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ORIGINAL ARTICLE

Edraianthus × *lakusicii* (Campanulaceae) a new intersectional natural hybrid: morphological and molecular evidence

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Abstract The genus *Edraianthus* A.DC. has a center of distribution in the Balkans and it is one of the most prominent groups of endemic plants in this region. During our recent fieldtrip to Mt. Lovćen (Montenegro), putative hybrid individuals were encountered, morphologically intermediate between two sympatric taxa, *E. tenuifolius* (*E.* sect. *Edraianthus*) and *E. wettsteinii* subsp. *lovcenicus* (*E.* sect. *Uniflori*). Multivariate morphometric and molecular analyses were carried out to investigate the occurrence of hybridization between these two species. As a result, a new nothospecies is described here, *Edraianthus* × *lakusicii* V. Stevanović & D. Lakušić, a natural hybrid between *E. tenuifolius* and *E. wettsteinii* subsp. *lovcenicus*. At present, this hybrid is known only from the single locality of Mt. Lovćen. Its population size is estimated to be <50 mature individuals and the estimated "area of occupancy" is smaller than 1 km^2.

Keywords *Edraianthus* · Campanulaceae · Balkan Peninsula · Hybridization · Morphometrics · Phylogeny · cpDNA · AFLP

Introduction

Rare in animals but common in plants, hybridization is one of the key elements in plant evolution (Stebbins 1950; Abbott 1992; Arnold 1997; Thompson 2005) because it stimulates genetic recombination and hence has a potential to increases the level of variability within hybrid taxa (Grant 1981; Rieseberg 1997; Rieseberg and Carney 1998; Barton 2001; Marhold and Lihová 2006). Recently, series of molecular studies have demonstrated that hybridization can promote adaptive evolution and speciation (e.g., Matthew and Hiscock 2005). Occasional natural hybridization has always been regarded as the rule rather than the exception in plants, but the frequency of spontaneous natural hybridization varies considerably between different plant genera and families (Ellstrand et al. 1996). Rieseberg (1997) showed that natural hybridization occurs in about 34% of plant families, with 16% of genera recorded as having one or more records of natural hybrids.

In Campanulaceae, hybridization is recorded in genera from all of its main clades (sensu Eddie et al. 2003). This includes examples from the Campanulaceae s.str. clade (e.g., *Campanula* s. l. and *Edraianthus*; Damboldt 1965b; Wettstein in Murbeck 1891; Gusmus 1904; Degen 1938), *Rapunculus* clade (e.g., *Campanula* s. l. and *Triodanis*; Crook 1951; Böcher 1960; Bielawska 1964, 1972; Gadella 1964, 1967; Damboldt 1965a, b; Musch and Gadella 1972;

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Bradley 1975; Shetler 1982; Kovanda and Ančev 1989; Lewis and Lynch 1998; Ančev 1994; Kovanda 1999; Kovačić 2004, 2006; Kovačić and Nikolić 2006; Park et al. 2006; Liber et al. 2008; Roquet et al. 2008), "Transitional taxa" group (e.g., *Wahlenbergia*; Ricci and Eaton 1994; Lammers 1996). This extends further into closely related Lobeliaceae (e.g., *Pratia*; Murray et al. 2004).

Edraianthus DC. has its center of distribution in the Balkans (SE Europe) and it represents one of the most prominent groups of endemic plants in this region. Additional disjunct parts of this group's range are found in the Apennines, Sicily, and southern Carpathians (Wettstein 1887). *Edraianthus* was the subject of multiple monographs early on (e.g., Wettstein 1887; Beck 1893; Janchen 1910). The most prominent was the one by Janchen (1910), who recognized 11 taxa within this genus (ten species, one with two subspecies). The most recent monograph was offered by Lakušić (1974). In this monograph, combined with two subsequent contributions (Lakušić 1987, 1988), he recognized altogether more than 45 taxa (28 species plus a number of subspecies and/or varieties). The most significant contemporary floristic works covering SE Europe, Flora Europaea (Kuzmanov 1976) and Med-Checklist (Greuter et al. 1984), recognize between 10 and 14 species within *Edraianthus*, respectively, basically accepting Janchen's (1910) concepts regarding taxonomy of this genus. Also, according to Lammers (2007a, b) *Edraianthus* includes approximately 13 species.

Further complicating its taxonomy, three hybrid species were also described in *Edraianthus*. First, based on material collected by S. Murbeck on Mt. Veliki Velež (Herzegovina), Wettstein (in Murbeck 1891) described hybrid *E.* × *murbeckii* Wettstein. According to Wettstein, this species originated from the natural cross-breeding between *E. kitaibelii* (A. DC.) A. DC. and *E. serpyllifolius* (Vis.) A. DC. Second, Gusmus (1904) described a hybrid, *E.* × *linifolius* Gusmus, based on a single specimen collected at Sv. Jure summit (Mt. Biokovo; C. Dalmatia). However, he simply pointed out that *E.* × *linifolius* is a natural hybrid between *E. pumilio* (Schultes) A. DC. and *E. serpyllifolius* without providing any protologue for the proposed hybrid. The third *Edraianthus* hybrid has been described from Mt. Velebit (Croatia) by A. Degen under the name *E.* × *intermedius* Degen (Degen 1938). He considered this hybrid to originate from natural crosses between *E. caricinus* Schott, Nyman & Kotschy and *E. croaticus* Kern. Taxonomic significance of these three previously described *Edraianthus* hybrids was not confirmed by any morphometric or molecular data analyses.

Many of the *Edraianthus* taxa occur sympatrically (Lakušić 1974). Some of them flower simultaneously for certain period of time and frequently share the same pollinators, all of which are factors that can potentially facilitate the occurrence of natural hybridization. Thus, it may be hypothesized that the taxonomic complexity and many difficulties in the delimitation of some taxa within this genus are a direct consequence of natural hybridization between the species in sympatric zones.

In his Ph.D. thesis, Međedović (1981) made an initial observation on potential hybridization within the sympatric populations of *E. tenuifolius* (Waldst. & Kit.) A. DC. (*E.* sect. *Edraianthus*) and *E. wettsteinii* Haláscy & Baldaccii subsp. *lovcenicus* E. Mayer & Blečić (*E.* sect. *Uniflori*) from Mt. Lovćen (Montenegro). He found individuals with intermediate features in their gross morphological characters as well as pollen morphology and chromosome characteristics. Međedović (1981) was also the first to propose the possibility of hybridization between these two taxa, but, he did not further analyze these populations nor did he formally describe this species of putative hybrid origin from Mt. Lovćen.

During our recent fieldtrip to Mt. Lovćen individuals with intermediate morphology between sympatric taxa *E. tenuifolius* and *E. wettsteinii* subsp. *lovcenicus* were noted again and collected. The main aim of the present study is to demonstrate and document for the first time the case of natural hybridization between species from different sections in *Edraianthus*. Hence, we provide here the results of multivariate morphometric and molecular phylogenetic analyses, as well as a formal description of this new hybrid species.

Materials and methods

Taxon sampling

Studies were carried out on the plants collected in the field, fixed in the ethanol–glycerol mixture (50:50) for morphological studies and dried in silica gel for molecular studies. The herbarium specimens are deposited at BEOU (Table 1). Because of the small population sizes of *E. tenuifolius* and the putative hybrid in their narrow hybridization zone, and their presumed endangered status, destructive sampling was limited to a minimum, which resulted in a relatively limited number of analyzed individuals. A total of 43 specimens were selected and scored for analyses. Those include 16 individuals of *E. tenuifolius*, 18 specimens of *E. wettsteinii* subsp. *lovcenicus*, and nine specimens of putative hybrid, all from the same locality on Mt. Lovćen (Branjevine; above Mirac village) in Montenegro (Table 1). Molecular analyses were carried out using a subset of individuals sampled for the morphometric analysis. The taxon sampling strategies used originally to delimit major lineages within *Edraianthus* and to infer

Table 1 Populations and number of individuals of *Edraianthus wettsteinii* subsp. *lovcenicus*, *E. tenuifolius* and putative hybrid occurring in the Lovćen mountain range, Montenegro, used in this study

Taxon	Origin of material	Individuals	Voucher	GenBank accession numbers (*trn*L-F; *rbc*L-*atp*B spacer)
E. wettsteinii subsp. *lovcenicus*	Štirovnik, Branjevine above village Mirac, 42°23.285 N, 18°48.206 E, 1,340 masl	18	*Stevanović, V., Lakušić, D. 20946*	EF213338; EF213541
E. tenuifolius	Štirovnik, Branjevine above village Mirac, 42°23.285 N, 18°48.206 E, 1,340 masl	16	*Stevanović, V., Lakušić, D. 20947*	EF213333; EF213536
Putative hybrid	Štirovnik, Branjevine above village Mirac, 42°23.285 N, 18°48.206 E, 1,340 masl	9	*Stevanović, V., Lakušić, D. 20948*	EF213334; EF213537

Vouchers are deposited in the herbarium of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOU)

overall relationships among those lineages are detailed in Surina et al. (2007) and Stefanović et al. (2008). To the relevant portions of these backbone phylogenies (i.e., the *E. tenuifolius* complex and its closest relatives) we added here the representative individuals collected in the hybridization zone.

Morphometric analysis

The character states measured in this study are listed in Table 2. Descriptive statistics were calculated for each character state and multivariate analysis was performed to identify the structure of variability [principal component analysis (PCA)] and to measure the distances between groups [canonical discriminant analysis (CDA)]. Statistical analyses were performed using the package Statistica 5.1 (StatSoft 1996).

Amplified fragment length polymorphism markers

The amplified fragment length polymorphism (AFLP) procedure followed Vos et al. (1995) and Gaudeul et al. (2000) but with polymerase chain reaction (PCR) volumes halved. An initial screening of selective primers using twelve primer combinations with three nucleotides was performed. The three final primer combinations for the selective PCR were (fluorescent dye in brackets): *Eco*RI (6-Fam)-ACA/*Mse*I-CAT; *Eco*RI (VIC)-ACG/*Mse*I CAA; and *Eco*RI (NED)-ACC/*Mse*I-CAG. The selective PCR product was purified using Sephadex G-50 Superfine (GE Healthcare Bio-Sciences, Uppsala) applied to a Multi Screen-HV plate (Millipore, Molsheim, France) in three steps of 200 µl each and packed at 600 × *g* for 1, 1, and 5 min, respectively. The same rotation was used for centrifugation of the samples (5 µl of each selective PCR product), again for 5 min. 1.2 µl of the elution product was combined with 10 µl formamide and 0.1 µl GeneScan ROX (Applied Biosystems) and run on an ABI 3130x automated capillary sequencer. Raw AFLP data were collected and aligned with the internal size standard using ABI Prism GeneScan analysis software 3.7.1 (Applied Biosystems). Subsequently, the GeneScan files were imported into Genographer v. 1.6.0 (available at http://hordeum.oscs.montana.edu/genographer) for scoring of the fragments. Fragments in the size range 60–500 bp were scored and the results were exported as a presence/absence matrix for 25 accessions including replicates belonging to different taxa thriving in close proximity: *E. tenuifolius* from Mt. Lovćen (Lvc1520) and Njeguši (Njg4-5), *E. serpyllifolius* from Mt. Orjen (Orj1-5), *E. graminifolius* from Mts Terzin bogaz-Durmitor (Tb1, 3) and Lovćen (Krst1-3), and *E. wettsteinii* subsp. *lovcenicus* (Lvc2, Lvc1512), *E. wettsteinii* subsp. *wettsteinii* from Mt. Rumija (Rmj5), and a putative hybrid from Mt. Lovćen (*E.* × *lakusicii*), all from Montenegro. A neighbor-net network (Bryant and Moulton 2004), well suited to depicting the reticulate relationships, was produced using SplitsTree 4 (Huson and Bryant 2006).

Chloroplast DNA sequencing and phylogenetic reconstruction

Protocols for DNA extractions, PCR conditions, amplicon purifications, as well as sequencing procedures are outlined in Stefanović et al. (2007, 2008). PCR primers described by Taberlet et al. (1991) and by Hoot et al. (1995) were used to target chloroplast *trn*L-F and *rbc*L-*atp*B spacer regions, respectively. Two closely related species from the *E. tenuifolius* complex, *E. serbicus* and *E. dalmaticus*, were used as outgroups. The chloroplast sequences were obtained from 20 *Edraianthus* individuals in total, and include multiple accessions of *E. tenuifolius*, geographically the most widespread. Sequences obtained for this study are deposited in GenBank (see Table 1). Alignment was done manually, using Se-Al v.2.0a11 (Rambaut 2002). Gaps in the alignments were scored as missing data, coded as binary characters, and appended to the sequence matrix (Simmons and Ochoterena 2000). Parsimony searches, along with accompanying clade support estimations, were

Table 2 Descriptive statistics and factor loadings of principal component analysis (PCA) of morphometric data related to morphological characteristics of *Edraianthus tenuifolius*, *E. wettsteinii* subsp. *lovcenicus*, and putative hybrid *E.* × *lakusicii* (all measures in mm)

	E. tenuifolius						*E.* × *lakusicii*						*E. wettsteinii* subsp. *lovcenicus*						Principal component analysis		
	No	Mean	Min	Max	Std. dev.	Std. error	No	Mean	Min	Max	Std. dev.	Std. error	No	Mean	Min	Max	Std. dev.	Std. error	Factor 1	Factor 2	Factor 3
Stem height	16	19.2	6.8	41.5	11.9	3.0	9	68.0	29.2	121.6	34.2	11.4	18	38.1	20.5	67.8	12.6	3.0	−0.161	0.699	−0.049
Cauline leaves—BW	15	1.7	1.2	2.9	0.5	0.1	9	1.6	1.1	2.6	0.5	0.2	18	1.1	0.8	1.8	0.3	0.1	0.289	−0.031	−0.541
Cauline leaves—MW	15	2.6	1.9	3.5	0.6	0.1	9	1.9	1.3	2.6	0.4	0.1	18	1.5	1.1	2.1	0.3	0.1	−0.315	0.551	−0.058
Cauline leaves—WUQ	15	1.0	0.7	1.3	0.2	0.0	9	1.3	1.0	1.6	0.2	0.1	18	1.3	0.8	1.8	0.3	0.1	0.364	0.368	−0.041
Cauline leaves—TL	15	30.6	19.8	48.6	8.7	2.2	9	19.8	14.5	25.5	3.8	1.3	18	8.2	4.5	12.5	2.2	0.5	0.677	0.251	0.093
Cauline leaves—DLB	15	1.2	0.1	2.0	0.7	0.2	9	4.6	0.6	17.2	6.2	2.1	18	3.9	0.4	8.4	7.2	0.5	**0.789**	0.062	0.017
Rosette leaves—MW	15	3.6	1.7	8.6	2.2	0.6	8	4.1	3.1	5.1	0.6	0.2	16	2.4	1.6	3.1	0.5	0.1	−0.410	0.527	0.115
Rosette leaves—WUQ	15	1.1	0.9	1.5	0.2	0.0	9	1.6	1.1	2.7	0.5	0.2	17	1.4	1.1	1.7	0.2	0.1	**0.914**	−0.071	0.034
Rosette leaves—TL	15	34.3	24.4	46.8	7.9	2.0	9	53.3	30.8	84.4	14.9	5.0	17	21.0	14.4	30.6	4.5	1.1	−0.362	0.446	0.014
Inner involucral bracts—BW	16	1.8	1.0	3.1	0.6	0.2	9	1.0	0.8	1.3	0.1	0.0	18	1.0	0.6	1.6	0.3	0.1	**0.785**	−0.185	0.204
Inner involucral bracts—MW	16	4.1	2.7	6.0	1.0	0.2	9	2.4	1.2	3.3	0.7	0.2	18	1.9	1.0	2.4	0.3	0.1	**0.872**	−0.261	0.002
Inner involucral bracts—WUQ	16	2.9	1.3	4.3	0.9	0.2	9	1.8	0.8	2.4	0.5	0.2	18	1.4	0.7	1.8	0.3	0.1	**0.703**	−0.270	−0.089
Inner involucral bracts—TL	16	10.3	6.6	17.6	3.4	0.8	9	8.9	5.7	11.3	1.7	0.6	18	6.3	5.3	7.4	0.7	0.2	**0.861**	0.173	0.019
Inner involucral bracts—DLB	16	4.6	2.3	7.8	1.6	0.4	9	4.2	1.2	6.0	1.6	0.5	18	1.7	0.7	4.4	0.9	0.2	**0.722**	0.116	−0.172
Inner involucral bracts—DAB	16	7.9	5.8	10.8	1.5	0.4	9	8.3	5.7	10.9	1.6	0.5	18	6.3	5.3	7.4	0.7	0.2	0.684	0.477	−0.002
Central involucral bracts—BW	16	2.8	1.5	4.9	0.9	0.2	9	2.0	1.4	2.6	0.3	0.1	18	1.2	0.7	1.7	0.3	0.1	**0.881**	−0.017	0.136
Central involucral bracts—MW	16	6.1	5.1	7.9	0.9	0.2	9	4.2	3.0	5.2	0.7	0.2	18	2.0	1.6	2.7	0.3	0.1	**0.920**	−0.201	−0.012
Central involucral bracts—WUQ	16	1.8	0.8	3.4	0.6	0.1	9	1.8	1.5	2.2	0.2	0.1	18	1.6	1.2	2.2	0.3	0.1	0.340	0.091	0.485
Central involucral bracts—TL	16	13.2	8.6	20.4	3.3	0.8	9	10.6	8.1	13.0	1.6	0.5	18	6.7	5.6	9.2	0.9	0.2	**0.937**	0.052	0.106
Central involucral bracts—DLB	16	4.4	2.2	8.4	1.7	0.4	9	2.6	2.1	3.2	0.3	0.1	18	1.9	0.7	4.4	1.1	0.3	**0.715**	−0.075	0.436
Central involucral bracts—DAB	16	7.5	5.2	11.3	1.8	0.5	9	6.3	4.3	10.0	1.6	0.5	18	6.7	5.6	9.2	0.9	0.2	0.481	0.155	0.528
Outer involucral bracts—BW	16	3.2	1.9	5.0	1.1	0.3	9	2.0	1.8	2.3	0.2	0.1	18	1.2	0.8	1.7	0.3	0.1	**0.901**	−0.033	0.210
Outer involucral bracts—MW	16	5.6	3.6	9.5	1.5	0.4	9	3.8	2.5	4.5	0.7	0.2	18	2.0	1.4	2.6	0.4	0.1	**0.863**	−0.105	0.280
Outer involucral bracts—WUQ	16	1.3	0.7	1.9	0.1	0.1	9	1.6	1.3	2.4	0.3	0.1	18	1.6	1.1	2.3	0.3	0.1	−0.322	0.324	0.277
Outer involucral bracts—TL	16	18.5	11.2	30.8	5.1	1.3	9	12.2	9.6	15.7	2.1	0.7	18	7.5	5.9	9.4	1.0	0.2	**0.933**	−0.083	−0.007
Outer involucral bracts—DLB	16	2.7	1.1	5.8	3.1	0.3	9	2.1	0.7	4.5	1.2	0.4	18	2.2	0.8	4.7	1.3	0.3	0.300	0.285	0.539
Outer involucral bracts—DAB	16	5.0	0.1	9.1	3.1	0.8	9	4.1	0.1	11.7	4.3	1.4	18	7.5	5.9	9.4	1.0	0.2	−0.307	0.086	0.638
Calyx diameter	12	2.6	1.9	3.0	0.4	0.1	8	2.5	2.1	2.7	0.2	0.1	15	2.3	1.8	2.6	0.2	0.1	0.495	0.423	−0.240
Calyx tooth—BW	12	1.3	0.6	1.7	0.3	0.1	8	1.4	1.1	1.8	0.2	0.1	15	1.3	0.9	1.6	0.2	0.1	0.120	0.498	0.159
Calyx tooth—WUQ	12	0.3	0.2	0.5	0.1	0.0	8	0.8	0.6	1.2	0.2	0.1	15	1.0	0.9	1.2	0.1	0.0	**−0.711**	0.351	0.328
Calyx tooth—TL	12	5.0	3.3	6.3	1.0	0.3	8	5.3	4.6	5.8	0.4	0.1	15	4.6	3.7	5.4	0.5	0.1	0.467	0.600	0.006
Corolla—BW	11	3.3	2.5	4.1	0.5	0.2	8	2.5	1.9	3.3	0.5	0.2	14	2.4	2.2	2.8	0.2	0.0	0.632	−0.084	−0.281
Corolla—MW	11	8.6	7.0	11.5	1.3	0.4	8	8.4	6.9	9.7	0.8	0.3	14	8.1	6.4	9.2	0.8	0.2	0.434	0.305	−0.321

Table 2 continued

	E. tenuifolius						*E.* × *lakusicii*						*E. wettsteinii* subsp. *lovcenicus*						Principal component analysis		
	No	Mean	Min	Max	Std. dev.	Std. error	No	Mean	Min	Max	Std. dev.	Std. error	No	Mean	Min	Max	Std. dev.	Std. error	Factor 1	Factor 2	Factor 3
Corolla—WB	11	4.1	3.6	5.3	0.5	0.2	8	3.9	2.6	4.4	0.6	0.2	14	3.4	2.5	4.0	0.4	0.1	0.559	0.317	−0.355
Corolla—TL	11	15.7	13.1	19.4	1.8	0.5	8	15.6	13.4	18.6	1.7	0.6	14	14.3	11.5	16.9	1.7	0.4	0.433	0.359	−0.422
Corolla—LH	11	6.0	4.8	7.5	1.0	0.3	8	6.9	5.8	8.0	0.7	0.3	14	6.4	5.4	7.9	0.7	0.2	−0.027	0.494	−0.240
Stylus length	10	12.1	8.1	15.1	2.3	0.7	4	13.3	8.3	15.8	3.4	1.7	22	11.5	6.7	15.1	1.9	0.4	0.041	0.045	−0.222
Anthers length	11	5.0	3.8	6.9	0.8	0.3	5	5.3	4.0	6.1	0.8	0.4	23	4.4	3.5	5.7	0.7	0.1	0.497	0.098	−0.141
Stamens—FL	11	0.9	0.4	1.6	0.3	0.1	2	0.9	0.8	1.1	0.2	0.1	23	1.0	0.7	1.7	0.2	0.1	−0.309	−0.015	0.026
Stamens—BL	4	1.8	1.3	2.3	0.4	0.2	0	–	–	–	–	–	18	1.6	1.3	2.0	0.2	0.1	0.151	−0.145	−0.206

Bold variable with factor loadings > 0.7

No number of measured cases, *Mean* mean value of measured character, *Min* minimum value of measured character, *Max* maximum value of measured character, *Std. dev.* standard deviation of measured character, *Std. error* standard error of measured character, *Factors 1, 2 and 3* factor loadings of principal component analysis, *BL* base length, *BW* base width, *DAB* distance between bract base and abruptly narrowed part of a bract, *DLB* distance between the largest leaves width point and the leaves base, *FL* filaments length, *LH* lobe height, *MW* maximal width, *TL* total length, *WUQ* width in the upper quarter, *WB* width of lobe base

conducted for each region separately and concatenated. Given the moderate number of terminal units, we used Branch-and-Bound search algorithm with PAUP* v.4.0b10 (Swofford 2002), ensuring that all of most parsimonious trees will be recovered. Relative support for clades was inferred by nonparametric bootstrapping (Felsenstein 1985), using 500 repetitions with Branch-and-Bound algorithm.

Results

Morphometric analyses

Results of morphometric analysis are summarized in Table 2, showing the parameters of descriptive and multivariate analysis. In the PCA, most of the variation was explained by the first three axes, 36.67, 9.33, and 7.20%, respectively. However, because the first three PCA axes account only for 53.21% of total variability, it appears that the structural variability of the studied populations is quite complex. The PCA separated three entities on the first two axis, with the putative hybrids occupying an intermediate position between the presumed parents (Fig. 1). The individuals of *E. tenuifolius* stand clearly separated from the remainder of the plants included in the analysis. It is also apparent that this species shows substantial morphometrical variations for the characters scored in this study. Also, a differentiation between plants recognized as *E. wettsteinii* susbp. *lovcenicus* and intermediate plants recognized as putative hybrid is noticeable. Although these individuals are for the most part separated on both the first and the second axis, there is a region of overlap among them as

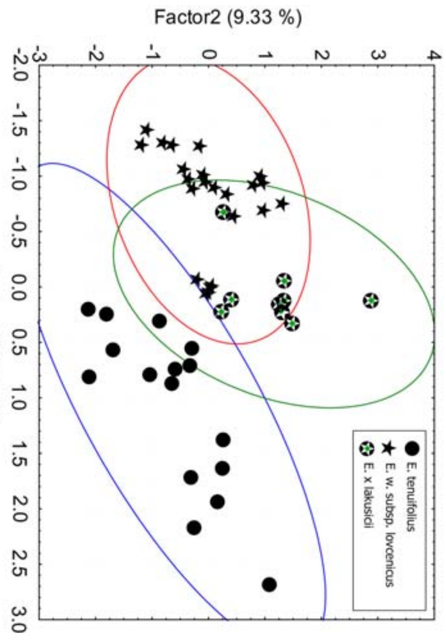

Fig. 1 Principal component analysis (PCA) of morphometric data of *Edraianthus tenuifolius*, *E. wettsteinii* subsp. *lovcenicus* and their putative hybrid *E.* × *lakusicii* (*ellipses* are constructed with coefficient 0.95)

Table 3 Comparison of some qualitative diagnostic morphological characters of putative hybrid *Edraianthus* × *lakusicii* with its parents *E. wettsteinii* subsp. *lovcenicus* and *E. tenuifolius*

	E. wettsteinii subsp. *lovcenicus*	*E.* × *lakusicii*	*E. tenuifolius*
Leaves	Narrowly lanceolate, with more or less involute slightly ciliate margin; densely appressed-hirsute and silvery above	Narrowly linear, with flat margin, more or less densely appressed-hirsute and grayish above, ciliate up to the apex	Narrowly linear, with flat or slightly involute margin, glabrous, ciliate up to the apex with very prominent toward leaf apex reversed ciliae
Involucral bracts	Leaf like, short lanceolate, linear-attenuate, much shorter than the flowers, densely appressed-hirsute and silvery above, glabrous beneath	Subovate-lanceolate, slightly attenuate, as long as or shorter than the flowers, ciliate from base to the apex, bracts base glabrous beneath and above, bract apex more or less densely appressed-hirsute and grayish above, glabrous beneath	Broadly ovate, abruptly long-attenuate, as long as or longer then flowers, ciliate, glabrous
Inflorescence	Flowers solitary, rarely 2–3 (–5)	With 3–5 (–7) subsessile flowers in terminal cluster	With 3–6 (–15) subsessile flowers in terminal cluster
Calyx	Densely appressed-hirsute	Sparsely appressed-hirsute	Glabrous or sparsely hairy
Calyx teeth	Triangular-lanceolate	Lanceolate	Linear
Corolla	Light-blue, more or less densely hirsute	Light-blue, more or less sparsely hirsute	Bluish-violet, glabrous or more or less ciliate on keel

well (Fig. 1). The most important variables on the first axis (factor loadings > 0.7) are cauline leaves (DLB), rosette leaves (WUQ), inner involucral bracts (BW, MW, WUQ, TH, DLB), central involucral bracts (BW, MW, TH, DLB), outer involucral bracts (BW, MW, TH), and calyx tooth (WUQ). All of these variables except the last one were positively correlated with the first axis. However, on the second and third axis, they were not variable with factor loadings > 0.7.

The intermediate morphological position of the hybrid between *E. tenuifolius* and *E. wettsteini* subsp. *lovcenicus*, can be accounted for in large part by the size and shape of bracts and calyx teeth, as well as by a number of leaf characters (see Table 2 for details). The morphological differences among the two analyzed species and their putative hybrid are summarized in Table 3, showing the qualitative diagnostic morphological characters.

The CDA has shown that *E. tenuifolius*, *E. wettsteinii* subsp. *lovcenicus*, and the putative hybrid are completely differentiated from each other (Fig. 2). The plants of *E. tenuifolius* are clearly separated along the first axis, while those of *E. wettsteinii* subsp. *lovcenicus* and the putative hybrid are separated along the second axis. The CDA based on four sets of morphological characters (habitus, rosette leaves, involucral bracts, flowers) indicated that in almost all cases *E. wettsteinii* subsp. *lovcenicus* and the putative hybrid show higher level of similarity (Mahalanobis distance) between them than either has with *E. tenuifolius*. The only exception to these results is found within the group of characters related to rosette leaves, which indicate higher similarity of the putative hybrid to *E. tenuifolius*, and not to *E. wettsteinii* susbp. *lovcenicus* (Table 4).

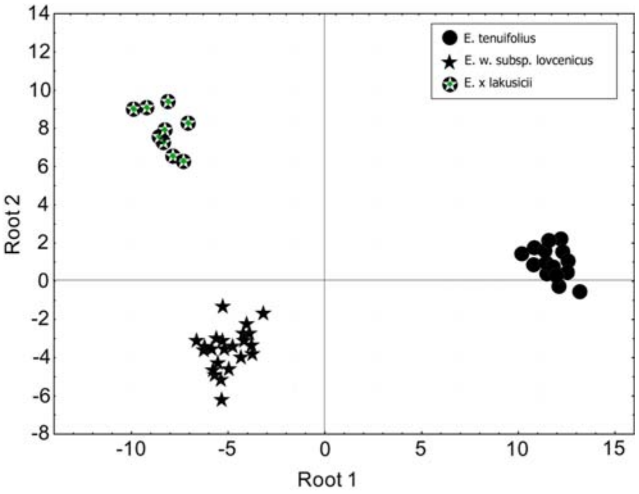

Fig. 2 Canonical discriminant analysis (CDA) of morphometric data of *Edraianthus tenuifolius*, *E. wettsteinii* subsp. *lovcenicus* and their putative hybrid *E.* × *lakusicii*

Phylogenetic analyses

AFLP markers

A total of 163 of reproducible bands were produced for the accessions in study, of which 4 were monomorphic. The error rate (Bonin et al. 2004) before the exclusion of unreliable characters was <1%. In the neighbor-net network (Fig. 3), four well supported groups were formed: *E. serpyllifolius*, *E. graminifolius* (both as outgroups), *E. tenuifolius*, and *E. wettsteinii* s. l. The putative hybrid nests within the *E. wettsteinii* s. l. group, supporting further the results based on morphological data.

Table 4 Mahalanobis distance calculated in canonical discriminant analysis (CDA) for four sets of morphological characters (habitus, rosette leaves, involucral bracts, flowers) of the *Edraianthus wettsteinii* subsp. *lovcenicus*, *E. tenuifolius* and putative hybrid *E.* × *lakusicii*

	E. tenuifolius	*E. w. lovcenicus*	*E.* × *lakusicii*
Habitus			
E. tenuifolius	0.000	17.001	18.993
E. w. lovcenicus	17.001	0.000	5.646
E. × *lakusicii*	18.993	5.646	0.000
Rosette leaves			
E. tenuifolius	0.000	2.693	7.719
E. w. lovcenicus	2.693	0.000	12.662
E. × *lakusicii*	7.719	12.662	0.000
Involucral bracts			
E. tenuifolius	0.000	30.931	35.761
E. w. lovcenicus	30.931	0.000	21.046
E. × *lakusicii*	35.761	21.046	0.000
Flowers			
E. tenuifolius	0.000	8.860	6.686
E. w. lovcenicus	8.860	0.000	4.800
E. × *lakusicii*	6.686	4.800	0.000

cpDNA phylogeny

To the genus-wide molecular phylogenetic analyses based on chloroplast sequence data (Stefanović et al. 2008) here we included putative hybrid together with, among others, the representatives of its sympatric populations belonging to *E. tenuifolius* and *E. wettsteinii* subsp. *lovcenicus* from Mt. Lovćen. Even though the putative hybrid was found to be morphologically more similar to *E. wettsteinii* subsp. *lovcenicus* than to *E. tenuifolius* (Fig. 1; Table 2), a result also supported by the AFLP data (Fig. 3), the parsimony analysis of its chloroplast sequence data strongly supports its grouping with *E. tenuifolius* (Fig. 4).

Discussion

Edraianthus tenuifolius is broadly distributed in Mediterranean and submediterranean parts of Adriatic coast, from Istria (SW Slovenia) southward to N. Albania (see Fig. 5c in Stefanović et al. 2008). This is mainly a lowland species growing in rocky grasslands and pastures, but it is also sometimes observed on much higher altitudes (e.g., upper montane and even subalpine vegetation belt). On the other hand, *E. wettsteinii* is a narrow endemic of Mts Lovćen, Sutorman, and Rumija in Montenegro, and is differentiated into two subspecies (Mayer and Blečić 1969; see Fig. 5d in Stefanović et al. 2008). The typical subspecies, *E. wettsteinii* subsp. *wettsteinii*, occurs on Mts Sutorman and Rumija. This

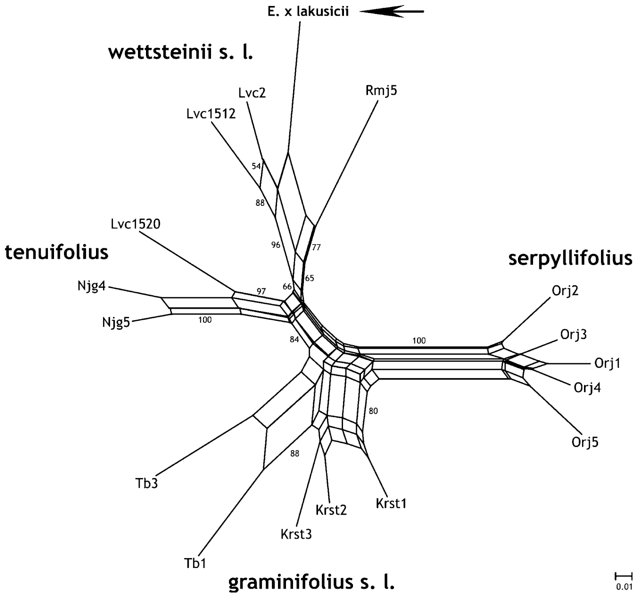

Fig. 3 A neighbor-net of AFLP phenotypes belonging to four groups of taxa of the genus *Edraianthus*. *Numbers* are bootstrap values higher than 50% (1,000 replicates); accession are labeled according to their localities of origin: *Lvc* Mt. Lovćen, *Orj* Mt. Orjen, *Rmj* Mt. Rumija, *Tb* Mt. Terzin bogaz (Durmitor), *Njg* Njeguši, *Krst* Mt. Lovćen, all Montenegro. *Open rectangle* indicates all the samples collected from Mt. Lovćen (Montenegro). The *arrow* indicates the phylogenetic position of the putative hybrid, nested within the clade belonging to one of its putative progenitor species (compare with Fig. 4)

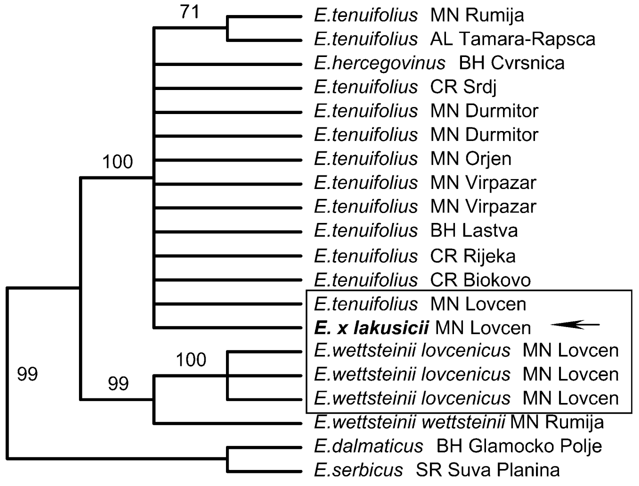

Fig. 4 The strict consensus of 19 equally parsimonious trees from the combined plastid analysis (*trn*L-F region and *rbc*L-*atp*B spacer). *Numbers above branches* indicate bootstrap support. Names of species are followed by two-letter country abbreviation as well as the mountain/region from which the specimen originates. *Open rectangle* indicates all the samples collected from Mt. Lovćen (Montenegro). The *arrow* indicates the phylogenetic position of chloroplast haplotype belonging to the putative hybrid (in *boldface*), nested within the clade of its putative maternal progenitor species. *AL* Albania, *BH* Bosnia and Herzegovina, *CR* Croatia, *MN* Montenegro, *SR* Serbia

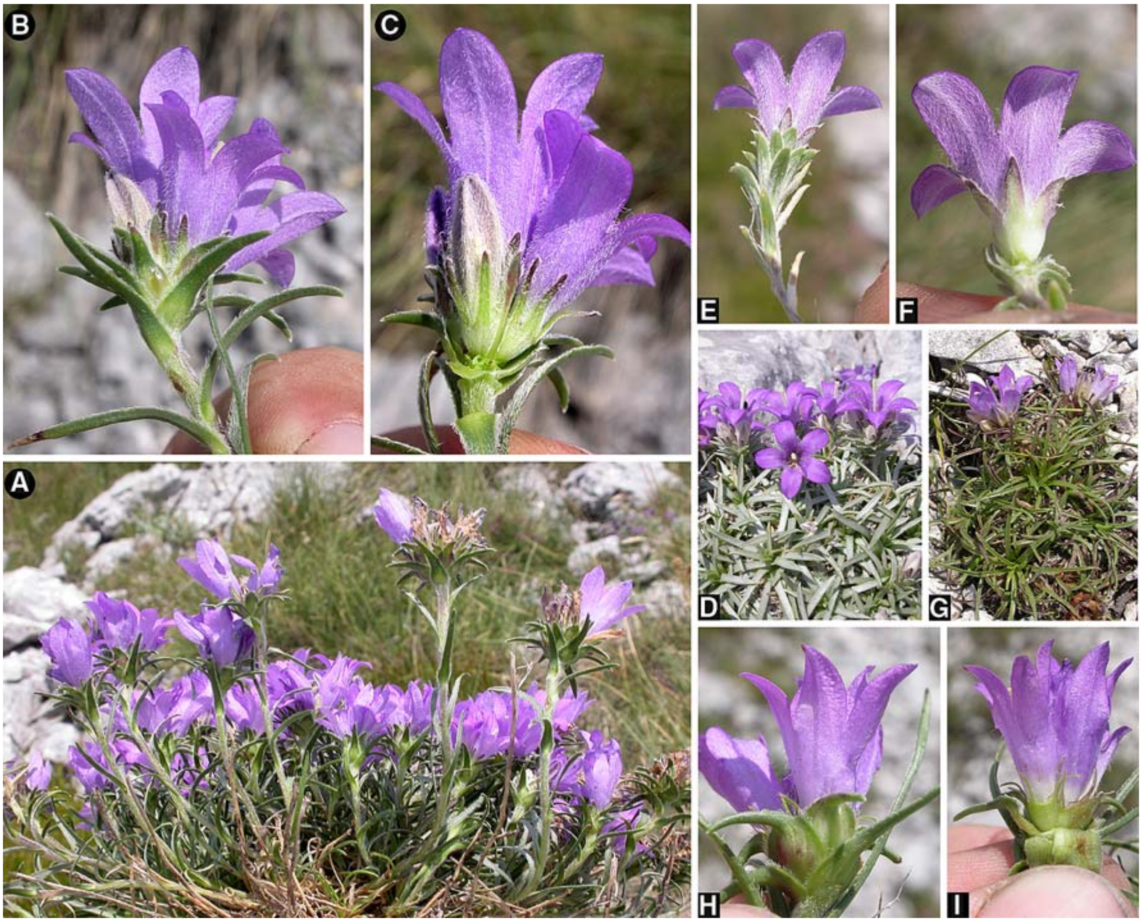

Fig. 5 Differences between **a–c** *Edraianthus* × *lakusicii* nothosp. nova, Holotypus (**a** habitus, **b** inflorescence with involucral bracts, **c** flowers with calyx teeth), **d–f** *E. wettsteinii* subsp. *lovcenicus* (**d** habitus, **e** inflorescens with involucral bracts, **f** flowers with calyx teeth), **g–i** *Edraianthus tenuifolius* (**g** habitus, **h** inflorescens with involucral bracts, **i** flowers with calyx teeth). All from locus classicus, Montenegro, Mt. Lovćen, Branjevine above village Mirac, 42°23.285 N, 18°48.206 E

subspecies inhabits rock crevices at altitudes from 1,100 to 1,600 m. The other subspecies, *E. wettsteinii* subsp. *lovcenicus*, is found only on Mt. Lovćen where it inhabits limestone rocks and rocky slopes at altitudes from 1,300 to 1,400 m.

Edraianthus tenuifolius and *E. wettsteinii* were treated in more detail in several studies (Međedović 1980, 1981; Šoljan 1987; Stefanović et al. 2008). Because *E. tenuifolius* has long, linear to linear-lanceolate, ciliate basal leaves, and sessile flowers arranged in terminal cluster closely subtended by large leaf-like bracts, while *E. wettsteinii* has short, linear, more or less densely hirsute, grayish above and glabrous beneath basal leaves, and solitary flowers closely subtended by small leaf-like bracts (flowers are only rarely arranged in terminal cluster), these two species can be easily morphologically differentiated (Fig. 5). According to traditional taxonomic concepts, *E. tenuifolius* was placed into sect. *Edraianthus* (= *Capitati*), while *E. wettsteinii* was placed into sect. *Uniflori* (= *Strigosi*) (Janchen 1910; Mayer and Blečić 1969; Lakušić 1974). However, results of our genus-wide molecular analyses based on chloroplast sequence data have shown that *E. tenuifolius* and *E. wettsteinii* form a strongly supported clade (Stefanović et al. 2008) and are phylogenetically much more closely related to each than it has been previously suspected.

The results of our analyses confirmed the occurrence of natural hybridization between the populations of *E. tenuifolius* and *E. wettsteinii* subsp. *lovcenicus* on Mt. Lovćen. Taken together, results of morphological and molecular analyses are consistent with the hybrid origin of intermediate individuals, described in this paper as a new nothospecies, *Edraianthus* × *lakusicii.* Morphometric analyses have shown that this natural hybrid shares more morphological similarities with *E. wettsteinii* subsp. *lovcenicus* than with *E. tenuifolius* (Fig. 1). This result is also supported by the AFLP fingerprinting analysis (Fig. 3), according to which the hybrid is distinct from *E. tenuifolius*, and nests within *E. wettsteinii* group. However, the phylogenetic analysis of chloroplast haplotypes have shown that the hybrid belongs to the clade with

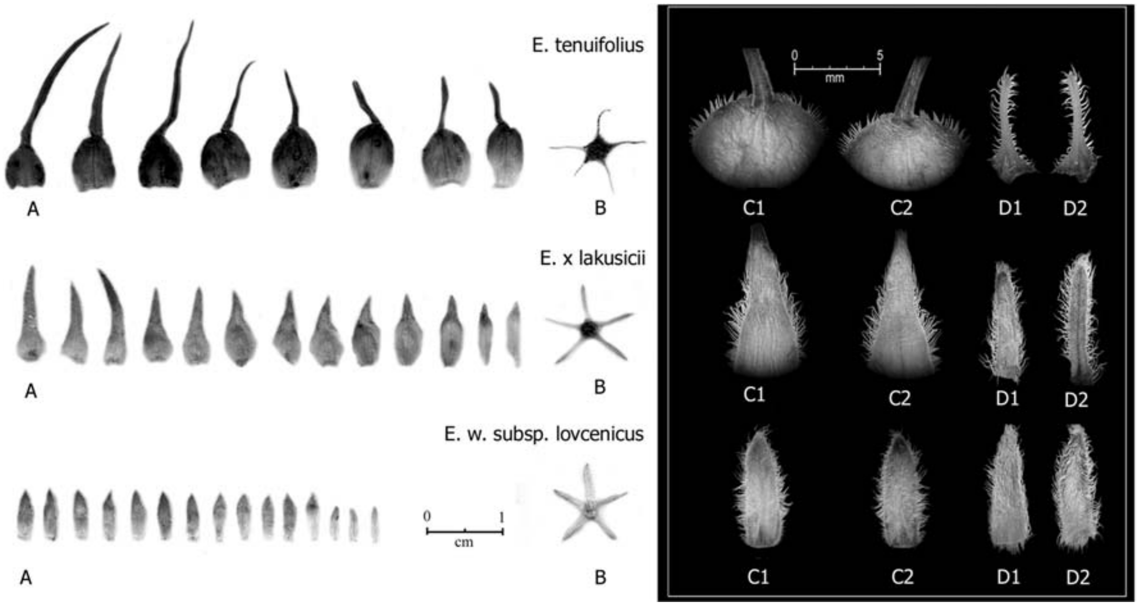

Fig. 6 Differences in involucral bracts, calyx and indumentum characteristics between *Edraianthus tenuifolius*, putative hybrid *E.* × *lakusicii* and *E. wettsteinii* subsp. *lovcenicus* (from locus classicus, Montenegro, Mt. Lovćen, Branjevine above village Mirac, 42°23.285 N, 18°48.206 E). *A* involucral bracts, *B* calyx, *C1* indumentum of involucral bracts—adaxial side, *C2* indumentum of involucral bracts—abaxial side, *D1* indumentum of calyx teeth—adaxial side, *D2* indumentum of calyx teeth—abaxial side

E. tenuifolius, a species whom it shares less morphological similarities with (Fig. 4). These discrepancies between chloroplast-based findings and those based on morphometric studies and AFLP phylogenies are consistent with the hybrid origin of *E.* × *lakusicii*. Furthermore, these findings bear also on directionality of hybridization. Because the chloroplast genome is maternally inherited in the majority of flowering plants (Reboud and Zeyl 1994; Mogensen 1996), the chloroplast-derived phylogeny will most likely trace maternal genealogy. Therefore, our chloroplast data support maternal parentage of the hybrid species by *E. tenuifolius*. Finally, our preliminary results of genome sizes (S. Šiljak-Jakovljev et al. unpublished data) also showed an intermediate position of *E.* × *lakusicii*, consistent with its hybrid origin. Namely, the DNA amount in *E. wettsteinii* subsp. *lovcenicus* was estimated at 4.12 pg, in *E. tenuifolius* at 3.59 pg, and in *E.* × *lakusicii* at 3.93 pg which is an intermediate value. Further investigations are necessary to elucidate the genetic viability and fertility of these hybrids.

Information about hybridization in *Edraianthus* is generally rare (Wettstein in Murbeck 1891; Gusmus 1904; Degen 1938), and, up to now, its occurrence was never confirmed using modern molecular tools. Presently recorded and described hybrid, *E.* × *lakusicii*, represents the first hybrid in *Edraianthus* whose morphological intermediacy is formally described through the results of morphometric comparisons, and whose hybrid origin is further supported by molecular phylogenetic studies. Existence of this natural hybrid strongly indicates the possibility of hybridization or/and introgression between well differentiated *Edraianthus* taxa, which shows us in new light the nature of speciation and evolution within this endemic Balkan group. For the first time the case of reticulate evolution in *Edraianthus* is demonstrated and documented in present study.

From the taxonomic point of view, the presence of rare hybrids within natural populations can cause problems for the delimitation of taxa (Conceição et al. 2008). Like in some other plant groups (Conceição et al. 2007), the taxonomic problems in genus *Edraianthus* could possibly be related to the occurrence of natural hybrids between species of this group and the much conserved floral morphology of these species.

Edraianthus × *lakusicii* V. Stevanović & D. Lakušić [*Edraianthus tenuifolius* (Waldst. & Kit.) A. DC. × *E. wettsteinii* Halácsy & Baldacci subsp. *lovcenicus* E. Mayer & Blečić], nothosp. nov. (Figs. 5, 6).

Type

Montenegro, Lovćen mountain range, southern slopes of Štirovnik, Branjevine above village Mirac, 42°23.285 N, 18°48.206 E, limestone, rocky slopes exposed to bora, in grassland community with predominating *Sesleria robusta* Shott, Nyman & Kotschy, 1,340 masl, 14. July 2006, *Stevanović, V., Lakušić, D. 20948* (Holotype: BEOU, Isotypes: WU, NHMR).

Diagnosis

Planta hybrida inter E. tenuifolius et E. wettsteinii subsp. *lovcenicus. Affinis E. wettsteinii* subsp. *lovcenicus sed caulis alteoribus 30–100 (–121) mm (non 20–50 (–68) mm); folia basalia et caulina linearia vel angustae-lanceolata (non lanceolata), basalia longioribus (31–) 38–68 (–84) × 3–4.7 mm (non 16–25 (–30) × (1.5–) 2–3 mm); capitulum semper multiflorum (non uniflorum vel multiflorum); dentibus calycis longioribus et angustioribus 4.6–5.7 × 0.5–1.0 (–1.2) mm (non 3.7–5.1 (–5.4) × (0.8–) 0.9–1.1 (1.2) mm). A E. tenuifolius folia basalia et caulina argenteo-pilosa (non glabra); folia involucralia subovata vel subovato-lanceolata, in parte superiore infirme contracta, breviter acuminata (non in parte inferiore lata, in parte superiore subito contracta, breviter vel longe acuminata), 9.6–14 (15.6) × (2.5–) 3–4.5 mm (non 11.2–23.5 (–31) × 3.5–7 (–9.5) mm); calyx argenteo-pilosus (non glabrus); dentibus calycis latioribus 4.6–5.7 × 0.5–1.0 (–1.2) mm (non 3.3–6.2 × 0.2–0.4 (0.5) mm); corolla pilosa (non glabra), recedit.*

Description

Dwarf, caespitose perennials. Rhizome stout, woody, branched. Stem simple 3–10 (–12) cm, ascending to erect, densely leafy, hirsute. Leaves narrowly linear, with flat margin, more or less densely appressed-hirsute and grayish above, glabrous beneath, entire, ciliate, ciliae strongly reversed toward leaf apex; rosette leaves (31–) 38–68 (–84) × 3–4 (–5) mm; cauline leaves 14.5–25 × 1–2 (–2.6) mm. Involucral bracts subovate-lanceolate, slightly attenuate, shorter than the flowers, ciliate from base to the apex with toward leaf apex reversed ciliae, bract base glabrous beneath and above, bract apex more or less densely appressed-hirsute and grayish above, glabrous beneath, entire, interior bracts (5.7–) 7–11 × (1.2–) 1.6–3.2 mm, exterior bracts 9.6–15.6 × (2.5–) 3–4.5 mm. Inflorescence with 3–5 (–7) subsessile flowers in terminal cluster. Calyx appressed-hirsute (2.1–) 2.3–2.7 mm in diameter; calyx teeth narrowly lanceolate, two to three times as long as the ovary, (4.6–) 5–6 × 0.5–1 (–1.2) mm. Corolla campanulate, light-blue, hirsute, (13–) 14–16 (–17) × 7–9.5 mm; corolla lobes (5.8–) 6–7.6 (–8) × (2.6–) 3.2–4.4 mm. Stylus 8–12.5 (–16) mm long. Anthere (4–) 4.5–6 mm long.

Eponymy

This taxon is dedicated to the last monographer of the genus *Edraianthus* to Montenegrin botanist Prof. Radomir Lakušić.

Distribution and ecology

Edraianthus × *lakusicii* represents a stenoendemic of Mt. Lovćen, and it might be classified as dinaric (illyrian) endemic, i.e., local endemic of S. Adriatic Dinarides. Its population size is estimated to be <50 mature individuals and the estimated “area of occupancy” is smaller than 1 km^2. The new hybrid inhabits mountain pastures on limestone bedrock, at the altitudes between 1,300 and 1,400 m, in rocky grassland communities where *Sesleria robusta* is a dominant species and other frequent taxa include *Edraianthus wettsteinii* subsp. *lovcenicus*, *E. tenuifolius, E. graminifolius* L. s. l., *Stachys recta* L. s. l., *Scabiosa crenata* Cirillo, *Astragalus vesicarius* L. s. l., *Dianthus sylvestris* Wulfen, *Silene petraea* Waldst. & Kit., *Petrorhagia saxifraga* Link, *Amphoricarpos neumayeri* Visiani, *Teucrium arduinii* L., *Fumana ericoides* Pau, *Asperula cynanchica* L., *Viburnum maculatum* Pantoc. etc.

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