New Microsatellite Markers for Campanula scheuchzeri (Campanulaceae), with Cross-Amplification in C. rotundifolia

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PRIMER NOTE

NEW MICROSATELLITE MARKERS FOR *CAMPANULA SCHEUCHZERI* (CAMPANULACEAE), WITH CROSS-AMPLIFICATION IN *C. ROTUNDIFOLIA*[1]

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- *Premise of the study:* We developed new microsatellite primers for the alpine bellflower *Campanula scheuchzeri*. Allelic polymorphisms will be used to study differentiation along elevation gradients of *C. scheuchzeri* populations and in the co-occurring sister-species *C. rotundifolia* in the Alps.
- *Methods and Results:* We analyzed *C. scheuchzeri* from three high-elevation sites and *C. rotundifolia* from two low-elevation sites in Switzerland. *Campanula scheuchzeri* was found to be tetraploid ($2n = 68 = 4x$), and up to 22 alleles were found per locus and population. Of the 15 polymorphic loci developed for *C. scheuchzeri*, 10 loci were tested, all of which amplified in *C. rotundifolia*, with similar amplicon length. *Campanula rotundifolia* individuals also showed tetraploid signals.
- *Conclusions:* We speculate that *C. scheuchzeri* and *C. rotundifolia* share a common gene pool and evolve under vicariance. This presents a testable hypothesis that will be evaluated through future work. Our developed primers might also amplify in other related *Campanula* taxa.

Key words: bellflowers; *Campanula scheuchzeri*; Campanulaceae; simple sequence repeat (SSR); tetraploid plants.

With nearly 500 accepted species, *Campanula* L. is the largest Campanulaceae genus (Roquet et al., 2008). *Campanula scheuchzeri* Vill. is distributed in meadows and pastures of European mountains and is primarily tetraploid ($2n = 68 = 4x$; Geslot, 1984; Lauber and Wagner, 2007), although some diploid populations have been identified in the Pyrenees (Geslot, 1984). *Campanula rotundifolia* L. is a sister species of *C. scheuchzeri* and is widespread across the northern hemisphere (Roquet et al., 2008; Stevens et al., 2012). In the Alps, both taxa may co-occur on mountain slopes, with *C. scheuchzeri* found in greatest abundance above c. 1200 m and *C. rotundifolia* below c. 1200 m (Aeschimann et al., 2004; Frei, 2007). The two species are morphologically similar, phenotypically highly variable, and some populations are difficult to classify (Böcher, 1936; Frei, 2007; Lauber and Wagner, 2007). No nucleotide differences were observed between the two taxa in an 850-bp chloroplast sequence (Roquet et al., 2008; aligning GenBank sequence EF088759 with EF088762). However, typical populations of *C. scheuchzeri* and *C. rotundifolia* can be easily distinguished by the number and size of flowers, leaf shape, and hair density (Frei, 2007). In the future, we will use the microsatellite loci developed in this study to evaluate phenotypic and molecular differentiation, gene flow, and local adaptation in *C. scheuchzeri* and *C. rotundifolia* in different regions of the Swiss Alps.

METHODS AND RESULTS

We sampled leaf material of *C. scheuchzeri* individuals in three populations in the Swiss Alps that were separated from one another by at least 60 km: Fondei (canton of Graubünden: 1950 m a.s.l.; $N = 20$ individuals), Niessen (canton of Bern: 1680 m a.s.l.; $N = 20$), and Furka (canton of Uri: 2420 m a.s.l.; $N = 20$). We also sampled leaf material from two lowland populations of *C. rotundifolia* in Switzerland for cross-amplification: Blauen (canton Basel-Land: 620 m a.s.l.; $N = 5$) and Bonaduz (canton Graubünden: 660 m a.s.l.; $N = 5$). Leaf samples were silica-dried, and reference samples (Appendix 1) were stored in the Botanical Institute, University of Basel, Switzerland. Extracted DNA was sent to Ecogenics GmbH molecular marker services (Zurich-Schlieren, Switzerland) to develop microsatellite markers. In brief, Ecogenics used a traditional approach with genomic library enrichment, M13-tailing of the forward primers (Schuelke, 2000; see Table 1), and fluorophore labeling of the M13 primer. ECO500 was used as a size standard in the electropherograms. Additional technical information is described in detail in Kesselring et al. (2013) and Hamann et al. (2014). Ecogenics used a standard PCR program for all loci, with 15-min denaturation at 95°C and PCR start at 95°C for 30 s, 56°C for 45 s, and 72°C for 45 s in 30 cycles followed by eight cycles of 95°C for 30 s, 53°C for 45 s, and 72°C for 45 s. Termination was set to 72°C for 30 min (Kesselring et al., 2013). Each locus was analyzed separately. The library was enriched for tetranucleotide motifs (Table 1). This strategy likely assists in allele scoring, because a maximum of four different allelic peaks in an individual may be stretched over a wide range of base pairs. Ecogenics also performed the allele scoring, which was conducted twice independently. Ten out of 15 polymorphic microsatellite loci were randomly chosen and gave clearly readable electropherograms (Table 1). We used a conservative approach of binning of 1-bp differences due to potential stuttering (Table 1). Sample replicates were processed from the point of DNA extraction for 10% ($N = 6$) of the *C. scheuchzeri* samples, and gave identical allele signals to the first run. The polished allelic data of *C. scheuchzeri* were written in a two-digit code for each allele to calculate average expected heterozygosity (H_e) in the ATETRA software package (version 1.2.a; Van Puyvelde et al., 2010). One thousand Monte Carlo

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TABLE 1. Newly developed microsatellite markers in *Campanula scheuchzeri*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Amplicon length (bp)[b]	Allelic binning[c]	GenBank accession no.
Scheuch1	F: AAAGTGCATTATACCTAAATTGCTG R: GTTGGCAAATGGGTTGACTTTC	$(TACA)_8$	123–147	Few binnings	KP342303
Scheuch2	F: TTAGGCTCAAAACTTACCACAC R: CGTTCTCAGATCCGTTACTGTTTC	$(ATAC)_8$	139–166	No binning	KP342304
Scheuch3	F: AGCAATCTTGGCCCCCTAAC R: TACTCGAACATGGCTTCACC	$(TGTA)_7$	138–184	Few binnings	KP342305
Scheuch4	F: TGCATCATAAGTGAGCACATCG R: CGAATCGCTGGGAGAAAAGG	$(TATG)_7$	84–130	No binning	KP342306
Scheuch5	F: TGGGGTGGTTTACTCTACTCG R: TGGAACCCCGTGATGAGATG	$(CTAT)_{11}$	145–193	Several binnings	KP342307
Scheuch6	F: TTATGTTTTTGGGGGCGTGG R: TCATGGGCTGATTATCTAGGGG	$(ATGT)_7$	119–199	Few binnings	KP342308
Scheuch7	F: GCAACTTAAACTGGGACAGAGG R: ACTTTACACATTTAAAAGGCATTGAGG	$(ATAC)_7$	127–220	No binning	KP342309
Scheuch8	F: TCAAATAGAGTGCCACCTTAGC R: TGGGGTATACAGTTGAAGAGG	$(CATA)_7$	127–168	Few binnings	KP342310
Scheuch9	F: TGACCAATGTTCTGGACTTGAC R: ACTAAAACATCATTATTTTGCAACGC	$(ATAC)_9$	107–170	No binning	KP342311
Scheuch10	F: CTCTCTCTCTATAACACACCGC R: GTTGGAGGAGTGACACAAGC	$(ATAC)_7$	83–122	Few binnings	KP342312
Scheuch11	F: GTGACCCTTTCTTATTATTTGCCC R: GCTTTGGAGAGGCTTGCATAC	$(TATC)_{10}$	153–226	NA	KP342313
Scheuch12	F: TCTAGTCATCCCTAGGCCCG R: TGCGCAGTTCACTTGGTTTG	$(AGAT)_7$	103–199	NA	KP342314
Scheuch13	F: TGTTGACTCGCCTCGACTTC R: ACAAGTCCTTCCTAGTTCCTAC	$(ATGT)_8$	153–195	NA	KP342315
Scheuch14	F: ATTACACGAGACGGAGGGAG R: TGGCCTTGTCAAAACGCTTC	$(GTAT)_7$	164–294	NA	KP342316
Scheuch15	F: ATGCCCTAATTTCCACTTGC R: GTGAATTTTTAGCACATTTAGTAGCAC	$(ATGT)_7$	143–220	NA	KP342317

[a]Ten randomly chosen loci (Scheuch1–10) were analyzed in three populations of *C. scheuchzeri*, in a total of 60 individuals (Table 2). Five other loci (Scheuch11–15) were tested in a preliminary analysis by Ecogenics and proved to be polymorphic in *C. scheuchzeri*, but were not included in our study (NA).

[b]Lengths of amplicons include the 18-bp M13 tail 5′-TGTAAAACGACGGCCAGT-3′ of the forward primer (see Methods and Results).

[c]Some allelic peaks of the 60 genotyped *C. scheuchzeri* individuals (see Table 2) were corrected for amplicon size. Due to potential stuttering, binning of 1-bp differences of few alleles was performed (Scheuch1, Scheuch3, Scheuch6, Scheuch8, Scheuch10). In locus Scheuch5, binning of 1-bp differences of several alleles was necessary. Allelic peaks of four loci were clear-cut, and binning was not necessary.

simulations were performed to calculate H_e for each locus in each of the three populations of *C. scheuchzeri* (Table 2).

All 10 primer pairs cross-amplified in *C. rotundifolia* without any PCR dropouts (Table 3), and the allelic range in both species is quite similar. For example, locus Scheuch1 shows alleles between 123 bp and 143 bp in *C. scheuchzeri*, and between 123 bp and 139 bp in *C. rotundifolia* (Tables 1, 3). In *C. scheuchzeri*, between five and 22 alleles were found per locus and population (Table 2). The number of alleles per population in *C. rotundifolia* was lower (2–9; Table 3), but this is probably a consequence of the lower sample size ($N = 20$ in *C. scheuchzeri*, $N = 10$ in *C. rotundifolia*). Despite the similarities, some alleles were only found in the 10 individuals of *C. rotundifolia* (Table 3). The frequency of these *C. rotundifolia* signals was particularly high (59%) at locus Scheuch7 (Table 3).

We confirm the tetrapolyploid nature of the study populations of *C. scheuchzeri*, as up to 60% of all individuals possessed four allelic peaks (locus Scheuch5 and Scheuch7 in the Furka population; Table 2). H_e was high in each locus, ranging from 0.67 to 0.90. Interestingly, we also observed high homozygosity values in some populations (Table 2). High HO_1 values between 0.35 and 0.50 were found in the Fondei population (loci Scheuch3 and Scheuch4) and in the Furka population (locus Scheuch9). We consider four possible explanations

TABLE 2. Population genetic parameters for three tetraploid populations of *Campanula scheuchzeri* from the Swiss Alps.

	Fondei ($N = 20$)				Niessen ($N = 20$)				Furka ($N = 20$)			
Locus	*A*	HO_1	HE_4	H_e	*A*	HO_1	HE_4	H_e	*A*	HO_1	HE_4	H_e
Scheuch1	6	0.05	0.20	0.76	7	0	0.25	0.76	7	0	0.10	0.78
Scheuch2	7	0.15	0	0.72	10	0.25	0.05	0.67	5	0.05	0	0.68
Scheuch3	6	0.35	0.05	0.68	12	0.15	0.05	0.82	7	0.15	0	0.67
Scheuch4	9	0.40	0	0.78	9	0.40	0	0.81	11	0.25	0.05	0.82
Scheuch5	15	0	0.35	0.86	13	0.05	0.25	0.80	13	0	0.60	0.88
Scheuch6	8	0	0.10	0.75	14	0.05	0.30	0.83	8	0	0.10	0.73
Scheuch7	13	0.05	0.40	0.84	20	0.05	0.35	0.90	22	0	0.60	0.88
Scheuch8	8	0.05	0.20	0.83	8	0.05	0.25	0.81	7	0.10	0.06	0.76
Scheuch9	14	0.10	0.35	0.87	10	0.20	0.06	0.78	7	0.50	0	0.74
Scheuch10	6	0.10	0.06	0.73	8	0.10	0.15	0.76	6	0.05	0.05	0.71

Note: *A* = number of alleles; H_e = mean expected heterozygosity based on ATETRA simulations; HE_4 = frequency of observed heterozygous individuals with maximum number of alleles (i.e., four different allele peaks visible); HO_1 = frequency of observed homozygous individuals (i.e., with just one visible allele peak); N = number of individuals genotyped.

TABLE 3. Cross-amplification of 10 microsatellite loci from *Campanula scheuchzeri* in 10 individuals of two populations of *C. rotundifolia*.[a]

Locus	*A*	Amplicon length (bp)[b]	*A* (rot)	% rot[c]	HO_1	HE_2	HE_3	HE_4
Scheuch1*	7	123–139	—	—	—	2	7	1
Scheuch2*	6	143–162	—	—	2	7	—	1
Scheuch3	4	142–159	—	—	6	4	—	—
Scheuch4	5	93–126	—	—	2	6	2	—
Scheuch5*	3	144–153	—	—	3	5	2	—
Scheuch6	2	124–130	—	—	6	4	—	—
Scheuch7	8	133–182	133, 137, 141	59	3	4	1	2
Scheuch8*	3	127–140	131	5	—	9	1	—
Scheuch9	9	107–146	—	—	—	6	2	2
Scheuch10	8	95–130	118, 126, 130	15	—	4	6	—

Note: % rot = percentage of these alleles in the *C. rotundifolia* data set; *A* = total number of alleles; *A* (rot) = alleles (in bp) only found in the 10 *C. rotundifolia* individuals (i.e., not found in the 60 individuals of *C. scheuchzeri*); HE_2 = number of heterozygous individuals with two allelic peaks; HE_3 = number of heterozygous individuals with three allelic peaks; HE_4 = number of heterozygous individuals with maximum number of alleles (i.e., four different alleles visible); HO_1 = number of homozygous individuals (i.e., with just one visible allele peak).

* Some binning of alleles might be necessary (see Table 1).

[a] Note that no PCR dropout occurred, i.e., the cross sum adds up to 10 individuals.

[b] Amplicon length includes the M13 tail.

[c] Calculation was as follows: number of allele peaks of *A* (rot) in the electropherograms of *C. rotundifolia* was divided by the total number of allele peaks, e.g., the three "exclusive" alleles 133, 137, and 141 of locus Scheuch7 were found with 13 allelic peaks in 10 individuals. In total, the electropherograms of *C. rotundifolia* showed 22 peaks at locus Scheuch7, hence 13/22 = 59%.

for the high HO_1 values: (1) null alleles are present at these loci; (2) increased homozygosity is due to selection of an unknown, linked locus; (3) the observed homozygosity results from autonomous self-fertilization, a scenario that was found in tetraploid individuals of *C. rotundifolia* (Stevens et al., 2012); or (4) half-sib mating occurred in these populations. Explanations 3 and 4, however, would also have led to higher than the observed HO_1 values at other loci (cf. Table 2). Consequently, we tentatively support either the null-allele scenario or the possibility of selection.

In *C. rotundifolia*, we also found signals for tetraploidy (see HE_4 in Table 3), which is common in Europe (Böcher, 1936; Hess et al., 1980; Stevens et al., 2012). Notably, Frei (2007), who scored populations of *C. rotundifolia* from several locations in Switzerland using flow cytometry, observed only tetraploid populations, although some authors reported other ploidy levels (Hess et al., 1980). In the current study, different combinations of one, two, three, or four allelic signals were found in the 10 selected individuals (Table 3).

CONCLUSIONS

Newly developed microsatellite markers confirmed that our focal populations of *C. scheuchzeri* are tetraploid. All 10 primer pairs cross-amplified in specimens of the widespread sister-species *C. rotundifolia*. The allelic divergence of *C. scheuchzeri* and *C. rotundifolia* at locus Scheuch7 has several possible explanations at this time including genetic drift due to isolation in space and time between the two taxa. However, artificial cross-fertilization was successful in both species, making genetic isolation a weak argument for the observed allelic divergence (Stevens et al., 2012). A clear genetic delimitation of the two species is probably not possible, and at intermediate elevations assigning populations to species may prove difficult due to overlapping variability. Nevertheless, our working hypothesis is therefore that both nominal species evolved under vicariance.

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Appendix 1. Voucher and location information for populations of *Campanula scheuchzeri* and *C. rotundifolia* used in this study. The voucher specimens are deposited in the Botanical Institute of the University of Basel, Switzerland.

Species and population	Geographic coordinates	Altitude (m a.s.l.)	Voucher no.
C. scheuchzeri			
Fondei	46°51′2.73″N, 9°45′46.53″E	1950	Sch-Fo (1–20)
Niessen	46°38′34″N, 7°40′0″E	1680	Sch-N (1–20)
Furka	46°34′33.85″N, 8°25′18.71″E	2420	Sch-Fu (1–20)
C. rotundifolia			
Blauen	47°27′30.52″N, 7°31′52.48″E	620	Rot-Bl (1–5)
Bonaduz	46°48′49.11″N, 9°23′13.53″E	660	Rot-Bo (1–5)