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TECHNICAL NOTE

Development of a microsatellite library for the flightless moth *Pringleophaga marioni* Viette (Lepidoptera: Tineidae)

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Abstract Fifteen microsatellite markers were isolated from an enriched microsatellite library developed specifically for *Pringleophaga marioni*, a flightless moth species from the sub-Antarctic Prince Edward Islands. Of these, 12 markers were polymorphic in a population of 44 individuals collected on Marion Island. Between 2 and 7 alleles were amplified per marker and the expected heterozygosities (H_E) for these ranged from 0.046 to 0.753. These microsatellite markers reported here are the first for *P. marioni* and will be used to investigate conservation genetic aspects of this species on the Prince Edward Islands.

Keywords Flightless moth · Microsatellite · Fiasco method · Sub-Antarctic · Marion Island

Pringleophaga marioni, a species endemic to the Prince Edward Islands (Marion and Prince Edward Island), is one of three flightless tineid Lepidoptera found exclusively on islands of the sub-Antarctic (Crafford et al. 1986). *Pringleophaga marioni* is characterized by several unique and unusual traits compared to other lepidopteran species, probably as a result of limited predation and the harsh physical environment. Specifically, it apparently lacks pheromones and is flightless, with highly reduced wings and strongly developed hind legs for jumping (Crafford 1990). Caterpillars of *P. marioni* are the major litter-dwelling detritivores on the Prince Edwards and play an essential role in nutrient cycling (Smith and Steenkamp 1992a) where they annually process ~1,500 tons of dead plant material (Crafford et al. 1986; Crafford 1990). Caterpillars also stimulate the mineralization of nutrients from plant litter in lowland plant communities (Smith 1985; Smith and Steenkamp 1992b; Smith and Steenkamp 1993). On Marion Island they are under considerable threat because they form the preferred prey of invasive house mice, which are absent from the neighbouring Prince Edward Island (Crafford 1990; Chown and Smith 1993). Establishing the extent of population differences in this species between the two islands is therefore essential for informing management actions, which include plans for mouse eradication on Marion Island (Davies et al. 2007; Wanless et al. 2010).

To do so, and to investigate various other components of population variation in *P. marioni*, species-specific microsatellite markers were developed. Caterpillars were collected at Swartkop Point on Marion Island within Wandering Albatross nests. Genomic DNA was extracted from 44 individuals (caterpillars) with the Qiagen DNeasy® Blood and Tissue Kit. An enriched microsatellite library was constructed in association with Inqaba Biotechnical Industries (Pty) Ltd (http://www.inqababiotec.co.za) by means of the FIASCO method (Zane et al. 2002). The library was constructed using the protocol from Zhang et al. (2008) with two oligonucleotides (5′-GACGATGAGT CCTGAG-3′ and 5′-TACTCAGGACTCAT-3′) as adapters and the adapter-specific primer *Mse*I-N (5′-GATGAG TCCTGAGTAAN-3′). Genomic DNA was enriched using $(AC)_{12}$, $(TA)_{12}$ and $(CT)_{12}$ di-nucleotide repeat probes.

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Table 1 The primer sequences, repeat motif, GenBank accession numbers and basic statistics of the microsatellite loci developed for *Pringleophaga marioni*

Locus name	Primer sequences (5′–3′)	Dye	GenBank accession number	Repeat motif	Size range	N	N_a	H_O	H_E
Pm01	F: CGTGAGGACCAGTTTCTTCC R: ACGGTTAGTGTTGGCACCAT	6-FAM	HM035496	$(TG)_4CG(TG)_6CT(TG)_5$	340–386	44	7	0.267	0.360
Pm04	F: AGTCGCCAGTGACAAGTGTG R: CCTCGCAGTGCGTCATAGT	NED	HM035497	$(CA)_{10}$	331–339	43	3	0.302	0.367
Pm05	F: AGCTGGCTTCATTGATACCG R: TCAAAGTGCCACCGCTAAGT	6-FAM	HM035498	$(CA)_6TA(CA)_8$	263–265	43	2	0.256	0.260
Pm06	F: GATGACGTGATGTGATGGCTA R: GAGATCACCAAATCCCACGA	PET	HM035499	$(TG)_9AG(TG)_{12}$	270–280	44	4	0.295	0.647
*Pm07	F: CATACGTCGAAGGCCACTTT R: AAGTTCCCAATCCACACTGG	VIC	HM035500	$(TG)_7$	246	44	1	n/a	n/a
*Pm08	F: TGTGTCTAGCAACTGCCAAAA R: ATGAGCCGCAAGAGTAGAGG	NED	HM035501	$(TG)_6GG(TG)_7$	244	43	1	n/a	n/a
Pm14	F: TTCCTGTAGCACCAACTATTATCAG R: GCGTTATACTCACACCAGCGTTA	PET	HM035502	$(TA)_6$	85–87	43	2	0.047	0.046
Pm15	F: GCCTATGGGTGCTCCTTTC R: AGATACAGAGGCACGAAGACAGT	VIC	HM035503	$(CT)_4CW(CT)_9$	74–86	44	5	0.167	0.407
Pm16	F: GCTTGCGTGCGTGTGTAA R: GCCTCTTACTCTGTTTCCATCC	NED	HM035504	$(TG)_{12}$	60–70	44	3	0.500	0.549
Pm20	F: CGATATGTGTTTGCGTACGTG R: AGCTGGTGTAATGATGATGGTG	VIC	HM035505	$(TG)_{11}$	379–401	44	5	0.568	0.568
Pm23	F: CCCAACCTCTGCACTAGACG R: GTTGTTCCAACTTTCTGCCTTA	VIC	HM035506	$(TG)_8AG(TG)_{10}$	244–278	44	5	0.727	0.753
*Pm26	F: GTGAGGAAATCCCGCACTT R: GGGTGGCCAGAGACATACAC	PET	HM035507	$(TG)_7$	160	44	1	n/a	n/a
Pm30	F: TACAGTTCGTGTGCGTGTGT R: AGCCGCAAGAGTAGAGGCTA	6-FAM	HM035508	$(TG)_6CG(TG)_7$	81–87	44	3	0.295	0.320
Pm31	F: GCAAAGCATGATAGCAAATAGG R: CATTACACACGCACAAACACTT	NED	HM035509	$(TG)_{13}$	250–277	44	4	0.568	0.577
Pm35	F: CGGAAGCTTGGCAAATGTAT R: TTGAGGATATAAGCGTGTGTGC	NED	HM035510	$(AC)_5CC(AC)_4$	171–179	44	3	0.091	0.130

N number of individuals genotyped, N_a number of alleles, H_O observed heterozygosity, H_E expected heterozygosity. Monomorphic markers indicated with asterisk

A total of 128 positive clones were sequenced of which 38 contained microsatellite motifs of five or more repeats which also had sufficient flanking regions for primer design. Primers were designed for these clones using Primer 3 Plus (Untergasser et al. 2007) and ordered from Applied Biosystems with a fluorescent dye (6-FAM, PET, VIC or NED) associated with the forward primers. Primers were tested for amplification and polymorphism. Sequences of the clones (Pm01–Pm35) were deposited in GenBank (accession numbers HM035496-HM035510). The QIAGEN Multiplex PCR Kit was used to amplify markers in a single PCR reaction following a protocol which included Q-solution (QIAGEN). Multiplex reactions (numbered 1–3) consisted of the following primer pairs: 1) Pm01, Pm05, Pm23, Pm30; 2) Pm04, Pm06, Pm14, Pm16 and 3) Pm15, Pm20, Pm31, Pm35. Amplifications were performed (GeneAmp 2700 Thermocycler; Applied Biosystems) in a final volume of 10 µl containing 2 µl (±80 ng) of DNA, 5 µl of 2X QIAGEN Multiplex PCR Master Mix, 1 µl of Q-solution (5X), 1 µl of primer mix (2 µM) and 1 µl of ddH_2O. Amplifications comprised an initial denaturation at 95°C for 15 min, 30 cycles at 94°C for 30 s, 60°C for 90 s and 72°C for 50 s. A final elongation step at 60°C for 30 min completed reactions. Genotyping was performed in an ABI 3730 automatic sequencer (Applied Biosystems) using 0.2 µl of GS500LIZ size standard (Applied Biosystems). Alleles were scored using GeneMapper 3.7 (Applied Biosystems).

Following the initial screening process, fifteen microsatellite markers were selected for further use given their successful amplification and ease of inclusion into a PCR multiplex. These primer sequences, repeat motifs and GenBank accession numbers are given in Table 1. Of these, 12 markers were polymorphic in our study population. The three monomorphic markers (Pm07, Pm08 and Pm26) are also listed, but excluded from subsequent analyses. Table 1 also includes summary statistics for the markers. The number of alleles per locus varied between 2 and 7 (average of 3.83 alleles per locus). GENEPOP 4.0.10 was used to perform Hardy–Weinberg equilibrium and linkage disequilibrium tests as well as to calculate the observed and expected heterozygosities. The population was in Hardy–Weinberg equilibrium for all the markers except for Pm06 (P-value = 0) and Pm15 (P-value = 0.0003). This deviation was attributed to the occurrence of null alleles as evidenced by an excess of homozygotes at a frequency of 0.2451 and 0.2524 respectively (MICROCHECKER; van Oosterhout et al. 2004; see also Okello et al. 2005). Lepidoptera species are notorious for the occurrence of null alleles (Chapuis and Estoup 2007; Meglecz et al. 2004). Even though two of the microsatellite markers showed signs of null alleles in the study population, it might still be useful in other *Pringleophaga* populations, and the occurrence of null alleles would have to be tested on a case-by-case basis. No significant linkage disequilibrium could be detected after Bonferroni corrections (P-value adjusted to 0.0012). The markers can therefore be considered as independent. Observed (H_O) and expected heterozygosities (H_E) were calculated according to Raymond and Rousset (1995) and Weir and Cockerham (1984). Observed and expected heterozygosities ranged from 0.047 to 0.727 and 0.046 to 0.753, respectively (Table 1). These markers will be useful in studies that hold implications for the management and conservation of this unique species on the Prince Edward Islands.

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