Random amplified polymorphic DNA (RAPD) markers for determination of genetic variation in wild populations of the black tiger prawn (*Penaeus monodon*) in Thailand

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Abstract

Random amplified polymorphic DNA (RAPD) analysis was used to amplify the genome of black tiger prawns (*Penaeus monodon*) to detect DNA markers and assess the utility of the RAPD method for investigating genetic variation in wild *P. monodon* in Thailand. A total of 200 ten-base primers were screened, and 84 primers yielded amplification products. Six positive primers that gave highly reproducible RAPD patterns were selected for the analysis of three geographically different samples of Thai *P. monodon*. A total of 70 reproducible RAPD fragments ranging in size from 200 to 2000 bp were scored, and 40 fragments (57%) were polymorphic. The RAPD analysis of broodstocks from three different locales, Satun-Trang, Trat, and Angsila, revealed different levels of genetic variability among the samples. The percentages of polymorphic bands were 48% and 45% in Satun-Trang and Trat, respectively, suggesting a high genetic variability of the two samples to be used in selective breeding programs. Only 25% polymorphic bands were found in the Angsila sample, indicating the lowest polymorphic level among the three samples examined. Primer 428 detected a RAPD marker that was found only in *P. monodon* originating from Satun-Trang, suggesting

the potential use of this marker as a population-specific marker in this species.

Introduction

The black tiger prawn (*Penaeus monodon* Fabricius) is successfully cultured for food in many Asian countries. In Thailand, this species is the most economically important cultured shrimp, providing a substantial income of approximately US$2 billion in 1994 (Lin and Nash, 1996). The shrimp production of Thailand has increased rapidly during the last decade. The total amount of farm-raised black tiger prawns increased from 33,000 metric tons in 1987 to 220,000 metric tons in 1994. However, the farming of *P. monodon* relies entirely on broodstocks caught from wild populations for the supply of juveniles. Attempts to domesticate this species are now being undertaken (Benzie, 1994). As a first step toward the genetic improvement of this species, genetic variations among populations are being investigated.

Allozyme variability has been extensively used in Penaied prawns, but a low level of variation has been reported (Nelson and Hedgecock, 1980). Although a significant difference in allozyme frequency among Australian populations of *P. monodon* has been found (Benzie et al., 1992), more genetic variation has been detected using mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis (Benzie et al., 1993) and mtDNA sequence analysis (Palumbi and Benzie, 1991). These studies suggested that the DNA technique would provide a better source of useful markers in Penaeid prawns.

The random amplified polymorphic DNA (RAPD) analysis, developed by Williams et al. (1990), utilizes a single short primer of arbitrary sequence to amplify random segments of genomic DNA. The RAPD markers allow the examination of genome variation without previous knowledge of DNA sequences. The method has been used successfully to detect genetic variation in several plants and animals (Devos and Gale, 1992; Brummer et al.,

1995; Gwakisa et al., 1995). It has been used to examine genetic diversity in *P. vannamei* (Garcia et al., 1994), revealing a high level of genetic variation among populations. In *P. monodon,* the inheritance of RAPD banding patterns in six fullsib families has been demonstrated (Garcia and Benzie, 1995), indicating the possibility of using RAPD markers to track the progeny of pair mating in selective breeding programs.

In this study, we used RAPD analysis to detect polymorphic DNA markers in *P. monodon* and assess the utility of RAPD for investigating genetic variation of wild *P. monodon* in Thailand.

Results and Discussion

Optimization of the polymerase chain reaction assay for P. monodon

Experiments were carried out to optimize polymerase chain reaction (PCR) program parameters for the reproducible amplification of discrete DNA patterns. The length of each phase of the PCR program and the number of cycles were evaluated. Inconsistent amplification was minimized by including three concentrations of template DNA (100-fold span) in the program evaluations. Optimal program parameters were selected on the basis of consistent amplification at all DNA concentrations in addition to increased intensity and clarity of the banding pattern, as suggested by Bielawski et al. (1995). The optimal program parameters identified for *P. monodon* were 35 cycles of 5 seconds at 94°C, 45 seconds at 36°C and 1.5 minutes at 72°C. This program is considerably different from the standard protocol recommended by Williams et al. (1990) and that used by Garcia and Benzie (1995) for developing RAPD markers from the Australian *P. monodon*. We found that reducing the length of each phase of the program increased the intensity of amplification products, as also has been shown by Yu and Pauls (1992). This supports the proposal that decreasing the length of each phase of the PCR program helps maintain the activity of *Taq* polymerase. We also observed that increasing the number of PCR amplification cycles above 35 cycles did not significantly increase band intensity but produced smear patterns.

RAPD analysis

Two hundred 10-base primers were screened for their ability to prime PCR amplification of *P. monodon* genomic DNA. Only 84 RAPD primers (42%)

yielded amplification products, while the rest of the primers did not amplify the DNA template or resulted in smear or faint bands. Six positive primers that gave reproducible RAPD patterns with resolvable fragments that stained intensely were selected for further analysis. A total of 70 scorable bands, ranging in size from 200 to 2000 bp, were produced from RAPD analysis of three geographically different samples of Thai *P. monodon.* Thirty of these bands were monomorphic, and 40 bands were polymorphic (Table 1). Each primer generated 8 to 15 scorable bands. An example of RAPD amplification patterns and the bands scored by primer 459 is shown in Figure 1. Only reproducible bands were scored for presence or absence. Primer 428 appears to identify more variable regions of the *P. monodon* genome, whereas primers 101 and 456 show less variable RAPD patterns.

To assess the usefulness of the RAPD markers in determining the genetic diversity of *P. monodon,* we studied genetic variation of three geographically different samples of *P. monodon* in Thailand. The most important shrimp fishing grounds in Thailand are the Gulf of Thailand and the Andaman Sea, and there are different locations along both coasts where broodstocks are caught for shrimp farming (Figure 2). In this study, we compared *P. monodon* of the Andaman Sea collected from Satun and Trang provinces with that of the Gulf of Thailand collected from Chon Buri (Angsila district) and Trat provinces. The tiger prawns collected from Satun-Trang and Trat are those most commonly used as spawners in *P. monodon* farming and are candidates for use in selective breeding. However, broodstocks from Satun-Trang tend to be much larger than those from Trat and exhibit different color. Moreover, although this is still controversial, the shrimp farmers claim that the Andaman Sea broodstocks produce more eggs and better quality seeds, making the price of the Andaman Sea broodstocks 5 to 10 times higher than those of the Gulf of Thailand.

RAPD analysis of 28 individuals in each of the Satun-Trang and Trat samples and 15 individuals of the Angsila sample revealed different levels of polymorphisms. The percentages of polymorphic bands were 47.8% for Satun-Trang, 45.3% for Trat, and 24.2% for Angsila (Table 2). The results suggest that *P. monodon* collected from Angsila are the least polymorphic among the three samples. The sample collected from Angsila was small because this population is rather homogeneous. Before farming activities, no *P. monodon* was found in this area, and not until recently was *P. monodon* present in fisheries

Table 1.   Nucleotide sequences of six selected primers and number of amplified bands shown in the RAPD analysis in *P. monodon*.

Primer No.	Sequence	No. of amplified bands	No. of polymorphic bands
101	GCGCCTGGAG	9	4
174	AACGGGCAGG	15	9
428	GGCTGCGGTA	8	6
456	GCGGAGGTCC	15	7
457	CGACGCCCTG	15	10
459	GCGTCGAGGG	8	4
Total		70	40
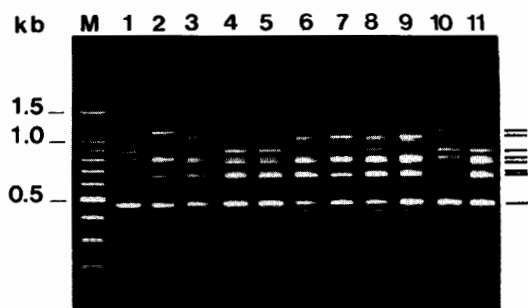
Figure 1. RAPD patterns using primer 459 of wild-caught individuals of *P monodon* collected from the Andaman Sea. Lane M: 100:bp DNA ladder. Lines indicate the bands that were scored.

catches, indicating that these shrimp may be escapees from farms in this area (K. Hassanai, personal communication). Although Angsila and Trat are a few hundred kilometers apart, prawns collected from these two areas are quite different. Broodstocks form Angsila will not be used for shrimp farming because of their lower fecundity and hatchability when compared with broodstocks from the Andaman Sea.

The percentages of polymorphic bands were comparable in Satun-Trang and Trat, suggesting similar levels of polymorphism of the two samples to be used for establishing selective breeding programs. This is in good agreement with mtDNA RFLP analysis of *P. monodon* collected from these two locations, which showed similar levels of mtDNA variations (Klinbunga, 1996). The RAPD analysis of the six fullsib families of *P. monodon* showed a much lower level of polymorphism (6.2%; Garcia and Benzie, 1995), which may be due the fact that wild-caught individuals would exhibit a higher level of genetic variation than that of the families. Studies of genetic variation in *P. vannamei* using the RAPD technique (Garcia et al., 1994) also indi-
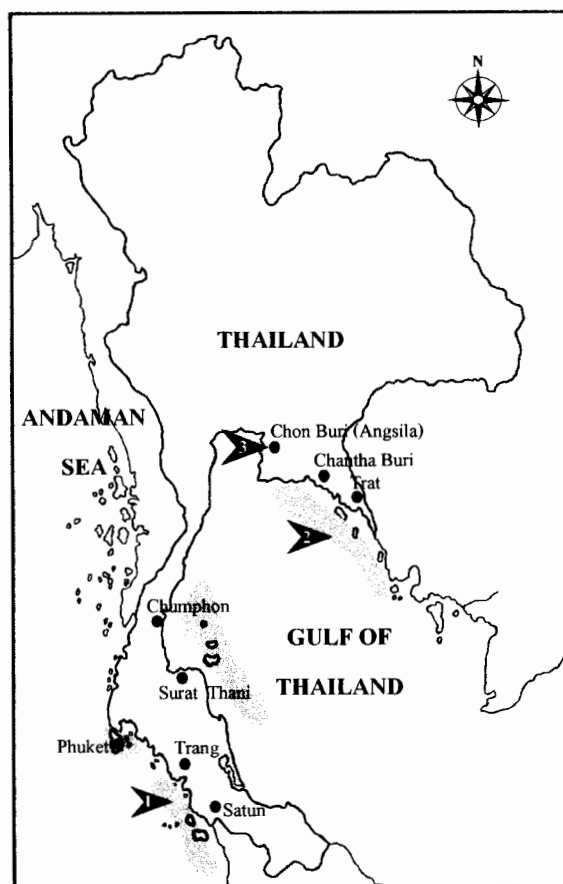
Figure 2. Map showing locations (shaded) where black tiger prawn broodstocks are commonly caught for shrimp farming in Thailand. Arrows indicate the three sample sites: 1, Satun-Trang: 2, Trat; and 3, Angsila.

cated a higher genetic variability among populations; percentages of polymorphic bands ranging from 39% to 77% have been found.
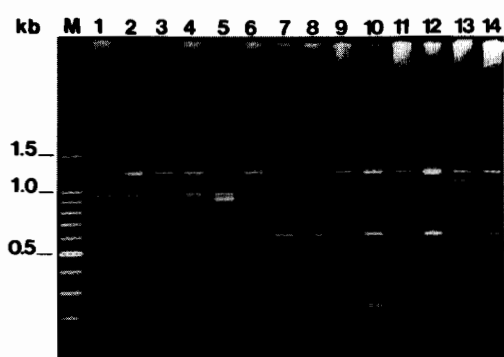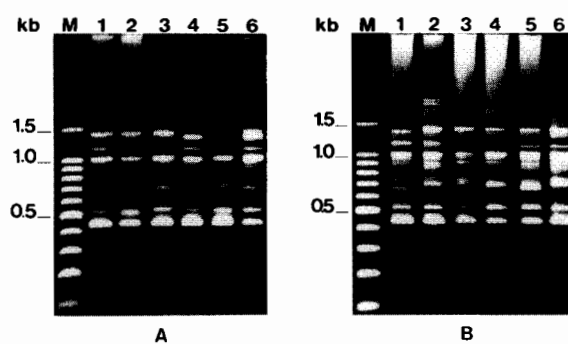
The six RAPD primers used in this study appear to detect genetic variability among the three sam-

Table 2. Total numbers of bands and percentages of polymorphic bands found in populations of *P. monodon*.

Sample	Total no. of bands present out of the possible total of 70 bands	No. of monomorphic/ polymorphic bands	Percentage of polymorphic bands
Satun-Trang	67	35/32	47.8
Trat	64	35/29	45.3
Angsila	62	47/15	24.2

ples at different levels. Figure 3 shows the results of amplification using primer 428. Different patterns were seen between Satun-Trang and Trat. A 950-bp band (indicated by the arrow) was present in more than 70% of individuals from Satun-Trang (lanes 1–7) but absent in all individuals from Trat (lanes 8–14). This band was also absent in the Angsila sample (data not shown), suggesting a population-specific marker detected by primer 428. RAPD patterns produced by other primers such as primer 174 shows less variation, and different patterns among different samples were not clearly observed (Figure 4).

The total proportion of shared bands was used to calculate the similarity coefficient (*F*) between samples (Nei and Li, 1979). As shown in Table 3, genetic similarity coefficients between samples indicated that Trat is more similar to Angsila than to Satun-Trang. However, to obtain a more stable data analysis, a larger number of primers as well as larger sample sizes are required. Liu et al. (1994) suggested that the number of primers required is correlated with the level of genetic variation of the species. Six to seven primers were sufficient to assess genetic

Figure 4. RAPD patterns using primer 174. Lane M: 100-bp DNA ladder; (A) lanes 1–6, individuals from Satun-Trang; (B) lanes 1–6 individuals from Trat.

variation within and among populations of highly polymorphic species, but at least 10 to 15 primers were required for species with a low level of genetic diversity (Demeke et al., 1992; Huff et al., 1993). For marine species, a larger number of sample sizes was required to accurately detect genetic diversity in populations than for terrestrial species. Grewe et al. (1993) caculated that to be 95% confident of detecting mtDNA haplotypes that occur in a population of lake trout (*Salvelinus namaycush*) with a frequency of 5%, 60 fishes would have to be sampled. However, O'Connel et al. (1995) showed that a sample size of 25 to 30 individuals was adequate to identify all genotypes present in Atlantic salmon populations but a larger number of individuals would be required to differentiate between closely related populations.

Figure 3. RAPD patterns using primer 428 Lane M: 100-bp DNA ladder; lanes 1–7, individuals from Satun-Trang; lanes 8–14, individuals from Trat. Lines indicate polymorphic bands; an arrow indicates the specific band that was found only in the Satun-Trang sample.

Table 3. Similarity coefficients, *F* (top diagonal), and genetic distance, *d* (bottom diagonal), for the wild populations of *P. monodon* using RAPD analysis.

Sample	Satun-Trang	Trat	Angsila
Satun-Trang	—	0.931	0.930
Trat	0.069	—	0.968
Angsila	0.070	0.032	—

This work shows the usefulness of RAPD analysis in studying genetic variation in *P. monodon*. Genetic variability found in the wild populations will be useful in selecting particular populations for establishing a selective breeding program. The RAPD method provides a simple way to detect genetic polymorphisms with a nanogram of DNA, which allows the animals to be used further in the selective breeding program. DNA extracted from the juveniles as small as PL5 was also more than enough for genetic analysis using this method. Moreover, the number of DNA polymorphisms that can be detected by the RAPD method seems virtually unlimited because the number of primers can be increased effectively as desired.

Experimental Procedures

Prawn samples and DNA extraction

Samples of black tiger prawn (*P. monodon*) were obtained from three different locations comprising Trat and Angsila in the Gulf of Thailand and Satun-Trang in the Andaman Sea from December 1994 to June 1996. Specimens were kept frozen in liquid nitrogen or alternatively stored in ethanol and transported to the laboratory for further analysis. Genomic DNAs were extracted from pleopods, which were ground with plastic pestles in microcentrifuge tubes containing 500 µl of extraction buffer (100 mM Tris-HCl, pH9.0; 100 mM NaCl; 200 mM sucrose; 50 mM EDTA; and 1% SDS). Samples were incubated at 65°C for one hour. Subsequently, proteinase K (500µg/ml) and RNase solution (100µg/ml) were added to the reaction tubes, and the samples were further incubated at 37°C for one hour. Proteins were precipitated from nucleic acids by adding 5 M potassium acetate to the final concentration of 1 M, chilled on ice for 30 minutes, and centrifuged at 12,000 rpm for 10 minutes. The supernantants were then extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). The samples were precipitated with absolute ethanol and washed twice with 70% ethanol and resuspended in TE buffer. DNA concentration was determined by spectrophotometric method using DU 650 Spectrophotometer (Beckman Instrument Inc.).

PCR amplifications and product analysis

DNA amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler (model 2400).

Amplification reactions were carried out in 25-µl reaction volume containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2 mM $MgCl_2$; 0.001% gelatin; 100 µM each dATP, dCTP, dGTP, and TTP; 0.2 µM primer; 25 ng genomic DNA; and 1 unit of *Taq* DNA polymerase (Williams et al. 1990). Ten-base oligonucleotides, numbers 101–200 and 401–500 (Biotechnology Laboratory, University of British Columbia), were used in the primer screening step. Amplification was perfomed for 35 cycles of 5 seconds at 94°C, 45 seconds at 36°C, and 1.5 minutes at 72°C.

Amplification products were analyzed by electrophoresis in 1.6% agarose gels and detected by ethidium bromide staining (Maniatis et al., 1982).

Data analysis

The RAPD patterns of individuals were scored based on band presence or absence. The index of similarity *(F)* between samples was calculated using the formula (Nei and Li, 1979):

$$F_{xy} = 2n_{xy}/(n_x + n_y),$$

where n_{xy} is the number of RAPD fragments shared by the two samples, and n_x and n_y are the numbers of RAPD fragments scored in each sample. The genetic distance (*d*) was calculated using the formula Hillis and Moritz, 1990):

$$d = 1 - F$$

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