

Species Identification of Five Penaeid Shrimps Using PCR-RFLP and SSCP Analyses of 16S Ribosomal DNA

Bavornlak Khamnamtong^{†,‡,§}, Sirawut Klinbunga^{†,§,*} and Piamsak Menasveta^{†,§}

[†]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Paholyothin Rd., Klong 1, Klong Laung, Pathumthani 12120

[‡]Program of Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok, 10330

[§]Center of Excellence for Marine Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand

Received 8 February 2005, Accepted 28 April 2005

DNA-based molecular markers for differentiation of five penaeid shrimps (*Penaeus monodon*, *P. semisulcatus*, *Feneropenaeus merguensis*, *Litopenaeus vannamei* and *Marsupenaeus japonicus*) were developed based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and single-stranded conformation polymorphism (SSCP) of 16S ribosomal (r) DNA. Differentiation of *P. monodon*, *P. semisulcatus* and *L. vannamei* can be unambiguously carried out by PCR-RFLP of 16S rDNA₅₆₀ whereas *P. semisulcatus* and *M. japonicus* shared a BABB mitotype. These shrimps were successfully discriminated by SSCP analysis of 16S rDNA₅₆₀. Nevertheless, the amplification success for *L. vannamei* and *F. merguensis* was not consistent when tested against larger sample sizes. As a result, 16S rDNA₅₆₀ of an individual representing the most common mitotype of each species was cloned and sequenced. The new primer pair was designed and tested against the large sample sizes (312 bp product, $N=185$). The amplification success was consistent across all species. PCR-RFLP of 16S rDNA₃₁₂ was as effective as that of 16S rDNA₅₆₀. Differentiation of all shrimp species were successfully carried out by SSCP analysis.

Keywords: Genetic marker, mtDNA, PCR-RFLP, SSCP, Penaeid shrimps

Introduction

World cultured shrimp production (mainly *Penaeus monodon* and *Litopenaeus vannamei*) has rapidly increased since the last two decades. It was estimated that approximately 341000 metric tons (MT) of farmed shrimps were produced by the shrimp industry in 1986 and this has more than doubled (638000-855500 MT) since 1993 (Rosenberry, 2001).

Thailand has been regarded as the leading shrimp producer (mainly *P. monodon*) for more than a decade with the production equal or exceed 200000 MT annually. However, the industry has consistently encountered production losses from infectious diseases, particularly from white spot syndrome virus (WSSV), yellow head virus (YHV) and *Vibrio* sp..

At present, the production cycle of *P. monodon* has yet to be complete because breeding of pond-reared *P. monodon* rarely produced enough quality of larvae required by the industry. Accordingly, aquaculture hatcheries are almost totally reliant on wild-caught broodstock resulting in overexploitation of natural *P. monodon*. Besides problems from diseases, the lack of high quality wild and/or domesticated broodstock of *P. monodon* has possibly caused an occurrence of a large portion of stunted shrimps at the harvest time (3-5 g body weight at 4 month cultivation period). As a result, *L. vannamei* has been introduced to Thailand as an alternative cultured species and initially contributed approximately 20000 MT of the production in 2002 and dramatically increased to 170000 and 220000 MT in 2003 and 2004, respectively (Limsuwan, 2004).

Owing to morphological similarity at the larval stages, larvae of *F. merguensis* are intentionally traded as those of *L. vannamei*. In addition, the external morphology of *P. monodon* and *P. semisulcatus* is resemble at all stages of development but the growth rate of *P. semisulcatus* is approximately 3 times lower than that of *P. monodon* (B.

*To whom correspondence should be addressed.
Tel: 66-2-2185279; Fax: 66-2-2547680
E-mail: sirawut@biotec.or.th

Withyachumnarnkul, personal communication). Once the shrimp is processed (e.g. leaving only the shrimp meat), species identification becomes problematic. Accordingly, species-diagnostic markers play important roles to prevent supplying incorrect shrimp larvae for the industry and for quality control of shrimps exported from Thailand.

Molecular phylogeny of penaeid shrimps has been reported based on nucleotide sequences of COI (Baldwin *et al.*, 1998), 16S rDNA and COI (Lavery *et al.*, 2004) and AFLP (Wang *et al.*, 2004). Phylogenetic trees revealed close genetic relationships between *P. monodon* and *P. semisulcatus* (subgenera *Penaeus*) but distant relationships were observed among economically important shrimps from different genera (*P. monodon*, *P. semisulcatus*, *F. merguensis*, *L. vannamei* and *Marsupenaeus japonicus*). Nevertheless, simple molecular markers for differentiation of these shrimps have not been reported.

The use of polymerase chain reaction (PCR) in combination with restriction enzymes digestion, RFLP (Thaewnon-ngiw *et al.*, 2004) or single-stranded conformation polymorphism, SSCP (Weder *et al.*, 2001) are favored for identifying species-origins of shrimp products due to their convenient and cost-effective.

Weder *et al.* (2001) used SSCP patterns of a 148 bp cytochrome *b* gene segment to identify species origins from raw materials of several fish and animal species. SSCP patterns of 2-4 bands were obtained from blue ling, carp, haddock, mackerel, mackerel shark, saithe, catfish, Alaska pollack and skipjack. The patterns were fish species-specific and the method could be used to identify Alaska pollack in surimi-based products. Inter-laboratory results suggested reproducibility of SSCP analysis for species identification purposes.

To date, there have been no publications concerning species-diagnostic markers of indigenously important shrimp species (*P. monodon*, *P. semisulcatus* and *F. merguensis*) and the introduced species (*L. vannamei*) in Thailand. The objective of this study was development of simple and reliable methods for identification species origin of five penaeid shrimps. Primers were designed from 16S rDNA of *P. monodon*, *P. semisulcatus*, *L. vannamei*, *F. merguensis* and *M. japonicus*. Species-specific PCR-RFLP and SSCP markers of these taxa were successfully developed and practically applied for tracing species origins of suspected shrimps.

Materials and Methods

Sampling Broodstock-sized penaeid shrimps including the giant tiger shrimp; *P. monodon* ($N=86$), the green tiger shrimp; *P. semisulcatus* ($N=15$), the banana shrimp; *F. merguensis* ($N=38$), the introduced white shrimp; *L. vannamei* ($N=30$) and the kuruma shrimp; *M. japonicus* ($N=16$) were collected (Table 1). Specimens were kept at -30°C until required.

DNA extraction Total DNA was extracted from a piece of pleopod of each shrimp using a phenol-chloroform-proteinase K method (Klinbunga *et al.*, 1996). The concentration of extracted

Table 1. Sample collection sites and sample sizes of penaeid shrimps used in this study

Species	Sample location	Sample size ^a
<i>P. monodon</i>	Chumphon (GOT)	15 (4)
	Trat (GOT)	15 (4)
	Satun (west of PT)	15 (4)
	Trang (west of PT)	15 (4)
	Phangnga (west of PT)	15 (4)
<i>P. semisulcatus</i>	Ranong (west of PT)	11 (9)
	Chumphon (GOT)	11 (11)
<i>F. merguensis</i>	Phuket (west of PT)	4 (4)
	Samyan Market ^b	7 (7)
<i>L. vannamei</i>	Chonburi (GOT)	17 (7)
	Indonesia (west of PT)	14 (0)
	Mexico*	6 (2)
<i>M. japonicus</i>	Ratchaburi (central Thailand)*	12 (2)
	Rangsit (central Thailand)*	12 (10)
	Japan*	16 (7)

^aNumber in parentheses are individuals analyzed by universal primers of 16S rDNA (16S rDNA₅₆₀)

^bunknown origin

*cultivated stocks. GOT = Gulf of Thailand, PT = peninsular Thailand

DNA was spectrophotometrically estimated. DNA was stored at 4°C until needed.

PCR using universal primers of COI-COII and 16S rDNA and restriction analysis The COI-COII gene segment (1550 bp) of each *P. monodon* ($N=29$) and *P. semisulcatus* ($N=15$) was amplified using primers and conditions described by Roehrdanz (1993) and Klinbunga *et al.* (2001b), respectively.

In addition, the 16S rDNA (560 bp, hereafter called 16S rDNA₅₆₀) fragment of *P. monodon* ($N=29$), *P. semisulcatus* ($N=15$), *F. merguensis* ($N=14$), *L. vannamei* ($N=14$) and *M. japonicus* ($N=7$) was amplified using 16S_{F1} (5' CGC CTG TTT AAC AAA AAC AT 3') and 16S_{R1} (5' CCG GTC TGA ACT CAG ATC ATG T 3'; Palumbi *et al.*, 1991) according to Klinbunga *et al.* (2001b).

The COI-COII and 16S rDNA amplification products were singly digested with *Dra* I (TTT/AAA), *Ssp* I (AAT/ATT) and *Vsp* I (AT/TAAT) and *Alu* I (AG/CT), *Mbo* I (/GATC), *Ssp* I and *Vsp* I, respectively. The restricted products were electrophoresed through 1.0% (COI-COII) and 1.5% (16S rDNA) agarose gels and visualized under a UV transilluminator after ethidium bromide staining (Maniatis *et al.*, 1982).

Cloning and sequencing of 16S rDNA₅₆₀ of penaeid shrimps The 16S rDNA₅₆₀ segment was amplified from an individual representing the most common mitotype of each species (AAAA, BABB, CBBC, BAAB and BABB for *P. monodon*, *P. semisulcatus*, *L. vannamei*, *F. merguensis* and *M. japonicus*; Tables 2 and 3), gel-eluted and ligated to pGEM®-T Easy vector (Hoelzel and Green, 1992). One-tenth of the volume of each ligation reaction was then electrotransformed into *E. coli* JM 109 (Dower *et al.*, 1988). Recombinant clones were selected using a *lac Z* system following a standard protocol (Maniatis *et al.*, 1982). The insert sizes were

verified by colony PCR (Srisuparbh *et al.*, 2003). Each insert was sequenced in both directions using a LI-COR automated DNA sequencer.

Primer design, PCR-RFLP and SSCP of 16S rDNA₃₁₂
Nucleotide sequences of 16S rDNA₅₆₀ were aligned using Clustal W (Thompson *et al.*, 1994). A pair of primers primed at the conserved regions of 16S rDNA₅₆₀ was designed and tested against 185 shrimp individuals (Table 1). PCR was performed involving predenaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and

extension at 72°C for 1 min. The final extension was performed at 72°C for 7 min.

Eight microlitres of the product was subjected to PCR-RFLP (*Alu* I, *Ssp* I and *Vsp* I) and electrophoretically analyzed. Additionally, 5 µl of gel-eluted 16S rDNA₃₁₂ of each shrimp was mixed with 4 volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured at 95°C for 5 minutes, immediately cooled on ice for 3 minutes and electrophoretically analyzed through 15.0% non-denaturing polyacrylamide gels (37.5 : 1) at 12.5 V/cm for 16 hours at 4°C. SSCP bands were visualized by silver staining.

Table 2. Restriction fragment patterns resulted from digestion of mitochondrial gene segments of *P. monodon*, *P. semisulcatus*, *F. merguensis*, *L. vannamei* and *M. japonicus* with various restriction enzymes

Gene/Enzyme	Pattern observed (bp)	Pm	Ps	FM	LV	MJ
COI-II						
<i>Dra</i> I	A: 1550	+	-	ND	ND	ND
	B: 1000, 500	-	+	ND	ND	ND
<i>Ssp</i> I	A: 1550	+	-	ND	ND	ND
	B: 1250, 280	+	-	ND	ND	ND
	C: 800, 600	-	+	ND	ND	ND
	D: 800, 500, 100	-	+	ND	ND	ND
	E: 960, 580	+	-	ND	ND	ND
<i>Vsp</i> I	A: 700, 380, 300	+	-	ND	ND	ND
	B: 700, 550, 350	-	+	ND	ND	ND
	C: 1550	-	+	ND	ND	ND
	D: 1100, 380	+	-	ND	ND	ND
16S rDNA₅₆₀*						
<i>Alu</i> I	A: 560	+	-	-	+	-
	B: 320, 160, 80	-	+	+	-	+
	C: 320, 240	-	-	-	+	-
	D: 260, 100, 70, 70	+	-	-	-	-
<i>Mbo</i> I	A: 380, 170	+	+	+	+	+
	B: 380, 160	-	-	-	+	-
	C: 280, 170, 110	+	-	-	-	-
	D: 320, 270	+	-	-	-	-
<i>Ssp</i> I	A: 340, 220	+	-	+	-	-
	B: 560	+	+	-	+	+
	C: 470, 70	+	-	-	-	-
<i>Vsp</i> I	A: 290, 270	+	-	-	-	-
	B: 560	+	+	+	+	+
	C: 370, 190	-	-	-	+	-
	D: 460, 100	+	-	-	-	-
16S rDNA₃₁₂**						
<i>Alu</i> I	A: 312	+	-	-	-	-
	B: 200, 90	-	+	+	+	+
	C: 200, 110	-	-	-	+	-
<i>Ssp</i> I	A: 220, 100	+	-	+	-	-
	B: 312	+	+	-	+	+
<i>Vsp</i> I	A: 170, 150	+	-	-	-	-
	B: 312	+	+	+	-	+
	C: 250, 70	-	-	-	+	-

*Analyzed by universal primers (Palumbi *et al.*, 1991)

**Analyzed by primers developed in this study.

Table 3 Mitotypes and SSCP patterns of 5 penaeid shrimps (*P. monodon*, *P. semisulcatus*, *F. merguensis*, *L. vannamei* and *M. japonicus*) analyzed by PCR-RFLP and SSCP of mitochondrial DNA gene segments

Gene	Mitotype	Frequency					SSCP Pattern (no. of individuals)
		PM	PS	FM	LV	MJ	
COI-II (1550 bp)							
	ABA	0.481	-	ND	ND	ND	ND
	AAA	0.407	-	ND	ND	ND	ND
	AEA	0.074	-	ND	ND	ND	ND
	AAD	0.037	-	ND	ND	ND	ND
	BCB	-	0.667	ND	ND	ND	ND
	BDB	-	0.200	ND	ND	ND	ND
	BDC	-	0.067	ND	ND	ND	ND
	BCC	-	0.067	ND	ND	ND	ND
16S rDNA₅₆₀*							
	AAAA	0.414	-	-	-	-	ND
	ACBA	0.345	-	-	-	-	ND
	ADCB	0.034	-	-	-	-	ND
	DDBD	0.034	-	-	-	-	ND
	AABB	0.069	-	-	-	-	ND
	DDCD	0.103	-	-	-	-	ND
	BABB	-	1.000	-	-	1.000	Fig. 1
	CBBC	-	-	-	0.643	-	ND
	CBBB	-	-	-	0.214	-	ND
	AABC	-	-	-	0.143	-	ND
	BAAB	-	-	1.000	-	-	ND
16S rDNA₃₁₂**							
	AAA	0.512	-	-	-	-	9/15 ^a (31), 10/15 (7), 4/14 (3), 9/14 (1), 10/14 (1), 10/17 (1)
	ABA	0.430	-	-	-	-	11/16 (32), 10/16 (2), 13/17 (2), 10/15 (1)
	ABB	0.058	-	-	-	-	13/17 (3), 11/16 (2)
	BBB	-	1.000	-	-	1.000	3/18 (15) for <i>P. semisulcatus</i> and 7/17 (16) for <i>M. japonicus</i>
	CBC	-	-	-	0.967	-	2/12 (28), 1/12 (1)
	BBC	-	-	-	0.033	-	2/12 (1)
	BAB	-	-	1.000	-	-	5/8 (17), 4/6 (16), 5/9 (2), 5/11 (2), 6/8 (1)

*Analyzed by universal primers (Palumbi *et al.*, 1991)

**Analyzed by primers developed in this study.

^aThe number of SSCP bands was orderly assigned from that showing the slowest mobility to that showing the fastest mobility in the gels. Numbers in parentheses indicated individuals possess a particular SSCP patterns. ND = not determined.

Results and Discussion

Differentiation of morphologically similar shrimps; *P. monodon* and *P. semisulcatus* by PCR-RFLP of COI-COII
PCR-RFLP of COI-COII (1550 bp) was initially applied for differentiation of *P. monodon* ($N=27$) and *P. semisulcatus* ($N=15$). Two, five and four restriction patterns were observed from digestion of COI-COII with *Dra* I, *Ssp* I and *Vsp* I, respectively. Eight composite restriction patterns (hereafter called mitotypes) were generated and exhibiting non-overlapping distribution between these taxa (Table 3). Both

single enzyme digestion patterns and mitotypes of COI-COII could discriminate *P. monodon* ($N=27$) and *P. semisulcatus* ($N=15$) unambiguously (Tables 2 and 3). The experiments were extended to cover *L. vannamei*, *F. merguensis* and *M. japonicus* but COI-COII primers did not generate the positive amplification product in those taxa.

Species-identification of penaeid shrimps by PCR-RFLP and SSCP analyses of 16S rDNA₅₆₀
The universal primers of 16S rDNA previously used for population genetic studies of *P. monodon* were applied (Klinbunga *et al.*, 2001b). The

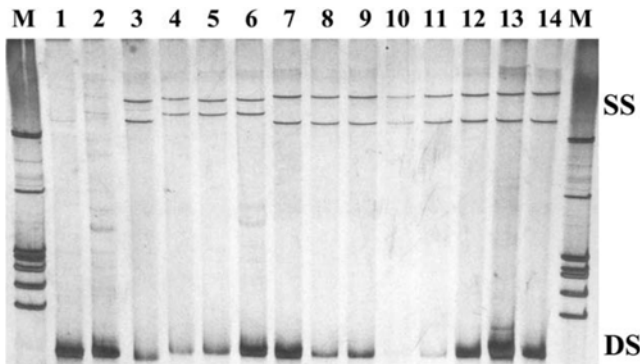


Fig. 1. SSCP patterns of *P. monodon* (lane 3) possessing a AAAA mitotype and *P. semisulcatus* (lanes 4-6) and *M. japonicus* (lanes 7-14) possessing a shared BABB mitotype fractionated through a 12.5% non-denaturing polyacrylamide gel (37.5:1). Lane M is a 100 bp DNA ladder. Lanes 1 and 2 are non-denatured 16S rDNA₅₆₀. SS and DS are single and double stranded DNA, respectively.

16S rDNA₅₆₀ gene segment was successfully amplified across small sample sizes of each shrimp ($N = 79$, Table 1). Fifteen restriction patterns (4, 4, 3 and 4 from *Alu* I, *Mbo* I, *Ssp* I and *Vsp* I, respectively) were found. Differentiation between *P. monodon* and *P. semisulcatus* and between *L. vannamei* and *F. merguensis* by single enzyme digestion patterns was interfered by shared restriction patterns from other species (Table 2).

A total of 11 mitotypes were observed. Non-overlapping mitotypes of 16S rDNA₅₆₀ were found in *P. monodon*, *L. vannamei* and *F. merguensis*. Three mitotypes were found in *L. vannamei* indicating that *L. vannamei* has possibly been introduced from more than a single stock. Nevertheless, *P. semisulcatus* and *M. japonicus* shared a BABB genotype. Phylogenetic relationships regard these shrimps to be distantly related taxa (Baldwin *et al.*, 1998; Lavery *et al.*, 2004). Therefore, a shared mitotype should be resulted from the use of limited number of restriction endonucleases. SSCP analysis of 16S rDNA₅₆₀ indicated clear distinction between *P. semisulcatus* and *M. japonicus* (Fig. 1).

Development of species-diagnostic markers by PCR-RFLP and SSCP analyses of 16S rDNA₃₁₂ Species identification is necessary for quality control of cultured species particularly when wrong species are intentionally supplied. Moreover, labeling and traceability of the cultured product are important matter owing to an increase in trade and the need to maintain confidence in the quality of the products.

Oysters have been classified based principally on morphology. However, they show ecomorphological variation. Therefore, two sympatric species or allopatric populations of a single species inhabit different habitats may be misidentified. Species-diagnostic markers of *Crassostrea belcheri*, *C. iredalei*, *Saccostrea cucullata*, *S. forskali* and *Striostrea (Parastriostrea) mytiloides* were examined using PCR-RFLP of 16S rDNA

(*Acs* I, *Alu* I, *Dde* I, *Dra* I, *Rsa* I and *Taq* I), 18S rDNA (*Hinf* I) and COI (*Acs* I, *Dde* I and *Mbo* I). A total of 54 mitotypes were found. Species-diagnostic PCR-RFLP markers were specifically found in *C. belcheri*, *C. iredalei* and *S. cucullata* but not in *S. forskali* and *Striostrea (Parastriostrea) mytiloides* (Klinbunga *et al.*, 2003a).

Likewise, species-diagnostic markers of the tropical abalone (*Haliotis asinina*, *H. ovina* and *H. varia*) in Thai waters were successfully developed based on PCR-RFLP of 16S rDNA (*Alu* I, *Bam* HI, *Eco* RI and *Hae* III). Non-overlapping mitotypes were found in *H. asinina* (AAAA and AAAE, $N = 115$), *H. ovina* (ABBB, AAAB and AABB, $N = 71$) and *H. varia* (BABG, BABC, BABD, BABF and AABG, $N = 23$), respectively. The 16S rDNA from an individual representing each mitotype was cloned and sequenced. Species-specific PCR was further developed in *H. asinina* and *H. varia* without any false negative or false positive results. The sensitivity of detection was approximately 25 pg and 50 pg of the DNA template, respectively (Klinbunga *et al.*, 2003b).

Species-diagnostic markers should be established from DNA segments exhibiting low genetic polymorphism within a particular species but showing high genetic divergence between different species (Thaewnon-ngiw *et al.*, 2004). Overlapping patterns (in this case, PCR-RFLP and SSCP) between different species should not be observed. Although the nature of 16S rDNA₅₆₀ fulfilled the requirement, the amplification success in *L. vannamei* and *F. merguensis* was quite low when sample sizes were increased. Therefore, more reliable primers were required.

The 16S rDNA₅₆₀ gene segment was then amplified from a representative individual possessing the most common mitotype of each species, cloned and sequenced (Fig. 2). The actual length of this fragment was 561 bp in *P. semisulcatus* and 562 bp in other species. Sequence divergence (Kimura, 1980) between pairs of 16S rDNA₅₆₀ was 5.76% (between *P. semisulcatus* and *F. merguensis*)-10.23% (between *M. japonicus* and *L. vannamei*). The divergence between taxonomically problematic species was 6.15% between *P. monodon* and *P. semisulcatus* and 9.80% between *L. vannamei* and *F. merguensis*, respectively.

Due to high intraspecific genetic diversity previously reported in *P. monodon* (Klinbunga *et al.*, 1999 and 2001b) and *F. merguensis* (Hualkasin *et al.*, 2003; Wanna *et al.*, 2004), we did not develop species-specific PCR (presence/absence of the amplification band) for each species because serious false negative may be occurred when specimens from new geographic samples are analyzed. Alternatively, a pair of primers primed at the conserved region of 16S rDNA providing a 312 bp fragments was designed.

Three, two and three restriction patterns were found from digestion of 16S rDNA₃₁₂ with *Alu* I, *Ssp* I and *Vsp* I, respectively. All restriction patterns observed for each enzyme could be related to one another by the loss or gain of a single or double restriction sites. Restriction patterns of *Alu* I- and *Vsp* I-digested 16S rDNA₃₁₂ clearly differentiate both *P.*

```

P. semisulcatus CGCCTGTTTAAACAAAAACATGCTCTATATGATTGTTTATATAAAGTCTAGCCTGCCACTGA
P. monodon CGCCTGTTTAAACAAAAACATGCTCTATATGATTGTTTATATAAAGTCTAGCCTGCCACTGA
F. merguensis CGCCTGTTTAAACAAAAACATGCTCTATATGATTGTTTATATAAAGTCTAGCCTGCCACTGA
M. japonicus CGCCTGTTTAAACAAAAACATGCTCTATATGATTGTTTATATAAAGTCTAGCCTGCCACTGA
L. vannamei CGCCTGTTTAAACAAAAACATGCTCTATATGATTGTTTATATAAAGTCTAGCCTGCCACTGA
*****

P. semisulcatus TTGAAATTTAAAGGGCCGCGGTATACTGACCGTGC GAAGGTAGCATAATCATTAGTCTTT
P. monodon ATTATTTTAAAGGGCCGCGGTATACTGACCGTGC GAAGGTAGCATAATCATTAGTCTTT
F. merguensis TTTAG-TTTAAAGGGCCGCGGTATACTGACCGTGC GAAGGTAGCATAATCATTAGTCTTT
M. japonicus TTTGT-TTTAAAGGGCCGCGGTATACTGACCGTGC GAAGGTAGCATAATCATTAGTCTTT
L. vannamei TTTAT-TTTAAAGGGCCGCGGTATACTGACCGTGC GAAGGTAGCATAATCATTAGTCTCT
* *****

16S312F
P. semisulcatus TAATTGAAGCCTGTATGAATGGTTGACAAAAAGTAAGTGTCTCAGTTATAATAATTG
P. monodon TAATTGAAGCCTGTATGAATGGTTGACAAAAAGTAATCTGTCTCAGTTATAATAGTTG
F. merguensis TAATTGAAGCCTGTATGAATGGTTGACAAAAAGTAAGTGTCTCATTATAATGATTG
M. japonicus TAATTGAAGCCTGTATGAATGGTTGACAAAAAGTAAGTGTCTCGATTATAATAATTG
L. vannamei TAATTGAAGCCTGTATGAATGGTTGACAAAAAGCAACTGTCTCAATTATATTATTG
*****

P. semisulcatus AATTTAACTTTTAAAGTAAAAGGCTTAAATGGATTAAGGGGACGATAAGACCTATAAAG
P. monodon AACTTAACTTTTAAAGTAAAAGGCTTAAATACTTTAAGGGGACGATAAGACCTATAAAG
F. merguensis AACTTAACTTTTAAAGTAAAAGGCTTAAATAAATTAAGGGGACGATAAGACCTATAAAG
M. japonicus AACTTAACTTTTAAAGTAAAAGGCTTAAATGTTTCAGGGGACGATAAGACCTATAAAG
L. vannamei AATTTAACTTTTAAAGTAAAAGGCTTAAATAAATTAAGGGGACGATAAGACCTATAAAG
** *****

P. semisulcatus CTTGACAATAAGTTAATTATATTATAAATGTTTAGTATAACTTGATTTTAAATTGACGTTT
P. monodon CTTAACAATAAATTTGATTAATTAATAAATGTTTAGTATAACTTGATTTTAAATTAATGTTT
F. merguensis CTTGACAATAAATTTAATTATACTATCAATGTTTAGTATAACTTGATTTTAAATTAAGATTT
M. japonicus CTTGACAATAAATTCGTTATATTATAAATGTTTAGTATAACTTGATTTTAAACGGGGTTT
L. vannamei CTTTACAATAAGTTACCTATATTATAAATGTTTAGTATAACTTGAGTTTAGTAAACGTTT
*** *****

P. semisulcatus GTTACGTTGGGGCGACGAGAATATAATAGTAACGTCTTAAATGTTT-ATTGACAAT
P. monodon GTTGCCTTGGGGCGACGGAATATAATAGTAACGTCTTAAATATTTTATTAACAAGT
F. merguensis GTTGCCTTGGGGCGACGAGAATATAATAGTAACGTCTTAAATATTT-AATAACAAT
M. japonicus GTTTCCTTGGGGCGACGGAATATAATAAATAACTGTTCTTTAAATAT-AATTACAAA
L. vannamei GTTGCCTTGGGGCGACGAGAATATAATAAGTAACGTCTTAAAGTTATTTAATGACAGAA
*** *****

P. semisulcatus ATAATTGGTATTTGAT-TGATCCCTTTATTAAGATTAAAAGATTAAAGTTACTTTAGGG-A
P. monodon ATAATTGAAGAATAAT-TGATCCCTTTATTAAGATTAAAAGATTAAAGTTACTTTAGGG-A
F. merguensis ATAATTGAAAATTAGTGTGATCCCTCTATTAGCGATTAAAAGATTAAAGTTACTTTAGGG-A
M. japonicus ATGTTTGGTAAATAAT-TGATCCCTCTATTAGAGATTAAAAGATTAAAGTTACTTTAGGGGA
L. vannamei ATTTCTGAAAATTAA-TGATCCCTCTACTAGAGATCATAAGATTAAAGTTACTTTAGGG-A
** * *****

16S312R
P. semisulcatus TAACAGCGTAATCTTCTTTGAGAGTTCATATCGACAAGAAGGTTTGCACCTCGATGTTG
P. monodon TAACAGCGTAATCTTCTTTGAGAGTCCCTATCGACAAGAAGGTTTGCACCTCGATGTTG
F. merguensis TAACAGCGTAATCTTCTTTGAGAGTCCCTATCGACAAGAAGGTTTGCACCTCGATGTTG
M. japonicus TAACAGCGTAATCTTCTTTGAGAGTCCACATCGACAAGAAGGTTTGCACCTCGATGTTG
L. vannamei TAACAGCGTAATCTTCTTTGAGAGTCCACATCGACAGGAAGGTTTGCACCTCGATGTTG
*****

P. semisulcatus AATTAAGGTATCCTTATGATGCAGCAGTTATAAAGGAAGGCTGTTTCGACCTTTAAATCC
P. monodon AATTAAGGTATCCTTATAATGCAGCAGTTATAAAGGAAGGCTGTTTCGACCTTTAAATCC
F. merguensis AATTAAGGTATCCTTATGATGCAGCAGTTATANAGGAAGGCTGTTTCGACCTTTAAATCC
M. japonicus AATTAAGGTATCCTTATAATGCAGCAGTTATAAAGGAGGGTCTGTTTCGACCTTTAAATCC
L. vannamei AATTAAGGGTTCCCTTATAATGCAGCAGTTATAAAGGAGGGTCTGTTTCGACCTTTAAATCC
*****

P. semisulcatus TTACATGATCTGAGTTCAGACCGG-
P. monodon TTACATGATCTGAGTTCAGACCGG-
F. merguensis TTACATGATCTGAGTTCAGACCGGA
M. japonicus TTACATGATCTGAGTTCAGACCGGA
L. vannamei TTACATGATCTGAGTTCAGACCGGA
*****

```

Fig. 2. Multiple sequence alignments of 16S rDNA₅₆₀ of *P. monodon* (AAAA), *P. semisulcatus* (BABB), *F. merguensis* (BAAB), *L. vannamei* (CBBC) and *M. japonicus* (BABB). Positions and sequences of the forward and those complementary to the reverse primers of 16S rDNA₃₁₂ are illustrated in boldface and underlined.

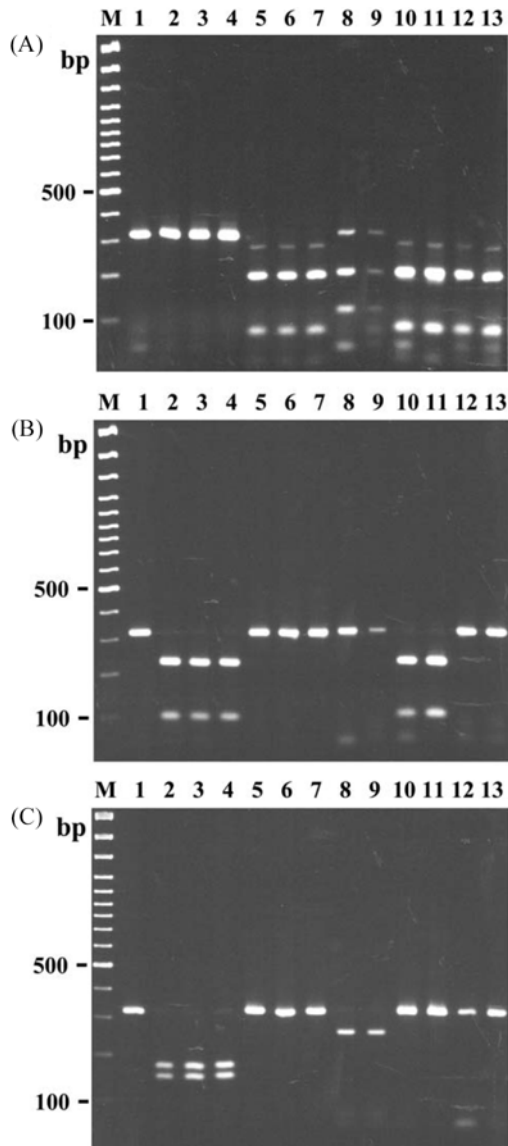


Fig. 3. PCR-RFLP patterns of 16S rDNA₃₁₂ digested with *Alu* I (A), *Ssp* I (B) and *Vsp* I (C) of *P. monodon* (patterns A for all restriction enzymes, lanes 2-4), *P. semisulcatus* (patterns B for all restriction enzymes, lanes 5-7), *L. vannamei* (pattern C for *Alu* I, B for *Ssp* I and C for *Vsp* I, lanes 8-9), *F. merguensis* (pattern B for *Alu* I, A for *Ssp* I and B for *Vsp* I, lanes 10-11) and *M. japonicus* (patterns B for all restriction enzymes, lanes 12-13). Lanes M and 1 are a 100 bp DNA ladder and undigested 16S rDNA₃₁₂, respectively.

monodon and *L. vannamei* from the remaining species (Table 2 and Fig. 3).

Seven mitotypes were found (Table 3). Like results from 16S rDNA₅₆₀, *P. monodon*, *L. vannamei* and *F. merguensis* can be unambiguously differentiated while a shared BBB mitotype was observed in *P. semisulcatus* and *M. japonicus*. Although nucleotide sequences of 16S rDNA₅₆₀ indicated that *Aha* III, *Apo* I, *Mae* II, *Mae* III, *Mse* I and *Tsp* EI could differentiate *P. semisulcatus* and *M. japonicus*, all restriction

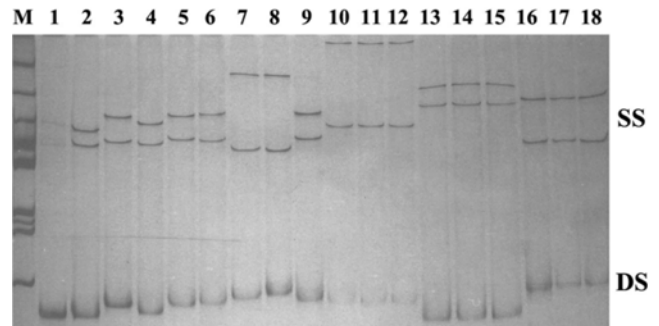


Fig. 4. SSCP patterns of 16S rDNA₃₁₂ of *P. monodon* (lanes 2-6 and 9), *P. semisulcatus* (lanes 7-8), *L. vannamei* (lanes 10-12), *F. merguensis* (lanes 13-15) and *M. japonicus* (lanes 16-18) fractionated through a 15.0% of non-denaturing polyacrylamide gel (37.5 : 1). Lanes M and 1 are a 100 bp DNA ladder and non-denatured 16S rDNA₃₁₂, respectively.

enzymes except *Mse* I are not commonly used. Moreover, the predicted restriction products of 16S rDNA₃₁₂ digested with *Mse* I are too small (7-83 bp) for conveniently analyzed by agarose gel electrophoresis.

Using SSCP analysis, non-overlapping SSCP patterns were found indicating successful development of species-diagnostic markers across all taxa (Table 3 and Fig. 4). Typically, a single mitotype from PCR-RFLP (e.g. AAA, ABA and ABB in *P. monodon* and BAB in *F. merguensis*) possessed several SSCP patterns. This indicated that SSCP analysis is more sensitive than PCR-RFLP when the same DNA fragment is analyzed. Accordingly, cost and time consuming based on restriction analysis particularly when a large number of restriction enzymes are needed, can be significantly reduced. Additional gene segments can then be included to the analysis favored population genetic studies and species identification based on SSCP analysis.

Applications for species identification of suspected shrimps and frozen shrimp meat

Two groups of cultured juvenile shrimps were sent to our laboratory. Farmer was told when purchased the larvae that the Group 1 sample was *F. merguensis* from Indonesia and the Group 2 sample was hybrid offspring between *P. monodon* (sire) and *M. japonicus* (dam). PCR-RFLP of 16S rDNA₃₁₂ indicated that both samples were *F. merguensis* (BAB mitotype). SSCP analysis of the amplified 16S rDNA₃₁₂ further confirmed results from PCR-RFLP analysis (Fig. 5A).

In the other case, the frozen shrimp meat was introduced to Thailand and claimed as the product of the white shrimp (*P. orientalis*) and the pink shrimp (*Metapenaeus affinis*) from fisheries. The suspected *P. orientalis* exhibited a BAB mitotype restrictively found in *F. merguensis* whereas the suspected *M. affinis* showed a DBB mitotype which were not found in our database. SSCP analysis of 16S rDNA₃₁₂ indicated that suspected *P. orientalis* was actually *F. merguensis* (Fig. 5B). In addition, suspected *M. affinis*

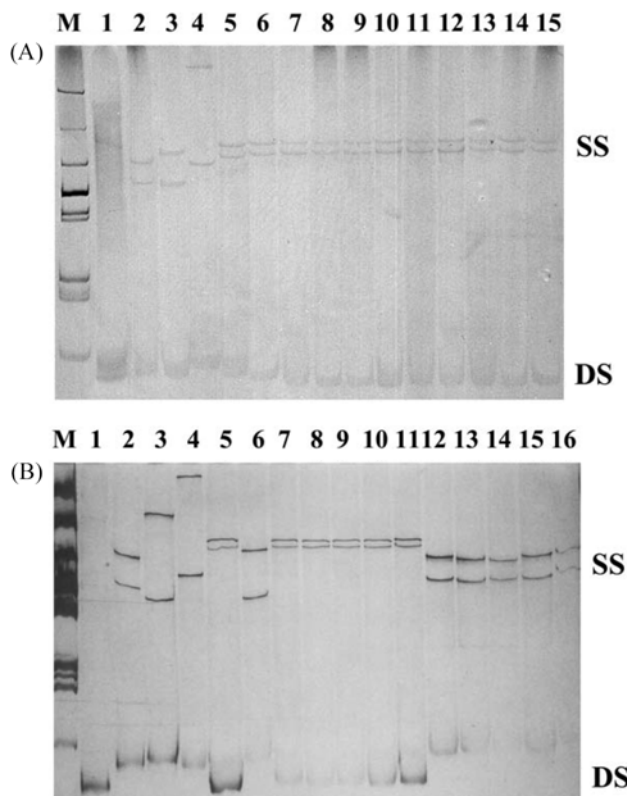


Fig. 5. Applications of SSCP analysis for species identification of suspected cultured shrimp; *F. merguensis* (lanes 6-10, panel A) and offspring claimed as hybrids between *P. monodon* (sire) and *M. japonicus* (dam) (lanes 11-15, panel A) and suspected frozen shrimp meat (claimed as *P. orientalis*, lanes 7-11 and *Metapenaeus affinis*, lanes 12-16; panel B). SSCP patterns of *P. monodon* (lanes 2, A and B), *M. japonicus* (lane 3, A and 6, B), *P. semisulcatus* (lane 3, B), *L. vannamei* (lanes 4, A and B) and *F. merguensis* (lanes 5, A and B) were also included. Lanes M and 1 are a 100 bp DNA ladder and non-denatured 16S rDNA₃₁₂, respectively (see text for discussion).

exhibited clearly different SSCP patterns with that of *P. semisulcatus*, *L. vannamei*, *F. merguensis* and *M. japonicus*. Its polymorphic SSCP patterns were similar, but not identical, to that of *P. monodon* exhibiting an AAA mitotype. Based on large interspecific sequence divergence of 16S rDNA₅₆₀ found in the present study (5.76-10.23%), we concluded that suspected *M. affinis* should be a member of *Penaeus* rather than *Metapenaeus*.

Applications for molecular phylogeny and population genetic studies of *Penaeus* shrimps Genetic diversity of *P. monodon* collected from 5 areas, Chumphon and Trat (Gulf of Thailand), and Phangnga, Satun, and Trang (Andaman Sea), was examined by mtDNA (16S rDNA₅₆₀ and COI-COII) polymorphism. In a total, 37 mitotypes were found. High haplotype diversity (0.855) and nucleotide diversity (3.328%) of Thai *P. monodon* were observed. However, population-specific markers based on mtDNA polymorphism were not

found (Klinbunga *et al.*, 1999; 2001b). Neither population-specific PCR-RFLP nor SSCP fragments/patterns of *P. monodon* were found in the present study. Genetic diversity and population differentiation based on polymorphism of mtDNA gene segments were also reported in *F. merguensis* (Hualkasin *et al.*, 2003), *L. vannamei* (Maggioni *et al.*, 2001) and *M. japonicus* (Tzeng *et al.*, 2004).

High genetic diversity of *P. monodon* was observed based on SSCP analysis of 16S rDNA₃₁₂ (haplotype diversity = 0.72). The percentage of polymorphic bands across overall samples was 60% which is as high as that from RAPD analysis (46.7-61.4%) (Klinbunga *et al.*, 2001b). Results were concordant to previous population genetic studies of *P. monodon* in Thai waters using microsatellites (observed heterozygosity = 0.71-0.82; Supungul *et al.*, 2000) and PCR-RFLP of 16S rDNA and COI-COII (haplotype diversity = 0.83-0.89; Klinbunga *et al.*, 2001b).

Relatively high genetic diversity was also observed in *F. merguensis* (haplotype diversity = 0.40). Five SSCP patterns were observed from individuals exhibiting a BAB mitotype. Hualkasin *et al.* (2003) sequenced a 558 bp fragment of COI from 26 individuals and reported the occurrence of 2 distinct clades of *F. merguensis* in Thai waters. The clade A mainly consisted of shrimps from the Gulf of Thailand whereas the clade B mainly included shrimps from the Andaman Sea. Divergence within and between clades was 0.00-2.25% and 0.18-1.49% and 5.00%, respectively.

Population genetic studies of *P. semisulcatus* have not been reported. Only single PCR-RFLP (BBB) and SSCP patterns were observed from shrimps collected from 2 different geographic samples; Chumphon (Gulf of Thailand, east) and Phuket (Andaman Sea, west). Nevertheless, 4 mitotypes were observed from restriction analysis of COI-COII amplified from the same individuals ($N=15$). Disregarding the fragment length, this suggested that COI-COII possesses greater polymorphism than does 16S rDNA. Limited genetic diversity was observed in farmed *L. vannamei* whereas only single PCR-RFLP and SSCP patterns were observed in *M. japonicus*.

In the present study, reliable PCR-based methods for identifying species origins of morphological similar shrimps (between *P. monodon* and *P. semisulcatus* and between *L. vannamei* and *F. merguensis*) were successfully developed based on PCR-RFLP and SSCP analyses of the amplified rDNA gene segment. These simple methods can be used to prevent supplying incorrect shrimp larvae for the industry and for quality control of shrimp products from Thailand. Population genetic studies of local *Penaeus* species (e.g. *P. semisulcatus* and *F. merguensis*) can be conveniently carried out. The techniques can be applied for rapid genotyping of captured shrimp released from the stock enhancement programs of *P. monodon* in Thai waters.

Acknowledgments This research is supported by a research grant from the National Center for Genetic Engineering and

Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) awarded to SK.

References

- Baldwin, J. D., Bass, A. L., Bowen, B. W. and Clark, Jr., W. H. (1998) Molecular phylogeny and biogeography of the marine shrimp *Penaeus*. *Mol. Phylogent. Evol.* **10**, 399-407.
- Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* **16**, 6127-6145.
- Hoelzel, A. R. and Green, A. (1992) Analysis of population-level variation by sequencing PCR-amplified DNA; in: *Molecular Genetic Analysis of Populations*, Hoelzel, A. R. (ed.), pp. 159-187, Oxford University Press, Oxford, UK.
- Hualkasin, W., Sirimontaporn, P., Chotigeat, W., Querci, J. and Phongdara, A. (2003) Molecular phylogenetic analysis of white prawns species and the existence of two clades in *Penaeus merguensis*. *J. Exp. Mar. Biol. Ecol.* **296**, 1-11.
- Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111-120.
- Klinbunga, S., Sodsuk, S., Penman, D. J. and McAndrew, B. J. (1996) An improved protocol for total DNA isolation and visualisation of mtDNA RFLP(s) in tiger prawn, *Penaeus monodon*. *Thai J. Aquat. Sci.* **3**, 36-41.
- Klinbunga, S., Penman, D. J., McAndrew, B. J. and Tassanakajon, A. (1999) Mitochondrial DNA diversity in three populations of the giant tiger shrimp *Penaeus monodon*. *Mar. Biotechnol.* **1**, 113-121.
- Klinbunga, S., Ampayup, P., Tassanakajon, A., Jarayabhand, P. and Yoosukh, W. (2001a) Genetic diversity and molecular markers of commercial oysters (Genera *Crassostrea*, *Saccostrea* and *Striosstrea*) in Thailand determined by RAPD analysis. *Mar. Biotechnol.* **3**, 133-144.
- Klinbunga, S., Siludjai, D., Wuthijinda, W., Tassanakajon, A., Jarayabhand, A. and Menasveta, P. (2001b) Genetic heterogeneity of the giant tiger shrimp (*Penaeus monodon*) in Thailand revealed by RAPD and mtDNA-RFLP analyses. *Mar. Biotechnol.* **3**, 428-438.
- Klinbunga, S., Khamnamtong, N., Tassanakajon, A., Puanglarp, N., Jarayabhand, P. and Yoosukh, W. (2003a) Molecular genetic tools for three commercial cultured oysters (*Crassostrea belcheri*, *C. iredalei* and *Saccostrea cucullata*) in Thailand. *Mar. Biotechnol.* **5**, 27-36.
- Klinbunga, S., Pripue, P., Khamnamtong, N., Puanglarp, N., Tassanakajon, A., Jarayabhand, P., Hirono, I., Aoki, T. and Menasveta, P. (2003b) Genetic diversity and molecular markers of the tropical abalone (*Haliotis asinina*) in Thailand. *Mar. Biotechnol.* **5**, 505-517.
- Lavery, S., Chan, T. Y., Tam, Y. K. and Chu, K. H. (2004) Phylogenetic relationships and evolutionary history of the shrimp genus *Penaeus s.l.* derived from mitochondrial DNA. *Mol. Phylogent. Evol.* **31**, 39-49.
- Limsuwan, C. (2004) Diseases of Pacific white shrimp (*Litopenaeus vannamei*) cultured in Thailand. *Proceeding of the JSPS-NRCT International Symposium Joint Seminar 2004: Management of Food Safety in Aquaculture and HACCP*. pp 36-41, Kasetsart University, Thailand.
- Maggioni, R., Rogers, A. D., Maclean, N. and D'Incao, F. (2001) Molecular phylogeny of western Atlantic *Farfantopenaeus* and *Litopenaeus* shrimp based on mitochondrial 16S partial sequences. *Mol. Phylogent. Ecol.* **18**, 66-73.
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, USA.
- Palumbi, S. R., Martin, A., Pomano, S., McMillan, W. O., Stice, L. and Grabowski, G. (1991) *The Simple Fool's Guide to PCR, Version 2*: Zoology Department, University of Hawaii, Honolulu, USA.
- Roehrdanz, R. L. (1993) An improved primer for PCR amplification of mitochondrial DNA in a variety of insect species. *Insect Mol. Biol.* **2**, 89-91.
- Rosenberry B (2001) World shrimp farming 2001. Shrimp News International, San Diego, USA
- Srisuparbh, D., Klinbunga, S., Wongsiri, S. and Sittipraneed, S. (2003) Isolation and partial characterization of royal jelly cDNA and proteins of the honey bee (*Apis cerana*). *J. Biochem. Mol. Biol.* **36**, 572-579.
- Supungul, P., Sootanan, P., Klinbunga, S., Kamonrat, W., Jarayabhand, P. and Tassanakajon, A. (2000) Microsatellite polymorphism and the population structure of the black tiger shrimp (*Penaeus monodon*) in Thailand. *Mar. Biotechnol.* **2**, 339-347.
- Thaewnon-ngiw, B., Klinbunga, S., Phanwichien, K., Sangduen, N., Lauhajinda, N. and Menasveta, P. (2004) Genetic diversity and molecular markers of introduced and native apple snails (Genera *Pomacea* and *Pila*) in Thailand. *J. Biochem. Mol. Biol.* **37**, 493-502.
- Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence weighting, position-specific gap penalties and weight metric choices. *Nucleic Acids Res.* **22**, 4673-4680.
- Tzeng, T-D, Yeh, S-Y. and Hui C-F. (2004) Population genetic structure of the kuruma prawn (*Penaeus japonicus*) in east Asia inferred from mitochondrial DNA sequences. *ICES J. Mar. Sci.* **61**, 913-920.
- Wanna, W., Rolland, J. L., Bonhomme, F. and Phongdara, A. (2004) Population genetic structure of *Penaeus merguensis* in Thailand based on nuclear DNA variation. *J. Exp. Mar. Biol. Ecol.* **311**, 63-78.
- Wang, Z. Y., Tsoi, K. H. and Chu, K. H. (2004) Applications of AFLP technology in genetic and phylogenetic analysis of penaeid shrimp. *Biochem. Sys. Ecol.* **32**, 399-407.
- Weder, J. K. P., Rehbein, H. and Kaiser, K-P. (2001) On the specificity of tuna-directed primers in PCR-SSCP analysis of fish and meat. *Eur. Food Res. Technol.* **213**, 139-144.