Campanula Skanderbegii: Molecular and Morphological Evidence of a New Campanula Species (*Campanulaceae*) Endemic to Albania

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Campanula skanderbegii: Molecular and Morphological Evidence of a New Campanula Species (Campanulaceae) Endemic to Albania

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Abstract—A new species of Campanula from Central Albania, named C. skanderbegii, belonging to isophyllous Campanula ser. Garganicae is illustrated and described. Morphologically and phylogenetically it is well differentiated from other species of this series. It is morphologically similar to C. portenschlagiana from Central Dalmatia (Croatia) based on corolla shape. Phylogenetic analyses, maximum parsimony, maximum likelihood, and Bayesian inference, based on nuclear ITS and chloroplast trnL–trnF data support C. skanderbegii as a clearly distinct taxon within the isophyllous species of Campanula. A distribution map for C. skanderbegii and a key to all species of Campanula ser. Garganicae are provided. Because of its rarity and highly restricted distribution, C. skanderbegii should be included in the IUCN red list as a critically endangered species.

Keywords—Balkan Peninsula, endemism, isophyllous bellflowers, ITS, phylogeny, trnL–trnF.

Campanula L., comprising ca. 580–600 species (Mansion et al. 2012), is the largest genus of Campanulaceae. Its distribution is in the Northern Hemisphere and is represented by annual and perennial taxa growing in various habitats, such as meadows, grasslands, garrigues, woodlands, and often in rocky sites (Kovačić 2004; Roquet et al. 2008). Within the genus, there is high variability in morphology (Kolakovsky 1986; Roquet et al. 2008), carpology (Kolakovsky 1986), paly-
genesis and revealed three well supported clades corresponding to
series Elatines Trin., ser. Fragiles Trinajstić, and ser. Garganicae
(Lovašen-Eberhardt and Trinajstić 1978). Ten taxa are cur-
tently included in ser. Garganicae: C. gargaricae Ten., C. rotia
Lucchese, C. fenisellata Feer, C. fenisellata subsp. istriaca (Feer)
Federov, C. portenschlagiana Roem. & Schult., C. poscharskyana
Degen, C. teutana Bogdanović & Brullo, C. cephallica Feer,
C. acarnanica Damboldt and C. de bacteris Rech. f. The sister spe-
cies of ser. Garganicae is Albanian endemic C. conosiformis (Hayek
& Janch.) Frajman & Schneew. (Frajman and Schneeweiss 2009;
Bogdanović et al. 2014). Morphologically, these species are charac-
terized by a monopodial growth form, isophyllous and long petiole leaves, with coriaceous ovate to oblong, elongated and rather unilaterial inflorescence, campanulate or rotate corolla, obtuse hairs at the base of filaments, and brown shiny seeds (Damboldt 1965). In contrast, C. conosiformis has a slender habit, densely clustered racemose inflorescences, ovate-rohmmic leaves, and dentate calyx teeth (Hayek 1921; Frajman and Schneeweiss 2009). The phylogenetic relationships of these species were recently inferred with plastid and ITS sequence data (Park et al. 2006; Frajman and Schneeweiss 2009; Bogdanović et al. 2014), but they remain unclear due to low clade support and conflicting signals between plastid and nuclear data.

During field trips in 2011 and 2012, an unknown population of Campanula was found in the town of Kruje, in central Albania. Based on some morphological features (e.g. habit, leaves, inflorescence, and flowers), the specimens belong to ser. Garganicae, and appear closely related to C. portenschlagiana. On the other hand, they differ from C. portenschlagiana in some
characters related to indumentum, leaf petiole, calyx, corolla, stamens, pollen colour, capsule, and seeds. In the Albanian flora (Markgraf 1931; Damboldt 1965; Qosja et al. 1996; Park et al. 2006; Frajman and Schneeweiss 2009), ser. Garganicae is represented by *C. debarensis* and a species that is closely related to this group, *C. comosiformis*. However, these species are morphologically well differentiated from the *Campanula* specimens collected in Kruje. To verify the taxonomic position and

![Distribution map of Campanula skanderbegii from Albania, showing the area of distribution of the species of Campanula series Garganicae.](image-url)
phylogenetic relationships of this newly discovered plant population from Albania, we carried out a molecular study using ITS and cpDNA sequences, as well as a morphological assessment of multiple species in Campanula ser. Garganicae and related groups.

**Materials and Methods**

**Plant Material**—The investigations were carried out on specimens collected during field trips in Albania, in June 2011 and July 2012. In total, only ten individuals were collected to avoid damage to the current small population in the town of Kruje. The plants of these collections were used for herbarium exsiccate, while fresh leaves were conserved in silica-gel for DNA analysis. Floral and vegetative parts were placed in 50% glycerine-ethyl alcohol solution for further morphological assessment. To obtain a denser sampling in Campanula ser. Garganicae, we used the data sets of Park et al. (2006), Frajman and Schneeweiss (2009), and Bogdanovic et al. (2014), extended with 22 new sequences from addi-

**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 GmbH, Hilden, Germany), following the manufacturer’s instructions. The reaction mix for polymerase chain reaction (PCR) of 50 μL contained 25 ng of DNA, 1 × PCR Buffer (TaKaRa Bio Inc., Shiga, Japan), 0.2 mM each dNTP (TaKaRa Bio), 0.2 μM of each primer (175E and 265E of Sun et al. (1994) for nuclear ITS; c and f of Taberlet et al. (1991) for plastid trnL–trnF) and 1.25 U of TaKaRa Taq™ HS polymerase (TaKaRa Bio). The PCR conditions for ITS were as described in Park et al. (2006) and for trnL–trnF as described in Bogdanovic et al. (2014). The PCR reactions were performed using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, California). The PCR products were purified with GenElute PCR clean-up kit (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) according to the manufacturer’s protocol. The products were sequenced by Macrogen Inc. (Seoul, Korea) using the BigDye™ terminator cycle sequencing kit (Applied Biosystems) and analyzed on an ABI PRISM 3730XL automated sequencer (Applied Biosystems). Sequences were edited and manually aligned using Geneious Pro 5.3.6 (Drummond et al. 2011). Sequence alignments are available from TreeBASE (study number S15431).

**Phylogenetic Analyses**—Three different datasets (ITS, trnL–trnF, ITS–trnL–trnF combined dataset) were analyzed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). The trees were rooted using Trachystemum L. as an outgroup. This species belongs to the Campanula s. s. clade while all other ingroup taxa belong to the Rapunculus clade (see Park et al. 2006).

To assess degree of phylogenetic congruence between the two different datasets, an incongruence length difference (ILD) test (Farris et al. 1994) implemented as partition-homogeneity test in PAU™ was performed using 1,000 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 1,000 random addition sequence replicates, and tree bisection reconnection (TBR) in the branch swapping option of PAU™ 4.0b10 (Swofford 2003). Bootstrap support values (MPB; Felsenstein 1985) from 1,000 replicates were generated using the heuristic search options as above except for random addition sequence with 100 replicates. The scores between 50 and 74 bootstrap percentages were defined as weak support, scores between 75 and 89% MPB as moderate support, and scores above 90% MPB as strong support.

The best-fit substitution models for each region were determined using the Akaike Information Criterion as implemented in MrModeltest (Nylander 2004). MrModeltest retrieved General Time Reversible model with a gamma distribution (GTR + G) as the most likely evolutionary model for the cpDNA and Symmetrical model with a gamma distribution (SYM + G) for the ITS region. For cpDNA region, GTR models with a gamma distribution (G) or with a proportion of invariable sites (I) or with both parameters (G + I) already had a cumulative Akaike weight of 1.00, while for ITS region, the best model, SYM + G, had an Akaike weight of 0.54 and three models (SYM + I + G, GTR + G, GTR + I + G) were included until the cumulative Akaike weight exceeded 0.95.

Maximum likelihood (ML) analyses were performed using RAXML 7.0.4 (Stamatakis 2006; Stamatakis et al. 2008) assuming the GTR + G model of substitution, GTR models (GTR + G, I + G) being the only ones implemented in RAXML. We did not use models including both invariable sites and gamma (I + G) due to the strong interaction between these parameters, making it impossible to estimate both parameters reliably (Ren et al. 2005; Yang 2006). The combined dataset was partitioned into two regions (ITS and trnL–trnF), with model parameters estimated and optimized individually for each partition. Bootstrap support (MLB) was estimated from 1,000 replicates.

BI was conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The analysis of the combined data set was carried out under partition-specific substitution models as selected for each partition separately using AIC scores in MrModeltest. 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, with the sample frequency set to 1,000. The first 2,500 trees (prior to the 2.5 × 10^7 generation), which was 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 (ESS) with the program Tracer ver. 1.4 (Rambaut and Drummond 2007). A majority rule consensus tree was constructed from the posterior set of 15,000 trees.

**Results**

The characteristics of the cpDNA, ITS and cpDNA–ITS combined datasets analysed using MP, ML and BI are summarized in Table 1. As the ILD test revealed no significant difference (p = 0.23) between the selected partitions (cpDNA and ITS), the phylogenetic tree of the combined data set obtained by ML and BI analyses is presented in Fig. 2.

In all three data sets, the Campanula individuals from Albania are inferred as distinct members of the Garganica clade (Fig. 2). Specifically, C. skanderbegii is resolved as sister to C. portenschlagiana with the cpDNA and combined cpDNA–ITS datasets, with low to high support (cpDNA: 0.96 PP, 71 MLB, 54 MPB; cpDNA-ITS: 0.92 PP, no support in ML and MP analyses), or as sister to a clade comprising C. reatina, C. poscharskyana, C. acarnanica, and C. garganica (0.88 PP, 62 MLB, 50 MPB) in the ITS phylogeny. Within the Garganica clade all species with the exception of C. acarnanica are congruently inferred as monophyletic albeit with partly conflicting relationships between plastid and ITS data. This is not only evident in the position of C. skanderbegii, but is also noticeable regarding the position of C. debarestensis, C. reatina and C. teutana (Suppl. Figs. 1, 2).

**Taxonomic Treatment**

Campanula skanderbegii Bogdanovic, Brullo & D. Lakušić, sp. nov. — TYPE: ALBANIA. Kruje, calcareous rocky cliff under the Skanderbeg’s castle, N 41.50787°, E 019.79527°, 14 July 2012, S. Bogdanovic & M. Jug-Dujaković s. n. (holotype: ZAGR!, isotypes: BEOU!, CATH!, ZAGR!).

Campanula portenschlagiana Roem. et Schult. similaris sed scapis semper pilosis, folii petiolo usque ad 8 cm longo, dentibus calycinis integris, corolla breviore, extus pilosa,
styro breviore, lamina basali staminorum semicirculare, 0.6–0.8 mm longa, filamentibus staminorum 1.2–1.3 mm longis, anthera albida, breviore, polline albo, capsula obovidea, dentibus calicis omnino reflexis, seminibus ellipsoideis, minoribus, lucidis, dinfert.

Plant perennial, densely hairy, with rigid and erect-patent hairs. **Rootstock** woody, branched, covered by leaf remains, with numerous ascending stems, simple or branched at the base. **Stems** herbaceous, 10–30 cm long, leafy, ending in many flowered racemes. **Leaves** arranged in basal rosettes,
Fig. 3. *Campanula skanderbegii*. A. Habit (CAT!, isotype). B. Leaves. Drawing by Salvatore Brullo.
densely covered by rigid hairs, 0.1–0.8 mm long; petiole 1–8 cm long; blade cordate to reniform, pale gray-green, 4–25 × 4–27 mm, cordate at the base, irregularly dentate at the margin (10–22 acute teeth), with palmate to palmate-pinnate venation; cauline leaves similar to the basal, gradually decreasing in size upwards, with petioles 3–30 mm long, blade 4–22 × 4–22 mm. **Inflorescence** a raceme. **Flowers** usually solitary or verticillate (2–3 at leaf axil); pedicel 5–22 mm long, densely hairy, with 1, rarely 2 flowers, with 1–2 bracteoles. **Calyx** green, densely hairy, with teeth entire, linear, 1-nerved, 3–4 × 0.3–0.4 mm, patent to reflexed, acute at the apex. **Corolla** lilac, turning blue-violet by drying, campanulate, 9–11 mm long, 12–14 mm in diameter, glabrous inside, outside densely hairy on the principal veins and sparsely hairy on the tube and lobes; tube subconic, 4.5–6 mm long; lobes 4.5–5 × 2.8 mm, ovate-lanceolate, patent-reflexed to completely reflexed, with 1 midrib and various secondary veins, apex subobtuse. **Style** with stigma exerted from corolla tube, 7.5–9 mm long, white on upper and lower parts, pale blue in the middle part, slightly verrucose above, with 3 stigmas, each 1.5–2 mm long. **Stamens** 5; filaments widened at base into a semi-circular blade (or disk), 0.6–0.8 × 0.6–0.8 mm, densely ciliate at the upper and marginal parts; filaments glabrous, slightly violaceous, 1.2–1.3 mm long; anthers white, 3.4–3.5 mm long, apiculate at the apex; pollen white. **Capsule** obovoid, 2.5 mm long, 5-ribbed, densely hairy (hairs 0.2–0.6 mm long), opening by basal pores, with completely deflexed calyx teeth, 5 mm long.

**Seeds** elliptical-rounded in outline, 0.40–0.45 × 0.35–0.38 mm, yellow-brownish to brown, shiny. Figures 3, 4.

**Additional Specimens Examined**—**ALBANIA.** Kruje tvrava, stene (Asplenietea trichomanes), krečnjak, 574 m n/v, 24 June 2011, Lakusić et al. 32470 (BEOU!, ZAGR!).

**Seed Micromorphology**—Different types of seed micromorphology have been described in Campanulaceae (Geslot 1980; Murata 1992, 1995; Toniuc 1999; Buss et al. 2001; Akcin 2009; Alçitepe 2010). In *Campanula*, the seed coat patterns are either reticulate or striate. Striate testa, of which cells may vary in shape, size and arrangement, are usually characteristic for isophyllus bellflowers (Bogdanović et al. 2014). The seeds of *C. skanderbegii* are well-differentiated from those of *C. portenschlagiana*, its most closely related species: the seeds of the former are ellipsoid with a striate testa characterized by very elongate fibriform cells, slightly raised and anastomosed radial walls, with distinct, wide and rugose lumen, which is essentially linear with fused anticlinal walls (Fig. 5A, B), whereas in the latter, seeds are elongated and flattened, with a markedly striate testa characterized by ever elongate fibriform cells, but well prominent radial walls and deeply incise linear lumen, giving to the surface a cerebriform aspect (Fig. 5C, D).

**Etymology**—The specific epithet honours George Kastrioti Skanderbeg (1405–1468), national hero of Albania, whose castle stands on the hill where the new species was found.

**Phenology**—Flowering and fruiting late June to early July.

**Distribution and Ecology**—*Campanula skanderbegii* is only known from the type locality, inside the town of Krujë in

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![Fig. 5. Scanning electron micrographs of seed surface of *Campanula skanderbegii* (A, B) from CAT! isotype, and *C. portenschlagiana* (C, D) from Dalmatia, Biokovo mountain ZAGR!.
A, C – seed (full view). B, D – testa detail.](image-url)
Central Albania (Fig. 1). It grows in the shade, in small rock cavities with northern exposure, in a calcareous cliff under the Skanderbeg’s castle (Fig. 6). It is a typical chasmophyte exclusive of fresher niches of rupestran community characterized by some rare or endemic species, such as Campanula versicolor Sibth. & Sm., C. ramossisima Sibth. & Sm., Moltkia petrea (Tratt.) Griseb., Ramonda serbica Pančić, Portenschlagiella ramosissima (Port.) Tutin, Asperula scutellaris Vis.,

Fig. 6. Campanula skanderbegii. A. Habitat. B. Natural population. C. Habit. D. Flower detail. (photos from type locality by Sandro Bogdanović).
Conservation Status—C. skanderbegii is only known from a small population (ca. 12,000 sq m, ca. 500 individuals), in peculiar habitat within an urban area. The species was not found during the field investigations of the surrounding mountains and similar limestone cliffs in the region. Therefore, this species is threatened of extinction by human activities and should be included in the IUCN red list of threatened plants as critically endangered – CR B2a(ii, v) (IUCN 2013).

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Appendix 1. Alphabetical list of taxa, collection details, voucher information, and GenBank accession numbers of Campanulaceae species analysed in the present study. In cases where multiple collections were included for one species, they are listed in numerical order following the designations in Fig. 2. GenBank accession numbers are listed in a fixed order (ITS and trnL–trnF). Asterisks indicate sequences published previously in Park et al. (2006), Roquet et al. (2008), Frijman and Schneeweiss (2009), and Bogdanović et al. (2014).


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