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Genetic population differentiation of the blue swimming crab *Portunus pelagicus* (Portunidae) in Thai waters revealed by RAPD analysis

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ABSTRACT. Genetic diversity and population differentiation of the blue swimming crab, *Portunus pelagicus*, in Thailand were analyzed by RAPD analysis. One hundred and twelve RAPD fragments were generated from 109 individuals of *P. pelagicus* using OPA02, OPA14, OPB10, UBC122, and UBC158 primers. The percentage of polymorphic bands in each geographic sample and that of each primer across overall samples were 72.7-85.0 and 92.0-100%, respectively. Large numbers of polymorphic bands found in the RAPD analysis suggested high genetic diversity of Thai *P. pelagicus*. The mean genetic distance between

samples across all primers was 0.0929-0.2471. Significant geographic heterogeneity was observed across samples overall and between all pairs of geographic samples ($P < 0.01$ for θ and $P < 0.0001$ for the exact test), indicating strong genetic differentiation of *P. pelagicus* in Thai waters, despite its high potential of dispersal. Limited gene flow levels (0.44-1.19 individuals per generation) of Thai *P. pelagicus* were also observed. A fine scale level of differentiation suggested that *P. pelagicus* from each geographic sample in Thai waters should be regarded as a separate genetic population and treated as a different exploited stock.

Key words: Genetic diversity; Population structure; RAPD; *Portunus pelagicus*; Species-specific markers

INTRODUCTION

The blue swimming crab, *Portunus pelagicus* (Linnaeus) is one of the most important marine species. It is distributed from the intertidal zone to approximately 50 m depth (Kangas, 2000). In Thailand, *P. pelagicus* is distributed along the coasts of both the Andaman Sea (west) and the Gulf of Thailand (east) (Naiyanetr, 1998). Commercial fishing of *P. pelagicus* has been reduced during the last few years. The catch volume of the blue swimming crab in the Gulf of Thailand was reduced from 37,219 metric tons (MT) in 2000 to 22,113 MT in 2004, though the catch in the Andaman Sea remained relatively constant during the same period (6652 to 7411 MT; Ministry of Commerce Thailand, 2005).

The blue swimming crab exhibits moderately long planktonic larval stages (26-45 days) and high mobility during the crab phase (Kangas, 2000). Therefore, high gene flow levels are expected in this species (Edgar, 1990). Currently, *P. pelagicus* can be cultivated commercially in Thailand.

An initial step to establish appropriate broodstock management and conservation programs and to avoid including inbred founder populations in the breeding programs of this species is the development of molecular genetic markers that can be used for determination of stock structure and evaluation of the levels of gene flow. This information will provide means for more effective management of natural stocks of *P. pelagicus* (Klinbunga et al., 2007).

Randomly amplified polymorphic DNA (RAPD) analysis has been used to determine genetic diversity and identify useful genetic markers of various marine organisms (Tyler-Walters and Hawkins, 1995; Tassanakajon et al., 1998; Klinbunga et al., 2000, 2003, 2007). RAPD analysis uses a random oligonucleotide primer, obviating the need for knowledge of the sequences of the genome under investigation (Welsh and McClelland, 1990; Williams et al., 1990). This advantage is particularly useful for a non-model species such as *P. pegalicus*, for which known nucleotide sequences of both coding and non-coding DNA in this species are rather limited. Moreover, RAPD analysis does not require expensive equipment and extensive steps of analysis (Weising et al., 1995). This makes RAPD analysis suitable for population genetic studies in many species (Klinbunga et al., 2003; Lui and Cordes, 2004; Tang et al., 2005).

Genetic population structure of Australian *P. pelagicus* was previously reported based on allozyme analysis at seven polymorphic loci (*ACYC*, *ALDH-2*, *ARGK*, *PEP-A*, *PEP-B2*, *PEP-D2*, and *PGM*) (Bryars and Adams, 1999), six microsatellite loci and polymorphism of a 342-bp fragment of *cytochrome oxidase subunit I* (*COI*) (Sezmis, 2004).

Recently, genetic diversity of *P. pelagicus* in various regions of Thailand (Ranong, Krabi, Chanthaburi, Prachuap Khiri Khan, and Suratthani) was reported based on AFLP analysis (Klinbunga et al., 2007). Although high genetic diversity and geographic differentiation of *P. pelagicus* were observed, the sample size was rather limited (N = 72). Therefore, estimated genetic diversity and degrees of geographic differentiation may not be accurate.

We examined the genetic diversity and intraspecific population differentiation of *P. pelagicus* in Thai waters, by RAPD analysis, using a larger sample size.

MATERIAL AND METHODS

Sampling

One hundred and nine individuals of blue swimming crab (*P. pelagicus*) were live-caught from Chanthaburi (12°35'60" N, 102°9'0" E, N = 23), Prachup Khiri Khan (11°49'0" N, 99°47'60" E, N = 20) and Suratthani (9°7'60" N, 99°19'0" E, N = 21) located in the Gulf of Thailand and Ranong (9°58'0" N, 98°37'60" E, N = 23) and Krabi (8°4'0" N, 98°55'0" E, N = 22) located in the Andaman Sea (Figure 1). The whole specimens or muscle dissected from the first walking leg of each crab were kept at -30°C until used.

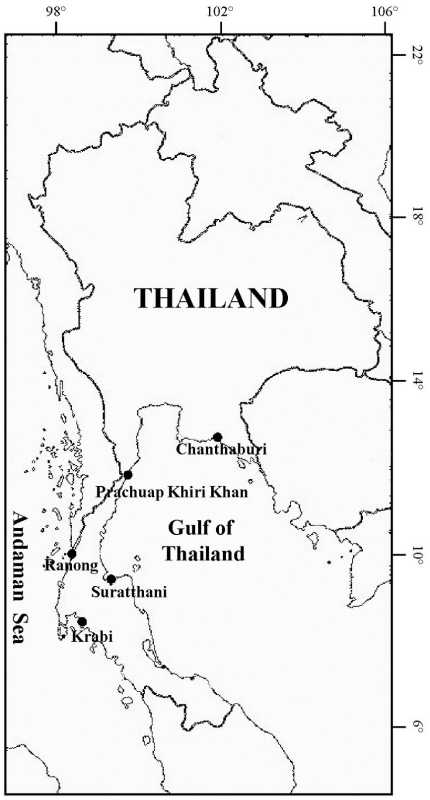

Figure 1. Sampling collection sites of *Portunus pelagicus* in Thailand.

DNA extraction

Genomic DNA was extracted from the muscle of the 1st periopod of each crab using a phenol-chloroform-proteinase K method (Klinbunga et al., 1996). The concentration of the extracted DNA was spectrophotometrically estimated. DNA was stored at 4°C until needed.

RAPD-PCR

Thirty-one RAPD primers were screened for the amplification success against genomic DNA of a representative individual of *P. pelagicus* from each geographic sample. Five primers (UBC122, UBC158, OPA02, OPA14, and OPB10) were selected for population genetic analysis of *P. pelagicus*. Polymerase chain reaction (PCR) was performed in a 25-μL reaction volume containing 10 mM Tris-HCl, pH 8.8, 0.01% Triton X-100, 50 mM KCl, 2 mM $MgCl_2$, 100 μM of each dNTP, 0.2 μM of an arbitrary primer, 1 U DyNazyme^{TM}II DNA polymerase (Finnzymes, Espoo, Finland) and 25 ng DNA template. The amplification profiles consisted of predenaturation at 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 36°C for 60 s and extension at 72°C for 90 s. The final extension was carried out at 72°C for 7 min. Five microliters of the amplification reaction was electrophoresed through 1.6% agarose gels and visualized under a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001).

Data analysis

RAPD bands were treated as dominant markers. The percentage of monomorphic (>95% of investigated specimens) and polymorphic (<95% of investigated specimens) bands was estimated for each geographic sample. Unbiased genetic distance between pairs of geographic samples was determined (Nei, 1978). Genetic heterogeneity in the allele distribution frequencies of overall samples and between paired geographic samples was examined using the exact test (Raymond and Rousset, 1995). The F_{ST}-based statistics (θ) between pairs of geographic samples, performing 10,000 iterations to generate the 95% bootstrapping confidence interval, was estimated. The χ^2 value was calculated and tested to determine whether θ was significantly different from zero (Weir and Cockerham, 1984), using $\chi^2 = 2N\theta(k - 1)$ and d.f. = (k - 1) (s - 1), where N = number of investigated individuals, k = number of allele per locus and s = number of geographic samples. These population genetic parameters were analyzed with Tools for Population Genetic Analysis (TFPGA) (Miller, 1997). The significance level of multiple comparisons was further adjusted using a sequential Bonferroni's method (Rice, 1989). Genetic exchange among population per generation was estimated using $N_em = 1 - \theta / 4\theta$ (Hudson et al., 1992).

RESULTS

Among 31 RAPD primers that were screened, 26 (UBC119, UBC122, UBC135, UBC138, UBC158, UBC169, UBC174, UBC191, UBC222, UBC228, UBC263, UBC268, UBC273, UBC299, UBC457, UBC458, UBC459, OPA01, OPA02, OPA06, OPA14, OPA15, OPA17, OPB10, OPZ09, and YNZ22) successfully amplified genomic DNA of representative individuals of *P. pelagicus* (N = 5). Five primers (UBC122, UBC158, OPA02, OPA14, and

OPB10) exhibiting reproducible and easy scoring results were selected for population genetic studies of *P. pelagicus* in Thai waters (Figure 2).

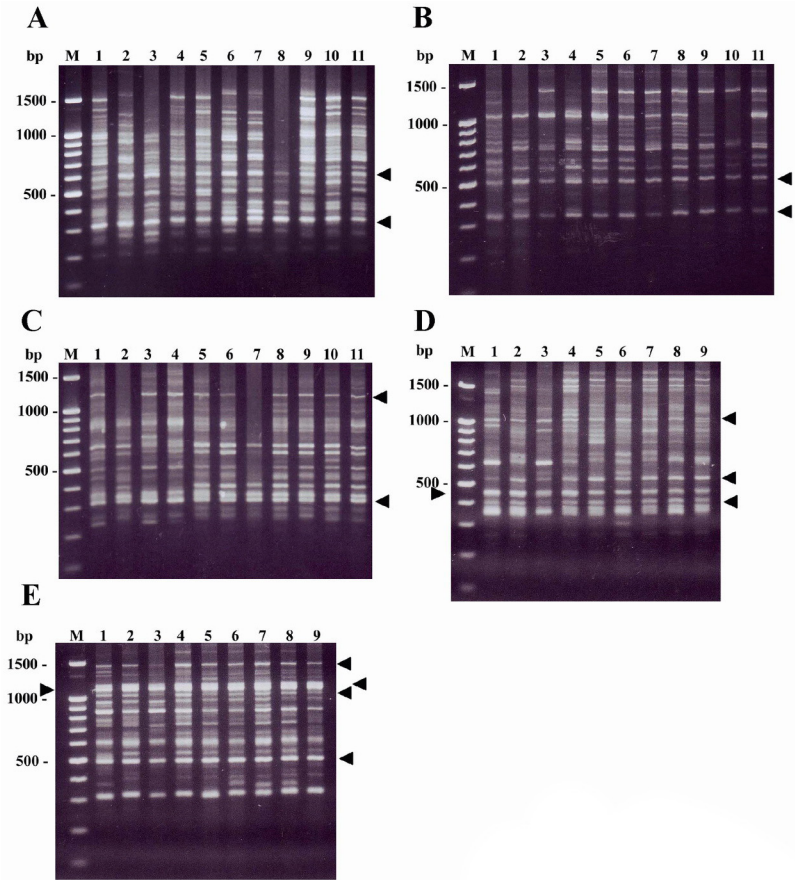

Figure 2. Examples of RAPD patterns of *Portunus pelagicus* originating from Chanthaburi (*lanes 1-2*, A-E), Ranong (*lanes 3-4*, A-E), Suratthani (*lanes 5-6*, A-E), Krabi (*lanes 7-8*, A-E), and Prachuap Khiri Khan (*lanes 9-11*, A-C and *lane 9*, D-E) generated by OPA02 (A), OPA14 (B), OPB10 (C), UBC122 (D), UBC158 (E). M = 100-bp DNA ladder. Arrowheads indicate candidate species-specific RAPD fragments for *P. pelagicus*.

One hundred and twelve RAPD fragments ranging from 220 to 1500 bp in length were generated (Table 1). High genetic polymorphism was observed in all geographic samples. The percentage of polymorphic bands for each primer across all geographic samples was 92.0-100% (Table 1). The Chanthaburi sample exhibited the highest percentage of polymorphic bands, followed by Prachuap Khiri Khan, Ranong, Krabi, and Suratthani (Table 2). Four, 5, 2, 2, and 2 candidate species-specific RAPD markers were generated from UBC122, UBC158, OPA02, OPA14, and OPB10, respectively (Figure 2 and Table 3). Population-specific RAPD fragments were not found in any of these geographic samples. The mean genetic distance between samples for all primers was 0.0929-0.2471 (Table 4).

Table 1. Sequences of arbitrary primers, sizes and number of amplified bands, and the percentage of polymorphic bands resulting from random amplified polymorphic DNA (RAPD) analysis of *Portunus pelagicus* using UBC122, UBC158, OPA02, OPA14, and OPB10 primers.

Primer	Sequence	Size range (bp)	No. of RAPD bands	Polymorphic bands (%)
UBC122	5'-GTAGACGAGC-3'	320-1200	22	21 (95.5%)
UBC158	5'-TAGCCGTGGC-3'	310-1500	24	23 (95.8%)
OPA02	5'-TGCCGAGCTG-3'	250-1500	25	23 (92.0%)
OPA14	5'-TCTGTGCTGG-3'	340-1400	22	22 (100%)
OPB10	5'-CTGCTGGGAC-3'	220-1500	19	18 (94.7%)
Overall primers	-	220-1500	112	107 (95.5%)

Table 2. Total number of bands, percentage of polymorphic and monomorphic bands of each geographic sample of *Portunus pelagicus* in Thai waters revealed by RAPD analysis using UBC122, UBC158, OPA02, OPA14, and OPB10 primers.

	UBC122	UBC158	OPA02	OPA14	OPB10	Total
Chanthaburi (N = 23)						
No. of bands	21	23	23	22	18	107
No. of polymorphic bands	18	19	19	19	16	91 (85.0%)
No. of monomorphic bands	3	4	4	3	2	16 (15.0%)
Ranong (N = 23)						
No. of bands	22	24	25	22	17	110
No. of polymorphic bands	17	15	23	21	15	91 (82.7%)
No. of monomorphic bands	5	9	2	1	2	19 (17.3%)
Suratthani (N = 21)						
No. of bands	21	24	24	22	19	110
No. of polymorphic bands	17	14	21	13	15	80 (72.7%)
No. of monomorphic bands	4	10	3	9	4	30 (27.3%)
Krabi (N = 22)						
No. of bands	19	22	24	16	19	100
No. of polymorphic bands	14	17	22	12	17	82 (82.0%)
No. of monomorphic bands	5	5	2	4	2	18 (18.0%)
Prachuap Khiri Khan (N = 20)						
No. of bands	19	20	23	13	16	91
No. of polymorphic bands	16	18	19	9	14	76 (83.5%)
No. of monomorphic bands	3	2	4	4	2	15 (16.5%)

Table 3. Candidate species-specific fragments of *Portunus pelagicus* in Thailand revealed by random amplified polymorphic DNA (RAPD) analysis.

Primer	RAPD marker (bp)
OPA02	300 and 590
OPA14	340 and 500
OPB10	310 and 1200
UBC122	400, 440, 510, and 1050
UBC158	500, 1050, 1150, 1200, and 1500

Table 4. Pairwise Nei's (1978) genetic distances (below diagonal) and genetic identity (above diagonal) between geographic samples of *Portunus pelagicus.*

	Chanthaburi (E)	Suratthani (E)	Prachuap Khiri Khan (E)	Ranong (W)	Krabi (W)
Chanthaburi (E)	-	0.9109	0.8974	0.8351	0.8645
Suratthani (E)	0.0933	-	0.8666	0.8842	0.8363
Prachuap Khiri Khan (E)	0.1083	0.1432	-	0.7811	0.9113
Ranong (W)	0.1802	0.1230	0.2471	-	0.8525
Krabi (W)	0.1456	0.1788	0.0929	0.1596	-

E = east; W = west.

Geographic heterogeneity analysis of all primers indicated significant differences for overall samples and between all pairs of geographic samples ($P < 0.0001$ for the exact test and $P < 0.01$ for θ; Table 5). The gene flow level (N_em) between pairs of geographic samples of *P. pelagicus* was 0.44-1.19 individual per generation.

Table 5. Geographic heterogeneity analysis of five conspecific samples of *Portunus pelagicus* based on F_{ST}-based statistics (θ) and the exact test.

Geographic sample	F_{ST}-based statistics		N_em
	Theta (θ)	χ^2	
CHN-RNG	0.2750	25.30	0.66
CHN-SUT	0.1732	15.24	1.19
CHN-KRB	0.2508	22.57	0.75
CHN-PKK	0.2026	17.42	0.98
SUT-PKK	0.2618	21.47	0.70
SUT-RNG	0.2215	19.49	0.88
SUT-KRB	0.3024	26.01	0.58
PKK-RNG	0.3617	31.11	0.44
PKK-KRB	0.1987	16.69	1.01
RNG-KRB	0.2758	24.82	0.66

CHN = Chanthaburi; RNG = Ranong; SUT = Suratthani; KRB = Krabi; PKK = Prachuap Khiri Khan. $\chi^2 = 2N\theta(k - 1)$, d.f. = (s - 1) (k - 1), where N is the number of individuals used in the analysis, k is the number of alleles per locus and s is the number of populations. Exact test: $P < 0.0001$. All comparisons significant at $P < 0.017$ based on a sequential Bonferroni's analysis (Rice, 1989).

DISCUSSION

Using RAPD-PCR, relatively high genetic diversity was found in *P. pelagicus*. The percentage polymorphic bands in each of the geographic samples of *P. pelagicus* ranged from 72.7-85.0%, which is slightly greater than that previously reported for the giant tiger shrimp, *Penaeus monodon* (51.1-57.7%; Tassanakajon et al., 1998), *Crassostrea* oysters (53.2-77.7%; Klinbunga et al., 2001) and the mud crabs (*Scylla serrata*, *S. oceanica*, and *S. tranquebarica*; Klinbunga et al., 2000) but lower than that of the tropical abalone, *Haliotis asinina* (81.3-90.5%; Tang et al., 2005) and *Saccostrea* oysters (86.2-99.4%; Klinbunga et al., 2001) in Thai waters.

Several unique RAPD fragments exhibiting fixed frequencies were identified. These fragments were considered as potential species-specific markers for *P. pelagicus*. Klinbunga et al. (2010) further developed species-specific markers of *P. pelagicus* and five of 15 candidate species-specific RAPD fragments (OPA14-500, UBC158-500, UBC122-510, UBC158-1200, and UBC158-1500; Table 3) identified in this study were cloned and sequenced. Three RAPD-derived sequence-characterized amplified region (SCAR) markers (called PP-$SCAR_{152}$, PP-$SCAR_{397}$ and PP-$SCAR_{262}$ derived from UBC122-510, UBC158-1200 and UBC158-1500, respectively) generated the expected product in 95, 100 and 100% of *P. pelagicus* (N = 174) and in all individuals of the three spot swimming crab, *P. sanguinolentus* (N = 10) but not in the swimming crab, *Charybdis crucifera* (N = 20) and mud crabs; *Scylla oceanica* (N = 18), *S. serrata* (N = 7), *S. tranquebarica* (N = 9). Single-strand conformational polymorphism (SSCP) analysis of PP-$SCAR_{397}$ and PP-$SCAR_{262}$ was applied and was sufficient to distinguish *P. pelagicus* from *P. sanguinolentus* unambiguously.

Large genetic distances between pairs of geographic samples were observed; these values indirectly reflected degrees of within species differentiation of Thai *P. pe-*

lagicus. Generally, paired geographic samples that were situated farther apart geographically did not exhibit greater genetic distance.

Our results from this study were drawn solely from sharing of RAPD-amplified fragments after electrophoresis. The possibility of comigration of RAPD fragments having different sequences but similar sizes cannot be excluded. Homology of comigrating diagnostic fragments should be further verified (Hadrys et al., 1992). This would result in more valid and stable data for subsequent applications in conservation and breeding programs of *P. pelagicus*.

Lu et al. (2000) analyzed three species of mitten crabs (*Eriocheir sinensis*, *E. japonicus* and *E. japonicus hepuensis*) and three geographic samples of *E. sinensis* by RAPD analysis. No species diagnostic markers were found, but significant genetic differences were found between taxa ($P < 0.001$) and geographic samples ($P < 0.001$); intraspecific similarities were larger than interspecific similarity, and intrapopulational similarity was larger than interpopulational similarities.

Intraspecific genetic subdivisions of the Pacific blue shrimp (*P. stylirostris*) on the east coast of the Gulf of California (Mexico) have also been studied with RAPD analysis. Seventy-eight individuals of *P. stylirostris* from six fishing grounds ($N = 13$ per location) were analyzed with eight informative primers (OPM04, 11, 12, 14, 16, 17, 19, and 20). Three hundred and twenty-four RAPD fragments were obtained. Analysis of molecular variance (AMOVA) and pairwise between-population Φ_{ST} indicated significant genetic differentiation of *P. stylirostris* ($P = 0.0001$; Aubert and Lighter, 2000).

Previously, intraspecific population differentiation of *P. pelagicus*, originating from the same geographic locations in this study, was reported based on amplified fragment length polymorphism (AFLP) analysis. High genetic diversity of *P. pelagicus* in Thai waters was found ($N = 72$). A total of 227 AFLP were generated from four primer combinations (P_{ACC}/M_{CAA}, P_{AGT}/M_{CAA}, P_{AGT}/M_{CAC}, and P_{ATC}/M_{CAA}). 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 ($N_e m$) was 0.26-0.76, suggesting restricted gene flow levels of *P. pelagicus* (Klinbunga et al., 2007).

Likewise, geographic heterogeneity analysis based on each RAPD primer indicated significant genetic heterogeneity among 43 of 50 possible comparisons, after a sequential Bonferroni's adjustment ($P < 0.01$; data not shown). The combined data confirmed a fine scale level of population subdivisions among all pairwise comparisons of *P. pelagicus* previously reported by AFLP analysis ($P < 0.0001$ for the exact test and $P < 0.01$ for θ). The lower bootstrapping 95% confidence interval of overall samples and paired geographic samples overall loci was also much greater than zero (0.2227 and 0.1226-0.3023). This result further supports the affirmation of genetic differentiation between conspecific samples of *P. pelagicus* ($P < 0.0001$).

Surprisingly, patterns of genetic differentiation (e.g., between pairs of geographic samples) based on RAPD analysis did not indicate long range migration of *P. pelagicus* in our study, and the patterns were different from those of other marine species locally found in Thai waters, including *P. monodon* (Khamnamtong et al., 2009), *P. merguiensis* (Hualkasin et al., 2003), *H. asinina* (Praipue et al., 2010) and *H. varia* (Klinbunga et al., 2003); in these previous studies, significant genetic heterogeneity was found between coastal regions (i.e., between the Andaman Sea and the Gulf of Thailand).

Generally, *P. pelagicus* is regarded as a potential vagile species; females migrate out of estuaries into the open ocean for spawning, and both females and males migrate out as a re-

action to lowered salinity (Meagher, 1971; Potter et al., 1983). However, the high level of population differentiation that we observed indicates that *P. pelagicus* experienced restricted gene flow levels (N_em <1 for almost all comparisons). Geographic distances between the samples (Bryars and Adams, 1999; Sezmis, 2004), non-concurrent male and female maturation periods (Pillay and Nair, 1971; Kailola et al., 1993; Kangas, 2000) and migratory behavior of *P. pelagicus* may have been responsible for the present differentiation of its gene pool. Migration distance and population dynamics of *P. pelagicus* in Thai waters are relatively unknown and should be further studied to help explain the unexpectedly low gene flow levels in this species.

Although analysis of genetic diversity and population genetics of organisms by AFLP is normally more reliable than with RAPD analysis, in our study the two approaches were sufficient to examine genetic differentiation of *P. pelagicus* in Thailand. The RAPD technique is simpler and more cost-effective than AFLP analysis for monitoring genetic diversity levels of *P. pelagicus*.

Molecular population genetic studies provide necessary information required for increasing the efficiency of management of *P. pelagicus*. The blue swimming crab in Thailand is presently perceived and managed as a single stock. Basic knowledge about high genetic diversity and large genetic divergence between geographic samples at a fine scale level lead us to suggest that *P. pelagicus* from each geographic sample in Thai waters should be regarded as a separate genetic population and treated as a different exploited stock (Carvalho and Hauser, 1994; Conover et al., 2006). A genetic-based stock enhancement program should be implemented to resolve problems of overexploitation, as illustrated by an increasing proportion of small sizes of captured *P. pelagicus* (Klinbunga et al., 2007) and to maintain the genetic diversity of Thai *P. pelagicus*.

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