



Microsatellite markers for barnacle studies: Isolation and characterization of polymorphic microsatellite *loci* from the invasive barnacle *Megabalanus coccopoma* (Darwin, 1854) and its cross-amplification in the Southern Atlantic endemic species *Megabalanus vesiculosus* (Darwin, 1854)

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ABSTRACT

Megabalanus coccopoma (Darwin, 1854), an important invasive species in the world scenario, is included in one of the most common barnacle genus known. Nine new polymorphic microsatellite *loci* from *M. coccopoma* and cross-amplification tests of these primers in the Brazilian endemic species *Megabalanus vesiculosus* (Darwin, 1854) are described. Allele numbers varied between seven and 21 in 23 samples of the invasive *M. coccopoma* collected in Brazil. Mean observed and expected heterozygosities ranged from 0.32 to 0.77, and between 0.78 and 0.91, respectively. Linkage disequilibrium was not observed between *loci*, but all of them presented high heterozygote deficiencies, possibly because of recent colonization events along the Brazilian coast. Five *loci* successfully amplified the co-generic species *M. vesiculosus*, but the low diversity described indicates a low transferability of this primers. The microsatellite markers developed herein can be used in future studies of this species that has been registered outside the natural distribution boundaries in several regions of the world, aiming at elucidating the dynamics of this important invasive species inside and outside its native geographic limits.

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1. Introduction

Biological invasions is one of the biggest concern about the Oceans in the last decades. Alien species can be vectors of new diseases, cause changes in the ecosystem and decrease biodiversity (Vitousek et al., 1996). History, diversity and effects of marine bioinvasions remains with a lack in our knowledge and some introductions that happened in the past still keep ignored (Carlton, 1996). There are different ways of dispersion in a potential invasive species, but the oldest and more problematic is the incrustation in ships, platforms and mobile substrates. Barnacles are sessile organisms that firmly attaches

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to the substrate, and especially those from the Family Balanidae who presents a calcareous basis that firmly connects the shell to a variety of substrate making them difficult to remove from the ship hulls.

Megabalanus genus is composed by circa of 25 species that lives mostly on wave-exposed shores on tropical seas (Newman, 1979; Henry and McLaughlin, 1986) but also its members are commonly found on ship hulls and other artificial substrates (Yamaguchi et al., 2009). The presence on mobile artificial substrate leads to some of its member to be found outside of their natural endemic area. Although studies on *Megabalanus* date back to Darwin's (1854) Monographies, there is still many uncertainties concerning species status and distribution (Ross, 1999; Cohen et al., 2014). Also, there is no evolutionary hypothesis for members of the genus, and this might be due to weak species delimitation as Ross (1999) already noted. Recent studies revealed a cryptic *Megabalanus* species among *Megabalanus coccopoma* (Darwin, 1854) invasive population along USA Eastern Coast (Cohen et al., 2014; Tyson, 2015), which might reinforces Ross (1999) concerns about species discrimination.

Megabalanus coccopoma, known as 'Titan Acorn Barnacle', has its natural distribution in the Tropical Eastern Pacific region, from Mexico to Peru (Henry and McLaughlin, 1986) and the first record out of its endemic area was Lacombe and Monteiro (1974), which described its presence in Guanabara Bay, Brazil. After that, several reports of *M. coccopoma* happened all over the world. The species is reported to be present in Eastern coast of USA (Jones et al., 2012; Perreault, 2004), Mexico (Celis et al., 2007), Gambia, on tropical East Africa (Kerckhof et al., 2010), Belgium (Kerckhof, 2002), Japan and Australia (Yamaguchi et al., 2009). Recently Cohen et al. (2014) on a Phylogeographic study based on COI analysis of *M. coccopoma* did not found any structuring pattern among several populations from its endemic and invaded areas (Panama, Mexico, USA and Brazil). Although the study showed no structuring among the studied populations, there is urgency to access how the interchange among its populations using different approaches. Along the Brazilian coast the populations of *M. coccopoma* occurs on natural and artificial substrate and on many places dominates the lower mid-littoral zone, occurring along circa 2000 km of the coast from 20°19'S (Vila Velha, ES) to 32°10'S (Rio Grande, RS) (Young, 1994). The range and abundance along the Brazilian shores, makes it as a natural reservoir for the species and as many of the ports are within trading-ships routes it can be considered a secondary source of introduction to other regions of the globe.

Megabalanus coccopoma can easily spread attached to ship hulls and can settle, grow and reproduce in many different places. The biggest concern on the spread of *M. coccopoma* beyond the natural distribution areas consists in the potential ecological impacts and local habitat changes including the competition with other native species. These impacts may also affect sea-farming, resulting in a reduction of seafood production and hence in economic losses.

Microsatellite data have been extensively used in population analyses and can be useful to estimate contemporary migrations, as well as in the study of recent bioinvasions (Sarre et al., 2014). Due to its highly polymorphic nature, microsatellites can help to elucidate possible routes of invasion, comparing native populations of the species with the individuals of the invaded areas, outside the natural distribution boundaries (source-sink). Recently, Reigel et al., 2015 described polymorphic tetranucleotide microsatellite for *M. coccopoma* occurring in two localities in the East coast of USA. In the present work, we developed and characterized polymorphic di and trinucleotide microsatellite *loci* that can be used in the studies of *M. coccopoma*. We also proceeded cross-amplification tests of the microsatellite *loci* with the co-generic species *Megabalanus vesiculosus* (Darwin, 1854), endemic to the Brazilian coast, which is possibly in risk by the presence of *M. coccopoma* in its natural habitat.

2. Material and methods

Microsatellite libraries were developed by Genetic Identification Services (Chatsworth, California, USA) from purified DNA of one individual of *M. coccopoma* following the methods of Jones et al. (2002) for library construction, microsatellite enrichment and screening. The libraries were enriched for four repeat motifs—(CA)_n, (GA)_n, (AAC)_n, and (ATG)_n. One hundred and twenty four clones containing microsatellites were sequenced and, using DesignerPCR version 1.03 (Research Genetics), ninety primer pairs were designed. Twelve primer pairs were selected for a screening across 23 individuals of *M. coccopoma* and 28 individuals of the native Brazilian barnacle *M. vesiculosus*, in order to test for amplification success and access polymorphism degrees for each *loci* in both species.

Genomic DNA for genotyping was extracted from the prossoma using a modified 2% CTAB protocol (Gusmão and Sole-Cava, 2002). Forward primers were synthesized with a M13 tail at their 5' end (5'-TGTAACGACGGCCAGT-3'), allowing the use of the tailed primer method (Schuelke, 2000). PCR mix consisted of 1 U GoTaq (Promega), 0.20 mM dNTPs, 2.5 mM MgCl₂, 15 µg BSA, 0.4 µM of labelled forward primer, 0.8 µM of reverse primer, and 0.2 µM of tailed primer, with a final volume of 15 µL per reaction containing approximately 30 ng of DNA template. Cycling conditions were: 95 °C, 3 min (min), 30 cycles at 94 °C, 45 s (sec); 56 °C–66 °C, according to each primer, 45 s; 72 °C, 45 s, 8 cycles at 92 °C, 45 s; 53 °C, 45 s; 72 °C, 45 s and 72 °C, 30 min. PCR products were pooled with GeneScan Rox-500 size standards (Applied Biosystems) and analysed by capillary electrophoresis. The software GeneMarker V.2.6.4 (SoftGenetics – demonstration version) was used for genotyping score and allele sizing. The occurrence of null alleles within *loci* and calculation of null allele frequencies for each *locus* (Oosterhout method) were performed with Micro-Checker software (Van Oosterhout et al., 2004). Genepop V. 4.2 (Rousset, 2008) was used to test for linkage disequilibrium between *loci* and calculation of inbreeding index (F_{IS}). Tests of Hardy–Weinberg equilibrium were performed using Genetix 4.05 software (Belkhir et al., 2004).

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