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1           **Microsatellite loci discovery from next-generation sequencing data and marker**  
2           **characterization in the epizoic barnacle *Chelonibia testudinaria* (Linnaeus, 1798)**

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## 10 Abstract

11 Microsatellite markers remain an important tool for ecological and evolutionary research, but are  
12 unavailable for many non-model organisms. One such organism with rare ecological and  
13 evolutionary features is the epizoic barnacle *Chelonibia testudinaria* (Linnaeus, 1758).  
14 *Chelonibia testudinaria* appears to be a host generalist, and has an unusual sexual system,  
15 androdioecy. Genetic studies on host specificity and mating behavior are impeded by the lack of  
16 fine-scale, highly variable markers, such as microsatellite markers. In the present study, we  
17 discovered thousands of new microsatellite loci from next-generation sequencing data, and  
18 characterized 12 loci thoroughly. We conclude that 11 of these loci will be useful markers in  
19 future ecological and evolutionary studies on *C. testudinaria*.

## 20 Introduction

21 Microsatellite loci are valuable tools in ecological and evolutionary studies (e.g. Jarne  
22 and Lagoda, 1996; Vignal et al. 2002; Selkoe and Toonen, 2006). Next-generation sequencing  
23 approaches have revolutionized microsatellite loci development, allowing the rapid discovery of  
24 thousands of potential microsatellite loci in the genome of non-model organisms (Castoe et al.,  
25 2012). However, a thorough characterization of potential microsatellite loci remains labor-  
26 intensive. It is nonetheless necessary to use these markers successfully in evolutionary and  
27 ecological studies.

28 A non-model organism for which genetic and genomic resources are lacking is the  
29 epizoic barnacle *Chelonibia testudinaria* (Linnaeus, 1758). *Chelonibia testudinaria* uses diverse

30 marine animals as substratum, such as sea turtles, manatees, swimming crabs, and horseshoe  
31 crabs. Host-specific morphotypes were previously described as distinct species (Darwin, 1854;  
32 Hayashi, 2013). Recent molecular analyses indicate that *C. testudinaria* is a host generalist, and  
33 *C. patula* (Ranzani, 1818) and *C. manati* (Gruvel, 1903) are now considered synonyms of *C.*  
34 *testudinaria* (Cheang et al., 2013; Zardus et al., 2014). However, a fine-scale genetic assessment  
35 of host-specificity based on highly polymorphic nuclear markers, such as microsatellite markers,  
36 is still lacking. Instead of host-specific divergence, genetic lineages are delineated by geographic  
37 affinities. The three major lineages are restricted to the Indo-West Pacific, Tropical Eastern  
38 Pacific and Atlantic Ocean, respectively (Rawson et al., 2003). These lineages likely represent  
39 separate species based on their levels of genetic differentiation (Zardus et al., 2014).

40 All lineages of *C. testudinaria* exhibit a rare sexual system: androdioecy. Androdioecy is  
41 characterized by the co-existence of hermaphrodites and males in the same reproductive  
42 population. Understanding mating success and mating patterns of both sexes would greatly  
43 advance our understanding of this rare sexual system. This would be most easily achieved with  
44 genetic parentage assignment – but its prerequisite, highly variable genetic markers, are not yet  
45 developed.

46 In order to overcome these shortcomings, we used next-generation sequencing to discover  
47 microsatellite markers for *C. testudinaria*. We characterized 12 promising markers using  
48 individuals of *C. testudinaria* from the Atlantic coast of the United States and from the  
49 Northeastern coast of Australia.

## 50 **Material & Methods**

### 51 Specimen collections

52 Specimen collections of the Atlantic lineage took place on Nannygoat Beach, Sapelo  
53 Island, GA, USA (31.48° N, 81.24° W) between 2012 and 2014 under the collection permit of  
54 the University of Georgia Marine Institute, and sanctioned by the Georgia DNR Wildlife  
55 Services. We chose to collect from the horseshoe crab *Limulus polyphemus* (Linnaeus, 1758)  
56 because it is relatively abundant and easy to sample: each spring and early summer, horseshoe  
57 crabs crawl onto beaches to mate and lay their eggs. During this process, we removed one  
58 individual of *C. testudinaria* per host individual with a sharp knife directly on the beach, and  
59 preserved it in 95% EtOH immediately after collection. We collected specimens of the Indo-  
60 West Pacific lineage in the vicinity of Townsville, Queensland, Australia (23° S, 143° E), in  
61 September 2012 from green turtles (*Chelonia mydas* Brongniart, 1800). Working with officials  
62 from the Queensland Department of Environment and Heritage Protection, turtles were captured  
63 in-water for routine tagging and release. During capture barnacles were collected and  
64 immediately preserved in 95% EtOH.

### 65 Microsatellite marker development

66 We extracted genomic DNA from the feeding appendage of a single large hermaphroditic  
67 *C. testudinaria* collected from a horseshoe crab with Gentra Puregene Tissue Kit (Qiagen), and

68 measured DNA concentration with a Qubit 2.0 Fluorometer (Life Technologies). Genomic DNA  
69 was fragmented into approximately 700bp lengths (insert size) and shotgun-sequenced on an  
70 Illumina MiSeq sequencer (PE250). We quality-checked paired-end reads with FastQC  
71 (Andrews, 2015). The software FASTQMC was used to trim adapters, cut low quality ends and  
72 remove low quality reads and their mate-pair read (Aronesty, 2011). We calculated the haploid  
73 genome size by mapping genomic reads to 52 nuclear single-copy gene fragments available from  
74 the acorn barnacle *Semibalanus balanoides* (Regier et al., 2010), and calculating the median  
75 coverage for all 52 gene fragments. We then took the grand median coverage of all gene  
76 fragments, and dividing the total number of amplified base pairs by the grand median coverage.

77 We executed the perl script PALFINDER to identify short sequence repeat regions (Castoe et  
78 al., 2012). The script also calls PRIMER3, version 2.0.0, to identify potential primer pairs that span  
79 the repeat region (Rozen & Skaletsky, 2000). The minimum number of repeat units was chosen as  
80 in Castoe et al. (2012). A repeat unit, also called kmer, is defined as the length of the short  
81 sequence repeat. For example, a dimer would be a repeat of two base pairs (e.g. GC), and a  
82 tetramer would be a repeat of four basepairs (e.g. AGGT). PRIMER3 parameters were the default  
83 values. The search resulted in a large number of potentially amplifiable loci (PALs), repeat  
84 regions for which primers were identified. We filtered the results by removing all PALs which  
85 occurred less than two times and more than the estimated genome coverage in the genomic reads  
86 based on the following reasoning: If the number of primer occurrences is low, the primer  
87 sequence may contain sequencing error. If the number of occurrences is higher than the expected  
88 genome coverage, the primer region may occur more than once in the genome, leading to  
89 amplification of multiple loci (genomic regions). Neither of these outcomes is desirable because

90 a good marker occurs only once in the genome, and has a primer sequence that matches the  
91 genomic sequence well. R scripts for screening PALFINDER output as well as calculating genome  
92 coverage are available as supplementary information.

93 Of the filtered PALs, we chose 48 PALs for trial amplification, which differed in kmer  
94 length, kmer motif (e.g. AG vs TG) and fragment size. We extracted and amplified DNA of 16  
95 *C. testudinaria* individuals for trials. DNA was extracted from feeding appendages of barnacles  
96 with the Chelex method (Walsh et al., 1991). Trials used the method of Schuelke (2000) to  
97 amplify fragments and simultaneously tag forward primers with a fluorescent dye. Loci that  
98 amplified and scored consistently in all individuals were fluorescently labeled with 6-FAM,  
99 NED or HEX (Applied Biosystems, Custom Oligo Synthesis Center), and used on a larger  
100 number of individuals to characterize the microsatellite markers.

#### 101 Microsatellite marker amplification

102 Genomic DNA was extracted from feeding appendages of barnacles with the Chelex  
103 method (Walsh et al., 1991). PCR amplifications were performed in 20ul volumes containing  
104 final concentrations of 1x PCR buffer (Bioline), 5% bovine serum albumin 10 mg/mL (Sigma),  
105 200 mM each dNTP, 2 nM MgCl, 0.5 mM each primer, 0.5 units of Promega GoTaq DNA  
106 Polymerase, and 1 µl template DNA. PCR conditions were as follows: 4 min initial denaturation,  
107 followed by 40 cycles with 45 sec denaturing at 94°C, 60 sec annealing at 55°C, 60 sec extension  
108 at 72°C and a final extension time of 10 min. The PCR were carried out in a MJ Research PCR  
109 Engine. HiDi and ROX500 size standard were added to each sample, and fragment length  
110 analysis was carried out at the Georgia Genomics Facility on an ABI 3730xl. Peaks were called

111 and binned with the microsatellite plugin of Geneious version 8.1 (Kearse et al., 2012).

112 Microsatellite marker characterization

113 We inspected peak calls for fragment size consistency, using the R package MSATALLELE  
114 (Alberto, 2009). MSATALLELE plots peak calls of a locus in histogram form, facilitating visual  
115 binning of alleles. If bins could not be clearly assigned, the locus was excluded from the  
116 subsequent analysis.

117 We tested whether loci were in Hardy-Weinberg Equilibrium (HWE) by using 1999  
118 Monte Carlo permutations, as implemented by the function HW.TEST in the R package PEGAS  
119 (Paradis, 2010). We recorded the number of alleles, range of fragment sizes, and allelic richness  
120 of each locus. The frequency of null alleles was computed based on the method of Brookfield  
121 (1996). Genotyping error rates were calculated by repeating genotyping for all individuals. In  
122 addition, we amplified the markers for 24 individuals from Queensland, Australia, to assess if the  
123 markers could be used in cross-lineage analysis. A R script detailing these analyses is available  
124 as supplementary information.

## 125 Results

126 Microsatellite marker development

127 The MiSeq run generated 15,324,079 paired-end reads (35-251 bp long) with 81.05% >  
128 Q30. Raw reads are available in NCBI's short read archive (SRA) under accession number XXX.



129 After quality control, 13,498,280 paired-end reads (19-251 bp long) remained, for a total of 6.2  
130 Gb. The median genome coverage was 8x (min = 3, max = 24) for 52 nuclear single-copy gene  
131 fragments, and the haploid genome size is therefore approximately 800 Mb (= 6.2 Gb / 8x). The  
132 PALFINDER script detected 629,990 microsatellite repeat regions, of which 29,627 (5.38%) were  
133 potentially amplifiable loci (PALs) with forward and reverse primer. A summary of detected  
134 microsatellite repeat regions is available as supplementary information. A list of all detected  
135 microsatellite regions (with primer sequences) is available on figshare ([www.figshare.com](http://www.figshare.com); DOI:  
136 10.6084/m9.figshare.2070070). After removing PALs with more than eight or less than two  
137 occurrences of either forward or reverse primer in the sequence read data, 17,265 PALs  
138 remained. We chose 48 loci for trial amplification, which differed in kmer length and repeat  
139 motif. Of those 48 loci, 12 loci amplified and scored consistently throughout the trials, and were  
140 tagged with fluorescently labeled dye (Table 1).

#### 141 Microsatellite marker characterization

142 We genotyped 42 individuals successfully at more than half of the 12 consistently scoring  
143 loci. Visual inspection of peak call histograms revealed that peak calls of Ctest2 did not have  
144 clearly defined bins, and were excluded from subsequent analyses. The number of alleles of the  
145 11 scorable loci ranged from six to 30 (Figure 1). Microsatellite genotype and collecting date for  
146 each individual are available as supplementary information. For the Atlantic population, six loci  
147 were not in HWE, and showed homozygote excess (Table 2). The estimated percentage of null  
148 alleles ranged from 0% to 20.3%. Allelic richness ranged from 3.6 to 20.7, and genotyping error  
149 rates ranged from 0 to 7.32% (Table 2).

150 All 11 loci amplified in some of the 23 Australian individuals, and had at least two  
151 alleles. For the Pacific lineage of *C. testudinaria*, none of the loci showed significant deviations  
152 from HWE. Allelic richness was significantly lower than in the Atlantic individuals (1.8-6.8).  
153 The percentage of null alleles ranged from 0% to 30% (Table 3).

## 154 Discussion

155 The present study developed and characterized 11 microsatellite markers for the epizoic  
156 barnacle *Chelonibia testudinaria*. Several loci are not in HWE, probably due to null alleles.  
157 Their high allelic diversity and scoring consistency should nonetheless make them useful in  
158 ecological and evolutionary studies. In addition, we provide the resources to evaluate thousands  
159 of additional potentially amplifiable loci (PALs) for *C. testudinaria*.

160 Several loci were not in HWE, and displayed homozygote excess. Homozygote excess  
161 can have several causes: selection on these loci, the presence of null alleles, inbreeding,  
162 population substructure or large variance in reproductive success. Inbreeding is unlikely because  
163 most barnacles are obligate outcrossers and *C. testudinaria* has a widely-dispersing planktonic  
164 larval phase. Selection cannot be excluded as an explanation, but selection on several markers  
165 appears unlikely. Population substructure may be present, but if so, is neither host-induced nor  
166 geographical. Large variance in reproductive success can cause homozygote excess (Hedgecock,  
167 1994), and has been invoked to explain homozygote excess in e.g. sea urchins (Addison & Hart,  
168 2004). If variance in reproductive success is present, the effective population size of *C.*  
169 *testudinaria* should be low (Hedgecock, 1994). We estimated a  $\Theta$  of 10 for the Atlantic *C.*

170 *testudinaria* population using Watterson's estimator (Watterson, 1975) on published COI data,  
171 which suggests a large effective population size (data not shown). These data do not support the  
172 variance-in-reproductive-success hypothesis. The most likely cause for homozygote excess is the  
173 presence of null alleles. Null alleles are ubiquitous in microsatellite loci, and are caused by  
174 mutations in the primer sequence, leading to the dropout of alleles. While the true genotype is  
175 heterozygous, the observed genotype is homozygous due to the non-amplification of one of the  
176 alleles. Null alleles become increasingly prevalent with increasing effective population size  
177 (Chapuis and Estoup, 2007). Chapuis and Estoup (2007) show that simulated null allele  
178 frequencies were larger than 0.2 for all loci when the population mutation rate ( $\theta$ ) was one,  
179 the largest value they simulated. We estimated null allele frequencies between zero and 0.3 for  
180 our microsatellite markers, well within the range of simulated data with large effective  
181 population size. Thus the observed homozygote excess can be explained by the presence of null  
182 alleles.

183         We were able to amplify all loci in both lineages, which was somewhat surprising given  
184 the combination of large effective population size and significant between-lineage divergence.  
185 Both factors increase the chance for primer sequences to differ between lineages. Further, results  
186 for the Indo-West Pacific lineage need to be evaluated with caution because primers were  
187 designed from an individual of the Atlantic lineage. Future studies should increase sample sizes  
188 for both lineages to compare and contrast genotypic diversity. In summary, we identified new  
189 genetic resources that can be used in future ecological and evolutionary studies on the epizoic,  
190 androdioecious barnacle *Chelonibia testudinaria*.

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## Captions

**Figure 1.** Allele frequencies for each microsatellite locus. Each barplot represents a locus, each bar an allele, and the height of each bar indicates the frequency of each allele in the data. Sample sizes are indicated in table 2.

**Table 1.** Microsatellite loci amplification information. All loci were amplified at 55°C annealing temperature. “Dye” refers to the fluorescent color label for each forward primer. Ned is yellow, 6-FAM is blue and HEX is green. Labeling forward primers with different colors allows multiplexing several primer sets in the same reaction. “Multiplex reaction” refers to the multiplexing PCR scheme, e.g. all loci with the same multiplex code are amplified in the same reaction.

**Table 2.** Microsatellite loci characterization for *Chelonibia testudinaria* of the Atlantic lineage. Range refers to the smallest (min) and largest (max) allele observed. Frequency of null alleles was estimated after Brookfield (1996). Genotyping error rates were based on re-genotyping of the all 42 Atlantic individuals. Abbreviations: n = number of individuals; Obs het. = observed heterozygosity; Exp het. = expected heterozygosity.

**Table 3.** Microsatellite loci characterization for *Chelonibia testudinaria* of the Indo-West Pacific lineage. Abbreviations: Obs het. = observed heterozygosity; Exp het. = expected heterozygosity.

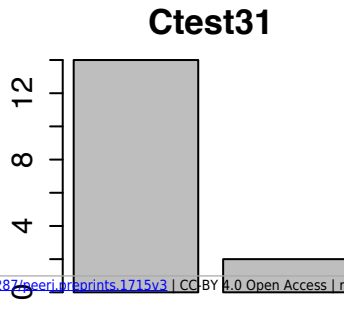
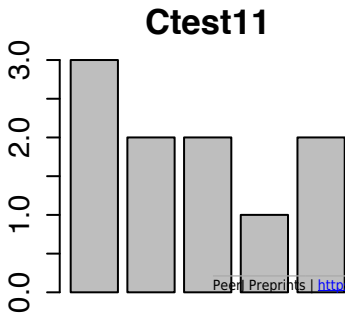
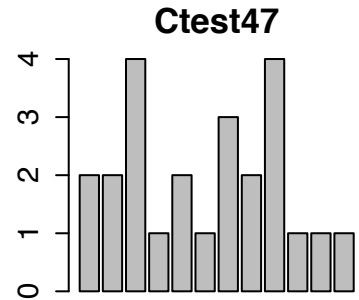
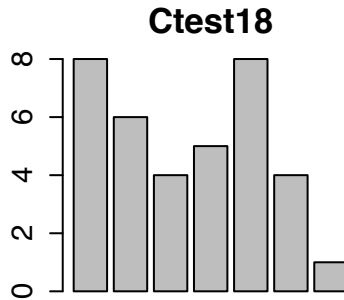
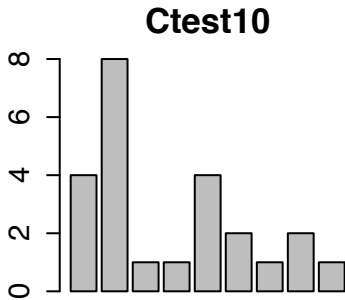
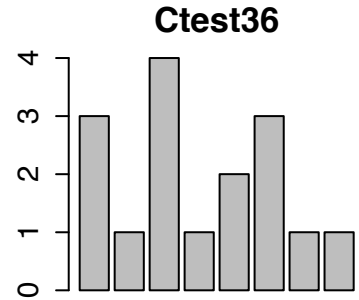
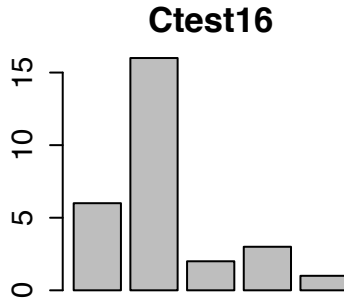
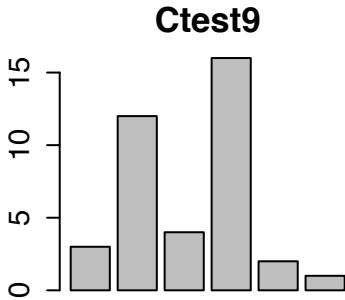
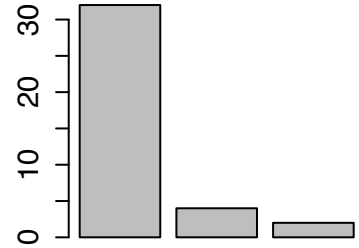
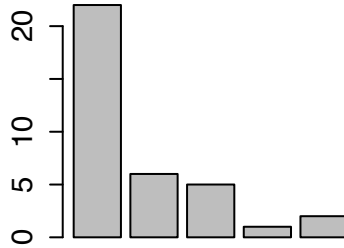
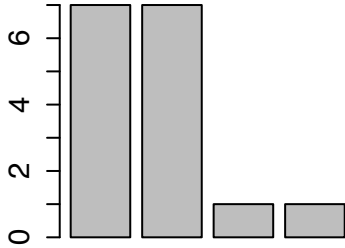




Table 1

<b>Locus</b>	<b>Kmer</b>	<b>Forward primer sequence</b>	<b>Reverse primer sequence</b>	<b>Dye</b>	<b>Multiplex reaction</b>
Ctest2		ACACACATCACTGGACTCG	CAGTAAGCAGCTCTGTTCCG	NED	BB
Ctest7	4	GTTATCCGTCATTCCATCC	GACGTAACCACCTTGTCCG	6-FAM	AA
Ctest9	4	AACAGATGTGACATTGATGC	TTGTACTGTCCTTGTAACGC	6-FAM	BB
Ctest10	2	ATACGCACAAACTCACACC	TGTCCTCTTACAGAGATCGG	HEX	BB
Ctest11	2	GTGTCCACCTTTATGTCTGG	AGTTGAAAATACGCACGC	HEX	CC
Ctest12	4	AACTGGTGGACAGTCTGG	CATCTTTATGAGTAGCGAGG	HEX	AA
Ctest16	4	TCAGGTACAGCATTATCGC	CAAGGACCATCAATTACCC	6-FAM	CC
Ctest18	5	TTCATGAATCACTTCCTGG	GTAATCAAATAAGGCGATGC	NED	AA
Ctest31	4	GTACGCCGAAAGTAAAGC	AGCTCTGACAAAGTTATGCC	6-FAM	DD
Ctest32	4	AGAAATCCATAATCGTCTGG	ATAACGACGTAATCAGCACC	NED	CC
Ctest36	4	AGATATTGGTGGAACGAGC	CACAACATACTCAACGAACG	HEX	DD
Ctest47	5	GTTGACACGATGACATAACG	ACAATTCCAGCTCTGTTAGC	NED	DD

Table 2

Locus	n	Range min	Range max	Number of alleles	Obs het.	Exp het.	HWE p-value	Allelic richness	Frequency null alleles	Genotyping error rate
Ctest7	34	206	314	18	0.559	0.817	0.007	16.113	0.142	0
Ctest9	34	388	432	8	0.618	0.69	0.022	7.449	0.043	0.021
Ctest10	34	264	278	8	0.647	0.766	0.141	7.453	0.067	0.032
Ctest11	38	140	318	27	0.868	0.93	0.559	22.678	0.032	0.073
Ctest12	34	388	476	23	0.735	0.917	0.006	20.261	0.095	0.024
Ctest16	35	355	367	4	0.371	0.39	0.137	3.703	0.014	0
Ctest18	33	450	485	7	0.606	0.776	0.033	6.984	0.096	0.061
Ctest31	36	292	336	8	0.556	0.664	0.001	6.542	0.065	0.068
Ctest32	35	316	464	4	0.571	0.518	0.493	3.647	0	0
Ctest36	33	336	524	23	0.455	0.891	0	20.072	0.231	0.025
Ctest47	37	255	385	10	0.622	0.662	0.259	8.356	0.024	0.017

Table 3

<b>Locus</b>	<b>n</b>	<b>Range min</b>	<b>Range max</b>	<b>Number of alleles</b>	<b>Obs het.</b>	<b>Exp het.</b>	<b>HWE p-value</b>	<b>Allelic richness</b>	<b>Frequency null alleles</b>
Ctest7	8	186	314	4	1	0.609	0.004	3.067	0
Ctest9	19	408	456	6	0.737	0.702	0.806	3.946	0
Ctest10	12	260	344	9	0.5	0.813	0.001	5.479	0.172
Ctest11	5	136	236	5	0.2	0.78	0.001	4.628	0.326
Ctest12	18	354	374	5	0.333	0.576	0	3.377	0.154
Ctest16	14	351	375	5	0.571	0.61	0.158	3.525	0.024
Ctest18	18	440	480	7	0.278	0.829	0	5.221	0.301
Ctest31	8	224	304	2	0.25	0.219	1	1.797	0
Ctest32	19	308	316	3	0	0.277	0	2.134	0.217
Ctest36	8	348	468	8	0.5	0.836	0.002	5.739	0.183
Ctest47	12	140	340	12	0.833	0.892	0.008	6.882	0.031