

Identification and characterization of microsatellite loci in the spiny spider crab *Maja brachydactyla*

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Abstract The spiny spider crab *Maja brachydactyla* is an important fishery resource throughout its distribution range (Northeast Atlantic). Here we describe the isolation of nine microsatellite loci for this species. These new markers were tested in 20 crabs from NW Spain. The number of alleles ranged from 3 to 20 and expected heterozygosity from 0.57 to 0.95. All loci followed Hardy–Weinberg expectations except mMb33 and mMb307, which could be affected by null alleles. Some of these loci seem to be better fitted by a multi-step substitution process.

Keywords microsatellites · *Maja brachydactyla*

The spiny spider crab *Maja brachydactyla* is a decapod crustacean that inhabits the Northeast Atlantic from the British Isles to Angola. This crab is an important fishery resource throughout its distribution range, enduring high exploitation levels. In order to assess the level of genetic diversity in this species we have developed primers for nine microsatellite loci. The genetic data presented here constitute the first population study in this crab. Understanding the genetic status of this species will be very important to develop rational conservation management plans.

Spider crabs were collected in Galician coasts (NW Spain) and samples of muscle tissue were stored in 100% ethanol. Genomic DNA was extracted by a standard phenol/chloroform protocol (Taggart et al. 1992). Microsatellites were isolated according to the following protocol

described by Bloor et al. (2001). Each DNA extract was digested with blunt-end cutting enzyme *Hae*III (Amersham Biosciences). Digests were run in 1.5% agarose gels stained with ethidium bromide. Fragments between 400 and 800 bp were excised from gels and purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). These fragments were ligated to double-stranded adaptors (Oligo A: 5'-CTCTTGCTTACGCGTGGACTA-3' and Oligo B: 5'-pTAGTCCACGCGTAAGCAAGAGCACA-3', Invitrogen) overnight at 4 °C using T₄ ligase (Promega), so they could be selected and enriched by PCR using Oligo A. Amplification was performed in a GeneAmp PCR system 9700 (Applied Biosystems) in a final volume of 20 µl: 1 µl of ligation reaction, 2 µl of 10× PCR buffer (160 mM (NH₄)₂SO₄, 670 mM Tris–HCl pH 8.8, 0.1% Tween 20), 1 µl of 50 mM MgCl₂, 2 µl of 200 pmol/µl Oligo A, 1 µl of 10 mM dNTP Mix (Applied Biosystems), 0.2 µl BIOTAQ polymerase (5 U/µl, Bio-line). Conditions were as follows: initial denaturing at 95 °C for 2 min, 25 cycles of 95 °C for 20 s, 60 °C for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Purified PCR products were denatured and incubated with 200 pmol of 5' biotinylated (CT)₁₂ and (GT)₁₂ probes (Invitrogen) attached to streptavidin-coated magnetic beads (Streptavidin MagneSphere Paramagnetic Particles, Promega). Hybridization was carried out in 6× SSC for 30 min at 60 °C, in the thermocycler. Specific fragments were recovered after washing beads suspension with solutions progressively desalted at 60 °C, and, subsequently amplified using Oligo A, in a final volume of 50 µl under the following conditions: initial denaturing at 95 °C for 3 min, 5 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, 30 cycles of 92 °C for 30 s, 60 °C for 30 s, 72 °C for 55 s, and a final extension step at 72 °C for 30 min. After purification, PCR products were ligated into

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a pGEM-T Vector System (Promega), and transformed, by heat-shocking, into *Escherichia coli* JM109 (High Efficiency Competent Cells, Promega). LB/ampicillin/IPTG/X-gal plates were incubated overnight at 37 °C to identify recombinant colonies by blue/white selection. White colonies were picked and inserts were released by PCR using oligonucleotides with sequences complementary to the T7 and M13 sites of the vector (5'-TAATACGACTCACTATAGG-3' and 5'-GGAAACAGCTATGACCATG-3', respectively, Invitrogen). Reactions were made in 50 µl with a profile of initial denaturing at 95 °C for 2 min, 30 cycles of 95 °C, 50 °C, 72 °C for 30 s each, and a final extension step at 72 °C for 7 min. Isolated clones in the range of selected sizes were sequenced using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and the T7 primer. Sequences were run in an ABI PRISM 310 Genetic Analyzer and visualized using the DNA Sequencing Analysis 3.7 software (Applied Biosystems). Repeat motifs were screened by eye and the clones containing appropriate repeat sizes for efficient amplification and appropriate flanking regions for primer design were selected.

A total of three enriched libraries were constructed, 367 colonies tested, and 193 clones sequenced, 127 of which were positive for microsatellite motifs. Primer pairs for 14 sequences were designed with the program Designer PCR 1.03 (Research Genetics). Nine of these were successfully amplified and showed polymorphism: mMb9, mMb12, mMb15FAM, mMb15NED, mMb23, mMb33, mMb307, mMb339, mMb403 (GenBank accession numbers: DQ398862–DQ398870). All loci contain dinucleotide repeats except mMb12, which presents a trinucleotide motif. In fact, (GT)₁₂ probe was the most successful, with only the compound locus mMb33 containing (CT) and (GT) repeats. The forward primer of each pair was 5' fluorescently labeled (FAM, HEX or NED, Applied Biosystems) for screening of alleles on the ABI 310 sequencer. The GeneScan Analysis

3.7 program (Applied Biosystems) was used to genotype individuals, with GeneScan 400HD ROX as internal size standard.

Amplifications were optimized separately for each locus in a GeneAmp PCR system 9700 or a 2270 Thermal Cycler (Applied Biosystems). In this case, genomic DNA from employed samples was extracted using Chelex resin (Estoup et al. 1996). For the six first loci, reactions were carried out in 20 µl while in 10 µl for the three remaining loci. Standard composition for 20 µl reactions was considered: 1 µl of DNA sample, 2 µl of 10× PCR buffer (160 mM (NH₄)₂SO₄, 670 mM Tris–HCl pH 8.8, 0.1% Tween 20), 1 µl of 50 mM MgCl₂, 1 µl of each primer (8.3 µM), 1 µl of 10 mM dNTP Mix (Applied Biosystems), 0.2 µl BI-OTAQ polymerase (5 U/µl, Bionline) and proportionally a half of each product for 10 µl reactions. Departures from these standards, as well as the specific PCR program for each locus, are indicated in Table 1. First and final steps were common for all loci: 95 °C for 5 min and 72 °C for 7 min.

The new markers were tested in 20 crabs from NW Spain. Genetic indexes were calculated with Genepop 3.4 (Raymond and Rousset 1995) (Table 2). Number of alleles ranged from 3 to 20; allele size ranged from 132 to 351 bp, and H_O and H_E ranged from 0.30 to 1 and from 0.57 to 0.95, respectively. After sequential Bonferroni correction (Rice 1989), only mMb33 and mMb307 showed significant departures from Hardy–Weinberg equilibrium. These heterozygote deficiencies could be due to the presence of null alleles, as suggested by Micro-Checker (Van Oosterhout et al. 2004). No linkage disequilibrium was detected among the different loci after Bonferroni correction (Fisher's exact test). The population parameter θ ($4N_e\mu$), where N_e is the population effective size and μ is the mutation rate, was estimated by maximum likelihood with the program MISAT (Nielsen 1997), ranging from 1.32 to 143.44 (Table 3). A likelihood ratio test contrasting

Table 1 PCR characteristics of isolated microsatellite loci in the spiny spider crab *M. brachydactyla*

Locus	Composition				Reaction volume	Cycle						Number of cycles
	DNA	Primers	MgCl ₂	Taq		D	t	A	t	E	t	
mMb12		0.5			20	95	20	50	20	72	20	35
mMb15FAM		0.7	0.5									
mMb23			0.75									
mMb33	0.7	0.5	0.75				10	45/48	10		12	10/20 ^a
mMb9							15	67	15		15	30
mMb15NED		0.5	0.5	0.1			10	52	10		12	
mMb307			0.25	0.08	10							
mMb339				0.05								
mMb403												

Reaction composition, volume is indicated in µl; Cycle, D, A, E, denaturing, annealing and extension temperatures in °C, respectively; t, time is indicated in seconds

^a PCR profile for mMb33 consists of 10 cycles at 45 °C followed by 20 cycles at 48 °C

Table 2 Characteristics of isolated microsatellite loci in the spiny spider crab *M. brachydactyla*

Locus	Primer sequence (5′–3′)	Repeat unit	Size	Range	A	H _O	H _E	HWE
mMb9	CACGGAGACAAGTCCTGTA AGTAGGCACCCTTTGAAC	(AC) ₁₆	350	347–351	3	0.65	0.57	0.4448
mMb12	TGGAACCTCTCTTTTCAGGTAAC TGGACTAAACAATCGTTGGTAA	(AAC) ₁₄	182	168–204	10	0.60	0.76	0.3235
mMb15FAM	CCCTCCAGTCTGTATGAG CGTTCTGTTTTCCAGTTAC	(TG) ₁₉	137	132–190	8	0.60	0.79	0.0209
mMb15NED	AACGGTGTGTTGATGTGTGG GGTCCCCCTCTGATTTTGAT	(AC) ₂₃	250	239–293	20	1	0.95	1
mMb23	TAACTACACAGCAGTAT GGTATTTGTTGTTGATAA	(AC) ₈ AAA (AC) ₅ AT(AC) ₆	242	238–244	4	0.45	0.58	0.1941
mMb33	GCTCGTGGTTTTGTCTTG TTACCTTATCCTTTGGGAGAGT	(CT) ₁₇ (CA) ₁₇	194	183–209	10	0.30	0.92	0.0000*
mMb307	GCCAGGCTACGAACATTGTA GTAACCCAGCATTGTCAG	(AC) ₁₇	178	162–180	7	0.53	0.82	0.0051*
mMb339	GGAATCTCAAAGCGTTTATCAG TTGCTCAAGAAGGACAGTGA	(AC) ₁₈	205	195–229	12	1	0.89	0.7825
mMb403	GAGGCAAGTCCATTTTACCT AGATGCTAACCGCTCGTC	(GT) ₃₆	278	230–284	13	1	0.88	0.9149

Locus name; Primer sequence, up forward and down reverse; Repeat unit and size in sequenced clone; Size range over all samples; A, number of alleles per locus; H_O, H_E, observed and expected heterozygosity; HWE, *P*-values of test for deviations from Hardy–Weinberg equilibrium, significant values (*P* < 0.05) are indicated with an asterisk

one-step and multi-step models of microsatellite evolution (Nielsen 1997) indicated that some loci are best fit by a multi-step substitution process (Table 3). In general, the high levels of polymorphism detected at these loci indicate their usefulness to study this species along its distribution range.

We also evaluated cross species amplification with other species of the genus that inhabit Mediterranean coasts: *M. squinado* (N=4), *M. crispata* (N=4) and *M. goltziana* (N=1). Six loci amplified successfully in all species (mMb9, mMb15FAM, mMb15NED, mMb23, mMb339 and mMb403), while mMb12 only amplified in *M. crispata*. Loci mMb33 and mMb307 did not amplify in any species.

Table 3 Estimates of the population mutation parameters and mutation model

Locus	One-step model		Multi-step model			LRT ^a
	$\hat{\theta}$	$l(\hat{\theta})$	$\hat{\theta}$	\hat{q}	$l(\hat{\theta}, \hat{q})$	
mMb9	1.32	-7.96	1.23	0.01	-8.01	-0.10
mMb12	15.25	-27.18	10.87	0.03	-25.64	3.08
mMb15FAM	32.85	-31.98	28.58	0.01	-28.66	6.64*
mMb15NED	143.44	-65.31	64.58	0.11	-56.64	17.34*
mMb23	2.10	-8.65	1.75	0.01	-8.75	-0.20
mMb33	35.13	-31.57	26.80	0.06	-29.28	4.58*
mMb307	6.47	-23.55	7.67	0.01	-23.84	-0.58
mMb339	61.08	-42.56	36.60	0.03	-33.32	18.48*
mMb403	65.20	-47.67	23.07	0.03	-37.13	21.08*

$\hat{\theta}$, population mutation parameter; \hat{q} , proportion of multi-step mutations; $l()$, log of the maximum likelihood; LRT, likelihood ratio test comparing the one-step (null hypothesis) and multi-step models; significant values (*P* < 0.05; χ^2 with 1 d.f.) are indicated with an asterisk

^aSome negative LRTs are due to imprecision on the likelihood calculations

Although variation wasn't assayed, we observed some differences in size between species for some loci.

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