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PERMANENT GENETIC RESOURCES NOTE
Characterization of polymorphic microsatellite markers in the brine shrimp Artemia (Branchiopoda, Anostraca)

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Abstract
The brine shrimp Artemia is a complex genus containing sexual species and parthenogenetic lineages. Artemia franciscana is native to America and its cysts (diapausing eggs) are used worldwide as a food source in aquaculture. As a consequence, this anostracan has become an invasive species in many hypersaline aquatic ecosystems of other continents. Parthenogenetic Artemia lineages occur only in the Old World. Ten and five microsatellite markers were developed to characterize two populations for A. franciscana and two populations for diploid parthenogenetic Artemia, respectively. For A. franciscana the number of alleles ranged from 11 to 58 per locus, while for parthenogens the number of alleles ranged from three to 10. The levels of heterozygosity in A. franciscana and in parthenogens ranged from 0.115 to 0.976 and from 0.000 to 0.971, respectively. These microsatellite loci showed a high population assignment power, which will be useful for future studies of population genetics and invasive processes in Artemia.

Keywords: Artemia franciscana, Artemia parthenogenetica, hypersaline ecosystem invasions, population assignment analysis, population genetics

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provided by the manufacturer (CPG, Inc.). Captured molecules with repetitive sequences were amplified and restricted with HindIII to remove the adapters. The resulting fragments were ligated into the HindIII site of the pUC19 plasmid and introduced into Escherichia coli strain DH5α by electroporation (ElectroMax, Invitrogen).

A total of 45 different microsatellite-containing clones were identified in A. franciscana, while 27 were identified in A. parthenogenetica. Only 31 and 19 polymerase chain reaction (PCR) primer pairs, respectively, could be designed using Primer 3 (Rozen & Skaletsky 2000) and oligo version 6.4 (Molecular Biology Insight, Inc.) software. Seventeen and eight of the primer pairs, respectively, yielded products of varying size, within the clonal expected size range, from 10 different brine shrimp samples and were selected for fluorescent labelling.

PCR amplifications were carried out in 20-μL total volume containing 2 μL of 10× PCR buffer (Bioline), 1.5–3.0 mM of MgCl2 (Bioline), 0.25–0.60 μM of each primer (see Table 1 for details), 250 μM of each dNTPs, 0.5 μL of 20 mg/mL bovine serum albumin (BSA) (Roche Diagnostics), and 0.5 U Taq DNA polymerase (Bioline). Reaction conditions were as follows: an initial denaturation step at 95 °C for 5 min, 16–21 cycles consisting of 60 s at 95 °C, 60 s at 60 °C decreasing 1 °C per cycle, and 60 s at 72 °C. Then, 24 additional cycles were performed consisting of a step of 30 s at 95 °C, 30 s at 40 °C, and 30 s at 72 °C, and a final supplementary extension step of 15 min at 72 °C.

Ten out of the 17 polymorphic markers were screened in a set of 88 individual cysts from two US populations [GSL (n = 42), and SFB (n = 46)] in A. franciscana. Five out of the eight loci for A. parthenogenetica were screened in a set of 50 individual cysts from two Spanish populations [Cabo de Gata, GAT (n = 15), and La Mata, MAT (n = 35)]. DNA was isolated from individual cysts using the Montero-Pau et al. protocol (2008). Individuals were genotyped by assessing allele size on an ABI 3130xl Genetic Analyser (Applied Biosystems) using forward primers labelled with FAM (Sigma), and NED, PET and VIC (Applied Biosystems) together with LIZ 500 size standard (Applied Biosystems). Due to some screened loci showing peaks with one base of difference, we added a palindromic sequence (5′-GTGTCTT-3′) at the 5′ end of their unlabelled reverse primer (see Table 1) in order to minimize the generation of stutter peaks (Brownstein et al. 1996). Allele scoring was carried out in two independent laboratories using GeneMapper version 3.7 software (Applied Biosystems). Sequences of loci were deposited in GenBank (EU888832–EU888846).

Observed and expected heterozygosities, deviations from Hardy–Weinberg equilibrium (HWE) expectations and linkage disequilibrium were calculated using Arlequin version 2.000 (Schneider et al. 2000). Sequential Bonferroni corrections for multiple tests were applied using a global P value of 0.05 (Rice 1989). The program Micro-Checker version 2.2.3 (van Oosterhout et al. 2004) was used to test for null alleles, large allele dropout and scoring errors due to stutter peaks.

The number of alleles detected at each locus ranged from 11 to 58 for A. franciscana, and from three to 10 for A. parthenogenetica, identifying frequent single-base alleles. Observed heterozygosity ranged from 0.115 to 0.976 for A. franciscana and from 0.000 to 0.971 for A. parthenogenetica showing in some loci excess of heterozygosity probably due to its reproduction mode (see Table 1). Six and four loci showed significant departures from HWE expectations after Bonferroni corrections in A. franciscana from SFB and GSL, respectively. Only two loci showed departures from HWE in both populations (Af_B117TAIL and Af_B105TAIL). One and two loci showed significant departures from HWE in A. parthenogenetica from GAT and MAT, respectively. Analyses performed with Micro-Checker indicated homozygote excess at loci with HWE departures was due to the presence of null alleles. Although two independent laboratories assigned identical genotypes, Micro-Checker identified scoring error in locus Af_A108 at A. franciscana from SFB population due to shortage of heterozygote genotypes in alleles of one repeat unit difference. For A. franciscana, there was significant linkage disequilibrium for two loci in SFB (Af_B9, Af_A136), two loci in GSL (Af_B117TAIL, Af_B139TAIL), and one locus in both populations (Af_B105TAIL) (see Table 1 for details), while no significant linkage disequilibrium was found in the parthenogens. Additionally, no evidence for large allele dropout was observed.

Finally, population assignment analysis (PAA) and principal coordinates analysis (PCA) (see Fig. S1) were assessed with GENALEX version 6 (Peakall & Smouse 2006) using the 10 loci described in Table 1, showing a high discriminatory power between populations, and 100% of assignment capacity in the A. franciscana specimens analysed. The same assignment capacity was observed for A. parthenogenetica populations (figure not shown). Although some loci need to be used with care given the presence of null alleles, these new microsatellite markers will provide useful tools to assess genetic variation and genetic structure in the populations of these two Artemia, and should be useful to identify the source of introduction events in the Mediterranean Basin and elsewhere. Thus, these results could assist in future conservation plans.

Acknowledgements

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Table 1  Characterization of polymorphic microsatellite marker isolates for Artemia showing name of locus with fluorescent labels used in primer sequences.

<table>
<thead>
<tr>
<th>Locus and GB Acc. No.</th>
<th>Primer sequences (5′–3′)</th>
<th>Repeat sequence</th>
<th>MgCl2 (mm)</th>
<th>N_A</th>
<th>Size range (bp)</th>
<th>San Francisco Bay population</th>
<th>Great Salt Lake population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ta (°C)</td>
<td></td>
<td></td>
<td></td>
<td>H_O</td>
<td>H_E</td>
</tr>
<tr>
<td>Artemia franciscana</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Af_A108 EUS88840</td>
<td>F: FAM-AGTGGCACAACATCCTTTTG</td>
<td>(CA)_13</td>
<td>60–40</td>
<td>3.0</td>
<td>12 150–177</td>
<td>0.357</td>
<td>0.804</td>
</tr>
<tr>
<td>Af_B117TAIL EUS88834</td>
<td>R: CACGACGACGATTTGTTTTGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Af_B10 EUS88838</td>
<td>F: VIC-CTTGCCTGCAACCTTAAA</td>
<td>(CT)_15</td>
<td>60–40</td>
<td>3.0</td>
<td>11 212–236</td>
<td>0.357</td>
<td>0.355</td>
</tr>
<tr>
<td>Af_A104TAIL EUS88841</td>
<td>R: AGAGCGTAAAGGAAAAGGCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Af_B139TAIL EUS88833</td>
<td>F: PET-AGAAGAGGAAGAGAGATTAG</td>
<td>(GA)_15</td>
<td>60–40</td>
<td>2.0</td>
<td>58 238–390</td>
<td>0.167</td>
<td>0.900</td>
</tr>
<tr>
<td>Af_B9 EUS88832</td>
<td>R: CACCCTCTACCCGACACACATAG</td>
<td>(GA)_13</td>
<td>60–40</td>
<td>2.0</td>
<td>24 236–281</td>
<td>0.579</td>
<td>0.926</td>
</tr>
<tr>
<td>Af_B109 EUS88836</td>
<td>F: PET-AGGAGAGAGAGAGAGAGATAC</td>
<td>(GA)_16</td>
<td>60–40</td>
<td>2.0</td>
<td>39 194–288</td>
<td>0.976</td>
<td>0.949</td>
</tr>
<tr>
<td>Af_B105TAIL EUS88837</td>
<td>R: AGCTGGCTGCTCCCTTACATGTC</td>
<td>(GA)_12</td>
<td>60–40</td>
<td>3.0</td>
<td>31 266–314</td>
<td>0.536</td>
<td>0.950</td>
</tr>
<tr>
<td>Af_B11 EUS88835</td>
<td>F: PET-GGGAATTGTTGTTGATTAG</td>
<td>(CT)_17</td>
<td>60–40</td>
<td>3.0</td>
<td>37 286–338</td>
<td>0.909</td>
<td>0.935</td>
</tr>
<tr>
<td>Af_A136 EUS88839</td>
<td>R: TCCCTGTTGAAACACTGTTTGGG</td>
<td>(TG)_10</td>
<td>60–40</td>
<td>3.0</td>
<td>19 215–317</td>
<td>0.189</td>
<td>0.814</td>
</tr>
<tr>
<td>Artemia parthenogenetica</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apdq01TAIL EUS88844</td>
<td>F: FAM-AGTGGCACAACATCCCTTTTG</td>
<td>(TG)_19</td>
<td>60–45</td>
<td>1.5</td>
<td>3 175–183</td>
<td>Mon.</td>
<td>Mon.</td>
</tr>
<tr>
<td>Apdq02TAIL EUS88845</td>
<td>R: TGTCTCCTGCTCCCTTTTCCCTTG</td>
<td></td>
<td>60–45</td>
<td>2.0</td>
<td>4 229–250</td>
<td>0.200</td>
<td>0.494</td>
</tr>
<tr>
<td>Apdq03TAIL EUS88842</td>
<td>F: VIC-AACGACACTCGCCGTTTGC</td>
<td>(ACT)_20</td>
<td>60–45</td>
<td>2.0</td>
<td>5 212–238</td>
<td>0.933</td>
<td>0.651</td>
</tr>
<tr>
<td>Apdq04TAIL EUS88843</td>
<td>R: GTCACTCCCTCGCGGCTTCCCGGT</td>
<td>(AG)_21</td>
<td>60–45</td>
<td>1.5</td>
<td>4 308–318</td>
<td>0.400</td>
<td>0.515</td>
</tr>
<tr>
<td>Apdq05TAIL EUS88846</td>
<td>F: PET-AGAAGAGGAAGAGAGATTAG</td>
<td>(GA)_11</td>
<td>60–45</td>
<td>2.0</td>
<td>10 85–181</td>
<td>0.800</td>
<td>0.660</td>
</tr>
</tbody>
</table>

Notes: GB Acc. No., GenBank Accession no.; T_a, annealing temperature (see text for details); N_A, number of alleles for each locus; H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; P, P value of exact test using Markov chains with a confidence interval of 95%. FNA, frequency of null alleles based on the Oosterhout method (Micro-Checker software); Mon., monomorphic locus. Asterisks indicate significant departure from Hardy–Weinberg equilibrium after Bonferroni correction.
References


Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Plot of principal coordinates analysis (PCA) performed with GENALEX software using the 10 polymorphic microsatellite loci described for Artemia franciscana in Table 1. Black squares and white triangles indicate individuals from the San Francisco Bay and Great Salt Lake populations, respectively.

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