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RESEARCH PAPER

Genetic Diversity and Population Structure of the Asian Green Mussel (Pernaviridis) in the Waters of Sabah, Malaysia Based on Mitochondrial DNA D-Loop Sequences

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Abstract

The Asian green mussel (*Pernaviridis*) is a bivalve species with a high economic value. The waters surrounding the Malaysian state of Sabah hosts a wide range of geographically distributed populations of *P. viridis* whose population structure is unknown. This study was conducted to elucidate the genetic diversity and population structure of Asian green mussel from six sites located along the coastline of Sabah based on the mitochondrial DNA control region (D-loop). The complete D-loop sequences of 197 individuals were recovered by amplification using PCR, followed by DNA sequencing. Interpretation of the results revealed that the *P. viridis* populations exhibited high haplotype diversity (H_d=0.912±0.0002) and low nucleotide diversity (π=0.00890±0.00066). The AMOVA analysis showed Φ_{ST} was 0.02322 (P value<0.05), which is indicative of low but significant structuring. PairwiseΦ_{ST} ranged from low to moderate indicating population differentiation. Five out of the fifteen population pairs showed significant population differentiation. Both AMOVA and pairwise Φ_{ST} suggest there were significant population subdivisions in Asian green mussel population in Sabah. Although the Asian green mussel population in Sabah has gone through a severe mass mortality, evidence for genetic bottleneck was not detected. However, a smaller population size after a severe demographic reduction can cause the population become vulnerable. Thus, regular monitoring of Asian green mussel population is required to keep track of fluctuations in population size and composition. This study provided new population genetic information which is crucial for establishing fisheries management strategies for this species.

Keywords: *Pernaviridis*, D-loop, control region, mitochondrial DNA, genetic diversity, high haplotype diversity, low nucleotide diversity, population structure, demographic history, Sabah, Malaysia.

Introduction

The Asian Asian green mussel (*Pernaviridis*) is a type of marine bivalve mollusk under the family Mytilidae. The genus *Perna* consists of three extant species, *Pernaperna*, *Pernaviridis* and *Perna canaliculus* (Siddall, 1980). Some researcher shave also included *Pernaindica* and *Pernapicta* under genus *Perna*. However, both of them have been synonymized with *Pernaperna* (Siddall, 1980; Vakily, 1989; Wood, Apte, Macvoy& Gardner, 2007).

The Asian green mussel has two hinged shells, which are connected with a posterior adductor muscle (Gosling, 2015). The native habitat of the Asian green mussel is in the Indo-Pacific region, which encompasses regions between Japan to New Guinea and from Persian Gulf to South Pacific Islands (FAO, 2013). Asian green mussel generally inhabits marine intertidal, subtidal and estuarine environments, which have high salinity and receive more nutrients from land run-off (Rajagopal,Venugopalan, van der Velde, & Jenner 2006).Asian green mussel is able to tolerate a wide range of salinities and temperatures (Sivalingam, 1977). It is a dioecious organism which means the male and female reproductive organs are in separate individuals. *P. viridis* employs external fertilization whereby males and females Asian green mussels release gametes directly into the water during spawning. The Asian green mussel adult is sessile throughout its life whereas the larvae of Asian green mussel have long pelagic duration, which last around 21 to 35 days after fertilization (Laxmilatha *et al*., 2011).

Asian green mussel is a popular type of seafood, especially in China, Philippines and Malaysia (FAO, 2013). Asian green mussel farming is considered one of the potential shellfish aquaculture in Malaysia. An experimental culture of Asian green mussel was initiated by the Fisheries Research Institute of Malaysia in Penang in 1977 (Mazuki, 1998). The aquaculture of this species has advanced since its first introduction; however, the production of Asian green

mussel in Malaysia lags behind as compared to countries such as Thailand and Philippines (FAO, 2013).There is a need to formulate fisheries management strategies for improving the aquaculture of the Asian green mussel aquaculture, but this is limited by the lack of knowledge with reference to the genetic diversity of the species. Therefore, there is a need to understand the population genetic of Asian green mussel in Sabah.

Genetic diversity and population structure of aquatic animals can be examined using a variety of molecular markers. The characteristics of mtDNA which include maternal inheritance and the absence of genetic recombination makesit the most popular molecular marker (Galtier, Nabholz, Glémin, & Hurst, 2009). Mitochondrial DNA is a super-coiled circular double stranded DNA located outside the of cell nucleus (Freeland, Kirk & Petersen, 2011). The control region of mtDNA, which also is known as D-loop region has the highest mutation rate (Wan, Wu, Fujihara, & Fang, 2004). This characteristic makes D-loop region a good marker for investigating genetic diversity in closely related species or within a species (Rosel, Haygood, & Perrin, 1995).

Several population genetic studies have been conducted on Asian green mussel populations in Thailand, India, Malaysia, China and Singapore (Yap, Tan, Ismail, & Omar, 2002; 2004; Ong, Yusoff, Yap, & Tan, 2009; Divya, Thomas, Gopalakrishnan, Sathianandan, &Paulton, 2012; Lin, Loong, & Gen, 2012; Yap, Cheng, Ong & Tan, 2013; Gilg, Johnson, Gobin, Bright, & Ortolaza, 2013; Ibrahim, 2014; Ye, Li, & Wu, 2015). As far as Malaysian Borneo is concerned, a preliminary study was conducted in Santubong, Sarawak (Ibrahim, 2014) but no studies have been reported from Sabah. This investigation was directed towards elucidation of the genetic diversity of the population along the coastal waters of Sabah.

Materials and Methods

Sample Collection

A total of 197 Asian green mussels (*P. viridis*) were collected from six locations in the coastal waters of Sabah (Figure 1). Detailed information regarding the sampling location has been summarized in Table 1. The adductor muscle of each specimen was kept at -20˚C prior to DNA extraction.

DNA Extraction

Doubly Uniparental Inheritance (DUI) had been discovered in several mollusc species. The F-type mtDNA exists in the somatic tissue whereas the M-type mtDNA is present mainly in the males' gonadal tissue and sperm (Chiesa *et al*., 2011; Plazzi & Passamonti, 2010). A study conducted by Wei, Kong, Wu, and Yu (2009) reported on the absence of doubly uniparental inheritance in *P. viridis* and based on this report DNA was only extracted from the adductor muscle to avoid the possibility of M-type mtDNA extraction. Total genomic DNA was extracted from the Asian green mussel adductor muscle with DNeasy DNA extraction kit (Qiagen) according to manufacturer's instructions. Concentration and purity of DNA were verified using agarose gel electrophoresis and DNA Spectrophotometry (Pharmacia GeneQuant pro UV-Visible Spectrophotometer). The genomic DNA were diluted to 25ng/µL and stored at -20°C in 100µL AE buffer (Qiagen).

PCR Amplification and DNA Sequencing

The D-loop region of the mitochondrial DNA was amplified using forward primer 5'-GGGAGGCTATGGTGAGTCAA-3' reverse primer 5'-TGCCACATAAACTACCCTCATC-3'. The primers were designed using Primer3 (Koressaar & Remm, 2007; Untergrasser *et al*., 2012). Polymerase chain reactions (PCR) were carried out in volumes of 25μL with 1x GoTaq Flexi Buffer (Promega), 0.2mM of each dNTP (Promega), 1.5mM of $MgCl_2$ (Promega), 0.1μM of each primer, 1U of *Taq* DNA polymerase (Promega) and 50ng/μL of DNA.PCR was carried out using the Applied BiosystemsVeriti^{TM} thermal cycler under the following conditions: one cycle of 95°C for 3min, followed by 30 cycles at 95°C for 30s, 60°C for 30s and 72°C for 30s and with a final extension for 5 min at 72°C. PCR products were purified with PCRquick-spin^{TM}PCR Product Purification Kit (iNtRON) according to manufacturer's instructions. Each of the PCR products was cloned into *pGEM®-T Easy* cloning vector (Promega). Bidirectional DNA sequencing was conducted by AITBiotech Pt. Ltd (Singapore).The sequencing reactions for the DNA samples were carried out with BigDye Terminator v3.1 Cycle Sequencing kit and resolved using ABI 3730XL DNA analyzers (Applied Biosystems) sequencer.

Statistical Analysis

Forward and reverse D-loop sequences were assembled and further checked manually using the SeqMan software (DNASTAR package version 7.1.0, 2006). VecScreen (Altschul *et al*., 1997) in the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/ was used to eliminate vector contamination sites from the sequences. The flanking region encoding afragment of the COI gene (136 base pair) and NADH dehydrogenase subunit4 gene (35 base pair) were edited out from the consensus sequences. The DNA sequences were then aligned using the ClustalW in MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, &

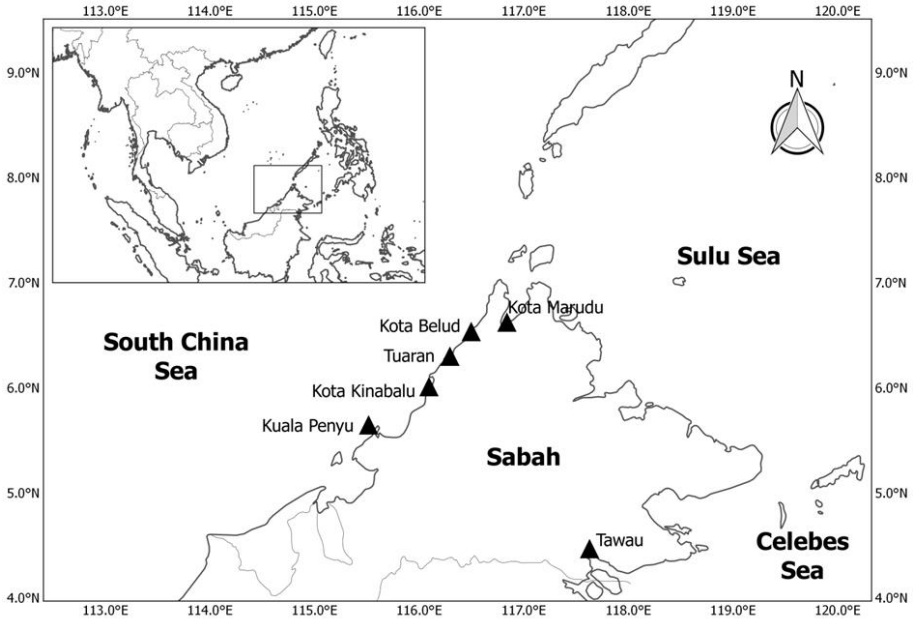

Figure 1. Map of Sabah, Malaysia with the six sampling locations (black triangles).

Table 1. Sampling details, genetic diversity indices and neutral tests of green mussel (*P. viridis*) populations in Sabah

Geographic Location	Collection Date	*N*	n	n_p	H_d (mean ± SD)	π (mean ± SD)	Tajima's D (P-value)	Fu's Fs (P-value)
Kota Belud	Sept 2015	35	15	10	0.881±0.002	0.00766±0.00156	-0.916(0.194)	-2.565(0.151)
Kota Kinabalu	Oct 2013	30	22	17	0.966±0.020	0.01216±0.00126	-0.861(0.203)	-5.786(0.062)
Kota Marudu	Sept 2013	32	21	16	0.919±0.040	0.01096±0.00156	-1.123(0.113)	-3.821(0.096)
Kuala Penyu	Sept 2014	34	12	8	0.779±0.064	0.00602±0.00166	-1.411(0.057)	-1.522(0.264)
Tawau	Jun 2014	35	21	16	0.919±0.034	0.00751±0.00131	-1.318(0.074)	-5.821(0.053)
Tuaran	April 2013	31	19	14	0.920±0.034	0.00839±0.00169	-1.214(0.105)	-4.948(0.078)
Total population	-	197	88	-	0.912±0.0002	0.00890±0.00066	-1.141(0.124)	-4.077(0.092)

Note: N, sample size; n, haplotype number; H_d, haplotype diversity and π, nucleotide diversity.

Kumar, 2013). The general genetic indices of mtDNA, including the haplotype number (*H*), haplotype diversity (H_d) and nucleotide diversity (π) were calculated using the DnaSP5.10.1 (Librado & Rozas, 2009).

The neighbor joining phylogenetic tree, maximum likelihood phylogenetic tree and maximum parsimony phylogenetic trees were constructed using the MEGA 6.0 to depict the genetic relationships among haplotypes. The statistical robustness of the nodes for all three phylogenetic trees was determined using bootstrap analysis with 1000 replicates. In addition, a haplotype network was created to depict the phylogenetic and geographical relationships of haplotypes using the median-joining method in PopART (Bandelt,Forster, &Röhl, 1999).

Pairwise Φ_{ST}, analysis of molecular variance (AMOVA) and Mantel test were used to examine Asian green mussel population structure using the Arlequin 3.5.2.1. The analysis of molecular variance (AMOVA) was conducted to quantify the partitioning of genetic variation present within population and among populations, and its significance was determined with 1000 permutations. Pairwise Φ_{ST}, an analog of F-statistic was also calculated to determine the genetic differentiation between sampling locations and the significance of pairwise Φ_{ST} values was determined with 100 permutations. Besides, Mantel test (Mantel, 1967) was used to determine if there was any significant correlation between pairwise Φ_{ST} and geographic distance. Geographic distance was referred to the shortest distance between two locations along the coast. Significance of the Mantel test was determined with 10000 permutations.

Demographic history of Asian green mussel populations was examined using the neutral tests including Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) incorporated in the Arlequin (Version 3.5.2.1). Both Tajima's D and Fu's F were used to determine whether the variation in DNA sequences deviate from the neutral model (Hamilton, 2009). Significance of the Tajima's D and Fu's F was determined with 1000

permutations.

Results

Sequence Variation and Genetic Diversity

The length of the D-loop fragment amplified of the mtDNA genome in the Asian green mussel in Sabah ranged from 657bp to 665bp.The actual size of the D-loop DNA fragment varied due to the presence of insertions and deletion (indels).All the DNA sequences were been deposited in the EMBL Nucleotide Sequence Database under accession numbers KP731638-KP731799 and KU555309-KU555343. The average base composition was as follows: *T*=30.4%, *C*=6.6%, *A*=33.8% and *G*=29.2%. The A/T base contents of the D-loop region were higher than the C/G base contents. This result was concordant with previous studies, which reported that the D-loop is an A-T rich region of the mitochondrial genome (Brown, Gadaleta, Pepe, Saccone,&Sbisà, 1986). In addition, 107 polymorphic sites were observed, including 73 singleton variable sites and 34 parsimony informative sites. Genetic diversity indices including haplotype number (n), haplotype diversity (H_d) and nucleotide diversity (π) are presented in Table 1.A total of 88 haplotypes were identified in the 197 samples. The number of haplotypes ranged from 15 in Kota Belud to 22 in Kota Kinabalu. The mean haplotype diversity (H_d) and nucleotide diversity (π) of the total population were 0.912±0.0002 and 0.00890±0.00066 respectively. The haplotype diversities (H_d) were very high, ranging from to 0.779±0.064 in Kuala Penyuto 0.966±0.020 in Kota Kinabalu. On the other hand, the nucleotide diversity (π) is very low, ranging from 0.00602±0.00166 in Kuala Penyu to 0.01216±0.00126 in Kota Kinabalu.

Phylogenetic and Network Analysis

The topologies produced from neighbor joining phylogenetic tree (Figure 2), maximum likelihood phylogenetic tree (Figure 3) and maximum parsimony phylogenetic tree (Figure 4) were similar. All three phylogenetic trees were divided into two haplogroups. One haplogroup contained most of the haplotypes and the second haplogroup contained another 18 haplotypes. The median joining network was used to further depict the phylogenetic and geographical relationships among haplotypes (Figure 3). Resultant network exhibited star-like patterns surrounding haplotype 2, haplotype 3 and haplotype 4. Haplotype 4 was considered the ancestral haplotype, which was the most common haplotype (53.4%) encompassing all six locations. Haplotype 3 (27.2%) encompassed all six locations whereas haplotype 2 (28.4%) encompassed all locations except Kota Kinabalu. The phylogenetic trees and median joining network provided some insight into the relationship among haplotypes of Asian green mussel in Sabah. Nevertheless, a clear spatial pattern was not able to be identified from phylogenetic trees or median joining network.

Population Structure Analysis

The AMOVA analysis based on haplotype frequencies revealed that 97.68% of the genetic variation occurred within populations while only 2.32% of the genetic variation occurred among populations (Table 2). The Φ_{ST} was 0.02322 ($P<0.05$), which indicate low but significant population structuring of the *P. viridis* populations in Sabah.

Population pairwise Φ_{ST} showed that the Φ_{ST} between Kuala Penyu and Tuaran was the highest 0.07048 whereas the Φ_{ST} between Kota Marudu and Tawau was the lowest, at -0.01311 (Table 3).Mantel test with correlation coefficient, r=-0.37, ($P>0.05$) showed there was no correlation between geographic distance and population pairwise Φ_{ST} between Asian green mussel populations

Neutral Tests

The results of Tajima's D and Fu's Fs neutral tests are presented in Table 1. Both Tajima's D and Fu's F test showed consistentnon significant negative D-value across populations, which indicate that, the demographic history of the D-loop region of *P. viridis* populations was in agreement with neutral expectation.

Discussion

Despite high haplotype diversity (H_d=0.912±0.0002) of the Asian green mussel populations in Sabah, its nucleotide diversity (π=0.00890±0.00066) was very low. High haplotype diversity but low nucleotide diversity of Asian green mussel population in Sabah could be attributed to the high mutational rate of the D-loop region (Wan, Wu, Fujihara, & Fang, 2004). The haplotype diversity of a relatively rapid evolving genome within a population often approach 1.0 as many individuals will tend to have unique haplotypes (Freeland, Kirk, & Petersen, 2011). Besides, low nucleotide diversity but high haplotype diversity may also be the indication of genetic bottleneck. Both haplotype and nucleotide diversities can be diminished in the event of genetic bottleneck (Frankham, Ballou, & Briscoe, 2004; Hamilton, 2009). High haplotype diversity and low nucleotide diversity can be observed in a population experiences rapid expansion from a low effective population size, assuming there is sufficient time for haplotype to increase through mutation but insufficient time for accumulation of large sequence differences. (Grant & Bowen, 1998; Lowe, Harris, & Ashton, 2004).A rapid population growth can enhance the retention of new mutations in the population (Grant & Bowen, 1998). High haplotype diversity and

low nucleotide diversity has been reported in many marine organisms such as *Cynoscionacoupa* (Rodrigues *et al*., 2008), *Architecuthis dux* (Winkelmann *et al*., 2013) and *Girellapunctata (Saito et al., 2008)* that have undergone a severe demographic reduction.

The population pairwise Φ_{ST} showed low to moderate population differentiation which ranged from -0.01311 to 0.07048. According to Wright (1978), fixation index,Φ_{ST} should range around 0.0 to 1.0. However, pairwise Φ_{ST} was negative between Tawau and Kota Marudu. Negative Φ_{ST} occurs when the genetic differentiation within population is higher than between populations, which indicate that gene

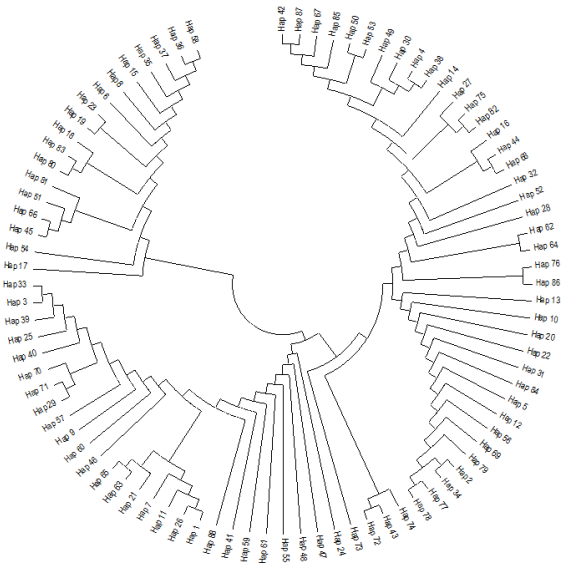

Figure 2. Neighbour-joining tree based on D-loop haplotypes of the green mussel (*P. viridis*). (Hap: Haplotype).

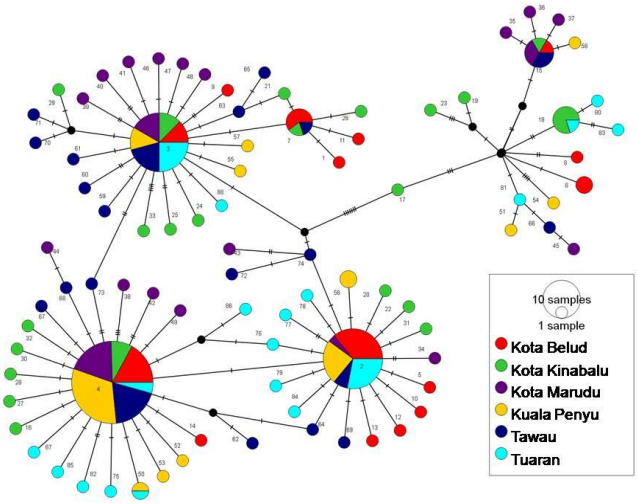

Figure 3. Median-joining network based on the D-loop haplotypes of the *P. viridis*. Circles represent different haplotypes with relative size proportionate to its observed frequency. The colors of the circles indicate the geographic region. The number labels represent haplotypes names. The hatch marks indicate the mutation steps between haplotypes and the black circles represent missing haplotypes.

Table 2. Analysis of molecular variance (AMOVA) for green mussel populations in Sabah based on D-loop region

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	F_{ST}	P value
Among populations	5	3.980	0.01063	2.32	0.02322	0.0098
Within populations	191	85.416	0.44720	97.68		
Total	196	89.396	0.45783			

Table 3. Population pairwise F_{ST} between green mussel populations in Sabah based on D-loop region

	KB	KK	KM	KP	TA	TU
KB	-					
KK	0.03591*	-				
KM	0.01994	0.00904	-			
KP	0.01931	0.06527*	0.01559	-		
TA	0.01121	0.00661	-0.01311	0.01695	-	
TU	0.01113	0.02563*	0.03550*	0.07048*	0.02437	-

*Significant values at P<0.05; Abbreviation: KB=Kota Belud; KK=Kota Kinabalu; KM=Kota Marudu; KP=Kuala Penyu; TA=Tawau; TU=Tuaran.

flow between the populations is very high (Bortolotto, Bucklin, Mezzavilla, Zane, &Patarnello, 2011; Roesti, Salzburger, & Berner, 2012). Most of the genetic variation of marine invertebrates is observed within rather than between populations (Burton, 1997). This is likely to be the result of the spawning behaviour of adult invertebrates and high dispersal ability of marine invertebrate larvae. The larvae of this species have very long pelagic duration, which is around 21 to 35 days (Laxmilatha *et al*., 2011). It is likely that the larvae of Asian green musseltend to drift away from their natal habitat and that this in turn increases the gene flow between populations and lowers the degree of differentiation between populations. This study detected significant genetic differentiation between KK-KB, KK-KP, KK-TU, KM-TU and KP-TU. The significant population differentiation could be attributed to oceanographic features or the current patterns that limit the gene flow between two sites. Oceanographic features such as oceanographic front can prevent two adjacent sites to exchange migrants (Gilg&Hilbish, 2003).The surface current around Sabah is remarkably complex. The dominant surface circulation is the Mindoro strait outflow which drives the sea current from South China Sea to Sulu Sea through Mindoro strait. The Mindoro strait flow outwards to the South China Sea through the Balabac Strait and out to Sulu Sea through the Sibutu Passage (Han *et al*., 2009). Nevertheless, we are not able to pinpoint the real reasons for the population subdivision in this study. Further studies will require identifying specific causative factors. The significant AMOVA and population differentiation indicated there was a significant subdivision among populations of Asian green mussels in Sabah. However, a clear spatial pattern could not be discerned. The significant subdivision among populations should be taken into consideration in the development and implementation of Asian green mussel aquaculture management strategies.

It is important to determine if the populations experienced bottleneck, as this would likely to lead to inbreeding depression, loss of genetic variation and deleterious allele fixation, any of which can ultimately compromise species' adaptive potential and the probability of population persistence (Frankham, Ballou, & Briscoe, 2004). The Asian green mussel populations in Sabah have been reported to have gone through a severe mass mortality from 2010 (Tan &Ransangan, 2015; Taib, Madin,&Ransangan, 2016).Studies carried out by researchers from several institutions of higher learning and fisheries department in Malaysia have not been able to determine the cause of the mass mortality. Although the green mussel experienced severe mass mortality for a long period of time, this study did not detect the evidence of genetic bottleneck in Asian green mussel populations. Under certain circumstances, a severe demographic bottleneck may not necessarily present itself as evidence in terms of a genetic signature. Several reasons could lead to this phenomenon. Firstly, effective population size that is large enough candiminish the bottleneck signature. Sastre *et al*. (2011) reported the mitochondrial DNA data can effectively detect bottleneck in the small isolated population but not in a large one. Secondly, small number of migrants can also diminish the effects of genetic bottleneck (Keller *et al*., 2001; Busch, Waser, & DeWoody, 2007). Despite the absence of evidence of a genetic bottleneck, the smaller population size after severe demographic reduction could cause Asian green mussel populations to become more sensitive to environmental perturbations and stochastic demographic processes. Thus, regular monitoring of Asian green mussel population is recommended in order to keep track of fluctuations in population size and composition.

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Conflict of Interest

The authors declare that they do not have any conflicting interests.

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