RESEARCH ARTICLE

Genetic characterization of *Perna viridis* L. in peninsular Malaysia using microsatellite markers

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Abstract

A total of 19 polymorphic microsatellite loci were used to analyse levels of genetic variation for 10 populations of *Perna viridis* L. collected from all over peninsular Malaysia. The populations involved in this study included Pulau Aman in Penang, Tanjung Rhu in Kedah, Bagan Tiang in Perak, Pulau Ketam in Selangor, Muar, Parit Jawa, Pantai Lido and Kampung Pasir Puteh in Johore, and Kuala Pontian and Nenasi in Pahang state. The number of alleles per locus ranged from two to seven, with an average of 3.1. Heterozygote deficiencies were observed across all the 10 populations. Characterization of the populations revealed that local populations of *P. viridis* in peninsular Malaysia were genetically similar enough to be used as a biomonitoring agent for heavy metal contamination in the Straits of Malacca. Cluster analysis grouped the *P. viridis* populations according to their geographical distributions with the exception of Parit Jawa. The analysis also revealed that *P. viridis* from the northern parts of peninsular Malaysia were found to be the most distant populations among the populations of mussels investigated and *P. viridis* from the eastern part of peninsular Malaysia were closer to the central and southern populations than to the northern populations.

[Ong C. C., Yusoff K., Yap C. K. and Tan S. G. 2009 Genetic characterization of *Perna viridis* L. in peninsular Malaysia using microsatellite markers. *J. Genet.* **88**, 153–163]

Introduction

The green-lipped mussel, *Perna viridis* L., is native to the Indo–Pacific region and currently they are being extensively cultured in many Asian countries; largely because of their value as a cheap source of animal protein for human consumption (Nicholson and Lam 2005). Besides being consumed as a protein rich food, they are also used as a biomonitoring agent for heavy metal contamination in various Asian countries (Monirith *et al*. 2003).

In Malaysia, this mussel is widely distributed along the Straits of Malacca and, to a lesser extent, in certain parts of Sabah state on Borneo Island and the east coast of peninsular Malaysia. This mussel has been proposed by Ismail *et al*. (2000) as a potential biomonitoring agent for heavy metal contamination in the Straits of Malacca; which is one of the busiest shipping lanes in the world. However, before this species can be used as a biomonitoring agent for heavy metal contamination in the Straits of Malacca, it needs to fulfill several recommended criteria. Among the criteria are that *P. viridis* collected from different geographical populations along the straits should have similar morphological characteristics for easy and correct species identification, and low-to-moderate degrees of genetic differentiation as they may genetically adapt to heavy metal stresses (Gyllensten and Ryman 1985; Rainbow 1995). Therefore, studies on the population genetic structure of *P. viridis* in Malaysia should be done to validate whether *P. viridis* populations collected from the coastal waters of peninsular Malaysia have low degree of genetic differentiation so that any differences in the biomonitoring parameters obtained from the tissues of mussels from different areas were not confounded by genetic factors rather

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Keywords. biomonitoring agent; green-lipped mussel; microsatellite markers; population structure; genetic variation; *Perna viridis*.

than being due to real differences in the environmental pollutants levels.

Until today molecular genetic markers such as allozymes, RAPD and RAM have been used to elucidate genetic information relating to local populations of *P. viridis*. Results based on allozymes support the use of *P. viridis* as a biomonitoring agent for heavy metal contamination in the straits (Yap *et al*. 2002). However, Yap *et al*. (2004) reported that there is a distinct genetic variation between *P. viridis* populations collected from contaminated and uncontaminated sites, in which a population from a contaminated site showed an excess of heterozygosity when compared to those of the populations from three uncontaminated sites. This in turn would put into question the genetic relationships among the eight *P. viridis* populations that were obtained by Yap *et al*. (2002), because the selective neutrality of all the allozymes that were used to estimate the genetic distance values had been assumed. Moreover, a study by Chua *et al*. (2003) based on RAPD and RAM markers showed clustering of populations that differed from those derived from the use of allozyme marker data.

In order to clarify the above, it is apparent that a more powerful marker system is required and a single locus DNA microsatellite markers appears to be the best choice because of reproducibility, codominant inheritance, high levels of polymorphism, assay ability by PCR, conformity to Mendelian inheritance and selective neutrality. Therefore, the objective of this paper is to validate whether local populations of *P. viridis* collected from the coastal waters of peninsular Malaysia are genetically similar enough to be used as a biomonitoring agent for heavy metal contamination in the Straits of Malacca by using the more informative single locus DNA microsatellite markers compared to the findings by using allozymes (Yap *et al*. 2002).

Materials and methods

Materials

P. viridis were collected from 10 different locations (figure 1) in peninsular Malaysia. The sample size for each locations was 20, except for Pulau Aman (Penang) only 10 individuals were obtained. Table 1 shows the sampling date, sample size

Figure 1. Map of peninsular Malaysia indicating the sampling sites of *P. viridis*.

Table 1. Sampling date, sample size (*N*), longitude and latitude of the sampling sites, method of sample collection and description of sampling sites for *P. viridis* from 10 locations in peninsular Malaysia.

No.	Location	State	Sampling date	*N*	Latitude (north)	Longitude (east)	Method of collection	Description of sampling site
1	Tanjung Rhu	Kedah	April 2002	20	6°25′	99°44′	Wild	Recreational and aquacultural areas
2	Pulau Aman	Penang	April 2002	10	5°17′	100°23′	Wild	Fish aquacultural area
3	Bagan Tiang	Perak	April 2002	20	5°07′	100°25′	Wild	Aquacultural area
4	Pulau Ketam	Selangor	June 2002	20	3°01′	101°16′	Wild	Fishing village
5	Muar	Johore	February 2002	20	2°02′	102°34′	Wild	Agricultural area
6	Parit Jawa	Johore	April 2004	20	1°57′	102°39′	Bought from roadside	Mussel aquacultural area
7	Pantai Lido	Johore	April 2002	20	1°27′	103°41′	Wild	Urban and agricultural areas
8	Kampung Pasir Puteh	Johore	April 2002	20	1°26′	103°55′	Wild	Industrial, shipping and urban runoff
9	Kuala Pontian	Pahang	April 2004	20	2°46′	103°32′	Cultured	Mussel aquacultural site; clean site
10	Nenasi	Pahang	April 2004	20	3°08′	103°27′	Near by lighthouse	A lighthouse; pristine waters

(*N*), longitude and latitude of the sampling sites, method of sample collection and description of sampling sites for the 10 locations. In the laboratory, the adductor muscle was excised from the mussel and kept at −80°C prior to DNA extraction and analysis.

Isolation of genomic DNA and microsatellite amplification

Genomic DNA from *P. viridis* adductor muscle was isolated by using a CTAB-based protocol described by Winnepennincks *et al*. (1993) with minor modifications. The modifications were omission of 0.2% v/v β-mercaptoethanol, which hinders DNA oxidation during the extraction, from the extraction buffer, and inclusion of phenol : chloroform : isoamylalcohol extraction step to remove proteins from the cell lysate before proceeding to the ethanol precipitation step.

PCR amplifications were performed in a 10 μL final reaction volume containing 25 ng of genomic DNA, 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton® X-100), 0.25 mM each of dNTPs, 0.15 μM of each reverse and forward primers, 1–3.75 mM of $MgCl_2$ and 0.5–1.5 U of *Taq* DNA polymerase (Promega, Madison, USA). Amplifications were performed in a Peltier Thermal Cycler PTC-220 (MJ Research, Waltham, USA) with an initial 3 min of pre-denaturation at 95°C, followed by 35–40 cycles of denaturation at 94°C for 30 s, an optimum annealing temperature (as shown in table 1 of appendix) for 30 s and extension at 68°C for 30 s. The amplifications were concluded with a 5 min final extension at 68°C.

The *P. viridis* specific primer pairs that were used in this study are presented in table 1 of appendix. Loci BP2-49-1, BP2-49-2, VJ1-12-2 and VJ1-18-1 were from Ong *et al*. (2005), loci BP2-35-2, BP9-7-1, BP9-13-2, BP9-16-2, BP9-19-2, BP9-27-1, BP14-7-1, VJ1-9-1, VJ1-15-1, VJ1-21-2 and VJ1-22-2 were from Ong *et al*. (2008), while loci BP10-5-1, BP10-16-1, BP10-17-2 and LR1-58-1 are reported here for the first time.

Electrophoresis of PCR products

A total of 10 μL of PCR product was mixed with 3 μL of gel-loadding buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% w/v sucrose in water). A 20 bp DNA ladder (200 ng/μL; BioWhittaker Molecular Applications, Rockland, USA) was used as the molecular weight standard. The PCR products were then electrophoresed on 4% (w/v) horizontal MetaPhor® agarose gel (BioWhittaker Molecular Applications, Rockland, USA) at 74 V for 3–4 h with 1× TBE (89 mM Tris-base, 89 mM boric acid and 2 mM EDTA) as running buffer. The gel was then stained in ethidium bromide (0.1 mg/mL) and photographed using the Alpha Imager Gel Documentation System (Siber Hegner, Zurich, Switzerland).

MetaPhor® agarose gel was used due to its high-resolution capabilities and its being easy to cast and handle. According to the manufacturer, the gel is capable of resolving PCR fragments differing in size by 4 bp. A 2–4% MetaPhor® agarose gel has approximately the resolution power of 4%–8% polyacrylamide gel. Comparative runs were initially done on 8% (w/v) polyacrylamide gel to confirm this. The PAGE gels were also stained in ethidium bromide (0.1 mg/mL) and photographed using the Alpha Imager gel documentation system (Siber Hegner, Zurich, Switzerland).

Data analysis

Genetic variability measures including mean number of alleles per locus and mean heterozygosity were calculated for all the populations. The *F*-statistics were calculated according to Wright (1978), providing a measure of the deficiency or excess of heterozygotes. Chi-square goodness-of-fit tests were used to determine whether the observed genotypic numbers were consistent with Hardy–Weinberg expectations for each population. Nei (1978) unbiased genetic distance (D_N), which takes small sample size into consideration, was calculated to assess the genetic distances among the populations. All the genetic data were analysed by using the POPGENE version 1.32 (Yeh and Boyle 1997), except for the hierarchical *F*-statistics analysis, which was done using the BIOSYS-1 computer package of Swofford and Selander (1989). By using the multivariate analysis software NT-SYS (Rohlf 1989), an UPGMA dendrogram was constructed based on Nei (1978) unbiased genetic distance estimates to depict the genetic relationships among the populations of *P. viridis*.

Results

Metaphor® agarose gel versus polyacrylamide gel

Comparisons of the electrophoresis gel resolutions between 4% Metaphor® agarose and 8% polyacrylamide gels revealed that there was no significant difference between the results produced by either gel type (figure 2). This result showed that the resolution for 4% Metaphor® agarose gel was as good as 8% polyacrylamide gel, as claimed by its manufacturer. However, polyacrylamide gel was still used whenever the separation of bands was unclear in Metaphor® agarose gel, for confirmation purposes.

Analysis of genetic variability

The genetic variability indices estimated for the 10 *P. viridis* populations are summarized in table 2 and table 2 of appendix. The range of number of alleles observed at each of the 19 loci across all the populations was two to seven. All the 10 populations showed lower mean observed heterozygosity values than expected. The highest mean observed heterozygosity was found in the Pulau Ketam population with

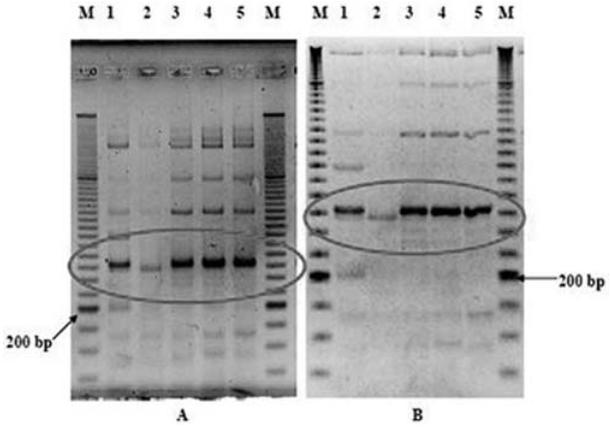

Figure 2. Comparison between 4% Metaphor® agarose gel and 8% polyacrylamide gel electrophoresis of the amplification products of primer pair BP2-35-2 from the Pantai Lido population: (A) the banding profiles when electrophoresed on 4% Metaphor® agarose gel, and (B) the same PCR products run on 8% polyacrylamide.

a value of 0.21, while the lowest was found in the Pulau Aman population with a value of 0.14. The Pantai Lido population had the highest difference between the means of observed and expected heterozygosity values (0.08) while the Parit Jawa and Kuala Pontian population had the least difference (0.02). Values of *F*-statistics for *P. viridis* are presented in table 2. The mean F_{IS}, F_{IT} and F_{ST} values were 0.174, 0.255 and 0.098, respectively. The positive values of both the mean F_{IS} and the mean F_{IT} indicated deficit of heterozygosity across all the populations and the mean F_{ST} value of 0.098 showed very moderate genetic differentiation among the populations of *P. viridis*. Two loci; namely BP2-35-2 and BP14-7-1 showed significant deviations from HWE in all the 10 populations.

Genetic differentiation

Wright's (1978) hierarchical *F*-statistics (table 3) shows that populations within the two, three and four regions accounted for 13.9%, 36.1% and 47.2%, respectively, of the total variance, while the between and among region variance components were 86.1%, 63.8% and 52.8% of the total, respectively, depending on which hierarchy was considered.

Table 2. Population genetics parameters for 19 polymorphic microsatellite loci in the 10 *P. viridis* populations.

Locus	N_O (N_E)	H_o	H_e	F_{IS}	F_{IT}	F_{ST}
BP2-35-2	4 (1.60)	0.050	0.380	0.855	0.869	0.096
BP2-49-1	5 (1.30)	0.194	0.234	0.134	0.169	0.040
BP2-49-2	4 (1.37)	0.281	0.268	−0.090	−0.050	0.037
BP9-7-1	2 (1.11)	0.103	0.098	−0.071	−0.057	0.013
BP9-13-2	2 (1.06)	0.058	0.056	−0.148	−0.031	0.102
BP9-16-2	2 (1.46)	0.390	0.315	−0.255	−0.240	0.012
BP9-19-2	2 (1.05)	0.045	0.044	−0.053	−0.024	0.027
BP9-27-1	3 (1.24)	0.220	0.197	−0.167	−0.114	0.045
BP10-5-1	2 (1.17)	0.154	0.142	−0.099	−0.085	0.013
BP10-16-1	2 (1.04)	0.042	0.042	−0.046	−0.020	0.024
BP10-17-2	4 (2.33)	0.475	0.573	0.106	0.155	0.055
BP14-7-1	7 (2.88)	0.087	0.654	0.822	0.863	0.229
LR1-58-1	4 (2.05)	0.183	0.514	0.591	0.638	0.115
VJ1-9-1	2 (1.57)	0.480	0.366	−0.395	−0.317	0.056
VJ1-12-2	3 (1.20)	0.148	0.165	0.129	0.143	0.017
VJ1-15-1	2 (1.06)	0.050	0.059	0.015	0.137	0.124
VJ1-18-1	4 (2.45)	0.484	0.593	−0.008	0.197	0.204
VJ1-21-2	2 (1.10)	0.097	0.093	−0.089	−0.049	0.037
VJ1-22-2	2 (1.08)	0.075	0.072	−0.059	−0.036	0.022
Mean	3.1 (1.48)	0.191	0.256	0.174†	0.255	0.098

N_O, observed number of alleles; N_E, effective number of alleles (Kimura and Crow 1964); H_o, observed heterozygosity; H_e, expected heterozygosity.

†The mean F_{IS} value based on 16 polymorphic microsatellite loci was −0.068 when three loci; namely, BP2-35-3, LR1-58-1 and BP14-7-1 were excluded from this analysis in order to determine whether the F_{IS} value would still show deficit of heterozygosity across the 10 *P. viridis* populations.

Therefore, the hierarchical F-statistics suggest that a greater amount of the genetic variation is due to differentiation between (northern and southern) or among (northern, central, southern and eastern) regions.

Genetic distance and cluster analysis

The analysis of Nei (1978) unbiased genetic distance (D_N) among the 10 populations showed high genetic similarity among the 10 populations of *P. viridis* with a range of D_N values from 0.0070 to 0.0785. The highest D_N value (0.0785) was found between the Pulau Aman and Kuala Pontian populations and while the Nenasi and Kuala Pontian populations had the lowest D_N value (0.0070) (table 4).

The UPGMA dendrogram constructed based on D_N estimates revealed two major clusters (figure 3). The first cluster consisted of *P. viridis* collected from the northern part of peninsular Malaysia (the Pulau Aman and Tanjung Rhu populations) while the second cluster consisted of populations collected from the central, southern and eastern parts of peninsular Malaysia. The second cluster was further

Table 3. Wright's (1978) hierarchical F-statistics of genetic differentiation for the 10 *P. viridis* populations grouped into two (northern and southern), three (northern, central and southern) and four (northern, central, southern and eastern) regions.

Contrast	Variance component	(%)	F_{xy}
Populations in two regions	0.05	13.9	0.011
Populations in three regions	0.13	36.1	0.027
Populations in four regions	0.17	47.2	0.035
Between two regions	0.31	86.1	0.064
Among three regions	0.23	63.8	0.049
Among four regions	0.19	52.8	0.041
Among all populations	0.36	100.0	0.075

Note: The two regions were northern (Tanjung Rhu and Pulau Aman) and southern (Bagan Tiang, Pulau Ketam, Muar, Parit Jawa, Pantai Lido, Kampung Pasir Puteh, Kuala Pontian and Nenasi); the three regions were northern (Tanjung Rhu and Pulau Aman), central (Bagan Tiang, Pulau Ketam, Muar, Parit Jawa) and southern (Pantai Lido, Kampung Pasir Puteh, Kuala Pontian and Nenasi); the four regions were northern (Tanjung Rhu and Pulau Aman), central (Bagan Tiang, Pulau Ketam, Muar, Parit Jawa), southern (Pantai Lido and Kampung Pasir Puteh) and eastern (Kuala Pontian and Nenasi).

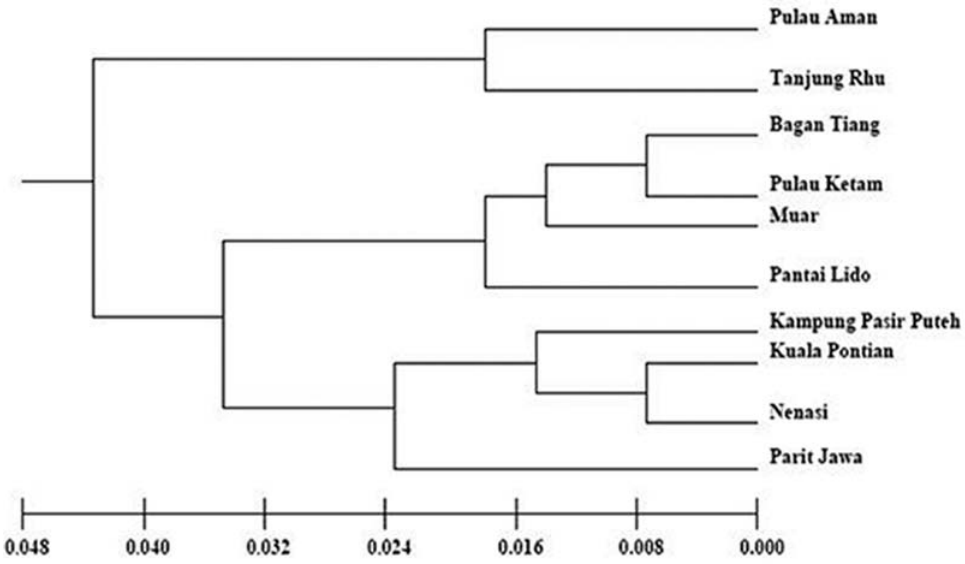

Figure 3. UPGMA dendrogram of genetic relationships among 10 populations of *P. viridis* based on Nei's (1978) unbiased genetic distance derived from 19 microsatellite loci.

Table 4. Nei's (1978) unbiased measures of genetic distance based on 19 microsatellite loci in the 10 populations of *P. viridis* from peninsular Malaysia.

Populations	Pulau Aman	Tanjung Rhu	Bagan Tiang	Pulau Ketam	Muar	Parit Jawa	Pantai Lido	Kampung Pasir Puteh	Kuala Pontian	Nenasi
Pulau Aman	******									
Tanjung Rhu	0.0172	******								
Bagan Tiang	0.0482	0.0306	******							
Pulau Ketam	0.0393	0.0296	0.0072	******						
Muar	0.0502	0.0394	0.0117	0.0134	******					
Parit Jawa	0.0584	0.0319	0.0176	0.0318	0.0356	******				
Pantai Lido	0.0210	0.0180	0.0143	0.0168	0.0212	0.0235	******			
Kampung Pasir Puteh	0.0486	0.0319	0.0274	0.0291	0.0360	0.0237	0.0168	******		
Kuala Pontian	0.0785	0.0432	0.0296	0.0368	0.0548	0.0213	0.0345	0.0173	******	
Nenasi	0.0772	0.0467	0.0348	0.0417	0.0571	0.0223	0.0329	0.0112	0.0070	******

differentiated into two subclusters, with the Bagan Tiang, Pulau Ketam, Muar and Pantai Lido populations in the first subcluster and the Kampung Pasir Puteh, Kuala Pontian, Nenasi and Parit Jawa populations in the second subcluster.

Discussion

In this study, 19 polymorphic microsatellite loci were used to analyse the levels of genetic variation for 10 populations of *P. viridis* collected from all over peninsular Malaysia. The analysis revealed low genetic variation within and among the 10 populations of *P. viridis* and this supports the use of local populations of *P. viridis* as a suitable biomonitoring agent for heavy metal contamination in the Straits of Malacca. From a biomonitoring point of view, it is very important to use a single species to act as a biomonitor. This single species should have similar morphological characteristic and low-to-moderate degrees of genetic differentiation because different species or subspecies have different rates of regulation, excretion and sequestration of contaminants (metals) in the mussel body which may render invalid the results of a biomonitoring programme.

The number of alleles at each of the 19 loci that ranged from two to seven per locus (average 3.1 per locus) was higher than those from a previous study using allozyme markers (Yap *et al*. 2002). However, this was relatively low when compared to the generally reported number of alleles per locus for microsatellite loci in the literature, which usually ranged from two to more than 10, suggesting low levels of allelic diversity for the local populations of *P. viridis*. The MetaPhor® agarose gels that we used to type the microsatellite loci in this study had been shown by Ochsenreither *et al*. (2006) to be as efficient as polyacrylamide gels and an automated capillary sequencer system (CEQ 8000; Beckman Coulter, Fullerton, USA) and by Kamara *et al*. (2007) to be comparable with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA) for microsatellite allele discrimination.

Heterozygote deficiency was observed across all the 10 populations. This finding was not uncommon as studies of marine bivalves often report lower values of observed heterozygosities than those expected under Hardy–Weinberg equilibrium (Zouros and Foltz 1984; Arnaud-Haond *et al*. 2003). Inbreeding, Wahlund effect, null alleles, natural selection, mutation, gene flow, genetic drift and aneuploidy are some of the reasons that have been offered to explain these phenomena (Lowe *et al*. 2004). O'Connell and Wright (1997) suggested that a minimum sample size of 50 individuals per population should be considered for loci showing between five and ten alleles. In this study, except for one site, 20 samples were typed due to both the limited number of samples available and the expense of the assay. The presence of null alleles could also affect our results as this is a common problem with microsatellite loci (Callen *et al*. 1993; Hare *et al*. 1996; O'Connell and Wright 1997). An individual heterozygous for a null allele would be scored as being homozygous for the alternative allele (Kalinowski and Taper 2006).

Analysis of F-statistics revealed that the overall F_{IS} value was largely influenced by three loci; namely BP2-35-2, LR1-

58-1 and BP14-7-1 which showed considerably higher positive F_{IS} values compared to the other loci and two loci, namely BP14-7-1 and BP2-35-2, showed significant deviations from HWE in all the 10 populations. Further statistical analysis excluding these three loci was carried out in order to determine whether the F_{IS} value would still show a deficit of heterozygosity across the 10 *P. viridis* populations. A negative mean F_{IS} value of −0.068, indicating excess of heterozygosity, was obtained when the three loci were excluded from the analysis (table 2). F_{ST} values can be used to determine the degree of genetic differentiation among populations of *P. viridis*. According to Wright (1978), there are four qualitative guidelines for the interpretation of F_{ST}: 0–0.05 for little genetic differentiation, 0.05–0.15 for moderate genetic differentiation, 0.15–0.25 for large genetic differentiation and above 0.25 for very large genetic differentiation. Based on these guidelines, the F_{ST} values showed that our samples belonged to the same species but with moderate genetic differentiation among the regional populations. Hence, this mussel is suitable to be used as a biomonitoring agent for the waters of peninsular Malaysia since the same species is found in the Straits of Malacca, the Straits of Johore and the South China Sea which surround the peninsula. Our F_{ST} values are also close to those reported by Gosling *et al*. (2008) for zebra mussel, *Dreissena polymorpha* (0.118), Holland (2001) for brown mussel *Perna perna* (0.007–0.042) and by Johnson *et al*. (1998) for four species of mussels namely *Amblema plicata* (0.082), *Plectomerus dombeyanus* (0.121), *Quadrula pustulosa* (0.108) and *Q. quadrula* (0.160).

The genetic distance values presented in table 4 showed high genetic similarity among the 10 populations of *P. viridis* with D_N values ranging from 0.0070 to 0.0785. Cluster analysis grouped the 10 populations according to their geographical distributions except for the Parit Jawa population. The dendrogram showed that *P. viridis* populations from the northern part of peninsular Malaysia (Tanjung Rhu and Pulau Aman) were the most distant populations while the central and southern populations, particularly those from the Straits of Malacca and the west side of the Johore Straits seemed to be closely related populations. This pattern of clustering showed agreement with the results obtained using allozyme data (Yap *et al*. 2002). It could be that the two major clusters observed were due to limited genetic exchange resulting from the movements of currents in the straits or to local selection pressure. Close proximity between localities will increase gene flow, which tends to result in genetic uniformity among the populations. Geographically, the Straits of Malacca are narrower in the southern part when compared to the northern part and this will encourage greater genetic exchange between *P. viridis* populations in the southern regions. The Kampung Pasir Puteh (east side of the Johore Straits) and Pantai Lido (west side of the Johore Straits) were grouped separately into different subclusters although both are located near to each other in the Straits of Johore, which separates the Malaysian state of Johore to the north from Singapore to the south. The most likely explanation for this observation is that the Johore causeway linking Johore to Singapore island blocked the gene flow by pelagic dispersal between these two sites which are on different sides of the causeway. Hence this physical barrier to the free flow of sea water has had a biological effect on the green-lipped mussel. Yap *et al*. (2004) reported higher concentrations of copper and cadmium in the total soft tissues of mussels collected from Kampung Pasir Puteh when compared to those from other geographical populations. Although the 19 polymorphic microsatellite loci used in this study did not specifically distinguish the mussels from this area from those of the other geographical populations we studied, they are useful to identify the geographical origins of the mussel populations since the dendrogram clearly grouped the populations into distinct geographical regions. The Parit Jawa population, the only samples used in this study that were bought from a road side stall rather than being collected by us, clustered together with the south eastern and eastern Kampung Pasir Puteh, Kuala Pontian and Nenasi populations. This was not in accordance with Parit Jawa's geographical location on the south west coast of the peninsula and showed the power of our molecular markers in revealing samples of doubtful origins. Hence, these commercially sold samples were probably not local samples as claimed by the vendor. Based on the dendrogram presented in figure 3, these samples most likely originated from an area east of the Johore causeway since it clustered with populations from that region rather than with those from west of the causeway where Parit Jawa is geographically located. The dendrogram (figure 3) also indicated that *P. viridis* from the eastern part of peninsular Malaysia (Kuala Pontian and Nenasi) were closer to the central and southern populations than to the northern populations. During our sampling, hardly any *P. viridis* were found along the east coast of peninsular Malaysia except in Kuala Pontian and Nenasi. The Kuala Pontian mussels were obtained from a mussel aquaculture site while the Nenasi population was collected from a nearby lighthouse.

In conclusion, the findings from this study confirmed that the local populations of *P. viridis* in peninsular Malaysia are genetically similar enough to be used as a biomonitoring agent for heavy metal contamination in the seas which surround peninsular Malaysia since they are of the same species and with only moderate differentiation among regional populations.

Appendix

Table 1. Primer sequences of 19 polymorphic microsatellite loci that were used to characterize the 10 *P. viridis* populations in peninsular Malaysia and their specific annealing temperature (T_a) of PCR amplification.

Locus	Primer sequence 5′ to 3′	T_a	Expected size (bp)	GenBank accession no.
BP2-35-2	F: CTC TTT CAT CTT TCA CCT C R: CGT CAG GTA CTC CAT ATC C	40	222	DQ010059
BP2-49-1	F: GGT ACT TTT CTC ACT TCA CA R: GGA GTG AAC CTC TTC GAC	44	229	AY850129
BP2-49-2	F: GTT AAA CAA CCA ACC AAC G R: GTC TTT TTG TCA TTG CAC AC	44	215	AY850129
BP9-7-1	F: GTA TAT CAG AGA GAG AGA G R: AGG AAC TGA ACA CTG TTT G	40	299	DQ112051
BP9-13-2	F: CTC CCT ACT AAT GAG GAC AT R: TTC TAT GTG AGA GAG AGA G	40	263	DQ112055
BP9-16-2	F: GGC AAC ATT AGA AGT TCT GT R: TTG TAT ACC AGA GAG AGA G	40	213	DQ112058
BP9-19-2	F: CTC CCT ACT AAT GAG GAC AT R: TTC TAT GTG AGA GAG AGA G	40	263	DQ112060
BP9-27-1	F: GTA TGT CAG AGA GAG AGA G R: CAC CCA TAG AGT ATG TCA TT	40	268	DQ112066
BP10-5-1	F: GGT AGG TTC TCT CTC TCT CTC R: TTT CAG TAT TCA GGG CAC TT	48	233	DQ112034
BP10-16-1	F: TGT GTG TTC TCT CTC TCT C R: CTG TCT TTG CTA GTT CCT C	40	207	DQ112044
BP10-17-2	F: ATA CAC TGG GCT ATT CTC TT R: TAT TCT CTC TCT CTC TCT C	40	199	DQ112045
BP14-7-1	F: TGA GGC GAT AGA TAG ATA G R: GAT CAA CTG TTA AGC GAT AG	45	169	AY254777
LR1-58-1	F: ACT GAC TGA TGA GGA AAT GG R: TGT AGC GGC TCT CTC TCT C	48	202	DQ010097
VJ1-9-1	F: TGC GTG TGG AGG CTC TCT R: TCA CCT CTT GGT TGA GGA CA	40	205	DQ010072
VJ1-12-2	F: ATA GGA TAG AGT CAC GTT AG R: TAA GAC CTC TCT CTC TCT C	41	201	AY850124
VJ1-15-1	F: GGT TGA GAG CCT CTC TCT CT R: AGG AGA ATC CTG CTC TCT TC	42	220	DQ010077
VJ1-18-1	F: GTA GCG GCT CTC TCT CTC T R: GCG TGA CAC TCT TTT TCT TT	55	258	AY850126
VJ1-21-2	F: CTA GTA GAA GCT CTC TCT CTC R: GAA GTT TTG CTC ACT CAT CT	40	224	DQ010081
VJ1-22-2	F: AGA CGG AAT GCA GTA AGA AG R: CAT AAG CAG AAT TCC CAG AG	51	198	DQ010082

Table 2. Estimates of genetic variability in the 10 *P. viridis* populations.

Locus	Parameter	Pulau Aman	Tanjung Rhu	Bagan Tiang	Pulau Ketam	Muar	Parit Jawa	Pantai Lido	Kampung Puteh	Pasir Kuala Pontian	Nenasi
BP2-35-2	N_O (N_E)	2 (1.98)	3 (1.65)	3 (1.31)	3 (1.56)	3 (1.23)	2 (1.22)	2 (1.63)	4 (2.19)	3 (1.59)	3 (1.74)
	H_o	0.00	0.10	0.05	0.05	0.10	0.00	0.00	0.11	0.05	0.05
	H_e	0.53	0.41	0.24	0.37	0.19	0.18	0.40	0.56	0.38	0.44
	χ^2	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
BP2-49-1	N_O (N_E)	2 (1.11)	3 (1.33)	2 (1.12)	2 (1.32)	3 (1.29)	2 (1.06)	3 (1.63)	4 (1.43)	3 (1.61)	3 (1.17)
	H_o	0.20	0.15	0.15	0.17	0.06	0.15	0.20	0.26	0.05	0.18
	H_e	0.19	0.14	0.14	0.16	0.06	0.14	0.18	0.23	0.05	0.17
	χ^2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BP2-49-2	N_O (N_E)	2 (1.11)	2 (1.47)	2 (1.17)	3 (1.46)	3 (1.35)	2 (1.60)	2 (1.31)	2 (1.16)	2 (1.34)	2 (1.64)
	H_o	0.10	0.40	0.16	0.16	0.30	0.50	0.17	0.15	0.30	0.53
	H_e	0.10	0.33	0.15	0.32	0.27	0.38	0.25	0.14	0.26	0.40
	χ^2	NS	NS	NS	(−)	NS	NS	NS	NS	NS	NS
BP9-7-1	N_O (N_E)	2 (1.22)	2 (1.11)	2 (1.11)	2 (1.22)	2 (1.05)	2 (1.05)	2 (1.12)	2 (1.05)	2 (1.11)	2 (1.11)
	H_o	0.20	0.11	0.11	0.20	0.05	0.05	0.11	0.05	0.10	0.11
	H_e	0.19	0.10	0.10	0.18	0.05	0.05	0.11	0.05	0.10	0.10
	χ^2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BP9-13-2	N_O (N_E)	2 (1.11)	1 (1.00)	1 (1.00)	1 (1.00)	1 (1.00)	2 (1.41)	1 (1.00)	1 (1.00)	1 (1.00)	2 (1.16)
	H_o	0.10	0.00	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.15
	H_e	0.10	0.00	0.00	0.00	0.00	0.30	0.00	0.00	0.00	0.14
	χ^2	NS	Homo	Homo	Homo	Homo	NS	Homo	Homo	Homo	NS
BP9-16-2	N_O (N_E)	2 (1.38)	2 (1.34)	2 (1.54)	2 (1.41)	2 (1.28)	2 (1.66)	2 (1.43)	2 (1.41)	2 (1.54)	2 (1.57)
	H_o	0.33	0.30	0.45	0.35	0.25	0.55	0.37	0.35	0.45	0.47
	He	0.29	0.26	0.36	0.30	0.22	0.41	0.31	0.30	0.36	0.37
	χ^2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BP9-19-2	N_O (N_E)	2 (1.11)	1 (1.00)	1 (1.00)	2 (1.11)	2 (1.11)	1 (1.00)	1 (1.00)	2 (1.05)	2 (1.12)	1 (1.00)
	H_o	0.10	0.00	0.00	0.11	0.11	0.00	0.00	0.05	0.11	0.00
	H_e	0.10	0.00	0.00	0.10	0.10	0.00	0.00	0.05	0.11	0.00
	χ^2	NS	Homo	Homo	NS	NS	Homo	Homo	NS	NS	Homo
BP9-27-1	N_O (N_E)	1 (1.00)	2 (1.47)	2 (1.47)	2 (1.28)	2 (1.17)	3 (1.23)	2 (1.05)	2 (1.28)	2 (1.33)	2 (1.11)
	H_o	0.00	0.40	0.40	0.25	0.16	0.20	0.05	0.25	0.29	0.10
	H_e	0.00	0.33	0.33	0.22	0.15	0.19	0.05	0.22	0.26	0.10
	χ^2	Homo	NS	NS	NS	NS	NS	NS	NS	NS	NS
BP10-5-1	N_O (N_E)	2 (1.22)	2 (1.16)	2 (1.16)	2 (1.18)	2 (1.06)	2 (1.16)	2 (1.22)	2 (1.30)	2 (1.05)	2 (1.19)
	H_o	0.20	0.15	0.15	0.17	0.06	0.15	0.20	0.26	0.05	0.18
	H_e	0.19	0.14	0.14	0.16	0.06	0.14	0.18	0.23	0.05	0.17
	χ^2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BP10-16-1	N_O (N_E)	1 (1.00)	2 (1.05)	2 (1.05)	2 (1.05)	1 (1.00)	2 (1.05)	1 (1.00)	2 (1.16)	1 (1.00)	2 (1.05)
	H_o	0.00	0.05	0.05	0.05	0.00	0.05	0.00	0.15	0.00	0.50
	H_e	0.00	0.05	0.05	0.05	0.00	0.05	0.00	0.14	0.00	0.50
	χ^2	Homo	NS	NS	NS	Homo	NS	Homo	NS	Homo	NS
BP10-17-2	N_O (N_E)	3 (1.78)	3 (1.60)	4 (2.09)	3 (1.83)	4 (2.67)	4 (2.96)	4 (2.67)	4 (2.61)	4 (2.25)	4 (1.95)
	H_o	0.56	0.47	0.37	0.60	0.63	0.53	0.42	0.30	0.65	0.26
	H_e	0.46	0.38	0.54	0.47	0.64	0.68	0.64	0.63	0.57	0.50
	χ^2	NS	NS	(−)	NS	(−)	(−)	(−)	(−)	(+)	(−)
BP14-7-1	N_O (N_E)	5 (3.13)	5 (2.97)	5 (3.64)	4 (3.56)	7 (4.88)	3 (1.75)	5 (3.50)	4 (1.61)	1 (1.00)	1 (1.00)
	H_o	0.20	0.20	0.00	0.05	0.25	0.05	0.11	0.05	0.00	0.00
	H_e	0.72	0.68	0.75	0.74	0.82	0.44	0.73	0.39	0.00	0.00
	χ^2	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	Homo	Homo
LR1-58-1	N_O (N_E)	2 (1.22)	4 (1.46)	3 (1.89)	3 (2.27)	3 (1.87)	3 (1.42)	4 (2.06)	3 (2.15)	3 (2.68)	2 (1.95)
	H_o	0.20	0.11	0.16	0.40	0.30	0.05	0.16	0.10	0.35	0.00

Table 2 *(contd.)*

	H_e	0.19	0.32	0.48	0.57	0.48	0.30	0.53	0.55	0.64	0.50
	χ^2	NS	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
VJ1-9-1	N_O (N_E)	2 (1.67)	2 (1.50)	2 (1.25)	2 (1.70)	2 (1.78)	2 (1.25)	2 (1.17)	2 (1.96)	2 (1.22)	2 (1.63)
	H_o	0.56	0.42	0.22	0.58	0.65	0.22	0.59	0.85	0.20	0.53
	H_e	0.42	0.34	0.20	0.42	0.45	0.20	0.43	0.50	0.18	0.40
	χ^2	NS	NS	NS	NS	(+)	NS	NS	(+)	NS	NS
VJ1-12-2	N_O (N_E)	2 (1.34)	2 (1.17)	2 (1.18)	2 (1.34)	2 (1.05)	2 (1.16)	2 (1.25)	2 (1.11)	2 (1.22)	3 (1.24)
	H_o	0.10	0.16	0.17	0.30	0.05	0.05	0.11	0.11	0.20	0.21
	H_e	0.27	0.15	0.16	0.26	0.05	0.14	0.20	0.10	0.18	0.20
	χ^2	(−)	NS	NS	NS	NS	(−)	(−)	NS	NS	NS
VJ1-15-1	N_O (N_E)	1 (1.00)	2 (1.49)	2 (1.05)	2 (1.11)	1 (1.00)	2 (1.05)	1 (1.00)	1 (1.00)	1 (1.00)	1 (1.00)
	H_o	0.00	0.41	0.05	0.00	0.00	0.05	0.00	0.00	0.00	0.00
	H_e	0.00	0.34	0.05	0.10	0.00	0.05	0.00	0.00	0.00	0.00
	χ^2	Homo	NS	NS	(−)	Homo	NS	Homo	Homo	Homo	Homo
VJ1-18-1	N_O (N_E)	1 (1.00)	1 (1.00)	4 (3.38)	4 (3.80)	3 (2.79)	2 (1.96)	3 (1.83)	3 (2.10)	2 (1.96)	3 (2.04)
	H_o	0.00	0.00	0.80	0.30	0.45	0.75	0.59	0.44	0.75	0.50
	H_e	0.00	0.00	0.72	0.76	0.66	0.50	0.47	0.54	0.50	0.52
	χ^2	Homo	Homo	(+)	(−)	(−)	(+)	NS	(−)	(+)	NS
VJ1-21-2	N_O (N_E)	1 (1.00)	2 (1.05)	2 (1.11)	2 (1.05)	2 (1.11)	1 (1.00)	2 (1.30)	2 (1.05)	2 (1.11)	2 (1.23)
	H_o	0.00	0.05	0.11	0.05	0.10	0.00	0.26	0.05	0.10	0.21
	H_e	0.00	0.05	0.10	0.05	0.10	0.00	0.23	0.05	0.10	0.19
	χ^2	Homo	NS	NS	NS	NS	Homo	NS	NS	NS	NS
VJ1-22-2	N_O (N_E)	1 (1.00)	2 (1.06)	2 (1.05)	2 (1.05)	2 (1.16)	2 (1.18)	1 (1.00)	2 (1.12)	2 (1.05)	2 (1.06)
	H_o	0.00	0.06	0.05	0.05	0.15	0.17	0.00	0.12	0.05	0.06
	H_e	0.00	0.06	0.05	0.05	0.14	0.16	0.00	0.11	0.05	0.06
	χ^2	Homo	NS	NS	NS	NS	NS	Homo	NS	NS	NS
Mean	N_O (N_E)	1.9 (1.33)	2.3 (1.36)	2.4 (1.50)	2.4 (1.59)	2.5 (1.57)	2.2 (1.38)	2.2 (1.51)	2.4 (1.46)	2.1 (1.38)	2.2 (1.36)
	H_o	0.14	0.19	0.17	0.21	0.20	0.20	0.18	0.20	0.20	0.19
	H_e	0.19	0.22	0.24	0.28	0.24	0.22	0.26	0.26	0.22	0.23

N_O, observed number of alleles; N_E, effective number of alleles (Kimura and Crow 1964); H_o, observed heterozygosity; H_e, expected heterozygosity; χ^2, chi-square tests for deviation from Hardy–Weinberg equilibrium (significant at $P < 0.05$); Homo, homozygous; NS, not significant; (+), significant excess of observed heterozygosity; (−), significant deficiency of observed heterozygosity.

Acknowledgements

This work was funded by IRPA grant 09-02-04-EA001 and eScience fund grant 05-01-04-SF0147 from the Ministry of Science, Technology and Innovation Malaysia.

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Received 2 September 2008, in final revised form 4 December 2008; accepted 28 January 2009
Published on the Web: 18 June 2009