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PRIMER NOTE

Characterization of microsatellite loci in neotropical *Heliconius* butterflies

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Abstract

The *Heliconius* butterflies offer exceptional opportunities for the study of the ecology and evolution of mimicry. Despite previous reports of difficulties in the development of microsatellite loci in Lepidoptera, we characterize 15 polymorphic loci in *H. erato* that show promise for genetic mapping and population studies in this and other species. Levels of variation were high, in both numbers and size ranges of alleles. The loci showed broad amplification success across the genus and in two other genera. All loci that amplified in a population of *H. melpomene* were polymorphic.

Keywords: Lepidoptera, linkage mapping, mimicry, Nymphalidae

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The *Heliconius* butterflies are characterized by an extraordinary degree of phenotypic radiation and mimetic convergence in their aposematic wing colour patterns. The Müllerian comimetic species, *H. erato* and *H. melpomene*, placed phylogenetically into two divergent clades within the genus, show extensive geographical variation in wing colour pattern. None the less, they exhibit an almost perfect wing colour pattern mimicry, changing in parallel across their sympatric ranges. Here we characterize 15 microsatellite loci in these and other Heliconiine species. Eleven loci have been developed in the laboratory of WOM as part of an ongoing project to understand the genetic architecture of wing colour patterns in both comimetic species. The remaining four loci were developed separately for *H. erato*, following protocols outlined in Armour *et al*. (1994) and Peter *et al*. (1998) (see Blum 2002 for details).

Loci HEL.01 to HEL.11 were developed in the following manner. Genomic DNA was extracted from individual butterflies using a standard protein digestion protocol (e.g. Milligan 1998). Enriched genomic libraries were constructed by Genetic Identification Services (GIS, http://www.genetic-id-services,com; Chatsworth, CA, USA), using 100 μg of DNA combined from both *H. erato* and *H. melpomene*. Insert DNA from individual clones was PCR amplified following GIS guidelines. The PCR products were cleaned using Qiaquick™ columns (Qiagen® Inc.) and sequenced with both forward and reverse universal M13 primers and ABI PRISM® BigDye™ Terminator chemistry. Sequencing products were resolved on the ABI PRISM® 377 Sequencer (PE Applied Biosystems).

PCR primers were designed using the program Primer 3 (Rozen & Skaletsky 1998). Polymerase chain reaction (PCR) amplifications of the microsatellite loci were carried out in 10 μL reactions consisting of 10–50 ηg of DNA, 0.3 U of *Taq* DNA polymerase (Qiagen), 200 μM each dNTP, 0.5 μM each primer, 1 × Qiagen PCR buffer (contains Tris Cl, KCl $(NH4)_2SO_4$ at unspecified concentrations, 1.5 mM $MgCl_2$, pH 8.7 at 20 °C). PCRs, performed on an PTC-100 thermal cycler (MJ Research Inc.), consisted of 3 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, T_{an} °C (see Table 1) for 30 s and 72 °C for 45 s, and finally 72 °C for 10 min. Fragment size analysis was performed on the ABI PRISM® 377 Sequencer, using GENESCAN 3.1 and the TAMRA-500 size standard. Levels of heterozygosity were calculated using ARLEQUIN v2.001 (Schneider *et al*. 2000).

Three genomic libraries were constructed, enriched for $(GA)_n$ $(CA)_n$ and $(TAGA)_n$ repeats. Primers were designed for 31, 18 and 11 unique repeat sequences from the three

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Table 1 Microsatellite locus characterization in *Heliconius erato* and *H. melpomene*. The reverse primers were fluorescently labelled. Loci HEL.12 to HEL.15 were characterized on different populations of both *H. erato* and *H. melpomene* to the preceding loci

					H. erato				*H. melpomene*			
Locus	Primer sequence (5′–3′)	Core repeat*	GenBank accession no.	T_a (°C)	Size range (bp)	No. of alleles/ no. of samples	H_O	H_E	Size range (bp)	No. of alleles/ no. of samples	H_O	H_E
HEL.01	F: TCGTAGATATCCATTACTCTGGTCTG R: AGGGCGTCGTTAGTTTGTGT	$(GA)_{21}$	AF481466	54	418–448	14/16	0.63†	0.95				
HEL.02	F: TCAAAATGTTGCAGACCGAG R: TGCACTTCATTGTAAGGCGT	$(GA)_{13}(GAAA)_2(GA)_2$	AF481467	55	158–178	9/12	0.92	0.89	161–175	6/14	0.64	0.70
HEL.03	F: CCAATTATGTCACATGGATCTGTT R: CTCTGTCGTCCTCTGCAGTC	$(GA)_{14}$	AF481468	54	364–656	16/15	0.60†	0.97				
HEL.04	F: CGTTGCCGCTTATACTTTCC R: GGAACGGAGTGCCCTAAAAC	$(GA)_{18}$	AF481469	55	178–234	11/15	0.73	0.86	214–284	12/14	0.29†	0.96
HEL.05	F: TGCTGTCCATACCCAACTCA R: CGAACTCACAACCATCAGTCA	$(GA)_{14}CA(GA)_4$	AF481470	55	246–346	17/17	0.35†	0.97	299–333	12/12	0.92	0.91
HEL.06	F: TAGCCTTCACTTTGAACCCG R: CCCACTCGAAGCAATGAAAT	$(GA)_{38}$	AF481471	55	294–416	12/12	0.17†	0.98				
HEL.07	F: CCCGAACCACTAAAGTCGAA R: GCGGGGACAACTACATAAGC	$(GA)_{24}(TA)_3$	AF481472	55	246–302	15/15	0.73†	0.95				
HEL.08	F: ACATCTCAGAACTGGTCGGC R: CTCGATCAGCCGGTGATTAT	$(CA)_{14}$	AF481473	53	280–300	9/16	0.88	0.87	294–300	4/14	0.36	0.49
HEL.09	F: GCGGATCGAAATTTAAGGTG R: GTATCGAGGTAAAGCGCGAC	$(CA)_2AA(CA)_7AA(CA)_{11}AA(CA)_2$	AF481474	55	241–267	10/15	0.47†	0.94				
HEL.10	F: TCTCACTTTCCCACACAGCA R: TGTGAAGAGACACATGGGGA	$(CA)_7TA(CA)_{10}$	AF481475	55	292–356	11/13	0.69	0.82				
HEL.11	F: TTTCTTTTGAGTCCCGATGG R: ATCTCAGAACTGGTCGGCAG	$(CA)_{12}$	AF481476	55	186–208	10/15	0.93	0.86	199–205	4/14	0.43	0.43
HEL.12	F: CGGCACTTCATGTTTCATTT R: GGCATTTGACTTCAGAATGG	$(TAG)_4$	AF481477	52	353–407	12/15	0.57†	0.91	368–374	4/5	N/M	N/M
HEL.13‡	F: ATTTCATAGTAACGCCCTCC R: TGACTTATCGCTAAGGTCAA	$(CA)_{13}$	AF481478	48	226–242	9/15	0.76	0.90				
HEL.14‡	F: GCACATTTACTTACACTAACGCC R: ATTTGTTTCGAACGACTGCC	$(AAT)_4$	AF481478	48	165–180	5/15	0.88	0.70	163–172	4/15	0.25†	0.73
HEL.15	F: CCCGCTCTTCCATAACACTT R: TGCAACGTTGAACTTGCTCT	$(GT)_{19}CC(GT)_{11}$	AF481479	52	275–287	7/15	0.70	0.78	279–291	6/15	0.50	0.70

*From sequenced clone.
T_a, optimized temperature of annealing.
†Heterozygote deficiency.
N/M, not measurable.
‡HEL.13 and HEL.14 were identified from the same clone.

Table 2 Number of alleles detected in 13 Heliconiine species (number of individuals tested per species)

Species	Locus														
	HEL.01	HEL.02	HEL.03	HEL.04	HEL.05	HEL.06	HEL.07	HEL.08	HEL.09	HEL.10	HEL.11	HEL.12	HEL.13	HEL.14	HEL.15
H. himera (1)	2	1	1	2	1	1	2	2	2	2	2	—	1	1	—
H. clysonimus (1)	—	1	—	—	2	1	2	1	—	—	1	—	1	—	—
H. charithonia (1)	2	2	—	2	1	1	1	1	—	—	1	—	2	—	—
H. sapho (1)	2	2	—	1	2	2	2	2	—	—	2	—	2	—	—
H. sara (1)	—	2	1	—	—	—	1	1	—	—	1	—	2	—	1
H. melpomene (14)	—	6	—	12	12	—	—	4	—	—	4	4 (5)*	—	4 (15)*	6 (15)*
H. cydno (5)	—	2	—	5	8	—	—	3	—	—	3	N/T	N/T	N/T	N/T
H. numata (1)	—	2	2	1	2	—	—	1	—	—	2	—	—	—	—
H. hecale (1)	1	1	—	2	2	2	1	1	—	—	2	—	2	—	—
H. wallacei (1)	—	1	—	1	1	—	2	2	—	—	2	—	2	—	1
H. hierax (1)	1	1	2	—	—	—	1	2	—	—	2	—	—	—	—
Euides aliphera (1)	—	2	—	2	—	—	—	1	—	—	1	—	1	—	—
Dryas iulia (1)	—	2	—	—	—	—	—	1	—	—	1	—	—	—	2

–Indicates no amplification product, or multiple bands amplified.
*From amplification on different set of individuals (*n* in parentheses).
N/T, not tested.

respective libraries. All 60 primer pairs were subject to a preliminary test amplification on three individuals each of *H. erato* and *H. melpomene*. From this screen, run on 1.8% agarose gels, we identified those loci that amplified a single or double band in either *H. erato* or *H. melpomene*, or both. Eighteen primer pairs amplified in each of the species, with seven amplifying in both. No TAGA loci amplified in *H. erato*. Of the 18 primer pairs that gave an amplification product in *H. erato*, 11 show promise for genetic mapping and population studies in this and other species.

Here we characterize more fully these 11 loci, and the additional four developed by MJB. Mendelian inheritance was seen at all loci in genetic linkage map construction in *H. erato* and *H. himera* (Tobler A *et al*. in prep.). All loci demonstrated considerable variation in size and number of alleles in a single population of *H. erato* (Table 1). Broad size ranges have also been seen in microsatellites isolated from other Lepidoptera (Meglécz & Solignac 1998; Keyghobadi *et al*. 1999; Harper *et al*. 2000). At seven loci significant heterozygote deficiency was observed in *H. erato* populations. Common explanations for such observations are undetected population subdivision or the presence of null alleles. An additional explanation at these loci may be the failure to detect alleles larger than the standard electrophoresis range. At one locus, HEL.03, such alleles were detected when our gel ran for an unexpectedly longer period. We re-ran the samples using the GENESCAN TAMRA-2500 size marker to score these big alleles. None the less, it may be that we still did not score all amplified alleles at all loci. The already broad size ranges of these loci within the resolution of a standard electrophoresis gel suggest that alleles larger than those normally detected may, in fact, be common. Interestingly, the two loci, HEL.04 and HEL.14, that showed significant heterozygote deficiency in *H. melpomene* were in Hardy–Weinberg equilibrium in *H. erato*.

Locus amplification was assessed broadly across other Heliconiine species (Table 2). Twelve loci amplified extensively, including species from the divergent clades within the genus. Three loci amplified in all 11 *Heliconius* species and in two other genera. All loci that amplified in *H. melpomene* were polymorphic.

These 15 loci add considerably to the number of published microsatellite markers for butterflies. Despite the observation of heterozygote deficit at some loci, it is likely that, with careful characterization, they will provide useful markers for molecular-based ecological and evolutionary studies within the Heliconiine butterflies.

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