

POLYMORPHIC MICROSATELLITE LOCI FOR PATERNITY ANALYSIS IN THE FIDDLER CRAB *UCA MJOEBERGI*

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ABSTRACT

Microsatellite markers were developed for the fiddler crab *Uca mjoebergi* for use in paternity assignment. Five highly variable loci were found to amplify reliably in *U. mjoebergi*, although one locus exhibited evidence for the presence of null alleles, and two loci exhibited evidence of linkage. The number of alleles, expected heterozygosity and probability of paternity exclusion ($P > 0.999$) suggest that these markers when combined provide sufficient power to determine paternity by exclusion in this species. Three primer pairs were found to also amplify PCR products from three additional fiddler crab species (*U. dampieri*, *U. flammula* and *U. seismella*), and will prove useful for phylogeography and behavioural ecology of fiddler crabs.

KEY WORDS: fiddler crab, microsatellite, paternity, polymorphism, *Uca*

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INTRODUCTION

There are two types of mating in most fiddler crabs: burrow mating and surface mating. In burrow mating, females are extremely selective and choose their mates based on male size, wave rate (Backwell et al., 1999), wave leadership (Backwell et al., 1998), burrow quality (Backwell and Passmore, 1996) and other male behaviours (Reaney and Backwell, 2007). In surface mating, a female appears to be non-selective and will mate with her male neighbours if they approach her. Surface mated females almost always go on to burrow mate with another male before releasing a clutch of eggs. The mating success of individual males can therefore not be determined by clutch size alone. In order to obtain a more direct measure of male mating success, we report the development of highly polymorphic microsatellite markers for the fiddler crab *U. mjoebergi* Rathbun, 1924, and demonstrate amplification of loci from sympatric *Uca* species, including *U. damperieri* Crane, 1975, *U. flammula* Crane, 1975, and *U. seismella* Crane, 1975.

MATERIALS AND METHODS

Starting with *U. mjoebergi* muscle tissue dissected from claws preserved in salt-saturated DMSO (Amos and Hoelzel, 1992) or 40% ethanol, we obtained ~15 µg of high molecular weight DNA by proteinase K digestion and phenol chloroform extraction (Sambrook et al., 1989). Genomic libraries enriched for fragments containing GAAA and GA repeats were created using the method of Gardner et al. (1999) with modifications (Adcock and Mulder, 2002). Clones containing repeat sequences were identified with a PCR-based method and sequenced using an ABI 3100 automated sequencer according to the manufacturer's instructions. Primers were designed and manufactured (Prologo) to amplify 39 microsatellite sequences with four repeats or more in length and having flanking sequence suitable for primer design. These primers were tested for PCR amplification from *U. mjoebergi* genomic DNA templates. Screening reactions (10 µL) contained 5 pmol 5'-fluorescently labelled primer, 5 pmol unlabelled opposite strand primer, 10–40 ng of genomic DNA, 1.5–3.0 mM MgCl₂ and 0.5 U Taq (Scientific, Aust). Amplification began with one cycle at 96°C for 150 s, followed by 11 cycles at 94°C for 20 s, 70–50°C touchdown annealing over 11 cycles for 20 s and 72°C for 60 s, then 35 cycles of 94°C 20 s, 56°C 20 s, 72°C 60 s, then 72°C for 12 min, and final hold at 10°C performed on an Eppendorf Mastercycler

Gradient thermocycler. Primer pairs that consistently produced specific products showing size variability on a set of 10 individuals visualised on 2% agarose gels were analysed for polymorphism on an ABI 3100 automated sequencer using the ABI 500 LIZ size standard and ABI GeneMapper v3.7 software. One primer from each pair of "good" loci was directly fluorescently labelled and tested for polymorphism on a set of 144 individuals from a single population of *U. mjoebergi* collected from East Point Reserve, Darwin Australia (Reaney and Backwell, 2007).

RESULTS AND DISCUSSION

The GAAA and GA enriched genomic libraries yielded 72 and 89 positive colonies respectively. Of these 11 (15.3%) and 41 (46.1%) respectively were found to contain at least one di-, tri- or tetra-nucleotide repeat greater than four repeat units, and thus considered to constitute microsatellite loci. Twelve clones containing microsatellite loci did not include sufficient flanking sequences for primer design. Primer pairs were designed to amplify 40 microsatellite loci, of which seven primer pairs consistently produced specific products showing size variability in a set of 10 individuals. Five of these primer pairs were variable and amplified reliably from the population sample yielding 17 to 33 alleles among 144 individuals (Table 1). The primer pairs for the loci C361 and UCTTT8 performed well in duplex PCR reactions using equal primer concentrations. Tests for divergence from Hardy-Weinberg equilibrium (HWE) were performed using GenAIX6 (Peakall and Smouse, 2006). One locus, UCTTT28 exhibited an excess of homozygotes and significant deviation from HWE indicating the presence of null alleles. The pattern of allele sizes for all five loci suggests that considerable variation occurs due to indels and short mono-, di and tri-nucleotide repeats within the regions flanking the targeted repeat sequences, and this variation may contribute to the occurrence of null alleles as variation could be expected in the nearby sequences where primers were designed. Tests for linkage disequilibrium implemented in GENEPOP version 3.1b (Raymond and Rousset, 1995), indicated linkage between the loci C359b and CT155 ($P < 0.01$). No linkage was found between the other pairs of loci.

Table 1. Locus name, GenBank Accession, repeat sequence in the sequenced clone, allele size range over 144 samples, primer sequences 5' to 3', optimised MgCl₂ concentration (mM), number of alleles (N_a), observed (H_o) and expected (H_e) heterozygosities, and P -values of tests for divergence from Hardy Weinberg Equilibrium (HWE) for five microsatellite loci generated from *Uca mjoebergi*.

Locus	GenBank accession	Cloned repeat	Amplified allele size (bp)	Primer sequence 5' to 3'	MgCl ₂ (mM)	N_a	H_o	H_e	HWE P val.
C359b	EU703142	(AAAG) ₆ (GAGG) ₂ GAAT(GAAA) ₂ AA(GAAA) ₂ (GGAA) ₅ GACAA GAA(AGGG) ₄ (AAGG) ₃	358-442	Fwd: AAATAAAGCTCTGGACTATA CGACTTGTGC Rev: AATAATGGTAATGTTACG TTCAGCCATCTC	1.5	26	0.90	0.94	0.164
C361	EU703141	(CTGC) ₅ CTC(TCTG) ₆	323-370	Fwd: CTCTTCACCACTTCACTC TTTGTCAGCC Rev: TGAGCCAGACAGGTAAC TACAAAACGAGAC	2.0	17	0.83	0.85	0.957
CT155	EU703139	(GT) ₂₂	130-251	Fwd: ACCGCTACACCAGCCATAAC Rev: TGGAAATGAAGACCAGAAAAGG	3.0	33	0.84	0.95	0.164
UCTTT8	EU703138	(AC) ₈ TA(CA) ₃	128-173	Fwd: TTCGGATCCTAGACGTCACA Rev: GGTGAGGGGAGAGGAGGTGTT	2.0	33	0.92	0.73	0.261
UCTTT28	EU703143	(GA) ₁₀ GG(GA) ₃ GG(GA) ₆	283-402	Fwd: GCTTCGGATCGATAGAAACAA Rev: GAAAACAAGGAGTCGTGACAAA	1.75	30	0.63	0.96	<0.01

These markers are intended for use in paternity exclusion where the maternal and potential paternal genotype is known. The probability of exclusion where one parental genotype is known (equation 2a in Jamieson and Taylor, 1997) was greater than 0.999 when calculated for three loci, (C361), (UCTTT8) and (CT155), suggesting that these loci provide sufficient power to exclude paternity. The locus UCTTT28 was not included in this analysis as it was found to diverge significantly from HWE.

The five primer pairs were tested for amplification on five males each of six additional species of *Uca*, also occurring on the north coast of Australia. Of these species only three showed amplification for any of the primer sets, *U. dampieri* (2 males), *U. flammula* (2 males), *U. seismella* (3 males), while the remaining three species (*U. hirsutimanus* George and Jones, 1982, *U. capricornis* Crane, 1975, and *U. polita* Crane, 1975) did not amplify. Primers for the locus C359b amplified a product corresponding in size to a common *U. mjoebergi* allele from *U. dampieri*, *U. flammula* and *U. seismella*. Primers for the locus UCTTT8 amplified a product of corresponding size to the most common *U. mjoebergi* allele from *U. dampieri* and *U. seismella*, and a product corresponding to a further novel allele from *U. seismella*. Primers for the locus CT155 amplified two alleles from *U. flammula*, corresponding to alleles found in *U. mjoebergi*.

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