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Tetranucleotide microsatellites for the barnacle *Megabalanus coccopoma* (Darwin, 1854)

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ABSTRACT

The barnacle *Megabalanus coccopoma* is indigenous to the tropical Pacific Ocean, but was recently introduced to the coastal waters of the southeastern U.S.A. As part of a larger effort to investigate the population dynamics of this introduction, we designed 13 microsatellite primers specific to *M. coccopoma* and developed the accompanying polymerase chain reaction (PCR) conditions. We tested these primers on 42 individuals of *M. coccopoma* collected from two sampling locations in coastal Georgia, USA. The 13 loci developed showed means of 24.5 alleles per locus, 0.93 expected heterozygosity, 0.67 observed heterozygosity, and 0.91 polymorphic information content. The high variation observed within these microsatellite loci makes them useful tools for testing hypotheses related to population genetics, including source-sink dynamics for range expansions and rates of self-fertilization and outcrossing.

1. Introduction

The barnacle *Megabalanus coccopoma* (Darwin, 1854) is indigenous to the tropical eastern Pacific Ocean where it ranges from Ecuador to northern Mexico and Baja California (Laguna, 1990; Newman and McConnaughey, 1987). In the 1970s the species was reported in the Atlantic waters of coastal Brazil, an area far outside of its native range (Lacombe and Monteiro, 1974). More recently *M. coccopoma* has invaded the Gulf shores of Louisiana (Perreault, 2004), Japan and eastern Australia (Yamaguchi et al., 2009), western Africa (Kerckhof, 2002) and sporadic populations have been noted in southern California (Newman and McConnaughey, 1987) and the North Sea (Kerckhof and Cattrijsse, 2001; Kerckhof, 2002). *M. coccopoma* was first documented in the southeastern U.S.A. simultaneously in St. Augustine, FL, Brunswick, GA, and Charleston, SC in 2006 (Tibbetts, 2007; Gilg et al., 2010; Spinuzzi et al., 2013). The current range of *M. coccopoma* in the southeastern U.S. extends from Fort Pierce Inlet, FL to Cape Hattaras, NC, where it primarily inhabits artificial structures including rock jetties, piers, buoys and offshore towers (Crickenberger and Moran, 2013; Cohen et al., 2014).

The combined life-history characteristics of *M. coccopoma* have heightened concerns that this species will outcompete native barnacles along the eastern seaboard (Tibbetts, 2007; Spinuzzi et al., 2013). *Megabalanus coccopoma* appears to grow rapidly, have high fecundity (Crickenberger pers. comm.), and can reach sizes far exceeding native southeastern barnacle species (Tibbetts, 2007). Specimens of *M. coccopoma* we have collected in Georgia waters have a maximum shell height of

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8.8 cm and a maximum basal diameter of 6.8 cm. Like many species of barnacles, *M. coccopoma* has the potential for long-range dispersal through planktonic larvae (Severino and Resgalla, 2005). Range retractions up to 825 km and subsequent re-expansions as large as 794 km have been documented along the southeastern U.S. coast in a single mating and settlement season (Crickenberger and Moran, 2013). The rapid range adjustments show that *M. coccopoma* can quickly colonize new substrates and may vary temporally, which makes it an ideal organism for studying the effects of these changes on population genetic variation during a biological invasion.

Little is known about the population structure of *M. coccopoma* in either its native or introduced ranges. Recent studies using the mitochondrial genes cytochrome c oxidase subunit I (COI) and 16S rRNA to assess genetic variation in the introduced populations of *M. coccopoma* indicated a single large population in the introduced range with high gene flow (Williamson, 2010; Cohen et al., 2014). Mitochondrial markers are valuable tools for investigating population structure and the influence of historical processes on the distribution of genetic variation. However, these markers can have limited utility when evaluating recent and more rapid evolutionary processes, including those acting on nuclear markers (Wang, 2010). Microsatellite markers, because of their higher mutation rates, can be more effective tools for studying contemporary evolutionary processes and associated demographics, including cases of recent and ongoing species introductions into non-native areas (Wang, 2010, 2011). In this study we describe 13 highly variable microsatellite markers developed to study contemporary evolutionary processes and their consequences on the population structure and demographics of the introduced barnacle *M. coccopoma*.

2. Materials and methods

Using morphological characters, we collected a single individual of *M. coccopoma* from the fishing pier at Tybee Island, GA (31°59′31″N, 80°50′42″W) in July 2013. Total genomic DNA was extracted from this individual using the DNeasy tissue kit (Qiagen) following manufacturer protocols, including RNAse treatment to remove RNA from the sample. The purified sample was sent to the Savannah River Ecology Lab to prepare an Illumina paired-end shotgun library. Paired-end sequencing returned 10 million reads with an average length of 100 bp. The genome size for *M. coccopoma* is unknown, but published genome size estimates in the family Balanidae range from 723 Mb to 1299 Mb (Bachmann and Rheinsmith, 1973; Rheinsmith et al., 1974). If *M. coccopoma* falls within this range our sequence data represents a possible 0.77× to 1.23× genome coverage. The resulting sequences were analyzed using the program *PAL_FINDER_v0.02.03* to find sequences containing simple repeats (Castoe et al., 2012). This analysis initially returned 140,413 loci that contained di-, tri-, tetra-, penta- or hexanucleotide repeats. In an attempt to avoid primers with multiple priming sites we selected loci where primer sequences occurred only once or twice in the 10 million total reads. In addition, if one of the primers in a pair occurred twice the other could only occur once within the genomic reads. Finally, we selected primer pairs that occurred only once within the set of paired reads. This increases the probability that the forward and reverse primer only occur in close proximity for the loci of interest even if individual primer sequences occur more than once in the genome (Castoe et al., 2012). We identified 7097 loci that met the designated criteria. Of these loci, we chose to test primer pairs for 30 loci with tetranucleotide repeats within the sequence data.

Initial primer screening of the 30 loci for amplification was completed on eight individuals collected in September 2013 from two locations within the introduced range of the barnacle: four specimens from Navy Tower R2, located approximately 50 km off the coast of Georgia, USA (31°22′30″N, 80°34′01″W), and four specimens from the St. Simons Island fishing pier, GA (31°08′02″N, 81°23′48″W). PCR was performed in 10 μl reactions including: 0.625 units of *Taq* DNA Polymerase (Apex), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 μmol each of forward and reverse primers, and 3.5 μl diH_20. Primers were not labeled with fluorescent dyes for this initial amplification screening. PCR reactions were performed using the following conditions for all thirteen loci: initial denaturing step at 95 °C for 5 min, followed by 30 cycles of 95 °C denaturing for 10 s, 60 °C annealing for 10 s, 72 °C extension for 10 s, and a 72 °C final annealing step for 5 min. The PCR products were separated using an agarose gel.

When the amplification product was viewed on the agarose gel thirteen of the initial 30 primer pairs tested showed consistently clean, clear bands for all 8 specimens and were chosen for further characterization using 36 to 42 individuals. Samples used for loci characterization were collected from Navy Tower R2 ($N = 18$) and St. Simons Island Fishing Pier, GA ($N = 24$). PCR was performed using the same conditions and protocol described above. All forward primers were fluorescently labeled and all reverse primers included a GTTT 'pigtail' to the 5′ end of the primer to standardize the addition or deletion of adenosine by *Taq* polymerase (Brownstein et al., 1996). Primers compatible in multiplex reactions are indicated in Table 1. Amplified PCR products were sized using an ABI 3500 Genetic Analyzer with an internal size standard. Alleles were scored manually using GENEMAPPER software (PE Applied Biosystems). To estimate the level of genetic differentiation between sampling locations, we calculated a pairwise F_{ST} value using FSTAT v 2.9.3 (Goudet, 1995). We calculated expected heterozygosity (H_E), observed heterozygosity (H_O), and polymorphic information content (PIC) using the Microsatellite Toolkit Add-in for Microsoft Excel (Park, 2001a, 2001b). PIC is the expected proportion of informative offspring that cosegregates by phenotype for the locus being examined. Tests for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using GENEPOP v4.0.10 (Rousset, 2008) with 10,000 dememorization steps, 500 batches and 10,000 iterations.

Table 1
Characterization of 13 primer pairs amplified from microsatellite loci for the barnacle, *Megabalanus coccopoma*, collected along the coast of Georgia, USA.

Locus	Primer sequence (5′–3′)	*GenBank* Accession numbers	Dye	Repeats in sequence	Amplified concurrently in a multiplex	*N*	*A*	Size range (bp)	H_O	H_E	P_{HW}	PIC
MC-1	F: GAGCCGGACTAGATCACATGG R: GACTTCAATCGGCTCGTGG	KT380925	FAM	$(ATAC)_{18}$	MC-15	42	20	195–291	0.60	0.94	<0.0001	0.92
MC-3	F: CCCTGAGAATCCAAACACGG R: AGATACGTTGCAGGAACCAGG	KT380926	FAM	$(AGTG)_{16}$	NA	36	23	185–317	1.0	0.95	0.95	0.94
MC-4	F: CCTTGGTTCCCGAAATAATCC R: AAGGTCACATTGCAAACAATAGC	KT380927	HEX	$(ATAC)_{16}$	NA	38	21	165–270	0.49	0.87	<0.0001	0.84
MC-5	F: GACGTAGACGACCATCAGCC R: GGTTGTTCTCAGTACATACGCCC	KT380928	FAM	$(ATAC)_{16}$	MC-28	36	33	160–324	0.59	0.97	<0.0001	0.96
MC-9	F: CAATCGTAGGAATCCAGCGG R: CTCAGGTCAGCTGGCAAGG	KT380929	HEX	$(ACTG)_{14}$	NA	30	27	509–729	0.43	0.97	<0.0001	0.95
MC-13	F: GCGTGCAATCCACTATCG R: CTAGATCGCGAGGCATCC	KT380930	HEX	$(ATAC)_{18}$	NA	42	45	144–416	0.76	0.98	<0.0001	0.97
MC-15	F: GGTTCGCGAGACAATTCTAAATACC R: CGCTCTGAAACACAAACATGG	KT380931	NED	$(ATAC)_{18}$	MC-1	41	26	213–417	0.76	0.95	<0.0001	0.94
MC-22	F: GCGTCATGTATTCAGGTTCAGG R: TAAGAATCGCAACCCGATGG	KT380932	HEX	$(ATAC)_{15}$	MC-24	41	20	167–233	0.66	0.94	<0.0001	0.92
MC-24	F: GAGCACATACAGCAGAGCGG R: GGGAGGACTAATTTCCGTTGC	KT380933	FAM	$(TCTG)_{15}$	MC-22	41	9	172–208	0.59	0.79	0.0106	0.75
MC-26	F: CTCCGGAGGGTCCAATCC R: ATGAATGCGCACATAAACGC	KT380934	NED	$(ATAC)_{15}$	NA	41	31	213–359	0.83	0.96	0.0044	0.95
MC-27	F: CCTCTGACCTCTGACCTATGACG R: ACGCGAAACACACTATTGCC	KT380935	HEX	$(ACTG)_{14}$	NA	41	23	265–397	0.81	0.93	0.0865	0.91
MC-28	F: CAGTACAGTACAGTTGAGATAGTTCACCC R: AAATCAGTCCTCCTGACAGTGC	KT380936	NED	$(ATAC)_{14}$	MC-5	39	20	286–450	0.42	0.91	<0.0001	0.88
MC-29	F: AGGAGCATCGACAGTGACTAGC R: TGCTAAAGCATTGCTCCTCC	KT380937	NED	$(ATAC)_{14}$	NA	41	20	164–244	0.85	0.93	0.2753	0.91

N, indicates the number of individuals out of the 42 tested that were successfully genotyped at each locus; *A*, number of alleles for each locus; H_O, is the observed heterozygosity; H_E, the expected heterozygosity; P_{HW}, the probability that the genotype proportions meet the expectation of Hardy–Weinberg equilibrium; and PIC, the polymorphic information content. NA, not applicable, indicates a locus that was not used in a multiplex reaction due to either allele size overlap with loci of the same dye color or better results when the locus was amplified individually. All loci were amplified successfully in at least one multiplex reaction over the course of the study.

3. Results and discussion

The pairwise F_{ST} value was low indicating no genetic differentiation between sampling locations ($F_{ST} = 0.0066$, $p = 0.08$) therefore all individuals were pooled into one group for the remaining genetic analyses. The number of alleles per locus ranged from 9 to 45 (mean ± SD = 24.46 ± 8.57, Table 1). Observed heterozygosity (H_O) values ranged from 0.42 to 1.0 (mean ± SD = 0.675 ± 0.021) and expected heterozygosity (H_E) values from 0.79 to 0.98 (mean ± SD = 0.93 ± 0.05, Table 1). The polymorphic information content (PIC) values ranged from 0.75 to 0.97 (mean ± SD = 0.91 ± 0.06, Table 1). All loci pairs were tested for linkage disequilibrium, but no significance was detected after Bonferonni corrections. All except three loci (MC-3, MC-27, MC-29) exhibit significant deviations from Hardy–Weinberg expectations due to heterozygote deficiency (Table 1).

Population admixture, non-random mating, and null alleles are all possible factors that can result in deviations from Hardy–Weinberg equilibrium. Although null alleles cannot be ruled out as an explanation in this study, the high number of loci showing homozygote excess and the ecology of *M. coccopoma* suggest admixture and/or non-random mating are likely driving forces. The samples used in this study are from recently introduced populations with the possibility of establishment from multiple sources. If several source populations contribute to an introduction, Wahlund Effect may be seen in the early stages before the introduced population becomes self-sustaining (Holland, 2000; Kolbe et al., 2008). However, Cohen et al. (2014) found little genetic structuring between two native *M. coccopoma* populations using mitochondrial COI data. The results of Cohen et al. (2014) do not rule out the possibility that population structuring exists that may be detected with more variable markers or in other regions of the species range. Highly variable markers such as microsatellites can be more powerful tools than mitochondrial marker to detect population structuring (Balloux and Lugon Moulin, 2002). Comparison of the microsatellite loci developed in this study with an established native population will add valuable insight to the conclusion of heterozygote deficiency as a result of admixture.

A second, but not necessarily mutually exclusive explanation for the homozygote excess observed in our sample is self-fertilization. Like many barnacles, *M. coccopoma* is a simultaneous hermaphrodite with the potential to self-fertilize but the details of *M. coccopoma* reproductive biology remain unpublished (Crickenberger, 2014). In the majority of balanoid barnacle species studied, outcrossing is preferred and self-fertilization is thought to be rare (Barnes and Crisp, 1956; Kelly et al., 2012). Facultative selfing may be a response to low densities during range introduction or expansion and could

contribute to the higher levels of homozygosity than expected from random mating in these introduced populations. *Megabalanus azoricus* populations in their native range demonstrated similar patterns of homozygote excess at microsatellite loci, suggesting the possibility that some fraction of self-fertilization may be more common in *Megabalanus* than other barnacles (De Girolamo et al., 2013).

The high variation observed in these microsatellite loci indicates that they are a useful tool for measuring genetic variation within and among introduced and native populations of *M. coccopoma*. In addition, little is known about the reproductive biology of this species. These loci can serve as a valuable tool to investigate the reproductive biology of *M. coccopoma* including rates of selfing and outcrossing.

Author contributions

The following are the author contributions to this paper. A.M. Reigel collaborated on the design of the research, performed the research for this study including collecting of specimens, creating and testing the polymerase chain reaction protocol, analyzed the data and wrote the manuscript. J.S. Harrison contributed to the design of the research, provided support for the molecular lab work and data analysis and collaborated on writing the manuscript. D.F. Gleason collaborated on the design of the research, helped collect specimens for the project and collaborated on writing the manuscript.

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