leaves and corollas, by smaller and broader calyx lobes, by different altitudinal distribution and by distinct distribution areas (Fig. 1). Yet, these taxa have nearly identical ITS sequences (this study) and similar electrophoretic profiles (Frizzi et al. 1987; Frizzi and Tammaro 1991) and this together with the observations that both subspecies can produce fully fertile hybrids (Damboldt 1965b), strongly supports their treatment on the subspecific level. In contrast, crosses of *C. fragilis* s.l. with *C. isophylla* produce either no seedlings (when *C. isophylla* is the pollen donor) or the F_1 is sterile (when any of the *C. fragilis* subspecies is the pollen donor; Damboldt 1965b), indicating a certain degree of genetic isolation between these geographically well isolated species (Fig. 1) despite the lack of differentiation on the level of ITS sequences. This together with morphological differences between *C. fragilis* s.l. and *C. isophylla* in growth form (monopodial vs. sympodial), calyx lobe width and leaf persistence supports the recognition at the specific level.

The two other clades of the *Isophylla* group differ from the *fragilis* clade by chromosome numbers of

$2n = 34$, which is a very common chromosome number within *Campanula* (Löve and Löve 1961; Gadella 1964; Contandriopoulos 1984; Lammers 1992). The small *elatines* clade consists only of the narrowly endemic Alpine *C. elatines* and *C. elatinoides* (Fig. 1) and is congruent with sect. *Elatines* ser. *Elatines* of Trinajstić (Lovašen-Eberhardt and Trinajstić 1978). Damboldt (1965b) treated them as members of his *garganica* group, a relationship clearly refuted by the results of our study. Instead, *C. elatines* and *C. elatinoides* appear to be more closely related to the *fragilis* clade than to the remainder of the *garganica* clade (Figs. 2–4). Electrophoretic data even suggest that *C. elatinoides* might be more closely related to the *fragilis* clade than to *C. elatines* (Frizzi and Tammaro 1991). These authors noted that the distinct phylogenetic position of *C. elatines* is supported by the peculiar ecological profile of this species: while *C. elatines* is restricted to granitic and gneiss rocks in the (sub)alpine zone, all other species including *C. elatinoides* grow on calcareous rocks in lower altitudes. In contrast, both ITS data (this study) and results of crossing experiments (Damboldt 1965b) support a close relationship of *C. elatines* and *C. elatinoides*, which produce hybrid offspring, although with reduced fertility. Further taxon sampling will be necessary to test if the suggested, but only weakly supported, sister relationship of the *elatines* clade to *C. lusitanica* is confirmed. The group of *C. elatines* and *C. elatinoides* is characterized by sympodial growth form (like in *C. isophylla* of the *fragilis* clade), elongate more or less unilateral inflorescences and rotate flowers with narrow corolla lobes (as in some members of the *garganica* clade), long acuminate hairs at the filament basis (as in the *fragilis* clade), reddish-brown pollen (as in the *fragilis* clade), and black-brownish dull shiny seeds.

The third clade, the *garganica* clade, comprises all species of the *garganica* group of Damboldt (1965b) after the exclusion of *C. elatines* and *C. elatinoides*, and is thus congruent with sect. *Elatines* ser. *Garganicae* of Trinajstić (Lovašen-Eberhardt and Trinajstić 1978). This amphi-Adriatic group is characterized by a monopodial growth form, elongate more or less unilateral inflorescences, broadly funnel-shaped to rotate (only in *C. portenschlagiana* campanulate) corollas with mostly narrow corolla lobes, mostly short obtuse hairs at the filament basis, blue or yellow pollen, and reddish-brown to dark-brown strongly shiny seeds. The phylogenetic relationships of the *garganica* clade are unclear. The inferred well supported sister relationship to the clade including the majority of *Campanula* species of the *Rapunculus* clade included in this study (including the *fragilis* clade and the *elatines* clade, Figs. 2–4) might be altered by the inclusion of other *Campanula* species.

Phylogeny and Biogeography of the* Garganica *Clade. *Campanula fenestrellata* subsp. *fenestrellata* and subsp. *istriaca* are clearly separated from the remaining species of this clade (Figs. 2–4). They occupy northern Dalmatian distribution areas, which only overlap in a small area in the northern Velebit Mountains (Fig. 1), where morphologically intermediate forms occur. The presence of these intermediates and the fact that their ITS sequences are virtually identical supports their recognition on the subspecific level. In contrast, the Albanian-Macedonian endemic *C. fenestrellata* subsp. *debarensis* is phylogenetically distinct, as suggested by the morphological differences and the distribution gap spanning the distribution areas of other species of the *garganica* group (Fig. 1). *Campanula fenestrellata* subsp. *debarensis* differs from the other two subspecies by its generally larger habit (larger leaves on longer petioles, longer flowering stems, larger upper cauline leaves), pronouncedly deflexed calyx lobes (instead of erect) and pollen grains twice as large. Taxonomically, this taxon should again be treated as separate species *C. debarensis*, as was done by Damboldt (1965b), who transferred it to the subspecific rank only in a note added in proof to his publication, and Lovašen-Eberhardt and Trinajstić (1978).

The second species divided into subspecies by Damboldt (1965b) is *C. garganica*, which includes three subspecies. Interestingly, this species is not monophyletic either (Figs. 2–4). Instead, *C. garganica* subsp. *garganica* and subsp. *acarnanica* group with *C. poscharskyana* and *C. reatina*, although only with moderate statistical support. The results of reciprocal crossing experiments agree with this inferred relationship, because *C. garganica* subsp. *garganica* and *C. poscharskyana* can produce fully fertile hybrid offspring (Damboldt 1965b, 1968a; *C. garganica* subsp. *acarnanica* was not investigated in this respect). This is noteworthy, because crosses including other species of Damboldt's *garganica* group do not produce viable offspring, if *C. poscharskyana* is a parent, while the autogamous *C. garganica* subsp. *garganica* can also produce viable but mostly sterile hybrids with *C. fenestrellata* subsp. *fenestrellata* and subsp. *istriaca*.

The phylogenetic position of *C. garganica* subsp. *acarnanica* as sister to subsp. *garganica* is somewhat surprising. Based on the mode of capsule dehiscence (by pores instead of slits as in *C. garganica* subsp. *garganica*), the shared ecological profile (rock crevices in *Abies cephallonica*-forests at higher

elevations vs. rock-crevices at low elevations) and the close proximity to the distribution area (Fig. 1), a closer relationship of subsp. *acarnanica* to subsp. *cephallenica* (see below) was expected. A morphological synapomorphy of *C. garganica* subsp. *acarnanica* and subsp. *garganica* is provided by the short obtuse hairs on the filament bases (vs. long acute hairs in *C. garganica* subsp. *cephallenica*). The clear morphological differences between *C. garganica* subsp. *garganica* and subsp. *acarnanica* (calyx teeth reflexed vs. erect; corolla divided for 2/3–3/4 vs. 1/2–2/3; capsule dehiscence via slits vs. via pores), which are of similar magnitude as among other species in the *garganica* clade, together with the geographic distinctness argue in favor of the treatment as separate species, as initially anticipated by Damboldt (1965b).

Campanula reatina was described after Damboldt's taxonomic treatment of this group (Lucchese 1993), and no data from crossing experiments are therefore available. As in *C. garganica* subsp. *garganica*, the capsules do not open by pores, as in the other species of this group, but apparently via valves (slits in *C. garganica* subsp. *garganica*). On the other hand, it shares blue pollen with *C. fenestrellata* s.l., while the other species of the *garganica* clade have yellow pollen. The molecular results suggest a moderately to well supported relationship to *C. garganica* subsp. *garganica*/subsp. *acarnanica* and *C. poscharskyana* (Figs. 2–4).

The phylogenetic positions of *C. portenschlagiana*, unique within the *garganica* clade by its campanulate corollas, and of *C. garganica* subsp. *cephallenica* are unresolved, or if any are suggested, these are poorly supported. The lack of support for a monophyletic *C. garganica* suggests the treatment of *C. garganica* subsp. *cephallenica* as a separate species, as was done by Lovašen-Eberhardt and Trinajstić (1978). The morphological evidence in *C. garganica* is ambiguous because *C. garganica* subsp. *cephallenica* shares characters with *C. garganica* subsp. *garganica* (reflexed calyx lobes, more deeply divided corollas, style pubescent in lower half) and with *C. garganica* subsp. *acarnanica* (capsule dehiscence via pores). Most striking, however, is the geographic proximity and ecological congruence of *C. garganica* subsp. *cephallenica* with *C. garganica* subsp. *acarnanica*, which obviously evolved in parallel in these taxa.

The *garganica* clade exhibits an amphi-Adriatic distribution with the majority of species on the East Adriatic side (Fig. 1). To obtain age estimates for the clade involved in the trans-Adriatic distribution (*C. garganica* subsp. *garganica*, *C. garganica* subsp. *acarnanica*, *C. reatina*, *C. poscharskyana*), we used two different approaches. One relies on a molecular clock by translating branch lengths into divergence times using a range of ITS substitution rates proposed for other herbaceous groups with the potential pitfall that incorrect substitution rates will lead to grossly wrong ages (assuming correct branch lengths). ITS sequences do not evolve in a strict clock-like manner in our group of interest, which can additionally bias the age estimates. The second approach does not require strict clock-like evolution of sequences, but relies on the availability of external calibration points. Obviously, the lack of sound calibration points for the Campanulaceae in general and the *garganica* clade in particular strongly hampers this approach. We believe, however, that the obtained age estimates can still be used in a meaningful way, if they are not taken as estimates of the actual divergence times but as approximations.

While the evolution of the *garganica* clade might have already started in the Miocene, the diversification of the clade including the Western Adriatic species probably took place in the late Miocene to Pleistocene from a widely distributed ancestor (Fig. 5). Although the range of obtained age estimates is too great (essentially spanning 6 to less than 1 mya) to make any strong conclusions, this temporal setting does not disagree with the hypothesis that the climatic fluctuations of the late Pliocene and the Pleistocene were involved in a trans-Adriatic exchange. In cold periods sea regression led to formation of partly continuous landbridges especially in the northern half of the Adriatic Sea basin (Colantoni et al. 1979), supporting exchange between areas east and west of the Adriatic coast (dispersals inferred from dispersal-vicariance analysis) as suggested for the rock partridge (Randi et al. 2003). In warmer periods, the northern Adriatic basin would be flooded and thus act as a barrier supporting vicariance events. Repeated cycles of isolation in climatically more favorable periods with higher sea levels might have reinforced speciation of these *Campanula* taxa.

Phylogenetic Relationships of the Isophylloids. In contrast to the morphologically well-circumscribed *Isophylla* group, the delimitation of the isophylloids is much less clear, and if taken in the broad circumscription envisaged by Damboldt (1965b), they do not form a monophyletic group. Morphologically, they occupy an intermediate position between the *Isophylla* group and subsect. *Heterophylla*. Morphological characters resembling members of the *Isophylla* group include triangular to oblong-ovate calyx teeth, rotate corollas (except *C. tommasiniana*, see below), and erect capsules. On the other hand, characters otherwise typical for members of subsect. *Heterophylla* are heterophylly

(the basal leaves are often already withered during anthesis) and the presence of lateral sterile shoots in some species.

Although only weakly supported, the following two aggregates might form a monophyletic group, the core isophylloids (Figs. 3, 4). The *waldsteiniana*-aggr. (sensu Geslot in Greuter et al. 1984) includes *C. tommasiniana* and *C. waldsteiniana*. Despite the pronounced differences in their corolla shape (narrowly funnel-shaped vs. broadly campanulate), these species have always been considered very closely related (Damboldt 1965a). This is supported by the results of reciprocal crosses, which yield fully fertile offspring (Damboldt 1965a), and by molecular results from this study. The *pyramidalis*-aggr. (sensu Geslot in Greuter et al. 1984) includes *C. pyramidalis*, *C. versicolor*, and *C. secundiflora*. The close relationship of *C. pyramidalis* and *C. versicolor* is supported by their ability to hybridize (Crook 1951; Lewis and Lynch 1999; the rare *C. secundiflora* is not investigated in this respect). A potential morphological synapomorphy of this group is the presence of verrucose netted ridges on the testa (Damboldt 1965b). Interestingly, *C. versicolor* has seeds similar to *C. waldsteiniana*, supporting a closer relationship between these two aggregates. On the other hand, similar seeds are also found in *C. zoysii*, which clearly does not belong to the core isophylloids (Figs. 2–4). A careful re-evaluation of the seed characters in this group in the light of the molecular phylogenetic hypothesis is required. The phylogenetic relationships of the core isophylloids to other species are not clear. Some evidence comes from crossing experiments: Crosses between *C. tommasiniana* and *C. morettiana* produced only very few viable but sterile plants in several trials (Damboldt 1965a), while those between *C. pyramidalis* and *C. isophylla* produced few plants reaching the flowering stage, but those remained completely sterile (Musch in Musch and Gadella 1972). These results suggest closer relationships between the *waldsteiniana*-aggr. and *C. morettiana* or the *pyramidalis*-aggr. and the *fragilis* clade, but none of them finds sufficient support from the molecular data. Instead, a weakly supported clade of *C. pulla* and *C. carpatica* is congruently inferred as sister group to the core-isophylloids.

The third taxon of the isophylloids included in this study is *C. morettiana*, which groups together with *C. raineri*, another steno-endemic of the Southern Alps. This relationship was suggested by Pitschmann and Reisigl (1959), but based on the position of the capsule pores Damboldt (1965b) explicitly excluded *C. raineri* from the group including the *Isophylla* group and the isophylloids. The phylogenetic relationships of the clade comprising *C. morettiana* and *C. raineri* suggested by the molecular data are inconclusive, as well as the singular successful cross with *C. tommasiniana* (see above). In overall appearance, *C. raineri* is more rapunculoid and not unlike *C. carpatica*, and indeed both species are often confused in seed collections (Good 1986, S. Kovačić, pers. obs.). Generally, the clade including the core isophylloids appears very heterogenous by comprising species traditionally assigned to other groups, such as *C. raineri* (see above) or *C. pulla* (see below).

There are several other species probably related to the groups investigated in this study, which could not be included, but might change some of the inferred relationships. Among the relevant taxa are the Eurasian *C. cymbalaria* Sibth. & Sm. and other species of the problematical subsect. *Saxicolae* (Boiss.) Charadze., such as *C. acutiloba* Vatke from Near Asia; *C. sartorii* Boiss. & Heldr. ex Boiss. from the Aegean Islands; *C. piperi* Howell from Pacific North America; the western North American *C. aurita* Greene, and the northeast Asian segregate genus *Astrocodon*. Other species are somewhat transitional between typically rapunculoid and isophylloid, such as *C. lasiocarpa* Cham. in the North Pacific, *C. reverchoni* A. Gray in Texas, or *C. arvatica* and *C. lusitanica* in Spain, the latter two being included in our analyses, but with unclear phylogenetic relationships.

Phylogenetic Relationships of Isophyllous to Heterophyllous Species. All species of subsect. *Heterophylla* included in this study fall in the same clade as the *fragilis* clade, the *elatines* clade, and the isophylloids. Nyman (1878–1882) considered *C. pulla* and *C. cespitosa* as closely related to the *waldsteiniana*-aggr., while Kovanda (1970b) grouped them as series *Alpicolae* in the group *Rotundifolia* of the subsect. *Heterophylla*. The molecular data show that these two taxa belong to different groups. *Campanula pulla*, an endemic of the northeastern European Alps, belongs to the same well supported clade as the *waldsteiniana*-aggr. and the *pyramidalis*-aggr. (Figs. 2–4). *Campanula cespitosa* groups with the majority of subsect. *Heterophylla* included in this study, although with uncertain phylogenetic affinities. The third species of series *Alpicolae* included in this study, *C. stenocodon*, groups with *C. rotundifolia* and *C. scheuchzeri* among others and thus falls in the same clade as *C. hercegovina*, which has some overall morphological similarity with the *waldsteiniana*-aggr. due to similar gross habit and similar shapes of both basal and cauline leaves. This core group of heterophyllous species seems to form a natural group, although a denser sampling and the use of

better resolving markers will be necessary to convincingly address this question.

Implications for Classification. Recent molecular phylogenetic results suggest that *Campanula* in its current circumscription is paraphyletic with numerous smaller genera nested within (Eddie et al. 2003). Although this is based on the use of a single marker only with known possible problems (Álvarez and Wendel 2003; Bailey et al. 2003), analyses of further molecular markers are not expected to fundamentally change this outcome. There is an ongoing debate on whether to recognize paraphyletic taxa or not (e.g., Nordal and Stedje 2005 vs. Dias et al. 2005; Potter et al. 2005; Williams et al. 2005), and clearly *Campanula* could serve well as an exemplar in this discussion. In the taxonomic treatment by Kolakovsky (1994), which is based primarily on fruit characters, *Campanula* is split into numerous small genera, which are grouped together with traditionally recognized genera such as *Phyteuma* or *Edraianthus* into different tribes. Although not all of these genera are supported (e.g., *C. zoysii* as separate genus *Favratia* Feer) and the circumscription of others would require to be redefined (e.g., *C. carpatica*, *C. lambertiana* and *C. stevenii* are, among others, members of *Neocodon* Kolak. & Serd.; Figs. 2–4), it could serve as a starting point for a revised classification of *Campanula* and related genera. As shown in the present study for the *Isophylla* group, the lack of predictability for phylogenetic relationships of the currently used classifications is a big obstacle rendering changes in classifications based on a limited sampling at best premature. Therefore, a broad scale phylogenetic study of *Campanula* and its allies would be rewarding to elucidate the relationships within this taxonomically controversial group and to get a better understanding of character evolution.

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APPENDIX 1. List of taxa, collection details, voucher information (or reference in case of already published sequences) and GenBank accession numbers. All vouchers are deposited at WU unless otherwise noted.

Outgroup

Jasione crispa (Pourr.) G. Sampaio; France, Eastern Pyrenees, Carnigou; *Schönswetter & Tribsch 6423*; DQ304567. – *J. montana* L.; Italy, Alpi Pennini; *Schönswetter & Tribsch 4787*; DQ304566. – *J. perennis* Lam.; France, Eastern Pyrenees, Carnigou; *Schönswetter & Tribsch 6400*; DQ304568.

***Campanula* s. str. clade**

Campanula alpina Jacq.; Austria, Niedere Tauern; *Schönswetter & Tribsch 6552*; DQ304573. – *C. bononiensis* L.; Austria, Leithagebirge; *Gutermann 22620*; DQ304571. – *C. dichotoma* L.; Italy, Calabria, NW of Nicótera; *Gutermann 36025*; DQ304579. – *C. drabifolia* Sibth. & Sm.; Greece, Ionian islands, Atokos; *Gutermann 34079*; DQ304578. *C. erinus* L.; Greece, Ionian islands, Kalamos; *Gutermann 31606*; DQ304580. – *C. spicata* L.; Italy, Southern Alps; *Schönswetter & Tribsch 6334*; DQ304574. – *C. thyrsoides* L. subsp. *carniolica* (Sünderm.) Podl.; Austria, Lavanttal; *Gutermann 19118*; DQ304575. – *C. trachelium* L.; Austria, Vienna; *Schneeweiss 6284*; DQ304572.

Edraianthus tenuifolius DC.; Croatia, Vratnik pass; *Schönswetter & Tribsch 6275*; DQ304576.

Symphyandra hofmanni Pant.; Bosnia-Herzegovina, Bosut river banks; *Kovačić 767* (ZA); DQ304577.

Trachelium caeruleum L., accession 1; cultivated as ornamental (Italy); no voucher; DQ304569. – *T. caeruleum* L., accession 2; Spain, N of Malaga; *Schönswetter & Tribsch 8736*; DQ304570.

***Rapunculus* clade**

Adenophora liliifolia (L.) DC.; Austria, Vienna Basin; *Schönswetter & Tribsch 6554*; DQ304581.

Asyneuma campanuloides Bornm.; Georgia, Greater Caucasus; *Schönswetter & Tribsch 4469*; DQ304586. – *A. limonifolium* Bornm.; Greece, Ionian Islands, Lefkada; *Gutermann 35549*; DQ304587. – *A. japonicum* (Miq.) Briq.; Kim et al. (1999); AF183437 & AF183438.

Campanula arvatica Lag.; Eddie et al. (2003); AY322010 & AY331423. – *C. beckiana* Hayek; Austria, Northeastern Alps; *Schönswetter & Tribsch 6289*; DQ304619. – *C. carpatica* Jacq.; Eddie et al. (2003); AY322013 & AY331426. – *C. cenisia* L., accession 1; Austria, Lechtaler Alps; *Gutermann 22598*; DQ304622. – *C. cenisia* L., accession 2; France, Savoie, Mont Cenis; *Gutermann 33433*; DQ304623. – *C. cespitosa* Scop.; Austria, Northeastern Alps; *Gutermann 13266*; DQ304621. – *C. elatines* L.; Italy, Alpi Cozie; *Schönswetter & Tribsch 6349*; DQ304624. – *C. elatinoides* Moretti; Italy, Southern Alps, Lago d'Iseo; *Gutermann 1879*; DQ304625. – *C.* (*fenestrellata* Feer subsp.) *debarensis* (Rech. f.) Damboldt; FYR Macedonia, Crni Drin; *Kovačić 1097* (ZA); DQ304595. – *C. fenestrellata* Feer subsp. *fenestrellata*, accession 1; Croatia, Velebit, Velika Paklenica; *Kovačić 920* (ZA); DQ304592. – *C. fenestrellata* Feer subsp. *fenestrellata*, accession 2; Croatia, Velebit, Velika Paklenica; *Gutermann 36526*; DQ304593. – *C. fenestrellata* Feer subsp. *istriaca* (Feer) Fedorov; Croatia, Krk, Uvala Oprna; *Schönswetter & Tribsch 6272*; DQ304594. – *C. fragilis* Cyr. subsp. *fragilis*; Italy, Calabria, city of Scalea; *Gutermann 36164*; DQ304626–DQ304628. – *C. fragilis* Cyr. subsp. *cavolinii* (Ten.) Damb.; Italy, Abruzzo; *M. Iberite & A. Pavesi 15573* (B); DQ304629. – *C.* (*garganica* Ten. subsp.) *acarnanica* Damb.; Greece, Acarnania, Mt. Boumistos; *Damboldt Ca1 1058* (B); DQ304598. – *C.* (*garganica* Ten. subsp.) *cephallenica* (Feer) Hayek; Greece, Ionian Islands, Kefallinía; *Gutermann 28945*;

DQ304597. – *C.* (*garganica* Ten. subsp.) *garganica*; cult. in Botanical Garden Zagreb (material from Italy); *Kovačić 1012* (ZA); DQ304596. – *C. hawkinsiana* Hausskn. & Heldr.; Eddie et al. (2003); AY322019 & AY331432. – *C. hercegovina* Degen & Fiala; Bosnia & Herzegovina, Blidinje; *Kovačić 1076* (ZA); DQ304616–DQ304618. – *C. herminii* Hoffmanns. & Link; Eddie et al. (2003); AY322020 & AY331433. – *C. isophylla* Moretti; cultivated in Botanical Garden Zagreb (material from Italy); *Kovačić 1013* (ZA); DQ304630. – *C. justiniana* Witasek; Croatia, Čabranka river; *Kovačić 746* (ZA); DQ304613. – *C. lambertiana* DC.; Azerbaijan, Talysh; *Schönswetter & Tribsch 6764*; DQ304609. – *C. lusitanica* L.; Eddie et al. (2003); AY322025 & AY331438. – *C. marchesettii* Witasek; Croatia, Učka; *Kovačić 781* (ZA); DQ304612. – *C. morettiana* Reichenb.; Italy, Dolomites; *Festi s. n.* (ROV); DQ304602. – *C. persicifolia* L.; Austria, Northeastern Alps; *Schönswetter & Tribsch 6288*; DQ304590. – *C. portenschlagiana* Schultes; Croatia, Biokovo; *Kovačić 692* (ZA); DQ304600. – *C. poscharskyana* Degen; Croatia, Dubrovnik region; *Kovačić 690* (ZA); DQ304601. – *C. pulla* L.; Austria, northeastern Alps; *Schönswetter & Tribsch 6555*; DQ304605. – *C. pyramidalis* L.; Croatia, Vratnik pass; *Schönswetter & Tribsch 6243*; DQ304606. – *C. raineri* Perpenti; Italy, Alpi Bergamaschi; *Gutermann 2553*; DQ304604. – *C. reatina* Lucchese; Italy, Turano Valley; *Kovačić 768* (ZA); DQ304599. – *C. rotundifolia* L.; Croatia, Platak-Rijeka region; *Kovačić 784* (ZA); DQ304615. – *C. scheuchzeri* Vill.; Croatia, North Velebit; *Kovačić 807* (ZA); DQ304614. – *C. secundiflora* Vis. & Panc.; Serbia-Crna Gora, Panjica river banks; *Kovačić 1109* (ZA); DQ304608. – *C. stenocodon* Boiss. & Reuter; Italy, Alpi Cozie; *Schönswetter & Tribsch 6340*; DQ304620. – *C. stevenii* Bieb.; Georgia, Minor Caucasus; *Schönswetter & Tribsch 6976*; DQ304591. – *C. tommasiniana* Koch; Croatia, Učka; *Kovačić 775* (ZA); DQ304611. – *C. uniflora* L.; Norway, Sor-Trondelag; *Schönswetter & Tribsch 7090*; DQ304588. – *C. versicolor* Andrews; Greece, Ionian Islands, Kefallinía; *Gutermann 30067*; DQ304607. – *C. waldsteiniana* Schultes; Croatia, Velebit Mtns.; *Schönswetter & Tribsch 6302*; DQ304610. – *C. zoysii* Wulfen; Slovenia, Kamniške Alps; *Schönswetter & Tribsch 9708*; DQ304603.

Legousia falcata Fritsch; Greece, Ionian Islands, Meganisi; *Gutermann 30418*; DQ304589.

Petromarula pinnata DC.; Greece, Crete; *Schönswetter & Tribsch* 7821; DQ304582.

Physoplexis comosa Schur; Italy, Southern Alps; *Schönswetter & Tribsch 3902*; DQ304585.

Phyteuma globulariifolium Sternb. & Hoppe; Austria, Niedere Tauern; *Schönswetter & Tribsch 4551*; DQ304583. – *Ph. spicatum* L.; Croatia, Gorski kotar; *Schönswetter & Tribsch 6233*; DQ304584.