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Brief report
Isolation and characterization of microsatellite markers from the endangered Karner blue butterfly *Lycaeides melissa samuelis* (Lepidoptera)

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Nuclear microsatellite repeats have increasingly become the marker of choice in studies of the population genetics and social structure of a wide variety of insect species (e.g. GERTSCH et al. 1995; ESTOUP et al. 1995; BOGDANOWICZ et al. 1997; BOURKE et al. 1997; MÉGLECZ et al. 1998; KEYGHOBADI et al. 1999; DONNELLY and TOWNSON 2000). As these markers can be readily PCR-amplified from limited quantities of DNA, they also represent important conservation genetic tools (BRUFORD and WAYNE 1994). Although butterflies are one of the most well studied of arthropod groups, very few studies have been able to successfully make use of microsatellite loci as population genetic markers (MÉGLECZ and SOLIGNAC 1998). To date, microsatellite loci have been isolated in butterfly species from several families including the Papilionidae, Nymphalidae, Satyridae, Lymantriidae and Lycaenidae (PALO et al. 1995; BOGDANOWICZ et al. 1997; ROSE 1997; MÉGLECZ and SOLIGNAC, 1998 and work cited therein; SACCHERI et al. 1998; KEYGHOBADI et al. 1999; HARPER et al. 2000; Jiggins and Mallet, in prep.). However, in many cases few usable loci have been isolated. Furthermore, many authors have reported difficulties obtaining reliable results because of frequent PCR failure and/or frequent deviations from Hardy-Weinberg equilibria (MÉGLECZ and SOLIGNAC 1998; MÉGLECZ et al. 1998; KEYGHOBADI et al. 1999; HARPER et al. 2000).

MATERIALS AND METHODS

Four microsatellite markers were developed to complement a phylogeographic study of mitochondrial and morphological variation in the North American butterfly genus *Lycaeides* (Nice et al. submitted). These markers were also used to assess patterns of genetic variation in a range of key populations of the federally endangered Karner blue butterfly *Lycaeides melissa samuelis*. Microsatellite loci were isolated from a genomic library constructed from DNA extracted from the non-endangered Melissa blue butterfly *L. m. melissa*, using methods similar to those previously described (RASSMAN et al. 1991; HUGHES and QUELLER 1993; PAETKAU et al. 1995). Briefly, the library was based on randomly-cleaved genomic fragments resulting from a *Sau3A1* digest of intact genomic DNA and cloned into the *Bam*H1 site of the plasmid vector BSKS + (Stratagene, La Jolla, California). Recombinant clones where then plated at high density and screened with a synthetic ^{32}P labeled $(AC)_N$ probe that was complementary to the corresponding $(GT)_N$ repeat in the Melissa blue genome. As this strategy proved highly successful, further screens were carried out using either tri- $(TAA)_N$ or tetra- $(GATA)_N$ nucleotide repeats. Primers were designed in regions flanking the microsatellite core repeat and one primer pair was 5′ labeled with one of two fluorescent dye groups (6-FAM or HEX). Automatic analysis of microsatellite length polymorphisms was then carried out using an ABI 373A DNA sequencer and sized using an internal size standard labeled with a third fluorescent dye (ROX). Data collection, analysis and automatic sizing of PCR-amplified microsatellite loci was carried out using the GENESCAN 672 and GENOTYPER software (Applied Biosystems, Perkin-Elmer Corporation, California).

PCR reactions were carried out in 50 µl reaction volumes containing 10–100 ng DNA, 0.2 mM of each dNTP and 0.2 µM of each primer. Amplification conditions were optimized for each locus and were as follows. Msat 4 was amplified in 1X Promega

Table 1. *Primers and core sequences for four microsatellite loci isolated from Lycaedies melissa melissa. H_O: observed heterozygosity, H_E: excepted heterozygosity calculated for a population sample of L. m. samuelis from Fort McCoy, WI, N: the number of sampled haploid genomes; Total number of alleles and size range in base pairs is shown for 27 populations of Lycaeides across the continental United States. Genbank accession numbers for each locus are as follows: Msat4: AF372522; Msat6, AF372523; MsatZ12-1, AF372524; Msat201, AF372521*

Microsatellite	Primer sequence (5′ to 3′)	Core sequence	H_O	H_E	N	Total number alleles	Size range (base pairs)
Msat4	F:TGGACTTCATAACAG-GGG R:TAGTCGATCAGCTGT-TCGCCATG	$(CA)_5$ $AA(CA)_{14}$-$AA(CA)_2$	0.56	0.48	50	34	206–280
Msat6	F:GCTTGCACTGGAATAA-CGAG R:ACGCAGACAGACAAA-CAGC	$(GT)_2$ GCGT-$(GT)_8$ CTGA$(GT)_2$	0.32	0.64	44	11	189–229
MsatZ12-1	F:CTACGCGCAGAGACA-GTACAGTA R:ACATACCACGGAATTG-GACGTCG	$(GT)_{15}$	0.13	0.21	46	13	190–214
Msat201	F:CTCTTCTGGGTAATTATG R:TCGTTTCAGTGGTTGAGG	$(CTGT)_5$	0.12	0.12	50	5	109–117, 138

reaction buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 % Triton® X-100.], 2 mM $MgCl_2$, 1 M betaine, and 2.5 U of Taq DNA polymerase (Promega, WI). A "touchdown" protocol was used as follows: 94°C for 3 min, then 95°C for 40 s denaturation, 57°C 45 s annealing, 57°C 90 s extension. The last three steps were repeated with a 1°C drop in the annealing and extension temperature every two cycles until 42°C was reached, then 17 cycles of 95°C 40 s denaturation, 46°C 45 s annealing and 46°C 90 s extension. Alternatively, the extension temperature was held at 60°C throughout or lowered to 51°C and thereafter held constant whilst decreasing the annealing temperature. Reduced extension temperature greatly diminished stutter artifacts at this locus and at other loci that we examined. Msat6 was amplified using 2.5 U Pfu (exo-) DNA polymerase (Strategene, La Jolla, CA) in 1X commercial buffer [10 mM KCl, 10 mM $(NH_4)_2$ SO_4 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$ 0.1 % Triton® X-100, 0.1 mg/ml Bovine Serum Albumin], supplemented with 0.5 mM $MgCl_2$ and 5 % DMSO. The touchdown protocol was the same as described for Msat 4 with the exception that extension temperature was held constant at 72°C. Msat 201 was amplified using 1X Promega reaction buffer, 1.5 mM $MgCl_2$ using the following conditions: 94°C for 3 min, then 94°C 1 min denaturation, 53°C 1 min annealing and 72°C 1 min extension temperature for 35 cycles. Cycling conditions for Msat Z12-1 were identical to those used for Msat 201 except the annealing temperature was 68°C. In cases where amplification proved difficult, buffers were supplemented with 0.8 mM spermidine. Values for observed heterozygosity (H_O) and unbiased estimates of gene diversity (equivalent to expected heterozygosity H_E) (NEI 1987) were calculated for all loci.

RESULTS AND DISCUSSION

A total of 14 microsatellite loci were isolated from the Melissa blue genome. Ten of these consisted of imperfect dimeric $(AC)_N$ repeats and the remaining four consisted of a perfect dimeric $(AC)_N$, a complex trimeric $(AAT)_N$, a perfect tetrameric $(GATA)_N$ and a perfect octameric repeat $(ACACATCC)_N$. The high incidence of imperfect repeats reflects findings in other lepidoptera (MÉGLECZ and SOLIGNAC 1998). Of all of the loci isolated in the present study, only 4 microsatellite loci (Msat 201, Msat 4, Msat 6 and Msat Z12-1) could be reliably amplified. With the exception of the tetrameric Msat 201, the other three loci successfully used in this study all consisted of $(AC)_N$ repeats. Other microsatellite loci were abandoned because of problems with optimization of the PCR amplification process or because of a high frequency of apparent null alleles. Table 1 illustrates the core sequences for each microsatellite repeat and the primers used to amplify the corresponding locus from *L. m. samuelis* populations. Results from one of the Karner blue butterfly populations (Fort McCoy, WI) are also presented in Table 1. Observed heterozygosity was much lower than the expected heterozygosity

for two (Msat6, MsatZ12-1) of the loci examined. Heterozygote deficiencies at these two loci were also observed in other populations sampled in this study (data not shown) suggesting either allelic drop out or competitive amplification of alternate alleles at these loci. This result is consistent with similar devations from Hardy Weinberg equilibrium found in other butterfly studies (PALO et al. 1995; MÉGLECZ and SOLIGNAC 1998; KEYGHOBADI et al. 1999; HARPER et al. 2000).

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REFERENCES

Bruford MW and Wayne RK, (1994). The use of molecular genetic techniques to address conservation questions. In: Molecular environmental biology (Ed SJ Garte). Lewis Publishers.

Bogdanowicz SM, Mastro VS, Prasher DC and Harrison RG, (1997). Microsatellite variation among Asian and North American gypsy moths (Lepidoptera: Lymantriidae). Ann. Entomol. Soc. Am. 90: 768–775.

Bourke AFG, Green HAA and Bruford MW, (1997). Parentage, reproductive skew and queen turnover in a multiple-queen ant analyzed with microsatellites. Proc. Royal Soc. London Set. B 264: 277–283.

Donnelly MJ and Townson H, (2000). Evidence for extensive genetic differentiation among populations of the malaria vector (Anopheles arabiensis) in Eastern Africa. Insect Mol. Biol. 9: 357–367.

Estoup A, Garnery L, Solignac M and Cornuet JM, (1995). Microsatellite variation in honey bee (Apis mellifera) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. Genetics 140: 679–695.

Gertsch P, Pamilo P and Varvio SL, (1995). Microsatellites reveal high genetic diversity within colonies of Camponotus ants. Mol. Ecol. 4: 257–260.

Hughes CR and Queller DC, (1993). Detection of highly polymorphic microsatellite loci in a species with little allozyme polymorphism. Mol. Ecol. 2: 131–137.

Harper GL, Piyapattanakorn S, Goulson D and Maclean N, (2000). Isolation of microsatellite markers from the Adonis blue butterfly (Lysandra bellargus). Mol. Ecol. 9: 1919–1952.

Keyghobadi N, Roland J and Strobeck, (1999). Influence of landscape on the population of the alpine butterfly Parnassius smintheus (Papilionidae). Mol. Ecol. 8: 1481–1495.

Méglecz E and Solignac M, (1998). Microsatellite loci for Parnassius mnemosyme (Lepidoptera). Hereditas 128: 179–180.

Méglecz E, Pecsenye K, Varga Z and Solignac M, (1998). Comparison of differentiation pattern at allozyme and microsatellite loci in Parnassius mnemosyne (Lepidoptera) populations. Hereditas 128: 95–103.

Nei M, (1987). Molecular evolutionary genetics. Colombia University Press, New York.

Paetkau D, Calvert W, Stirling I and Strobeck C, (1995). Microsatellite analysis of population structure in Canadian polar bears. Mol. Ecol. 4: 347–354.

Palo J, Varvio S-L, Kanski I and Väinölä R, (1995). Developing microsatellite markers for insect population structure: complex variation in a checkerspot butterfly. Hereditas 123: 295–300.

Rassman K, Schlotterer C and Tautz D, (1991). Isolation of simple-sequence loci for use in polymerase chain reaction-based DNA fingerprinting. Electrophoresis 12: 113–118.

Rose OR, (1997). The evolution of microsatellites. Ph.D. Thesis, University of London, UK.

Saccheri IJ, Kuussaari M, Kankare M, Vikman P, Fortelius W and Hanski I, (1998). Inbreeding and extinction in a butterfly metapopulation. Nature 392: 491–494.