Universal primer design for crustacean and bivalve-mollusc authenticity based on cytochrome-\textit{b} gene

DWIYITNO DWIYITNO$^{1,2,*}$, STEFAN HOFFMAN$^3$, KOEN PARMENTIER$^1$, CHRIS VAN KEER$^2$

$^1$Research and Development Center for Marine and Fisheries Product Processing and Biotechnology. Jl. K.S. Tubun Pettamburan VI, Jakarta Pusat 10260, Jakarta, Indonesia. Tel./fax.: +62-21-53650157/58, *email: dwiyitno.brkp@kkp.go.id
$^2$KU Leuven Technologie Campus Gent. Gebroeders Desmetstraat 1, Gent 9000, Belgium
$^3$Institute for Agricultural and Fisheries Research (ILVO). Ankerstraat 1, Oostende 8400, Belgium

Abstract. Dwiyitno D, Hoffman S, Parmentier K, Keer CV. 2021. Universal primer design for crustacean and bivalve-mollusc authenticity based on cytochrome-\textit{b} gene. Biodiversitas 23: 17-24. Fish and seafood authenticity is important to support traceability practices and protect the public from economic fraud and adulteration. Molecular-based techniques of PCR are known as the most common methods for identifying seafood species. Nevertheless, these techniques rely on the appropriate primer set designed to amplify specific DNA fragments on targeted species. For efficiency application on a wide range of species, a universal primer set is more valuable than a specific primer. The present study developed universal primers, especially for identifying crustaceans and molluscs based on the cytochrome \textit{b} mitochondrial DNA (Cyt \textit{b}). The initial primer pair of CytBL1/CytBH originally designed for fish species was applicable to amplify the Cyt \textit{b} gene on most selected fish samples, but not for crustacean and mollusc samples. Based on annealing profile, sequence evaluation (92-100% similarity), and RT-PCR analysis, the universal primer couple of CytBL1/CytBHW designed in the present study potentially applied to identify crustacean and mollusc samples, especially shrimp and bivalve-mollusc.

Keywords: Authenticity, bivalve, molluscs, polymerase chain reaction, sequencing, RT-PCR, universal primer

INTRODUCTION

Fish and seafood authentication is important, especially when visual identification is difficult to distinguish the species. Species authenticity is also beneficial to prevent mislabeling of food and protect consumers from fraud and adulteration practices (EU Regulation 2013, Ceruso et al. 2020). Specifically, seafood authentication will prevent the public from the risk of food intoxication from toxic species as well as illegal trading of endangered/conserved species or Illegal Unreported Unregulated Fishing (IUUF) practices (Helyar et al. 2014; Cardenosa et al. 2018; But et al. 2020).

Protein separation by electrophoresis and nucleic acid-based methods are the two practical approaches for fish authenticity identification (Martinez and Friis 2004). However, molecular-based identification showed more effective throughput and has been globally accepted for detecting seafood species, including in foods products for quality control and authentication (Rasmussen Hellberg and Morrissey 2011, Horreo et al. 2013). The Fish Barcode of Life Initiative (BOLD), for example, has been developed to facilitate a global taxonomic identification database for animal species, including seafood (Becker et al. 2011).

DNA fragments of mitochondria have been widely used for species identification due to accuracy and sensitivity (Barth et al. 2017). Cyt \textit{b}, cytochrome oxidase subunit I (COI), and 16S ribosomal RNA (16SrRNA) genes are among the most targeted mitochondrial genes for species identification (Armani et al. 2011; Nicolè et al. 2012; Shokralla et al. 2015; Giusti et al. 2017; Zanzi and Martinsohn 2017). Fingerprinting techniques (such as RFLP, AFLP, SSCP, RAPD, DGGE), real-time PCR, and sequencing have been employed as alternative tools for evaluating PCR results. Regardless of the appropriate gene target, other factors are crucial for a successful identification based on the molecular method, such as DNA purity, reagent concentration and combination, and polymerase enzyme. The successfulness of species discrimination based on DNA approach including polymerase chain reaction (PCR) and sequencing remarkably rely on the primer couple specificity (Armani et al. 2016).

In order to overcome the limitation of molecular identification with specific primers for a wide range of seafood groups, a universal primer set could be an alternative (Miya et al. 2015; Kim et al. 2019). However, many universal primer couples fail to identify aquatic species (Giusti et al. 2017). This study aimed to design a universal primer couple based on Cyt \textit{b} gene, potentially applicable for crustacean and mollusc-bivalve. To date, there are no applicable universal primers for crustacean and bivalve species designed for Cyt \textit{b} gene, except that for mollusc as reported by Merritt et al. (1998). The Cyt \textit{b} gene shows a high variation of interspecies but low variation among species (Aranishi et al. 2005). This gene has also been successfully applied to identify processed fish species (Cutarelli et al. 2018).
MATERIAL AND METHODS

Primer design
At first, a typical specific primer couple for seafood identification based on Cyt b was evaluated for universal primer suitability. We chose the CytBL1/CytBH primer couple, which was designed to amplify the Cyt b of fish species (Céspedes et al. 1998). Since our test showed not optimal for crustaceans, mollusc, and some fishes, these primers were modified by introducing some wobbles (Merritt et al. 1998). Multiple alignment analysis was assessed to Cyt b genes of selected seafood samples from gene banks (https://www.ncbi.nlm.nih.gov/genbank/) or FishTrace (https://fishtrace.jrc.ec.europa.eu/) as drawn in Figure 1. The analysis was employed based on the pairwise alignment formula by BioEdit version 5.0.6 (https://bioedit.software.informer.com/5.0/).

We selected three candidates of forwarding primers and a reverse primer as presented in Table 1. Primer couple was designed with Oligo software following several primer design rules (Quellhorst and Rulli 2012). Table 1 summarizes the properties of the designed primers. Selected primer candidates were then synthesized, and the reliability of designed universal primers was confirmed to amplify the same region as the initial primers on selected seafood samples.

Table 1. Set of primers targeted for the amplification of seafood mitochondrial Cyt b gene

| Primer code   | Sequence (5’-3’)                  | Position* (bp) | GC (%) | Tm (°C) | Reference          |
|---------------|-----------------------------------|----------------|--------|---------|--------------------|
| CytBL1(F)     | CCATCCAACATCTCCAGCATGATAAGAA      | 115            | 42     | 74      | Céspedes (1998)    |
| CytBL1A(F)    | CGGCGWAAYATATGCGAACTTTG         | 115            | 37     | 66      | This study         |
| CytBL1B(F)    | CGGCGWAAYATATGCGAACTTTG         | 115            | 44     | 72      | Céspedes (1998)    |
| CytBL1C(F)    | CGGCGWAAYATATGCGAACTTTG         | 115            | 88     |         | This study         |
| CytBH (R)     | TGAAGGCAATAATCATTGCAAGG         | 115            | 44     | 74      | Céspedes (1998)    |
| CytBH1(F)     | CCATCCAACATCTCCAGCATGATAAGAA      | 115            | 42     | 74      | Céspedes (1998)    |
| CytBH2(F)     | CCATCCAACATCTCCAGCATGATAAGAA      | 115            | 42     | 74      | Céspedes (1998)    |
| CytBL1A(R)    | CGGCGWAAYATATGCGAACTTTG         | 115            | 37     | 66      | This study         |
| CytBL1B(R)    | CGGCGWAAYATATGCGAACTTTG         | 115            | 44     | 72      | This study         |
| CytBL1C(R)    | CGGCGWAAYATATGCGAACTTTG         | 115            | 88     |         | This study         |
| CytBH (R)     | TGAAGGCAATAATCATTGCAAGG         | 115            | 44     | 74      | Céspedes (1998)    |

Note: *Based on G. morhua (Fishtrace); Wobble: Y: T/C; R: G/A; W: A/T (Merritt et al. 1998)
Primer evaluation on seafood samples

Selected seafood samples for primer evaluation were provided from the North Sea and selected fish markets in Belgium. 5-10 individual fresh samples were pre-identified through morphological features. They included 14 fish species, including rounded fish, flatfish, and smelt fish (sand lances). The evaluation also involved seven crustaceans and six molluscs. The species detail is explained in Table 2. Prior to DNA extraction, samples were cleaned and filleted.

Total DNA was extracted using Wizard Promega purification kit (Promega Corporation 2019). The DNA quality was measured by NanoDrop spectrophotometry (ND-1000) to calculate the absorbance ratio at 260 and 280 nm according to the manufacturer's instruction (Thermo Scientific 2019). A ratio of A260/280 1.80-2.00 is considered pure DNA (Brescia and Banks 2012) and used for further PCR assay.

PCR assay and direct sequencing

The reliability of the designed primers to amplify the ~357bp of the Cyt b gene was employed by PCR assay. The reaction was performed in a mix composed of 10µL Jump Start RED Taq Ready Mix (Sigma P-0982) containing 10mM Tris-HCl pH 8.3, 50mM KCl, 2mM MgCl₂, 0.2mM of each dNTP, and 0.03U/µL Taq DNA polymerase. Two µL of each primer (10mM) and 5-20 ng/20µL of DNA templates were added to the mix.

Amplification was carried out in a PCR-express thermal cycler (Hybaid) at the condition of five min initiation at 94°C, 35 cycles of amplification at 94°C for 30 sec; 50°C for 30 sec; 72°C for 1 min and five min final extension at 72°C (Richardson et al. 2007). Amplified PCR products were analyzed by agarose gel electrophoresis 2.0% (w/v). The amplification reaction was performed at least in triplicates.

Direct sequencing was performed on the optimal DNA fragments in order to ensure the designed primers able to amplify the targeted DNA fragment. Sequence analysis was carried out in an ABI 3730xl DNA analyzer. Sequence chromatograms were viewed and evaluated by using ChromasPro and BioEdit software. The selected sequences were then assembled to analyze the overlapping bases (Tamura et al. 2004).

Real-Time PCR application

The reliability of designed primers for real-time PCR (RT-PCR) was also evaluated on selected crustacean and mollusc samples using QuantiTech SYBR Green RT-PCR Master Mix. PCR reaction was performed in triplicate of DNA samples in MicroAmp Optical 96-well reaction plates (Roche). The RT-PCR amplification was carried out in a LightCycler® 480 (Roche Diagnostics 2006) with the condition of initial activation at 95°C for 10 min; 40-50 cycles at 95°C for 10 sec, and 45-60°C annealing for 20 sec; and 72°C elongation for 30 sec. The melting points (T_m) generated from the melting curve (60-90°C) were used to discriminate between closely related species.

Table 2. Detail of fish, crustacean, and mollusc samples

| Common name              | Scientific name               |
|--------------------------|-------------------------------|
| Fish                     |                               |
| Cod                      | Gadus morhua                 |
| Fanea                    | Trisopterus luscus            |
| Grey gurnard             | Eutrigla gurnardus            |
| Common sole              | Solea solea                  |
| Lemon sole               | Microstomus kitt             |
| Tarbot                   | Scopthalmus maximus          |
| Smooth skate             | Malacorpus senta             |
| Thornback ray            | Raja clavata                 |
| Tuna                     | Thunnus sp.                  |
| Small sandeel            | Ammododites tobianus         |
| Great sandeel            | Hyperolus lanceolatus        |
| Unknown smelt            |                               |
| Crustacean               |                               |
| Giant tiger shrimp       | Peneaus monodon              |
| King shrimp              | Peneaus latiuscutus          |
| Brown shrimp             | Crangon crangon              |
| Giant freshwater shrimp  | Macrobrachium rosenbergii    |
| Mollusc                  |                               |
| Green mussel             | Perna. canalicus             |
| Blue mussel              | Mytilus edulis               |
| Illex squid              | Illex argentinus             |
| Unknown mussel           |                               |

Data analysis

The primer couple's reliability was evaluated based on the annealing profiles, showed by a strong band of targeted Cyt b fragment, the variation of annealing temperature, and the similarity index of sequencing analysis. The obtained sequences were identified utilizing the Basic Local Alignment Search Tool/BLAST (www.ncbi.nlm.nih.gov/blast). The similarity was compared to the references/libraries obtained via NCBI Genbank.

RESULTS AND DISCUSSION

PCR amplification

The initial primer (CytBL1/CytBH) was employed previously for the identification of tuna species and for flatfish (Céspedes et al. 1998). In the present study, this primer set was suitable to amplify the Cyt b fragment of flatfish (T. solea) and rounded fish (T. luscus) and M. edulis. On the other hand, multiple fragments were found on H. lanceolatus. A. tobianus, and S. maximus (Figure 2). Further PCR evaluation on crustacean and mollusc samples showed that the amplification was not optimum, especially on P. monodon. Solenocera sp., C. crangon, and M. edulis (Figure 3).

The present study revealed that the initial primer of CytBL1/CytBH couple was not optimal to amplify crustacean and mollusc species. Intense bands of low weight fragments (less than 100 bp) are seen on the electrophoresis gel indicated non-optimal amplification on those species (Figure 3). It can result from residues of primers, primer dimers, DNA templates, or nucleotides due to inappropriate PCR amplification (Mubarak et al. 2020).
In order to increase the annealing efficiency of CytBL1 and CytBH primers on various groups of species, some wobbles were introduced on three forward primers (CytBL1A, CytBL1B, and CytBL1C) and a reverse one (CytBHW). CytBL1A is a base shorter than the initial primer (CytBL1) with wobbles modification on six bases; CytBL1B is a base longer with eight wobble modifications, while the CytBL1C is six bases longer than CytBL1 and consists of 7 wobbles. The forward primer CytBHW has the same length as the initial primer (CytBH) with a modification of six wobbles (Table 1). To reduce the possibility of misbinding, we only use double wobble, i.e. Y(T/C), R(G/A), and W(A/T).

![Figure 2](image1.png)

**Figure 2.** Amplification result of CytBL1/CytBH primers on fish samples. Ote: 1-3: S. solea; 4-6: T. luscus; 7: Thunnus sp.; 8-10: G. morhua; 11: R. lanceolatus; 12: A. tobianus; 13-15: S. maximus; 16-17: M. kitt; 18-19: E. gurnardus; 20-21: M. senta; 22-23: R. clavata; M: DNA marker

![Figure 3](image2.png)

**Figure 3.** Amplification result of CytBL1/CytBH primers on crustaceans and molluscs. Note: 1: P. monodon; 2: Solenocera sp.; 3-4: P. laitisulcatus; 5-7: C. crangon; 8-10: M. edulis; 11-12: Perna canaliculus; 13-14: Illex argentinus; M: DNA marker

![Figure 4](image3.png)

**Figure 4.** Amplification result of the degenerate primers on selected samples. Note: 1: C. crangon; 2: M. edulis; 3: M. kitt; 4: T. luscus; M: DNA ladder 100bp; B: Blank

Evaluation of designed primers (CytBL1C and CytBHW) on selected samples (C. crangon, M. edulis, M. kitt, and T. luscus) showed that a combination of these primers showed the most optimum result, mainly on *M. edulis* (Figure 4). However, further evaluation on the different fish species showed this primer couple failed to produce a single band on the samples, except *S. maximus*. Reducing a base on CytBL1A may reduce the primer specificity, while the double wobbles (RR) may also produce a lower priming efficiency on CytBL1B. The relatively low GC content (<40%) may also contribute to reducing the priming efficiency of CytBL1A and CytBL1B (Mallona et al. 2011).

The further evaluation of CytBL1C and CytBHW showed the primer set effectively amplified crustacean samples of *P. monodon, P. laitisulcatus, Solenocera sp.*, and *M. rosenbergii* (Figure 4). A similar result was performed on mollusk samples of *M. edulis, Perna canaliculus*, and unknown mussel samples. Not only was it performed by a single strong band on electrophoresis result, but a relatively wide range of annealing temperature (45-60°C) indicates that the designed primer is effective for targeted species.
However, the primer set produced a smeared band on C. crangon, suggesting the amplification was not optimal for this particular species (Lorenz 2012; Mubarak et al. 2020).

Sequencing evaluation
The sequencing analysis on selected samples was also attempted to evaluate primer effectiveness for species identification by assessing the similarity index compared to the online references. Sequence results proved that the designed primer couple effectively amplified the desired DNA fragment of 356-358 bp of Cyt b region. Further BLAST analysis showed various similarity indexes between 92% (M. rosenbergii) and 100% (L. vannamei and M. edulis) as presented in Table 3. Pearson (2013) suggested that more than 80% of identity is accepted as significant similarity in sequence analysis.

Identities between 98 and 100% were obtained from BLAST analysis, indicating the designed primers produced species-specific specific discrimination to differentiate between selected crustaceans and mollusc samples. However, BLAST analysis of M. rosenbergii showed a relatively low similarity (92-93%) to NCBI database. This result could be associated with the fact that genus Macrobrachium has shown high genetic divergence, especially between eastern and western species of Indo-Pacific origin (Ng and Wowor 2011). Another reason might relate to the limitation of the available reference as a comparison. So far, there is no available reference of partial Cyt b gene either from NCBI or other sources. The only available reference is a complete mitochondrial genome from NCBI (Li et al. 2019). Therefore, the present study's sequence is the first reported partial Cyt b gene of M. rosenbergii.

BLAST analysis on unknown mussels identified similarly (100%) as M. edulis and M. galloprovincialis. This double identity could be explained that M. edulis and M. galloprovincialis are known as closely related taxa due to either hybridization within the species or doubly uniparental inheritance (Skibinski et al. 1994). This phenomenon results in heteroplasmy in sea mussels that hamper their interspecific divergences (Hilbish et al. 2000). This result indicates the designed primers could identify the variation of closely related species.

Sequencing test of Thunnus sp. sample with CytBL1/CytBH primer produced a 99% similarity as T. albacares based on NCBI databank (Table 3). Additionally, BLAST analysis of the CytBL1C/CytBHW primer set was able to differentiate between two unknown shrimps, i.e. the shrimp-1 was identified as M. rosenbergii (92%), and the shrimp-2 was identified as L. vannamei (100%) in comparison to the gene bank of NCBI. Meanwhile, all unknown mussels were identified with the same primers as M. edulis, whereas the mussel-2 was also recognized as M. galloprovincialis.

Real-Time PCR analysis
RT-PCR analysis was carried out to discriminate between different species based on their melting temperatures (T_m). Figure 5A-B shows that RT-PCR was applicable to differentiate selected crustacean and mollusc species. RT-PCR with CytBL1C/CytBHW primer set could differentiate two crustaceans (P. monodon and P. latisulcatus), and two mussels (M. edulis and Perna canaliculus). In the present study, we evaluated the initial CytBL/CytBH primers for the RT-PCR amplification of fish species between G. morhua and Thunnus sp., as presented in Figure 5C.

The RT-PCR quantitation cycle (C_q) was achieved between 20-30 cycles out of 50 cycles, except for crustacean (30-40 cycles). The melting temperature (T_m) variation among different species was between 82.48 and 86.37°C for selected fish samples, while for crustacean and mollusc were 78.71-83.54°C (Table 4). A different Tm of shrimp species was reported by Sharma et al. (2020) on an HRM RT-PCR study with SYBR Green-I dye i.e. between 71 and 76°C. Nevertheless, GC content of 3 samples (M. senta, Perna canaliculus, and P. latisulcatus) could not be evaluated as the sequencing analysis was not conducted on these samples.

Table 3. Sequencing analysis of selected samples

| Sample         | Primer couple | Fragment length (bp) | Library identity | Similarity (%) | GenBank number  |
|----------------|---------------|----------------------|------------------|----------------|----------------|
| Fish Thunnus sp. | CytBL1/CytBH | 357                  | T. albacares     | 99             | NCBI_EU250986.1 |
| Crustacean     |               |                      |                  |                |                |
| P. monodon     | CytBL1C/CytBHW | 358                 | P. monodon       | 98             | NCBI_EU069440.1 |
| M. rosenbergii | CytBL1C/CytBHW | 358                 | M. rosenbergii   | 93             | NCBI_AY659990.1 |
| Shrimp-1       | CytBL1C/CytBHW | 358                 | M. rosenbergii   | 92             | NCBI_AY659990.1 |
| Shrimp-2       | CytBL1C/CytBHW | 358                 | L. vannamei      | 100            | NCBI_EF584003.1 |
| Mollusc        |               |                      |                  |                |                |
| M. edulis      | CytBL1C/CytBHW | 358                 | M. edulis        | 100            | NCBI_AY484747.1 |
| Mussel-1       | CytBL1C/CytBHW | 358                 | M. edulis        | 100            | NCBI_AY484747.1 |
| Mussel-2       | CytBL1C/CytBHW | 358                 | M. edulis        | 100            | NCBI_AY484747.1 |
| Mussel-3       | CytBL1C/CytBHW | 358                 | M. edulis        | 100            | NCBI_AY484747.1 |
RT-PCR analysis showed that the designed primers (CytBL1/CytBHW) successfully differentiate the selected crustacean and mussel samples. A similar result was performed on the initial primer of CytBL1/CytBH on selected fish species i.e., between *G. morhua* and *Thunnus* sp., and between *R. clavata* and *M. senta* as performed by significant different of melting temperature profile (Figure 5). The melting point difference of approx. 1°C is sufficient to discriminate among different species. A single peak of each melting curve indicates the applicability of the designed primers for RT-PCR analysis and successfully amplified a single targeted DNA fragment (Taylor et al. 2017). The fact of annealing efficiency was also indicated by the Cq value between 20-30 cycles. This result is comparable to other studies on RT-PCR assay of penaeid shrimps with a Cq value of 17-30 (Sharma et al. 2020). In general, Tm profiles of selected samples (Table 4) correspond to their "GC" contents, as the more GC, the higher the melting temperature is (Bonab et al. 2015; Mubarak et al. 2020). RT-PCR has many advantages for species identification, such as higher sensitivity and specificity, rapid analysis, and direct identification compared to conventional PCR. However, only limited studies reported RT-PCR application for seafood identification, mainly based on the mitochondrial gene. RT-PCR has been applied for fish authentication on 16S rRNA of tuna species (Liu et al. 2016), salmon and trout (Feng et al. 2017), penaeid shrimp (Sharma et al. 2020), as well as European sole on ITS-1 fragment (Herrero et al. 2012). Therefore, the present study would be the first RT-PCR application on crustacean and mollusc identification based on Cyt b gene.

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