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海洋高階掠食魚類的市場調查與檢驗方法改進 – 鯊與鮪
Market status & detection methods improvement of top marine predatory fishes – case study for shark and tuna

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海洋高階掠食魚類的市場調查與檢驗方法改進 – 鯊與鮪

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本論文係呂仲軒（R10241201）在國立臺灣大學海洋研究所完成之碩士學位論文，於民國 112 年 7 月 25 日承下列考試委員審查通過及口試及格，特此證明。

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摘要

鯊魚和鮪魚等頂級掠食性魚類在維持海洋食物網穩定性扮演至關重要角色。然而，商業漁撈與高市場需求已對它們的生態功能構成威脅。因此，需要有效的物種檢測方法來建立有效的漁業管理。本研究使用粒線體細胞色素 c 氧化酶次單元Ⅰ（Cytochrome c oxidase subunit I, COI）DNA 條形碼來確定魚翅乾貨的市場分布情況，提供乾貨市場中鯊魚物種組成的重要資訊。此外，本研究也利用粒線體基因來開發檢測南方黑鮪（*Thunnus maccoyii*）的恆溫式圈環形核酸增幅法（LAMP）。乾魚翅抽樣結果顯示，鋸峰齒鮫（*Prionace glauca*）佔逾六成乾魚翅貨源。雖然 2017 年起臺灣實施了更嚴格的漁業管理法律，但受保護和瀕危物種製成的乾魚翅仍在乾貨店出售，如：平滑白眼鮫（*Carcharhinus falciformis*）、污斑白眼鮫（*Carcharhinus longimanus*）、紅肉丫髻鮫（*Sphyrna lewini*），佔逾兩成。而在針對平滑白眼鮫（*Carcharhinus falciformis*）的抽樣結果顯示約有兩成贋品率，顯示管理政策仍有可改善空間。本研究儘管測試了不同基因、引子與 DNA 黏合溫度、引子組和反應時間，但南方黑鮪 LAMP 法未能區分不同鮪魚物種。這顯示基於粒線體基因開發的 LAMP 方法可能不適用於南方黑鮪的物種鑑定。

關鍵字：魚翅、物種鑑定、南方黑鮪、恆溫式圈環形核酸增幅法、粒線體基因、市場調查

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Abstract

Top predatory fishes, such as sharks and tuna, play a crucial role in maintaining the stability of marine food webs. However, their ecological function is threatened by fishing activity and high market demand. Therefore, it is imperative to employ effective species detection methods to enhance fishery management. In this study, mitochondrial Cytochrome c oxidase subunit I (*COI*) DNA barcoding was used to identify the market status of dried shark fins, providing insights into the composition of shark species in the market. Furthermore, an attempt was made to develop loop-mediated isothermal amplification (LAMP) as a detection tool for southern bluefin tuna (*Thunnus maccoyii*) using mitochondrial genes i.e. ND5 (NADH dehydrogenase 5) and D-loop regions. Despite the implementation of stricter fishing laws, the sale of shark fins derived from protected and endangered species, e.g. silky shark (*Carcharhinus falciformis*), oceanic whitetip shark (*Carcharhinus longimanus*), scalloped hammerhead (*Sphyrna lewini*) continues in retail stores, highlighting the necessity for policy revisions. In the case of the LAMP assay for southern bluefin tuna, various approaches were explored, including testing different genes, annealing temperatures, primer sets, and reaction times. Nevertheless, these efforts failed to distinguish between different tuna species. This outcome suggests that the LAMP assay based on mitochondrial genes may not be suitable for accurately identifying southern bluefin tuna.

Key words: Shark fin, Species identification, Southern bluefin tuna, Loop-mediated isotheral amplification (LAMP), Mitochondria gene, Market survey

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Introduction

High-level predators, such as sharks and tuna, play an important role in maintaining the stability and biodiversity of the marine ecosystems through top-down regulation. They also serve as indicators of the ocean health (Atwood & Hammill, 2018; Lynam et al., 2017). However, the increasing demand and exploitation rates for certain shark species and shark products have raised significant concerns about the status of many shark stocks and their exploitation in global fisheries (Pacoureau et al., 2021; Yagnesh B et al., 2020). The potential overfishing puts additional pressure on fragile marine food webs particularly in the face of climate change (Frisch et al., 2014; Hobday et al., 2013). Chondrichthyans, sought after for their fins, are highly-valued in markets for their fins, making them prime targets for fisheries (Zhou et al., 2021). The intensive fishing and finning of chondrichthyans, especially sharks, are major factors contributing to their population decline and raise concerns about long-term sustainability (Ferretti et al., 2010; Jaiteh et al., 2017). Taiwan's involvement in finning and the entire shark fishery trade chain underscores its responsibility to actively manage fisheries for these organisms (Bonaccorso et al., 2021; Lack & Sant, 2011).

The European Union designated Taiwan engaging in Illegal, unreported, unregulated (IUU) fisheries in 2015. This prompted Taiwanese government to amend the Tri-Act on fisheries, consisting of the Act for Distant Water Fisheries, the Act to Govern Investment in the Operation of Foreign Flag Fishing Vessels, and the Fisheries Act in 2016. These amendments come into effect in 2017 (https://law.moj.gov.tw/). Several shark and ray species listed in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES Appendix II) are now subject to catch and trade restrictions. However, though previous works point out the trading of shark fins, especially dried fins plays an essential role in shark fisheries, there is no market survey conducted to assess the species composition in the retail market since the amendment event in Taiwan (Chuang et al., 2016; Shea & To, 2017). Furthermore, the official recording of IUU

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violation events in Taiwan began in 2017 (Figure 1), leaving the effectiveness of the Tri-Act on fisheries uncertain (Fisheries Agency, 2022).

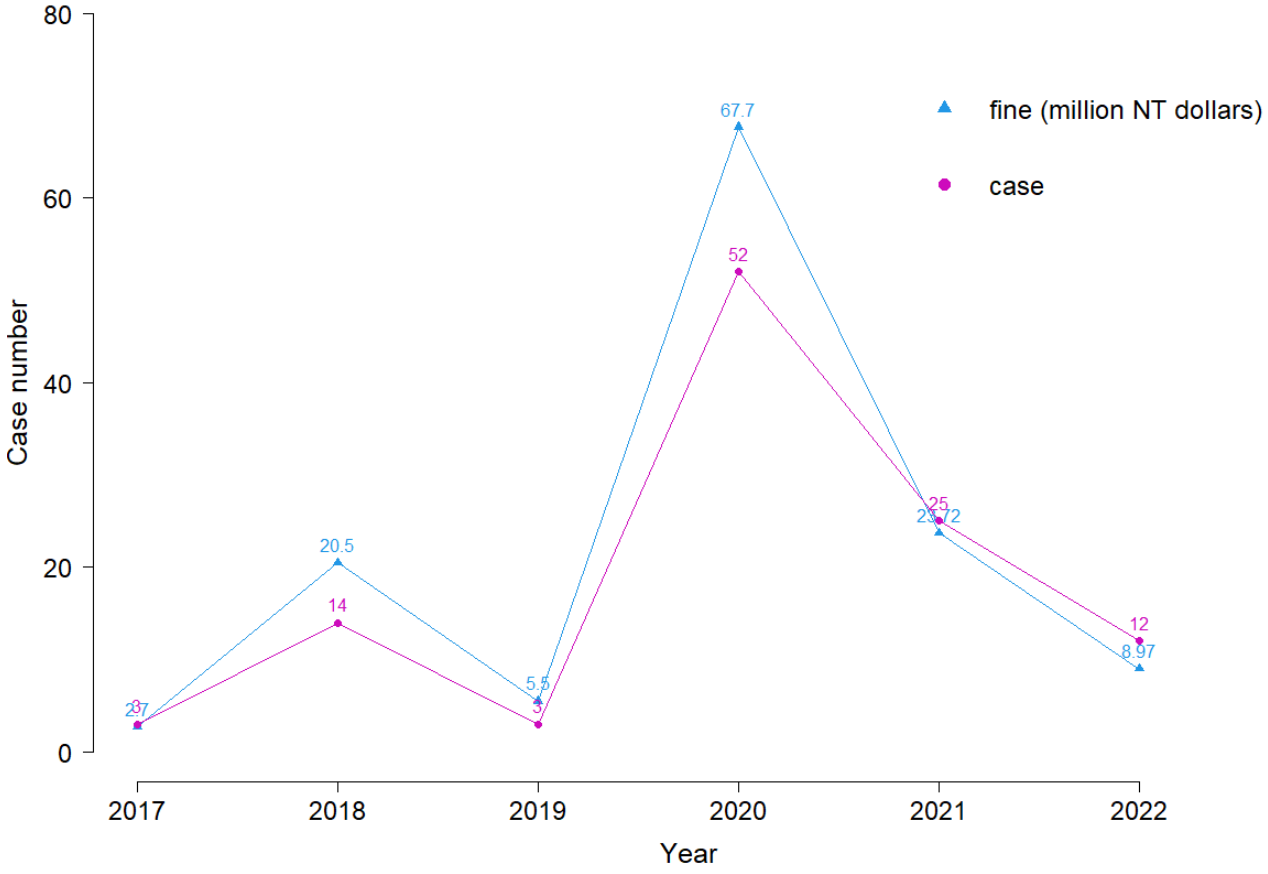

Figure 1. The fine and case number of shark fin-related IUU from 2017 to 2022.

To establish baseline information on the species composition of dried and processed fins sold in retail markets, this study conducted market surveys at retail markets on Dihua Street in Taipei, Taiwan using DNA barcoding. DNA barcoding is a powerful taxonomic tool for species identification and discovery. It utilises one or more standardised short DNA regions for taxa identification. Rapid species identification through DNA barcoding has applications in a various fields, including forensic science, food supply chain control, and disease understanding (Antil et al., 2023; Gostel & Kress, 2022). The Consortium for Barcode of Life (CBOL) oversees several working groups aimed at identifying universal barcode genes, such as COI in metazoans; rbcL, matK, and ITS in plants, ITS in fungi, and 16S rRNA gene in bacteria and archaea. These efforts contribute to the

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creation of reference DNA barcode libraries (Antil et al., 2023; DeSalle & Goldstein, 2019). Therefore, DNA barcoding proves to be a reliable method to identify the source species of the shark fins.

On the other hand, tunas, also confront significant overfishing pressure due to their high economic value (Ellis & Kiessling, 2016; Escalle et al., 2021; Gopalakrishna Pillai & Satheeshkumar, 2012).

In particular, southern bluefin tuna (*Thunnus maccoyii*) is an economically important marine species known for its high quality meat. Southern bluefin tuna can grow up to 2.45 m in length and 260 kg in weight. However, this tuna species has faced a substantial decline in population due to overfishing and other human activities. As a result, southern bluefin tuna has been listed as endangered species on the IUCN Red List since 2021 (Adams, 2014; Zhao et al., 2022). Moreover, the varying commercial value across different tuna species leads to widespread fraudulent seafood mislabelling in both domestic and international markets (Yao et al., 2020).

To address the conservation and regulatory issues, the development of a rapid molecular identification method is crucial for accurately identifying the species and preventing mislabelling. DNA-based molecular identification approaches have been widely used to detect species commonly mislabelled as tuna (Chuang et al., 2012). Common molecular identification techniques include PCR, qPCR, and isothermal amplification techniques with shorter reaction times such as nuclear acid sequence-based amplification (NASBA), Signal mediated amplification of RNA technology (SMART), strand displacement amplification (SDA), Single primer isothermal amplification (SPIA), and Loop-mediated isothermal amplification (LAMP). The reaction temperatures for NASBA, SMART, and SDA are around 40 degrees, which can easily cause non-specific amplification and produce false-positive results. SPIA cannot use crude DNA for the reaction and is not suitable for field testing. LAMP PCR can react with multiple primers and proprietary polymerase at a constant temperature (60-65◦C) without the need for purified DNA, greatly reducing the threshold for field

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operation and has the advantages of high specificity, quick, and low equipment requirements (Gill & Ghaemi, 2008). Loop-mediated isothermal amplification (LAMP), utilise a nucleic acid amplification technique using four-to-six specially designed primers to recognise six regions on the target sequence, ensuring high specificity and sensitivity (Supplementary figure 1) (Foo et al., 2020; Xu et al., 2021). LAMP stand out due to its isothermal and energy-efficient amplification requirements, making it suitable for low-cost diagnostics and on-site analysis (Becherer et al., 2020; Nagamine et al., 2001). The advantages of LAMP are its ability to detect products rapidly and its compatibility with other methods (Soroka et al., 2021). The LAMP technique can provide results within an hour, and the results can be interpreted with the naked eye (Mori et al., 2013; Notomi et al., 2000). This simple and cost-effective method can be performed in laboratories, or even in the field, reducing the time between sample collection and diagnosis (Foo et al., 2020; Wong et al., 2018). The equipment required for methods such as polymerase chain reaction (PCR) and real-time quantitative polymerase chain reaction (qPCR) is relatively precise and expensive, and the reaction time is relatively long, making it difficult to perform directly in the field. Loop-mediated isothermal amplification (LAMP), with its short operating time, low equipment requirements, high accuracy and sensitivity, and the ability of LAMP to determine experimental results directly without instruments, can even be operated with minimal resources using only experimental consumables and a car heater. It is a rapid screening technology for on-site species identification (Centeno-Cuadros et al., 2017).

The assessment of the shark species' market status could aid the Taiwanese government in assessing policies and resolving the conflicts amongst stakeholders (Simpfendorfer et al., 2021). By obtaining fundamental data on the species composition of shark fins in the retail market, it becomes feasible to conduct a comprehensive evaluation of the Tri-Act on Fisheries' implementation. Additionally, this study seeks to develop a LAMP assay for detecting southern bluefin tuna, thereby assisting in the identification of fraudulent or mislabelling products.

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Materials and methods

I. Sample collection

All processed and dried shark fin samples were purchased from Dihua Street, Taipei, Taiwan between April and July, 2022. To investigate the species composition of shark fins in the retail market, a total of 99 shark fins were randomly purchased from five stores on Dihua Street. The other 119 samples were purchased based on the preliminary results from seven stores, targeting fins labelled as '黑鯊' (silky shark, i.e. *Carcharhinus falciformis*) on Dihua Street. These fins have length ranging from 5 to 30 cm, with the majority being intact (Supplementary figure 2 & Supplementary figure 3).

Southern bluefin tuna samples were purchased from Shuu Chang Oceanic Enterprise Co., Ltd. Longtail tuna (*Thunnus tonggol*) samples were collected from the fishing porting in Magong City, Penghu County by the Fisheries Research Institute, COA. The other samples were obtained from Donggang port samplers. All tunas were collected in 2022, except for yellowfin tuna (*Thunnus albacares*) in 2020. All the samples are frozen under -20°C for preservation.

II. Species identification (DNA barcoding)

DNA extraction:

DNA extraction was conducted on all samples using a DNA Mini Kit (Cat No./ID: LGD480-220, TAIGEN, Taiwan). From each sample, 1 mm^3 of tissue was excised with sterile scissors and digested in Proteinase K solution (250 µl LTL buffer + 30 µl Proteinase K) overnight at 65°C. After centrifugation at 1200 rpm for 5 minutes, 180 µl of the suspension was transferred to the Labturbo 48C. Follow the manufacturer's protocol to automatically obtain purified DNA.

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Polymerase Chain Reaction:

The partial mitochondrial cytochrome oxidase subunit 1 gene (COI, approximately 650 bps) was amplified through PCR with 5-10 ng of genomic DNA, 0.2 µmol forwards and reverse primers (Genomics, Taiwan; Supplementary table 5), 2X Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark) and adjusted to a final volume of 25 µl. The PCR conditions were as follows: initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 30s, 50°C for 30s 72°C for 1 min, and final extension at 72°C for 5 min.

Afterwards, the PCR products were sequenced by Applied Biosystems 3730xl DNA Analyser (MB MISSION BIOTECH, Taipei, Taiwan).

Species identification:

Identification was performed using the Bold system v4 (Barcode of Life Data System, https://www.boldsystems.org/) and BLAST search against all available sequences on GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with 100% similarity.

To test whether the COI fragment used in this study can distinguish the ocean origin of silky sharks, the *COI* sequences would be retrieved from Genbank and analysed by RAxML, plotted by Evolviewer (Stamatakis, 2014; Subramanian et al., 2019). A total of 34 silky shark sequences from the Atlantic, Pacific and Indian Oceans were used, and the out group is blue shark (*Prionace glauca*).

Estimation of species diversity of sharks on the retail market:

All resulting species counts from the random sampling were then used to estimate the total number of shark species in the fin trade using iNEXT (Hsieh et al., 2016). Parameters were set as follows: q = 0, endpoint = 500, bootstraps = 10,000, confidence interval level = 0.95.

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III. Species-specific LAMP primer set design and assay development for southern bluefin tuna

The mitochondrial genomes of all tuna species (*Thunnus*) were retrieved from Genbank, aligned using Clustal Omega (Sievers et al., 2011), and the most species-specific fragment for southern bluefin tuna was automatically obtained using MorphoCatcher (Shirshikov et al., 2019). The species-specific sequences were then submitted to PrimerExplorer V5 (https://primerexplorer.jp/) for primer design (The conditions for primer design are provided in the Supplementary table 6). The primers were subsequently synthesised by Genomics Inc., New Taipei, Taiwan. Primer sets were generated for the ND5 (NADH dehydrogenase 5) and D-loop regions of southern bluefin tuna due to their highest variation between tuna species (Primer sequence in the Supplementary table 7 & Supplementary table 8).

The LAMP assays contain 1× Isothermal Amplification Buffer (New England BioLabs), 8 mM $MgSO_4$ (New England BioLabs), 1.4 mM each dNTP (New England BioLabs), 1.6 μM forwards inner primer/backwards inner primer (FIP/BIP), 0.2 μM outer primers (F3/B3), 0.4 μM loopB primer. Additionally, at least 3.2U of Bst 2.0 DNA polymerase (New England BioLabs) and 0.8 ng of DNA template were included.

The LAMP assay reactions were carried out under the following conditions: amplification (ranging from 50°C to 69°C for 60 min), polymerase denaturation (80°C for 20 min), then held at 4°C. Results were verified by capillary electrophoresis (Qiaxcel advanced system, Qiagen, Germany). Results were considered positive if multiple dark bands were present in the electrophoresis.

LAMP performance evaluation:

The sensitivity and specificity of each primer set were then evaluated. Sensitivity and specificity are defined as follows (Carvajal & Rowe, 2010):

Sensitivity: True positive / (True positive + False negative)

Specificity: True negative / (True negative + False positive)

In this context, 'true positive' refers to positive results for southern bluefin tuna, while 'false

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positive' stands for positive results for the other tuna species. 'True negative' represents negative results for the tuna samples other than southern bluefin tuna, and 'false negative' indicates negative results for southern bluefin tuna.

The meaning of the sensitivity aforesaid is that given the samples are southern bluefin tuna, the proportion of successfully identified samples; while the specificity refers to the ratio of correctly unamplified results given the samples are not southern bluefin tuna.

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Results

Species composition of shark fins in the retail market:

Of 99 randomly sampled shark fins, 72.7% (72 out of 99) were successfully identified to species or genus level. The species composition from random sampling of 72 shark fins shows that the major source species of dried shark fins is blue shark (*Prionace glauca*), accounting for approximately 60%. The second most abundant one is silky shark (*Carcharhinus falciformis*), constituting 13% of shark fins. The remainder are scalloped hammerhead (*Sphyrna lewini*) with 8.33%, *Centrophorus spp.* with 6.94%, Angular angel shark (*Squatina Guggenheim*) with 4.17%, while the Australian blackspot shark (*Carcharhinus coatesi*), oceanic whitetip shark (*Carcharhinus longimanus*), spot-tail shark (*Carcharhinus sorrah*), smooth-hound (*Mustelus spp*.) each accounts for 1.39% (Figure 2). In the targeted sampling of silky sharks, 97.5% (116 out of 119) of the shark fins were successfully identified. The remaining failures include three fins identified as *Aspergillus luchuensis* species (in random sampling), and the rest be amplified through PCR.

The back-calculation of species involved in the fin trade suggests that the species number is estimated to be between five and 20 based on extrapolation (Figure 3). The vertical axis in the Figure 3 represents the species number for the given sample size. The solid line stands for the calculated value while the dashed line indicates the extrapolation. The shaded area is the 95% confidence interval.

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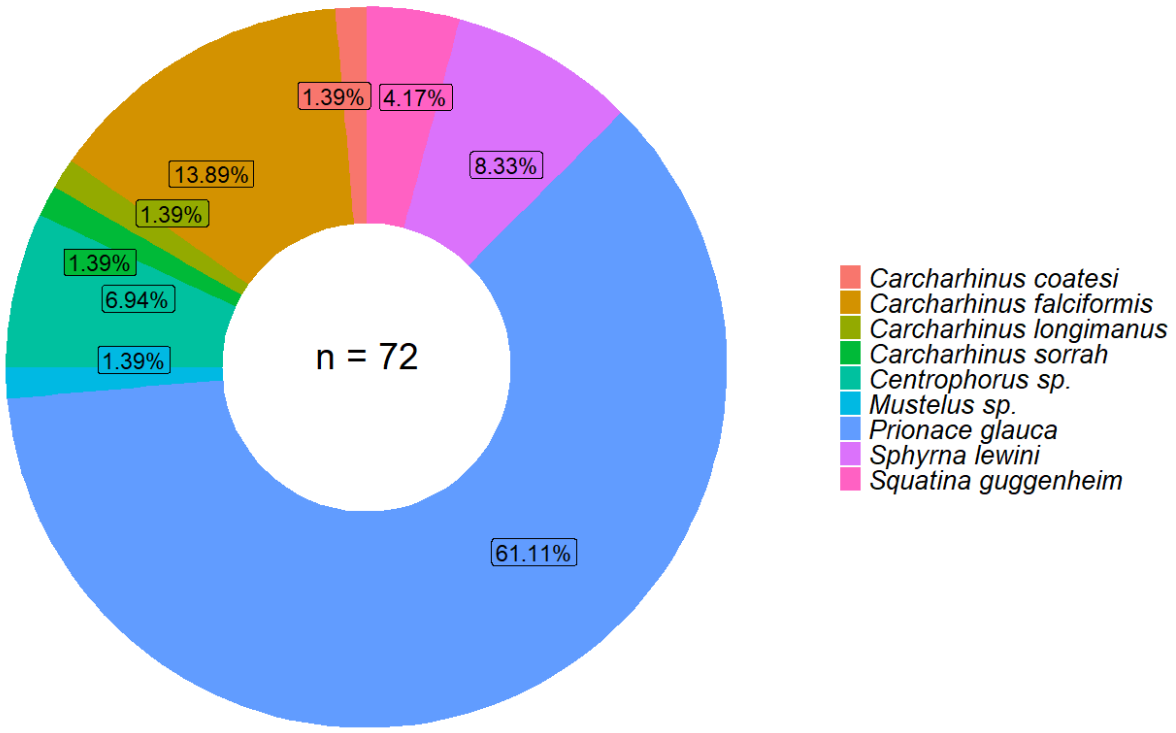

Figure 2. Pie chart with the species composition of shark fins randomly sampled from retail stores.n = sample size, colours represent taxa and the number on each piece represents the percentage of certain species.

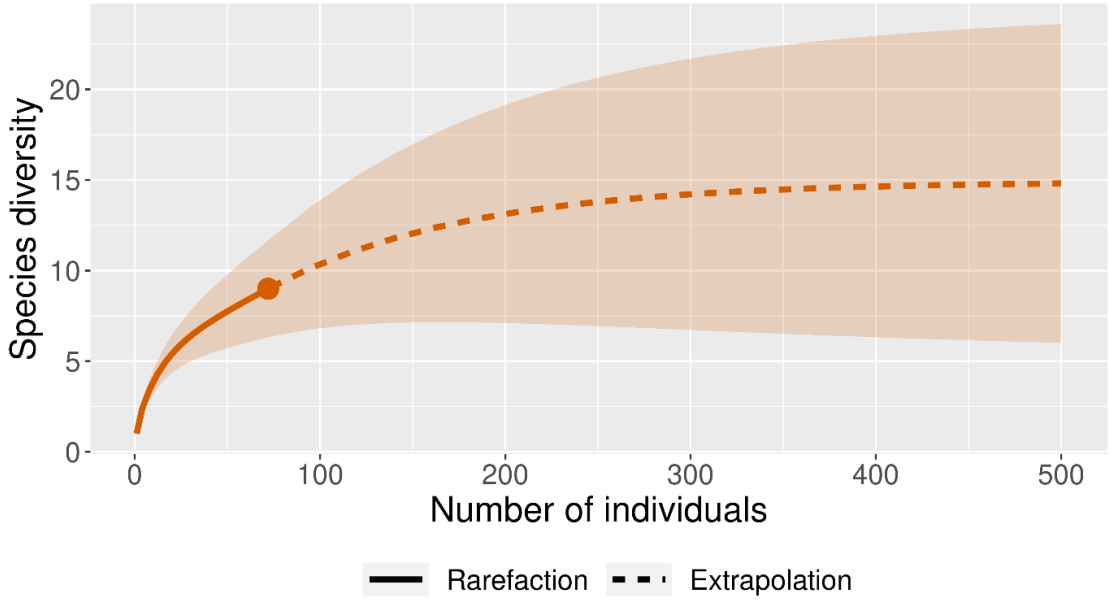

Figure 3. The estimated source species number of shark fins randomly sampled from retail stores.The solid curve is the rarefaction of given data, and the dashed curve is extrapolation. The orange -shaded area is 95% confidence interval.

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The species composition of the targeted sampling for silky shark shows that over 80% of the shark fins labelled as silky shark are indeed silky sharks. Amongst the remainder, oceanic whitetip shark (*Carcharhinus longimanus*) has the highest proportion but only 5.17%; scalloped hammerhead (*Sphyrna lewini*) 3.45%; blacktip shark (*Carcharhinus limbatus*) 2.59%; pelagic thresher (*Alopias pelagicus*), blue shark (*Prionace glauca*), and spot-tail shark (*Carcharhinus sorrah*) all 1.72% (Figure 4).

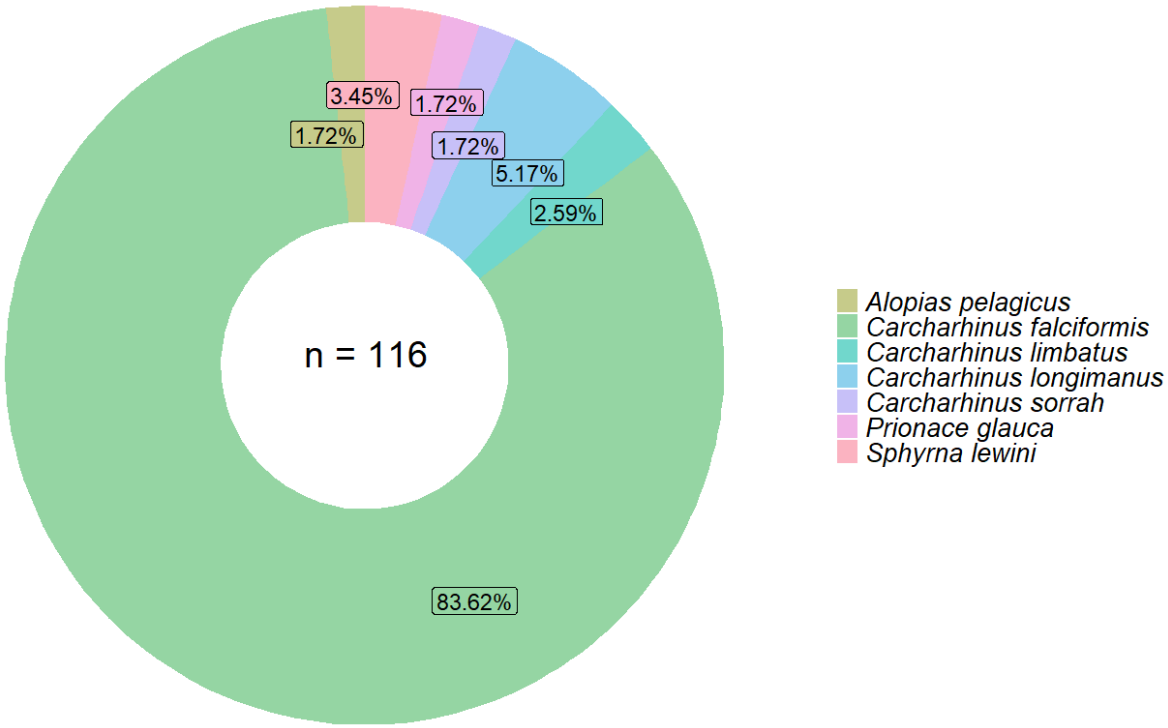

Figure 4. Pie chart with the species composition of the shark fins from silky-shark-targeting sampling amongst retail stores.n = sample size, colours represent taxa and the number on each piece represents the percentage of certain species.

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Species differentiation of tunas using LAMP assays with ND5 and D-loop primer sets:

The ND5 gene primer set has a total number of mismatches between southern bluefin tuna and albacore (*Thunnus alalunga*) with 5 mismatches, Atlantic bluefin tuna (*Thunnus thynnus*) 3, bigeye tuna (*Thunnus obesus*) 7, blackfin tuna (*Thunnus atlanticus*) 8, longtail tuna (*Thunnus tonggol*) 8, Pacific bluefin tuna (*Thunnus orientalis*) 6, and yellowfin tuna (*Thunnus albacares*) 8 (Supplementary table 9).

On the other hand, the D-loop gene primer set has a total number of mismatches between southern bluefin tuna and albacore with 8 mismatches, Atlantic bluefin tuna 50, bigeye tuna 10, blackfin tuna 13, longtail tuna 13, Pacific bluefin tuna 13, and yellowfin tuna 9 (Supplementary table 10).

The 23 samples subjected to LAMP with the ND5 primer set at 65°C did not yield clear species differentiation. Only half of the southern bluefin tuna samples were successfully amplified, while the rest failed. Similar results were observed for the other tuna species (Supplementary table 1 & Supplementary figure 5).

All six samples subjected to LAMP assays with the ND5 primer set at 55, 57, 60°C showed positive results regardless of the species (southern bluefin tuna, Pacific bluefin tuna, albacore, bigeye tuna, yellowfin tuna, Atlantic bluefin tuna, with one sample per species), resulting in 100% sensitivity but 0% specificity. On the other hand, all four LAMP samples at 69°C tested negative (including two samples each of southern bluefin tuna and bigeye tuna) (Figure 5 and Supplementary figure 6), leading to 0% sensitivity and 100% specificity. To further assess its performance, a larger sample size of 34 samples was used at 65°C, but the results remain inconclusive, with sensitivity below 50% and specificity around 10% (Figure 6, Supplementary table 2 and Supplementary figure 7).

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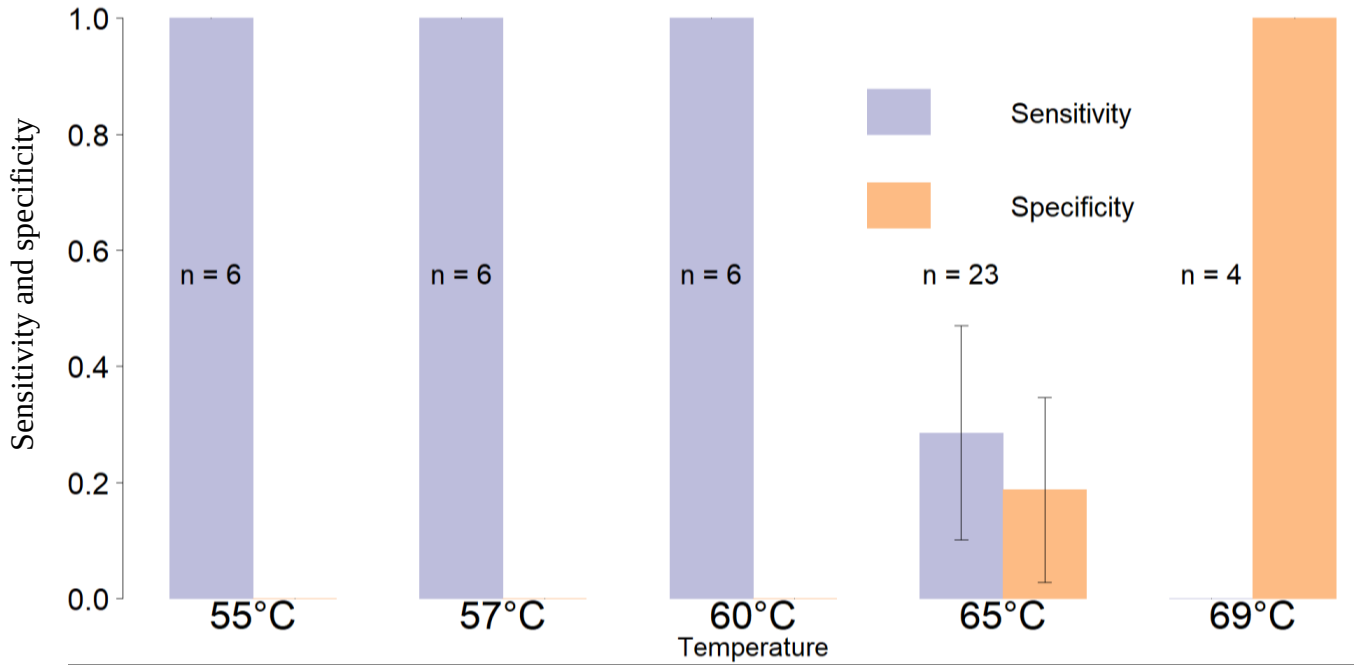

Figure 5. Bar plot of preliminary ND5 LAMP results across different temperatures. The bar plot showing the LAMP results using the primer set for mitochondrion ND5 gene at different reaction temperatures across tunas. n = sample size, purple and orange bar represents sensitivity and specificity, respectively.

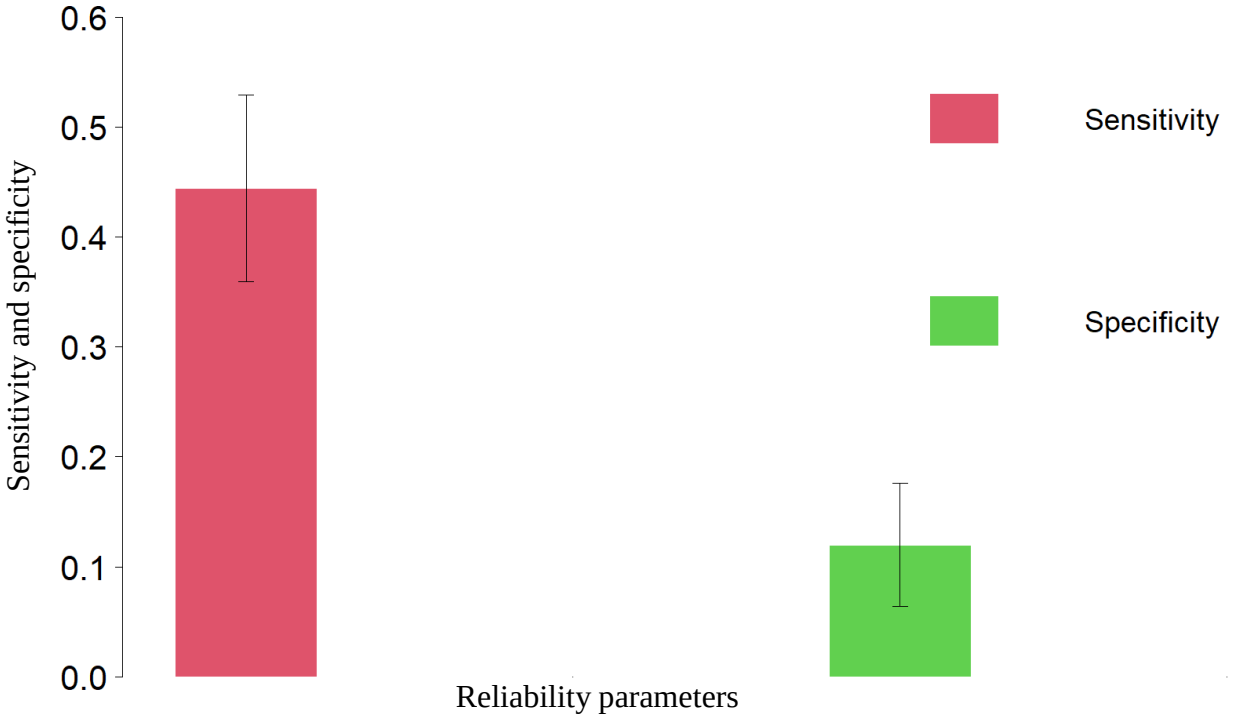

Figure 6. Bar plot of ND5 LAMP performance at 65°C.The bar plot showing the LAMP results using the primer set for mitochondrial ND5 gene at 65°C with sample size = 34. Magenta and green bar represents sensitivity and specificity, respectively.

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The results of the pre-test for the D-loop reaction temperature indicate that 55°C is a suitable temperature for the subsequent experiments (Figure 7).

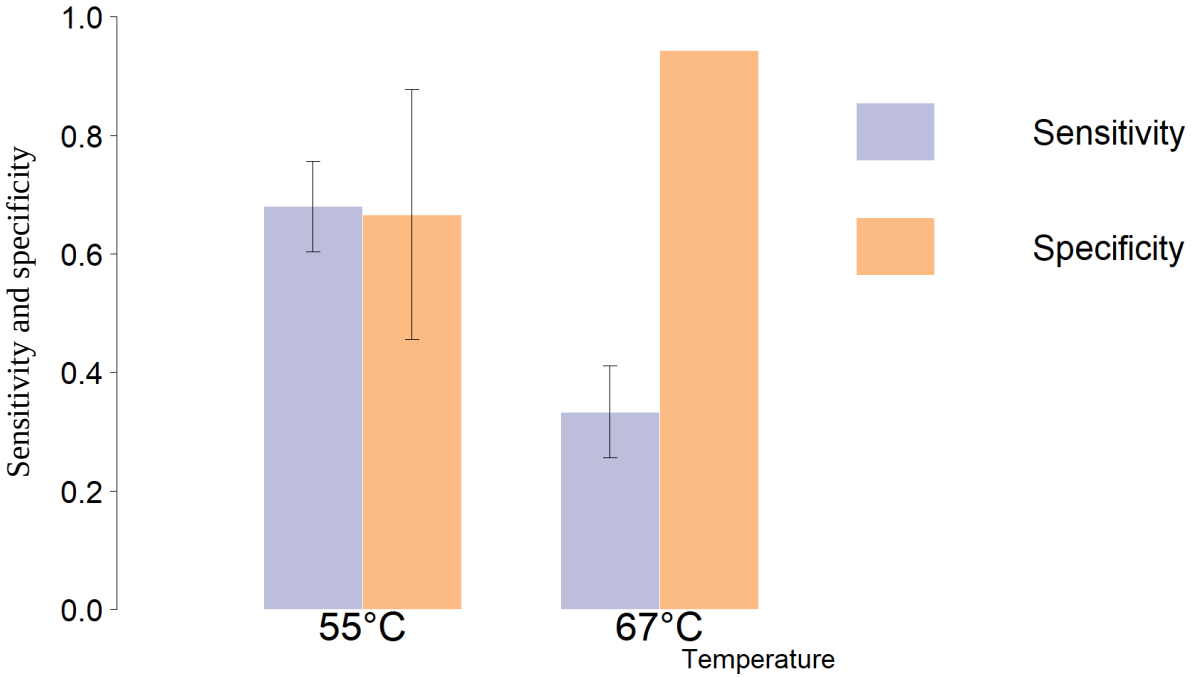

Figure 7. Bar plot of the preliminary D-loop LAMP results between different temperatures. The bar plot showing the preliminary LAMP test between 55°C and 67°C using the D-loop primer set. Sample size = 21, purple and orange bar represents sensitivity and specificity, respectively.

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The LAMP assays with the D-loop primer set at 55°C and 82 samples reveal that a quarter of the southern bluefin tunas fail to be amplified, while some non-specific amplification occurs in other species, particularly in bigeye tuna, yellowfin tuna, and Pacific bluefin tuna. This results in a sensitivity of 0.75, and a specificity of 0.67 (Supplementary table 3, Supplementary figure 8 and Figure 8). Considering the unsatisfactory performance of both primer sets, and the possibility of the low specificity due to the non-specific loop primer, further experiments using the same primer sets except for the loop primer, would be conducted.

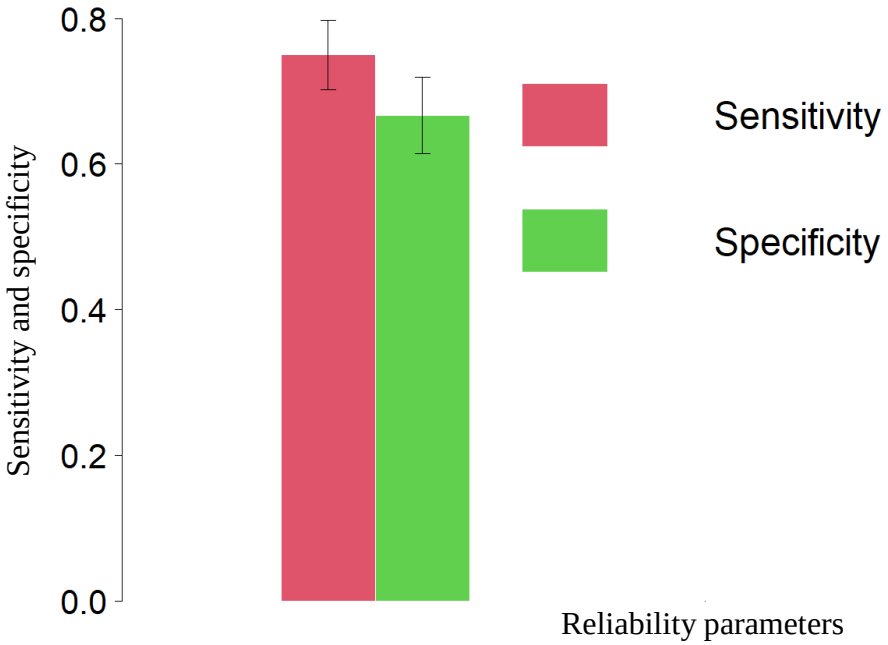

Figure 8. Bar plot of D-loop LAMP performance at 55°C.The bar plot showing the LAMP results using the D-loop primer set at 55°C, sample size = 82. Magenta and green bar represents sensitivity and specificity, respectively.

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Performing LAMP assays of the ND5 gene without the loop primer increases its specificity to 100% across all 12 samples, but results in 0% sensitivity (Figure 9). During this reaction, no southern bluefin tuna samples are successfully amplified.

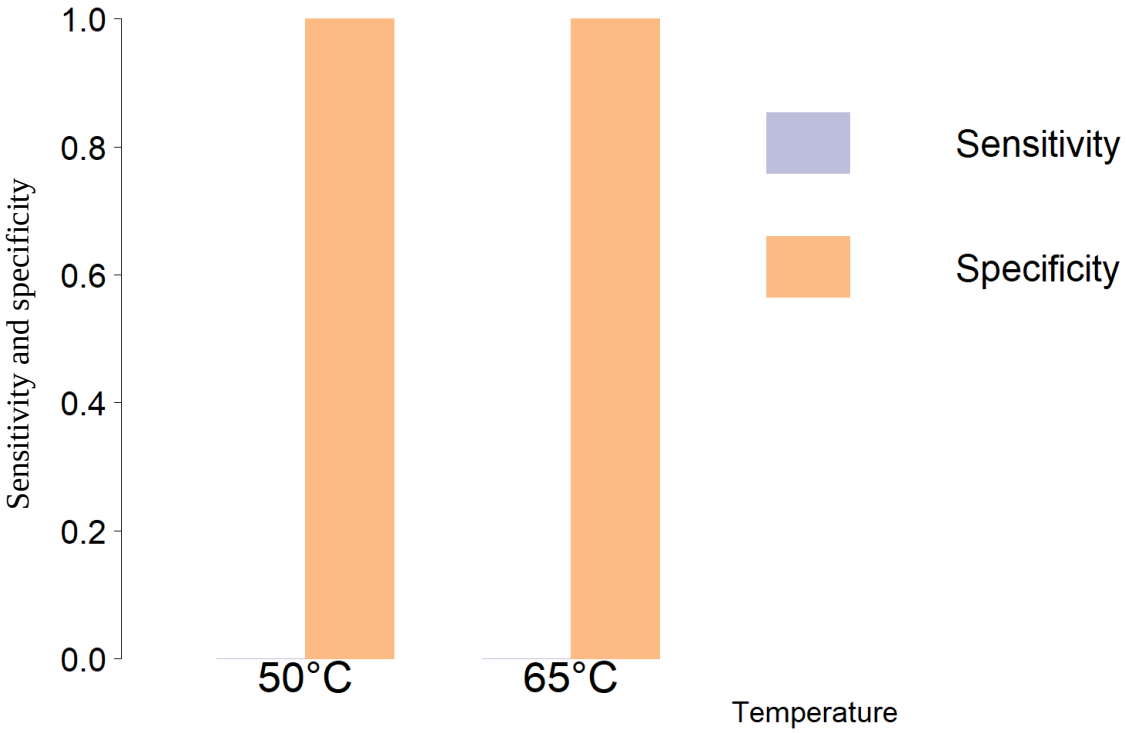

Figure 9. Bar plot of the preliminary LAMP results using ND5 primer set without loop primer across different temperaturesThe sensitivity and specificity of the LAMP assays using the ND5 primer set without the loop primer between 55℃ and 65℃. Sample size = 12, purple and orange bar represents sensitivity and specificity, respectively.

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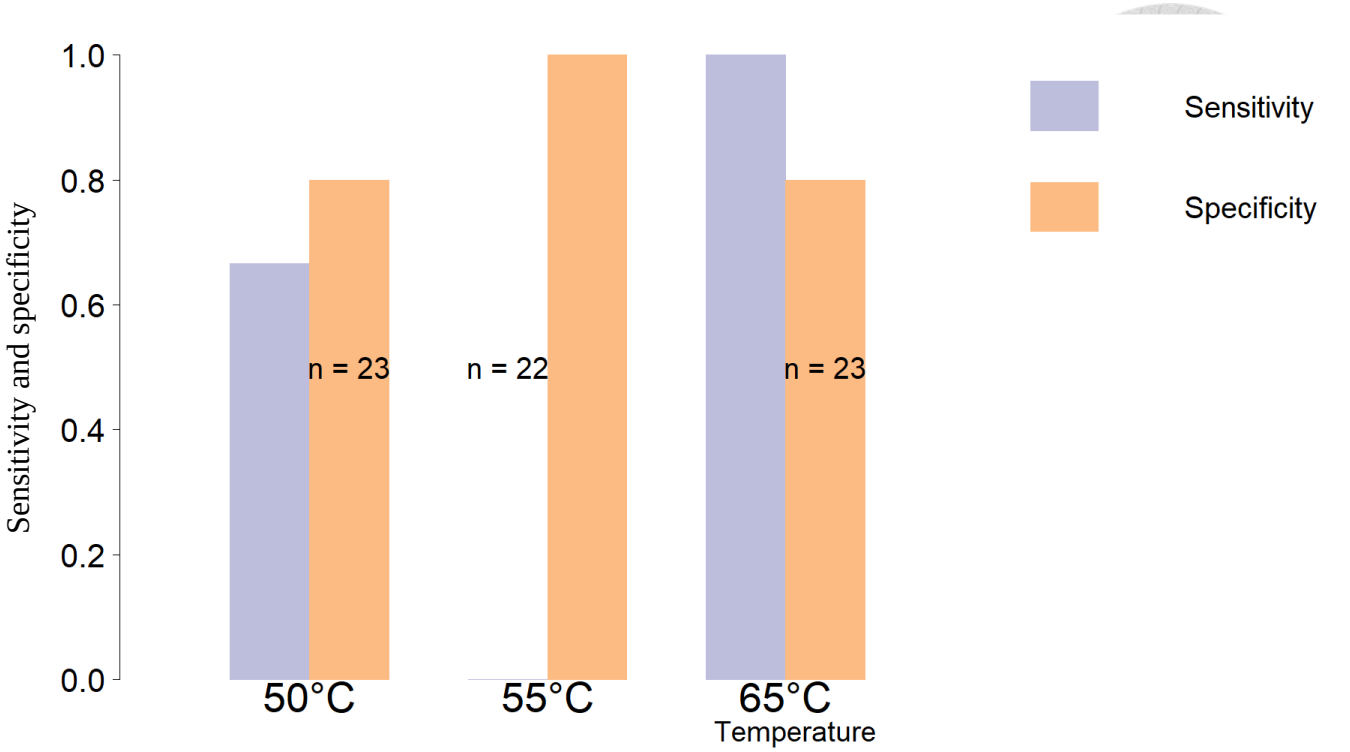

Figure 10. Bar plot of the preliminary LAMP results using D-loop primer set without loop primer across different temperatures.The bar plot showing the preliminary LAMP test amongst 50°C, 55°C and 67°C using the D-loop primer set without loop primer. n = sample size, purple and orange bar represents sensitivity and specificity, respectively.

On the other hand, the LAMP reaction of the D-loop without loop primer exhibits high sensitivity and specificity of 100% and 80% respectively at 65°C (

Figure 10). All southern bluefin tuna samples in this reaction at 65°C are successfully amplified, with only few false positives (Supplementary table 11).

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However, when tested with larger sample sizes, LAMP using D-loop primers without the loop primer at 65°C demonstrates poor sensitivity and specificity, around 30% and 60% respectively. Only four out of 11 southern bluefin tuna samples yield positive results, and all samples of the other species except albacore produce at least one false positive result (Supplementary table 4, Supplementary figure 9 and Figure 11).

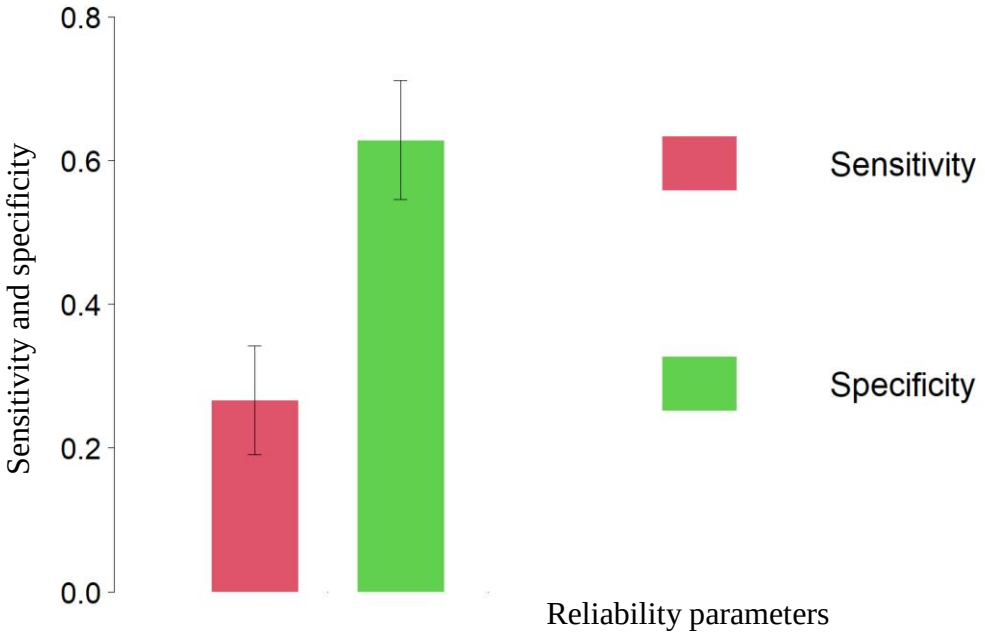

Figure 11. Bar plot of the LAMP performance using D-loop primer set without loop primer at 65°C

The bar plot showing the LAMP results using the D-loop primer set without the loop primer at 65°C, sample size = 50. Magenta and green bar represents sensitivity and specificity, respectively.

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Discussion

Species composition of shark fins in the retail market:

The sampling effort of the shark fins from retail stores has some shortcomings regarding the randomness. The sampling targets are not focused on the fins at the designated position on the shark. This means that some of the fins used in this study may come from the same individual, with the potential risk of repeated sampling. The result may therefore underestimate the species number in the retail market and the original source.

The results of the random sampling of shark fins indicate that the blue shark is the primary source of dried fins in the retail market, accounting for approximately 61% of the sample fins. Silky shark follows with over 13% of the fins, aligning with the findings of a previous study conducted in Hong Kong and Guangzhou (Cardeñosa et al., 2020; Fields et al., 2018). Previous studies also found the concordance amongst the retail markets in Taiwan (Chuang et al., 2016; Liu et al., 2013). This suggests similar major species distribution of shark fins between different retail markets. However, the back-calculation reveals that the number of potentially traded species is likely to be less than 20, which is substantially lower than the reported 50-60 species in Hong Kong and Guangzhou. This discrepancy may be due to the different import source, or more likely the sample sizes.

Given that the silky shark is listed in the Appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES Appendix II) and yet remains the second most abundant source of shark fins, another survey specifically focusing on this species was conducted. The survey conducted on the second most abundant species, the silky shark, reveals that at least 80% of the sampled fins are genuine, with only a few cases of fraudulence detected. These results suggest that despite the implementation of the Tri-Act on fisheries (consisting of the Act for Distant Water Fisheries, the Act to Govern Investment in the Operation of Foreign Flag Fishing Vessels, and the Fisheries Act), certain species restricted from fishing and trade, such as silky shark (*Carcharhinus falciformis*), oceanic whitetip shark (*Carcharhinus longimanus*), scalloped hammerhead (*Sphyrna*

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lewini), are still making their way into the market. Possible reasons for this phenomenon include shopkeepers acquiring restricted shark products prior to the amendment of the laws, inadequate dissemination of information regarding the Tri-Act on fisheries to shopkeepers or a perception amongst shopkeepers that they are unlikely to be apprehended by the competent authority. It is worth noting that the silky shark is only banned from fishing in the Atlantic and Mid-West Pacific by the Act for Distant Water Fisheries, but not in the Indian Ocean. This may also be a reason for the appearance of silky shark in the retail market, whereas the DNA barcoding method used in this study is incapable of tracing back the geological origin of the shark fins in the retail market (Supplementary figure 4).

The fraud rate of approximately 16% in the silky shark targeting survey may be attributed to widespread issue of seafood mislabelling globally (Kroetz et al., 2020; Warner et al., 2016). This mislabelling could be unintentional due to the ambiguous common name (Hasan et al., 2023) or the misidentification between species with similar morphology, particularly in the case of processed shark fin (Barendse et al., 2019). Furthermore, the intricate supply chain of seafood can also contribute to these discrepancies (Luque & Donlan, 2019). In spite of the enforcement difficulties proposed by previous studies, the urgency to manage shark fin fisheries are crucial for a sustainable economy (Cardeñosa et al., 2018; Miller et al., 2019; Van Houtan et al., 2020). As a previous study indicates that reducing the gap between stakeholder and decision-maker may help the management reach the consensus and the needs of society (Ho, 2022).

Species differentiation of tunas using LAMP assays with ND5 and D-loop primer sets:

In order to develop an efficient method for identifying southern bluefin tuna, LAMP assays are designed and tested under various conditions in this study. Firstly, the primer set targeting the ND5 gene yields 0% specificity at 55°C, 57°C, and 60°C as it successfully amplify the DNA fragments across all tuna species. In contrast, this primer set fails to amplify any tuna sample at 69°C, resulting

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in 0% sensitivity. The diminished performance of the ND5 gene primer set below 60°C is likely due to the poor selectivity of the primers at low melting temperatures. Additionally, the high temperature of 69°C might hinder the efficient binding of the primers to the DNA templates. Nevertheless, the LAMP assays exhibit partial discrimination between southern bluefin tuna and the other tuna species when tested at 65°C, concordant with the optimal LAMP condition suggested in previous studies (Notomi et al., 2000; Parida et al., 2008). Nevertheless, the sensitivity and specificity of the larger sample size at 65°C remain indecisive.

Hence, the D-loop primer set would be employed for LAMP assays aiming to improve the performance. The temperature test reveals that the 55°C produced satisfactory outcomes in terms of sensitivity and specificity. Therefore, a subsequent experiment was conducted with a larger sample size under this condition. The LAMP assays utilising the D-loop primer set at 55°C show improved performance compared to previous attempts, yet the sensitivity and specificity remain indecisive. These results indicate that temperature adjustments alone is insufficient to achieve satisfactory performance of the LAMP assay in this study. Consequently, the primer set, another adjustable component, was evaluated.

To assess primer selectivity, each primer was aligned to the targeted gene fragments of all tuna species using Primer Map (https://www.bioinformatics.org/sms2/primer_map.html). The results indicate the backwards loop primers used in either ND5 or D-loop primer set are complementary to the target region in all tunas, i.e. the backwards loop primer are able to bind and contribute to the amplification across all tuna species in LAMP experiments. As a result, a series of LAMP assays were conducted using both the ND5 and D-loop primer sets without the addition of the loop primer afterwards. The LAMP assays carried out with the ND5 primer set but without the loop primer failed to amplify any sample of southern bluefin tuna. This suggests that the non-species-specific amplification observed the complete ND5 primer set is likely due to the loop primer. On the other hand, despite the satisfactory performance of the LAMP assay with the D-loop primer set without

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the loop primer at 65°C, the same experiment conducted with a larger sample size yields poor results.

The sensitivity and specificity of the LAMP assays conducted in this study fall short in comparison to previous studies that focused on different taxa (Li et al., 2017; Rizzo et al., 2022). Several potential reasons could explain the subpar performance of the LAMP assays in this context. It is possible that there is insufficient variation across the mitochondrial genomes of different tuna species, or the existing variants may not be densely enough distributed for the LAMP assay to effectively discriminate between the tunas. Consequently, the LAMP assays developed based on the mitochondrial genome proves to be unreliable for southern bluefin tuna detection. Therefore, future studies aiming to identify southern bluefin tuna using LAMP assays may consider alternative options such as nuclear genes. However, it is important to note that using nuclear DNA as a template for primer design is unlikely to yield satisfactory result in the LAMP assay for southern bluefin tuna detection. This is because nuclear DNA undergoes slower mutation rates compared to mitochondrial DNA, resulting in less frequent and more sparse variations in the genome (Allio et al., 2017). It should be acknowledged that the LAMP assay developed in this study, utilising the D-loop primer set may be more suitable for surveillance screening purposes rather than precise identification (Kollenda et al., 2018). Although only few previous studies have developed LAMP for certain tuna species, such as yellowfin tuna (*Thunnus albacares*) and skipjack tuna (*Katsuwonus pelamis*), there are still some problems in applying the technique. On the one hand, the former uses phylogenetically distant fishes as control group, e.g. Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), etc. The difference between the genomes of these species is greater than intra-genus variation in this study, so they are more likely to be distinguishable. However, when it comes to practical use, the phylogenetically distant taxa are less likely to be misidentified, the demand for closely related species identification therefore more necessary. On the other hand, the 'skipjack tuna' used in the latter study does not belong to the tuna genus (*Thunnus*), nor are their identification taxa closely related (Ali et al., 2022; Xiong et al., 2021).

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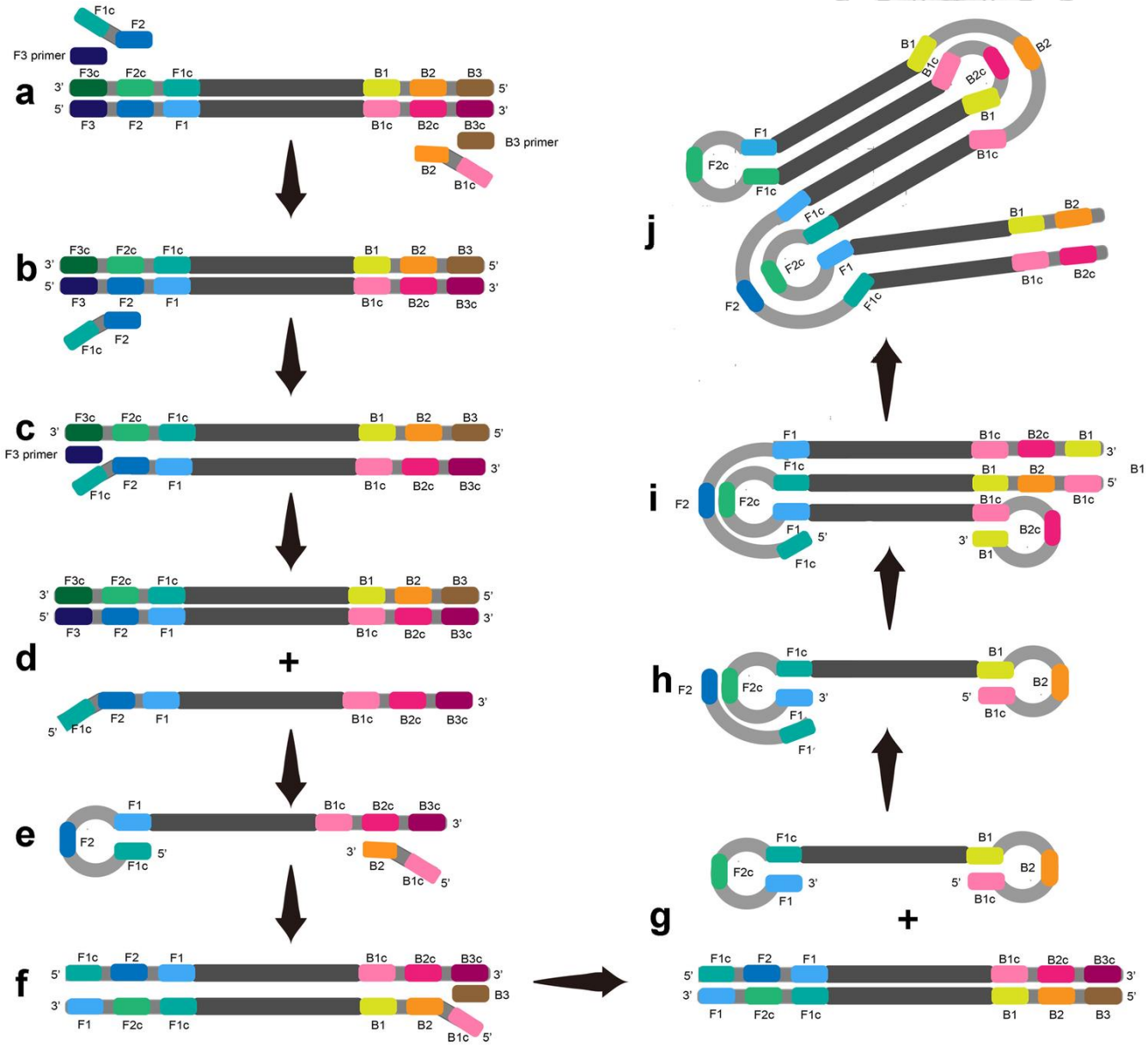
Supplementary



Supplementary figure 1. Principle of loop-mediated isothermal amplification(Zeng et al., 2022). Forwards inner primer (FIP) consisting of F1c and F2 sequences can attach to F2c region on the template DNA by F2 then generating a reverse complementary one by Bst, the DNA polymerase used in LAMP. Notice that the F1c region is unable to pair with F3c region of the template, thus remaining as a hanging head. The F3 primer, being capable of pairing with the F3c region, will attach to it and synthesise a reverse complementary one simultaneously shovelling the product generated by FIP with the help of Bst, which also has exonuclease activity (a-f). The generated DNA can forming loop structure by the F1 and F1c reverse complementation, apart from recognised by FIP, the forwards loop primer LF can also help to speed up the reaction process (g-j). The backwards primers work in the same way.

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Supplementary figure 1. An example of dried fin in random sampling.

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Supplementary figure 2. An example of dried fin in silky-shark-targeting sampling.

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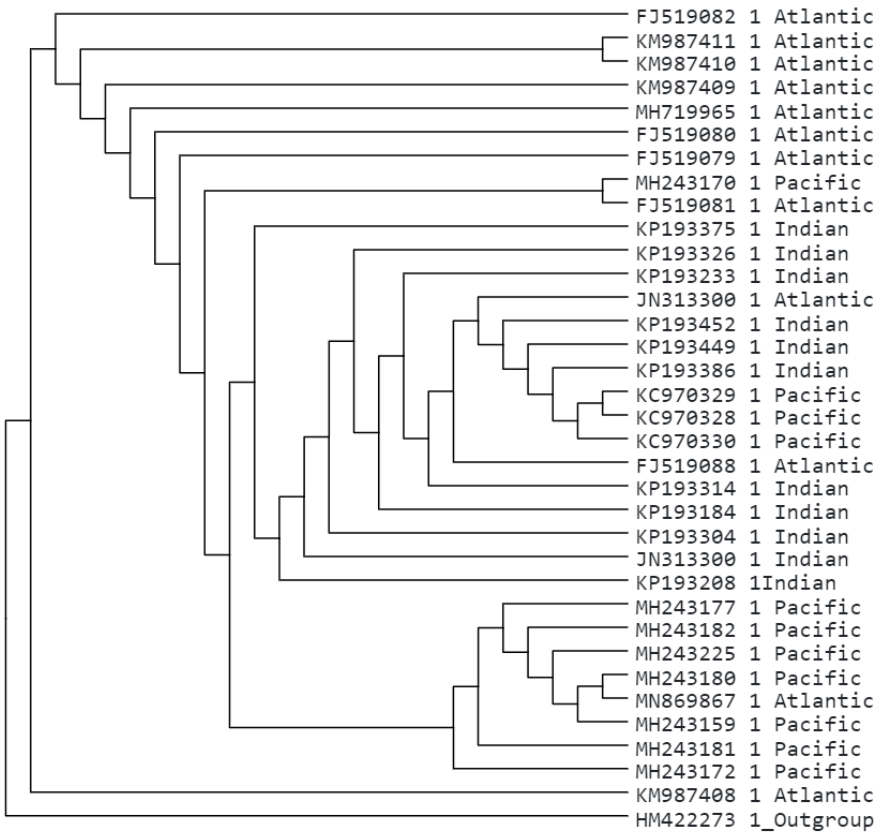

Supplementary figure 4. Phylogenetic tree of 34 silky sharks from different oceans.All bootstrap values are less than 0.04 and are therefore not shown. The silky sharks in different oceans are unable to be separated by the *COI* gene.

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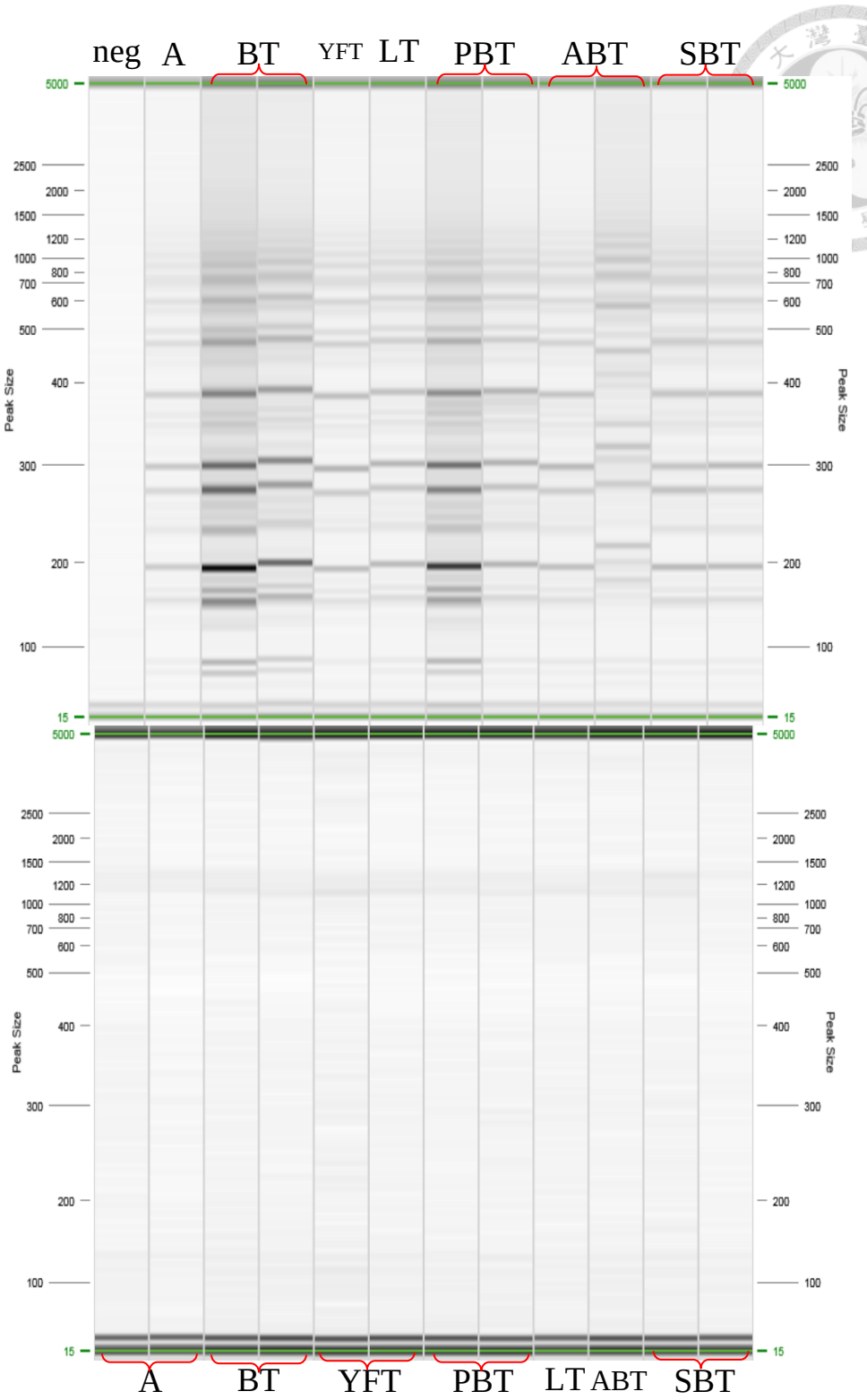

Supplementary figure 5. Capillary electrophoresis of ND5 LAMP results at 65°C.The results of capillary electrophoresis of the channels with multiple dark bands are positive results of the LAMP assay using ND5 primer set at 65°C, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*). Supplementary

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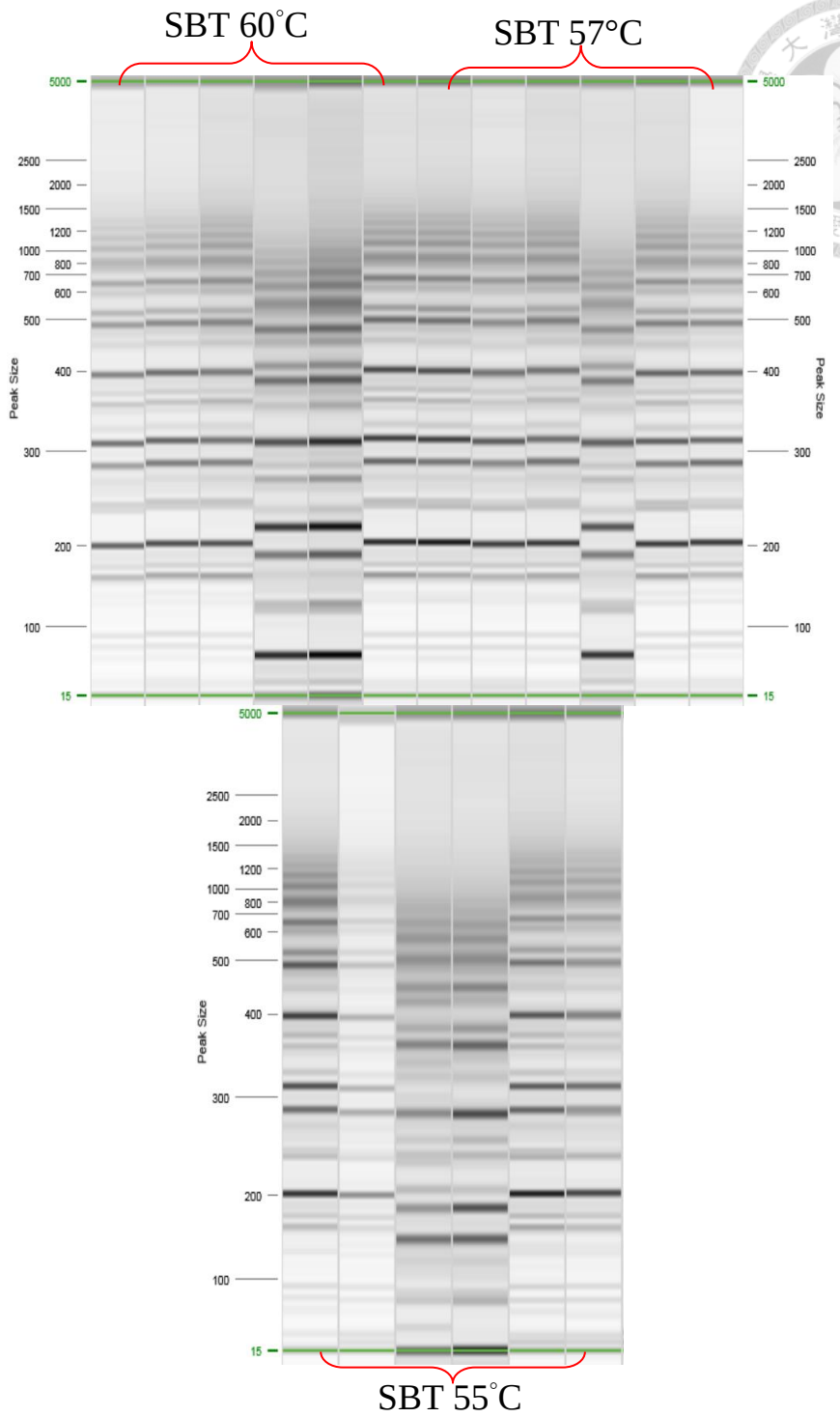
table 1.The positive and negative result numbers of the LAMP assay using ND5 primer set at 65°C (n = 23).SBT: southern bluefin tuna (*Thunnus maccoyii*); PBT: Pacific bluefin tuna (*Thunnus orientalis*); Bigeye: bigeye tuna (*Thunnus obesus*); YFT: yellowfin tuna (*Thunnus albacares*); ABT: Atlantic bluefin tuna (*Thunnus thynnus*); LT: longtail tuna (*Thunnus tonggol*).

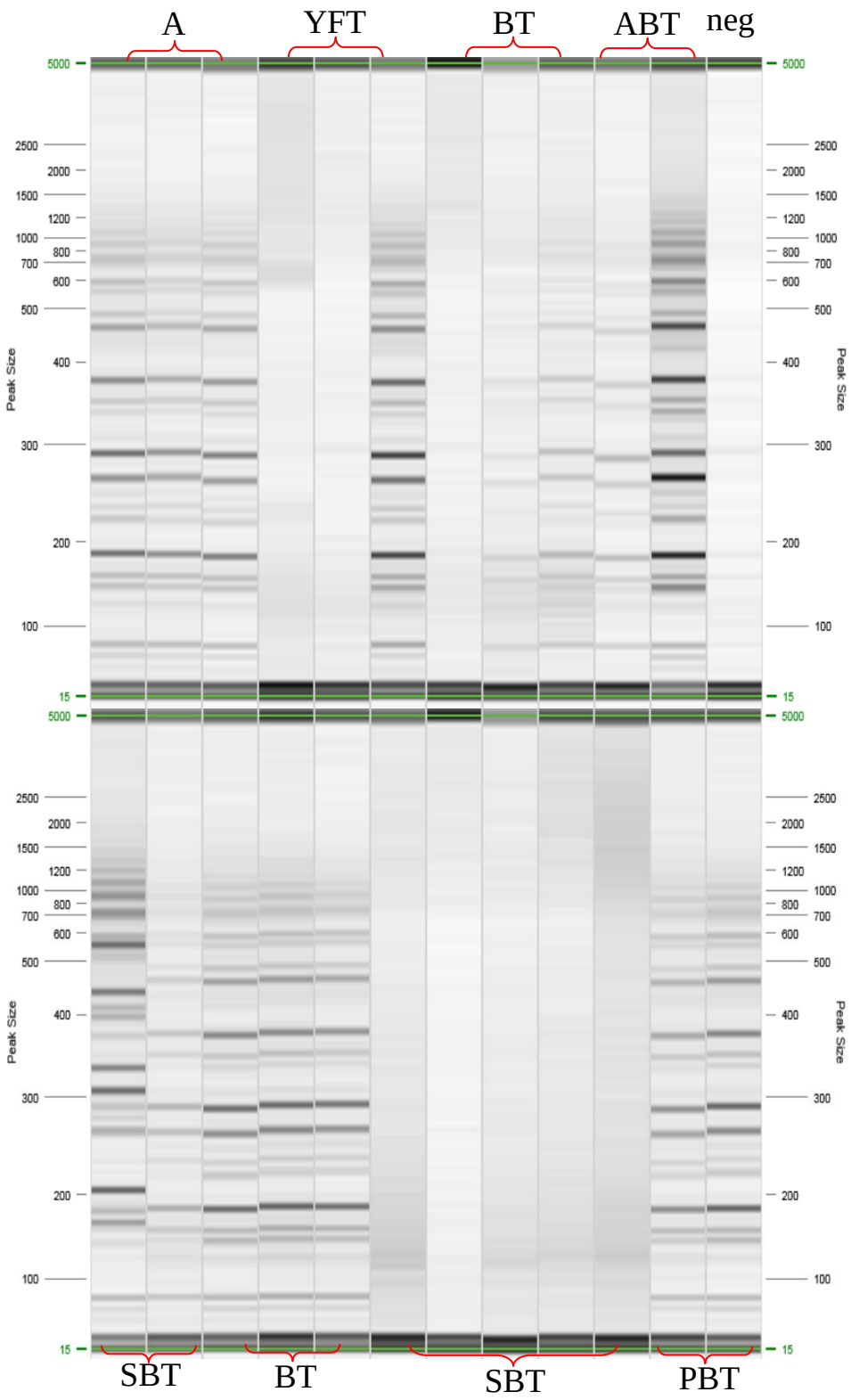
ND5 65°C	SBT	PBT	Albacore	Bigeye	YFT	ABT	LT
Positive	2	2	1	2	1	2	1
Negative	2	2	2	2	2	1	1

doi:10.6342/NTU202302785



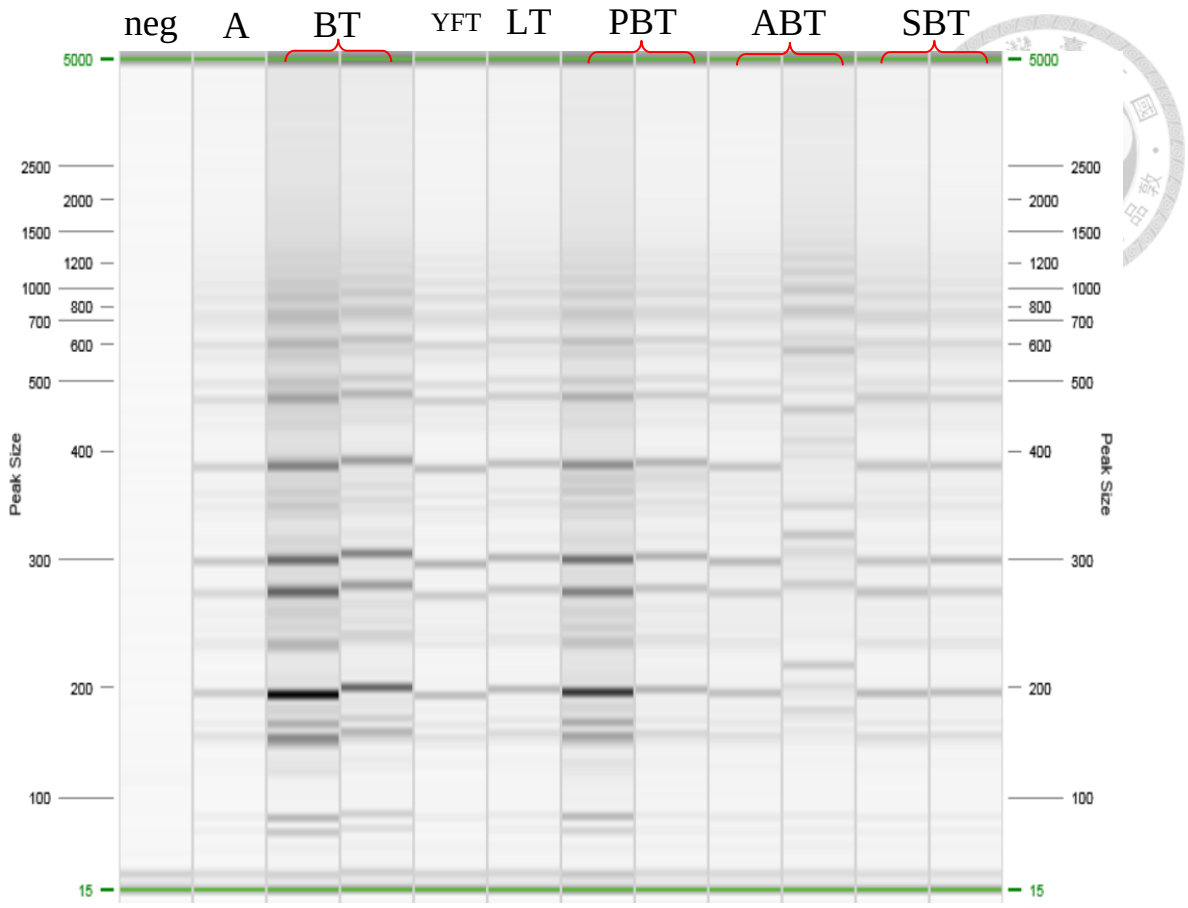
Supplementary figure 6.The capillary electrophoresis of the LAMP assays using the ND5 primer set across different temperatures.The channels with multiple dark bands are positive results. SBT means southern bluefin tuna (*Thunnus maccoyii*).

doi:10.6342/NTU202302785



Supplementary figure 7.The capillary electrophoresis of the LAMP assays using the ND5 primer set at 65°C.The channels with multiple dark bands are positive results, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*).

doi:10.6342/NTU202302785

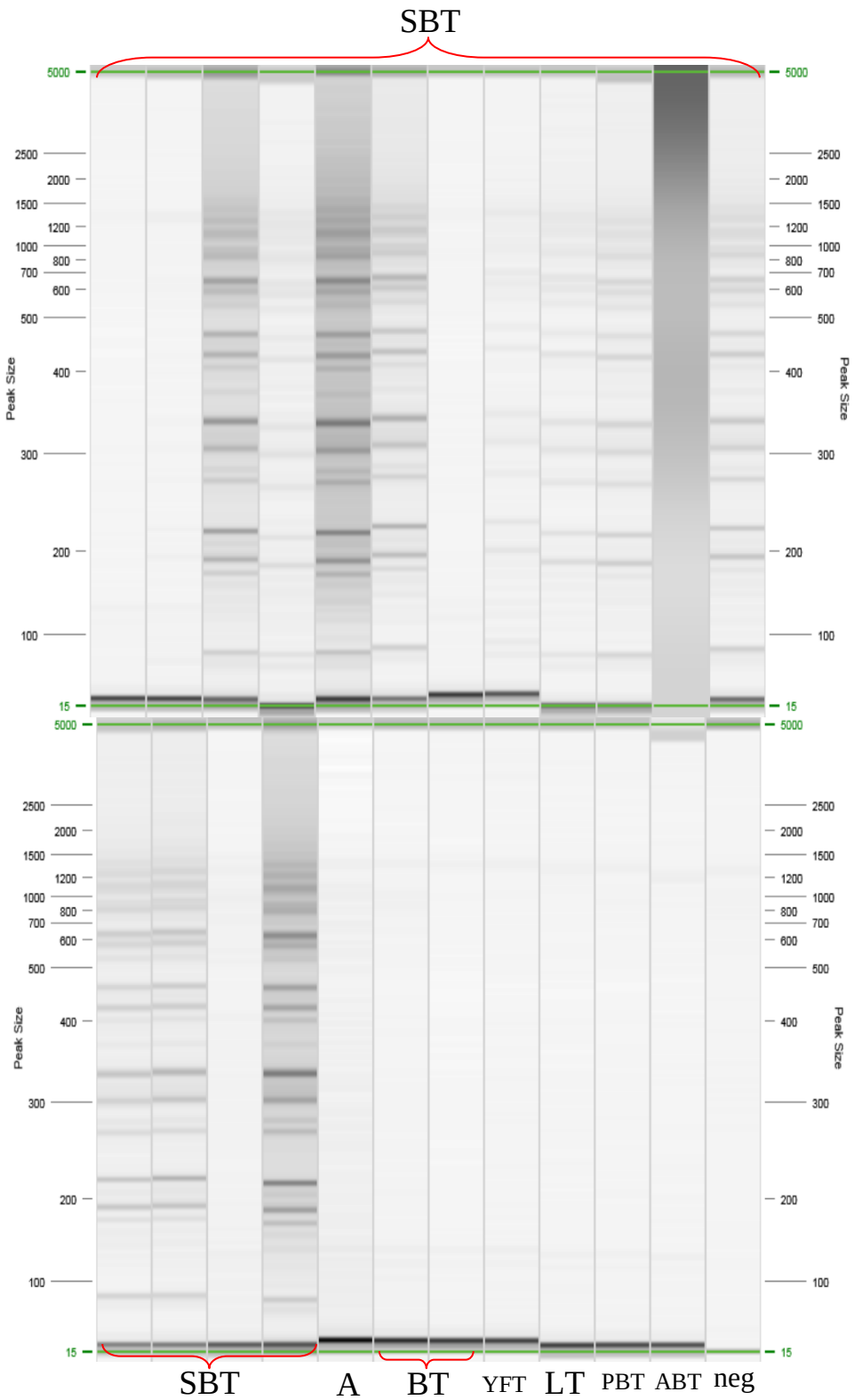


Supplementary figure 7 continued, the capillary electrophoresis of the LAMP assays using the ND5 primer set at 65°C. The channels with multiple dark bands are positive results, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*).

Supplementary table 2.The positive and negative result numbers of the LAMP assay using ND5 primer set at 65°C (sample size = 34).SBT: southern bluefin tuna (*Thunnus maccoyii*); PBT: Pacific bluefin tuna (*Thunnus orientalis*); Bigeye: bigeye tuna (*Thunnus obesus*); YFT: yellowfin tuna (*Thunnus albacares*); ABT: Atlantic bluefin tuna (*Thunnus thynnus*); LT: longtail tuna (*Thunnus tonggol*).

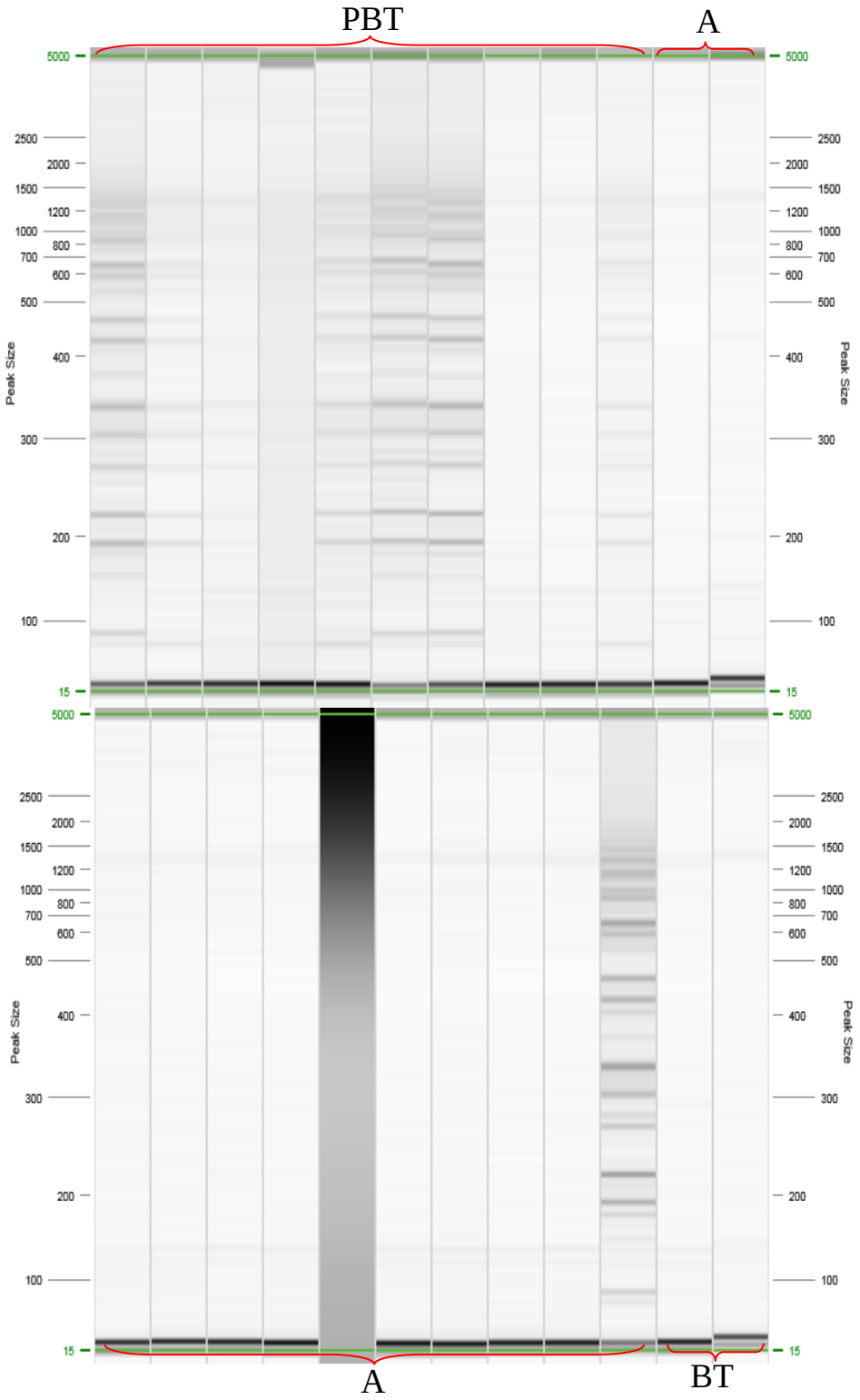
ND5 65°C	SBT	PBT	Albacore	Bigeye	YFT	ABT	LT
Positive	4	4	4	7	2	4	1
Negative	5	0	0	1	2	0	0

doi:10.6342/NTU202302785



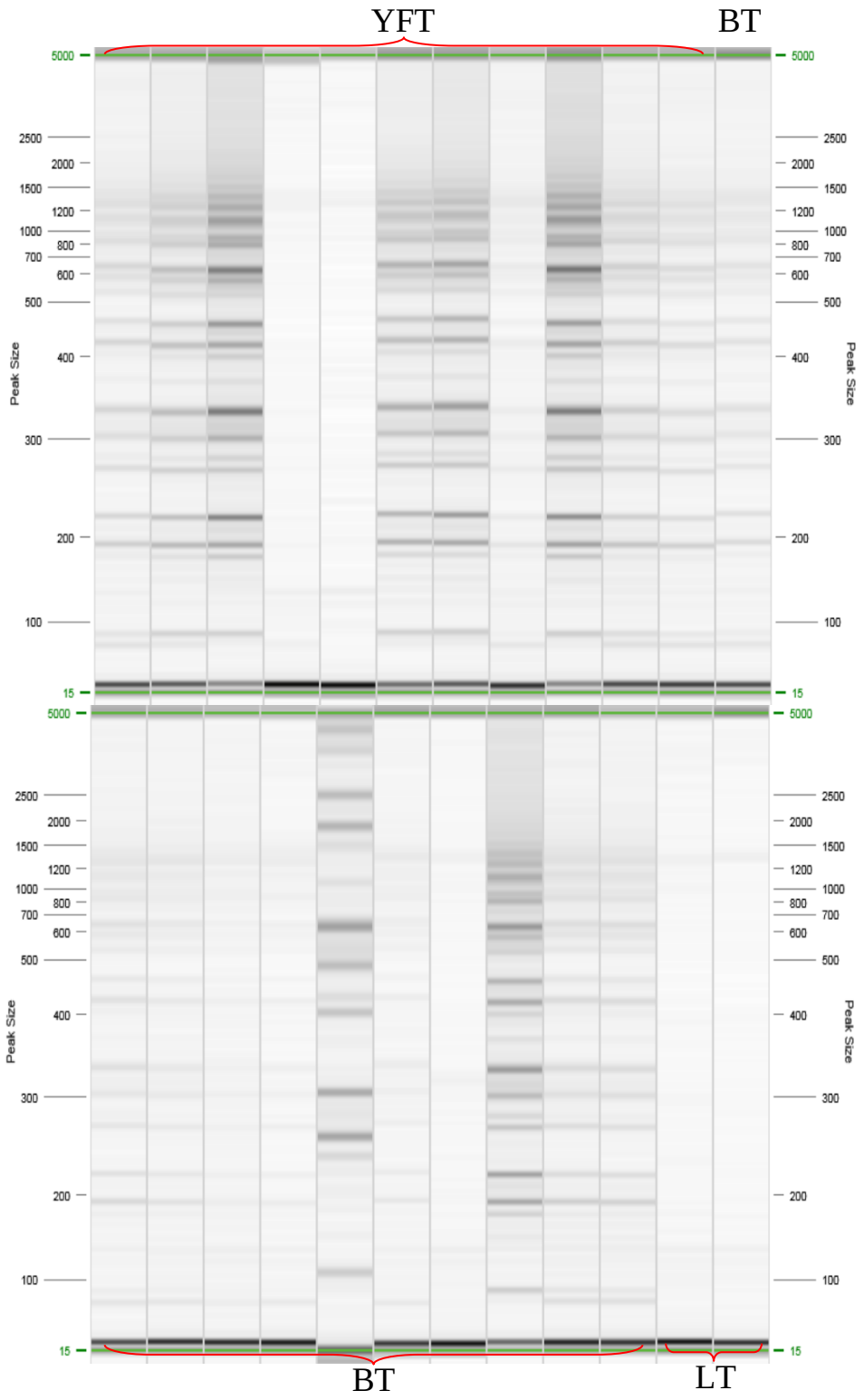
Supplementary figure 8.The capillary electrophoresis of the LAMP assays using the D-loop primer set at 55°C.The channels with multiple dark bands are positive results, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*).

doi:10.6342/NTU202302785



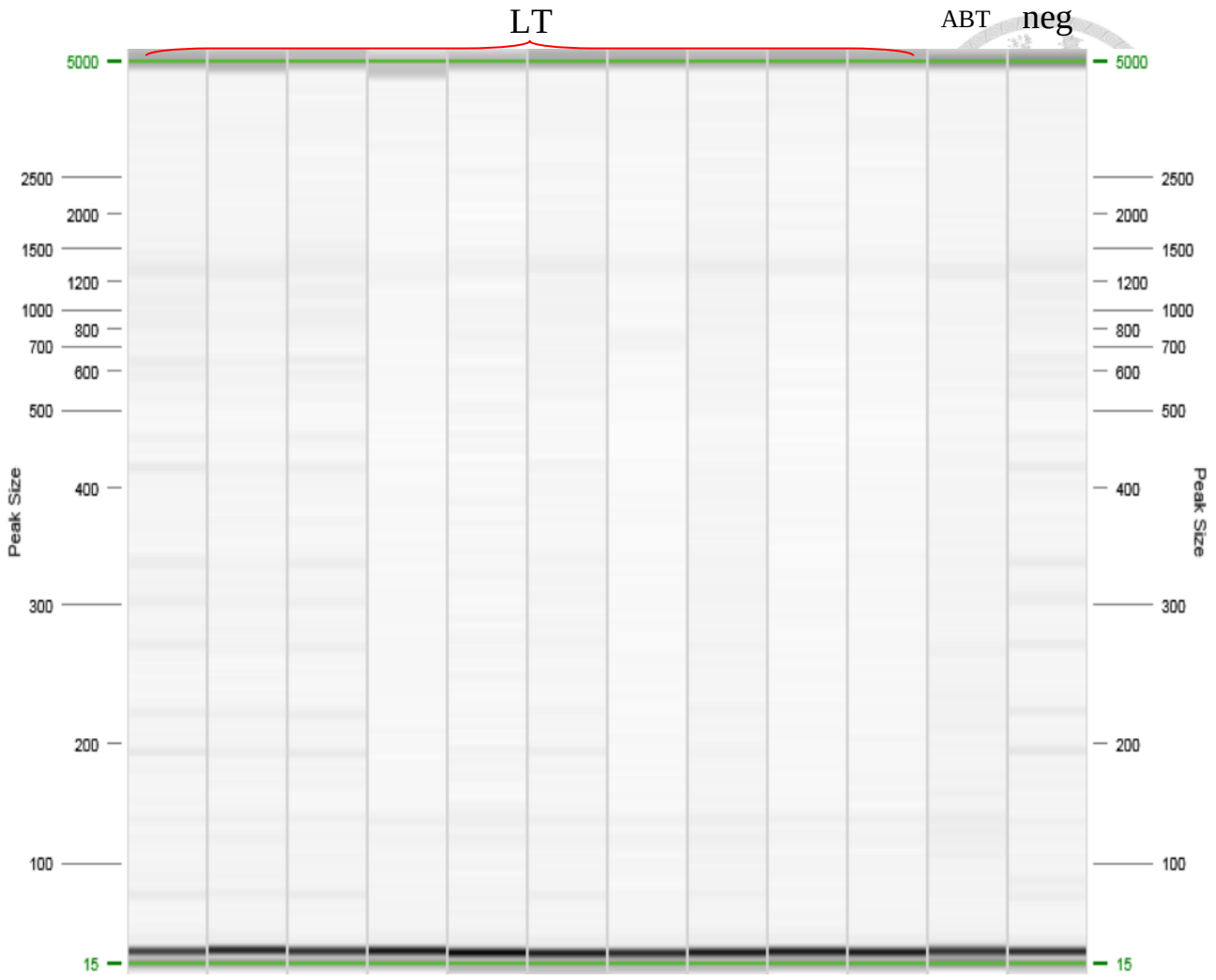
Supplementary figure 8 continued. The capillary electrophoresis of the LAMP assays using the D-loop primer set at 55°C. The channels with multiple dark bands are positive results, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*).

doi:10.6342/NTU202302785



Supplementary figure 8 continued. The capillary electrophoresis of the LAMP assays using the D-loop primer set at 55°C. The channels with multiple dark bands are positive results, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*).

doi:10.6342/NTU202302785

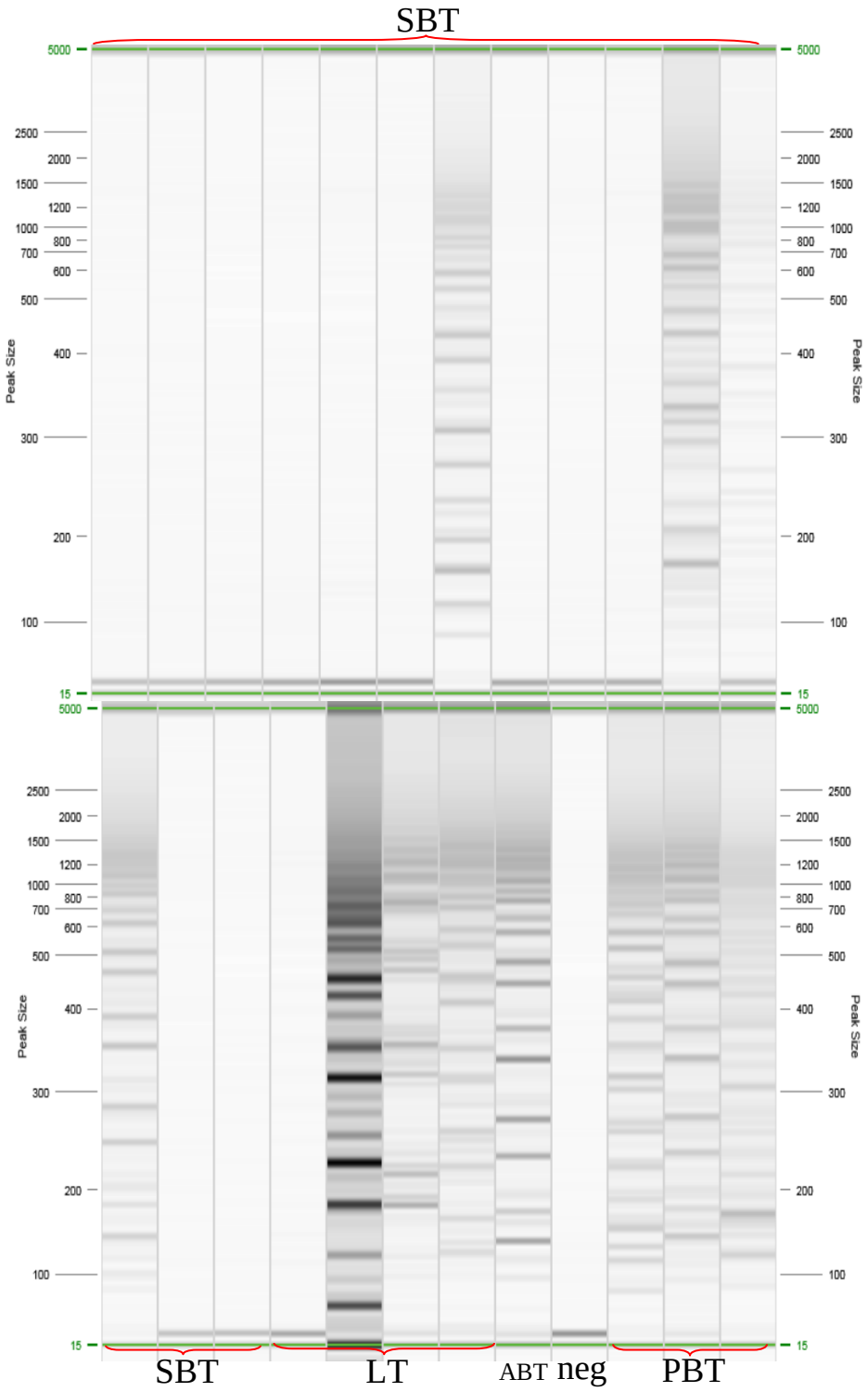


Supplementary figure 8 continued. The capillary electrophoresis of the LAMP assays using the D-loop primer set at 55°C. The channels with multiple dark bands are positive results, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*).

Supplementary table 3.The positive and negative result numbers of the LAMP assay using the D-loop primer set at 55°C (sample size = 82).SBT: southern bluefin tuna (*Thunnus maccoyii*); PBT: Pacific bluefin tuna (*Thunnus orientalis*); Bigeye: bigeye tuna (*Thunnus obesus*); YFT: yellowfin tuna (*Thunnus albacares*); ABT: Atlantic bluefin tuna (*Thunnus thynnus*); LT: longtail tuna (*Thunnus tonggol*).

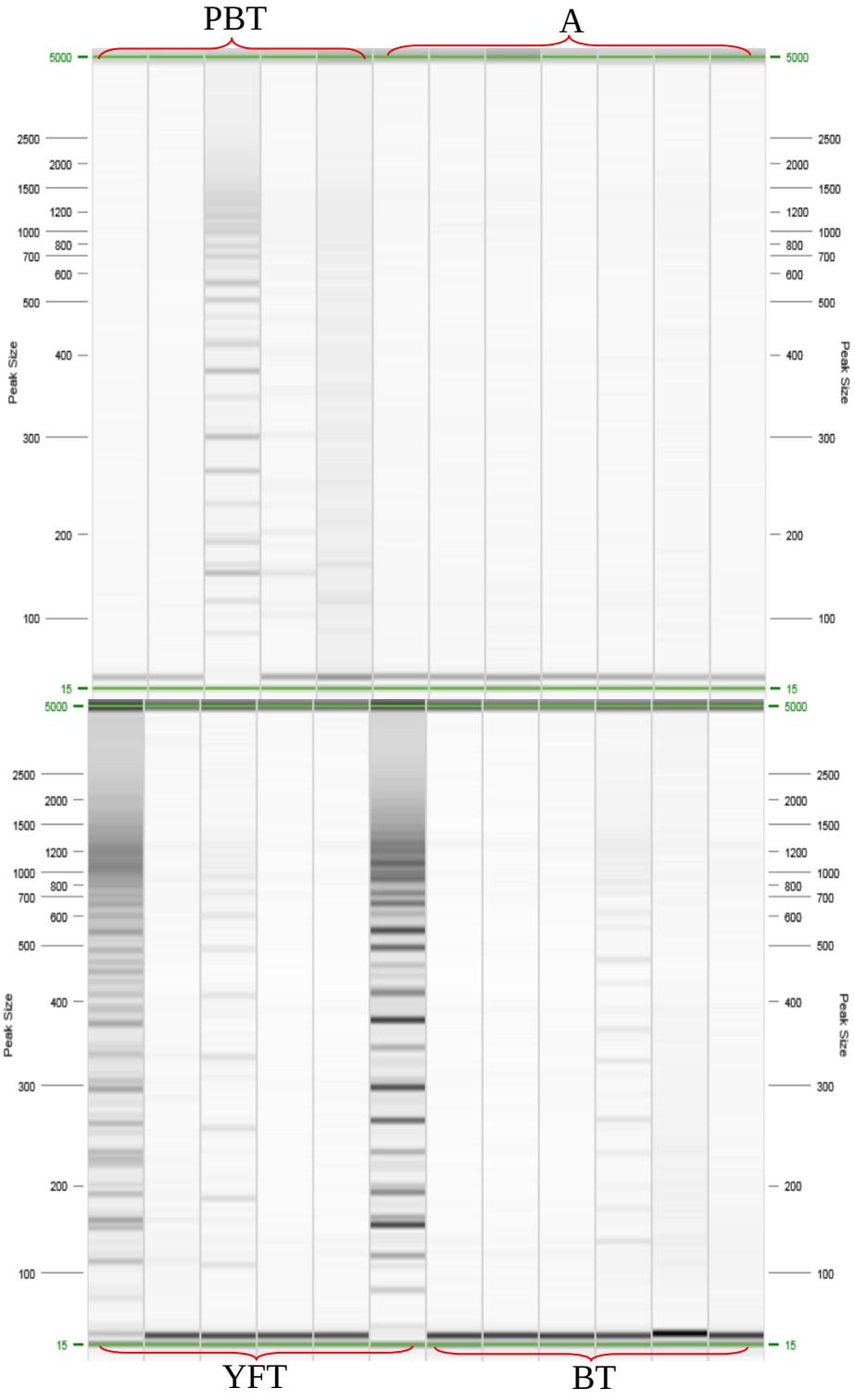
D-loop 55°C	SBT	Albacore	Bigeye	YFT	LT	PBT	ABT
positive	12	1	7	8	0	6	0
negative	4	12	6	6	13	5	2

doi:10.6342/NTU202302785



Supplementary figure 9. The capillary electrophoresis of the LAMP assays using the D-loop primer set without loop primer at 55°C.The channels with multiple dark bands are positive results, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*).

doi:10.6342/NTU202302785



Supplementary figure 9 continued. The capillary electrophoresis of the LAMP assays using the D-loop primer set without loop primer at 55°C. The channels with multiple dark bands are positive results, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*).

doi:10.6342/NTU202302785

Supplementary figure 9 continued. The capillary electrophoresis of the LAMP assays using the D-loop primer set without loop primer at 65°C. The channels with multiple dark bands are positive results, while the blank ones are negative results. neg: negative control, A: albacore (*Thunnus alalunga*), BT: bigeye tuna (*Thunnus obesus*), YFT: yellowfin tuna (*Thunnus albacares*), LT: (*Thunnus tonggol*), PBT: Pacific bluefin tuna (*Thunnus orientalis*), ABT: Atlantic bluefin tuna (*Thunnus thynnus*), SBT: southern bluefin tuna (*Thunnus maccoyii*).

doi:10.6342/NTU202302785

Supplementary table 4. The positive and negative result numbers of the LAMP assays using D-loop primer set without loop primer at 65°C (sample size = 50).SBT: southern bluefin tuna (*Thunnus maccoyii*); PBT: Pacific bluefin tuna (*Thunnus orientalis*); Bigeye: bigeye tuna (*Thunnus obesus*); YFT: yellowfin tuna (*Thunnus albacares*); ABT: Atlantic bluefin tuna (*Thunnus thynnus*); LT: longtail tuna (*Thunnus tonggol*).

D-loop 65°C	SBT	Albacore	Bigeye	YFT	LT	ABT	PBT
positive	4	0	1	3	3	1	5
negative	11	7	5	3	4	0	3

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Supplementary table 5. PCR primers for shark fin barcoding.

Forwards:	
	FishF1: 5'-TCAACCAACCACAAAGACATTGGCAC-3'
	FishF2: 5'-TCGACTAATCATAAAGATATCGGCAC-3'
Reverse	
	FishR1: 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'
	FIshR2: 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'
	HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3'

Supplementary table 6. Designing conditions for LAMP primer sets.

Tm:
F1c---65°C (64-66)
B1c---65°C (64-66)
F2---60°C (59-61)
B2---60°C (59-61)
F3---60°C (59-61)
B3---60°C (59-61)
loop---65°C (64-66)
ΔG:
-4kcal/mol (or lower)
GC content:
50%-60% (40%-65%)
primer distance:
F2-B2---120-160bps (120-180)
F2-F1c---40-60bps
F2-F3---0-60bps (0-20)

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Supplementary table 7. The sequences of the LAMP primer for the ND5 gene. Both F3 and FIP (forwards inner primer) are forwards primers, while B3, BIP (backwards inner primer) and LB (backwards loop primer) are backwards primers.

F3: 5'-ACCGTTTCTTTAAATACCTTCT-3'
B3: 5'-GTCCGATATCCCCCACTC-3'
FIP:
5'-CTTCCCACCCGATAAAGAGTTGCATCGCTATAATTATTCTAGTCACA-3'
BIP: 5'-GCGTAGGAATCATATCCTTCCTCCTACTACCGCTTGTAGAGCA-3'
LB: 5'-TCGGCTGATGGTATGGTCG-3'

Supplementary table 8. The sequences of the LAMP primer for the D-loop gene. Both F3 and FIP (forwards inner primer) are forwards primers, while B3, BIP (backwards inner primer) and LB (backwards loop primer) are backwards primers.

F3: 5'-ACACAAACCTAAATCGTCTAAGCC-3'
B3: 5'-CTTATGCAAGCGTCGATGAAAG-3'
FIP:
5'-CCGTGTGCATTAAGAAATGGACTGGCCTCATTCCTGAGGTCTGGTA-3'
BIP: 5'-GGTGAGGGACAATAATTGTGGGGGCCAAGTCATGGCCCTGAAG-3'
LB: 5'-CAGTGAATTATTCCTGGCATTTGGT-3'

doi:10.6342/NTU202302785

Supplementary table 9. The mismatch numbers of ND5 primer set across tuna species. Both F3 and FIP (forwards inner primer) are forwards primers, while B3, BIP (backwards inner primer) and LB (backwards loop primer) are backwards primers. SBT: southern bluefin tuna (*Thunnus maccoyii*); PBT: Pacific bluefin tuna (*Thunnus orientalis*); Bigeye: bigeye tuna (*Thunnus obesus*); YFT: yellowfin tuna (*Thunnus albacares*); ABT: Atlantic bluefin tuna (*Thunnus thynnus*); LT: longtail tuna (*Thunnus tonggol*).

name	F3	B3	FIP	BIP	LB	sum of mismatches
Albacore	0	0	3	2	0	5
ABT	0	2	1	0	0	3
BET	1	1	4	1	0	7
BT	1	3	2	2	0	8
LT	1	2	3	2	0	8
PBT	0	1	3	2	0	6
YFT	1	2	3	2	0	8

Supplementary table 10. The mismatch numbers of D-loop primer set across the tuna species.Both F3 and FIP (forwards inner primer) are forwards primers, while B3, BIP (backwards inner primer) and LB (backwards loop primer) are backwards primers. SBT: southern bluefin tuna (*Thunnus maccoyii*); PBT: Pacific bluefin tuna (*Thunnus orientalis*); Bigeye: bigeye tuna (*Thunnus obesus*); YFT: yellowfin tuna (*Thunnus albacares*); ABT: Atlantic bluefin tuna (*Thunnus thynnus*); LT: longtail tuna (*Thunnus tonggol*).

name	F3	B3	FIP	BIP	LB	sum of mismatches
Albacore	1	0	7	1	0	9
ABT	1	0	8	0	0	9
BET	1	0	7	2	0	10
LT	4	0	8	1	0	13
PBT	1	0	8	3	0	12
YFT	1	0	5	2	0	8

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Supplementary table 11. The positive and negative result numbers of the LAMP assay using the D-loop primer set but without the loop primer at 65°C (n = 23). SBT: southern bluefin tuna (*Thunnus maccoyii*); PBT: Pacific bluefin tuna (*Thunnus orientalis*); Bigeye: bigeye tuna (*Thunnus obesus*); YFT: yellowfin tuna (*Thunnus albacares*); ABT: Atlantic bluefin tuna (*Thunnus thynnus*); LT: longtail tuna (*Thunnus tonggol*).

D-loop 65°C	SBT	Albacore	Bigeye	YFT	LT	ABT	PBT
positive	3	2	0	1	0	0	1
negative	0	1	4	3	3	2	3

doi:10.6342/NTU202302785