

Tree hole odonates as environmental monitors: Non-invasive isolation of polymorphic microsatellites from the neotropical damselfly *Megaloprepus caerulatus*

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Abstract

Because of their complex mating behaviour and life cycle (alternating aquatic and terrestrial stages) odonates provide important model systems for environmental monitoring, evolutionary ecology, and conservation genetics. Many odonate species are endangered and call for the use of non-invasive molecular studies. In the neotropical damselfly *Megaloprepus caerulatus* we have identified polymorphic microsatellite loci by means of the randomly amplified microsatellite technique (RAMS; Ender et al. 1996). Using the DNA from each a single leg of three unrelated individuals we screened 63 RAPD primers for small size banding patterns. A total of 95 RAPD profiles was hybridized with digoxigenin labelled di- and trinucleotide repeats (GA_n, GT_n, CA_n and AAT_n) and 36 RAPD fragments harbouring microsatellite motifs were isolated. Cloning and sequencing of positive fragments revealed five polymorphic microsatellite loci. Since *Megaloprepus caerulatus* is a viable bio-indicator for primary rainforests the microsatellite system can be used to study the effects of forest fragmentation on population viability.

Many odonate species are endangered and call for the use of non-invasive molecular studies (Hadrys 1992; Fincke and Hadrys 2001). Larvae of the neotropical damselfly *Megaloprepus caerulatus* develop in water-filled tree holes of secondary and primary rainforests. These discrete habitats constitute an important aquatic ecosystem in neotropical forests (Fincke 1992). As one of the two top predators in this small habitat, *M. caerulatus* is a keystone species, in that its presence or absence determines in large part the possible array of other species (Fincke 1999). Because it avoids crossing large light gaps, *M. caerulatus* behaviour may effectively limit its dispersal ability between separated forest fragments. This raises the general question how

fragmentation and habitat specificity affects population dynamics and viability in this species. In this study, we use the RAMS technique (Randomly Amplified Microsatellites; Ender et al. 1996) to isolate microsatellite loci from very small amounts of DNA, provided by a single leg of a *M. caerulatus* individual (Fincke and Hadrys 2001). The RAMS protocol is a PCR-based strategy for isolation of microsatellite motifs from small amounts of tissue/DNA samples without construction and screening of genomic libraries.

DNA from three unrelated individuals was isolated from single legs in 400 µl homogenizing buffer containing proteinase-K; a single phenol–chloroform extraction was performed

(Hadrys et al. 1993). Total DNA was resuspended in 30 μ l low TE buffer containing 2 μ l RNase (0.5 mg/ml). Using 63 random 10 mer primers (Kits A, B, C, F, M, and AB; Operon Technologies Inc., Alameda, CA) we amplified some 1000 RAPD fragments for each of the three individuals. PCR reactions were performed in a total volume of 25 μ l containing 0.5 ng template DNA, 2 mM MgCl₂, 5 pmol random primer, 0.3 U Taq polymerase (Silverstar), 0.25 mM each dNTP, 1 \times buffer (Eurogentec). The amplification conditions (Perkin Elmer 9600) were 2 min at 90 °C followed by 40 cycles 20 s at 92 °C, 15 s at 38 °C, a ramp of 0.5 °C/s, 15 s at 72 °C followed by 2 min at 72 °C (e.g. Hadrys et al. 1993). Following the original RAMS protocol RAPD profiles were blotted onto positively charged nylon membranes and hybridised overnight to digoxigenin-labelled oligonucleotides (GAn, GTn, CAn and ATTn). In contrast to the original RAMS protocol, positive RAPD fragments were re-amplified directly from the membrane. A total of 36 fragments, which were of suitable size (200 to 1000 bp) and revealed a positive hybridisation signal, were cut out of the membrane and placed directly into a PCR tube. For the re-amplification, the same PCR conditions and primers were used as in the original amplification. Re-amplification of five fragments resulted in a smear of multiple banding patterns. Consequently we excluded these fragments from further analyses. To confirm that the re-amplified RAPD fragments indeed harbour microsatellite motifs, 10–30 ng of the amplification products were

directly cloned into the pGEM-T-Vector (Promega). DNA mini-preparations were made from positive clones of transformed JM109 *E. coli* cells and sequenced following Ender et al. (1996). This way we identified a total of 31 “positive” RAPD fragments which we cloned and sequenced. All of the 31 clones sequenced harboured a tandem repeat unit. Only 12 out of the 31 clones harboured microsatellite motifs of promising length (>6 repeat units). In sum, the major advantage of the above strategy was the direct cloning of positive RAPD-fragments instead of genomic DNA. No construction and screening of genomic libraries was necessary, which allowed non-invasive tissue sampling.

We designed primers for PCR typing for the 12 potentially polymorphic loci. Approximately 2 ng of genomic DNA were amplified in a total of 25 μ l reaction volume containing 1 \times buffer, 2 mM MgCl₂, 0.2 μ M of each primer, 100 μ M dNTPs and 0.35 U Silverstar Taq Polymerase (Eurogentec). Reaction conditions were as follows: 2 min denaturation at 92 °C followed by 40 cycles of 90 °C for 30 s, primer-specific annealing temperatures (see Table 1) for 30 s, a ramp of 3 s/1 °C to 72 °C for 15 s followed by a final elongation of 2 min at 72 °C. The PCR products were separated on 6% polyacrylamide gels and visualised by colorimetric detection following Ender et al. (1996).

The utility of the 12 new primer sets was tested by genotyping (i) 126 individuals from an island population in the Panama Canal (Barro Colorado Island, n = 104), an adjacent mainland popula-

Table 1. Locus name, primer sequences, annealing temperatures, allele, size range (bp), repeat motif, number of alleles per locus (N_A) and observed (H_O) and expected (H_E) heterozygosity rates of five non-invasively isolated microsatellite loci from *Megaloprepus caerulatus*. The loci have been tested by typing 126 individuals

Locus	Primer sequences (5'-3')	Ta (°C)	Allele size range (bp)	Repeat motif	N_A	H_O	H_E	GenBank Accession
MeAB 3/11	f: TGTAATGGTCTCTAGCC r: AAACGCAGACGAAGG	51	191–219	(TC) ₄ (GC) ₂ (TC) ₂ (TG) ₈	11	0.65	0.69 n.s.	AY507676
MeM 12/15	f: TATATGGACACACTGTGC r: TCGGTACAGTAGTTTGG	48	160–206	(CA) ₈	13	0.71	0.75 n.s.	AY484972
MeAB 3/16	f: GAATCTTCGCCAAAGC r: TTACCGCATTAACCTGG	52	176–198	(GA) ₈	8	0.40	0.58**	AY507677
MeAB 5/19	f: AAAGGCATTCTAATCG r: AAATGTTACAGTATAGGC	50	143–179	(GA) ₁₈	10	0.68	0.81**	AY507675
MeAB 3/12	f: CTTCCTCAAACCTTTTAT r: GCGTGTGTGAGAGGAATA	51	291–301	(GA) ₄ GT (GA) ₂	2	1.00	ND*	AY507674

Significance level = ** $P < 0.01$; ND* = not determined.

tion (Gigante, n22) and (ii) 72 individuals constituting 10 families (mother, father and 5–6 offspring per family) of known pedigree. Four loci appeared to be monomorphic, three loci produced unclear amplification patterns and five loci showed different degrees of polymorphisms. Table 1 summarizes the outcome of the PCR-typing for the five polymorphic loci. The number of alleles per locus ranged from 2 to 13 with an overall number of 44 alleles. The genotype ratios of the loci were tested for significant departures from Hardy–Weinberg expectations and for linkage disequilibrium using exact tests employing the Markov Chain method in GENPOP 3.1 c (Raymond and Rousset 1995). No significant linkage disequilibrium was found between any paired loci ($P > 0.05$). Two of the loci deviate significantly from Hardy–Weinberg expectations for genotype ratios (with P -values of 0.006 for locus MeAB5/19 and 0.002 for locus MeAB3/16). An excess of homozygotes was found for one (MeAB5/19) and two (MeAB3/16) of the most common alleles. The observed heterozygosity ranged from 0.40 to 0.71. The F_{is} estimates denote also a significant deficiency in heterozygous genotypes for the loci (MeAB3/16 = +0.31 and MeAB5/19 = +0.16; $P < 0.05$; Weir and Cockerham 1984). More extensive genotyping will indicate whether the excess of homozygotes is attributable to null alleles, non-random sampling, the mating system (limited dispersal ability) and/or inbreeding. None of the tested 126 individuals failed in PCR amplification of any of the loci. Furthermore, initial results from paternity testing indicate that loci conform to standard Mendelian segregation. 10 families of known pedigree (5–6 larvae per family) assigned all larvae correctly to their parents (scoring each adult/larvae combination). The estimated frequency of null alleles by using the program CERVUS 2.0 (Marshall et al. 1998) was also low and ranged from –0.064 to 0.031. The parental exclusion power of the loci combined when neither of the parents is known was 0.981 (CERVUS 2.0; Marshall et al. 1998). Furthermore we tested each of the primer pairs of the five loci in three other species of the Pseudostigmatidae (*Mecistogaster linearis*, *M. ornata* and *Coryphagrion grandis*). None of the primers amplified a product, even after various changes in PCR conditions.

Despite the low abundance of microsatellites in Odonata the presented non-invasively generated microsatellite system proved to be a sensitive tool for studying mating system correlates and conservation genetic issues in *M. caerulatus*. Our long-term goal is to understand how populations behave in areas of increasing fragmentation and possibly assess the potential that *M. caerulatus* offers as “indicator” species reflecting the health of forest communities more generally.

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References

- Ender A, Streit B, Städler T, Schwenk K, Schierwater B (1996) RAPD identification of microsatellites in *Daphnia*. *Mol. Ecol.*, **5**, 437–441.
- Fincke OM (1992) Interspecific competition for tree holes: Consequences for mating systems and coexistence in neotropical damselflies *The Am. Nat.*, **139**, 80–101.
- Fincke OM (1999) Organisation of predator assemblages in Neotropical tree holes: Effects of abiotic factors and priority *Ecol. Entomol.*, **24**, 13–23.
- Fincke OM, Hadrys H (2001) Unpredictable offspring survivorship shapes parental strategies, constrains sexual selection and challenges traditional fitness measures. *Evolution*, **55**, 762–772.
- Hadrys H, Balick M, Schierwater B (1992) Applications of Random Amplified Polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.*, **1**, 55–63.
- Hadrys H, Schierwater B, DeSalle R, Dellaporta SL, Buss LW (1993) Determination of paternity in dragonflies by Random Amplified Polymorphic DNA fingerprinting. *Mol. Ecol.*, **2**, 79–87.
- Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Mol. Ecol.*, **7**, 639–655.
- Raymond M, Rousset F (1995) GENPOP: Population genetics software for exact tests and ecumenicism. *J. Hered.*, **86**, 248–249.
- Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.