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DNA Barcoding Application Using CYTB (Cytochrome-B) Marks for Authentication of Patin Fish (*Pangasius* sp.) Products in Surabaya Area

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ABSTRACT

DNA barcoding is a practical and accurate molecular technique for identifying processed fish products that lack morphological characteristics. However, its application is limited by the availability of reference databases, DNA quality, and primer suitability. This study aims to determine the genetic identity of pangasius catfish (Pangasius sp.) marketed in Surabaya using the Cytochrome b (CytB) gene and to evaluate PCR-RFLP as an alternative to sequencing. A total of twenty samples (fresh and frozen fillets) were analyzed through DNA extraction and PCR amplification of the CytB gene, followed by in silico and in vitro analyses.The results show that CytB demonstrates high amplification success and provides sufficient resolution for species-level identification based on Neighbor-Joining analysis with the Kimura 2-parameter model. The study confirms that the samples were genetically identified as *Pangasius pangasius* and *Pangasius hypophthalmus*. This research highlights the novelty of integrating in silico prediction with laboratory validation to improve marker evaluation in DNA barcoding studies. Furthermore, it demonstrates that CytB-based PCR-RFLP is a rapid and cost-effective alternative to sequencing for detecting species mislabeling. Overall, the CytB gene is proven to be a reliable molecular marker for species authentication, supporting traceability systems and strengthening seafood authenticity monitoring in commercial markets.

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INTRODUCTION

Indonesia is an archipelagic country with a total marine area of approximately 6.32 million km² and a coastline of about 81,000 km, making it one of the countries with the highest fisheries potential in the world. Indonesia hosts around 8,500 fish species, representing approximately 37% of global fish biodiversity (Handayani & Murniati, 2020). Fish and fish-based products are major sources of animal protein widely consumed due to their relatively low cost compared to other animal protein sources. Among processed fish products, fish fillets have high commercial potential because they are boneless, easy to process, and can be stored for longer periods (FAO, 2022; Gephart et al., 2021). Striped catfish (Pangasius sp.) is a freshwater species that is highly favored due to its affordable price and wide utilization in processed products. This species is easy to cultivate, does not require flowing water, and exhibits rapid growth, reaching 35-40 cm in length within six months. Morphologically, Pangasius sp. has a forked caudal fin, symmetrical fins, a dorsal spine with serrated edges, no scales, and a silvery-white body with a bluish dorsal surface (Hassan et al., 2022).

Processed fish products generally have higher economic value and greater consumer acceptance; however, they are also vulnerable to product adulteration and mislabeling (Jacquet & Pauly, 2008). Mislabeling has been reported in 50% of fish products in Germany (Kappel & Schröder, 2016), 24% of seafood in southern Brazil (Carvalho et al., 2015), and 82% of commercial fish fillets in Italy (Pinto et al., 2013). Such practices are difficult to detect using morphological identification due to the loss of diagnostic traits during processing (Zhao et al., 2013). Therefore, molecular identification and genetic analysis of *Pangasius S*p., particularly in the Surabaya region, are essential to support fisheries conservation and product authentication. This research is expected to contribute genetic data relevant to fisheries conservation policies, as outlined in Government Regulation of Indonesia No. 60/2007 and the Ministerial Decree No. 79/KEPMEN-KP/2016 on Fisheries Management Areas (WPPNRI 712).

One widely accepted approach for seafood authentication is DNA barcoding, which has been globally recognized as a reliable molecular identification system since 2005 through the Barcode of Life Data System (BOLD) (Hebert et al., 2003). DNA barcoding has been extensively applied in ecology, conservation, biomedicine, and food safety, offering a rapid, accurate, and cost-effective method for species identification, including in processed products (Chen et al., 2003; Cutarelli et al., 2018). This method has also been adopted by regulatory agencies such as the US-FDA to combat seafood fraud (Naaum & Hanner, 2016). In DNA barcoding, in silico analysis is commonly employed to design specific primers and target sequences prior to in vitro PCR experiments, ensuring high specificity and sensitivity. Among mitochondrial DNA markers, Cytochrome b (CytB) is widely used for fish species identification due to its relatively high mutation rate and ability to discriminate closely related species. Standard barcode fragments of CytB range from approximately 300–500 bp. These partial sequences are also useful for taxonomic analysis, genetic diversity assessment, divergence time estimation, and population structure analysis (Drummond et al., 2012). Despite its effectiveness, DNA barcoding still faces several limitations, including suboptimal DNA extraction, unsuitable primer design, and limited availability of mitochondrial sequence data in public databases, which may affect the accuracy of species identification.

MATERIALS AND METHOD

A total of 10 whole fish samples and 10 processed samples (fillets) in the form of fish fillets were used for species identification using a DNA barcoding approach targeting the mitochondrial Cytochrome B (CytB) gene. Fresh samples were obtained from Traditional market in Surabaya, while fillet samples were purchased from a local supermarket. Sample preservation was carried out using 98% ethanol prior to DNA extraction. Genomic DNA was extracted using a commercial silica column-based extraction method (Qiagen DNeasy Kit) following the manufacturer's protocol. The CytB gene was amplified using the Polymerase Chain Reaction (PCR) method with specific primers, and the PCR mixture consisted of ddH_2O, Taq buffer, $MgCl_2$, dNTP mix, BSA, Taq DNA polymerase, and template DNA. PCR products were visualized using agarose gel electrophoresis with SYBR Safe staining, and successful amplicons were subsequently subjected to Sanger sequencing. Sequence data were analyzed using BLAST (NCBI) for species identification and further evaluated through phylogenetic analysis using the Neighbor-Joining method with the Kimura 2-parameter model.

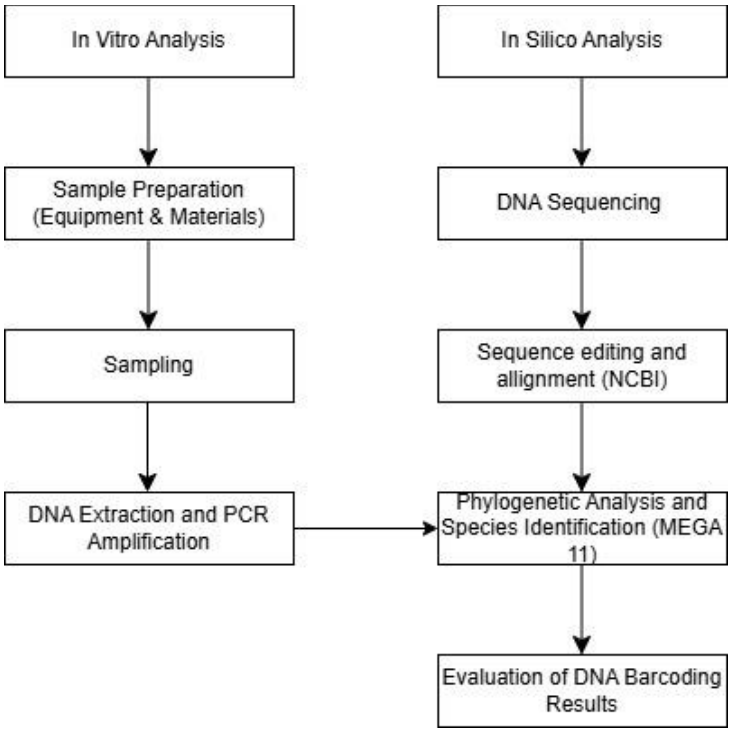

Figure 1. Workflow of DNA Barcoding

The equipment used in this study included a laptop for *in silico* DNA barcode exploration. Laboratory instruments for DNA extraction comprised a water bath, 2.5 mL microtubes, micropipettes, vortex mixer, refrigerator, and centrifuge. PCR amplification was performed using a thermal cycler, followed by gel electrophoresis to evaluate amplification results, and visualization was conducted using a UV illuminator. DNA extraction was performed according to the Zymo DNA Extraction Kit protocol with minor modifications. Fresh fish and fish fillet samples were initially weighed at approximately 10–25 mg and cut into small pieces prior to extraction. For each extraction, a total of 50 mg of tissue sample was used as input material**.** The sample was mixed with 1 mL of food lysis buffer and 5 µL of Proteinase K. The mixture was vortexed until homogeneous and incubated in a shaking water bath at 60 °C for 12 h, with intermittent vortexing every hour to ensure complete homogenization and tissue digestion.

After incubation, the samples were centrifuged at 2,500 × g for 5 min. A volume of 700 µL of the supernatant was transferred to a new microcentrifuge tube, followed by the addition of 500 µL chloroform. The mixture was vortexed thoroughly and centrifuged at 14,000 × g for 15 min, resulting in phase separation. Subsequently, 350 µL of the aqueous supernatant was transferred to a new microtube and mixed with BP buffer, followed by homogenization. The mixture was then loaded onto a QIAquick spin column and centrifuged at 16,300 × g for 1 min. The flow-through was discarded, and the column was centrifuged again at 16,300 × g for 1 min to remove residual liquid. The spin column was transferred to a new microtube, and 200 µL of EB buffer was added directly to the membrane. The column was incubated for 5 min at room temperature (15–25 °C) and centrifuged at 16,300 × g for 2 min to elute the DNA. The extracted DNA was subsequently assessed for concentration and purity and visualized using 1% agarose gel electrophoresis prior to use as a template for PCR amplification.

PCR analysis was performed using a reaction mixture consisting of 19.7 µL ddH_2O, 3 µL Taq buffer, 3 µL $MgCl_2$, 0.6 µL dNTP mix, 0.5 µL BSA, 1 µL of each forward and reverse CytB primer, 0.2 µL Taq DNA polymerase, and 1 µL of DNA template (approximately 100 ng), resulting in a total reaction volume of 30 µL. PCR amplification of the mitochondrial cytochrome b (CytB) gene was carried out using a step-down PCR protocol. The amplification conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. The PCR process was completed with a final extension at 72 °C for 7 min. PCR products were visualized by electrophoresis on a 2% agarose gel using a 1 kb DNA ladder as a molecular size marker. The gel was observed

under a UV transilluminator to confirm the presence and size of the amplified fragments. PCR products that showed successful amplification were subsequently subjected to DNA sequencing. Sequencing was performed using the Sanger sequencing method (Sanger et al., 1977) on purified PCR products to obtain nucleotide sequence data for further genetic and phylogenetic analysis.

Table 1. CytB primer used in this study, adopted from (Pepe *et al.*,2007)

Primer	Sequence (5'-3')	Amplikon (bp)	Object
CytB Forward	CCATCCAACTCTCAGCATGATGAAA	358	Fish
CytB Reverse	GCCCCTCAGAATGATATTTGTGCTCA	358	Fish

RESULTS AND DISCUSSION

Based on the *in silico* PCR analysis using universal CytB primers (Pepe et al., 2005), amplification products with a fragment length of 358 bp were obtained for most of the tested Pangasidae species. However, not all species showed optimal amplification results. In *Pangasius sanitwongsei*, only the reverse primer successfully annealed to the target sequence, while the forward primer did not show sufficient complementarity. Conversely, in *Pangasius mekongensis*, only the forward primer annealed effectively, whereas the reverse primer failed to bind. Therefore, forward trace and reverse trace analyses of 358 bp were required to obtain the corresponding target sequences.

The failure of one primer to anneal properly to the target DNA is presumably caused by mutations or nucleotide variations within the primer binding sites. Changes in nucleotide sequences in these regions can reduce primer–template complementarity, resulting in inefficient annealing and amplification failure (Chuang et al., 2013). In addition, this phenomenon may also be influenced by several primer design pitfalls, such as the formation of primer dimers and hairpin structures. Primer dimers occur due to hybridization between primers at complementary 3′ ends, which interferes with primer binding to the target DNA. Meanwhile, hairpin structures are formed when a primer contains self-complementary sequences, which can also reduce PCR efficiency (Das et al., 1999). Another important factor affecting amplification success is the melting temperature (Tm). Primers with low Tm values may not remain stable at annealing temperatures, whereas a large difference in Tm between forward and reverse primers (greater than 5°C) can lead to reduced amplification efficiency or complete PCR failure (Chuang et al., 2013). Therefore, primer accuracy and compatibility with the target sequence play a crucial role in determining the success of PCR analysis.

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Consensus                                 CATCYAATATCTCCGCATGATGAAATTTYGGCTCCCTCCTAYTAYTATGTCTTATAGTAC   60
Pangasianodon hypophthalmus_KC846907.1 cytb ....C.....T.............C..T..T.....A...T..T....C..........   60
Pangasianodon hypophthalmus_NC021751.1 cytb ....C.....T.............C..T..T.....A...T..T....C..........   60
Pangasius bocourti_MN842723.1 cytb          ....C.....T.............C..T..T.....A...T..T....C..........   60
Pangasius bocourti_NC046590.1 cytb          ....C.....T.............C..T..T.....A...T..T....C..........   60
Pangasius conchophilus_NC067896.1 cytb      .G..T..C.....T.............C............C..T..............   60
Pangasius conchophilus_OP23603.1 cytb       .G..T..C.....T.............C............C..T..............   60
Pangasius larnaudii_AP01208.1 cytb          ....T..C.....................C............T..C..............   60
Pangasius larnaudii_NC015839.1 cytb         ....T..C.....................C............T..C..............   60
Pangasius mekongensis _NC065096.1 cytb      ....C........T.............T............C..C..........G.C..   60
Pangasius mekongensis_MZ272451.1 cytb       ....C........T.............T............C..C..........G.C..   60
Pangasius nasutus_NC066440.1 cytb           .G..T..C.....T.............C............C..T..............   60
Pangasius nasutus_OP236030 cytb             .G..T..C.....T.............C............C..T..............   60
Pangasius pangasius_KX950698.1 cytb         ....C.....................C..T............C..C..............   60
Pangasius pangasius_NC023924.1 cytb         ....C.....................C..T............C..C..............   60
Pangasius sanitwongsei_MN809630.1 cytb      ....T.....T...A.............C............T..C..............   60
Pangasius sanitwongsei_NC057256.1 cytb      ....T.....T...A.............C............T..C..............   60

Consensus                                 ARATCCTAACAGGACTCTTCCTAGCCATACACTATACCTCAGACATCTCAACTGCCTTCT  120
Pangasianodon hypophthalmus_KC846907.1 cytb .G..............T..............T..................T..........  120
Pangasianodon hypophthalmus_NC021751.1 cytb .G..............T..............T..................T..........  120
Pangasius bocourti_MN842723.1 cytb          .G..............T..............T..................T..........  120
Pangasius bocourti_NC046590.1 cytb          .G..............T..............T..................T..........  120
Pangasius conchophilus_NC067896.1 cytb      .A.................................C........T.................  120
Pangasius conchophilus_OP23603.1 cytb       .A.................................C........T.................  120
Pangasius larnaudii_AP01208.1 cytb          .A..T..............T..................T..G..............T..T.  120
Pangasius larnaudii_NC015839.1 cytb         .A..T..............T..................T..G..............T..T.  120
Pangasius mekongensis _NC065096.1 cytb      .G..............T...........G.....C..T........T..G..C..T....  120
Pangasius mekongensis_MZ272451.1 cytb       .G..............T...........G.....C..T........T..G..C..T....  120
Pangasius nasutus_NC066440.1 cytb           .A.................................C..T.....T.................  120
Pangasius nasutus_OP236030 cytb             .A.................................C..T.....T.................  120
Pangasius pangasius_KX950698.1 cytb         .G..T.....................................................C..T....  120
Pangasius pangasius_NC023924.1 cytb         .G..T.....................................................C..T....  120
Pangasius sanitwongsei_MN809630.1 cytb      .A..............................T..............................  120
Pangasius sanitwongsei_NC057256.1 cytb      .A..............................T..............................  120
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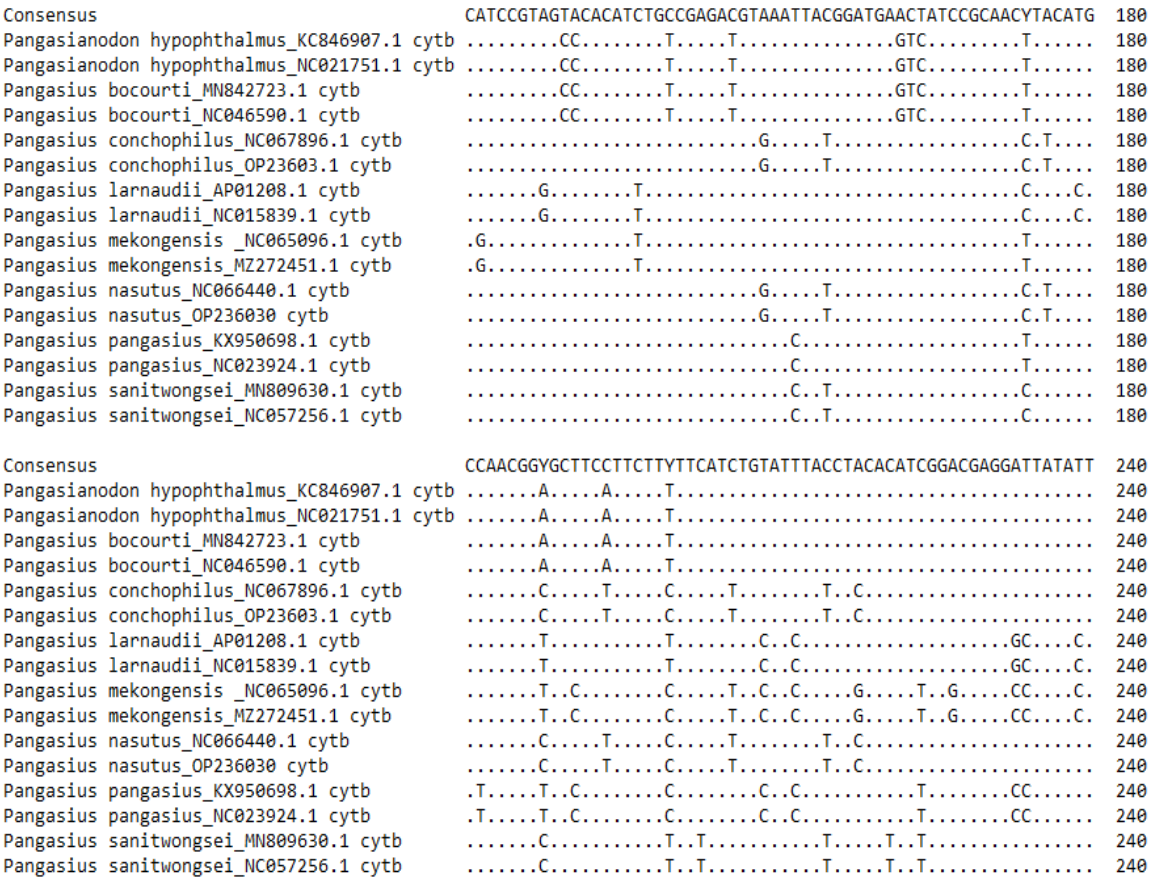

Figure 2. Results of In Silico Assay

After obtaining the amplified sequences from the *in silico* PCR, PCR-RFLP analysis was performed using four restriction enzymes, namely HinfI, NlaIII, TaqI, and BsaXI, with the assistance of the NEBcutter software. The results indicated that all four restriction enzymes were able to generate distinct restriction patterns among Pangasidae species using the CytB marker, although some patterns appeared relatively similar at first glance. Nevertheless, differences in the number of fragments and fragment sizes were still observed, enabling molecular discrimination among species.

However, in *Pangasius nasutus*, *Pangasius larnaudii*, and *Pangasius conchophilus*, no restriction cleavage was observed when using TaqI and BsaXI enzymes. As a result, the RFLP profiles of these species exhibited a single band corresponding to the full length of the CytB amplicon, which was 358 bp. The absence of restriction sites indicates that the target sequences in these species do not contain recognition sites for TaqI and BsaXI enzymes. This finding suggests the presence of nucleotide sequence variation among Pangasidae species within the CytB gene region, which affects the distribution of restriction enzyme recognition sites.

Overall, these results demonstrate that the CytB marker has strong potential as a molecular marker for PCR-RFLP analysis in discriminating Pangasidae species. Although the amplified fragment is relatively short, sequence variations within the CytB gene are sufficient to generate species-specific restriction patterns. Therefore, the combination of CytB primers and appropriate restriction enzymes can serve as an efficient and cost-effective alternative method for species identification without requiring more complex sequencing techniques (Fatchiah *et al*., 2011; Zhu *et al*., 2011).

Table 2. Results of PCR RLFP In Silico Assay

RLFP (RE) Fragment Size (In Silico) CytB							
No	Common Name	Scientific Name	Mitochondrion NCBI Access	HinfI	NlaIII	TaqI	BsaXI
1	Siamese shark	*Pangasianodon hypophthalmus*	**NC021751.1**	160; 198	154; 161	140; 218	358
2	Siamese shark	*Pangasianodon hypophthalmus*	**KC846907.1**	160; 198	154;161	140;218	358
3	Pangas catfish	*Pangasius pangasius*	***NC023924.1***	62; 285	161; 178	358	154; 159;358
4	Pangas catfish	*Pangasius pangasius*	**KX950698.1**	62; 285	154;161	358	154;159;358
5	Basa catfish	*Pangasius bocourti*	***NC046590.1***	160; 198	154;161	140; 218	358
6	Basa catfish	*Pangasius bocourti*	**MN842723.1**	160; 198	154;161	140; 218	358
7	Giant catfish	*Pangasius sanitwongsei*	**NC057256.1**	73; 285	154;161	358	304; 358
8	Giant catfish	*Pangasius sanitwongsei*	**MN809630.1**	73; 286	154;161	358	304; 359
9	Mekong catfish	*Pangasius mekongensis*	**NC065096.1**	358	91; 178	108; 250	154; 204; 358
10	Mekong catfish	*Pangasius mekongensis*	**MZ272451.1**	358	91; 178	108; 250	154; 204; 358
11	Seladang catfish	*Pangasius nasutus*	**NC066440.1**	73;285	154;161	358	358
12	Seladang catfish	*Pangasius nasutus*	**OP236030**	385	154;161	358	358
13	Black ear catfish	*Pangasius larnaudii*	**NC015839.1**	73;285	315	358	358
14	Black ear catfish	*Pangasius larnaudii*	**AP01208.1**	73;285	315	358	358
15	Shark catfish	*Pangasius conchophilus*	**NC067896.1**	73;285	154;161	358	358
16	Shark catfish	*Pangasius conchophilus*	**OP23603.1**	73;285	154;161	358	358

Phylogenetic Tree Reconstruction Based on CytB Marker

Genetic distance calculation and phylogenetic tree reconstruction were performed using MEGA 11 software (Kumar et al., 2018). A total of 16 mtDNA sequences from the Pangasidae family that were successfully amplified using universal CytB primers were used as the *in-group*, while one species, *Xiphias gladius*, was used as the *out-group*. All sequences were first aligned using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm to obtain homologous nucleotide positions. Phylogenetic tree reconstruction was carried out using the Neighbour-Joining (NJ) method based on a genetic distance matrix. Based on the phylogenetic analysis using the CytB marker, five main clusters were obtained (Figure X). Cluster A consisted of six sequences forming three sub-clusters, indicating relatively close evolutionary relationships among the members of this cluster. This clustering pattern suggests that nucleotide variations within the CytB gene are sufficient to represent phylogenetic relationships among Pangasidae species. Cluster B consisted of *Pangasius bocourti* and *Pangasianodon hypophthalmus*, which exhibited a very close genetic relationship with a genetic distance value of 0.065. These two species formed a polyphyletic taxonomic group with two apomorphic strains and one plesiomorphic strain. Apomorphy refers to derived characters resulting from evolutionary adaptations, whereas plesiomorphy refers to ancestral or primitive characters inherited from a common ancestor. The close genetic relationship indicates that the CytB gene is relatively conserved in both species, resulting in only minor nucleotide differences.

Cluster C consisted of *Pangasius sanitwongsei*, which formed a distinct sub-cluster and exhibited a polyphyletic pattern with apomorphic characteristics. This indicates that this species has undergone significant genetic divergence compared to other Pangasidae species, leading to its separation into a distinct cluster. This divergence may be associated with differences in habitat, geographical isolation, or varying evolutionary selection pressures. Cluster D consisted of *Pangasius conchophilus* and *Pangasius nasutus*, which showed a very close genetic relationship with a genetic distance of 0.01. This extremely small genetic distance suggests a high level of nucleotide sequence similarity in the CytB gene, indicating that these two species can be considered *sister taxa*. This relationship implies that both species likely originated from a relatively recent common ancestor and have undergone a relatively recent speciation process. Cluster E consisted of *Xiphias gladius*, which was used as the *out-group*. This species was clearly separated from the Pangasidae clusters, showing a very large genetic distance. This confirms that *X. gladius* has a very distant evolutionary relationship with Pangasidae, thus validating its use as an appropriate out-group in phylogenetic reconstruction.

Overall, the phylogenetic reconstruction based on the CytB marker demonstrates that this gene is capable of representing evolutionary relationships among Pangasidae species. Although the analyzed fragment length was relatively short (358 bp), the nucleotide variations within this region were sufficient to separate species

into distinct phylogenetic clusters. These results indicate that the CytB marker has strong potential as a molecular marker for phylogenetic analysis and species identification in the Pangasidae family, particularly using mitochondrial DNA-based approaches.

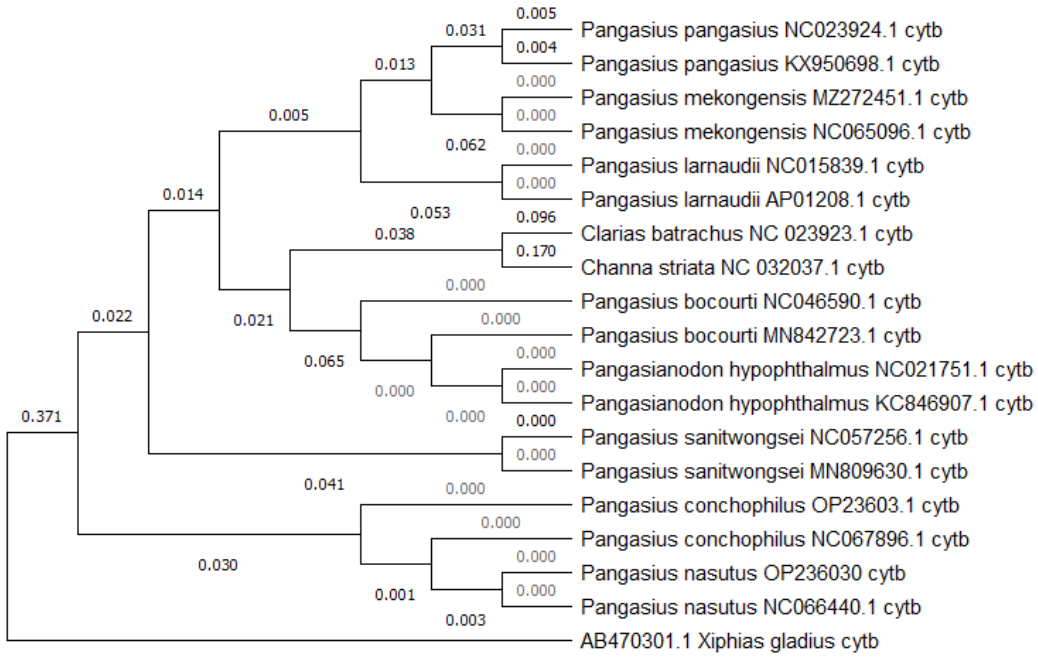

Figure 3. Phylogenetic tree based on *in silico* analysis

In Vitro PCR Analysis

The initial stage of this study involved the collection of Pangasius catfish samples sold in the Surabaya area. A total of 10 fresh Pangasius fish (I1-I10) samples and 10 Pangasius fillet (F1-F10) samples were used in this study. Detailed information regarding sample identity is presented in Table 1. Subsequently, DNA isolation and analysis were performed on all 20 samples to evaluate their genetic performance.

Table 1. Detailed information of Pangasius samples used for in vitro analysis.

Sample code	Information
F1, F2,F3,F4,F5,F6,F7,F8,F9,F10	Pangasius fillet (processed sample)
I1,I2,I3,I4,I5,I6,I7,18,19,I10	Fresh Pangasius (whole fish)

Total genomic DNA was extracted from fresh Pangasius fish tissue and Pangasius fillets using the Geneaid extraction kit without the addition of proteinase K. The quality and quantity of the extracted DNA were measured using a Nanodrop spectrophotometer at wavelengths of 260 nm and 280 nm. The results showed that DNA concentrations ranged from 1.0 to 88 ng/µL, with DNA purity values (A260/A280 ratio) ranging from 1.8 to 1.9. These values indicate that the extracted DNA had relatively good purity and was minimally contaminated by proteins. The extracted DNA was visualized using 1.5% agarose gel electrophoresis with a 1 kb DNA ladder (Thermo Scientific) and observed under a UV transilluminator. The total genomic DNA was then used as a template for Polymerase Chain Reaction (PCR) to amplify the CytB gene.

The PCR amplification results using the *Cytochrome b* (CytB) primer on 25 Pangasius samples showed that the majority of samples were successfully amplified. Visualization by agarose gel electrophoresis revealed clear DNA bands at approximately 500 bp, corresponding to the expected target fragment of the CytB gene. Out of the 25 samples analyzed, 23 samples showed successful amplification, as indicated by the presence of distinct bands at the expected size, while 2 samples did not produce any visible bands, suggesting amplification failure. Samples i6 to i10 were analyzed in duplicate to assess the reproducibility of the PCR results. The duplicate samples exhibited consistent banding patterns at the same fragment size, indicating that the PCR process was stable and reproducible. Variations in band intensity among samples were likely influenced by differences in DNA template concentration and quality. This fragment size is consistent with the reported CytB

amplicon length, which typically ranges between 300-500 bp (Sarmah et al., 2022). These findings indicate that the CytB primers were effective in amplifying the target DNA from Pangasius samples. The successful amplification of the CytB gene in this study suggests that the DNA concentration obtained from the extraction process was sufficient to serve as a PCR template. Moreover, the relatively short length of the CytB fragment contributes to higher amplification efficiency, particularly in samples with suboptimal DNA quality, such as processed fish fillets. Shorter DNA fragments are generally more stable and easier to amplify than longer fragments (Katevatis et al., 2017). Although proteinase K was not used during DNA extraction, CytB amplification still yielded satisfactory results. This indicates that the CytB gene is relatively tolerant to DNA samples with low to moderate quality. However, the absence of proteinase K may still affect DNA yield and integrity, as this enzyme plays an important role in degrading proteins and facilitating DNA release from cellular components (Frazer et al., 2020). Therefore, the use of proteinase K is strongly recommended in future studies to improve DNA concentration and quality, especially when targeting longer gene fragments.

Overall, the results demonstrate that the CytB marker shows high amplification success and can be effectively applied for molecular analysis of Pangasius catfish, even in fillet samples with relatively low DNA quality. This highlights the strong potential of the CytB gene as a robust molecular marker for species identification and genetic analysis using PCR-based approaches in fisheries research.

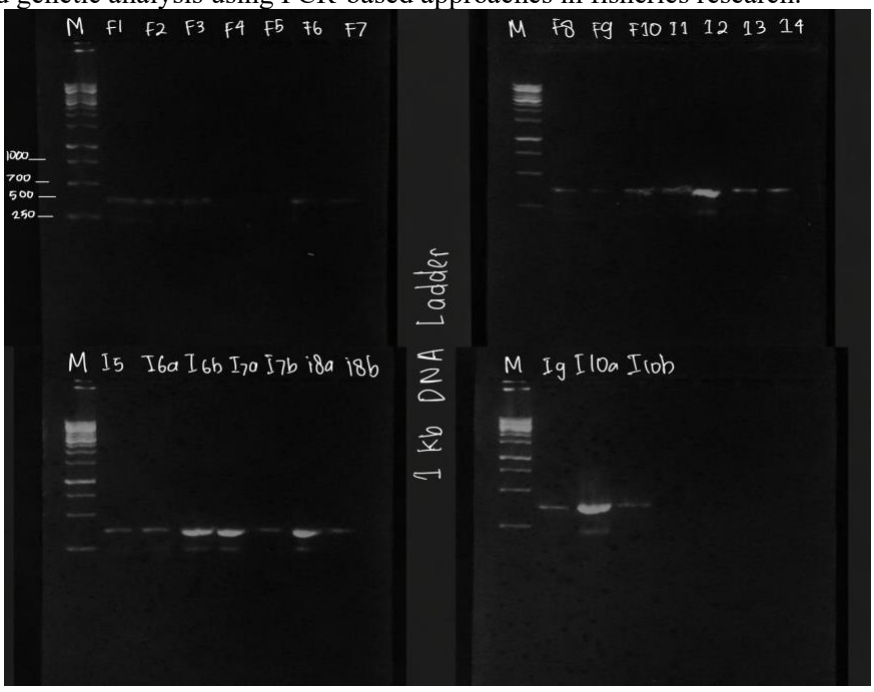

Figure 4. Gel electrophoresis results of PCR amplification using the cytochrome *b* (Cyt *b*) marker with a 1 kb DNA ladder.

The PCR amplification results using the *Cytochrome b* (CytB) primer on 25 Pangasius samples showed that the majority of samples were successfully amplified. Visualization by agarose gel electrophoresis revealed clear DNA bands at approximately 500 bp, corresponding to the expected target fragment of the CytB gene. Out of the 25 samples analyzed, 23 samples showed successful amplification, as indicated by the presence of distinct bands at the expected size, while 2 samples did not produce any visible bands, suggesting amplification failure. Samples i6 to i10 were analyzed in duplicate to assess the reproducibility of the PCR results. The duplicate samples exhibited consistent banding patterns at the same fragment size, indicating that the PCR process was stable and reproducible. Variations in band intensity among samples were likely influenced by differences in DNA template concentration and quality. Overall, these results demonstrate that the CytB primer used in this study has good specificity and efficiency in amplifying the target DNA fragment (~500 bp) of Pangasius samples.

Species Identification Based on Phylogenetic Analysis. BLAST analysis of the CytB PCR products searched against the GenBank database through the National Center for Biotechnology Information (NCBI) revealed that three Pangasius samples collected from the Surabaya area were successfully identified at the species level. Sample F6 was identified as *Pangasius pangasius*, while samples I1 and I10 were identified as *Pangasianodon hypophthalmus*. All three samples showed an E-value of 0.0 with a sequence homology of

98% compared to their closest reference sequences in GenBank. The E-value (Expectation value) is a statistical parameter used to evaluate the significance of sequence alignment between a query sequence and a subject sequence in BLAST searches. It represents the number of expected matches with a similar or better alignment score that could occur by chance in a database of a given size. The lower the E-value, the more significant the alignment result. An E-value of 0.0 indicates that the similarity between the query sequence and the reference sequence is highly significant and unlikely to occur by random chance (Fawley & Fawley, 2020). Species identification based on BLAST results was conducted following standard sequence identity thresholds. If the identity level between the query sequence and the reference sequence is ≥99.0%, the species name can be assigned with high confidence. If the identity level is between 98.0% and 99.0%, the species name is assigned using the term "cf." (conferre), indicating some uncertainty in species identification. If the identity level is <98.0%, the sequence can only be identified to the genus level and is designated as "sp." (Fawley & Fawley, 2020). Based on these criteria, the three samples in this study were considered validly identified at the species level.

The CytB sequences obtained from sequencing were further used for phylogenetic tree reconstruction to confirm the BLAST-based identification. The phylogenetic analysis showed that sample F6, identified as Pangasius pangasius, clustered within the same branch as Pangasius sanitwongsei with a genetic distance of 0.003. This extremely small genetic distance indicates a very close evolutionary relationship and a high degree of nucleotide similarity in the CytB gene region. Meanwhile, samples I1 and I10, identified as *Pangasianodon hypophthalmus*, clustered in the same clade as *Pangasius bocourti* and the reference *Pangasianodon hypophthalmus* sequences, with a genetic distance of 0.08. This result indicates that both samples have phylogenetic positions consistent with their reference species in GenBank and further supports the accuracy of the BLAST-based identification using the CytB marker. Overall, the combination of BLAST analysis and CytB-based phylogenetic reconstruction provided reliable species identification for Pangasius samples collected from the Surabaya area. The CytB marker proved to be effective for molecular discrimination of Pangasidae species and can be applied as a valid molecular barcode for species authentication, particularly in fisheries product monitoring and food traceability studies.

Table 3. BLAST analysis results of Pangasius samples collected from the Surabaya region based on the mitochondrial CytB gene

Sample code	Gene	Description	E-value	Query Cover (%)	Identity (%)
F6	CytB	*Pangasius pangasius*, Partial coding sequence (cds), mitochondrial DNA (OM938456.1)	0.0	99%	98%
I1	CytB	*Pangasianodon hypophthalmus*, Partial coding sequence (cds), mitochondrial DNA (KY586022.1)	0.0	100%	98%
I10	CytB	*Pangasianodon hypophthalmus*, Partial coding sequence (cds), mitochondrial DNA (MT441543)	0.0	100%	98%

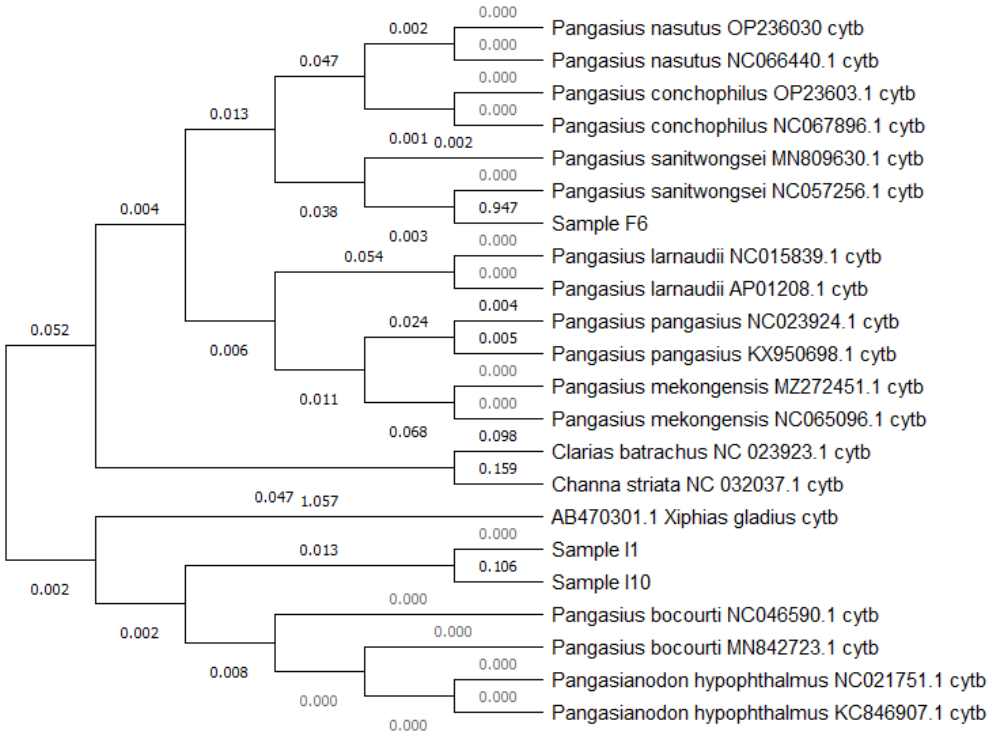

Figure 5. Phylogenetic tree based on in vitro analysis

The phylogenetic tree constructed using the mitochondrial CytB gene demonstrates clear clustering patterns among *Pangasius* species, indicating strong genetic relationships within the genus. Most reference sequences of *Pangasius* spp. are grouped into well-defined clades, such as *Pangasius nasutus*, *P. conchophilus*, *P. sanitwongsei*, *P. larnaudii*, *P. pangasius*, and *P. mekongensis*. These clusters are supported by relatively low genetic distances (e.g., 0.000–0.047), suggesting high sequence similarity within species and confirming the reliability of CytB as a marker for species-level identification. Sample F6 is positioned within the *Pangasius sanitwongsei* clade, showing very close genetic proximity to the reference sequences (distance ≈ 0.000–0.003). This indicates that Sample F6 can be confidently identified as *P. sanitwongsei*. The tight clustering and minimal branch length further support accurate species assignment and demonstrate the effectiveness of CytB in resolving closely related taxa. In contrast, Samples I1 and I10 form a separate cluster closer to *Pangasianodon hypophthalmus* and *Pangasius bocourti*. Their placement suggests a strong genetic affinity with these commercially important species, which are commonly found in aquaculture and fish markets. This may indicate correct labeling or, alternatively, reflects the dominance of these species in processed fish products. The low genetic divergence (near 0.000) supports high sequence similarity with the reference database.

Interestingly, outgroup species such as *Clarias batrachus*, *Channa striata*, and *Xiphias gladius* are clearly separated from the *Pangasius* clades, with higher genetic distances (up to ~1.057). This confirms the robustness of the phylogenetic reconstruction and validates the tree topology, as these species belong to different taxonomic groups. Their distinct clustering reinforces that the analyzed samples are correctly placed within the *Pangasius* lineage. Overall, the phylogenetic analysis highlights that the CytB gene provides sufficient resolution to discriminate among *Pangasius* species and to identify unknown samples. The clustering pattern also suggests relatively low intraspecific variation and clear interspecific divergence, supporting the applicability of CytB for molecular authentication of fish products in the Surabaya market.

CONCLUSION

Based on the results of this study, it can be concluded that the CytB marker shows strong performance as a molecular marker for genetic analysis and species identification of Pangasius catfish. The *in silico* analysis demonstrated that CytB sequences were able to generate distinct PCR-RFLP patterns among Pangasidae species, allowing molecular discrimination through variations in restriction fragment profiles produced by different restriction enzymes. The *in vitro* PCR results indicated that the CytB gene was amplified more easily and consistently, with a high amplification success rate, as evidenced by the presence of a single DNA band of approximately 358 bp in most samples. This suggests that the CytB gene is relatively stable and tolerant to variations in DNA quality, including in processed fish fillet samples that have undergone post-harvest handling. BLAST analysis of CytB sequences successfully identified several Pangasius samples marketed in the Surabaya area as *Pangasius pangasius* and *Pangasianodon hypophthalmus*, with an E-value of 0.0 and sequence homology of ≥98%. These results were further supported by CytB-based phylogenetic analysis using the Neighbour-Joining method, which showed that the samples clustered within the same clades as their reference species in the GenBank database, with relatively small genetic distances.

Overall, the CytB marker proved to be effective and reliable for molecular identification of Pangasius species. The combination of PCR amplification, BLAST analysis, and CytB-based phylogenetic reconstruction provides an efficient, accurate, and practical approach for species authentication, particularly for fisheries product monitoring and DNA-based food traceability applications. Future studies are recommended to integrate CytB with additional mitochondrial or nuclear markers to enhance species resolution and phylogenetic robustness.

AUTHORS CONTRIBUTION

Wina Intan Nur Aulia, S.Si was responsible for the conceptualization of the study, methodology design, data collection, data analysis, and drafting the initial manuscript. Prof. Dr. rer. nat. Edwin Setiawan, S.Si., Bio.Cur., M.Sc. provided overall supervision, validated the results, and contributed to the critical review and editing of the manuscript. Radestya Triwibowo, S.Pi., Ph.D. contributed to the research design, interpretation of the results, and manuscript revision. Triono Bagus Saputro, S.Si., M.Biotech, Ph.D. was involved in statistical analysis, data interpretation, and provided technical and scientific input to improve the manuscript. Prof. Dr. Awik Puji Dyah Nurhayati, S.Si., M.Si. and Muhammad Ainur Rosyid Ridho, S.Si., M.Si. contributed to the evaluation of the research framework, final manuscript editing, and overall scientific validation.
All authors have read and approved the final version of the manuscript for publication.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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