Authentication of commercially available frozen shrimp meats using DNA barcoding

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ABSTRACT

Shrimps and prawns are quite nutritious and provide quality protein, omega-3 fatty acids, certain antioxidants, vitamins, and minerals like iodine. Therefore, they are healthy food for human consumption. In order to find out fish fraud in value-added products, in this study, six frozen shrimp species packed and sold by different commercial companies were purchased, correctly identified using DNA barcoding of mt-COI gene, and the sequences were authenticated properly with the GenBank. The genomic DNA isolated from frozen shrimp samples showed greater than 10 kb. Their amplified products showed 564–770 bp of mt-COI gene. The BLAST results for the labeled frozen shrimp sample *Litopenaeus vannamei* showed maximum similarity (99%–100%) with the same species sequences available with NCBI database. Five unlabeled sample sequences (two samples taken from two independent packets, and three samples taken from one mixed shrimp packet) showed maximum similarity with the sequences available in the NCBI database and exactly identified as *Fenneropenaeus merguiensis*, *Penaeus monodon*, *Fenneropenaeus indicus*, *Penaeus semisulcatus*, and *P. monodon*, respectively. These six sequences showed more variable amino acid sites than the identical and similar amino acid residues within themselves. The AT biases (61.5%–67.51%, *P. semisulcatus* and *P. monodon* taken from unlabeled mixed packet) and GC biases (32.9%–38.6%, *P. monodon* and *P. semisulcatus*) varied among these six species sequences, which indicated less NUMTs gene. The interspecies divergence rate calculated for these six species ranged between 0.434 and 3.401 (*L. vannamei* vs. *F. indicus* and *F. merguiensis* vs. *P. monodon*, taken from unlabeled independent packet). Thus, it is recommended that DNA barcoding of mt-COI gene can be used to identify any unlabeled or labeled shrimp/prawn/seafood available in the market to avoid fish fraud.

INTRODUCTION

Seafood including fish, prawn, shrimp, lobster, crayfish, crab, and clam has become a favorite nutritious delicacy for mankind [1]. They are a good source of complete protein and act as the building blocks necessary for the production of new proteins. Seafood products are frequently mislabeled and misrepresented food items. Mislabeling is sometimes by human error but mostly by intention. Usually, the superior valued species are substituted by substandard ones, which are causing serious health concern in addition to economic implications and financial risks on consumer [2–5]. According to OCEANA, 33% of seafood is mislabeled, which was revealed through DNA-based investigations [6–8]. The mislabeled seafood may be dangerous due to the presence of unknown toxic/allergenic substances. It may cause species loss if an endangered species is commercialized [9,10]. Under sliced, packed, and marketed stage, most of the prawns, shrimps, and fishes are similar in texture and it is very difficult to differentiate the right species without diagnosing either by body parts or DNA analysis.

There are several brands of frozen seafood available in India that are being exported. The majority of brands do not specify the species used. There is a mixing of species in packages. In such cases, for example, minced meat, swapping can be hidden as the seafood is highly processed. The nomenclature of commercially important seafood is not globally standardized; varied species are recognized by the same vernacular name, and a single species may be labeled with different names in different regions of the same country. Therefore, the Food and Drug Administration (FDA) of US (1988) and the EU (CE, 2065/2001) have stressed the scientific community and commercial industries for using adequate tools...
to confirm species authenticity, products labeling, and avoiding commercial fraud in seafood products labeling [11–16].

DNA barcoding is applied in biodiversity monitoring (taxonomy, ecology, and conservation) to identify organisms that lack distinctive morphological features due to homoplasy and phenotypic plasticity [17,18]. By using this technique, the illegal trading of protected and endangered wildlife species could also be checked [19,20]. It is also used to identify the species origin in commercially processed food [21]. This is being used as a forensic tool in food safety to check the labeling, substitutions, adulteration, genetically modified organisms, food contamination, and food poisoning [22–31]. It could also be used in identifying seafood that causes health issues in human [32].

India is one of the major exporters of seafood. Any fraudulent substitution in the export is considered as a threat to our economy. There is a possibility of mixing of low priced meat in the place of high priced one. The problem lies in the fact that mislabeled meat is sold at the same price as that of the original meat and the inferior quality meat can cause allergies. In addition to getting substandard foods, consumers are at risk of getting sick as well. The food processing industry must mention the scientific name of the species packed and marketed. In case of any forgery, DNA-based identification always provides a helping hand to identify the exact species. Therefore, in the present study, the frozen shrimp/prawn meat packets marketed by four different leading export companies were chosen to check for any mislabeling or fish fraud. Actually, the aim of the present study was to identify the exact shrimp/prawn species, which have been packed in these company products, by applying the DNA barcoding of mt-COI gene. In fact, this gene was sequenced and compared with sequences available in the NCBI database for similarity check. This method of identification is absolutely trustworthy and thus it is recommended for all food processing and aquaculture industries.

2. MATERIALS AND METHODS

2.1. Sample procurement

The frozen shrimp were obtained from the Nilgiri’s Super Market at R.S. Puram, Coimbatore, India, and a local marketing factory located at Saibaba Colony, Coimbatore, India (Plate 1). The samples belonged to four major food processing brands and exporters in India. They are Sumeru, Innovative Foods Limited, Cochin, India (Sample-A, unlabeled independent species packet), Buffet (Sample-B, unlabeled independent species packet), Cambay Tiger, Mumbai, India (Sample-C, labeled independent species packet), and Britte Seafood, Britto Seafood Exports Pvt. Ltd. (Samples D, E, and F unlabeled mixed-species packet). The sample pockets contained only small-sized prawn meat. The collected samples were stored in a deep freezer. They were subjected to molecular analysis.

2.2. Molecular analysis

2.2.1. DNA isolation

The shrimp meats were subjected to isolation of genomic DNA by adopting the method of Sambrook et al. [33]. Agarose gel electrophoresis (1% AGE) was performed and the genomic DNA was viewed under a gel documentation system (Medicare, India).

2.2.2. DNA amplification

Amplification of DNA of mt-COI gene was done using a Thermo Cycler (Applied Biosys, USA) with universal primers (LCO1490 & HCO2198) [34]. These primers have worked well with crustacean [35–39]. The amplification process is as given below: total volume, 100 µl (3 µl of DNA template; 1 µl of each primer; 25 µl of 2× PCR Master Mix (MBI Fermentas), and 70 µl of water. The PCR condition was as follows: pre-running (at 95°C for 8 minutes); denaturation (at 95°C for 35 cycles of 30 seconds each); annealing (at 57°C for 30 seconds); extension (at 72°C for 1 minute); and final extension (at 72°C for 8 minutes). 2% AGE was performed to resolve the amplified sequence of mt-COI gene.

2.2.3. DNA sequencing

Sequencing was done with the following conditions: the reaction mixture (20 µl total volume) containing template DNA (3 µl), primers (3.2 pM/µl of forward (0.50 µl) and reverse (0.50 µl), 5× big dye sequencing buffer (2 µl) and 2.5× ready reaction premix (4 µl, containing Tris-HCL, (pH, 9.0) and MgCl2) and DNase-RNase free water (10 µl). The PCR sequencing condition was as follows: pre-running at 96°C (25 cycles for 1 second each); denaturation at 96°C (25 cycles for 10 seconds each); annealing at 50°C (25 cycles for 5 seconds each); and elongation at 60°C (30 cycles of 4 minutes each). After completion of the PCR process, the samples were processed for ethanolic precipitation: the samples were transferred to 96-well microtiter plate, to each well 125 mM EDTA (Ethylene Diamine Tetra-Acetic Acid) (5 µl) and ice cold 100% ethanol (60 µl) stored at −20°C were added, the plate was sealed and mixed by vortexing for 20–30 seconds and incubated at room temperature for 15 minutes. The plate was spun at 3,000×g at 4°C for 30 minutes. The supernatant was carefully removed and again spun at 180×g for 1 minute. 60 µl ice cold 70% ethanol (stored at −20°C) was added to the pellet and centrifuged at 1,650×g at 4°C for 15 minutes. The plate was inverted again and spun up to 180×g for 1 minute. The samples were re-suspended in Hi-Di formamide (10 µl) and incubated at room temperature for 15 minutes. The re-suspended samples were transferred to the appropriate wells of the sample plate. The samples were denatured with snap chill at 95°C for 5 minutes and sequenced. The data were analyzed using ABI 3500 XL Genetic Analyzer (Chromous Biotech, Bangalore, India).

2.2.4. Sequence analysis

The sequences were aligned pairwise using CAP3. The similar sequences deposited in the NCBI database were retrieved, internal stop codons were removed through BLAST (Basic Local Alignment Search Tool). The reading frameshift within the sequence was identified and trimmed using ORF finder. The sequences were authenticated with GenBank (L. vannamei, MK792401; Fenneropenaeus merguiensis, MK792399; P. monodon, MK792400; Fenneropenaeus indicus, MK792402; Penaeus semisulcatus, MK792403; and P. monodon, MK792404). The other molecular analysis, such as multiple sequence alignment and highlighting of identical, similar, and variable sites of amino acids.
was done through the software T-Coffee and multiple align show (MAS), respectively. The phylogenetic information, AT and GC biases, nucleotide divergence (K2P model) [40], and reconstruction of the phylogenetic tree were performed using MEGA (Molecular Evolutionary Genetics Analysis) software (v.6.01).

3. RESULTS AND DISCUSSION

3.1. Genomic DNA and its amplification

The isolated genomic DNA from shrimp samples showed >10 kb nucleotides (Plate 2) and their respective PCR amplified products showed >500 bp length (Fig. 1). Actually, the aligned sequences have 546, 770, 770, 700, 630, and 552 bp for samples A, B, C, D, E, and F, respectively. These sequences showed 99%–100% similarity with the retrieved sequences (Table 1). The sample-A showed 99% similarity with *F. merguiensis* from Thailand, sample-B showed 100% similarity with *P. monodon* from Thailand, sample-C showed 98% similarity with *L. vannamei* from China, sample-D showed 100% similarity with *F. indicus* from Tanzania, sample-E showed 100% similarity with *P. semisulcatus* from Sri Lanka, and sample-F showed 100% similarity with *P. monodon* from India.

3.2. Amino acid residues

These sequences showed 112 identical, 53 similar, and 595 variable amino acids. Therefore, the variable amino acid sites were
higher than that of the identical and similar amino acid residues (Table 2 and Fig. 2). This indicated that these shrimp species are genetically discriminated.

3.3. Nucleotide composition

The base composition of mt-COI gene sequences varied among these species. Actually, AT bias varied from 61.5% to 67.51% (P. semisulcatus and P. monodon, respectively) and the GC bias varied from 32.9% to 38.6% (P. monodon and P. semisulcatus). In this study, the more AT bias observed suggests that lower abundance of nuclear copies in the mt-DNA of COI gene (NUMTs), known as a pseudogene (Table 3). Similar to the present study, the higher AT bias have also been reported in marine crabs, Portunus sanguinolentus, Charybdis natator, Portunus pelagicus, Portunus trituberculatus, and Travancoriana napaea [41]; freshwater crabs, Spiralothelphusa hydrodroma and Barytelphusa jacquemontii, and freshwater prawns, Macrobrachium rosenbergii, Macrobrachium malcolmsonii, Macrobrachium lamarrei, Macrobrachium lamarrei lamarroids, and Caridina gracilipes [36,37]; freshwater zooplankton, Asplanchna intermedia, Moina micrura,
Mesocyclops edax, and Cypris protubera [38,39]. The higher A+T content and lower G+C content have also been reported by Wang et al. [42] in Vitis vinifera.

3.4. Nucleotide divergence
The mean inter species divergence of these frozen shrimp samples were 1.709 with a maximum of 3.401 (between F. merguiensis and P. monodon) and a minimum of 0.434 (between L. vannamei and F. indicus (Table 4). However, the divergent value was >3% in the following two combinations, F. merguiensis (A) versus P. monodon (B) and P. monodon (B) versus L. vannamei (C), and the intraspecies divergence of P. monodon also showed >3% which may occur due to the geographical variation of the species.

3.5. Phylogenetic tree topology
The phylogenetic tree topology of frozen shrimp samples appeared with three clades. At the base, F. indicus formed a single distinct clade and P. semisulcatus also formed a single distinct clade. The rest of the four species, F. merguiensis, L. vannamei, P. monodon (taken from mixed packet), and P. monodon (taken from an independent packet) formed as a cluster in another clade (Fig. 3). Thus, the phylogenetic tree topology indicates clear discrimination between these frozen shrimp samples.

The Telegraph Calcutta reports that more than one-fifth of the seafood sold in markets and restaurants is mislabeled [43]. Mixing of fish species can lead to that with contaminants, toxins, and allergens to be consumed making the consumer sick [44]. The study stresses the importance of labeling marketed seafood, be it in whole meat form or deveined, de-shelled frozen form. The novelty of the work lies in the fact that DNA bar-coding is a suitable tool to exactly pinpoint the species that are marketed in case the manufacturers haven’t labeled the package or in times where we suspect fraudulent substitutions. The samples used in this study consisted of four major food processing brands, out of which only one brand had specified the shrimp that had been packaged (Sample C—L. vannamei). Samples A and B were found to be F. merguiensis and P. monodon, respectively, whereas the packaged frozen meat from Britte Seafood, Britto Seafood Exports Pvt. Ltd. consisted of mixed shrimps D—F. indicus, E—P. semisulcatus, F—P. monodon which was identified using DNA barcoding. These unlabeled packages that are sold in supermarkets with a high market value are sometimes of a fraudulent nature as shrimp meats are mixed. This mixing of species is often due to fraud or even human error. This has an impact on the international markets with meats of high value being substituted with the meat of low economic value [45]. DNA barcoding can be a suitable solution as the species used can be easily and accurately identified and the fraud averted. This study is novel as it combines a good and reliable technology and a common problem in the seafood industry and brings about an effective solution that can be employed. The food processing industries must compulsorily label their products in case if distinguishable parts have been removed and even in packages that are imported or sold in the local markets so that consumers are aware of what they eat.
Table 1: BLAST identification of COI gene sequences generated for frozen shrimp.

| Queried/subjected unknown shrimp sequences | Identity (%) | Gap (%) | Matched strand | Matched species | Reported country |
|------------------------------------------|-------------|--------|----------------|-----------------|-----------------|
| A                                        | 99          | 0      | Plus           | *F. mergiensis*  | Thailand        |
| B                                        | 100         | 0      | Plus           | *P. monodon*     | Thailand        |
| C                                        | 99          | 0      | Plus           | *L. vannamei*    | China           |
| D                                        | 100         | 0      | Plus           | *F. indicus*     | Tanzania        |
| E                                        | 98          | 0      | Plus           | *P. semisulcatus*| Sri Lanka       |
| F                                        | 100         | 0      | Plus           | *P. monodon*     | India           |

Table text continues with DNA sequences for each shrimp species.
Table 2: Number of identical, similar, and variable sites of amino acid residues in the COI gene sequences generated for different subjected frozen shrimp species.

| Species Name                  | Number identical amino acid residues | Number of similar amino acid residues | Number of variable amino acid sites |
|-------------------------------|--------------------------------------|---------------------------------------|------------------------------------|
| P. monodon                   | 112                                  | 53                                    | 595                                |

Figure 2: Multiple sequence alignment of COI gene sequences generated for subjected frozen shrimp species (A, *F. merguiensis*; B, *P. monodon*; C, *L. vannamei*; D, *F. indicus*; E, *P. semisulcatus*; F, *P. monodon*). An alignment is formatted by using multiple align show (MAS) with colored background and a consensus setting of 100%. Identical residues are indicated by amino acid color and similar residues are black in color. Gaps and other residues are given in white background.

Table 3: Nucleotide composition percentage of independent subjected frozen shrimp.

| Species Name | A   | T   | G   | C   | AT  | GC  |
|--------------|-----|-----|-----|-----|-----|-----|
| *F. merguiensis* | 28.8 | 35.7 | 16.5 | 19.0 | 64.5 | 35.5 |
| *P. monodon* | 26.2 | 38.7 | 17.4 | 17.7 | 64.9 | 35.1 |
| *L. vannamei* | 26.1 | 38.1 | 16.9 | 19.0 | 64.2 | 35.9 |
| *F. indicus* | 37.9 | 26.3 | 18.9 | 17.0 | 64.2 | 35.9 |
| *P. semisulcatus* | 27.5 | 34.0 | 18.1 | 20.5 | 61.5 | 38.6 |
| *P. monodon* | 28.8 | 38.3 | 16.8 | 16.1 | 67.1 | 32.9 |
| Average      | 29.2 | 35.2 | 17.5 | 18.2 | 64.4 | 35.69 |

A: Adenine, T: Thymine, G: Guanine, C: Cytosine.
4. CONCLUSION
This study concluded that the unknown shrimp species in frozen meat samples were clearly identified and discriminated through DNA barcoding of mt-COI gene, a tool for identifying the unknown seafood.

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