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Title: Characterization of polymorphic microsatellite loci in the western rock lobster (Panulirus cygnus)

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Abstract: Nine microsatellite loci were identified in the western rock lobster (Panulirus cygnus) using two different methods. The first method involved the screening of a small, fragment, partial genomic library with a radioactive (CA)6 probe. The second method, was based upon an enrichment method and used biotinylated, tetranucleotide microsatellite oligonucleotide capture probes. The nine loci described are all very polymorphic, with 11 to 34 alleles observed for each locus and heterozygosities ranging from 0.58 to 0.86. These microsatellite loci will be useful in analysing both the population structure and the mating systems used by this species and will add important information for the management of the wild stocks of this economically important species.

Characterization of polymorphic microsatellite loci in the western rock lobster (*Panulirus cygnus*)

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Running title: Microsatellite loci in western rock lobster

Abstract

Nine microsatellite loci were identified in the western rock lobster (*Panulirus cygnus*) using two different methods. The first method involved the screening of a small, fragment, partial genomic library with a radioactive $(CA)_6$ probe. The second method, was based upon an enrichment method and used biotinylated, tetranucleotide microsatellite oligonucleotide capture probes. The nine loci described are all very polymorphic, with 11 to 34 alleles observed for each locus and heterozygosities ranging from 0.58 to 0.86. These microsatellite loci will be useful in analysing both the population structure and the mating systems used by this species and will add important information for the management of the wild stocks of this economically important species.

The western rock lobster (*Panulirus cygnus*) is Western Australia's most important single species fishery with annual harvests over the last decade ranging between approximately 9 and 14.5 thousand tonnes per annum. The elucidation and understanding of population and mating structures for this species will allow the information obtained to be incorporated into an improved management strategy for the brood stock. In order to understand the population structure and mating systems of the western rock lobster, polymorphic, microsatellite genetic markers have been isolated and characterised. Two strategies were used to develop these microsatellites, the first consisting of the construction of a Hae III small fragment library which was screened with a (CA)n probe and the second library was made using an enrichment technique followed by screening with tetranucleotide microsatellite motifs. Nine microsatellite loci have been fully characterised and these are described in this report.

A DNA genomic library was made using Qiagen Tissue Kit (Qiagen) to isolate DNA extracted from tail muscle tissue. The DNA was digested with HaeIII restriction endonuclease (Promega, CA, USA) followed by excision of the 100-500bp region after agarose (1%) gel electrophoresis. Fragments were ligated into Sma I (Promega) digested, dephosphorylased pUC18 plasmid vector and transformed into competent cells (DH5α Invitrogen). Colonies were transferred to Hybond N+ membranes and screened with radiolabelled (CA)10 probe. Thirty clones were identified after the first round of screening. Following successive screening rounds to further isolate the clones, twenty of these were sequenced. Plasmid DNA was isolated from the positive clones and sequenced using cycle-sequencing combined with IRD_{800} labelled primers on a Li-Cor gene sequencer. Primers were designed using Mac Vector (Eastman-Kodak, USA) or PRIMER 3 software (Rozen and Skaletsky, 2000). Many of the loci identified contained insufficient flanking DNA sequences or produced microsatellites containing few repeat units. Three loci (WRL 1, WRL 2 and WRL 3) identified by using this method were deemed to be useful.

A second library enriched for tetranucleotide microsatellites was prepared using genomic DNA. Briefly, rock lobster DNA (20ug) was digested with either Hae III or RsaI restriction endonuclease and ligated to super SNX linkers (Glenn and Schable, 2005). The ligated DNA was amplified by polymerase chain reaction (PCR) using the super SNX 24 primer. The PCR product was heat denatured and hybridised to a pool of 5' biotinylated microsatellite motif ($GATA_5$, $GGAT_5$, $GAAA_5$ and $GACA_5$) oligonucleotides at $45^{O}C$. The hybridising DNA was captured on magnetic streptavidin beads and unbound or weakly bound amplified lobster DNA washed away using a series of stringency washes (final wash at 1 x X SSC at $50^{O}C$). The remaining captured DNA was recovered by heating the beads to $95^{O}C$ for 5 mins in 0.1X SSC buffer and removal of the magnetic beads using a magnetic tube station. The eluted DNA was then subjected to an additional round of PCR amplification using super SNX 24 primer as described by Glen and Schable, (2005). Following PCR amplification, the product was ligated into the plasmid (pGEM) using the pGEM T easy cloning kit (Promega) and used to transform *TOPO10 E. coli* by electroporation. *E. coli* colonies were grided onto 82 mm petri plates, lifted and screened using 5' end labelled (^{32}P) oligonucleotides. The genomic library enriched for microsatellites was screened using radiolabelled oligonucleotides identical to those used during the capture process. The result of this enrichment and screening process was the identification of over 70 clones containing identifiable microsatellite motifs. All four of the repeat motifs used in the enrichment process were identified in

P. cygnus. However, a relatively high proportion of the loci identified showed little or no flanking sequences and hence were unsuitable for further primer design. Furthermore, several clones had very large-sized repeat elements, which were not amenable to PCR amplification and so were not developed any further.

Tissue collected into 70% ethanol from a population of lobsters collected from the Juren Bay area north of Perth representing one age group (late stage juvenile) were used for analysis. DNA was extracted lobster muscle tissue using Qiagen Tissue Extraction kit according to the manufacturer's instructions (Qiagen). The PCR primers used in this study were made by Geneworks (Adelaide, Australia). PCR reactions were performed in an Eppendorf thermal cycler and comprised the following reaction mixture; approximately 20 ng of lobster DNA, 1 x PCR buffer (Invitrogen), 0.5 U Platinum Taq (Invitrogen), 1.5 mM $MgCl_2$, 2 mM each dNTP, xxug of BSA (Roche Molecular Systems) and 1 pmol of labelled forward primer (IRD_{800} LiCor, USA or Beckman), 5 pmol of forward and reverse primers as described in Table 1. The PCR cycling parameters were as follows; denaturation at 95^OC for 5 min followed by 35 cycles of 95^OC for 20 sec, T_{ann} for 30 sec and 72^OC for 1 min, followed by a final incubation at 72^OC for 5 mins. The cycling protocol was as follows: 5 min at 95^0C, 10 sec at annealing temperature, followed by 72^0C for 30 sec, next 8 cycles at 94^0C for 30 sec, annealing temperature for 10 sec and 72^0C for 30 sec, then 24 cycles of 94^0C for 20 sec, annealing temperature for 10 sec and 72^0C for 30 sec. Next, 92^0C for 20 sec, annealing temperature for 10 sec and a final extension of 72^0C for 5 min completed the run. Fragment sizes were determined with reference to standard base pair ladder or an in-house generated allelic ladder. Genepop 4.0 (Raymond and Rousset 1995) was used to calculate heterozygosities, Fis and observed deviations from Hardy-Weinberg equilibrium.

Primers were designed for twelve loci, for which nine loci gave consistently reliable amplification and displayed a relatively high degree of polymorphism. The three loci identified from the screening of the small insert library with the (CA)10 oligonucleotide probe (WRL 1, WRL 2 and WRL 3) and six tetranucleotide motif containing loci identified through specific sequence capture showed robust amplification reactions. The primer sequences for these nine loci (WRL 1, WRL 2, WRL 3, S3, S8, S28, S36, S50 and W25) are described in Table 1 together with their associated GenBank accession details. The number of observed alleles for each locus ranged from 11 to 34 and the observed heterozygosity varied from 0.58 to 0.86. Deviations from Hardy-Weinberg equilibrium were detected in 8 of the 9 loci tested. The majority of these deviations were also observed in three other geographically independent populations (data not shown). Analysis of the data using MICROCHECKER (Van Oosterhout *et al.,* 2004), predicted "null" alleles for all of the loci that showed deviations from HWE. The predicted "null" allele gene frequency estimates (Chakraborty calculation) was 0.14, 0.21, 0.12, 0.16, 0.22, 0.3, 0.10, 0.23 for the loci WRL 1, WRL 2, WRL 3, S3, S8, S36, S50 and W25 respectively. However, it is interesting, that apart from WRL1, no other homozygous "null" genotypes were observed in this data set. Furthermore an additional 283 samples genotyped with S50 showed no homozygous "nulls". Based upon the estimated gene frequency (excluding WRL 1) approximately 14 homozygous "null" genotypes would be expected in the first data set and an additional two in the second data set (S50). In addition, when these nine loci were used to genotype eggs and parental tissue derived from a tank based mating experiment comprising four

independent family groups a simple autosomal Mendelian inheritance was shown for all loci. No significant linkage disequilibrium was observed between the loci in the populations studied.

Deficiency of heterozygotes in marine invertebrates is not uncommon (Maggioni and Rogers, 2001; Puebla, Parent and Sevigny, 2003; Shearer and Coffroth 2004; Damodaran, Karunasagar and Karunasagar, 2008; Feng and Li, 2008; Costantini, Fauvelot and Abbiati, 2007). The reason for the observed departure from HWE seen in this species may be a result of unrecognised polymorphisms in one or both of the primer sequences leading to the presence of an apparent "null" allele. However, as we did not observe any homozygous "null" genotypes for most of the loci, together with the high estimated "null" allele frequency, there may be an alternative reason for the departure from HWE in this species such as undetected Walhund effects. One possibility is that the population studied is not randomly mating or is under some form of selective pressure, such as selection for zygote viability, resulting in an apparent increase in homozygotes. This phenomenon has been observed in other marine species (Reece *et al*., 2004; Launey and Hedgecock, 2001; Malmquist *et al*., 2002). As these microsatellites will be used to understand the mating system in this species through the identification of spermatophores deposited on females during the mating process, we have calculated that the probability of two samples being identical at all nine loci is less than 1 in 10,000 and in most cases is considerably lower.

Acknowledgments

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table

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Table 1. *Panulirus cygnus* microsatellite loci showing name of locus, GENBANK accession number, sequences of forward (F) and reverse (R) primers with labeled primer indicated *; the repeat motif, the annealing temperature (^{o}C), size of the cloned PCR product in base pairs, the number of individuals genotyped (N), and the number of alleles detected (Na). Observed (H_o) and expected heterozygosities (H_e) and probablility of deviation from Hardy-Weinberg equilibrium (HW) using Weir and Cockerham (1984), Fis Nei's (1987) *F*-statistics are presented.

Locus ID	GenBank Accession number	Primer sequence (5'-3')	Repeat motif	Annealing Temp (^{o}C)	Size (bp)	N	Na	H_o	H_e	HW	Fis
WRL 1	FJ001813	F- CCACAGTCACACGTGCATGG* R- GAACCCAAACCTGGGGTGTT	(CA)	55	143	41	15	0.61	0.82	<0.01	0.273
WRL 2	FJ001814	F- CAACTTCCGACATTACGTCT* R - GGATACCTGGCTCAGGTTGG	(CA)	55	116	41	21	0.58	0.93	<0.01	0.335
WRL 3	FJ001815	F – GCTCACAACAGAAAATGAATATCGG* R-GAAAGAGTTGAACGTTGGTGAGTATG	(GT)	55	125	41	21	0.73	0.93	<0.01	0.138
S3	EU152080	F:TTGCGTCGAGAAGCATTAGA* R:CATCCACCTAGCCAACCAAA	(GGAT)	58	248	64	22	0.78	0.89	0.01	0.246
S8	EU152081	F:CATTCTTCGAACAGTCCATATAA* R: TGGAAGGAGCAACTGGAAGT	(GATA)	50	226	64	34	0.77	0.97	0.02	0.391
S28	EU152082	F:GGAAGGGGTGGGTTTACTGTAATG* R: CATCCGCTCTGTTATGAGTCATC	(GACA)	55	251	64	12	0.78	0.81	ns	0.071
S36	EU152083	F: GGTCTGTCGACCTCTTTTCCT* R: TCGAACACGGGACCTTATGT	(GATA)	50	165	64	14	0.79	0.91	<0.01	0.333
S50	EU152084	F: GAGGTGTGCGTCATGACAAG* R: TCTTCGGTTACACCCGTCTCT	(GACA)	48	217	64	30	0.86	0.97	<0.01	0.213
W25	EU152085	F: TCGTCCGTCACCGCCACCGA* R: CCAGTGAGCAATTACCAG	(GAAA)	53	205	64	11	0.64	0.72	<0.01	0.294

3rd June 2009

Dr Goossens (Editor-in-Chief)
Conservation Genetics Resources
Conservation Genetics Resources Editorial Office,
Springer Netherlands

Dear Dr Goossens,

Please find attached for submission in Molecular Ecology Resources a paper describing the identification and analysis of nine microsatellite loci in the Western Rock Lobster. This paper describes nine loci of which, 8 are showing deviation from HWE and we believe reflects a biological phenomenon in this species, Walhund effects or more controversially inbreeding due to distorted sex ratios and extensive fishing pressure. The loci will be used for more extensive studies on the population genetics of this species as well as being used to analyse the mating system in this species.

Yours sincerely

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