# AUTHENTICATION OF PROCESSED BLUE SWIMMING CRAB MEAT (*PORTUNUS PELAGICUS*) USING PCR BASED MOLECULAR TECHNIQUE

Thesis submitted in part fulfillment of the requirements for the Degree

**Master of Fisheries Science in Fish Quality Assurance and Management**

to the Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam


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# CERTIFICATE

This is to certify that the thesis entitled, **"Authentication of processed blue swimming crab meat products (*Portunus pelagicus*) using PCR based molecular technique",** submitted in part fulfillment of the requirements for the award of the degree of **Master of Fisheries Science in Fish Quality Assurance and Management** to the Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam is a record of bonafide research work carried out by **Mr. DINESH KUMAR, ID No. MFT 16092 (FQM)** under my supervision and guidance and that no part of this thesis has been submitted for the award of any degree, diploma, fellowship or other similar prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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Dedicated to

My Parents

And

Teachers

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## ACKNOWLEDGEMENT

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***(DINESH KUMAR)***

# ABSTRACT

# ABSTRACT


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|---|---|---|
| **Title** | **:** | Authentication of processed blue swimming crab meat (*Portunus pelagicus*) using PCR based molecular technique |
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The development of reliable and specific analytical methods for the simultaneous detection and quantification of different crab species in food is an urgent need for seafood industries, restaurants and quality control laboratories. There is a growing need for rapid, reliable, and reproducible test to verify species in commercial fish and seafood products. In international trade and global seafood consumption, along with fluctuations in the supply and demand of different fish and seafood species have resulted in intentional product mislabeling. The effects of species substitution are far-reaching and include economic fraud, health

hazards, and illegal trade of protected species. Portunid crabs of the genus, *Portunus* viz. *P. pelagicus* and *P. sanguinolentus* and the genus, *Scylla* viz. *S. serrata* and genus, *Charybdis* viz. *C. natator* are economically important species in India. An ongoing problem in the crab industry is mislabeling due to the difficulty of species identification after processing. There is a chance of food substitution with the cheaper crabmeat for the more expensive ones by manufacturers. Blue swimming crab (*Portunus pelagicus*) is an economically important species fetching more demand in export market in USA and Europe due to its savory flavour and high nutritional value. There has been reports of possible substitution of meat from three spot swimming crab (*Portunus sanguinolentus)* that is of low commercial value with blue swimming crab (*Portunus pelagicus*), which is of high value. To overcome this commercial crab meat fraud, a DNA-based multiplex PCR assay was developed for rapid detection of crab meat product. Species-specific primer sets were designed based on the nucleotide variation in mt cyt b and mt 16S rRNA regions for *P. pelagicus* and *P. sanguinolentus*, respectively. The primer set specifically amplified fragments of 298 bp in *P. pelagicus*, and 16S rRNA fragment of 383 bp in *P. sanguinolentus*. The MPCR assay was specific and rapid, as it did not amplify the DNA of other crab species tested and detected the species specific crab within 2 h. The MPCR assay was also detected the species of the crab meat that has undergone different processing treatments like freezing, cooking and frying. The Real Time PCR (qPCR) assay based on SYBR Green was developed for the quantification of mixed meat of crab species belonging to *P. pelagicus* and *P. sanguinolentus*. The developed qPCR assay was sensitive and rapid, as it detected 0.0003 pg to 0.024 ng of genomic DNA within 150 min. A sharp defined melting curve with narrow

peak was found indicating that there was no primer dimers in the amplicons and the efficiency of amplification was excellent. The developed MPCR and qPCR assays was also validated with the commercial pasteurized crab meat products obtained from five crab processing industries located in the Thoothukudi region of Tamil Nadu in the in-house and inter-house laboratories, and found efficient and reproducible. The developed MPCR and qPCR assays could be used by the Food Regulatory Authorities for authenticating *Portunus pelagicus* meat.

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# INTRODUCTION

# I. INTRODUCTION

Seafood is the globally traded food commodity all over the World, following a long, complex and non-transparent supply chain. However, seafood fraud and species substitution lead to cheating of the consumer putting public health at risk. In addition, it allows illegally caught fish to be laundered into the legal seafood trade. In order to increase profits, consumers are being mislead about their seafood by fraudulent practices. The type of seafood frauds include substituting one species for another without changing the label, selling threatened species as more sustainable products, adding more ice to seafood in package than is indicated on the label, and shipping products through different countries in order to avoid duties and tariff.

Recent studies have indicated seafood mislabeling as often as 25 to 70% for fish like red snapper, wild salmon and Atlantic cod, disguises species that are less desirable and cheap. According to US Food and Drug Administration (FDA) Guidelines, Oceana in 2013 found that more than 1,200 fish samples were mislabeled. The USFDA also found 30 % of shrimp samples were misrepresented in a similar study in 2014. In most cases, mislabeling involves passing off a cheap fish as a more expensive type forcing consumers to overpay in stores. Seafood fraud has been a major issue in USA and 55 other countries from fish to crustaceans (Warner et al., 2015).

In a recent study, over one third of blue crab cakes tested were found to contain different imported crab species instead of the local blue crab (Chesapeake) found printed on the label. For instance, the iconic Chesapeake Bay blue crab is considered a best choice due to their relative scarcity keeping the

prices higher than imported crab. The imported crabs are often the swimming crab, which can come from illegal or unreported fisheries in India and South East Asia (Warner et al., 2015).

In India, shrimp and crab are species having greater economical value. Crabmeat of portunid crabs has high value and is considered as an important raw material in the seafood industry. It is processed into several types of food products using various processes, which can result in species identification of the crabs based on morphological characteristics a difficult task. Blue swimming crab (*Portunus pelagicus*) is a large edible species of Portunid crab, inhabiting near shore and estuarine waters and is one of the most economical species. This is the major species for processing as pasteurized crabmeat having very high export potential. There are few exclusive crab processing industries in Tamil Nadu producing 10 metric tonnes of processed crabmeat monthly (MPEDA, 2015-16). Other marine crab species viz. *Portunus sanguinolentus*, *Scylla serrata* and *Charybdis natator* are of less commercial importance, however, are often substituted for blue swimming crab.

Species identification becomes problematic, once the crab is processed by removing shells. *Portunus pelagicus, P. gladiator* and *P. sanguinolentus*, *Charybdis natator* and *C. feriatus* are economically important portunid species. As all species are sold at different prices, they are prone to being both intentionally and accidentally substituted in both fresh and processed crabmeat products (Ng, 1998; Soundarapandian et al., 2013).

In early 1990s, USFDA used protein to identify seafood and frozen tissue was kept as standard for comparison. In 2015, USFDA shifted towards DNA

barcoding as a reliable tool as molecular patterns do not change for species. The FDA's fish SCALE (Seafood Compliance & Labeling Enforcement) have reference libraries of DNA sequences for more than 1000 shrimp, crab and lobster species. The USA currently imports 90% of its seafood and less than 2% inspected at the Border especially for fraud. Seafood Import Monitoring Program has been effective from January 2018 as a new requirement for preventing Illegal fishing and seafood fraud.

Molecular genetic markers (species diagnostic markers) play important roles in preventing the supply of unwanted crab species. DNA barcoding has been identified as a vital tool for molecular identification by targeting a partial sequence of cytochrome oxidase (COI) gene of seven commercial brachyuran species (Haye et al., 2011), which did not include blue swimming crab. Mandal et al. (2014) have attempted to resolve taxonomic uncertainty of the mud crabs available in Indian market using genetic markers (ITS -1 and COI). Besides, there has been a study on the development of species specific marker for the blue swimming crab (*P. pelagicus*) based on DNA and mt DNA (COI and 12S DNA) polymorphism (Klinbunga et al., 2010). Most of the above studies have been carried out on fresh crab species to establish population genetic diversity and brood stock management.

No study is available on the authentication of crab species in processed condition and for establishing the extent of mixing of less economic crab species with highly priced species in India. The major problem faced by crab processing industries in India is the non-availability of a reliable analytical tool to authenticate their crabmeat products to ensure better traceability. So, the development of molecular markers based on PCR particularly MPCR assay shall certainly help to

evolve a reliable analytical tool for the crab processing industries as well as the food safety authorities of the Government to prevent seafood fraud and to ensure the traceability of the crab products.

Real time PCR (qPCR) is an excellent tool that amplifies the target sequence region of the specific species and enables quantification of the DNA products to derive the proportion of adulteration. Hence, the development of such molecular markers involving qPCR shall certainly help to evolve a reliable analytical tool for the seafood industries and in a long run for the food safety authorities to prevent seafood fraud. The present study was undertaken with the following objectives:

1. To design species specific primer for the authentication of commercially important crab species available along Thoothukudi coast.
2. To standardize a PCR based technique for authentication of blue swimming crab (*Portunus pelagicus*) from other important processed crab meat.
3. To validate the developed PCR protocol through in-house and Inter-laboratory validation methods.

# REVIEW OF LITERATURE

# II. REVIEW OF LITERATURE

Food authentication is more important to detect commercial fraud due to species substitution. The increasing demand for fishery products over the current years has increase the output of processed products, which has made the common practice of fraudulent substitution of species. Authentication of commercial seafood products has become market priority. The adulteration rates of processed meat product are higher than that of raw meat. Seafood fraud refers to cases of deliberate species substitution, claiming expensive fish for cheaper ones or farmed fish for wild-caught fish (Armani et al*.,* 2012; Cutarelli et al*.,* 2014). Hence, it is an important task for food control laboratories to be able to carry out species differentiation of raw material to be used for industrial food preparation and detection of species in processed food in order to protect consumers from fraud and adulteration.

## 2.1. Need for seafood authenticity

Fish is the wild protein hunted at a global scale, and seafood is the most highly traded food commodity in the World. But, identifying fish to the species level can be challenging in the processed state. There are scores of species including cod, pollock, haddock and hake that are collectively marketed as “whitefish” and, once cooked; a fillet of halibut from coast of British Columbia can resemble a cut of mahi mahi from Hawaii, or Patagonian toothfish from the Southern Ocean. The situation is further complicated as many species can also go into different products depending on where you live. Dolphin or Dorado is the other term for mahi mahi. The Chilean seabass is used instead of Patagonian toothfish, as the original species name is believed to sound unappetizing and foreign. In many cases, the use of indistinct product names can mask the impact of

overexploitation, as different fish can be substituted without consumer knowledge of ecological impact. Yet, while consistent product terminology is one reason why traceability is important. Having proper information available to consumer is essential for a host of other reasons as well, ranging from human rights abuse and illegal fishing, to the health and safety implications of particular products. The detection of species substitution has become an important aspect within the food industry and there is a growing need for rapid, reliable and reproducible tests to verify species in commercial fish and seafood products. Major concern is the prevention of commercial fraud and the assessment of safety risks derived from non-declared introduction of any food ingredients that might be harmful to human health such as potential allergenic or toxic compounds, or others that might alter the consumption habits of a certain group of consumers. Hence, Authenticity testing of species present in food is important for economic, safety, legal, religious and health reasons.

### 2.2. Techniques for seafood authentication

Development in food preservation, processing technologies and liberalization of trade have contributed significantly to the globalization of fish trade and to the diversification of seafood, both in terms of species and products. Authentication of fish and seafood species has become an important issue within the seafood industry to protect the consumers from fraudulent and misleading practices whereby low valued species are substituted for high valued species. Species identification is traditionally based on external morphological features, including body shape, pattern of colours, scale size and count, number and type of fin and rays and various relative measurements of body parts. Yet, in some cases morphological features are of limited value for identification and

differentiation purposes, even with whole specimens, because they can show either considerable intra-specific variations or small differences between species (Teletchea, 2009). Moreover, the respect for labeling regulations becomes complicated in processed food such as frozen fillets and precooked seafood because the original identifying morphological characteristics are absent. Therefore, the development of protocols for assurance and control of seafood safety is currently a major challenge. To improve detection of commercial seafood fraud, a variety of protein and DNA-based techniques have been developed (Rasmussen and Morrissey, 2008).

### 2.2.1. Protein-based methods for seafood identification

The identification is based on the different sarcoplasmic proteins or amino acids profiles between meats from different species (Knuutinen and Harjula, 1998; Armstrong et al., 1992). Isoelectric focusing (IEF) technique has been used to identify four freshwater fish commercially labeled as “perch” (Berrini et al., 2006), and puffer fish species (Chen et al., 2003). Puffer fish proteome was also analyzed by two-dimensional electrophoresis technique (2DE), which resulted in useful discrimination of harmless species from the ones that accumulates lethal level of tetrodotoxin in their muscle (Chen et al., 2003). In general, 2DE is a powerful tool to distinguish among closely related species, as reported by Pineiro et al. (1998). At present, there are only few examples of rapid immunoassay, such as a strip test or enzyme-linked immunosorbent assay (ELISA) kit, capable of identifying species of fish, even if this technique could be easy and fast to apply. The issue is obtaining species specific antibodies for each fish product liable to fraud and it is essential that antibodies don’t cross-react with non-target species. Gajewski et al. (2009) developed monoclonal antibodies for rapid identification of

two species of *Pangasius*, both commercially labeled as catfish, but with considerable differences in their meat quality.

### 2.2.2. DNA based methods for seafood identification

Genetic species identification is based on the principle of DNA polymorphism, or genetic variations that take place as a result of naturally occurring mutations in the genetic code. Determination of fish and seafood can be carried out using either nuclear DNA (nDNA) or mitochondrial DNA (mt DNA). Some major advantages of mt DNA over nDNA are that it is relatively small compared to nDNA, because it lacks features such as large noncoding sequences (introns), pseudogenes, repetitive DNA and transposable elements; it is relatively easy to extract; it does not undergo genetic rearrangements such as recombination and sequence ambiguities resulting from heterozygous genotypes. Moreover, mtDNA presents a higher copy number and a faster rate of mutation, making it generally more appropriate in the study of evolutionary genetics and inter and intra-species variability. However, high intraspecies variation can become a disadvantage to species diagnostic methods and the maternal inheritance pattern of mtDNA may produce misleading results in the event of species hybridization. The most common mtDNA genes exploited in species identification research have been cytochrome b, 12S and 16S rRNA (Rasmussen and Morrissey, 2008). The species identification is basically based on PCR carried out with universal primer or species specific primers designed on the basis of single nucleotide polymorphisms. Following PCR amplification, the resulting DNA fragments must be properly analyzed to verify the presence or absence of species-specific genetic markers. Some methods include restriction fragment length polymorphism (RFLP), forensically informative nucleotide sequencing

(FINS), amplified fragment length polymorphism (AFLP) or single strand conformational polymorphism (SSCP) (Rasmussen and Morrissey, 2008). Several online resources have also been developed for specific use in the field of DNA based identification of fish and seafood species, like the FISH-BOL (http://www.fishbol.org/), which is part of the Consortium for the Barcoding of Life (CBOL; http://www.barcoding.si.edu/). The purpose of CBOL is sequencing the mt COI gene in all biological species.

#### 2.2.2.1. DNA chips

DNA chips (also known as DNA microarrays or DNA macroarrays) may prove to be a valuable tool, as they have the potential to simultaneously identify up to hundreds or thousands of species (Teletchea et al., 2005). On a smaller scale, a DNA chip was developed that allowed for differentiation of six animal species commonly consumed in Europe (Peter et al., 2004). Universal primers were used to amplify a 377 bp fragment of the mt cyt b gene, and the resulting fragments could then be identified in a microarray with species-specific oligonucleotide probes. This DNA chip was able to detect species present at only 0.1% in an admixture and could identify up to four different species simultaneously in mixed commercial food samples. Interestingly, a commercial DNA chip-based product called the FoodExpert-ID was launched in France in 2004 by the biological diagnostics company, BioMerieux (http://www.biomerieux.com). According to the company, this product is first high density DNA chip for use in species identification from food and animal feeds, and it was able to detect 33 different species of vertebrates, including 15 species of fish. Despite their potential advantages, array based methods have not yet been heavily exploited for species identification in foods; they are still fairly inaccessible due to high costs and long

start-up times. A DNA microarray was recently developed to differentiate 11 commercially important fish species based on a 600 bp fragment of the 16S rDNA gene (Kochzius et al., 2008). Based on these results, a “Fish Chip” for identification of approximately 50 species found in European seas is developed for the authentication and research purposes in the fisheries industry.

#### 2.2.2.2. Electrochemical DNA sensors

An innovative method for the detection of PCR products was recently described by Lai et al. (2006). This method was based on the use of electrochemical DNA (E-DNA) sensors to detect *Salmonella typhimurium*. An advantage of E-DNA technology is its potential for use in a field-portable, hand-held species identification device. This application is not as feasible in other emerging techniques such as Lab-on-a-chip capillary electrophoresis (CE) and fluorescence based methodologies due to analytical needs such as power intensive laser light sources, high numerical aperture optics, and use of relatively high voltages. Despite the potential for the use of E-DNA sensors in the detection of mislabeled fish and seafood products, analytical protocols for this purpose have not yet been fully developed.

#### 2.2.2.3. Single stranded conformational polymorphism (SSCP)

SSCP is an alternative to methods such as FINS or RFLP for the detection of interspecies polymorphisms, especially when closely related species are being analyzed. Analysis with SSCP begins with PCR amplification of a specific DNA fragment in all species being examined (Lockley and Bardsley, 2000). The resulting amplicon is then denatured into a fragment of single-stranded DNA that has a secondary structure dependent on its sequence. Variations in sequence, which may be as small as a single nucleotide, can be detected by differences in

electrophoretic mobility with PAGE. SSCP patterns are visualized by silver staining and then compared with the profiles of authentic species to correctly identify an unknown sample (Mackie et al., 1999).

#### 2.2.2.4. Amplified fragment length polymorphism (AFLP)

The amplified (restriction) fragment length polymorphism (AFLP) technique is a method for DNA profiling that is now widely applied for assessing diversity among various organisms with varying genomic complexity, from small bacterial to large plant genomes. A study was conducted to assess the potential use of the AFLP technology to determine fish species in processed commercial products and domestic stocks (Jerome et al., 2003). A species database of fish, molluscs and crustaceans has been created with the aim to identify species of origin of seafood products by previously defined AFLP patterns. However, AFLP fails to identify species from highly degraded food product, and so it is not suitable for tracing the authenticity of highly processed canned and freeze dried products.

#### 2.2.2.5. Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) analysis is based on amplification of discrete regions of the genome by polymerase chain reaction (PCR) with short oligonucleotide primers of arbitrary sequence (El-Jaafari et al., 2008) to detect polymorphisms in the respective primer sites of the genome. Such polymorphisms inherit in a Mendelian fashion and can be used as genetic markers in discriminating different populations (Dahle et al., 1997). PCR-RAPD has been used for discrimination of species in Cyprinidae family (Mansoori et al., 2014), analysis of four coral reef fish species in the genus Cephalopholis (Abied et al., 2014) and for finding the genetic variations and phylogenetic relationship among snakehead fish, *Channa striatus* population (Marimuthu et al., 2016). The main

advantage of RAPD is that it does not target predetermined DNA fragments. Instead, an arbitrary primer is designed without previous knowledge of the target DNA sequence, and during PCR this primer randomly amplifies segments of DNA (Williams et al., 1990). Due to variations in the genetic code, RAPD analysis on different species results in unique patterns of DNA fragments.

#### 2.2.2.6. Forensically informative nucleotide sequencing (FINS)

FINS is a DNA-based procedure first described by Bartlett and Davidson, (1992). To identify a species using FINS, a specific DNA fragment is amplified by PCR, its nucleotide sequence is determined, and the sequence is then compared with related sequences in a database using phylogenetic analysis. The sequence with the lowest genetic distance or numbers of nucleotide substitutions, from the target fragment represents the species group to which the original sample belongs (Bartlett and Davidson, 1992). Lago et al. (2011) employed FINS methodology to assure the correct labeling of sardine and sardine-type products and they proved that this methodology can be applied to all kinds of processed products, including those which have been subjected to intensive processing treatments, such as canned foods. The method was also used for genetic identification of mussel species belonging to the genera *Mytilus*, *Perna* and *Aulacomya* (Santaclara et al., 2006); distinguishing eight tuna species belonging to the subfamily Neothunnus that are very closely related, and identification of skates species belonging to the family Rajidae (Lago et al., 2012).

#### 2.2.2.7. Multiplex PCR

In multiplex PCR, multiple species can be analyzed in a single run by using a combination of species specific primers and universal primers resulting in DNA

fragment lengths that vary with species (Veneza et al., 2017). The length of the fragments can be predicted, if the complete sequence is known, and a given species can be identified by the appearance of an amplicon of appropriate size on an agarose gel or PAGE. Multiplex PCR is a practical alternative for routine use in fishing industries and the retail sector, as well as by surveillance agencies for controlling quality and protecting the consumer (Armani et al., 2012). Veneza et al. (2014) developed an authentication protocol employing multiplex PCR targeting COI gene for three species of snappers (*Lutjanus purpureus, L. synagris,* and *Ocyurus chrysurus*). The protocol yielded a distinct triple-banding pattern for *L. purpureus*, whereas *L. synagris* and *O. chrysurus* showed a double banding pattern of different sizes, thereby allowing differentiation of the three species. Further advantage of multiplex PCR is that real time PCR probes such as TaqManTM can also be applied, which allows for a rapid, quantitative analysis that does not require the use of gel electrophoresis (Nguyen et al., 2017). Armani et al. (2011) developed a multiplex PCR to detect fraudulent substitutions of Bianchetto (juvenile form of *Sardina philcardus*) and Rossetto (*Aphia minuta*) with Icefish (*Neosalanx* spp.). They designed specific primer on the mitochondrial cytochrome b gene to amplify sequences of different lengths by multiplex PCR. Suwannarat et al. (2017) developed a convenient and accurate multiplex PCR assay for species specific primer set KUGEN_PORTspec based on the nucleotide variation of cyt b and 28S rRNA for portunid species identification from both high and low quality DNA obtained from processed food.

#### 2.2.2.8. Real time PCR (qPCR)

Real Time PCR is a technique that allows for the quantification of target DNA. It can quantitatively measure DNA, which uses fluorescent probes to obtain

results during the reaction and do not require gel electrophoresis (Fig. 1). The qPCR machines differ in sample capacity, up to 96-well (ABI Step One Plus) and 384- well standard format, others process 72 (RotorGene) or only 32 samples and require specialized glass capillaries (LightCycler), excitation method (lasers and others broadspectrum light sources with various filters), and fluorescence acquisition channels. There are also platform-specific differences in how the software processes data with focus on absolute or relative quantification strategies. Among the real time PCR detection chemistry, SYBR Green I and TaqMan assays produce comparable dynamic range and sensitivity, while SYBR Green I detection is more precise and produce a more linear decay plot than the TaqMan probe detection (Pfaffl, 2004). For single PCR product reactions with well-designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles. SYBR green based assays involve the use of SYBR Green I, a nonspecific double stranded binding dye for real-time detection of PCR. This dye has virtually no fluorescence when free in solution but become highly fluorescent when bound to the double stranded DNA (Nygren et al., 1998) and therefore in qPCR, the fluorescent signal increases with each amplification step (Fig. 1). SYBR green assays, when optimized, are cheaper and simpler than TaqMan probe assays. These methods are advantageous not only in their speed and simplicity, but also in the ability to quantify targeted genetic material. In fact, SYBR Green probes have been investigated for their ability to detect and quantify DNA from Crustacean Allergen in Foods (Herrero et al., 2012) and in tropical shellfish (Tyagi et al., 2009). Zhou et al. (2007) reported the successful development of a real-time PCR assay with SYBER Green probes for effective quantification of *V. alginolyticus* in seawater

and seafood. The application of real-time PCR to multiplex assays has been reported to be effective for the differentiation of three species of gadoids (Taylor et al., 2002), two eel species (Itoi et al., 2005), and two tuna species (Lopez and Pardo, 2005). Armani et al. (2011) developed a real time PCR for detecting substitution of Bianchetto (juvenile form of *Sardina philcardus*) and Rossetto (*Aphia minuta*) with Icefish (*Neosalanx* spp.), even in marinated products.

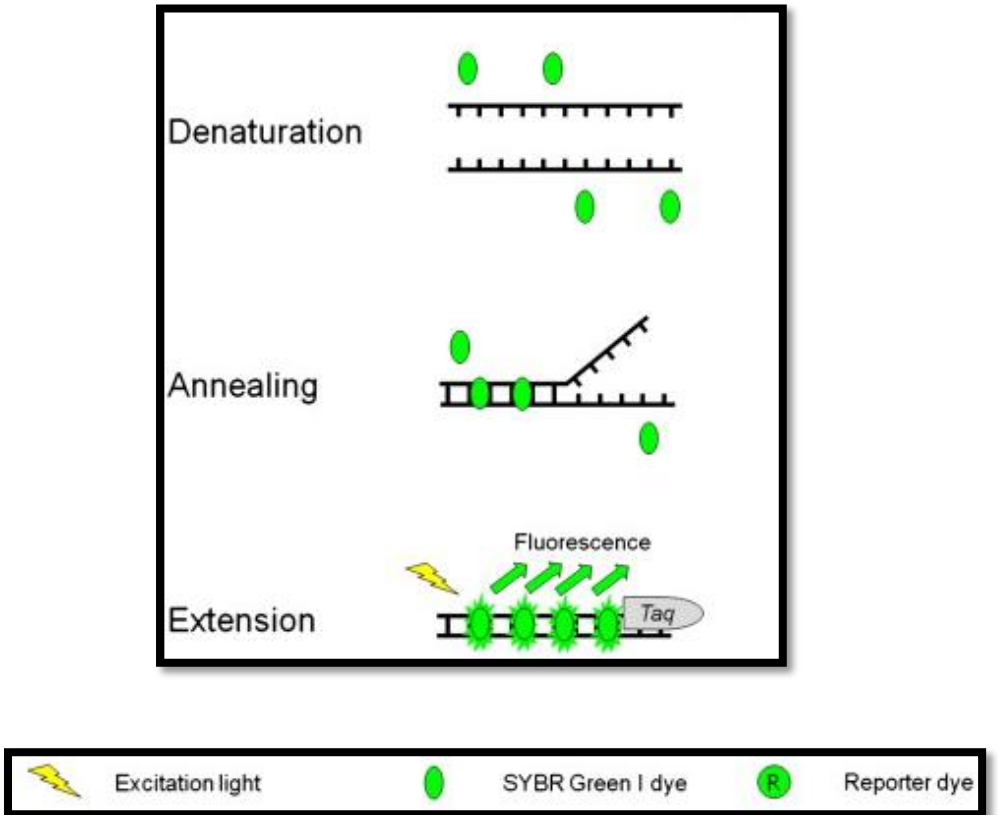


**Fig. 1.** Real time PCR using SYBR green. Following DNA denaturation, the SYBR Green probe hybridizes to the target DNA during primer extension. The result is emission of a specific fluorescent signal that can be detected and quantified.

Real-time PCR was recently utilized in the development and design of a "ready-to-use" reaction plate for the detection of small fragments (≤ 212 bp) of DNA from seven different animal species commonly found in processed foods (Laube et al., 2007). Despite the advantages of real-time PCR, some limitations

remain, multiplex real-time reactions are generally restricted to four fluorogenic probe colours per tube; the size of PCR products cannot be monitored in a closed system; and some systems are not compatible with the chemical properties of fluorogenic probes (Arya et al., 2005).

### 2.3. Seafood mislabeling and substitution incidence

Seafood mislabeling is a global issue. Inadequate product labeling can have serious consequences in terms of human health, as well as having ecological and economical implications. In addition to entailing potential risk for the consumer (van Leeuwen et al., 2009) including financial cost (Marko et al., 2004), it may impact management programmes that are designed for the conservation of the stocks of certain species (Ward, 2005). As Oceana's nationwide study and others demonstrate that seafood may be mislabeled as often as 26 to 87 % of the time for commonly swapped fish such as Grouper, Cod and Snapper, disguising fish that are less desirable, cheaper or more readily available (USFDA Guidelines, 2012). Fraudulent labels may indicate higher weight than actually contained in the package, false points of origin, missing ingredients, and species substitution, all with risk to consumer health and safety (Spink and Moyer, 2011). The practice could severely impact conservation efforts, particularly protected or critically endangered species. Once seafood products are processed and packaged, it is difficult to accurately identify them to species because the morphological characteristics used to identify them with traditional methods are lacking. New DNA based tools can circumvent this weakness and enhance the accuracy of seafood labeling by validating the identity of the species in the package with market name under which a product is being sold.

### 2.3.1. Seafood mislabeling and substitution in finfish

Evidence of seafood mislabeling has been confirmed as far back as 1915 a newspaper article that reported excess catches of sharks were to be sold as swordfish (Anonymous, 1915). This still continues to be an issue today as documented by market surveys, which have been increasing in number over the past years, as outlined in a 2014 global review (Golden and Warner, 2014). The most commonly substituted types of seafood in the database are those labeled as snapper, caviar, grouper, cod, crab, tuna, salmon, and hake. Snapper and cod are the most studied types of fish in terms of total number of mislabeling. The most common substituted fish across multiple studies are Asian catfish (*Pangasius*), hake, farmed salmon, shark/ ray, tilapia, cod and escolar/ oil fish. Many lutjanids such as red snapper (*Lutjanus vivanus, L. purpureus, L. campechenus, L. bucanella* and *L. peru*) are highly similar morphologically (Carvigon et al., 1993; Nelson, 2006) and are difficult to reliably identify based on external characteristics. This problem is exacerbated by the industrial processing of catches, which typically involves the removal of the head and fins. Based on the analysis of a fragmented mitochondrial cytochrome b gene, Marko et al. (2004) discovered that approximately 80% of the fillets sold in the United States as red snapper (*L. campechanus*) were actually from other lutjanus species. Blatant seafood species substitution for economic gain has been confirmed in numerous instances in the last few years. Intentional substitution is the labeling of farm raised freshwater pangas species from Vietnam as grouper, which are wild caught marine fish. Such seafood species substitution also includes tilapia labeled as snapper and Pollock labeled as Cod.

### 2.3.2. Seafood mislabeling and substitution in shrimps

Shrimp is the most commonly consumed and highly traded seafood in the World. However, this high demand has led to many environmental and human rights abuse in the fishing, farming and processing of shrimp. Maximum intra-species specific divergence was calculated as 0.8% in *Fenneropenaeus indicus* of India, Thailand and China. Inter-species specific divergence was found to be maximum of 3.95% between *Solenocera crassicornis* and *S. koelbeli* of India and China. No divergence was observed between two Indian haplotypes, *Metapenaeus dobsoni* and *M. brevicornis,* which overlap each other (Rajkumar et al., 2015). Pacific white shrimp (*Litopenaeus vannamei*) is being replaced with the blue shrimp (*Litopenaeus stylirostris*) (Pascoal et al., 2011). Oceana In 2014, found 30% of shrimp samples to be misrepresented according to US Food and Drug Administration Guidelines (Warner et al., 2015). They were either mislabeled (15%), misleading (10%), or unidentifiable with at least two different species in the same bag (5%). Mislabeling can occur when farmed shrimp is marketed as a wild caught species. Misleading labeling could consist of shrimp sold as Gulf Shrimp, presumably wild caught, when the shrimp were actually farmed in the Gulf.

### 2.3.3. Seafood mislabeling and substitution in crabs

Various food processing techniques have been undertaken on crabmeat including steaming, freezing, and canning to supply various product forms to the expanding markets (Samuel and Soundarapandian, 2009). An ongoing problem in the crab industry is food labeling or food fraud in which many food manufacturers may have substituted the cheaper crabmeat for the more expensive ones due to the difficulty of species identification after food processing. Klinbunga et al. (2007) verified species origins of various forms of the blue swimming crab products from

Thailand to prevent intentional use of the wrong species in canning. Their study was development of a reproducible species-diagnostic marker for *P. pelagicus* in Thailand. They tested specificity of BSCSCAR1 and BSCSCAR2 primer pairs. The amplification success of BSCSCAR1 (164 bp) in *P. pelagicus* was low (30%). Conversely, BSCSCAR2 generated the expected product (188 bp) in 97 % of *P. pelagicus,* but not in the mud crabs, *S*. *serrata*, *S*. *oceanica* and *S. tranquebarica*. Nevertheless, cross-species amplification was found in *C. crucifera.* Haye et al. (2011) used DNA Barcoding targeting a partial sequence of the Cytochrome Oxidase I (COI) gene of seven commercialized brachyuran species for authentication of commercial crabmeat in Chile. They reported species in crabmeat market as *Metacarcinus edwardsii* (68.4%), followed by *Romaleon polyodon* (21.1%). The other three species detected were present in less than 6% of the authenticated samples. Vartak et al. (2014) identified 11 edible crab species from India by classical taxonomy and developed molecular barcodes with the cytochrome coxidase I (COI) gene. They found 100% improper labeling of restaurant samples to cover-up inferior quantity crab meat. Suwannarat et al. (2017) developed species-specific primer set KUGEN_PORTspec based on the nucleotide variation of cytochrome b and 28S rRNA genes. They reported that cyt b variations showed a number of inter-species specific substitution sites, which could be utilized in simultaneous identification of the five target species, three species of the genus *Portunus* and two species of the genus *Charybdis.*

### 2.4. Commercial DNA based species identification kits

Some of the DNA-based methods discussed previously for the identification of fish and seafood species have been utilized by various companies to provide food testing services or products. One example is the U.S.-based molecular

diagnostics company, Applied Food Technologies (http://www.appliedfoodtechnologies.com/), which uses AUTHENTI-KITSM DNA technology to identify animal species in food products, including fish and seafood species: channel catfish (*Ictalurus punctatus*), basa (*Pangasius bocourti*), tra (*Pangasius hypophthalmus*), Atlantic blue crab (*Callinectes sapidus*), and blue swimming crab (*Portunus pelagicus*). Applied Food Technologies is currently working in collaboration with the USFDA and the Fish Barcoding of Life Initiative (FISH-BOL) to standardize DNA sequencing methods for the identification of fish and seafood species (Applewhite and Bennett, 2008). Another species identification company that offers testing services for fish and seafood products is Therion Intl., LLC (http://www.theriondna.com/). With analyses such as mt DNA sequencing and amplification of species-specific microsatellite loci, Therion Intl. is able to identify commonly substituted species in food products, including grouper, red snapper, mahi mahi, tuna, Chilean seabass, walleye, and zander. On the other hand, a number of companies offer commercial test kits for fish species identification. The biotechnology company, Bionostra (http://www.bionostra.net/), located in Madrid, Spain, offers the Fish ID Kit, which is a fish species identification kit based on amplification and analysis of mt DNA. Another Spanish biotechnology company, Biotools (www.biotools.net), offers two kits based on genetic markers for the detection of fish species in fresh and processed samples: the BIOFISH cod Kit, which utilizes RFLP analysis to identify cod (*Gadus morhua*), Alaska cod (*Gadus macrocephalus*), pollack (*Pollachius pollachius*), and Arctic cod (*Arctogadus glacialis*), and the BIOFISH salmon Kit, which allows for identification of Atlantic salmon and two trout species (*Oncorhynchus mykiss* and *Salmo trutta*). Biotools also offers a series of BIOFISH SEQ kits that allow for species

identification based on DNA sequencing for the following groups of fish: flatfish (7 species), sardines (7 species), hake (10 species), and tuna (10 species). The U.K. based company, Tepnel Life Sciences (www.tepnel.com), also offers a series of fish species identification kits that allow the detection of cod, hake, coley, haddock, pollock, trout, and salmon in raw and processed products. Tepnel Life Sciences utilizes magnetic bead technology for DNA extraction, followed by a multiplex PCR and analysis of the results with gel electrophoresis. In addition to the above-mentioned diagnostic methods, a DNA microarray chip has also been utilizes commercially for fish species identification by the European company, BioMerieux.

# MATERIALS AND METHODS

# III. MATERIALS AND METHODS

## 3.1. MATERIALS

### 3.1.1. Crab samples

Four commercially important crab species viz. *Portunus pelagicus* (Blue swimming crab), *Porutunus sanguinolentus* (Three spot swimming crab), *Scylla serrata* (Mud crab) and *Charybdis natator* (Ridged swimming crab) were collected from fish landing center of Vellapatti, and Fishing Harbour of Thoothukudi, Tamil Nadu and brought to the laboratory in chilled condition (Plates 1 to 4).

**Plate 1.** *Portunus pelagicus* (Blue swimming crab)

**Plate 2.** *Portunus sanguinolentus* (Three spot swimming crab)

**Plate 3**. *Scylla serrata* (Mud crab)

**Plate 4.** *Charybdis natator* (Ridged swimming crab)

### 3.1.2. Insulated boxes

Milton insulated boxes with a drain valve was used for icing and transportation of crabs from the fish landing center to the laboratory.

### 3.1.3. Ice

Fresh flake ice was used for icing the crab samples during transportation and handling at the laboratory**.**

### 3.1.4. Chemicals for molecular biology work

DNA markers (50 & 100 bp ladder), ethidium bromide, ethylene diamine-tetra acetate (EDTA), formamide, gel loading buffer (6X), glycerol, proteinase K, sodium carbonate, Tris-HCl, Tris base, N,N,N',N'- tetramethylethylenediamine (TEMED) and xylene cyanol were purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Ammonium acetate, isoamyl alcohol, isopropanol, phenol and

sodium dodecyl sulphate (SDS) were obtained from Merck Specialties Pvt. Ltd., Mumbai, India. Agarose was obtained from Medox, Chennai, India. Primers for PCR amplification, selective primers, 2X PCR master mix, SYBR Green master mix were obtained from Synergy Scientific Services, Chennai, India. Other chemicals such as absolute alcohol and sodium chloride were of analytical grade.

### 3.1.5. Reagents for DNA extraction (Phenol-Chloroform method)

i. Lysis buffer consisting 200mM of Tris-HCl (pH 8.0), 100mM EDTA (pH 8.0) and 250 mM NaCl

ii. Proteinase k (10mg/ml)

iii. SDS, 20%

iv. Phenol

v. Chloroform

vi. Isoamyl alcohol

vii. ammonium acetate (10M)

viii. Absolute alcohol (70%)

ix. Isopropanol

### 3.1.6. Reagents for Polymerase Chain Reaction (PCR)

i. 2X PCR Master mix

ii. DNA markers of 100 bp and 50 bp (0.5 µg/µl) (Thermo Fisher Scientific Inc., Massachusetts, USA)

iii. Gel loading dye (6X) consisting of Glycerol (60%), Tris-HCl (pH 7.6) 10

mM, EDTA (60 mM), Bromophenol Blue (0.03%) and Xylene Cyanol FF (0.03 %)

### 3.1.7. Reagents for Agarose Gel Electrophoresis

I. Tris acetate buffer (TAE buffer) (50X) consisting of Tris base-242 g; glacial acetic acid-57 ml; and disodium EDTA. $2H_2O$- 37.2 g in 1 L of deionised water. The pH was adjusted to 8.5.

II. TAE Buffer (1X) was diluted from 50X TAE buffer by 20 ml of buffer to 980 ml of deionised water

III. Agarose solution (2%) was prepared by melting 2g agarose in 100 ml of 1X TAE buffer

IV. Ethidium bromide solution was prepared by mixing 5 mg of Ethidiumbromide and 30µl TEMED in 1 ml of deionized water

### 3.1.8. Reagents for Real Time PCR (qPCR)

i. SYBR Green Master mix (Fisher Scientific Inc., Massachusetts,USA)

ii. Deionised water

### 3.1.9. Equipments

#### 3.1.9.1. Gradient Master Cycler

Gradient Master Cycler (Eppendorf AG, Hamburg, Germany) was used for the standardization of PCR conditions and further amplification.

#### 3.1.9.2. Real time PCR (qPCR) Machine

Applied Biosystems Step One Plus$^{TM}$Real Time PCR machine (available in our laboratory), Applied Biosystems®7500 Real Time PCR machine (available in

the Dept. of Animal Biotechonoly, Madras Veterinary College, TANUVAS, Chennai), Quant studio Real Time PCR machine (available in the Molecular Biology Laboratory of Thermo Scientific, Bangalore) were used for performing quantitative PCR assay of crabmeat. All the machines had 96 wells thermal cycling block.

#### 3.1.9.3. Biosafety Cabinet Type II

Biosafety cabinet (Class II, Type B2, Clean Air Systems, Chennai, India) was used for carrying out DNA extraction from crab meat and mixing of PCR reagents.

#### 3.1.9.4. Biophotometer

Biophotometer (Eppendorf AG, Hamburg, Germany) was used to quantify both the extracted DNA and the amplified DNA

#### 3.1.9.5. Submarine electrophoresis system

Submarine electrophoresis system (Medox, Biotech India Pvt., Ltd.) was used for running the PCR products on agarose gels at 150V.

#### 3.1.9.6. Gel documentation system

Gel documentation system having UV Transilluminator (Alpha Innotech Co., San Leandro, USA) was used for capturing and documenting gel images using Alpha imager EC software.

#### 3.1.9.7. Molecular grade water purification system

Molecular grade water obtained from Water Purification System (SartoriousStedim Biotech, Germany) was used in this study.

### 3.1.9.8. Refrigerated microfuge

Refrigerated microfuge (Model 5415R, Eppendorf AG, Hamburg, Germany) was used for the DNA extraction.

### 3.1.9.9. Serological water bath

Serological water bath (INLAB Equipments Pvt. Lt., Madras, India) set at 55ºC was used for the inactivation of the restriction enzyme.

### 3.1.9.10. Incubator

An incubator (Laboratory Instruments Scientific Engineering Corporation, Delhi, India) was used for the restriction digestion of PCR product.

### 3.1.9.11. Other instruments

Other instruments used in this study included ultrafreezer (Elcold, Hobro, Denmark), chill cabinet (Sri Ganapathi Enterprises, Salem, India), deep freezer (Bluestar, Chennai, India), pH meter (Model 707, Digisun, Hyderabad, India), high precision electronic balance (Model CP 2250, Sartorius Mechatronicss India Pvt Ltd, Chennai, India) and microwave oven (LG electronics, Seoul, South Korea).

## 3.2. METHODS

### 3.2.1. Identification of crab species

Four commercially important crab species were morphologically identified as per FAO catalogue (Heemstra and Randall, 1993) as *Portunus pelagicus* (Blue swimming crab), *Porutunus sanguinolentus* (Three spot swimming crab), *Scylla serrata* (Mud crab) and *Charybdis natator* (Ridged swimming crab), all species Samples were packed and transported in iced condition to the laboratory using insulated boxes.

### 3.2.2. Preparation of processed crab products

Four species of crabs were used for the multiplex PCR study. Each species was divided into four different lots as whole crab with shell. The first lot was frozen at -40°C for 3 h in ultra-freezer and designated as “Frozen” crab (FC). The second lot was cooked at 100°C for 20 min and designated as “Cooked” crab (CC). The third lot was shallow fried at 180°C for 10 min and designated as “Fried” crab (FrC). The final lot was not given any processing treatment and was designated as “Raw” crab (RC), which served as control. Meat was separately picked from each lot and stored in 70% ethanol in deep freezer at -20ºC until used for DNA extraction.

### 3.2.3. DNA extraction

Total DNA was extracted from the crab samples as per the method described by Kumar et al. (2007). For which, 50 mg of tissue was taken in a 2 ml microfuge tube, to which 950 µl of lysis buffer, 20 µl of proteinase K and 30 µl of 20% SDS were added and subjected to homogenization, the tubes were

incubated at 48ºC for 50min 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 13000 rpm for 10 min at $4^0$C. The top transparent aqueous layer was then transferred to a new 1.5 ml microfuge tube. The collected supernatant was precipitated by the addition of an equal volume of chilled isopropanol and 0.2 volume of 10 M ammonium acetate. This tube was again subjected to centrifugation at 13,200 rpm for 10 min at 4ºC. The supernatant was removed using a micropipette and the pellet was then washed in 500 µl of 70% ethanol, air-dried and re-suspended in 100 µl of sterile water. Extracted DNA were labeled and preserved at - 20° C and used for PCR analysis.

### 3.2.4. Quantification of extracted DNA

The extracted DNA from the selected crab species samples was quantified using the Biophotmeter at 260 nm.

### 3.2.5. Preparation of samples for qPCR

The meat tissue of *P. sanguinolentus* was spiked with *P. pelagicus* meat at different ratios as detailed given in Tables 1, 2 and 3. The tissues were homogenized well and further DNA was extracted as per the method given in 3.2.3.

**Table 1. Spiking of *P. sanguinolentus* meat with *P. pelagicus* meat**

| ***P. pelagicus*** **(g)** | ***P. sanguinolentus*** **(g)** | **Level of spiking (%)** |
|---|---|---|
| 10 | - | 0 |
| 5 | 5 | 50 |
| 6 | 4 | 40 |
| 7 | 3 | 30 |

**Table 2. Spiking of *P. pelagicus* meat with *P. sanguinolentus* meat**

| ***P. sanguinolentus*** **(g)** | ***P. pelagicus*** **(g)** | **Level of spiking (%)** |
|---|---|---|
| 10 | - | 0 |
| 5 | 5 | 50 |
| 4 | 6 | 60 |
| 3 | 7 | 70 |

**Table 3. Spiking of *P. sanguinolentus* meat with *P. pelagicus* meat for third calibration curve**

| ***P. sanguinolentus*** **(g)** | ***P. pelagicus*** **(g)** | **Level of spiking (%)** |
|---|---|---|
| 10 | - | 100 |
| 5 | 5 | 50 |
| 4 | 6 | 40 |
| 3 | 7 | 30 |

### 3.2.6. Designing of species specific primers

Whole sequences of the five mitochondrial regions viz. cyt b, 16S rRNA, 12S rRNA, D-loop and COI of the crab species available in GenBank were chosen from NCBI data base and aligned using BioEdit software to design species specific primers for *P. pelagicus* and *P. sanguinolentus,* with the help of Primer Stat online software (www.bioinformatics.org). A total of ten primer sets were designed from five mitochondrial regions viz. cyt b, 16S rRNA, 12S rRNA, D-loop and COI for *P. pelagicus* and seven sets for *P. sanguinolentus*. The nucleotide sequences of the designed primers are given in the Tables 4 and 5.

**Table 4. List of designed species specific primer sets for *P. pelagicus***

| Target region | Primer | Sequence (5' to 3') | Product size | Annealing temperatures |
|---|---|---|---|---|
| 16S rRNA | Forward | GTAGGTGTCTAATTCCGTA | 160 bp | 48 to 54 $^0$C – 30 s |
| | Reverse | AAAGCTCGTATGACATCTC | | |
| 12S rRNA | Forward | TTGGCGGTGGTTTAGTCTTG | 312 bp | 53.5 to 60.5$^0$C-30 s |
| | Reverse | CGGGCGATGTGTACATGCTT | | |
| cyt b | Forward | CGTACTATGCATGCCAAT | 298 bp | 49.5 to 55.5$^0$C-30 s |
| | Reverse | AAGAACCGTGTTAGTGTAG | | |
| D-loop | Forward | AAGAGGAGTAAAGGACAC | 310 bp | 48.4 to 54.4$^0$–30 s |
| | Reverse | GTCTGTGAGGATTAAAGGG | | |
| COI | Forward | AGGAACCTCACTAAGTCT | 585 bp | 48.4 to 54.4$^0$C-30 s |
| | Reverse | AGGATACACCAGCTAAATG | | |
| 12S rRNA | Forward | GTTGATTGTTAGGTGGACT | 246 bp | 48 to 54.5$^0$C – 30s |
| | Reverse | GTTCCTCTGACAAGACTA | | |
| cyt b | Forward | CCTTTTAATCCTATCCCTTCTT | 186 bp | 52.6 to 58.5$^0$C-30s |
| | Reverse | AATGGCAACAGAGGCGAC | | |
| D-loop | Forward | CACCGCCTGTAATAATAG | 107 bp | 48.4 to 54.4$^0$C-30 s |
| | Reverse | TAGGCTACAACTTGGTTATG | | |
| 16S rRNA | Forward | AGTAGGTGTCTAATTCCGTA | 247 bp | 49.3 to 55.3$^0$C-30 s |
| | Reverse | TAGGAGGGATTAGCTTCTTA | | |
| cyt b | Forward | CGTACTATGCATGCCAAT | 70 bp | 49.5 to 62.5$^0$C-30 s |
| | Reverse | TAGATGCCACGCCCGATGTG | | |

**Table 5. List of designed species specific primer sets for *P. sanguinolentus***

| Target region | Primer | Sequence (5' to 3') | Product size | Annealing temperatures |
|---|---|---|---|---|
| 16S rRNA | Forward | GAAGTAAGGATAGCTAGTAG | 383 bp | 49.3 to 54.3$^0$C -30 s |
| | Reverse | TCTACACTTGCACTGTTAC | | |
| 16S rRNA | Forward | GAAGTAAGGATAGCTAGTAG | 91 bp | 50.5 to 59.5$^0$C- 30 s |
| | Reverse | TATTGCAAGGCTCGTATGACG | | |
| cyt b | Forward | CACTTCTTCTCATTTTGTCTC | 240 bp | 50.9 to 60.9$^0$C- 30 s |
| | Reverse | CTCCTAACTTGTTTGGAATGGAG | | |
| D-loop | Forward | TGGCGAGTGGATATATTAG | 270 bp | 49.4 to 53.4$^0$C -30 s |
| | Reverse | TCACCACTTCCTATTGGA | | |
| D-loop | Forward | TTACATGGCGAGTGGATA | 91 bp | 49.4 to 55.4$^0$C- 30 s |
| | Reverse | GAAATAGAGGGAGGGGTAT | | |
| 12S rRNA | Forward | GTAGCATAAGGGCTATGA | 240 bp | 49.4 to 54.4$^0$C- 30 s |
| | Reverse | TAGTAGTTCACACTCTGAC | | |
| cyt b | Forward | GTAGCATAAGGGCTATGAA | 240 bp | 49.3 to 54.3$^0$C-30s |
| | Reverse | TTAGTAGTTCACACTCTGAC | | |

### 3.2.7. PCR analysis

Optimization of PCR conditions for the amplification of different mitochondrial regions of *P. pelagicus* and *P. sanguinolentus* were done by using the above mentioned sets of primer individually. The PCR conditions consisted of initial denaturation at $94^0$C for 2 min. followed by denaturation at $94^0$C for 30 sec; annealing temperatures varied from each primer to primer as given in Table 1; extension at $72^0$C for 30 sec for 35 cycles and final extension at $72^0$C for 30 sec for 35 cycles and final extension at $72^0$C for 1 min.

### 3.2.8. Optimization of PCR conditions

Based on the preliminary PCR analysis using the designed primer sets, the primer sets that amplified the species specific genes were chosen for further development of multiplex PCR assay. The PCR reaction assay mixture consisted of 3 µl of template DNA, 10µl of a master mix (consisting of reaction buffer, dNTPs, magnesium chloride, Taq DNA polymerase), 10µl of molecular grade water and 1µl (10 pmol) of each forward and reverse oligonucleotide primer in a final volume of 25µl. Amplification conditions and primer sequences are given in Tables1 and 2.

### 3.2.9. Agarose gel electrophoresis

On completion of PCR reaction, 5 µl of PCR product was mixed with 1 µl of 6X gel loading dye. Agarose gel (2%) was prepared with 1% TAE buffer containing ethidium bromide (0.5 mg/ml). PCR product was electrophoresed using 0.5% TAE buffer. A 100 bp or 50 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).

### 3.2.10. Development of multiplex PCR (MPCR) assay

A MPCR assay was developed for the simultaneous detection of *P. pelagicus* and *P. sanguinolentus* using the species specific primers sets viz. cyt b and 16S rRNA, respectively. The optimized MPCR condition was similar to that of uniplex PCR, except that the total reaction mixture volume was 50 µl. The developed MPCR assay was also tested on differently treated crab meat products (freezing, cooking and frying).

### 3.2.11. Optimization of qPCR conditions

The qPCR amplification was performed in 10 µl reaction mixture consisting of 1 µl template DNA of selected crab sample, 5µl of a SYBR Green master mix Thermo (Fisher Scientific Inc., Massachusetts,USA), 3 µl of molecular grade water and 0.5 µl (10 pmol) of each of forward and reverse oligonucleotide primer. The optimized cyclic condition is given in Table 5.

#### 3.2.11.1. Development of qPCR assay for quantification of mixed crab species

Three calibration curves were generated to quantify the proportion of mixed meat in *P. pelagicus*. First calibration curve were generated for *P. pleagicus* using mt cyt b gene and second for *P. sanguinolentus* using mt 16S rRNA gene. Initial copy number for *P. pleagicus* and *P. sanguinolentus* was calculated through online software (www.scienceprimer.com) then targeted their specific regions using mt cyt b and mt 16S rRNA species specific primer under the optimized

condition, respectively. Total DNA was serially diluted at 10 fold and obtained threshold cycle (Ct) values were used for construction of first and second calibration curve (Tables 4.3 & 4.5). The third calibration curve was generated for *P. sanguinolentus* using mt 16S rRNA gene. Spiked *P. sanguinolentus* meat at different ratio used to generate calibration curve (Table 4.6). Unknown quantity of samples were taken at the laboratory and compared separately with standardized calibration curve obtained for *P. sanguinolentus* at different quantities.

The No. of copies of DNA in unknown sample was calculated using the following equation (Godornes et al., 2007).

$$\text{Number of Copies/ µl} = \frac{\text{X (ng)} \times 6.02\times10^{23}\text{(molecules/mole)}}{(\text{N} \times 660\ \text{g/ mole}) \times 1 \times 10^{9}\ \text{ng/g}}$$

Where,

**X** **=** Amount of amplicon (ng)

**N** **=** Length of double strand amplicon

**660 mole** **=** Average mass of 1 base paire double stranded DNA (dsDNA)

### 3.2.12. Validation of developed assays

Processed crab products obtained from five crab processing industries located in the Thoothukudi region of Tamil Nadu were subjected to validation process using the developed MPCR assay. The developed MPCR assay was also subjected to inter- laboratory validation in the laboratories of Dept. of Animal

Biotechnology, Madras Veterinary College (MVC), Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai, and Dept. of Fisheries Biotechnology, Fisheries College & Research Institute (FC & RI) of our University (TNJFU), Thoothukudi.

The developed qPCR assay was performed in the Laboratory of Dept. of Animal Biotechnology, MVC, TANUVAS, Chennai due to calibration error in our real time PCR machine and assay was subjected to inter-laboratory validation in Molecular Biology Laboratory of Thermo Scientific, Bangalore.

### 3.2.13. Statistical analysis

Statistical analysis was performed using single factor ANOVA, where the individual data sets were subjected to an estimation of the significant difference in the mixed meat percentage (Pfaffl, 2004).

# IV. RESULTS

## 4.1. Extraction of DNA from crab

Total genomic DNA of all four species was individually extracted from raw, frozen, cooked and fried *Portunus pelagicus, P. sanguinolentus*, *Scylla serrata* and *Charybdis natator*. The extracted DNA quality and concentration was determined by measuring the absorbance at 260 nm using a Biophotometer and the results are given in Table 4.1.

**Table 4.1. DNA concentration (ng/µl) of different crab species**

| Species | Raw | Frozen | Cooked | Fried |
|---|---|---|---|---|
| *P. pelagicus* | 850 | 789 | 763 | 728 |
| *P. sanguinolentus* | 901 | 795 | 697 | 687 |
| *C.natator* | 881 | 823 | 789 | 784 |
| *S. serrata* | 862 | 819 | 784 | 771 |

The DNA concentration of raw crab ranged from 850 to 901 ng/µl, frozen from 789 to 823 ng/µl, cooked from 697 to 789 ng/µl and fried from 687 to 784 ng/µl, respectively.

## 4.2. Species specific primers for *P. pelagicus* and *P. sanguinolentus*

Out of ten sets of primers designed for *P. pelagicus*, only one set of primer from cyt b gene gave species specific pattern for *P. pelagicus*. Out of seven sets of primers designed for *P. sanguinolentus*, only one set of primer from 16S rRNA

gene gave species specific pattern for *P. sanguinolentus*. The successful primer sets are given in Table 4.2. The selected primer sets did not amplify the DNA of the other three crab species.

**Table 4.2. Species specific primers for *P. pelagicus* and *P. sanguinolentus* and their optimized PCR conditions**

| Species | Target region | Primer | Sequence (5' to 3') | Product size | Cyclic conditions |
|---|---|---|---|---|---|
| *P. pelagicus* | cyt b | Forward | CGTACTATGCATGCCAAT | 298 bp | $94^0$C- 2 min<br>$94^0$C- 30 s<br>$53.5^0$C- 30 s<br>$72^0$C- 30 s<br>$72^0$C- 1 min |
| | | Reverse | AAGAACCGTGTTAGTGTAG | | |
| *P. saguinolentus* | 16S rRNA | Forward | TCTACACTTGCACTGTTAC | 383 bp | |
| | | Reverse | AAAGCTCGTATGACATCTC | | |

## 4.3. Optimized PCR conditions for mt cyt b gene of *P. pelagicus* and mt 16S rRNA gene of *P. sanguinolentus* for species specificity

The optimized PCR conditions for mt cyt b gene of *P. pelagicus* and mt 16S rRNA gene of *P. sanguinolentus* are given in Table 4.2. It showed that the optimum annealing temperature for the PCR amplification of cyt b region of *P. pelagicus* and 16S rRNA region of *P. sanguinolentus* was same as $53.5^0$C for 30 sec.

## 4.4. Amplification of mt cyt b gene in *P. pelagicus* using PCR

The designed species specific primer for *P. pelagicus* of cyt b region of mt DNA successfully amplified the target region of *P. pelagicus*, but it did not amplify the DNA from the other species of crabs viz. *P. sanguinolentus, S. serrata* and *C. natator* (Fig. 4.1). The PCR product produced an intense band of the expected product size of 298 bp for cyt b gene of *P. pelagicus* in the agarose gel.

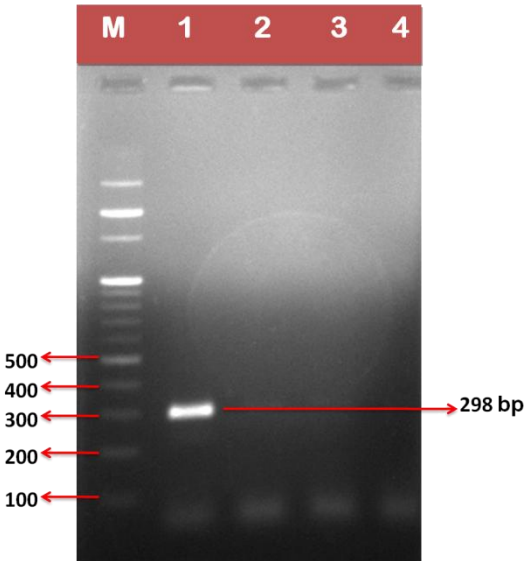


**Fig. 4.1.** Agarose gel (2 %) showing results of electrophoretic pattern of amplified mitochondrial 16S rRNA for the specificity of *Portunus pelagicus* with other species. Lane M- 100 bp DNA Marker; Lane 1- *P. pelagicus*; Lane 2- *P. sanguinolentus;* Lane 3- *S. serrata*; Lane 4- *C. natator*

## 4.5. Amplification of mt 16S rRNA gene in *P. sanguinolentus* using PCR

The designed species specific primer for *P. sanguinolentus* of 16S rRNA region of mt DNA successfully amplified the target region of *P. sanguinolentus,* but it did not amplify the DNA from the other species of crabs viz. *P. pelagicus, S. serrata* and *C. natator* (Fig. 4.2).The PCR product produced an intense band of the expected product size of 383 bp for 16S rRNA gene of *P. sanguinolentus* in the agarose gel.

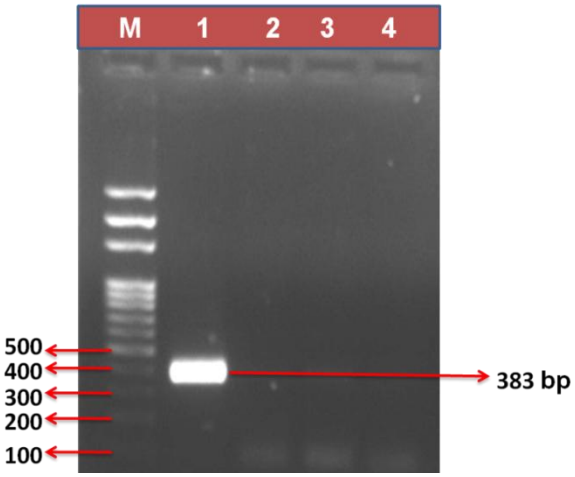


**Fig. 4.2.** Agarose gel (2 %) showing results of electrophoretic pattern of amplified mitochondrial 16S rRNA for the specificity of *Portunus sanguinolentus* with other species. Lane M- 100 bp DNA Marker; Lane 1- *P. sanguinolentus*; Lane 2- *P. pelagicus;* Lane 3- *S. serrata*; Lane 4- *C. natator*

## 4.6. Amplification of mt cyt b in processed crab product of *P. pelagicus*

The processed products of *P. pelagicus*, which had undergone different treatments like freezing, cooking and frying, showed similar pattern as that of raw crab, emphasizing that the above processes did not alter the selected DNA target region of crab species after different processing treatments (Fig. 4.3).

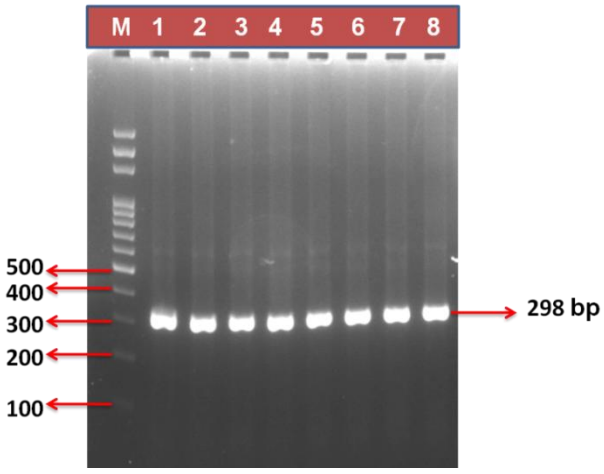


**Fig. 4.3.** Agarose gel (2 %) showing results of electrophoretic pattern of amplified mitochondrial cyt b for processed products of *P. pelagicus*. Lane M- 100 bp DNA Marker; Lanes 1 & 2- Raw crab (RC); Lanes 3 & 4- Frozen crab (FC); Lanes 5 & 6- Cooked crab (CC); Lanes 7 & 8 Fried crab (FrC)

## 4.7. Amplification of mt 16S rRNA in processed crab product of *P. sanguinolentus*

The processed products of *P. sanguinolentus*, which had undergone different treatments like freezing, cooking and frying, showed similar pattern as that of raw crab, emphasizing that the above processes did not alter the selected DNA target region of crab species after different processed treatments (Fig. 4.4).

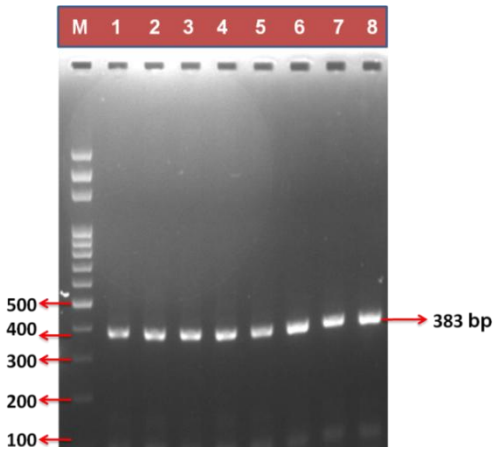


**Fig. 4.4.** Agarose gel (2 %) showing results of electrophoretic pattern of amplified mitochondrial 16S rRNA for processed products of *P. sanguinolentus*. Lane M- 100 bp DNA Marker; Lanes 1 & 2- Raw crab (RC); Lanes 3 & 4- Frozen crab (FC); Lanes 5 & 6- Cooked crab (CC); Lanes 7 & 8- Fried crab (FrC)

The developed MPCR assay simultaneously detected both the targeted crab species viz. *P. pelagicus* and *P. sanguinolentus* in a single reaction by amplifying the targeted cyt b and 16S rRNA genes at 298 bp and 383 bp, respectively. The optimized annealing temperature for the MPCR assay was $53.5^0$C for 30 sec with the reaction volume of 50 µl (Fig. 4.5).

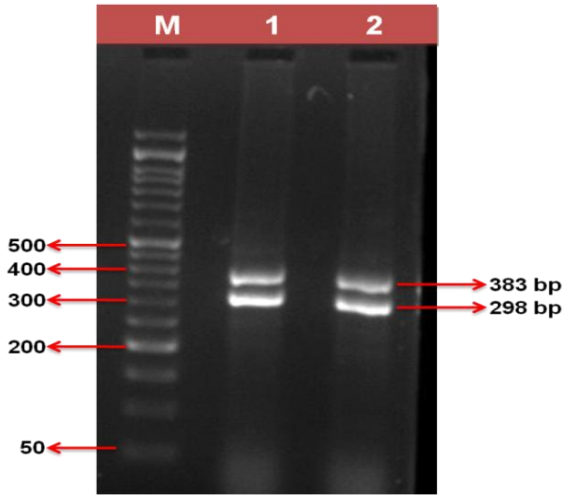


**Fig. 4.5.** Agarose gel (2 %) showing results of simultaneous detection of mitochondrial cyt b of *P. pelagicus* (298 bp) and 16S rRNA of *P. sanguinolentus* (383 bp). Lane M- 50 bp DNA Marker; Lanes 1 & 2- Mixed meat of *P. pelagicus* and *P. sanguinolentus*

The developed MPCR assay detected both the species of crabs, *P. pelagicus* and *P. sanguinolentus*, in frozen, cooked and fried products in a single reaction (Fig. 4.6).

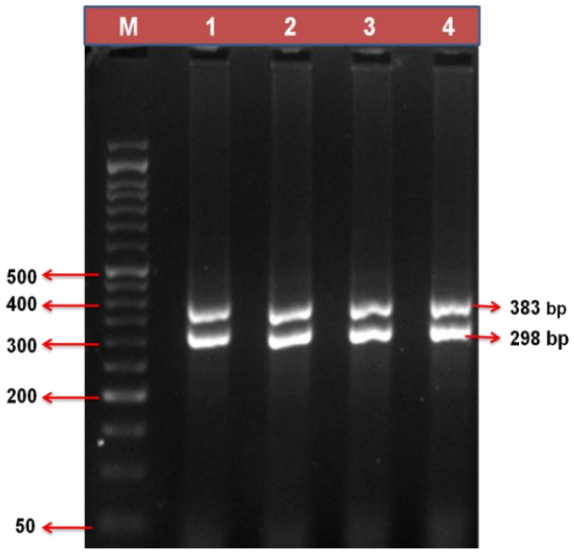


**Fig. 4.6.** Agarose gel (2 %) showing results of simultaneous detection of mitochondrial cyt b of *P. pelagicus* (298 bp) and 16S rRNA of *P. sanguinolentus* (383 bp). Lane M - 50 bp DNA Marker; Lane 1- Raw crab (RC); Lane 2 -Frozen crab (FC); Lane3 - Cooked crab (CC);

### 4.9. Optimization of qPCR assay

The optimized annealing temperature for the qPCR assay was $53.5^0$C for 30 sec. with the reaction volume of 10µl. The developed qPCR assay was found to detect and quantify the meat from both the species of crabs viz. *P. pelagicus* and *P. sanguinolentus*.

#### 4.9.1. Construction of calibration curve for *P. sanguinolentus*

A standard calibration curve was constructed for *P. sanguinolentus* to investigate the proportion of *P. sanguinolentus* adulteration with *P. pelagicus* (Figs. 4.9). Initial copy number for *P. sanguinolentus* was calculated as $7.171*10^{10}$ in the total DNA through online software (www.scienceprimer.com) and then targeted their specific regions using species specific primer under the optimized condition. The qPCR was standardized with 10 fold serial dilution of total DNA of *P. sanguinolentus* and the corresponding Ct values were obtained (Table 4.3). This calibration curve was used to investigate the adulteration of *P. sanguinolentus* with *P. pelagicus.*

**Table 4.3. Ct values for different concentration of DNA of *P. sanguinolentus***

| Serial Dilution of DNA (ng) | Ct value |
|---|---|
| 3.1 | 9.305 |
| 0.31 | 15.095 |
| 0.031 | 17.525 |
| 0.003 | 21.215 |


**Fig. 4.7**. The real- time PCR assay amplification plot obtained for *P. sanguinolentus* when plotted as fluorescence intensity against the cycle number from triplicates of 10 fold serial dilution of genome DNA. This figure was generated by Real-Time PCR system.

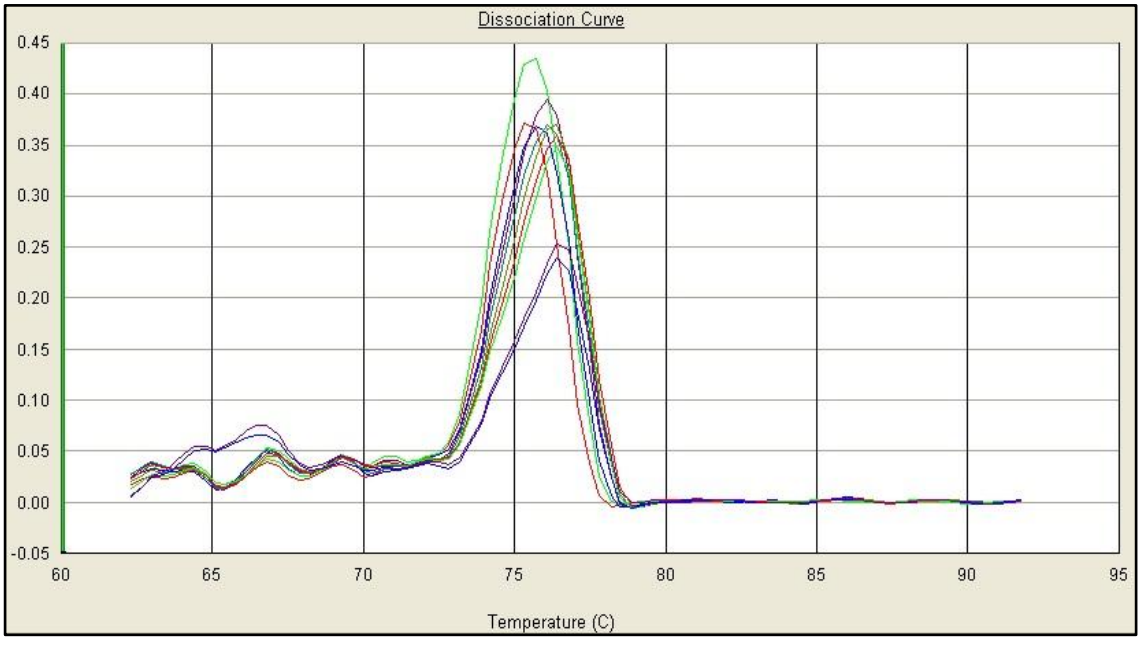


**Fig. 4.8.** Melting curve analysis of *P. sanguinolentus* by plotting first derivative of fluorescence versus dissociation temperature after amplification in the SYBR Green based real time PCR

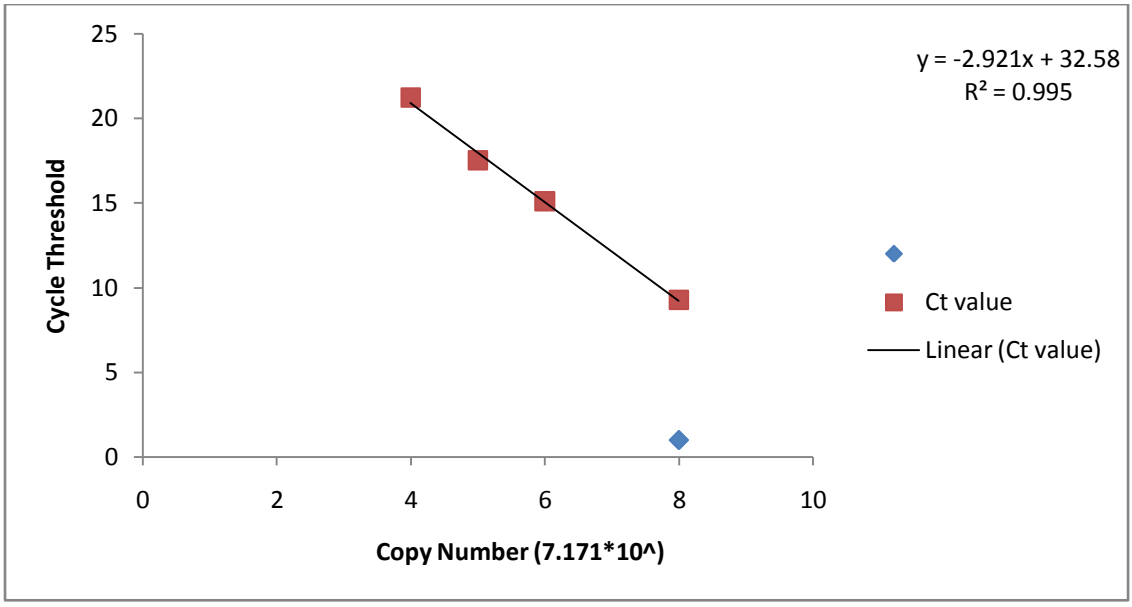


**Fig. 4.9.** Calibration curve for *P. sanguinolentus* generated from 10 fold serial dilutions amplified by real time PCR. The mean Ct values were plotted against copy numbers.

### 4.9.2. qPCR for detection of *P. sanguinolentus* adulterant in *P. pelagicus*

Adulteration of *P. sanguinolentus* with *P. pelagicus* was simulated by mixing *P. sanguinolentus* meat with *P. pelagicus* at different quantities. The qPCR was performed with the selected primer to amplify the 16S rRNA mitochondrial gene of *P. sanguinolentus* and the total DNA copy number was calculated for the different mixed proportion with the obtained Ct value. The Ct values and DNA copy number for the mixed meat of *P. sanguinolentus* with *P. pelagicus* are given in Table 4.4.

The standard curve drawn showed significant relationship between the copy number and the Ct values returned. The optimal linear range for each target as well as the $R^2$ and reaction efficiency values for that linear range has been observed (y = -2.921x + 32.58 and $R^2$ = 0.995). It is clear that more the adulteration, there will be an increase the copy numbers of DNA of adulterated

*P. sanguinolentus*. Statistical analysis of these optimized data was performed using single factor ANOVA, where the individual data sets were subjected to an estimation of the significant difference. The result of this study showed that there is a significant difference ($P<0.05$) between the concentrations of *P. sanguinolentus* adulterated with *P. pelagicus*. A significant difference was also found between the Ct values and adulterated DNA copy numbers of *P. sanguinolentus*. This indicated that the quantification of adulterated *P. sanguinolentus* would return data with a similar degree of accuracy.

**Table 4.4. Ct values and DNA copy numbers of different proportion of mixed meat of *P. pelagicus* and *P. sanguinolentus***

| Mixed meat (%) | | Ct value | DNA Copy number | Corresponding DNA concentration |
|---|---|---|---|---|
| ***P. plelagicus*** | ***P. sanguinolentus*** | | | |
| - | 100 | 16.08 | $7.171*10^{5.65}$ | 0.00013 ng |
| 70 | 30 | 17.545 | $7.171*10^{5.15}$ | 0.00011 ng |
| 60 | 40 | 16.815 | $7.171*10^{5.4}$ | 0.00075 pg |
| 50 | 50 | 16.32 | $7.171*10^{5.57}$ | 0.00042 pg |

### 4.9.3. Construction of calibration curves for *P. pelagicus*

A standard curve was constructed for *P. pelagicus* to investigate the proportion of adulteration of *P. sanguinolentus* with *P. pelagicus* meat (Fig. 4.12). Initial copy number for *P. pelagicus* was calculated as $2.633*10^{11}$ in the total DNA through online software and then targeted their specific regions using species

specific primer under the optimized condition. The qPCR was standardized with the 10 fold serial dilution of total DNA of *P. pelagicus* and the corresponding Ct values were obtained (www.scienceprimer.com) (Table 4.5). Standard curve was made in order to verify the linearity of the assay within a particular concentration range. Standard curves were constructed with EXCEL (Microsoft, Redmond, WA) software (Figs. 4.12).

**Table 4.5. Ct values for different concentration of DNA of *P. pelagicus***

| Serial Dilution of DNA (ng) | Ct value |
|---|---|
| 8.6 | 11.79 |
| 0.86 | 14.025 |
| 0.086 | 17.955 |
| 0.008 | 21.69 |

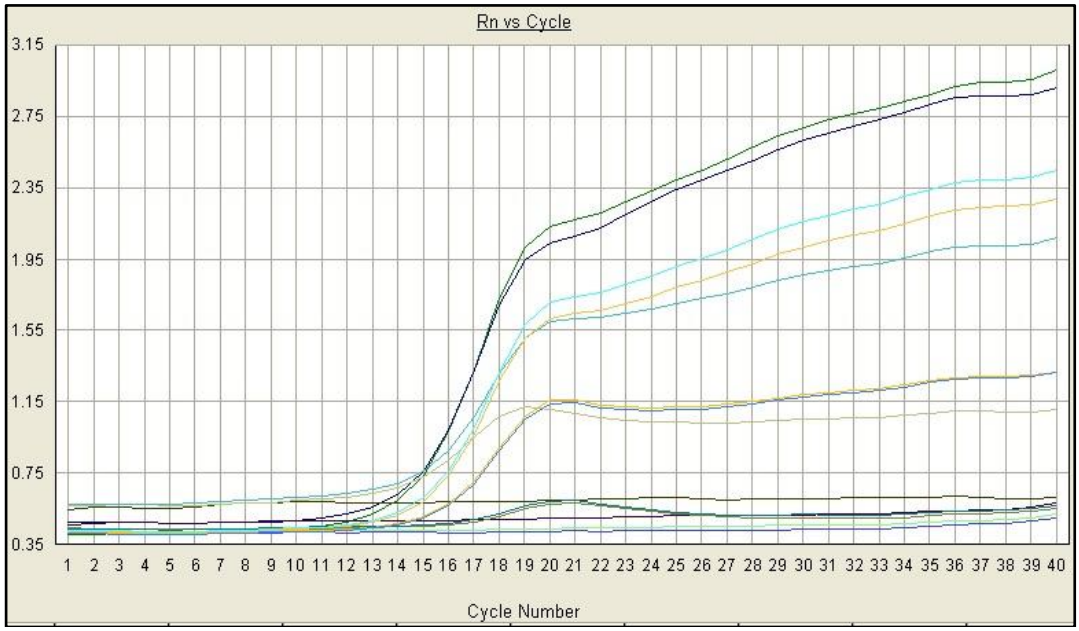


**Fig. 4.10.** The real- time PCR assay amplification plot obtained for *P. pelagicus* when plotted as fluorescence intensity against the cycle number from 10 fold serial dilution of genome DNA.

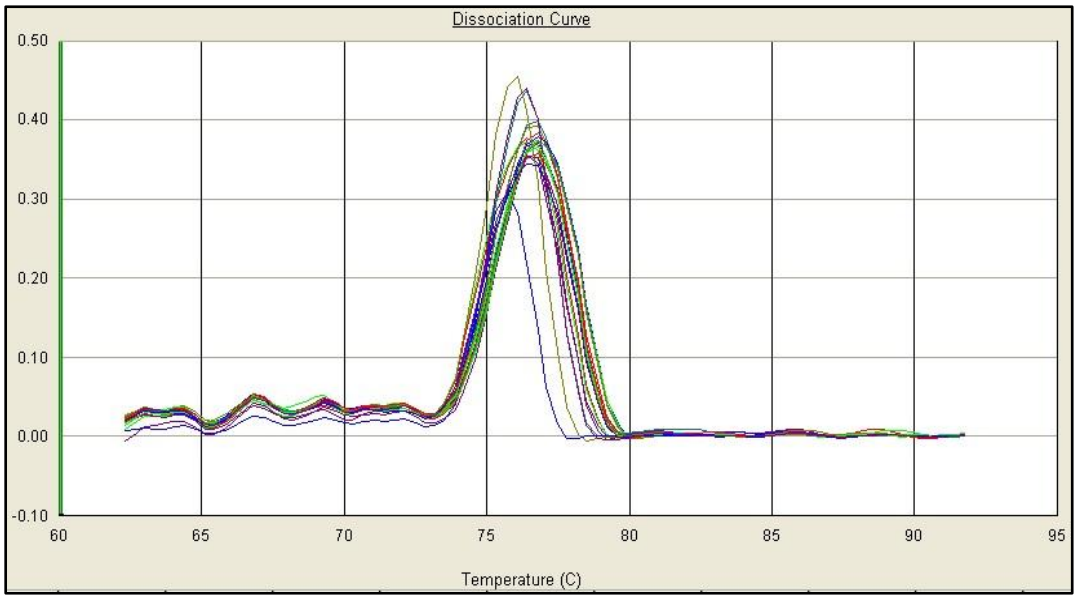


**Fig. 4.11.** Melting curve analysis of *P. pelagicus* by plotting first derivative of fluorescence versus dissociation temperature after amplification in the SYBR Green based real time PCR

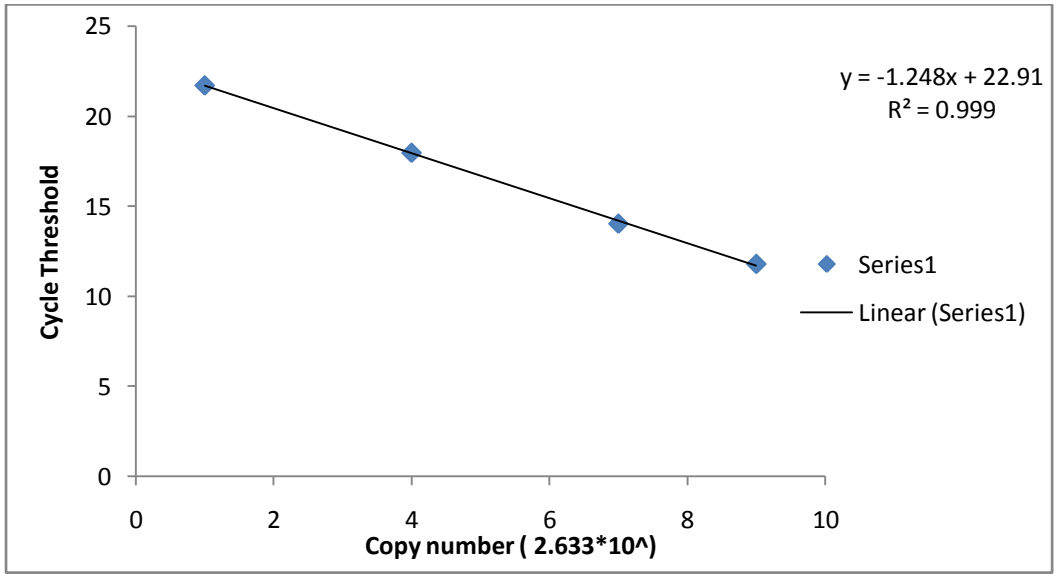


**Fig. 4.12.** Calibration curve for *P. pelagicus* generated from 10 fold serial dilutions amplified by real time PCR. The mean Ct values were plotted against copy numbers.

### 4.9.4. qPCR for detection of adulterant in *P. pelagicus*

Adulteration in *P. pelagicus* was simulated by mixing *P. sanguinolentus* meat with *P. pelagicus* at different quantities. The qPCR was performed with the selected primer to amplify the cyt b mitochondrial gene of *P. pelagicus*. Total DNA copy number was calculated for the different mixed proportion. The Ct values and DNA copy numbers for the mixed meat of *P. pelagicus* with *P. sanguinolentus* are given in Table 4.6. The standard curve drawn showed significant relationship between the copy number and the Ct values returned. The optimal linear range for each target as well as the Correlation coefficients ($R^2$) and reaction efficiency values for that linear range has been observed ($y = -1.248x + 22.91$ and $R^2 = 0.999$). Statistical analysis of these optimized data was performed using one way ANOVA, where the individual data sets were subjected to an estimation of the significant difference. The result of this study showed that there is a significant difference ($P<0.05$) between the concentrations of adulterant with *P. pelagicus*. A significant difference was also found between the Ct values and DNA copy numbers of *P. pelagicus.* This indicated that the quantification of *P. pelagic* meat would return data with a similar degree of accuracy.

**Table 4.6. Ct values and DNA copy numbers of different proportion of mixed meat of *P. pelagicus* and *P. sanguinolentus***

| Mixed meat (%) | | Ct value | DNA Copy number | Corresponding DNA concentration |
|---|---|---|---|---|
| ***P. plelagicus*** | ***P. sanguinolentus*** | | | |
| 100 | - | 14.85 | $2.633*10^{6.46}$ | 0.0024 ng |
| 90 | 10 | 15 | $2.633*10^{6.33}$ | 0.0018 ng |
| 80 | 20 | 16.46 | $2.633*10^{5.17}$ | 0.0001 ng |
| 50 | 50 | 19.64 | $2.633*10^{2.62}$ | 0.0003 pg |

### 4.9.5. Construction of calibration curve for *P. sanguinolentus*

The construction of calibration curve using *P. sanguinolentus* meat added to *P. pelagicus* meat was done as shown in Figs. 4.12. The DNA was extracted from the samples, then analysed using the mt 16S rRNA primer set under the optimised conditions (Table 5). The calibration curve drawn for each matrix showed a good relationship between the proportion of *P. sanguinolentus* meat added and the Ct values returned (Table 4.7)

**Table 4.7. Ct values for *P. sanguinolentus s* meat added to *P. pelagicu* meat**

| ***P. pelagicus*** **(g)** | ***P. sanguinolentus*** **(g)** | **Spiked meat (%)** | **Ct value** |
|---|---|---|---|
| 0 | 10 | 100 | 33.565 |
| 5 | 5 | 50 | 36.129 |
| 6 | 4 | 40 | 36.748 |
| 7 | 3 | 30 | 37.227 |

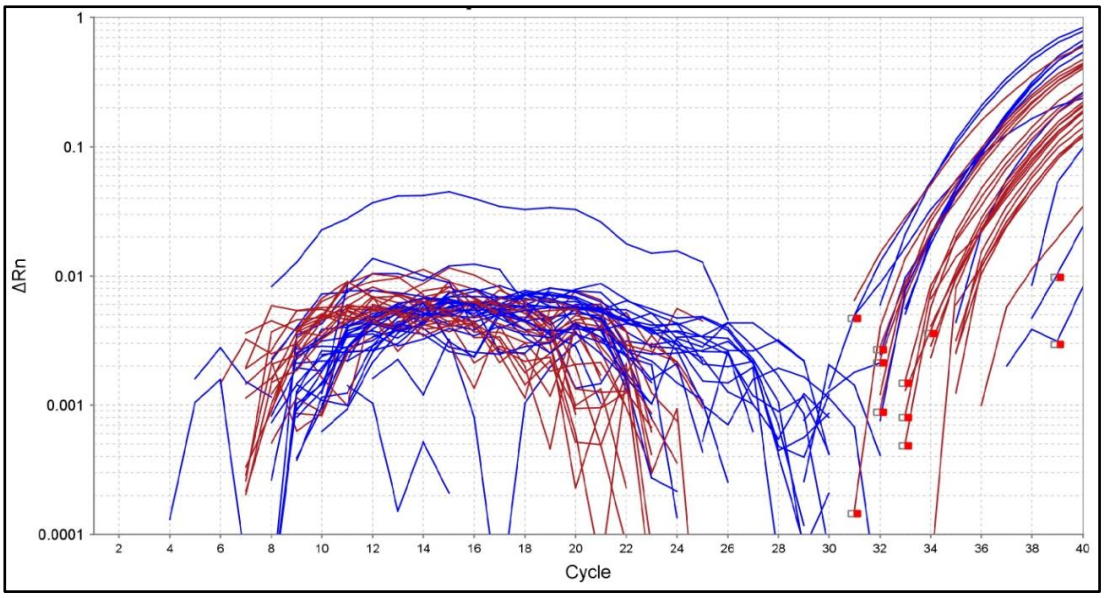


**Fig. 4.13.** The real- time PCR assay amplification plot obtained for *P. sanguinolentus* when plotted as fluorescence intensity against the cycle number from 10 fold serial dilution of genome DNA. This figure was generated by Real-Time PCR

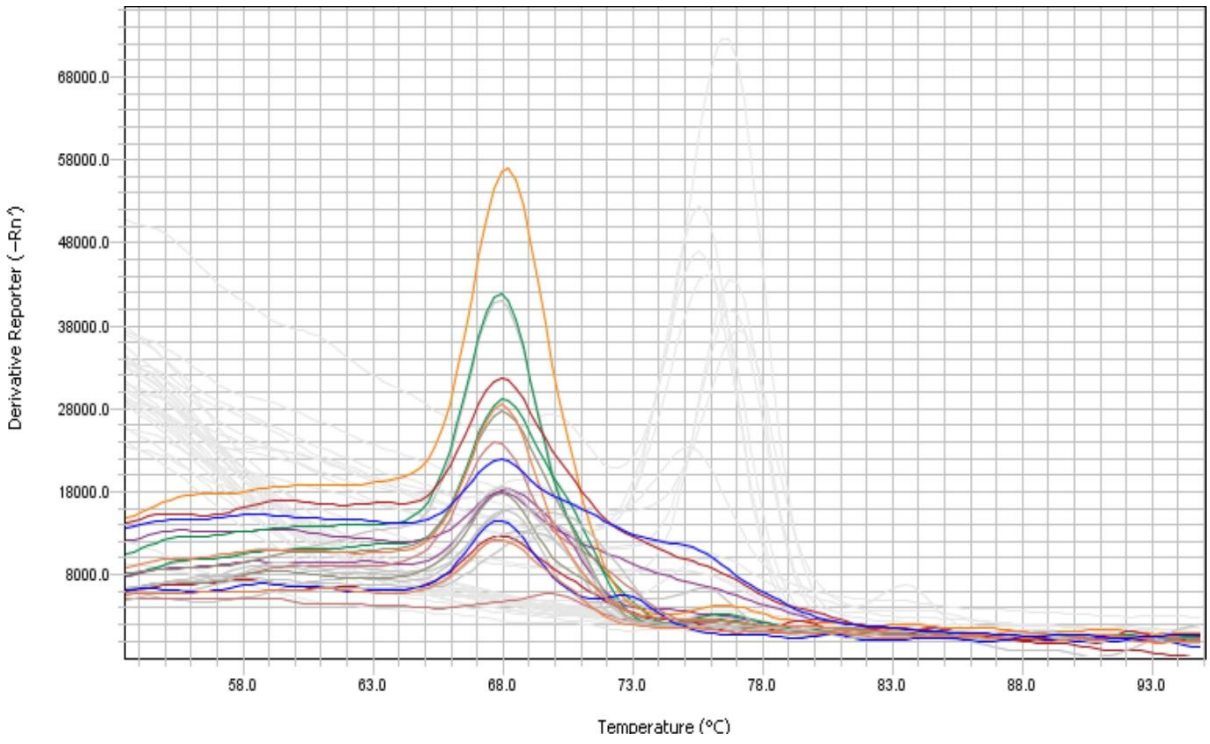


**Fig. 4.14.** Melting curve analysis of *P. sanguinolentus* by plotting first derivative of fluorescence versus dissociation temperature after amplification in the SYBR Green based real time PCR

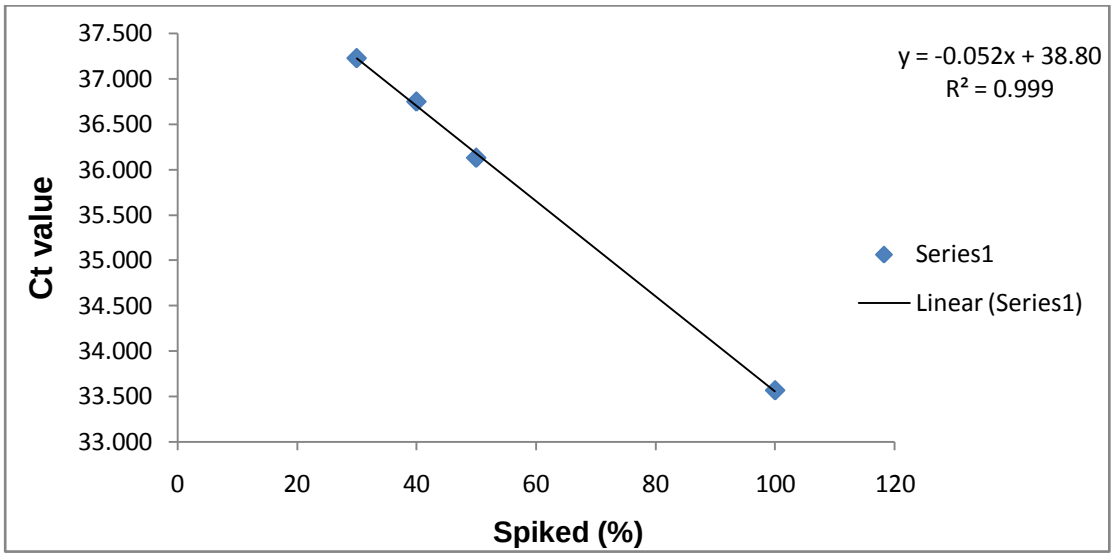


**Fig. 4.15.** Calibration curve for the spiked sample contained the meat of *P. pelagicus* and *P. sanguinolentus* amplified by real time PCR. The mean Ct value was plotted against spiked percentage of *P. sanguinolentus* meat.

## 4.10. Validation of developed MPCR and qPCR assays in species authentication of commercial crab products

The developed MPCR assay was validated with nine samples of commercial crab products procured from five crab processing industries of Thoothukudi region in Tamil Nadu. The results are given in Table 4.8, and their respective patterns are shown in Fig. 4.16 (a and b).

**Table 4.8. Validation of developed MPCR assay in species authentication of commercial crab meat products**

| Sample No. | Commercial products | Confirmation of both the species of *P. pelagicus* and *P. sanguinolentus* (Yes or No) |
|---|---|---|
| 1 | Pasteurized crab meat products | No |
| 2 | | No |
| 3 | | No |
| 4 | | No |
| 5 | | No |
| 6 | | Yes |
| 7 | | Yes |
| 8 | | Yes |
| 9 | | Yes |

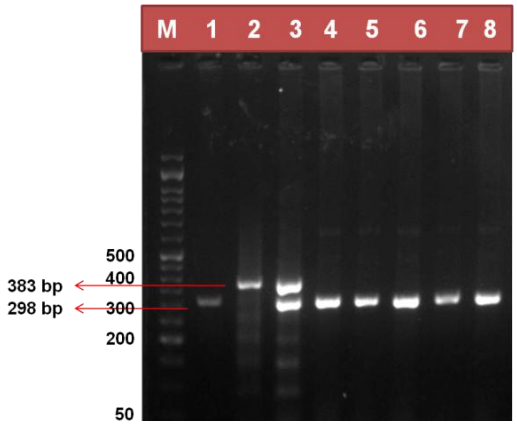


**Fig. 4.16a.** Agarose gel (2 %) showing results of simultaneous detection of mitochondrial cyt b *of P. pelagicus* (298 bp) and 16S rRNA of *P. sanguinolentus* (383 bp). Lane M - 50 bp DNA Marker; Lane 1- *P. pelagicus*: Lane 2 - *P. sanguinolentus*; Lane 3 - *P. pelagicus* and *P. sanguinolentus*; Lanes 4 to 8 - Pasteurized crab meat products (Sample Nos. 1, 2, 3, 4 and 5)

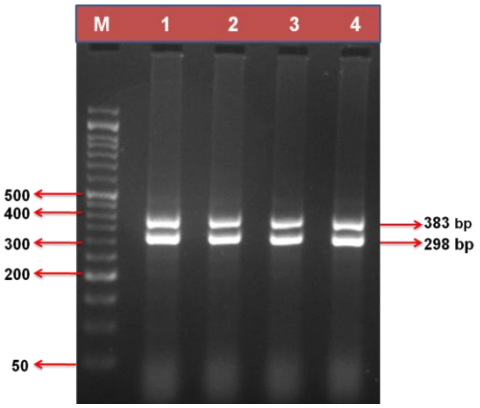


**Fig. 4.16b.** Agarose gel (2 %) showing results of simultaneous detection of mitochondrial cyt b of *P. pelagicus* (298 bp) and 16S rRNA of *P. sanguinolentus* (383 bp). Lane M - 50 bp DNA Marker; Lanes 1 to 4 - Pasteurized crabmeat products (Sample Nos.6, 7, 8 and 9)

Raw samples were also validated in the Laboratory of Dept. of Animal Biotechnology, MVC, Chennai (Fig. 4.17).

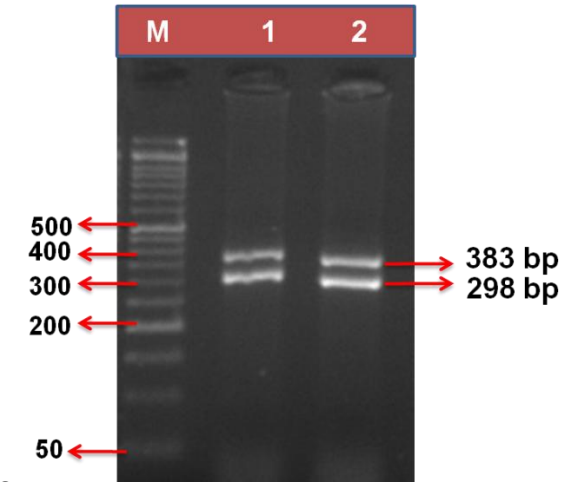


**Fig. 4.17.** Agarose gel (2 %) showing results of simultaneous detection of mitochondrial cyt b of *P. pelagicus* (298 bp) and 16S rRNA of *P. sanguinolentus* (383 bp). Lane M- 50bp DNA Marker; Lanes 1 & 2 – Mixed meat of *P. pelagicus* & *P. sanguinolentus*

Crab meat samples that were subjected to different processing were also validated in the Laboratory of Dept. of Fisheries Biotechnology, FC&RI, Thoothukudi (Fig. 4.18).

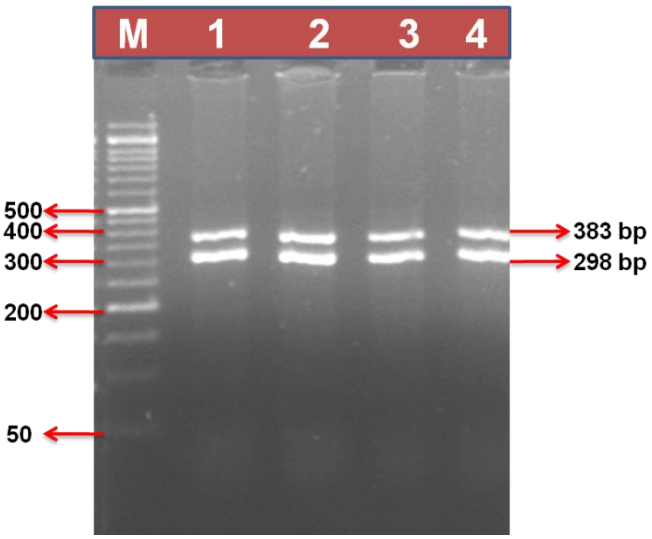


**Fig. 4.18.** Agarose gel (2 %) showing results of simultaneous detection of mitochondrial cyt b of *P. pelagicus* (298 bp) and 16S rRNA of *P. sanguinolentus* (383 bp). Lane M - 50 bp DNA Marker; Lane 1- Raw crab (RC); Lane 2 -Frozen crab (FC); Lane 3- Cooked crab (CC); Lane 4- Fried crab (FrC)

The constructed standard curve in the developed qPCR assay was used to investigate the quantity of *P. sanguinolentus* adulterated with *P. pelagicus* (Fig.4.15). The qPCR assay was validated in the Molecular Biology Laboratory of Thermo Scientific, Bangalore in eight unknown samples with the selected primer to amplify the 16S rRNA gene of *P. sanguinolentus* to detect the quantity of adulteration of *P. sanguinolentus* with *P. pelagicus.* The Ct values of unknown samples were compared with the Ct values of standard curve of *P. sanguinolentus* to quantify the level of adulteration, which ranged from 38 to 80 % (Table 4.9).

**Table 4.9. Ct values of unknown samples and observed quantity (%) of *P. sanguinolentus* meat adulterated with *P. pelagicus* meat**

| Unknown sample Ct values | Observed quantity of adulterated meat (%) | Actual quantity of adulterated meat (%) | Percentage recovery |
|---|---|---|---|
| 36.803 | 38.41 | 55 | 69.83 |
| 37.177 | 31.22 | 45 | 69.37 |
| 34.771 | 77.47 | 80 | 96.83 |
| 34.913 | 74.75 | 75 | 99.66 |
| 35.753 | 58.59 | 65 | 90.13 |
| 36.147 | 51.02 | 60 | 85.03 |
| 37.199 | 30.79 | 35 | 87.97 |
| 34.635 | 80.10 | 85 | 94.23 |
| Average recovery percentage | | | 86.63 |

# DISCUSSION

# V. DISCUSSION

Seafood mislabeling is one of the most articulated socioeconomic concerns, which contributed to increase in awareness of human beings on what they eat and how and where it is produced (Ali et al., 2014). Seafood mislabeling is also a major problem in value added crab products around the World. The authenticity and certification of crab products is highly important in raw, frozen, cooked and fried crabmeat products. In this study, PCR based molecular techniques were developed for the detection of commercial seafood fraud in processed crab products. Ali et al. (2014) have reported that DNA-based authentication schemes are reliable due to the superior stability and universality of DNA in all tissues. Multiplex PCR and Real Time PCR (qPCR) are well known assays for the detection of the adulteration in seafood products. Armani et al. (2012) developed multiplex and real-time PCR as convenient, simple and rapid tools to support both the industry and regulatory agencies in the detection and prevention of species substitution in fresh, marinated and cooked products. When species-specific primers are utilized, analysis would become as simple as visualization of the amplicons with gel electrophoresis. Real time PCR technique uses fluorescent probe to obtain results during the each cycle of reaction and does not contain post-gel electrophoresis, for quantification of target DNA, which considerably reduces contamination problem (Rasmussen and Morrissey, 2008). Hence, it is possible to produce more authentic results with less chance of error.

## 5.1. Genomic DNA from crab

High quality DNA is prerequisite for studying the molecular systematic of any organism. Several protocols have been established for the extraction of nucleic acids. Crab naturally retains some inhibitory compounds that interfere not

only with the extraction of high molecular weight DNA's quality, but also with the process of PCR (Schander and Kenneth, 2003). Inhibitory compounds like mucopolysaccharides, pigments, and chitin are normally secreted from or embedded within the tissues when they are live, while in preserved condition they are present in high concentrations all over the body. During extraction of DNA from the preserved specimen, these factors may be challenging to obtain high quality genomic DNA.

For DNA isolation, numerous protocols are being used to separate protein molecules and debris from the genomic DNA, which is, in practical, toxic and hazardous to extracted DNA. These protocols are quite expensive and require special control facilities to maximize personnel safety and minimize environmental concern (Sahu et al., 2012). Therefore in our study, a reliable and rapid phenol-chloroform method, devoid of costly and toxic chemicals, was used for the extraction of high quality nucleic acids using small quantity of tissue (50 mg) from crab species. The present protocol produced high yield of nucleic acids from fresh tissue and high quality of extracted DNA, which was confirmed through sectrophotometric analysis. Though commercial kits are available, their high cost limits their use (Kumar et al., 2007). Our results showed that the DNA concentration of raw crab ranged from 850 to 901ng/µl, frozen from 789 to 823 ng/µl, whereas cooked from 687 to 784 ng/µl and fried ranged from 697 to 789 ng/µl (Table 4.1). The extracted DNA was found sufficient for the amplification by PCR method, as Rehbein et al. (2005) have also reported that the minimum concentration of DNA required for efficient PCR amplification is 100-1000 ng/µl.

Extraction of the pure DNA is a crucial step in DNA based analysis. Several factors such as temperature and pH can affect the DNA and cause degradation,

depurination, and hydrolysis. Since seafood are mostly sold in processed forms, severe processing methods such as frying, canning and freezing may cause damage in the integrity of DNA leading to shorter sequences when compared to fresh samples. Therefore, a common challenge in the application of DNA based methods in the authentication of seafood products is to obtain quality DNA in sufficient quantity for further downstream analysis (Hsieh et al., 2007). In this study, from the results, it is clear that the cooking and frying had caused considerable damage to DNA resulting in lower yield of DNA than freezing (Table 4.1). Earlier report also state that the heat processing methods such as cooking and frying degraded the DNA and gave less DNA yield when compared to the fresh and frozen samples (Infante et al., 2004).

### 5.2. PCR amplification of newly designed species specific primers

DNA is more resistant and thermostable than proteins and it is possible to amplify very small fragment by PCR with sufficient information to allow identification. As DNA molecule is present in all cells of an organism, it can be recovered from any substrate. DNA provides more information because of the degeneracy of the genetic code and the presence of many non-coding regions (Teletchea, 2009). Moreover, mt DNA presents a higher copy number and a faster rate of mutation, making it generally more appropriate in the study of evolutionary genetics and inter- and intra-species variability. The most common mt DNA genes exploited in species identification are cyt b, 12S and 16S rRNA (Rasmussen and Morrissey, 2008). Cox I and D-loop region in mt DNA are also often employed for authentication of species (Diniz et al., 2005). Designing of species specific primers

and predicting the amplicon length of the product is the most important step. Analysis of sequences is particularly delicate because of the need to choose amplicons with different lengths and different melting temperatures, thereby avoiding all possible cross-reactions. In this study, *Labeo rohita* and *Litopenaeus vannamei* sequences for inter-species variation, and *S. serrata* and *C. natator* sequences for intra-species variation were chosen to designed species specific primers for targeted species viz. *P. pelagicus* and *P. sanguinolentus*. Total 17 sets of species specific primers were designed from the five mitochondrial regions of cyt b, 16S rRNA, 12S rRNA, D-loop and COI of selected species based on the sequences available on NCBI databases with the help of BioEdit software for authentication of *P. pelagicus* and *P. sanguinolentus* (Tables 4 and 5). Out of 17 sets of primer, ten sets were designed for *P. pelagicus* and among ten sets only mt cyt b fragment of 298 bp gave species specific pattern for *P. pelagicus* (Table 4.2). Out of 17 sets, seven sets of primer were designed for *P. sanguinolentus* and among seven only mt 16S rRNA gave species specific pattern for *P. sanguinolentus* (Table 4.2).

### 5.3. Amplification of mt cytochrome b gene for *P. pelagicus*

Cytochrome b (cyt b) is one of the most important protein encoding genes on the heavy strand of the mt DNA molecule. It has sufficient inter-specific variation in the nucleotide sequence to allow for differentiation between closely related species (Infante et al., 2004). This gene has been frequently used in the identification of several crab species (Zhang et al., 2009; Eischeid et al., 2013; Suwannarat et al., 2017). It is used as a molecular marker in many applications to access intra or inter-species genetic diversity, genetic variation, phylogeography, species and hybrid identification, phylogeny in numerous species and genera,

population genetic structure, conservation, and demographic history information (Li et al., 2009; Ma et al., 2010; Thangaraj and Lipton, 2010). It is considered as a good marker to infer the impact of genetic differentiation among species within the same genus or the same family (Hsu et al., 2009).

The cytochrome b region of crab possessed molecular bases of ~1135 bp. In our study, the newly designed forward primer for *P. pelagicus* is 5'-CGTACTATGCATGCCAAT-3', located at the position of 241-258 bp and the reverse primer for *P. pelagicus* is 5'AAGAACCGTGTTAGTGTAG 3,' at the position of 521-539 bp (Fig. 2). This new primer amplified a target region of 298 bp in *P. pelagicus* without showing any non-specificity

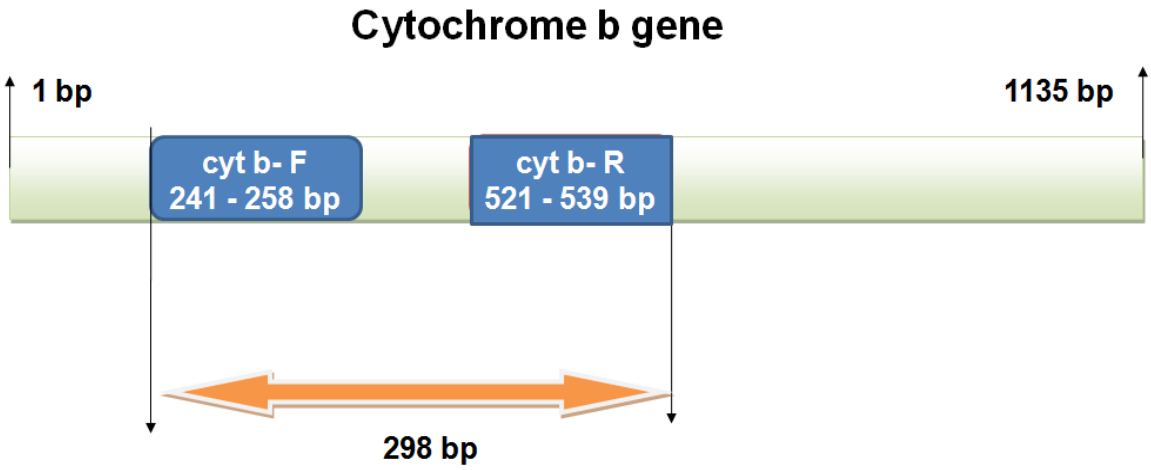


**Fig. 2.** Location and size of the DNA fragments of cyt b gene amplified and position of the designed primer set for *P. pelagicus*

### 5.4. Amplification of mt 16S rRNA gene for *P. sanguinolentus*

The mitochondrial 16S rRNA gene occupies 1/10 of the entire mitochondrial genome. It is used to study the evolutionary relationships of fishes at different taxonomic levels (Palumbi, 1996; Vences et al., 2005; Di Finizio et al., 2007). Several studies have employed partial sequences of the 16S rRNA gene

as standard markers to discriminate between groups of fishes and crabs (Ali et al., 2014; Sumathi et al., 2015; Zagon et al., 2017; Sivaraman et al., 2018; Wilwet et al., 2017).

The 16S rRNA region of crab possessed molecular bases of ~1500 bp. PCR method was developed for the identification of *P. sanguinoletus* by using the newly designed forward primer, 5'TCTACACTTGCACTGTTAC3', which is located at the position of 225-244 bp and the reverse primer, 5'AAAGCTCGTATGACATCTC 3', at 590-608 bp (Fig. 3). This new primer amplified a target region of 383 bp in *P. sanguinoletus* without showing any non-specificity.

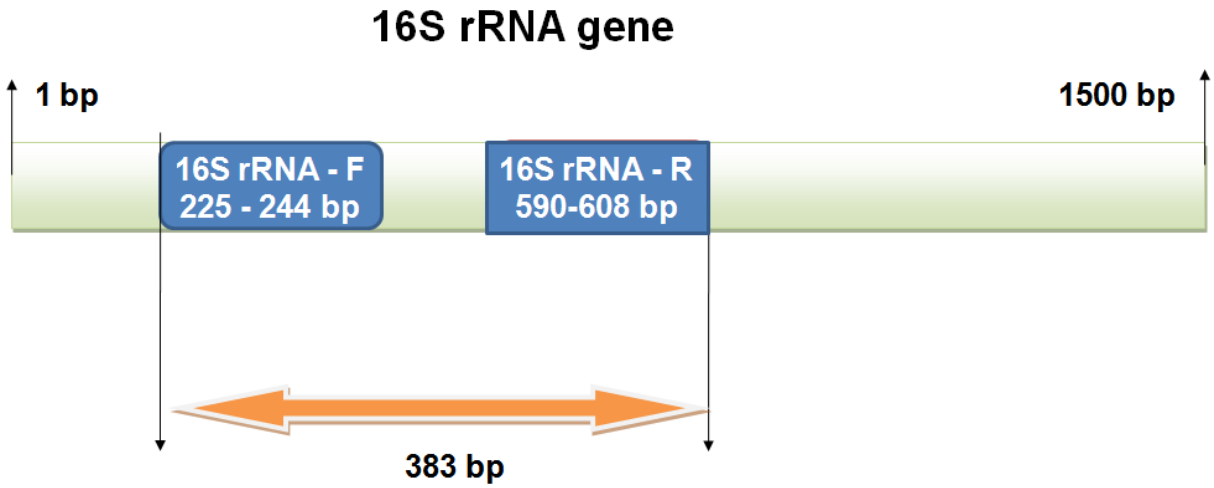


**Fig. 3.** Location and size of the DNA fragments of 16S rRNA gene amplified and position of the designed primer sets for *P. sanguinolentus*

### 5.5. MPCR assay for authentication of crab products

In MPCR assay, considerable time and effort can be saved by simultaneously amplifying multiple sequences in a single reaction (Markoulatos et al., 2002). In this assay, primers lead to amplification of unique regions of DNA, both in individual pairs and in combinations of many primers, under a single set of

reaction condition. Success results of a MPCR depend on the ability of the primers to be selectively annealed with their respective targets under a single reaction of PCR conditions (Rojas et al., 2010). Thus, it demands unique or specific primer design for multiple species and multiple primers for a single species. In fact, designing of species specific primer is the most crucial step to develop a multiplex PCR system. This is because of difficulties in optimizing melting, annealing, and elongation temperatures as well as preventing the formation of secondary structures and primer-dimers, which could affect the efficiency of MPCR. The MPCR assays have been earlier developed for the authentication of species, food and food products (Ali et al., 2014; Suwannarat et al., 2017).

A genomic DNA particularly mitochondrial genomic region has been used for the authentication studies. Though processing of seafood could damage DNA, the MPCR assay targeting appropriate fragments can be still effective on amplifying DNA from processed products. The PCR products from MPCR assay for species identification in processed seafood in earlier studies have been reported to be in the range of 100- 500 bp (Lin and Hwang, 2008; Catanese et al., 2010; Armani et al., 2012; Suwannarat et al., 2017). In this study, cyt b and 16S rRNA regions were chosen for the authentication of crab meat. PCR products in target species sample was amplified using the designed primer set for *P. pelagicus* and *P. sanguinolentus* at 298 bp and 383 bp, respectively (Table 4.2). The short product size allowed the designed primer set to be used on frozen, cooked and fried crab meat and without any alteration it showed similar pattern as that in fresh (raw) crab meat (Figs. 4.3 and 4.4). Hence, the developed MPCR assay can be

convenient to apply for food authentication by food regulatory authorities to protect the consumers against seafood fraud in crab processing industries.

### 5.6. MPCR for authentication of *P. pelagicus* targeting cyt b region and of *P. sanguinolentus* targeting 16S rRNA region

In mitochondrial genome, cyt b and 16S rRNA regions are widely used in crab authentication due to its high inter-and intra-species specific variation within closely related species (Suwannarat et al., 2017). Jain et al. (2007) successfully used a highly sensitive and reproducible multiplex PCR for detection of animal species in meat samples by using cyt b gene variability. It is a highly conserved region for DNA amplification in processed products. Armani et al. (2012) used 16S rRNA to provide a positive control of DNA amplification in processed products due to highly conserved region among other regions. In this study, MPCR assay was developed for detecting two crab species viz. *P. pelagicus* and *P. sanguinolentus* (Fig. 4.5) by targeting their cyt b and 16S rRNA genes, respectively. Both the species were simultaneously detected in the mixed meat using species specific primers in a single reaction with the product size of 298 bp and 383 bp, respectively. The processed crab meat viz., frozen, cooked and fried products were also checked for species authentication and found the MPCR patterns as that in raw crab meat (Fig. 4.6). This result clearly indicated that different processing methods did not affect the amplification of selected cyt b and 16S rRNA genes.

### 5.7. qPCR assay for quantification of adulterated crab meat

The qPCR assay does not require post-PCR sample handling, thus avoiding potential PCR carryover contamination and saving time (Cai et al., 2006). It can provide sensitive, species specific and quantitative detection. This

technique is especially suitable for heated products because small fragments of DNA can still be amplified and identified (Hird et al., 2005). It is proved to be a highly specific method among all other methods as it even detects minor amounts of DNA in products (Farouk et al., 2006). The qPCR assay is beneficial for the fair identification of fish in a fish product, where substitution is suspected (Prado et al., 2013). Zagon et al. (2017) developed six real time PCR assays for the crustacean species such as shrimp (*Crangon crangon*), lobster (*Homarus* sp.), river prawn (*Macrobrachium* sp.) and Chinese mitten crab (*Eriocheir sinensis*) based on mt 16S rRNA gene sequence with a universal annealing temperature of $60^0$ C. Upon validation, it was reported to have a sensitivity of 0.01-0.1 genome copies or 0.04 to 0.5 pg of genomic DNA. But, in our study, qPCR was developed for the quantification of mixed meat proportion in *P. pelagicus* meat and sensitivity was found better with 0.0003 pg to 0.024 ng of genomic DNA (Tables 4.3 and 4.5).

The threshold cycle (Ct) is the most important parameter of a real-time PCR system (Herrero et al., 2011), which is defined as the cycle at which fluorescence is first detected at a statistically significant level that is above the baseline or background signal (Heid et al.,1996). The Ct value is set above the amplification baseline within the exponential phase, which is inversely correlated to the logarithmic value of the initial copy number. An early detection is indicative of more copies of target DNA templates present in the sample (Ali et al., 2012; Mohamad et al., 2013). In PCR products, there is a chance of primer dimers, formation of primer dimers competes with formation of specific PCR product and leading to reduced amplification efficiency. To distinguish primer dimers from the specific amplicon, a melting curve analysis can be performed. The pure and homogeneous qPCR products produce a single, sharply defined melting curve

with a narrow peak (Pfaffl, 2004). Armani et al. (2012) analysed a melting curve for possibility of evaluating the specificity of the products by an automated post qPCR melting curve analysis, without gel electrophoresis. Trotta et al. (2005) also analyzed melting curve in their study. In our study also, a sharp defined melting curve with narrow peak was found and indicating there was no primer dimers in the amplicons (Figs. 4.8, 4.11 and 4.14). A SYBR Green based qPCR assay was developed to quantify the adulteration of *P. sanguinolentus* meat in *P. pelagicus* meat by individually targeting mt cyt b and mt 16S rRNA genes, respectively.

## 5.8. qPCR assay for quantifying the proportion of adulteration in *P. pelagicus*

Quantification methods in qPCR generally depend upon calibration curves to return accurate data (Hird et al., 2005). The qPCR data was used to generate linear calibration curves, and qPCR assays were evaluated with respect to linear range and reaction efficiency. Calibration curve allows the generation of highly specific, sensitive and reproducible data due to its highly reproducibility. The calibration curves used in absolute quantification can be based on known concentrations of DNA standard molecules. Eischeid et al. (2012) generated linear standard curves using 10 fold serial dilutions of target (shrimp or crab). The calibration curves drawn for each matrix in our study showed a good relationship between percentage of *P. sanguinolentus* meat added to *P. pelagicus* meat and Ct values returned (Figs. 4.9, 4.12 and 4.15). Hird et al. (2005) also found a similar relationship between the percentage of haddock added to cod and the Ct values returned. In our study, three calibration curves were generated to quantify the proportion of adulteration in *P. pelagicus* meat. First and second calibration curves were generated using 10 fold serial dilution of target DNA (*P. pelagicus*

and *P. sanguinolentus*) (Tables 4.3 and 4.4; Figs. 4.9 and 4.12), and the third calibration curve was generated by spiking different ratio of *P. sanguinolentus* meat in *P. pelagicus* meat (Table 4.5.; Fig. 4.15). The increasing DNA copy numbers in *P. sanguinolentus* and decreasing DNA copy numbers in *P. pelagicus* in first and second calibration curves indicated adulteration proportion. It is clearly stated that more the adulteration more the DNA copy numbers of adulterant and vice versa (Mohamad et al., 2013). The third calibration curve was used to detect the adulteration of mixed proportion of *P. sanguinolentus* meat in the *P. pelagicus* meat compared with the Ct values of calibration curve and unknown sample. The efficiency of the assay ranged between 90 and 99 % with $R^2$ = 0.995 and $R^2$=0.999 deduced from the respective first, and second and third regression curves, respectively (Figs. 4.9, 4.12 and 4.15). Hird et al. (2005) also developed a qPCR assay with efficiency from 90 to 99 % and R2 > 0.98 for the detection of adulteration of haddock with cod. According to European Network of GMO Laboratories (ENGL), the performance of the PCR system shall meet the minimum performance criteria as laid down for GMO detection methods demanding efficiencies between 90 and 110%, and $R^2$ > 0.98 (ENGL, 2011). The qPCR assay developed in this study for detecting the adulteration of crab meat also fulfilled the criteria suggested by ENGL.

## 5.9. Validation of developed MPCR and qPCR assays

In the nine samples obtained from crab processing industries and checked for the validation of the developed MPCR assay, the extracted DNA from four samples simultaneously amplified to obtain the 298 bp fragment of mt cyt b and 383 bp fragments of mt 16S rRNA regions (Figs. 4.16a and b). From the above results, it is clear that only four crab meat samples were adulterated by having the

meat from *P. pelagicus* and *P. sanguinolentus.* In the MPCR assay, the DNA sample of both the species were validated in the laboratory of Dept. of Animal Biotechnology, MVC, Chennai, and in the laboratory of Dept. of Fisheries Biotechnology, FC&RI, Thoothukudi (Figs. 4.17 and 4.18) and results are similar to the results obtained in our laboratory. The developed qPCR assay was validated in Molecular Biology Laboratory of Thermo Scientific, Bangalore and similar results were obtained. When the qPCR assay was validated with the unknown sample Ct value, it detected the adulteration of P*. sanguinolentus* meat with *P. pelagicus* meat even at a lower level of 38% (Table 4.9).

## VI. SUMMARY

Four commercially important crab species collected from fish landing center of Vellapatti and Fishing Harbor of Thoothukudi, Tamil Nadu were taxonomically identified by FAO species catalogue as *Portunus pelagicus* *Portunus sanguinolentus*, *Scylla serrata* and *Charybdis natator.* Authentication of crab species was made by developing MPCR method targeting the mt cyt b and mt 16S rRNA regions of *P. pelagicus* and *P. sanguinolentus*, respectively. Crabs subjected to freezing, cooking and frying were also examined to know whether the developed MPCR method had helped in authentication of processed products. Quantification of adulterated crab species was made by developing qPCR method targeting the mt cyt b and mt 16S rRNA regions of *P. pelagicus* and *P. sanguinolentus,* respectively. Calibration curve was constructed to know the proportion of adulteration in crab products. The salient findings of this study are summarized below:

- Total DNA was extracted from all the crab species as per Phenol-chloroform method. The method resulted in high yield of nucleic acids from fresh tissue (850 to 901 ng/µl) and high quality of extracted DNA, which was confirmed through spectrophotometric analysis. The DNA yields of frozen, cooked and fried crab species were slightly lower (687 to 823 ng/µl) than the raw crabs indicating partial damage of DNA occurred in the products when it was subjected to different technological treatments.
- Two sets of species specific primer were newly designed of mt cyt b region and mt 16S rRNA region of the crab species tested. The first set of primer mt cyt b targeting with product size of 298 bp was species specific for

*P. pelagicus* and successfully amplified in raw as well as in processed *P. pelagicus* without any alteration.

- The second set of primer targeting mt 16S rRNA gene with product size of 383 bp was species specific for *P. sanguinolentus* and successfully amplified in raw as well as in processed *P. sanguinolentus* without any alteration.
- The developed MPCR assay was found specific, and rapid in the identification of *P. pelagicus* and *P. sanguinolentus*, as it did not amplify in the other crab species belonging to family Portunidae crabs (*S. serrata* and *C. natator*). It amplified the correct species within 2h.
- The unique MPCR pattern was also obtained for processed crab products tested without any degradation or alteration in the major fragments.
- Since mt cyt b and 16S rRNA regions were found to contain more informative restricted sites that allow intra-species differentiation of crab species, the developed MPCR assay was more reliable in authenticating frozen, cooked and fried crab products.
- The developed MPCR assay was validated by analyzing the authenticity of commercial crab products of the five different crab processing industries. Totally nine 9 crab product samples were analyzed. Among that, five samples were authenticated as not having adulteration of *P. sanguinolentus* with *P. pelagicus* and remaining four having mixed meat of both the species.
- The developed SYBR Green based qPCR assay was found to quantify the proportion of adulteration in crab products. Calibration curve was

constructed to analyze the mixed meat percentage of *P. sanguinolentus* in *P. pelagicus* meat.

- Adulteration of *P. sanguinolentus* in *P. pelagicus* meat was quantified using mt cyt b region and the proportion of *P. sanguinolentus* meat mixed with *P. pelagicus* meat was quantified using mt 16S rRNA region. Calibration curve was used to compare Ct value of unknown sample with the standard Ct value to find out the mixed proportion of unknown sample in crab meat.
- The developed qPCR assay was sensitive and rapid, as it detected 0.0003 pg to 0.024 ng of genomic DNA within 150 min.
- The results indicated that the qPCR assay conducted targeting mt cyt b and mt 16S rRNA genes successfully quantified the adulteration of crab species in *P. pelagicus*. The qPCR assay detected the adulteration of *P. sanguinolentus* meat with *P. pelagicus* meat even at a lower level of 38%.
- The inter house laboratory validation of developed MPCR assay was done in the laboratories of TANUVAS, Chennai and TNJFU, Thoothukudi and qPCR assay was done in the Molecular Biology laboratory of Thermo Scientific, Bangalore and the results were similar to results obtained in our study.
- The developed MPCR and qPCR assays can be conveniently adopted by Food Regulatory Authorities to protect the consumer from fraudulent species substitution in the commercially important crab products.

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# VI. REFERENCES


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