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SHORT COMMUNICATION

Development and multiplexing of microsatellite markers using pyrosequencing in a tetraploid plant, *Vaccinium uliginosum* (Ericaceae)

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Background and aims – We developed a new set of microsatellite primers in the tetraploid perennial shrub, *Vaccinium uliginosum* L., to investigate genetic diversity and population genetic structure.
Methods – Using pyrosequencing, we identified and designed primers for PCR amplification of microsatellite loci in *V. uliginosum*. The primers were first screened for amplification and polymorphism by PCR and agarose gel electrophoresis. PCR products of selected primers were then ligated into a vector, amplified with a universal fluorescently labelled forward primer and a specific reverse primer, and electrophoresed on a capillary sequencer to check the scorability of the peaks. Finally, multiplexes were designed and tested on ninety individuals sampled in three Belgian populations.
Key results – We designed and tested a total of 52 primer pairs, of which nine yielded scorable peaks, i.e. eight di- and one tri-nucleotide loci with seven to fourteen alleles per locus in three Belgian populations. The expected heterozygosity was high, ranging from 0.52 to 0.87 (mean = 0.77). Genetic diversity (Shannon's diversity, *H'*) ranged from 1.27 to 1.42 and was much higher than that observed by Albert et al. (2005) using RAPD-analyses in the same populations. This could be due to the higher polymorphism retrieved with microsatellite markers.
Conclusions – The microsatellite markers we developed showed enough polymorphism to investigate genetic diversity and structure even at small spatial scales, gene and pollen dispersal (through paternity inference) or outcrossing rates.

Key words – Clonal growth, genetic structure, multiplex PCR, pyrosequencing, tetraploidy, *Vaccinium uliginosum*.

INTRODUCTION

Vaccinium uliginosum L. (Ericaceae, bog whortleberry) is a perennial shrub with a circumboreal distribution. It grows on acid, poorly drained and wet soils and is found in open biotopes like wet heaths and bogs (Jacquemart 1996). The species propagates clonally via subterranean rhizomes with a typical phalanx growth leading to highly structured populations of distinct clumps composed of ramets belonging to the same clone as shown with RAPDs by Albert et al. (2005). *V. uliginosum* is considered as an arctic-montane relict, occurring only at high altitudes and latitudes (Jacquemart 1996). Thus, global warming but also habitat destruction threaten its populations, which could have critical consequences for associated flower visiting insects. In the Upper Ardennes in Belgium, *V. uliginosum* is an important pollen and nectar resource for bumble bees in early spring (Jacquemart 1992, 1993). Bumble bee abundances have been observed to decline with decreasing *V. uliginosum* population size in fragmented areas (Mayer et al. 2012). The species is therefore of high interest for the conservation of species diversity. The measurement of genetic diversity and gene flow within and among populations is helpful for the identification of appropriate management strategies for its long-term persistence. To identify microsatellite loci *de novo* for *Vaccinium uliginosum*, we used the high-throughput genomic sequencing technology of pyrosequencing. This method allows quick and cheap sequencing of genomic DNA (Abdelkrim et al. 2009, Arif et al. 2010). First, this method had been mainly used for microsatellite isolation in animal species, but has recently become a routinely used method for plant species as well (e.g. Csencsics et al. 2010, Buehler et al. 2011).

MATERIAL AND METHODS

DNA extraction

We extracted genomic DNA from c. 100 mg fresh leaf tissue of one *Vaccinium uliginosum* individual from a Belgian population (Massotais, 50°14'13"N 5°45'34"E) using the E.Z.N.A. HP Plant DNA kit (Omega Bio-Tek, Norcross, GA, USA) with RNase treatment. The purified DNA was subjected to pyrosequencing by Macrogen (Seoul, Korea) using 1/8 of a plate on a GS FLX Titanium platform (454 Sequencing; Roche Applied Science). No enrichment step was realized prior to pyrosequencing to reduce costs and to avoid bias towards particular microsatellite repeat motifs.

Primer development

We used MsatCommander (Faircloth 2008) to screen the pyrosequencing reads for microsatellite motifs (di-nucleotide and tri-nucleotide loci with at least ten or eight repeats, respectively) and to design primers. To avoid designing primers for any locus twice, we compared all sequences of the same SSR repeat (including also the reverse complement repeats, e.g. $(CTT)_n$ and $(AAG)_n$), in BioEdit ver. 7.0.5 (Hall 1999). We designed both forward and reverse primers for the amplification of 52 microsatellite loci. In order to facilitate later multiplex development, we redesigned some primers to obtain a better size distribution of loci using Primer3 ver. 0.4.0 (Rozen & Skaletsky 2000) using the following criteria: optimal primer size of 20 bp (min = 18, max = 25 bp), optimal primer TM of 60°C (min = 57, max = 63°C), optimal GC content of 55% (min = 30, max = 70%) and product size of 100–300 bp.

To test for amplification consistency and specificity of the primer pairs, we conducted PCR amplification with ten samples, each from a different Belgian population. DNA was isolated from ~75 mg leaf tissue using a CTAB method (Doyle & Doyle 1990). We estimated the concentration of genomic DNA extracts with the Qubit Quantitation Platform (Invitrogen) and standardized samples to a concentration of 2 ng/µL. Each PCR reaction contained 4 µL of DNA, 0.2 µL of each primer (20 µM), 2 µL of Buffer, 1.6 µL of BSA (2.5 mg/mL; Fermentas, Burlington, Canada), 1.6 µL of 2 mM dNTPs, 0.1 µL of DreamTaq™ DNA Polymerase (Fermentas) and 10.3 µL of RNase-free water. Thermocycling consisted of one denaturation step of 5 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72° C; and finally one elongation step of 7 min at 72°C. All PCR reactions were done on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). We checked amplification success on 2% agarose gels with ethidium bromide.

Pre-screening of promising primers

Primer pairs that resulted in consistent amplification without unspecific bands, and that showed polymorphism on agarose gels were selected for further screening. To assess quality and scorability of the peaks on the capillary sequencer at low cost, we used a procedure that allows screening a large number of candidate loci with a single, universal fluorescently labelled primer and unlabelled specific primers (Somme et al. 2012). The most promising loci can then be selected and specific labelled primers may be ordered. PCR products of the selected primer pairs were ligated into a pJET1.2/blunt cloning vector containing the T7 promoter sequence using the CloneJET PCR Cloning Kit (Fermentas, Burlington, Canada). First, PCR products were purified with the MinElute PCR purification kit (Qiagen, Hilden, Germany); 8 µL of each PCR product was eluted in 40 µL of EB buffer. Then, we performed four ligations with mixed PCR products: for each ligation, two to eleven loci were mixed before the ligation. Each of the four ligations was repeated five times with a different individual (individuals may differ among loci). PCR amplification of the ligation product was performed using the T7 forward primer labelled with FAM fluorescent dye (Eurogentec, Liège, Belgium) and the specific unlabelled reverse primers. Each PCR reaction contained 7 µL of DNA, 10 µL of HotStarTaq Plus Master Mix (QIAGEN, Hilden, Germany), 0.2 µL of each primer and 2.5 µL of RNase-free water. Thermocycling consisted of one denaturation step of 5 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C; and finally one elongation step of 7 min at 72°C.

PCR products were then electrophoresed on an ABI 3100 capillary sequencer with GS400HD size standard (Applied Biosystems). Peak analysis was done with Peak Scanner v1.0 (Applied Biosystems) and nine markers with good quality profiles were selected (i.e. one to four sharp peaks, low stuttering, low background or unspecific amplification signals; table 1).

Refinement of PCR conditions for combination in multiplexes

For the nine selected markers, forward labelled primers were specifically designed with FAM, HEX or Dragon Fly Orange (DFO) fluorescent dyes (Eurogentec).

We used the same ten samples as above to estimate the allelic range of each marker, refine PCR protocols and combine the identified loci into multiplex PCR primer sets. We amplified each locus separately using a mix of 5 µL of DNA (at 2 ng/ µL), 0.5 µL of each primer (20 µM) and a mix of: 4 µL of Buffer, 2 µL of 2 mM dNTPs, 0.2 µL of Phusion Hot Start II Polymerase (Finnzymes, Espoo, Finland; resulting in lower stuttering) and 7.8 µL of RNase-free water. For some primers (table 1), we added 3 µL of 5 M betaine solution (Sigma-Aldrich, St. Louis, USA; at the same time reducing the amount of RNase-free water to 4.8 µL), which reduced unspecific amplification. Thermocycling consisted of one denaturation step of 30 s at 98°C; 30 cycles of 30 s at 98°C, 30 s at 65°C (62°C when betaine was used) and 30 s at 72°C; and finally one elongation step of 10 min at 72°C. As above, PCR products were electrophoresed on an ABI 3100 capillary sequencer with GS400HD size standard (Applied Biosystems) and peaks were analysed using Peak Scanner v1.0 (Applied Biosystems).

Multiplexing

The initial criteria for multiplexing were at least a 25 bp gap between allele size ranges of loci with the same dye, and Autodimer was used with default settings to test for primer

Table 1 – Characteristics of nine microsatellite markers developed in *Vaccinium uliginosum*.
For each marker, the forward and reverse sequence are given, repeat type, size of the original fragment (bp), number of alleles (*A*), and allele size range estimated in ten individuals from different populations (within brackets, the increased values if additional alleles were found in the ninety individuals from three populations), annealing temperature when run individually (T_a), multiplex number - fluorescent dye, and GenBank accession number.

Marker	Sequence	Repeat	Size	*A*	Size range	T_a	Betaine	Multiplex-dye	GenBank accession number
Vu-CT-1	F: CGGGAGGAAGAAATGACAAG R: GGTGGAGTTTTGTTCGTGGT	$(CT)_{12}$	255	7	230–256	62	yes	0- FAM	KJ454407
Vu-CT-10	F: GCCATCATCTAACGCCATTT R: TGGATTGAGTTACGGCGATT	$(CT)_{11}$	116	12	83–129	65	no	1- FAM	KJ454408
Vu-CT-2	F: AGAGGTGCGAACGAAGTCC R: ACCATCAAATGTGAATGCAAACC	$(CT)_{10}$	255	14	244–308	65	no	1-HEX	KJ454409
Vu-AG-4	F: CCTTCGGCAGCAGGTATAG R: TGTTTCTTTGAGCTGGACTCG	$(AG)_{15}$	230	7(9)	221–237	65	no	1-DFO	KJ454410
Vu-AG-18	F: CGTGGTGGAAAGAAGATTCAA R: CCAAACTCCTCCTGCTTCAT	$(AG)_{11}$	130	10(12)	114–140(144)	62	yes	2-FAM	KJ454411
Vu-CTT-1	F: TCTGGAGCTTCAAAGCCATAA R: AGGAGAAACAGCACGAGAGC	$(CTT)_{16}$	219	9(10)	194–244	62	yes	2- HEX	KJ454412
Vu-CT-7	F: AAACATGTGGTGGGGTTTTC R: AACCCTATTCTCTTTTGGACTCC	$(CT)_{11}$	198	9(14)	178–218(220)	62	yes	2- DFO	KJ454413
Vu-AG-22	F: GGCCAAGTCCAACGCTTAC R: GCTTTCATCACAGCATAGGTG	$(AG)_{10}$	192	7	183–203	62	yes	3-FAM	KJ454414
Vu-CT-6	F: TCTGGCATAAATTCCCACCT R: AATCTCAGAGAGAACGGTTGG	$(CT)_{12}(CA)_6$	107	8(10)	90–110(116)	62	yes	3- HEX	KJ454415

dimer formation (National Institute of Standards and Technology 2005).

The multiplex PCR reactions contained 5 µL of DNA, 2 µL of the primer mix (each primer at 2 µM in TE pH 8.0), and 13 µL of the mix as described above for single primer pairs. Thermocycling conditions were the same as for single primer pairs, with annealing temperature depending on the use of betaine (table 1). Again, PCR products were electrophoresed on the capillary sequencer. We verified if the peaks obtained from multiplex reactions corresponded to those from single-locus PCRs with Peak Scanner v1.0.

Multiplex testing

We tested the usefulness of the markers for population genetic studies on ninety individuals sampled in three Belgian populations (thirty individuals per population, sampled every 2–5 m following a transect; Grande Fange = 50°14'40"N 5°46'45"E; Fange aux Mochettes = 50°13'22"N 5°40'54"E; Sacrawé = 50°14'40"N 5°45'32"E). To bin the allele sizes, we used GeneMapper version 3.7 (Applied Biosystems). We estimated expected heterozygosity with Atetra, which takes into account all possible combinations of allele copy numbers in populations with partial heterozygotes (Van Puyvelde et al. 2010).

RESULTS

Pyrosequencing yielded a total of 63,220 reads, for a total of 18,572,523 bases, which represents an average read length of 294 bases. Among these reads, MsatCommander found 788 reads containing a total of 828 microsatellites of at least ten repeats for dinucleotide motifs and eight repeats for trinucleotide motifs. The $(AG)_n/(CT)_n$ motif was the most common (table 2). We tested 52 primer pairs (designed to amplify 47 dinucleotide microsatellites and five trinucleotide microsatellites) for amplification consistency and specificity, and polymorphism. Of these, 28 consistently amplified and showed bands of expected size with polymorphism. However, after amplification with a fluorescently labelled T7 primer and a specific reverse primer, and electrophoresis on the capillary sequencer, only nine markers showed good quality profiles with one to four sharp peaks per individual, low stut-

Table 2 – Diversity of microsatellite motifs obtained by pyrosequencing.
The criterion for the motifs was a minimum of ten repeats for dinucleotide and eight repeats for trinucleotide motifs.

Repeat motif	Frequency	Proportion (%)
Dinucleotide		
$(AC)_n/(GT)_n$	105	12.7
$(AG)_n/(CT)_n$	596	72.0
$(AT)_n$	71	8.6
Trinucleotide		
$(AAC)_n/(GTT)_n$	5	0.6
$(AAG)_n/(CTT)_n$	13	1.6
$(AAT)_n/(ATT)_n$	26	3.1
$(ACC)_n/(GGT)_n$	3	0.4
$(ACT)_n/(AGT)_n$	3	0.4
$(AGG)_n/(CCT)_n$	1	0.1
$(ATC)_n/(GAT)_n$	3	0.4
$(CCG)_n/(CGG)_n$	2	0.2
TOTAL	828	

Table 3 – Results of primer screening in three populations of *Vaccinium uliginosum*.
Shown for each primer pair are the number of alleles (A), mean values of expected (H_E) heterozygosity, and the Shannon-Wiener Diversity Index corrected for sample size (H'_{corr}). The sample size for each population was thirty individuals.

	Grande Fange			Fange aux Mochettes			Sacrawé		
Primer	A	H_E	H'_{corr}	A	H_E	H'_{corr}	A	H_E	H'_{corr}
Vu-CT-10	8	0.83	1.52	7	0.70	1.40	9	0.83	1.34
Vu-CT-2	7	0.69	1.26	6	0.74	1.08	8	0.67	0.95
Vu-AG-18	11	0.85	1.31	10	0.86	1.78	10	0.87	1.22
Vu-CTT-1	8	0.76	1.49	8	0.74	1.81	8	0.79	1.42
Vu-CT-7	9	0.81	1.50	10	0.82	1.25	14	0.85	1.66
Vu-AG-4	9	0.83	1.30	8	0.82	1.13	9	0.84	0.95
Vu-CT-6	9	0.76	1.54	7	0.52	1.23	8	0.69	1.33
Mean	8.7	0.79	1.42	8.0	0.74	1.38	9.4	0.79	1.27

tering, amplification of a single locus and low background or unspecific amplification signal (table 1). Specific fluorescently labelled primers were ordered for these nine loci.

Eight primer pairs could be combined in three multiplexes (table 1). We combined three primer pairs of different fragment length and with different fluorescent dyes into each of two PCR multiplexes and another two primer pairs into one PCR multiplex (table 1). One primer pair (Vu-CT-1) could not be combined with others in the PCR. Due to unknown reasons, the peaks of this marker could no longer be identified after combination. This primer pair was not used for further analyses. Though multiplex I and III have different PCR conditions, their PCR products could be mixed for electrophoresis in the capillary sequencer.

When testing the usefulness of the markers for population genetic studies on ninety individuals, the number of alleles per locus ranged from nine to fourteen over the three populations, with a mean of 11.3 (table 1). Against previous results, peaks produced by one primer pair (Vu-AG-22) could not be allocated to single alleles. The remaining seven loci all showed a minimum of two and maximum of four alleles per individual confirming the tetraploid nature of the species. The large majority of individuals had three (69%) or four alleles (17%) per locus indicating that all four copies of the loci are equally likely to amplify. This would suggest that *V. uliginosum* is of autotetraploid origin, which has been previously assumed (Grant 1981). Among the ninety individuals tested, few clonal repeats were identified in only two out of the three populations studied: In the Fange aux Mochettes population, seven samples were attributed to three different genotypes. In the Grande Fange population, fifteen samples belonged to five genotypes. The expected heterozygosity was high and ranged from 0.52 to 0.87 with a mean of 0.77 (table 3). Genetic diversity (Shannon's diversity, H'_{corr}) ranged from 1.27 to 1.42 in the three populations (table 3).

DISCUSSION

The observed polymorphism of the developed microsatellite markers is high enough to allow for precise genetic analyses at population level. The genetic diversity we found was much higher than that observed by Albert et al. (2005) using RAPD-analyses in the same populations (on average 0.647). These authors identified a higher incidence of clonality, with 34 genotypes among 104 samples in Sacrawé and 27 genotypes among 115 samples in Fange aux Mochettes. In our study, we only sampled two genets twice and one genet three times in the Fange aux Mochettes population, whereas in Sacrawé, all samples we analysed corresponded to distinct genotypes. This difference between our results and those in Albert et al. (2005) may be a consequence of the sampling scheme they employed. While we sampled along transects, Albert and colleagues sampled in patches, which could explain why they sampled the same genets more often. Our observation of higher diversity with microsatellites could also be due to the higher polymorphism usually retrieved with this type of markers. These markers are thus optimal to be used for population studies (especially at small spatial scales) and allow precise studies on pollen flow and gene dispersal. Alternative methods, e.g. fluorescent dye as a pollen analogue, which is effective for other species (e.g. Mayer et al. 2012), do not work well for *Vaccinium* species that form patches with thousands of flowers (Van Rossum et al. 2013). The microsatellite markers we isolated can be used for studying the breeding system (outcrossing rate and paternity analysis), genetic diversity, clonal growth patterns, and population genetic structure. They might be transferable and could be used for other *Vaccinium* species as well.

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