Isolation and characterization of 16 polymorphic microsatellite loci for the Asian green mussel *Perna viridis* (Mollusca, Mytilidae)

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**Abstract**

The Asian green mussel *Perna viridis* is an abundant and important ecological and economical species across its native range. However, outside its native range, this species has been considered invasive and concerns have been raised worldwide regarding its potential impacts. Despite this, little work has been done to investigate the genetics of native and/or introduced populations of this species. In the present study, we developed 16 new polymorphic microsatellite markers using the Illumina MiSeq Platform. Four to 15 alleles per locus were detected. There was no evidence of linkage disequilibrium between pairs of loci and all loci were in Hardy-Weinberg equilibrium.

**Key words:** marine pest, biosecurity, genetic markers, *Perna viridis*, population genetics

**Introduction**

The Asian green mussel *Perna viridis* (Linnaeus, 1758) occurs extensively throughout the Indo-Pacific region from the Persian Gulf, throughout India and South East Asia, to China and Japan (Baker et al. 2007; Siddall 1980). This species’ native and introduced range within Asian waters is, however, unclear. *Perna viridis* are fouling organisms that are likely to have increased their range through centuries of shipping in the region (Baker et al. 2007; Hanyu and Sekiguchi 2000; Siddall 1980). They are also an important food source and have been intensively farmed, and translocated for farming, in South East Asia since the 1950’s (Ye 1997; Vakily 1989). Intentional (aquaculture) and unintentional (shipping) introductions of *P. viridis* outside of its Asian range have further occurred to numerous Pacific islands (Eldredge 1994), the Caribbean (Agard et al. 1993) and North and South America within the Atlantic Ocean (Benson et al. 2001; Power et al. 2004; Rylander et al. 1996).

The successful establishment of this species outside its native range has had concerning economic and ecological impacts in some areas e.g. south-east coast of the USA (Ingrao et al. 2001; Benson et al. 2001; Gilg et al. 2012). In Australia, *P. viridis* is among the most commonly identified target pest species within the biofouling community of vessels...
Genomic Research Facility (AGRF, Melbourne) for Mini Kit. The DNA was sent to the Australian Favorgen FavorPrep Tissue Genomic DNA Extraction tissue sample of a Genomic DNA (2.6 µg) was isolated from a 25 mg Next-generation sequencing

Materials and methods

Next-generation sequencing

Genomic DNA (2.6 µg) was isolated from a 25 mg tissue sample of a P. viridis using a Fisher Biotec Favorgen FavorPrep Tissue Genomic DNA Extraction Mini Kit. The DNA was sent to the Australian Genomic Research Facility (AGRF, Melbourne) for sequencing using an Illumina MiSeq Platform. Libraries were prepared with the TruSeq DNA Nano using the 550 base pair (bp) insert protocol, which includes shearing and bead size selection at the AGRF. Sequencing was performed at the AGRF using 300 bp paired end reads on the Illumina MiSeq. The data was de-multiplexed as part of the sequencing protocol options. The sequences were assembled into paired reads using PEAR v0.9.7 (Zhang et al. 2014), with a q-value cut-off of 20. These paired reads were scanned for Simple Sequence Repeats (SSRs) and a list of primer sequences and PCR conditions was generated for pure microsatellites using the open source QDD v1.3 (Meglécz et al. 2010) and Primer3 v2.3.3 (Rozen and Skaletsky 2000) software following Gardner et al. (2011).

Primer testing

We selected 39 di-, tri-, tetra-, and penta-base repeat microsatellite loci with a PCR product of 100–400 bp for further development. These loci were trialled for amplification separately in 5 µl reactions containing 10 ng of DNA, 1 x MyTaq reaction buffer (containing 5 mM dNTP and 15 mM MgCl2), 0.5 U MyTaq DNA polymerase (Bioline Reagents), and 0.2 µM of each primer. The following PCR conditions were used: 95 °C for 3 min followed by 30 cycles at 95 °C for 30 s, an optimal annealing temperature (Table 1) for 30 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. PCR products were visualized on 3 % agarose gels stained with GelRed (Biotium Inc.) alongside a 100 bp molecular weight marker (Axygen Biosciences) and visualised under UV light. Loci which generated a product of the expected size were tested for polymorphism using DNA extracted from eight individual P. viridis mussels collected by hand from along the Kaohsiung river (22º37′33.57″N, 120º16′10.02″E), a built up area in Taiwan with considerable boating activity.

Each of the forward primers for polymorphic loci selected for fragment analysis were labelled with a fluorescent tag: FAM (GeneWorks), NED, PET or VIC (Applied Biosystems) and screened for variation using 37 P. viridis collected by hand from mussel aquaculture farms in Jakarta Bay (06º04′00″S, 106º43′00″E), Indonesia. PCR products (2.5 µl) were analysed on an ABI 3730 Sequencer, sized using the GeneScan-500 LIZ internal size standard and scored using GENEMARKER software (SoftGenetics).

We used CERVUS (Kalinowski et al. 2007) to calculate the number and range of allele sizes, polymorphic information content, observed and expected heterozygosity and the frequency of null alleles for each locus. To test for deviation from Hardy-
Table 1. Primer sequences, GenBank accession numbers, annealing temperatures, repeat motif, and levels of diversity for 16 microsatellite loci in marine mussel *Perna viridis*. Number of alleles (Na), polymorphic information content (PIC), observed heterozygosity (Ho), expected heterozygosity (He), probability value from a test for deviation from Hardy-Weinberg Equilibrium (P) and null allele frequency (F). F, N, V, and P indicate dyes FAM, NED, VIC, and PET respectively.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequence (5'-3')</th>
<th>GenBank Acc. no.</th>
<th>Annealing Temp</th>
<th>Repeat Motif</th>
<th>Na</th>
<th>Size Range (bp)</th>
<th>PIC</th>
<th>Ho</th>
<th>He</th>
<th>P</th>
<th>F (Null)</th>
</tr>
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<tbody>
<tr>
<td>Pv02</td>
<td>F:ATGGAACATTCGAGTGCAA R:CGTTGACCTTCAACACCTCA</td>
<td>KX463418</td>
<td>53°C (AAAC)9</td>
<td>8</td>
<td>230-259</td>
<td>0.58</td>
<td>0.61</td>
<td>0.63</td>
<td>0.59</td>
<td>-0.01</td>
<td></td>
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<td>Pv04</td>
<td>F:CTAGCTGATTCGTTCTCG R:AGCAATTACCTCGAGAAGG</td>
<td>KX463419</td>
<td>53°C (AACT)7</td>
<td>11</td>
<td>333-372</td>
<td>0.83</td>
<td>0.81</td>
<td>0.86</td>
<td>0.87</td>
<td>0.02</td>
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<tr>
<td>Pv06</td>
<td>F:TTTTGAGATCCCTAACACCC R:AGCATAGCATGTCTGTTGT</td>
<td>KX463420</td>
<td>60°C (AATC)7</td>
<td>12</td>
<td>130-168</td>
<td>0.85</td>
<td>0.77</td>
<td>0.88</td>
<td>0.23</td>
<td>0.05</td>
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<td>Pv13</td>
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<td>KX463422</td>
<td>53°C (AAT)14</td>
<td>8</td>
<td>195-220</td>
<td>0.79</td>
<td>0.85</td>
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<td>F:TAGGAGTCAAGGATGCTAAAAG</td>
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<td>53°C (AAT)5</td>
<td>5</td>
<td>156-168</td>
<td>0.61</td>
<td>0.62</td>
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<td>0.05</td>
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<td>Pv18</td>
<td>F:TTCTGTGAAGCAAACAGTTACG R:TGGAGAAAACCAAGCTGAA</td>
<td>KX463424</td>
<td>53°C (AAT)15</td>
<td>12</td>
<td>127-164</td>
<td>0.85</td>
<td>0.78</td>
<td>0.88</td>
<td>0.19</td>
<td>0.05</td>
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<tr>
<td>Pv21</td>
<td>F:GTAGGTTTCTTGAACTTTAAGAGTAGTGAT</td>
<td>KX463425</td>
<td>53°C (AAT)4</td>
<td>5</td>
<td>130-146</td>
<td>0.59</td>
<td>0.69</td>
<td>0.65</td>
<td>0.59</td>
<td>-0.05</td>
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<tr>
<td>Pv22</td>
<td>F:TAGCAATGATGAGGAACATCTC R:CTATTGATACACGGTAATGAA</td>
<td>KX463426</td>
<td>53°C (AC)12</td>
<td>9</td>
<td>252-268</td>
<td>0.74</td>
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<td>0.78</td>
<td>0.10</td>
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<tr>
<td>Pv26</td>
<td>F:AGTCTTCTTCTGCTGCTGAT R:AAAGACATGTTAGGCTC</td>
<td>KX463427</td>
<td>60°C (AC)10</td>
<td>7</td>
<td>189-201</td>
<td>0.70</td>
<td>0.73</td>
<td>0.74</td>
<td>0.96</td>
<td>0.00</td>
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<tr>
<td>Pv30</td>
<td>F:GCGCAGAATGTCGCTTC</td>
<td>KX463428</td>
<td>53°C (AC)10</td>
<td>12</td>
<td>192-215</td>
<td>0.85</td>
<td>0.60</td>
<td>0.88</td>
<td>0.66</td>
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<tr>
<td>Pv31</td>
<td>F:GCTGATTACCTCACTCACAAG R:TGTATTTTGACCAAGTTGACAT</td>
<td>KX463429</td>
<td>53°C (AC)9</td>
<td>4</td>
<td>188-194</td>
<td>0.23</td>
<td>0.22</td>
<td>0.25</td>
<td>0.05</td>
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<td></td>
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<tr>
<td>Pv32</td>
<td>F:GGCCAGGTACATTTTGGGAG R:TCTCAACACTAACGATGAGG</td>
<td>KX463430</td>
<td>53°C (AC)8</td>
<td>4</td>
<td>127-134</td>
<td>0.46</td>
<td>0.59</td>
<td>0.47</td>
<td>0.07</td>
<td>0.18</td>
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<tr>
<td>Pv33</td>
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<td>KX463431</td>
<td>53°C (AG)9</td>
<td>6</td>
<td>214-227</td>
<td>0.64</td>
<td>0.70</td>
<td>0.68</td>
<td>0.72</td>
<td>-0.01</td>
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<tr>
<td>Pv34</td>
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<td>KX463432</td>
<td>53°C (AG)9</td>
<td>15</td>
<td>145-181</td>
<td>0.90</td>
<td>0.90</td>
<td>0.92</td>
<td>0.40</td>
<td>0.00</td>
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<td>Pv37</td>
<td>F:CCACATGTCATACATGGCTGCA R:GAAACAGTCTGTTCC</td>
<td>KX463433</td>
<td>60°C (AC)9</td>
<td>13</td>
<td>176-209</td>
<td>0.76</td>
<td>0.70</td>
<td>0.79</td>
<td>0.38</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

Weinberg equilibrium and linkage disequilibrium between pairs of loci, we used the online version of GENEPOP 4.0 (Raymond and Roussset 1995). All pairwise tests were adjusted for multiple tests by false discovery rate (FDR) correction (Benjamini and Yekutieli 2001).

Results and discussion

The sequence run yielded 890,475 quality paired reads with the sequences between 50–590 bp having a peak at approximately 550 bp. There were 14,404 pure microsatellites of >4 repeats for which primers were designed. Thirty four of the 39 targeted loci generated a product of the expected size. Of the 39 loci initially screened, 27 (69%) produced PCR products with clear bands and appeared polymorphic after agarose gel electrophoresis. From these, 16 loci produced genotypes that were consistently scoreable. The number of alleles per locus ranged from four to 15 and the observed and expected heterozygosities ranged between 0.22 to 0.91, and 0.25 to 0.92 respectively (Table 1). All loci were in Hardy-Weinberg equilibrium, and there was no evidence of linkage disequilibrium between any pair of loci.

Studies looking at differences between the enrichment technique and the next-generation sequencing technique (Gardner et al. 2011; Abdelkrim et al. 2009) have found the latter to recover more useable loci, as it targets all microsatellite repeat types (e.g. di-, tri-, tetra-, penta- and hexanucleotides). The high number of perfect and polymorphic loci developed in this study, should allow for a higher success when attempting to reproduce its application. Further, they will be useful to future genetic diversity and bioinvasion studies of *P. viridis*, supporting a science-based management approach to the future prevention and management of this species.
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References