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Research Article

Optimization of Genetic Material Extraction Techniques and Application of Isothermal Amplification Method for Field Authentication of Two Thresher Sharks (*Alopias pelagicus* and *Alopias superciliosus*)

Vatia Dwi Rizki Ramadhani Aldina[1], Asadatun Abdullah[1*], Puji Rianti[2], Agoes Mardiono Jacoeb[1]

[1]*Department of Aquatic Product Technology, Faculty of Fisheries and Marine Science, IPB University, Bogor 16680, Indonesia*
[2]*Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor 16680, Indonesia*

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ABSTRACT

The pelagic thresher shark (*Alopias pelagicus*) and bigeye thresher shark (*Alopias superciliosus*) are important shark species for Indonesia's consumption and finning industry. Both Alopias species are included in the CITES appendix II, thus requiring certain documents for trading. Regarding species identification for on-site application, the DNA isolation method is a crucial step. In this study, we developed a DNA isolation method suitable for on-site application based on isothermal amplification (LAMP) and species-specific COI gene markers. Three different extraction methods were applied, namely modified spin column kits and dipsticks. The quality of DNA was evaluated and tested for isothermal amplification using a reference sample, fresh fillet, and ethanol-preserved sample. The extracted sample concentration was in the range of 135.35-0.65 ng/µL. The LAMP test showed that three different DNA extraction methods successfully amplified the DNA fragments through the color changes at the end point of the LAMP reaction. The LAMP test was also sufficient to detect less than 10 ng of DNA from *A. pelagicus* and *A. superciliosus* within 30-50 min. The DNA from the modified spin column and dipstick extraction method combined with LAMP can potentially be used to detect Alopias pelagicus and Alopias superciliosus species on-site.

1. Introduction

The pelagic thresher shark (*Alopias pelagicus*) and bigeye thresher shark (*Alopias superciliosus*) are two species found in Indonesian waters and included in the endangered species list (White *et al.* 2006). Based on the International Union of Conservation of Nature (IUCN), the pelagic and bigeye thresher sharks are classified as endangered and vulnerable, respectively (IUCN 2023). Both sharks are listed under The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) appendix II, meaning their international trade is regulated (CITES 2023). In Indonesia, the capture or trade of thresher sharks (Alopiidae family) is prohibited (Permen KP 2012), and fishery products are prohibited (Permen KP 2019). However, shark trading in Indonesia can be formed as a shark product in various ways, such as grilling, salting, drying, or as an ingredient in various dishes, making it challenging to authenticate the type of shark species used (Ho *et al.* 2020). Therefore, comprehensive efforts are needed to overcome this problem, one of which is by applying the DNA approach (Hellberg *et al.* 2019).

DNA testing can be employed to identify the fish species used, even in processed products, since morphological features are lost during the processing (Neo *et al.* 2022). Several studies have been reported in authentication using the DNA approach in shark

* Corresponding Author
E-mail Address: asabdullah@apps.ipb.ac.id

products (Henderson *et al.* 2016; Cardeñosa *et al.* 2018; Abdullah *et al.* 2020). Thus, the methods generally rely on laboratory settings, meaning the samples must be transferred to a laboratory for analysis. In Indonesia, based on Permen KP (2021), every transportation of fish species resulting from domestic capture must be accompanied by SAJI-DN (*Surat Angkut Jenis Ikan Dalam Negeri*). Therefore, field-based authentication is urgently needed.

The loop-mediated isothermal amplification (LAMP) method is suitable for field detection. LAMP has gained significant attention due to its high specificity and simplicity. LAMP was first described by Notomi *et al.* (2000). The method applies the principle of strand displacement with an amplification reaction that works under isothermal conditions (60-65°C) for 1 hour. Due to its ability to work at isothermal temperatures, LAMP can be performed using portable equipment, such as a thermo-block, making it well-suited for point-of-care testing. In recent years, LAMP has been applied in various fields involving authentication seafood products, such as eel (Spielmann *et al.* 2019), salmon (Xiong *et al.* 2020), and tuna (Ali *et al.* 2022). A crucial step in molecular methods, including the LAMP technique, is to obtain high-quality genetic material. The quality of extracted DNA significantly impacts the success of downstream molecular techniques (Ruggieri *et al.* 2016).

The fundamental steps of isolating DNA, regardless of the technique and protocol used, are lysis, purification, and DNA recovery. The availability of commercial DNA extraction kits has played an essential part in obtaining high-quality DNA for specific types of samples and objectives (Lee dan Shewale 2017). The commercial DNA extraction kits have ready-to-use reagents and provide the protocol instructor's guidance. However, to achieve reliable and consistent results, it is crucial to carefully follow the specific protocol offered by the DNA extraction kit. It requires laboratory equipment, such as weighing the sample, incubating with constant time and temperature, and using a high-speed centrifuge.

Additionally, most commercial DNA extraction kits are not easily accessible and affordable (Goldberg and Goldberg 2015). Therefore, developing DNA extraction techniques that can be applied is crucial for field-based testing, making it simple and easily performed by an untrained technician. This research aims to optimize DNA extraction techniques that can be applied in field-based testing and use the LAMP technique to detect *Alopias pelagicus* and *Alopias superciliosus* in shark products.

2. Materials and Methods

2.1. Sample Collection

We obtained the collected samples from two different fishing grounds (FAO 71 and FAO 57). We collected samples from *Alopias pelagicus* and *Alopias superciliosus* as targeted detecting species, along with *Prionace glauca, Carcharhinus falciformis, Isurus oxyrinchus, Rhynchobatus australiae, Sphyrna lewini* as compared in this study (Table 1). The samples were stored at -20°C until further analysis.

2.2. DNA extraction

2.2.1. Commercial kit

We used a Genomic DNA mini kit (Geneaid, New Taipei City, Taiwan) to extract DNA samples following the manufacturer's protocols. Cut the sample (20-30 mg), place it in a 1.5 ml tube, and crush it with a micro pestle. 200 µL of GT Buffer and 20 µL of Proteinase K were added and incubated at 60°C for 30 minutes. Afterward, 200 µL of GBT buffer was added, vortexed, and set at 60°C for 20 minutes. After incubation, the sample was centrifuged at 16,000 g for 2 minutes, and then the clear supernatant was transferred into a fresh tube containing 200 µL of absolute ethanol. The mixture sample was transferred to the GS column in a 2 ml collection tube and centrifuged at 16,000 g for 2 minutes. GS column

Table 1. List of shark species used in this study

Sample code	Species	Common name	IUCN/CITES	Type of sample
APL	*Alopias pelagicus*	Pelagic tresher	En/II	Alcohol-preserved
ASC	*Alopias pelagicus*	Bigeye tresher	Vu/II	Alcohol-preserved
CAF	*Carcharhinus falciformis*	Silky shark	Vu/II	Alcohol-preserved
I7	*Isurus oxyrinchus*	Shortfin mako shark	En/II	Alcohol-preserved
RAS	*Rhynchobatus australiae*	Bottlenose wedgefish	CE/II	Alcohol-preserved
SL	*Sphyrna lewini*	Scalloped hammerhead	CE/II	Alcohol-preserved
PG	*Prionace glauca*	Blue shark	NT/-	Alcohol-preserved

was transferred to a new 2 ml collection tube, 400 µL of W1 buffer was added, centrifuged at 4,000 rpm for 2 minutes, and discard the flow-through. Then 600 µL of wash buffer was added, centrifuged for 2 minutes at 16,000 g, and discarded the flow-through. The GS column was centrifuged again at 16,000 g for 3 minutes. The GS Column was placed in a new 1.5 tube, adding 50 µL of pre-heated elution buffer. The tube was centrifuged at 4,000 rpm for 2 minutes to elute the purified DNA.

2.2.2. Commercial Modified kit

We used a Genomic DNA mini kit (Geneaid, New Taipei City, Taiwan) to extract DNA samples with modified several extraction steps based on the kit protocol instructor's guidance as mentioned in the previous paragraph, including sample dissociation, incubation, and centrifugation process. For sample dissociation, we cut the sample at 2 cm. We also used a mini-portable water bath for the incubation process, using the incubation condition according to the manufacturer's protocols. We employed a centrifugation tool with a speed of 4,000 rpm for the centrifugation process. Initially, we measured the rotor length using the centrifugation tool. Subsequently, the rotor's conversion of centrifugation speed from g force to revolutions per minute (RPM) was carried out using a G-force calculator (https://www.sigmaaldrich.com/ID/en/support/calculators-and-apps/g-force-calculator).

2.2.3. Dipstick Method

In this study, we used a dipstick DNA isolation protocol, using three different extraction and wash buffers (Table 2). The DNA isolation protocol was based on Zou *et al.* (2017) with some modifications. A 1.5 ml tube was filled with 800 µL of wash buffer, and a PCR tube was filled with 20 µL of TE buffer. A 30 mg sample (0.5 cm) was placed in a new 1.5 ml tube, crushed with a micro pestle, and filled with 100 µL of extraction buffer. The mixture was then crushed until the color of the solution changed. Added 400 µL extraction buffer and manually inverted the tube for 1 minute. The dipstick was dipped five times in the extraction buffer, ten times in the wash buffer, and three times in the TE buffer. The white part of the dipstick was cut and put in the elution buffer until submerged. The tube was vortexed for 1 minute.

Table 2. The extraction and wash buffer of a dipstick method was used in this study

Extraction buffer	Wash buffer	Ref.
20 mM Trish (pH 8), 25 mM NaCl, 2.5 mM EDTA, 0.05% (wt/vol) SDS, 2% (wt/vol) PVP-K30	Tris-base 10 mM Tris pH 8	Mason and Botella 2020
0.4 M Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 0.15 M NaCl, 0.1% (vol/vol) SDS	Steril water 100 ml (pH 7.0)	Hammouda *et al.* 2019
1% SDS (vol/vol) and 0.5 M NaCl	10 mM Tris (pH 8.0)	Margam *et al.* 2010; Zou *et al.* 2017

2.3. Species-specific LAMP Primer design

The primers were designed using the NEB LAMP Primer Design Tool (https://lamp.neb.com/#!/) based on the cytochrome oxidase I (COI) gene sequence of *Alopias pelagicus* and *Alopias superciliosus*. Initially, the DNA sequence was downloaded from Genbank NCBI (https://www.ncbi.nlm.nih.gov/). Primers were also evaluated by OligoAnalyzer tool (https://www.idtdna.com/pages/tools/oligoanalyzer) and NCBI Primer Basic Local Alignment Tool (BLAST) (www.ncbi.nlm.nih.gov/blast) to ensure the quality and specificity of primer pairs for LAMP. Each set of primers consisted of two outer primers (F3 and B3), two inner primers (FIP primer consisted of the complementary sequences of F1c and F2 and BIP primer consisted of B1c and B2), and or two loop primers (LF and LB) Table 3.

2.4. LAMP Reaction

For the colorimetric LAMP reaction, the WarmStart Colorimetric LAMP 2X Master Mix (NEB, England) was used. This kit contains phenol red, a pH indicator that changes color from pink to yellow. The LAMP reaction was performed in a mini portable water bath in the volume of 12.5 µL, consisting of 8 µL of WarmStart Colorimetric LAMP 2X Master Mix, 2.5 µL of Primer Oligo, 2 µL of DNA template. Each set of primers contained FIP (Forward Inner Primer) and BIP (Backward Inner Primer) at 1.6 µM each, F3 (Forward Outer Primer) and B3 (Backward Outer Primer) at 0.2 µM each, and LB (Forward Loop and Backward Loop) at 0.4 µM. Amplification was carried out at 70°C for around 30-50 minutes.

3. Results

3.1. Species-specific Primer of *Alopias pelagicus* and *Alopias superciliousus*

Six oligonucleotides recognize eight distinct regions on the target sequence as LAMP primers. These primers

consist of two outer primers (F3 and B3), two inner (FIP and BIP), and two loop primers (LF and LB). The designed primer LAMP for Alopias pelagicus and Alopias superciliosus applied in this study is presented in Table 1. The location of the LAMP primers' binding sites on COI for both species is shown in Figure 1.

3.2. DNA Isolation Protocols and Gel Electrophoresis

DNA was extracted using various isolation protocols listed in Table 4. The concentrations of DNA were comparable within the same isolation protocol, as shown in Table 4. Isolation using a commercially

Table 3. The LAMP primers of the thresher shark selected and used in this study

Species	Primers	Sequence (5'-3')
Alopias pelagicus	F3	TAAGCCTCTTAATTCGAGCC
	B3	AGGGGGGAAGGAGTCAAAAG
	FIP	GCATGGGCGGTTACAATAACATTGAATTAGGACAGCCAGGAT
	BIP	CCGTAATAATTGGCGGATTTGGCGTGGAAAAGCTATGTCTG
	LB	CTGACTAGTGCCATTAATAATTGG
Alopias superciliosus	F3	CCTAGGGGATGATCAGGTCTA
	B3	CTGTTCAACCAGTGCCAGCT
	FIP	CAAATCCCCCAATTATTACGGGTATTATCGTAACCGCCCATG
	BIP	GCCTTCCCGCGAATAAATAAGCTGAAGCTAGGAGTAAGAGA
	LB	CTTTTGACTCCTTCCCCCTTC

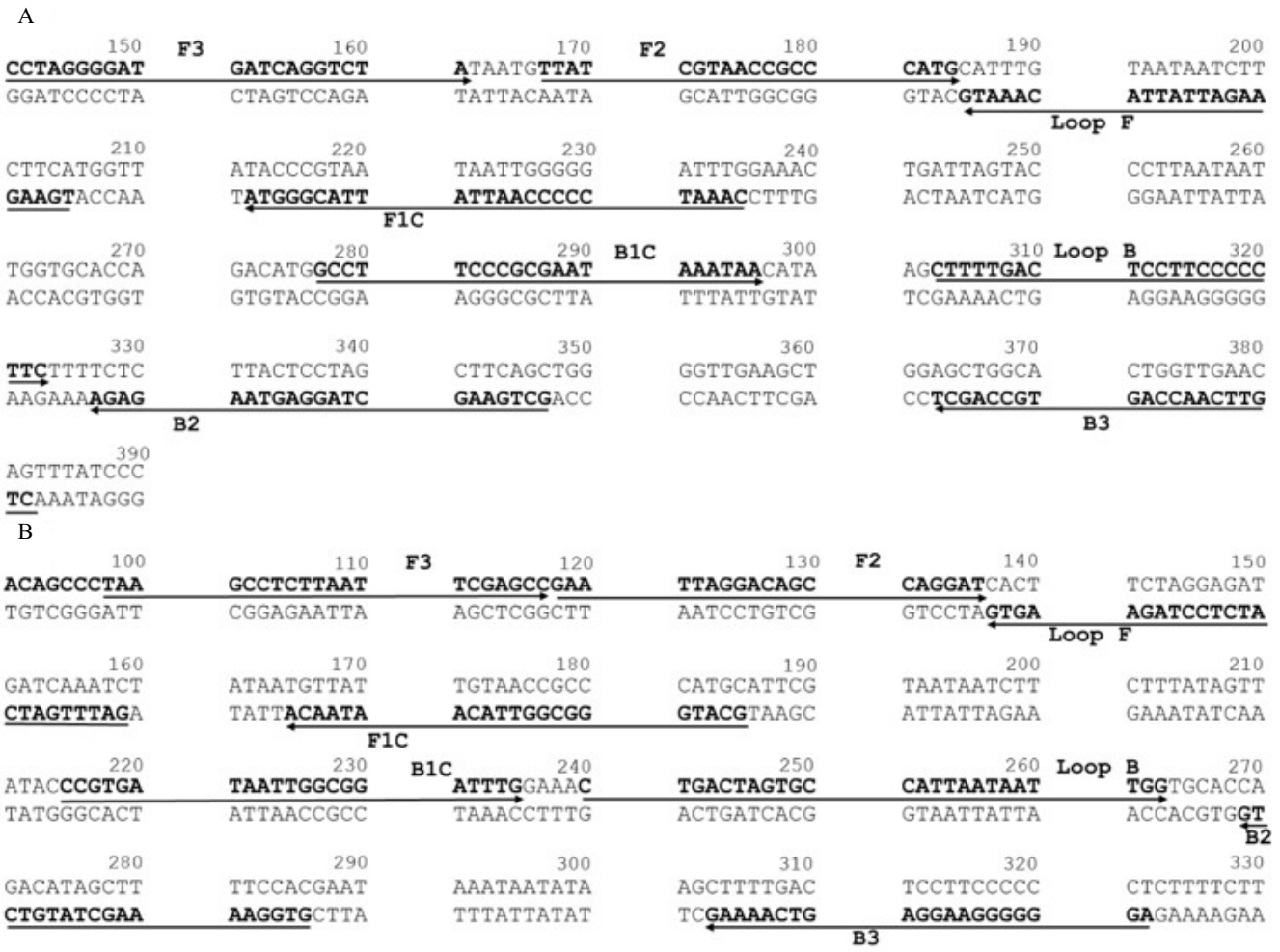


Figure 1. Oligonucleotide primers used for LAMP amplification of *Alopias supeciliosus* using mtCOI (GenBank accession number 021443.1) (A) Oligonucleotide primers used for LAMP amplification of *Alopias pelagicus* using mtCOI (GenBank accession number KF412639). (B) The underlined letters indicate the sequences of primerss

modified protocol was also carried out for *Isurus oxyrinchus, Rhynchobatus australiae, Carcharhinus falciformis*, and *Prionace glauca*, with concentration values of 13.60, 20.75, 63.10, and 5.05, respectively.

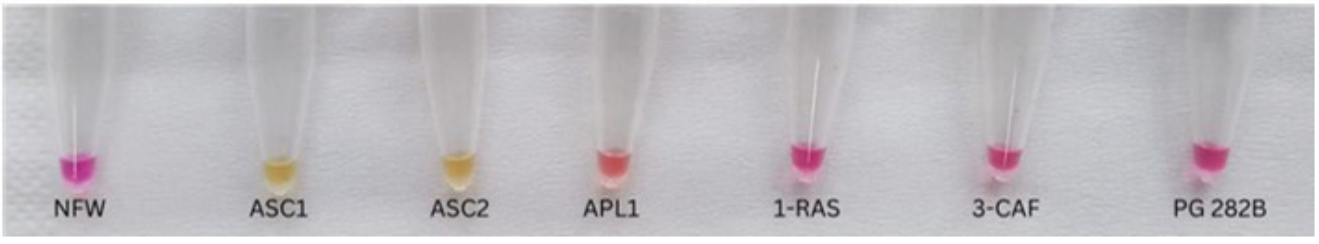
3.3. Identification of *Alopias superciliosus* and *Alopias pelagicus* using the Method of LAMP Reaction

The determination of the targeted species was carried out using the LAMP reaction method. In this study, primer sets were initially designed for the COI gene of *Alopias superciliosus* (Figure 1). The result showed that the optimal amplification of LAMP results obtained for the *Alopias superciliosus* varied depending on the extraction DNA method. The LAMP reactions were optimized for 30 minutes at 70°C for DNA templates from a commercial kit. LAMP amplified the DNA from the commercial modified kit with the optimal incubation results at 70°C for 40 minutes. Isothermal amplification of template DNA from dipstick method optimized at 70°C for 50 minutes. The results indicate that the primers for Alopias superciliosus successfully amplified the DNA template of *Alopias superciliosus*, as evidenced by the color change from red to yellow (Figure 2). Furthermore, to validate the specificity of the designed primers, the reaction was tested with DNA templates from various other species, including *Alopias pelagicus, Rhynchobatus australiae, Carcharhinus falciformis*, and *Prionace glauca*. Additionally, a negative control using nuclease-free water was included in the experiment. The result showed no color change in the tubes containing DNA templates from other shark species or the negative control (nuclease-free water), indicating that the designed primers did not cause amplification or the absence of DNA in these non-target species.

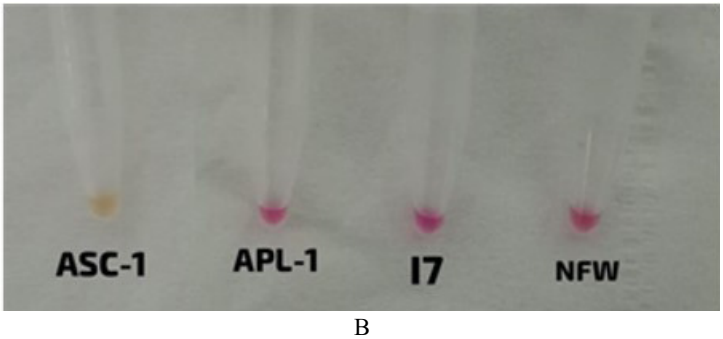
Table 4. DNA concentration measured with the different methods of DNA isolation protocols

Sample code	n	Commercial kit (ng/µL)	Commercial modified kit (ng/µL)	Dipstick (ng/µL)		
				Mason and Botella 2020	Hammouda *et al.* 2019	Margam *et al.* 2010; Zou *et al.* 2017
APL	4	132.50	132.50	-	-	2.200
ASC	6	111.6	111.6	-	0.750	5.150

n: number of specimens



A



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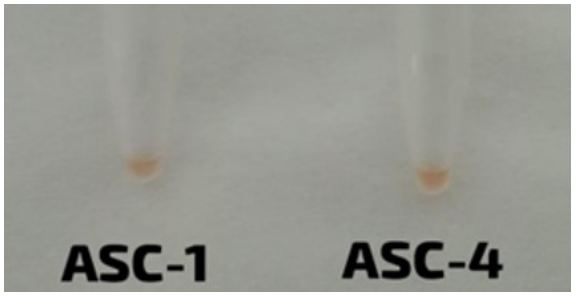

C

Figure 2. The results of specificity of LAMP Alopias supercilious using COI gene towards non-target sample using DNA templates from commercial kit (A) commercial modified kit (B) dipstick (C) methods. APL-1: *Alopias pelagicus*, 1-RAS: *Rhynchobatus australiae*, 3-CAF: *Carcharhinus falciformis*, PG 282B: *Prionace glauca*, I7: *Isurus oxyrinchus,* and negative control or NFW: Nuclease Free Water

The LAMP reaction method was used to determine the targeted species. Initially, primer sets were designed for the COI gene of Alopias pelagicus (Figure 1). The LAMP assays showed positive results for *Alopias pelagicus* DNA templates using various DNA extraction methods. However, the reference isolates yielded negative results. The LAMP Amplification was carried out at a constant temperature of 70°C in a dry bath. At the same time, the incubation time varied depending on the DNA extraction method used (Figure 3). The incubation times for LAMP were 30, 35, and 50 minutes when using DNA templates from commercial kits, commercial modified kits, and dipstick methods.

4. Discussion

Loop-mediated isothermal amplification (LAMP) is a well-suited method for species identification due to its simplicity, cost-effectiveness, and rapid analysis. This method utilizes specifically designed inner and outer primers, leading to high specificity in detecting and amplifying DNA sequences. This method likely relies on the unique DNA sequences specific to each species and has designed LAMP primers that target these specific sequences. As an isothermal amplification technique, the LAMP reaction works at a constant temperature and does not require a thermal cycler, making it advantageous in field applications or settings with limited equipment (Lee *et al.* 2017). In this study, we applied the LAMP method to authenticate *Alopias pelagicus* and *Alopias superciliosus* in fish products, which could be valuable in food authenticity verification and ensure accurate species identification in fish products. We also presented several rapid DNA isolation methods to provide suitable DNA extraction methods explicitly tailored for the LAMP reaction, intending to establish on-site applications.

The primers designed for the LAMP reaction for *Alopias pelagicus* and *Alopias superciliosus* sequence were tested for specificity using the NCBI BLAST bioinformatics application. Six types of primers were designed to target distinct regions within the DNA of *Alopias pelagicus* and *Alopias supercilious*. These regions were identified based on their suitability for amplification to the target DNA sequence (as shown in Figure 1). The primers were classified into three groups, each playing a specific role in the amplification process. The outer primers (F3 and B3) initiate the LAMP reaction by binding to the target DNA sequence. The inner primers (FIP and BIP) facilitate the synthesis of the new DNA strands. The loop primers (LF and LB) enhance the LAMP reaction, amplifying it faster. (Notomi 2000).

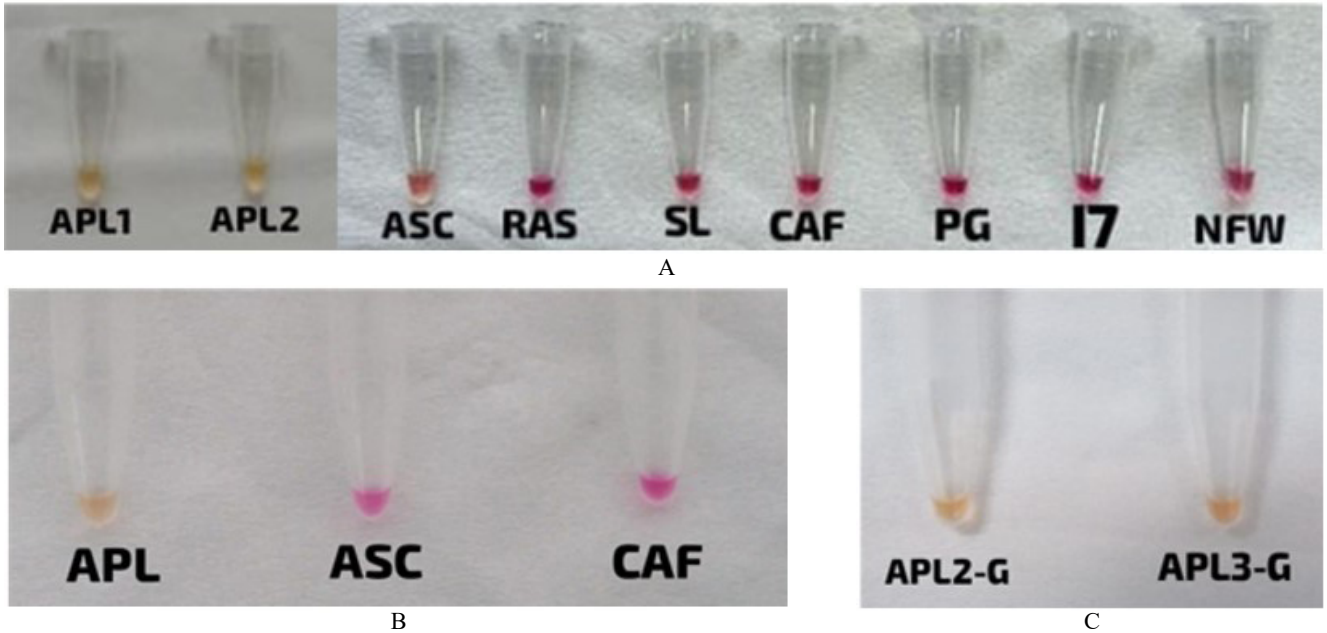

Figure 3. The results of specificity of LAMP Alopias pelagicus using COI gene towards non-target sample using DNA templates from commercial kit (A) commercial modified kit (B) dipstick (C) methods. (ASC: *Alopias superciliosus*; RAS: *Rynchobatus australiae*; SL: *Sphyrna lewini*; CAF: *Carcharhinus falciformis*; PG: *Prionace glauca*; I7: *Isurus oxyrinchus* and negative control or NFW: nuclease-free water)

All DNA extraction was used successfully in this study to amplify the fragment DNA using LAMP assay. The specific primer sets have been designed to target the COI gene of *Alopias pelagicus* and *Alopias superciliosus*. LAMP reaction. Figures 2 and 3 in the study depicted successful amplification reactions for the target sequences of *Alopias pelagicus* and *Alopias superciliosus* COI genes, respectively. To conduct the LAMP reaction, the researchers subjected the reaction product to 70°C within 30-50 minutes, which resulted in a color change. The change in color from red to yellow directly indicated a successful amplification reaction for the target sequences of these genes, enabling a straightforward and observable means of confirming positive LAMP results. Phenol red was an indicator of the amplification process, which changed color from red to yellow upon successful amplification. This color change provided a clear and easily distinguishable visual cue for amplification (Rabe and Cepko 2020).

In conclusion, this study successfully developed specific-species primers targeting the COI gene of *Alopias pelagicus* and *Alopias superciliosus*. These primers were used to detect the species using a phenol red colorimetric LAMP assay. Furthermore, the study also used various DNA extraction methods to isolate genomic DNA from tissue samples that will be useful in on-site applications for detecting and identifying these species.

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