# Genetic Heterogeneity of the Blue Swimming Crab (*Portunus pelagicus*) in Thailand Determined by AFLP Analysis

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**Abstract** Genetic diversity and population differentiation of the blue swimming crab (*Portunus pelagicus*) in Thailand, originating from Ranong and Krabi located in the Andaman Sea (west) and Chanthaburi, Prachuap Khiri Khan, and Suratthani located in the Gulf of Thailand (east), were examined by AFLP analysis. High genetic diversity of *P. pelagicus* in Thai waters was found ($N = 72$). The four primer combinations generated 227 AFLP fragments, and the percentage of polymorphic bands in each geographic sample was 66.19–94.38%. The mean genetic distance between pairs of samples was 0.1151–0.2440. Geographic heterogeneity analyses using the exact test and $F_{ST}$-based statistics between all pairwise comparisons were statistically significant ($P < 0.01$), indicating a fine-scale level of intraspecific population differentiation of Thai *P. pelagicus*. The estimated number of migrants per generation ($N_em$) was 0.26–0.76, suggesting restricted gene flow levels of *P. pelagicus* in Thai waters.

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**Keywords** Genetic diversity · Population differentiation · *Portunus pelagicus* · Amplified fragment length polymorphism (AFLP)

## Introduction

The blue swimming crab, *Portunus pelagicus* (Linnaeus 1758), is a large, edible species inhabiting coastal areas throughout the Indo-West Pacific region, from east Africa to Japan and northern New Zealand (Kailola et al. 1993). Within Thailand, *P. pelagicus* is distributed along the coastal lines of both the Andaman Sea (west) and the Gulf of Thailand (east) (Naiyanetr 1998).

Commercial fishing of *P. pelagicus* has rapidly expanded from 2,883 metric tonnes (MT) in 2002 to 6978 MT in 2005 (Ministry of Commerce 2005). An occurrence of an increasingly large proportion of small sizes of wild-caught *P. pelagicus* at present suggests overexploitation of this species. Currently, commercial cultivation of *P. pelagicus* has successfully been developed in Thailand (P. Jarayabhand, pers. comm.). This would significantly reduce the heavily commercial harvest of natural *P. pelagicus* that may result in stock collapse as previously reported in *P. pelagicus* from Streaky Bay in the west coast region of South Australia (Grove-Jones 1987).

The life cycle of *P. pelagicus* is composed of five larval stages, which last for 26–45 days, and the crab phase (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).

There have been no publications concerning genetic diversity and population subdivisions of *P. pelagicus* in Thailand. The recognition of reproductively isolated and/or genetically differentiated populations within a species is of importance for broodstock selection and breeding programs (Carvalho and Hauser 1994; Conover et al. 2006). Knowledge of the genetic diversity of *P. pelagicus* in Thai waters is essential for the construction of an appropriate management scheme in this taxon (Bryars and Adams 1999). This information would enhance aquaculture output without adversely affecting native populations, leading to sustainable farming of this species.

Amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1995) is a PCR-based multilocus fingerprinting technique that combines the strengths and overcomes the weaknesses of PCR-RFLP and RAPD-PCR (Welsh and McClelland 1990; Williams et al. 1990). AFLP analysis has been used for indirect examination of levels of genetic diversity in several species (Han and Ely 2002; Yue et al. 2004; Liu et al. 2005). The molecular basis of AFLP includes indels between restriction sites and base substitutions at restriction sites for RFLP as well as base substitutions at PCR primer binding sites and indels in the amplification regions for RAPD analyses (Lui and Cordes 2004).

The major strengths of the AFLP method include simultaneous screening of a large number of polymorphic loci, high reproducibility due to high stringency of PCR, and relative cost effectiveness (Lui and Cordes 2004). Moreover, it does not require any prior molecular information about sequences under investigation and is

thus especially applicable to species in which the genome sequences are not well characterized, like *P. pelagicus*.

The objectives of this study are determination of genetic diversity and intraspecific population differentiation of *P. pelagicus* in Thai waters using AFLP analysis for which no data are available at present. The basic information obtained can be applied to the construction of a genetic-based stock enhancement program and to avoid including inbred founder populations in breeding programs of *P. pelagicus*.

## Materials and Methods

### Sampling

The blue swimming crabs (*P. pelagicus*) were live-caught from Ranong (9°58′0 N 98°37′60 E, $N = 14$) and Krabi (8°4′0 N 98°55′0 E, $N = 14$) located in the Andaman Sea (west of peninsular Thailand) and Chanthaburi (12°35′60 N 102°9′0 E, $N = 12$), Prachuap Khiri Khan (11°49′0 N 99°47′60 E, $N = 16$), and Suratthani (9°7′60 N 99°19′0 E, $N = 16$) located in the Gulf of Thailand (east of peninsular Thailand; Fig. 1). Specimens were kept at –30°C until used.

### DNA Extraction

Genomic DNA was extracted from the muscle of the first pereiopod of each crab using a phenol–chloroform–proteinase K method (Klinbunga et al. 1996). The concentration of the extracted DNA was spectrophotometrically estimated. The DNA was stored at 4°C until needed.

### AFLP Analysis

Genomic DNA of each crab (500 ng) was digested with *Eco* RI (or *Pst* I) and *Mse* I prior to ligation with restriction site-specific adaptors (Vos et al. 1995). Preamplification was carried out utilizing adaptor-specific primers with a single selective base on each primer; $E_{+A}$ (5′-GAC TGA GTA CCA ATT CA-3′) and $M_{+C}$ (5′-GAT GAG TCC TGA GTA AC-3′), or $P_{+A}$ (5′-GAC TGC GTA CAT GCA GA-3′) and $M_{+C}$ (5′-GAT GAG TCC TGA GTA AC-3′). The preamplification product was diluted 25 fold and selectively amplified with $E_{+3}$ (or $P_{+3}$) and $M_{+3}$ primers or $E_{+2}$ (or $P_{+2}$) and $M_{+3}$ (or $M_{+2}$) primers, 1.5 units of DyNazymeII DNA Polymerase (Finnzymes), and 1 μl of the diluted preamplification product. PCR was performed in a Perkin Elmer 9700 thermocycler, consisting 2 cycles of denaturation at 94°C for 45 s, annealing at 65°C for 60 s, and extension at 72°C for 90 s, followed by 12 cycles of a touchdown phase with lowering of the annealing temperature 1°C in every cycle and additional 25–28 cycles of 94°C for 45 s, 53°C for 60 s, and 72°C for 90 s. The final extension was carried out at 72°C for 5 min. AFLP fragments were size-

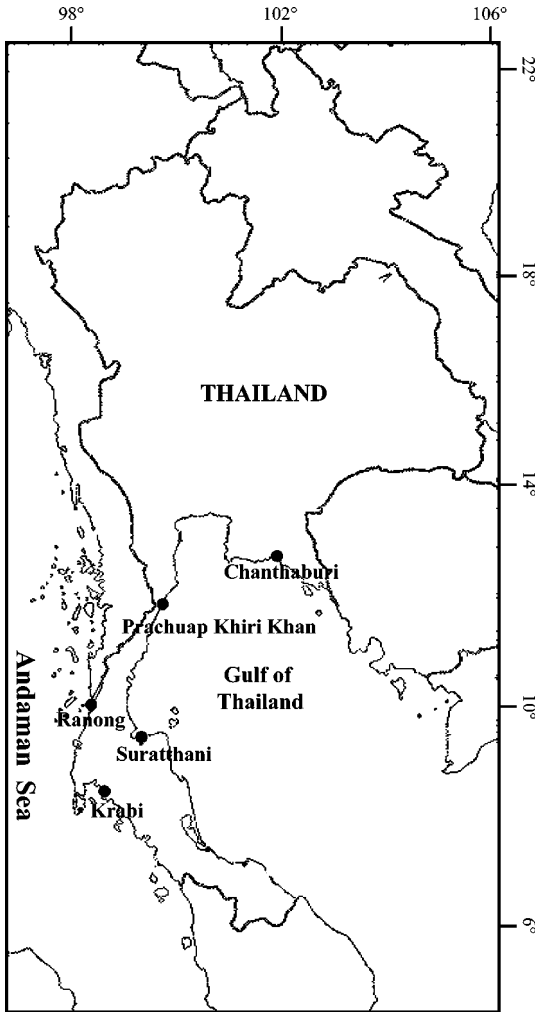


**Fig. 1** Sample collection sites of the blue swimming crab (*P. pelagicus*) used in this study

fractionated by 6.0% denaturing polyacrylamide gels and visualized by silver staining (Sambrook and Russell 2001).

## Data Analysis

AFLP bands were treated as biallelic dominant markers. The percentage of monomorphic (>95% of investigated specimens) and polymorphic (<95% of investigated specimens) loci 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. $F_{ST}$-based statistics ($\theta$) between pairs of geographic samples, performing 10,000 iterations to generate the

95% confidence interval (bootstrapping method), were estimated. The chi-square value was calculated and tested to determine whether $\theta$ was statistically different from zero (Weir and Cockerham 1984), using $\chi^2 = 2N\theta(k-1)$ and df = $(k-1)(s-1)$, where $N$ = number of investigated individuals, $k$ = number of alleles per locus, and $s$ = number of geographic samples. Population genetic parameters described above were computationally analyzed by TFPGA (Miller 1997). The significance level of multiple comparisons was further adjusted using a sequential Bonferroni method (Rice 1989). The number of migrants per generation was estimated using $N_e m = (1 - \theta/4\theta)$.

## Results

Among 52 primer combinations screened, only $P_{+3}$ and $M_{+3}$ primers, but not $E_{+3}$ (or $E_{+2}$) and $M_{+3}$ (or $M_{+2}$) primers, generated discrete amplification bands. Four primer combinations ($P_{ACC}/M_{CAA}$, $P_{AGT}/M_{CAA}$, $P_{AGT}/M_{CAC}$, and $P_{ATC}/M_{CAA}$) provided 227 amplification bands, ranging between 100 and 600 bp (Fig. 2), and were further applied for population genetic studies of *P. pelagicus* in Thai waters.

High genetic diversity was observed in all geographic samples (Table 1). The average polymorphic bands of all primers in each of the Andaman samples (94.38% and 90.20% in Krabi and Ranong) was greater than that of the Gulf of Thailand (73.24 and 66.19% in Chanthaburi and Prachuap Khiri Khan), and the Suratthani sample possessed a higher number of polymorphic bands (93.84%) than other east-coast samples.

The unbiased genetic distance between pairs of geographic samples was 0.1097–0.3786 for $P_{ACC}/M_{CAA}$, 0.0655–0.2577 for $P_{AGT}/M_{CAA}$, 0.1218–0.3086 for $P_{AGT}/M_{CAC}$, and 0.0180–0.2616 for $P_{ATC}/M_{CAA}$. Average genetic distances between samples across all primer combinations ranged from 0.1151 to 0.2440 (Table 2).

Significant geographic heterogeneity was observed between all pairwise comparisons of samples analyzed ($P < 0.0001$), except $P_{ATC}/M_{CAA}$, which did not reveal significant genetic difference between *P. pelagicus* from Chanthaburi and Prachuap Khiri Khan ($P = 0.6812$). The combined data across overall primer combinations indicated significant genetic differences between all comparisons ($P < 0.0001$ for the exact test and $P < 0.01$ for $F_{ST}$-based statistics, Table 3). The gene flow level between pairs of geographic samples of Thai *P. pelagicus* was less than one migrant per generation, suggesting restrictive gene flow levels in this species.

## Discussion

### Genetic Diversity of *P. pelagicus* in Thailand

Analysis of genetic diversity and population differentiation is essential for genetic research (population genetics, phylogenetics, molecular taxonomy and systematics, and evolutionary studies) of various organisms (Avise 1994). High genetic diversity

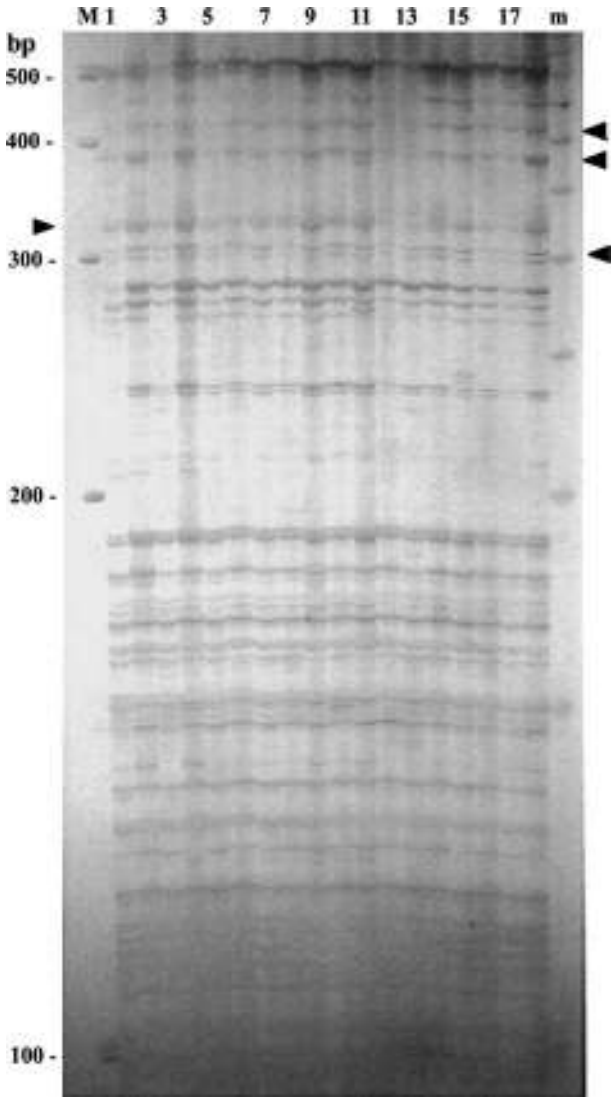


**Fig. 2** AFLP products of *P. pelagicus* originating from Ranong (lane 1), Suratthani (lane 2), Krabi (lane 3), Prachuap Khiri Khan (lane 4), and Chanthaburi (lanes 5–18) amplified by $P_{AGT}/M_{CAC}$ were size-fractionated through a 6% denaturing polyacrylamide gel. Lanes M and m are 100 and 50 bp DNA markers, respectively. Arrowheads indicate candidate species-specific AFLP fragments (found in at least 95% of overall investigated specimens) in *P. pelagicus*

within each geographic sample of Thai *P. pelagicus* was observed from AFLP analysis (66.19–94.38%), suggesting that inbreeding is not a major concern for this economically important species. The percentage of polymorphic bands in *P. pelagicus* was greater than that of penaeid shrimp (*Penaeus monodon, P. chinensis, P. merguiensis, P. latisulcatus, P. canaliculatus*, and *P. japonicus*, with $N = 3–8$

**Table 1** Genetic diversity of geographic samples of *P. pelagicus* in Thai waters revealed by AFLP analysis

| Primer | Chanthaburi ($N$ = 12) | | | Ranong ($N$ = 14) | | | Suratthani ($N$ = 16) | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. of bands | No. of polymorphic bands | No. of monomorphic bands | No. of bands | No. of polymorphic bands | No. of monomorphic bands | No. of bands | No. of polymorphic bands | No. of monomorphic bands |
| $P_{AAC}/M_{CAA}$ | 26 | 12 | 14 | 27 | 19 | 8 | 29 | 25 | 4 |
| $P_{AGT}/M_{CAA}$ | 44 | 41 | 3 | 47 | 46 | 1 | 36 | 35 | 1 |
| $P_{AGT}/M_{CAC}$ | 23 | 4 | 19 | 32 | 26 | 6 | 33 | 30 | 3 |
| $P_{ATC}/M_{CAA}$ | 49 | 47 | 2 | 47 | 47 | 0 | 48 | 47 | 1 |
| Total | 142 | 104 (73.24%) | 38 (26.76%) | 153 | 138 (90.20%) | 15 (9.80%) | 146 | 137 (93.84%) | 9 (6.16%) |

| Primer | Krabi ($N$ = 14) | | | Prachuap Khiri Khan ($N$ = 16) | | |
|---|---|---|---|---|---|---|
| | No. of bands | No. of polymorphic bands | No. of monomorphic bands | No. of bands | No. of polymorphic bands | No. of monomorphic bands |
| $P_{AAC}/M_{CAA}$ | 27 | 26 | 1 | 24 | 12 | 12 |
| $P_{AGT}/M_{CAA}$ | 46 | 44 | 2 | 44 | 33 | 11 |
| $P_{AGT}/M_{CAC}$ | 36 | 36 | 0 | 33 | 19 | 14 |
| $P_{ATC}/M_{CAA}$ | 51 | 45 | 6 | 38 | 28 | 10 |
| Total | 160 | 151 (94.38%) | 9 (5.62%) | 139 | 92 (66.19%) | 47 (33.81%) |

**Table 2** Genetic distance between pairs of geographic samples of *P. pelagicus*

| | CHN | SUT | PKK | RNG | KRB |
|---|---|---|---|---|---|
| CHN | – | 0.8889 | 0.7963 | 0.8611 | 0.8087 |
| SUT | 0.1177 | – | 0.8290 | 0.8913 | 0.8883 |
| PKK | 0.2278 | 0.1876 | – | 0.7898 | 0.7834 |
| RNG | 0.1495 | 0.1151 | 0.2360 | – | 0.8890 |
| KRB | 0.2123 | 0.1184 | 0.2440 | 0.1176 | – |

*Note*: CHN, Chanthaburi; PKK, Prachuap Khiri Khan; SUT, Suratthani located in the Gulf of Thailand (east); RNG, Ranong; and KRB, Krabi located in the Andaman Sea (west)

Above the diagonal: Average genetic identity. Below the diagonal: Nei's (1978) genetic distance

**Table 3** Geographic heterogeneity of five geographic samples of *P. pelagicus* based on AFLP analysis

| Geographic sample | $F_{ST}$-based statistics | | $N_em$ | Exact test (*P*-value) |
|---|---|---|---|---|
| | Theta ($\theta$) | $\chi^2$ | | |
| CHN-SUT | 0.3052 | 17.09* | 0.57 | <0.0001* |
| CHN-PKK | 0.4050 | 27.50* | 0.37 | <0.0001* |
| CHN-RNG | 0.3437 | 17.87* | 0.48 | <0.0001* |
| CHN-KRB | 0.2627 | 21.06* | 0.70 | <0.0001* |
| SUT-PKK | 0.4076 | 26.09* | 0.36 | <0.0001* |
| SUT-RNG | 0.2480 | 15.76* | 0.76 | <0.0001* |
| SUT-KRB | 0.4474 | 16.12* | 0.31 | <0.0001* |
| PKK-RNG | 0.4910 | 26.84* | 0.26 | <0.0001* |
| PKK-KRB | 0.4421 | 26.53* | 0.32 | <0.0001* |
| RNG-KRB | 0.2686 | 13.89* | 0.68 | <0.0001* |

*Note*: Geographic abbreviations as in Table 2

$\chi^2 = 2N\theta(k-1)$; df = $(s-1)(k-1)$; $N$ 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

*Significant at $P < 0.01$ following the sequential Bonferroni approach

for each species) analyzed with three AFLP primer combinations, where the level of polymorphic bands ranged from 24.6% in *P. canaliculatus* to 60.8% in *P. japonicus* (Wang et al. 2004), but was comparable to that (60.92–65.78%) of the flounder (*Paralichthys olivaceus*) analyzed by 10 AFLP primer combinations (Liu et al. 2005).

Although sample size from each geographic site in this study was limited, specimens were collected from different geographic locations on either side of peninsular Thailand. This should be sufficient to generate the preliminary data on genetic diversity and population differentiation of *P. pelagicus* in Thailand.

## Intraspecific Population Differentiation and Limited Gene Flow Levels of *P. pelagicus*

Theoretically, the extended planktonic larval stages of *P. pelagicus* suggest high dispersal potential and the possibility of extensive gene flow between conspecific samples, at least on a geographic mesoscale of tens to hundreds of kilometers (Yap et al. 2002). Nevertheless, Bryars and Adams (1999) illustrated population subdivisions of *P. pelagicus* in Australia based on allozyme polymorphism. Initially, 57 individuals from eight geographic sites were screened for 35 allozymatic loci. Subsequently, 609 individuals of *P. pelagicus* from 11 different locations covering three regions in South Australia and two regions in the Northern Territory were analyzed at seven polymorphic loci (*ACYC*, *ALDH-2*, *ARGK*, *PEP-A*, *PEP-B2*, *PEP-D2*, and *PGM*). Four differentiated subpopulations could be recognized, including West Coast, Spencer Gulf, and Gulf St. Vincent in South Australia and Darwin-Gove in the Northern Territory. Population substructuring among sites within each subpopulation was also found.

Yap et al. (2002) isolated and characterized eight microsatellites in *P. pelagicus* (seven dinucleotide and one tetranucleotide). All eight loci were polymorphic when tested against genomic DNA of *P. pelagicus* collected throughout Australia ($N$ = 85–864). The mean observed heterozygosity ($H_o$) at each locus was 0.30–0.78, which was not significantly different from the expected heterozygosity ($H_e$), except at pPp05, where homozygote excess was observed.

More recently, Sezmis (2004) investigated population genetic structure of *P. pelagicus* collected from 16 different assemblages throughout the geographic range of this species in Australian waters ($N$ = 4–57 per sample site), using six microsatellite loci and a 342 bp fragment of *cytochrome oxidase subunit I* (*COI*). Significant genetic heterogeneity of Australian *P. pelagicus* was found ($F_{ST}$-based statistics for microsatellite and *COI* data = 0.098 and 0.375, respectively).

Large genetic distances between pairs of geographic samples (0.1176–0.2440) indirectly reflected strong intraspecific genetic differentiation of *P. pelagicus*. Generally, the levels of genetic distance between paired geographic samples did not reveal larger genetic distance with greater geographic distance.

Geographic heterogeneity of 49 of 50 possible comparisons was statistically significant ($P < 0.0001$ based on the exact test). The combined data indicated significant population differentiation among all pairwise comparisons of Thai *P. pelagicus* based on the exact test ($P < 0.0001$) and $F_{ST}$-based statistics ($\theta$, $P < 0.01$). Moreover, the lower 95% confidence limit of overall samples and paired geographic samples bootstrapped over all loci was much greater than zero (0.3329 and 0.1980–0.4230, respectively). This further suggested the existence of significant genetic divergence at a fine-scale level between conspecific samples under investigation.

The present study indicated that the gene pool of *P. pelagicus* was not homogeneous but was microgeographically fragmented intraspecifically. Patterns of genetic differentiation at the fine-scale level in *P. pelagicus* (e.g., between geographic samples approximately a few 100 km apart) were different from those of other marine species. For example, significant genetic heterogeneity was

previously reported for the giant tiger shrimp (*P. monodon*; Supungul et al. 2000; Klinbunga et al. 2001), the banana shrimp (*P. merguiensis*; Hualkasin et al. 2003), and the abalone (*Haliotis asinina* and *H. ovina*; Klinbunga et al. 2003), between geographic samples from different coastal regions (i.e., between the Andaman Sea and Gulf of Thailand) in Thai waters.

Marine species with long larval phases are believed to have high levels of genetic variation within populations (Féral 2002). In addition, a long-duration planktonic larval stage influences the opportunity for a high degree of gene flow as evidenced by an absence or lack of genetic differentiation over vast geographic areas in several taxa, for example, in the sea urchins *Strongylogentrotus purpulatus* and *S. droebachiensis* (Palumbi and Wilson 1990) and the red rock lobster, *Jasus edwardsii* (Ovenden et al. 1992).

Basically, *P. pelagicus* is regarded as a potential vagile species because adults are able to travel approximately 20 km daily (Kangas 2000). Both adult and juvenile *P. pelagicus* inhabit sheltered benthic coastal environments, and females migrate outward into the open ocean for spawning and return into the estuaries for a time after spawning. Both males and females migrate from the estuaries as a reaction to lowered salinity (Meagher 1971; Potter et al. 1983, 1991). Nevertheless, strong population differentiation was observed in this species as reflected by the large genetic divergence and restricted levels of gene flow ($N_em < 1$) between pairs of geographic samples of Thai *P. pelagicus*. Geographic distance between investigated samples *per se* and migratory behavior of *P. pelagicus* might have promoted degrees of genetic differentiation of this species.

On the basis of the present study, five *P. pelagicus* samples were regarded as different genetic populations. From the management point of view, these genetically isolated populations should be treated as separate management units. Stock enhancement to resolve consequent effects of overexploitation of natural *P. pelagicus* may be carried out using a fine-scale level of local populations as the founders.

In terms of aquaculture, domestication and subsequently selective breeding programs should be established for *P. pelagicus* using the advantage of strong intraspecific genetic differentiation between geographically different samples of *P. pelagicus* found in the present study. The proper source to be used as the founder stock for breeding programs of *P. pelagicus* should be established from different genetic populations that are maintained separately. Interpopulational crosses may be carried out, possibly, to promote heterosis of economically important traits in this species.

Molecular population genetics provide information required for elevating culture and management efficiency of *P. pelagicus*. Knowledge on genetic diversity and population differentiation of *P. pelagicus* found in this study yields not only critical information on biogeographic and evolutionary aspects of *P. pelagicus* in Thailand but also allows the construction of effective breeding programs and restocking projects in this species.

**Acknowledgments** This research is supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand, awarded to SK and BK.

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