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Original Research Article

Polymerase Chain Reaction-Amplification of COI Gene of Stingray and Shark Using Four Primer Pairs

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ABSTRACT

The polymerase chain reaction (PCR)-based technique is essential for the successful amplification of the COI gene for the molecular identification of species. The present study was conducted to explore the amplification of the COI gene in stingray and shark samples using four primer pairs: FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R. Five species of stingrays and two types of sharks were obtained from TPI Tasik Agung Rembang. DNA was isolated from the fish samples using the TIANamp Marine Animal DNA Kit. The concentration and quality of the DNA extracts were determined using the Nanodrop spectrophotometer and gel electrophoresis, respectively. The amplification results showed that the COI gene of *Himantura gerrardi* stingray was successfully amplified by the primer pair FishF1-FishR1; *Himantura uarnocoides* by the primer pairs FishF1-FishR1 and FishF2-FishR2; *Himantura walga* by the primer pairs FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R; *Neotrygon kuhlii* by the primer pairs FishF1-FishR1, FishF2-FishR2, and FishF1-HCO2198R; *Rhinobatos penggali* by the primer pairs FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R. *Chiloscyllium punctatum* and *Carcharhinus sealei* are shark species that were amplified successfully using the four primer pairs FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R. The findings of this study reveal that the test primer pairs can be used for the molecular identification of stingrays and sharks.

Keywords: Amplification, COI gene, PCR, Primer, Shark, Stingray

Introduction

Stingrays and sharks are members of the class Chondrichthyes, or cartilaginous fish, which have been extensively exploited for trade and human use. For example, stingray skin has been used to make industrial products, handcrafted leather bags, shoes, bracelets, wallets, belts, and so on. Stingray meat can be utilized as a pharmaceutical raw material, and the bones can be used as raw materials for glue in addition to being a food source.[1,2] A total of 116 species from 25 shark families are found in Indonesia. According to research, up to 60 species of sharks are classified as threatened or vulnerable.[3] If stingray use is not balanced with conservation measures, populations and species of stingrays may decline rapidly and take a long time to recover.[4] Based on data from the International Union for Conservation of Nature and Natural Resources (IUCN), there are 28 species of stingrays in Java, of which three species are critically endangered, six are endangered, seven are vulnerable, three are near threatened, three are least concern, and six are in the deficient data category species.[5] This reality is made worse by its biological characteristics, where the growth rate and maturity are slow, and the fecundity of stingrays is relatively low.[6] Hence, conservation efforts need to be made to maintain its sustainability.

TPI Tasik Agung is a fish auction site in Rembang Regency, on the north coast of Central Java, where the likelihood of encountering stingrays and sharks is higher than on the south coast. This is because the Java Sea's local wind (wind sea) causes low wave energy conditions in the waters along the north shore.[7] Storms (swells) from the Indian Ocean cause the southern coastal region to experience high wave energy waters.[8] From August 2019 to December 2019, the number of stingrays and sharks taken by fishermen and traded at TPI Tasik Agung Rembang consistently increased.[9] Conservation efforts are required to safeguard the sustainability of nature through molecular identification since it is expected that the growing output will lead to a decline in availability.

The cytochrome C oxidase 1 (COI) gene is the most popularly used gene in mitochondrial DNA for molecular identification. Aquatic species' phylogeny, genetic diversity, and species identity have all been demonstrated to be revealed by COI genes.[10] The success of the COI gene in identifying species cannot be separated from the amplification of the gene in the polymerase chain reaction (PCR) technique. The PCR technique is based on a primer-mediated enzymatic method.[11] Using a universal primer frequently fails to amplify the COI genes of various animal species. Using FishF1-FishR1 and FishF2-FishR2 primer pairs, the stingray CO1 gene was amplified, but only in 4 of the 5 samples tested.[12] As a result, modifications were made using HCO2198R primers, which had never been done in earlier investigations, especially in sharks, to amplify the COI gene of stingrays traded at TPI Tasik Agung Rembang. The use of this primer variant builds on the success of these primers in amplifying the stingray CO1 gene that has been made by several researchers, including studies on stingrays in Australasia.[13] The primer pair also amplified stingrays in Australian waters successfully.[14] In the North Minahasa village of Tumbak, four shark samples were successfully amplified using the FishF1-HCO2198R primer pair.[15]

The present study was aimed at amplifying the COI genes of stingrays and sharks using four pairs of primers.

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Materials and Methods

Sample collection

Samples were obtained from fishermen who traded fish at TPI Tasik Agung on March 26, 2020, by 3 PM. Five species of stingrays were identified: *Himantura gerrardi* (A), *Himantura uarnocoides* (B), *Himantura walga* (C), *Neotrygon kuhlii* (D), and *Rhinobatos penggali* (E). Also, two types of sharks including *Carcharhinus sealei* (H1) and *Chiloscyllium punctatum* (H2) were collected. The identification was carried out by Ning Setiati and Partaya based on the books, Economically Important Sharks and Rays of Indonesia (2006); Sharks and Rays of Borneo (2010). The samples were obtained from the pectoral fins about 5 cm away using scissors and tweezers. TPI Tasik Agung is a fish auction site in Rembang Regency, located on the north coast of Central Java. Geographically, it is located at 111,000–111,030 East Longitude and 6 30-7 00 South Latitude in Tasik Agung Village, Rembang District, Rembang Regency. TPI Tasik Agung has the potential to produce stingrays and sharks, which are quite high compared to the south coast.

DNA isolation

DNA isolation was carried out using the TIANamp Marine Animal DNA Kit. Shark and stingray fins weighing 30 mg each were chopped into 1.5 ml microfuge tubes, to which 200 µl of GA buffer was added to obtain samples. After 15 seconds of vortexing, 4 µl of RNAse was added to the sample, and the process was repeated for 15 seconds. The sample was then incubated at room temperature for five minutes. After adding 20 µl of proteinase K, the sample was vortexed for 15 seconds again. The sample was incubated at 56ºC for an hour until it was completely lysed. The sample was spun down and vortexed for 15 seconds every 15 minutes. After adding 200 µl of GB buffer, the sample was vortexed for 15 seconds. Following the previous step, 200 ml of absolute ethanol was added, and the sample was spun down after being vortexed for 15 seconds. The pipetted sample was placed in the collection tube's spin column and centrifuged for 30 seconds at 12,000 rpm. The pipetted sample was centrifuged for 30 seconds at 12,000 rpm after receiving 500 µl of GD buffer. The collection tube was re-attached to the spin column after the supernatant in the tube was discarded. After adding 600 µl of PW buffer, the supernatant was centrifuged for 30 seconds at 12,000 rpm. The pellet was then dehydrated by centrifugation for two minutes at 12,000 rpm. The collection tube was removed, the spin column was placed in a 1.5 ml microfuge tube, 50 µl of TE buffer was added, and the microfuge tube was incubated at room temperature for 2 to 5 minutes. It was finally centrifuged for two minutes at 12,000 rpm.

Polymerase chain reaction-amplification of COI gene

FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R were the PCR primer pairs utilized to amplify the DNA. The primer annealing temperature was initially optimized using the gradient PCR method with eight temperature variations. The total volume of the sample used was 12.5 µl with a composition of 6.25 µl 2x Taq PCR Master Mix (with loading dye), 1 µl forward primer, 1 µl reverse primer, 3.25 µl ddH_2O, and 1 µl DNA sample. The PCR was performed using a thermal cycler machine with a 35-cycle program that included pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. The melting temperature (T_m) is at which 50% of the DNA double strands separate. The selection of the T_m of a primer is essential because the T_m of the primer will affect the selection of the annealing temperature of the PCR process. The T_m relates to the primer composition and the primer's length. Theoretically, primer T_m can be calculated using the formula [2(A+T) + 4(C+G)]. Preferably, primer T_m ranges from 50–65 ºC. The sequence and T_m of each primer are presented in Table 1.

Gel electrophoresis

Gel electrophoresis analysis is required after the amplification step to evaluate the effectiveness of the primer pair utilized. At the end of the PCR program, the products were electrophoresed on a 2% agarose gel at 50 volts for 1 hour. The gel was visualized with a UV transilluminator gel documentation system. The data from the study in the form of COI gene fragment bands that appeared in the visualization results of 2% agarose gel were then analyzed descriptively to make inferences.

Table 1: Nucleotide sequence and melting temperature (T_m) of primers utilized for PCR amplification

Primer	Sequence	T_m
Fish F1	5’ - TCA ACC AAC CAC AAA GAC ATT GGC AC- 3’	66.3
Fish R1	5’ - TAG ACT TCT GGG TGG CCA AAG AAT CA - 3’	66.3
Fish F2	5’ - TCG ACT AAT CAT AAA GAT ATC GGC AC - 3’	63.2
Fish R2	5’- ACT TCA GGG TGA CCG AAG AAT CAG AA - 3’	66.3
HCO2198R	5’-TAA ACT TCA GGG TGA CCA AAA AAT CA-3	61,6

Results and Discussion

The purity of the extracted DNA was evaluated using 0.8% agarose gel electrophoresis. The results of the gel electrophoresis are shown in Figures 1 and 2. Some bands were thick, some were thin, and some samples showed smears contaminated with RNA. *Himantura gerrardi* and *Himantura uarnocoides* have thin DNA bands and smears. The *Himantura walga* DNA sample showed thin DNA bands and very few smears. *Neotrygon kuhlii* DNA samples produced thick DNA bands, but there were still many smears. The DNA samples from *Rhinobatos penggali* produced thick DNA bands and very few smears. High-quality DNA is indicated by thick DNA bands and little to no smears. Thus, the *Rhinobatos penggali* DNA sample has better DNA quality than other stingray samples. The DNA samples of *Carcharhinus sealei* (H1) and *Chiloscyllium punctatum* (H2) produced thick DNA bands but many smears. DNA can absorb ultraviolet (UV) light at 260 nm due to purine and pyrimidine bases. Protein contaminants or phenols absorb light at 280 nm, so DNA purity can be measured by calculating the absorbance value at 260 nm divided by the absorbance value at 280 nm. DNA purity values range from 1.8 to 2.0, and a value below 1.8 indicates that there are contaminants in the form of proteins. Meanwhile, DNA purity values above 2.0 indicate that there are contaminants in the form of RNA.[16]

Technical issues with the isolation stages, such as improper sample destruction that prevents DNA in cells from lysing during isolation, can have an impact on the purity and concentration of DNA. As a result, the resulting DNA has a low purity index. Another factor is that the cell may not fully lyse and may create insufficient amounts of DNA as well as impurities in the DNA if the temperature and time at the incubation stage are not appropriate.[17] DNA in the supernatant is located in the uppermost layer, while proteins form the middle layer, and the organic component is found below because it has a high specific gravity.[18] Inaccurate and careless supernatant removal can cause unexpected material other than DNA to be taken. In addition, contaminants in the form of RNA can be caused by the addition of RNase solutions at the isolation stage that are not suitable. In contrast, protein contaminants are caused by adding inappropriate proteinase K.[19]

The purity of the extracted DNA was evaluated using 0.8% agarose gel electrophoresis. Samples with high-quality DNA exhibit thick DNA bands and little to no smears. The results of the electrophoresis on the seven samples revealed that DNA from each sample was successfully isolated. This is evident from the DNA bands that emerge on the gel,

some of which are thick and others thin, and there is a visible smear. The quantity of separated DNA concentration determines how differently the results behave in each sample.[20] The thicker the DNA band, the higher the concentration, and the thinner the DNA band, the lower the concentration. Smears that contain DNA bands have low DNA purity levels.[21] The presence of smears at the very bottom of each column indicates the presence of contaminants in the form of RNA, while smears that appear below the DNA band on the electrophoresis results indicate that the isolated DNA is not intact. These DNA fragments were formed as a result of physical treatment during the isolation process, such as sample storage in the freezer and repeated thawing of samples.[16] RNA contaminants are present, as shown by the smear at the bottom. In this study, successfully isolated samples could still be used for PCR-based amplification.

Optimal primer annealing temperature

Primer optimization was performed with eight temperature variations on the best-quality *Rhinobatos penggali* DNA sample. The primer range T_m employed and carried out in a gradient method during the PCR process indicated the usage of annealing temperature variations to identify the optimal annealing temperature.

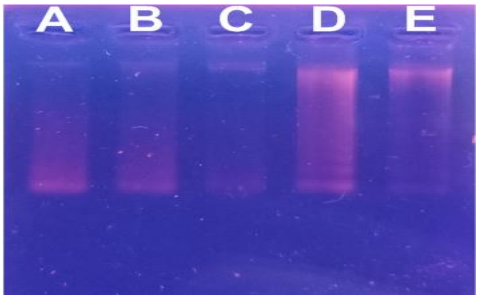

Figure 1: Gel electrophoresis of isolated DNA from stingray.
A: *Himantura gerrardi*; B: *Himantura uarnocoides*; C: *Himantura walga;* D: *Neotrygon kuhlii*; E: *Rhinobatos penggali*.

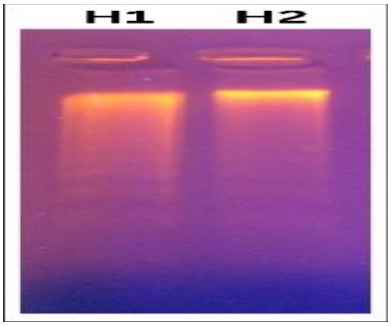

Figure 2: Gel electrophoresis of isolated DNA from shark.
H1: *Chiloscyllium punctatum;* H2: *Carcharhinus sealei*

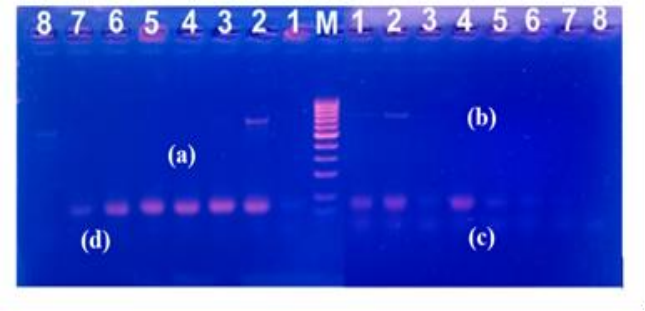

Figure 3: Gel electrophoresis of primer annealing temperature optimization.
a: FishF1-FishR1; b: FishF2-FishR2; c: FishF1-HCO2198R; d: FishF2-HCO2198R; M: DNA *ladder*; 1: 51°C; 2: 54°C; 3: 56°C; 4: 59°C; 5: 61°C; 6: 63°C; 7: 66°C; 8: 68°C; The primer annealing temperature was conducted using the DNA isolated from *Rhinobatos penggali*.

During optimization, primer pairs of FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R with different annealing temperatures (51, 54, 56, 59, 61, 63, 66, and 68°C) were used. The optimal primer annealing temperature was detected by 2% agarose gel electrophoresis after the PCR, as shown in Figure 3. The best annealing temperature is the one that produces the thickest and clearest DNA bands as well as the smallest on-target PCR product size. The PCR gradient revealed that the thickest and clearest DNA bands were observed at an annealing temperature of 54°C in the FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R primer pairs. The optimal annealing temperature was chosen in four primer combinations to amplify all DNA samples.

The choice of primer has a significant impact on the success of the amplification. It is critical because the primer triggers the synthesis of the target DNA. The primers used in this investigation were chosen based on their past performance in amplifying the stingray COI gene.[23] An accurate primer annealing temperature is also required, hence, the primer can attach specifically to both ends of the target DNA. A too-low annealing temperature will cause miss-priming, while a too-high temperature will cause the PCR product not to form because the primer attached to the DNA template is rereleased. The optimal annealing temperature was determined using a PCR gradient. The best quality *Rhinobatos penggali* DNA sample was used for optimization. The annealing temperature was chosen based on the band that appears most clearly and thickly on the gel electrophoresis results.

The gel electrophoresis analysis showed that the primer pairs FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R with an annealing temperature of 54°C produced the thickest bands compared to others. At different temperatures, thinner bands were evident; at others, no bands were seen on the gel. A mismatch between the annealing temperature and the primer used led to the thin band because the primer did not adhere properly, and the amplification was insufficient. No bands emerged on the gel due to the wrong temperature, which prevented the primer from adhering. The polymerase enzyme did not insert the complementary DNA sequence into the DNA template. Finally, no new DNA was produced. Based on these findings, FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R primer pairs at an annealing temperature of 54°C can be used to amplify all samples.

Polymerase chain reaction-amplified products

All DNA samples were amplified by PCR using four primer pairs at an annealing temperature of 54°C. Successfully amplified samples were recognized by the presence of DNA bands on the gel, with the size of the DNA bands varying according to the location of the primer attachment to the sample COI gene.[11] The DNA band was about 655 bp. The thickness depends on the sample DNA's concentration, while the smear depends on the sample DNA's purity. Furthermore, the thickness of the band is affected by the small and less uniform DNA samples used. When DNA samples amplify slightly and consistently, narrow bands can be created even if the DNA's anticipated concentration and purity are appropriate. The results of the COI gene amplification products from stingrays and sharks using four primer pairs are presented in Figures 4-11. As shown in Figure 4, the FishF1-FishR1 primer pair successfully amplified *Himantura gerrardi*, *Himantura uarnocoides*, *Neotrygon kuhlii*, and *Rhinobatos penggali* DNA samples. The *Himantura walga* DNA sample, in contrast, did not amplify. The band size for each sample seemed different depending on the position of the primer attachment to the sample COI gene, but it was still around 655 bp. The thickness of the bands for each DNA sample was also slightly different. The bands in *Himantura gerrardi* and *Neotrygon kuhlii* were slightly thinner than those observed in *Himantura uarnocoides* and *Rhinobatos penggali*. It was possible because the concentration and DNA samples were smaller and less homogeneous.

The results of the amplification by the primer pair FishF1-FishR1 in sharks are depicted in Figure 5. It was observed that the PCR product visualization of H1 and H2 shark DNA samples was successfully amplified by the primer pair of Fish F1–Fish R2 using 2% agarose gel, characterized by the appearance of DNA bands. The position and thickness of the tape appear to be the same for each sample. Figures 6

and 7 highlight the amplification results using the FishF2-FishR2 primer pair in stingray and shark DNA samples. Figure 6 demonstrates the effective amplification of *Himantura uarnocoides, Himantura walga, Neotrygon kuhlii*, and *Rhinobatos penggali* using the FishF2-FishR2 primer pair. Based on the primer attachment point on the COI gene of each DNA sample, which was around 655 bp, the amplification produced different DNA band sizes. The *Himantura gerrardi* sample, in contrast, was not amplified. The bands in *Neotrygon kuhlii* and *Rhinobatos penggali* were thinner than those in *Himantura uarnocoides* and *Rhinobatos penggali* due to the smaller and less homogeneous concentration of DNA samples.

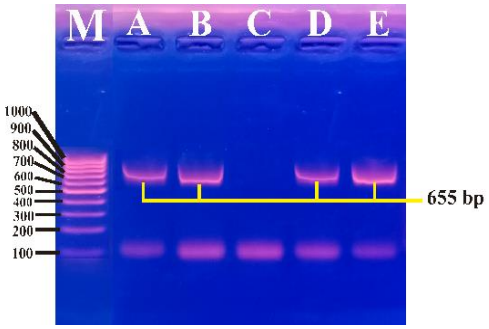

Figure 4: Visualization of PCR product from stingray DNA using FishF1-FishR1 primer pair. M: DNA ladder; A: *Himantura gerrardi*; B: *Himantura uarnocoides*; C: *Himantura walga*; D: *Neotrygon kuhlii*; E: *Rhinobatos penggali*.

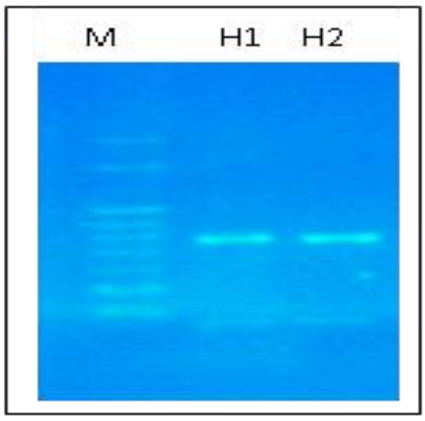

Figure 5: Visualization of PCR product from shark DNA using FishF1-FishR1 primer pair.
M: DNA ladder; H1: *Chiloscyllium punctatum*; H2: *Carcharhinus sealei*

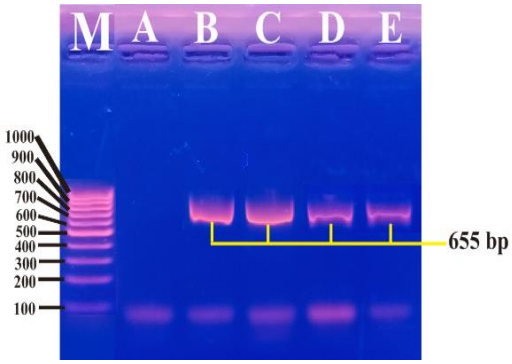

Figure 6: Visualization of PCR products from stingray DNA using FishF2-FishR2 primer pairs. DNA ladder; A: *Himantura gerrardi*; B: *Himantura uarnocoides*; C: *Himantura walga;* D: *Neotrygon kuhlii*; E: *Rhinobatos penggali*

In Figure 7, the results of gel electrophoresis analysis showed that the FishF2-FishR2 primer pair successfully amplified the COI gene from DNA samples of *Chiloscyllium punctatum* and *Carcharhinus sealei* sharks. The results were marked by the appearance of DNA bands on the H1 and H2 pathways with PCR products of 650-655 bp. Furthermore, the amplification results by the primer pairs of the FishF1-HCO2198R COI gene of stingrays are shown in Figures 8 and 9. As observed in Figure 8, the primer pair FishF1-HCO2198R successfully amplified three samples: *Himantura walga, Neotrygon kuhlii*, and *Rhinobatos penggali*. In contrast, DNA samples *of Himantura gerrardi* and *Himantura uarnocoides* did not amplify. The three samples all produced almost the same-sized bands (655 bp), but the bands produced by the FishF1-FishR1 and FishF2-FishR2 primer pairs were thinner than the amplified tape. The results of the amplification by the primer pair FishF1-HCO2198R COI gene in sharks are presented in Figure 9. It was observed that the primer pair FishF1-HCO2198R successfully amplified two samples: *Chiloscyllium punctatum* and *Carcharhinus sealei*. The size of the bands in the two PCR product samples is about identical (655 bp), however, the band generated by the FishF1-FishR1 and FishF2-FishR2 primer pairs is much thinner. Similarly, the results of the amplification by the primer pair FishF2-HCO2198R COI gene are shown in Figure 10. The FishF2-HCO2198R primer pair was only able to amplify two stingray DNA samples, *Himantura walga*, and *Rhinobatos penggali*, with a nearly identical band size of about 655 bp. On the other hand, samples *of Himantura gerrardi, Himantura uarnocoides*, and *Neotrygon kuhlii* failed to amplify. Similar to the amplification results of the FishF1-HCO2198R primer pair, the bands produced by the FishF2-HCO2198R primer pair were also thinner than those of the FishF1-FishR1 and FishF2-FishR2 primer pairs. As a result, it was affected by the primer pair employed. Similarly, the results of the amplification by the FishF2-HCO2198R primer pair are also shown in Figure 11. H1 and H2 shark DNA samples were successfully amplified by FishF2-HCO2198R primer pair, which was characterized by the appearance of DNA bands (650-652 bp). Each sample's tape size, which ranges from 650 to 652 bp and was relatively thick, appeared to be of identical positional quality.

Based on the results of COI gene amplification, the proper primer selection significantly affects the success of amplification. This is important because primers are initiators in the synthesis of target DNA.[24] FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R were chosen as the primers for this investigation based on their success in amplifying the COI genes of stingrays and sharks in previous studies.[11,14,25] Primer annealing temperature accuracy is also required so that the primer can attach specifically to both ends of the target DNA. Annealing temperatures that are too low cause mispriming, while temperatures that are too high can cause PCR products not to form due to the failure of primer attachment to the DNA template. The right annealing temperature was obtained from optimization using a PCR gradient. Optimization was carried out on DNA samples with the best quality, namely *Rhinobatos penggali* samples. The annealing temperature selected was determined based on the most evident and thickest visible band on the electrophoresis gel.

The results of gel electrophoresis analysis showed that the primer pairs FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R with an annealing temperature of 54°C produced the thickest band compared to other bands at different temperatures. Thinner bands were visible at other temperatures. In some cases, there were no bands visible on the gel. Because the annealing temperature did not match the primer temperature employed, the primer did not adhere specifically, and the amplification process did not proceed as intended, resulting in a thin band. The absence of bands on the gel due to inappropriate temperatures causes no primer attachment, and polymerase enzymes cannot catalyze the insertion of complementary DNA sequences into the DNA template, which in turn does not form new DNA. Based on these results, the primer pairs FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R with an annealing temperature of 54°C can then be used for amplification of all samples. The primer attachment position in the COI gene for the FishF1-FishR1 primer pair is base 6475 to base 7126; the primer pair FishF2-FishR2 is base 6474 to base 7127; the primer pair FishF1-HCO2198R is base 6475 to base 7123; and the primer pair FishF2-

HCO2198R is base 6475 to base 7123.[22] The amplified base of each primer is approximately 655 bp.[9]

Based on the amplification results, the primer pair FishF1-FishR1 successfully amplified DNA samples from *Himantura gerrardi*, *Himantura uarnocoides*, *Neotrygon kuhlii*, *Rhinobatos penggali*, *Chiloscyllium punctatum*, and *Carcharhinus sealei* sharks. Also, the FishF2-FishR2 primer pair successfully amplified DNA samples from *Himantura uarnocoides*, *Himantura walga*, *Neotrygon kuhlii*, *Rhinobatos* penggali, *Chiloscyllium punctatum*, and *Carcharhinus sealei shark* samples. These outcomes are consistent with previous studies that amplified the same stingray DNA samples used in this study.[12] The electrophoresis results in this study also showed that the primer pair FishF1-HCO2198R successfully amplified samples of *Himantura walga*, *Neotrygon kuhlii*, *Rhinobatos penggali*, *Chiloscyllium punctatum*, and *Carcharhinus sealei* sharks. In contrast, the FishF2-HCO2198R primer pair successfully amplified samples of *Himantura walga* and *Rhinobatos penggali*, *Chiloscyllium punctatum*, and *Carcharhinus sealei* sharks. For samples that successfully amplified, the DNA sequence in the primer is complementary to or homologous to the DNA sequence in the sample's COI gene. DNA samples that fail to amplify can be due to genetic variations in the COI gene in the sample, which make it difficult for the primer to connect by not finding an appropriate base pair.[26] Due to the existence of one or more primer bases that did not match the target gene sequence, the study's results for samples did not successfully amplify suspected genetic variants in the sample.[27]

The amplification results show that the size of the DNA band of each DNA sample in a pair of primers produced is different. It depends on the position of the primer attachment and the presence of a primer DNA sequence that complements the DNA sequence in the sample's COI gene. As earlier stated, the thickness of the DNA band produced by each sample is also different depending on the results of measuring the number of DNA samples. Thick bands indicate high DNA concentrations and thin bands indicate low DNA concentrations.[28] The DNA sample's purity also affects the appearance of the smear on the gel. If the purity of the DNA is within the acceptable limit, there won't be any smears, while smears show that there are still contaminants if the purity of the DNA is smaller or greater than its effective range.

Although the results of measuring DNA concentration and purity are good when the amplification of DNA samples taken is small and less homogeneous, it is still possible to produce thin DNA bands. In addition, the thickness of different DNA bands in each sample can also be caused by little or no homogeneity in the DNA samples taken.[29]

The amplification in each primer pair resulted in different DNA bands, even on the same sample. In contrast to the primer pairs FishF1-FishR1 and FishF2-FishR2, which produced thicker DNA bands, HCO2198R was used in the primer pairs FishF1-HCO2198R and FishF2-HCO2198R to produce thinner DNA bands. This can be due to the %GC in HCO2198R primer being only 34.62%. A primer should have a %GC between 40% and 60%. Because HCO2198R primers have a low percentage of GC, the PCR process is less effective because primers cannot successfully compete for attachment to the DNA sample.[23] In this study, molecular identification was successful due to the amplification of DNA obtained from the various samples. The future directive of this study is to determine the genetic distance as well as the phylogenetic tree. The identification of stingrays and sharks is related to fish species protection and the determination of their conservation status. In accordance with Government Regulation of the Republic of Indonesia Number 60 of 2007, maintaining fish species diversity, ecosystem balance, and stability are the main goals of fish protection and conservation. Also, natural resources, such as fish, are sustainably utilized.

Conclusion

The findings of the study show that the COI gene of *Himantura gerrardi* stingray was successfully amplified by the primer pair FishF1-FishR1; *Himantura uarnocoides* by the primer pairs FishF1-FishR1 and FishF2-FishR2; *Himantura walga* by the primer pairs FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R; *Neotrygon kuhlii* by the primer pairs FishF1-FishR1, FishF2-FishR2, and FishF1-HCO2198R; *Rhinobatos penggali* FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R. *Chiloscyllium punctatum* and *Carcharhinus sealei* are shark species that have been amplified successfully using the four primer pairs FishF1-FishR1, FishF2-FishR2, FishF1-HCO2198R, and FishF2-HCO2198R. Therefore, these primers can be employed for the molecular identification of stingrays and sharks.

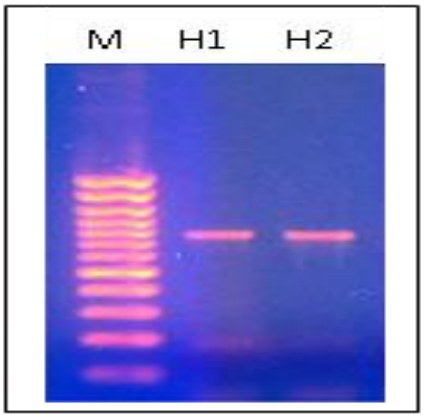

Figure 7: Visualization of PCR product from shark fish COI gene using FishF2-FishR2 primer pair. M: DNA ladder; H1: *Chiloscyllium punctatum* ; H2: *Carcharhinus sealei*

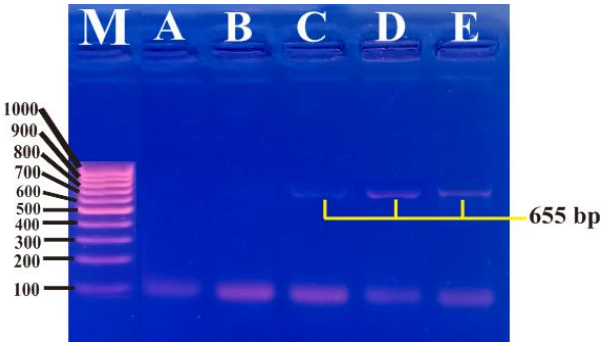

Figure 8: Visualization of PCR product from stingray DNA using FishF1-HCO2198R primer pair. M: DNA ladder; A: *Himantura gerrardi*; B: *Himantura uarnocoides*; C: *Himantura walga;* D: *Neotrygon kuhlii*; E: *Rhinobatos penggali*.

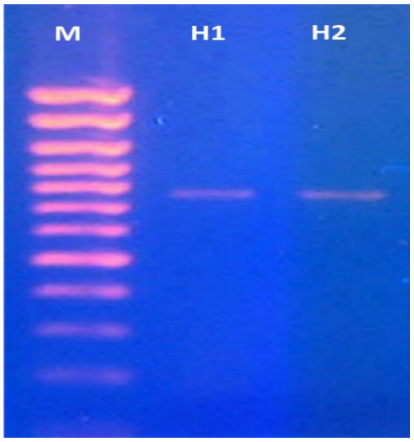

Figure 9: Visualization of PCR product from shark DNA using FishF1-HCO2198R primer pair. M: DNA ladder; H1: *Chiloscyllium punctatum* ; H2: *Carcharhinus sealei*

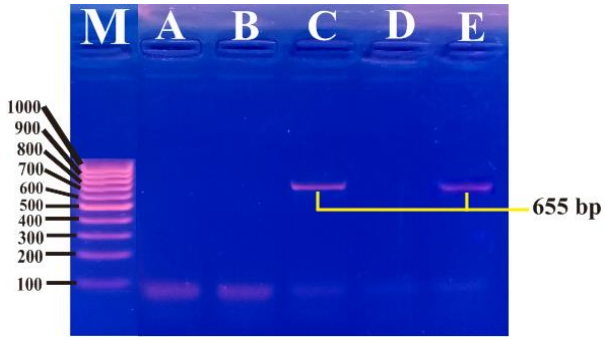

Figure 10: Visualization of PCR product from stingray DNA using FishF2-HCO2198R primer pair. M: DNA ladder; A: *Himantura gerrardi*; B: *Himantura uarnocoides*; C: *Himantura walga;*
D: *Neotrygon kuhlii*; E: *Rhinobatos penggali*.

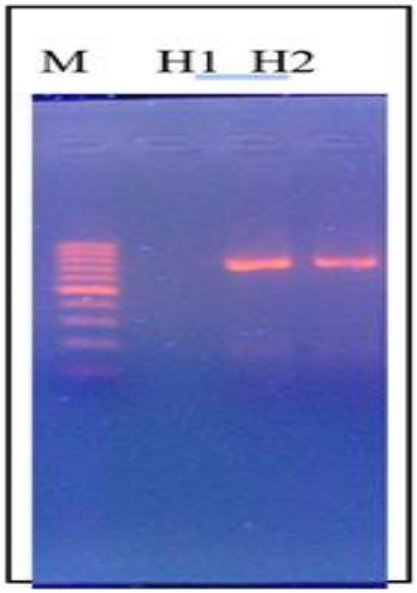

Figure 11: Visualization of PCR product from shark DNA using FishF2-HCO2198R primer pair.
M: DNA ladder; H1: *Chiloscyllium punctatum;* H2: *Carcharhinus sealei*

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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