Eight Novel Microsatellite Loci Developed from Vernal Pool Fairy Shrimp

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Abstract

We developed primer sets for eight di-, tri-, and tetranucleotide microsatellite loci for the threatened vernal pool fairy shrimp Branchinecta lynchi from CA, CAA, ATG, CAGA, and TAGA enriched genomic libraries. We tested primers in 74 individuals of B. lynchi from California, as well as 19 individuals from six other Branchinecta species: B. coloradensis, B. constricta, B. dissimilis, B. lindahli, B. mesovalensis, and B. oriena. The eight loci showed sufficient variability for investigations of B. lynchi evolution, population structure, and genetic diversity and may provide a new tool for assisting in delineation of management areas.

Keywords: Branchinecta lynchi; evolution; fairy shrimp; microsatellite; population; vernal pool


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Introduction

The vernal pool fairy shrimp Branchinecta lynchi is adapted to the ephemeral aquatic conditions of vernal pool habitats of southern Oregon and central and southern California (Figure 1). Although widely distributed, B. lynchi is uncommon throughout its range (Eng et al. 1990), and it is listed as threatened pursuant to the U.S. Endangered Species Act (ESA 1973, as amended) and has an IUCN ranking of vulnerable/A2c (IUCN 2011). Primary threats to B. lynchi populations include habitat degradation, habitat loss and fragmentation, altered hydrology, and invasive plant and animal species (USFWS 1994, 2005).

Management of B. lynchi and other closely related species of conservation concern may benefit from an understanding of the evolutionary and population genetic relationships among vernal pool complexes at a range-wide scale, as well as from a better understanding of the landscape features that structure populations on a more local level. Here we describe the development, characterization, and genetic diversity of eight novel microsatellite loci for B. lynchi. We tested loci for diversity from samples from throughout the species’ range, and we present results from tests of cross-amplification in six other Branchinecta species.

Methods and Results

Genetic Identification Services (Chatsworth, CA) created eight libraries enriched with microsatellite motifs (CA, CAA, ATG, CAGA, and TAGA) by using DNA from B. lynchi that came from Riverside County, California. A cocktail of seven blunt-end cutting enzymes (RsaI, HaeIII, BsrB1, PvuII, StuI, ScaI, EcoRV) partially restricted genomic DNA. We adapted fragments in the size range of 300 to 750 bp and subjected them to magnetic bead capture (CPG, Inc., Lincoln Park, NJ) using biotinylated capture molecules. We prepared libraries in parallel with Biotin-CA, Biotin-CAA, Biotin-ATG, Biotin-CAGA, and Biotin-TAGA as capture molecules. We amplified captured molecules and used HindIII to remove the adapters. We then ligated the resulting fragments into the HindIII site of pUC19 and electroporated recombinant molecules into Escherichia coli DH5α. We selected random recombinant clones for sequencing and expressed enrichment levels as the fraction of sequences that contained a microsatellite.
Genotyper, using ABI Prism Taq dye terminator cycle sequencing methodology (Applied Biosystems, Grand Island, NY). From the eight libraries, we sequenced a total of 768 clones. The clone screening produced quality sequence reads but resulted in a low success rate of 12% (89 sequences) that had inserts with a repeat region and enough flanking sequence from which to design primers for amplification of the loci.

We used default settings in Primer3 (Rozen and Skaletsky 2000) to design 89 primer sets with melting temperatures between 59 and 61°C. We used the Qiagen DNeasy kit (Qiagen, Inc. Valencia, CA) following the manufacturer’s recommended protocol to extract DNA from Branchinecta tissue samples. Preliminary screening reactions consisted of testing a series of polymerase chain reaction (PCR) cycling protocols starting with protocol 1 and moving to the next if it did not amplify a product (up to protocol 5, Table 1). We tested loci in a set of seven individuals from across the species range in California: Butte County, N = 1; Shasta County, N = 1; Fresno County, N = 1; Tuolumne County, N = 1; Tehama County, N = 2; and Riverside County, N = 1, which was the same sample that we used to create libraries from which we discovered the loci.

Screening reactions underwent electrophoresis at 120 V for 30 min on a 3% agarose gel in a 1 x sodium borate buffer, after which we visually inspected the results. Loci were accepted as working if a PCR produced banding patterns expected based on di-, tri-, and tetranucleotide repeats. Only these working loci underwent further analysis. Among the 89 primers that we tested, 19 amplified a PCR product, and we considered 8 to be working loci (Tables 1 and 2).

To assess the utility of these eight loci, we tested 74 Branchinecta lynchi samples from many pools in three additional regions in California (Carrizo N = 24; San Joaquin Valley N = 13; and southeastern Sacramento Valley, N = 37) and estimated allele range and number of alleles at each locus (Data S1, Supplemental Material). Additionally, we tested these loci in a total of 19 individuals from six other Branchinecta species that are also present in North America: B. coloradensis (N = 3), B. constricta (N = 3), B. dissimilis (N = 3), B. lindahli (N = 3), B. mesovalensis (N = 1), and B. oriena (N = 6). All PCRs were carried out in 10-µL volumes with final concentrations of 1 x supplied buffer (Faststart TAQ, Roche, Inc., Basel, Switzerland) 1 x bovine serum albumin, 0.2 mM dNTPs, 1.5 mM MgCl₂ for all loci except Blynchi-C11.

Figure 1. Branchinecta lynchi specimens from the collection at the California Academy of Sciences (CAS) in San Francisco, California (CA). Top is a male (CAS161073) collected from Placer County, California, and bottom is a female (CAS180431) collected from Solano County, California. Photo credit: Kristy Deiner.
Table 1. Polymerase chain reaction protocols for eight microsatellite loci optimized in Branchinecta lynchi. Numbers in cells indicate (in order) temperature in degrees Celsius/time in seconds/number of cycles at that temperature and time. Protocol 1 included a ramp protocol starting at a low temperature that increased by 0.1°C at each cycle to a final temperature. Protocols 2 through 5 included a touch-down (TD) in which the cycle started at a high temperature and decreased to a final temperature.

<table>
<thead>
<tr>
<th>PCR protocol</th>
<th>Total cycles</th>
<th>Cycling regime 1</th>
<th>Cycling regime 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Denature</td>
<td>Annealing</td>
<td>Extension</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>94/40/35</td>
<td>Ramp 56.5–60/40/35</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>94/30/15</td>
<td>TD 65–50/60/15</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>94/40/15</td>
<td>TD 65–60/54/15</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>94/30/15</td>
<td>TD 65–50/60/15</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>94/40/5</td>
<td>TD 70–65/40/5</td>
</tr>
</tbody>
</table>

a All PCRs had an initial denature of 94°C for 4 min and a final extension of 72°C for 7 min and 60°C for 20 min.

Table 1. Extended.

<table>
<thead>
<tr>
<th>Cycling regime 3</th>
<th>Loci that worked for protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denature</td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>94/40/10</td>
<td>TD 60–55/40/10</td>
</tr>
</tbody>
</table>

and Blynchi-H8, which had a concentration of 2.0 mM MgCl₂, 0.05 U/μL Taq DNA polymerase (Faststart T AQ, Roche, Inc., Basel, Switzerland). Fluorescently labeled forward and unlabeled reverse primers were at a concentration of 0.54 μM. We added 1 μL of template DNA, which ranged in concentration from 10 to 70 ng/μL. We used a 3730 DNA Analyzer to separate PCR products and visualized and scored the products with GENEMAPPER, (Applied Biosystems, Grand Island, New York) version 4.0 (Applied Biosystems, Grand Island, NY).

The number of alleles per locus ranged from 3 to 14 with an average of 7.9 in pooled sample sites (Table 2). We tested Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) within a single vernal pool from the southeastern Sacramento Valley (N = 26) with ARLEQUIN version 3.5.1.3 (Excoffier and Lischer 2010). One locus, Blynchi-A273, was monomorphic for this sampling site and therefore we could not execute tests. Expected heterozygosity for all seven polymorphic loci ranged from 0.28 to 0.73, while their observed heterozygosity ranged from 0.00 to 0.57. We found that five loci significantly deviated from HWE (all had heterozygosity deficiencies, P < 0.05, Table 2) after Bonferroni correction for multiple tests (Rice 1989). One locus pair (C11 × B205) was significantly out of linkage equilibrium (P < 0.05, Table 2) after Bonferroni correction for multiple tests (Rice 1989).

To rule out potential genotyping errors we regenotyped all southeastern Sacramento Valley (N = 26) samples three times from the same DNA extract using the multiltube approach and calculated the mean error rate per locus following that of Pompanon et al. (2005). The mean error rate per locus was zero for all loci except Blynchi-C11, which had an error rate of 2.6%.

Using PCR protocols established for B. lynchi, cross-amplification in six sister taxa was successful for two loci. We were successful in amplifying the Blynchi-B205 locus in B. coloradensis (three of three individuals), B. oriena (five of six individuals), and B. dissimilis (one of three individuals). Similarly, we amplified the Blynchi-C11 locus in B. lindahli (one of three individuals) and B. constricta (two of three individuals).

Discussion

The eight microsatellite loci that we developed in this study showed diversity across B. lynchi sample sites and may be useful for studies of population genetic structure and range-wide genetic diversity to aid in identification of areas of conservation importance. These loci may also be helpful for testing hypotheses related to the molecular evolution and reproductive biology of this diverse group of Anostraca crustaceans. However, data from these loci require careful interpretation in population genetic studies because they deviate from HWE. Loci that are out of HWE may indicate that their populations of origin violate assumptions of many population genetic models. Therefore use of such loci has the potential to bias measures of population substructure.

Wild populations may deviate from HWE for several reasons, including population subdivision (spatial or temporal), assortative mating, inbreeding, and selection. Other studies have found Anostraca shrimp in natural populations to deviate from HWE. For example, previous studies using other genetic loci indicated that Branchinecta coloradensis (Bohonak 1998), Branchinecta sandiegensis (Davies et al. 1997), and Branchipodopsis wolfi (Brendonck et al. 2000) all showed departures from HWE in natural
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Table 2.  Characterization of eight microsatellite loci examined in 74 vernal pool fairy shrimp, Branchinecta lynchi sampled from Carrizo (24), San Joaquin Valley (13), and Sacramento Valley (37). Motif indicates the microsatellite repeat for each locus, number of gene copies indicates the number of alleles successfully amplified from the total sample (this number is not 148 in all cases because some loci in some individuals failed to amplify), and $N_a$ is the number of alleles observed for each locus among all samples. Observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium (LD) were calculated from a subset of 26 individuals sampled from a single pool in southeastern Sacramento Valley. Of these, $N$ indicates the number of individuals that were successfully amplified for each locus and subsequently used to estimate $H_O$, $H_E$, HWE, and LD.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequences</th>
<th>GenBank accession no.</th>
<th>Motif</th>
<th>No. gene copies</th>
<th>Allele size range (bp)</th>
<th>$N$</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>HWE $P$ value</th>
<th>LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blynchi-A273</td>
<td>F: GCCTCAAGGAAAACGTTTACTCAA R: ATCACAGAACCCTGTTGGAGT</td>
<td>KC170306 CA</td>
<td>144</td>
<td>3</td>
<td>132–136</td>
<td></td>
<td></td>
<td></td>
<td>0.01* n.s.</td>
<td></td>
</tr>
<tr>
<td>Blynchi-B107</td>
<td>F: CGAAGATTTATCTGTTTACAG R: ATTCGGTTGAAGGTGGTTACCC</td>
<td>KC170307 ACC</td>
<td>138</td>
<td>12</td>
<td>155–227</td>
<td>24</td>
<td>0.250.55</td>
<td>&lt;0.01* n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blynchi-B205</td>
<td>F: TTTATTGATAACCTCTAGGGTAAGA R: GGGGATAGGAAGAAAATGTTAA</td>
<td>KC170308 ACC</td>
<td>132</td>
<td>5</td>
<td>117–131</td>
<td>24</td>
<td>0.540.49</td>
<td>0.68 w/C11*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blynchi-B263</td>
<td>F: CCTGGGCATGATAGATAGATA R: CTTCCAGATAGGGTTAGCACAC</td>
<td>KC170309 ACC</td>
<td>112</td>
<td>11</td>
<td>130–169</td>
<td>24</td>
<td>0.750.66</td>
<td>0.59 n.s.</td>
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<tr>
<td>Blynchi-C11</td>
<td>F: TACAGATGCCAGACACAGTA R: TTTCCACTTGTAGAACCAC</td>
<td>KC170310 ATG</td>
<td>110</td>
<td>7</td>
<td>249–267</td>
<td>23</td>
<td>0.350.62</td>
<td>0.01* w/B205*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blynchi-C219</td>
<td>F: TTTGGAAGAGCCCTTGGAGGA R: GGGGAGGAGTCTGTGTATTTT</td>
<td>KC170311 ATG</td>
<td>138</td>
<td>14</td>
<td>142–172</td>
<td>24</td>
<td>0.000.54</td>
<td>&lt;0.01* n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blynchi-D210</td>
<td>F: ATGGTACGGCTCAACCAAGG R: TTAGTTTGGTCGGGTTCCTG</td>
<td>KC170312 CAGA</td>
<td>116</td>
<td>6</td>
<td>173–233</td>
<td>25</td>
<td>0.320.57</td>
<td>&lt;0.01* n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blynchi-H8</td>
<td>F: ACTGCAATTTTGTGACTGTTGATTT R: GCCGTACAGGATGGATCAAT</td>
<td>KC170313 TAGA</td>
<td>114</td>
<td>5</td>
<td>138–192</td>
<td>25</td>
<td>0.080.27</td>
<td>&lt;0.01* n.s.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a HWE and LD were not tested for Blynchi-A273 because it was monomorphic for this subsample from a single pool.
b n.s., not significant.
c Significant LD between this pair of loci after Bonferroni correction with a $P$-value of <0.01.
d Significant at the 0.05 alpha level after Bonferroni correction for multiple tests.

Although these eight microsatellite loci are not in HWE, they provide a tool that may be used to advance conservation efforts in this endemic and threatened North American species of fairy shrimp through the study the genetic diversity, evolution, and spatial and temporal isolation on population genetic structure.

Supplemental Material

Please note: The Journal of Fish and Wildlife Management is not responsible for the content or functionality of any supplemental material. Queries should be directed to the corresponding author for the article.

Data S1. Microsatellite genotypes for 74 individual Branchinecta lynchi from three regions (Carrizo = 24, San Joaquin Valley = 13, Sacramento Valley = 37) for the eight microsatellite loci developed in this study collected. Found at DOI: http://dx.doi.org/10.3996/112012-JFWM-096.51 (16 KB XLSX).

Acknowledgments

The work described in this article was conducted under a USFWS 10(a)(1)(A) research permit (permit number TE-FWSSFWO). We would like to thank Dave Kelly who provided significant technical support. We also thank the Journal Subject Editor and anonymous reviewers who greatly improved the manuscript. Financial support was provided by the U.S. Fish and Wildlife Service, UC Davis Dissertation Year Fellowship, The Crustacean Society: Denton Belk Research Award, and the Achievement Rewards for College Scientists Fellowship.

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References


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