PRIMER NOTE

Identification of microsatellite loci in *Collinsia verna* (Veronicaceae)

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Abstract

We developed eight polymorphic microsatellite loci for *Collinsia verna* (Veronicaceae). In a sample of 18–35 individuals from a single population, we found two to 15 alleles per locus (mean 8.3). We also tested these loci for cross-amplification in all 22 species in the tribe Collinseae. Overall, more than half the species in the tribe amplified one microsatellite while three species most closely related to *C. verna* (*Collinsia violacea, Collinsia parviflora* and *Collinsia grandiflora*) amplified multiple microsatellite loci. These microsatellite loci will be used in future studies of mating system in this tribe and other quantitative genetic and population genetic studies.

Keywords: Veronicaceae, Scrophulariaceae, *Collinsia*, *Tonella*, population genetics, microsatellite

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Introduction

The genus *Collinsia* (Veronicaceae) includes 20 self-compatible winter or spring annual species native to North America. These species express a dramatic range of floral size, shape and colour (Neese 1993), and occupy a wide array of habitats from serpentine soils, costal sand dunes and flood plain forests. Interest in *Collinsia* began with the work of Garber (1956) and collaborators who for the next 40 years investigated the cytogenetics of interspecific hybrids. Recently, a wealth of evolutionary and ecological studies with this genus have investigated within and among species variation in floral size and development (Kalisz *et al*. 1999; Armbruster *et al*. 2002; Elle & Carney 2003; Parachnowitsch & Elle 2004) pollination biology (Kalisz *et al*. 1999, 2004; Kalisz & Vogler 2003) inbreeding depression (Kalisz 1989, 2004; Mayer *et al*. 1996), outcrossing rates (Weil & Allard 1964; Kalisz *et al*. 2004), selection, population differentiation and local adaptation (Kalisz 1986 1991; Wright *et al*. 2006) and quantitative genetics and maternal effects (Charlesworth & Mayer 1995; Thiede 1998). In addition, a well-resolved phylogenetic tree for the tribe Collinseae was recently published (Armbruster *et al*. 2002). This phylogeny shows that the tribe is monophyletic with *Collinsia* and *Tonella* as sister taxa. Microsatellite markers would be extremely useful for advancing future studies with *Collinsia* and the phylogeny provides excellent new opportunities for further exploration of evolutionary and population genetic processes in this group. Here we describe the isolation and characterization of microsatellite markers for *Collinsia verna* Nutt. and their cross-amplification in all other species in the tribe Collinseae.

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To develop primers to amplify microsatellite markers, genomic DNA was extracted from wild collected, frozen tissue samples of *Collinsia verna* using the Gentra PUREGENE Extraction Kit. GA-, AAG-, AAT- and AAAT-enriched libraries were created by Genetic Identification Services (GIS) following a protocol previously described in Jones *et al*. (2002). The genome was partially digested using several blunt-end cutting enzymes (*Rsa*I, *Hae*III, *Bsr* B1, *Pvu*II, *Stu*I, *Sca*I, *Eco*RV). Fragments were subjected to magnetic bead capture, using biotinylated capture molecules biotin-AAC_{12}, biotin-CAG_{10}, biotin-$CATC_8$ and biotin-$TAGA_8$ (CPG, Inc.). Captured molecules were restricted with *Hin*dIII, and fragments were ligated into the *Hin*dIII site of the pUC19 plasmid. Recombinant plasmids were transformed into *Escherichia coli* strain DH5α, and clones were selected by incubation on X-gal/IPTG/ampicillin plates. One hundred clones were directly sequenced and approximately half (48%) of them

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Table 1 Microsatellite loci isolated from *Collinsia verna*. The GenBank Accession no., repeat motif of the sequenced clone, the range of allele sizes, fluorescent label, annealing temperature (T_a) and $MgCl_2$ concentration used in PCR, the number of samples genotyped (*N*), the number of alleles identified (N_A), expected heterozygosity (H_E) and the observed heterozygosity (H_O). Starred H_O values represent significant heterozygote deficiency

Locus	GenBank Accession no.	Primer sequences (5′–3′)	Repeat motif	Size range	Fluorescent label	T_a (°C)	$MgCl_2$ (mM)	N	N_A	H_E	H_O
CoveA107	DQ518248	F: TTTTTGCCTCTGGTATTTTTGC R: AAAAAGGAGTTAATGTTGAAACGTG	(CT)x17	196–221	6-FAM	56	2.0	17	8	13.5	3*
CoveA119	DQ518250	F: CTATTAGGCCCCAAAGATGATG R: CAAAACAAGGTCACACATGAGG	(CT)x31	207–262	6-FAM	62	1.5	24	16	20.8	9*
CoveA125	DQ518251	F: TTCTAGGTCCGAACAAAAGCTG R: CCCCCTTAGAATTTCCAGTTAG	(TC)x30	171–220	VIC	55	2.0	29	10	24.5	10*
CoveA134	DQ518252	F: GAGCAGATGGACGAACAGTAAG R: CGTCGATCAAGTTGCAATACTC	(CT)x23	154–178	VIC	57	1.5	35	8	27.7	29
CoveB105	DQ518253	F: ACAGCACCACATGAACAAAAAC R: TACCTACCCTCGAAAGATGATG	(CTT)x8	295–298	PET	56	1.5	25	2	12.7	7
CoveB116	DQ518254	F: GGCTGTTGAAGATGATTAGG R: TTAGGACTGAGCCAACACAATC	(AGA)x8	162–201	PET	59	1.5	32	8	9.2	8
CoveB2	DQ518249	F: TAACAAAATGAAATGGGACTGC R: TATAGAGCACATACAACCGC	(AGA)x18	156–183	NED	52	1.5	23	7	17.1	4*
CoveC8	DQ518255	F: TCGTTGTTCTTTTCGATTCTTG R: CTAGCCGTAGGTTCCAAATCC	(ATT)x10	189–213	NED	60.5	2.0	25	8	15.3	10*

contained microsatellites. Of these 48 sequences, 24 had flanking regions that were appropriate for primer design. We designed 24 unique primer pairs using PRIMER 3 software (Rozen & Skaletsky 1998) and AMPLIFY 3.1 (Engels 2005).

Primer pairs were screened for amplification using polymerase chain reaction (PCR) and agarose gel electrophoresis to identify primers that produced one or two bands using DNA from a single *C. verna* individual. Eleven of the tested primer pairs met these criteria. PCR conditions were optimized using two to four DNA samples extracted from individual *C. verna* plants. PCRs contained 15 μL each of 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 1.5–2.0 mM $MgCl_2$ (see Table 1), 0.25 mM of each dNTP, 0.13 μM–0.4 μM of each primer (IDT DNA), and 1 U of *Taq* DNA polymerase (Invitrogen). In general, thermocycler conditions consisted of an initial 4 min denaturation at 94 °C followed by 25 cycles of denaturing for 1 min at 94 °C, annealing for 1 min at primer specific annealing temperatures (T_a) and extension for 2 min at 72 °C, and a final extension period of 7 min at 72 °C. We optimized the concentration of primer, Tris-HCl, MgCl and the annealing temperature for each primer (Table 1). PCR products were visualized on a 3% agarose gel containing 0.0005% ethidium bromide. For each of the 11 primer pairs, we fluorescently tagged each of the forward primers with either 6-FAM, NED, PET, or VIC labels (Applied Biosystems).

We tested the amplification of these microsatellite loci on 35 individuals from a single wild population of *C. verna* located along Wheeling Creek in Greene County, Pennsylvania (39°53.80′N, 80°29.12′W). We used fluorescently tagged primers and the PCR conditions described above (Table 1). The fluorescent-tagged PCR products were sized using LIZ 500 size standard (Applied Biosystems) on an ABI PRISM 3730 DNA Analyser. Alleles for each microsatellite locus and individual were called using GENEMAPPER 4.0 software (Applied Biosystems). Of these 11 primer pairs, eight loci yielded genotype data that could be scored.

All markers were highly polymorphic in the Wheeling Creek population, except for CoveB105 (Table 1). The number of alleles per locus ranged from two to 15 (mean 8.3). An additional 96 samples from 12 other *C. verna* populations (7 PA, 2 MI, 2 MO, 1 IL) were genotyped for the CoveB105 marker to estimate its polymorphism across populations. We found a total of nine alleles for CoveB105 locus across all populations tested.

We tested for linkage disequilibrium using FSTAT version 2.9.3.2 (Goudet 2002), and found all loci to be unlinked after applying the Bonferroni correction. We calculated H_E, the expected number of heterozygotes, and H_O, the observed number of heterozygotes using GENEPOP version 3.4. Five loci exhibit significant heterozygote deficiency (Table 1) that is common in mixed mating species (Nassar 2001) such as *C. verna* (Kalisz *et al*. 2004). Heterozygote deficiency could also be the result of small sample size, the presence of null alleles, or population subdivision.

We tested the cross-species amplification success of these microsatellite loci in the 22 other species from tribe Collinseae (19 other species of *Collinsia*, 2 species of

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Table 2 Cross-species amplification and allele sizes of *Collinsia verna* microsatellite primers in the tribe Collinseae*. Two individuals were tested for each of the 22 species of *Collinsia* and one individual in the two *Tonella* species. PCR products resolved on agarose gels indicated +, strong amplification; +/–, weak amplification; or –, no amplification. Loci with + and +/– amplification were genotyped for seven to 42 individuals. Genotypes were obtained only from the + amplifying loci

	Microsatellite locus amplification (allele sizes)			
Species	CoveA107	CoveA134	CoveB105	CoveB116
C. antonina	–	–	+/–	–
C. bartsiifolia	–	–	+/–	–
C. corymbosa	–	–	+/–	–
C. concolor	–	–	+/–	–
C. grandiflora	–	+ (158–160)	+ (280)	–
C. linearis	–	–	+/–	–
C. multicolor	–	–	+/–	–
C. parviflora	–	+ (154–160)	+ (274–280)	–
C. parryi	–	–	+/–	–
C. sparsiflora vs. *arvensis*	–	–	+/–	+/–
C. sparsiflora vs. *collinsa*	–	–	+/–	+/–
C. violacea	+ [187–211]	+ (154)	+ (302)	+/–

**All species in the tribe* Collinseae *were tested*: *Collinsia greenei*, *C. heterophylla*, *C. sparsiflora* vs. *arvensis*, *C. sparsiflora* vs. *collina*, *C. sparsiflora* vs. *sparsiflora*, *C. bartsiifolia*, *C. corymbosa*, *C. tinctoria*, *C. multicolor*, *C. parryi*, *C. concolor*, *C. antonina*, *C. callosa*, *C. childii*, *C. parviflora*, *C. grandiflora*, *C. violacea*, *C. linearis*, *C. rattani*, *C. torreyi* vs. *wrightii*, *C. torreyi* vs. *torreyi*, *Tonella tenella*, *Tonella floribunda*.

Tonella) to determine if they were conserved across the tribe. We assayed two individuals grown from wild-collected seed per *Collinsia* species and one individual of each *Tonella* species. The number of microsatellite loci that amplified varied among species. Four of the primer pairs amplified only in *C. verna;* eight species of *Collinsia* and both species of *Tonella* failed to amplify any loci. Cross-amplification was seen in 13 species for at least one locus, but with variable intensity (Table 2). Species with weak amplification exhibited bands in the correct size region on an agarose gel, but when we attempted to genotype these samples, we were not able to do so. For these cases, re-designed primers will likely improve amplification. Multiple loci amplified in *Collinsia violacea,* the sister species to *C. verna* and in both members of the sister taxa pair closest to *C. verna (Collinsia parviflora*, *Collinsia grandiflora).* We genotyped seven to 42 individuals for each microsatellite locus for these three species to verify that these PCR products were actually orthologous to the *C. verna* microsatellite loci. The PCR products were orthologous — genotypes obtained were consistent in sizes and nucleotide repeats to the loci in *C. verna* and revealed one to five alleles per locus per species. In one instance (CoveB105 in *C. violacea*), only two individuals amplified. We interpret this as likely the result of a high frequency of null alleles for this locus in *C. violacea*.

Our results indicate the presence of conserved microsatellite loci in closely related species of *Collinsia*. Together, these markers will be useful for future investigations of mating system evolution, population-level processes and mapping studies. In addition, other laboratories studying species related to *C. verna* will benefit from the microsatellite loci we have identified.

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