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TECHNICAL NOTE

# Isolation of novel microsatellite markers for the clouded Apollo (*P. mnemosyne* Linnaeus, 1758; Lepidoptera, Papilionidae)

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**Abstract** Five novel polymorphic microsatellite loci were isolated and characterized using an enriched genomic DNA library for *Parnassius mnemosyne*, a European butterfly of conservation concern, and a valuable model for the study of metapopulation dynamics. Allele numbers ranged from 4 to 12 and observed and expected heterozygosities from 0.17 to 0.74 and from 0.26 to 0.835, respectively. Two samples from geographically close populations were analyzed, demonstrating that the new markers can be successfully employed to investigate fine-scale population structure.

**Keywords** Microsatellites · Lepidoptera · *Parnassius* · Primers · Clouded Apollo · SSR

*Parnassius mnemosyne* is a Centroasian–European Papilionid butterfly. Its wide distribution and specialized ecology make it a valued model organism to investigate metapopulation dynamics and the effects of past and present climate change on the distribution of species and ecosystems (Araujo and Luoto 2007; Välimäki and Itämies 2003; Gratton et al. 2008). Population decrease has been observed through most of the range (Konvička and Kuras 1999) and *P. mnemosyne* is regarded as a species of primary conservation concern (CEE Habitat directive 43/92, annex IV). Genetic consequences of habitat fragmentation are regarded as one of the main threats to *P. mnemosyne* populations (Descimon and Napolitano 1993). Despite the interest raised by this species, only three microsatellite loci were developed so far (Meglécz and Solignac 1998), in part due to intrinsic impediments posed by the structure of Lepidopteran genomes (Meglecz et al. 2004; Nève and Meglécz 2000). This paper presents five novel polymorphic microsatellites in *P. mnemosyne*, considerably increasing the availability of highly variable DNA markers for this species.

Characterization of microsatellite DNA markers was performed following Zane et al. (2002) with modifications according to Hammond et al. (1998). Genomic DNA of two *P. mnemosyne* individuals was digested with *MboI* endonuclease (New England Biolabs, Ipswich, MA, USA) and ligated to complementary adaptors SAUL-A (5′-GCGGT ACCCGGGAAGCTTGG-3′) and SAUL-B (5′-GATCCCA AGCTTCCCGGGTACCGC-3′) (Hammond et al. 1998). Twenty-five pmoles of SAUL-A were used as bi-directional primers to increase the DNA amount. Enrichment was carried out by hybridizing PCR products with biotinylated $(AC)_{13}$ and $(AC)_{17}$ DNA probes: first, the amplified DNA (500 ng) was mixed with 50–80 pmol of biotinylated oligonucleotide in a total volume of 100 µl (SSC 4.2×, SDS 0.07%); second, it was denatured (95°C, 3 min) and annealed at room temperature for 15 min. Biotinylated probes were captured on streptavidin coated magnetic beads (MagneSphere™, Promega).

The beads-probe-DNA complex was separated from hybridization buffer by applying a magnetic field, the buffer was discarded, and non-hybridized DNA removed by three washes with $TEN_{100}$ and two washes with 400 µl of SSC 0.2×, 0.1% SDS. DNA was separated from the beads-probe complex by a denaturation step in 12 µl of 0.15 M NaOH. The solution was neutralized with 0.1667 M $CH_3COOH$, and TE was added to a final volume of 50 µl. DNA was precipitated with one volume of

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isopropanol/sodium acetate, and re-suspended in 50 µl of sterile water. A final amplification with SAUL-A as primer restored the DNA double strand and added poli-A tails, that were used for the following cloning (TOPO-TA cloning kit, Invitrogen).

One hundred and twenty positive colonies were lysated picking up a small amount of bacterial matter and incubating it for 1 min at 95°C in 20 µl sterile water. Bacterial lysate of 1 µl was amplified and sequenced using vector primers.

Primers were designed using PRIMER3 (Rozen & Skaletsky, available at http://primer3.sourceforge.net) for 22 sequences containing suitable microsatellite sequences. Nine primer pairs provided efficient amplification of genomic DNA, but four of them had to be excluded: two pairs amplified multiple bands (>2 per individual); one pair failed to amplify on some individuals and showed strong homozygote excess (suggesting high frequency of "null alleles"); one pair proved to be highly selective for short alleles ("large alleles dropout").

Genetic variation of the five selected markers was assayed on a total of 53 individuals, sampled in two different sites in the Monti Simbruini Natural Park (Central Italy: Livata, 41.93N; 13.15E and Viglio, 41.91N; 13.38E). PCR reactions were performed in 12 µl solution containing 1.5 mM $MgCl_2$ and 25 pmol of each primer. Optimized annealing temperatures ($T_a$) are given in Table 1. One primer of each pair was 5′-labeled with fluorescent dyes and PCR products were analyzed on ABI Prism® 3100 Genetic Analyzer.

All individuals were successfully genotyped at all five loci. The total number of alleles per locus ranged from 4 to 12. Observed and expected heterozygosity varied from 0.173 to 0.736 and from 0.259 to 0.835, respectively. FSTAT 2.9.3 (Goudet 2001) was used to assess Hardy–Weinberg equilibrium by testing the significance of $F_{IS}$ on 200 randomizations and test for linkage disequilibrium and genotypic differentiation between the two samples (*G*-test based on 1,000 randomizations, not assuming random mating within samples). Evidences of homozygosity excess ($P < 0.05$) were observed at loci *m30* and *m100* in the Livata sample and at loci *m100* and *m172* in the Viglio sample. The same pattern was also evidenced when the data have been analyzed using MICROCHECKER (Van Oosterhout et al. 2004). Consistent disequilibrium at locus *m100* indicates the existence of rare null alleles, which might be also present at loci *m30* and *m172*. Association between loci *m30* and *m111* ($P < 0.05$) was detected in the sample from Livata. The hypothesis of panmixia was rejected ($P = 0.007$), thus showing that the new markers may can detect fine-scale differentiation of geographically close (ca 19 km) populations not separated by any obvious barrier.

**Table 1** Primer sequences, PCR conditions and characteristics for five microsatellite loci in *Parnassius mnemosyne*

| Locus | GB Acc. Number | Primer sequence (5′–3′) | Allele size range (bp) | Repeat motif | $T_m$(°C) | $T_a$(°C) | Global ($N = 53$) | | | Livata ($N = 28$) | | | Viglio ($N = 25$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *A* | $H_O$ | $H_E$ | *A* | $H_O$ | $H_E$ | *A* | $H_O$ | $H_E$ |
| *m30* | FJ375770 | F: CCACCAGATGGTAAGTGGCTA<br>R: TCTGTTCTACCCAAGGTTAGCTG | 233–280 | $(TG)_9…(TGCG)_4$ | 60 | 55 | 12 | 0.654 | 0.835 | 9 | 0.607 | 0.845 | 11 | 0.708 | 0.795 |
| *m96* | FJ375771 | F: GAGATACCACATTCACCCATGA<br>R: TGAAGGATTTGTTGCCTTCC | 95–100 | $(TG)_9$ | 60 | 55 | 4 | 0.358 | 0.452 | 4 | 0.379 | 0.458 | 3 | 0.333 | 0.446 |
| *m100* | FJ375769 | F: GGGCTCCATTTGTGAATTTG<br>R: TCACGACCCCGTGTTCTAGT | 69–164 | $(CA)_4CT(CA)_9$ | 60 | 55 | 10 | 0.481 | 0.729 | 8 | 0.448 | 0.707 | 7 | 0.522 | 0.749 |
| *m111* | FJ375767 | F: TCACGACCCCGTGTTCTAGT<br>R: AGGGTACCGCTGCTAAGACA | 116–124 | $(TG)_8$ | 60 | 55 | 8 | 0.736 | 0.750 | 6 | 0.690 | 0.782 | 7 | 0.792 | 0.682 |
| *m172* | FJ375768 | F: TAACCAAGCCAAGGTTGACA<br>R: CGCTTTTCATTGAAGACTCGT | 105–118 | $(CA)_7$ | 59 | 53 | 4 | 0.173 | 0.259 | 3 | 0.214 | 0.2 | 4 | 0.125 | 0.333 |

A, number of alleles; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity in the two samples from Monte Livata and Monte Viglio, and in the global sample

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