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Primer Note

New microsatellite markers for *Campanula pyramidalis* (Campanulaceae) and cross-amplification in closely related species[1]

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- *Premise of the study:* Microsatellite markers were identified and characterized to study the genetic diversity and structure, conservation status, taxonomy, and biogeography of subspecific taxa and populations of *Campanula pyramidalis* (Campanulaceae).
- *Methods and Results:* Eleven microsatellite markers were developed from genomic libraries enriched for di- and trinucleotide repeats. A total of 80 alleles were observed in the tested natural population. The number of alleles per locus, observed heterozygosity, and expected heterozygosity ranged from four to 13, 0.217 to 0.913, and 0.521 to 0.895, respectively.
- *Conclusions:* The new microsatellite markers will be useful for studying genetic diversity and structure as well as for better assessing the conservation status of subspecific taxa and populations of *C. pyramidalis*. Furthermore, a set of seven loci was successfully cross-amplified in *C. secundiflora* and *C. versicolor* and will be of great value for addressing unsolved taxonomic and biogeographic issues within the *C. pyramidalis* species complex.

Key words: *Campanula pyramidalis*; Campanulaceae; cross-amplification; microsatellites; simple sequence repeat (SSR).

The *Campanula pyramidalis* L. species complex (Campanulaceae) is naturally distributed across the Balkan Peninsula and in a small part of the southern Apennines. It is also established in horticulture worldwide. This species complex is usually found in rocky habitats with specific edaphic and microclimatic conditions. Although 21 taxa have been described within the *C. pyramidalis* complex, only three species have been generally accepted: *C. pyramidalis*, *C. versicolor* Sibth. & Sm., and *C. secundiflora* Vis. & Pančić (Fedorov and Kovanda, 1976; Lammers, 2007; Lakušić et al., 2013). All these taxa have narrow geographic distributions and form phylogenetically closely related groups (Park et al., 2006; Liber et al., 2008; Lakušić et al., 2013).

To elucidate the complicated relationships within this species complex, a broad molecular phylogenetic study based on DNA sequences was performed by Lakušić et al. (2013). Although this work led to many new insights and the description of a new species (*C. austroadriatica* D. Lakušić & Kovačić), the relationships and evolutionary patterns at the subspecific level remain poorly understood. Our current research focuses on expanding the sampling and developing faster-evolving molecular markers that can discriminate among and within closely related and recently diverged taxa. In accordance with these objectives, 11 new microsatellite markers were developed in the current study.

METHODS AND RESULTS

Total genomic DNA was isolated from 25 mg of silica-dried leaves with the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, Missouri, USA). New microsatellites were identified and characterized from genomic DNA libraries enriched for di- and trinucleotide repeats according to Radosavljević et al. (2011, 2012). Enriched fragments containing microsatellite regions were ligated to the pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA) followed by transformation of XL-10 Gold Competent Cells (Agilent Technologies, Santa Clara, California, USA). After overnight incubation at 37°C, white bacterial colonies were transferred into 384-well plates containing Luria–Bertani (LB) freezing media (LB broth + 13 mM KH_2PO_4, 6.8 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, 36 mM K_2HPO_4, 4.4% v/v glycerol). Libraries were transferred onto nylon membranes and screened by Southern hybridization using Cy5-labeled and Cy3-labeled 30-bp oligonucleotides with GA, GT, AGA, ACT, and ATC repeats (Eurofins MWG Operon, Huntsville, Alabama, USA). Positives were detected by scanning the blots using an Ettan DIGE Imager (GE Healthcare Biosciences, Pittsburgh, Pennsylvania, USA). A total of 192 positive clones were selected from the libraries, and plasmid isolations were performed using Wizard Plus SV Minipreps (Promega Corporation). The plasmids were sequenced from both ends using T7 and SP6 universal PCR primers, BigDye chemistry, and an ABI 3130xL DNA analyzer (Applied Biosystems,

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TABLE 1. Characteristics of 11 new microsatellite markers for *Campanula pyramidalis*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	GenBank accession no.
CpUZ001	F: AAGTATTGGACCCCGAGCTT R: ATCACCCTAGCCATGCAATC	$(ACT)_8$	130–163	KF926847
CpUZ002	F: CCGAATGCACCGTATACTCA R: AGCAGTAAATTGGACGGTCTG	$(AGA)_9$	155–167	KF926848
CpUZ003	F: CCTCTTCCGAATGACGCTTA R: CACTCTTGCTTACACCTTGTGC	$(GA)_{12}$	167–259	KF926849
CpUZ004	F: GCGAGACTTCTGTGATGTGG R: TGTGGAGAAATGGACGTTCT	$(AGA)_{12}$	133–181	KF926850
CpUZ005	F: ATGTTTGCCCTTTTCACTGC R: TTGGGATGTTGGAACACAAA	$(GT)_{12}$	146–160	KF926851
CpUZ006	F: CAGCAATGCAGAAATCGAAA R: AAACCCCTTCCTCCTAAATCA	$(GT)_{14}$	212–228	KF926852
CpUZ007	F: TGTGGGAGGTTCATTGGTTT R: ACTGCATGCGACAAGATCAA	$(GT)_{16}$	200–248	KF926853
CpUZ008	F: ATGCAGGGAGCATTGAAGAT R: CCCCAGGAGTCTTTCTCTCC	$(GT)_{15}$	196–204	KF926854
CpUZ009	F: CAAATTTGAACGGGTTTTCG R: ACTCTTCCCCTCCACATTCC	$(GA)_{19}$	181–207	KF926855
CpUZ010	F: TCCACCCACCAATAATCTCC R: ATCCAGAACCCGAAAATTCC	$(ATC)_{10}$	163–199	KF926856
CpUZ011	F: ACACTGCCGATATGTGCGTA R: CCATGTTCGTGTGTAATCATCA	$(GT)_{16}$	220–246	KF926857

[a]A PCR protocol with initial touchdown cycles was used (annealing temperature 55°C).

Foster City, California, USA). Geneious 5.6.4 (Biomatters Ltd., Auckland, New Zealand; http://www.geneious.com/) was used to edit and assemble the sequences. Microsatellite repeats were localized using MISA Perl script (Thiel et al., 2003). PCR primers flanking the microsatellite repeats were designed for 48 sequences using Primer3 version 4.0 (Untergasser et al., 2012), with the optimum conditions set at a length of 20 bp (18–27 bp), a temperature of 60.0°C (57–63°C), a GC content of 50% (20–80%), and a product size range of 140–210 bp.

A preliminary study using five *C. pyramidalis* individuals from a natural population from Mt. Velebit (Croatia) (Appendix 1) resulted in the selection of 11 microsatellite loci (Table 1) that were polymorphic, had low levels of stutter bands, and did not yield evidence of nonspecific amplification. These 11 microsatellite loci were subsequently used in a wider analysis using 24 individuals from the same population. The sequences of microsatellite loci were deposited into GenBank (accession no. KF926847–KF926857; Table 1). PCR amplification was performed using a tailed PCR primer approach (Schuelke, 2000) that involved a two-step PCR protocol with an initial touchdown cycle (94°C for 5 min; 5 cycles of 45 s at 94°C, 30 s at 60°C, which was lowered by 1°C in each cycle, and 90 s at 72°C; 25 cycles of 45 s at 94°C, 30 s at 55°C, and 90 s at 72°C; and an 8-min extension step at 72°C). The 20-μL total volume of the PCR mix contained 8 pmol each of reverse and FAM-M13(–21) primers, 2 pmol of the forward primer, 1× PCR buffer, 0.2 mM dNTPs, 1 unit TaKaRa *Taq* Hot Start DNA Polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan), and 5 ng of template DNA. The PCR products were genotyped on an ABI 3730xL DNA analyzer (Applied Biosystems) by the Macrogen DNA service (Seoul, Korea) and analyzed using GeneMapper 4.0 (Applied Biosystems).

PowerMarker 3.25 (Liu and Muse, 2005) software was used to calculate the average number of alleles per locus (A), the observed heterozygosity (H_o), and the expected heterozygosity (H_e) of each microsatellite locus. Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were tested using GENEPOP 3.4 (Raymond and Rousset, 1995). Sequential Bonferroni corrections (Holm, 1979) were applied when conducting multiple statistical tests using SAS 8.02 (SAS Institute, Cary, North Carolina, USA). Each locus was evaluated for the presence of null alleles, scoring errors, and allelic dropout using MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004).

All 11 loci were polymorphic, with an average of 7.27 alleles per locus, H_o ranging from 0.217 to 0.913, and H_e from 0.521 to 0.895 (Table 2). Three out of the 11 newly developed microsatellite loci (CpUZ001, CpUZ004, and CpUZ008) showed significant deviations from HWE (Table 2). Deviations from HWE may have been related to the presence of null alleles, although we found no null homozygotes. The null allele frequencies, estimated using Brookfield's formula (Brookfield, 1996), were 0.178 (CpUZ001), 0.119 (CpUZ004), and 0.267 (CpUZ008). One out of the 55 tests for linkage disequilibrium was significant ($P < 0.01$) after applying sequential Bonferroni corrections (CpUZ003/CpUZ009).

The 11 new microsatellite markers from *C. pyramidalis* were also tested on 24 individuals from natural populations of the closely related *C. secundiflora* and *C. versicolor* (Appendix 1). Seven out of the 11 microsatellite loci were successfully cross-amplified in both species (Table 2). The total number of alleles observed at each locus in *C. secundiflora* ranged from two to eight, the H_o from 0.136 to 0.833, and the H_e from 0.509 to 0.852. All loci were in accordance with HWE, and no linkage disequilibrium was detected between any pair of loci. The total number of alleles observed at each locus in *C. versicolor* ranged from two to six, the H_o from 0.167 to 0.625, and the H_e from 0.156 to 0.763. Two out of seven loci (CpUZ003 and CpUZ004) deviated from HWE. Based on the results from MICRO-CHECKER, the occurrence of null alleles was suggested for both loci at frequencies of 0.296 (CpUZ003) and 0.136 (CpUZ004). No evidence of linkage disequilibrium was detected across any pairwise comparisons except between CpUZ009 and CpUZ010.

TABLE 2. Genetic properties of the 11 newly developed microsatellites in natural populations of *Campanula pyramidalis*, *C. secundiflora*, and *C. versicolor*.

	C. pyramidalis (n = 24)			*C. secundiflora* (n = 24)			*C. versicolor* (n = 24)		
Locus	A	H_o	H_e[a]	A	H_o	H_e	A	H_o	H_e[a]
CpUZ001	5	0.458	0.798*	3	0.136	0.548	3	0.625	0.524
CpUZ002	5	0.478	0.629	—	—	—	—	—	—
CpUZ003	13	0.864	0.870	8	0.714	0.852	6	0.217	0.763***
CpUZ004	8	0.542	0.770**	4	0.833	0.703	5	0.500	0.758*
CpUZ005	7	0.727	0.850	4	0.500	0.521	2	0.167	0.156
CpUZ006	8	0.708	0.790	6	0.792	0.808	5	0.500	0.660
CpUZ007	7	0.454	0.521	—	—	—	—	—	—
CpUZ008	4	0.217	0.686***	—	—	—	—	—	—
CpUZ009	7	0.652	0.857	2	0.227	0.509	4	0.542	0.650
CpUZ010	5	0.409	0.734	5	0.792	0.762	6	0.375	0.607
CpUZ011	11	0.913	0.895	—	—	—	—	—	—

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals analyzed.

[a]Significant deviations from Hardy–Weinberg equilibrium after sequential Bonferroni corrections: *** represents significance at the 0.1% nominal level; ** represents significance at the 1% nominal level; * represents significance at the 5% nominal level.

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CONCLUSIONS

The 11 new microsatellite markers developed here will be useful for studying genetic diversity and structure as well as for better assessment of the conservation status of subspecific taxa and populations of *C. pyramidalis*. A set of seven loci was successfully cross-amplified in *C. secundiflora* and *C. versicolor*. Because the three species are very closely related, a high level of homoplasy is not likely. We expect these seven microsatellite loci to be of great value in addressing unsolved taxonomic and biogeographic issues in the *C. pyramidalis* species complex.

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Appendix 1. Voucher information for *Campanula* species used in this study.

Species	Voucher specimen accession no.[a]	Collection locality	Geographic coordinates	*n*
C. pyramidalis L.	ZA-30825	Mt. Velebit, Croatia	44°20′24.38″N, 15°38′58.76″E	24
C. secundiflora Vis. & Pančić	BEOU-25034	Panjica Gorge, Serbia	43°40′04.00″N, 20°05′44.00″E	24
C. versicolor Sibth. & Sm.	BEOU-28150	Tempe Vale, Greece	39°52′41.16″N, 22°35′05.64″E	24

Note: *n* = number of individuals.
[a]Herbarium codes: BEOU = Herbarium of the University of Belgrade; ZA = Herbarium Croaticum, University of Zagreb.

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