NEW MICROSATELITE MARKERS FOR *Campanula pyramidalis* (Campanulaceae) AND CROSS-AMPLIFICATION IN CLOSELY RELATED SPECIES

**IVAN Radosavljević**2, JERNEJ JAKSE3, ZLATKO SATOVIC4, BRANKA JAVORNIK3, IVANA JANKOVIĆ5, AND ZLATKO LIBER2,6

2Division of Botany, Department of Biology, Faculty of Science, University of Zagreb, Marulićev trg 9a, 10000 Zagreb, Croatia; 3Centre for Plant Biotechnology and Breeding, Agronomy Department, Biotechnical Faculty, University of Ljubljana, Jammnikarjeva 101, 1000 Ljubljana, Slovenia; 4Department of Seed Science and Technology, Faculty of Agriculture, University of Zagreb, Svetošimunska cesta 25, 10000 Zagreb, Croatia; and 5Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia.

- **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 & Kovanda), 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 KH₂PO₄, 6.8 mM (NH₄)₂SO₄, 1.7 mM sodium citrate, 36 mM K₂HPO₄, 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 Etan 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 Miniprep (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, Applied Biosystems).
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*<sub>o</sub> from 0.136 to 0.833, and the *H*<sub>e</sub> 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*<sub>o</sub> from 0.167 to 0.625, and the *H*<sub>e</sub> 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.

Foster City, California, USA). Geneious 5.6.4 (Biometers 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 nested 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 FM-M13(–21) primers, 2 pmol of the forward primer, 1× PCR buffer, 0.2 mM dNTPs, 1 unit TaKaRa 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*<sub>o</sub>), and the expected heterozygosity (*H*<sub>e</sub>) of each microsatellite locus. Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were tested using GENEPOP 4.0 (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*<sub>o</sub> ranging from 0.217 to 0.913, and *H*<sub>e</sub> from 0.521 to 0.895 (Table 1). 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 homzygotes. 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 53 tests for linkage disequilibrium was significant (*P* < 0.01) after applying sequential Bonferroni corrections (CpUZ003/CpUZ009).

**Table 1.** Characteristics of 11 new microsatellite markers for *Campanula pyramidalis*.

| Locus  | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | GenBank accession no. |
|--------|-------------------------|--------------|------------------------|----------------------|
| CpUZ001 | F: AAGTAGTTGAGCAGGAGCTT | (ACT)<sub>4</sub> | 130–163 | KF926847 |
| CpUZ002 | R: ATCACCTGAGCAGTGAACTC | (AGA)<sub>3</sub> | 155–167 | KF926848 |
| CpUZ003 | F: CCTGTTGAGACCTTCTTA | (GA)<sub>12</sub> | 167–259 | KF926849 |
| CpUZ004 | F: GCGAGACCTTCTGGAGGTTG | (AGA)<sub>12</sub> | 133–181 | KF926850 |
| CpUZ005 | F: ATGTGTCCTCTTTCCTACGTC | (GT)<sub>12</sub> | 146–160 | KF926851 |
| CpUZ006 | F: TAGGAGGGTGGAGAATGAAA | (GT)<sub>14</sub> | 212–228 | KF926852 |
| CpUZ007 | F: TGTGGGAGTTCCTGTTGTTT | (GT)<sub>16</sub> | 200–248 | KF926853 |
| CpUZ008 | F: ATGGACGGAGGATCTGAGAT | (GT)<sub>15</sub> | 196–204 | KF926854 |
| CpUZ009 | F: CAAATTGGAACGGTTTCTCG | (GA)<sub>10</sub> | 181–207 | KF926855 |
| CpUZ010 | F: TCCACCCACCAATATTCC | (ATC)<sub>10</sub> | 163–199 | KF926856 |
| CpUZ011 | F: AGACTGCCGATATGCTGTA | (GT)<sub>16</sub> | 220–246 | KF926857 |

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

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

| Locus   | CpUZ001 | CpUZ002 | CpUZ003 | CpUZ004 | CpUZ005 | CpUZ006 | CpUZ007 | CpUZ008 | CpUZ009 | CpUZ010 | CpUZ011 |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| A       | 5       | 5       | 13      | 8       | 8       | 5       | 5       | 4       | 7       | 7       | 5       |
| H<sub>o</sub> | 0.458 | 0.478 | 0.864 | 0.542 | 0.727 | 0.708 | 0.708 | 0.708 | 0.708 | 0.652 | 0.409 |
| H<sub>e</sub> | 0.798 | 0.629 | 0.870 | 0.770 | 0.850 | 0.790 | 0.790 | 0.790 | 0.790 | 0.686 | 0.734 |

Note: *A* = number of alleles; *H*<sub>o</sub> = observed heterozygosity; *H*<sub>e</sub> = expected heterozygosity; *n* = number of individuals analyzed.

* 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.
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 *Lakua* taxa and populations of *Campanula 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.

LITERATURE CITED

Brookfield, J. F. Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. *Molecular Ecology* 5: 453–455.

Fedotov, A., and M. Kovanda. 1976. *Campanula*. In T. G. Tutin, V. H. Heywood, N. A. Burges, D. M. Moore, D. H. Valentine, S. M. Walters, and D. A. Webb [eds.], Flora Europaea, vol. 4, 74–93. Cambridge University Press, Cambridge, United Kingdom.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* 6: 65–70.

Lukić, D., Z. Liber, T. Nikolić, B. Suren, S. Kovačić, S. Bogdanović, and S. Stefanović. 2013. Molecular phylogeny of the *Campanula pyramidalis* species complex (Campanulaceae) inferred from chloroplast and nuclear non-coding sequences and its taxonomic implications. *Taxon* 62: 505–524.

Lammers, T. G. 2007. *Campanula*. In T. G. Lammers [ed.], World checklist and bibliography of *Campanulaceae*, 86–221. Royal Botanical Gardens, Kew, Richmond, Surrey, United Kingdom.

Notes:

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

| Species                      | Voucher specimen accession no. | 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* Sibh. & Sm.  | BEOU-28150                    | Tempe Vale, Greece  | 39°52′41.16″N, 22°35′05.64″E | 24 |

*Note: n = number of individuals.*

*Herbarium codes: BEOU = Herbarium of the University of Belgrade; ZA = Herbarium Croatianum, University of Zagreb.*