Mini DNA-barcode as molecular marker for heavily processed hairtail fish products authentication

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Abstract. Fish species mislabeling, wrongful description, and substitution by species with a lower value are problems that occur due to globalization of fish markets. Establishment of fishery products and seafood species identification methods becomes an important task for Indonesian fishery research laboratories and seafood control authorities. Therefore, various DNA barcode markers must be tested to determine which markers are suitable for rapid, simple, and applicable methods. This study aimed to determine the reliability of full-length DNA barcode and mini DNA-barcodes for raw fillets and numerous cooked Indonesian hairtails. The results showed all DNA barcode markers were successfully applied to all of the samples with the species level assignment of 97-100%. Our study proposed a practical molecular marker for the monitoring of quality and food safety assurance of important hairtail products.

Keywords: COI, DNA mini-barcode, hairtail, processed food, Trichiurus

1. Introduction

Hairtail fishery is an important commodity with high economic value as an export commodity. The export value trend of hairtail fishery increased yearly. In 2014 the value of hairtail exports experienced an increase of 128% which was recorded from US $12,053 to US $27,517. The main export destinations for Indonesian hairtail fishery are Vietnam, China, Korea, Malaysia and Hong Kong [1]. Most of the hairtails exported were in the form of frozen fish (fillet or headless). Hairtail fish found in Indonesian waters are quite diverse, namely Benthodesmus tenuis, Benthodesmus tuckeri, Eupleurogrammus glossodon, Eupleurogrammus muticus, Lepturacanthus savala, Trichiurus auriga, and T. lepturus [2]. Trichiurus sp. is a common fish species used for export trading. The exported products of the hairtails are not only in the form of fresh fish, but also processed fish products in order to increase their selling value.

The consumers interest of hairtail products may raise the possibility of cheating which is done by producers, known as seafood fraud [3]. Food fraud usually found is the use of substituted fish species. Fish species substitution is when fish used is different from the types of fish that are mentioned on the label [4]. Mislabeling and fraud has been detected in 50% of fish products in Germany [5], 24% of seafood in southern Brazil [6], 22% of seafood in India [7] and 82% of commercial fish fillets in Italy [8]. Consumers will experience losses such as more expensive prices for cheaper substituted fish species and health problems.
Efforts that can be done to overcome seafood fraud are applying species authentication [9]. Authentication of food products can be done using various methods, one of which is a method based on protein or DNA analysis. DNA approach method or known as DNA barcoding gives accurate, stable and specific species identification results. DNA barcoding is the basic principle for determining nucleotide sequences of short DNA segments from target specimens and compared with online repositories for species identification [10].

Fragments of 650 bp from the tip of the mitochondrial cytochrome oxidase subunit I (COI) gene have been recommended to develop a uniform animal species identification method or have close kinship [10-12]. Many previous researches had used COI or cyt b mitochondrial DNA barcoding so far to identify seafood products and investigate the problem of fish mislabeling [8,11,13]. Identification with DNA full length has a success rate of 20.5%, whereas with DNA mini-barcodes has a greater level [14]. Fishery products that have undergone processing in addition to experiencing morphological changes also contain additional substances that can affect the DNA produced. Therefore, the aim of this study was to authenticate hairtail fish using a mini-DNA barcoding with shorter DNA fragments such as 200-300 bp as an alternative to processed products that have undergone changes in the quality and quantity of DNA.

2. Materials and Methods

2.1. Fish sample preparation
Sample preparation consists of two processes: gutting and washing of the fish. Hairtail fish is cut into fillet form with a knife that has been given alcohol. The next stage is the process of hairtail fish fillets (sample code L1-L5) consisting of smoking (LA), broiled (LB), sterilization (LC), frying (LD), and baking (LE). The raw materials used for processed products LA, LB, LC, LD, and LD are respectively L1, L2, L3, L4, and L5. Smoking uses liquid smoke with a long soaking sample with liquid smoke (1:3) for 15 minutes then put it in the oven at 140°C for 30 minutes. Burning over the stove at a temperature of 100°C for 3-5 minutes. The sterilization process uses autoclaves with a temperature of 121°C and a pressure of 1 atm for 45 minutes. Palm oil and Teflon are used to fry the samples with a temperature of 175°C for 2-3 minutes, and baking with an oven at 150°C for 10 minutes.

2.2. Isolation of fresh sample DNA
Isolation of DNA samples of fresh fish (SA1-SA5 and SM1-SM5) were performed using muscle tissue. The initial stage is sample thawing. The next step is weighing 0.0125 g of sample meat, then mashing using a mortar. The crushed samples were then put into micro tubes and were isolated by commercial KIT TIANamp Genomic DNA KIT [15].

2.3. Isolation of processed sample DNA
DNA isolation for processed fish using a sample of muscle tissue as much as 0.2 g. The sample was mashed using a mortar and then put into a micro tube. The next stage of the sample was isolated using a commercial KIT Qiagen Dneasy Food Mericon [16].

2.4. DNA amplification
DNA amplification in the COI gene segment was carried out using PCR (Polymerase Chain Reaction) technique using the FishF1R1 universal primer for fresh samples. PCR temperature conditions were consecutive, i.e. pre-denaturation at 95°C for 3 minutes, denaturation of 95°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 30 seconds, followed by the final extension at 72°C for 5 minutes. Processed samples were amplified using mini-F mini-R primers with annealing temperatures respectively 59°C [17].

2.5. Sequences data analysis
The obtained sequences were aligned using ClustalW integrated in MEGA version 6 [18]. Moreover, all of the sequences were compared to sequences available in databases by BLAST analysis (GenBank/NCBI). Sequence similarity of at least 98% was used as the threshold to determine the
potential species identification [19,20]. The genetic distances among sequences were calculated using the Kimura 2-parameter model [21] and the construction of the phylogenetic tree was carried out in Mega 6.0 [18] with the Neighbor-joining (NJ) method using 2,000 bootstrap replications.

3. Results and Discussion

DNA amplification in this study uses two primers, namely the full length COI primer and the mini-barcodes COI primer. Visualization of PCR products was carried out using a UV-Transilluminator. The reaction in gel electrophoresis consists of breaking the molecule into fragments that will be dispersed on an agarose gel medium which has been applied to the electric field. Electricity and different molecular weights in fragments cause a transfer to different places through the gel until the process stops [22]. DNA amplification in hairtail fish samples can be seen in figure 1.

![Electropherogram of full length DNA barcodes and mini-DNA barcode from hairtail fish.](image)

**Figure 1.** Electropherogram of full length DNA barcodes and mini-DNA barcode from hairtail fish.

PCR electropherogram shows that all samples have been amplified well in the range of 500-700 bp which is indicated by the appearance of the target band and can be seen in Figure 1a. The range is in accordance with COI’s primer target of 650 pb. Amplification of processed samples of hairtail fish (LA-LE) using specific fish hairtail primers (*T. lepturus*) and FishF1R1. Primer pairs of mini-barcodes show higher amplification capabilities than full length primer pairs (FishF1R1).

The DNA of hairtail fish was detected using a universal primer fish and was successfully amplified. The success of target amplification is largely determined by the primer conditions, namely the primer attachment of the sample genome DNA used [23]. Single DNA bands have shown that the primer pairs used are specific and attached to the expected position (annealing conditions used) [24]. This is consistent with the results of amplification using specific primers of hairtail fish which have a single DNA band.

Sequencing results demonstrate all samples have homology level that ranged from 91-100%. The level of similarity of the sequence which is used as the threshold for identifying potential species is at least 98% [19, 20]. The similarity percentage of sequences stated with the database sequence of 97% to 100% is said to be significant, 92% to 96% is enough, and less than 91% is not significant that the sequence is a species in the GenBank database [25]. All samples were identified as *T. lepturus*.

Measurement of genetic distance of a DNA sequence will be compared to one nucleotide with other nucleotides. The genetic distance can be calculated from the number of differences in polymorphic bases of a gene locus in each population based on DNA sequences [26]. The results of genetic distance analysis showed that genetic distance between samples identified as fish hairtail ranged from 0.000 to 0.177.

The lowest genetic distance among all samples is L1, L2, and LC (0.000) with *T. lepturus* and the highest genetic distance is at 1 (0.177) with *Benthodesmus tenuis*. The lower the genetic distance between the two organisms, the closer the kinship relationship is [27]. *T. lepturus* has a closer relationship with *T. auriga* compared to *T. japonicus*. The genetic distance of *T. lepturus* with two species of hairtail fish are 9.9% and 11.0% respectively. The relationship between *Lepturacanthus*
saval and T. Japonicus is closer, while with T. lepturus is not. This can be seen in the genetic distance with T. japonicus which is 11.0% and T. lepturus by 12.7%.

Phylogenetics is a method used to determine the kinship between taxa, for example molecular phylogenetics that utilize nucleotide sequence data to regulate kinship relationships in evolutionary structures in taxa [28-30]. Hairtail fish sequences are equated with various other hairtail fish species or in groups and Portunus trituberculatus and Holothuria leucosplota species as out groups. Group out groups are needed to provide polarization of characters or characteristics, namely apomorphic and plesiomorphic characters [31]. Phylogenetic tree construction uses the Neighbor Joining Tree (NJT) method with 1000 bootstrap values and two parameter Kimura models.

A bootstrap value of 1,000 is a possibility in making a tree with 1,000 repetitions [32] and shows the closeness of homology [18]. Groups and branches on the phylogeny tree supported by bootstrap values determine the stability of group formation [33]. Fresh samples of L1 and L2 form groups with T. lepturus. The T. auriga group, T. japonicus with L6 and L7, while the Benthodesmus tenuis species are separated from the other genera of hairtail fish. The highest bootstrap value in the COI (full length) phylogeny tree is 100% in the L6, L7, T. auriga, T. japonicus, and L. savala groups. L2 has a bootstrap value of 97% and L1 is 99%.

The phylogeny tree COI (Mini DNA-barcodes) visualizes that the highest bootstrap value is found in T. japonicus which is 81%. Samples of LC and LD formed groups with T. lepturus, T. auriga, and Trichiurus sp. with a bootstrap value of 65%. Phylogenetic tree construction based on the full-length COI gene has a higher stability compared to COI mini DNA-barcodes. This is based on the bootstrap value of the two phylogenetic trees. The COI (full length) bootstrap value is 46-100% and the bootstrap value in the COI (Mini DNA-barcoding) is 7-81%. Bootstrap analysis with a value of 70% or more indicates a reliable grouping [34]. Bootstrap values above 95% indicate a stable branch and a branch is said to be unstable if the bootstrap value is below 70% [35].

4. Conclusion

Mini-Barcode primers can be used as molecular markers for processed fish products with a target of 295 bp in specific primers. Authentication in other hairtail fish processed products, namely high fat content, salt, and emulsions using DNA mini-barcodes molecular markers needs to be done and can be applied for strengthening traceability of fishery products. Subsequent research needs to be done on hairtail fish with the development of molecular markers based on Single Nucleotide Polymorphism (SNPs) without sequencing.

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