A multiple microsatellite assay to evaluate the mating behavior of the intensively exploited marine gastropod *Concholepas concholepas* (Bruguïère, 1789) (Gastropoda: Muricidae)

Kennia Morales  
Roland Sánchez  
Paulina Bruning  
Leyla Cardenas1  
Instituto de Ciencias Ambientales y Evolutivas  
Facultad de Ciencias, Universidad Austral de Chile  
Isla Teja S/N, Casilla 567  
Valdivia, CHILE

Patricio H. Manríquez  
Laboratorio de Ecología y Conducta de la Ontogenia Temprana (LECOT)  
Centro de Estudios Avanzados en Zonas Áridas (CEAZA)  
Avenida Ossandón 877  
Coquimbo, CHILE

Antonio Brante  
Departamento de Ecología  
Facultad de Ciencias  
Universidad Católica de la Santísima Concepción  
Alonso de Ribera, 2550  
Concepción, CHILE  
and  
Centro de Investigación en Biodiversidad y Ambientes Sustentables (CIBAS)  
Universidad Católica de la Santísima Concepción  
Concepción, CHILE

ABSTRACT

The study of reproduction and mating behavior constitutes a main issue in biology, ecology and evolution, given its relation with fitness traits. Here, we developed a simple microsatellite multiple assay to evaluate the mating strategy and male reproductive success of the marine gastropod *Concholepas concholepas* (Bruguïère, 1789), an important fishery resource and a key predator species of Chilean rocky shore communities. *Concholepas concholepas* is a dioecious species with internal fertilization, encapsulation, and long larval phase. In laboratory, adult males and females were cultivated in tanks, and 37 larvae from 5 different clutches were genotyped to run paternity analyses using seven microsatellite loci. Results showed that promiscuity is a common mating behavior in *C. concholepas* displaying an exceptionally high level of multipaternity and males participating as fathers in clutches from more than one female. This microsatellite multiple assay helped to improve our understanding of the reproductive behavior of this ecological key species with high economic importance.

INTRODUCTION

Given its importance in determining fitness, studying reproductive success is crucial to understanding an organism’s biology (Stearns, 2000; Avise et al., 2011). Also, the study of the reproductive strategies of commercially relevant species is crucial for developing appropriate conservation and management policies (Deleo and Castilla, 2005; Hobday et al., 2010). One of the main challenges in estimating reproductive success in sexual species is determining an accurate method to quantify reproductive success; this is especially challenging for males. Here, we developed a simple multiple microsatellite assay to study the reproductive strategy and the reproductive success of the marine gastropod *Concholepas concholepas* (Bruguïère, 1789) (Figure 1). *Concholepas concholepas* is locally known as “loco” and is one of the main target species of small-scale artisanal fisheries operating along the southeastern coast of Chile (Deleo and Castilla, 2005). We first tested a set of previously developed microsatellite loci for use in paternity analyses. Secondly, we inferred some general aspects of the mating behavior of this species.

MATERIALS AND METHODS

Analyses were run on mature adult individuals obtained from a single 50 L tank containing an experimental aggregation of 18 females and 35 males. Individuals were sexed following methodologies described by Castilla (1974). All of the reared individuals used in this study were sexually mature and capable of mating (Manríquez et al., 2008). From this experimental aggregation, five clutches laid by five females identified as potential mothers were studied. Between five and 11 veliger larvae pre-hatching from one or two ovicapsules were collected at random, and the paternity of each clutch analyzed. When two ovicapsules were sampled, larvae from the two capsules were mixed before..."
choosing which would be analyzed. Simultaneously, a small piece of the muscular foot tissue (less than 1 cm$^3$) was removed from each adult individual of the experimental aggregation.

DNA extraction for adults was performed using the E.Z.N.A.® Tissue DNA kit (OMEGA) following the manufacturer's protocol. To each larva we added 200 μL of 5% Chelex solution and 2 μL of proteinase K (0.2 mg/mL). Then, this mixture was incubated at 56°C for 2 hours followed by 8 minutes at 100°C. A total of 37 veliger larvae (from 6 ovicapsules) were successfully genotyped. We selected seven microsatellite loci (Cc2A11-1, Cc1D8,
RESULTS AND DISCUSSION

Throughout the entire dataset, we found that none of the loci showed preferential amplification of short alleles or any evidence of scoring errors or linkage disequilibrium. All loci were highly polymorphic with 159 alleles detected in the 53 adults inspected and 112 alleles detected in the 37 larvae analyzed (Table 1). After Bonferroni corrections, no significant deviations from Hardy-Weinberg equilibrium were observed. Altogether, the probability of exclusion of the seven loci was 0.999, and the probability of exclusion was high for each of the seven loci analyzed (Table 1).

From a total of 37 larvae examined, 34 (91.9%) were unequivocally assigned to only one adult present in the aquarium. In the remaining cases, analyses could not discriminate between two males as potential fathers. The paternity analysis showed that a total of 18 out of 35 males (51.4%) participated in the clutches as fathers. The number of sires per clutch ranged from three to seven, and the number of fertilized females per sire ranged from one to three (Table 2); additionally, sire M19 had the highest percentage of assignments, and this was achieved with two different female mates (Table 2). In general, the relatedness analysis indicated a wide range of kinship between adults in the aquarium. Most adults were not related (relatedness level = 0). However, some pairs of individuals were identified as potential half siblings (relatedness level close to 0.25), and in two cases, some evidence of parental relationship was detected (relatedness level close to 0.5). With the exception of one case in which half siblings were identified, the potential mates assigned by parentage were not related.

In summary, the seven loci selected here were highly useful to perform parentage analyses in *C. concholepas*. We found evidence of promiscuity and a high level of multiple paternity in this species which is in concordance with reports for other gastropod species (Dupont et al., 2006; Mäkinen et al., 2007; Brante et al., 2011; Xue et al., 2014). In addition, 94.4% of males that participated as fathers contributed in more than one brood. From the information presented here, it will be possible to design new experiments using the present multiplex microsatellite assay to fully understand the mating behavior and reproductive strategy of *C. concholepas*.

Table 1. Summary statistics of the microsatellite loci used for the paternity analysis of *Concholepas concholepas*. Locus name, number of individuals genotyped at the specific locus (N), number of observed alleles (Na), and unbiased expected genetic diversity (He) are indicated for each locus; adults and larvae were used for calculations. The table shows the probability of the exact test for Hardy-Weinberg equilibrium (PHW) and the probability of exclusion (P_{excl} of 95% confidence level) defined as the probability of excluding a randomly chosen unrelated candidate parent from the parentage analysis.

<table>
<thead>
<tr>
<th>Locus</th>
<th>N</th>
<th>Na_{adult}</th>
<th>Na_{larvae}</th>
<th>He_{adult}</th>
<th>He_{larvae}</th>
<th>PHW</th>
<th>P_{excl}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cc2A11-1</td>
<td>94</td>
<td>13</td>
<td>12</td>
<td>0.688</td>
<td>0.782</td>
<td>0.036</td>
<td>0.779</td>
</tr>
<tr>
<td>Cc1D8</td>
<td>95</td>
<td>26</td>
<td>19</td>
<td>0.873</td>
<td>0.903</td>
<td>0.905</td>
<td>0.943</td>
</tr>
<tr>
<td>Cc1E5</td>
<td>94</td>
<td>13</td>
<td>12</td>
<td>0.850</td>
<td>0.816</td>
<td>0.073</td>
<td>0.868</td>
</tr>
<tr>
<td>Cc2A5</td>
<td>95</td>
<td>13</td>
<td>12</td>
<td>0.642</td>
<td>0.459</td>
<td>0.444</td>
<td>0.618</td>
</tr>
<tr>
<td>Cc709</td>
<td>93</td>
<td>8</td>
<td>7</td>
<td>0.702</td>
<td>0.775</td>
<td>0.271</td>
<td>0.741</td>
</tr>
<tr>
<td>CcQVC</td>
<td>90</td>
<td>10</td>
<td>7</td>
<td>0.835</td>
<td>0.806</td>
<td>0.548</td>
<td>0.831</td>
</tr>
<tr>
<td>Cc1H2</td>
<td>91</td>
<td>58</td>
<td>32</td>
<td>0.974</td>
<td>0.946</td>
<td>0.023</td>
<td>0.993</td>
</tr>
<tr>
<td>TOTAL</td>
<td>95</td>
<td>159</td>
<td>112</td>
<td>0.806</td>
<td>0.793</td>
<td>0.034</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Cc2E5, Cc2A5, Cc1H2, Cc709 and CcQVC) previously developed to study the spatial population genetics of loco (Cárdenas et al., 2007, 2011). The criteria to select loci were: (1) level of polymorphism, (2) repeat motif and (3) amplification pattern. Although amplification patterns were unambiguous, we checked the microsatellite data for evidence of null alleles and technical artifacts such as stuttering and large allele dropout using MICROCHECKER 2.2.3 (Van Oosterhout et al., 2004). Genetic diversity analyses of larvae and adults were performed using Genetix v. 4.05 (Belkhir et al., 2004). Tests for genotypic linkage disequilibrium and genetic differentiation were computed using Genepop v. 3.3 (Raymond and Rousset, 1995).

Paternity analyses as well as estimations of the expected probability of exclusion of each locus and across loci were performed using the software CERVUS 2.0 (Marshall et al., 1998). This procedure employs maximum likelihood calculations previously proposed by Meagher (1986). Given the genotypes of the embryos and the known genotype of the mother, paternity was assigned to the male in the tank with the highest log-likelihood ratio (LOD). In order to take into account potential misidentifications of sex, the remaining 53 individuals in the aquarium were considered as potential fathers. One advantage of using CERVUS 2.0 is the possibility to evaluate the statistical significance of the LOD through computer simulations (here 95% confidence level; Marshall et al., 1998). Computations were carried out using 10,000 iterations of the population allelic frequencies estimated using the genotypes of the 53 adults studied. We estimated the number of potential fathers per clutch and whether or not a single male individual participated in more than one brood. Posteriorly, we estimated the level of relatedness between all of the adults (males and females) in the tank using the ML relate software (Kalinowski et al., 2006).
Acknowledgments

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