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Isolation and Characterization of New Polymorphic Microsatellite Markers from the Invasive Worm *Branchiomma Luctuosum* (Grube, 1870) (Annelida)

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Abstract

Introduction of exotic species in new areas through anthropic action is one of the major problems that can affect biodiversity. *Branchiomma luctuosum* is known for its highly invasive potential and the actual occurrence of species commonly associated with port activity areas is an extra evidence that this anthropogenic activity should not be underestimated. In order to develop suitable molecular markers for future studies on colonization routes and population dynamics of the invading individuals of *B. luctuosum*, nine highly polymorphic microsatellite *loci* were isolated and their polymorphism levels were evaluated. These *loci* showed a range of number of alleles per *locus* from five to ten and all *loci* had a high level of genetic diversity, and exhibited significant heterozygote deficiencies probably due to the presence of null alleles. Significant deviations from the Hardy-Weinberg equilibrium were detected at several *loci* and most of them were related to a heterozygous deficit. Heterozygous deficiency can be expected in this case due to the biology and history of this invasive species, in relation to its recent introduction in the Brazilian coast and possible action of multiple introductory events.

Keywords: Bioinvasion; Molecular Markers; Fanworm; Biofouling

Introduction

Introduction of exotic species in new areas through anthropic action is one of the major problems that can affect biodiversity. In marine environments, an introduction of exotic species may occur accidentally, through transportation by ships and boats, or intentionally, in order to control pests, provide food or create new aquaculture products [1]. However, the increasing maritime traffic due to expanding

globalization, introduction of alien species has occurred more often, especially with ballast water and biofouling [2]. Exotic organisms usually do not have natural predators in the new settled environment, so they can exponentially increase their population, which can lead to a decline in natural populations. Therefore, the presence of exotic organisms can cause ecological, economic, and health risk impacts, and the competition with native species can lead to environmental imbalance, extinctions, and consequently, loss of biodiversity. Understanding about biology and ecology of alien species colonization is crucial to determine the effects of introduction and dissemination, as well as to evaluate their interaction with native species [2].

The genus *Branchiomma* is composed by about 30 widely distributed species of sessile marine sabelids, mostly found in sheltered waters (*e.g.*: bays, lagoons, and ports). *Branchiomma* specimens are usually found in cracks of rocks, corals, and among the incrustating fauna [3]. The genus presented several invasive species, such as *B. bairdi, B. boholense, B. coheni, and B. luctuosum,* which have been reported as exotic for many areas [4-6]. Originally described for the Red Sea by Grube in 1870, *B. luctuosum* is known for its highly invasive potential [1]. The actual occurrence of the species commonly associated with port activity areas is an extra evidence that anthropogenic activity should not be underestimated [7, 8]. The first record of *B. luctuosum* occurring outside the Red Sea was made in 1989 [9] for Mediterranean Sea. Thereafter, the distribution rapidly expanded and invaded several ports on eastern Iberian coast [1] and most of Italian coast, spreading throughout the Mediterranean and being considered a pest for above mentioned areas [8]. In addition, recent records indicated that *B. luctuosum* successfully crossed the Strait of Gibraltar and colonized the Atlantic coast of Africa [2]. Concerning the Atlantic coast of America, the first record was made by Nogueira, et al. [7] for Brazilian coast, in a port region of São Paulo and was likely to be a case of recent introduction. Subsequently, the species spread rapidly and have been recorded occurring along the entire Brazilian coast from Santa Catarina to Paraíba [10,11].

Besides the invasive nature, *B. luctuosum* could be potentially used in medical therapy, since it presents a blood pigment, an extracellular globin called chlorocruorin, which has been investigated as a possible artificial oxygen carrier for mammalian systems [12,13]. To develop suitable molecular markers for future studies on colonization routes and population dynamics of *B. luctuosum*, nine highly polymorphic microsatellite *loci* were isolated and their polymorphism levels were evaluated and described in the present study. Microsatellite markers will be a valuable tool in assessing the demographic processes associated with invasion of the exotic *B. luctuosum* from a genetic point of view.

Material and Methods

Twenty-five specimens of *B. luctuosum* were collected in the southern region of Brazilian coast, in Bay of Florianópolis, Santa Catarina, Brazil (23°38'29,9"S 048°31'34,1"W). Genomic DNA was extracted from the body wall using a modified guanidine and phenol-chloroform extraction protocol from Hillis, et al. [14]. Microsatellite libraries were developed by Macrogen Inc., (Seoul, Korea) from purified DNA of one specimen following the methods of Jones, et al. [15] for library construction, microsatellite enrichment and screening. Fifty-seven pairs of primers were designed from the enriched library using WebSat software [16]. Posteriorly, 20 primer pairs were selected to test for amplification success and evaluate polymorphism levels for each *locus*. Forward primers were synthesized with a M13 tail at their 5′end allowing use of the tailed primer method [17].

Gradient PCR was used to define optimal annealing temperature for each *locus* (Table I). Then, all *loci* were amplified using one of the four fluorescent dyes (6-FAM™, PET®, NED™ and VIC®, Applied Biosystems®). During all the procedure, each *locus* was amplified with the same fluorescent dye. PCR mix consisted of 1 U GoTaq (Promega), 0.20 mM dNTPs, 2.5 mM $MgCl_2$, 1 mM BSA, 0.5 µM of reverse primer, 0.25 mM of oligo marked with a dye (tail) primer, and 0.13 of mM forward primer, with a final volume of 15 µL per reaction containing 30 ng of DNA template. Cycling conditions were: 94°C, 3 min, 30 cycles at 94°C, 45 sec (s); 52°C-67°C, according to each primer, 45 s; 72°C, 45 s, 8 cycles at 94°C, 45 s; 53°C, 45 s; 72°C, 45 s and 72°C, 30 min. Samples were pooled with a size standard (GeneScan 500-LIZ; Applied Biosystems), and genotyped using the automated platform ABI3500.

The GeneMarker® software V 2.6.3 (SoftGenetics LLC) was used for genotyping score and allele sizing and Excel macro Autobin V 0.9 was employed to establish the fragment size values of each allele in discrete units. The possibility of occurrence of genotyping errors, such as the presence of null alleles, was calculated using Micro-Checker V 2.2.3 [18]. Moreover, null allele frequencies for each *locus* were calculated with Cervus V 3.0.7 [19] and number of alleles, allele frequencies, observed and expected heterozygosities, and inbreeding index (Fis)

were evaluated using Fstat V 2.9.3 [20]. The 4.2 online version of GENEPOP [21] was used to test for linkage disequilibrium between *loci* and for tests of Hardy-Weinberg equilibrium (HWE).

Results and Discussion

From 20 tested *loci*, five were discarded due to amplification failure and, then, another seven *loci* were discarded due to inconsistencies in amplification or size patterns. Genotypes were obtained for the remaining nine *loci*, however, Bluc25 *locus* was subdivided into two *loci*, since results amplifications of two distinct (and, thus, not linked) regions of microsatellites (Table 1). The analyzed *loci* showed a range of number of alleles per *locus* from five to ten with expected and observed heterozygosity ranged from 0.650 to 0.874 and from 0.000 to 0.909, respectively (Table 2). All nine *loci* had a high level of genetic diversity and exhibited significant heterozygote deficiencies probably due to the presence of null alleles, as suggested by Micro-Checker analysis, which indicated no evidence for scoring error due to stuttering or evidence for large allele dropout. Moreover, the occurrence of null alleles has been commonly reported during characterization of microsatellite *loci* and population genetics studies, being described in many studies with annelids [22-24]. Significant deviations ($P<0.05$ after Bonferroni correction) from HWE were detected at several *loci*, as shown in Table II. Most of deviations were related to a heterozygous deficit, evidenced by the significantly positive values of the Fis index. Our results did not observed any significant linkage disequilibrium between any pair of *loci*. Observed heterozygous deficiency can be expected considering the biology and history of this invasive species, specially its recent introduction in the Brazilian coast [7] and the possibility of multiple introductory events, which characterizes Wahlund effect. The presence of null alleles and consequent heterozygous deficiency, as well as multiple recent introduction events are determining factors for deviations from HWE.

Our findings are consistent with previous studies with different invasive marine species, including other annelid species, where Wahlund effect, recent introduction events, and presence of null alleles contributed to the deviations from HWE [22-24]. Therefore, the nine microsatellite markers developed represent effective molecular tools for population analysis of *B. luctuosum*, and may be very useful for studies on population dynamics of this highly invasive species.

Locus	Motif	Primer Sequence (5'-3')		T (°C)
Bluc07	GT(12)	F	GACAATTCAAACTGCGACTGAC	67
		R	GTGTTTAGGGTTCTAGGGCAAA	
Bluc08	CA(8)	F	CAACTGCCATACAAAACTACACTGA	67
		R	AGGGACAGCCAGGGTTTG	
Bluc23	AC(15)	F	GGAGACAACATTCAAAAAGTGA	52
		R	GGATTCAAACTGACGACTCTG	
Bluc25	GT(10)	F	AATTCAATGGCTAGGTCTATCC	52
		R	GGCTGAGAAATACAGATTTTTG	
Bluc25a	GT(10)	F	AATTCAATGGCTAGGTCTATCC	52
		R	GGCTGAGAAATACAGATTTTTG	
Bluc27	TG(9)...TG(12)	F	GTTCGTTTGTCCGTTATTCAG	61
		R	TATGGCTTCAGCATTAAATCTG	
Bluc32	TTG(7)	F	TGTTGCTGCTGTTGTTGTTGTA	58
		R	CTGACTGACACCTGACACCTATG	
Bluc34	TTG(6)	F	CCCAACTCAAGTAACCAGTCCT	52
		R	CGAAACAACACTAATTCAACGC	
Bluc36	GTT(4)	F	TTCCTTGCTGGACACTGAGATA	67
		R	ACAATTAGGCCAGATGAGTGCT	

Table 1: Characteristics of nine polymorphic microsatellite markers developed for B. luctuosum. Locus name; Motif: Repeat motif; F: Forward Primer Sequence; R: Reverse Primer Sequence; T: Annealing Temperature in °C.

Locus	N	Na	Size range (bp)	H(o)	H(e)	Null freq.	Fis	HWE
Bluc07	22	6	284-298	0.636	0.771	0.086	0.178	0.1247
Bluc08	24	8	270-284	0.583	0.867	0.1844	0.332	0.0024
Bluc23	25	9	299-319	0.64	0.723	0.0503	0.117	0.0233
Bluc25	25	6	197-207	0.76	0.809	0.0189	0.062	0.0001*
Bluc25a	25	5	348-356	0	0.65	1	1	0.0001*
Bluc27	22	5	232-240	0.909	0.754	(-)0.1073	(-)0.212	0.1435
Bluc32	20	5	393-411	0.3	0.755	0.4213	0.609	0.0001*
Bluc34	25	10	331-370	0.44	0.811	0.2947	0.463	0.0001*
Bluc36	25	9	267-291	0.72	0.874	0.0857	0.179	0.0001*

Table 2: Genetic variability of nine polymorphic microsatellite markers developed for B. luctuosum. Locus Name; N: Number of individuals genotyped; Na: Number of alleles; Size ranges in base pairs; H(o): Observed heterozygosity; H(e): Expected heterozygosity; Null freq.: Frequency of Null Alleles; Fis: Inbreeding Index; HWE: p-values of Hardy-Weinberg equilibrium test (Bonferroni corrected alfa=0.00714). Asterisks indicate loci with significant deviation from HWE (p<0,05).

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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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