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PRIMER NOTE

A set of microsatellite markers for *Heliconius melpomene* and closely related species

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Abstract

The butterflies in the genus *Heliconius* offer an exceptional opportunity for the study of the ecology and genetics of an adaptive radiation due to their extensive intra- and interspecific variation in wing colour patterns and mimetic associations. Here, we characterize 22 polymorphic microsatellite loci in *Heliconius melpomene* that have been shown to be useful for linkage mapping and population studies in this and other species. Levels of variation were high, although heterozygosity deficiencies were found in most loci, probably due to null alleles. The loci showed broad amplification success on six other species across the genus.

Keywords: *Heliconius*, Lepidoptera, microsatellites, Nymphalidae

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Introduction

The butterflies in the genus *Heliconius* have long been recognized as excellent models for the study of the ecology and genetics of an adaptive radiation due to their extensive intra- and interspecific variation in wing colour patterns and mimetic associations (Sheppard *et al.* 1985). Interestingly, most of the variation in *Heliconius* colour patterns seem determined by a handful of Mendelian loci with major effect. For example, a single genomic region in *Heliconius melpomene* controls the expression of a yellow hindwing bar, a white submarginal hindwing band and a yellow forewing band (Naisbit *et al.* 2003). Similar patterns are known for *H. melpomene*'s comimic; *Heliconius erato*. These simple genetic determination patterns have motivated a comparative linkage mapping project aiming to describe the genetic architecture of colour pattern evolution in *Heliconius* (Jiggins *et al.* 2005). Here we provide a set of 22 polymorphic microsatellite loci useful for linkage mapping and population genetics studies in *H. melpomene* and some closely related species.

A CA-rich microsatellite library was constructed using *H. melpomene*'s DNA extracted from a French Guiana individual, using the QIAmp Tissue Kit (QIAGEN). In short, genomic DNA was digested with Sau-3A1 (Promega), size-selected (400–800 bp) from a 1% agarose gel and purified (Gel Purification Kit, QIAGEN). Restriction fragments were ligated with 21 and 25 bp adapters and amplified by polymerase chain reaction (PCR) using the 21 bp adapter as primer. PCR products were purified (QIAquick PCR Purification Kit, QIAGEN), hybridized with 3'-biotiniled $(CA)_{10}$ oligonucleotides and eluted using Vectrex Avidin D (Vector Laboratories) following manufacturer's protocol. A PCR was effectuated with the eluted DNA as template and used to transform supercompetent cells (pGEM-T Easy Vector System II, Promega). Recombinant colonies were screened for microsatellites following Waldbieser (1995), using pUC19 forward or reverse universal primers and a $(CA)_{10}$ primer. Positive clones were sequenced on an ABI 377 automatic sequencer using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Primer pairs on flaking regions of 10 loci were designed with PRIMER 3 software (Rozen & Skaletsky 2000) and tested in 24 *H. melpomene* individuals from Pointe Macouria, French Guiana (04.9138 N, 52.3595 W). Additionally, primers pairs for 12 new loci (marked with * on Table 1) were designed from the *Heliconius* microsatellite library developed by Flanagan *et al.* (2002) and tested in 24 individuals from Pipeline Road, Panama (09.1714 N, 79.7573 W).

PCRs (10 μL) were as follow: 1× PCR buffer (composition not provided, QIAGEN), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.025 U/mL *Taq* polymerase (QIAGEN) and 10–40 ng genomic DNA. Primers were tested using either of two different conditions: (i) by using 0.1 μM dye-labelled (6-FAM,

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Table 1 Characterization of microsatellite loci in *Heliconius melpomene*. Loci marked with * belong to the Flanagan *et al.* (2002) microsatellite library and were analysed in individuals from Panama. The other loci belong to the library developed here and were analysed in individuals from French Guiana. The core sequence, primer sequences, size range in bp and linkage group (Jiggins *et al.* 2005) for each locus is given. n, H_O, H_E and AR represent the number of individuals analysed, observed heterozygosity, expected heterozygosity and allele richness (for 15 diploid individuals), respectively, per locus and population. Observed heterozygosities in bold are different from Hardy–Weinberg expectations with $P < 0.01$ (†) and $P < 0.05$ (‡)

Locus	Primer sequences	Core	Size range (bp)	Linkage Group	n	H_O	H_E	AR	GenBank Accession nos
Hel17*	F: GGCGCACAGACGAGACTAC R: ACTGCCGCACGAATAATAAC	$(CA)_{19}$	174–210	LG06	24	0.42	0.49	3.6	DQ020071
Hm01*	F: CGCGGTAGAAATAGCACAAG R: CGAGAAGCCCTACAAGTGTG	$(CA)_{34}$	157–215	LG15	24	**0.50**†	0.66	10.5	DQ020072
Hm02*	F: TATTTGCACGATGGAAACCC R: GCGAGGTGGAGACAAAAGAC	$(GA)_{20}$	163–177	LG03	24	0.71	0.68	4.0	DQ020073
Hm03*	F: GACGTCACAGCGGGGAAC R: AGAGGGGAACGGAGTGTCAT	$(CA)_{16}$	286–352	LG10	21	**0.48**†	0.89	12.1	DQ020074
Hm04*	F: CCTGGCTTATCTACGACGACA R: ATGCAGCTTACTCGCTGGTT	$(CA)_{12}$	379	Z	24	0.00	0.00	1.0	DQ020075
Hm05*	F: GCGGTAAGGTAAAACCGTGA R: CAGAAGAAAATGGTTGGATGG	$(GA)_{18}$	293–357	LG07	24	**0.58**†	0.80	9.8	DQ020076
Hm06*	F: AAATAGTGTGCGGCGGAATA R: TGGAGTAGAAATGCGGGTTTA	$(CA)_{7}$	219–227	LG03	24	0.79	0.73	4.6	DQ020077
Hm07*	F: GCAGAGGGAACCTCGTGTTA R: CGCAGTTTGTGCGAATTACA	$(CA)_{9}$	260–280	LG02	23	**0.43**†	0.77	6.3	DQ020078
Hm08*	F: AAAGCCTGAGTGCCGTATTG R: GCAATGTCAGCATCGAATGT	$(CA)_{17}$	291–369	LG15	24	0.67	0.73	8.4	DQ020079
Hm09	F: CAACTGCAATGACCCATCAC R: AATGTCGTGCTCCCATGAAG	$(GA)_{14}$	196–204	LG12	14	**0.14**†	0.67	6.4	DQ020080
Hm10	F: GGCCGCTTTGTAAGAATGTC R: TGTGTAAATGAAATCCATAATTGGTC	$(CA)_{37}$	212–244	LG09	21	**0.57**‡	0.79	11.5	DQ020081
Hm11	F: TTCTGGTGTCTAGCGGTTATG R: AATAGCGACCATGCTGAGAG	$(CA)_{24}$	375–411	LG06	22	**0.36**†	0.83	11.5	DQ020082
Hm13	F: TCACTAGTTTTCGGCTTATCG R: AAGGCTAAATGATGCCTAAAG	$(CA)_{18}$	185–199	LG19	20	**0.55**†	0.81	6.6	DQ020083
Hm14	F: ATGCTGTAACCCGCATAGC R: TGCATTTATGATGTAAAAGTTCG	$(CA)_{59}$	144–158	LG18	22	**0.05**‡	0.13	3.4	DQ020084
Hm15	F: TTTCGCCACCATAATCTTTC R: CACATCGCAGGTATTCCATC	$(CA)_{26}$	240–294	LG05	17	**0.35**†	0.94	18.8	DQ020085
Hm16	F: CGGATAGACATTTGTTAAAGTGTG R: ACGAGGATGCGGACTACG	$(CA)_{14}$	235–299	LG19	18	0.89	0.93	17.7	DQ020086
Hm18*	F: AATTACATATCGTTTCATTA R: CTACGACAAGACCCTCCTGA	$(CA)_{28}$	204–302	LG02	20	**0.55**†	0.78	9.0	DQ020087
Hm19*	F: CGCTAATTCAAAGGAAAGAGGA R: AGTGCTGTCATGGCTAACGA	$(CA)_{13}$	180	LG20	15	0.00	0.00	1.0	DQ020088
Hm21	F: GAACTCCAGAAGGTTACCCCA R: GCCGGTCTTTGTCTATTGGA	$(GATA)_{8}$	144–346	LG02	23	**0.35**†	0.66	5.2	DQ020089
Hm22*	F: CCTCGTCCAAACTCCAAAAC R: AACAATGTCACAACCATCGC	$(GA)_{16}$	230–262	–	24	0.79	0.88	8.9	DQ020090
Hm23	F: AGTGGACATGCTGGTAGACG R: CGATTTCACAACATCTGAACG	$(CA)_{16}$	174–198	–	20	**0.40**†	0.73	8.1	DQ020091
Hm24	F: AGGGCATATACTCGCACTG R: GACTGGTTTCAGATAGAGAAAGAATC	$(CA)_{13}$	192–252	–	15	**0.00**†	0.73	6.7	DQ020092

HEX or TET) forward primers and 0.5 μM reverse primers or (ii) by using 0.01 μM M13-tailed forward primer, 0.4 μM dye-labelled (6-FAM, HEX or TET) M13 primer (5′CACGACGTTGTAAAACGAC3′) and 0.4 μM reverse primer. The PCR was performed in a PTC-100 or PTC-200 thermal cycler (MJ Research) using a first incubation at 96 °C for 6 min, followed by 30 cycles at 92 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s. A final extension of 20 min

Table 2 Number of alleles observed in cross amplifications of 19 *Heliconius melpomene* microsatellite loci on six closely related *Heliconius* species. Two individuals assayed per species. — indicates no amplification or inconsistent amplification

Locus	*H. cydno*	*H. heurippa*	*H. timareta*	*H. numata*	*H. hecale*	*H. ismenius*
Hel17*	2	1	3	3	1	2
Hm01*	2	1	1	4	1	2
Hm02*	2	1	1	4	—	2
Hm03*	4	3	2	1	2	1
Hm04*	4	3	2	3	—	—
Hm05*	3	2	2	4	3	4
Hm06*	2	3	2	3	2	2
Hm07*	2	1	2	3	1	1
Hm08*	2	1	—	3	—	1
Hm09	1	1	1	1	1	—
Hm10	4	2	3	2	3	—
Hm11	—	3	2	1	1	—
Hm13	1	2	1	—	2	2
Hm14	1	3	3	2	2	—
Hm15	1	—	1	—	1	—
Hm16	2	1	3	2	3	2
Hm22*	3	2	—	—	—	—
Hm23	—	1	1	3	—	—
Hm24	—	2	4	—	2	2

at 72 °C was added. Reaction products were diluted (usually 1 : 20, 1 : 30 and 1 : 40 for HEX, TET and 6-FAM labelled products respectively) and resolved in 6% Long Ranger (BioWhittaker Molecular Applications) denaturing polyacrylamide gels on a GeneSys BaseStation DNA Fragment Analyser (MJ Research) following manufacturer's protocols. Allele sizes were determined using CARTOGRAPHER analysis software (MJ Research) with GeneScan 400HD ROX (Applied Biosystems) as size standards.

Details on diversity of the 22 loci are shown in Table 1. Although loci Hm04* and Hm19* appear monomorphic, they are included here because they are polymorphic in other populations/species. Allelic richness (15 diploid individuals) varied from 3.4 in locus Hm11–18.8 in locus Hm15 (average = 7.9, s.d. = 4.7), while observed heterozygosities varied from zero in locus Hm24 to 0.89 in locus Hm16 (average = 0.48, s.d. = 0.24). Departures from Hardy–Weinberg were evaluated using exact tests as implemented in the GENEPOP 3.4 package (Raymond & Rousset 1995). A very high number of loci showed significant heterozygote deficiencies (Table 1), which is certainly due to the presence of null alleles at the loci concerned. Null alleles appear to be a common feature of butterflies' microsatellites and *Heliconius* seems not to be an exception (Flanagan *et al.* 2002).

These microsatellite loci, together with 219 amplified fragment length polymorphisms (AFLP) markers and 19 single copy nuclear genes have been scored in 73 individuals from a single F2 family, offspring of a cross between two races of *H. melpomene*. The resulting linkage map has 21 linkage groups corresponding to the 21 chromosomes of *H. melpomene*, and the colour-pattern loci Yb and Sb (Jiggins *et al.* 2005). Nineteen of the microsatellite loci described here have been successfully assigned to 13 linkage groups, including one at the Z-sex chromosome (Table 1). This map represents the first step towards a future comparative linkage analysis of colour pattern evolution in *Heliconius*.

Nineteen loci were also tested in two individuals of six related *Heliconius* species, three in the closely related cydno clade: *H. cydno*, *H. heurippa* and *H. timareta* and three in the slightly more distant silvaniform clade: *H. numata*, *H. hecale* and *H. ismenius* (Table 2). Overall amplification success in the cydno clade is 89.5%; while in the silvaniform clade is 71.9%, which confirms relationships among the six species and *H. melpomene* (Beltrán *et al.* 2002).

In conclusion, 22 polymorphic microsatellite loci suitable for population genetics studies in natural populations of *H. melpomene* have been obtained. These loci, together with those previously described by (Flanagan *et al.* 2002) have been shown to be useful for linkage analyses studies of colour patterns in *H. melpomene* and probably in some closely related species.

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