New Microsatellite Markers for Anthyllis vulneraria (Fabaceae), Analyzed with Spreadex Gel Electrophoresis

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PRIMER NOTE

NEW MICROSATELLITE MARKERS FOR *ANTHYLLIS VULNERARIA* (FABACEAE), ANALYZED WITH SPREADEX GEL ELECTROPHORESIS[1]

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- *Premise of the study:* New microsatellite primers were developed for the diploid herb *Anthyllis vulneraria*. These primers will be used in upcoming studies focusing on random genetic variation, local adaptation, and phenotypic plasticity in alpine plants.
- *Methods and Results:* The new primers were adjusted to separate PCR amplicons (70 to 170 bp) on precast Spreadex gels using horizontal gel electrophoresis. No capillary sequencer was needed. Three to twelve alleles were found per locus depending on the population studied.
- *Conclusions:* Our preliminary results showed that the three studied alpine populations are predominantly outcrossing, but include variable levels of self-fertilization.

Key words: alpine plants; *Anthyllis vulneraria*; ethidium bromide; Fabaceae; horizontal electrophoresis; microsatellites.

Alpine environments are considered to be particularly heterogeneous. Two fundamental survival strategies for heterogeneous environments can be contrasted: local adaptation or specialization vs. phenotypic plasticity, a generalist strategy. A major hypothesis suggests that phenotypic plasticity is favored over local adaptation when the spatial scale of dispersal spans several environmental states (Sultan and Spencer, 2002). Reciprocal transplantation experiments (RTE) are suitable to study both local adaptation and the reaction norm of plant phenotypes at different transplantation sites (Kawecki and Ebert, 2004). In the near future, we will apply RTE using populations from two spatial scales (representing fine-grained vs. coarse-grained environmental variation) of four alpine species including *Anthyllis vulneraria* L. (Fabaceae). The degree of neutral genetic differentiation will be estimated using microsatellites and will be compared to phenotypic differentiation (e.g., F_{ST}–Q_{ST} analysis).

METHODS AND RESULTS

In our laboratory, we used Spreadex gels and the ORIGINS electrophoresis unit (Elchrom Scientific AG, Cham, Switzerland) for microsatellite analyses. Spreadex gels resolve PCR amplicons with size differences of 2 bp in an electrophoresis time of 1 to 2 h. Amplicons should not be longer than ca. 170 bp. In nearly all cases, heterozygous character states show a 'third' top band in the gel (i.e., a heteroduplex) because the gels consist of a nondenaturing matrix (see Fig. 1, and Armbruster et al., 2005). Homozygous individuals show a single prominent PCR band. For *A. vulneraria*, we checked the five microsatellite loci AV2, AV3, AV7, AV12, and AV23 and the respective primers described by van Glabeke et al. (2007). These loci promised to be suitable for Spreadex electrophoresis because the amplicons are between 60 and 170 bp. Despite the infraspecific taxonomic uncertainties of *A. vulneraria* (Nanni et al., 2004), the above microsatellites finally proved to be useful for our populations from the Swiss Alps (data not shown). However, to study spatial genetic variation with greater power we needed additional polymorphic microsatellite sequences from the genome of *A. vulneraria*. The development of 10 additional microsatellite primer pairs was outsourced to Ecogenics GmbH (Schlieren, Zurich, Switzerland; see Matter et al., 2012).

Ecogenics started with leaf material of *A. vulneraria* from the alpine region of Davos, Switzerland. Size-selected fragments from genomic DNA were enriched for simple sequence repeats (SSRs) by using streptavidin-coated magnetic beads and biotin-labeled CT and GT repeat oligonucleotides. The SSR-enriched library was analyzed on a Roche 454 platform (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) using the GS FLX titanium reagents (Microsynth AG, Balgach, Switzerland). A total of 23,720 reads resulted, with an average length of 188 bp. Of these, 574 contained a microsatellite insert with a tetra- or trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. One prerequisite was that the newly developed amplicons should be in the size range from 70 to 170 bp (see above). Suitable primer design was possible in 120 reads. Subsequently, 10 loci (Table 1) provided allelic polymorphisms in 15 individuals (using a 48-capillary ABI 3730 sequencer [Applied Biosystems, Carlsbad, California, USA]; data not shown). Ecogenics used M13-tailing at the 5′ end of each forward primer for PCR. Hence, PCR conditions of Ecogenics were different from our protocol in the running phase (below). The 10-µL PCR mix of Ecogenics consisted of 1 µL of PCR stock buffer (QIAGEN, Hilden, Germany) with 15 mM $MgCl_2$, 200 µM dNTPs, 0.04 µM forward primer (with M13-tail), 0.16 µM reverse primer, 0.16 µM M13 primer (5′-TGTAAAACGACGGCCAGT-3′, labeled with a fluorescent dye for multiplexing), 0.5 U HotstarTaq polymerase (QIAGEN), and 10 ng DNA. Cycling conditions were: denaturation at 95°C for 15 min; start PCR at 95°C for 30 s, 56°C for 45 s, and 72°C for 45 s in 30 cycles; continued with 95°C for 30 s, 53°C for 45 s, and 72°C for 45 s in eight cycles. Termination was set to 72°C for 30 min.

In the running phase, we checked the 10 loci with Spreadex electrophoresis. Three distinct populations of *A. vulneraria* that were geographically close to Davos, Switzerland, were selected (each with $N = 20$): Schiahorn

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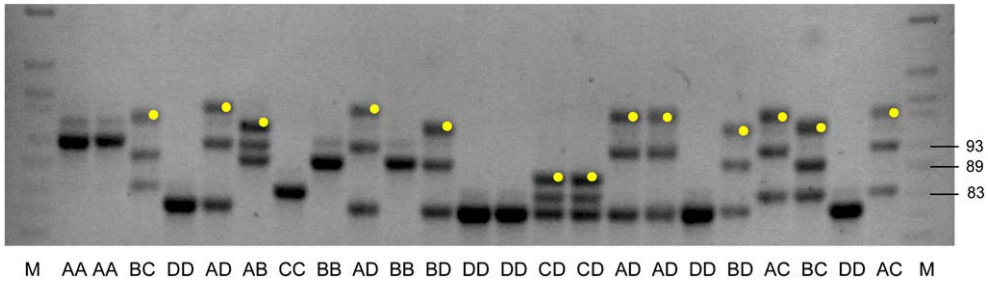

Fig. 1. Spreadex EL 400 gel with electrophoretic resolution of 8 to 9 µL of microsatellite amplicons at locus AV-005692. Fingerprints of 23 diploid individuals of *Anthyllis vulneraria* are shown. M = 7 µL of M3 marker (Elchrom Scientific; see bp at right margin). Genotypes are labeled in capital letters. Alleles (A, B, C, D) are coded by size (A = 79 bp, B = 83 bp, C = 89 bp, D = 93 bp). Note that heterozygous individuals show a prominent heterduplex signal (yellow dots).

(46°48′59.64″N, 9°48′16.80″E), Monstein (46°41′16.92″N, 9°47′15.84″E), and Casanna (46°51′26.88″N, 9°49′37.74″E). Voucher specimens and seeds (sampled by H.K.) are stored in the collection of the University of Basel, section of Population Biology of Plants. DNA was extracted with the DNeasy Plant Mini Kit (QIAGEN). We used self-dissolving illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Forward and reverse primer (25 pmol each), ddH_2O, and 5 ng of DNA were added to the beads (e.g., Steiner et al., 2012). PCR was run in a Mastercycler Gradient (Eppendorf, Hamburg, Germany), with denaturation at 95°C for 2 min, and start PCR at 95°C for 30 s, at the locus-specific annealing temperature (Table 1) for 45 s, and at 72°C for 45 s in 35 cycles. Termination was set to 72°C for 8 min. Samples were loaded on EL 400 or EL 600 gels (Elchrom Scientific; Table 1, Fig. 1), and an M3 ladder from Elchrom Scientific was used as size marker. Finally, gels were stained with ethidium bromide. Nine loci provided PCR amplicons, and their alleles were identical in size (bp) to those reported by Ecogenics (Table 1). We tested the observed allelic signals for repeatability. Repetition comprised DNA extraction of nine individuals (= 15% of the 60 individuals; Table 2), PCR, and electrophoresis. In the 81 microsatellite lanes on the gels (9 samples · 9 loci), two lanes gave unclear genotype reassignment (i.e., an error rate of ca. 2.5%).

Three to 12 alleles were found per locus depending on the population studied (Table 2). Observed and expected heterozygosities (Table 2), linkage equilibrium, and Weir and Cockerham values of inbreeding within populations (F_{IS}) were calculated with GENEPOP version 4.2 (Raymond and Rousset, 1995; Rousset, 2008; http://genepop.curtin.edu.au/). *P* values for each locus pair across all populations yielded no significant linkage (all $P > 0.07$). The mean F_{IS} values over all loci were positive (Schiahorn = 0.12, Monstein = 0.33, and Casanna = 0.34). We tested for null alleles using MICRO-CHECKER (van Oosterhout et al., 2004), with maximum expected allele size set to 200 bp and a confidence interval of 95%; no unusual observations were found. MICRO-CHECKER suggested null alleles for loci including AV-021012, AV-021049, and AV-021224 (Table 2). However, in the 60 individuals tested

Table 1. Characteristics of the newly developed microsatellite markers in *Anthyllis vulneraria*.

Locus	GenBank accession no.	Primer sequences (5′–3′)[a]	Repeat motif[b]	Amplicon length (bp)[c]	T_a (°C)	Spreadex gel type[d]
AV-000290	KF379737	F: GCAGAGAAGTTATAGTAGCTGTGTG R: CAGCCTGAAAGTATTGGTGGG	$(GA)_{13}$	89–123	52	EL 400
AV-002128	KF379738	F: GCATCTAGCCTCGTTTGTTTTATG R: CACTCTTGCGATACGAGAGC	$(TG)_{13}$	77–101	52	EL 400
AV-004868	KF379739	F: GTCTGTTTATATGCAATGCGTGC R: CAGCATAGCTGCTTCTGTGAG	$(TG)_{12}(AG)_{12}$	114–147	50	EL 600
AV-005692	KF379740	F: TGAAATCAACCCACTAGACAACG R: AACAATCTGGAAACCCTCGC	$(GTT)_7$	77–93	52	EL 400
AV-015354	KF379741	F: GACTATGGTGGGTGGGTGG R: TGCGCATACACGAAGAAACC	$(TC)_{11}$	89–117	50	EL 400
AV-020270	KF379742	F: ATGAAGGAGGTGGGGCATAG R: TGGGCCATTTGCTTCTATATATGTG	$(CA)_{12}$	136–155	52	EL 600
AV-021012	KF379743	F: ACCAGCACCCAAGACCATAG R: TGGAATCGGAGATTGATTCTGG	$(AGT)_8$	82–98	50	EL 400
AV-021049	KF379744	F: GGAGCTGCTTTTAGCGAGAG R: GGTCCTCTATGGCAATCCTCC	$(AG)_{17}$	88–120	52	EL 400
AV-021224	KF379745	F: TGCATTGTTAAATTGAAGCTAGGTG R: CAGTCGATTCTCCACCCCTC	$(AC)_{18}$	133–170	52	EL 600
AV-021803[e]	KF379746	F: TCTTACTTTCTCACAAGAATGCTATC R: TTTGCTAGTGTTGGACCTGC	$(AC)_{12}$	74–104[e]	—	—

Note: T_a = annealing temperature in the running phase of our project (see text).

[a] Primers used for PCR and subsequent Spreadex gel electrophoresis with the ORIGINS electrophoresis chamber (Elchrom Scientific). Note that Ecogenics used forward primers with an M13 tail at the 5′ end and fluorescent-labeled M13 primers in their developmental phase (see text).

[b] Protocol of Ecogenics, based on genomic DNA sequences analyzed on a Roche 454 GS FLX platform (454 Life Sciences, a Roche Company).

[c] In the 60 individuals from three populations in Davos, Switzerland (see Table 2), except locus AV-021803 (see footnote e).

[d] We recommend an electrophoresis time of 1.5 to 2.0 h, a temperature of 55°C, and 10 V/cm (i.e., 120 V in the ORIGINS Elchrom electrophoresis chamber). Our preferred precast Spreadex gels are the Mini S-2×25 with a loading capacity of 25 samples per gel (loading volume ca. 9 µL per slot). EL 400 and EL 600 differ in the density of the gel matrix. For longer amplicons, we used EL 600, for shorter amplicons EL 400.

[e] This locus worked according to the protocol of Ecogenics (with M13 tailing; see text) but could not be established in the running phase in our laboratory. Amplicon size is based on 15 individuals checked by Ecogenics.

TABLE 2. Details on the three populations of *Anthyllis vulneraria*.

	Schiahorn (n = 20)			Monstein (n = 20)			Casanna (n = 20)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
AV-000290	5	0.600	0.601	6	0.600	0.715	8	0.650	0.750
AV-002128	7	0.750	0.695	8	0.650	0.820	8	0.600*	0.802
AV-004868	8	0.850	0.827	8	0.500*	0.729	12	0.600*	0.917
AV-005692	4	0.750	0.675	4	0.500	0.602	6	0.500	0.601
AV-015354	7	0.800	0.770	6	0.550	0.673	8	0.700	0.715
AV-020270	6	0.500*	0.764	5	0.550	0.689	7	0.700	0.764
AV-021012	4	0.700	0.714	4	0.300*	0.670	3	0.150*	0.678
AV-021049	4	0.300*	0.610	8	0.250*	0.720	10	0.200*	0.803
AV-021224	6	0.350*	0.667	6	0.300*	0.635	12	0.400*	0.769

Note: A = number of alleles found; H_e = expected heterozygosity; H_o = observed heterozygosity.
* Indicates excess of homozygotes and/or potential null alleles based on MICRO-CHECKER analysis (see text).

only four blank lanes appeared—all at AV-021049. We believe that actual null alleles are therefore only likely for that particular locus. Hence, we suppose that the excess of homozygosity is mostly due to self-fertilization (e.g., three of the 60 specimens were homozygous in all nine loci). Inbreeding is also indicated by the positive F_{IS} values. Autogamy has been reported as the predominant mode of reproduction for French populations of *A. vulneraria* (see Couderc, 1971), whereas Navarro (1999) found that strong protandry constrained self-fertilization in an Iberian population. The molecular analysis of van Glabeke et al. (2007) of two Belgian populations indicated that they were predominantly outcrossing. As all flowers of an individual plant do not develop synchronously, it is very likely that insects transfer pollen from late flowers to stigmata of early flowers of the same plant (i.e., geitonogamy). Based on our results, we suppose that there is variation in the degree of outcrossing and inbreeding among our study populations from the Swiss Alps.

CONCLUSIONS

The newly developed microsatellite markers are suitable for horizontal Spreadex gel electrophoresis with simple ethidium bromide staining and a considerably short electrophoresis time. No sequencer is needed to resolve the allelic patterns. Multiplex of two loci can also be tested, e.g., if the locus-specific amplicons differ in their respective length (e.g., 80–100 bp vs. 110–130 bp). Central alpine populations of *A. vulneraria* seem to be predominantly outcrossing with variable levels of self-fertilization.

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