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Original article

Isolation and characterization of 17 polymorphic microsatellite loci for a sea urchin (*Echinometra lucunter*: Echinometridae)

Aislamiento y caracterización de 17 microsatélites polimórficos en un erizo de mar (*Echinometra lucunter*: Echinometridae)

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Abstract

As a first step to establish the genetic structure of the sea urchin *Echinometra lucunter lucunter* throughout the Caribbean Sea, 26 microsatellite loci were isolated using Illumina paired-end sequencing, Next Generation Sequencing (NGS). We successfully optimized 17 loci for genotyping and the variation tested for 23 individuals from the Caribbean Sea and Tropical Eastern Atlantic Ocean. The allele number per locus (Na) ranged from four to 24, the observed heterozygosity (Ho) from 0.682 to 1, and the expected heterozygosity (He) from 0.609 to 0.9304. We detected no linkage disequilibrium between pairs of loci. These microsatellites will be used for the first time to detect the influence of marine barriers to genetic flow in the sea urchin *E. lucunter lucunter* throughout the Caribbean Sea. These new validated markers will be essential for current conservation and connectivity studies across the Caribbean Sea and the Atlantic Ocean.

Keywords: Marine connectivity; Echinodermata; Caribbean Sea; Microsatellites; Nuclear markers; *Echinometra lucunter.*

Resumen

Como primer paso para establecer la estructura genética del erizo de mar *Echinometra lucunter lucunter* a lo largo del Caribe, se aislaron 26 microsatélites usando secuenciación de extremo pareado (*Next Generation Sequencing*, NGS) Illumina. Se optimizaron exitosamente 17 marcadores y se probó su variación alélica en 23 individuos recolectados a lo largo del mar Caribe y en Cabo Verde, Atlántico oriental tropical. El número de alelos por locus (Na) fluctuó entre cuatro y 24, la heterocigosidad observada (Ho) entre 0,682 y 1 y la heterocigosidad esperada (He), entre 0,609 y 0,9304. No hubo desequilibrio de ligamento entre los pares de locus detectados. Los microsatelites aislados e identificados se usarán por primera vez para detectar la influencia de las barreras marinas en el flujo génico del erizo *E. lucunter lucunter* a lo largo del mar Caribe. Estos nuevos marcadores serán esenciales para la conservación y los estudios de conectividad a través del mar Caribe y el océano Atlántico, área donde se distribuye la especie.

Palabras clave: Conectividad marina; Echinodermata; Mar Caribe; Microsatelites; Marcadores nucleares; *Echinometra lucunter.*

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Introduction

Sea urchins play an ecologically important role in marine coastal ecosystems because of their high abundance and herbivorous feeding preference. *Echinometra lucunter* (Linnaeus, 1758) is often the dominant herbivore of benthic algae in shallow intertidal areas making it an excellent model to identify connectivity patterns for benthic organisms of the Atlantic Ocean.

E. lucunter is the most widely distributed species of the genus *Echinometra*. It has two subspecies, one in the Eastern Atlantic (*Echinometra lucunter polypora* Pawson, 1978) and the other in the Greater Caribbean (*E. lucunter lucunter* Linnaeus, 1758) which are separated from each other by 200000-250000 million years (**McCartney, *et al.,*** 2000). The subspecies *E. lucunter polypora* was proposed by **Pawson** (1978) based on morphological differences found in specimens that live in the Central Atlantic islands of Ascension and St. Helena. Molecular analyses based on the CO1 gene (**McCartney, *et al.,*** 2000) show that the closest approach is found between populations present in the African Atlantic, the Central Atlantic Islands (Ascension and St. Helena), and Brazil, suggesting that these populations belong to the subspecies *E. lucunter polypora*. The subspecies *E. lucunter lucunter* is present only in the Greater Caribbean.

Researchers have designed and used several types of molecular markers in the past to study genetic diversity within a species and analyse population distribution. Marker choice depends on the study, the particular species, and the questions to be answered (**Sunnucks,** 2000), therefore, it is necessary to use the correct molecular marker in line with the goals of the research and the available budget.

Microsatellites, or simple sequence repeats of loci (SSR), are tandem repeats of up to six nucleotides in the nuclear genome. The number of repeat units commonly ranges from around 5 to 40, with the number of repeats (and therefore sequence length) corresponding to different alleles (**Selkoe & Tonnen,** 2006).

As a first step to establish the genetic structure and the influence of the marine barriers to the sea urchin *Echinometra lucunter lucunter* throughout the Caribbean Sea, we present the design, development, and characterization of 17 microsatellite markers in the sea urchin *E. lucunter*.

Materials and methods

Development of microsatellites

We selected eight *E. lucunter lucunter* individuals from three sites: Venezuela, Honduras, and Puerto Rico to develop microsatellite markers (**Table 1**).

Table 1. Samples used to design the simple sequence repeats (SSRs) of *Echinometra lucunter*

Country	Coordinates	Collection date	Sites/Number of samples	Approval organization samples / approval number
Venezuela	10°41’20.7” N 63°52’29.11” W	15/02/2016	Islote Lobos/2	*Universidad Simón Bolívar*/ by-product of the artisanal fishery of the Araya Peninsula in Venezuela
Honduras	15°58’17.92” N 86°28’14.36” W	14/05/2015	Cayos Cochinos/3	*Instituto Nacional de Conservación y Desarrollo Forestal Áreas Protegidas y Vida Silvestre* (ICF)/ Resolution-DE-MP-080-2015
Puerto Rico	17°57’08.61” N 67°03’24.30” W	15/06/2016	Cayo Enrique/3	*Departamento de Recursos Naturales y Ambientales*/ DRNA: 2016-IC-052

Genomic DNA was extracted from tube feet using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) (**Qiagen,** 2006). The resulting DNA extractions were indexed and pooled to create a paired-end library using the Illumina Nextera® DNA Library Preparation Kit (Illumina, California, USA). Sequencing was carried out using an Illumina MiSeq platform (Illumina, California, USA) at the University of Salford, Salford, UK.

Microsatellite PCR markers were developed in-house using a previously published microsatellite design method (**Fox, *et al.,*** 2019). The **Griffiths, *et al.*** (2016) workflow was used to detect microsatellites in the sequence data and design PCR primers. Multi-individual microsatellite identification (MiMi) (**Fox, *et al.,*** 2019) was used subsequently as further quality control on potential markers.

To check for amplification success, we tested potential markers with the same eight DNA extractions used to design the microsatellites and extractions from additional individuals from the Colombian Caribbean Sea and the Tropical Eastern Atlantic Ocean. For the amplification steps we used the specifications of the Type-it® Microsatellite PCR kit (Qiagen, Hilden, Germany), (**Qiagen,** 2009) in a reaction volume of 5µl and the following thermal cycle: Step 1: 95°C/5 minutes; step 2: 28 cycles x (95°/30 seconds for denaturation, 61°C/90 seconds for annealing, and 72°C/30 seconds for elongation); step 3: 60°C/30 minutes, and step 4: hold at 4°C. We checked the final 5µl of PCR product solution on 1.8% agarose electrophoresis gel against a Hyperladder IV size standard (Bioline, London, UK) to confirm successful PCR amplification and the approximate size range of the fragments. Twenty-six SSR's were successfully visualized with these steps. To facilitate multiplexing, a universal tail PCR approach (**Blacket, *et al.,*** 2012; **Culley, *et al.,*** 2013) was used to add a fluorochrome to each of the markers (6FAM, TAMRA, HEX, and PET). Amplification success, thermal cycles, PCR features, and confirmation of successful gels were used as in the earlier step.

Microsatellite screening

In order to assess polymorphism of SSR's, we analysed 23 DNA samples from Cape Verde (Africa) and Caribbean localities (**Table 2**) using six multiplex (three-four markers each) and seven singleplex reactions (**Table 3**) using the PCR thermal cycles described previously. Confirmation of amplified SSR was again through visualizing on 1.8% agarose gels.

Table 2. Populations used to observe behaviour and polymorphism of microsatellites loci developed for *Echinometra lucunter*

Country	Coordinates	Collection date	Sites / number of samples	Approval organization samples / approval number
Honduras	15°58'17.92" N 86°28'14.36" W	14/05/2015	Cayo Cochinos/3	*Instituto Nacional de Conservación y Desarrollo Forestal Áreas Protegidas y Vida Silvestre* (ICF)/ Resolution-DE-MP-080-2015
Puerto Rico	17°57'08.61" N 67°03'24.30" W	15/06/2016	Cayo Enrique/3	*Departamento de Recursos Naturales y Ambientales*/ DRNA: 2016-IC-052
Colombia	12°27'3.6" N 72°7'17.4" W	01/07/2016	Cabo de la Vela/1	*Ministerio de Ambiente y Desarrollo Sostenible, República de Colombia, Autoridad Nacional de Licencias Ambientales* (ANLA): Resolution number 255, 12 of March of 2014 Export permit #00848
	8°41'8.2" N 77°19'12.7" W	15/06/2016	Capurganá/2	
	12°32'34" N 81°44'45" W	14/08/2016	San Andrés/3	
	14°17'4.52" N 80°22'55" W	18/08/2016	Isla Cayo Serrana /1	
Venezuela	10°41'20.7" N 63°52'29.11" W	15/02/2016	Islote Lobos/2	*Universidad Simón Bolívar*/ by-product of the artisanal fishery of the Araya Peninsula in Venezuela
Cape Verde-Africa	16°35'39.4"N 22°53'42.6" W	11/08/2011	Santa María, Sol Island/8	Donation by Owen Wangensteen, Department of Animal Biology at University of Barcelona.

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Capillary electrophoresis of PCR products was performed using an Applied Biosystems 3730 DNA Analyzer at the Genomic Technologies Facility, University of Manchester (UK). Length and allele scoring of PCR products were estimated using the GeneMapper 5 (ThermoFisher Scientific) and Peakscanner v1.0 software (Applied Biosystems). Seventeen primers showed successful amplification, variable microsatellite loci, and were successfully genotyped in all 23 samples used.

Data analysis

We used the microsatellite genotypes of 23 individuals from several localities in the Caribbean and at Cape Verde (Africa) for analysis. Data were edited in Excel (Microsoft, Washington, USA) tables and converted to input files (Microsatellite Toolkit) to produce the genepop data. To detect and estimate genotyping error, data were probed using the Microchecker (**Van Oosterhout, *et al.,*** 2004) software. We then tested for Hardy-Weinberg Equilibrium (HWE), calculated the number of alleles per loci (Na) and population, and observed heterozygosity (Ho) and expected heterozygocity (He) with the GenAlEx platform (http://biology.anu.edu.au/GenAlEx/Welcome.html). We estimated

Table 3. Characteristics of 26 nuclear microsatellite markers developed for *Echinometra lucunter*. F: Forward. R: Reverse. F.L: Fluorescent label. Grey: No development

Reactions	Locus		Universal tail used	Primer sequences	F.L	Repeat motif	Allele size tange (bp)
Singleplex	EL1	F:	GCCTCCCTCGCGCCA	AATGCTCCCCAAATTTCTCG	6-FAM	AAG*81	
		R:		GCCTGTTACAATTTCCTGGGG			
Singleplex	EL2	F:	GCCTCCCTCGCGCCA	ATGGCTTTTGGGACAGATGG	6-FAM	AAG*111	394-434
		R:		TCCTCAGAAATTATGCCCGC			
Singleplex	EL3	F:	GCCTCCCTCGCGCCA	CGATGTGAGACTAGAAATGTACGG	6-FAM	AAC*93	390-407
		R:		CGACAACTGGTACCTGGACG			
Singleplex	EL4	F:	GCCTCCCTCGCGCCA	GCATGTTCACGATTTGAAGG	6-FAM	AAG*90	241-269
		R:		GCAGATGGTGGAGAAGAGG			
Singleplex	EL5	F:	GCCTCCCTCGCGCCA	CTTTCAAAGACCCGATACAACG	6-FAM	AG*90	296-324
		R:		TGCACCGAATAATGATGAGC			
Singleplex	EL6	F:	GCCTCCCTCGCGCCA	TGATCGAAACGGTGACATCC	6-FAM	AAAC*104	209-253
		R:		AGTCAGATCACCGCCATGC			
Singleplex	EL7	F:	GCCTCCCTCGCGCCA	TGGGACAAAGAGAGAGCTTGG	6-FAM	AG*72	162-171
		R:		AGCGGATGTTGATTTACGGC			
Multiplex1	EL8	F:	CAGGACCAGGCTACCGTG	GGCTTATGGTCACAGGACTGG	6-FAM	AG*68	
		R:		GGAAGAGCTCGCTTGATTCG			
	EL14	F:	CACTGCTTAGAGCGATGC	TCTTCTCGATCCCTCTTTGTCC	PET	AAG*84	333-345
		R:		TGAAGGTGCACTGATGGAGG			
	EL20	F:	CGGAGAGCCGAGAGGTG	CGCCATTAATGTCAACACCG	HEX	ATC*69	249-318
		R:		AGCATCTGAATCCCCACCC			
Multiplex2	EL9	F:	CAGGACCAGGCTACCGTG	CCGTCTTGAGAGCTATCGGC	6-FAM	AGT*72	
		R:		GCGTTTAAGATTCCCTTTGCC			
	EL15	F:	CACTGCTTAGAGCGATGC	GTTTCACATCGGTCCGTCG	PET	AAG*93	346-400
		R:		CTCCATAGCAACATGACGGG			
	EL21	F:	CGGAGAGCCGAGAGGTG	GCAGTATCATCATCGCCAGC	HEX	ATC*126	315-561
		R:		TCAGGGATTGTGTCTTTGCG			

Reactions	Locus		Universal tail used	Primer sequences	F.L	Repeat motif	Allele size tange (bp)
Multiplex3	EL10	F:	CAGGACCAGGCTACCGTG	TCAGCCTAAGTGTGTTGGAAGC	6-FAM	AG*114	
		R:		CTCTGTGCAAAGAAGGTTAAGTGC			
	EL16	F:	CACTGCTTAGAGCGATGC	TGAAGACGTTCGTTGATTGC	PET	AG*52	182-272
		R:		GCACTTACATCAGTGGATTGC			
	EL22	F:	CGGAGAGCCGAGAGGTG	ACATTGAAAACACTGGGGCG	HEX	AAG*129	156-294
		R:		ATGCATTTTCATGTGCACCC			
Multiplex4	EL11	F:	CAGGACCAGGCTACCGTG	TGATGCTCTACATTTGAGCCC	6-FAM	AG*68	
		R:		TCTCCCTAGCGTTACAGGGG			
	EL17	F:	CACTGCTTAGAGCGATGC	AAGTGGGTAGGCCAACGCTACACG	PET	AG*96	217-247
		R:		ACGCGCATACTATAGCGTGGTGG			
	EL23	F:	CGGAGAGCCGAGAGGTG	GAGACAGAATGAAAATCGCTGC	HEX	TG*38	297-367
		R:		CGAAGGGACTATGAAACGGG			
Multiplex5	EL12	F:	CAGGACCAGGCTACCGTG	TGGTGCAAATGTAGCAGTCG	6-FAM	AGT*75	
		R:		CGTGGTACATTGCTTACAGCC			
	EL18	F:	CACTGCTTAGAGCGATGC	GCTCATTGGCAGATCAAACG	PET	AG*60	217-399
		R:		CTTGCTCTCTTGTTCTATTCCCC			
	EL24	F:	CGGAGAGCCGAGAGGTG	CGGTGACCCTACAGTAAACTTGC	HEX	AG*54	300-474
		R:		TCCCTCTTTCTCCCACGC			
Multiplex6	EL13	F:	CAGGACCAGGCTACCGTG	CCATGCCCAATAATAACGCC	6-FAM	AG*128	
		R:		ATCCTTGGGCCATCTTTCC			
	EL19	F:	CACTGCTTAGAGCGATGC	CAAACTAAAGACGCCCAGCC	PET	AG*40	156-186
		R:		ATGAGGCTTCCCTGAATCCC			
	EL25	F:	CGGAGAGCCGAGAGGTG	TAAACCAGGCCAGAATGACG	HEX	TC*72	
		R:		TGGGAGCTTTTAGTCCGAGC			
Singleplex	EL26	F:	CGGAGAGCCGAGAGGTG	TTCATCCAACTCTGTCACATCC	HEX	AAG*147	

the Polymorphic Information Content (PIC) of each locus using the program Cervus 3.0.7 (**Kalinowski, *et al.*,** 2007), and the genotypic linkage disequilibrium using GenPop (http://genepop.curtin.edu.au/). We used the Bonferroni correction method to adjust the significance levels for HWE and linkage disequilibrium tests.

Results

The Illumina MiSeq run generated 27,043,607 paired-end reads with the eight DNA samples (Honduras, Puerto Rico, and Venezuela). We obtained a further filtered list of 308 potentially amplifiable loci (PAL) with the pal_filter from the Galaxy platform and we selected 26 PAL's for screening: 14 di-, 11 tri- and one tetra-nucleotide (**Table 3**).

After several assays, we obtained 17 successful microsatellite markers: Nine di-, seven tri-, and one tetra nucleotide (**Table 4**). The allele number per locus (Na) ranged from four to 24 while the observed (Ho) and expected (He) heterozygosities ranged from 0.682 to 1 and 0.609 to 0.9304, respectively.

Two out of the 17 loci deviated from HWE (EL22- present in 16 of 23 samples during microsatellite screening and EL14- present in all samples except one in San Andres Island) after Bonferroni correction for multiple testing ($p<0.05$). Homozygote excess, related to the presence of null alleles or stochasticity due to the different locality sources for one

Table 4. Characteristics of 17 markers developed for *Echinometra lucunter*

Locus name	Forward and reverse primer	Fluor	R. motif	Locus size range	N	Na	Ne	Ho	He	PIC	pHWE	pHWEbc
EL2	ATGGCTTTTGGGACAGATGG TCCTCAGAAATTATGCCCGC	6-FAM -	$(AAG)_{111}$	394-434	7	7	4.900	0.857	0.796	0.772	0.817	1.000
EL3	CGATGTGAGACTAGAAATGTACGG CGACAACTGGTACCTGGACG	6-FAM -	$(AAC)_{93}$	390-407	8	6	4.414	1.000	0.773	0.744	0.509	1.000
EL4	GCATGTTCACGATTTGAAGG GCAGATGGTGGAGAAGAGG	6-FAM -	$(AAG)_{90}$	241-269	8	6	4.000	1.000	0.750	0.712	0.559	1.000
EL5	CTTTCAAAGACCCGATACAACG TGCACCGAATAATGATGAGC	6-FAM -	$(AG)_{90}$	296-324	6	10	9.000	1.000	0.889	0.878	0.600	1.000
EL6	TGATCGAAACGGTGACATCC AGTCAGATCACCGCCATGC	6-FAM -	$(AAAC)_{104}$	209-253	8	4	2.844	0.875	0.648	0.592	0.204	1.000
EL7	TGGGACAAAGAGAGAGCTTGG AGCGGATGTTGATTTACGGC	6-FAM -	$(AG)_{72}$	162-171	8	4	2.560	0.875	0.609	0.530	0.495	1.000
EL14	TCTTCTCGATCCCTCTTTGTCC TGAAGGTGCACTGATGGAGG	PET -	$(AG)_{68}$	333-345	22	5	3.625	0.682	0.724	0.681	0.002**	0.034*
EL15	GTTTCACATCGGTCCGTCG CTCCATAGCAACATGACGGG	PET -	$(AAG)_{93}$	346-400	20	16	10.256	0.800	0.903	0.895	0.014*	0.238
EL16	TGAAGACGTTCGTTGATTGC GCACTTACATCAGTGGATTGC	PET -	$(AG)_{52}$	182-272	16	19	15.059	0.875	0.934	0.930	0.144	1.000
EL17	AAGTGGGTAGGCCAACGCTACACG ACGCGCATACTATAGCGTGGTGG	PET -	$(AG)_{96}$	217-247	19	13	8.022	0.789	0.875	0.864	0.590	1.000
EL18	GCTCATTGGCAGATCAAACG CTTGCTCTCTTGTTCTATTCCCC	PET -	$(AG)_{60}$	217-399	21	22	11.919	0.810	0.916	0.911	0.024*	0.408
EL19	CAAACTAAAGACGCCCAGCC ATGAGGCTTCCCTGAATCCC	PET -	$(AG)_{40}$	156-186	11	10	5.261	0.909	0.810	0.792	0.275	1.000
EL20	CGCCATTAATGTCAACACCG AGCATCTGAATCCCCACCC	HEX -	$(ATC)_{69}$	249-318	20	16	10.000	0.850	0.900	0.892	0.021*	0.357
EL21	GCAGTATCATCATCGCCAGC TCAGGGATTGTGTCTTTGCG	HEX -	$(ATC)_{126}$	315-561	22	24	13.444	1.000	0.926	0.921	0.229	1.000
EL22	ACATTGAAAACACTGGGGCG ATGCATTTTCATGTGCACCC	HEX -	$(AAG)_{129}$	156-294	15	13	9.783	0.733	0.898	0.889	0.001***	0.017*
EL23	GAGACAGAATGAAAATCGCTGC CGAAGGGACTATGAAACGGG	HEX -	$(TG)_{38}$	297-367	19	18	11.108	0.842	0.910	0.904	0.017*	0.289
EL24	CGGTGACCCTACAGTAAACTTGC TCCCTCTTTCTCCCACGC	HEX -	$(AG)_{54}$	300-474	10	13	10.000	0.800	0.900	0.892	0.130	1.000

Fluor: Fluorescent dye, R. motif: Motif of repetition, N: Sample size, Na: Allele number, Ne: Allele effective number, Ho: Observed heterozygosity, He: Expected heterozygosity, PIC: Polymorphic information content, pHWE: Hardy-Weingberg Equilibrium, pHWEbc: Hardy-Weingberg Equilibrium with Bonferroni Correction method. *: p<0,05, **: p<0,01; *** p<0,001

population, might explain these deviations. Polymorphic information content (PIC) ranging from 0.530 to 0.930 shows normal polymorphism behavior (**Table 4**). We did not detect linkage disequilibrium between any locus pair after the Bonferroni correction, which means that in general, all the loci were independent.

Discussion

Microsatellite development is useful for a variety of genetic analyses of population structure. In this case, these markers will be used for the first time to confirm the influence of marine barriers to genetic flow among populations of *E. lucunter lucunter* throughout the Caribbean Sea.

For many years, based upon the biological characteristics of marine organisms and barriers such as mouths of rivers, marine currents, physicochemical parameters, and others in the Caribbean Sea, several authors have wondered if the organisms of this area are genetically homogeneous or segregated. While few marine barriers have been identified (**Carlin, *et al.,*** 2003, **Baums, *et al.,*** 2005, **Cowen, *et al.,*** 2006, **Taylor & Hellberg,** 2003, 2006), several biophysical models have been proposed to identify patterns of connectivity and potential barriers to larval dispersal of reef fish (**Schultz & Cowen,** 1994; **Paris & Cowen,** 2004; **Cowen, *et al.,*** 2006). There are few genetic evaluations of other marine groups providing evidence of both connectivity and phylogeographic breaks for the Caribbean (**Avise,** 1992; **Carlin, *et al.,*** 2003; **Taylor & Hellberg,** 2003; **Baums, *et al.,*** 2005).

According to **Fox, *et al.*** (2019), ecological and conservation studies often focus upon non-model species for which genetic markers are not available, and *Echinometra* species are an example. The methodology used to isolate and characterize the *E. lucunter* loci followed the method of multi-individual microsatellite identification (MiMi) proposed by **Fox, *et al.*** (2019) with the combination of affordable Next Generation Sequencing (NGS) and freely available bioinformatics tools. Our positive results support once again the effectiveness and speed of this method.

The application of the microsatellite markers developed herein to additional *E. lucunter* populations will allow understanding how the genetic diversity is distributed in this benthic species throughout the Caribbean Sea considering its biological features and the marine barriers described for the region.

Conclusions

Seventeen (17) new microsatellites were developed and characterized for the sea urchin *Echinometra lucunter lucunter*. The markers fill up the gap of information regarding tools to characterize the genetic structure of this species in the Caribbean Sea. Due to the absence of microsatellites within the species of the genus *Echinometra,* it was necessary to develop and characterize a number of this kind of markers to determine the genetic structure of *Echinometra lucunter lucunter* in the Caribbean Sea. The successful isolation and characterization of these microsatellites will contribute to genetic and marine connectivity studies of *Echinometra lucunter lucunter* and *Echinometra lucunter polypora* through their broad range of distribution in the Atlantic Ocean. Information on population genetics of this species is crucial for the assessment of the ecological situation of their populations and the development of management strategies when necessary.

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Author contributions

MBS was the person in charge of the results and their respective analyses, and she wrote and sent the manuscript to the journal. YFC worked with the principal author in the obtention of results and their respective analysis. LMB directly advised the work and supported the sample collection and laboratory processes, and participated in the manuscript writing. GF collaborated directly to laboratory results and actively participated in the edition of the manuscript. TDH collaborated in the laboratory work. NHCC advised the manuscript writing, and RP participated in the editing of the document.

Conflict of interests

We have no conflicts of interest to report.

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