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DNA BARCODING PRIMER DESIGN FOR SPECIES IDENTIFICATION OF SKIPJACK TUNA, YELLOWFIN TUNA, AND MACKEREL: DEVELOPMENT AND VALIDATION OF PRIMERS FOR GENETIC RESEARCH

DESAIN PRIMER DNA BARCODING UNTUK IDENTIFIKASI SPESIES IKAN TONGKOL, TUNA SIRIP KUNING, DAN CAKALANG: PENGEMBANGAN DAN VALIDASI PRIMER UNTUK PENELITIAN GENETIK

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Abstract

The DNA barcoding technique is primarily utilized to rapidly identify species, particularly when samples are damaged and cannot be identified accurately through morphological methods. This approach utilizes polymerase chain reaction (PCR) technology to amplify DNA fragments from the targeted species, with its success largely due to the design of the primers employed. The cytochrome c oxidase subunit I (COI) gene, a mitochondrial gene, is frequently targeted in DNA barcoding and has been proven effective in distinguishing species. At Pondokdadap Port, over 90% of the fish caught consist of skipjack tuna, yellowfin tuna, and mackerel (T2C). This study aimed at designing silico DNA barcoding primers for these three species. The successful development of these primers may facilitate the documentation and understanding of the genetic diversity of the species under study, which is crucial for efficient and effective fisheries management. The primer design process applied Primer-BLAST software from the NCBI website, followed by additional testing with OligoAnalyzer. The selected primer pairs were the forward primer 5'-GGCCCATGCCTTCGTAATGA-3' and the reverse primer 5'-GCAGGGTCGAAGAAGGTTGT-3'. These primers successfully amplified the DNA of T2C fish, with PCR results indicating that the optimal annealing temperature for these primers was 55 °C.

Keywords: DNA barcoding; Polymerase chain reaction; Primer design

Abstrak

Teknik DNA barcoding merupakan metode yang umum digunakan untuk identifikasi spesies secara cepat, terutama pada sampel yang mengalami kerusakan dan sulit diidentifikasi secara morfologis. Metode ini memanfaatkan teknologi polymerase chain reaction (PCR) untuk memperbanyak fragmen DNA spesies target, dengan keberhasilan yang sangat bergantung pada desain primer yang digunakan. Gen cytochrome c oxidase subunit I (CoI) adalah gen mitokondrial yang sering dijadikan target dalam DNA barcoding dan terbukti efektif dalam membedakan spesies. Lebih dari 90% ikan yang tertangkap di TPI Pondokdadap didominasi oleh ikan tongkol, tuna sirip kuning, dan cakalang (T2C). Penelitian ini bertujuan untuk merancang primer DNA barcoding secara in silico untuk ketiga spesies ikan tersebut. Keberhasilan desain primer ini diharapkan dapat membantu dokumentasi dan pemahaman keanekaragaman hayati genetik dari spesies yang diteliti. Informasi genetik ini diperlukan sebagai salah satu dasar untuk pengelolaan perikanan yang efektif dan efisien, khususnya untuk ikan tongkol, tuna sirip kuning, dan cakalang (T2C). Desain primer dilakukan menggunakan perangkat lunak Primer-BLAST di situs web NCBI, diikuti dengan pengujian lebih lanjut menggunakan OligoAnalyzer. Pasangan primer yang terpilih adalah primer depan 5'-GGCCCATGCCTTCGTAATGA-3' dan primer belakang 5'-GCAGGGTCGAAGAAGGTTGT-3'. Primer yang dirancang berhasil memperbanyak DNA ikan T2C, dengan hasil amplifikasi PCR menunjukkan bahwa suhu annealing yang optimal untuk primer yang dirancang adalah 55 ℃.

***Kata Kunci**: Desain primer; DNA barcoding; Polymerase chain reaction*

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INTRODUCTION

DNA barcoding techniques are extensively utilized to rapidly identify species, particularly when the samples under investigation are damaged, making morphological identification challenging. The information derived from DNA barcoding is essential for documenting genetic biodiversity within the studied species, predicting species distribution, and monitoring changes in the genetic diversity of populations. In the industrial sector, this technique plays a crucial role in ensuring food safety and regulatory compliance, verifying that the species of fish used in products correresponds to the labels provided (Wu et al., 2021). In the realms of ecology and conservation, DNA barcoding facilitates the assessment of species presence in their environments by employing environmental DNA (eDNA) collected from habitats, thus negating the need to capture individual specimens.

Researchers also employ this technique in biogeographic studies of terrestrial and marine species. DNA barcoding utilizes polymerase chain reaction (PCR) technology to amplify DNA fragments from the species under investigation, and its success is contingent upon the effective design of primers. Researchers frequently target the cytochrome c oxidase subunit I (COI) gene in DNA barcoding studies. This method employs specific sequences from targeted DNA regions to identify and differentiate among species. The COI gene has proven to be an effective target for species differentiation due to its advantageous characteristics for barcoding analysis (Pasha et al., 2016; Suparman et al., 2016; Sihotang et al., 2021).

Using inappropriate primers in DNA barcoding can lead to inefficient DNA amplification, resulting in no amplification or non-specific results. Such complications hinder the identification process, which is the primary objective of DNA barcoding. Errors in obtaining the expected DNA target can result in misidentification, thereby reducing the accuracy of the analysis (Wu et al., 2021). The overly universal primer may yield non-specific PCR results, complicating the separation of the desired target fragment from other DNA fragments. Consequently, the identification process of the species under study becomes increasingly difficult due to these non-specific PCR results, which can lead to errors in species identification.

Over 90% of the fishing catch landed at TPI Pondokdadap, dominated by skipjack tuna, yellowfin tuna, and mackerel (Agustina et al., 2020). Effective management of fish resources is essential to ensure the sustainability of target fish populations in the event of fishing activities. Sustainable resource management for target fish species is necessary to maintain their populations. Accurately identifying species and populations is crucial for efficient and effective management purposes. The study objective was to design primers for DNA barcoding in-silico for the earlier fish-mentioned species. The success of primer design may enhance the documentation and understanding of the genetic biodiversity of skipjack tuna, yellowfin tuna, and mackerel (T2C). The development of specific primers can significantly improve the success of PCR amplification for species and population identification.

Primers are short nucleotide sequences that serve as the starting point for DNA synthesis, defining the boundaries of the target DNA fragments to be amplified (Pradnyaniti et al., 2014). An effective universal primer for the T2C fish species must adhere to ideal primer design criteria. The cytochrome c oxidase subunit I (COI) gene is one of the genes suitable for molecular identification. This gene was selected due to its widespread application in speciation, population genetics, and phylogeny studies. The COI gene is particularly suitable for species identification research due to its rapid mutation rate, which allows for better differentiation at lower taxonomic levels, such as species (Pasha et al., 2016; Suparman et al., 2016; Sihotang et al., 2021). The COI gene sequence can be obtained from GenBank, a nucleotide database available on the NCBI website.

GenBank is a dedicated NCBI platform for storing nucleotide sequences, synchronized with the DNA Databank of Japan (DDBJ) and the European Molecular Biology Laboratory (EMBL). Gene sequences can be retrieved by entering the gene name and desired species into the search bar, after which primer design can be conducted using Primer-BLAST. The function of BLAST in primer design is to perform in silico simulations that demonstrate the DNA sequence produced when the primers (forward and reverse) are used for PCR amplification, according to the specified primer design parameters.

MATERIALS AND METHODS

T2C fish samples were collected from fishermen's landings at Pondokdadap Port in Malang Regency. The sampling location is illustrated in Figure 1. DNA amplification using polymerase chain reaction (PCR) was conducted to evaluate the success of primer attachment at the Fish Cultivation Laboratory, Division of Fish Disease and Health, Faculty of Fisheries and Marine Sciences, Brawijaya University.

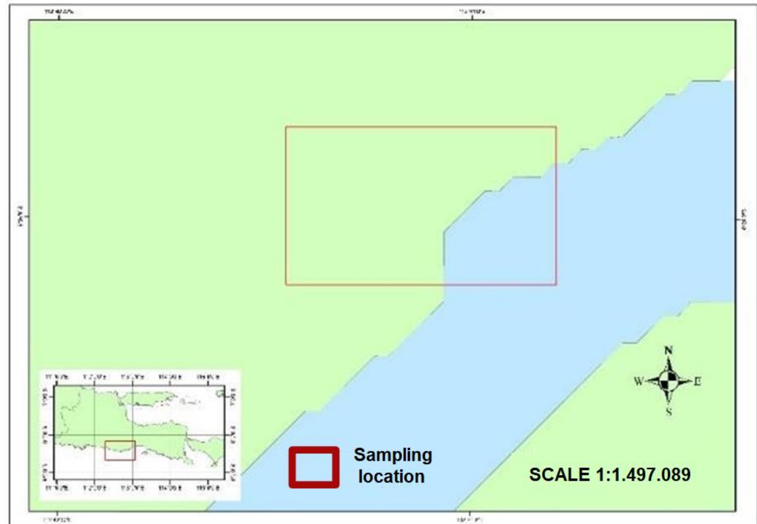

Figure 1. T2C fish sampling location (map base source: ESRI, 2023)

DNA Extraction and Amplification

Tissue samples were taken from the area near the pectoral fins of yellowfin tuna, skipjack tuna, and mackerel. These samples were placed in bottles containing 96% ethanol to maintain the integrity of the DNA structure and stored in a freezer at -20 °C before extraction. Extraction was carried out using the G-Spin Total DNA Extraction Kit. The quality and concentration of the extracted DNA were assessed using the Maestrogen Nanodrop Spectrophotometer. The extraction results were then used as DNA templates in DNA amplification that was performed using the Biorad thermal cycler. The DNA amplification procedure consists of three stages: denaturation (double-strand separation), annealing (primer attachment), and extension. The amplification process was conducted for a total of 30 cycles.

DNA Visualization

Visualization of DNA amplification results was performed using electrophoresis using the Gelato Electrophoresis and Visualization System apparatus, allowing for the direct visualization of DNA bands as they progress through the 1.5% agarose gel. DNA was stained with Biotium gel green and run with 1× TAE buffer. Gel Green was selected due to its compatibility with this visualization system that operates blue light, which is optimal for visualizing the green fluorescence emitted by Gel Green. The electrophoresis was conducted for 45 minutes at a voltage of 75 V.

RESULTS

Results of Fish T2C COI Gene Primer Design

The design and in silico testing of primers for the COI gene were conducted using Primer-BLAST from NCBI, generating ten pairs of primers. The graphical representation of primer pairs derived from the Primer-BLAST results is presented in Figure 2. This graphical view indicates that most of the primers generated produced short product lengths. Only primer pairs 1, 2, and 5 yielded product lengths exceeding 400 base pairs. Primer pairs 2 and 5 exhibited a greater Tm difference, notable disparities in the lengths of the forward and reverse primers, and self-complementarity values reaching 5. Consequently, these characteristics led to the exclusion of primer pairs 2 and 5, as they did not satisfy the ideal primer criteria. The specific characteristics of all primer pairs generated by Primer-BLAST are detailed in Figure 3.

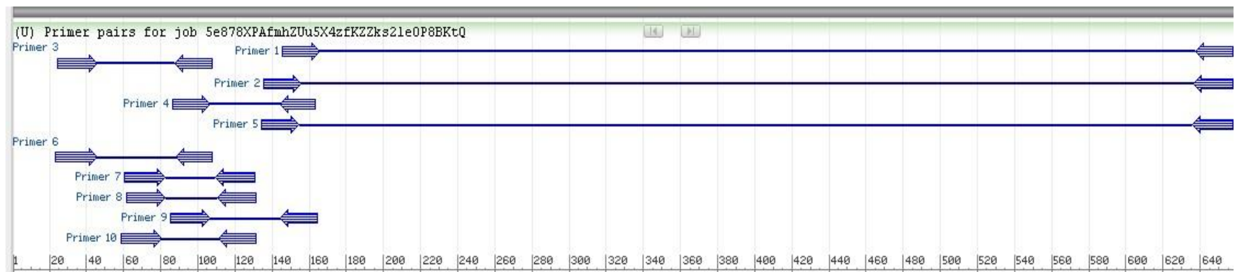

Figure 2. Graphical display of each primer pair encoding the COI gene of T2C fish

The graphical representation in Figure 2 indicates that the start codon for primer pair one is located between 146–165 bp, while the stop codon is found between 638–657 bp. The length of the PCR product, as estimated from the Primer-BLAST results at NCBI, is 512 bp (Figure 3). The melting temperature (Tm) of primers can be calculated manually using the formula, Tm= 2(nA + nT) + 4(nG + nC). According to this formula, the Tm is calculated to be 62 °C, while the Tm determined by NCBI's Primer-BLAST is 60 °C.

The results of secondary structure analysis of the primers are presented in Figures 4 and 5. Heterodimer testing conducted using OligoAnalyzer by IDT shows that all three primer pairs tested for the possibility of primer dimers produced delta G values of less than -9 (Figure 4), and the temperature that allows the highest occurrence of primer hairpin is at 49 °C (Figure 5). Based on the fulfillment of the ideal primer criteria and the results of the secondary structure testing, primer pair one was selected. The forward and reverse base sequences of primer pair one and their characteristics are detailed in Table 1. For ease of comparison with other primer pairs, this primer design will be designated the code name Primer T2C. Subsequently, amplification testing and optimization of the annealing temperature were performed.

Table 1. Selected primer pairs

Primer TC2	Sequence (5'→3')	Tm (°C)	GC (%)	Self 3' comp.
Forward	GGCCCATGCCTTCGTAATGA	60,47	55	3
Reverse	GCAGGGTCGAAGAAGGTTGT	60,25	55	2

Primary Analysis Test Results with PCR Method

DNA amplification testing was conducted using the first PCR program, following the Tiangen Biotech PCR Mix 2× Taq Plus manual guidelines. This PCR mix comprises several optimized components, offering advantages such as ease of use, specificity, and stability. The use of this PCR mix reagent is expected to minimize human error during the amplification process. The DNA template for this electrophoresis test was derived from the G-Spin Total DNA Kit extraction.

PCR testing was also conducted using Folmer primers, which consist of LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') as the forward primer and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') as the reverse primer. The purpose of using and subsequently comparing two primer pairs (Folmer's primer and T2C primer) was to assess whether the designed T2C primer demonstrated greater specificity in amplifying the COI gene in yellowfin tuna, skipjack tuna, and mackerel. The PCR temperature settings aligned with the Tiangen Biotech PCR Mix 2× Taq Plus protocol. The temperature settings and duration of the PCR program are illustrated in Figure 6.

The results of DNA extraction using the G-spin Kit are presented in Table 2. However, the extraction concentrations obtained with the G-spin Kit show notable differences: yellowfin tuna yielded a concentration of 0.73 ng/µL, skipjack tuna 0.52 ng/µL, and mackerel significantly higher at 87.68 ng/µL. Given the substantial difference in DNA concentrations, particularly the lower concentrations in yellowfin and skipjack tuna compared to mackerel, optimization of the reagent composition was necessary for the DNA (primer) amplification tests. Therefore, the DNA template of the yellowfin and skipjack tuna was increased from 2 µL to 5 µL, as their extraction concentrations were below 1 ng/µL. Deionized water (ddH2O) was added to the DNA amplification assay for yellowfin tuna and skipjack tuna, but instead, a DNA template volume was added. Therefore, the total

reagent volume was 12.5 µL (Table 3). Optimization was implemented in wells 2, 3, 6, and 7 for yellowfin and skipjack tuna samples, whereas skipjack tuna (wells 4 and 8) remained unoptimized, as its DNA concentration was within the recommended standard.

Primer pair 1

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GGCCCATGCCTTCGTAATGA	20	146	165	60.47	55.00	4.00	3.00
Reverse primer	GCAGGGTCGAAGAAGGTTGT	20	657	638	60.25	55.00	4.00	2.00
Product length	512							

Primer pair 2

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TAATCGTTACGGCCCATGCC	20	136	155	60.53	55.00	5.00	2.00
Reverse primer	GCAGGGTCGAAGAAGGTTGTA	21	657	637	60.00	52.38	4.00	2.00
Product length	522							

Primer pair 3

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGTATTCGGTGCATGAGCTGG	21	25	45	60.47	52.38	4.00	0.00
Reverse primer	GGCACCTGGTTGGCTTAGTT	20	107	88	60.54	55.00	5.00	0.00
Product length	83							

Primer pair 4

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GAACTAAGCCAACCAGGTGC	20	87	106	59.12	55.00	5.00	2.00
Reverse primer	ATTACGAAGGCATGGGCCG	19	163	145	60.53	57.89	4.00	2.00
Product length	77							

Primer pair 5

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GTAATCGTTACGGCCCATGC	20	135	154	59.42	55.00	6.00	2.00
Reverse primer	GCAGGGTCGAAGAAGGTTGTAT	22	657	636	60.35	50.00	4.00	2.00
Product length	523							

Primer pair 6

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TAGTATTCGGTGCATGAGCTGG	22	24	45	60.22	50.00	4.00	0.00
Reverse primer	GGCACCTGGTTGGCTTAGT	19	107	89	59.98	57.89	5.00	1.00
Product length	84							

Primer pair 7

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTTAAGCACCATTGCTCATCCG	22	61	82	59.97	50.00	6.00	2.00
Reverse primer	TAGATCTGGTCGTCCCCAAGA	21	130	110	59.71	52.38	6.00	3.00
Product length	70							

Primer pair 8

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTAAGCACCATTGCTCATCCG	21	62	82	58.98	47.62	4.00	2.00
Reverse primer	GTAGATCTGGTCGTCCCCAAG	21	131	111	59.59	57.14	6.00	1.00
Product length	70							

Primer pair 9

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGAACTAAGCCAACCAGGTGC	21	85	105	60.82	52.38	5.00	2.00
Reverse primer	CATTACGAAGGCATGGGCCG	20	164	145	61.79	60.00	4.00	2.00
Product length	79							

Primer pair 10

	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCCTTAAGCACCATTGCTCATC	22	59	80	60.22	50.00	6.00	1.00
Reverse primer	GTAGATCTGGTCGTCCCCAA	20	131	112	58.52	55.00	6.00	0.00
Product length	73							

Figure 3. BLAST primer generation results

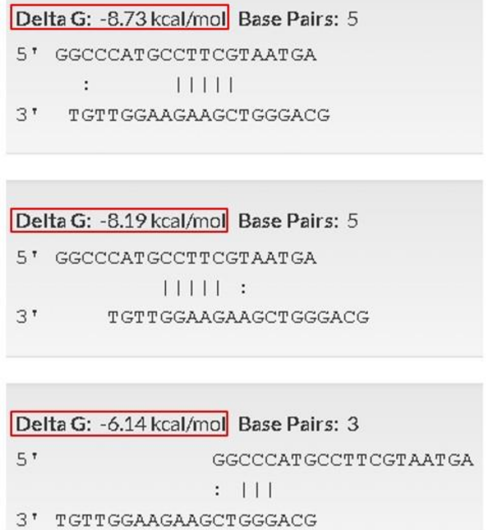

Figure 4. Dimer test results from OligoAnalyzer

Structures

structure	Image	ΔG (kcal.mole^{-1})	T_m (°C)
1		-1.89	49
2		-1.14	35.2
3		-0.9	33

Figure 5. Hairpin test results from OligoAnalyzer

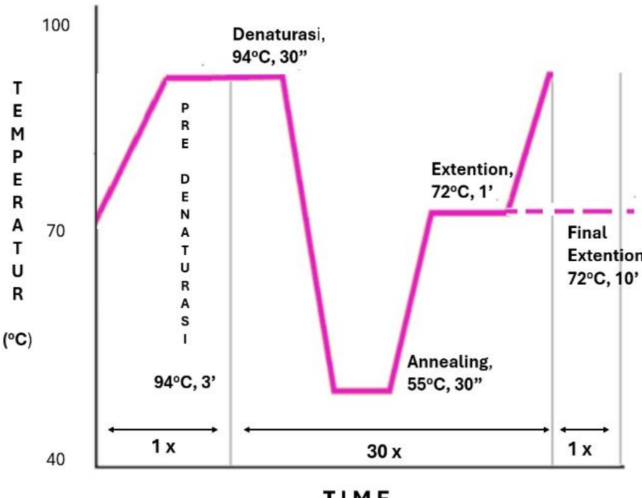

Figure 6. Polymerase chain reaction (PCR) programs and cycles

Table 2. DNA extraction results

Sample	Purity (A260/280)	Concentration (ng/ µL)
Yellowfin tuna	1.896	0.73
Skipjack tuna	1.859	0.52
Mackerel	1.948	87.68

The electrophoresis results from the amplification DNA (primer) test using PCR demonstrated the formation of DNA bands in all samples utilizing the T2C fish COI gene design primer. The DNA band observed in the samples amplified with the T2C primer was notably thick, indicating successful amplification at the expected length of around 512 bp, as estimated by Primer-BLAST; however, some smearing was also present. In contrast, the sample amplified with Folmer primer produced a single thin DNA band in well 7, located below 500 bp. The results of the initial DNA test visualization are illustrated in Figure 7. The graphical representation of the annealing segments for both primer T2C and Folmer primer on the COI (cytochrome c oxidase subunit 1) gene is illustrated in Figure 8.

Table 3. DNA amplification reagent composition

Reagen	Tunas (µL)	Mackarel (µL)
DNA template	5	2
Primer forward	0.5	0.5
Primer reverse	0.5	0.5
PCR mix	6.5	6.5
ddH_2O	-	3

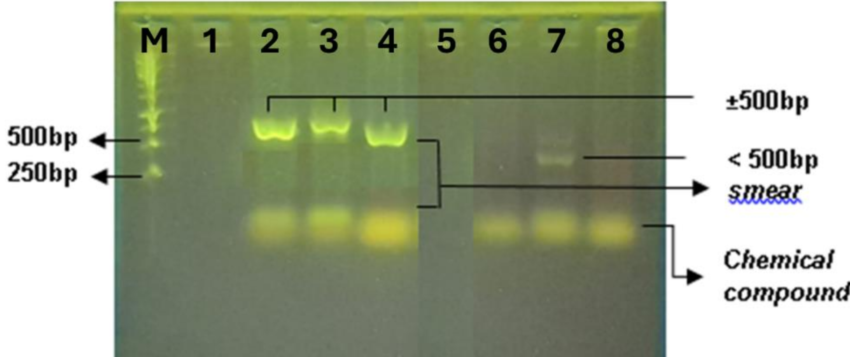

Figure 7. DNA visualization results. Note: M is a DNA Marker 1,000 bp; no. 2–3 are the results of the first PCR test using primer T2C and are sequential samples of skipjack tuna, yellowfin tuna, and mackerel; no. 6–8 are the results of PCR using primer LCO1490/HCO2198 and are sequential samples of skipjack tuna, yellowfin tuna, and mackerel

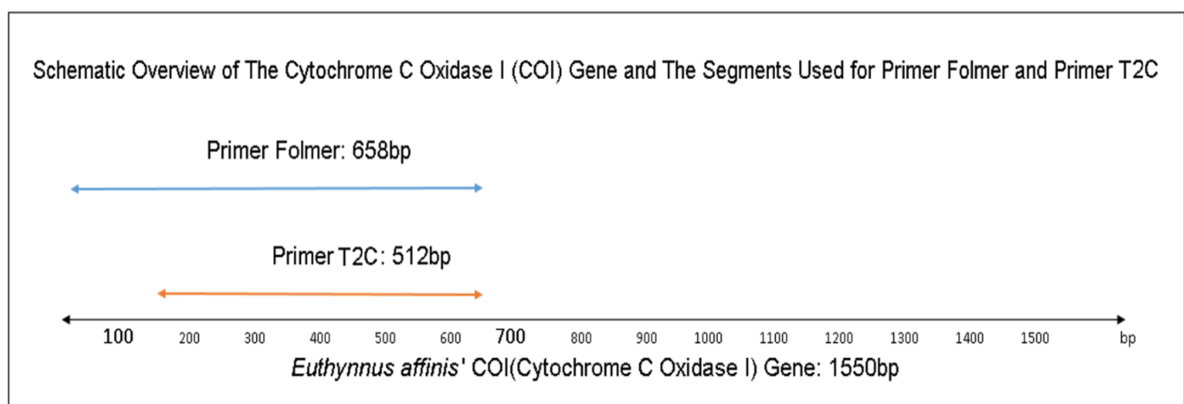

Figure 8. Graphic of annealing segment for primer T2C and primer Folmer on the COI gene

DISCUSSION

Some issues to consider for the ideal primer are an optimal primer length between 18–30 bp, with a melting temperature (Tm) difference of less than 5 °C (Pradnyaniti et al., 2014). Furthermore, the GC content of the TC2 primers was found to be 55% (Table 1), which falls within the recommended range of 40–60%. The values for self-complementarity and 3' self-complementarity were both less than 5, indicating the low potential for undesirable interactions among primer pairs. The complete cytochrome c oxidase subunit I (COI) gene sequence length is 1,535 bp (Doorenweerd et al., 2020). Given that the typical length of the COI gene is approximately 1,500 bp, the suitable product length for primers based on the COI gene is approximately one-third of the gene length, or around 400–600 bp. In the hairpin test, the Tm for hairpin formation was determined to be below 49 °C, while the Tm for the selected primer pair one was found to be 60 °C (Figure 5), suggesting a low likelihood of hairpin formation (Anika et al., 2019). The secondary structure test, as represented in Figure 4, also revealed that primer pair one exhibited the highest delta G value of -8.73 kcal/mol, which remains above the critical threshold for stable primer dimer formation (-9 kcal/mol). These results, together with the balanced Tm values and ideal GC content, indicate that primer pair one fulfills the key criteria of optimal primer design (Pradnyaniti et al., 2014).

Furthermore, the detailed analysis of Figures 4 and 5 shows that primer pair 1 was less likely to form 3′-dimers compared to primer pairs 2 and 5, which displayed slightly more stable predicted structures. The predicted hairpin Tm (49 °C) is well below the annealing temperature used (55 °C), indicating that any potential secondary structure would not persist under PCR conditions. Together with the successful in vitro amplification results (Figure 7), these findings justify the selection of primer pair 1 (designated T2C primer) as the most suitable for COI amplification in skipjack tuna, yellowfin tuna, and mackerel (Delghandi et al., 2022).

The results of DNA extraction using the G-spin Kit (Table 2) showed no contamination in all DNA samples. The optimal purity range determined by spectrophotometer analysis at a wavelength of 260/280 is approximately 1.8–2.0 (Setiyawan et al., 2015). Based on the DNA purity ratio values, the samples from yellowfin tuna, skipjack tuna, and mackerel all fall within this optimal range. Although the purity of the DNA samples was quite good, the extraction results showed that only the mackerel samples had the appropriate concentration for DNA amplification. The template concentration for PCR amplification is between 10–100 ng/μL (Barbeyrac & Bebear, 1996). Therefore, the amount of DNA template was increased in the yellowfin tuna and skipjack tuna samples, as presented in Table 3.

The DNA amplification testing for the designed primer was carried out using the Tiangen Biotech PCR Mix 2× Taq Plus. This extraction kit was selected for its ability to quickly and easily extract genomic DNA from animal culture cells, blood, and tissues while minimizing contamination during the process. It has been successfully employed to extract DNA from human appendicular tissue and porcine peritoneal tissue (stomach lining) (Sengal et al., 2016; Kim et al., 2022). Apart from optimizing the amount of DNA template used in the PCR process, annealing temperature optimization was also carried out. The annealing temperature range for PCR amplification is calculated based on the primer's nucleotide sequence, specifically Tm ±5 °C, resulting in an optimization range of 55–65 °C (Setyawati & Zubaidah, 2021). Based on these calculations, annealing temperature optimization was conducted at 55, 58, and 60 °C. From this optimization, the annealing temperature that amplifies the TC2 primer is 55 °C, with the time and PCR program as presented in Figure 6.

Amplification testing with T2C primers (Figure 7) resulted in a relatively thick DNA band compared to the PCR outcomes using LCO1490 and HCO2198 (Folmer) primers. The LCO1490 and HCO2198 primers were designed based on three coding strands and six anti-coding strands, focusing on the most conserved regions of the cytochrome c oxidase subunit 1 (COI) gene across 15 taxa. This primer pair has successfully amplified a 710 base pair fragment of the COI gene in over 80 invertebrate species from 11 phyla (Folmer et al., 1994). The DNA band formed in samples amplified with Folmer primers was less pronounced. This difference is likely due to a less optimal annealing temperature for the LCO1490 and HCO2198 primers and possibly to primer-template mismatches in the fish species studied. The melting temperature (Tm) of the LCO1490 primer is 50.5 °C, while that of the HCO2198 primer is 55 °C; however, the annealing temperature employed was set at 55 °C. Previous studies utilizing Folmer primers have reported variations in annealing temperatures ranging from 46–56 °C, depending on the polymerase used. The optimal annealing temperature for a primer is generally considered to be approximately 5 °C below its Tm. An annealing temperature exceeding this optimal range may reduce the number of amplicons produced (Yuenleni, 2019).

The Folmer primers (LCO1490, HCO2198) are considered universal primers capable of amplifying the COI gene across many taxa. However, subsequent studies have indicated that the purported conserved nature of these primers may not be entirely accurate. A comparison of 725 COI gene sequences revealed more than 130,000 variations, with LCO1490 showing only four conserved regions, suggesting that sequence mismatches may reduce amplification efficiency in some taxa. In addition, the specifically designed primer T2C demonstrated better performance in amplifying the COI gene of the target fish samples (yellowfin tuna, skipjack tuna, and mackerel). This result supports the conclusion that Folmer primers may be too 'universal,' producing suboptimal amplification in these species compared to the T2C primer, which was designed for species-specific amplification (Sharma & Kobayashi, 2014).

The schematic comparison in Figure 8 highlights the difference between the newly designed T2C primer and the Folmer primer pair. The T2C primers amplify a 512 bp fragment near the 5′ region of the COI gene, while the Folmer primers amplify a longer 658–710 bp fragment in the central region. This shorter T2C fragment is advantageous for partially degraded DNA, resulting in higher PCR efficiency, as evidenced by the more intense bands in Figure 7 (Delghandi et al., 2022). Another benefit of targeting a slightly different region of the COI gene is that it may reduce the impact of potential polymorphisms or primer-template mismatches reported for universal primers such as LCO1490/HCO2198 (Sharma & Kobayashi, 2014). This specificity improvement is particularly

important for skipjack tuna, yellowfin tuna, and mackerel populations in the study area, where sequence divergence could compromise the universality of the Folmer primer set.

The Folmer primer is a standard primer used for DNA barcoding in metazoans. However, its universal nature can pose challenges during the PCR amplification process. Although designed to amplify a wide range of species, the Folmer primer is incompatible with certain species, necessitating modifications to enhance specificity (Geller et al., 2013). Universal primers are commonly used in forensic applications to amplify mitochondrial DNA sequences even when the nucleotide sequence is unknown (Verma & Singh, 2002). However, their use requires careful consideration to maintain specificity and prevent misidentification, particularly in wild-type species (Sarre et al., 2014). The success of DNA amplification using the PCR method primarily depends on primer design. Primers derived from closely related species tend to perform more effectively due to lower sequence mismatches with the target species. Studies in poultry have demonstrated a positive correlation between genetic similarity and PCR success (Primmer et al., 2005; Housley et al., 2006).

Primers with a length of 16–20 bases, particularly those targeting regions containing tetranucleotide repeats, are more effective in amplifying polymorphic sequences across multiple species (Gupta et al., 1994). Additionally, the designed primer's melting temperature (Tm) plays a crucial role in ensuring successful amplification and minimizing non-specific amplification (Delghandi et al., 2022). The quality of the sample also affects amplification success (Vidya & Sukumar, 2005). DNA fragmentation may occur in degraded samples, potentially leading to the loss of alleles from the target gene. The amplification process may fail if this occurs in the primer binding region. Therefore, proper sample collection and storage methods must be carefully considered.

The alignment of sequences from multiple target species during primer design is another critical factor influencing DNA amplification success. Primers should be designed from conserved sequences within the target species. In this study, global alignment was conducted using Primer-BLAST software, which ensures primer specificity and reduces the likelihood of non-specific amplification. Moreover, this software minimizes the risk of primer dimer formation, thereby increasing the probability of success in subsequent experimental stages (Banaganapalli et al., 2019).

CONCLUSION

The selected primer design, based on the results generated from NCBI Primer-BLAST, is Primer Pair one, with the forward primer sequence (5'–3') is GGCCCATGCCTTCGTAATGA, and the reverse primer sequence (5'–3') is GCAGGGTCGAAGAAGGTTGT. This primer pair was chosen due to its adherence to the ideal primer criteria and its minimal likelihood of secondary structure formation (such as dimers and hairpins). The start codon for primer pair one is located at 146–165 bp, and the stop codon is at 657–638 bp. The estimated length of the PCR product, as determined by the Primer-BLAST process at NCBI, is 512 bp. The melting temperatures for the forward and reverse sequences are 60.47 °C and 60.25 °C, respectively, indicating that an appropriate annealing temperature for PCR is 55 °C. The amplification results suggest that this temperature is optimal; however, the presence of smearing indicates a need for optimization of the PCR reagent components. It is important to note that optimization of components such as dNTP or MgCl cannot be performed, as the PCR Mix product is pre-mixed in a single vial.

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