Arch. Biol. Sci., Belgrade, 67(4), 1369-1376, 2015 DOI:10.2298/ABS141205115A

ISOLATION AND IDENTIFICATION OF A NEW SET OF MICROSATELLITE LOCI FROM *UCIDES CORDATUS* GENOME

Erlane S. Araújo[1], Fábio B. Britto[1], Adriana M. F. Carvalho[2], Gleice R. Orasmo[1] and Fábio M. Diniz[3,*]

[1] *Department of Biology, Federal University of Piauí, Campus Agrícola da Socopo*, Teresina, Brazil.

[2] *Department of Animal Sciences, Federal University of Piauí, Campus Agrícola da Socopo*, Teresina, Brazil

[3] *EMBRAPA Meio-Norte*, Teresina, Brazil

*__Corresponding author__: fabio.diniz@embrapa.br

Abstract: A new set of microsatellite loci (simple sequence repeats, SSRs) from the overexploited mangrove crab *Ucides cordatus* is described in this study. Microsatellite isolation used a highly simplified and inexpensive protocol based on (i) multiple enzyme digestion/ligation; (ii) mixed biotin-labeled probes and streptavidin-coated magnetic bead hybridization capture strategy, and (iii) a double-repeat-enrichment procedure. A genomic library, double-enriched for inserts containing tetranucleotide repeat motifs [$(GACA)_6$, $(GATA)_7$, $(GGAT)_5$ and $(GTAT)_5$], was constructed to increase the chance of recovering SSR-containing sequences within DNA fragments. Amplified enriched DNA was cloned and transformed into competent *E. coli*. Then, positive clones were identified by 'white/blue plaque selection'. One hundred and five colonies were PCR-screened for sequencing, and 72 of these were found to have unique SSR inserts. Microsatellite motifs contained more than five repeats, and most loci were found to have perfect tandem repeats (51.4%), of which 94.4% were dinucleotide and 5.5% trinucleotide. Only 20% of all loci were compound and 28.6% were imperfect repeats containing di-, tri- and/or tetranucleotides. The high frequency of perfect repeat motifs after enrichment is additional evidence of the importance of adopting this procedure for the isolation of SSR. The novel 34 SSRs described in this study are expected to be highly polymorphic and, therefore, useful in population/stocks discrimination of this valuable mangrove species throughout its range, currently subjected to excessive fishing efforts.

Key words: microsatellite loci; short tandem repeats; double enrichment; molecular markers; genetic diversity

Received: December 5, 2014; **Revised:** February 21, 2015; **Accepted:** February 23

INTRODUCTION

The mangrove crab *Ucides cordatus* (Crustacea: Decapoda: Brachyura), popularly known as *Uçá*, is among the most important invertebrates of estuarine (mangrove) habitats of the Americas, distributed from the east coast of the USA (Florida) to Southern Brazil (Santa Catarina) (Amaral et al., 2014; Melo, 1996; Coelho and Ramos 1972).

Due to its wide geographical distribution, the different populations of this crab species may become adapted to the specific environmental conditions of the regions they inhabit. Therefore, intense exploitation (overfishing) and habitat degradation may threaten these separate populations with a serious risk of extinction. The human impact on its natural habitat can lead to a decline in population in terms of abundance, and a possible loss of distinct fishing stocks (Oliveira Neto et al., 2007; Avise and Hamrick, 1996).

Studies on genetic variation and population structuring of estuarine invertebrate populations are important to assist programs in conservation and to maintain the sustainable management of fishery resources. Population genetic parameters estimated by the use of molecular markers can be applied in the conservation of a threatened species and in the development of a fishery regulation aimed at sustainable use of fishing stocks. These markers are useful in the detection of populations that have different magni-

tudes of genetic variability and thus require different management strategies (Avise and Hamrick, 1996). In this context, DNA-based molecular markers are being more often used in the conservation of genetic resources of commercial interest (Carvalho et al., 2013; Larraín, 2012; Diniz et al., 2005a).

Microsatellite loci, or simple sequence repeats (SSRs), are highly informative molecular markers and can be of great use for population and conservation genetic studies. SSRs are ideal for the analysis of fine local genetic structures, due to their high levels of intraspecific genetic variability in many taxa, nuclear codominant inheritance and high reproducibility. Moreover, these markers are also important in assessing the impact of the reproductive behavior, social structure and dispersal in endangered species (Beaumont and Bruford, 1999). Microsatellites are short, tandemly repeated nucleotide sequences, usually 2 to 6 base pairs varying in the number of repeats, e.g. $(CA)_n$, $(ATA)_n$, or $(GATA)_n$, and are found throughout eukaryote genomes (O'Connell and Wright, 1997; Wright and Bentzen, 1994; Tautz et al., 1986). Despite their usefulness for stock discrimination and for determining the amount of intermixing that takes place among those stocks, few microsatellite loci have been described for the mangrove crab *U. cordatus* (Baggio et al., 2011; Britto et al., 2009; Varela et al., 2009).

In this study, we described the isolation and identification of a new set of microsatellite DNA loci from the overexploited mangrove crab *Ucides cordatus* using a highly simplified and inexpensive isolation protocol, based on (i) multiple enzyme digestion/ligation; (ii) mixed biotin-labeled probes and streptavidin-coated magnetic beads hybridization capture strategy, and (iii) a double-repeat-enrichment procedure.

MATERIALS AND METHODS

Tissue preparation and DNA extraction

Genomic DNA was extracted from the *Ucides cordatus* pereiopod muscle tissue (approx. 30 mg) using the phenol/chloroform-isoamyl alcohol (25:24:1; v/v) protocol described by Sambrook et al. (1989). DNA was checked for quality and quantity on a 1% agarose gel electrophoresis with ethidium bromide. Extracts were maintained at -20°C until further analyses.

Genomic library construction and SSR isolation

Microsatellite isolation was carried out as outlined in Glenn and Schable (2005) and Diniz et al. (2005b), with modifications. Genomic DNA from a single individual (~2 µg) was digested separately with *Rsa*I (0.4 U/µL) and *Bst*UI (0.4 U/µL (New England Biolabs – NEB, Beverly, MA) in the presence of bovine serum albumin (BSA; 0.025 µg/µL) overnight in a PTC-225 DNA Engine Tetrad thermocycler (MJ Research). Optimal enzyme concentration (2 µL) was selected based on the majority of DNA fragments of desired size (500-3000 bp). Genomic DNA digests were recovered using a QIAquick® PCR Purification Kit (Qiagen) and, subsequently, fragments were dephosphorylated at their 5' ends using Calf Intestinal Phosphatase (CIP; 0.17 U/µL; New England Biolabs). Dephosphorylation took place at 37°C for 2 h. Then, an additional purification step using QIAquick® PCR Purification Kit (Qiagen) was carried out. DNA fragments were ligated to double stranded SuperSNX linkers (SuperSNX24For: 5′-GTTTAAGGCCTAGCTAGCAGAATC-3′ and SNX24+4PRev: 5′-PGATTCTGCTAGCTAGGCCTTAAACAAAA-3′) with T4 ligase (400 U/µL; New England Biolabs) in the presence of *Xmn*I (0.7 U/µL; New England Biolabs) to avoid adaptor dimerization. The ligation reaction (30 µL) consisted of 20 µL digested-*g*DNA, 7.0 µL of adaptors (5 µM), 1.0 µL of 1× NEB buffer and 2.0 µL of T4 ligase (400 U/µL).

Linker-ligated inserts were amplified by an asymmetric PCR with SuperSNX24For as primer. Cycle number was optimized to minimize overamplification of products. PCR conditions were as follows: 1× 95°C for 5min, 20-30× (95°C for 45 s, 62°C for 1 min, and 72°C for 2 min), 1× 72°C for 30 min. Amplifications were carried out in 50-µL reaction volume containing 20-100 ng DNA, 1× Thermopol buffer (+1.5 mM $MgCl_2$), 0.8 mM each dNTP, 5.0 U *Taq* DNA poly-

merase (Thermopol, New England Biolabs), 0.8 μM of Super SNX-Forward on an MJ Research DNA Engine Tetrad PTC-225 thermocycler.

PCR products (purified linker-ligated DNA) were recovered using Qiaquick PCR Purification columns (Qiagen) and enriched for repeat motifs using subtractive hybridization with biotinylated probes (i.e. Mix: 2,5 μM $(GACA)_6$, 2,5 μM $(GATA)_7$, 2,5 μM $(GGAT)_5$ and 2,5 μM $(GTAT)_5$; Operon Technologies, Alameda, Calif.) at 55°C, and bound to magnetic beads (Dynabeads M-280 Streptavidin; Dynal Biotech Inc., NY).

Following hybridization, according to Glenn and Schable (2005), the streptavidin-coated beads were washed with decreasing concentrations of saline sodium citrate buffer (SSC) as follows: four washes in 2×SSC, four washes in 1×SSC, and then four washes in 0.5×SSC. All washes were carried out for 5 min in the presence of 0.5 ng/μL SuperSNX24For primer. After the final wash, 30 μL TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) was added to the beads, and the mixture was incubated at 98°C for 15 min to release the DNA from the probes; tubes were spun briefly, and the supernatant containing the heat-released single-stranded DNA was transferred to a clean tube. The enriched DNA recovered from the beads was amplified once again with SuperSNX24For linker to generate double-stranded DNA using the following PCR conditions: 1× 95°C for 5min, 10× (95°C for 45 s, 62°C for 1 min, and 72°C for 2 min), 1× 72°C for 30 min. Amplified enriched DNA was cleaned with Qiaquick PCR purification columns (Qiagen) and a second round of hybridization was performed using a small fraction of the post-enrichment amplified inserts, with the same tetranucleotide probes and hybridization conditions described previously.

Enriched and double-enriched fragments were recovered and amplified in 50-μL reactions using 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 25 μg/μL BSA, 1.0 mM dNTP mix, 0.8 μM SuperSNX-F, 5.0 U of *Taq* DNA polymerase and 10 μL of the enriched DNA. The following program was used for amplification on an MJ Research DNA Engine Tetrad PTC-225 thermocycler: 95°C for 5 min, 10-30× (95 °C for 45 s, 62 °C for 1 min, 72 °C for 2 min) and 72 °C for 30 min.

Amplified enriched DNA from the double-enrichment procedure was cleaned with Purelink™ PCR purification kit (Invitrogen) and ligated into Qiagen pDrive™ Vector (Qiagen PCR Cloning Kit), which was used to transform NEB 5-alpha competent *Escherichia coli* (DH5α™ derivative; New England Biolabs). Transformation was achieved by heat shocking at 42°C for 30 s according to the manufacturer's instructions. Transformed bacteria were initially grown for 1.5 h on sterile liquid media on a shaking incubator at 37°C. After the initial growth phase, 100 mL of the culture was spread on culture plates containing 50 μg/mL of ampicillin, and incubated overnight at 37°C. Transformed bacteria were grown in white colonies, while untransformed bacteria yielded blue colonies. Plasmid inserts were amplified directly from bacterial colonies with M13F and M13R and size-fractionated on agarose gels (1.5%). Plasmid DNA of appropriate length (500-1000 bp inserts) was prepared using a QIAprep Spin Miniprep Kit (Qiagen) and cycle-sequenced (96°C for 3 min, 40× (96°C for 20s, 52°C for 20s and 60°C for 4 min) in one direction with a universal primer (T7/M13F) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Electrophoresis of sequencing products was performed on an ABI 3130 automated DNA analyzer (Applied Biosystems). Confirmed positives were further sequenced for the opposite strand using primers SP6/M13R.

SSR primers designing sequence analysis

DNA sequences (forward and reverse sequences for each individual) were inspected by eye with the aid of the sequence editor, Chromas Version 2.23 (Technelysium Pty Ltd.) and corrected when necessary. Homologous sequences (forward sequence and its reverse complement) containing repeat motifs were aligned on the sequence alignment editor, Bioedit (Hall, 1999). Alignments were double-checked by eye and refined manually if needed. The online software Websat (Martins et al., 2009) was used to identify di-, tri- and tetranucleotides with five or more repeats, and primer pairs were designed on the unique flanking regions of each microsatellite locus using Primer3 (Rozen and Skaletsky, 2000). Microsatellites were classified as perfect, imperfect or compound (Weber, 1990).

RESULTS AND DISCUSSION

Both restriction enzymes (RE), *Rsa*I and *Bst*UI, generated products that included fragments between 300 and 3000 bp, under different concentrations. *Rsa*I-digested DNA, however, produced mainly 300-3000 bp fragments with no specific banding pattern on the agarose gel electrophoresis profile (Fig. 1A), and was subjected to further experiments. The selection of RE for microsatellite development is more related to the features presented by the genome than the enzyme itself. Therefore, the occurrence of enzyme recognition sites (5'-GT$^{\blacktriangledown}$AC-3' for *Rsa*I) on the studied genome determines at which frequency such enzyme will digest *g*DNA and, consequently, determines the restriction pattern for a specific enzyme (Lynn et al., 1980). Amplification of ligated DNA, with SuperSNX-*Forward* as primer in the PCR reaction, was optimized at 20 cycles, to avoid the possible formation of *chimeric* DNA fragments (Fig. 1B).

The best results for the amplification of enriched DNA recovered from the beads after the first hybridization step were reached at 25 cycles (Fig. 2A). After the second round of hybridization, minimum overamplification of double-enriched fragments was warranted when PCR was carried out with 15 cycles (Fig. 2B). The use of a double-enrichment protocol provides an inexpensive approach for increasing the number of loci that can be screened, and decreases the costs of genotyping. Even though no recombination problem in the PCR following the enrichment procedure was detected, care should be taken to avoid possible complications. Keeping PCR cycles to a minimum seems to be an effective way to overcome any recombination problem or *chimeric* DNA fragments.

After cloning and transformation of amplified enriched DNA from the double-enrichment procedure, 105 positive colonies were identified by 'white/blue plaque selection'. PCR amplification of target sequences and subsequent agarose gel electrophoresis revealed 77 clones (73.3%) containing fragments between 500-1000 bp, 22 (21.0%) outside this range and 6 (5.7%) unsuccessful amplifications (Fig. 3). After sequencing, some repeat regions were found to be too short

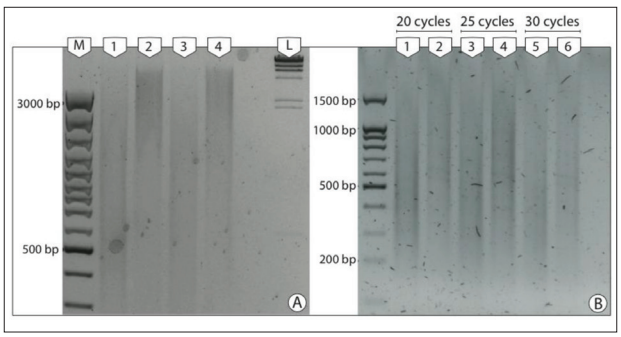

Fig. 1. (**A**) Agarose (1.5%) gel electrophoresis of digested *U. cordatus* *g*DNA with restriction enzymes (RE). 1: *g*DNA from individual UcCAN84 digested with *Rsa*I; 2: *g*DNA from individual UcCAN84 digested with *Bst*UI; 3: *g*DNA from individual UcCAN83 digested with *Rsa*I; 4: *g*DNA from individual UcCAN83 digested with *Bst*UI. M: GeneRuler™ 100 bp Plus DNA Ladder (Fermentas); L: λ*Hin*dIII (first band is 23130 bp); (**B**) PCR products of digested-ligated *g*DNA. 1, 3, 5: UcCAN84 digested with *Rsa*I; 2, 4, 6: UcCAN84 digested with *Bst*UI. *Ladder*: 100 bp DNA ladders (Bio Basic Inc, Canada).

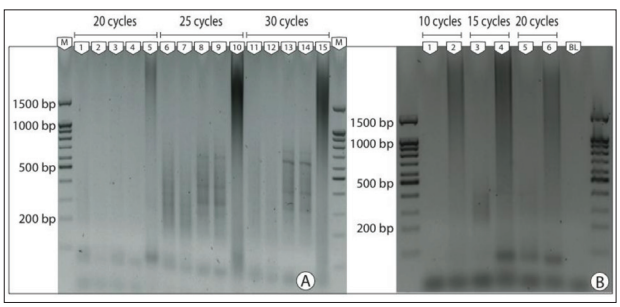

Fig. 2. PCR products (10 µL) of enriched DNA for tandem repeat arrays. (**A**) Single enrichment (1×), 1-2, 6-7, 11-12: UcCAN84 *Rsa*I, 3-5, 8-10, 13-15: UcCAN84 *Bst*UI; (**B**) double enrichment (2×). 1, 3, 5: UcCAN84 *Rsa*I, 2, 4, 6: UcCAN84 *Bst*UI; *Ladder*: 100 bp DNA ladders (Bio Basic Inc, Canada). Agarose gel Electrophoresis (1.5%).

or had a low GC content to have primers designed on their flanking regions. The number of sequences with repeat motifs not suitable for primer designing was high (49.4%), which might be a consequence of the restriction enzyme used to digest high molecular-weight DNA, as well as the choice of probes. Out of 77 confirmed clones, 72 (93.5%) were non-redundant, of which 7 (9.1%) contained sequences with no repeat motifs. Microsatellite motifs contained more than 5 repeats, and most loci were found be of perfect repeats (51.4%), of which 94.4% were dinucleotide and 5.5%

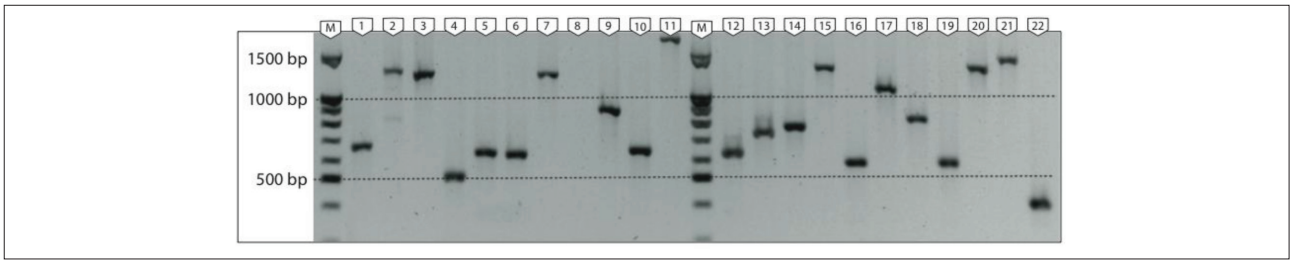

Fig. 3. An example of the PCR screening for positive colonies (1-22). Insert size variation of plasmid library clones resolved in 1.5% agarose gel electrophoresis. L: 100 bp DNA ladder (Bio Basic Inc, Canada).

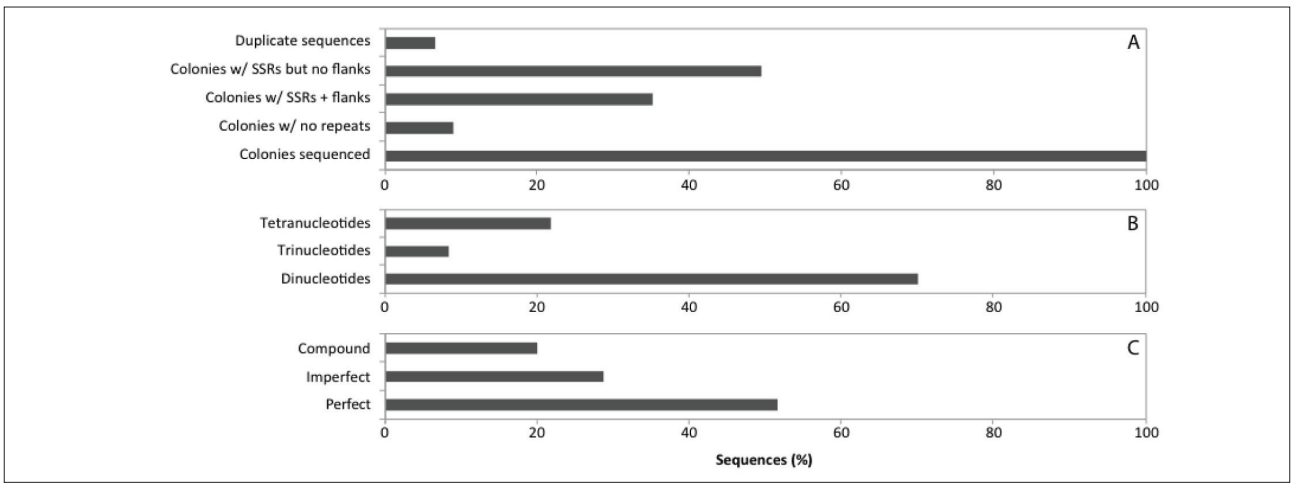

Fig. 4. Evaluation of the success in obtaining microsatellite repeats using a double-enriched genomic library procedure (A); percentage of sequences by the type of repeat motif (B); and according to Weber's (1990) classification (C).

trinucleotide. Only 20% were compound and 28.6% of imperfect repeats contained di-, tri- and/or tetranucleotides (Fig. 4). The locus (UcSSR-29) with the largest number of repeats was a $(GA)_{31}$ dinucleotide (Table 1). The fragment sizes ranged from 81 (UcSSR-37) to 293 bp (UcSSR-20a). The high frequency of perfect repeat motifs after enrichment is additional evidence of the importance of adopting this procedure for the isolation of short tandem repeats.

Despite the fact that the constructed genomic library was probed with tetranucleotide repeats, most microsatellite loci obtained were dinucleotides (Fig. 5). Similar results were reported in the literature for marine invertebrates (Kaukinen et al., 2004). The most frequent dinucleotides, $(TC/AG)_n$, $(CT/GA)_n$, $(AC/TG)_n$ and $(CA/GT)_n$, seem to be abundant in other invertebrate species, such as penaeid shrimps (Freitas

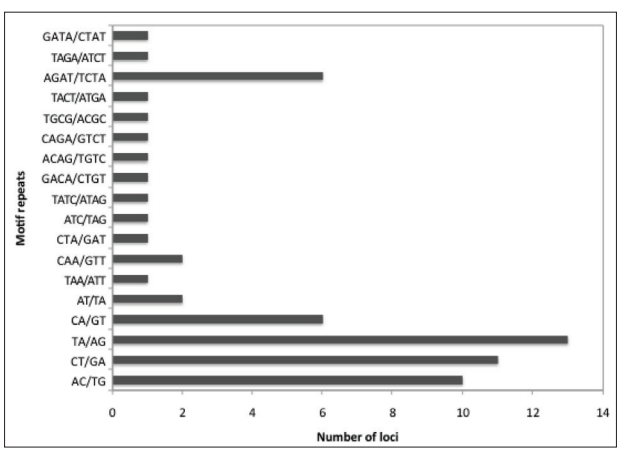

Fig. 5. Distribution of simple sequence repeat (SSR) nucleotide classes based on the number of loci isolated from the mangrove crab *Ucides cordatus*.

Table 1. Characteristics of microsatellite loci in the mangrove crab *Ucides cordatus*.

Clone name	Core motif	Forward primers (5' – 3')	Tm (°C)	Reverse primers (5' – 3')	Tm (°C)	Product length (bp)	Repeat type*
UcSSR-09	$(AC)_5...(AC)_5$	F: GATTAAAGGTGCTGGGAGAGAA	F: 59.7	R: CAGGTAGAGAGAGGTAGCGGAA	R: 60.0	156	Compound
UcSSR-10	$(CT)_{23}(TATC)_8...(TC)_9$	F: ACATCACATCACTCTTGCCTCA	F: 60.7	R: ATGCTGTAAAGGCGCACTAAA	R: 59.9	267	Imperfect
UcSSR-11	$(GA)_{22}$	F: CGTTAGATACTTTGCTTGCGTT	F: 58.7	R: ACACGTCACACCGAGGAA	R: 58.6	208	Perfect
UcSSR-12	$(CA)_7$	F: CAGAATCACTGCCAAAGT	F: 51.7	R: CTACGTCAGAAAAGGTGTTT	R: 52.1	117	Perfect
UcSSR-13	$(AC)_9$	F: TAAAGGATGCAGCTTACGAACA	F: 59.9	R: CTATGACCATGATTACGCCAAG	R: 59.5	225	Perfect
UcSSR-14	$(GA)_6...(AG)_{12}$	F: ACTGTCCGTCCGTCTGTCT	F: 58.2	R: CCGGGTCTTTGGGTCTAT	R: 57.4	242	Compound
UcSSR-15	$(AG)_5T(GACA)_3$	F: TTACGTGAAGAAGGGGAGGATA	F: 60.0	R: GTGGTGCATGTTTGTAGCTGTT	R: 60.1	193	Imperfect
UcSSR-16	$(GT)_6$	F: TTCTACACCCACCCGTCTC	F: 58.5	R: CTAGCAGAATCACATGAAGGC	R: 57.6	193	Perfect
UcSSR-17	$(GA)_5...(ACAG)_4...(CAGA)_4$	F: TCACAAACACAGCTAAAGAGGG	F: 59.4	R: ACGTCTTCACAATAATCGCCTT	R: 60.0	195	Imperfect
UcSSR-18	$(TG)_5$	F: CTTTCTTCTTGCTTTCCAGGC	F: 60.5	R: AACTGAGCTTGACCCAATCCTA	R: 60.1	150	Perfect
UcSSR-19	$(TG)_8...(TG)_5...(TG)_8CT(TGCG)_3...(TAA)_5$	F: GTGTATGTGCGTGTATGCGTG	F: 61.1	R: CCCTCTTTATTTCCAGTCGTCA	R: 60.5	179	Imperfect
UcSSR-20a	$(CAA)_6C(TACT)_{14}T(CTA)_{10}...(AG)_{24}$	F: GCTGAGGTCACAGAGACCTATTT	F: 58.9	R: AAGGCCTAGCTAGCAGAATCAC	R: 59.2	293	Imperfect
UcSSR-20b	$(CAA)_6C(TACT)_{14}T(CTA)_{10}$	F: TTCATACAAAAGTTGAGAATTG	F: 52.4	R: CTCTCTCTGTAGAAGTAGAAGTAGTTG	R: 54.0	158	Imperfect
UcSSR-21	$(GA)_5A(AG)_{25}$	F: CTTGAGAGATTGCTTGACGCT	F: 59.8	R: GGCCTAGATAGCAGAATCACCTT	R: 60.1	125	Compound
UcSSR-22	$(AC)_{18}$	F: GTTTATGGATTGGTGCTTGTCC	F: 60.6	R: CTCTCTCATGCAGCGCAA	R: 60.0	128	Perfect
UcSSR-23	$(CA)_9A(AGAT)_4AGG(TAGA)_6$	F: GGCAACGACTTTCAGTTTCTCT	F: 60.0	R: TGACCGATCCTTTCTCTCTCTG	R: 60.9	191	Imperfect
UcSSR-24	$(AGAT)_3...(GATA)_5...(AG)_5$	F: GTGTGTGTACCTTCGCTCGTTC	F: 60.6	R: AATCCACTCGCCATTTATTCTG	R: 60.3	181	Imperfect
UcSSR-25	$(AT)_5$	F: AGGAGGAAGGGAAAGAAGAAGA	F: 59.8	R: CGCTGCTGCTACTACCGAAT	R: 60.6	145	Perfect
UcSSR-26	$(GT)_7$	F:ATCTGGCATGAGTTTTCGTGT	F: 59.6	R:TATTCTCCTCTGTAGCCCTGGA	R: 60.2	109	Perfect
UcSSR-27	$(GT)_5$	F: TATGCTTGAGTCTACGCCTGT	F: 57.7	R: GGAGACAGAGAGACAAACGG	R: 57.4	140	Perfect
UcSSR-28	$(TC)_6$	F: TCTGTCTCCACTTCTTCTTCCC	F: 59.9	R: ATTTTCTCTTCCAGCCTCGTCT	R: 60.7	105	Perfect
UcSSR-29	$(GA)_{31}$	F: GAATCCGTCAGGTGATGTCC	F: 60.3	R: TTTCACATTTCACTGTCCCTTG	R: 60.0	149	Perfect
UcSSR-30	$(AC)_{11}...(AG)_{28}$	F: AGCTTGTCGACGAATTCAGATT	F: 60.3	R: TTTTCTCACACAGATTATATTGGGAT	R: 59.3	267	Compound
UcSSR-31	$(TA)_6$	F: TCCCATATTTCCTCTCCACTG	F: 59.0	R: CGAGGAGCACCCTTGTATTTT	R: 60.5	100	Perfect
UcSSR-32	$(TC)_6$	F: ACCCCTCGCCCCAATATC	F: 62.0	R: GCGGGAAGAAAGAGCATGTAT	R: 60.6	100	Perfect
UcSSR-33	$(CT)_{25}...(TG)_{10}$	F: ATCACCTATGTATCTCTCTGTC	F: 50.0	R: ATCTCTTTATTTTTGTATGTGT	R: 47.8	198	Compound
UcSSR-34	$(GGT)_5$	F: AATGTAGAGAAGCAGCGAGACC	F: 60.0	R: TAAACCACATGCACCACCAC	R: 60.2	114	Perfect
UcSSR-35	$(AG)_{11}AT(AG)_{25}$	F: TCGATAATACACGACAAATGCC	F: 59.9	R: GGGGTAAAAGTGAGAGGAGAAAA	R: 60.0	142	Compound
UcSSR-36	$(GA)_{18}$	F: AGACAGTGAGTGAAAGGCAAAA	F: 59.0	R: GATCCATTTCTCCGGTTTCTTA	R: 59.4	178	Perfect
UcSSR-37	$(AG)_{10}$	F: GGAAAAGGGCTGCTGTAAACT	F: 59.8	R: CCTAGATAGCAAAATCACCCGA	R: 60.5	081	Perfect
UcSSR-38	$(GT)_9...(AGAT)_7...(AGAT)_5$	F: CGAATTCAGATTGTTTAAGGCC	F: 60.0	R: AGCTGTCTATCTATCCATCTGTCT	R: 55.5	165	Imperfect
UcSSR-39	$(GA)_{25}$	F: CGAAATTATCAGTAAAGACAATATGAA	F: 57.3	R: TAGGTAGAAATCCGGGCG	R: 58.7	150	Perfect
UcSSR-40	$(AG)_5GGG(GA)_{20}$	F: GGAGAGTGAGTAGCTGGCGAT	F: 60.9	R: TGTGCGTGTGTGTAGGGAAATA	R: 61.4	138	Compound
UcSSR-41	$(GA)_6$	F: CGTTGTATCGCTGTCCGTTAT	F: 60.0	R: TTTTCCCTTTTCGTCTTTAGGC	R: 60.9	169	Perfect
UcSSR-42	$(TCTA)_8...(ATC)_7$	F: CAGACGTTGTTGCCAGTTATT	F: 57.8	R: TTAACATCAGACAGAGAGGGGT	R: 57.8	196	Imperfect

*According to Weber (1990).

et al., 2007) and *Drosophila melanogaster* (Bachtrog et al., 2000). However, the probe sequence composition used for the enrichment protocol, to some extent, may also be likely to increase the ease of capture for repeats containing TC/CT/AG/GA. The nucleotide composition of the repeat sequence is an important factor in understanding the abundance and evolution of microsatellites (Schlötterer and Harr, 2001).

The success rate of the enrichment protocol using *U. cordatus* as the target genome was lower when compared to other decapods (e.g. *Panulirus argus*; Diniz et al., 2005a); nevertheless, it was high enough to identify 34 microsatellite loci within the 77 positive clones. Many factors may have influenced the success of the protocol, which may include an excessive concatemer formation caused by the polymerase during

PCR steps within the protocol as well as the presence of fewer microsatellites than other invertebrates (Ji et al., 2003).

The new simple sequence repeats described in this study are expected to be polymorphic and therefore useful in population/stock discrimination of this valuable mangrove species, currently subjected to excessive fishing, throughout its geographical range.

Acknowledgments: This study was supported by the Brazilian Government (FAPEPI/CNPq-PPP program), the Bank of Northeastern Brazil (BNB/ETENE/FUNDECI) and the Brazilian Agricultural Research Corporation (Embrapa). We thank two anonymous referees and the editor for their remarks and suggestions.

Authors' contributions: Conceived and designed the experiments: G.R.O., F.B.B., F.M.D. Performed the experiments: E.S.A., F.B.B., A.M.F.C. Analyzed the data: E.S.A., A.M.F.C., F.M.D. Contributed reagents/materials/analysis tools: F.B.B., F.M.D. Wrote the paper: E.S.A., F.B.B., F.M.D.

Conflict of interest disclosure: The authors declare no conflict of interest.

REFERENCES

Amaral, K. D. S., Vieira, I. M., Osório, F. M., Rocha, J. D. M. and *J. F. Lima* (2014). Bioecology of the crab *Ucides cordatus* (Crustacea, Decapoda) in mangroves influenced by the Amazon River, Brazil. *Acta Amaz.* **44**, 213-222.

Avise. J. C. and *J. L. Hamrick* (1996). Conservation Genetics: Case Histories from Nature. Chapman and Hall, New York.

Bachtrog, D., Agis, M., Imhof, M. and *C. Schlötterer* (2000). Microsatellite variability differs between dinucleotide repeat motifs – Evidence from *Drosophila melanogaster*. *Mol. Biol. Evol.* **17**, 1277-1285.

Baggio, R. A., Pil, M. W., Boeger, W. A., Patella, L. A., Ostrensky, A. and *M. R. Pie*. (2011). Genetic evidence for multiple paternity in the mangrove land crab *Ucides cordatus* (Decapoda: Ocypodidae). *Mar. Biol. Res.* **7**, 520-524.

Beaumont, M. A. and *M. W. Bruford* (1999). Microsatellites in conservation genetics. In: *Microsatellites: Evolution and Applications* (Eds. Goldstein, D. B. and C. Schlötterer), 165-182. Oxford University Press, New York.

Britto, F. B., Diniz, F. M., Paterson, I. and *P. Bentzen* (2009). Polymorphic microsatellite DNA markers in the mangrove crab *Ucides cordatus* (Brachyura: Ocypodidae). *Mol. Ecol. Resour.* **9**, 1249-1252.

Carvalho, F. L., Pileggi, L. G. and *F. L. Mantelatto* (2013). Molecular data raise the possibility of cryptic species in the Brazilian endemic prawn *Macrobrachium potiuna* (Decapoda, Palaemonidae). *Lat. Am. J. Aquat. Res.* **41**, 707-717.

Coelho, P. A. and *M. A. Ramos* (1972). A constituição e a distribuição da fauna de decápodes do litoral leste da América do Sul, entre as latitudes 5ºN e 39ºS. *Trabalhos Oceanográficos da Universidade Federal de Pernambuco.* **13**, 133-236.

Diniz, F. M., Maclean, N., Ogawa, M., Cintra, I. H. A. and *P. Bentzen* (2005a). The hypervariable domain of the mitochondrial control region in Atlantic spiny lobsters and its potential as a marker for investigating phylogeographic structuring. *Mar. Biotech.* **7**, 462-473.

Diniz F. M., Maclean, N., Ogawa, M., Paterson I. and *P. Bentzen* (2005b). Microsatellites in the overexploited spiny lobster, *Panulirus argus*: isolation, characterization of loci and potential for intraspecific variability studies. *Conserv. Genet.* **6**, 637-641.

Diniz, F. M., Iyengar, A., Lima, P. S. C., Maclean, N. and *P. Bentzen* (2007). Application of a double-enrichment procedure for microsatellite isolation and the use of tailed primers for high throughput genotyping. *Genet. Mol. Biol.* **30**, 380-384.

Freitas, P. D., Pinheiro, A. P., Silva, T. B. and *P. M. Galetti* (2007). *In silico* analysis of polymorphic microsatellites in penaeid shrimp and construction of a free-access database. *Genet. Mol. Biol.* **30**, 1194-1197.

Glenn T. C. and *N. A. Schable* (2005). Isolating microsatellite DNA loci. *Method. Enzymol.* **395**, 202-222.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98NT. *Nucleic Acids Sym. Ser.* **41**, 95-98.

Ji, Y. J., Zhang, D. X., Hewitt, G. M., Kang, L. and *D. M. Li* (2003). Polymorphic microsatellite loci for the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) and some remarks on their isolation. *Mol. Ecol. Notes.* **3**, 102-104.

Kaukinen K. H., Supernault, K. J. and *K. M. Miller* (2004). Enrichment of tetranucleotide microsatellite loci from invertebrate species. *J. Shellfish Res.* **23**, 621-626.

Larraín, M. A., Díaz, N. F., Lamas, C., Vargas, C. and *C. Araneda* (2012). Genetic composition of *Mytilus* species in mussel populations from southern Chile. *Lat. Am. J. Aquat. Res.* **40**, 1007-1084.

Lynn S. P., Cohen, L. K., Kaplan, S. and *J. F. Gardner* (1980). *Rsa*I: a new sequence-specific endonuclease activity from *Rhodopseudomonas sphaeroides*. *J. Bacteriol.* **142**, 380-383.

Martins, W. S., Lucas, D. C. S., Neves, K. F. S. and *D. J. Bertiolli* (2009). WebSat: A web software for microsatellite marker development. *Bioinformation.* **3**, 282-283.

Melo, G. A. S. (1996). Manual de Identificação dos Brachyura (caranguejos e siris) do Litoral Brasileiro. Plêiade/FAPESP Press, São Paulo, 603 p.

O'Connell, M. and *J. M. Wright* (1997). Microsatellite DNA in fishes. *Rev. Fish Biol. Fisher.* **7**, 331-363.

Oliveira-Neto, J. F., Boeger, W. A., Pie, M. R., Ostrensky, A. and *D. B. Hungria* (2007). Genetic structure of populations of the

mangrove crab *Ucides cordatus* (Decapoda: Ocypodidae) at local and regional scales. *Hydrobiologia.* **586**, 69-76.

Rozen, S. and *H. J. Skaletsky* (2000). Primer 3 on the WWW for general users and biologists programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (Eds. S. Krawetz and S. Misener), 365–386. Humana Press, Totowa, NJ.

Sambrook J., Fritsch, E. F. and *T. Maniatis* (1989). Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schlötterer, C. and *B. Harr* (2001). Microsatellite instability. In: *Encyclopedia of life sciences* (eLS), 1-4. J. Wiley and Sons, New York.

Tautz, D., Trick, M. and *G. A. Dover* (1986). Cryptic simplicity in DNA is a major source of genetic variation. *Nature.* **322**, 652-656.

Varela, E. S., Goncalves, E. C., Santos, M .O., Sampaio, I. and *H. Schneider* (2009). Isolation and characterization of 11 microsatellite loci in the mangrove crab, *Ucides cordatus* (Ocypodidae: Ucidinae). *Mol. Ecol. Notes* **9**, 1395-1397.

Weber, J. L. (1990). Informativeness of human (dC-dA)n.(dG-dT) polymorphisms. *Genomics.* **7**, 524-530.

Wright, J. M. and *P. Bentzen* (1994). Microsatellites – genetic markers for the future. *Rev. Fish Biol. Fisher.* **4**, 384-388.