

Microsatellite loci for the blue swimming crab (*Callinectes bellicosus*) (Crustacea: Portunidae) from the Gulf of California, Mexico

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Abstract Twelve microsatellite markers (six di-nucleotides, four tri-nucleotides and two tetra-nucleotides) were isolated and characterized for *Callinectes bellicosus*, a commercial crustacean species from the Gulf of California, Mexico. One locus was monomorphic and 11 loci were polymorphic in 32 individual samples from a single location. Overall polymorphic loci, the number of alleles per locus ranged from 2 to 24 (average 10.0), the observed heterozygosity ranged from 0.094 to 0.969 (average 0.603), and the expected heterozygosity varied from 0.089 to 0.935 (average 0.597). One locus deviated significantly from Hardy–Weinberg equilibrium due to an excess of heterozygotes, while another locus showed evidence for the presence of a null allele. No evidence of linkage disequilibrium was found

among pair of loci. These markers will be helpful to estimate the level of genetic connectivity over a small spatial and temporal scale in order to identify stocks for the management of this small-scale fishery in the Gulf of California.

Keywords Gulf of California · Fisheries · *Callinectes bellicosus* · Blue swimming crab · Connectivity · Microsatellites

The blue swimming crab, *Callinectes bellicosus*, is a decapod crustacean with a geographic distribution from San Diego (California) to Oaxaca (Mexico), including the Gulf of California (Hendrickx 1995). The species reaches up to 17 cm of carapace length and 0.5 kg of fresh weight, and is relatively abundant in shallow coastal lagoons up to 55 m in depth with sandy and muddy bottoms, and eelgrass (*Zostera marina*) and mangrove zones.

The blue crab fishery, one of the main fisheries in the US (Rosenfield 1998), has experienced significant fluctuations due to overexploitation and habitat deterioration (Engel and Thayer 1998; Jordan 1998). This has produced a growing demand for crab products from other countries. Crab exports (*Callinectes* sp. and *Portunus* sp.) have increased mainly from Asia and Latin America to the US (Oesterling 1998). In 1995, México was first in exports from Latin America, and second in the world, with 1,500 t of crabmeat valued at US \$16 million. In Mexico, the crab fishery started in the Gulf of Mexico, and developed in the Gulf of California on a larger scale in the mid 1980s (Gonzalez-Ramirez et al. 1996; Molina-Ocampo 2000). In order to accomplish a better management of *C. bellicosus*, which constitutes 95% of the capture in the Gulf of California, it is important to identify the spatial distribution of distinct stocks. We isolated 12 microsatellite loci to

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estimate the level of genetic connectivity over a small spatial and temporal scale relevant for management of this commercial fishery.

Genomic DNA was extracted from five individuals using the DNeasy Blood & Tissue Kit (Qiagen). These DNAs were pooled and used to create microsatellite-enriched libraries following a modified version of Glenn and Schable (2005). Approximately 5 µg of genomic DNA was digested with *RsaI* (NEB), and the resulting fragments ligated to SuperSNX-24 linkers. Linker-ligated fragments ranging 300–1,200 bp were recovered by the polymerase chain reaction (PCR), using the SuperSNX-24 forward primer and Platinum high-fidelity Taq DNA polymerase (Invitrogen). Linker-ligated fragments were then hybridized to 5'-biotinylated microsatellite oligonucleotide probes (GT)₁₅, (CT)₁₅, (GTAT)₁₀ and (GTCT)₁₀ (1 µM each). Enriched fragments were isolated with streptavidin-coated magnetic beads (Dyna, Invitrogen). Polymerase chain reactions (PCR) were carried out as described above to recover

fragments containing potential microsatellites. Libraries were ligated into the vector PCR4-TOPO (Invitrogen), transformed into TOP10 chemically competent *Escherichia coli* cells (Invitrogen) and plated on Invitrogen imMedia™ Amp agar. One-hundred and ninety-six clones were amplified by PCR using M13 primers under standard PCR conditions, visualized on 2% agarose gels and suitably sized inserts (>500 and <1,500 bp) were selected for sequencing in both directions on an Applied Biosystems 3730XL DNA Analyzer using the BigDye Terminator Cycle Sequencing Kit. One-hundred and sixty-three (83.1%) of the clones had recognizable microsatellite sequences, but from those only 40 represented unique clones and had adequate flanking regions to design primers using PRIMER3 (Rozen and Skaletsky 2000). The universal M13 primer was added at the 5' end of the forward primers to allow fluorescent labeling of the amplicons using M13 labeled oligonucleotides (Schuelke 2000). Reverse primers were designed with a "pig-tail" at

Table 1 Twelve microsatellite loci cloned from *Callinectes bellicosus*, including: locus name, GenBank accession number, repeat motif found in the cloned allele, primer sequences, clone size, size range of

observed allelic variation, number of alleles (N_A), and observed (H_O) and expected (H_E) heterozygosities

Locus genbank	Repeat motif	Primer sequences (5'-3')	Clone size (bp)	Size range (bp)	N_A	H_O	H_E
<i>Cabe28</i> GU338216	(GT) ₃₆	F: GAATGTGAGAAGCAGTGATGC R: GAGACGGAGGGAGGAAGAAG	265	255–273	4	0.710	0.575
<i>Cabe30</i> GU338217	(CA) ₁₉	F: AACGCAGCAGGATAATACCG R: CATTTCAGCTTGCGTGAGTG	179	157–169	6	0.387	0.384
<i>Cabe31B</i> GU338218	(CCA) ₇	F: CGTGCAGATCAAGGGTGAAT R: ACGGGCAGGTTGTAGTGGTA	135	132	1	0.0	–
<i>Cabe44</i> GU338219	(TAG) ₃₂	F: TGGAACAGTAGTAGGTAGTAGCTGTA R: GGTGTCATCATTACTATTATCAATATT	197	171–213	14	0.733	0.893
<i>Cabe45B</i> GU338220	(CA) ₉	F: CAGCAGCAGCGGTAGTAGTG R: GCTAGCCTTCGTTCAACAAGC	178	176–178	2	0.094	0.089
<i>Cabe55</i> GU338221	(GT) ₁₁	F: GATTCTCATCATCTATATGTATGTATG R: TCCACCTCAAGGGACTCA	194	196–198	2	0.097	0.092
<i>Cabe78</i> GU338222	(GA) ₃₃	F: TCCTCTTTCTCTCTTCACGA R: AGTAACGCTACCACGCCAAC	182	149–205	21	0.903	0.935
<i>Cabe80B</i> GU338223	(GTT) ₁₈	F: TGGTTGGGAAATGTGATCTG R: TGAGGGTGAATAATTTACAGAAAAGA	179	156–222	17	0.696	0.896
<i>Cabe2</i> GU338224	(GTAT) ₉ (GT) ₃	F: GAGTCTCTTTCTCTCTGTGTGTATG R: TGATAGAAGGTCGAAATTCAAATG	263	191–203	5	0.406	0.470
<i>Cabe73</i> GU338225	(GCCT) ₃ (GTCT) ₁₀	F: TGGCAAGATACTTCATGATTCC R: CTGGCTAAGGGCAACAAAAA	266	239–305	13	0.767	0.781
<i>Cabe81</i> GU338226	(CAA) ₅	F: CTACCCTTCCACCCATC R: AGGTCTAACGGCCTCTTGCT	128	123–135	3	0.969	0.514*
<i>Cabe90</i> GU338227	(CA) ₁₄	F: AGGCAGACGGCTAGACTGAC R: TGAGCGTGACCAGAGTGAAG	217	250–308	24	0.875	0.933

* Locus that deviated significantly from HWE after Bonferroni correction (adjusted critical $P < 0.0041$)

the 5' end to reduce variability in adenylation of amplification products (Brownstein et al. 1996).

PCRs were performed in 15 µl volumes containing ~50 ng genomic DNA, 1× PCR buffer, 0.2 mM each dNTP, 0.02 µM of the unlabeled M13-tailed forward primer, 0.2 µM of the reverse and 0.2 µM of the fluorescently-labeled M13 primers, 1.5 mM MgCl₂ (except loci *Cabe28* and *Cabe55* for which 3 mM MgCl₂ were used), 0.5 U taq DNA polymerase (Invitrogen), and 0.2% BSA. All loci, except locus *Cabe28* and *Cabe55*, were amplified with a touchdown protocol that included an initial denaturation at 94°C for 5 min, 15 cycles at 94°C for 30 s, annealing at 65–50°C for 30 s (1°C decrease in each cycle), 72°C for 30 s, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension of 72°C for 5 min. Loci *Cabe28* and *Cabe55* were amplified by an initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension of 72°C for 5 min. Microsatellite genotyping was performed on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Allele sizes were estimated by GENOTYPER 3.7 (Applied Biosystems) and classified into bins with FLEXIBIN (Amos et al. 2007). Data were analyzed using GENALEX 6.2 (Peakall and Smouse 2006) to calculate observed and expected heterozygosities, deviations from Hardy–Weinberg equilibrium (HWE) and probability of identity. Tests for linkage disequilibrium (LD) between pairs of loci were conducted in FSTAT (Goudet 1995). MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) was used to check for scoring errors and null alleles. Adjusted P values were obtained using a sequential Bonferroni test for multiple comparisons (Rice 1989). The experiment-wise error rate was predefined at 0.05.

Twelve loci successfully amplified and were scored on 32 individuals collected from San Jorge island, Sonora, in the Gulf of California, Mexico (Table 1). One locus (*Cabe31B*) was monomorphic. Overall polymorphic loci, the number of alleles per locus ranged from 2 to 24 (average 10.0). Observed heterozygosity ranged from 0.094 to 0.969 (average 0.603), and expected heterozygosity varied from 0.089 to 0.935 (average 0.597). A significant deviation from HWE was detected only at locus *Cabe81* using a Chi-square test, due to an excess of heterozygotes. The distribution of homozygote-size classes suggested the presence of a null allele at locus *Cabe80B* with an estimated frequency of 0.111 (Van Oosterhout et al. 2004). No significant linkage disequilibrium was detected among the tests for each pair of loci (all adjusted P values > 0.0007). The probability of identity calculated combining all loci was 1.65×10^{-11} .

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