

PRIMER NOTE

Dinucleotide repeat microsatellite markers for buck's-horn plantain (*Plantago coronopus*)

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Abstract

Eleven polymorphic microsatellite loci were obtained from a GA enriched genomic library, constructed from DNA of buck's-horn plantain (*Plantago coronopus*). The microsatellite loci were tested on 24 genotypes. These plants were collected from meadows along the coast, located on 11 sites ranging from the southwest to the northeast of the Netherlands. In this set of plants the isolated microsatellites were highly polymorphic with 3–24 alleles per locus and a maximum observed heterozygosity of 0.91. Some of the microsatellite loci also showed amplification in two other plantain species (*P. lanceolata* and *P. maritima*).

Keywords: fully informative markers, microsatellite loci, *Plantago coronopus*, quantitative trait locus (QTL)

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Buck's-horn plantain (*Plantago coronopus*) is a small, short living perennial. In the Netherlands the species grows mainly in the coastal area. The flowers are protogynous with an overlap in the sexual phase. It is a wind pollinated, gynodioecious and predominantly outcrossing species. The mean outcrossing rate, measured with allozyme markers, was 0.77 (Wolff *et al.* 1988). Cytoplasmic male sterility (CMS) variation (Koelewijn & Van Damme 1995a), nuclear restorer gene variation for two CMS-types (Koelewijn & Van Damme 1995b) and sex allocation have been investigated (Koelewijn & Hunscheid 2000). To unravel the genetics of those ecologically important characteristics in more detail, a Quantitative Trait Locus (QTL) mapping strategy is currently applied. For this reason a molecular marker map in an outbred mapping cross will be constructed. To integrate both parental maps, fully informative markers are necessary. Because microsatellites can be highly polymorphic they are good candidates for fully informative markers in the QTL cross. In addition, they can be useful in population genetic projects. Squirrell & Wolff (2001) isolated microsatellite loci for *P. major* and *P. intermedia* and tested cross amplification for six plantain

species. One of the ten tested loci showed amplification in *P. coronopus* at an equivocal allele size but data were insufficient to determine whether the loci were polymorphic (Squirrell & Wolff 2001).

To generate more simple sequence repeat (SSR) loci for buck's-horn plantain, microsatellite loci were isolated using an enrichment procedure (Karagyozov *et al.* 1993), modified by Van de Wiel *et al.* (1999) and Arens *et al.* (2000). DNA from the parents of the QTL mapping cross was extracted from freeze-dried leaves using a DNeasy Plant Mini Kit (Qiagen). Genomic DNA was digested with *AluI*, and size-fractionated by agarose gel electrophoresis. DNA fragments between 300 and 1000 bp were recovered by electro-elution, enriched by hybridization to synthetic GA-oligonucleotides, cloned in the PCR2.1-TOPO vector (Invitrogen) and transformed to *Escherichia coli* TOP10F' (Invitrogen). Colonies were transferred onto Hybond N+ membranes (Amersham) and screened by hybridization to GA-oligonucleotides. Forty-seven percent of the clones were positive. These positive clones were sequenced using a Ready Reaction Big Dye Terminator Cycle Sequencing Kit (PE Biosystems) and an ABI 3700 sequencer (PE Biosystems). Twenty-six primer pairs were designed for sequences flanking the microsatellite region using PRIMER3 (Rozen & Skaletsky 1998). Primers and Cy5 labelled primers were synthesized by Amersham Pharmacia Biotech (Roosendaal,

Table 1 Characterization of 12 polymorphic microsatellite loci in *P. coronopus* including locus name, EMBL nucleotide sequence database accession number, primer sequence, specific annealing temperature, repeat motif, expected allele size from sequencing, number of alleles and observed size range (in bp.) among 24 genotypes and observed heterozygosity (H_O)

Microsatellite locus	Accession number	Primer sequence 5'-3'	Annealing temperature	Repeat motif	Allele size	Number of alleles (Observed range)	H_O
PCM07	AJ440953	*GAGCGTCCGATCTAAACGAT GACTAACGTGCATTGCCTAGC	59.0	(CT) ₃₂	190	13 (169–203)	0.17
PCM09	AJ440954	*TCGCCAACCATCACCTTACT GATCCCACTCAAGGTTGGTC	55.0	(CT) ₇ (CC)(CT) ₃₈	203	21 (143–203)	0.61
PCM10	AJ440955	*CTCCGCCTTACGCCATCT TTCCACGCAGATTCCCTTCTT	55.0	(GA) ₈ (N) ₇ (GA) ₇ (N) ₁₇ (GA) ₄₃	211	20 (174–240)	0.45
PCM12	AJ440956	*TCGTGGGGAGGCTTATTAGA GAGAGGGGAGAAGGAGTGCT	55.0	(CT) ₂₈	139	24 (115–209)	0.52
PCM13	AJ440957	*TCCACCCCTTCTTTCTCTCA TAACCAGAGGGAGGAAACCA	57.0	(CT) ₄₂	158	23 (73–198)	0.91
PCM15	AJ440958	*ACAAACTTGCAAACCCAAA GCATGCAAAAAGGCTAAGGAT	50.0	(GA) ₄ (GGGA) ₃ (GA) ₃₉	146	17 (105–153)	0.46
PCM18	AJ440959	*ATAGGCAAGGGGAAGGTAGG CGGATTTTGTGAATGTAGGA	60.0	(GA) ₃₇	156	24 (130–194)	0.39
PCM20	AJ440960	*GCCCTTAAACACGCTTTTTC AAATTTCAGGAGGCAGTCTCG	57.0	(CT) ₂ C(CT) ₂₇	195	3 (196–202)	0.67
PCM22	AJ440961	*GATGTGAGATGGTGGTGGTG CTCTAAACCTCGGGCCAATC	59.0	(GA) ₄₀	194	22 (122–222)	0.42
PCM23	AJ440962	ATATTCAGCTTCGGCACTCG *TCGTTGCGTACACAGATGCT	60.0	(GA) ₃₅	211	3 (211–215)	0
PCM26	AJ440963	CACATGGCCTACCGAAAAGT *ACAAAGGTGTCAAGCGAAGG	56.0	(CT) ₂₁	199	19 (183–228)	0.71

*=Cy5 Labeled primer.

the Netherlands). Microsatellite analysis was setup in total volumes of 20 μ L containing: polymerase chain reaction (PCR)-buffer [2 mM Tris-HCl, pH 7.5 (25 °C), 10 mM KCl, 0.1 mM dithiothreitol (DTT), 0.01 mM EDTA, 0.05% Tween 20 (v/v), 0.05% Nonidet P40 (v/v), 5% glycerol (v/v), 1.5 mM MgCl₂], 0.25 mM dNTP, 1.5 pmol each primer, 0.5 U Expand™ High Fidelity PCR system enzyme mix (Boehringer Mannheim) and approximately 2 ng DNA. All loci were amplified on a PTC-200 (MJ-Research) according to the terminal profile: an initial denaturing step of 94 °C (2 min) followed by 35 cycles of 94 °C (15 s), 30 s at annealing temperature (for specific temperatures per locus see Table 1) and 72 °C (45 s), followed by a final step of 72 °C for 7 min PCR products were mixed with 5 mg/mL blue dextran in formamide and three molecular sizers, not interfering with microsatellite bands. Samples were denatured for 5 min at 95 °C and analysed on ALFexpressII high resolution gels (Pharmacia).

Primersets were tested first on plants from which they were isolated. When amplification was successful PCR reactions were applied to 22 genotypes of *P. coronopus* collected from meadows along the coast located on nine sites ranging from the midwest to the northeast of The Netherlands. The average distance between these sites was 17 km with a maximum of 50 km. Allele sizes were measured using

IMAGE MASTER® 1D Version 3.0 (Amersham pharmacia biotech) and internal size markers.

Eleven loci (Table 1) showed amplification of bands with appropriate sizes in the two QTL cross parents, from which microsatellites were developed. Those parents originated from the southwest of the Netherlands. The loci PCM07, PCM20 and PCM23 did not always produce amplification products when applied to other genotypes from meadows 100–250 kilometers away. Absence of amplification is likely due to mutational changes in the regions flanking the microsatellite repeats. The other eight microsatellites showed amplification in all 24 tested genotypes, which were highly polymorphic (between 17 and 24 alleles per locus) and had observed heterozygosities from 0.39 to 0.91. Markers with these characteristics have a high chance of being fully informative in the QTL mapping cross and are useful in population genetic studies, like parentage analyses.

Five microsatellite loci showed cross-amplification in *P. lanceolata* and *P. maritima* (Table 2) under conditions developed for *P. coronopus*. For both species eight unrelated plants were tested. Allele sizes were within or close to the range found in buck's-horn plantain. Only PCM15 showed amplification in all plants for both species, but polymorphism was low. Alleles were not sequenced, which is necessary to verify microsatellite identity.

Table 2 Cross species amplification using primers developed for *Plantago coronopus* in *P. lanceolata* and *P. maritima*. Only loci showing amplification are presented including microsatellite locus name, number of alleles and observed range (in bp) in eight genotypes for both species

Microsatellite locus	<i>P. lanceolata</i>		<i>P. maritima</i>	
	Alleles	Range	Alleles	Range
PCM07	5	184–193	5	182–192
PCM10	2	167–209	4	175–240
PCM15	2	143–145	3	135–145
PCM18	3	141–143	3	140–143
PCM20	2	193–194	—	—

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