Development of SSR Markers by 454 Sequencing in the Endemic Species Gentianella praecox subsp. bohemica (Gentianaceae)

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Primer Note

Development of SSR markers by 454 sequencing in the endemic species *Gentianella praecox* subsp. *bohemica* (Gentianaceae)[1]

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- *Premise of the study:* Polymorphic microsatellite loci were developed and used to genotype individuals of *Gentianella praecox* subsp. *bohemica* (Gentianaceae), a highly protected taxon in Europe, to study the genetic structure of the remaining populations.
- *Methods and Results:* Thirty-eight primer pairs were successfully amplified; of these, 12 polymorphic microsatellite loci were developed using a 454 sequencing approach and used to genotype 180 individuals of *G. praecox* subsp. *bohemica* from six populations. Allelic richness ranged between one and nine alleles per locus. We detected a high frequency of polyploid individuals (77.8%). The highest average percentage of heterozygous genotypes was identified for samples from the Hroby population (75.5%). All loci can also be amplified in the congeneric species *G. praecox* subsp. *praecox*, *G. amarella* subsp. *amarella*, and *G. obtusifolia* subsp. *sturmiana*.
- *Conclusions:* These markers will provide knowledge on patterns of gene flow and population genetic structure, which is necessary for current protection actions and for effective conservation of this species in the future.

Key words: genotyping; Gentianaceae; *Gentianella praecox* subsp. *bohemica*; microsatellites; polyploidy.

Gentianella praecox (A. Kern. & Jos. Kern.) Dostál ex E. Mayer subsp. *bohemica* (Skalický) Holub (IUCN: e.T161825A5500524) is a strictly biennial herb endemic to the Bohemian Massif, with most populations occurring in the Czech Republic but extending to Bavaria (Germany), Upper and Lower Austria, and Poland. *Gentianella* Moench (Gentianaceae) is a highly diverse and taxonomically complicated genus due to seasonal dimorphism, introgression, and hybridization between closely related species (Winfield et al., 2003; Greimler and Jang, 2007; Plenk et al., 2016). It is expected that *G. praecox* subsp. *bohemica* is tetraploid (Oberdorfer, 1983), but cytotype distribution is unknown. It occurs in seminatural, nutrient-poor grasslands. Strong reduction of population size was recorded during the 20th century, probably due to land-use intensification or abandonment of traditional land use, which led to the disintegration of large habitats and fragmentation of original populations. *Gentianella praecox* subsp. *bohemica* is highly protected in Europe (Annexes II and IV of the Habitats Directive; Council of the European Community, 1992). By using amplified fragment length polymorphism, Königer et al. (2012) studied the genetic structure of 11 *G. praecox* subsp. *bohemica* populations, but this taxon is known from 99 localities (Brabec, 2010). For effective protection of this subspecies, it is necessary to identify populations with high genetic diversity so these populations can be prioritized for protection. Moreover, knowledge about the genetic structure of all remaining populations will reveal patterns of gene flow among populations and the potential for inbreeding depression.

METHODS AND RESULTS

Microsatellite development—Total genomic DNA of 14 individuals (two individuals per population collected across the whole distribution range) of *G. praecox* subsp. *bohemica* was extracted from dehydrated leaves using the cetyltrimethylammonium bromide (CTAB) method of Lodhi et al. (1994), with all amounts downscaled 10×. The sequencing facility GenoScreen (Lille, France) was used to prepare libraries and design primers. Extracted DNA was pooled for microsatellite library preparation. The fragmented DNA was hybridized with eight probes (TG, TC, AAC, AAG, AGG, ACG, ACAT, and ACTC) to enrich the DNA library. Sequencing was performed using a GS FLX sequencer (Roche, 454 Life Sciences, Branford, Connecticut, USA). A total of 19,152 reads were obtained. Raw sequencing data were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession no. SRR5113067). QDD software (Meglécz et al., 2009) with default settings was used to identify microsatellite loci and for design of the microsatellite primers. A total of 3017 reads contained microsatellite motifs, and 373 candidate microsatellite loci were identified (Appendix S1), with an average sequence length of 325 bp. Markers belonged to di-, tri-, tetra-, penta-, and hexanucleotide

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repeats (40.2%, 52.8%, 5.4%, 0.8%, and 0.8%, respectively). Across all candidate loci, 3378 primer pairs (3–15 primer pairs per locus) were designed using Primer3, as implemented within QDD (Malausa et al., 2011) with amplicon lengths ranging between 90 and 319 bp. For each microsatellite candidate locus, one primer pair was selected for further analysis. Of these, we selected 50 primer pairs (Appendix S1) recommended by GenoScreen to identify polymorphic markers. Primers were synthesized (Sigma-Aldrich, St. Louis, Missouri, USA) with M13 tails preceding the 5′ end of the forward primer sequences (Schuelke, 2000). Six individuals from six populations of *G. praecox* subsp. *bohemica* (Appendix 1) were used to test amplification efficiency and polymorphism. DNA amplification was performed in 10-μL reactions consisting of 5 μL of QIAGEN Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.25 μL of each M13-labeled forward, reverse, and fluorolabeled (5′-FAM) M13 primer (10 μM each in initial volume), 20 ng of DNA dissolved in 1 μL TE buffer, and 3.25 μL of H_2O.

The following PCR protocol was performed using an Eppendorf Mastercycler pro S Thermal Cycler (Eppendorf, Hamburg, Germany): an initial denaturation step at 95°C for 15 min; followed by 25 cycles of denaturation (95°C for 20 s), annealing (59°C for 30 s), and extension (72°C for 20 s); followed by 10 cycles of denaturation (95°C for 30 s), annealing (53°C for 45 s), and extension (72°C for 45 s); and a final extension at 72°C for 10 min. Thirty-eight primer pairs (76%) were successfully amplified. Due to allele dosage uncertainty in polyploid individuals, preliminary statistics included determination of polymorphic information content (PIC) for each locus by PICcalc (Nagy et al., 2012). Based on PIC, 20 (53%) of the 38 primer pairs were selected for detailed variability screening on 36 individuals of *G. praecox* subsp. *bohemica* (two individuals from each population). Based on the multiplex PCR performance and variability screening, 12 polymorphic primer pairs were identified.

To confirm primer specificity for these 12 loci, we ran PCRs for each primer pair separately under the same conditions described in the next paragraph. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) and cloned using pGEM-T Vector Systems II (Promega Corporation, Madison, Wisconsin, USA) in accordance with the manufacturer's instructions, but downscaled to half reactions. Approximately 10 colonies per sample were transferred into 20 μL of ddH_20 and denatured at 95°C for 10 min. These served as templates for subsequent PCR amplifications for sequencing. Sequencing was performed by the commercial company SEQme (Dobříš, Czech Republic), and the resulting sequences were aligned using MAFFT 7.017 (Katoh et al., 2002) as implemented in Geneious 8.1.6 (Kearse et al., 2012). Repeat motifs with variation in number of repeats were confirmed in the obtained sequences. GenBank accession numbers of identified sequences for 12 loci of *G. praecox* subsp. *bohemica* are provided in Table 1.

Genotyping—Total DNA was extracted from 180 *G. praecox* subsp. *bohemica* individuals from six populations and from 114 individuals from eight populations of three closely related taxa (Appendix 1) for initial primer screening. DNA amplification was carried out in three multiplex reactions consisting of 2.5 μL of QIAGEN Multiplex PCR Master Mix and 10 ng of DNA dissolved in 0.5 μL of TE buffer. For multiplex mix I (MM I), the PCR contained 1.1 μL of primer mix (10 μM each in initial volume) and 0.9 μL of H_2O, for MM II the PCR consisted of 1.1 μL of primer mix (10 μM each in initial volume) and 0.9 μL of H_2O, and for MM III the PCR contained 0.7 μL of primer mix (10 μM each in initial volume) and 1.3 μL of H_2O. The sequence, labeling, motif information, final volumes, and PCR product size range are given in Table 1. The following PCR protocol was performed using an Eppendorf Mastercycler pro S Thermal Cycler: an initial denaturation step at 95°C for 15 min; followed by 35 cycles of denaturation (95°C for 20 s), annealing (59°C for 30 s), and extension (72°C for 20 s); and a final extension at 72°C for 10 min. PCR products were diluted with ddH_2O in these ratios: 1 : 2 (PCR product of MM I and MM II PCRs : ddH_2O), 1 : 9 (PCR product of MM III PCR : ddH_2O). Each PCR product (1 μL) was mixed with 11 μL of a 120 : 1 solution of formamide : size standard (GeneScan 500 LIZ; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Fragment lengths were determined by capillary gel electrophoresis with an ABI 3130 Genetic Analyzer using GeneMapper 4.0 (Thermo Fisher Scientific). Using SPAGeDi (Hardy and Vekemans, 2002), we calculated the number of alleles per locus, which ranged between one and nine (Table 2). All markers were polymorphic in all *G. praecox* subsp. *bohemica* populations, except marker GbM48, which was monomorphic in the Zidkovi population. The highest average percentage of heterozygous genotypes was identified for individuals from the Hroby population (75.5%) and the lowest percentage for individuals from the Zidkovi population (50.5%). We detected a high frequency of polyploid individuals (77.8%). The observed heterozygote excess is likely caused by the fact that the species is tetraploid.

We also tested cross-amplification of these loci in three other *Gentianella* taxa: *G. praecox* subsp. *praecox*, *G. amarella* (L.) Börner subsp. *amarella*, and *G. obtusifolia* (F. W. Schmidt) Holub subsp. *sturmiana* (A. Kern. & Jos. Kern.) Holub. We tested 114 individuals from eight populations (Appendix 1). DNA amplification was carried out in three multiplex reactions as described above. Tests for cross-amplification in the three congeneric taxa resulted in successful amplification of up to seven of the 12 polymorphic loci (Table 2). These results (Table 3) demonstrate that these primer pairs may be of broad utility throughout *Gentianella*.

Table 1. Characteristics of 12 polymorphic loci designed for genotyping of *Gentianella praecox* subsp. *bohemica*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	PCR product size range (bp)	Fluorescent label	Volume of forward primer (μL)	Multiplex	GenBank accession no.
GbM46	F: CAACCACAAGAAGCTTCCAA R: GCATTGCCAACAGATGCAG	$(CTT)_4$	81–129	PET	0.1	I	KX420610
GbM11	F: TGGTTTGATTTCAGACCCTTG R: CAGGTTGCCCTACCAAGATG	$(TTG)_{16}$	138–180	PET	0.25	I	KX420608
GbM34	F: GAAGCGTCCGTTTCAGTTTC R: GCTTAGAGCCCAAGATACCTAGA	$(TGT)_5$	119–152	NED	0.075	I	KX420611
GbM3	F: AGTTGAGAATTGGCCTGGAG R: GATGCATTGGAAGCAGGATT	$(GTAT)_5$	134–174	VIC	0.125	I	KX420606
GbM12	F: ATCAGGCATTGCCATTAAGC R: GAGATTCATAGGTGGCGAGG	$(AC)_5$	96–108	VIC	0.15	II	KX420604
GbM19	F: GGAATTCCTTGTGAAGCCAG R: TTGCTGCTTCTTTTCCATGA	$(GAG)_8$	136–202	VIC	0.225	II	KX420609
GbM38	F: TTTCAAGGTTGCTTTTGGCT R: GCCTTGTGTTAAATTAGTTGCAG	$(AGA)_6$	129–162	NED	0.075	II	KX420612
GbM5	F: CTCCTTCCCTTTTCCCAAAC R: GCTTATGTCGCAGTGCAGAA	$(AG)_8$	158–180	PET	0.1	II	KX420615
GbM2	F: GGGAGAAGCGAGTTCAAAAG R: AAGCTGCTAAACTTCAATACTTGC	$(GGA)_{13}$	147–180	VIC	0.075	III	KX420607
GbM48	F: ACCGAAGGCAGTTTCAACAC R: CCAACAAACTTAGCTACCTTAGCA	$(GGA)_3$	84–93	NED	0.175	III	KX420613
GbM39	F: AACAGAGCAAAAACAAAAACAGG R: CAAGAAAGCAATGAATCCCC	$(AGA)_8$	79–94	VIC	0.05	III	KX420614
GbM43	F: AATCATGTCCAGCTCAGCCT R: GCCGACGTAGAATGTTTGGT	$(CCT)_4$	158–185	NED	0.05	III	KX420605

[a] Optimal annealing temperature was 59°C for all loci.

Table 2. Results of initial primer screening of 12 microsatellite loci developed in *Gentianella praecox* subsp. *bohemica* and congeners.

Species	Population code[a]	Primer/Indexes[b]	GbM46	GbM11	GbM34	GbM3	GbM12	GbM19	GbM38	GbM5	GbM2	GbM48	GbM39	GBm43
G. praecox subsp. *bohemica*	HROBY	A	3	6	4	7	5	9	4	4	4	4	5	6
		A_{ind}	2.3	2.3	1.43	1.73	2.7	2.8	1.63	2.53	1.43	1.77	1.5	2.6
		% Het	96.7	86.7	40	73.3	100	96.7	60	96.7	43.3	63.3	50	100
	PODVORI	A	6	4	4	3	5	5	4	4	5	2	3	5
		A_{ind}	2.3	1.9	1.43	1.47	1.7	1.9	1.27	2.57	1.33	1.2	1.3	2.57
		% Het	86.7	86.7	36.7	46.7	56.7	70	26.7	100	33.3	20	30	100
	POLNA	A	4	5	4	4	4	5	4	4	6	3	2	5
		A_{ind}	1.43	2.07	1.83	1.5	2.17	2	1.43	2.47	1.5	1.3	1.13	2.43
		% Het	40	80	73.3	50	83.3	80	43.3	100	50	30	13.3	100
	VYSNY	A	7	3	2	3	5	6	3	5	3	3	3	4
		A_{ind}	1.73	1.73	1.5	1.13	2.13	2.63	1.53	2.6	1.5	1.27	1.5	2.23
		% Het	73.3	73.3	50	13.3	83.3	100	53.3	100	50	26.7	50	100
	ZIDKOVI	A	5	2	4	6	2	4	2	3	3	1	2	3
		A_{ind}	2.7	1.77	1.1	1.37	1.53	2.3	1.03	2.03	1.13	1	1.17	2.1
		% Het	100	76.7	10	36.7	53.3	100	3.3	100	13.3	0	16.7	96.7
	VANIC	A	9	4	3	3	4	5	4	4	3	3	3	4
		A_{ind}	2.8	2.27	1.03	1.53	1.63	2.77	1.67	2.47	1.43	1.53	1.57	2.53
		% Het	96.7	100	6.7	53.3	60	96.7	66.7	100	43.3	46.7	56.7	100
G. amarella subsp. *amarella*	VANIC	A	4	3	2	3	4	4	4	3	4	2	3	7
		A_{ind}	1.63	1.67	1.17	1.33	2.17	2.17	1.17	2.17	2	1.83	1.17	2.83
		% Het	60	50	16.7	33.3	100	100	16.7	100	83.3	83.3	16.7	100
	ČER.S.	A	4	1	1	1	2	2	1	2	2	2	1	3
		A_{ind}	2.53	1	1	1	2	2	1	2	2	2	1	3
		% Het	96.7	0	0	0	100	100	0	100	100	100	0	100
G. obtusifolia subsp. *sturmiana*	PP PILA	A	4	5	4	6	2	7	4	2	2	3	3	4
		A_{ind}	1.43	2.3	1.6	1.5	1.7	2.6	1.6	1.2	1.1	1.4	1.4	2.6
		% Het	43.3	90	60	50	70	90	60	20	10	40	40	100
	KOCEL	A	4	4	3	3	4	7	4	3	1	1	2	4
		A_{ind}	1.77	2.5	1.7	1.6	2.3	2.6	1.8	1.8	1	1	1.1	2.8
		% Het	63.3	100	70	60	100	100	80	70	0	0	10	100
	RANK	A	5	3	1	2	3	2	1	2	1	1	1	2
		A_{ind}	1.5	1.1	1	1.3	2.3	2	1	2	1	1	1	2
		% Het	50	10	0	30	100	100	0	100	0	0	0	100
G. praecox subsp. *praecox*	BUBE	A	6	2	3	4	4	3	2	2	4	5	3	2
		A_{ind}	2.6	1.71	1.21	1.29	1.54	1.71	1.21	2.29	1.25	1.21	1.17	2.46
		% Het	100	62.5	20.8	29.2	45.8	66.7	20.8	100	25	20.8	16.7	100
	GIEE	A	1	1	2	2	2	1	1	2	3	3	3	2
		A_{ind}	1.8	1.25	1	1	1.05	1	1.4	2	1	1.25	1.15	2.3
		% Het	75	25	0	0	5	0	40	20	0	25	15	20
	LEOE	A	2	2	2	1	2	1	1	2	3	4	2	2
		A_{ind}	1.79	1	1	1.13	1.83	1	1.04	2	1.13	1.04	1.08	2.13
		% Het	79.2	0	0	12.5	83.3	0	4.2	100	12.5	4.2	8.3	100

Note: A = number of alleles; A_{ind} = mean number of alleles per individual; % Het = percentage of heterozygous genotypes.
[a] Population code refers to collecting locality. Detailed information is provided in Appendix 1.
[b] Due to allele ambiguity in polyploids, the $H_o:H_e$ ratio is replaced by the percentage of heterozygous genotypes.

Table 3. Allele size ranges obtained during cross-amplification trials of microsatellite loci isolated from *Gentianella praecox* subsp. *bohemica* and tested in three additional taxa.

Locus	*G. amarella* subsp. *amarella*	*G. praecox* subsp. *praecox*	*G. obtusifolia* subsp. *sturmiana*
GbM46	111–126	114–117	114–126
GbM11	159–168	150–165	138–168
GbM34	131–134	128–131	119–131
GbM3	142–170	134–158	134–170
GbM12	100–102	100–106	100–108
GbM19	142–154	142–157	142–178
GbM38	137–143	137–146	134–153
GbM5	168–176	168–176	168–178
GbM2	150–177	150–156	147–153
GbM48	84–87	87–90	84–90
GbM39	85–91	82–91	79–85
GbM43	167–179	164–176	167–176

CONCLUSIONS

We developed and successfully multiplexed 12 polymorphic markers in several taxa of *Gentianella*. These polymorphic loci will be valuable for the future management of the extremely rare *G. praecox* subsp. *bohemica*.

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Appendix 1. Accession information for *Gentianella* species used in this study.[a]

Species name	Population code	Collection locality	Country	*n*	Latitude	Longitude
G. praecox (A. Kern. & Jos. Kern.) Dostál ex E. Mayer subsp. *bohemica* (Skalický) Holub	HROBY	Hroby	CZ	30	49.3932222	14.85622
G. praecox subsp. *bohemica*	PODVORI	Podvoří	CZ	30	48.8356111	14.20819
G. praecox subsp. *bohemica*	POLNA	Polná in the Šumava Mountains	CZ	30	48.7928056	14.14997
G. praecox subsp. *bohemica*	VYSNY	Vyšný	CZ	30	48.8266944	14.30211
G. praecox subsp. *bohemica*	ZIDKOVI	Olešnice in the Orlické Mountains	CZ	30	50.3619444	16.28389
G. praecox subsp. *bohemica*	VANIC	Nature Reserve Opolenec	CZ	30	49.0866667	13.79706
G. amarella (L.) Börner subsp. *amarella*	VANIC	Nature Reserve Opolenec	CZ	6	49.0866667	13.79706
G. amarella subsp. *amarella*	ČER.S.	Kouty nad Desnou	CZ	10	50.1225	17.16111
G. obtusifolia (F. W. Schmidt) Holub subsp. *sturmiana* (A. Kern. & Jos. Kern.) Holub	PP PILA	Pila u Karlových Varů	CZ	10	50.1747222	12.92694
G. obtusifolia subsp. *sturmiana*	KOCEL	Kocelovice	CZ	10	49.475	13.82444
G. obtusifolia subsp. *sturmiana*	RANK	Rankovice	CZ	10	50.0067778	12.84208
G. praecox subsp. *praecox*	BUBE	Buchberg, Lower Austria	AU	24	48.376944	15.39722
G. praecox subsp. *praecox*	GIEE	Gießhübl, Lower Austria	AU	20	48.320833	15.36306
G. praecox subsp. *praecox*	LEOE	Leopolds, Lower Austria	AU	24	48.427778	15.28611

Note: AU = Austria; CZ = Czech Republic; *n* = number of individuals.

[a]Because all investigated species are rare and highly protected, it was not possible to sample whole plants for herbarium vouchers. Leaf samples were collected in the field for up to five individuals per population and were dried in silica gel before performing DNA extraction. The leaf samples and DNA extracts were deposited at the Institute of Botany of the Czech Academy of Sciences, Průhonice, Czech Republic.