Food Chemistry 239 (2018) 369–376

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

A single enzyme PCR-RFLP protocol targeting 16S rRNA/tRNA^{val} region to authenticate four commercially important shrimp species in India

Lidiya Wilwet [a], Geevaretnam Jeyasekaran [a,*], Robinson Jeya Shakila [a], Balasubramanian Sivaraman [a], Pandurengan Padmavathy [b]

[a] Department of Fish Quality Assurance and Management, Fisheries College and Research Institute, Tamil Nadu Fisheries University, Thoothukkudi 628 008, India
[b] Department of Fisheries Environment, Fisheries College and Research Institute, Tamil Nadu Fisheries University, Thoothukkudi 628 008, India

ARTICLE INFO

Article history:
Received 1 March 2017
Received in revised form 12 May 2017
Accepted 22 June 2017
Available online 23 June 2017

Keywords:
PCR-RFLP
mtDNA
Single enzyme
Penaeid shrimps
Species authentication
Seafood adulteration

ABSTRACT

Food authenticity is an issue of major concern for food authorities, as mislabeling represents one of the major commercial frauds. In this study, a novel PCR-RFLP protocol was developed as a tool to authenticate four shrimp products of commercial importance belonging to the family, Penaeidae, *viz. Litopenaeus vannamei, Penaeus monodon, P. semisulcatus* and *Fenneropenaeus indicus*. PCR amplification was performed targeting 16S rRNA/tRNA^{val} region having an amplicon size of 530 bp using the specific primers for shrimps, 16S-Cru4/16S-Cru3. Subsequent restriction analysis with a single restriction enzyme, Tsp5091, yielded distinct RFLP pattern for each species of shrimps having fragment sizes below 150 bp. The unique RFLP patterns were also obtained in processed shrimp products without any degradation or alteration in the major fragments. The method was also validated with commercial shrimp products. Thus, the developed protocol can be performed within 8 h using a single enzyme to authenticate four shrimp products of commercial significance.

1. Introduction

Shrimps are an important resource for both commercial fisheries and aquaculture in many countries, accounting for more than 30% of global consumption of seafood Worldwide (Rosenberry, 2001). Out of the total seafood export of 5.50 billion US$ from India during 2014–15, frozen shrimps contributed to a share of 67.19% in terms of the total USD earnings (Marine Product Export Development Authority India. Press Release Export statistics., 2015). Additionally, in recent years, the shrimp aquaculture is witnessing a tremendous growth recording the highest production of 4.34 lakh MT in 2014–2015. Among the shrimp species under culture in India, Pacific white shrimp (*Litopenaeus vannamei)* dominates by 41%, followed by scampi (32%), while the production of black tiger shrimp remains stagnant. Also, frozen shrimps continued to be the major export item in terms of quantity and value, and the USA is the largest market (1, 12,702 MT) for shrimp exports in quantity terms, followed by European Union (81,952 MT), South East Asia (69,068 MT) and Japan (30,434 MT) (Marine Product Export Development Authority India. Press Release Export statistics., 2015). Shrimps are marketed mainly in raw, headless with shell on, peeled and undeveined (PUD), peeled and deveined (PD), tail on, butterfly and cooked forms.

The high demand and popularity of shrimp products have paved the way for species substitution in the commercial market. Pacific white shrimp (*Litopenaeus vannamei)* is being replaced with the blue shrimp (*Litopenaeus stylirostris).* Even though both species are identical to the consumers visually, they have a different odour and taste. Likewise, the giant tiger shrimp (*Penaeus monodon)* is marketed together with the flower shrimp (*Penaeus semisulcatus)* without any specific labeling (Rosenberry, 2001). In 2014, a report by the Ocean Conservation Group (2015), Oceana, revealed that 15% of shrimps are mislabeled in terms of either the method of production (farm-raised or wild-caught) or species. Yet another way of marketing is labeling the farmed species as "Gulf shrimps," which actually contains a mixture of different species. There is a report that a sample of frozen shrimp salad contained a type of aquarium pet shrimp that is not intended for human consumption (www.mercola.com). So, the authentication of shrimp species has become a serious concern in the seafood industry.

In many countries, the enforcement of correct labeling is emerging as a mandatory requirement. Fresh seafood must disclose whether the food is farmed or caught. However, in the case of processed foods, including seafood that is steamed, breaded, canned or fried, it becomes highly impractical for such disclosure.

* Corresponding author.
E-mail address: jeyasekarang@gmail.com (G. Jeyasekaran).

http://dx.doi.org/10.1016/j.foodchem.2017.06.132
0308-8146/

In developed nations, nearly 50% of the shrimps sold are in processed form and often, they do not bear the appropriate label regarding the species, or farming method. In order to enforce labeling regulations and to prevent product substitution, there is a need for a fast and reliable method to authenticate the shrimp species and to protect the consumers from economic fraud.

Molecular methods targeting protein and DNA have been proposed as suitable strategies for species identification in crustaceans (Brzezinski, 2005; Ortea, Canas, Barros-Velazquez, Calo-Meta, & Gallardo, 2010). Among which, DNA based methods are preferred due to their stability, specificity, sensitivity and reliability, than that of protein based methods. Mitochondrial DNA is more commonly employed than genomic DNA for species authentication because of its maternal inheritance, relatively fast evolutionary rate, lack of intermolecular genetic recombination and also the presence of higher copy number (Chow, Okamoto, Uozumi, Takeuchi, & Takeyama, 1997; Rasmussen & Morrissey, 2008). The 12S rDNA and 16S rDNA regions of mitochondrial DNA are considered as the better DNA targets than Cytochrome Oxidase and Cytochrome *b* regions for peneaid shrimp species identification (Baldwin, Bass, Bowen, & Clark, 1998; Pascoal, Barros-Velazquez, Cepeda, Gallardo, & Calo-Mata, 2008a).

There are several molecular methods available for species authentication utilizing PCR. They are restriction fragment length polymorphism (RFLP), forensically informative nucleotide sequencing (FINS), amplified fragment length polymorphism (AFLP), or single-stranded conformational polymorphism (SSCP) (Lockley & Bardsley, 2000). Apart from that, there are several modern approaches for species authentication, such as DNA barcoding, Simple sequence repeats (SSR) and new generation sequencing (NGS), which are all based on part or whole sequencing of target region (Armani, Castigliego, Tinacci, Gianfaldoni, & Guidi, 2011; Angelica et al., 2014; Galal-Khallaf et al., 2017). Among these, RFLP is a well-studied method and widely accepted in seafood authentication because of its simplicity, speed, resolving power, low cost and no prior sequence knowledge as compared with other methods (Cespedes et al., 2000; Hisar, Aksakal, Hisar, Yanik, & Suhendan, 2008). In this study, four important exportable shrimp species are selected to develop a single enzyme PCR-RFLP method targeting the mtDNA gene to authenticate them in processed forms.

2. Materials and methods

2.1. Raw material

Four commercially important shrimp species were procured and brought to the laboratory in chilled condition. All species were morphologically identified by following the keys to the identification as per FAO Species Catalogue (Heemstra & Randall, 1993). All shrimp species belonged to the phylum; Crustacea, order; Decapoda and family Penaeidae. They were further identified as *Litopenaeus vannamei* (Pacific white shrimp), *Penaeus monodon* (Tiger shrimp), *P. semisulcatus* (Flower shrimp) and *Fenneropenaeus indicus* (Indian white shrimp) and were designated as PS, TS, FS and WS, respectively. Each species was divided into five different groups. The first group was "frozen at −40 °C" for 3 h in ultra-freezer and designated as "Frozen" shrimps (Fo). The second group was cooked at 100 °C for 20 min and designated as "Cooked" shrimps (Co). The third group was canned at 15 psi for 15 min and designated as "Canned" shrimps (Cn). The fourth group was shallow fried at 180 °C for 10 min and designated as "Fried" shrimps (Fr). The final group was not given any processing treatment and was designated as "Raw" shrimps (Ra).

2.2. DNA extraction

Total DNA was extracted from all the shrimp species as per the method described by Sumathi et al. (2015). About 50 mg of tissue was taken in a 2 ml microfuge tube, to which 940 μl of lysis buffer, 30 μl of proteinase K and 30 μl of 20% SDS were added. After homogenization, the tubes were incubated at 48 °C for 50 min in a water bath. Then, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added to the lysed tissue in the tube. The contents were mixed gently and centrifuged at 9200*g* for 10 min (5415R, Eppendorf, Germany). The top aqueous layer was then transferred to a new 1.5 ml microfuge tube. The DNA was precipitated by the addition of an equal volume of isopropanol and 0.2 vol of 10 M ammonium acetate. The tube was again centrifuged at 13,200*g* for 10 min at 4 °C. The supernatant was removed using a micropipette. The pellet was then washed again in 500 μl of chilled 70% ethanol, air-dried and re-suspended in 100 μl of sterile water and used for PCR analysis.

2.3. Amplification of mitochondrial 16S rDNA gene fragment

The PCR amplification of 16S rRNA/tRNA^{val} region of the mtDNA was done with the set of primers (Table 1) already designed by Pascoal et al. (2008a). The PCR amplification assay comprised of 3 μl of template DNA, 25 μl of a master mix (consisting of reaction buffer, dNTPs, magnesium chloride, Taq DNA polymerase), 20 μl of molecular grade water and 25 pmol of each oligonucleotide primer in a final volume of 50 μl. Amplification condition had an initial denaturing step at 95 °C for 1 min 30 s coupled to 35 cycles of denaturation (94 °C for 20 s), annealing (50 °C for 20 s), and extension (72 °C for 30 s), and with a final extension step at 72 °C for 15 min. The size of the amplified product was 530 bp. The PCR products (5 μl) were analyzed by gel electrophoresis on 2% agarose gel using 0.5% TAE buffer containing ethidium bromide (0.5 mg/ml). A 100 bp DNA ladder (Thermo Fisher Scientific Inc., Massachusetts, USA) was used as standard marker. Amplified DNA fragment was visualized under UV transilluminator and photographed using gel documentation system (Alpha Innotech Co., San Leandro, USA).

2.4. PCR-RFLP analysis

The PCR-RFLP analysis was performed with the restriction enzyme, *Tsp5091*. This enzyme was obtained from an *E. coli* strain that carries the *Tsp5091* (Thermo Fisher Scientific Inc., Massachusetts, USA) gene of *Thermus* sp and it recognizes ^AATT sites and cuts best at 65 °C in Tango buffer. The digestion reaction mixture (30 μl) comprised of 10 μl of PCR product, 2 μl of Tango assay buffer (10X), 1 μl (10 U) of restriction enzyme (*Tsp5091*), along with 17 μl of molecular grade water. The tubes were incubated at 65 °C in a water bath for 2 h. The reaction was stopped by adding 1.2 μl of 20 mM EDTA. The digested products (10 μl) were loaded onto 7% polyacrylamide gel and electrophoreses with 1% TBE buffer consisting of 8.9 mM Tris-Borate and 2 mM EDTA at pH 8.3. Silver staining was performed as per the method of Sumathi et al. (2015),

Table 1
Primers used for the amplification of mitochondrial 16S rRNA gene.

Target gene	Primer	Sequence	Product size
Mt16S rRNA	16 S-CruC4 (Forward)	5′-AATATGGCTGTTTTTAAGCCTAATTCA-3′	530 bp
	16 S-CruC3 (Reverse)	5′-CGTTGAGAAGTTCGTTGTGCA-3′	