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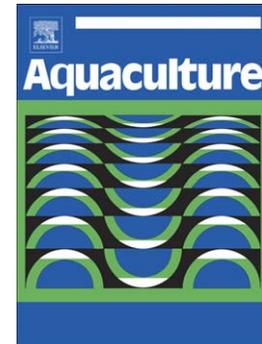
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**Species identification of the blue swimming crab *Portunus pelagicus* in Thai waters using mtDNA and RAPD-derived SCAR markers**

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## ABSTRACT

Species-specific markers of the blue swimming crab (*Portunus pelagicus*) were developed based on nuclear DNA (RAPD) and mtDNA (*COI* and *12S rDNA*) polymorphism. Initially, RAPD was carried out across 109 individuals of *P. pelagicus* originating from different geographic locations in Thai waters using 5 primers (OPA02, OPA14, OPB10, UBC122 and UBC158) and 5 of 15 candidate species-specific RAPD fragments were cloned and sequenced. Three RAPD-derived sequence-characterized amplified region (SCAR) markers (PP-SCAR<sub>152</sub>, PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub>) generated the expected product in 95%, 100% and 100% of *P. pelagicus* and in all individuals of the three spot swimming crab, *P. sanguinolentus* ( $n = 10$ ) but not in the swimming crab, *Charybdis crucifera* ( $n = 20$ ) and mud crabs; *Scylla oceanica* ( $n = 18$ ), *S. serrata*, ( $n = 7$ ), *S. tranquebarica*, ( $n = 9$ ). SSCP analysis of PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub> could differentiate *P. pelagicus* and *P. sanguinolentus* unambiguously. In addition, *COI* (706 bp) and *12S rDNA* (406 bp) gene segments of *P. pelagicus* were amplified and sequenced. Gene-specific primers (PP-COI<sub>270</sub>-F/R and 12S rDNA<sub>312</sub>-F/R, respectively) were designed. PP-COI<sub>270</sub> was specifically found in *P. pelagicus* ( $n = 174$ ) but not in other non-target species. In contrast, 12S rDNA<sub>312</sub> was successfully amplified in all species (100%). Nevertheless, SSCP patterns of the amplified 12S rDNA<sub>312</sub> gene segment of each species did not overlap. Authenticating species-origin of canned crab meat was also successfully carried out by PP-COI<sub>270</sub> and 12S rDNA<sub>312</sub> markers.

**Keywords:** SSCP, RAPD, SCAR, blue swimming crab, *Portunus pelagicus*, species-specific markers

## 1. Introduction

The blue swimming crab, *Portunus pelagicus* is a large, edible species inhabiting nearshore and estuarine waters (Edgar, 1990, Potter et al., 1991; Kailola et al., 1993; Kangas, 2000). It is one of the most important economically important species in Thailand with the production value of at least 2,000 million baht annually (Ministry of Commerce Thailand, 2005).

Appropriate genetic markers can be used to elevate culture and management efficiency of *P. pelagicus* (Awise, 1994; Klinbunga et al., 2007). Sustainable aquaculture of commercially important species requires the basic knowledge on stock structure as well as the use of suitable molecular genetic markers to establish broodstock management programs of exploited species (Awise, 1994; Calvalho and Hauser, 1994).

The blue swimming crab is the major species for the canned crab meat industry. Once the crab is processed (e.g. leaving only the crab meat), species identification becomes problematic. Accordingly, species-diagnostic markers play important roles to prevent supplying incorrect crab species for the industry and for quality control of canned crab meat exported from Thailand.

Recently, genetic diversity and population differentiation of *P. pelagicus* were carried out using AFLP analysis. A total of 13 AFLP fragments were found in more than 95% of investigated specimens ( $n = 72$ ). Sequence-characterized amplified region (SCAR) markers were developed. BSCSCAR2 generated the expected product (188 bp) in 97.0% of *P. pelagicus* ( $n = 100$ ) but not in mud crabs; *S. serrata* ( $n = 8$ ), *S. oceanica* ( $n = 19$ ) and *S. tranquebarica* ( $n = 11$ ). Nevertheless, cross-species amplification was found in *C. crucifera* ( $n = 6$ ). Single strand conformational polymorphism (SSCP) analysis was applied and

revealed a monomorphic pattern in *C. crucifera*, whereas non-overlapping SSCP patterns were observed in *P. pelagicus* (Klinbunga et al., 2007).

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. Overlapping patterns between different species should not be observed (Thaewnon-ngiw et al., 2004), nor should false positive and negative results (e.g. BSCSCAR2) be observed. Accordingly, additional species-diagnostic markers in *P. pelagicus* are required.

In this study, species-specific markers of *P. pelagicus* were successfully developed based on polymorphism of RAPD-derived fragment (PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub>) and mtDNA (*COI* and *12S rDNA*) in marine crabs commonly found in Thai waters. Species specificity and stability of these markers in wild samples and canned crab meat allow simple verifying species origins of various forms of the blue swimming crab products from Thailand to prevent intentional use of the wrong species in canning.

## 2. Materials and methods

### 2.1 Sampling

One hundred and seventy-four individuals of the blue swimming crabs (*P. pelagicus*) were live-caught from Chanthaburi ( $n = 29$ ), Prachup Khiri Khan ( $n = 40$ ) and Suratthani ( $n = 35$ ) located in the Gulf of Thailand (east) and Ranong ( $n = 35$ ) and Krabi ( $n = 35$ ) located in the Andaman Sea (west, Fig. 1). In addition, mud crabs; *S. oceanica* ( $n = 9$  and  $9$ ), *S. serrata* ( $n = 3$  and  $4$ ) and *S. tranquebarica* ( $n = 4$  and  $5$ ) were collected from Chanthaburi and Rayong (Gulf of Thailand), respectively. Taxonomic identification of mud crabs were carried out according to Estampador (1949). The swimming crab, *Charybdis crucifera* ( $n = 20$ ) and the three spot swimming crab, *P. sanguinolentus* ( $n = 10$ ) were collected from Chonburi (Gulf of Thailand). The whole specimens or muscle dissected out from the first walking leg of each crab was kept at  $-30^{\circ}\text{C}$  until used.

In addition, canned crab meat produced by four local companies in Thailand was also purchased from the supermarkets and subjected to species specificity analysis.

### 2.2 DNA extraction

Genomic DNA was extracted from the muscle of the 1<sup>st</sup> periopod of each crab using a phenol-chloroform-proteinase K method (Khamnamtong et al., 2005). The concentration of the extracted DNA was spectrophotometrically estimated. Alternatively, a piece of the muscle tissue was homogenized in 200  $\mu\text{l}$  of a 5% Chelex<sup>®</sup> (w/v) and incubated at  $55^{\circ}\text{C}$  for 30 min (Walsh et al., 1994). The supernatant was collected after centrifugation at 12000 rpm for 5 min at room temperature. The extracted DNA was stored at  $4^{\circ}\text{C}$  until needed.

### 2.3 RAPD analysis and cloning of candidate species-specific RAPD fragments

Genomic DNA of *P. pelagicus* originating from geographically different locations in Thai waters ( $n = 109$ ) was amplified by 5 arbitrary primers (OPA02, OPA14, OPB10, UBC122 and UBC158, Fritsch et al., 1993). PCR was performed in a 25  $\mu$ l reaction volume containing 10 mM Tris-HCl; pH 8.8, 0.01% Triton X-100, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 100  $\mu$ M each of dNTPs (dATP, dCTP, dGTP and dTTP), 0.2  $\mu$ M of a random primer, 1 U of DyNzyme™II DNA Polymerase (Finnzymes) and 25 ng of genomic DNA. The amplification profiles were composed of predenaturation at 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 36°C for 60 s and extension at 72°C for 90 s. The final extension was carried out at 72°C for 7 min. Five microlitres of the amplification reaction were electrophoretically analyzed (Sambrook and Russell, 2001). Five candidate species-specific fragments (RAPD14-500, RAPD158-500, RAPD122-510, RAPD158-1200 and RAPD158-1500, Table 1) were reamplified with the original primers. Then the gel-eluted PCR product was ligated to pGEM-T Easy vector (Promega) and transformed to *E. coli* JM109. Recombinant clones were selected by the *lacZ* system following standard protocols (Sambrook and Russell, 2001). Plasmid DNA was extracted and sequenced for both directions. DNA sequence of each insert was searched against previously deposited data in the GenBank using BlastN and BlastX (<http://ncbi.nlm.nih.gov>, Altschul et al., 1990). A pair of primers of each RAPD fragment (hereafter called PP-SCAR<sub>270</sub>-F/R, PP-SCAR<sub>295</sub>-F/R, PP-SCAR<sub>152</sub>-F/R, PP-SCAR<sub>397</sub>-F/R and PP-SCAR<sub>262</sub>-F/R, respectively) was designed (Table 2).

### 2.4 Cloning and sequencing of COI and 12S rDNA and primer design

*COI* (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3', Palumbi et al., 1991) and *12S rDNA* (F: 5'-AAACTAGGATTATATACCCTATTA-3' and R: 5'-AAGAGGGACGGGCGATTTGT-3', Roehrdanz, 1993) gene segments of *P. pelagicus* were amplified using the conditions described in Klinbunga et al. (2003a). The gel-eluted PCR product was cloned and sequenced for both directions. Nucleotide sequence of each insert was searched against data in the GenBank. A pair of primers of each gene (PP-COI<sub>270</sub>-F/R and 12S rDNA<sub>312</sub>-F/R) was designed (Table 2).

### 2.5 Species-specific PCR and SSCP analysis

Seven primer pairs for amplification of PP-SCAR<sub>270</sub>, PP-SCAR<sub>295</sub>, PP-SCAR<sub>152</sub>, PP-SCAR<sub>397</sub>, PP-SCAR<sub>262</sub>, *PP-COI*<sub>270</sub> and *12S rDNA*<sub>312</sub>, were initially tested for species-specificity against genomic DNA of 5 individuals of each species (excluding *P. sanguinolentus*). Subsequently, PP-SCAR<sub>152</sub>, PP-SCAR<sub>397</sub>, PP-SCAR<sub>262</sub>, *PP-COI*<sub>270</sub>, and *12S rDNA*<sub>312</sub> were further tested using larger sample sizes of *P. pelagicus* ( $n = 174$ ) and non-target species; *P. sanguinolentus* ( $n = 10$ ), *S. serrata* ( $n = 7$ ); *S. oceanica* ( $n = 18$ ), *S. tranquebarica* ( $n = 11$ ) and *C. crucifera* ( $n = 20$ ). PCR was carried out in a 25  $\mu$ l reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1.5 (PP-SCAR<sub>152</sub>, PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub>) or 2 mM (*PP-COI*<sub>270</sub> and *12S rDNA*<sub>312</sub>) MgCl<sub>2</sub>, 100  $\mu$ M of each dNTP, 0.2  $\mu$ M of each primer, 1 U of DyNazyme DNA polymerase (Finnzymes), and 25 ng of genomic DNA. The amplification reaction was performed by predenaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30s (PP-SCAR<sub>152</sub>, PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub>), 45 s (*12S rDNA*<sub>312</sub>) or 1 min (*PP-COI*<sub>270</sub>), annealing at 55 (*PP-COI*<sub>270</sub>, PP-SCAR<sub>152</sub> and PP-SCAR<sub>262</sub>) or 58°C (*12S rDNA*<sub>312</sub> and PP-SCAR<sub>397</sub>) for 45 s (PP-SCAR<sub>152</sub>, PP-SCAR<sub>397</sub>

and PP-SCAR<sub>262</sub>) or 1 min (*PP-COI*<sub>270</sub> and *I2S rDNA*<sub>312</sub>), and extension at 72°C for 30 s (PP-SCAR<sub>152</sub>, PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub>), 45 s (*I2S rDNA*<sub>312</sub>) or 1 min (*PP-COI*<sub>270</sub>). The final extension was carried out at 72°C for 7 min. Five microlitres of each amplification product was electrophoretically analyzed through 1.8% agarose gel.

For SSCP analysis (Orita et al., 1989), 5 µl of the amplified PP-SCAR<sub>397</sub>, PP-SCAR<sub>262</sub> and *I2S rDNA*<sub>312</sub>, of each crab 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 min, immediately cooled on ice for 3 min and electrophoretically analyzed by 15.0-17.5% non-denaturing polyacrylamide gels (crosslink = 37.5:1) at 12.5-15 V/cm for 13.5-19 hr at 4°C. SSCP bands were visualized by silver staining.

#### 2.6 Verification of *I2S rDNA*<sub>312</sub> polymorphism by DNA sequencing

*I2S rDNA*<sub>312</sub> amplified from an individual exhibiting each SSCP pattern of *P. pelagicus* and non-target species were cloned, sequenced and multiple-aligned using Clustal W (Thompson et al., 1994). Nucleotide divergence between pairs of sequences was calculated using the two parameter method (Kimura, 1980).

#### 2.7 Stability test

PP-SCAR<sub>152</sub>, PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub> were amplified using genomic DNA extracted from frozen (2 years at -20°C), boiled (10 min) and saline-preserved (2 weeks at 4°C) meat of *P. pelagicus* by typical phenol-chloroform (Khamnamtong et al., 2005) or chelex-based methods (1 µl, Walsh et al., 1994). Bands were visualized under a transilluminator after ethidium bromide staining (Sambrook and Russell, 2001).

### 2.8 Species identification of canned crab meat using PP-COI<sub>270</sub> and 12S rDNA<sub>312</sub>

Pieces of the crab meat were randomly sampled from three different positions in the same can. Genomic DNA was extracted from each specimen using the phenol-chloroform and chelex-based methods. PP-COI<sub>270</sub> and 12S rDNA<sub>312</sub> were amplified and electrophoretically analyzed in 1.8% agarose gels. SSCP profiles of the amplified 12S rDNA<sub>312</sub> from canned crab meat were examined as described above.

## 3. Results

### 3.1 RAPD analysis and development of SCAR markers

Fifteen RAPD fragments generated from five arbitrary primers (UBC122, UBC155, OPA02, OPA14 and OPB10, Fig. 2) were found in > 95% of investigated specimens ( $n = 109$ , Table 1). Five of which (RAPD14-500, RAPD158-500, RAPD122-510, RAPD158-1200 and RAPD158-1500) were cloned and sequenced. Nucleotide sequences of these fragments did not match any sequence in the GenBank ( $E$ -value >  $1e-04$ ) and were regarded as anonymous nuclear DNA segments.

A preliminary species-specificity test ( $n = 5$  for each species excluding *P. sanguinolentus*, Table 2) revealed that PP-SCAR<sub>295</sub> (derived from RAPD158-500) did not generate the positive amplification product in any species whereas PP-SCAR<sub>270</sub> (RAPD14-500) yielded cross-species amplification in *S. serrata*, *S. oceanica*, *S. tranquebarica* and *C. crucifera*. PP-SCAR<sub>152</sub> (RAPD122-510), PP-SCAR<sub>397</sub> (RAPD158-1200) and PP-SCAR<sub>262</sub> (RAPD158-1500) were only observed in *P. pelagicus* and further tested covering larger sample sizes (including *P. sanguinolentus*,  $n = 10$ ).

The expected product of PP-SCAR<sub>152</sub> was successfully amplified in 95.4% of *P. pelagicus* along with a 300 bp band in several individuals (data not shown). In contrast, PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub> were successfully amplified in all individuals of *P. pelagicus*. These primers did not generate the expected product in *S. serrata*, *S. oceanica*, *S. tranquebarica* and *C. crucifera* but cross-species amplification was found in *P. sanguinolentus* (Table 2 and Fig. 3A and 4A).

### 3.2 Species-specific PCR of PP-COI<sub>270</sub>

Nucleotide sequences of the amplified *COI* (706 bp) and *12S rDNA* (406 bp) significantly matched previously deposited *COI* ( $E$ -value = 0.0) and *12S rDNA* ( $E$ -value = 0.0) of *P. pelagicus*, respectively. PP-COI<sub>270</sub> primers provided the expected product (270 bp) in all individuals of *P. pelagicus* ( $n = 174$ ) without any false positive result in non-target species; *S. oceanica* ( $n = 18$ ), *S. serrata* ( $n = 7$ ), *S. tranquebarica* ( $n = 9$ ), *C. crucifera* ( $n = 20$ ) and *P. sanguinolentus* ( $n = 10$ ) (Fig. 5).

### 3.3 Species-specific SSCP patterns of PP-SCAR<sub>397</sub>, PP-SCAR<sub>262</sub> and 12S rDNA<sub>312</sub>

PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub> of all individuals of *P. pelagicus* and *P. sanguinolentus* were further genotyped. SSCP patterns of PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub> did not overlap and could unambiguously differentiate *P. pelagicus* and *P. sanguinolentus* in this study (Fig. 3B and 4B).

Unlike PP-COI<sub>270</sub>, 12S rDNA<sub>312</sub> was observed in all investigated species (Fig. 6A). Five SSCP genotypes of 12S rDNA<sub>312</sub> were found in *P. pelagicus* and these patterns did not overlap with that of other species. The SSCP genotype I was the most common genotype ( $n =$

159 accounting for 91.4% of overall *P. pelagicus* specimens). A fixed SSCP pattern was found in each of the non-target species (Fig. 6B).

### 3.4 Accuracy of SSCP genotyping and sequence divergence between crab species

Nucleotide sequence of five different SSCP genotypes of *12S rDNA*<sub>312</sub> was different by one or a few substitutions (Fig. 7). This suggested the accuracy of SSCP analysis of *12S rDNA*<sub>312</sub> on rapid genotyping of *P. pelagicus*. Nucleotide sequence divergence within *P. pelagicus* was relatively low (0.32-1.29%). Three morphologically similar mud crab species were genetically closely related as reflected by relatively low intrageneric nucleotide divergence (2.85-6.99%). Large nucleotide divergence was clearly observed between *P. pelagicus* and *P. sanguinolentus* (9.65-10.38%), and mud crabs (15.48-16.65%) and *C. crucifera* (17.70-18.11%), respectively.

### 3.5 Stability of *P. pelagicus*-specific SCAR markers

Positive amplification products of PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub> were consistently amplified from genomic DNA of frozen, boiled and saline-preserved meat of *P. pelagicus* extracted by the phenol/chloroform and 5% Chelex-based methods. This indicated stability and reliability of the developed species-specific SCAR markers (Fig. 8).

### 3.6 Species identification of canned crab meat

The amplified PP-COI<sub>270</sub> was consistently observed in canned crab meat from different companies disregarding the quality of genomic DNA isolated by different extraction methods

(Fig. 9). In addition, *12S rDNA<sub>312</sub>* was successfully amplified from all examined canned crab meat. SSCP patterns of these specimens and wild *P. pelagicus* were identical but different from other crab species (Fig. 10).

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## 4. Discussion

### 4.1 *P. pelagicus*-specific PCR based on PP-COI<sub>270</sub> polymorphism

Both *P. pelagicus* and *P. sanguinolentus* are distributed from the intertidal zone to approximately 50 m depth along the coastlines (Kangas 2000; <http://www.fao.org/fishery/species/2629/en>) while *C. crucifera* inhabits on sandy to muddy substratum at 10-30 m depth (Wee and Ng, 1995). In contrast, mud crabs (*S. serrata*, *S. oceanica* and *S. tranquebarica*) inhabit mangrove areas and estuaries (Fuseya and Watanabe, 1996). Although taxonomic difficulties were only reported in mud crabs but not in portunid crabs (Fuseya and Watanabe, 1996; Klinbunga et al., 2000, 2007), *P. sanguinolentus* and *C. crucifera* are used along with *P. pelagicus* for the production of canned crab meat. Accordingly, species-specific markers play important roles for quality control of products from economically important species like *P. pelagicus* (Sweijd et al. 1998; Khamnamtong et al., 2006).

Results from population genetic studies indicated that *COI* (710 bp) and *12S rDNA* (416 bp) exhibited relatively low polymorphism in *P. pelagicus* (Thamniemdee, 2007). This suggested the possible applications for authenticating species-origin of *P. pelagicus* based on polymorphism of these gene regions.

Previously, species-specific PCR based on 16S rDNA polymorphism for detection of the tropical abalone; *Haliotis varia* (100% amplification success without any false positives,  $n = 20$ ) and *H. asinina* (100%,  $n = 75$  with a false positive from a single individual of *H. varia*) was reported. The mtDNA-based markers were able to eliminate misidentification problems of abalone larvae and processed products of Thai abalone (Klinbunga et al., 2003b).

Likewise, *PP-COI<sub>270</sub>-F/R* provided the positive amplification product in all investigated individuals of *P. pelagicus* without any false negative result. Cross species amplification in other crab species (*S. oceanica*, *S. serrata*, *S. tranquebarica*, *C. crucifera* and *P. sanguinolentus*) were not observed. Therefore, reliable and convenient species-specific PCR based on *COI* polymorphism (presence/absence of the amplification band) was successfully developed in *P. pelagicus*.

#### 4.2 Species-diagnostic markers of *P. pelagicus* based on SSCP analysis of RAPD-derived SCAR fragments

RAPD is a rapid technique for identification of molecular markers at different taxonomic levels (Welsh and McClelland 1990, Williams, et al. 1990, Hadrys et al., 1992). Klinbunga et al. (2000) identified species-specific markers to distinguish three mud crab species, *S. serrata*, *S. oceanica*, and *S. tranquebarica*, in eastern Thailand based on RAPD-PCR. Several RAPD fragments generated from UBC456, UBC457 and YNZ22 were fixed in each mud crab species and used to construct a molecular taxonomic key in those taxa.

Using the same approach, 15 candidate species-specific fragments were observed in *P. pelagicus* ( $n = 109$ ). Nevertheless, RAPD-PCR is sensitive to reaction conditions (Liu and Cordes, 2004). This technique also requires high-quality DNA template for reliable and consistent amplification results, which may not be possible for field specimens. As a result, RAPD markers may cause significant false-negative results from suspected specimens. Therefore, SCAR markers which are more reliable for species authentication of *P. pelagicus* were developed.

Three RAPD-derived SCAR markers (*PP-SCAR<sub>152</sub>*, *PP-SCAR<sub>397</sub>* and *PP-SCAR<sub>262</sub>*) generated the amplification product in both *P. pelagicus* and *P. sanguinolentus*. The false

negative amplification result found in PP-SCAR<sub>152</sub> reduced the potential of this marker for accurate authentication of *P. pelagicus*. Therefore, PP-SCAR<sub>152</sub> was not further characterized.

SSCP analysis, which is favored for identifying species origins of various species due to its convenience and cost-effectiveness, was then applied to verify whether nucleotide sequences of PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub> in *P. pelagicus* were different from *P. sanguinolentus*. Non-overlapping SSCP patterns between these closely related species were observed. Accordingly, authentication of *P. pelagicus* was simply carried out by PCR-SSCP of these markers.

Previously, 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.

Recently, a SCAR marker (BSCSCAR2) for identification of *P. pelagicus* was successfully developed but SSCP patterns of *P. pelagicus* and *C. crucifera* were difficult to differentiate from one another (Klinbunga et al., 2007). In this study, more efficient *P. pelagicus*-diagnostic SCAR markers were successfully developed using species-specific PCR of PP-COI<sub>270</sub> and PCR-SSCP analysis of PP-SCAR<sub>397</sub> and PP-SCAR<sub>262</sub>. The numbers of species-diagnostic markers developed in this study should be sufficient for identifying species origin of *P. pelagicus* in Thai waters accurately.

For rapid species-identification of *P. pelagicus*, the tedious and time-consuming genomic DNA isolation based on the phenol/chloroform extraction method was simplified to a rapid

5% chelex-based method. The positive fragments of *PP-COI*<sub>270</sub>, *12S rDNA*<sub>312</sub>, *PP-SCAR*<sub>397</sub> and *PP-SCAR*<sub>262</sub> were consistently amplified from frozen, saline-preserved and boiled meat of *P. pelagicus* disregarding the low quality of DNA template extracted from a chelex-based method. This reduces the operation time and effort for authentication of *P. pelagicus* products particularly when dealing with a large number of specimens.

#### *4.3 Molecular markers successfully amplified in both target and non-target species but exhibit interspecific sequence polymorphism are required for authentication of P. pelagicus in canned products*

Species-diagnostic markers were previously developed in five species of penaeid shrimp; *Penaeus monodon*, *P. semisulcatus*, *P. (Fenneropenaeus) merguensis*, *P. (Litopenaeus) vannamei* and *P. (Marsupenaeus) japonicus* (Khamnamtong et al., 2006), three species of abalone; *Haliotis asinina*, *H. ovina* and *H. varia* (Klinbunga et al., 2003b, 2004) and three species of oysters; *Crassostrea belcheri*, *C. iredalei* and *Saccostrea cucullata* (Klinbunga et al., 2003a) in Thailand. In contrast to other commercially important species where the major part or whole individuals of wrong species are supplied, *P. pelagicus* meat in cans may be mixed with that of one or a few non-target crab species. This readily limited the application of *PP-COI*<sub>270</sub>, *PP-SCAR*<sub>397</sub> and *PP-SCAR*<sub>262</sub> markers for the quality control of canned crab meat in practice. Therefore, a molecular marker that is consistently amplified across species but exhibit sequence polymorphism among species is required.

Apparently, *12S rDNA*<sub>312</sub> fulfilled the requirement on the ability to amplify an orthologous gene segment from all investigated species. Non-overlapping SSCP genotypes were found between *12S rDNA*<sub>312</sub> of *P. pelagicus* and other crab species. Therefore, a species-diagnostic marker for identification of contaminated crab meat using SSCP analysis

was successfully developed. Practically, the suspected specimens should be collected from different positions in the same can to ensure that specimens are appropriately sampled. This sampling strategy would promote the practical application of a species-specific *12S rDNA*<sub>312</sub> along with *PP-COI*<sub>270</sub>, *PP-SCAR*<sub>397</sub> and *PP-SCAR*<sub>262</sub> markers.

Five SSCP genotypes of *12S rDNA*<sub>312</sub> were found in *P. pelagicus* whereas only one pattern was observed in each of the non-target species. Nucleotide sequencing confirmed that SSCP is sufficiently reliable and cost-effective for identification of species origin and genetic differences of marine crabs in this study. In addition, large nucleotide sequence divergence was found between *P. pelagicus* and other crabs (2.85-18.11%) suggesting that *12S rDNA*<sub>312</sub> polymorphism can also be applied for molecular systematics of marine crabs.

#### *4.4 Application of PP-COI<sub>270</sub> and 12S rDNA<sub>312</sub> for species authentication of canned crab meat*

The amplification success of *PP-COI*<sub>270</sub> and *12S rDNA*<sub>312</sub> was tested using poor genomic DNA extracted from canned crab meat. The expected amplification product of *PP-COI*<sub>270</sub> and *12S rDNA*<sub>312</sub> was found against genomic DNA of all specimens isolated by both phenol/chloroform and chelex extraction methods. This reflected consistency and reproducibility of the developed markers for simple and rapid authenticating *P. pelagicus* products to prevent intentional use of the wrong species in canning.

In this study, we successful develop species-specific SCAR markers of *P. pelagicus* in Thai waters. These markers can be used to authenticate various forms of *P. pelagicus* products. Additional species-diagnostic SCAR markers could be developed from the remaining candidate species-specific RAPD markers to ensure reliable results when the species-origin of new populations of *P. pelagicus* is examined. The ability to identify

broodstock and seed species of *P. pelagicus* is crucial for both broodstock management and conservation programs in *P. pelagicus*. Polymorphic SSCP genotypes of *12S rDNA*<sub>312</sub> allow population genetic studies of *P. pelagicus* in Thai waters. The information can be applied for conservation of the key stock (s) and selection of a particular genetic stock for aquacultural purposes. Both *PP-COI*<sub>270</sub> and *12S rDNA*<sub>312</sub> can be further used for determining patterns of larval distribution and recruitment in this species.

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Table 1.

Candidate species-specific markers of *P. pelagicus* in Thailand revealed by RAPD analysis.

Primer	Size of RAPD fragment (bp)
OPA02	300 and 590
OPA14	340 and <b>500*</b>
OPB10	310 and 1200
UBC122	400, 440, <b>510*</b> and 1050
UBC158	<b>500*</b> , 1050, 1150, <b>1200*</b> and <b>1500*</b>

\*Fragments converted to SCAR markers.

Table 2.

Sequences of primers and amplification success of SCAR markers derived from candidate species-specific RAPD markers, *COI* and *12S rDNA* of *P. pelagicus*.

SCAR marker (RAPD fragment)	Primer sequence	Amplification success (%)						Species differentiation by SSCP
		PP (n=174)	PS (n = 10)	SS (n = 7)	SO (n = 18)	ST (n = 9)	CC (n = 20)	
<b>PP-SCAR<sub>270</sub>*</b> (RAPD14-500)	F: 5'-CACAGGTCTGGCACAACCTCT-3' R: 5'-AACATTTGCTGCGAATCACA-3'	80	ND	20	20	20	20	ND
<b>PP-SCAR<sub>295</sub>*</b> (RAPD158-500)	F: 5'-CTTGCTGCTTTCAGAACGAC-3' R: 5'-AGCATTAGCATCTCACTGGA-3'	-	ND	-	-	-	-	ND
<b>PP-SCAR<sub>152</sub></b> (RAPD122-510)	F: 5'-CACGAAACCTCCACGACCAA-3' R: 5'-TCGGTCGTCACTGTTCGTTG-3'	95.4	100	-	-	-	-	ND
<b>PP-SCAR<sub>397</sub></b> (RAPD158-1200)	F: 5'-GACGATGGTGGTCTGGTGAA-3' R: 5'-GCACTCTGTTCCCTCTCCACG-3'	100	100	-	-	-	-	Yes**

<b>PP-SCAR<sub>262</sub></b>	F: 5'-CCTTCCATTGCCTCCATCTA-3'	100	100	-	-	-	-	Yes**
(RAPD158-1500)	R: 5'-GACGAACTGGGGTGTGGAA-3'							
<b>PP-COI<sub>270</sub></b>	F: 5'-TTCAGCAGCCATCGCTCACG-3'	100	-	-	-	-	-	ND
	R: 5'-AGGGTCAAAGAATGAAGTAT-3'							
<b>12S rDNA<sub>312</sub></b>	F: 5'-TTGGCGGTGGTTTAGTCTTG-3'	100	100	100	100	100	100	Yes**
	R: 5'-CGGGCGATGTGTACATGCTT-3'							

PP = *P. pelagicus*, PS = *P. sanguinolentus*, SS = *S. serrata*, SO = *S. oceanica*, ST = *S. tranquebarica* and CC = *C. crurifera*, + = positive amplification products, - = no amplification products, ND = not determined. \* = preliminary tested in five individuals of each species. \*\*SSCP was further carried out for distinguishing species that could not be differentiated based on PCR analysis.

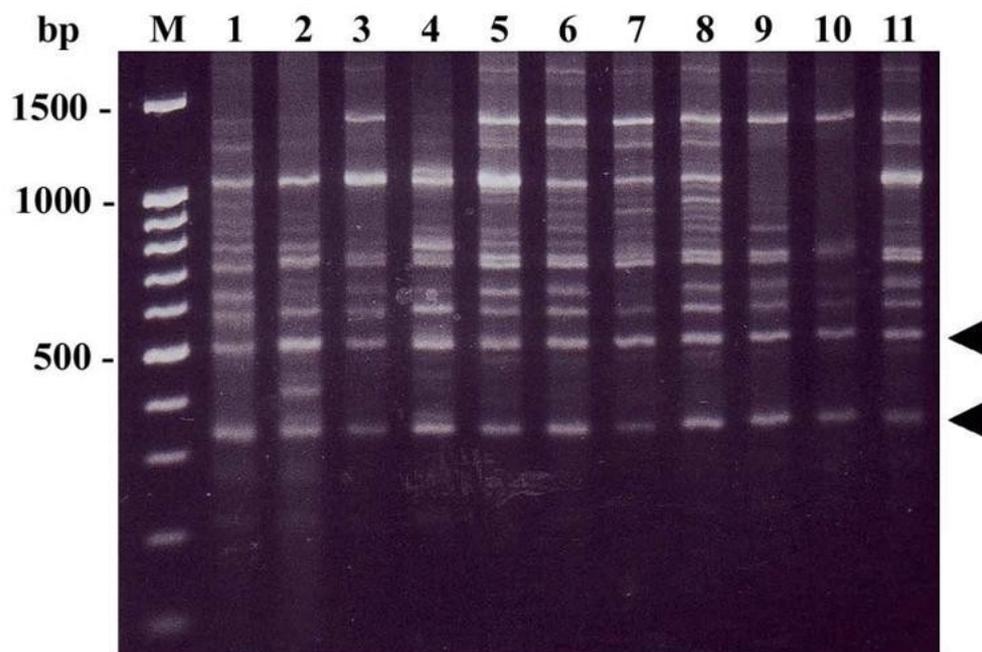
preliminary tested in five individuals of each species. \*\*SSCP was further carried out for distinguishing species that could not be differentiated based on PCR analysis.

### Figure legends

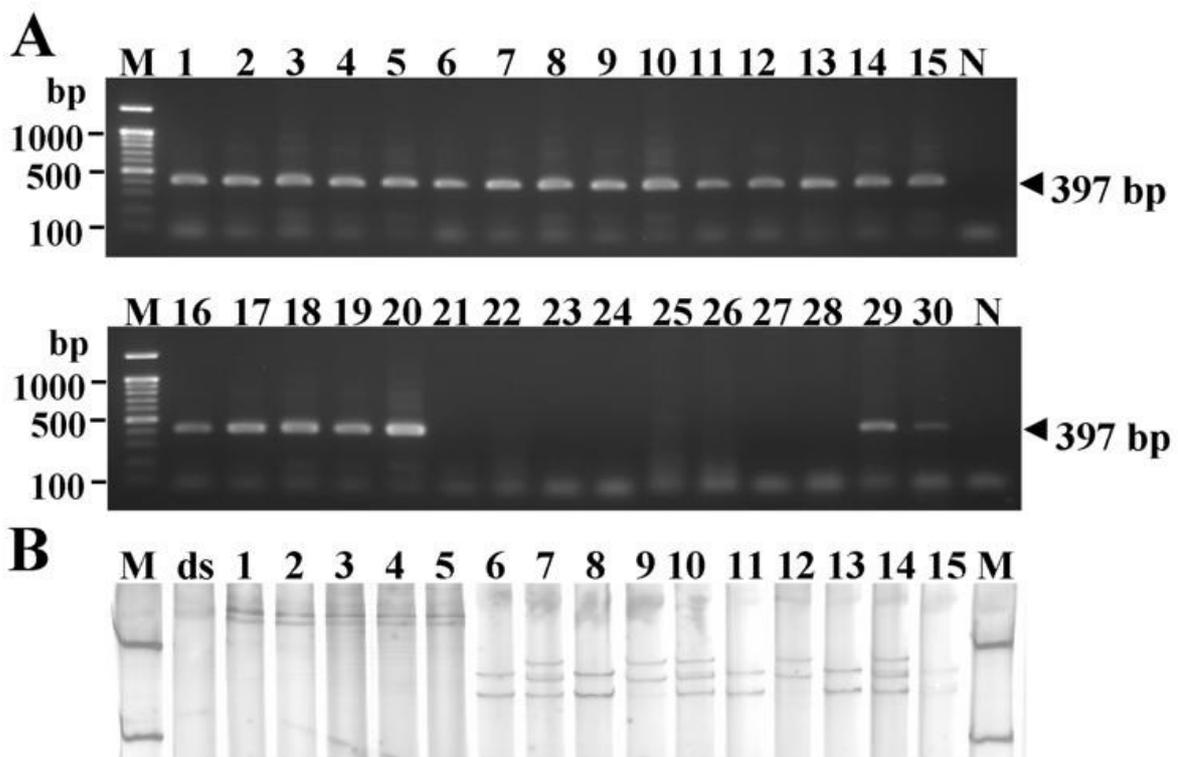
**Fig. 1.** Sampling sites of *P. pelagicus* used in this study.



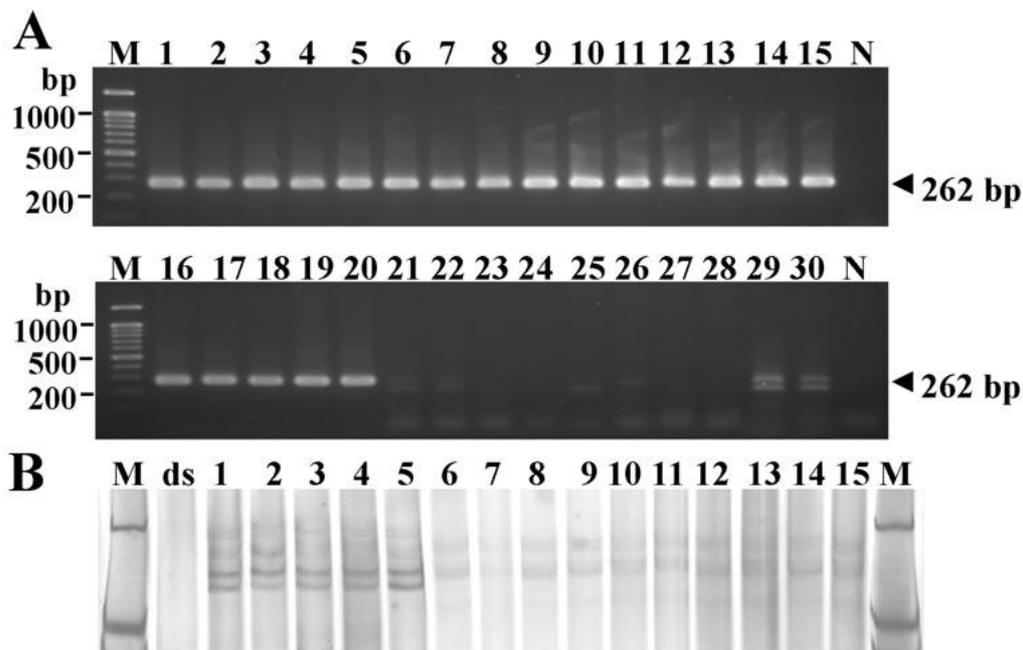
**Fig. 2.** An example of RAPD patterns of *P. pelagicus* originating from Chanthaburi (lanes 1-2), Ranong (lanes 3-4), Suratthani (lane 5-6), Krabi (lanes 7-8) and Prachuap Khiri Khan (lanes 9-11) generated by OPA14. Lanes M are a 100 bp ladder. Arrowheads indicate candidate species-specific RAPD fragments for *P. pelagicus*.



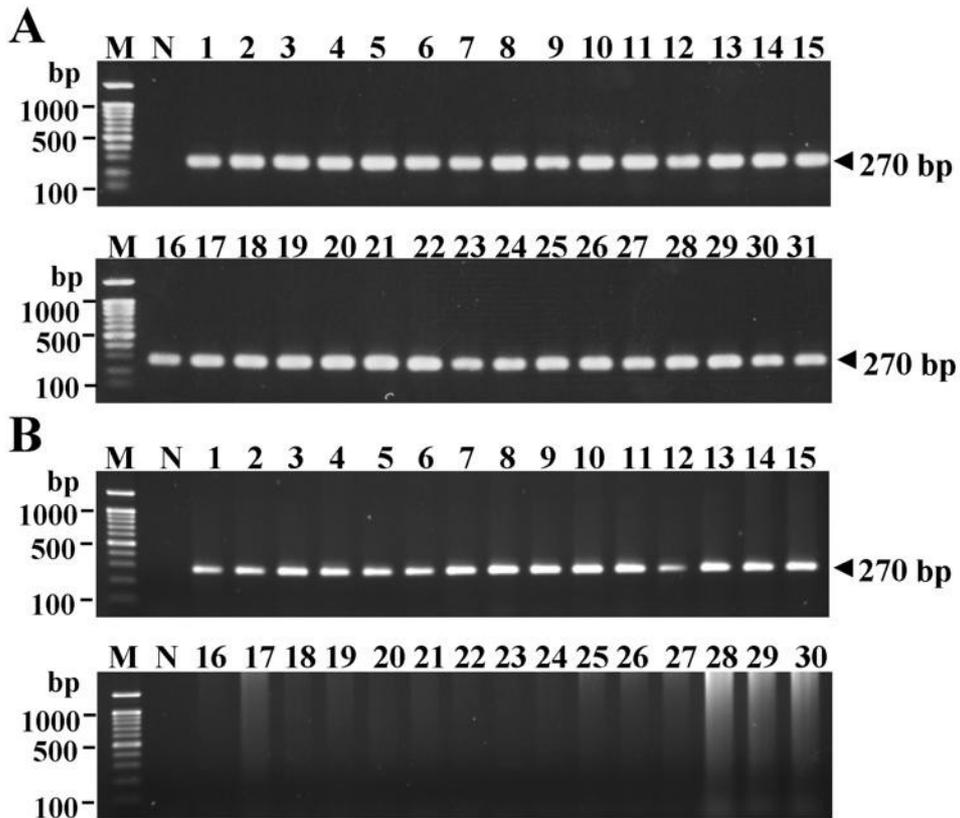
**Fig. 3.** (A) Amplification results of PP-SCAR<sub>397</sub> using genomic DNA of *P. pelagicus* (lanes 1-20), *S. oceanica* (lanes 21-22), *S. serrata* (lanes 23-24), *S. tranquebarica* (lanes 25-26), *C. crucifera* (lanes 27-28) and *P. sanguinolentus* (lanes 29-30). Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively. (B) SSCP patterns of PP-SCAR<sub>397</sub> of *P. pelagicus* (lanes 1-5) and *P. sanguinolentus* (lanes 6-15) electrophoretically analyzed through a 15% non-denaturing polyacrylamide gel (crosslink = 37.5:1; 250 V at 4°C for 19 hr) and silver-stained. The non-denatured PCR product was included as the double strand (ds) control.



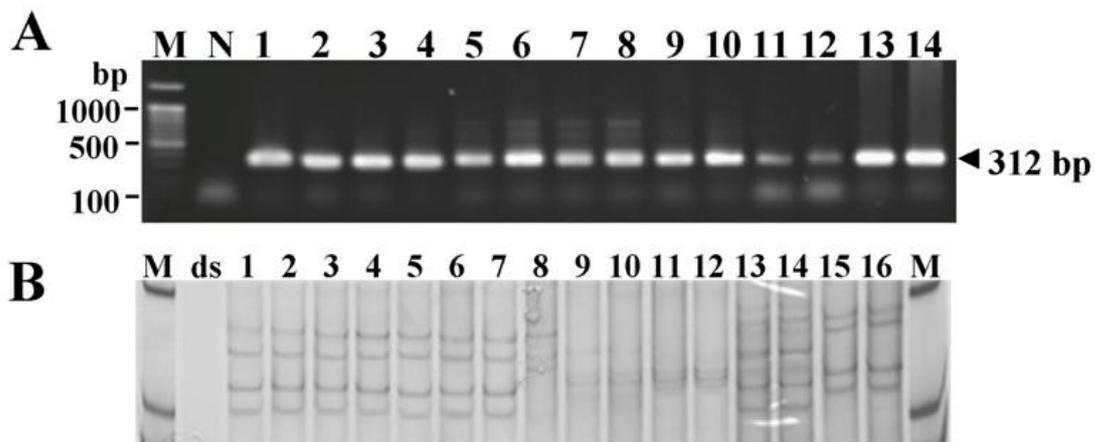
**Fig. 4.** (A) Amplification results of PP-SCAR<sub>262</sub> using genomic DNA of *P. pelagicus* (lanes 1-20), *S. oceanica* (lanes 21-22), *S. serrata* (lanes 23-24), *S. tranquebarica* (lanes 25-26), *C. crucifera* (lanes 27-28) and *P. sanguinolentus* (lanes 29-30). Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively. (B) SSCP patterns of PP-SCAR<sub>262</sub> of *P. pelagicus* (lanes 1-5) and *P. sanguinolentus* (lanes 6-15) electrophoretically analyzed through a 15% non-denaturing polyacrylamide gel (crosslink = 37.5:1; 250 V at 4°C for 19 hr) and silver-stained. The non-denatured PCR product was included as the double strand (ds) control.



**Fig. 5.** Species-specific PCR of *PP-COI*<sub>270</sub> against genomic DNA of *P. pelagicus* (lanes 1-31, A and lanes 1-15, B), *S. oceanica* (lanes 16-18, B), *S. serrata* (lanes 19-21, B), *S. tranquebarica* (lanes 22-24, B), *C. crucifera* (lanes 25-27, B) and *P. sanguinolentus* (lanes 28-30, B). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.



**Fig. 6.** (A) Amplification results of *12S rDNA*<sub>312</sub> using genomic DNA of *P. pelagicus* (lanes 1-4), *S. oceanica* (lanes 5-6), *S. serrata* (lanes 7-8), *S. tranquebarica* (lane 9-10), *C. crucifera* (lanes 11-12) and *P. sanguinolentus* (lanes 13-14). Lanes M and N are a 100 bp DNA marker and the negative control (without DNA template), respectively. (B) SSCP pattern of *12S rDNA*<sub>312</sub> of *P. pelagicus* (lanes 1-7), *S. oceanica* (lane 8), *S. serrata* (lanes 9-10), *S. tranquebarica* (lanes 11-12), *P. sanguinolentus* (lanes 13-14) and *C. crucifera* (lanes 15-16) electrophoretically analyzed through a 15% non-denaturing polyacrylamide gel (crosslink = 37.5:1; 300 V at 4°C for 13.5 hr) and silver-stained. The non-denatured PCR product was included as the double strand (ds) control.



**Fig. 7.** Multiple alignments of *12S rDNA*<sub>312</sub> of *P. pelagicus* (PP), *S. oceanica* (SO), *S. serrata* (SS), *S. tranquebarica* (ST), *P. sanguinolentus* (PS) and *C. crucifera* (CC) (GenBank accession no. GU906281 – GU906290). The location and sequence of a forward primer (*12S rDNA*<sub>312</sub>-F) and those complementary to a reverse primer (*12S rDNA*<sub>312</sub>-R) are boldfaced and underlined.

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PP-12S rDNA312-1 TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTGCTCTTTAAA-CGATACACCAAGAAATA
PP-12S rDNA312-2 TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTGCTCTTTAAA-CGATACACCAAGAAATA
PP-12S rDNA312-3 TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTGCTCTTTAAA-CGATACACCAAGAAATA
PP-12S rDNA312-4 TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTGCTCTTTAAA-CGATACACCAAGAAATA
PP-12S rDNA312-5 TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTGCTCTTTAAA-CGATACACCAAGAAATA
PS-12S rDNA TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTGCTCTTTAAA-CGATACACCAAGAAATA
SO-12S rDNA TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTGGTTTTAAATCGATACACCAAGAAA
ST-12S rDNA TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTGGTTTTAAATCGATACACCAAGAAA
SS-12S rDNA TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTG-TTTTTGAATCGATACACCAAGAAA
CC-12S rDNA TTGGCGGTGGTTAGTCTTGTCAGAGGAACCTG-TTTTTGAATCGATAAACCAGAAATA
*****

PP-12S rDNA312-1 TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
PP-12S rDNA312-2 TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
PP-12S rDNA312-3 TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
PP-12S rDNA312-4 TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
PP-12S rDNA312-5 TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
PS-12S rDNA TCTTACTTAAGTTAGTAG---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
SO-12S rDNA TCTTACTTAAGCTTGTATA---TAGTATGTATACCATCATTATTAGGTCATTTTTATAGAAT
ST-12S rDNA TCTTACTTAAGCTTGTATA---TAGTATGTATACCATCATTATTAGGTCATTTTTATAGAAT
SS-12S rDNA TCTTACTTAAGCTTGTATA---TAGTATGTATACCATCATTATTAGGTCATTTTTATAGAAT
CC-12S rDNA TCTTACTTAAGCTTGTATA---TAGTATGTATACCATCATTATTAGGTCATTTTTATAGAAT
*****

PP-12S rDNA312-1 AAATTACTGGAA---AGTT-TCGATAATGTGAATATATTAGATCAAGGTGCAGCTGATA
PP-12S rDNA312-2 AAATTACTGGAA---AGTT-TCGATAATGTGAATATATTAGATCAAGGTGCAGCTGATA
PP-12S rDNA312-3 AAATTACTGGAA---AGTT-CCGATAATGTGAATACATTAGATCAAGGTGCAGCTGATA
PP-12S rDNA312-4 AAATTACTGGAA---AGTT-TCGATAATGTGAATATATTAGATCAAGGTGCAGCTGATA
PP-12S rDNA312-5 AAATTACTGGAA---AGTT-TCGATAACGTTGAATATATTAGATCAAGGTGCAGCTGATA
PS-12S rDNA AAATTACTGTAT---TAAGATTAAATGTAAATATATTAGATCAAGGTGCAGCTTATA
SO-12S rDNA AAATTACTAAAA-ATAAATTAAATAGTGTAAATATATTAGATCAAGGTGCAGCTAATG
ST-12S rDNA AAATTACTGAAAGATAGTTAATAATAGTGTAAATATATTAGATCAAGGTGCAGCTAATG
SS-12S rDNA AAATTACTGTA---TATTTGTTAAATACGTTAAATATATTAGATCAAGGTGCAGCTAATG
CC-12S rDNA AAATTACTGAA----TTTGCTAATPATGTAAATATATTAGATCAAGGTGCAGCTTATA
*****

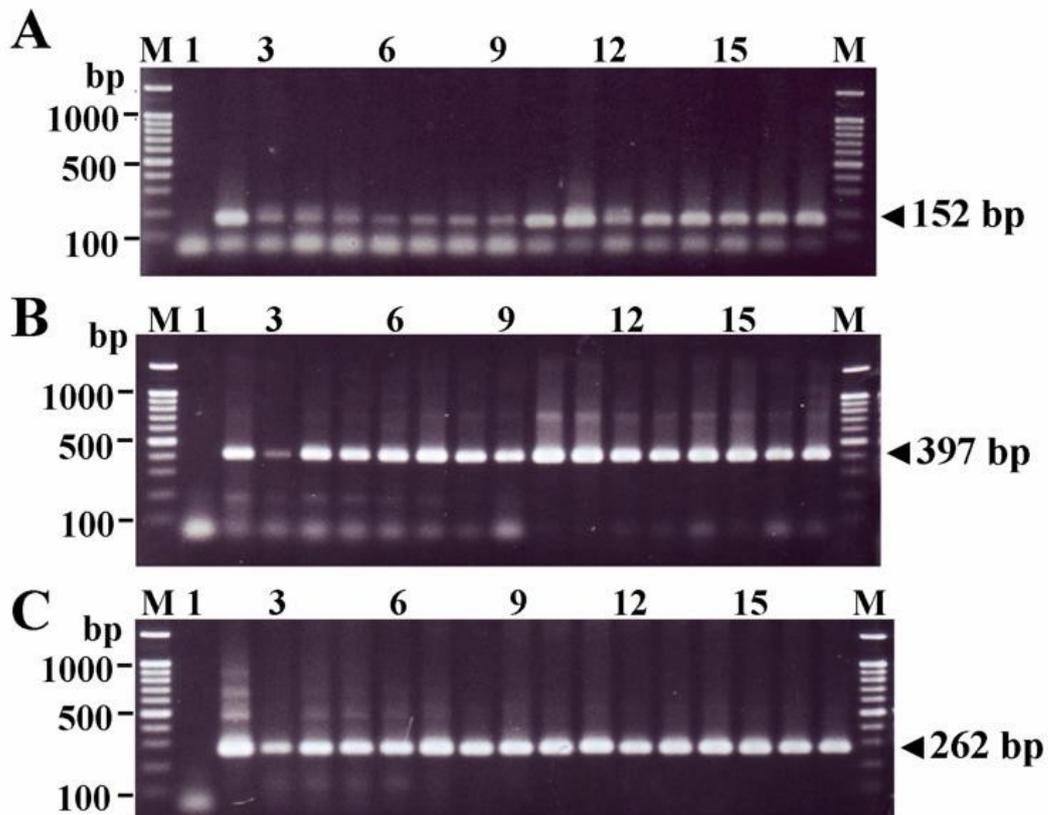
PP-12S rDNA312-1 ----CTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAA
PP-12S rDNA312-2 ----CTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAA
PP-12S rDNA312-3 ----CTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAA
PP-12S rDNA312-4 ----CTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAA
PP-12S rDNA312-5 ----TTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAA
PS-12S rDNA ----CTTAAGTCAAGATGGGTTACAATAGAATTTATGCTAT-AOGAATGAGTAACTGAAA
SO-12S rDNA ----CTTAAGTCAAAATGGGTTACAATAAAATTTATTTAATAOGGAATATTAATTTAAA
ST-12S rDNA ----TTTAAGTTAAAATGGGTTACAATAAAATTTATTTAATAOGGAATATTAATTTAAA
SS-12S rDNA AATGCTCAAGTTAAAATGGGTTACAATAAAA-TTCATTTAATAOGGAATATTAATTTAAA
CC-12S rDNA ----GTTGAGTTAAAATGGGTTACAATAAGT-TTTATTTAATAOGGAGATATAGTTTAAA
*****

PP-12S rDNA312-1 CTTT-ACTTTGAAGGAGGATTTGATTGTAATTTAGTTTAAATAAGCT-AATTTGATATAA
PP-12S rDNA312-2 CTTT-ACTTTGAAGGAGGATTTGATTGTAATTTAGTTTAAATAAGCT-AATTTGATATAA
PP-12S rDNA312-3 CTTT-ACTTTGAAGGAGGATTTGATTGTAATTTAGTTTAAATAAGCT-AATTTGATATAA
PP-12S rDNA312-4 CTTT-ACTTTGAAGGAGGATTTGATTGTAATTTAGTTTAAATAAGCT-AATTTGATATAA
PP-12S rDNA312-5 CTTT-ACTTTGAAGGAGGATTTGATTGTAATTTAGTTTAAATAAGCT-AATTTGATATAA
PS-12S rDNA TTGTGACTCGGAAGGAGGATTTGATTGTAATCTAGTTTAAATAAGCT-AGTTGATATAA
SO-12S rDNA TAATTAATTAGAAGGAGGATTTGATTGTAATTTAAATTTAATATGTTTAGTTAGATATAA
ST-12S rDNA TAGTTAATTAGAAGGAGGATTTGATTGTAATTTAAATTTAATATGTTTAAATTAGATATAA
SS-12S rDNA TAGTTAATTAGGAGGAGGATTTGATTGTAATAAAGTTAATAAGCTTAGTTAGATATAA
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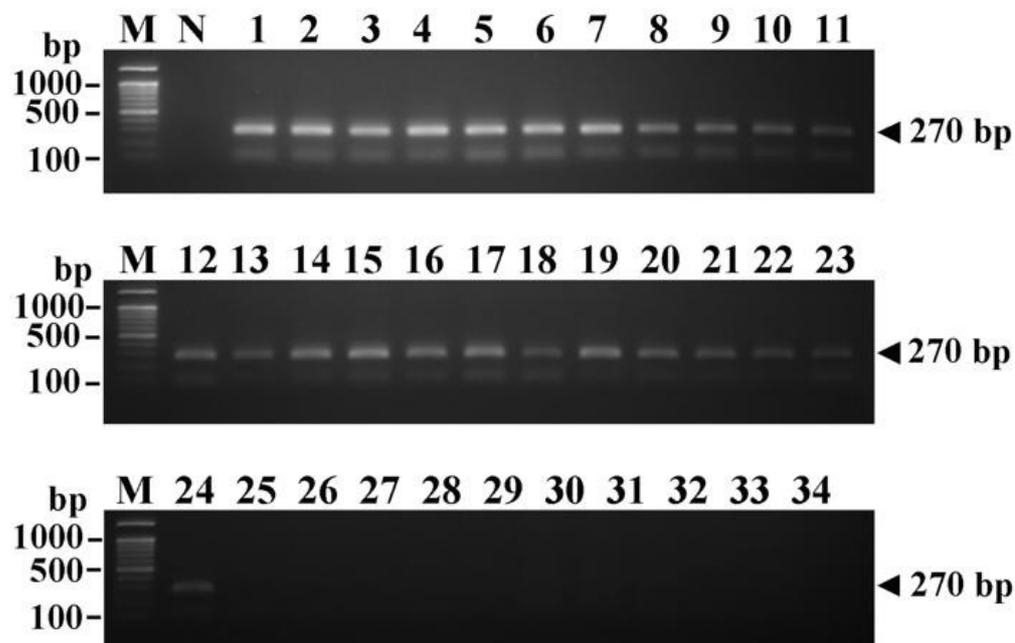
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PP-12S rDNA312-2 GCTCTAAGCATGTACACATCGCCCG
PP-12S rDNA312-3 GCTCTAAGCATGTACACATCGCCCG
PP-12S rDNA312-4 GCTCTAAGCATGTACACATCGCCCG
PP-12S rDNA312-5 GCTCTAAGCATGTACACATCGCCCG
PS-12S rDNA GCTCTAAGCATGTACACATCGCCCG
SO-12S rDNA GCTCTAAGCATGTACACATCGCCCG
ST-12S rDNA GCTCTAAGCATGTACACATCGCCCG
SS-12S rDNA GCTCTAAGCATGTACACATCGCCCG
CC-12S rDNA GCTCTAAGCATGTACACATCGCCCG
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**Fig. 8.** Amplification of genomic DNA isolated by phenol/chloroform (lanes 2-9) and 5% chelex extraction (lanes 10-17) methods of frozen (lanes 2-3 and 10-11), boiled (lanes 4-7 and 12-15) and saline-preserved (lane 8-9 and 16-17) meat of *P. pelagicus* with PP-SCAR<sub>152</sub> (A), PP-SCAR<sub>397</sub> (B) and PP-SCAR<sub>262</sub> (C) primers. Lane 1 is the negative control (without genomic DNA template). A 100 bp ladder (lanes M) was used as the DNA marker.



**Fig. 9.** Species-specific PCR of *PP-COI*<sub>270</sub> against genomic DNA of canned crab meat from companies A (lanes 1-3 and 13-15), B (lanes 4-6 and 16-18) C (lanes 7-9 and 19-21) and D (lanes 10-12 and 22-24) isolated by phenol/chloroform (lanes 1-12) and 5% chelex extraction (lanes 13-24) and that against phenol/chloroform-extracted genomic DNA from different individuals of wild *S. oceanica* (lane 25-26), *S. serrata* (lane 27-28), *S. tranquebarica* (lanes 29-30), *C. crucifera* (lanes 31-32) and *P. sanguinolentus* (lanes 33-34). Lanes M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.



**Fig. 10.** SSCP patterns of the amplified *12S rDNAA<sub>312</sub>* using genomic DNA isolated by phenol/chloroform (A) and 5% chelex extraction (B) methods of canned crab meat from companies A (lanes 1-2), B (lanes 3-4) C (lanes 5-6) and D (lanes 7-8) and those using phenol/chloroform-extracted genomic DNA from different individuals of wild *P. pelagicus* (lanes 9-11), *S. oceanica* (lane 12), *S. serrata* (lane 13), *S. tranquebarica* (lane 14), *C. crucifera* (lanes 15-16) and *P. sanguinolentus* (lanes 17-18). The non-denatured PCR product was included as the double strand (ds) control.

