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RESEARCH PAPER

Genetic Differentiation of the Blue Swimming Crab *Portunus pelagicus* Along the Coastal Thai Waters Revealed by SSCP Analysis of *Cytochrome c Oxidase Subunit I*

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Abstract

The basic information on genetic diversity and population structure is essential for the construction of appropriate management schemes leading to sustainable fisheries of the blue swimming crab (*Portunus pelagicus*). Here, genetic heterogeneity of *P. pelagicus* (N=174) was examined by single-strand conformational polymorphism (SSCP) analysis of mitochondrial *cytochrome c oxidase subunit I* ($PpCOI_{270}$). Seven SSCP genotypes were found across all investigated samples. The average genetic distance between pairs of geographic samples was 0.0014-0.7247. Significant geographic heterogeneity ($P<0.05$) and restricted levels of female gene flow between paired samples (0.03-1.60 individuals per generation) were observed except between Chanthaburi - Prachuap Kriri Khan and Ranong - Krabi ($P>0.05$; 6.54 and 16.17 individuals per generation) located in the same coastal regions. Therefore, the gene pool of *P. pelagicus* in Thai waters was genetically differentiated to different stocks even though it is biologically regarded as a potential dispersal species. Five geographic samples of *P. pelagicus* in Thai waters could be differentiated to three genetic stocks; Chanthaburi and Suratthani (stock A), Prachuap Khiri Khan (stock B) and Ranong and Krabi (stock C).

Introduction

The blue swimming crab, *Portunus pelagicus* is one of the commercially important species distributed from the eastern Mediterranean to east Africa in the Indian Ocean, and to Japan and Tahiti in the western and southern Pacific Ocean (Edgar 1990; Kailola *et al*. 1993). The information on intraspecific genetic diversity and population differentiation is essential for the construction of broodstock management schemes of this commercially important species (Avise 1994; Carvalho and Hauser 1994; Chai *et al*. 2017).

Genetic diversity of *P. pelagicus* in Australian waters was first reported based on allozyme analysis (Bryars and Adams 1999). Subsequently, Yap *et al*. (2002) isolated and characterized eight microsatellites in *P. pelagicus* and genetic polymorphism was examined in crabs collected covering the western seaboard of Australia. The mean observed heterozygosity (H_o) at each locus was 0.30–0.78. All loci did not deviate from Hardy-Weinberg expectations except at pPp05 where homozygote excess was observed.

Sezmis (2004) further investigated population genetic structure of Australian *P. pelagicus* collected

from 16 different assemblages (*N*=4-57 per each sample site) throughout the Australia waters using 6 microsatellite loci (pPp02, pPp04, pPp08, pPp09, pPp18 and pPp19) and *cytochrome c oxidase subunit I* (*COI*, 342 bp) polymorphism. Significant differentiation of Australian *P. pelagicus* was found (F_{ST}=0.098 for microsatellites and ϑ_{ST}=0.375 for *COI*, respectively) with varying degrees of genetic heterogeneity.

Commercial aquaculture of *P. pelagicus* is successfully developed (Azra1 and Ikhwanuddin 2015). Accordingly, identification of genetic diversity and presently different crab stocks in Thai waters is essential as the first step for sustainable fisheries of *P. pelagicus*. This information is also important for the establishment of selective breeding programs and selection of appropriate broodstock leading to sustainable aquaculture of blue swimming crab in Thailand.

Genetic diversity of a small sample size of *P. pelagicus* in Thai waters (Ranong, Krabi, Chanthaburi, Prachuap Khiri Khan, and Suratthani; *N*=72) was reported based on amplified fragment length polymorphism (AFLP) (Klinbunga *et al*. 2007). The average genetic distance between samples across overall primers was 0.1151-0.2440. Geographic heterogeneity analysis indicated statistically significant differences between all pairs of samples suggesting strong population subdivisions of Thai *P. pelagicus* (pairwise ϑ_{ST}=0.2480–0.4910; P<0.01). In contrast, weak degrees of population subdivision were found in *P. pelagicus* in east and west coast areas of Peninsular Malaysia (mean F_{ST}=0.0413, *N*=87) based on polymorphism of 4 microsatellite loci. Significant population differentiation was observed between each of the east coast populations (Terengganu, Negeri Sembilan and Sarawak; P<0.05) but not between the west coast populations (Perak, and Johor; P>0.05) and among different coastal regions (P>0.05) (Chai *et al* 2017). Therefore, intraspecific population structure of *P. pelagicus* in Thai-Malaysian peninsula need to be confirmed using larger sample sizes.

Single-strand conformational polymorphism (SSCP) is convenient and cost-effective for examining DNA polymorphism. The major advantage of SSCP is that a large number of individual could be simultaneously genotyped (Khamnamtong *et al*. 2005) and variations according to one or a few substitutions could be detected (Orita *et al*. 1989). Therefore, SSCP is one of the potential techniques for detection of genetic polymorphism at different taxonomic levels.

In this study, genetic diversity and intraspecific population differentiation of a larger sample size of *P. pelagicus* in Thai waters (*N*=174) were determined using SSCP analysis of the mitochondrial *COI* gene segment. Genetic diversity within samples and differentiation between geographic samples were examined.

Materials and Methods

Sampling

The blue swimming crabs (*P. pelagicus*) were live-caught from Chanthaburi (12°35′60 N 102°9′0 E, *N*=29), Prachuap Khiri Khan (11°49′0 N 99°47′60 E, *N*=40) and Suratthani (9°7′60 N 99°19′0 E, *N*=35) located in the Gulf of Thailand and Ranong (9°58′0 N 98°37′60 E, *N*=35) and Krabi (8°4′0 N 98°55′0 E, *N*=35) located in the Andaman Sea (Figure 1). Taxonomic identification of specimens

Figure 1. Sampling collection sites of *P. pelagicus* in this study.

was performed following Lai *et al*. (2006). The whole specimens or muscle dissected out from the first pereiopod of each crab were kept at -30ºC until used.

DNA Extraction

Genomic DNA was extracted from the pereiopod muscle of each crab using a phenol-chloroform-proteinase K method (Klinbunga *et al*. 1996). Briefly, the muscle tissue (20-30 mg) was placed in a mortar containing liquid N_2 and ground to fine powder. The tissue powder was transferred to a centrifuge tube containing 500 µl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH 8.0) and briefly homogenized with a micropestle. SDS (10%) and RNase A (10 mg/ml) solutions were added to final concentrations of 1.0% (w/v) and 100 µg/ml, respectively. The resulting mixture was then incubated at 37ºC for 1 h. Subsequently, a proteinase K solution (10 mg/ml) was added to the final concentration of 300 µg/ml and further incubated at 55ºC for 3 h. An equal volume of buffer-equilibrated phenol was added and gently mixed for 15 min. The solution was centrifuged at 10,000 *g* for 10 min at room temperature. The upper aqueous phase was transferred to a new sterile microcentrifuge tube. This extraction process was repeated once with phenol and twice with chloroform: isoamyalcohlo (24:1). The aqueous phase was transferred into a sterile microcentrifuge. One-tenth volume of 3 M sodium acetated, pH 5.2 was added. DNA was precipitated by an addition of two volumes of prechilled absolute ethanol and mixed thoroughly. The mixture was incubated at -80ºC for 30 min. The precipitated DNA was washed twice with 1 ml of 70% ethanol (10 min each). After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 30-50 µl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0). The DNA solution was incubated at 37ºC for 1-2 h and kept at 4ºC for immediately used or stored at -20°C for long storage.

Amplification of *18S, 12S* and *16S Ribosomal (r) DNAs* and *NADH Dehydrogenase Subunit 5 (ND5), Cytochrome c Oxidase Subunits I and II (COI-COII)* and *COI*

Universal primers for amplification of *18S rDNA* (Klinbunga *et al*. 2003), *COI* (Folmer *et al*. 1994), *COI-COII* (Sihanuntavong *et al*. 1999), and *12S rDNA, 16S rDNA* and *ND5* (Small and Chapman 1997) were tested. The amplification reaction was performed in a reaction volume of 25 µl contained 75 mM Tris-HCl (pH 8.8 at 25ºC), 20 mM $(NH_4)_2SO_4$, 0.1 % Tween 20, 1.5-2.0 mM $MgCl_2$, 200 µM each dATP, dTTP, dCTP and dGTP, 0.25-0.5 µM each primer, 1 unit *Taq* DNA polymerase (Fermentus) and 50 ng genomic DNA. PCR was carried out using conditions described in Khamnamtong *et al*. (2005). Five microliters of the PCR product was size-fractionated through a 1% agarose gel to determine whether the amplification reaction was successful.

Cloning of the Amplified *COI* and *12S rDNA* Gene Segment

The amplified *COI* and *12S rDNA* **(**706 and 406 bp**)** fragments were reamplified with the original primers and size-fractionated through a 1.5% agarose gel and excised. The electrophoresed fragment was eluted from the gel using a Hiyield™ Gel Extraction Kit following the protocol recommended by the manufacturer (Real Genomics). The eluted DNA was ligated to pGEM-T easy vector (Promega) in a total volume of 10 µl containing 3 µl of the gel-eluted PCR product, 25 ng of pGEM®-T easy vector, 5 µl of 2X rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10 % PEG 8000) and 3 Weiss units of T4 DNA ligase. The ligation mixture incubated at 4°C for 16 h. One-tenth volume of each ligation was electrotransformed to *Eschericha coli* JM109 (Dower *et al*., 1988). Recombinant clones were selected by the *lacZ* system following standard protocols (Sambrook and Russell 2001). Briefly, the mixture was incubated on ice for 30 min. The cells were heat-shock for 45 s at 42°C in a water bath without shaking. The tube was returned immediately to ice for 5 min. The cells transformed with ligation reactions were transferred to the tube containing 1 ml of room temperature SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37°C for 90 min before centrifuged at 6,000*g* for 1 min at room temperature. The pellet was gently resuspended in 100 µl of SOC medium and spread on a LB agar plate (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1.0 % NaCl and 1.5% agar) containing 50 µg/ml of ampicillin, 25 µg/ml of IPTG and 20 µg/ml of X-gal. The spread agar plate was further incubated at 37°C overnight. The colonies containing inserted DNA are white while those without inserted DNA are blue.

Plasmid DNA Extraction and Sequencing of Recombinant Clones

Recombinant plasmids containing inserted DNA were extracted using a Plasmid Mini Kit (ATP Biotech Inc.). A recombinant clone was inoculated into 3 ml of LB broth (1% tryptone, 0.5% yeast extract, 1.0 % NaCl) containing 50 µg/ml of ampicillin and incubated at 37°C with constant shaking at 250 rpm overnight. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 12,000 *g* for 1 min. The supernatant was discarded. The bacterial cell pellet was collected and resuspended with 200 µl of the PD1 buffer containing RNaseA and thoroughly mixed by vortexed. The resuspended cells were lysed by the addition of 200 µl of the PD2 buffer and mixed gently by inverting the tube 10 times. The mixture was left for 2 min at room temperature. Subsequently, 300 µl of the buffer PD3

was added to neutralize the alkaline lysis step and mixed immediately by inverting the tube for 10 times. To separate the cell debris, the mixture was centrifuged at 12,000 *g* for 15 min. The supernatant was transferred into a collection tube containing the PD column and centrifuged at 6,000*g* for 1 min. The flow-through was discarded. The PD column was placed back in the collection tube. The column was washed by adding 400 µl of the W1 buffer and centrifuged at 6,000*g* for 1 min. After discarding the flow-through, 600 µl of the ethanol-added Wash buffer was added and centrifuged as above. The flow-through was discarded. The spin tube was centrifuge for an additional 2 min at 12,000 *g* to remove the residual Wash buffer. The dried PD column was placed in a new 1.5 ml microcentrifuge tube and 30-50 µl of the Elution buffer or water was added at the center of the column to elute the extracted plasmid DNA. The column was left at room temperature for 2 min and centrifuge at 12,000 *g* for 2 min. The concentration of extracted plasmid DNA was spectrophotometrically measured. The insert size of each recombinant plasmid was examined by digestion of the plasmid with *Eco* RI. The digest was carried out in a 15 µl containing 1X restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM $MgCl_2$), 1 µg of recombinant plasmid and 3 units of *Eco* RI. The reaction was incubated at 37°C for 3-4 h before analyzed by agarose gel electrophoresis. Nucleotide sequences of recombinant plasmids were examined in both directions by automated DNA sequencer using M13 forward and/or M13 reverse primer as the sequencing primer. Inserted sequences were compared with those previously deposited in GenBank using Blast*N* (Altschul *et al*. 1990). Multiple sequence alignments were performed using Clustal W (Thompson *et al*, 1994).

Primer Design, PCR and SSCP Analysis

A pair of primers was designed from the amplified *COI* sequence and initially tested against genomic DNA of a representative individual from each geographic sample (*N*=5). A *PpCOI*$_{270}$ marker derived from the *COI* gene segment of *P. pelagicus* (primers PpCOI$_{270}$-F: 5'-TTCAGCAGCCATCGCTCAC-3' and PpCOI$_{270}$-R: 5'-AGGGTCAAAGAATGAAGTAT-3'), was used for population genetic studies of *P. pelagicus* (*N*=174, Table 1). PCR was performed composing of predenaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min. The final extension was performed at 72°C for 7 min. Five microliters of the product was mixed with 4 volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured at 95°C for 5 min, immediately cooled on ice for 3 min and electrophoretically analyzed through 15.0% nondenaturing polyacrylamide gels (37.5:1 crosslink) at 12.5 V/cm for 16 h at 4°C.

SSCP bands were visualized by silver staining following a modification of the protocol described for SILVER SEQUENCE™ DNA Sequencing System (Promega). After electrophoresis, the gel plates were carefully separated apart. The long glass plate with the electrophoresed gel was placed in a plastic tray containing 1.5 l of the fix/stop solution (10% glacial acetic acid) and agitated well for 25-30 min. The gel was briefly soaked in deionized water three times for 3 min each with shaking. The gel was transferred to 0.1% silver nitrate solution (1.5 l) and incubated with agitation at room temperature for 30 min. The gel was soaked in 1.5 l of deionized water with shaking (10 forward and 10 backward steps) and immediately placed in the tray containing 1.5 l of the chilled developing solution (chilled 0.03% sodium carbonate, 2.25 ml of formaldehyde and 300 µl of 10 mg/ml sodium thiosulfate). The gel was well agitated until the first bands are visible (1.5-2 min) and transferred to another tray containing 1.5 l of chilled developer and shaken until bands from all examined samples were observed (2-3 min). One liter of the fix/stop solution was directly added to the developing solution and continued shaking for 3 min each. The gel was placed in the plastic bag and air-dried.

Sequence Polymorphism of Different SSCP Genotypes of *PpCOI*$_{270}$

The PCR product of representative individuals of *P. pelagicus* exhibiting different SSCP genotypes of *PpCOI*$_{270}$ (*N*=2 each except genotype VI which was found in only one individual) was gel-eluted and direct-sequenced for both directions. Nucleotide sequences of

Table 1. SSCP genotypes resulted from analysis of *PpCOI*$_{270}$, of *P. pelagicus* originating from different geographic locations in Thai waters

SSCP genotype	Gulf of Thailand			Andaman Sea		Total
	CHN (N=29)	PKK (N=40)	SUT (N=35)	RNG (N=35)	KRB (N=35)	(N=174)
I	27	15	26	-	2	70
II	-	4	-	-	-	4
III	-	18	-	34	31	83
IV	-	-	2	-	1	3
V	1	1	7	1	1	11
VI	1	-	-	-	-	1
VII	-	2	-	-	-	2

Abbreviations: CHN = Chanthaburi, PKK = Prachuap Kriri Khan, SUT = Suratthani, RNG = Ranong, KRB = Krabi

different SSCP genotypes were multiple aligned using Clustal W (Thompson *et al*., 1994). An unrooted maximum parsimony network was constructed from sequence polymorphism between different genotypes of $PpCOI_{270}$.

Data Analysis

The gene diversity (Nei 1987) was estimated for each geographic sample. Unbiased genetic distance between pairs of geographic samples was determined (Nei 1978). Genetic heterogeneity in allele distribution frequencies between compared geographic samples was examined using the exact test. The F_{ST}-based statistics (ϑ) between pairs of geographic samples, bootstrapping 10000 iterations to generate the 95% confidence interval, was estimated. The chi-square value was calculated and tested to determine whether ϑ was statistically different from zero (Weir and Cockerham 1984) using $\chi^2=2N\vartheta(k-1)$ and df=(k-1) (s-1) where N=number of investigated individuals, k=number of allele per locus and s=number of geographic samples. Population genetic parameters described above were computationally analyzed by TFPGA (Miller 1997). Female genetic exchanges among populations per generation were estimated using $N_{ef}m=(1-\vartheta/2\vartheta)$. A UPGMA dendrogram (Sneath and Sokal 1973 was constructed to illustrate the relationships among geographic samples using PHYLIP (Felsenstein 1993).

Results

Development of DNA Markers for Population Genetic Studies of *P. Pelagicus*

Primers for *18S rDNA*, *COI-COII*, *16S rDNA* and *ND5* did not generate the amplification products against genomic DNA of representative individuals of blue swimming crabs (N=3 for each geographic samples). Two primer pairs (*COI* and *12S rDNA*) provided positive amplification products of 706, and 406 bp, respectively. Nucleotide sequences of the amplified fragments significantly matched *COI* of *P. pelagicus* voucher MaPrt001 (E-value=0.0) and *12S rDNA* of *P. pelagicus* voucher flh040815A (E-value=0.0). A pair of primers was designed from each sequence and tested against genomic DNA of wild *P. pelagicus*. However, low polymorphism of *12S rDNA* was found following SSCP analysis (data not shown). Therefore, only *COI* (hereafter called $PpCOI_{270}$) which was consistently amplified and showed relatively high polymorphism was further used for population genetic analysis.

Polymorphism of $PpCOI_{270}$ Revealed by SSCP Analysis

A total of 7 SSCP genotypes of $PpCOI_{270}$ were found from SSCP analysis (Figure 2A and Table 1). SSCP genotypes I predominated in crabs from the Gulf of Thailand (68/104 accounting for 65.38%). This genotype was found in only two individual of the Andaman samples (2/70, 2.86%). In contrast, the genotype III was found in 65 individuals (92.86%) of the Andaman samples but it was observed in only 18 individuals of *P. pelagicus* from Prachuap Khiri Khan (17.31% of the Gulf of Thailand samples). The genotype VI was found in a single individual from Chanthaburi while genotypes II and VII were found only in *P. pelagicus* originating from Prachuap Kriri Khan (N=4 and 2).

The $PpCOI_{270}$ gene segment of *P. pelagicus* exhibiting each SSCP genotype was sequenced (N=2 except genotype VI). Nucleotide sequences were aligned and indicated that different SSCP genotypes of each marker can be distinguished by one or a few substitutions (Figure 2B). A maximum parsimony network based on $PpCOI_{270}$ sequence polymorphism indicated 3 lineages of genotypes (A, B and C; Figure 3A). The blue swimming crab from Ranong (west) possessed the lineage B genotypes (1.000). The distributions of lineages A and B overlapped in the remaining geographic samples with different proportions. The highest frequency of lineage A genotypes was observed in Chanthaburi (0.931) followed by Suratthani (0.743), Prachuap Khiri Khan (0.475), and Krabi (0.057). The distribution of lineage B genotypes in a particular sample was in the opposite direction. The genotype VII, an only member of lineage C, was found in 2 individuals of *P. pelagicus* from Prachuap Khiri Khan (Figure 3B).

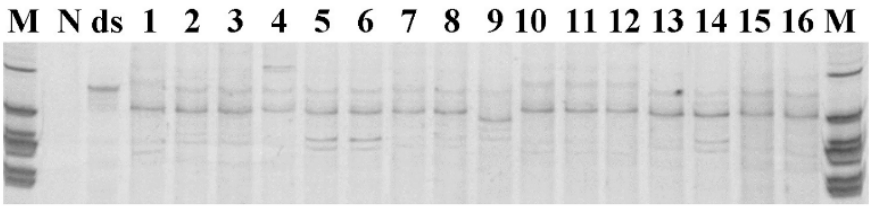
Genetic Diversity and Population Differentiation of *P. pelagicus* in Thai Waters

The average gene diversity in each and overall geographic sample were 0.0063 (Ranong)-0.2847 (Prachuap Kriri Khan) and 0.2860. The genetic distance between pairs of geographic samples was 0.0014 (between Ranong and Krabi; west coastal samples)-0.7247 (between Chanthaburi and Ranong located in different coastal regions; Table 2).

F_{ST}-based statistics (ϑ) for overall samples (0.5713) and paired geographic samples (0.0710-0.9357) bootstrapped overall loci was statistically significant ($P<0.05$). Similar differentiation was also observed between all pairwise comparisons of *P. pelagicus* sample except between Ranong-Krabi ($P>0.05$) following χ^2 analysis ($P<0.001$). Geographic differentiation was observed across overall samples and between almost all of the paired samples ($P<0.0001$) except between Chanhaburi-Prachuap Khiri Khan and Ranong-Krabi ($P>0.05$, Table 3). Accordingly, investigated samples could be differentiated to three genetic stocks including Chanthaburi and Suratthani (stock A), Prachuap Khiri Khan (stock B) and Ranong and Krabi (stock C).

The estimated female gene flow levels of Thai *P. pelagicus* in this study were 0.03–16.67 individuals per generation. The levels between different genetic stocks were less than 1.60 individuals per generation which was lower than those within the same stocks

A



B

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PP-COI270_I   TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_II  TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_III TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_IV  TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_V   TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_VI  TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_VII TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
              **************************************************************
PP-COI270_I   GGCAGGTGTTTCCTCTATTTTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_II  AGCAGGTGTTTCCTCTATTTTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_III GGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_IV  GGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTCATTAACATGCG
PP-COI270_V   GGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_VI  GGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_VII AGCAGGTGTTTCCTCTATTTTAGGTGCAGTAAATTTCATAACCACCGTTATTAACATACG
              ****************** ****************** ******** ******** **
PP-COI270_I   ATCTTTTGGTATAAGAATGGACCAAATACCATTATTCGTTTGATCAGTTTTTATCACTGC
PP-COI270_II  ATCTTTTGGTATAAGAATGGACCAAATACCATTATTCGTTTGATCAGTTTTTATCACTGC
PP-COI270_III ATCTTTTGGTATAAGAATAGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
PP-COI270_IV  ATCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
PP-COI270_V   ATCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
PP-COI270_VI  GTCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
PP-COI270_VII ATCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
              ****************** ******** ******************* ***********
PP-COI270_I   TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_II  TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_III TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_IV  TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_V   TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_VI  TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_VII TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
              ************************************************************
PP-COI270_I   CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_II  CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_III CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_IV  CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_V   CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_VI  CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_VII CCGAAATCTAAATACTTCATTCTTTGACCCT
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Figure 2. (A) Examples of SSCP patterns of *PpCOI*$_{270}$ of *P. Pelagicus* originating from Ranong (lanes 1-8), Suratthani (lanes 9-12), Krabi (lanes 13-16). Lane M, N and ds are a 100 bp DNA marker, the negative control (without genomic DNA template) and the non-denatured PCR product, respectively. (B) Nucleotide sequences of *P. pelagicus* representing seven SSCP genotypes of *PpCOI*$_{270}$. The location and sequence of a forward primer and those complementary to a reverse primer are underlined. Variants at polymorphic sites are highlighted.

Table 2. Pairwise Nei's (1978) genetic distance (below diagonal) between pairs of geographic samples and gene diversity (diagonal) within samples of Thai *P. pelagicus* based on SSCP analysis of *PpCOI*$_{270}$

	CHN	PKK	SUT	RNG	KRB
CHN	0.0667				
PKK	0.1926	0.2847			
SUT	0.0124	0.1099	0.1947		
RNG	0.7247	0.1105	0.5002	0.0063	
KRB	0.6220	0.0814	0.4243	0.0014	0.0898

Abbreviations: CHN = Chanthaburi, PKK = Prachuap Kriri Khan, SUT = Suratthani, RNG = Ranong, KRB = Krabi

(6.54 and 16.67 individuals per generation for stocks A and C) (Table 3).

A UPGMA dendrogram constructed from the average unbiased genetic distance between pairs of geographic samples of Thai *P. pelagicus* allocated 5 investigated samples to 2 evolutionarily related groups; Chanthaburi and Suratthani (cluster A) and Prachuap Kriri Khan, Ranong and Krabi (cluster B) (Figure 4).

Discussion

The estimation and partition of the level of intraspecific genetic variation and population subdivisions are fundamental for establishing rational management of natural resources of commercially important species (Avise 1994; Carvalho and Hauser 1994). Previously, genetic diversity and population structure of *P. pelagicus* in Thailand and Peninsular Malaysia were reported based on AFLP (Klinbunga *et al*. 2007) and microsatellite polymorphism (Chai *et al* 2017). Contradictory results on degrees of intraspecific population differentiation in those studies were clarified by the present study.

Theoretically, mitochondrial DNA evolves 5-10 times faster than single copy nuclear DNA (Brown *et al*. 1979). Polymorphism of *COI* sequences has been applied for evaluation of genetic diversity and population differentiation of several crab species, for example, coconut crab *Birgus latro* (Yorisue *et al*. 2020), mud crabs *Scylla tranquebarica* (Sharif *et al*. 2016) and *S. paramamosain* (Wang *et al*. 2020), Chinese mitten crab *Eriocheir sinensis* (Zhang *et al*. 2018) and sesarmid

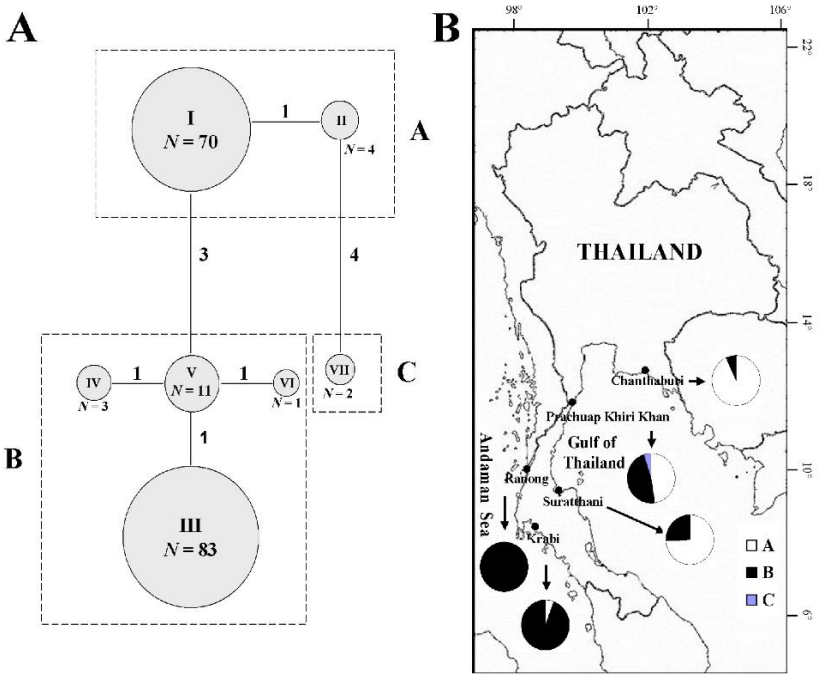

Figure 3. (A) An unrooted maximum parsimony network inferred from nucleotide sequence polymorphism of *P. pelagicus COI* (*PpCOI*$_{270}$). Numbers along the branches indicated the numbers of point mutation steps required for interconnections between genotypes. (B) Data from nucleotide sequences and the unrooted maximum parsimony network were used to infer distributions of *COI* lineages A, B and C in five geographic samples of *P. pelagicus*.

Table 3. Genetic heterogeneity of five geographic samples of *P. pelagicus* based on SSCP analysis of *PpCOI*$_{270}$

Geographic Sample	F_{ST}-based statistics		$N_{ef}m$	Exact test
	Theta (*ϑ*)	χ^2		(*P*-value)
CHN-PKK	0.4282*	29.55***	0.67	< 0.0001***
CHN-SUT	0.0710*	4.54*	6.54	0.0826^{ns}
CHN-RNG	0.9357*	59.88***	0.03	< 0.0001***
CHN-KRB	0.8407*	53.80***	0.09	< 0.0001***
PKK-SUT	0.2381*	17.86***	1.60	< 0.0001***
PKK-RNG	0.3825*	28.69***	0.81	< 0.0001***
PKK-KRB	0.2518*	18.89***	1.49	< 0.0001***
SUT-RNG	0.7771*	54.40***	0.14	< 0.0001***
SUT-KRB	0.6719*	47.03***	0.24	< 0.0001***
RNG-KRB	0.0300*	2.10^{ns}	16.17	0.9472 ^{ns}

$\chi^2 = 2N\vartheta(k\text{-}1)$; df = $(s\text{-}1)(k\text{-}1)$; *N* is the number of individuals used in the analysis, *k* is the number of alleles per locus, and *s* is the number of geographic samples. ns = not significant; * = Significant at P<0.05; ** = P<0.01; *** = P<0.001, respectively.

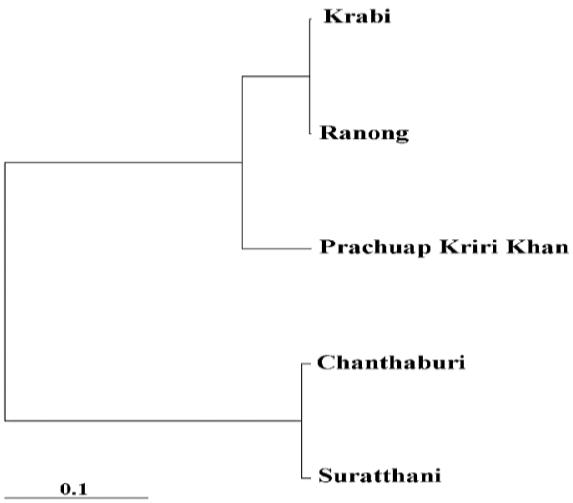

Figure 4. A UPGMA dendrogram indicating relationships of *P. pelagicus* in Thai waters based on genetic distance between pairs of geographic samples. A scale bar indicates genetic distance of 0.1.

crab *Perisesarma bidens* (Zhou *et al*. 2015). In *P. pelagicus*, relative high polymorphism in a 382 bp segment of *COI* sequences (53 haplotypes with haplotype and nucleotide diversity of 0.885±0.0076 and 0.01448±0.0078; *N*=400, respectively) were found in Australian populations (Sezmis 2004). More recently, population genetic differentiation of *P. pelagicus* from southeastern sea of China were also reported based on *COI* polymorphism (Ren *et al* 2016). In this study, polymorphic markers were developed from the positive amplification product of mitochondrial *COI* (*PpCOI*$_{270}$) and tested against Thai *P. pelagicus* (*N*=174). Seven SSCP genotypes of *PpCOI*$_{270}$ were found in Thai *P. pelagicus*. SSCP bands of mtDNA segments were treated as dominant markers and statistically analyzed.

Technically, SSCP is applied for detection of polymorphism based on conformational analysis but it is sensitive to several analysis parameters such as sizes of the product, temperature during electrophoresis and the residual primers in the PCR products (Chen *et al* 1995; Cai and Touitou 1993; Li *et al*. 2003). In the present study, the PCR products of crabs that exhibit different SSCP patterns of *PpCOI*$_{270}$ were further sequenced and all examined genotypes could be distinguished by one or a few nucleotide substitutions. This indicated that SSCP analysis is comparably potential but more cost-effective for population genetic studies of *P. pelagicus* comparing to direct DNA sequencing of the *COI* gene segment.

Previously, population genetic differentiation of *P. trituberculatus* from six locations: Changjiang Estuary (CJ), Shengsi Islands (SS), Zhoushan Islands (ZS), Dongtou Islands (DT), Dinghai Bay (DH), and Quanzhou Bay (QZ), in the East China Sea (*N*=213) was reported based on polymorphism of *COI* sequences (787 bp). A total of 27 haplotypes and 21 variable sites were found. Relatively high haplotype diversity (*h*=0.787±0.026) but limited nucleotide sequence divergence between haplotypes was observed (π=0.00241±0.00098). Analysis of molecular variance (AMOVA) indicated low degrees of population differentiation (7.64% of the variation occurred between populations, P<0.01). When genetic divergence among haplotypes but not haplotype frequencies were taken into the account, significant genetic differentiation among all examined populations (F_{ST}=0.03409-0.16748) of *P. trituberculatus* except between CJ-SS (F_{ST}=-0.00926) and CJ-ZS (F_{ST}=0.02437) was found. The moderate female gene flow levels between pairs of populations (N_{ef}m=2.5-20.0 individuals per generation) except between CJ-SS (large number of individuals) were observed (Liu *et al*. 2009).

Unlike *P. trituberculatus*, common SSCP genotypes I and III of *PpCOI*$_{270}$ showed different genotype frequencies distribution between *P. pelagicus* from the Gulf of Thailand and Andaman Sea samples. An unrooted maximum parsimony network inferred from *PpCOI*$_{270}$ sequences clearly suggested strong genetic differentiation of *P. pelagicus* from different coastal regions (i.e. Andaman and Gulf of Thailand). Apparently, polymorphism of mtDNA provided a prominent figure of population subdivisions of Thai *P. pelagicus* as previously reported in Australian *P. pelagicus* based on *COI* sequences (Sezmis 2004).

Population genetic studies of Thai *P. pelagicus* using a larger sample size were carried out in this study. Limited gene diversity (0.0063-0.2847) but relatively large genetic distance between pairs of geographic samples of *P. pelagicus* was found based on SSCP analysis (0.0014-0.7247). Larger genetic distance between samples from different coastal regions (0.4243-0.7247) than that between geographic samples within coastal regions (0.0124-0.1926 and 0.0014 for the east and west coast regions) was clearly observed. This circumstance supported strong degrees of genetic differentiation of *P. pelagicus* in Thai waters.

Both adult and juvenile *P. pelagicus* inhabited sheltered benthic coastal environments and females migrate outwards into the open ocean for spawning and return into the estuaries after spawning (Potter *et al*. 1983 and 1990). The larval stages of *P. pelagicus* last for

26-45 days (Kangas 2000). On the basis of moderately long planktonic larval stages and high potential mobility during the crab phase, a high gene flow level is expected in this species (Edgar 1990). Typically, degrees of population differentiation (inverse proportion to estimated gene flow levels) are inferred from F_{ST}-based statistics. Theoretically, F_{ST} estimates can range from 0 to 1 where 0 indicates no genetic divergence (complete sharing of genetic materials) and 1 indicates fixation for alternative alleles (no sharing) between populations (Hartl, 1988). Quantitative guideline for F_{ST} estimates is that great genetic differentiation is occurred if the value is > 0.25 (Wright, 1978). In the present study, significant population differentiation among almost pairwise comparisons (F_{ST}-based statistics, ϑ=0.2381-0.9357 except between Chanthaburi-Prachuap Kriri Khan and Ranong-Krabi) of Thai *P. pelagicus* was observed. This indicated that the gene pool of *P. pelagicus* in Thai waters is not panmictic but differentiated to local genetic populations (stocks). Results in the present study revealed clearer population differentiation of *P. pelagicus* in Thai waters than those previously inferred from a limited sample size analyzed by AFLP (Klinbunga *et al*. 2007).

Typically, significant genetic differentiation of marine invertebrates in Thai waters was found between the Andaman Sea (west) and Gulf of Thailand (east) but not within the east coastal region. Examples of these species are the giant tiger shrimp, *P. monodon* (Klinbunga *et al*., 2001), the banana shrimp, *P. merguiensis* (Hualkasin *et al*., 2003), the abalone, *Haliotis asinina* (Tang *et al*., 2004) and the Asian moon scallop, *Amusium pleuronectes* (Mahidol *et al*., 2007). In contrast, strong geographic differentiation between investigated samples located within and/or between coastal areas and low female gene flow levels ($N_{ef}m$<1.60 individuals per generation between different genetic stocks) was found in Thai *P. pelagicus*:

Recently, genetic diversity and differentiation of *P. pelagicus* in different Fishery Management Area (FMA) of Indonesia were reported using *COI* sequence polymorphism. Significant genetic differentiation between pairwise comparisons of populations (F_{ST}=0.954; P<0.001) and the fisheries management areas (F_{ST}=0.964; P<0.001) were found. Genetic disconnectivity was observed between populations in a distance of at least 60 km (Madduppa *et al*. 2021). Population differentiation at a microgeographic scale found in Indonesian *P. pelagicus* strongly supports results from AFLP (Klinbunga *et al*. 2007) and SSCP analysis of the *COI* gene (this study) for *P. pelagicus* in Thai waters

Genetic differentiation between *P. pelagicus* from the Andaman Sea and the Gulf of Thailand could be explained by a major physical barrier as the main current in the Straits of Malacca moves from the south to the north throughout the year. The major reversals in the monsoon driven surface current systems of the Gulf of Thailand (Dale 1956) may involve in differentiation of *P. pelagicus* within the Gulf of Thailand. In addition, the migratory behavior may have promoted degrees of genetic differentiation of *P. pelagicus* in Thai waters. Moreover, seasonal variation on gonad maturation between *P. pelagicus* from different geographic locations was reported (Shields and Wood 1993). This may have promoted the fragmentation of *P. pelagicus* gene pools.

A UPGMA dendrogram constructed from interpopulation genetic distance owing to $PpCOI_{270}$ polymorphism surprisingly allocated the Prachuap Kriri Khan sample to the west- coast samples (Ranong and Krabi). Biogeographic patterns and phylogenetic analysis of *P. pelagicus* suggested that the Gulf of Thailand samples may experience an ancient separation and might have undergone a recent population expansion afterwards.

The blue swimming crab in Thailand is currently managed as a single stock. On the basis of this study, five *P. pelagicus* samples should be regarded as three distinct genetic stocks (see above). From management points of view, these genetically different stocks should be treated and manage separately. Currently, commercial cultivation of *P. pelagicus* has successfully been developed (Azra and Ikhwanuddin 2015). This would significantly reduce the heavily harvest of natural *P. pelagicus*. The ability to identify high genetic diversity and strong population differentiation of *P. pelagicus* is crucial for selection of appropriate broodstock for cultivation of *P. pelagicus*, comparison on the performance of commercially important traits between different stocks and genetic improvement of *P. pelagicus*. An overexploitation of wild *P. pelagicus* reflected by the occurrence of the increasing proportion of small sizes of captured *P. pelagicus* at present may be compensated by enhancement of natural *P. pelagicus* in Thai waters where local genetic stocks are required as founders.

Ethical Statement

The experiments were carried out to conform to the protocols for animal care and use of the National Center for Genetic Engineering and Biotechnology (BIOTEC) animal welfare committee.

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Authors' Contributions

BK planed the experiments, carried out genetic diversity studies and wrote the first draft of the manuscript. SP and SJ screened primers for amplification of mitochondrial genes and performed DNA cloning and sequencing. SK supervised the project and edited the manuscript. All authors agree to publish the manuscript.

Conflict of Interest

BK, SP, SJ and SK declare that they have no conflict of interest.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., & Lipman, D.J. (1990). Basic local alignment search tool. Journal of Molecular Biology, 215(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Avise, J.C. (1994). Molecular Markers, Natural History and Evolution. Chapman and Hall, New York. http://dx.doi.org/10.1007/978-1-4615-2381-9

Azra, M.N., & Ikhwanuddin, M. (2015). Larval culture and rearing techniques of commercially important crab, *Portunus pelagicus* (Linnaeus, 1758): Present status and future prospects. Songklanakarin Journal of Science and Technology, 37 (2), 135-145.

Brown, W.M., George, Jr. M., & Wilson, A.C. (1979). Rapid evolution of animal mitochondrial DNA. Proceeding of the Natural and Academic Science of United State of America, 76, 1967-1971. https://doi.org/10.1073/pnas.76.4.1967

Bryars, S.R., & Adams, M. (1999). An allozyme study of the blue swimming crab, *Portunus pelagicus* (Crustacean: Portunidae), in Australia: stock delineation in southern Australia and evidence for a cryptic species in northern waters. Marine and Freshwater Research, 50, 15-26. https://doi.org/10.1071/MF98075

Cai, Q.Q., & Touitou, I. (1993). Excess PCR primers may dramatically affect SSCP efficiency. Nucleic Acids Research, 21, 3909-3910. https://doi.org/10.1093/nar/21.16.3909

Carvalho, G.R., & Hauser, L. (1994). Molecular genetics and the stock concept in fisheries. Reviews in Fish Biology and Fisheries 4, 326-350. https://doi.org/10.1007/978-94-011-1218-5_3

Chai, C.J., Esa, Y.B., Ismail, M.F.S., & Kamarudin, M.S. (2017). Population structure of the blue swimmer crab *Portunus pelagicus* in coastal areas of Malaysia inferred from microsatellites. Zoological Studies, 56, 26. DOI: 10.6620/ZS.2017.56-26

Chen, X., Baumstark, T., Steger, G., & Riesner, D. (1995). High resolution SSCP by optimization of the temperature by transverse TGGE. Nucleic Acids Research, 23, 4524-4525. https://doi.org/10.1093/nar/23.21.4524

Dale, W.L. (1956). Wind and drift currents in the South China Sea. The Malaysian Journal of Tropical Geography, 8, 1-31.

Dower, W.J., Miller, J.F., & Ragsdale, C.W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Research, 16, 6127–6145.

Edgar, G.J. (1990). Predator-prey interactions in seagrass beds II Distribution and diet of the blue manna crab *Portunus pelagicus* Linnaeus at Cliff Head, Western Australia. Journal of Experimental Marine Biology Ecology, 139, 23-32. https://doi.org/10.1016/0022-0981(90)90035-B

Felsenstein, J. (1993). Phylip (Phylogenetic Inference Package) Version 356c Department of Genetics, University of Washington, Seattle.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3, 294-299.

Hartl, D.L. (1988). A Primer of Population Genetics (2nd ed.). Sinauer Associates, Massachusetts. 305 p.

Hualkasin, W., Sirimontaporn, P., Chotigeat, W., Querci, J., & Pongdara, A. (2003). Molecular phylogenetic analysis of white prawn species and the existence of two clades in *Penaeus merguiensis*. Journal of Experimental Marine Biology Ecology, 296, 1-11. https://doi.org/10.1016/S0022-0981(03)00299-5

Kailola, P.J., Williams, M.J., Stewart, P.C., Reichelt, R.E., McNee, A., & Grieve, C. (1993). Australian Fisheries Resources (Bureau of Resource Sciences, Department of Primary Industries and Energy, and the Fisheries Research and Development Corporation, Canberra, Australia.

Kangas, M.I. (2000). Synopsis of the Biology and Exploitation of the Blue Swimmer Crab, *Portunus pelagicus* Linnaeus, in Western Australia. Fisheries Western Australia, Perth, Western Australia. Fisheries Research Report (121). 22 pp.

Khamnamtong, B., Klinbunga S., Menasveta P. (2005). Species identification of five penaeid shrimps using PCR-RFLP and SSCP analyses of 16S ribosomal DNA. J Biochem Mol Biol 38, 491-499. https://doi.org/10.5483/BMBRep.2005.38.4.491

Klinbunga, S., Khetpu, K., Khamnamtong, B., & Menasveta P. (2007). Genetic heterogeneity of the blue swimming crab (*Portunus pelagicus*) in Thailand determined by AFLP analysis. Biochemical Genetics, 45, 725-736. https://doi.org/10.1007/s10528-007-9110-1

Klinbunga, S., Praipue, P., Khamnamtong, N., Tassanakajon, A., Jarayabhan, P., Hirono, I., Aoki, T., Menaseveta, P. (2003). Genetic diversity and molecular markers of the tropical abalone (*Haliotis asinina*) in Thailand. Marine Biotechnology, 5, 505-517. https://doi.org/10.1007/s10126-002-0108-8

Klinbunga, S., Siludjai, D., Wudthijinda, W., Tassanakajon, A., Jarayabhand, P., & Menasveta, P. (2001). Genetic heterogeneity of the giant tiger shrimp (*Penaeus monodon*) in Thailand revealed by RAPD and mitochondrial DNA RFLP analyses. Marine Biotechnology 3, 428-438. https://doi.org/10.1007/s10126-001-0055-9

Klinbunga, S., Sodsuk, S., Penman, D.J., & McAndrew, B.J. (1996). An improved protocol for total DNA isolation and visualisation of mtDNA-RFLP(s) in tiger prawn, *Penaeus monodon*. Thai Journal of Aquatic Science 3, 36-41.

Lai, J.C.Y, Ng, P.K.L., & Davie, P.J.F. (2010). A Revision of the *Portunus pelagicus* (Linnaeus, 1758) species complex (Crustacea: Brachyura: Portunidae) with the recognition of four species. The Raffles Bulletin of Zoology, 58, 199-237.

Li, W., Gao, F., Liang, J., Li, C., Zhang, H., Tang, Z. *et al*. (2003). Estimation of the optimal electrophoretic temperature of DNA single-strand conformation polymorphism by DNA base composition. Electrophoresis, 24, 2283-2289. https://doi.org/10.1002/elps.200305506

Liu, Y., Liu, R., Ye, L, Liang, J., Xuan, F., & Xu, Q. (2009). Genetic differentiation between populations of swimming crab *Portunus trituberculatus* along the coastal waters of the East China Sea. Hydrobiologia, 61, 125-137. https://doi.org/10.1007/s10750-008-9570-2

Mahidol, C., Na-Nakorn, U., Sukmanomon, S., Taniguchi, N., & Nguyen, T.T.T. (2007). Mitochondrial DNA diversity of the Asian moon scallop, *Amusium pleuronectes* (Pectinidae), in Thailand. Marine Biotechnology, 9(3), 352-359. https://doi.org/10.1007/s10126-006-6137-y

Miller, M.P. (1997). Tools for population genetic analyses TFPGA 13: A window program for the analysis of allozyme and molecular population genetic data. Department of Biological Sciences, Northern Arozona University.

Madduppa, H., Martaulina, R., Zairion, Z., Renjani, R.M., Kawaroe, M., Anggraini, N.P., Subhan, B., Verawati, I., & San, L.M.I. (2021). Genetic population subdivision of the blue swimming crab *(Portunus pelagicus)* across Indonesia inferred from mitochondrial DNA: Implication to sustainable fishery. PLoS ONE, 16(2), e0240951. https://doi.org/10.1371/journal.pone.0240951

Nei, M. (1987). Molecular Evolutionary Genetics. Columbia University Press, New York. https://doi.org/10.1002/ajpa.1330750317

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89(3), 583-590.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., & Sekiya, T. (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proceedings of the Natural Academic Science of United State of America, 86, 2766–2770. https://doi.org/10.1073/pnas.86.8.2766

Potter, I.C., Chrysta, P.J., Loneragan, N.R. (1983). The biology of the blue manna crab *Portunus pelagicus* in an Australian estuary. Mar Biol 78, 75-85. https://doi.org/10.1007/BF00392974

Potter, I.C., Sumpton, W.D., Smith, G.S. (1991). Movement, fishing sector impact, and factors affecting the recapture rate of tagged sand crabs, *Portunus pelagicus* (L) in Moreton Bay, Queensland. Australian Journal of Marine and Freshwater Research 42, 751-760. https://doi.org/10.1071/MF9910751

Ren, G.J., Miao, G.D., Ma, C.Y., Lu, J.X., Yang, X.L., & Ma, H.Y. (2016). Genetic structure and historical demography of the blue swimming crab (*Portunus pelagicus*) from southeastern sea of China based on mitochondrial *COI* gene. Mitochondr DNA. https://doi.org/10.1080/24701394.2016.1261855

Sambrook, J., & Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, New York.

Sharif, N.A.M., Kahar, N.A.S., Rodrigues, K., Ransangan, J., & Kian, A.Y.S. (2016). Genetic diversity of mud crabs, *Scylla tranquebarica* in Sabah, Malaysia based on Cytochrome C Oxidase (COI) gene sequence. Songklanakarin Journal of Science and Technology, 38(4), 365-372.

Sezmis, E. (2004). The Population Genetic Structure of *Portunus pelagicus* in Australian Waters. PhD thesis, Murdoch University, Perth, Australia.

Shields, J.D., & Wood, F.E.I. (1993). Impact of parasites on the reproduction and fecundity of the blue sand crab *Portunus pelagicus* from Moreton Bay, Australia. Marine Ecology Progress Series, 92, 159-170. https://doi.org/10.3354/meps092159

Sihanuntavong, D., Sittipraneed, S., & Klinbunga, S. (1999). Mitochondrial DNA diversity and population structure of the honey bee, *Apis cerana*, in Thailand. Journal of Apiculture Research, 38, 211-219. https://doi.org/10.1080/00218839.1999.11101012

Small, M.P., & Chapman, R.W. (1997). Intraspecific variation in the 16S ribosomal gene of *Crassostrea virginica*. Molecular Marine Biology and Biotechnology, 6, 189–196.

Sneath, P.H.A. & Sokal, R.R. (1973). Numerical Taxonomy. WH Freeman and Co, San Francisco. https://doi.org/10.2307/2412767

Tang, S., Tassanakajon, A. Klinbunga, S., Jarayabhand, P., & Menasveta, P. (2004). Population structure of tropical abalone (*Haliotis asinina*) in coastal waters of Thailand determined using microsatellite markers. Marine Biotechnology, 6, 604-611. https://doi.org/10.1007/s10126-004-2300-5

Thompson, J.D., Higgins, D.G., & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence weighting, position-specific gap penalties and weight metric choices. Nucleic Acids Research, 22, 4673-4680. https://doi.org/10.1093/nar/22.22.4673

Wang, W., Ma, C., Chen, W., Jin, Z., Zhao, M., Zhang, F., Liu, Z., & Ma, L. (2020). Population genetic diversity of mud crab (*Scylla paramamosain*) from southeast coastal regions of China based on mitochondrial COI gene sequence. Gene, 751, 144763. https://doi.org/10.1016/j.gene.2020.144763

Weir, B.S., & Cockerham, C.C. (1984). Estimating *F*-statistics for the analysis of population structure. Evolution, 38, 1358-1370. https://doi.org/10.2307/2408641

Wright, S. (1978). Evolution and the Genetics of Populations. Vol. 4, Variability within and among Natural Populations. Univ. Chicago Press, Chicago. 580 p.

Yap, E.S., Sezmis, E., Chapin, J.A., Potter, I.C., & Spencer, P.B.S. (2002). Isolation and characterization of microsatellite loci in *Portunus pelagicus* (Crustacea: Portunidae). Molecular Ecology Notes, 2, 30-32. https://doi.org/10.1046/j.1471-8286.2002.00136.x

Yorisue, T., Iguchi, A., Yasuda, N., Yoshioka, Y., Sato, T., & Fujita, Y. (2020). Evaluating the effect of overharvesting on genetic diversity and genetic population structure of the coconut crab. Scientific Reports, 10, 10026. https://doi.org/10.1038/s41598-020-66712-4

Zhang, C., Li, Q., Wu, X., Lin, Q., & Cheng, Y. (2018). Genetic diversity and genetic structure of farmed and wild Chinese mitten crab (*Eriocheir sinensis*) populations from three major basins by mitochondrial DNA *COI* and *Cyt b* gene sequences. Mitochondrial DNA Part A DNA Mapping, Sequencing, and Analysis, 29(7), 1081-1089. https://doi.org/10.1080/24701394.2017.1404048

Zhou, H., Xu, J., Yang, M., Wu, B., Yan, B., & Xiong, Y. (2015). Population genetic diversity of sesarmid crab (Perisesarma bidens) in China based on mitochondrial DNA. Mitochondrial DNA, 27(5), 1-8. https://doi.org/10.3109/19401736.2015.1015002