Isolation and characterization of microsatellites in two tropical butterflies, Drupadia theda and Arhopala epimuta (Lepidoptera: Lycaenidae)

Cécile Fauvelot

Molecular Ecology Notes (2005) **5**, 724–726 doi: 10.1111/j.1471-8286.2005.01045.x

PRIMER NOTE

Isolation and characterization of microsatellites in two tropical butterflies, *Drupadia theda* and *Arhopala epimuta* (Lepidoptera: Lycaenidae)

CÉCILE FAUVELOT

Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands

Abstract

Little is known about the effect of El Niño Southern Oscillation-induced fires on the genetic diversity of tropical rainforest species. Here, I report on the isolation and characterization of 10 microsatellite loci, five loci each, for two lycaenid butterfly species in East Kalimantan, Indonesia, namely *Drupadia theda* and *Arhopala epimuta*, which will be used to specifically study the impact of disturbance on genetic diversity. Microsatellite enrichment was carried out using streptavidin-coated magnetic beads. Positive colonies were identified with the three-primer polymerase chain reaction (PIMA). Cross-species amplifications conducted both within and between genera were successful in 16 out of 20 tests.

Keywords: *Arhopala epimuta*, *Drupadia theda*, El Niño Southern Oscillation (ENSO), Kalimantan, Lycaenidae, microsatellites

Received 22 February 2005; revision accepted 6 April 2005

Wildfires pose a major threat to extant tropical biodiversity (Laurance 2003). The province of East Kalimantan (Indonesian Borneo) was heavily affected by the 1997/98 El Niño Southern Oscillation (ENSO) event, the most severe and widespread ever recorded in Borneo (Harrison 2000). The 97/98 ENSO-induced fires had a major impact on ecosystem dynamics and species diversity (Holmgren *et al*. 2001; Cleary 2003; Laurance 2003; Cleary & Genner 2004), but little is known about the effect on genetic diversity. We are currently investigating the genetic composition of two species of butterflies within landscapes differentially affected by ENSO-induced drought and fires. *Drupadia theda* and *Arhopala epimuta* are two common lycaenid butterfly species of the tropical rainforests of East Kalimantan. Polymorphic microsatellite markers will make it possible to assess fine-scale population genetic structure within fragmented areas, as well as temporal variation of genetic diversity within disturbed areas.

Genomic DNA was isolated from legs of two individuals per species, using a cetyltrimethyl ammonium bromide (CTAB) protocol (Hillis & Moritz 1990). Microsatellite enrichment was achieved using streptavidin-coated magnetic spheres, according to the protocol described in Boneh *et al*. (2003), with minor modifications. The positive colonies were detected using a three-primer polymerase chain reaction (PCR) (PIMA, Lunt *et al*. 1999), with pGEMT-F (TCACTATAGGGCGAATTGGG) and pGEMT-R (CTCAAGCTATGCATCCAAGG) primers (designed according to the pGEM®-T Easy vector sequence) and the biotin-labelled microsatellite sequences. Inserts were reamplified by direct PCR of the colony with pGEMT-F and -R, sequenced using a BigDye Terminator sequencing kit and an ABI PRISM 3100 genetic analyser (Applied Biosystems).

Out of 384 colonies selected after cloning the enriched fragments, 172 positive ones were detected for *D. theda* and 96 for *A. epimuta*. Of these, approximately 75% contained a microsatellite sequence for both species. Forty and 29 primer pairs were designed for *D. theda* and *A. epimuta*, respectively, using the PRIMER 3 program (Rozen & Skaletsky 1998). Each primer pair was tested using PCRs with a total volume of 10 μL containing 1 × PCR buffer (HT Biotechnology), 1.25 mM $MgCl_2$, 0.1 mM of each dNTP, 0.3 mg/mL bovine serum albumin, 0.2 μM of each primer, 0.2 U *Taq* polymerase (HT Biotechnology) and approximately 3 ng

Correspondence: C. Fauvelot, Fax: +31(0)20-5257878; E-mail: fauvelot@science.uva.nl

Table 1 Primer sequences, repeat motif, annealing temperature (T_a) and variability measures resulting from the amplification of 51 and 76 individuals in *Drupadia theda* and *Arhopala epimuta*, respectively, at five microsatellite loci each

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	Accession no.	N_a	H_E	H_O
Drupadia theda							
DtC1	F: TCTGTGCGAGACGGTGAGTA	$CCAACACCA_{(7)}$	58	AY974053	8	0.797	0.882
	R: GAACTTCCTGGACAGCCTGA						
DtD10	F: GGCATGACATTTTTGTGATGTT	$CT_{(12)}$	58	AY974054	9	0.754	0.529*
	R: TCCTTGTTACTGCCGATTTG						
DtE6	F: GGTACACCTACATAACGATAAAATGC	$TC_{(13)}$	58	AY974055	3	0.129	0.098
	R: TGACTATGTGTGTAAACTTTGTATGGA						
DtF6	F: GCGGTATCATGATTCAAGGTG	$CAGA_{(7)}CGGA_{(3)}$	58	AY974056	7	0.705	0.824
	R: AGTCGTTCTTGCAAAATCACTTA						
DtN10	F: GAAATGACGATCTCTACGCAAA	$GACGTT_{(14)}$	58	AY974057	9	0.833	0.941
	R: TGTTAAGCCAGTTGGTGAGC						
Arhopala epimuta							
AeA12	F: TTTGTCTCAATTGGTTGATTCG	$CA_{(9)}$	58	AY974048	8	0.733	0.284*
	R: CCGCTTTGAAAATCGGAAAT						
AeB9	F: TACTGCTGCTTAACGGCTGA	$CT_{(12)}$	58	AY974049	32	0.894	0.581*
	R: CTGCCTGCCTTTACCTCATC						
AeF10	F: CAGGCCTAACGATCCACAGT	$GAT_{(6)}$	58	AY974050	13	0.684	0.274*
	R: TTGCATAGGCCCTGAAAATC						
AeG5	F: ACGGGAAAAAGTGTCTGACC	$TCA_{(8)}$	58	AY974051	12	0.514	0.186*
	R: AGGCCTATGTTCAGCAGTGG						
AeH5	F: GCGGTGACGGCTAGATGATA	$GAT_{(7)}$	58	AY974052	11	0.673	0.693
	R: TGACGTAGATGTGATAAACGTATGC						

N_a, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; *, significant heterozygote deficiency.

DNA. PCRs were carried out in PTC 100 Thermocycler (MJ Research): 3 min denaturation at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C, and a final extension of 5 min at 72 °C. PCR products were run on Spreadex® gels (Elchrom Scientific). After examining patterns on gel, 18 and 20 microsatellites were selected for *D. theda* and *A. epimuta*, respectively. PCRs were conducted under the same conditions with the F-primers labelled (IRD700), loaded on a 6.5% acrylamide gel, and run on a Li-Cor 4200 automatic sequencer, with a size standard (50–350 bp, Li-Cor). Of these microsatellite loci, five produced interpretable and repeatable amplification products without PCR optimizations (Table 1). The reasons for abandoning the other microsatellites were multiband patterns, monomorphic loci or loci awaiting optimization of the PCR conditions.

Variability was tested in 51 and 76 individuals of *D. theda* and *A. epimuta*, respectively, collected in a primary forest in East Kalimantan. Genotypes and allele sizes were estimated by eye. The program, GENETIX (Belkhir *et al*. 2002), was used to determine the number of alleles and heterozygosity estimates per locus. Tests for linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium were performed using GENEPOP version 3.4 (Raymond & Rousset 1995). For *D. theda*, three to nine alleles per locus were observed, and eight to 32 for *A. epimuta* (Table 1). Expected heterozygosity values ranged from 0.129 to 0.833 for *D. theda*, and 0.514–0.894 for *A. epimuta*. No significant LD was found for any pair of loci ($P > 0.01$ for all comparisons) for both species. Significant heterozygote deficiencies were found in locus Dt10 for *D. theda* ($P < 0.01$) and in all loci for *A. epimuta* (all $P < 0.001$), with the exception of AeH5. Inbreeding, undetected population subdivision (both affecting all loci) and/or the presence of null alleles (locus-specific) might create such deficiencies. The population structure of these two species will be assessed at a finer geographical scale and on more individuals in order to test these possible hypotheses.

The results of cross-species amplification tests of the 10 loci are presented in Table 2. Five individuals each of the following additional species were used, *Drupadia ravindra*, *Arhopala muta*, and *Arhopala atosia*. Cross-species amplifications conducted both within and between genera were successful in 16 out of 20 tests. Between genera, *A. epimuta* successfully amplified four loci isolated from *D. theda*. Within genus, *D. ravindra* successfully amplified three loci isolated for *D. theda* (viz., DtC1, DtD10, and DtF6). The five loci isolated for *A. epimuta* were successfully amplified for both *A. muta* and *A. atosia*, with the exception of AeF10 for *A. muta*.

Table 2 Allele size range (bp) of generated fragments resulting from cross-species amplifications at 10 microsatellite loci; –, failure, n. t., not tested

Locus	*Drupadia theda*	*Drupadia ravindra*	*Arhopala epimuta*	*Arhopala atosia*	*Arhopala muta*
DtC1	136–199	145–181	118–158	n. t.	n. t.
DtD10	80–100	80–86	78–92	n. t.	n. t.
DtE6	136–140	—	138–140	n. t.	n. t.
DtF6	99–123	103–123	111–131	n. t.	n. t.
DtN10	150–254	—	—	n. t.	n. t.
AeA12	n. t.	n. t.	144–175	146–182	120–204
AeB9	n. t.	n. t.	114–256	146–178	114–238
AeF10	n. t.	n. t.	121–163	121–139	—
AeG5	n. t.	n. t.	73–103	80–98	86–111
AeH5	n. t.	n. t.	167–188	170–185	179–185

The small number of loci developed for *D. theda* and *A. epimuta* (five loci each) is due to the presence of highly conserved flanking regions shared between microsatellite sequences, detected within both species. This phenomenon appears to be a common problem for developing microsatellite markers in Lepidoptera (Meglécz *et al*. 2004; see Zhang 2004 for a review). Nevertheless, microsatellites remain markers of choice for assessing fine-scale genetic structure.

Acknowledgements

I am grateful to my two collaborators on this project, Steph B. J. Menken and Daniel F. R. Cleary. I thank Peter Kuperus and Betsie Voetdijk for valuable help in the laboratory. This work was supported by grant 895.100.029 of the Netherlands Foundation for the Advancement of Tropical Research (NWO-WOTRO), within the priority program, 'Biodiversity in disturbed ecosystems'.

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