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

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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

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

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.

loop-mediated isothermal The 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 μ L of GT Buffer and 20 μ L of Proteinase K were added and incubated at 60°C for 30 minutes. Afterward, 200 μ L 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 μ L 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

	1 5			
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 μ L of W1 buffer was added, centrifuged at 4,000 rpm for 2 minutes, and discard the flow-through. Then 600 μ L 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 μ L 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-forcecalculator).

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 μ L of wash buffer, and a PCR tube was filled with 20 μ L of TE buffer. A 30 mg sample (0.5 cm) was placed in a new 1.5 ml tube, crushed with a

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

micro pestle, and filled with 100 μ L of extraction buffer. The mixture was then crushed until the color of the solution changed. Added 400 μ L 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.

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 μ L, consisting of 8 μ L of WarmStart Colorimetric LAMP 2X Master Mix, 2.5 μ L of Primer Oligo, 2 μ L of DNA template. Each set of primers contained FIP (Forward Inner Primer) and BIP (Backward Inner Primer) at 1.6 μ M each, F3 (Forward Outer Primer) and B3 (Backward Outer Primer) at 0.2 μ M each, and LB (Forward Loop and Backward Loop) at 0.4 μ M. 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

Alopias pelogicus F3 TAAGCCTCTTATTCGAGCC B3 AGGGGGAGAGGAGTCAAAAG FIP GCATGGGCGTTACAATAACATTGAGACAGCAGCCAGGAT BIP CCTGACTAATAATTGGCGGATTGGCCGTGAAAAGCTATGTCTG LB CTGACTAGGCGGATATAATTGG Alopias supercitiosus F3 CCTAGGGGGATGACCAGTCA BIP CCTAGCGGCAGTTAACCGGCCACGT FIP CAAATCCCCCCAATAAATAGCGGAACGAGGCAACGAGGAGAAAGGAAGG	Species		Pr	Primers Sequence (5'-3')								
B3 AGGGGGAAGGAGTCAAAAG FIP GCATGGGCGGTAAATAACATTGAATTAGGACAGCCAGGAT BIP CCGTAATAATTGGCGGGTGAAAAAGGAGCAGCAGCAGGAT Alopias superciliosus F3 CCTGGCGGATGACGGGTGTAAGGGTTTGGCAGGCTA F1P CCAAATCCCCCCATTATAATATTGG CCTAGGGGAT F3 GATCAGGTCT F1P CCAATCCCCCCATTATGACTGGTAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG	Alopias pelagicus F3				TAAGCCTCTTAATTCGAGCC							
FIP GCATGGGCGGTTACAATAACTGGCGGATTGACATAAGCAGCCGCGGAA BIP CCGTAATAATTGGCGGATTGGCGGAAAAGCTATGTCTG LB CTGACTAAGTGCCATTAATAATGGCGGATTGGCCGGAAAAGCTAGGTGTCG Alopias superciliosus F3 CCTAGGGGATGACAGGTGTA B3 CTGTTCAACCAGTGCCAGGT F1P CAAATCCCCCCAATTATTACGGGATTACGTAACCAGGCCAGG F1P CAAATCCCCCCAATTATTACGGGATAACAGGGGAGTAAAGAGAGAA LB CTTTTGACTCCTCCCCGGAATAATAAGGGTGTAACCAGCCCCAGG TAATAACCGCCCAATTAT GGTCAGGGGAT GATCAGGTCT ATAATGTTAT GCATGGCGG CCTAGGGGGAT CTAGTGCGAGA TAATTGTAT GCATGGCGG GGATCAGGTCT ATAATGGGGG ATATTAGAATA GCATGGCGG CTAGGGGAT ATACCCGTAA TAATTGGGGA ATATATGAATA GGATCAGGTCA TAATGGCGAT ATATAGGGGG ATTTGGAACCTAG GATCAGGTA TAATGCCGTAA TAATGGGGA ATATATAGGGG CTTCATGGT ATAACCCGTAA TAATTGGGGG ATTTGGAAACTAG GATCAGGTGT ATAATGGGGGA TGGTGCACCA GACTTGCCGAA GACCACCA GACTGCCT TCCCCCGAAT TTTAGCCTG GACCACCA GACTGCCC AGGCCCTTA TTTATTGGAA GTGTACCGGA AGGCCCTAA TTAGCCCCGAA AGGCTTGAACC GACATAGCGCT TACGGCGCAA TCCCGCGAA TCCACGACC	1 1 0]	B3		AG	GGGGGGAAGGAG	GTCA.	AAAG			
BIP CCGTATATATTGGCGGATTTGGCGGGAAAAGCTATGTCTG LB CCGTATGGGGATGATCAGGTCTA B3 CCTAGGGGATGATCAGGTCTA B3 CCTGTCCACCGGGCGC FIP CCAACCGCCAGGCCCAGGGCGCCCAGGAGGAGAAAGCTAGGAGGAGAAAGGA LB CTTTGGCCCCCCATTATGCGGGATATATAGCGGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGA]	FIP		GC	ATGGGCGGTTA	CAAT	AACATTGAAT	TAGGA	ACAGCCA	.GGAT
LB CTGACTAGTGCCATTAITAATGG Alopias superciliosus F3 CCTAGGGGATGCATGATCAGGTCTA B3 CTGTTCAACCAGTGCCAGCT FIP CAAATCCCCCATTAITAGGGGATATAATGGAAGCAGGGAGAGAGAGAG BIP GCCTTCCCCGCAATAAATAAAAGCTGAAGCTAGGAGTAAGAGAG LB CTTTTGACTCCTTCCCCCCATTAT A CCTAGGGGAT ATAAGACTA ATAATGTATA GAACCAGGTA CACAGGCCT ATAATGTATA CGTAGGGGAT CACAGGCCT ATAATGTATA CGTAGGGGAT CACAGGCCT ATAATGATAA CCTAGGGGAT TACCCGTAA TAATGCTATA CGTAGGGGAT TACCCGTAA TAATGGGGG ATTTGGAAAC TATGGGCAT TATCACAATA GCATTGGCAG GTACGTAAC CATTAGTAC CTTCAAGGTT ATACCCGTAA TAATGGGGG ATTTGGAAAC CCTTAAGGAT TATGGGCAT TATTAACAATA GCATTGGAAC CTTCAAGGT TATCCCGTAA TAATGGGGG ATTTGGAAAC TGATGGCAA TATGGGCAT TATTAACCCCC TAAACCTTTG ACTAATCATG GGAATTATTA CGAGTACCCAA TATGGCGCT TCCCCGCGAAT ATTATGGAAC CCTCGGGCAT GACTGGCCC TCCCCGCGAA AGGCGCCTTA TTATTGTAT TGGTGCACCCA GACATGGCCC TCCCGCGAAT ATTATGGAAC CCCACGTGGT GTGTACCGGA AGGCGCCTA TTATGTGATA CCCACGTGGT GTGTACCGGA AGGCGCGCTA TTATGTGATA CCCACGTGGT GTGTACCGGA AGGCGCGCTA TTATGTGATA CCCACGTGGT GTGTACCGGA AGGCGCGCTA TTATGTGATA CCCACGTGGT GTGTACCGGA AGGCGCGCTA TTTATGTATA CGGAAACGCCC TCCCCGCGAA AGGCGCGCTA TTTATGTATA CGGAAACGCCC CCCACTTCGA CCCCCGCGAACGCC CCGGTGGAAC AGGTTGAACGCA AATGGGCCC CCAACTTCGA CCCTGGACGCA CTGGTGGAAC AGGTTGAACGG BC TGTCGGGGATT CCGGGGATTA AGCCGGCG CATGCTTCGA CCCTGGACGCA CTGGTGGAAC CCTGGGCCCTAA GCCCTTAAT TCGAACCTG GGCCCCCCCCCGCCCCTGGCA CTGGTGGAAC CCTGGCCCCTAA GCCCTTAAT AGCCCGC CATCCTCGA CCCTGGCCC CCGGGCCA AGGACATACCCC CCAACTTGCCC TAATGGCGC CATGCTTCGA CCCTGGTCGAAC ATGGGCCCTAA AATGGCGC CATGCTTCG CATTATATA AGCCCCCTA AGCCTTACCCCGGA TAATGGCGC CATGCTTGG CCATGCATCT TCTTGGGCGC CCTGGTGAACC ATATGGCGCC TAATGGCGC CATGCTTACC ATTATTAGAAACCTT CTTATGGAAACC TATGGGCCCT TAATGCCGC CATGATAACCTT CCTTCCCCCC CATTATATA ACCACCGGC CATGAAAACCTT CCTTCTCT CTGTATCCACGAAT AAACCTTG AACCTTTG ACTGACCG GTAATATATA ACCACCGGC GAAAAAACCC CTCTCTCCCCC CCTCTTTCT CTGTATCCACGAAT AAATAATATATA AGCTTTGGAAACC TCCTTCCCCC CCTCTTTCT CTGTATCCACAAAACCTT TCCCACGAAT AAATAATATATA AGCTTTGGAAAACC TCCTTCCCCCC CTCTTTCTC CTGTATCCACGAAAAACC CAAAACCTTG ACGACG]	BIP		CCO	GTAATAATTGGC	GGA	TTGGCGTGG	AAAA	GCTATGT	CTG
Alopias superciliosus B3 CCGTCACCGGATGATCACCTCTA B1P GCCTTCACCCGGATGACCAGCT FIP CAAATCCCCCAGTTATTACGGGATGATACGGCCCATG B1P GCCTTCCCGGGATAAAAAAAGCGGAAGCAAGAGAGA LB CTTTGGACCCCTTC GATCACCCTA GATCACGTCA ATAATACTTA GATCACCCTA CTAGGGGAT ATACCCTA GAACTCCCCAGA TATGGGGGAT ATACCCCTA CTCATGGCAGA ATACCCCCAA ATATACATA FIC CTCATGGGATT ATACCCCTAA CGAACTCCCCAA AGCTTTGAAC CCTCATGGCAA AGCTTTATACAA ACCACTGGC ATACCCCTAA CGAACTCCCCAA CCTCATGGCAA CCTCCGCCAA CCTCCGCCAA CCTCCGCCAA CCTCCGCCCAA CCTCATGGCAA CCTCCGCCCAA CCTCATGGCAA CCTCCGCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCAA CCTCCGCCCCAA CCTCCGCCCCAA CCTCCGCCCCAA CCTCCGCCCCAA CCTCCGCCCCAA CCTCCGCCCCAA CCTCCGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC]	LB		CTO	GACTAGTGCCAI	TAAT	CAATTGG			
A CTGTICCACCAGTGCTACCATTATACGGGGTATATCGTAACCGCCCATG BIP CCAAATCCCCCAGAATTATACGGGTATATCGTAACCGCCCATG BIP CCTTAGGGGAT GATCAGGTCT GATCAGGTCT CTAGGGCGAT ATAATCGTTATA GATCAGGTCT ATAACCGTA CTTCAGGTCAGA CTTCAGGTCAGA CTTCAGGTCAGA CTTCAGGTCAGA CTTCAGGTCAGA CTTCAGGTCAGA CTTCAGGTCAGA CTTCAGGTCAGA CTTCAGGTCA CTCAGGCGAT ATAACCGTA CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCAGGCGAT CTCCAGCGGA CTCCAGGCGA CTCCAGCGGA CTCCAGGCGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CTCCAGCGGA CCACCGCGGAA CCGGGGCGCA CCACCGCGGAA CCGGGGGCAT CCACGCCGC CCACCTTCGA CCCCGCCCGC CCACCTTCGA CCCCCCCGC CCACCTTCGA CCCCCCCGC CCACCTTCGA CCCCCCCCC CCACCTTCGA CCCCCCCCC CCACCTTCGA CCCCCCCCCCC CCACCTTCGA CCCCCCCCCC CCACCTTCGA CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	Alopias supercili	iosus]	F3				CAGG	ТСТА			
A LB CTTAGGGGAT A LB CTTTGACCCCTACCATTACTACAGCAGGAGTAAGAGA LB CTTTGACCCCTACCATTACTGAGCAGGAGTAAGAGA LB CTTTGACCCCTACCATTAG CAACCCCCTACCATTAG CAACCCCCTACCATTAG CATAGGGGAT A CAACCCCTAC CATCAGTCCAGA CATCAGGTCT ATAACCGTAA CAACCCCCTAC CATCAGTCCAGA CAACCCCCTACCATTAG CATAGGGGAT ATAACCGTAA CAACCCCTACCATTAG CATCAGGCAT ATAACCCGTAA CAACCCCTAC CATCAGTCCAGA CATCAGGCAT ATAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCCTAC CATCAGTCAGC CATCAGTCAGC CATCAGTCAGC CATCAGTCAGCA CATCAGTCA CAACCCCTAC CATCAGTCAGCAT ATAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CAACCCGTAA CACCCCTAA CGGAGAATTAA CGGAGAATAA CGCTCTAAC CGGAGAATAA CGCTCTAAC CGGAGAATAA CGCTCTAAC CGGAGAATAA CGGAGCTGCA CGGAGAATAA CGGAGCTGCA CGGAGAATAA CGGAGAATAA CGGAGAATAA CGGAGAATAA CGCTCTAAC CGGAGAATAA CGGAGAATAA CGCCCTAA CGGAGAATAA CGGAGAATAA CGCCCTAA CGGAGAATAA CGGAGAATAA CGCCCTAA CGGAGAATAA CGGAGAATAA CGCCCTAA CGGAGAATAA CGGAGAATAA CGCCCTAA CGGAGAATAA CGGAGAATAA CGCCCTAA CGGAGAATAA CGGAGAATAA CGCCCTAA CGGAGAATAA CGGAGAATAA CGCCCCTAA CGGAGAATAA CGCCCCTAA CGGAGAATAAA CGCCCCTAA CGGAGAATAA CGCCCCTAA CGGAGAATAA CGCCCCTAA CGGAGAATAA CGCCCCTAA CGGAGAATAA CGCCCCTAA CGGAGAATAAA CGCCCCTAA CGGAGAATAAAA CGCCCCTAA CGGAGAATAAAA CGCCCCCCC CATCAAACCG CATTAATAATCT CGTAACCGCC CATCAAACCG CATTAATAATAA CCCCCCCC CATCAAACCG CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC				B3		CTC	GTTCAACCAGT(JCCA	GCT			ATC
A CTTTGACTCCCCCCCTTC GATCAGGGAT GATCAGGTCT GATCAGGTCA CTAGGGCAT CTAGGCCCTA CTAGGTCCCCA CTTCAGGCAT CTAGTCCCCAGA CTAGTCCCCAGA CTAGTCCCCAGA CTAGTCCCCAGA CTAGTCCCCAGA CTTCAGGTT ATACCCGTAA CTAGTCCCCAGA CTTCAGGTT ATACCCGTAA CTAGTCCCCAGA CTTCAGGTT ATACCCGTAA CTTCAGGCACA CTTCAGGCACA CACACCGCA CTCCAGCCA CTTCCAGGCACA CACACCGCA CTTCCAGCCA CTTCCAGCCA CTTCCAGCCA CTTCCAGCCACA CTTCCAGCCACA CTTCCAGCCACA CTTCCAGCCACA CTTCCAGCCACA CTTCCAGCCACA CTTCCAGCCACA CTTCCAGCCACA CTTCCAGCCACA CTTCCACCGACA CTTCCAGCCACA CTTCCAGCCCA CCACCTCGAC CTTCCAGCCCA CCACCTCGA CTTCCAGCCCA CCACCTCGA CTTCCAGCCCA CCACCTCGACCCA CCACCTCGA CCACCTCGACCCA CCACCTCGA CCACCCCCA CCACCTCGACCCA CCACCCCCA CCACCCCCA CCACCCCCA CCACCCCCA CCACCCCCA CCACCCCCCA CCACCCCCCA CCACCCCCCA CCACCCCCCA CCACCCCCCA CCACCCCCCA CCACCCCCCA CCACCCCCCCA CCACCCCCCCC						CA				CGIAA		
A 150 F3 GATCAGGTCT ATATATGTTAT F2 180 190 200 CTAGGGGAT CAGACGTCA ATATATACTATAT GCGACCCC CATCCATTG TAATAAATCTT GATCACCTA CTAGTCCAGA TATTACAATA GCATTGGCGG GTACGTAAAC ATTATACAA CLOOP F 210 ATACCCGTAA TATATCGGGG ATTTGGAAAC TGATTAGTAC CCTTAATAAT CAAGTAACCAA TATACCCGTAA TATATCGGGG ATTTGGAAAC TGATTAGTAC CCTTAATAAT TGGTGCACCA GACATGGCCAT ATATAACCCC TAAACCTTTG ACTAATCATG GGAATTATA ACCACGTGGT GTGTACCGGA AGGGCGCTTA TTTATATGTAT TCGAAAACTG AGGAACTGA ACCACGTGGT GTGTACCGGA AGGGCGCTTA TTTATATGTAT TCGAAAACTG AGGAACTG AGGAAGGGGG 330 340 350 360 350 360 370 380 TTCTTTTCC TTACCCTAG CTTCAGCTGG GGTGGAAGCT GCACACTTG ACCAACTG AGGAACTG AGGAAGGGGG TCCAAATAGGGATC GAAGTCGACC CCAACTCGA CCTCGGACGCA CTGGTGAACG AAGAAAAGAAG AATGAGGATC GAAGTCGACC CCAACTTCGA CCTCGGACCGT GACCAACTTG B2 83 AGTTTATCCCCT B2 83 AGTTTATCCCCTA CCGGGAA TAGCCGCGAA TTAGGACACC CAGGATCACT TCTAGGAGAT GATCCAAATCG AGGAAATTA AGCTCGGCTT AATCCTGTCG GTCCTAGTCA ACAACCTTA ACACCCCTAA CCCTTAAT TCGAGCCGGAA TTAGGACACC CAGGATCACT TCTAGGAGAT TGTCGGGAT CGGAGAATTA AGCTCGGCT AATCCTGTCG GTCCTAGTCA ACAACCTCTA 160 110 F3 120 130 F2 140 150 CAACCCCTAA CCCTTAAT ACCTGGCCG CATGCGTTCG TAATAATCTT CTAGGAGAT TGTCGGGAT CGGAGAATTA AGCTCGCCC CATGCGTTCG TAATAATCTT CTATAGGAGA ACAATCGCCTA ACAATCGCC CAACGCCTTAATTATAGAA GAAATATCAA 160 170 180 CATGCGCTT AATCGCCC CATGCGTTCG TAATAATCTT CTATAGGAA ACAATCGCCTA ACAATCGCC CAACGCCTTAATTATAGAA GAAATATCAA 160 200 200 210 TAACCCCTGAA TAATGCCCC TAAACCTTTG ACCACCC CATGCATTCG TAATAATATCTT CTTATAGAA 160 ATAATGTAT ACAATGGCCC CAACGCCTTAACC ATTATTAGAA GAAATATCAA 160 ATTATGCGCG TTAATTGGAAAC TGCATAACG GTAATTATTA ACCACCGCC TAAATATCAA 170 CGAAAACG ATTAATGCCG CATGCATAGC ATTATTAGAA GAAATATCAA 170 GGCCACT ATTAACCGCC TAAACCTTTG ACTGACCCCC CATTATATATA ACCACCGCC TAAATATCAA 170 CGAAAACGT AATTAACCGC TAAATATTAA ACCACCGC CATGAACGG GTAACTATATTA ACCACCGCC TAAATATCAA ACCACCCCC CATTATTATAA ACCACCGCC CATTATTATA ACCACCGCC CATTATTATAA ACCACCGC CATTATTATA ACCACCGCC CATTATATTA ACCACCGCC CATTATTATAA			1			CT	TTGACTCCTTC		TAAUC I UAAU YTTC	CIAO	JAUIAAC	JAUA
A 150 F3 GATCAGGTCT ATATGTATT GATAGGTCT ATATGTATT GATAGGTCA CTATTGG GATCACTT GATAGGTC ATATAGTATT GATAGGTG CTA CTATAGGTCAGA TATATAGATA GCATTGGCGG GTACATAGG ATATATAGATA 210 220 230 240 CATGCATAGC TGATAGGTC CCTTAATAAT GAATTATAGGGGG ATTTGGAAAC TGATAGGTC CCTTAATAAT 210 220 230 240 CATGCATAGC TGATAGGTC CCTTAATAAT 310 LOOP F 200 F				LD		CI	IIIOACICCIIC					
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CCTAGOUGAT GARCAGOTET ATATTGUAT COTAGEOGC CARCHART ATATAGAT GGATCCCCTA CTAGTCCAGA TATTACAATA GCATTGCGC GTACGTAAC ATTATAGAA 210 220 230 240 250 CCTTAATAGA GAAGTACCAA TATACCCGTAA TAATGGGGG ATTATAGAA GACAATCACCA GGAATTATAAC TGGTGCACCA TATACCCGTA TATAACCTT ATAATACCTT ACTAATCATA GGAATTATAAC TGGTGCACCA GACAATGGCCT TCCCGCGGAAT BIC AAATAACATA ACCATTTGAC TCCTTCCCCCC 300 ACCACGTGGT GTACGTACCGAA AGGGGCGTTA TTTATTGAC TCCAAACTG AGGAAAGGGG 330 340 350 350 350 370 380 AGAAAAGAG AATGAGGATC GAAGTCGACC CCAACTTCGA CCTGGACCACT CTGGTGACAGCA AAGAAAAGAG AATGAGGATC GAAGTCGACC CAACACTTCGA CCTGGACCACT CTGGTGACAGCA CTGGTGACAGCA CTGGTGACAGCA CTGGTGACCGGA AGGAACGGAG 370 380 370 380 370 380 370 150 370 150	150	F3	160		170 ATAATCTTAT	F2	180		190		TAATAA	200
Schredeerik Charlesis Antrikesis Schribbes Antrikesis Antrikesis <td>CCIAGGGGAI</td> <td></td> <td>CTACTCCACA</td> <td></td> <td>TATTACAATA</td> <td></td> <td>CGIMACCGCC</td> <td></td> <td>GTACGTAAAC</td> <td></td> <td>ATTATT</td> <td>ACAA</td>	CCIAGGGGAI		CTACTCCACA		TATTACAATA		CGIMACCGCC		GTACGTAAAC		ATTATT	ACAA
210 220 230 ATATTGGGGG 240 250 260 CTTCATGGTT ATACCCGTAA TAATTGGGGG ATTTGGAAAC ACTAATCATC CCTCATATAATA GAAGTACCAA TATGGGCATT ATTAACCCCC TAAAACCTTG ACTAATCATC GGAATTATTA TGGTGCACCAA GACATGGCCT CCCCCGCGAAT TTTATATCATC ACTAATCATC AGGATGGCCCCCCCCCCCC ACCACGGGGT GGACTACCGGA AGGGCGCTTA TTTATTGTGAA AGGCTTTTGAC AGGAAAGGGGG 330 340 350 360 370 380 AAAAAAAGAG AAAGAAGCC CACACTGGCCA CTGGACACTG GGACAACTG AGGAAAAAGAG AAATGAGGATC GAAGTCGACC CCACACTTCGA CCTCGGACACT B2 TCCAAATGGCCAA TTAGGGCAACT CCAGGATCACT TCTGGAGGCA B3 GGCCTCTAAA AGCTCGGCAA TAAGGACACCC CAGGATCACT TCTAGGAGGA B3 100 110 F3 120 130 F2 140 150 ACGACCCTAA GCCTCTAAAT TCGAGCCGAA TAAGGACCACG CAGGACCACT TCTAGGAGACT TGTCGAGATT	GGAICCCCIA		CINGICONGA		INTINCANIA		GCAIIGGCGG		GIACGIAAAC	Loop	F	HOAA
CTTCATGGTT ATACCCCGTAA TAATTGGGGG ATTTGGAAAC TGATTAGTAC CCTTAATAAT GAAGTACCAA TATGGGCATT ATTAACCCCC TAAACCTTTG ACTAATCATG GGAATTAATA 270 GACATGGCCT TCCCGCGAAT 300 AATTAACCATG ACTAATCATG GGAATTAATA ACCACGTGGT GTGTACCGGA AGGGCGCTTA TTTATTGTAT TCGAAAACTG AGGAAGGGGG 330 340 350 360 370 380 TTGTTTTCTC TTACTCCTAG CTTCAGGGCACC CCAACTTCGA CTGGTGCACCG AGGAAGGGGG AAAAAAAGAG AATGAGGATC GAAGTCGGACC CCAACTTCGA CCTCGGACCGT GGACAACTG 390 AATGAGGATC GAAGTCGACC CCAACTTCGA CCTGGTCGAC CTGGTGCACCT 390 AATGAGGATC GAAGTCGACC CCAACTTCGA CCTCGGACCACT TCTGGGGCGCG GACCAACTT 390 AATAAGGGG AATGAGGACC CCAACTTCGA CCTCGGACCACT TCTGGGGCACT TCTGGGGCACT TCTGGGGCACT TCGGGGCGCAA TTAGGGACACC TCAGGACACT TCTAGGGAACACT TCTAGGGACACT TCTAGGGAAGACACT TCTAGGGACACT TCTAGGGAATA AGCTCGGGCA	210		220		230		240		250			260
GAAGTACCAA TATGGGCATT ATTAACCCCC TAAACCTTTG ACTAATCATG GGAATTATTA 270 280 280 290 BLC 300 AAATAACATA AGCTTTTGAC B 320 ACCACGTGGT GTGTACCGGA AGGGCGCTTA TTTATTGTAT TCGAAAACTG AGGAAGGGGG 330 340 350 360 370 380 AAATAACAG AATGAGGAC GAAGTCCTAG GGAACTCGAC CCACGTGGCA CTGGTGAACG AAAAAAGAG AATGAGGATC GAAGTCGACC CCACACTTCGA CCTGGACCGT GGACCAACTG B2 B3 B3 B3 B3 B3 B3 B3 ACGCCCTAA GCCTCTTAAT TCGAGCCCAA TTAGGACAGC CAGGATCACT TCTAGGAGAGT B3 CGGAGAATTA AGCTCGCCCAA TTAGGACAGC CAGGATCACT TCTAGGAGAGT ACAGCCCTAA GCCTCTTAAT TCGAGCCCAA TTAGGACAGC CAGGATCACT TCTAGGAGAGT GAGTACCAA GCCTCTTAAT TCGAGCCGCAA TTAGGACAGC CAGGATCACT TCTAGGAGAT ACAGCCCCCAA GCCCTCTAAT AGCTCGGCCAA TTAGGACAGC CAGGATCACT TCTAGGAGAT ACAGCCCCTAA GCCCTCTTAAT AGCTCGCCAA TTAGGACAGC CAGGATCACT TCTAGGAGAT	CTTCATGGTT		ATACCCGTAA		TAATTGGGGG		ATTTGGAAAC		TGATTAGTAC		CCTTAA	FAAT
270 280 290 B1C 300 AAATAACATA ACCACGTGGT GACATG <u>GCCT TCCCGCGCAAT TTTATTGTAT AGG<u>CTTTTGAC MGGAAGGGGG</u> 330 TGGTGCACCA GTGTACCGGA AGGGCGCTTA TTTATTGTAT TCGAAAACTG AGGAAGGGGG 330 TTACTCCTAG CTTCAGCTGG GGTTGAAGCT GGAGCTGGCA CTGGTTGAAC AAATAAGAG AATGAGGATC GAAGTCGACC CCAACTTCGA CCTCGACCGT GACCAACTTG B2 B3 B3 B3 B3 B3 B3 AGTTTATCCC TCGAAATAGGG GGCTCTTAAT TCGAGGCCGAA CTGGTGGCAC CCAACTTGGACC B3 B3 AGTTTATCCC GACCTCTTAAT TCGAGGCCGAC CCGAGGATCACT GACCACCTGA B3 B3 CGCTCTTAAT TCGAGCCCGAA TTAGGACAGC CAGGATCACT TCTAGGAGAT ACGCCCTAA GCCTCTTAAT TCGAGCCGCAA TTAGGACAGC CAGGATCACT TCTAGGAGAA TGCGGGGATT CGGAGAATTA AGCTCGCCGAA TTAGGACAGC CAGGATCACT TCTAGGAGAACT TGCGGGGATT CGGAGAATTA AGCTCGCCGAA TAGGCCCTA TCTAGGACACC AGATCCTCA GATCAATCC ATATGGCGGATTA AGCTCGCCGC AATATGACCGCC CATGGACATACCT TCTATAGGACACC </u>	GAAGTACCAA		TATGGGCATT	FIC	ATTAACCCCC		TAAAC CTTTG		ACTAATCATG		GGAATT	ATTA
TGGTGCACCA GACATG <u>GCCT TCCCGCGAAT BIC ANATALCATA AGC<u>TTTTGAC BOP TCCTTCCCCC ACCACGTGGT GTGTACCGGA AGGGCGCTTA TTTATTGTAT AGGAAAGGGGG AGGAAAGGGGGG 330 340 350 360 370 380 TTCTTTTCTC TTACTCCTAG CTTCAGCTGG GGTTGAAGCT GGAGCTGGCA CTGGTTGAAC AAGAAAAGAG AATGAGGATC GAAGTCGACC CCAACTTCGA CCTCGACCGT GACCAACTTG B2 B3 B3 B3 B3 B3 B3 AGTTTATCCC TCCAAAATAGGG GCCTCTTAAT TCGAGCCGAA TTAGGACAGC CAGGATCACT GACCAACTTG B3 B3 B3 B3 B3 B3 B3 B3 B3 AGTTTATCCC TCCAAATGGGG GCCTCTTAAT TCGAGCCGAA TTAGGACAGC CAGGATCACT TCTAGGAGAT AGCACCCCTAA GCCTCTTAAT TCGAGCCGGAA TTAGGACAGC CAGGATCACT TCTAGGAGAT AGCTCTCTCA AGCAGGGGATT CGGAGAATTA AGCTCGGCCT AATCGGCC CAGGATCACT TCTAGGAGAC CAGGATCACT GATCAAATCCT ATAATGGGG 120 130 F2 140 TCTTAGGAGAC CTTAGGAGAC GATCAAATGCGCT ATAATGGGG</u></u>	270		290	FIC	290	P1C	300		310	Loon	в	320
ACCACGTGGT GTGTACCGGA AGGGCGCTTA TTTATTGTAT TCGAAAACTG AGGAAGGGGG 330 340 350 360 370 380 TTCTTTTCTC TTACTCCTAG CTTCAGCTGG GGTTGAAGCT GGAGCTGGCA CTGGTTGAAC AAGAAAAGAG AATGAGGATC GAAGTCGACC CCACTTCGA CCTCGACCGT GACCAACTTG B2 B3 AGTTTATCCC TCAAATAGGG B AGTCTAAT BC B3 AGTTTATCCC TCAAATAGGG B AGTCTAAT CGAGAAGTAGA B AGTCTATATCCC TCAAATAGGG B C CAACTTCGA C CAGGATCACT CGAGAACTGA C CAGGATCACT CGAGAATTA AGCTCGGCTT AATCCGC CAGGATCACT TCTAGGAGAT TGTCGGGATT CGGAGAATTA AGCTCGGCTT AATCCTGTCG GTCCTAGTGA AGATCCTCTA CGGAGAATTAT AGCTCGGCT AATCCTGTCG GTCCTAGTGA AGATCCTCTA COOP F C CTAGTTTAGA TATTACAATA ACATTGGCGG GTACCTAAGC ATTATTAGAA GAAATATCAA F1C ATACCCCCGGA ATTA ACATTGGCGG GTACCTAAGC ATTATTAGAA GAAATATCAA C CATGATCAAAAT C CTAGTTAGGCACT ATTAACCGCC TAAATGGCGG GTACCTAAGC ATTATTAGAA GAAATATCAA C CATGATCGGCACT ATTAACCGCC TAAACCTTG ACTGATCAG GTAATTATTA ACCACGTGGT ATACCCCCGGA ATTA ACATTGGCAGAC TGACTAAGC ATTATTAGAA GAAATATCAA C C CATGATCAAAACCTTG ATAATGGCGG CTAATTAGCA CCACTAAGC ATTATTAGAA GAAATATCAA C C CATGATCAGGCACT ATTAACCGCC TAAACCTTG ACTGATCACG GTAATTATTA ACCACGTGGT C C C C C C C C C C C C C	TGGTGCACCA		GACATGGCCT		TCCCGCGAAT	BIC	AAATAACATA		AGCTTTTGAC	цоор	TCCTTC	CCCC
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TTCTTTTCTCTTACTCCTAGCTTCAGCTGGGGTTGAAGCTGGAGCTGGCACTGGTTGAACAAAGAAAAGAGAATGAGGATCGAAGTCGACCCCAACTTCGACCTCGACCGTGAACAACTTGB2B3AGTTTATCCCTCCAAATAGGGB3AGTTTATCCCTCCAAATAGGGB3B0100110F3120130F2140150ACAGCCCTAAGCCTCTTAATTCGAGCCGAATTAGGACAGCCAGGAT_CACTTCTAGGAGATACAGCCCTAAGCCTCTTAATTCGAGCCGAATTAGGACAGCCAGGAT_CACTTCTAGGAGATACAGCCCTAAGCCTCTTAATTCGAGCCGCAATTAGGACAGCCAGGAT_CACTTCTAGGAGATACAGCCCTAAGCCTCTTAATTCGAGCCGCAATTAGGACAGCCAGGAT_CACTTCTAGGAGATACAGCCCTAAGCCTCTTAATTCGAGCGGGTTAGGACAGCCAGGAT_CACTTCTAGGAGATACAGCCCGTGATATAATGTTATTGTAACCGCCCATGCTTCGTAATAATCTTCTTTATAGTTCTAGTTTAGATATTACAATAACATTGGCGGGTACCAAGTCCCATTAATATAATGAAATATCAAF1C220230B1C240250260LoopB270ATACCCCGTGATAATTGCGCGB1C240250260LoopB270ATACCCCGTGATAATTGGCGGB1C240250260LoopB270ATACCCCGTGATAATTGGCGGB1C240250260LoopB270ATACCCCGTGATAATTGGCGGB1C300310320330GACATACCGCA	330		340		350		360		370			380
AAGAAAAGAG AATGAGGATC GAAGTCGACC CCAACTTCGA CCTCGACCGT GACCAACTTG B2 B3 B3 B3 B3 B3 B3 B3 AGTTTATCCC TCAAATAGGG B3 B3 <td>TTC TTTTTCTC</td> <td></td> <td>TTACTCCTAG</td> <td></td> <td>CTTCAGCTGG</td> <td></td> <td>GGTTGAAGCT</td> <td></td> <td>GGAGCTGGCA</td> <td></td> <td>CTGGTT</td> <td>GAAC</td>	TTC TTTTTCTC		TTACTCCTAG		CTTCAGCTGG		GGTTGAAGCT		GGAGCTGGCA		CTGGTT	GAAC
B2 B3 AGTTTATCCC TCANATAGGG B 100 110 F3 120 130 F2 140 150 ACAGCCCTAA GCCTCTTAAT TCGAGCCGAA TTAGGACAGC CAGGATCACT TCTAGGAGAT TGTCGGGATT CGGAGAATTA AGCTCGGCT AATCCTGTCG GTCCTAGTGA AGAATCCTCTA CGGAGAATTA AGCTCGGCC CATGCATCG GTCCTAGTGA AGAATCTT CTAGTTAGA TATATGTTAT TGTAACCGCC CATGCATCG TAATAATCTT CTTATAGTT CTAGTTAGA TATTACAATA ACATTGGCGG GTACGTAAGC ATTATTAGAA GAAATATCAA FIC 220 TAATAGCGG ATTGGAAACC TGACTAGTGC CATGCATCAG GTAATTAATAT P CTAGGCCCT ATTAACGCC TAAACCTTG ACCGATGCG GTACGTAAGC ATTATTAGAA GAAATATCAA FIC 220 TAATGCGGG ATTGGAAAC TGACTAGTGC CATTAATAAT P CGATCACGTGA TAATGCCGG AATTGGAAAC TGACTAGTGC CATTAATAAT P 220 CATGCACCA TAAACCTTG ACCGATCAG GTAATTATTA ACCACGGGT TATGGGCACT ATTAACCGCC TAAACCTTG ACTGATCACG GTAATTATTA ACCACGTGGT TATGGGCACT ATTAACCGCC TAAACCTTG ACTGATCACG GTAATTATTA ACCACGTGGT 280 290 300 310 320 330 GACATAGCTT TTCCACGAAT AAATAATATA AGCTTTTGAC TCCTTCCCCC CTCTTTTCTT CTGTATCGAA AAGGTGCTTA TTTATTATAT TCGAAAACG AGGAAGGGGG GAGAAAAGAA	AAGAAA	-	AATGAGGATC		GAAGTCG ACC		CCAACTTCGA		CCTCGACCGT		GACCAA	CTTG
AGTTTATCSC TCAAATAGGG B 100 110 F3 120 130 F2 140 150 ACAGCCCTAA GCCTCTTAAT TCGAGGCCGAA TTAGGACAGC CAGGATCACT TCTAGGAGAT TGTCGGGATT CGGAGAATTA AGCTCGGCTT AATCCTGTCG GTCCTAGTGA AGATCCTCTA TGTCGGGATT CGGAGAATTA AGCTCGGCC CATGCATTCG TAATAATCTT CTTTATAGTA GATCAAATCT ATAATGTTAT TGTAACCGCC CATGCATTCG TAATAATCTT CTTTATAGTA GATCAAATCT ATAATGTTAT ACAATA ACATTGGCGG GTACGTAAGC ATTATTAGAA GAAATATCAA FIC 220 230 B1C 240 250 CATGATCG CATTAATAAT TGTGGCACCA TAATGGCGCA TAATGGCGG BACCTTTG ACTGATCG GTAATTATTA ACCACGTGGT TAATACCCGTGA TAATGCCGC TAAACCTTG ACTGATCG GTAATTATTA ACCACGTGGT TATGGGCACT ATTAACGCC TAAACCTTG ACTGATCACG GTAATTATTA ACCACGTGGT 280 290 300 310 320 330 GACATAGCTT TTCCACGAAT AAATAATATA AGCTTTTGAC TCCTTCCCCC CTCTTTCTT CTGTATCGAA AAGGTGCTTA TTTATTATAT TCGAAAACTG AGGAAGGGGG GAGAAAAGAA	200	B2								В3		
TCAAATAGGG B100110F3120130F2140150ACAGCCCTAAGCCTCTTAATTCGAGCCGAATTAGGACAGCCAGGATCACTTCTAGGAGATTGTCGGGATTCGGAGAATTAAGCTCGGCTAATCCTGTCGGTCCTAGTGAAGATCCTCTAGATCAAATCTCGGAGAATTAAGCTCGCCCATGCATTCGGTCCTAGTGAAGATCCTCTAGATCAAATCTATAATGTTATTGTAACCGCCCATGCATTCGTAATAATCTTCTTTATAGTTGATCAAATCTATAATGTTATTGTAACCGCCCATGCATTCGATAATAATCTTCTTTATAGTTCTAGTTTAGATATTACAATAACATTGGCGGGTACGTAAGCATTATTAGAAGAAATATCAAFIC220230B1C240250260LoopB CGTGCACCATGGTGCACCATATGGGCACTATTACCGCCTAAACCTTTGACTGATCACGGTAATTATAAACCACGTGGT280290300310320330GACATAGCTTTTCCACGAATAAATAATATAAGCTTTTGACTCCTTCCCCCTCTTTCTTCTGTATCGAAAAGGTGCTTATTTATTATATATAGCTTTTGACTCCTTCCCCCCTCTTTCTT	AGTTTATCCC											
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100110F3120130F2140150ACAGCCCTAAGCCTCTTAATTCGAGCCGAATTAGGACAGCCAGGATCACTTCTAGGAGATTGTCGGGATTCGGAGAATTAAGCTCGGCTTAATCCTGTCGGTCTACTAGTGAAGATCCTCTA160170180190200210GATCAAATCTATAATGTTATTGTAACCGCCCATGCATTCGTAATAATCTTCTTTATAGTTCTAGTTAGATATTACAATAACATTGGCGGGTACGTAAGCATTATAATCTTCTTTATAGTTCTAGTTAGATATTACCAATAACATTGGCGGGTACGTAAGCATTATATAGAAGAAATATCAAFIC220230B1C240250CATTAATAATAGAAATATCAATGGCTGCACCATATGGGCACTATTAACCGCCTAAACCTTGACTGATCACGGTAATTATAAACCACGGGGT280290300310320330GACATAGCTTTTCCACGAATAAATAATATAAGCTTTGGCCCTTCTCCCCCCTCTTTCTTCTGTATCGAAAAGGTGCTTATTTATTATATTCGAAAACTGAGGAAGGGGGGAAAAAGAA	В											
ACAGCCCTAAGCCTCTTAATTCGAGCCGAATTAGGACAGCCAGGATCACTTCTAGGAGATTGTCGGGATTCGGAGAATTAAGCTCGGCTTAATCCTGTCGGTCCTAGTGAAGATCCTCTA160170180190200210GATCAAATCTATAATGTTATTGTAACCGCCCATGCATTCGTAATAATCTTCTTTATAGTTCTAGTTAGATATTACAATAACATTGGCGGGTACGATTATATATATCTTCTTTATAGTTCTAGTTAGATATTACAATAACATTGGCGGGTACGATTATATATAATCTAGAAATATCAAFIC220230B1C240250260Loop B270ATACCCGTGATAATTGGCGGATTTGGAAACTGACTAGTGCCATTAATAATTGGTGCACCATATGGGCACTATTAACCGCCTAAACCTTTGACTGATCACGGTAATTATAACCACGTGGT280290300310320330GACATAGCTTTTCCACGAATAAATAATATAAGCTTTTGACTCCTTCCCCCCTCTTTCTTCTGTATCGAAAAGGTGCTTATTTATTATATTCGAAAACTGAGGAAGGGGGGAAAAAAGAA	100		110	F3	120		130	F2	140			150
TGTCGGGATT CGGAGAATTA AGCTCGGCTT AATCCTGTCG GTCCTAGTCA AGATCCTCTA 160 170 180 190 200 210 GATCAAATCT ATAATGTTAT TGTAACCGCC CATGCATCG TAATAATCTT CTTTATAGTT CTAGTTAGA TATTACAATA ACATTGGCGG GTACG ATTATAATCTT CTTTATAGTT CTAGTTAGA TATTACAATA ACATTGGCGG GTACG ATTATAATCTT CTTTATAGTT 220 230 B1C 240 250 260 Loop B 270 ATACCCGTGA TAATTGGCGG B1C 240 250 CATGATAAT TGGTGCACCA ATACCCGTGA TAATGGCCGC TAAACCTTTG ACTGATCACG GTAATTATA ACCACGTGGT ATACCACGTC ATTAACCGCC TAAACCTTTG ACTGATCACG GTAATTATA ACCACGTGGT 280 290 300 310 320 330 GACATAGCTT TTCCACGAAT AAATAATATA AGCTTTTGAC TCCTTCCCCC CTCTTTTCTT CTGTATCGAA AAGGTGCTTA TTATTATATA AGCTTTGAC TCCTTCCCCC CTCTTTTCTT	ACAGCCCTAA		GCCTCTTAAT		TCGAGCCGAA		TTAGGACAGC		CAGGATCACT		TCTAGGA	AGAT
160170180190200210GATCAAATCTATAATGTTATTGTAACCGCCCATGCATTCGTAATAATCTTCTTTATAGTTCTAGTTTAGAACATTGGCGGGTACGGAACATACCAACATTAGCAAAGAAATATCAAFIC220230B1C240250260LoopB270ATACCCGTGATAATTGGCGGATTTGGAAACTGACTAGTGCCATTAATAATGGACTAGTGCCATTAATAATTATGGGCACTATTAACCGCCTAAACCTTTGACTGATCACGGTAATTATAAACCACGTGGT280290300310320330GACATAGCTTTTCCACGAATAAATAATATAAGCTTTTGACTCCTTCCCCCCTCTTTCTTCTGTATCGAAAAGGTGCTTATTTATTATATTCGAAAACTGAGGAAGGGGGGGAAAAAGAA	TGTCGGGATT		CGGAGAATTA		AGCTCGGCTT		AATCCTGTCG		GTCCTAGTGA	Loop	AGATCC:	TCTA
GATCAAATCTATAATGTTATTGTAACCGCCCATGCATTCGTAATAATCTTCTTTATAGTTCTAGTTTAGATATTACAATAACATTGGCGGGTACGAATAATCTACATTAGTAGAAATATCAAFICImage: Control of the state of t	160		170		180		190		200			210
CTAGTTTAGATATTACAATAACATTGGCGGGTACGATTATTAGAAGAAATATCAAF1C	GATCAAATCT		ATAATGTTAT		TGTAACCGCC		CATGCATTCG		TAATAATCTT		CTTTATA	AGTT
220230B1C240250260LoopB270ATACCCGTGATAATTGGCGGATTTGGAAACTGACTAGTGCCATTAATAATTGGTGCACCATATGGGCACTATTAACCGCCTAAACCTTTGACTGATCACGGTAATTATTAACCACGTGGT280290300310320330GACATAGCTTTTCCACGAATAAATAATATAAGCTTTTGACTCCTTCCCCCCTCTTTCTTCTGTATCGAAAAGGTGCTTATTTATTATATTCGAAAACTGAGGAAGGGGGGGA	CTAGTTTAGA		TATTACAATA	10	ACATTGGCGG		GTACG TAAGC		ATTATTAGAA		GAAATA	rcaa
ATACCCGTGA TAATTGGCGG BIC ATTTGGAAAC TGACTAGTGC CATTAATAAT TGGTGCACCA TATGGGCACT ATTAACCGCC TAAACCTTTG ACTGATCACG GTAATTATTA ACCACGTGGT 280 290 300 310 320 330 GACATAGCTT TTCCACGAAT AAATAATATA AGCTTTTGAC TCCTTCCCCC CTCTTTCTT CTGTATCGAA AAGGTGCTTA TTTATTATAT TCCGAAAACTG AGGAAGGGGGG GAGAAAAGAA	220		£. 230	IC B1/	240		250		260	Loop	в	270
TATGGGCACTATTAACCGCCTAAACCTTTGACTGATCACGGTAATTATTAACCACGTGGT280290300310320330GACATAGCTTTTCCACGAATAAATAATATAAGCTTTTGACTCCTTCCCCCCTCTTTCTTCTGTATCGAAAAGGTGCTTATTTATTATATTCGAAAACTGAGGAAGGGGGGGAAAAAGAA	ATACCCGTGA		TAATTGGCGG	ы	ATTTGGAAAC		TGACTAGTGC		CATTAATAAT	тоор	TGGTGC	ACCA
280 290 300 310 320 330 GACATAGCTT TTCCACGAAT AAATAATATA AGCTTTTGAC TCCTTCCCCC CTCTTTCTT CTGTATCGAA AAGGTGCTTA TTTATTATAT TCGAAAACTG AGGAAGGGGGG GAGAAAAGAA	TATGGGCACT		ATTAACCGCC		TAAACCTTTG		ACTGATCACG		GTAATTATTA		ACCACG	TGGT
GACATAGCTT TTCCACGAAT AAATAATATA AGCTTTTGAC TCCTTCCCCC CTCTTTCTT CTGTATCGAA AAGGTGCTTA TTTATTATAT TCGAAAACTG AGGAAGGGGG GAGAAAAGAA	280		290		300		310		320			330
CTGTATCGAA AAGGTGCTTA TTTATTATAT TCGAAAACTG AGGAAGGGGGG GAGAAAAGAA	GACATAGCTT		TTCCACGAAT		AAATAATATA		AGCTTTTGAC		TCCTTCCCCC		CTCTTT	FCTT
	CTGTATCGAA		AAGGTGCTTA		TTTATTATAT		TCGAAAACTG		AGGAAGGGGG		GAGAAAA	AGAA

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 (nucleasefree 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		Commercial kit (ng/µL)	Commercial modified kit (ng/uL)	Dipstick (ng/µL)			
	11		Commercial modified kit (ng/µL)	Mason and Botella 2020	Hammouda <i>et al.</i> 2019	Margam <i>et al.</i> 2010; Zou <i>et al.</i> 2017	
APL	4	132.50	132.50	-	-	2.200	
ASC	6	111.6	111.6	-	0.750	5.150	

n: number of specimens



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*; 17: *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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