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RESEARCH ARTICLE

Identification and characterization of microsatellite loci in West Atlantic sea cucumber *Holothuria grisea* (Selenka 1867)

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Abstract. The sea cucumber *Holothuria grisea* has become the subject of intense and unregulated fishing in northeastern Brazil due to their growing demand in Asian market. However, there is little knowledge about the dynamics and genetics of *H. grisea* wild populations on the South American coast. In this study, we present the first set of *H. grisea* microsatellite markers, identified and characterized using Illumina paired-end reads of whole genome shotgun sequencing. From 50 strictly selected candidates, eight novel microsatellite markers were successfully developed. We then genotyped 30 individuals to evaluate the degree of polymorphism and validate the markers. The number of alleles ranged from three to 14, while observed and expected heterozygotes ranged from 0.156 to 0.906 and from 0.283 to 0.774, respectively. After correcting for multiple tests, we found no evidence of linkage disequilibrium in all pairwise combinations between the loci. One locus (*Hgr*15607) revealed deviation from the Hardy–Weinberg equilibrium, as well as the presence of null alleles. However, we observed significant differences in frequency distribution between males and females at locus *Hgr*15607. We believe that the markers described here will be useful for conservation efforts and management of *H. grisea* fisheries and for prospective aquaculture of these organisms.

Keywords. microsatellite; next-generation sequencing; genetic diversity; sea cucumber; *Holothuria grisea*.

Introduction

Sea cucumbers are marine invertebrates of the phylum Echinodermata, and members of the class Holothuroidea. They are dioecious, present external fertilization, yet a clear sexual dimorphism is absent. Sea cucumbers in general feed on organic debris and materials from the sediment. They play important ecological roles in nutrient recycling, sediment bioturbation, biodiversity enhancement and contribute to reducing localized acidification, which are important processes for ensuring the survival of corals and other benthic species (Purcell *et al.* 2016).

Sea cucumbers are widely consumed in Asia, mainly in China and Japan, where they are used as food and for traditional medicine (Uthicke and Conand 2005). In recent years, the growing demand for sea cucumber has led to increase in international market prices, which, in turn, seem to be leading to overfishing and depletion of natural stocks of many commercial species (Robinson and Lovatelli 2015).

In Brazil, the most abundant species of sea cucumber is *Holothuria grisea* Selenka 1867, which exist from southern Santa Catarina state to the northeast of the country (Tommasi 1969; Mendes *et al.* 2006). With the growing demand for sea cucumber in Asian markets, *H. grisea* has

become a source of additional income for fishermen in certain Brazilian coastal villages and an important alternative species for commercial fisheries among local communities (Leite-Castro *et al.* 2016). However, the lack of specific management policies has allowed intense and unhindered exploitation of the wild populations of *H. grisea* throughout its range in the Brazilian territory. Appropriate and effective management of sea cucumber fisheries is even more important because this species also shows biotechnological potential as a source of new bioactive molecules for the pharmaceutical industry (Mourão 2004; Sun *et al.* 2008; Moura *et al.* 2013; Melo *et al.* 2014).

The sea cucumber *H. grisea* is a typical holothurian, with a soft and cylindrical body, tube feet to move over the seabed and, at one of its extremities, a rounded mouth surrounded by tentacles (Dias 2012). *H. grisea* are dioecious animals with no sexual dimorphism and no evidence of asexual reproduction (Leite-Castro *et al.* 2016). In this species, sex can only be determined by gonadal inspection; therefore, dissection is necessary for sex identification.

Bueno *et al.* (2015) and Leite-Castro *et al.* (2016) have conducted studies on captive breeding of *H. grisea*. However, little is known about genetic diversity and population structure of this species, which occurs in the Gulf of Mexico, the Caribbean and along most of the Brazilian coast. As a potential aquaculture species, it is also important to assess genetic information about *H. grisea* wild populations. In aquaculture, inadequate management of breeding stocks can lead to loss of genetic diversity, thus affecting characteristics such as disease resistance and growth rate (Liao *et al.* 2011).

Microsatellite molecular markers have been widely used to estimate genetic diversity and population structure of marine organisms, and for genetic monitoring of aquacultured stocks in breeding programmes (Chistiakov *et al.* 2006). Microsatellites, or short-sequence repeats (SSRs), are tandem repeats of two to six base pair motifs in DNA sequences. Microsatellites tend to be highly polymorphic and are widely distributed in the eukaryotic genome (Zane *et al.* 2002). Microsatellite loci are in general non-coding and multi-allelic, which are easily and consistently detected by polymerase chain reaction (PCR). At present, the strategies for discovery and characterization of microsatellite loci have evolved to the use of next-generation sequencing (NGS), which allows rapid and efficient production of large quantities of sequences (Mardis 2008; Morozova and Marra 2008; Yang *et al.* 2012).

The objective of this work was to identify, characterize and develop microsatellite markers for the sea cucumber *H. grisea* from paired-end reads of whole genome NGS, as well as to explore correlations between these markers and sex. This is the first set of microsatellite markers made available for *H. grisea*, a species of growing commercial and environmental interest on the South American coast.

Materials and methods

Collection of animals and DNA extraction

The research was authorized by the System of Authorization and Information on Biodiversity (SISBIO), Ministry of Environment (License no. 22742-1). We collected 32 adult *H. grisea* individuals, with total body weight above 90 g from a single population at Bitupitá beach (Barroquinha, Ceará, Brazil, 02°47′43.38″S, 41°14′33.02″W), in November 2015. The animals were anaesthetized and dissected to collect tissue samples, and for direct inspection of the gonads to identify sex. Later, we extracted genomic DNA from muscle tissue preserved in 95% analytical grade ethanol (Sigma-Aldrich) with the Wizard SV Genomic DNA Purification System (Promega). After extraction, we measured DNA concentration using a Nanodrop microvolumetric spectrophotometer and kept the samples at −20°C until further processing.

Preparation of the genomic library and sequencing

For next-generation genome sequencing, we selected samples from one male (BT28M) and one female (BT30F). DNA from these two samples was reextracted with the Wizard SV Genomic DNA Purification System (Promega) and quantitated using a QUBIT 2.0 fluorometer (Life Technologies). We separately submitted these samples to the preparation of genomic libraries with the Nextera XT DNA Library Prep kit (Illumina), according to the manufacturer's instructions. The libraries were sequenced on an Illumina MiSeq platform (Illumina) using the MiSeq Reagent kit v3 (PE 2 × 300 bp). We initially evaluated the quality of the obtained readings with FastQC 0.11.15 (Babraham Institute). We then used PRINSEQ v. 0.20.4 (Schmieder and Edwards 2011) to filter low quality (average QC < 25) and duplicated sequences, to trim low quality ends (QC < 30), and to generate pair-matched FASTA files.

Identification of microsatellites and primer design

After preprocessing, we used PRINSEQ to refilter sequence files with the following criteria: sequences between 100 and 600 bp, 40–60% GC content and no poly-A/T/C/G. We then used SSR_pipeline (Miller *et al.* 2013) to identify sequences containing SSRs with the following parameters: trinucleotides ≥ six repeats, tetranucleotides ≥ five repeats, pentanucleotides ≥ four repeats, hexanucleotides ≥ four repeats. We selected only microsatellite sequences with a flanking region of at least 40 bp on each side. As the final step of this stage, we used WebSat (Martins *et al.* 2009) to visualize and discard imperfect microsatellites. We designed 50 primer pairs using Batch-Primer3 (Frank *et al.* 2008) and applying the following

criteria: amplification products between 100 and 500 bp, melting temperature ranging from 50 to 60°C and at least one C/G clamp. An oligonucleotide tail (5′-TGT AAA ACG ACG GCC AGT-3′) corresponding to the M13(-21) universal primer was added to the 5′-end of one primer of each pair to allow fluorescent labelling of the PCR products during amplification. This is a versatile approach that involves the amplification of microsatellite loci in the presence of an additional M13-tail oligonucleotide fluorescently labelled with phosphoramidites, either PET, 6-FAM, HEX or NED (Schuelke 2000; Diniz *et al.* 2007).

Primer validation and genotyping

Among the 50 primer pairs designed, we selected 15 for validation. We conducted validation assays in 10 μL PCRs containing 20 μg of genomic DNA, 1× PCR buffer, 2 mM $MgCl_2$, 250 μM of each dNTP, 1 μM of each primer and 0.25 U of *Taq* DNA polymerase (Invitrogen). All PCRs were run on a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, USA) using the following cycling conditions: 94°C for 4 min; 25 cycles of 94°C for 30 s, 50–60°C for 90 s, 72°C for 60 s; and a final extension of 72°C for 45 s. All assays included a negative control containing all components, except for template DNA. The genotyping PCRs contained the same components mentioned above plus 0.06 μM of labelled M13-tail, and were run in the optimized thermocycling conditions. We separated labelled PCR amplicons through capillary electrophoresis on an ABI3500 automated sequencer (Applied Biosystems) using GeneScan ROX 500 as the internal standard. Fragment sizes were determined using GeneMapper v4.0 (Applied Biosystems).

Data analysis

For descriptive statistics and disequilibrium tests, we used GenePop v. 4.2 (Raymond and Rousset 1995) and GenAlex v. 6.5 (Peakall and Smouse 2006). The number of alleles (A), number of effective alleles (A_e), expected (H_e) and observed (H_o) heterozygosity, as well as Wright's inbreeding index (Cockerham and Weir 1993) were calculated. We conducted Fisher's exact tests for departure from the Hardy–Weinberg equilibrium (HWE) and for linkage desequilibrium. Differentiation between allelic and genotype frequency distributions of males and females was assessed by Fisher's exact tests as well. We assessed the presence of null alleles using Micro-Checker v. 2.2.3 (van Oosterhout *et al.* 2004). Significance levels were corrected following the Bonferroni correction for multiple tests (Rice 1989).

Results

Sequencing data and SSR loci identification

We obtained a total of 764,601 and 920,260 paired reads from BT28M (male) and BT30F (female) library sequencing, respectively, with a mean paired size of 301 bp. After the filtering steps 80.19 and 81.22% of BT30F and BT28M sequences remained in the database, respectively. The GC content was 38% for BT28M and 39% for BT30F. From the SSR identification pipeline, BT28M revealed 143,588 sequences containing short repeats, while BT30F produced 182,326. Between these two samples, 1315 sequences contained trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide repeats within the established criteria (table 1).

SSR loci description

After optimization, eight primer pairs were found to be polymorphic, producing repeatable and clearly defined PCR products of the expected size. Genotyping of 32 male and female of *H. grisea* revealed 54 different alleles across eight loci (table 2). Locus *Hgr*15269 showed the lowest number of alleles (3), while locus *Hgr*10123 showed the highest (14). We observed the maximum effective number of alleles (A_e) at locus *Hgr*10123 (8.2) and the minimum at locus *Hgr*6685 (1.4). Average A_e (3.4) was half the value of the average number of alleles for the entire population (6.8) because three loci (*Hgr*6207, *Hgr*6685 and *Hgr*15607) had strong unimodal distributions, and five loci presented two or more alleles at low frequency

Table 1. Distribution and frequencies of different repeat motifs in *H. grisea*.

Microsatellite repeat type	Minimum number of repeats	Number of SSRs in male	% In male	Number of SSRs in female	% In female
Trinucleotide	6	158	27.4	201	27.2
Tetranucleotide	5	210	36.4	282	38.2
Pentanucleotide	4	126	21.8	163	22.1
Hexanucleotide	4	83	14.4	92	12.5
Total	–	577	–	738	–

Table 2. Characteristics and genetic variability of eight polymorphic microsatellite loci in *H. grisea*.

Locus name (accession)	Primer sequence (5′ → 3′)	Repeat Motif	T_a (°C)	Allele size range (bp)	H_o	H_e	F_{IS}	P_{HWE}	$A(A_e)$
*Hgr*6207 MH286899	F: ‡AAGTATGTGCATGTGAAAAGGAG R: CCTAGGCCTATGAGATTTGTGG	$(CAGA)_5$	50	♀151–167 ♂147–175	0.688	0.570	−0.226	0.510	6 (2.3)
*Hgr*6685 MH286900	F: ‡AGTTGGGCAGGTCAACTGG R: TCTTTGCATTGGAGCGATG	$(ATCAC)_4$	50	♀168–183 ♂168–183	0.321	0.283	−0.145	1.000	4 (1.4)
*Hgr*6916 MH286901	F: ‡TTTAGTATGGGGTGATAATTGATTTG R: TGGACAAAAGTGACGTGAGC	$(ATGGT)_5$	50	♀158–178 ♂163–178	0.688	0.718	0.014	0.417	6 (3.5)
*Hgr*10123 MH286902	F: ‡ACCCCAGTGTGCCAGTATTC R: TGTTTGTAAATCGTCGGTGTG	$(CCTAGT)_4$	50	♀270–304 ♂270–310	0.906	0.878	−0.048	0.173	14 (8.2)
*Hgr*15269 MH286903	F: ‡GCTGCTGCTTCTGCTCATAG R: TCTGATGACCTGATGAGTTTCG	$(TCCTTC)_4$	60	♀282–288 ♂276–288	0.417	0.531	0.216	0.028	3 (2.1)
*Hgr*15607 MH286904	F: ‡CTACGGCGTGGTTGTTCC R: CGGAAATGCAGTTCACTGAG	$(TCG)_7$	55	♀288–303 ♂282–295	0.156	0.488	0.666	0.000*	7 (2.0)
*Hgr*18815 MH286905	F: ‡TTGTGAAGAATTCAACCCTTGC R: CCCTGACAGAGTTCTGATTGTG	$(GCAC)_8$	50	♀161–181 ♂157–181	0.625	0.774	0.186	0.041	8 (4.4)
*Hgr*21832 MH286906	F: ‡AATGGTGAGCGAAACAAGC R: GCACCAAAGGCGAGTCTG	$(GCCT)_{14}$	55	♀118–150 ♂118–130	0.813	0.707	−0.160	0.116	6 (3.4)
Mean (SD)		–	–		0.577 (0.26)	0.619 (0.19)	0.063 (0.29)	–	6.8 (3.4) (3.4) (2.2)

T_a, annealing temperature; N, number of individuals genotyped; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, Wright inbreeding index; A, number of alleles detected; A_e, effective number of alleles; P_{HWE}, probability associated with the HWE (*significant after Bonferroni sequential correction for multiple tests). ‡Presence of the M13 tail, 5′-TGTAAAACGACGGCCAG-3′.

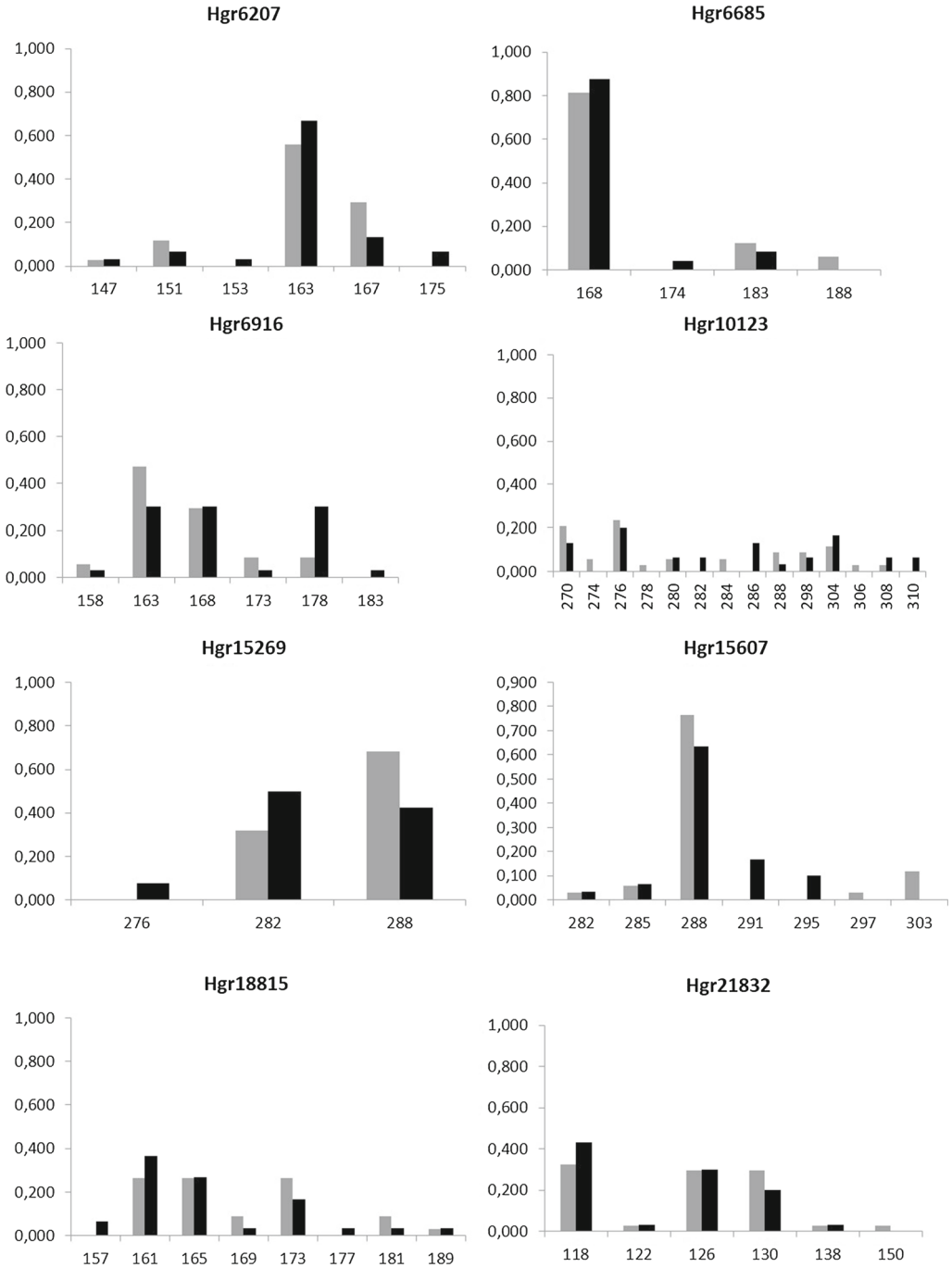

Figure 1. Distribution of allele frequencies at the eight microsatellite loci for male (black bars) and female (grey bars) *H. grisea*. The *X*-axis indicates allele sizes.

(<0.05). The expected heterozygosity ranged from 0.283 (*Hgr*6685) to 0.878 (*Hgr*10123), with an average value of 0.619 (table 2).

We have not found any evidence of linkage disequilibrium on pairwise loci comparisons. After Bonferroni corrections, only locus *Hgr*15607 showed a departure from Hardy–Weinberg proportions (table 2), as a result of strong heterozygote deficit ($F_{IS} = 0.666$). In addition, *Hgr*15607 was the only locus that indicated null allele presence, possibly at high frequency (estimated 0.274). However, we also noticed that this was the only locus where significant differences ($P < 0.05$) in frequency distribution between males and females occurred. In locus *Hgr*15607, we observed alleles with a frequency above 0.1, either in females (allele 303) or in males (alleles 291 and 295), exclusively (figure 1). The remaining seven loci showed a similar frequency distribution between males and females ($P > 0.05$).

Discussion

Here, we have described the first set of microsatellite markers for the sea cucumber, *H. grisea*. The markers' diversity and polymorphism fall within the range previously described for Holothuroidea. For instance, the average number of alleles (*A*) found in other species of *Holothuria* was 3.5 for *H. leucospilota* over eight loci and 30 individuals (Dai *et al.* 2015), 9.6 for *H. scabra* over 18 loci and 50 individuals (Fitch *et al.* 2012) and 16.6 for *H. mammata* over nine loci 15 individuals (Henriques *et al.* 2016). Similarly, the range of average expected heterozygosities recorded were 0.359–0.503 for *H. leucospilota* (Dai *et al.* 2015; Shangguan *et al.* 2015), 0.431–0.625 for *H. scabra* (Fitch *et al.* 2012; Li *et al.* 2015) and 0.786 for *H. mammata* (Henriques *et al.* 2016).

Among the described loci, *Hgr*15607 showed the strongest departure from the HWE. The presence of null alleles, a common artefact of microsatellite-based analyses (Oosterhout *et al.* 2004), could have caused the heterozygote deficiency we observed. Heterozygosity deficits due to null alleles have been recorded many times among sea cucumbers (Kanno *et al.* 2005, 2006; Chang *et al.* 2009; Peng *et al.* 2009, 2012; Kang *et al.* 2011; Fitch *et al.* 2012; Shangguan *et al.* 2015). However, we should not immediately exclude other reasons for departure from HWE proportions, such as selection or population effects. In this work, we were able to separate male and female genotypes, and locus *Hgr*15067 was the only one where we observed significant frequency differences between sexes. The frequency distribution for the locus was strongly unimodal and the differences were accounted for by alleles around or below the 0.1 frequency mark. Therefore, this is a preliminary observation that requires further investigation. Increasing the sample size and the number of populations might allow a better understanding of this feature.

In conclusion, this study provides a set of polymorphic microsatellite loci for the study of *H. grisea*, a species of growing commercial interest in the Southwest Atlantic. To our knowledge, this is the first set of microsatellite markers developed for this important species. These markers will be useful for studies on spatial and temporal genetic structure, gene flow, stock enhancement and management, which will consequently add valuable information for proper conservation of the species and development of sustainable fishery and aquaculture. Additionally, differences in allele frequencies suggest that marker *Hgr*15607 may be linked to sex in these sea cucumbers; further studies should be carried out to clarify this observation.

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