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Population Structure of the Blue Swimmer Crab *Portunus pelagicus* in Coastal Areas of Malaysia Inferred from Microsatellites

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Chuan Jian Chai, Yuzine Bin Esa, Muhammad Fadhil Syukri Ismail, and Mohd. Salleh Kamarudin (2017) *Portunus pelagicus*, distributed throughout the Indo-West Pacific region, is one of the large and edible species of blue swimmer crabs. Increasing demand for the frozen and canned crabmeat industry worldwide has now relied mainly on *P. pelagicus* which in turn generates splendid income for the fisherman communities. In the present study, the population genetic structure of *P. pelagicus* was examined using six pairs of microsatellite loci. A total of 87 crab samples were collected from five different coastal areas of Malaysia. Genomic DNA was extracted from each sample for polymerase chain reaction (PCR) amplification and fragment analysis. Four out of six microsatellite primers revealed polymorphic loci in *P. pelagicus* sampled. The number of alleles per locus in *P. pelagicus* ranged from 14 to 34. Microsatellites analyses indicated low levels of genetic differentiation among the *P. pelagicus* populations. The average observed heterozygosity (H_O = 0.48) obtained was lower than the standard heterozygosity found in most marine populations (H_O = 0.79). The high F_{IS} values (mean F_{IS} = 0.4756) and low F_{ST} values (mean F_{ST} = 0.0413) also suggested the existence of inbreeding among different populations of *P. pelagicus*. In conclusion, this study was able to shed light on the population structure of *P. pelagicus* in coastal areas of Malaysia.

Key words: *Portunus pelagicus*, Blue swimmer crabs, Population genetic structure, Microsatellites.

BACKGROUND

In Malaysia, the population genetic structure of the blue swimmer crabs, *Portunus pelagicus* has not been well-studied unlike countries such as Australia and Thailand (Yap et al. 2002; Klinbunga et al. 2007). However, the increasing demands of *P. pelagicus* in the fisheries industry of Malaysia currently have led to a growing interest on the broodstock of this particular crab species. The knowledge on the genetic differentiation of *P. pelagicus* is no doubt useful for the effective management of this edible crab species with wide distribution range and long planktonic larval stages (Klinbunga et al. 2007).

Microsatellites, also termed as short tandem repeats (STRs) or simple sequence repeats (SSRs) are tandemly repeating motifs of DNA (1-6 bases long) which are widely distributed throughout the nuclear genomes of eukaryotes (Putman and Carbone 2014; Senan et al. 2014). With high levels of allelic polymorphism and codominant inheritance, they have become the mainstay of population genetics, conservation management, parentage identification and fingerprinting (Nolan et al. 2000; Putman and Carbone 2014).

Lately, Yap et al. (2002) identified eight microsatellites in *P. pelagicus* (seven dinucleotides and one tetranucleotide). All eight microsatellite loci were polymorphic when inspected against

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the genomic DNA of *P. pelagicus* collected throughout Australia. In general, the mean observed heterozygosity (H_O) was not significantly different from the expected heterozygosity (H_E). To date, Sezmis (2004) investigated the population genetic structure of *P. pelagicus* from 16 diverse assemblages in Australia via six microsatellite loci. Large genetic distances between pairs of geographic samples indirectly reflected strong intraspecific genetic differentiation of *P. pelagicus*.

Recent studies have verified that microsatellites are more variable and informative than dominant markers like RAPD and AFLP (He et al. 2003; Senan et al. 2014). Bryars and Adams (1999) on the other hand reported that *P. pelagicus* shows relatively high polymorphisms when tested with allozymes. Nevertheless, microsatellite markers which exhibit higher levels of polymorphism than the allozyme loci are ideal for this research. Thus, the present study examined the population structure of *P. pelagicus* in coastal areas of Malaysia using microsatellites. The detailed information on the genetic diversity of *P. pelagicus* populations is necessary for the breeding programs of this exploited taxon.

MATERIALS AND METHODS

Sampling Location and Sample Collection

A total of 87 *Portunus pelagicus* samples were collected from selected sites on both east coast (South China Sea) and west coast (Strait of Malacca) of Peninsular Malaysia including Perak (Pantai Remis and Kuala Sepetang), Johor (Pendas), Negeri Sembilan (Port Dickson), and Terengganu (Besut) and Sarawak (Bako) of Borneo (Fig. 1). Samples were identified using taxonomic keys provided in Ng (1998) and Lai et al. (2010). The crab samples (muscle tissue from the chelipid manus or whole crab) were preserved in 95% ethanol. These samples were then stored at -20°C until further analyses.

DNA Extraction, Polymerase Chain Reaction Amplification (PCR), Agarose Gel Electrophoresis, DNA Screening and Fragment Analysis

Total genomic DNA extraction was performed using the DNeasy® Blood and Tissue Kit (QIAGEN). Alternatively, cetyl-trimethylammonium bromide (CTAB) method in the presence of proteinase K

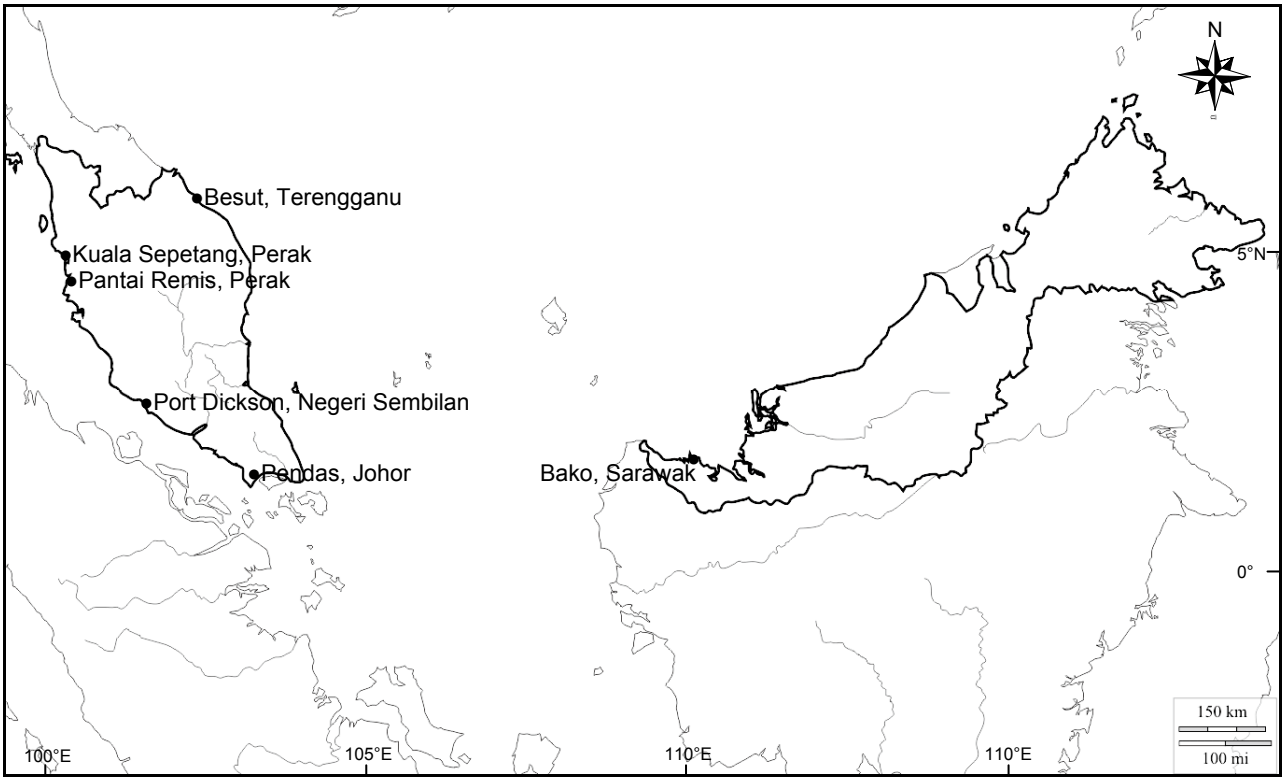

Fig. 1. Sampling locations of *Portunus pelagicus* in coastal areas of Malaysia.

was modified and applied (Grewe et al. 1993).

Microsatellite amplifications were carried out with six pairs of microsatellite loci (Table 1) developed by Yap et al. (2002) and Xu and Liu (2011). Approximately 1.0 μl of DNA template was amplified in a reaction mixture containing 7.5 μl of 5X MyTaq™ Red Mix (Bioline, USA) and 1.0 μl of each primer. The reaction mixture was then adjusted to a final volume of 15 μl with ddH_2O. The thermal cycling parameters included initial denaturation at 94°C for 2 minutes followed by 25 cycles of denaturation at 94°C for 20 seconds, primer annealing at T_a°C (Table 1) for 20 seconds, extension at 72°C for 40 seconds and a final extension at 72°C for 3 minutes. The completed amplification process hold at a routine 10°C. All amplifications were carried out with negative controls to check for contamination throughout the experiment.

The PCR products of the microsatellites were viewed under 2% high resolution agarose gel. A total of 1.2 grams of HR agarose powder (HydraGene, USA) was mixed with an exact amount of 60 ml of 1X TBE buffer (Promega, USA) to prepare the gel. BenchTop 50bp DNA ladder was used as a standard DNA size marker. PCR products with multiple fluorescence bands indicated the presence of DNA polymorphisms.

The samples were then subjected to microsatellite screening for estimation of the expected size of PCR products. Colourless MyTaq Red Mix and labelled primers with appropriate fluorescent dyes (FAM and HEX) were employed for PCR amplification at this stage. Also, loading dye was added into the PCR products for agarose gel electrophoresis.

All the PCR products were wrapped with aluminium foils and sent for fragment analysis through Applied Biosystems Genetic Analyzer. Fragment sizes were interpreted according to the 500-ROX DNA size standard using GeneMapper® version 5.0 (Chatterji and Pachter 2006). The results of fragment analysis obtained were then applied in the statistical analyses of microsatellites.

Statistical Analyses

The CONVERT 1.31 software (Glaubitz 2004) was used to translate the genotypic data into required formats for microsatellite analyses. These included the GENEPOP, ARLEQUIN and STRUCTURE formats. Allelic frequencies of the microsatellite loci found among the *Portunus pelagicus* populations were also computed through the CONVERT software. Besides, MicroChecker 2.2.3 software (Van Oosterhout et al. 2004) was utilised to check for genotyping errors, particularly due to null alleles and allele dropouts.

In addition, the exact tests for both Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium among pairs of loci with 10,000 permutations were conducted via GENEPOP version 4.4 (Rousset 2008). The inbreeding coefficient (F_{IS}), allelic richness (A_R), observed (H_O) and expected heterozygosity (H_E) on the other hand were calculated through FSTAT version 2.9.3.2 (Goudet 1995). Furthermore, ARLEQUIN version 3.0 (Excoffier et al. 2005) was used to estimate the significance of spatial variation in genetic diversity of *P. pelagicus* populations implemented in AMOVA. The fixation index (F_{ST}) was also measured using the ARLEQUIN software

Table 1. Primer sequences of six microsatellite loci

Locus	Sequences	Repeat motif	T_a (°C)	Size range (bp)
pPp2	F: GTGACCAGTAGGCGACCGAG R: ACGACTGCTTGTACGACCTTCA	$(CA)_{16}$	59	69-141
pPp5	F: GCTACGACAGTCCAATAACAACGT R: GATAGACCGACCTCACCTCAAAA	$(AG)_{35}$	57	87-151
pPp9	F: GACTTGAGCGATGCTGAAAG R: ATGGATAGATGGAATGCAAAAT	$(TG)_{19}$	52	133-187
pPp10	F: CCTGTATTGTCATGTGTTTGATTTT R: CTACGACCAACTTTACCGCC	$(TG)_{34}$	52	91-155
Ptri1	F: ACGCGTCTGGTAGTCATC R: TGTTCCCAAAGTTAGCAG	$(TGC)_{12}$	57	367-454
Ptri2	F: CAATGGCGGGTATGGTA R: TAAATGAAGGAAGCTAAAGACAAA	(TC)28	52	257-359

(Source: Yap et al. 2002; Xu and Liu 2011).

to examine the genetic differentiation among *P. pelagicus* populations (Weir and Cockerham 1984).

The STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used to allocate each individual to their genetic groups (*K*). This was done by admixing the ancestry within individuals. Ten independent runs were normally achieved with a burn-in period of 10,000 iterations and 10,000 replications. The best value of *K* was selected through the ad hoc statistic recommended by Evanno et al. (2005) and the log posterior probability of the data for a given *K*, In Pr (X|*K*) proposed by Pritchard et al. (2000).

Moreover, assignment tests of each individuals were performed via GeneClass version 2.0 (Piry et al. 2004). The probability of an individual belonged to a specific population was identified. The Bayesian approach by Rannala and Mountain (1997) was then employed to determine the likelihood of inherited population with 10,000 simulations and threshold value of 0.05. Finally, the BOTTLENECK version 1.2.02 (Piry et al. 1999) was implemented to test for the occurrence of current bottlenecks in each population. The two phase mutation (TPM) model (Di Rienzo et al. 1994) was introduced with different percentages (60-80%) of the stepwise mutation model (SMM) and a variance of 12 in 5,000 replications. All bottleneck analyses were derived from the Wilcoxon sign rank test (Maudet et al. 2002).

RESULTS

Microsatellite Genotyping

Of the six microsatellite primer pairs selected, only five primer pairs were successfully amplified. Of these five microsatellite loci, one (Ptri1) was monomorphic while the others were polymorphic (pPp2, pPp9, pPp10, Ptri2) and yielded PCR bands (Fig. 2) consistently. Ptri1 and Ptri2 were chosen for the cross-species amplification of *Portunus pelagicus* as these two primers were initially designed for *P. tritubeculatus* populations in China. The sizes of all the microsatellite loci used ranged from 69-454 bp. Most of the microsatellite primers contained dinucleotide repeat units except for Ptri1 with trinucleotide repeats. However, both pPp5 and Ptri1 loci were excluded from the statistical analyses to avoid scoring errors. The pPp5 primer had no PCR product at temperature higher or lower than the annealing temperature.

Microsatellite Variations

The allele frequencies of four microsatellite loci obtained from five different populations of *Portunus pelgicus* across the coastal areas of Malaysia were depicted in table 2. The highest and lowest number of alleles were acquired by loci pPp2 and Ptri2 with an amount of 34 alleles and 14 alleles respectively. On the other hand, the mean

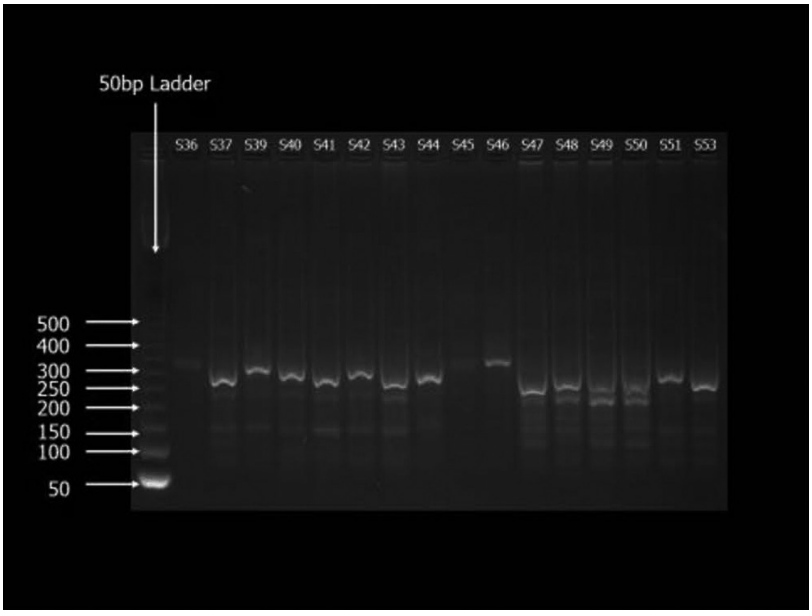

Fig. 2. Gel image of *Portunus pelagicus* samples obtained using microsatellite primers (Ptri2). S36-S37: Sample 36-Sample 37; S39-S51: Sample 39-Sample 51; S53: Sample 53.

Table 2. Allele frequencies of five *Portunus pelagicus* populations through four pairs of microsatellite loci

Locus	Allele	Size	Perak	Johor	Negeri Sembilan	Terengganu	Sarawak	Overall
pPp2	1	74	0.0000	0.0625	0.0000	0.0000	0.0000	0.0172
	2	76	0.0455	0.0000	0.0000	0.0000	0.0385	0.0115
	3	78	0.0000	0.0208	0.0000	0.0000	0.1538	0.0287
	4	80	0.0000	0.0000	0.0000	0.0000	0.3077	0.0460
	5	82	0.0000	0.0000	0.0357	0.0000	0.0000	0.0057
	6	84	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	7	86	0.0455	0.0833	0.1429	0.0800	0.0000	0.0747
	8	88	0.0000	0.0000	0.0357	0.0000	0.0000	0.0057
	9	90	0.0000	0.0000	0.0000	0.1000	0.0000	0.0287
	10	92	0.0000	0.0000	0.0000	0.0200	0.0000	0.0057
	11	94	0.0000	0.0208	0.0000	0.0400	0.0769	0.0287
	12	96	0.0909	0.0625	0.0000	0.0000	0.0000	0.0287
	13	98	0.0909	0.0208	0.1429	0.0000	0.0385	0.0460
	14	100	0.1364	0.0208	0.0357	0.1600	0.1538	0.0977
	15	102	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	16	104	0.0000	0.0417	0.0000	0.0400	0.0000	0.0230
	17	106	0.3182	0.0625	0.0000	0.1400	0.0000	0.0977
	18	108	0.0455	0.0417	0.0000	0.0000	0.0000	0.0172
	19	110	0.1818	0.0000	0.0000	0.1200	0.0000	0.0575
	20	112	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	21	114	0.0455	0.1042	0.0000	0.0200	0.0000	0.0402
	22	116	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	23	118	0.0000	0.0208	0.1429	0.0000	0.0769	0.0402
	24	120	0.0000	0.1875	0.0000	0.0000	0.0000	0.0517
	25	122	0.0000	0.0000	0.0000	0.0400	0.0000	0.0115
	26	124	0.0000	0.0625	0.1071	0.0400	0.0000	0.0460
	27	126	0.0000	0.0000	0.0714	0.1200	0.0000	0.0460
	28	128	0.0000	0.0000	0.1429	0.0000	0.0000	0.0230
	29	130	0.0000	0.0000	0.0714	0.0000	0.0000	0.0115
	30	132	0.0000	0.0208	0.0357	0.0000	0.0000	0.0115
	31	134	0.0000	0.0000	0.0000	0.0200	0.0385	0.0115
	32	136	0.0000	0.0000	0.0357	0.0200	0.0385	0.0172
	33	138	0.0000	0.0000	0.0000	0.0200	0.0000	0.0057
	34	140	0.0000	0.0000	0.0000	0.0200	0.0769	0.0172
pPp9	1	134	0.0000	0.2917	0.0357	0.0400	0.0769	0.1092
	2	136	0.0000	0.1250	0.0000	0.0800	0.1923	0.0862
	3	138	0.2727	0.0000	0.1071	0.1000	0.0000	0.0805
	4	140	0.0000	0.0208	0.0714	0.0000	0.0000	0.0172
	5	142	0.1364	0.0417	0.2857	0.2800	0.1538	0.1782
	6	144	0.0909	0.0000	0.0000	0.0000	0.0000	0.0115
	7	146	0.0455	0.0000	0.0357	0.0000	0.0000	0.0115
	8	148	0.0455	0.0000	0.0357	0.0000	0.0000	0.0115
	9	150	0.0000	0.0417	0.1429	0.0400	0.1923	0.0747
	10	152	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	11	154	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	12	156	0.2727	0.0625	0.0000	0.0800	0.0385	0.0805
	13	158	0.0000	0.0208	0.0000	0.0000	0.0000	0.0057
	14	160	0.0000	0.0000	0.1071	0.0000	0.1154	0.0345
	15	162	0.0909	0.0417	0.0357	0.0800	0.0769	0.0632
	16	164	0.0000	0.0208	0.0000	0.0400	0.0000	0.0172
	17	166	0.0000	0.0208	0.0000	0.0400	0.0000	0.0172
	18	168	0.0000	0.0833	0.0000	0.0800	0.0000	0.0460
	19	170	0.0000	0.0833	0.0357	0.0600	0.0769	0.0575

Table 2. (continued)

Locus	Allele	Size	Perak	Johor	Negeri Sembilan	Terengganu	Sarawak	Overall
	20	172	0.0000	0.0417	0.0357	0.0000	0.0000	0.0172
	21	174	0.0000	0.0208	0.0714	0.0400	0.0769	0.0402
	22	176	0.0000	0.0000	0.0000	0.0400	0.0000	0.0115
	23	178	0.0455	0.0000	0.0000	0.0000	0.0000	0.0057
pPp10	1	92	0.0000	0.0000	0.0000	0.0200	0.0000	0.0057
	2	94	0.0000	0.0000	0.0000	0.0200	0.0000	0.0057
	3	96	0.0000	0.0000	0.0000	0.0800	0.0000	0.0230
	4	98	0.0000	0.0208	0.0000	0.0000	0.0000	0.0057
	5	100	0.0000	0.0833	0.0714	0.0000	0.0000	0.0345
	6	102	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	7	104	0.0000	0.0417	0.0714	0.1200	0.0000	0.0575
	8	106	0.0000	0.0000	0.0357	0.0000	0.0000	0.0057
	9	108	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	10	110	0.0000	0.0625	0.0357	0.0000	0.0000	0.0230
	11	112	0.0000	0.0417	0.0000	0.0200	0.0000	0.0172
	12	114	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	13	116	0.0000	0.0000	0.0000	0.0200	0.0385	0.0115
	14	118	0.0000	0.0000	0.1429	0.0400	0.0000	0.0345
	15	120	0.0909	0.0000	0.0714	0.0400	0.0000	0.0345
	16	122	0.0455	0.0208	0.1071	0.1400	0.2692	0.1092
	17	124	0.0000	0.0000	0.0714	0.0400	0.0000	0.0230
	18	126	0.0455	0.0833	0.0000	0.0000	0.0000	0.0287
	19	128	0.3636	0.1250	0.0714	0.0000	0.0000	0.0920
	20	130	0.0000	0.1042	0.0000	0.0000	0.0385	0.0345
	21	132	0.0000	0.0417	0.0000	0.0000	0.0769	0.0230
	22	134	0.2273	0.0625	0.0357	0.1000	0.0769	0.0920
	23	136	0.0000	0.0417	0.1071	0.0000	0.0769	0.0402
	24	138	0.0000	0.0625	0.1429	0.0400	0.0385	0.0575
	25	140	0.0000	0.0417	0.0000	0.1800	0.0769	0.0747
	26	142	0.0455	0.0000	0.0000	0.0000	0.0000	0.0057
	27	144	0.0000	0.0000	0.0357	0.0200	0.0000	0.0115
	28	146	0.0909	0.0000	0.0000	0.0600	0.0000	0.0287
	29	148	0.0000	0.0417	0.0000	0.0000	0.0000	0.0115
	30	150	0.0000	0.0000	0.0000	0.0400	0.0385	0.0172
	31	152	0.0000	0.0000	0.0000	0.0000	0.0385	0.0057
	32	154	0.0909	0.0000	0.0000	0.0200	0.2308	0.0517
Ptri2	1	258	0.3182	0.1250	0.2143	0.3200	0.0769	0.2126
	2	260	0.0000	0.2917	0.0000	0.0200	0.0769	0.0977
	3	262	0.0455	0.1667	0.2857	0.1000	0.1923	0.1552
	4	264	0.0000	0.1250	0.0357	0.2800	0.2692	0.1609
	5	266	0.0909	0.0000	0.1071	0.0000	0.0769	0.0402
	6	268	0.0000	0.0000	0.0714	0.0000	0.0385	0.0172
	7	270	0.2273	0.0417	0.0000	0.0000	0.0000	0.0402
	8	272	0.0455	0.0000	0.0000	0.0000	0.0000	0.0057
	9	274	0.1818	0.0417	0.2143	0.1000	0.0000	0.0977
	10	276	0.0000	0.1250	0.0714	0.0200	0.1154	0.0690
	11	278	0.0000	0.0417	0.0000	0.1200	0.0769	0.0575
	12	280	0.0000	0.0417	0.0000	0.0200	0.0000	0.0172
	13	282	0.0000	0.0000	0.0000	0.0200	0.0000	0.0057
	14	284	0.0909	0.0000	0.0000	0.0000	0.0769	0.0230
Number of samples			11	24	14	25	13	87

allelic frequencies ranged from 0.0057 to 0.2126. *P. pelagicus* of the Terengganu and Negeri Sembilan populations displayed the highest and the lowest percentage of allele discrepancies, each with 12 and seven distinct allele sizes.

Hardy-Weinberg Equilibrium and Linkage Disequilibrium among *Portunus pelagicus* Populations

All four microsatellite loci assigned to characterise the HWE and linkage disequilibrium of *Portunus pelagicus* populations showed significant genetic differentiations (Table 3). The allele richness ranged from 6.643 (locus Ptri2 of the Terengganu population) to 13.662 (locus pPp10 of the Johor population). In addition, the observed heterozygosity (H_O) values over all loci extended from 0.182 (loci pPp10 and Ptri2 of the Perak population) to 1.000 (locus pPp2 of the Perak population). On the contrary, the highest and the lowest expected heterozygosity (H_E) was gleaned from locus pPp10 of the Johor population (0.951) and locus Ptri2 of the Terengganu population (0.799) respectively. In all *P. pelagicus* populations, the inbreeding coefficient (F_{IS}) values were significantly different from zero ($p < 0.05$). However, a negative F_{IS} value was detected for locus pPp2 of the Perak population, indicating a loss of heterozygosity in this particular population.

The informative contents illustrated that all four pairs of microsatellite loci applied were highly polymorphic. There was no linkage disequilibrium found within the microsatellite loci tested through the sequential Bonferroni correction (data not shown). Nevertheless, significant deviations from the HWE expectations in *P. pelagicus* populations except for locus Ptri2 of the Negeri Sembilan population reflected the occurrence of heterozygote deficiency. This phenomenon in turn

Table 3. Genetic diversity of *Portunus pelagicus* populations at four microsatellite loci

Locus	Perak	Johor	Negeri Sembilan	Terengganu	Sarawak
N	11	24	14	25	13
pPp2					
N_α	9	19	12	16	10
A_R	9.000	13.303	10.840	11.205	9.329
H_O	1.000	0.750	0.357	0.606	0.462
H_E	0.861	0.938	0.923	0.919	0.868
F_{IS}	-0.170	0.204	0.622	0.352	0.478
P	0.002	0.000	0.000	0.000	0.000
pPp9					
N_α	8	16	12	13	9
A_R	8.000	11.240	10.622	10.579	8.771
H_O	0.272	0.375	0.643	0.360	0.385
H_E	0.848	0.887	0.889	0.890	0.898
F_{IS}	0.689	0.583	0.284	0.601	0.582
P	0.000	0.000	0.000	0.000	0.000
pPp10					
N_α	8	18	13	17	11
A_R	8.000	13.662	11.931	11.756	10.157
H_O	0.182	0.542	0.500	0.560	0.462
H_E	0.848	0.951	0.939	0.922	0.878
F_{IS}	0.787	0.436	0.477	0.397	0.484
P	0.000	0.000	0.000	0.000	0.000
Ptri2					
N_α	7	9	7	9	9
A_R	7.000	7.787	6.700	6.643	8.752
H_O	0.182	0.208	0.786	0.160	0.769
H_E	0.831	0.851	0.833	0.799	0.880
F_{IS}	0.789	0.759	0.059	0.803	0.130
P	0.000	0.000	0.137	0.000	0.003

N: Sample size; N_α: Number of allele; A_R: Allele richness; H_O: Observed heterozygosity; H_E: Expected heterozygosity; F_{IS}: Inbreeding coefficient; *P*: *p*-value ($p < 0.05$).

was typically caused by the events of inbreeding and the presence of null alleles.

Genetic Differentiation among *Portunus pelagicus* Populations

Hierarchical results of AMOVA revealed that the individuals of *Portunus pelagicus* had a major contribution on the genetic variations of this species, with approximately 50% of total variance (Table 4). In contrast, only 4.13% of variance was accounted for the inter-population differentiations. Besides, the pairwise F_{ST} values unveiled significant genetic variations among the *P. pelagicus* populations (Table 5). The Sarawak population exhibited the highest degree of differentiations with the other populations, ranging from 0.04239 to 0.10237 ($p < 0.05$). The F_{ST} demonstrated between the Terengganu and Negeri Sembilan populations was relatively lower at 0.03569, signifying sufficient gene flow between these two populations.

Moreover, assignment tests of *P. pelagicus* disclosed that nearly all individuals were correctly assigned to their original populations (Table 6). The assignment scores ranged from 19.599-133.195, relating both the Perak and Johor populations. The Bayesian structure analysis on the other hand suggested that the most suitable *K* identified for *P. pelagicus* was *K* = 4 (Ln P(D) = -4265.5; Var[Ln P(D)] = 5096.5). The clustering roughly corresponded to geographic locations, with majority of individuals from Perak assigned to Cluster 1, Johor and Negeri Sembilan to Cluster 2, Terengganu to Cluster 3 and Sarawak to Cluster 4 (Fig. 3). Lastly, the bottleneck analyses elucidated that the Sarawak population involved in current population reduction (Table 7). The decrease

Table 4. Hierarchical analysis of molecular variance (AMOVA) in *Portunus pelagicus*

Source of variation	Sum of squares	Variance components	Percentage of variation
Among populations	21.069	0.07735	4.13
Among individuals within populations	217.500	0.85495	45.60
Within individuals	82.000	0.94253	50.27

Table 5. Estimation of F_{ST} among *Portunus pelagicus* populations via four microsatellite loci

Populations	Perak	Johor	Negeri Sembilan	Terengganu	Sarawak
Perak	-				
Johor	0.07921	-			
Negeri Sembilan	0.06698	0.05137	-		
Terengganu	0.05406	0.05358	0.03569*	-	
Sarawak	0.10237	0.05199	0.04808*	0.04239*	-

*$p < 0.05$.

Table 6. Assignment tests of *Portunus pelagicus* based on four microsatellite loci frequencies

Assigned population	CA (%)	Origin				
		Perak	Johor	Negeri Sembilan	Terengganu	Sarawak
Perak	100	19.599	76.998	68.729	68.432	77.209
Johor	100	133.195	42.384	124.708	128.956	121.687
Negeri Sembilan	100	89.446	89.228	27.875	84.894	83.774
Terengganu	100	113.494	117.821	109.239	37.891	105.700
Sarawak	100	89.364	77.645	75.213	72.793	24.665

CA: Correct assignment.

in population size of *P. pelagicus* from Sarawak was further evidenced by the infinite allele model (IAM) and the two phase model (TPM). However, the stepwise mutation model (SMM) strongly implied the absence of bottleneck incidents in all populations. In accordance, mode shift allele frequency distributions were perceived in all five *P. pelagicus* populations.

DISCUSSION

Genetic Differentiation of *Portunus pelagicus*

Four out of six microsatellite loci including pPp2, pPp9, pPp10 and Ptri2 showed favourable polymorphic results for microsatellite analyses. Of the four microsatellite loci applied, Ptri2 acted as a pair of primers for cross-species amplification of *Portunus pelagicus*. The positive polymorphic bands acquired were comparable to the results established in other cross-amplification studies of blue swimmer crabs. Yap et al. (2002) tested the microsatellite loci developed for *P. pelagicus* on an unidentified species, *Portunus* sp. in the northern Australia and recognized considerable levels of polymorphisms. Similar outcomes were also achieved by Xu and Liu (2011) where cross-species amplification of *P. trituberculatus* primers was carried out in two other portunid species, *P. sanguinolentus* and *P. pelagicus*.

Undeniably, the flanking regions of microsatellite sequences within related taxa were highly conservative (Scribner et al. 1996). Cross-species amplification was further enhanced when homologous loci in one species were successfully amplified in another species via a single primer pairs developed (Zardoya et al. 1996). Thus, the cross-amplification between *P. trituberculatus* and *P. pelagicus* loci in the current study was plausible. However, the cross-amplification of *P. pelagicus* using Ptri2 exhibited lower annealing temperature than the one reported by Xu and Liu (2011), from 57°C to 52°C. General assumption that cross-species amplification inclines to have lower annealing temperature than the amplification of the primitive species was practically supported (Zane et al. 2002; Esa et al. 2011). Besides, moderate allele frequencies were derived from this study through 87 samples and four microsatellite loci, ranging from 34 to 14 alleles. The allele frequencies obtained were further implemented in the exact probability tests. These tests are not biased by low allele frequencies, signifying low mutation rates and hence are suitable for microsatellite analyses (Raymond and Rousset 1995; Chakraborty et al. 1997).

The mean observed heterozygosity (H_O) at

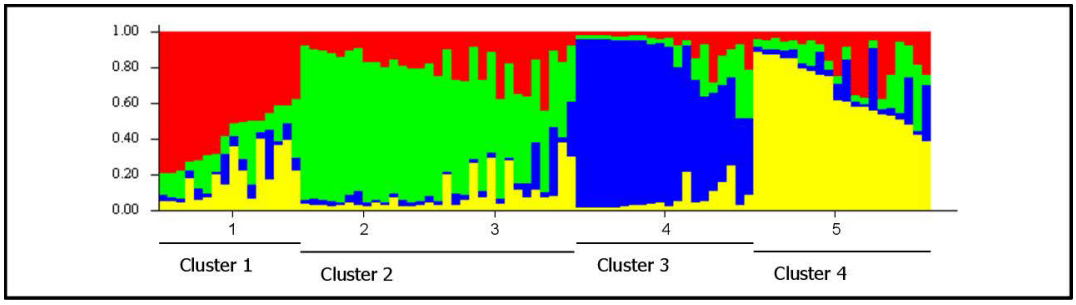

Fig. 3. Bar plot of *Portunus pelagicus* populations. 1: Perak; 2: Johor; 3: Negeri Sembilan; 4: Terengganu; 5: Sarawak.

Table 7. Current bottleneck evidences within populations of *Portunus pelagicus*

	I.A.M.	T.P.M.			S.M.M.	Mode shift
		60%	70%	80%		
Perak	0.562500	0.562500	0.843750	0.906250	0.906250	Y
Johor	0.437500	0.906250	0.906250	0.906250	0.968750	Y
Negeri Sembilan	0.093750	0.156250	0.156250	0.562500	0.906250	Y
Terengganu	0.156250	0.562500	0.562500	0.843750	0.906250	Y
Sarawak	0.031250*	0.031250*	0.031250*	0.031250*	0.562500	Y

I.A.M.: Infinite allele model; T.P.M.: Two phase model; S.M.M.: Stepwise mutation model; Y: Yes; N: No. *$p < 0.05$.

four microsatellite loci was 0.478 (data not shown). This value was much lower than the standard heterozygosity value (H_O = 0.79) identified in most marine populations (DeWoody and Avise 2000). Several factors that might lead to the heterozygote deficiencies in *Portunus pelagicus* populations examined have to be taken into account. Firstly, the Wahlund effect as a result of population subdivision and genetic drift (Wahlund 1928). Under the Wahlund principle, fewer heterozygotes than estimated were observed in a population. In other words, the frequency of homozygotes increased in a subdivided population (Balloux and Lugon-Moulin 2002; Kumar et al. 2006). Secondly, the restriction in genetic variation of *P. pelagicus* as a consequence of non-random mating behaviour or inbreeding (Brook et al. 2002).

In addition, sampling errors might contribute to low heterozygosity among the *P. pelagicus* populations. Small sample size and limited sampling locations studied did not fully represent the total heterozygosity of the exact population (Sezmis 2004). Moreover, the presence of null alleles caused excessive homozygotes in populations inspected (Esa et al. 2011; Xu and Liu 2011). This in turn affects the investigation of inbreeding coefficient (F_{IS}). According to Callen et al. (1993), null alleles are constantly reported at microsatellite loci. Hence, the application of higher polymorphic loci provide better resolution of population structure for *P. pelagicus*. Last but not least, overexploitation of *P. pelagicus* throughout the coastal area of Malaysia and anthropogenic disturbances such as water pollution diminished the effective breeding population size of this particular species (Esa et al. 2011). However, there is no precise record on the recent fishing volume of *P. pelagicus* available (Ikhwanuddin et al. 2014).

The outcomes of microsatellite analyses revealed the inconsistent patterns of genetic differentiation among *P. pelagicus* populations studied. Significant F_{IS} values in line with substantial HWE probability test values were obtained in all populations of *P. pelagicus* except for the Perak population. In this case, the potential loss of heterozygosity among *P. pelagicus* populations was apparent. The pairwise F_{ST} values on the other hand ranged from 0.03569 to 0.10237, indicating high levels of interactions among the populations analysed. Sezmis (2004) displayed similar results while trying to resolve the population structure of *P. pelagicus* in Australian waters. This was further evidenced by the assignment tests which showed high percentage of correctly assigned individuals, reflecting robust genetic divergence among populations.

Furthermore, the AMOVA results elucidated low inter-population variations (4.13%) among the *P. pelagicus* populations. This particular scenario might be influenced by high gene flow among the populations. Therefore, the dogma stating that long planktonic larval stages of *P. pelagicus* proposed high dispersal potential and extensive gene flow in this particular edible crab species seems to be supported by the current study (Klinbunga et al. 2007). Likewise, the bottleneck analyses exhibited a population reduction in the Sarawak population through the infinite allele model (IAM) and the two phase model (TPM). Nonetheless, this upshot was rejected by the stepwise mutation model (SMM). In the IAM, identical alleles share the same ancestry as homoplasy is inhibited (Kimura and Crow 1964). TPM on the other hand is developed to detect large proportion of mutation events (Di Rienzo et al. 1994). Unlike the other two models, SMM has a memory of allele sizes where alleles with distinct size differences will be distantly related (Kimura and Otha 1978). However, it is noteworthy that neither of these three models designed appears to ideally account for patterns of microsatellite variations (Balloux and Lugon-Moulin 2002).

CONCLUSIONS

To sum up, this research provided useful insights on the population structure of *Portunus pelagicus* throughout the coastal areas of Malaysia. Positive polymorphic results obtained from the cross-species amplification of *P. pelagicus* suggested that the microsatellite loci involved were highly informative. Accordingly, these loci were suitable for the evaluation of genetic differentiations among *P. pelagicus* populations. Nevertheless, low levels of microsatellite variations found among these populations implied that the sample size studied might be insufficient. Henceforth, microsatellite analyses on *P. pelagicus* ought to involve larger population size with multiple genetic markers in order to gain more reliable outcomes. Ultimately, the information on the population structuring of *P. pelagicus* is crucial for the future aquaculture planning of this edible crab species in Malaysia.

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REFERENCES

Balloux F, Lugon-Moulin N. 2002. The Estimation of Population Differentiation with Microsatellite Markers. Mol Ecol **11:**155-165.

Brook BW, Tonkyn DW, O'Grady JJ, Frankham R. 2002. Contribution of Inbreeding to Extinction Risk in Threatened Species. Conserv Ecol **6(1):**16.

Bryars SR, Adams M. 1999. An Allozyme Study of the Blue Swimmer Crab, *Portunus pelagicus* (Crustacea: Portunidae), in Australia: Stock Delineation in Southern Australia and Evidence for a Cryptic Species in Northern Waters. Mar Freshwater Res **50:**15-26.

Callen DF, Thompson AD, Shen Y, Philips HA, Richards RI, Mulley JC, Sutherland GR. 1993. Incidence and Origin of "Null" Alleles in the (AC)n Microsatellite Markers. Am J Hum Genet **52:**922-927.

Chakraborty R, Kimmel M, Stivers DN, Davison LJ, Deka R. 1997. Relative Mutation Rates at Dinucleotide, Trinucleotide and Tetranucleotide Microsatellite. Proc Natl Acad Sci USA **94:**1041-1046.

Chatterji S, Pachter L. 2006. Reference Based Annotation with GeneMapper. Genome Biol **7(4):**R29.

Dewoody JA, Avise JC. 2000. Microsatellite Variation in Marine, Freshwater and Anadromous Fishes Compared with Other Animals. J Fish Biol **56:**461-473.

Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, Freimer NB. 1994. Mutational Processes of Simple-sequence Repeat Loci in Human Populations. Proc Natl Acad Sci USA **91(8):**3166-3170.

Esa YB, Siraj SS, Rahim KAA, Daud SK, Ho GH, Tan SG, Syukri MF. 2011. Genetic Characterization of Two Mahseer Species (*Tor douronensis* and *Tor tambroides*) Using Microsatellite Markers from Other Cyprinids. Sains Malays **40(10):**1087-1095.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the Number of Clusters of Individuals using the Software Structure: A Stimulation Study. Mol Ecol **14:**2611-2620.

Excoffier L, Laval G, Schneider S. 2005. Arlequin ver. 3.0: An Integrated Software Package for Population Genetics Data Analysis. Evol Bioinform **1:**47-50.

Glaubitz JC. 2004. CONVERT: A User-friendly Program to Reformat Diploid Genotypic Data for Commonly Used Population Genetic Software Packages. Mol Ecol Notes **4:**309-310.

Goudet J. 1995. FSTAT version 1.2: A Computer Program to Calculate *F*-statistics. Heredity **86(6):**485-486.

Grewe PM, Krueger CC, Aquadro CF, Bermingham E, Kincaid HL, May B. 1993. Mitochondrial DNA Variation among Lake Trout (*Salvenilus namaycush*) Strains Stocked into Lake Ontario. Can J Fish Aquat Sci **50:**2397-2403.

He GH, Meng RH, Newman M, Gao GQ, Pittman RN, Prakash CS. 2003. Microsatellites as DNA Markers in Cultivated Peanut (*Arachis hypogaea* L.). BMC Plant Biol **3:**3.

Ikhwanuddin M, Liyana AN, Azra MN, Bachok Z, Abol-Munafi AB. 2014. Natural Diet of Blue Swimming Crab, *Portunus pelagicus* at Strait of Tebrau, Johor, Malaysia. Sains Malays **43(1):**37-44.

Kimura M, Crow JF. 1964. The Number of Alleles That Can Be Maintained in a Finite Populations. Genetics **49:**725-738.

Kimura M, Otha T. 1978. Stepwise Mutation Model and Distribution of Allelic Frequencies in a Finite Populations. Proc Natl Acad Sci USA **75:**2868-2872.

Klinbunga S, Khetpu K, Khamnamtong B, Menasveta P. 2007. Genetic Heterogeneity of the Blue Swimming Crab (*Portunus pelagicus*) in Thailand Determined by AFLP Analysis. Biochem Genet **45:**725-736.

Kumar S, Gupta J, Kumar N, Dikshit K, Navani N, Jain P, Nagarajan M. 2006. Genetic Variation and Relationships among Eight Indian Riverine Buffalo Breed. Mol Ecol **15:**593-600.

Lai JCY, Ng PKL, Davie PJF. 2010. A Revision of the *Portunus pelagicus* (Linnaeus, 1758) Species Complex (Crustacea: Brachyura: Portunidae) with the Recognition of Four Species. Raffles B Zool **58(2):**199-237.

Maudet C, Miller C, Bassano B, Breitenmoser-Wursten C, Gauthier D, Obexer-Ruff G, Michallet J, Taberlet P, Luikart G. 2002. Microsatellite DNA and Recent Statistical Methods in Wildlife Conservation Management: Applications in Alpine Ibex [*Capra ibex* (*ibex*)]. Mol Ecol **11(3):**421-436.

Ng PKL. 1998. Crabs. *In*: Carpenter KE, Niem VH (Eds) FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Pacific. Volume 2. Cephalopods, Crustaceans, Holothurians and Sharks. Rome: Food and Agriculture Organisation, pp. 1045-1155.

Nolan DV, Martin SAM, Kelly Y, Glennon K, Palmer R, Smith T, McCormack GP, Powell R. 2000. Development of Microsatellite PCR Typing Methodology for the Sea Louse, *Lepeophtheirus salmonis* (Kroyer). Aquac Res **31:**815-822.

Piry S, Alapetite A, Cornuet JM, Paetkau D, Baudouin L, Estoup A. 2004. GENECLASS2: A Software for Genetic Assignment and First-generation Migrant Detection. J Hered **95(6):**536-539.

Piry S, Luikart G, Cornuet JM. 1999. BOTTLENECK: A Computer Program for Detecting Recent Reductions in the Effective Population Size Using Allele Frequency Data. J Hered **90(4):**502-503.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of Population Structure Using Multilocus Genotype Data. Genetics **155(2):**945-959.

Putman AI, Carbone I. 2014. Challenges in Analysis and Interpretation of Microsatellite Data for Population Genetic Studies. Ecol Evol **4:**4399-4428.

Rannala B, Mountain JL. 1997. Detecting Immigration by Using Multilocus Genotypes. Proc Natl Acad Sci USA **94(17):**9197-9201.

Raymond M, Rousset F. 1995. GENEPOP: Population Genetic Software for Exact Test Ecumenicism. J Hered **86:**248-249.

Rousset F. 2008. Genepop'007: A Complete Reimplementation of the Genepop Software for Windows and Linux. Mol Ecol Resour **8:**103-106.

Scribner KT, Gust JR, Field RL. 1996. Isolation and Characterization of Novel Salmon Microsatellite Loci: Cross Species Amplification and Population Genetics Application. Can J Fish Aquat Sci **53:**833-841.

Senan S, Kizhakayil D, Sasikumar B, Sheeja TE. 2014. Methods for Development of Microsatellite Markers: An Overview. Not Sci Biol **6:**1-13.

Sezmis E. 2004. The Population Genetic Structure of *Portunus pelagicus* in Australian Waters. PhD dissertation, Murdoch University.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. 2004. MICRO-CHECKER: Software for Identifying and Correcting Genotyping Errors in Microsatellite Data. Mol Ecol Notes **4(3):**535-538.

Wahlund S. 1928. The Combination of Populations and the Appearance of Correlation Examined from the Standpoint of the Study of Heredity. J Hered **11:**65-106.

Weir BS, Cockerham CC. 1984. Estimating *F*-statistics for the Analysis of Population Structure. Evolution **38(6):**1358-1370.

Xu QH, Liu R. 2011. Development and Characterization of Microsatellite Markers for Genetic Analysis of the Swimming Crab, *Portunus trituberculatus*. Biochem Genet **49:**202-212.

Yap ES, Sezmis E, Chaplin JA, Potter IC, Spencer PBS. 2002. Isolation and Characterization of Microsatellite Loci in *Portunus pelagicus* (Crustacea: Portunidae). Mol Ecol Notes **2:**30-32.

Zane L, Bargelloni L, Patarnello T. 2002. Strategies for Microsatellite Isolation: A Review. Mol Ecol **11:**1-16.

Zardoya R, Vollmer DM, Craddock C, Streetman JT, Karl S, Meyer A. 1996. Evolutionary Conservation of Microsatellite Flanking Regions and Their Use in Resolving the Phylogeny of Cichlid Fishes (Pisces: Perciformes). Proc Roy Soc Lond B Bio **263:**1589-1598.