

# Identification of *Penaeus merguensis* and *Penaeus indicus* by RAPD-PCR Derived DNA Markers

Amornrat Phongdara<sup>1</sup>, Wilaiwan Chotigeat, Ausa Chandumpai, Chanpa Tanthana and Piyanan Duangtong

Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkla 90112, Thailand.

\* Corresponding author: Tel: 66-74-211 030 ext 2618 Fax: 66-74-446 656 E-mail: pamornra@ratree.psu.ac.th

Received 2 March 1999

**ABSTRACT** Banana prawns found in Thailand consist of two species, *Penaeus merguensis* and *Penaeus indicus*, which are very similar in morphology. The samples can have confused morphology suggestive of the occurrence of hybridization. In this study, DNA patterns obtained from random amplified polymorphic DNA (RAPD) were compared between *P. merguensis* and *P. indicus*. Several species-specific DNA fragments were isolated and sequenced. Three pairs of primer were designed for the PCR reactions generating single- and multi-locus profiles which could be used as genetic markers to differentiate between the two species. At least 7 haplotypes were obtained with type 1-2 found only in *P. indicus*, whereas haplotype 3-5 were found in *P. merguensis* and haplotype 6-7 occurred in samples with confused morphology, possibly hybrids.

**KEYWORDS:** RAPD, *P. merguensis*, *P. indicus*, genetic marker.

## INTRODUCTION

Marine shrimp farming is the most important aquaculture sector in the world, with 1996 production of 693,000 tonnes worth more than US \$  $4 \times 10^9$ .<sup>1</sup> In South East Asia, Penaeoid shrimp have contributed greatly to the economy of Indonesia, Philippines, Vietnam and Thailand. Thailand in particular managed to maintain its title of "the world's largest cultured shrimp producer" for many years. From 1982 to 1998 around 127,643 to 210,000 metric tonnes of shrimp were harvested. The total export value was US \$ 2,520 - US \$ 2,800  $\times 10^6$ . Shrimp exports included fresh and frozen products, and salted, dried and smoked products. Thailand has also expanded its shrimp farming from 3,943 to 26,000 farms, with the harvested area increasing from 30,793 to 500,000 hectares.<sup>2-3</sup>

Over 90% of the shrimp produced in Thailand are cultured. Sea-caught shrimp are mostly white and pink shrimps. The black tiger shrimp, *Penaeus monodon* Fabricius, is the only species in Thailand which has been selected for intensive farming. As a consequence of the large scale and high density of farmed shrimp, the risk for disease has increased and the shortage of wild caught broodstock has started to emerge. One approach to reduce these problems is to have an alternative species for farming which also has a high international market price.

The penaeoid shrimp found in Thailand consist

of 3 families, 9 genera and 56 species; the common large shrimp species are mostly in the genera *Penaeus* and *Metapenaeus*.<sup>2</sup> White or Banana prawn is one of the indigenous species in South East Asia and its natural population is large. Several lines of evidence have suggested a high potential for intensive farming of Banana prawn.<sup>4-5</sup> This prawn includes two species which are very similar in morphology, namely *Penaeus merguensis* and *Penaeus indicus*. *P. merguensis* de Man, 1888 or Banana prawn (Australian and FAO) has the following principal taxonomic features: rostrum with high blade and teeth above and below, hepatic ridge absent and gastro-orbital ridge absent or very feebly defined. Their size for females is up to 240 mm total length and males to 200 mm. This species can be mistaken for *Penaeus indicus*, but generally can be distinguished by the higher rostral crest in adult *P. merguensis* and the presence of a distinct gastro-orbital ridge in *P. indicus*. *P. merguensis* is one of the most important commercial species in the Indo-Pacific region, being the basis of extensive fisheries in Australia, New Guinea, Indonesia, the Philippines and to a lesser extent, in Malaysia, India, Pakistan and the Persian Gulf. *P. indicus* forms the basis of major commercial fisheries in East Africa, India, Malaysia, Thailand and Indonesia.<sup>6</sup>

Apart from morphology, which is sometimes ambiguous, there is no other reliable method to identify these two species. Moreover, with the available keys it is not possible to identify larval,

postlarval and juvenile penaeid prawns to species level. Therefore, there is a need to develop an identification technique, which is easy and reliable. Such a technique would be useful for studying genetic variations of wild populations and in broodstock selection for intensive farming.

Isozymes have been widely used as genetic markers to analyze the species and variability among marine animals.<sup>8-9</sup> However, systems based on DNA markers provide more detailed genetic information suitable for identifying species, subspecies, individuals and determining their relatedness to others. Recently Williams *et al*<sup>10</sup> and Caetano-Anolles *et al*<sup>11</sup> have described a method called random amplified polymorphic DNA (RAPD) as a tool for the differentiation of genetically distinct organisms. The technique has been applied successfully in a variety of genetics, phylogenetic and population genetic studies.<sup>12-16</sup> It employs random primers in a polymerase chain reaction (PCR) to rapidly generate polymorphic bands that can serve as genetic markers to phenotypic properties such as disease resistance,<sup>17</sup> to estimate the genetic relatedness between closely related organism,<sup>18-22</sup> and to assist in breeding programs and gene mapping.<sup>23-25</sup> Also, recent studies with RAPD have identified unique, species-specific DNA fragments between *Metapenaeus ensis* and *P. japonicus*.<sup>26</sup> The technique was also used to study the genetic structure in wild populations of *Penaeus monodon* in Thailand.<sup>27-28</sup>

The study presented here was therefore undertaken to assess the RAPD patterns of *P. merguensis* and *P. indicus*, with the ultimate aim to develop a specific DNA marker for the species identification.

## MATERIAL AND METHODS

### Prawn samples and DNA extraction

Two groups of forty wild-caught prawns were collected from Andaman Sea and the Gulf of Thailand respectively. The samples were identified to species by morphology and twenty-five samples were selected. Each individual was frozen and stored at -80°C prior to DNA extraction and amplification.

Total genomic DNA was isolated from 0.3 mg of prawn muscle. The tissue was chopped into small pieces and suspended in microcentrifuge tubes that contained 0.7 M NaCl, 50 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.5% SDS, 20 mM dithiothreitol and 1 µg/ml Proteinase K. Samples were incubated at 55°C for 2 h and extracted once with phenol/chloroform (25:24v/v) solution and then with chloroform/

isoamyl alcohol (24:1v/v) mixture. The DNA was precipitated with 2/3 volumes of isopropanol and sedimented in a microcentrifuge for 2 min. The DNA pellet was dried and dissolved in water.

### Preparation of tissue extract and isozyme analysis

Approximately 1 g of tissue (muscle) was dissected out into microcentrifuge tubes. 1-2 ml of chilled homogenizing buffer (0.1 M Tris-HCl pH 7.0, 1 mM EDTA and 7 mM 2-mercaptoethanol) were added and the mixture homogenized thoroughly with a chilled steel pestle. The mixture was centrifuged at 4°C at 10,000 g for 20 min, and the clear supernatant was kept in storage tubes. Protein concentration was determined by Lowry's method. The appropriate amount of protein was loaded onto 5% acrylamide gel and electrophoresed at 200V for 1.5 hr for (80 mm long gel). The gel was stained in staining buffer (modified from Pasteur *et al*<sup>29</sup>) containing substrate (ethanol, D-glucose-6-phosphate, 6-phosphogluconate or succinate), cofactor (NAD<sup>+</sup> or NADP<sup>+</sup>), nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

### RAPD amplification

Kit-C (OPC) RAPD primers were purchased from Operon Technologies, Inc, and UBC primer from University of British Columbia. RAPD PCR amplification reactions (12.5 µl) contained 25 ng of DNA, 200 µM each dNTP, 3.0 mM MgCl<sub>2</sub>, 0.2 µM 10-mer primer, and 1.0 unit of AmpliTaq DNA polymerase (Perkin Elmer). RAPD amplifications were performed in HYBAID Thermal Cycler using the following times and temperatures: 1 cycle at 94°C, 4 min; 35 additional cycles consisting of 94°C 5 sec, 37°C or 44°C 20 sec, and 72°C 20 sec.

Following amplification, the reaction products were electrophoresed on 10x14 cm 1.4%-1.8% agarose gel for 4 h using Tris-borate-EDTA Buffer.<sup>30</sup> The gel was stained with 0.5 µg/ml of ethidium bromide.

### Isolation and cloning of RAPD products

RAPD products selected as species-specific markers were recovered from agarose gels by using Agarose Gel DNA Extraction Kit (Boehringer Mannheim). Purified DNA fragment was cloned into pGEM-T Easy (Promega) and transformed into bacterial host strain DH5α [F<sup>-</sup> φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 phoA hsdR17* (r<sub>k</sub><sup>-</sup>, m<sub>k</sub><sup>-</sup>) *sup44*λ-*thi-1 gyrA96relA1*]. Plasmid clones containing inserts were purified and the insert sizes were checked by *EcoRI* digestion. The restriction enzyme digestion was performed

according to the manufacturer's instruction (Amersham). The clones were sequenced using automated ABI 370A sequencer.

### Specific marker assay

Primers were designed using nucleotide sequence information of the insert in pGEM-TEasy vector and synthesized by GIBCO BRL Custom Primer. PCR was performed in 12.5 µl reaction solution containing 50 ng of genomic DNA, 200 µM each dNTPs, 3 mM MgCl<sub>2</sub>, 0.5 µM of forward and reverse specific primers and 1 unit of AmpliTaq (Perkin Elmer), in HYBAID Thermal Cycler under the following conditions: initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30sec, 60°C for 30 sec and 72°C for 1 min. PCR products were analyzed on 1.4% agarose or 5% polyacrylamide gel electrophoresis.

## RESULTS

### 1. Collection of Prawn Samples and Species Identification by Morphology

Twenty-five wild caught prawns were obtained from Andaman Sea and the Gulf of Thailand. The species of the samples were identified according to the taxonomic keys.<sup>2,6</sup> Samples were separated into three categories, *P. merguensis*, *P. indicus* and mixed morphology between *P. merguensis* and *P. indicus* as characterized by the appearance of rostrum, petasma or thelycum and gastro-orbital ridge. Table 1 lists the morphology and species of each sample; letter "M" or "I" represents the morphology of each part that was indicated by the keys for *P. merguensis* and *P. indicus*, respectively. It was noticed that a number of samples were difficult to identify because of the aberration of the morphology. Therefore, these samples were indicated as "mixed morphology" (Mix).

### 2. Isozyme Patterns of *Penaeus* sp.

Because of the ambiguity in morphology seen in some samples in Table 1, the species of individual specimens were further determined by isozymes analysis. Proteins were separated by electrophoresis and gel was incubated in staining solutions containing appropriate substrate and cofactor for the activity staining. The zymograms of alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G-6-PDH), succinate dehydrogenase (SCDH), 6-phosphogluconate dehydrogenase (6-

PDH), were obtained (data not shown). The isozyme pattern of ADH showed three isoforms, ADH1, ADH2 and ADH3 (in order of increasing mobility in the gel). Individuals which were identified by morphology as *P. indicus* had isoform ADH2 and ADH3 whereas individuals of *P. merguensis* had ADH1, ADH2 and ADH3. *P. merguensis* also had extra slow-moving isoforms of SCDH, G-6-PDH and 6-PDH. Therefore, prawn samples were classified into *P. merguensis* and *P. indicus* according to the presence and absence of slow moving isoform of ADH, SCDH, G-6-PDH and 6-PDH (Table 1).

### 3. Screening of RAPD Primers

One hundred and twenty three primers were used in the screening experiments. The PCR conditions used for screening were 94°C for 5 sec, 37°C for 20 sec and 72°C for 20 sec. Primers were chosen for further studies based on the criterion that the DNA patterns were consistent in several trials and there were polymorphs among the samples tested. *Metapenaeus* sp. and *P. monodon* were included in the screening, as the primers which gave patterns that could differentiate *P. merguensis* and *P. indicus* from *P. monodon* and *Metapenaeus* sp. were chosen in the first round of screening. At the same time, the primers that gave the band specifically found either in *P. merguensis* or *P. indicus* were also considered.

Using the above criteria, 3 primers, OPC 06, UBC 701 and UBC 787, were found to be suitable for further investigation.

### 4. Optimization of PCR for Analysis with Selected RAPD Primers

The optimal PCR conditions of the four RAPD primers were investigated by varying the annealing temperature from 37°C to 50°C. It was found that the RAPD patterns obtained from these primers were more distinct (low smear background) when the annealing temperatures were between 42 - 44°C. At higher annealing temperature, though several bands disappeared, the major bands remained the same. Therefore, the annealing temperature was chosen at 44°C and the RAPD patterns obtained (see Fig 1) are analyzed as follows.

#### 4.1 Primer OPC-06

The bands appearing on 1.4% agarose gel electrophoresis ranged from 8-14 bands. The banding at region close to 500 bp (Fig 1, indicated by arrow) was of interest because from 1 to 4

**Table 1.** A list of samples used in this study with their species identification specified by morphology, isozyme patterns and DNA patterns.

Sample	Sex	Morphology			Species Identified by Morphology	Species Identified by Isozymes	Haplotypes
		P/T	R	GO			
A1	female	ND	ND	ND	<i>Metapenaeus</i> sp.	ND	-
A2	male	ND	ND	ND	<i>Metapenaeus</i> sp.	ND	-
A3	male	ND	ND	ND	<i>Metapenaeus</i> sp.	ND	-
A4	male	ND	ND	ND	<i>Metapenaeus</i> sp.	ND	-
A5	female	M	I	I	Mixed	<i>P. indicus</i>	1
A6	male	I	I	I	<i>P. indicus</i>	<i>P. indicus</i>	2
A7	female	I	I	I	<i>P. indicus</i>	<i>P. indicus</i>	2
A8	female	I	I	I	<i>P. indicus</i>	<i>P. indicus</i>	1
A9	female	I	I	I	<i>P. indicus</i>	<i>P. indicus</i>	1
A10	male	M	I	I	Mixed	<i>P. indicus</i>	1
A11	female	I	I	I	<i>P. indicus</i>	<i>P. indicus</i>	2
A12	female	I	I	I	<i>P. indicus</i>	<i>P. indicus</i>	2
A13	female	?	I	I	Uncertain	<i>P. indicus</i>	1
A14	female	I	I	?	Uncertain	<i>P. indicus</i>	1
D1-D6					<i>P. monodon</i>	ND	-
E1	female	M	M	M	<i>P. merguensis</i>	<i>P. merguensis</i>	3
E2	male	M	M	M	<i>P. merguensis</i>	<i>P. merguensis</i>	4
E3	female	M	M	M	<i>P. merguensis</i>	<i>P. merguensis</i>	4
E4	female	I	I	I	<i>P. indicus</i>	<i>P. merguensis</i>	4
E5	female	I	I	I	<i>P. indicus</i>	<i>P. merguensis</i>	2
E6	female	M	M	M	<i>P. merguensis</i>	<i>P. merguensis</i>	4
E7	female	M	M	I	Mixed	<i>P. merguensis</i>	4
E8	female	M	M	M	<i>P. merguensis</i>	<i>P. merguensis</i>	6
E9	female	I	I	I	<i>P. indicus</i>	<i>P. merguensis</i>	5
E10	female	M	M	M	<i>P. merguensis</i>	<i>P. merguensis</i>	4
E11	male	?	I	I	Uncertain	<i>P. indicus</i>	7
E12	male	?	M	I	Mixed and uncertain	<i>P. merguensis</i>	6
E13	female	I	I	I	<i>P. indicus</i>	Uncertain	7
E14	female	M	I	M	Mixed	<i>P. merguensis</i>	4
E15	male	?	I	I	Uncertain	<i>P. merguensis</i>	6

Abbreviation: P = Petasma or Thelycum, R = Rostrum, GO = Gastro-orbital, ND = not determined, ? = uncertain, M = the morphology is follow the taxonomic key as *P. merguensis*, I = the morphology is follow the taxonomic key as *P. indicus*.

fragments were obtained with *P. merguensis* and *P. indicus* individuals whereas no amplified products were observed in *Metapenaeus* sp. and *P. monodon* specimens. This result suggested the potential of using 500 bp DNA fragments as specific markers for *P. merguensis* and *P. indicus*.

4.2 Primer UBC-701

This primer gave a complicated DNA pattern and high polymorphism. Each band was not easily separated on 1.8% agarose gel electrophoresis. However, there was a fragment at 200 bp (Fig 1, indicated by arrow) which was present or absent in some samples.

4.3 Primer UBC-787

This primer produced a strong DNA band at 600 bp. (Fig 1, indicated by arrow). The fragment was present (A5, A6, A9), absent (E1) or appeared at another position on the gel (data not shown).

5. Isolation and cloning of RAPD Products

The four RAPD products selected as described in section 4 were recovered from agarose gel. The products were purified and cloned into pGEM-TEasy (Promega). Both strands of the insert in each clone were sequenced and the sequences were then used to design PCR primers(Fig 2). The sequences of designed primer pairs are shown in Fig 2 as bold and italic letters

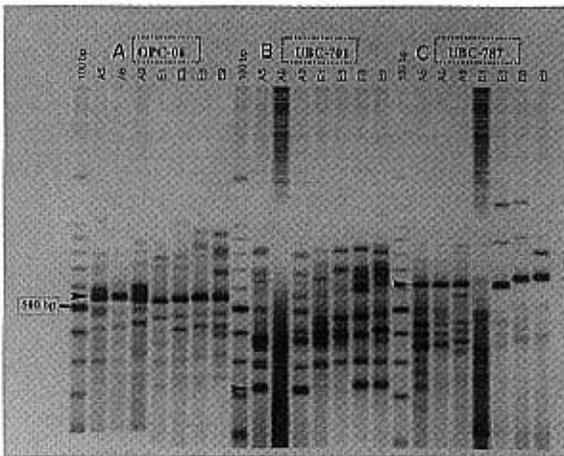
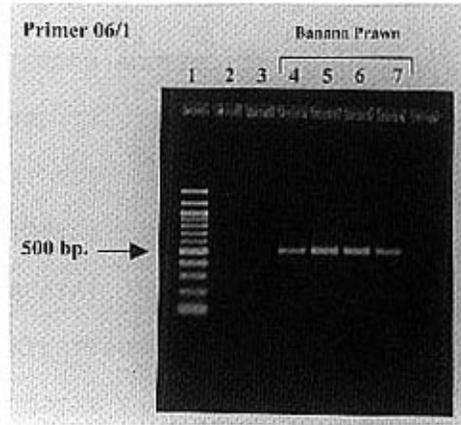
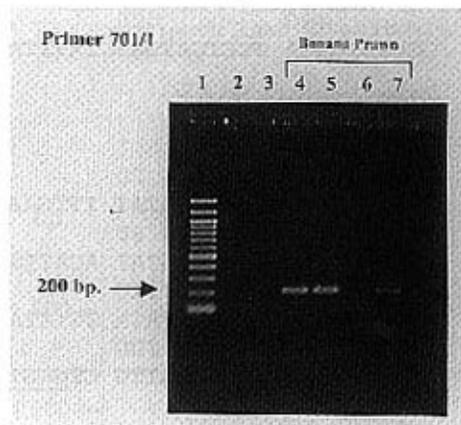


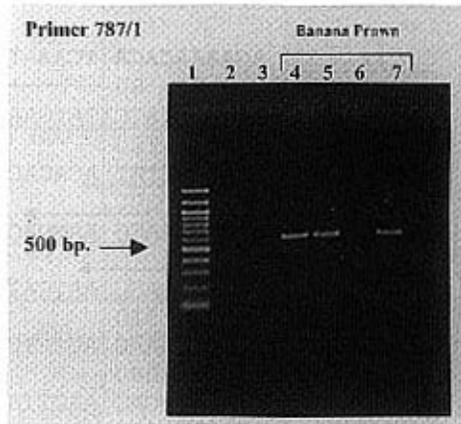
Fig 1. Image of RAPD patterns of seven individuals of Banana prawn acquired from Gel Documentation system (Bio-Rad). Total DNA was amplified using primers: (A) OPC 06 (B) UBC 701 and (C) UBC 787 under condition given in "Materials and Methods". Arrows indicate the specific bands that were cloned and sequenced. Fragments of 100 bp ladder (Promega) were used as molecular weight markers.



(A)



(B)



(C)

Fig 3. Amplification of genomic DNA of four individuals of Banana prawn (lane 4, A6; lane 5, A9; lane 6, E1; lane 7, E9), on 1.4 % agarose gel electrophoresis, compared with the amplification of two additional species, *P. monodon* (lane 2, D1) and *Metapenaeus* sp. (lane 3, A1). Three specific primers were used in the PCR reaction: (A) 06/1, (B) 701/1 and (C) 787/1. These primers are specific to Banana prawn as no PCR product appeared in lane 2 and 3. The size of PCR product obtained from each primer is the same as those observed in RAPD profiles (indicate by arrow in Fig 1). Additional details are given in the text.

## (A)

10	20	30	40	50	60
<u>GAACGGACTC</u>	<u>TCTCCTTCGA</u>	<u>CTCTGGAATG</u>	TCCTTGACCA	TCAATTTAGC	TCGGGATAAG
70	80	90	100	110	120
TTTATTTATC	TGTTTATCTG	TTTGTTTATT	TATTTATGTA	TCTATCTGTC	TGTGAACCTA
130	140	150	160	170	180
TCTATATCTG	TTTATCTATC	TGTCTATCTG	TTAATCTATC	AATTATATAT	TGTTTATGTA
190	200	210	220	230	240
TCTATTTATC	TATTTGATTT	TATTGTAGTA	AAGTTAGGAA	TTTCAGATC	TCATTTATTA
250	260	270	280	290	300
AGTTCAAATT	TCAACAATAG	TATACATTTT	ATAATCTTTT	GTTAGTAATA	TTGGAAGACA
310	320	330	340	350	360
AGTTCTCAAT	AATTGCATAG	TTATTAGACG	ATAAGTTTGA	AATTTGATA	TAAATATATA
370	380	390	400	410	420
TATATCTGTT	TATGACTAAT	TATGGTTTGA	GACTATTTCA	CTGTGTATTA	GTGGTGTATT
430	440	450	460	470	480
AATGACTTTT	TGGTSTTATC	AAGGCGACTG	TTAAACCATC	ACGAVIAATC	CGCTAGGTTT
490	500	510	520		
GCCTCTCGTA	<u>TCCTGATGAT</u>	<u>TATTTACGCA</u>	<u>TGAGTCCGTT</u>	<u>C</u>	

## (B)

10	20	30	40	50	60
<u>CCCACAAACC</u>	<u>TCAGAGATAT</u>	<u>TTTGAATTGG</u>	GAGTTACGAA	CCTCCTGCAA	TAAGGTAGCG
70	80	90	100	110	120
AATATGGTAT	ATATGTATAT	ACATTATATA	TTTTTTTCTT	CTTTTTTAC	TTTTCTTCAT
130	140	150	160	170	180
TAATGCTGTT	ACAGTATATA	TGTATAGATG	TAACACGATA	TAACTGATTT	GTATAACCAG
190	200	210			
TAAATAACCT	<u>AATCTATTG</u>	<u>CTGGGTTGTG</u>	<u>GG</u>		

## (C)

10	20	30	40	50	60
<u>CCCTTCTTCC</u>	<u>AGAAAACACA</u>	<u>TCAAATGCAT</u>	CAGTTCCCTAA	TAAGCAATAA	TCATTCACAC
	→ 80	→ 100	→ 110	→ 180	
ACGAAGTAAT	<u>GAAGCATATC</u>	<u>TATGAAGCAG</u>	<u>CGATCATTCA</u>	<u>CACACGTAGT</u>	<u>AATGAAGCAT</u>
	→ 140	→ 150	→ 160	→ 170	→ 180
<u>ATATATGAAG</u>	<u>CAATGATTAT</u>	<u>ACACACACAA</u>	AGTAATGAAG	CATATATATG	AAGCAATGAC
190	200	210	220	230	240
TATACACACA	<u>CAAAGTAATG</u>	<u>AAGCACATCT</u>	<u>ATGAAGCAGC</u>	<u>GATCATTAC</u>	<u>ACACGTAGTA</u>
	→ 270	→ 280	→ 300		
<u>ATGAAGCATA</u>	<u>TATATGAAGC</u>	<u>AATGATTATA</u>	CCGCACACCG	AAGTAATGAA	GCATATCTAT
→ 310	→ 320	→ 330	→ 340	→ 350	→ 360
GAAGCACAAA	GACAAGGAAC	ACTATCCACG	ACCATTAAAA	TTAACTAAGA	AGAGAAATAA
370	380	390	400	410	420
ACAGAAAGCA	GTATAAACAC	AACTTAGGTA	TGAATCCTAC	TACAACGTGA	TTAAAGATGA
430	440	450	460	470	480
GATGGTGGAC	ATAAATATCC	ATGACTGAAC	CAGTTGTGGG	TGGGATTATG	AATGAAAAAT
490	500	510	520	530	540
AAATGGACAA	CGTACAATAC	TGAAAACGTT	TTAAGGGACG	AGACAGAATA	ACACGAAACA
550	560	570	580	590	600
CATTCATCAT	CAGAATTGTA	AAATGAAAAA	AAAGAAAAAA	<u>AAAAATACTG</u>	<u>AGGAAAGAAG</u>

Fig 2. Nucleotide sequences of clone (A) 06/1, (B) 701/1 and (C) 787/1. Computer-assisted homology searches of the GeneBank data base revealed no significant matches with known nucleotide sequences. The ten base RAPD primer sites are underlined. Sequence of repeated units are underlined with arrow head.

Three primer pairs were tested against the sample DNA templates. The results demonstrated that the primers amplified fragments with the same molecular weight as the fragment from which were originally isolated and that they only amplified products from homologous DNA templates (see Figs 3A-3C). The primers were also tested for their specificity to Banana prawn by the lack of amplification when *Metapenaeus* sp. and *P. monodon* DNA were used (Fig 3A-3C, lanes 2 and 3). Among these three specific primer pairs, only the pair from clone 06/1 could reproducibly distinguish *P. merguensis* from *P. indicus*. Specifically, the 06/1 primer pair amplified a single 521-bp DNA fragment from *P. indicus* DNA (Fig 4, samples 2-5) whereas an additional 650-bp or > 1,000 bp fragment was amplified in *P. merguensis* (Fig 4, samples 6-9). None of these two bands was obtained in the samples of mixed morphology.

The 701/1 and 787/1 specific primer pairs also were used to differentiate between *P. merguensis* and *P. indicus*. (Fig 3). No obvious correlation could be made linking these two markers with the morphology or zymograms of the species. When the patterns of the amplified DNA fragments were grouped together several polymorphisms within each species were identified and at least 7 haplotypes were revealed (Fig 5). Haplotypes 1 and 2 were found in *P. indicus*, haplotypes 3-5 and 6-7 were found among *P. merguensis* and samples with mixed morphology, respectively (Table 1).

**DISCUSSION**

The goal of this work was to develop techniques that will allow for identification of the two species of Banana prawns, *Penaeus merguensis* and *Penaeus indicus*. RAPD technique was used to identify DNA markers that were specific to the two species. Cloning and sequencing of the species-specific DNA markers were performed in order to design primer pairs that can be used in PCR-based analysis.

Clone 06/1 was an important marker, with amplification of *P. indicus* genomic DNA using the designed primer pairs giving a single-band PCR product on 5% polyacrylamide gel electrophoresis. In contrast, the PCR products obtained from *P. merguensis* were two bands and the locus was polymorphic. Faint bands (ranging from 1-3 bands) were produced from samples with mixed morphology. The results of the identification of Banana prawn species by clone 06/1 primer pairs correlated with the results from morphology and zymograms.

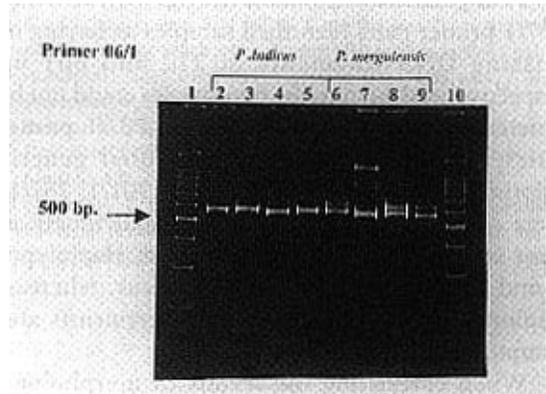


Fig 4. Amplification of genomic DNA of eight individuals of Banana prawn (lane 2-5, A5, A6, A8, A9; lane 6-9, E1, E2, E3, E9) using specific primer 06/1 on 5 % Polyacrylamide gel electrophoresis

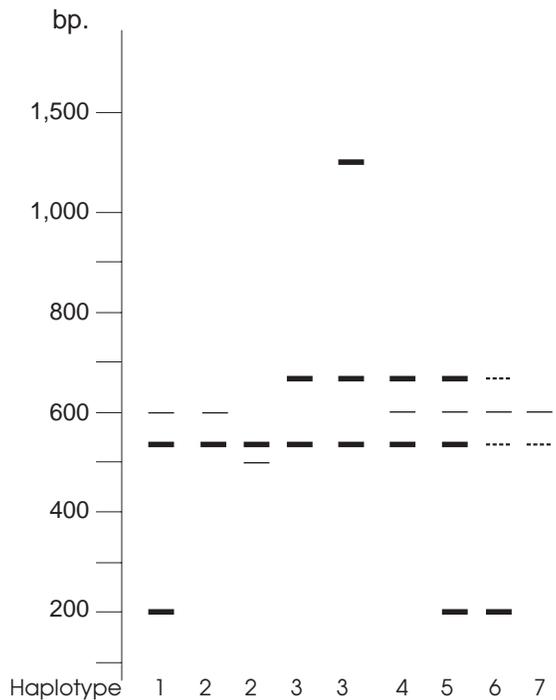


Fig 5. DNA patterns of seven haplotypes of samples from *P. merguensis* and *P. indicus*. Drawing was obtained from PCR products of the PCR reaction with three specific primers 06/1 (—), 701/1 (—, at 212 bp.) and 787/1 (—). (.....) represent faint band obtained from primer 06/1.

PCR-generated fragments from clone 701/1 and 787/1 primer pairs identified samples as having or missing DNA fragments of 212 and 601 bp respectively. Although, the two species could not be differentiated directly by 701/1 and 787/1 primer pairs, these primers gave additional genetic information. When three loci (06/1, 701/1, 787/1) were compared collectively among individuals, at least seven haplotypes were revealed. Haplotypes 1 and 2 were only found in *P. indicus*, whereas, haplotypes 3-7 were found in *P. merguensis* and samples with mixed morphology.

When correlating the results of morphology, zymogram and DNA fingerprint (Table 1), it seems that we could identify *P. indicus* easily by using our three specific primers. To identify *P. merguensis* was more complicated because of the limited number of samples, which could be identified confidently by morphology as *P. merguensis*. Therefore, we do not know which DNA patterns should be chosen to represent true *P. merguensis*. However, haplotypes 3-5 are likely candidates. Haplotypes 6 and 7 were found mostly in samples with mixed or confused morphology. These individuals are probably morphological deviants of *P. indicus* or *P. merguensis* or hybrids of the two species. In order to make a final conclusion, we need to develop more primers and test with more samples. Moreover, the results of abundant samples of haplotypes 1 and 2 from the Andaman Sea and those of haplotypes 3-7 in the Gulf of Thailand confirmed our former observation of penaeoid shrimp distribution in Thailand.<sup>2</sup> Therefore these markers are certainly useful for specific identification of the two species. To confirm this specificity (Fig 3), these primer pairs were tested against *Metapenaeus* sp. and *P. monodon* DNA. None of these genomes gave PCR products with our primers.

To further investigate the nature of the cloned RAPD-derived DNA fragments, their sequences data were analyzed using DNASIS program (Hitachi Software Engineering, Co Ltd). Two potential open reading frames (ORFs) of significant size were identified from the insert of clone 06/1. One ORF started at position 28 and extended to position 283. The other started at position 96, and contained 98 bp. Further studies by Northern analysis are required to demonstrate the presence of the transcript. Nucleotide sequences of the other two clones, 701/1 and 787/1, did not indicate any ORF. However, both clones appeared to be derived from repetitive DNA segments. In particular, two 73 bp repeats with at least 12 sets of internal directed repeats of ATGAAGCA were found in the sequence of 787/1

clone (Fig 2, indicate by an arrow above the sequences). Although, there were no perfect repeats similar to satellite or microsatellite observed in the sequences of 701/1 and 787/1, these clones will be useful for further investigation of repetitive DNA segments of the Banana prawn genomes.

These results confirm that the approach of isolating DNA fragments generated by RAPD allowed taxonomic studies in Banana prawns. Together with other primer pairs developed in the future, multi-locus markers will be available which can be used as a genetic analysis tool to study genetic diversity in natural populations and to manage breeding programs of cultured species.

## ACKNOWLEDGMENT

This work was supported by grant BT-38-06-API-18-45 from National Science and Technology Development Agency, Ministry of Science, Technology and Environment, Thailand. We would like to thank Professor Dr Vichai Boonsang, Mahidol University and Associate Professor Anchalee Tassanakajon, Chulalongkorn University for advice at the beginning of the project. Thanks are also due to Dr Allan Geater for reading the manuscript.

## REFERENCES

- Wyban J (1997) Recent progress in domestication of marine shrimp. In: Abstracts of the 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology, 24, 7-10 May 1997, Phuket, Thailand.
- Chaitiamvong S and Supongpan M (1992) A Guide to Penaeoid Shrimps Found in Thai Waters. Australian Institute of Marine Science Townsville, Australia.
- Lin CK and Nash GL (1996) Asian Shrimp News, Collected Volume, 1989-1995. Published by the Asian Shrimp Culture Council, Bangkok, Thailand.
- Sangpradab S, Krilrum S and Laitim W (1987) Pond culture of banana prawn (*Penaeus merguensis* de Man) at various stocking densities. Technical Paper, 4, Dept Fisheries, Thailand.
- Chandumpai A (1998) Development of techniques for intensive farming of banana prawn. Seminar in Biotech Forum, National Science and Technology Development Agency. March 9th, 1998, Bangkok, Thailand.
- Grey DL, Dall W, and Baker A (1983) A Guide to the Australian Penaeid Prawns. Northern Territory Government Printing Office, Australia.
- Heales DS, Polzin HG and Staples DJ (1985) Identification of the postlarvae of the commercially important *Penaeus* species in Australia. Second Australian National Prawn Seminar, NPS2, Cleveland, Australia, 41-6.
- Lavery S and Staples D (1990) Use of allozyme electrophoresis for identifying two species of *Penaeid* prawn postlarvae. *Australian Journal of Marine and Freshwater Research* 41, 259-66.
- Shaklee JB (1983) The utilization of isozymes as gene markers in fisheries management and conservation. In: *Isozymes*.

- Current Topics in Biological and Medical Research. Vol II. Eds. Rattazzi, JG, Alan R Liss, Inc, New York.
10. Willaims JGK, Kubelik R, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18, 6531-5.
  11. Caetano-Anolles G, Bassam BJ and Gresshoff PM (1991) DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology* 9, 553-7.
  12. Caetano-Anolles G, Bassam BJ and Gresshoff PM (1991) DNA amplification fingerprinting: a strategy for genome analysis. *Plant Mol Biol Rep* 9, 294-307.
  13. Bassam BJ, Caetano-Anolles G and Gresshoff PM (1992) DNA amplification fingerprinting and its potential application for genome analysis. In: *Current Topics in Plant Molecular Biology*, 1, pp 8-16. Chapman and Hall, New York.
  14. Hardrys H, Balick M and Schierwater B (1992) Amplifications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol Ecol* 1, 55-63.
  15. Tingey SV and del Tufo JP (1993) Genetic analysis with random amplified polymorphic DNA markers. *Plant Physiol* 101, 349-52.
  16. Lynch M and Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Mol Ecol* 3, 91-9.
  17. Martin GB, Williams GK and Tanksley SD (1991) Rapid identification of markers linked to *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. *Proc Natl Acad Sci USA* 88, 2336-40.
  18. Jones CS, Okamura B and Noble LR (1994) Parent and larval RAPD fingerprints reveal outcrossing in freshwater bryozoans. *Mol Ecol* 3, 193-9.
  19. Joachim A, Tenter AM, Jeffries AC and Johnson AM (1996) A RAPD-PCR derived marker can differentiate between pathogenic and non-pathogenic *Sarcocystis* species of sheep. *Molecular and Cellular Probes* 10, 165-72.
  20. Salimath SS, de oliveira AC, Godwin ID and Bennetzen JL (1995) Assessment of genome origins and genetic diversity in the genus *Eleusine* with DNA markers. *Genome* 38, 757-63.
  21. de Oliveira AC, Richter T and Bennetzen JL (1996) Regional and racial specificities in sorghum germplasm assessed with DNA markers. *Genome* 39, 579-87.
  22. Berretta M F, Lecuona RE, Zandomeni RO and Grau O (1998) Genotyping isolates of the entomopathogenic fungus *Beauveria bassiana* by RAPD with fluorescent labels. *J Invertebr Pathol* 71, 145-50.
  23. Garcia DK, Faggart MA, Rhoades L, Wyban JA, Carr WH, Sweeney JN, Ebert KM and Alcivar-Warren AA (1994) Genetic diversity of cultured *Penaeus vannamei* shrimp using three molecular genetic techniques. *Mol Mar Biol Biotechnol* 3, 270-80.
  24. Garcia DK and Benzie JAH (1995) RAPD markers of potential use in penaeid prawn (*Penaeus monodon*) breeding programs. (1995). *Aquaculture* 130, 137-44.
  25. Garcia DK, Dhar AK and Alcivar-Warren AA (1996) Molecular analysis of a RAPD marker (B20) reveals presence of two microsatellites and differential mRNA expression in *Penaeus vannamei*. *Mol Mar Biol Biotechnol* 5, 71-83.
  26. Meruane J, Takagi M and Taniguchi N (1987). Species identification and polymorphisms using RAPD-PCR in penaeid prawns *Penaeus japonicus* and *Metapenaeus ensis*. *Fish Sci* 63, 149-50.
  27. Tassanakajon A, Pongsomboon S, Rimphanitchayakit V, Jarayabhand P and Boonsaeng V (1997) Random amplified polymorphic DNA (RAPD) markers for determination of genetic variation in wild populations of the black tiger prawn (*Penaeus monodon*) in Thailand. *Mol Mar Biol Biotechnol* 6, 110-5.
  28. Tassanakajon A, Pongsomboon S, Jarayabhand P, Klinbunga S and Boonsaeng V (1998) Genetic structure in wild populations of black tiger shrimp (*Penaeus monodon*) using randomly amplified polymorphic DNA analysis. *J Mar Biotechnol* 6, 249-54.
  29. Pasteur N, Pasteur G, Bonhomme F, Catalan J and Britton-Davidian J (1988) *Practical Isozyme Genetics*. Ellis Horwood Limited, UK.
  30. Sambrook J, Fritsch E and Maniatis T (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed, Vol. 1-3. New York: Cold Spring Harbor Laboratory Press.