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

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DNA-based molecular markers for differentiation of five penaeid shrimps (*Penaeus monodon*, *P. semisulcatus*, *Feneropenaeus merguiensis*, *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 rDNA$_{560}$ whereas *P. semisulcatus* and *M. japonicus* shared a BABB mitotype. These shrimps were successfully discriminated by SSCP analysis of 16S rDNA$_{560}$. Nevertheless, the amplification success for *L. vannamei* and *F. merguiensis* was not consistent when tested against larger sample sizes. As a result, 16S rDNA$_{560}$ 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 rDNA$_{312}$ was as effective as that of 16S rDNA$_{560}$. Differentiation of all shrimp species were successfully carried out by SSCP analysis.

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

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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. merguiensis* 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.

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. merguiensis*, *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. merguiensis*) 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. merguiensis* 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. merguiensis* ($N = 38$), 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 DNA was spectrophotometrically estimated. DNA was stored at 4° C until needed.

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

Species	Sample location	Sample size[a]
P. monodon	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)
	Ranong (west of PT)	11 (9)
P. semisulcatus	Chumphon (GOT)	11 (11)
	Phuket (west of PT)	4 (4)
F. merguiensis	Samyan Market[b]	7 (7)
	Chonburi (GOT)	17 (7)
	Indonesia (west of PT)	14 (0)
L. vannamei	Mexico*	6 (2)
	Ratchaburi (central Thailand)*	12 (2)
	Rangsit (central Thailand)*	12 (10)
M. japonicus	Japan*	16 (7)

[a]Number in parentheses are individuals analyzed by universal primers of 16S rDNA (16S $rDNA_{560}$)
[b]unknown origin
*cultivated stocks. GOT = Gulf of Thailand, PT = peninsular Thailand

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 $rDNA_{560}$) fragment of *P. monodon* ($N = 29$), *P. semisulcatus* ($N = 15$), *F. merguiensis* ($N = 14$), *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 $rDNA_{560}$ of penaeid shrimps The 16S $rDNA_{560}$ 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. merguiensis* 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 rDNA$_{312}$ Nucleotide sequences of 16S rDNA$_{560}$ were aligned using Clustal W (Thompson *et al*., 1994). A pair of primers primed at the conserved regions of 16S rDNA$_{560}$ was designed and tested against 185 shrimp individuals (Table 1). PCR was performed involving predenaturation at 94ºC for 3 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 53ºC for 1 min, and extension at 72ºC for 1 min. The final extension was performed at 72ºC 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 rDNA$_{312}$ 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 95ºC 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 4ºC. 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. merguiensis*, *L. vannamei* and *M. japonicus* with various restriction enzymes

Gene/Enzyme	Pattern observed (bp)	Pm	Ps	FM	LV	MJ
COI-II						
Dra I	A: 1550	+	-	ND	ND	ND
	B: 1000, 500	-	+	ND	ND	ND
Ssp 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
Vsp 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 rDNA$_{560}$*						
Alu I	A: 560	+	-	-	+	-
	B: 320, 160, 80	-	+	+	-	+
	C: 320, 240	-	-	-	+	-
	D: 260, 100, 70, 70	+	-	-	-	-
Mbo I	A: 380, 170	+	+	+	+	+
	B: 380, 160	-	-	-	+	-
	C: 280, 170, 110	+	-	-	-	-
	D: 320, 270	+	-	-	-	-
Ssp I	A: 340, 220	+	-	+	-	-
	B: 560	+	+	-	+	+
	C: 470, 70	+	-	-	-	-
Vsp I	A: 290, 270	+	-	-	-	-
	B: 560	+	+	+	+	+
	C: 370, 190	-	-	-	+	-
	D: 460, 100	+	-	-	-	-
16S rDNA$_{312}$**						
Alu I	A: 312	+	-	-	-	-
	B: 200, 90	-	+	+	+	+
	C: 200, 110	-	-	-	+	-
Ssp I	A: 220, 100	+	-	+	-	-
	B: 312	+	+	-	+	+
Vsp 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. merguiensis*, *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 rDNA$_{560}$*							
	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 rDNA$_{312}$**							
	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 *P. semisulcatus* and 7/17 (16) for *M. japonicus*
	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.
[a]The 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. merguiensis* 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 rDNA$_{560}$ The universal primers of 16S rDNA previously used for population genetic studies of *P. monodon* were applied (Klinbunga *et al*., 2001b). The

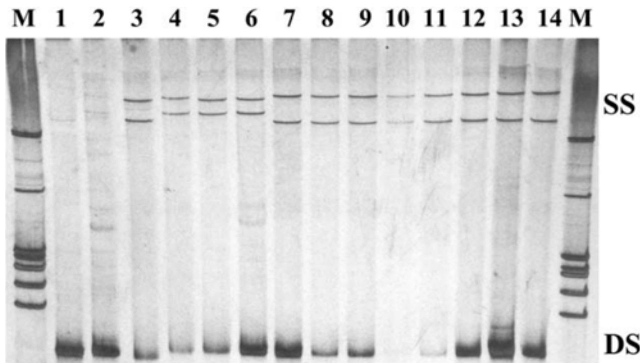

Fig. 1. SSCP patterns of *P. monodon* (lane 3) possessing a AAAA mitotype and *P. semisulcatus* (lanes 4-6) and *M. japonicus* (lanes 7-14) possessing a shared BABB mitotype fractionated through a 12.5% non-denaturing polyacrylamide gel (37.5 : 1). Lane M is a 100 bp DNA ladder. Lanes 1 and 2 are non-denatured 16S $rDNA_{560}$. SS and DS are single and double stranded DNA, respectively.

16S $rDNA_{560}$ 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. merguiensis* 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 $rDNA_{560}$ were found in *P. monodon*, *L. vannamei* and *F. merguiensis*. 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 $rDNA_{560}$ indicated clear distinction between *P. semisulcatus* and *M. japonicus* (Fig. 1).

Development of species-diagnostic markers by PCR-RFLP and SSCP analyses of 16S $rDNA_{312}$ 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 (*Hin*f 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 $rDNA_{560}$ fulfilled the requirement, the amplification success in *L. vannamei* and *F. merguiensis* was quite low when sample sizes were increased. Therefore, more reliable primers were required.

The 16S $rDNA_{560}$ 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 $rDNA_{560}$ was 5.76% (between *P. semisulcatus* and *F. merguiensis*)-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. merguiensis*, respectively.

Due to high intraspecific genetic diversity previously reported in *P. monodon* (Klinbunga *et al*., 1999 and 2001b) and *F. merguiensis* (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 $rDNA_{312}$ 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 $rDNA_{312}$ clearly differentiate both *P.*

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P. semisulcatus CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGA
P. monodon      CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGA
F. merguiensis  CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGA
M. japonicus    CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGA
L. vannamei     CGCCTGTTTAACAAAAACATGTCTATATGATTGGTATGTAAAGTCTGGCCTGCCCACTGA
                ********************************* *** ******** *************

P. semisulcatus TTGAAATTTAAAGGGCCGCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCTTT
P. monodon      ATTATTTTTAAAGGGCCGCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCTTT
F. merguiensis  TTTAG-TTTAAAGGGCCGCGGTATATTGACCGTGCGAAGGTAGCATAATCATTAGTCTTT
M. japonicus    TTTGT-TTTAAAGGGCCGCGGTATATTGACCGTGCGAAGGTAGCATAATCATTAGTCTTT
L. vannamei     TTTAT-TTTAAAGGGCCGCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCTCT
                 *    ******************* ******************************** *

                     16S312F
P. semisulcatus TAATTGAAGGCTTGTATGAATGGTTGGACAAAAAGTAAGCTGTCTCAGTTATAATAATTG
P. monodon      TAATTGAAGGCTTGTATGAATGGTTGGACAAAAAGTAATCTGTCTCAGTTATAATAGTTG
F. merguiensis  TAATTGAAGGCTTGTATGAATGGTTGGACAAAAAGTAAGCTGTCTCAATTATAATGATTG
M. japonicus    TAATTGGAGGCTTGTATGAATGGTTGGACAAAAAGTAAGCTGTCTCGATTATAATAATTG
L. vannamei     TAATTGGAGGCTTGTATGAATGGTTGGACAAAAAGCAAACTGTCTCAATTATATTTATTG
                ****** **************************** ** *******  ***** *  ***

P. semisulcatus AATTTAACTTTTAAGTGAAAAGGCTTAAATGGATTAAGGGGACGATAAGACCCTATAAAG
P. monodon      AACTTAACTTTTAAGTGAAAAGGCTTAAATACTTTAAGGGGACGATAAGACCCTATAAAA
F. merguiensis  AACTTAACTTTTAAGTGAAAAGGCTTAAATAAATTAAGGGGACGATAAGACCCTATAAAG
M. japonicus    AACTTAACTTTTAAGTGAAAAGGCTTAAATGTTTCAGGGGGACGATAAGACCCTATAAAG
L. vannamei     AATTTAACTTTTAAGTGAAAAGGCTTAAATAAATTAAGGGGACGATAAGACCCTATAAAG
                ** ***************************   * * ********************** 

P. semisulcatus CTTGACAATAAGTTAATTATATTATAAATTGTTAGTATAACTTGATTTAATTGACGTTT
P. monodon      CTTAACAATAATTTGATTAAATTATAAATTGTTAGTATAACTTGATTTAATTAATGTTT
F. merguiensis  CTTGACAATAATTTAATTATACTATCAATTGTTAGTATAACTTGGTTTAATTAAGATTT
M. japonicus    CTTGACAATAACTTCGTTATATTATAAATTGTTAGTATAACTTGATTTAACGGGGGTTT
L. vannamei     CTTTACAATAAGTTACCTATATTATAAATTGTTAGTATAACTTGAGTTTAGGTAACGTTT
                *** ******* **    ** * *** *****************  ****        ***

P. semisulcatus GTTACGTTGGGGCGACGAGAATATAATGAGTAACTGTTCTTAAATGTTT-ATTGACAAAT
P. monodon      GTTGCGTTGGGGCGACGGGAATATAATTAGTAACTGTTCTTAAATATTTTATTAACAAGT
F. merguiensis  GTTGCGTTGGGGCGACGAGAATATAATAGGTAACTGTTCTTAAATATTT-AATAACAAAT
M. japonicus    GTTTCGTTGGGGCGACGGGAATATAATAAATAACTGTTCTTTTAAATAT-AATTACAAAA
L. vannamei     GTTGCGTTGGGGCGACGAGAATATAATAAGTAACTGTTCTTAAGTTATTTAATGACAGAA
                *** ************* *********    ***********         * * * ***

P. semisulcatus ATAATTGGTATTTGAT-TGATCCTTTATTAAAGATTAAAAGATTAAGTTACTTTAGGG-A
P. monodon      ATAATTGAAGAATAAT-TGATCCTTTATTAAAGATTAAAAGATTAAGTTACTTTAGGG-A
F. merguiensis  ATAATTGAAAATTAGTGTGATCCTCTATTAGCGATTAAAAGATTAAGTTACTTTAGGG-A
M. japonicus    ATGTTTGGTAAATAAT-TGATCCTCTATTAGAGATTAAAAGATTAAGTTACTTTAGGGGA
L. vannamei     ATTTCTGGAAAATTAA-TGATCCTCTACTAGAGATCATAAGATTAAGTTACTTTAGGG-A
                **   **     *     ******* ** **   *** * ******************* *

                       16S312R
P. semisulcatus TAACAGCGTAATCTTCTTTGAGAGTTCATATCGACAAGAAGGTTTGCGACCTCGATGTTG
P. monodon      TAACAGCGTAATCTTCTTTGAGAGTCCTCATCGACAAGAAGGTTTGCGACCTCGATGTTG
F. merguiensis  TAACAGCGTAATCTTCTTTGAGAGTCCCTATCGACAAGAAGGTTTGCGACCTCGATGTTG
M. japonicus    TAACAGCGTAATCTTCTTTGAGAGTCCACATCGACAAGAAGGTTTGCGACCTCGATGTTG
L. vannamei     TAACAGCGTAATCTTCTTTGAGAGTCCACATCGACAGGAAGGGTTGCGACCTCGATGTTG
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P. semisulcatus AATTAAGGTATCCTTATGATGCAGCAGTTATAAAGGAAGGTCTGTTCGACCTTTAAATCC
P. monodon      AATTAAGGTATCCTTATAATGCAGCAGTTATAAAGGAAGGTCTGTTCGACCTTTAAATCC
F. merguiensis  AATTAAGGTATCCTTATGATGCAGCAGTTATANAGGAAGGTCTGTTCGACCTTTAAATCC
M. japonicus    AATTAAGGTATCCTTATAATGCAGCAGTTATAAAGGAGGGTCTGTTCGACCTTTAAATCC
L. vannamei     AATTAAGGGTTCCTTATAATGCAGCAGTTATAAAGGAGGGTCTGTTCGACCTTTAAATCC
                ********  ******* ************** **** **********************

P. semisulcatus TTACATGATCTGAGTTCAGACCGG-
P. monodon      TTACATGATCTGAGTTCAGACCGG-
F. merguiensis  TTACATGATCTGAGTTCAGACCGGA
M. japonicus    TTACATGATCTGAGTTCAGACCGGA
L. vannamei     TTACATGATCTGAGTTCAGACCGGA
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Fig. 2. Multiple sequence alignments of 16S $rDNA_{560}$ of *P. monodon* (AAAA), *P. semisulcatus* (BABB), *F. merguiensis* (BAAB), *L. vannamei* (CBBC) and *M. japonicus* (BABB). Positions and sequences of the forward and those complementary to the reverse primers of 16S $rDNA_{312}$ are illustrated in boldface and underlined.

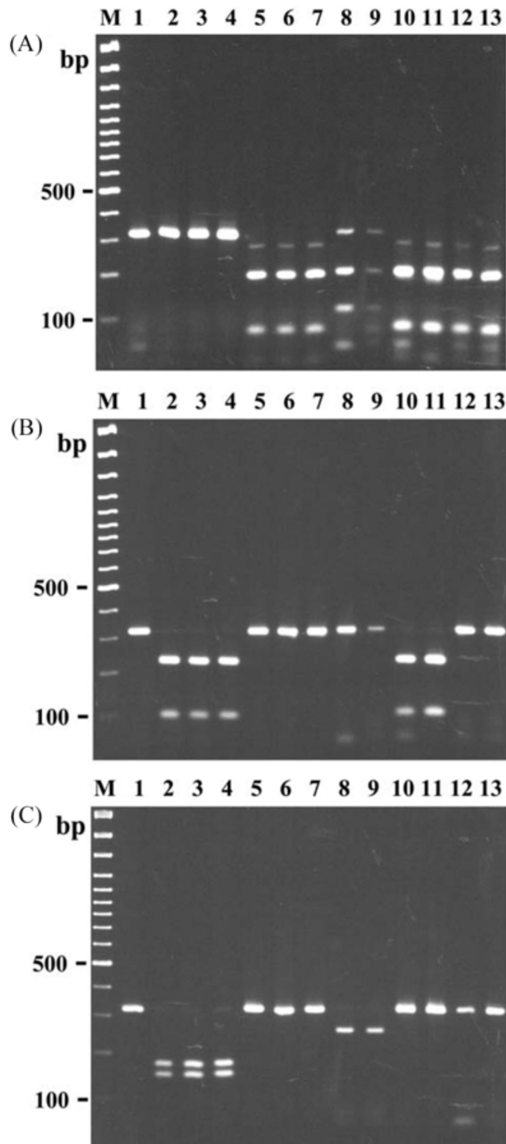

Fig. 3. PCR-RFLP patterns of 16S rDNA$_{312}$ digested with *Alu* I (A), *Ssp* I (B) and *Vsp* I (C) of *P. monodon* (patterns A for all restriction enzymes, lanes 2-4), *P. semisulcatus* (patterns B for all restriction enzymes, lanes 5-7), *L. vannamei* (pattern C for *Alu* I, B for *Ssp* I and C for *Vsp* I, lanes 8-9), *F. merguiensis* (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 rDNA$_{312}$, respectively.

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

Seven mitotypes were found (Table 3). Like results from 16S rDNA$_{560}$, *P. monodon*, *L. vannamei* and *F. merguiensis* can be unambiguously differentiated while a shared BBB mitotype was observed in *P. semisulcatus* and *M. japonicus*. Although nucleotide sequences of 16S rDNA$_{560}$ indicated that *Aha* III, *Apo* I, *Mae* II, *Mae* III, *Mse* I and *Tsp* EI could differentiate *P. semisulcatus* and *M. japonicus*, all restriction

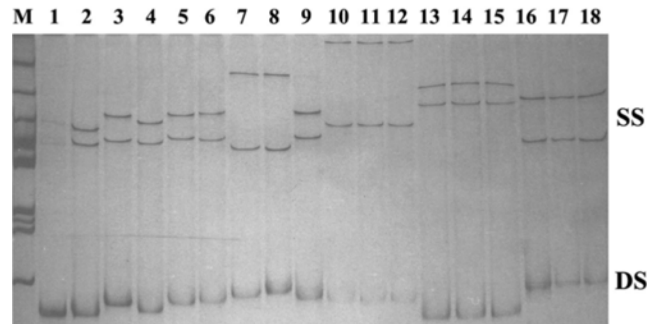

Fig. 4. SSCP patterns of 16S rDNA$_{312}$ of *P. monodon* (lanes 2-6 and 9), *P. semisulcatus* (lanes 7-8), *L. vannamei* (lanes 10-12), *F. merguiensis* (lanes 13-15) and *M. japonicus* (lanes 16-18) fractionated through a 15.0% of non-denaturing polyacrylamide gel (37.5 : 1). Lanes M and 1 are a 100 bp DNA ladder and non-denatured 16S rDNA$_{312}$, respectively.

enzymes except *Mse* I are not commonly used. Moreover, the predicted restriction products of 16S rDNA$_{312}$ 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. merguiensis*) 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. merguiensis* from Indonesia and the Group 2 sample was hybrid offspring between *P. monodon* (sire) and *M. japonicus* (dam). PCR-RFLP of 16S rDNA$_{312}$ indicated that both samples were *F. merguiensis* (BAB mitotype). SSCP analysis of the amplified 16S rDNA$_{312}$ 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. merguiensis* whereas the suspected *M. affinis* showed a DBB mitotype which were not found in our database. SSCP analysis of 16S rDNA$_{312}$ indicated that suspected *P. orientalis* was actually *F. merguiensis* (Fig. 5B). In addition, suspected *M. affinis*

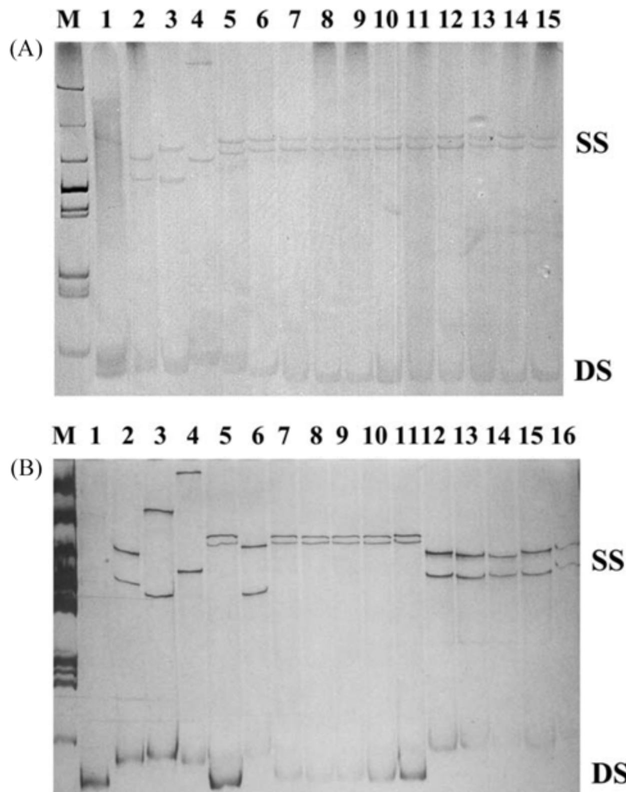

Fig. 5. Applications of SSCP analysis for species identification of suspected cultured shrimp; *F. merguiensis* (lanes 6-10, panel A) and offspring claimed as hybrids between *P. monodon* (sire) and *M. japonicus* (dam) (lanes 11-15, panel A) and suspected frozen shrimp meat (claimed as *P. orientalis*, 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. merguiensis* (lanes 5, A and B) were also included. Lanes M and 1 are a 100 bp DNA ladder and non-denatured 16S $rDNA_{312}$, respectively (see text for discussion).

exhibited clearly different SSCP patterns with that of *P. semisulcatus*, *L. vannamei*, *F. merguiensis* 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 $rDNA_{560}$ 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 $rDNA_{560}$ 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. merguiensis* (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 $rDNA_{312}$ (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. merguiensis* (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. merguiensis* 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. merguiensis*) 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. merguiensis*) 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.

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