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ORIGINAL ARTICLE

Molecular and morphological survey on *Campanula cremnophila* (Campanulaceae), a new isophyllous species from Croatia

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Abstract

Within the isophyllous group of *Campanula* ser. *Garganicae*, represented by species of prevalent Adriatic and Ionian distribution, a rare new endemic species named *C. cremnophila* is described and illustrated from Pelješac Peninsula and the nearby Elaphite islands of Olipa and Jakljan (southern Croatia). It is a chasmophyte growing on calcareous rocky crevices near the sea, showing close relationship with *C. garganica* from southern Italy. Phylogenetic analyses based on nuclear ITS and chloroplast *trn*L–*trn*F data emphasized that *C. cremnophila* is distinct from other species of this group, forming a subclade with *C. garganica* and *C. poscharskyana*. The morphology, SEM microstructure of seeds and pollen grains, taxonomical relationships, ecology, conservation status of *C. cremnophila*, as well as an identification key including the species of the ser. *Garganicae*, are provided.

Keywords *Campanula* · Dalmatia · Endemic · Phylogeny · Series *Garganicae* · Taxonomy

Introduction

Within the genus *Campanula* L., about 580–600 species are currently included (Mansion et al. 2012). The genus is represented by annual and perennial taxa occurring in different types of habitats such as grasslands, garrigues, meadows, woodlands and rupestrian habitats (Kovačić 2004; Roquet et al. 2008; Bogdanović et al. 2014a, b) distributed in the Northern Hemisphere. About 250 species are distributed in the Mediterranean area (Damboldt 1965; Podlech 1965; Kovanda 1970a, b, 1977; Fedorov and Kovanda 1976; Geslot 1984; Park et al. 2006) that can be considered as one of the principal centres of diversity (Myers et al. 2000). According to the literature, the genus shows marked variability in the habit, leaf and flower morphology (Kolakovsky 1986; Roquet et al. 2008), carpology (Kolakovsky 1986), seed micromorphology (Geslot 1980; Akcin 2009; Alçitepe 2010), palynology (Dunbar 1975; Dunbar and Wallentinus 1976) and karyology (Gadella 1964; Damboldt 1965, 1968; Kovanda 1970a, b, 1977), making the natural hierarchical arrangement of the genus challenging. In fact, the taxonomic treatments proposed in the past (Fedorov 1957; Rechinger and Schimann-Czeika 1965; Gadella 1966a, b; Damboldt 1978; Contandriopoulos 1984; Kolakovsky 1994) are not always supported by recently available phylogenetic studies (Eddie et al. 2003; Park et al. 2006; Roquet et al. 2008, 2009;

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Borsch et al. 2009; Cellinese et al. 2009; Haberle et al. 2009; Mansion et al. 2012; Crowl et al. 2014). Nevertheless, the most comprehensive phylogenetic inference for the genus resolved 17 well-supported and circumscribed sub-lineages (Mansion et al. 2012).

In the Western Balkan and amphi-Adriatic region, there are several endemic species of the genus *Campanula*, represented usually by chasmophytes belonging to different phyletic lineages (Park et al. 2006; Liber et al. 2008; Frajman and Schneeweiss 2009; Lakušić et al. 2013; Janković et al. 2016, 2019). In particular, a group very rich in endemics is represented by *Campanula* ser. *Garganicae* recognized by Trinajstić in Lovašen-Eberhardt and Trinajstić (1978), which is clearly monophyletic as shown by recent phylogenetic studies based on nuclear and plastid sequences (Park et al. 2006; Liber et al. 2008; Frajman and Schneeweiss 2009). According to Damboldt (1965, 1968) and Bogdanović et al. (2014a, b, 2015), this group includes numerous endemic species, often showing very restricted distribution, among them *C. reatina* Lucchese, *C. teutana* Bogdanović & Brullo, *C. acarnanica* Damboldt, *C. skanderbegii* Bogdanović, Brullo & D.Lakušić and *C. aureliana* Bogdanović, Rešetnik, Brullo & Shuka. All these taxa are localized along the eastern Adriatic coast of Croatia, Italy and Albania, as well as in the Republic of North Macedonia, Ionian Islands and western Greece. Additionally, Frajman and Schneeweiss (2009) revealed that the Albanian endemic *C. comosiformis* (Hayek & Janch.) Frajman & Schneew. is a sister to a *C. garganica* clade confirmed also by Bogdanović et al. (2014a, b, 2015).

The members of the *Campanula* ser. *Garganicae* share specific morphological features, such as monopodial growth form, isophyllous and long petiolate leaves, with cordate to ovate blades, elongated and more or less unilateral inflorescence, campanulate or rotate corolla, staminal filaments hairy at the base, brown shiny seeds and uniform diploid (2n = 34) chromosome number (Damboldt 1965; Lovašen-Eberhardt and Trinajstić 1978).

Within the frame of taxonomic investigation of the *Campanula* ser. *Garganicae*, the populations of a peculiar and unknown *Campanula* were discovered in circumscribed rupestrian habitats along the southern part of the Pelješac Peninsula and in nearby Elaphite islands of Olipa and Jakljan in southern Dalmatia (Croatia). To assess phylogenetic position and morphological relationships of unknown populations of *Campanula*, three individuals from Pelješac Peninsula were included in the broad data sets of nuclear ITS and plastid *trn*L–*trn*F sequences, and phylogenetic analyses were performed.

Materials and methods

Plant material

During field trips on Pelješac Peninsula and Elaphite islands of Olipa and Jakljan in 2016, 2017 and 2018, the investigation on collected specimens informally called "*Campanula cremnophila*" was carried out. In total, only 15 individuals were collected to avoid damage to the current small population. These specimens were used for herbarium exsiccata (CAT, ZA and ZAGR), and fresh leaves from three individuals were conserved in silica gel for DNA analysis, while ten floral and vegetative parts (flowers, buds, capsules, bracts and leaves) were placed in 50% glycerine–ethyl alcohol solution for further morphological assessment. For the phylogenetic analysis, in order to obtain better representation of the *Campanula* ser. *Garganicae*, we used the data sets of Park et al. (2006), Frajman and Schneeweiss (2009) and Bogdanović et al. (2014a, b, 2015), extended with ten new sequences from additional accessions. Voucher data and GenBank accession numbers of the newly sequenced taxa, as well as GenBank accession numbers from previous studies, are given in Table 1. Herbarium specimens of isophyllous species of *Campanula* were studied from B, BEOU, BM, BP, CAT, CNHM, FI, K, MKNH, NHMR, P, RO, TIR, W, WU, ZA, ZAGR and ZAHO (abbreviations follow Thiers 2019).

Scanning electron microscopy (SEM)

In total, ten mature capsules were collected from the field, and dried seeds were removed and prepared for the micromorphological seed testa analyses by using a scanning electron microscope (Zeiss EVO LS10). Terminology of seed coat sculpturing follows Barthlott (1981) and Gontcharova et al. (2009). The preparation of the seeds of "*C. cremnophila*", collected from Pelješac Peninsula in Croatia, and *C. garganica*, collected from Mt. Gargano in Italy, was done according to Huttunen and Laine (1983). Pollen grain morphology was examined on dried herbarium materials removed from type specimens (CAT and ZAGR). Dried pollen was mounted on stubs without any preparation. Pollen terminology follows Walker and Doyle (1975), Punt et al. (1994, 2007) and Hesse et al. (2009).

DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted from silica-gel-dried leaves or herbarium specimens using the DNeasy plant mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Polymerase chain reactions (PCR) were performed as described in Bogdanović et al. (2015) using the 17SE and 26SE primers (Sun et al. 1994) for the nuclear

Table 1 Checklist of taxa, collection details, voucher information and GenBank accession numbers of Campanulaceae species analysed in this study. Asterisks (*) indicate new sequences produced in this study

No.	Taxon	Collection details	Voucher information	ITS GenBank number	trnL–F GenBank number
1	*Asyneuma campanuloides* Bornm.	Georgia, Greater Caucasus	*Schönswetter* and *Tribsch 4469* (WU)	DQ304586	FJ426570
2	*Asyneuma limoniifolium* Bornm.	Greece, Ionian Islands, Lefkada	*Gutermann 35549* (WU)	DQ304587	FJ426571
3	*Campanula acarnanica* Damboldt 1	Greece, Acarnania, Mt., Akarnanika Ori	*Karamplianis Th. 1692* (ATHU)	KF957752	KF957763
4	*Campanula acarnanica* Damboldt 2	Greece, Acarnania, Kandila, Pighadia	*Bogdanović* and *Rešetnik s.n.* (ZAGR)	MK981222*	MN013847*
5	*Campanula aureliana* Bogdanović, Rešetnik, Brullo & Shuka 1	Albania, Tomori Mt, western part of village Kapinovë	*Bogdanović* and *Jug Dujaković* (ZAGR)	KM215787	KM215789
6	*Campanula aureliana* Bogdanović, Rešetnik, Brullo & Shuka 2	Albania, Tomori Mt, Tomori village	*Bogdanović, Rešetnik* and *Temunović s.n.* (ZAGR)	KM215788	KM215790
7	*Campanula cephallenica* Feer 1	Greece, Ionian Islands, Kefallinía	*Gutermann* 28945 (WU)	DQ304597	FJ426576
8	*Campanula cephallenica* Feer 2	Greece, Isola Cephalonia, Mt. Aivos	*S. Brullo* and *G. Giacalone s.n.*	KF957753	KF957764
9	*Campanula comosiformis* (Hayek & Janch.) Frajman & Schneew. 1	Albania, Gjallica, Mustafe	*Bogdanović* and *Jug Dujaković s.n.* (ZAGR)	KF957754	KF957765
10	*Campanula comosiformis* (Hayek & Janch.) Frajman & Schneew. 2	Albania, Šija gorge E of Bicaj	*Frajman 11089* (WU)	FJ426592	FJ426572
11	*Campanula cremnophila* Bogdanović, Rešetnik, M. Jeričević, N.Jeričević & Brullo 1	Pelješac peninsula, Marčuleti Bay, Rt Vrba	*M. Jeričević, N. Jeričević* and *Bogdanović s.n.* (ZAGR)	MK940906*	MK934576*
12	*Campanula cremnophila* Bogdanović, Rešetnik, M. Jeričević, N.Jeričević & Brullo 2	Pelješac peninsula, Marčuleti Bay, Rt Vrba	*M. Jeričević, N. Jeričević* and *Bogdanović s.n.* (ZAGR)	MK940907*	MK934574*
13	*Campanula cremnophila* Bogdanović, Rešetnik, M. Jeričević, N.Jeričević & Brullo 3	Pelješac Peninsula, Marčuleti Bay, Rt Vrba	*M. Jeričević, N. Jeričević* and *Bogdanović s.n.* (ZAGR)	MK940908*	MK934575*
14	*Campanula debarensis* Rech.f. 1	FYR Macedonia, Crni Drin	*Kovačić* 1097 (ZA)	DQ304595	FJ426575
15	*Campanula debarensis* Rech.f. 2	FYR Macedonia, Crni Drim	*K. Micevski s.n.* (MKNH 031830)	KF957738	KF957745
16	*Campanula elatines* L.	Italy, Alpi Cozie	*Schönswetter* and *Tribsch 6349* (WU)	DQ304624	FJ426577
17	*Campanula elatinoides* Moretti	Italy, Southern Alps, Lago d'Iseo	*Gutermann 1879* (WU)	DQ304625	FJ426578
18	*Campanula fenestrellata* Feer subsp. *fenestrellata* 1	Croatia, Velebit, Velika Paklenica	*Kovačić 920* (ZA)	DQ304592	FJ426579
19	*Campanula fenestrellata* Feer subsp. *fenestrellata* 2	Croatia, NP Krka, Roški slap	*Šegota* and *Hršak s.n.* (ZAGR)	KF957755	KF957766
20	*Campanula fenestrellata* subsp. *istriaca* (Feer) Damboldt 1	Croatia, Krk, Uvala Oprna	*Schönswetter* and *Tribsch 6272* (WU)	DQ304594	FJ426584
21	*Campanula fenestrellata* subsp. *istriaca* (Feer) Damboldt 2	Croatia, Istra, Plomin	*Bogdanović* and *Ljubičić s.n.* (ZAGR)	KF957756	KF957767
22	*Campanula fragilis* Cirillo	Italy, Calabria, city of Scalea	*Gutermann 36164* (WU)	DQ304626	FJ426580
23	*Campanula garganica* Ten. 1	Cult. in Botanical Garden Zagreb (material from Italy); Italy,	*Kovačić 1012* (ZA); *Aldobrandi 12-VII-96* et al. (MA 625685)	DQ304596	EF088725
24	*Campanula garganica* Ten. 2	Italy, Gargano, Vieste	*Brullo* and *Signorello s.n.* (CAT 037.237/7)	KF957739	KF957746
25	*Campanula garganica* Ten. 3	Italy, Gargano, St. Angelo, S. Maria di Pulsano	*Bogdanović s.n.* (ZAGR)	MK981223*	MN013848*
26	*Campanula isophylla* Moretti	Cult. in Botanical Garden Zagreb (material from Italy)	*Kovačić 1013* (ZA)	DQ304630	FJ426583

Table 1 (continued)

No.	Taxon	Collection details	Voucher information	ITS GenBank number	trnL–F GenBank number
27	*Campanula persicifolia* L.	Austria, Northeastern Alps	*Schönswetter* and *Tribsch 6288* (WU)	DQ304590	FJ426573
28	*Campanula pollinensis* Podlech	Italy, Monte Pollino	*Brullo, Signorello, Spampinato s.n.* (CAT 037.066/30)	KF957740	KF957747
29	*Campanula portenschlagiana* Roem. & Schult. 1	Croatia, Biokovo	*Kovačić 692* (ZA)	DQ304600	FJ426587
30	*Campanula portenschlagiana* Roem. & Schult. 2	Croatia, otok Brač, Vidova gora	*Ruščić s.n.* (ZAGR 26291)	KF957741	KF957748
31	*Campanula portenschlagiana* Roem. & Schult. 3	Bosnia and Herzegovina, Ljubuški	*Šiljeg s.n.* (ZAGR)	KF957757	KF957768
32	*Campanula portenschlagiana* Roem. & Schult. 4	Croatia, Island Hvar, Pitve	*Rimac s.n.* (ZAGR)	KF957758	KF957769
33	*Campanula poscharskyana* Degen 1	Croatia, Dubrovnik region	*Kovačić 690* (ZA)	DQ304601	FJ426588
34	*Campanula poscharskyana* Degen 2	Croatia, Radovčići	*Kovačić* (ZAGR)	KF957759	KF957770
35	*Campanula pyramidalis* L.	Croatia, Vratnik pass; Croatia, Rijeka	*Schönswetter* and *Tribsch 6243* (WU); *Vitek 99440* (MA 641379)	DQ304606	EF088754
36	*Campanula reatina* Lucchese 1	Italy, Turano Valley	*Kovačić 768* (ZA)	DQ304599	FJ426589
37	*Campanula reatina* Lucchese 2	Italy, Valle del Salto, Ponte Figureto	*Kirin s.n.* (ZAGR)	KF957760	KF957771
38	*Campanula rotundifolia* L.	Croatia, Platak—Rijeka region; Andorra	*Kovačić 784* (ZA); *Sa´ez 6134* (BCB)	DQ304615	EF088759
39	*Campanula scheuchzeri* Vill.	Croatia, North Velebit	*Kovačić 807* (ZA)	DQ304614	KF957749
40	*Campanula skanderbegii* Bogdanović, Brullo & D.Lakušić 1	Albania, Kruje	*Lakušić, Kuzmanović, Lazarević* and *Alegro s.n.* (ZAGR)	KF957761	KF957772
41	*Campanula skanderbegii* Bogdanović, Brullo & D.Lakušić 2	Albania, Kruje	*Lakušić, Kuzmanović, Lazarević* and *Alegro s.n.* (ZAGR)	KF957762	KF957773
42	*Campanula stevenii* Bieb.	Georgia, Minor Caucasus; Armenia, Vayk	*Schönswetter* and *Tribsch 6976* (WU); *Oganessian s.n.* (ERE 154865)	DQ304591	EF088770
43	*Campanula teutana* Bogdanović & Brullo 1	Croatia, Island of Vis, Oključina, calcareous cliffs near Kraljičina špilja	*Bogdanović s.n.* (ZAGR 32628)	KF957742	KF957750
44	*Campanula teutana* Bogdanović & Brullo 2	Croatia, otok Vis, Oključina	*Bogdanović s.n.* (ZAGR)	KF957743	KF957751
45	*Campanula tommasiniana* Koch	Croatia, Učka	*Kovačić 775* (ZA)	DQ304611	FJ426590
46	*Campanula versicolor* Andrews	Greece, Ionian Islands, Kefallinía	*Gutermann 30067* (WU)	DQ304607	FJ426591
47	*Petromarula pinnata* DC.	Greece, Crete	*Schönswetter* and *Tribsch 7821* (WU)	DQ304582	FJ426585
48	*Physoplexis comosa* Schur	Italy, Southern Alps	*Schönswetter* and *Tribsch 3902* (WU)	DQ304585	FJ426586
49	*Phyteuma globulariifolium* Sternb. & Hoppe	Austria, Niedere Tauern	*Schönswetter* and *Tribsch 4551* (WU)	DQ304583	FJ426582
50	*Phyteuma spicatum* L.	Croatia, Gorski Kotar; Spain, Barcelona, Aiguafreda	*Schönswetter* and *Tribsch 6233* (WU); *Roquet 8-V-05* (BC)	DQ304584	EF088787
51	*Trachelium caeruleum* L.	Spain, N of Malaga; Spain, Santander, Liencres	*Schönswetter* and *Tribsch 8736* (WU); *Aldasoro 3503* (MA)	DQ304570	EF088791

ITS and c and f primers (Taberlet et al. 1991) for the plastid *trn*L–*trn*F region. The PCR products were purified with the GenElute PCR clean-up kit (Sigma-Aldrich Chemie, Steinheim, Germany), according to the manufacturer's protocol. The products were sequenced on both strands by the Macrogen Inc. (Seoul, Korea) using the BigDyeTM terminator cycle sequencing kit (Applied Biosystems, Foster City, California) and analysed on an ABI PRISM 3730XL automated sequencer (Applied Biosystems, Foster City, California). Sequence alignments and data sets used for phylogenetic analyses are available at TreeBase study number 24483.

Phylogenetic analyses

Contigs were assembled and edited and sequences were manually aligned using Geneious Pro 5.3.6 (Drummond et al. 2011). Base polymorphisms in the ITS sequences were coded using NC-IUPAC ambiguity codes. Three different data sets (ITS, *trn*L–*trn*F, ITS–*trn*L–*trn*F combined data set) were analysed using maximum parsimony (MP) and Bayesian inference (BI). The trees were rooted using *Trachelium caeruleum* L. as an outgroup. To assess the degree of phylogenetic congruence between the two different data sets, an incongruence length difference (ILD) test (Farris et al. 1994) implemented as partition homogeneity test in PAUP* 4.0b10 (Swofford 2003) was performed using 1000 partition replicates, each comprising 100 random sequence addition replicates, and TBR branch swapping. Invariant characters were removed from the data sets prior to performing the ILD test (Cunningham 1997). Unweighted MP analyses were conducted using heuristic search, with 1000 random addition sequence replicates, and tree bisection reconnection (TBR) branch swapping, as implemented in PAUP* 4.0b10 (Swofford 2003). Bootstrap support values (MPB; Felsenstein 1985) from 1000 replicates were generated using the heuristic search options as above, except for random addition sequence with 100 replicates. BI was conducted using MrBayes 3.2.2 (Ronquist et al. 2012) applying the substitution models proposed by the Akaike information criterion implemented in MrModelTest (Nylander 2004; Table 2) as selected for each partition separately. The analysis of the combined data set was carried out under partition-specific substitution models (Nylander 2004), and thus, all substitution model parameters were allowed to vary across partitions. The Markov chain Monte Carlo (MCMC) settings consisted of two runs with four chains each for 10^7 generations, sampling trees every 1000th generation. The first 2500 trees (prior to the 2.5×10^6 generation), which were well after the chains had reached stationarity as judged from plots of the likelihood and from the average standard deviation of split frequencies being < 0.01, were discarded as burn-in. Convergence of the MCMC procedure was assessed further by calculating the effective sample sizes (ESSs) with the program Tracer 1.4 (Rambaut and Drummond 2007). A majority-rule consensus tree was constructed from the posterior set of 15,000 trees.

Results

On the basis of living and herbarium materials, *Campanula cremnophila* is well differentiated from the other species of the *Campanula* ser. *Garganicae* in several and significant features regarding the vegetative and reproductive structure. In particular, it is characterized by small leaves, minutely dentate, compact racemes and very small flowers. Some characters of this species make it more similar to *C. garganica*, mainly due to common calyx teeth linear-lanceolate, patent-reflexed at anthesis, corolla rotate, with tube subequal to lobes, petals externally hairy on the midrib, and capsule without pores. However, *C. garganica* differs from *C. cremnophila* in several diagnostic characters, as stems up to 35 cm long, leaves usually glabrous (sometimes hairy), petiole up to 10 cm long, blade cordate, crenate–serrate, with 10–22 teeth, flower pedicel up to 3 cm, calyx glabrous (sometimes hairy), calyx teeth often denticulate, up to 6 mm long, and 2 mm wide, corolla 10–13 mm long, 20–22 mm in diameter, with lobes 6–8 mm long, stamen filaments 3.5 mm long, anthers 4 mm long, pollen yellow, style 9–12 mm long, capsule subglobose, 4 mm long, seeds subglobose, red brown dark, 0.5–0.53 × 0.38–0.45 mm (Fig. 1).

Table 2 Characteristics of cpDNA, ITS and cpDNA–ITS combined data sets analysed using maximum parsimony (MP) and Bayesian inference (BI); substitution models proposed by MrModelTest and used in BI

Region	cpDNA	ITS	Combined
Alignment length	978	766	1744
Number/% of parsimony-informative characters	118/12.06	209/27.28	327/18.75
Length of MP trees	282	589	876
CI/RI	0.7629/0.9348	0.6116/0.8448	0.7272/0.8753
Substitution model used in BI	SYM + G	SYM + G	SYM + G
Harmonic mean of the posterior likelihood scores (*ln* L) of BI trees	− 3285.86	− 4307.55	− 7704.73
Effective sample size (ESS)	13,420.18	15,064.31	13,791.97

CI consistency index (excluding uninformative characters); *RI* retention index

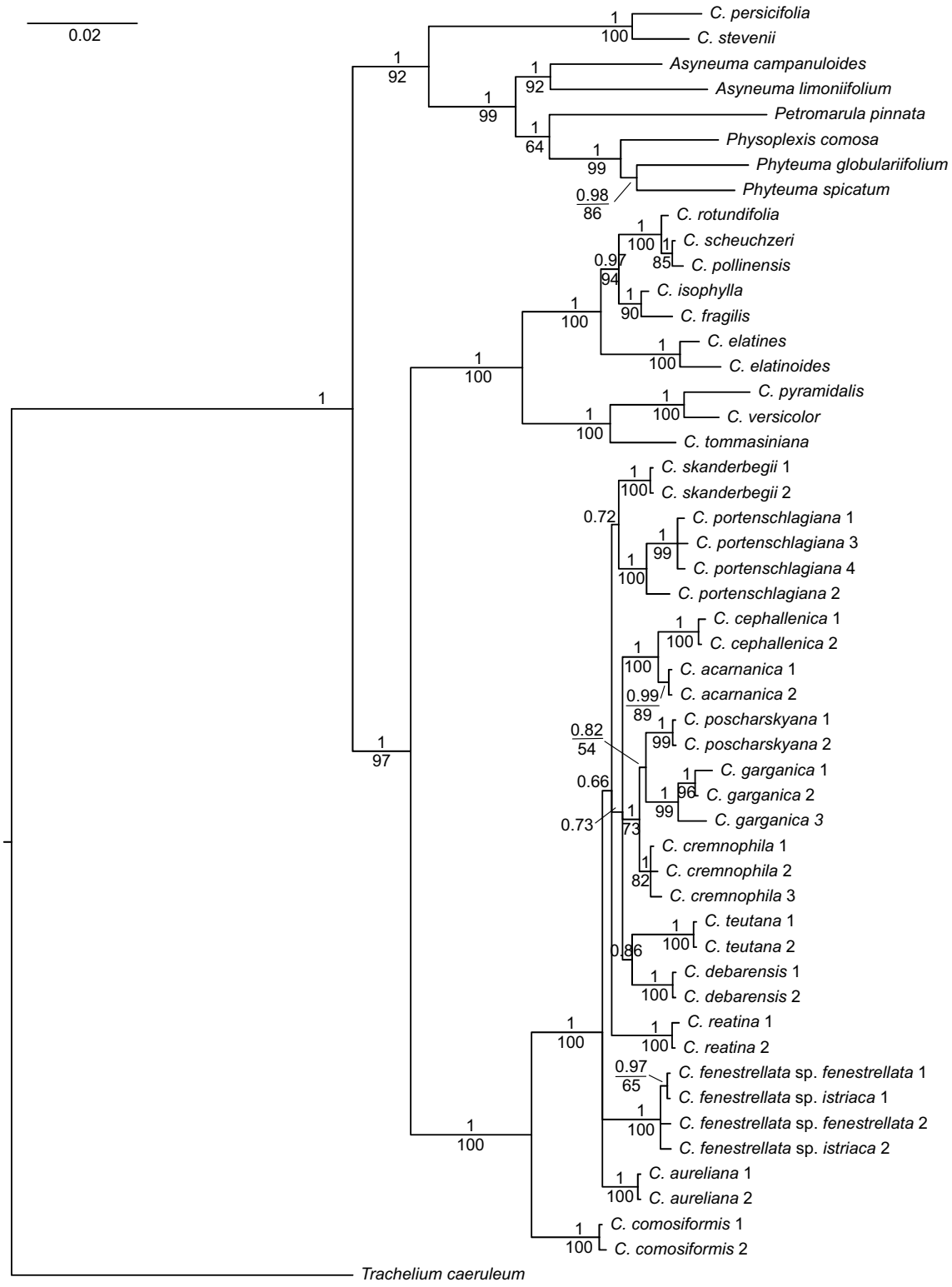

Fig. 1 Phylogenetic tree of the species belonging to *Campanula* ser. *Garganicae* based on combined data set (ITS + *trn*L–*trn*F) from Bayesian analysis. Values above branches are Bayesian posterior probabilities (PP), and values below branches are maximum parsimony (MPB) bootstrap percentages (only shown if at least 50%)

Pollen morphology

The dry yellow pollen grain of *C. cremnophila* is spheroidal, medium sized (24–25 µm), zonoaperturate, with circular outline in equatorial view, 4-porate, microechinate and rugulate (Fig. 2), while according to Halbritter (2016) the pollen of *C. garganica* differs in size (more than 25 µm), number of pores (5-porate, sometimes 4-porate) and striate–microreticulate.

Seed micromorphology

Campanula cremnophila shows seeds ovoid to ellipsoid, quite flat, pale brown, 0.45–0.5 × 0.3 mm, seed testa markedly striate, with short, large and fibriform cells, very anastomosed, 28–50 µm long, 6–7.5 µm wide, with periclinal walls showing a deeply incise linear lumen, while the anticlinal walls quite prominent, and slightly channelled on the beck (Fig. 3a–c). The seeds were compared with those ones of closely related *C. garganica*, which are ellipsoid to subglobose, swollen, bigger, red brown dark, 0.5–0.53 × 0.38–0.45 mm, seed testa weakly striate, with long, thin and fibriform cells, laxly anastomosed, 40–65 µm long, 1.5–2.5 µm wide, with periclinal walls wide, smooth and slightly raised, while the anticlinal walls are slightly prominent and smooth (Fig. 3d–f).

Phylogenetic analyses

The characteristics of the ITS, *trn*L–*trn*F, ITS–*trn*L–*trn*F combined data sets analysed using MP and BI are reported in Table 2. As the ILD test revealed no significant difference ($p = 0.25$) between the selected partitions (cpDNA and ITS), the phylogenetic tree of the combined data set obtained by BI analyses is presented in Fig. 1. In all three data sets, *Campanula* individuals from the Pelješac Peninsula are inferred as distinct member of the *C. garganica* clade (Fig. 1, Online Resource 1). In particular, *C. cremnophila* is resolved as sister to the subclade grouping *C. garganica* and *C. poscharskyana*, with moderate to high support (1 PP, 73 MPB, Fig. 1). As previously emphasized by Bogdanović et al. (2014a, b 2015), all species belonging to *Campanula* ser. *Garganicae* form in the phylogenetic trees a well-supported lineage that confirm the monophyletic origin of this group, although the relationships within the group are not resolved.

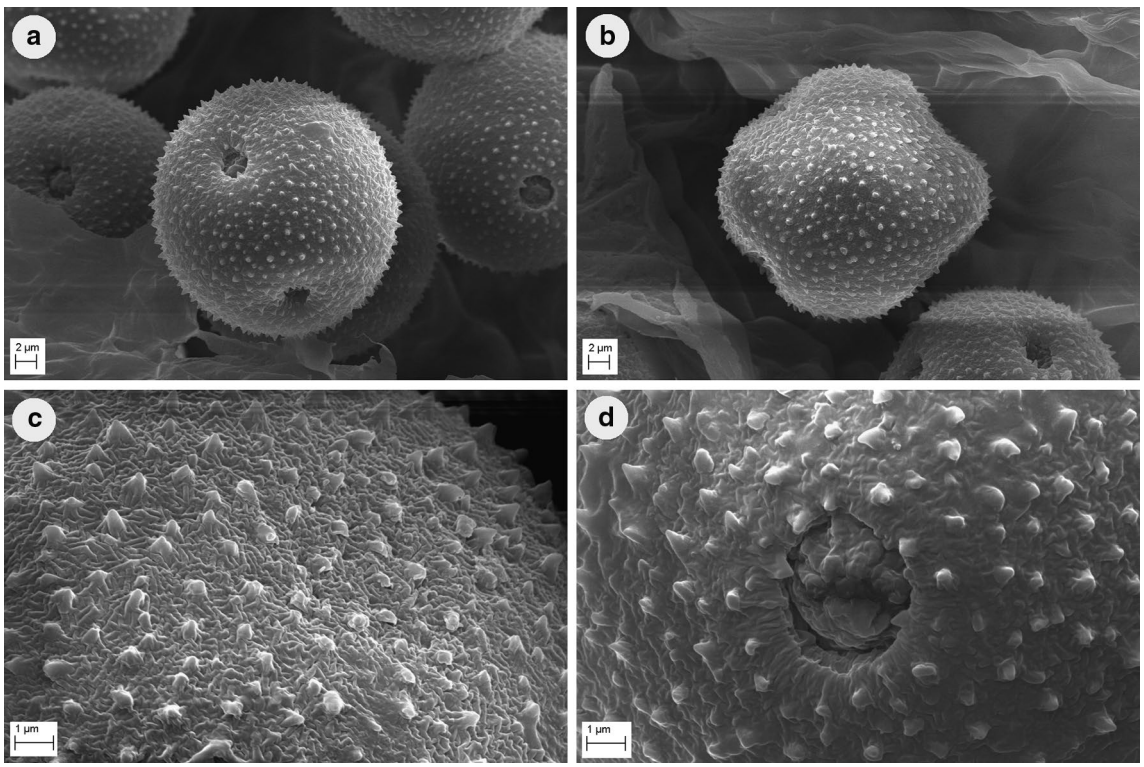

Fig. 2 SEM micrographs of *Campanula cremnophila* dry pollen grains from type material (Pelješac Peninsula). **a** equatorial view; **b** polar view; **c** exine detail; **d** pore detail

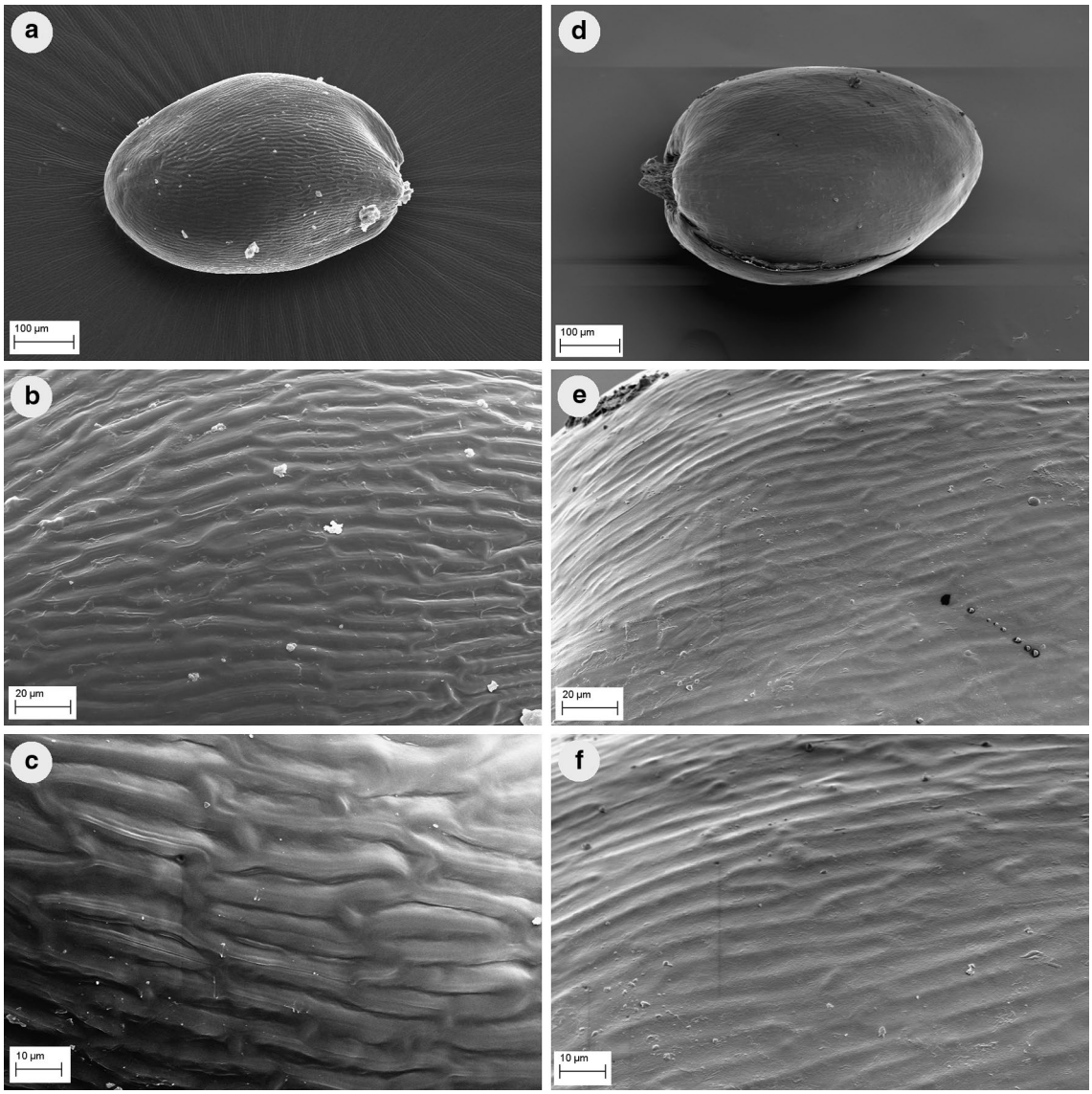

Fig. 3 SEM micrographs of seed of *Campanula cremnophila* (**a–c**) from type material (Pelješac Peninsula) and *C. garganica* (**d–f**) from Mt. Gargano. **a**, **d** seed full view; **b–f** testa detail (two different magnifications)

Discussion

The detailed morphological and molecular analyses carried out on populations of unknown Dalmatian *Campanula* allowed to putting in evidence that it is a well distinct and isolated member of the *Campanula* ser. *Garganicae*. From the phylogenetic point of view, it is nested within a subclade together with *C. garganica* and *C. poscharskyana* (Fig. 1). Morphologically the former species is similar to *C. cremnophila*, mainly due to the shape of the flowers and the absence of pores in the capsule; however, C. *cremnophila* is well differentiated in having habit prostrate–reptant, dense indumentum, reniform leaves, with fewer teeth at the margin, smaller flowers, capsules and seeds. The differences with *C. poscharskyana* are evident in inflorescence length (up to 7 cm in *C. cremnophila* vs. up to 30 cm in *C. poscharskyana*), flower size (8–13 mm vs. 3–4 cm in diameter), calyx teeth length (2.5–3 mm vs. up to 7 mm), length of rigid hairs of basal leaves (up to 0.6 mm vs. more than 1 mm) and basal leaves (smaller with irregularly dentate margin vs. larger with 2-serrate margin).

Other remarkable differences are the micromorphology of seed testa, the character recognized in the literature for having an important diagnostic value to differentiate the species of the genus *Campanula* (Geslot 1980; Shetler and Morin 1986; Murata 1992; Toniuc 1999; Buss et al. 2001; Akcin 2009; Alçitepe 2010; Mehrvarz and Kashi 2015). The testa seed ornamentations analysed by SEM can be used as a

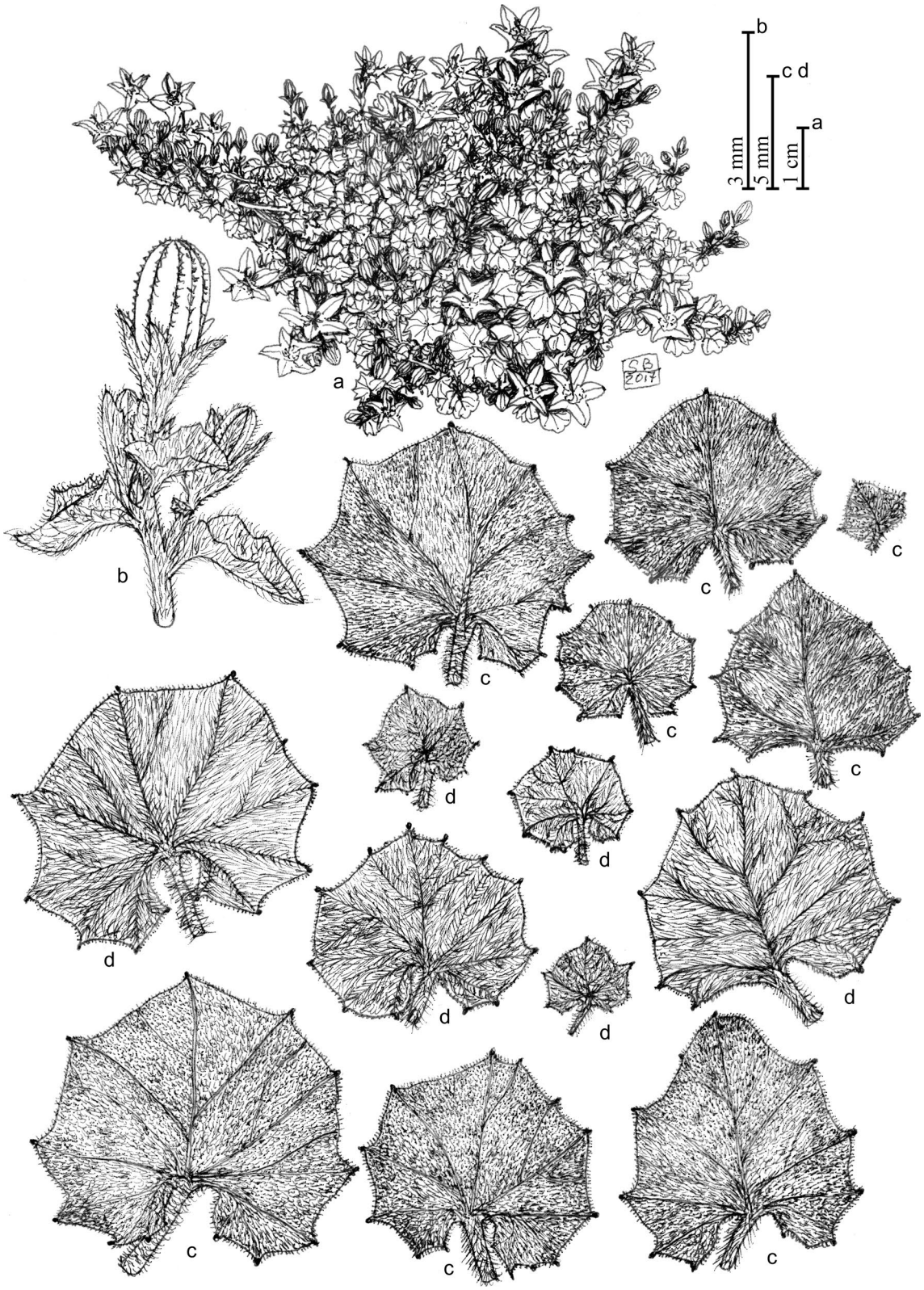

Fig. 4 *Campanula cremnophila*. **a** habit; **b** buds in raceme; **c** leaf adaxial face; **d** leaf abaxial face (ZAGR!, holotype). Drawing by Salvatore Brullo

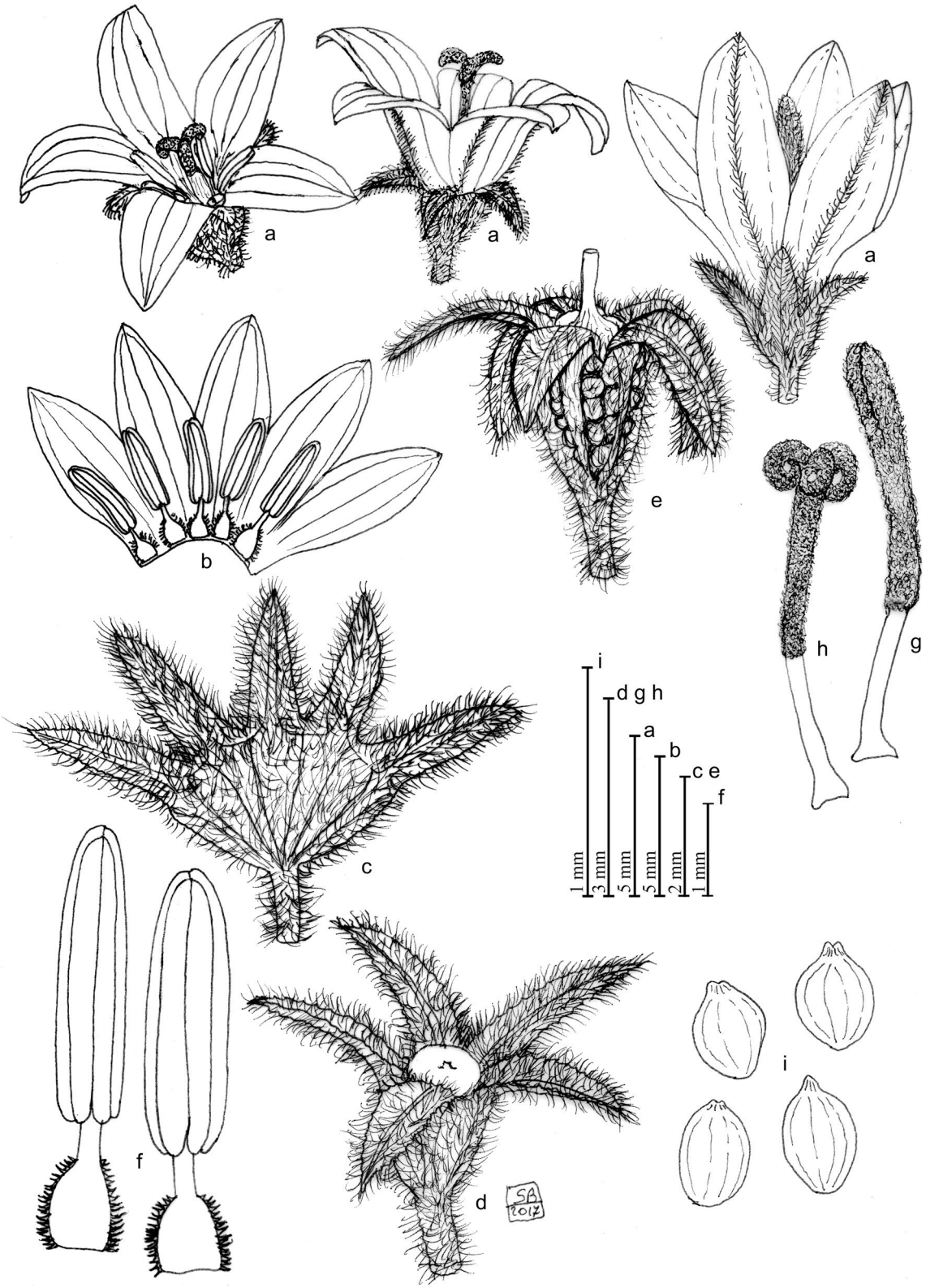

Fig. 5 *Campanula cremnophila*. **a** flowers; **b** corolla open and stamens (ventral face); **c** calyx open (dorsal view); **d** calyx (lateral view); **e** capsule with fructiferous calyx; **f** stamens (ventral face); **g** style with closed stigma; **h** style with open stigma; **i** seeds (ZAGR!, holotype). Drawing by Salvatore Brullo

support in the taxonomy of a given group of *Campanula*. In particular, within the *Campanula* ser. *Garganicae*, including a part of isophyllous taxa, the seed coat is usually striate, with elongated cells and lumen essentially linear, but quite variable in shape, size and arrangement (Bogdanović et al. 2014a, b, 2015). In *C. cremnophila* and allied *C. garganica*, there are also significant differences in the seed coat sculptures that confirm further the separation at specific level of the two taxa (Fig. 3). Moreover, the pollen grains of *C. cremnophila* are well differentiated from those of *C. garganica*, mainly by the size, exine microstructure and number of pores (Fig. 2).

Although the DNA sequence data are inconclusive regarding the relationships among species in *Campanula* ser. *Garganicae,* the amphi-Adriatic garganica–poscharskyana–cremnophila clade received moderate support (Fig. 1). Previous investigations (Park et al. 2006; Frajman and Schneeweiss 2009; Bogdanović et al. 2014a, b 2015; Surina et al. 2014) pointed out that the trans-Adriatic connection favoured the diversification of *C. garganica* clade. These palaeogeographic events regarding the East Adriatic coast and the Apennine Peninsula occurred probably during the climatic fluctuations in different periods from late Miocene to Pleistocene. As a consequence of this, dispersal–vicariance phenomena which happened within the region did not concern only *Campanula* genus but also other genera belonging to several families, e.g. *Cardamine*, *Edraianthus*, *Knautia*, *Centaurea*, *Allium*, *Limonium*, *Asperula*, *Euphorbia*, *Viola*, etc. (Korica et al. 1992; Bogdanović et al. 2008; Stefanović et al. 2008; Kučera et al. 2010; Mereďa et al. 2011; Rešetnik et al. 2014; Hilpold et al. 2014; Bogdanović and Brullo 2015; Frajman and Schönswetter 2017; Janković et al. 2019). The separate and highly supported position of second Apennine species within the group, *C. reatina*, provides further evidence for multiple trans-Adriatic dispersal which probably occurred in different temporal intervals as suggested by Park et al. (2006). The somewhat lower support for the monophyly of *C. cremnophila* and short branch lengths between it and the *C. garganica*-*C. poscharskyana* clade could indicate a more recent speciation event between these taxa which is nevertheless supported with robust morphological evidence.

Taxonomic treatment

Campanula cremnophila Bogdanović, Rešetnik, M.Jeričević, N.Jeričević & Brullo, **sp. nov.**—HOLOTYPE: CROATIA. Pelješac Peninsula, Marčuleti Bay, Rt Vrba, calcareous rocky cliffs along the road to Pržina, 27 May 2017, 20 m a. s. l., 42°47′16″ N, 17°44′58″ E, *M. Jeričević, N. Jeričević* and *S. Bogdanović s.n.* (ZAGR!; isotypes: CAT!, ZA! and ZAGR!) (Figs. 4, 5, 7).

Etymology: The Greek epithet "*cremnophila*" refers to the ecological requirement of this species, which is a chasmophyte growing in the rocky crevices.

Description: Plant perennial, densely hairy (rarely subglabrous), with patent or subrigid to rigid hairs. Rootstock woody, 2–5 mm in diameter, shortly branched in the upper part and covered by petiole remains. Stems numerous, up to 30, prostrate–reptant, simple or branched, herbaceous, 5–15 cm long, leafy, ending in more or less lax racemes. Basal leaves are arranged in rosettes, densely covered by rigid hairs 0.1–0.6 mm long, with petiole 1–5 cm long, blade greyish-green, often tinged with purplish, reniform, cordate at the base, 4–15 mm long, 4–21 mm wide, irregularly dentate at the margin with 5–15 obtuse teeth, with venations pinnate to pinnate–radiate. Cauline leaves similar to the basal ones, gradually decreasing upwards, with petioles 1–10 mm long. Raceme terminal, 10–70 mm long, with flowers arranged solitarily at leaf axil. Floral pedicel 3–15 mm long, hairy, with 0–2 bracteoles. Calyx teeth entire, linear–lanceolate, 1-nerved, green-purplish, densely hairy, 2.5–3 mm long, 1 mm wide, acute at the apex, subpatent in bud, and patent-reflexed at anthesis and in fruit. Corolla pale blue–lilac, white in the throat, rotate, 8–9 mm long, 8–13 mm in diameter, glabrous with hairs outside only along the five principal veins, tube 3.5–5 mm long, lobes 3.5–5 mm long, 2.2–2.6 mm wide, lanceolate, divaricate, with one midrib and two secondary veins, apex subobtuse. Stamens 5, with filaments widened at base into an elliptical blade, 0.6–0.8 mm long, 0.5–0.6 mm wide, lax ciliated at the margin, with cilia 0.1–0.15 mm long, upper part of the filaments glabrous, ca. 0.5 mm long, anthers whitish–lilac, 3–3.2 mm long, rounded at the apex; pollen yellow-whitish. Style exerted from tube, ca. 5 mm long, white below and pale blue–lilac above, from more than half of the upper part lanuginous, glabrous below, with three stigmas 1.5 mm long. Capsule subconic, 2.5 mm long, 5-ribbed, densely hairy (hairs 0.3–1 mm long), dehiscing for splitting of the wall, with patent-reflexed calyx teeth. Seeds ovoid to ellipsoid, 0.45–0.5 × 0.3 mm, pale brown, shiny.

Diagnosis: A *Campanula garganica* scapis usque ad 15 cm longis, pilosis, rare subglabris, prostrato–reptantibus, foliis semper pilosis, petiolo 1–5 cm longo, lamina reniforme, usque ad 15 mm longa, denticulata margine, pedicello florali 3–15 mm longo, calice semper piloso, dentibus calycinis integris, 2,5–3 mm longis, 1 mm latis, corolla 8–9 mm longa, 8–13 mm in diametro, lobis 3,5–5 mm longis, filamentibus staminorum 1–1,3 mm longis, anthera 3–3,2 mm longa, polline albo, stylo ca. 5 mm longo, capsula subconica, 2,5 mm longa, seminibus ovoideis vel ellipsoideis, 0,3 mm latis, differt.

Phenology: Flowering from early May to June and fruiting from late June to July.

Distribution, habitat and ecology: Campanula cremnophila is known from southern Dalmatia in Croatia, where it grows in three localities in southern part of the Pelješac Peninsula and on the Elaphite islands of Olipa and Jakljan (Fig. 6). It is exclusive of calcareous rocky crevices at 20–150 m of elevation in stands by the sea. Usually this species colonized the limestone outcrops within *Pinus halepensis* woodlands, where it is a member of a rupestrian community with western and eastern exposure (Fig. 7a). This vegetation is characterized by several endemic chasmophytes such as *Centaurea glaberrima* Tausch, *Edraianthus tenuifolius* (Waldst. et Kit.) A.DC., *Micromeria pseudocroatica* Šilić, *Genista sylvestris*

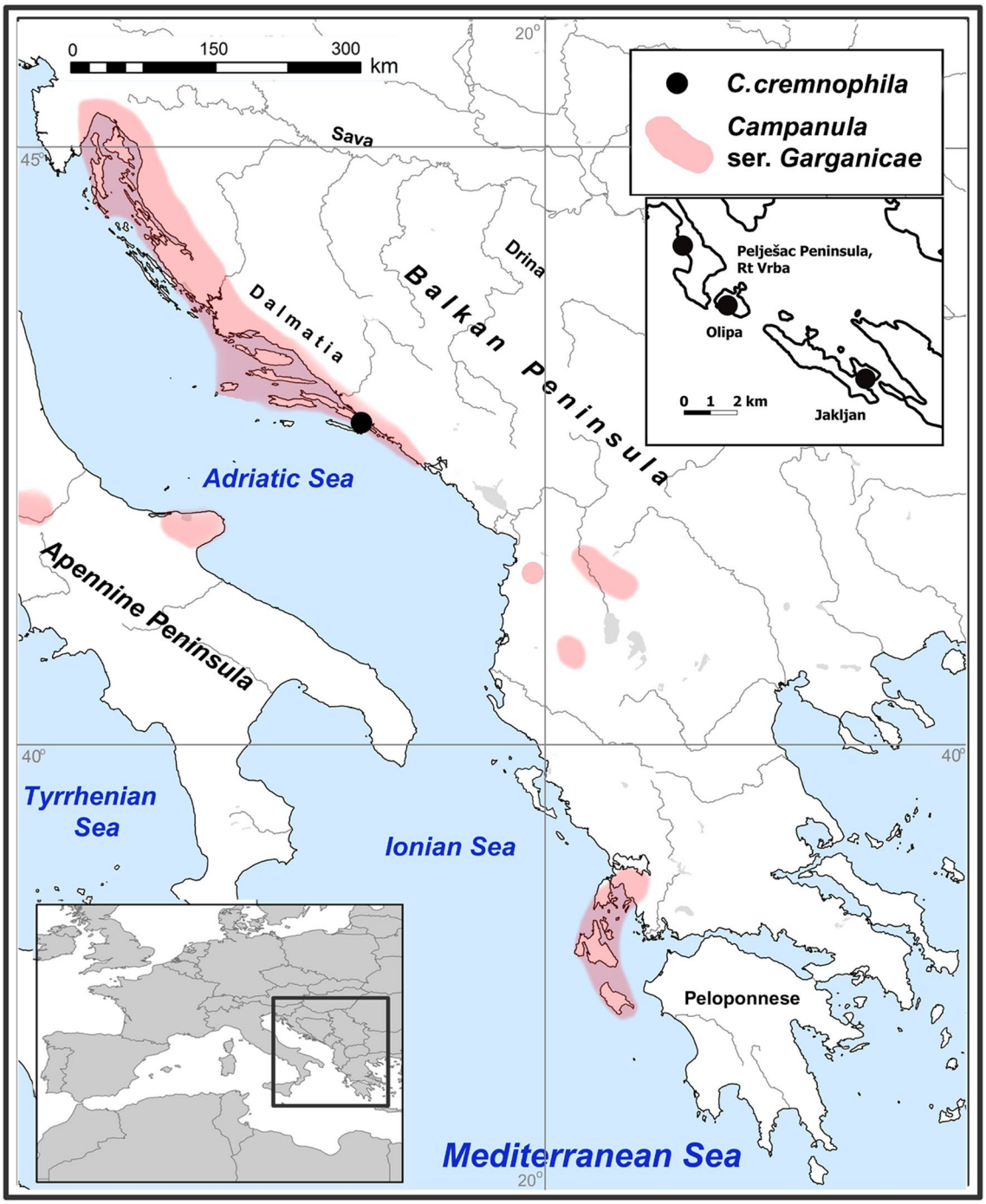

Fig. 6 Distribution map of *Campanula* ser. *Garganicae* and *Campanula cremnophila* (●) from southern Croatia

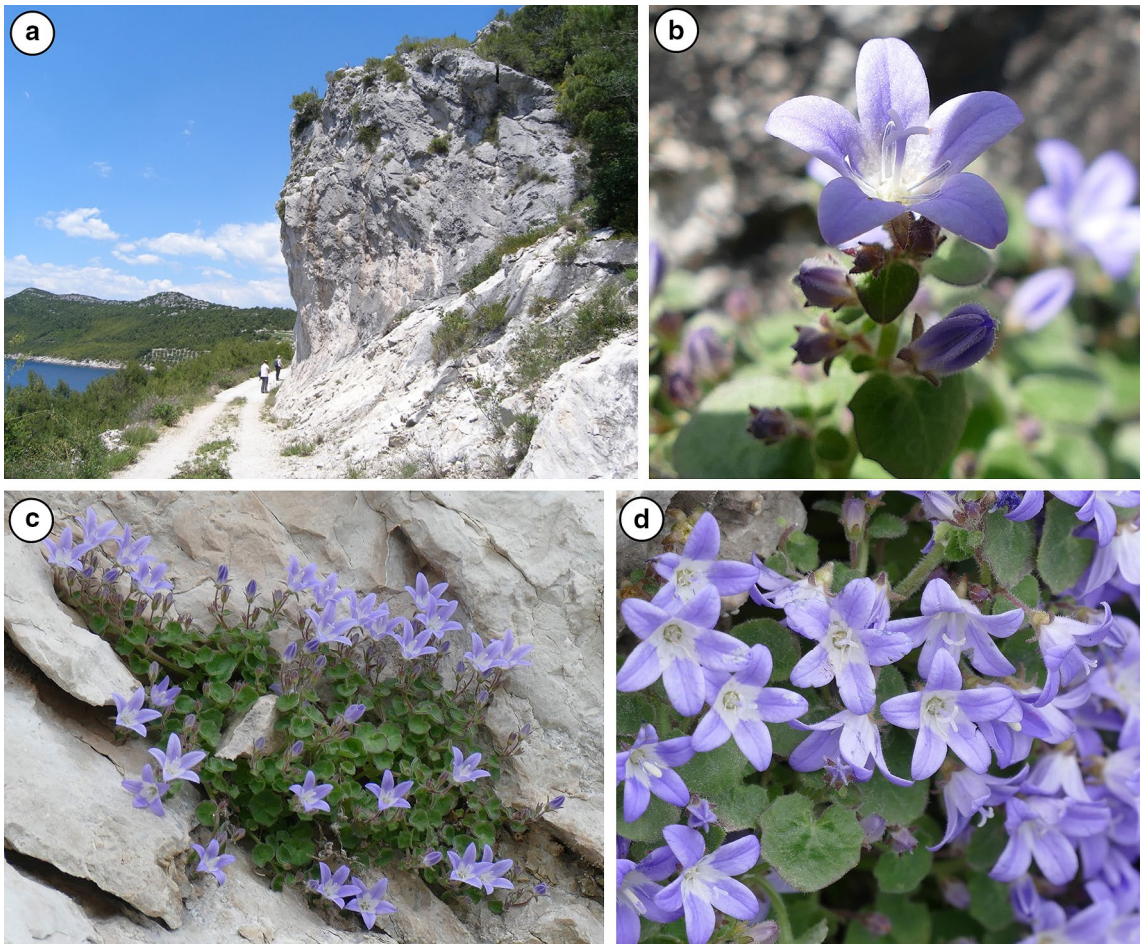

Fig. 7 *Campanula cremnophila*. **a** habitat; **b** inflorescence with flower and buds; **c** habit; **d** flowers and leaves (photographs by S. Bogdanović and N. Jeričević)

Scop. subsp. *dalmatica* (Bartl.) H. Lindb., *Inula verbascifolia* (Willd.) Hausskn., *Sesleria interrupta* Vis., and other more common species, among them *Erica manipuliflora* Salisb., *Saruteja montana* L., *Schoenus nigricans* L., *Globularia cordifolia* L., *Pinus halapensis* Mill., *Micromeria juliana* (L.) Benth. ex Rchb., *Fumana procumbens* (Dunal) Gren. & Godr., and *Asperula aristata* L.f. subsp. *scabra* (J.Presl & C.Presl) Nyman., etc.

Conservation status: Currently *C. cremnophila* is known from one locality distributed in extreme southern part of the Pelješac Peninsula and two insular localities (Olipa and Jakljan). On the whole, we have surveyed not more than 500 mature individuals growing in a very restricted area of 8 km^2. This rupestrian habitat is not directly influenced by human activities, although the pine forests can be occasionally subject to fires of various origins. According to the IUCN (2017), this species for its rarity, low number of mature individuals and restricted population distribution should be included in the category of threatened plants as Vulnerable (VU, D1 + 2).

Additional specimens examined: CROATIA. Pelješac, uvala Marčuleti kod Stona, okomite stijene, 15 May 2016, *N. Jeričević* and *M. Jeričević s.n.* (CAT, ZAGR); Pelješac, uvala Marčuleti, pukotine stijena, 7 May 2016, *Jeričević* and *M. Jeričević s.n.* (CAT, ZAGR); Pelješac, uvala Marčuleti, okomite stijene, 18 Jun 2016, *M. Jeričević s.n.* (CAT, ZAGR); Elaphite island Olipa, rocky crevices of vertical cliffs, 10 May 2018, *S. Bogdanović, I. Rešetnik, M. Jeričević* and *N. Jeričević s.n.* (CAT, ZA, ZAGR); Elaphite island Jakljan, rocky crevices of vertical cliff, 10 May 2018, *S. Bogdanović, I. Rešetnik, M. Jeričević* and *N. Jeričević s.n.* (CAT, ZA, ZAGR).

Key to the *Campanula* ser. *Garganicae* species (plus *C. comosiformis*) from Bogdanović et al. (2014a) modified

1a Corolla campanulate, with tube 1/2–3/4 its length … 2
1b Corolla rotate, more o less flattened or infundibular, with tube 1/6–1/2 it length 4
2a Corolla 9–11 mm long, 12–14 mm in diameter, outside totally hairy; calyx teeth in fruit deflexed, basal blade of stamen filament semicircular, 0.6–0.8 mm long ..... ***C. skanderbegii***
2b Corolla 14–25 mm long; 20–24 mm in diameter, outside glabrous or hairy along the midrib; calyx teeth in fruit erect to patent; basal blade of stamen filament ovate to elliptical, 1.2–1.7 mm long 3
3a Pedicel 5–12 mm long; calyx teeth 5–7 mm long; corolla with tube 1/2 its length; stamen filaments 2–2.5 mm long; anthers white tinged with pale blue, pollen whitish ..... ***C. teutana***
3b Pedicels 12–25 mm long; calyx teeth 3–4 mm long; corolla with tube 2/3–3/4 its length; stamen filaments 0.8–1 mm long; anthers and pollen yellowish ..... ***C. portenschlagiana***
4a Corolla infundibular, 30–40 mm in diameter; calyx teeth 7 mm long and 3 mm wide, twice as long as the capsule ..... ***C. poscharskyana***
4b Corolla rotate to infundibular, 7–20 mm in diameter; calyx teeth max. 6 mm long and max. 2 mm wide, subequal or shorter than capsule 5
5a Calyx teeth 1.5–2 (2.5) mm long 6
5b Calyx teeth 2.5–6 mm long 7
6a Plant glabrous; leaves reniform, corolla 10–15 mm long ..... ***C. reatina***
6b Plant densely hairy; leaves cordate; corolla 8–10 mm long ..... ***C. aureliana***
7a Pollen yellow; leaves serrate, rarely 2–serrate to dentate 8
7b Pollen whitish to blue; leaves markedly 2–serrate to dentate 11
8a Corolla lobes linear, 1.5 mm wide, fused near the base ..... ***C. comosiformis***
8b Corolla lobes never linear, more than 3 mm wide, with tube long 1/4–1/2 of their length 9
9a Calyx teeth erect to patent; corolla infundibular, with tube 1/3–1/2 its length; style hairy up to 2/3 its length ..... ***C. acarnanica***
9b Calyx teeth deflexed; corolla rotate to infundibular, with tube 1/4–1/3 its length; style hairy up to 1/2 its length 10
10a Stamen filament at the base with long and acute hairs; anther 3 mm long; calyx teeth up to 1 mm wide; capsule globose, 3 × 2.5 mm, opening by basal pores ..... ***C. cephallenica***
10b Stamen filament at the base with short and rounded hairs; anther 4 mm long; calyx teeth up to 2 mm wide; capsule flattened, 3 × 4 mm, without pores ..... ***C. garganica***
11a Leaves shortly dentate; corolla with tube 3.5–5 mm long, and lobes subequal; stamen filament 1.1–1.3 mm long; capsule 2.5 mm long, without pores ..... ***C. cremnophila***
11b Leaves 2–serrate to serrate–dentate; corolla with tube 2–3 mm long, and lobes very longer; stamen filament 3.5–4 mm long; capsule 3–4 mm long, with pores 12
12a Inflorescence loose; corolla dark blue, glabrous below, with lobes 3 mm wide at the base; calyx teeth deflexed, 0.5 mm wide, longer than capsule ..... ***C. debarensis***
12b Inflorescence compact; corolla pale blue, ciliate below, with lobes 3–4 mm long at the base; calyx teeth usually erect, 1.5 mm wide, a little exceeding the capsule 13
13a Plant glabrous, rarely pubescent above; corolla max. 15 mm in diameter; basal leaves with blade max. 4 × 3 cm, 2–serrate or dentate; capsule 2.5 × 3.5 mm ..... ***C. fenestrellata* subsp. *fenestrellata***
13b Plant densely tomentose–velvety; corolla up to 20 mm in diameter; basal leaves with blade up to 7 × 4 cm, serrate; capsule 4 × 4.5 mm ..... ***C. fenestrellata* subsp. *istriaca***

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Information on Electronic Supplementary Materials

Online Resource 1. Phylogenetic tree of the species belonging to *Campanula* ser. *Garganicae* based on Bayesian analyses of *trn*L-*trn*F (**a**) and ITS (**b**) datasets.

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