

## Short Communication

## 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

entering Australian waters (McDonald 2012; Piola and McDonald 2012) and is listed under the National System for Prevention and Management of Marine Pest Incursions (DAFF 2010). In Indonesia, concerns have been raised recently regarding the role of domestic shipping as vectors of introduction and dispersal across native (west) and non-native (east) bioregions (Huhn et al. 2015).

Despite the worldwide distribution, abundance and overall importance of this species, only a few studies have addressed its population genetics, with most occurring within its native range and using a small number of populations and/or markers (Gilg et al. 2012; Lin et al. 2012; Prakoon et al. 2010). Genetic markers can be used to characterise genetic diversity and are therefore able to provide important information needed to identify native and introduced ranges and investigate potential frequency and routes of colonisation and dispersal (Cristescu 2015; Holland 2000). The success of being able to track invasion routes however depends on a balance between sampling effort, type and number of markers used and, ultimately, the species native range genetic structure (Estoup and Guillemaud 2010; Geller et al. 2010; Holland 2000). Fast evolving mitochondrial markers such as COI are commonly used to investigate taxonomy, phylogeography and population genetics of metazoan species (Estoup and Guillemaud 2010). While the maternally transmitted mitochondrial COI marker can be more sensitive to genetic drift, abundant and hypervariable nuclear microsatellite markers tend to give higher population structure resolution (Darling et al. 2008; Holland 2000).

A total of 37 microsatellite markers developed specifically for *P. viridis* are publicly available in the scientific literature (10 by Lin et al. 2007, 19 by Ong et al. 2005, 2008 and 2009, and 8 by Cao et al. 2013). However, these markers were developed using the enrichment technique and only Lin et al. 2007 used a DNA sequencer to obtain allelic data. Upon testing, we found only six of these 37 loci to be consistently scoreable. Therefore, we developed 16 new markers because a greater number of loci are needed to support population and bioinvasion genetic studies on *P. viridis* at native and introduced locations.

## 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 with 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 (Megléczy 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 × MyTaq reaction buffer (containing 5 mM dNTP and 15 mM MgCl<sub>2</sub>), 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 45 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 (22°37'23.57"N, 120°16'10.02"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 (06°04'0"S, 106°43'0"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 ( $H_o$ ), expected heterozygosity ( $H_E$ ), 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.

Locus	Primer Sequence (5'-3')	GenBank Acc. no.	Annealing Temp	Repeat Motif	Na	Size Range (bp)	PIC	$H_o$	$H_E$	$P$	F (Null)
Pv02 <sup>P</sup>	F:ATGGAACATCTCGAGTGCAA R:CGTTTGACTTTCCAACCTTCA	KX463418	53°C	(AAAC)9	8	230-259	0.58	0.61	0.63	0.59	-0.01
Pv05 <sup>N</sup>	F:TACTGCATGCTGCTCTCTG R:AGCAATTAACCTCGGAACAGTTTCT	KX463419	53°C	(AATC)7	11	333-372	0.83	0.81	0.86	0.87	0.02
Pv06 <sup>N</sup>	F:AATTTAGATCTTCTCAATCGCCC R:AGCATAGCATGTCTGTGTCTTTC	KX463420	60°C	(AATC)9	7	224-248	0.62	0.61	0.67	0.56	0.04
Pv13 <sup>F</sup>	F:CTGCGTTAGCACTTGCTTTG R:ITCCCAATTAGTCATCGTTCA	KX463421	53°C	(AAT)15	12	130-168	0.85	0.77	0.88	0.23	0.05
Pv15 <sup>V</sup>	F:TCAATAGGGAAATGATATGAAGGA R:CAAATCGAACACCAGGATGA	KX463422	53°C	(AAT)14	8	195-220	0.79	0.85	0.83	0.30	-0.02
Pv17 <sup>F</sup>	F:TGAAAGATCAAAGGATAGCTTAAAGG R:CATGTGCATGTAATGACCAAAA	KX463423	53°C	(AAT)9	5	156-168	0.61	0.62	0.66	0.05	0.05
Pv18 <sup>F</sup>	F:TTTCTTGAAAGCAAACAGTTACG R:TGAGAAACCAAGACGCTGAA	KX463424	53°C	(AAT)15	12	127-164	0.85	0.78	0.88	0.19	0.05
Pv21 <sup>V</sup>	F:GCTAGGTTTCATCCTTAATAACATTG R:ATCCATGTCCAATGCACAAA	KX463425	53°C	(AAT)14	5	130-146	0.59	0.69	0.65	0.59	-0.05
Pv22 <sup>N</sup>	F:TGACATTATCATGTAGAACATCTCAA R:TCATTCAAACCTGTCTGTGCTT	KX463426	53°C	(AC)12	9	252-268	0.74	0.78	0.78	0.10	-0.02
Pv26 <sup>P</sup>	F:AGTCCCTTCTCCTCGCTGAT R:AAAGACAGTTTAGGCGTTCCA	KX463427	60°C	(AC)10	7	189-201	0.70	0.73	0.74	0.96	0.00
Pv30 <sup>V</sup>	F:GGCACCAGTAATGCTGTCTC R:TTTGAAGCATACCAATTACAGTGA	KX463428	53°C	(AC)10	12	192-215	0.85	0.91	0.88	0.66	-0.03
Pv31 <sup>P</sup>	F:TGCATATTATCATTACCCACAAG R:TGTATTTCAGCAAATGGCATT	KX463429	53°C	(AC)9	4	188-194	0.23	0.22	0.25	0.05	0.05
Pv32 <sup>N</sup>	F:GGCCGAGGTACATTGTGAG R:TCACCAAACCTAACATATCCGAGA	KX463430	53°C	(AC)8	4	127-134	0.46	0.39	0.57	0.07	0.18
Pv33 <sup>P</sup>	F:TGTCTCAATACCATGGCGAA R:TGCCTACTTGATACCATTCGAT	KX463431	53°C	(AC)9	6	214-227	0.64	0.70	0.68	0.72	-0.01
Pv34 <sup>N</sup>	F:TCAGACTGCACACTGAGTCAAAA R:TTGCAAACACATTTCAAGCA	KX463432	53°C	(AG)9	15	145-181	0.90	0.90	0.92	0.40	0.00
Pv37 <sup>F</sup>	F:CCACACCTGTACATAGCCTGA R:GAAAGCAGTTTCATGGGGT	KX463433	60°C	(AC)9	13	176-209	0.76	0.70	0.79	0.38	0.05

Weinberg equilibrium and linkage disequilibrium between pairs of loci, we used the online version of GENEPOP 4.0 (Raymond and Rousset 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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