



Analytical Methods

Molecular identification of the black tiger shrimp (*Penaeus monodon*), the white leg shrimp (*Litopenaeus vannamei*) and the Indian white shrimp (*Fenneropenaeus indicus*) by PCR targeted to the 16S rRNA mtDNA

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ABSTRACT

Polymerase chain reaction-based methodologies have been developed for the identification of three commercially-relevant penaeid shrimp species, these were: *Litopenaeus vannamei*, *Penaeus monodon* and *Fenneropenaeus indicus* in food products. Such three species represent more than 80% of the whole farmed shrimp production worldwide and may be fraudulently replaced by species exhibiting lower value such as *Litopenaeus stylirostris*, *Penaeus semisulcatus* and *Fenneropenaeus merguensis*, respectively. For it, preliminary sequencing of a mitochondrial sequence of ca. 530 bp in the 16S rRNA/tRNA^{Val} mitochondrial region was performed in nearly 20 penaeid shrimp species of commercial relevance. Careful analysis of such sequences allowed the design of primers PNVF/PNVR, which allowed the combined identification of *P. monodon* and *L. vannamei*, and PNIF/PNIR, which allowed the specific identification of *F. indicus*. In addition, *P. monodon* and *L. vannamei* could be easily differentiated by either restriction with *TspE1* or by amplification with novel primers MPNF/MPNR, specific for *P. monodon*. The proposed specific methods improve current general identification methods of these species based on more general RFLP analyses. In addition, these methods can be easily completed in less than 8 h.

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1. Introduction

The accurate labelling of food components avoids commercial fraud, also preventing potential safety risks caused by the introduction of any food ingredient that might be harmful to human health (Lockley & Bardsley, 2000; Prado, Pascoal, Calo-Mata, & Barros-Velázquez, 2005). Aquatic food products represent a significant market niche that includes a wide number of species of commercial interest. Among them, penaeid shrimps belonging to the Superfamily Penaeoidea, either harvested by extractive fishing or farmed in aquaculture facilities, account for more than 30% of the worldwide demand of crustacean species (Pérez-Farfante & Kensley, 1997; Rosenberry, 2001). Traditional identification of penaeid shrimps has been described to be complex and even impossible when the external anatomical parts are not present (Pascoal, Barros-Velázquez, Cepeda, Gallardo, & Calo-Mata, 2008b). Thus, the phenotypic similarities among penaeid shrimp species and the fact that in their processing their external carapace is often removed,

complicates this situation. In this scenery, either commercial fraud caused by the inadvertent substitution of species due to phenotypic similarities, or deliberate replacement of higher quality species by others of lower quality may occur, these leading to mislabelled adulterated products (Vondruska, Ottwell, & Martin, 1988).

The Pacific white shrimp *Litopenaeus vannamei*, also called white leg shrimp, is the leading farm-raised species in the Western hemisphere (Rosenberry, 2001). This species is native to the Pacific coast from Mexico to Peru, and accounts for 95% of the production in Latin America. *L. vannamei* can be replaced by the blue shrimp *Litopenaeus stylirostris*, since both species are almost identical for the consumer and both may be frequently mixed during commercialisation under the generic name of Western white shrimp (Rosenberry, 2001). Though often mixed together and sold under the same name, these two species have different sensory quality. Global suppliers of Pacific white shrimps are Belize, Colombia, Costa Rica, Ecuador, Honduras, Mexico, Nicaragua, Panama, Peru and the United States.

Likewise, the black tiger prawn *Penaeus monodon*, also known as giant tiger shrimp, occurs in the wild in the Indian Ocean and in the Pacific Ocean from Japan to Australia (Rosenberry, 2001). The black

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tiger shrimp dominates the major global share of shrimp products at 56% of the total world shrimp production. They are farmed everywhere in Asia especially Thailand, but also in other countries such as Ecuador, India, Indonesia, Bangladesh and Vietnam. The black tiger shrimp has a mild and sweet flavour and the cooked meat is firm and moist. Although this species is the most farmed among the penaeid shrimp species, its sensitivity to whitespot disease and the difficulty of breeding it in captivity has caused its gradual replacement by *L. vannamei* since 2001 (Rosenberry, 2001). From the commercial point of view, *P. monodon* may be marketed together with the green tiger shrimp *Penaeus semisulcatus* without any specific labelling, although recent studies have provided evidence of some divergence between both species (Pascoal, Barros-Velázquez, Cepeda, Gallardo, & Calo-Mata, 2008a; Pascoal et al., 2008b; Voloch, Freire, & Russo, 2005). In global numbers, *L. vannamei* and *P. monodon* are so relevant to the international markets that together represent about 80% of the whole farmed shrimp production worldwide (Rosenberry, 2001). Finally, *Fenneropenaeus indicus*, also known as “Indian white shrimp” is another commercially-relevant shrimp species farmed on extensive facilities in Southeast Asia, and widely cultured in India, the Middle East and Eastern Africa (Rosenberry, 2001). The commercialisation of this species may be complicated due to its anatomical similarities with respect to the banana shrimp *Fenneropenaeus merguensis*, the latter species exhibiting lower commercial value.

Molecular tools targeted to protein (Ortea, Cañas, Calo-Mata, Barros-Velázquez, & Gallardo, 2009, 2010) and DNA (Bellis, Ashton, Freney, Blair, & Griffiths, 2003; Brzezinski, 2005; Maggioni, Rogers, Maclean & D’Incao, 2001) biomarkers have been proposed as suitable strategies for species identification in crustacean species. Nevertheless, while electrophoretic and immunological methods may fail due to the lack of stability of the polypeptide targets when these products have been subjected to a processing step (Piñeiro, Vázquez, Figueras, Barros-Velázquez, & Gallardo, 2003), DNA-analysis may circumvent these problems due to its remarkable stability. Among the DNA targets considered for penaeid shrimp species identification, 12S rRNA, 16S rRNA, tRNA^{Val} and, to a lesser extent, cytochrome oxidase I (COI) and cytochrome *b* genes have been proposed as useful molecular markers in some crustacean species (Bellis et al., 2003; Brzezinski, 2005; Calo-Mata et al., 2009; Dharani et al., 2009; Maggioni, Rogers, Maclean & D’Incao, 2001; Pascoal, Barros-Velázquez, Cepeda, Gallardo, & Calo-Mata, 2008c), mainly through PCR–RFLP strategies using generic or universal oligonucleotide primers. However, methods aimed at specifically detecting the most relevant shrimp species in terms of commercial value have not been proposed up to date. Should they be developed, they would allow the direct detection of such species avoiding the potential complications of complex restriction patterns caused by the presence of more than one shrimp species, or by single nucleotide polymorphic events (SNPs) in the target sequence.

Accordingly, the main purpose of this study was to achieve the specific identification of the commercially-relevant *L. vannamei*, *P. monodon* and *Fen. indicus* species. For it, preliminary sequencing of a mitochondrial sequence of ca. 530 bp in the 16S rRNA/tRNA^{Val} mitochondrial region was performed in nearly 20 penaeid shrimp species of commercial relevance. Careful analysis of such sequences allowed the building of specific primers for the identification of the above-cited three commercially-relevant shrimp species.

2. Materials and methods

2.1. Penaeid shrimp species considered

Specimens belonging to the following five species: *L. vannamei*, *P. monodon*, *P. semisulcatus*, *Fen. indicus* and *Fen. merguensis* were

collected from either extractive fishing practices or aquaculture facilities from different fishing banks and continents worldwide (Table 1), all under the management and supervision of expert seafood technologists from the Centro Tecnológico del Mar (CETMAR, Vigo, Spain). Two different populations were considered for each species except for *Fen. indicus*, for which three populations were included in the study (Table 1). Different populations from different geographic origins were included to select 16S rRNA nucleotide targets that do not exhibit intra-specific variability. Three specimens from each population were also analyzed. Specimens were classed in their respective taxons according to their anatomical external features with the help of marine biologists from the Marine Sciences Institute (Mediterranean Centre for Marine and Environmental Research, Higher Council for Scientific Research, CMIMA-CSIC, Barcelona, Spain) with expertise in penaeid shrimp taxonomy. Besides, all specimens were identified by PCR and sequencing using 16ScruC4/16ScruC3 primers as described elsewhere (Pascoal et al., 2008a). Reference 16S rRNA sequences from *L. stylirostris* were retrieved from GenBank (Table 1), analyzed and aligned with sequences from the other five shrimp species.

2.2. Isolation and purification of DNA

Samples of 250 mg of skeletal muscle of each of the five penaeid shrimp species cited above were obtained. As stated above, three different specimens were considered for each population belonging to each species. DNA was extracted by means of a commercial kit (DNeasy Tissue kit, QIAGEN, Darmstadt, Germany), as previously described (Prado, Calo, Cepeda, & Barros-Velázquez, 2005). DNA concentrations in the extracts were determined by measuring the fluorescence developed by mixtures of the purified DNA extract and Hoechst 33258 reagent (Sigma, St. Louis, MO) in an LS 50 fluorimeter (Perkin Elmer, Wellesley, MA, USA), as described elsewhere (Downs & Wilfinger, 1983; Pascoal, Prado, Calo, Cepeda, & Barros-Velázquez, 2005). Two standard curves were made in order to verify the linearity of the assay within a particular concentration range. Both standard curves were constructed on calf thymus DNA (Sigma) standard solutions. Standard curves were constructed with EXCEL (Microsoft, Redmond, WA) software. Correlation coefficients (r^2) higher than 0.99 were achieved in all cases.

2.3. Primer design, PCR-amplification, electrophoretic analysis and DNA sequencing

As a preliminary phase of this study, specimens from all five species were amplified with primers 16ScruC4 (5′-AATATGGCTGT TTTAAGCCTAATTCA-3′)/16ScruC3 (5′-CGTTGAGAAGTTCGTTGTGCA-3′), these allowing the amplification of a ca. 530 bp fragment of the 16S rRNA/tRNA^{Val} genes in all the five species tested. Amplification conditions were as described elsewhere (Pascoal et al., 2008a). Nucleotide sequence alignment in the species considered (Fig. 1) allowed us to identify specific fragments of the 16S rRNA mitochondrial region that allowed the design of primers for *L. vannamei* + *P. monodon*, *P. monodon* and *Fen. indicus* (Table 2).

PCR-amplification assays comprised 100 ng of template DNA, 25 µl of a master mix (BioMix, Bioline Ltd., London, UK) – this including reaction buffer, dNTPs, magnesium chloride and *Taq* DNA polymerase–, PCR water (Genaxis, Montigny le Bretonneaux, France) and 25 pmol of each oligonucleotide primer in a final volume of 50 µl. Amplification conditions were as follows: a previous denaturing step at 94 °C for 1 min 30 was coupled to 35 cycles of denaturation (94 °C for 20 s), annealing (51 °C for 20 s), and extension (72 °C for 30 s), and with a final extension step at 72 °C for 15 min. All PCR assays were carried out on a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA).

Table 1

Penaeid shrimp species considered in this study.

Scientific name (FAO)	Commercial names	Origin	Accession number	Reference
<i>L. vannamei</i> (PNV)	Pacific white shrimp	Argentina	EF589702	Pascoal et al. (2008a)
	White leg shrimp	Costa Rica	EF589703	Pascoal et al. (2008a)
<i>L. stylirostris</i> (PNS)	Blue shrimp	Mexico	AY046913	Gutiérrez-Millan et al. (2002)
		Mexico	AJ297970	Unpublished
<i>P. monodon</i> (MPN)	Giant tiger prawn	Commercial	EF589684	Pascoal et al. (2008a)
	Black tiger shrimp	Malaysia	EF589685	Pascoal et al. (2008a)
<i>P. semisulcatus</i> (TIP)	Green tiger prawn	Indo-West Pacific	EF589706	Pascoal et al. (2008a)
		Tanzania	EF589705	Pascoal et al. (2008a)
<i>Fen. indicus</i> (PNI)	Indian white prawn	Commercial	EF589688	Pascoal et al. (2008a)
		Madagascar	EF589687	Pascoal et al. (2008a)
		Mozambique	EF589686	Pascoal et al. (2008a)
<i>Fen. merguensis</i> (PBA)	Banana shrimp	Western Central Pacific	EF589693	Pascoal et al. (2008a)
		Indo-West Pacific	EF589692	Pascoal et al. (2008a)

**Fig. 1.** Alignment and location of primers 16S rRNA-targeted PNVF/PNVR for *L. vannamei* + *P. monodon*, PNIF/PNIR for *Fen. indicus* and MPNF/MPNR for *P. monodon* designed in this study. Forward and reverse primers are indicated for each species with arrows pointing right or left, respectively.**Table 2**

Specific primers designed in this study, target species and amplicon sizes.

Target species	Primer	Sequence	Size	Accession no.
<i>L. vannamei</i>	PNVF	GGAGTATTATTGTTTCTAGTAGAGTGAATCGA	151 bp	EF589702/EF589703
+ <i>P. monodon</i>	PNVR	TTAATCCTCCCTAATACACAGGTACA		
<i>P. monodon</i>	MPNF	TCATTGTGCAATCAGTGTATCTTGA	362 bp	EF589684/EF589685
	MPNR	ACCAGATATGAAAAACGATTACGATT		
<i>Fen. indicus</i>	PNIF	ATCATTITGAGATTTCATTTTCTAGTAGAGT	213 bp	EF589687/EF589688
	PNIR	CTATATATTTACATTAGCTCTTTTCTTTCCG		

The position of primers in the 16S rRNA gene of the target sequences is shown in Fig. 1.

The differentiation of *P. monodon* and *L. vannamei* was performed by restriction analysis with endonuclease *TspEI* (Sigma), or by amplification with primers MPNF/MPNR, specific for *P. monodon*. Standard restriction assays were carried out for 2 h at 37 °C in a final volume of 20 µl. PCR products were processed in 2.5% horizontal agarose (MS-8, Pronadisa, Madrid, Spain) electrophoresis. PCR–RFLP analyses were carried out by SDS–PAGE in 15% ExcelGel homogeneous gels (GE Healthcare) at 15 °C in a Multiphor II electrophoresis unit (Amersham Biosciences, Uppsala, Sweden). The latter gels were stained using a standard silver staining protocol (Amersham Biosciences). When required, PCR products were purified from the agarose gels by means of the MinElute Gel Extraction kit (QIAGEN). Image analysis was performed using the 1-D Manager software (TDI, Madrid, Spain).

Prior to sequencing, the PCR products were purified by means of the ExoSAP-IT kit (GE Healthcare, Uppsala, Sweden). Direct sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The same primers used for PCR were considered for the sequencing of both strands of the PCR products, respectively. Sequencing reactions were analyzed in an automatic sequencing system (ABI 3730XL DNA Analyser, Applied Biosystems) provided with the POP-7 system. SNP events in DNA sequences were carefully reviewed by eye, using the Chromas software (Griffith University, Queensland, Australia). Alignment of sequences was accomplished using the CLUSTALW software (Thompson, Higgins, & Gibson, 1994).

3. Results

3.1. Design of primers for the identification of shrimp species

As a preliminary step performed in our laboratory, the previously-designed 16ScruC4/16ScruC3 primers were used to amplify a ca. 530 bp 16S rRNA/tRNA^{Val} mitochondrial region well conserved in penaeid shrimp species. Sequences for each species were subjected to intra- and inter-specific alignment and comparison with the aim of selecting specific primers for the identification of *P. monodon*, *L. vannamei* and *Fen. indicus*, respectively. Accordingly, oligonucleotide primers were selected for the identification of the white leg shrimp *L. vannamei* and the black tiger shrimp *P. monodon* (PNVF/PNVR) and the Indian white shrimp *Fen. indicus* (PNIF/PNIR). In addition, primers MPNF/MPNR, specific for *P. monodon*, were also designed. The nucleotide sequences of the selected primers are shown in Table 2. Thus, the sizes of the predicted amplification products were in the range of 151–362 bp. The number of mismatches between PNVF/PNVR primers for the white leg shrimp *L. vannamei* and the homologous 16S rRNA regions of *L. stylirostris* AY049613 were two and three, respectively, while the number of mismatches exhibited by *L. stylirostris* AJ297970 was higher than 10 for each primer. In the case of primers MPNF/MPNR for the black tiger shrimp *P. monodon* and the homologous 16S rRNA regions of the green tiger shrimp *P. semisulcatus*, the number of mismatches was four and four, respectively. Finally, the nucleotide mismatches of PNIF/PNIR primers for the Indian white shrimp *Fen. indicus* with respect to the homologous 16S rRNA regions of the banana shrimp *Fen. merguensis* were four and three, respectively.

3.2. Identification of *P. monodon*, *L. vannamei* and *Fen. indicus* in food products

Fig. 2a shows the results of the amplification of the species considered in this study with the primers described above. Thus, primers PNVF/PNVR allowed the amplification of the 151 bp target only in specimens belonging to the species *L. vannamei* (white leg

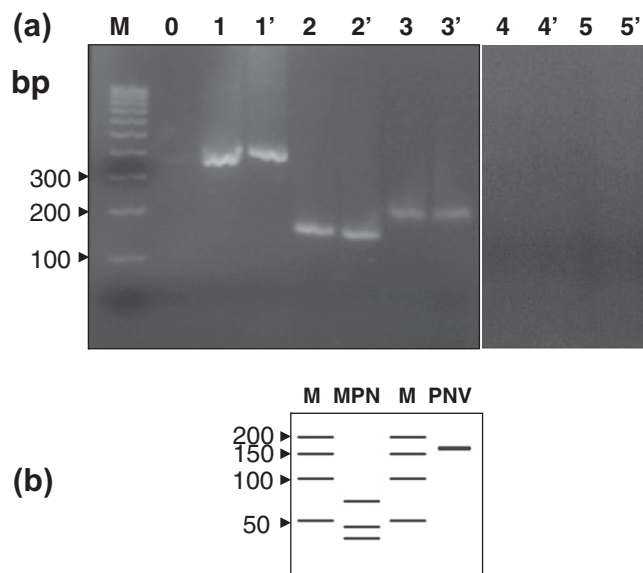


Fig. 2. (a) Amplification of the 16S rRNA mitochondrial targets in the three shrimp species. Lane M: molecular weight marker; lane 0: negative control; lanes 1 and 1' (primers MPNF/MPNR): *P. monodon*; lanes 2 and 2' (primers PNVF/PNVR): *L. vannamei*; lanes 3 and 3' (primers PNIF/PNIR): *Fen. indicus*; lanes 4 and 4' (primers MPNF/MPNR): *P. semisulcatus*; lanes 5 and 5' (primers PNIF/PNIR): *Fen. merguensis*. (b) Differentiation between *L. vannamei* and *P. monodon* by restriction analysis with endonucleases *TspEI* of the 151 bp PCR product amplified with PNVF/PNVR primers; lane M: molecular weight marker; MPN: *P. monodon*; PNV: *L. vannamei*.

shrimp) and *P. monodon* (black tiger shrimp), while none of the other shrimp species exhibited cross-amplification. Accordingly, this assay allowed us to detect the presence of the black tiger shrimp and the white leg shrimp, the two most commercialised penaeids, in a single assay. Interestingly, *L. stylirostris*, the penaeid shrimp species that may replace *L. vannamei* during the commercialisation of the Western white shrimp was discriminated with this assay (Fig. 2a). Likewise, the green tiger shrimp *P. semisulcatus*, the species that may complicate labelling of food products containing the black tiger shrimp *P. monodon*, neither gave cross-amplification.

The differentiation of the white leg shrimp *L. vannamei* with respect to the black tiger shrimp *P. monodon* could be easily achieved by two different strategies. On one hand, the 151 bp amplicon was cleaved with endonucleases *TspEI* in three restriction fragments of 69, 45 and 37 bp. only in the case of *P. monodon*, this allowing the specific identification of the black tiger shrimp with respect to the white leg shrimp *L. vannamei* (Fig. 2b). Moreover, PCR-amplification with primers MPNF/MPNR provided a 362 bp PCR product only in the case of *P. monodon*, with no cross-amplification being observed in the cases of all other shrimp species tested (Fig. 2a). This result allows the specific identification of the black tiger shrimp in food products, even in the case of the simultaneous presence of related species such as the white leg shrimp *L. vannamei* or the green tiger shrimp *P. semisulcatus* (Fig. 2a).

Finally, amplification with PNIF/PNIR primers gave a positive result only in the case of the Indian white shrimp *Fen. indicus*, no cross-reaction reaction being observed with the banana shrimp *Fen. merguensis*, a species of lower commercial value that may be fraudulently used to replace *Fen. indicus* during commercialisation (Fig. 2a).

4. Discussion

As stated above, the molecular identification of penaeid shrimp species of food interest has not been extensively considered up to now. Thus, a previous report by other authors described the useful-

ness of a 1.38 kb mitochondrial region that comprises fragments of the 16S rRNA and 12S rRNA genes and the entire tRNA^{Val} region for phylogenetic analysis of penaeid shrimps (Gutiérrez-Millán, Peregrino-Uriarte, Sotelo-Mundo, Vargas-Albores, & Yépiz-Plasencia, 2002). However, such study only considered three Eastern Pacific species, *Penaeus californiensis*, *Penaeus vannamei* and *Penaeus stylirostris*, and was focused on phylogenetic aspects, not on their specific identification. Likewise, another previous study provided a molecular method – based on COI, cytochrome oxidase II (COII) and 16S rRNA mitochondrial genes – for the identification of five shrimp species – *P. monodon*, *P. semisulcatus*, *L. vannamei*, *Fen. merguensis* and *Marsupenaeus japonicus* (Khamnamtong, Klinbunga, & Menasveta, 2005) – using restriction analysis of a 312 bp fragment of the 16S rRNA mitochondrial gene with the endonuclease *SspI*, *VspI* and *AluI*. However, *P. semisulcatus*, *Mars. japonicus* and *L. vannamei* could not be distinguished with this methodology since each exhibited identical restriction profiles. More recently, a method for the detection of crustacean DNA based on a PCR–RFLP approach has been proposed (Brzezinski, 2005). However, this method, aimed at the detection of potentially allergenic proteins, included only nine shrimp species and did not allow species identification, although it did permit their generic detection and differentiation with respect to crab, lobster and crawfish species. Recently, we have reported the identification of commercial shrimp species by two step PCR–RFLP methodologies targeted to 16S rRNA/tRNA^{Val} and cytochrome *b* mtDNA, respectively (Pascoal et al., 2008a, 2008c). However, such methods were based on a PCR–RFLP approach, these making them useful for a wide variety of penaeid shrimps, but not being focused on the specific identification of the few most relevant species that represent the majority of the international trade of decapoda crustaceans.

Accordingly, and as a complement of the above-cited methods, the specific PCR-based methodology described in this work represents a complement to the previously described methods based on universal PCR–RFLP approaches, and provides a novel authentication tool for the direct detection and identification of the three most relevant penaeid shrimp species in food products.

5. Conclusions

The PCR-based methods proposed in this study allow the specific identification of *L. vannamei*, *P. monodon* and *Fen. indicus* in food products, thus providing a tool to discriminate the presence of competing species such as *L. stylirostris*, *P. semisulcatus* and *Fen. merguensis*, respectively, and providing a valuable molecular tool for the administration and industry to check compliance with labelling and traceability regulations.

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