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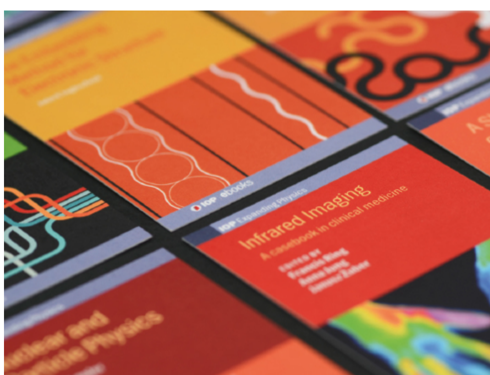

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The use of two pairs primer for CO1 gene amplification on traded stingray at fish auction Tasik Agung Rembang

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Abstract. Stingray provide a high economic contribution and increase in production and utilization. Conservation effort needed to identify molecularly using CO1 gene. The success of CO1 gene in identifying species can not be separated from amplification in polymerase chain reaction (PCR) technique. The use of primer FishF1, FishR1, FishF2, and FishR2 to amplify COI gene for stingray traded at fish auction Tasik Agung Rembang never been done, so it need to be researched. The purpose of this study is to amplify the COI gene of stingray traded at fish auction Tasik Agung Rembang using two pairs of primers. Five stingrays sample were obtained from fish auction Tasik Agung Rembang. Then its DNA was isolated using protocol from TIANamp Marine Animals DNA Kit and measured in quantity using nano spectrophotometer. Amplification of CO1 gene by PCR technique. Based on the results of the study showed that the five samples had good DNA purity, concentration, and quality. The 5 samples amplification result showed that the FishF1-FishR1 primer pair succeeded in amplifying samples a, b, d, and e, while the FishF2-FishR2 primer pair succeeded in amplifying samples b, c, d, and e with a PCR product length around 655 bp.

1. Introduction

Indonesia is a country with abundant natural resources, one of it is marine resource sector which has high biodiversity. One of the biota with high species diversity is stingray. Stingray is a fish from Chondrichthyes class or cartilaginous fish. In general stingray has a whip-like tail with a flat body shape and widen (depressed). Stingray has wide habitat distribution from shallow coastal water to deep oceans.

Based on Southeast Asian Fisheries Development Center (SEAFDEC) data (2018), total stingray production in Indonesia in 2016 reached 26,371 tons for Dasyatidae family, 3,711 tons for Myliopatidae family, and 5,436 tons for Mobulidae family [1]. Fishermen use stingray for consumption and trading purposes, such as the use of stingray skin for industrial materials, craft of leather bags, shoes, bracelets, wallets, belts, and so on [2]. Stingray meat, aside from being used as a consumption material, the oil can be used as pharmaceutical raw material as well as the bone for glue raw material [3]. According to [4]. Stingray cartilage tissue contains glycosaminoglycans which are used in osteoarthritis therapy or for joint health.

If utilization of stingrays not balanced with conservation efforts, it can lead rapid decline in population and takes long time to recover. Based on World Conservation Union (IUCN) data (2019), 109 species of stingrays, 1 species critically endangered, 9 species endangered category, 18 vulnerable species, 12 species including near threatened, 22 species least concern and 47 species data deficient categories. This fact is get worse due to its biological characteristics where the rate of growth and maturity is slow and also low fecundity [5].

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Fish auction Tasik Agung is one of fish auction in Rembang, Central Java. Stingray that is landed at fish auction Tasik Agung will be marketed to various regions to outside Java and even abroad. This is according to explanation from coordinator fish auction Tasik Agung Rembang that stingray is an export commodity and has given a high economic contribution to community, beside for various food products, stingray skin has high selling vallue for handicrafts. The high market demand and utilization of stingray cause increasing production. Stingray production is obtained from fishermen then auctioned at fish auction Tasik Agung. Based on data of stingray production at fish auction Tasik Agung Rembang in August 2019 to December 2019, it always increase. In August stingray production was 413 kg, 497 kg in September, 569 kg in October, 576 kg in November, and 648 kg in December. This increasing is skeptically because it can cause limited availability in nature, thus requiring conservation efforts to maintain its sustainability. One of main information needed is related to types of stingray. This aspect is very important, because conservation plan without adequate knowledge of its species leads to overexploitation and significant reduction in stingray diversity. The technique that can be used to find out the types of stingrays accurately and quickly is identification with molecular techniques.

Mitochondrial DNA molecular technique (mtDNA) is widely used for species identification. One of the most popular genes in mitochondrial DNA used for identification is Cytochrome c oxidase I (COI) gene. This CO1 gene proved to reveal species identity, phylogenetic pattern and genetic diversity of aquatic species [6]. The success of CO1 gene in identifying species cannot be separated from gene amplification in polymerase chain reaction (PCR) technique. Universal primer often succeed in amplifying many animal groups, but these primers fail to amplify COI gene of another animal groups as in Chondrichthyes class. In the research of [7] and [8] successfully used FishF1, FishR1, FishF2, and FishR2 primers for stingray identification in Australia and in [8] the primer was successfully used for identification of stingray in North Atlantic and Australasia. [9] also successfully used FishF2 and FishR2 primer pairs for stingray amplification COI gene western Australia.

Research and studies on the use of primer FishF1, FishR1, FishF2, and FishR2 for amplification of CO1 gene from traded stingray at fish auction Tasik Agung Rembang has never been done. Therefore, in this study the primer was used for amplification of CO1 gene from traded stingrays at fish auction Tasik Agung Rembang.

2. Methods

This research was conducted at Molecular Biology Laboratory and Research Laboratory of Faculty of Mathematics and Natural Sciences, Semarang State University. There are 5 different types of stingrays were collected from study site at fish auction Tasik Agung Rembang. Samples are taken at its fin.

DNA isolation was carried out using TIANamp Marine Animals DNA Kit method [10]. Stingray sample taken by cutting the fin as much as 30 mg. Samples were put in 1.5 ml microtube and added 200 µl GA buffer. The sample is vortexed for 15 seconds. After that, add 20 µl of proteinase-K and vortexed again for 15 seconds. Next, it was incubated at 56ºC for 1 hour until the lysis sample was complete, every 15 minutes the sample was vortexed for 15 seconds and spin downed. Added 200 µl buffer GB and vortexed for 15 seconds. Then incubated at 70ºC for 10 minutes and spin downed. Added 200 µl of absolute ethanol and vortexed for 15 seconds followed by spin downed. After that the sample is pipetted to be transferred to Spin Column in Collection Tube. Centrifuged at 12000 rpm for 30 seconds. Added 500 µl GD buffer and re-centrifuged at 12000 rpm for 30 seconds. The supernatant in Collection Tube is discarded and mounted to Spin Column again. Added 600 µl PW buffer and centrifuged at 12000 rpm for 30 seconds. The supernatant in Collection Tube is discarded and mounted to Spin Column again. Added 600 µl PW buffer and centrifuged at 12000 rpm for 30 seconds. The supernatant in Collection Tube is discarded and mounted to Spin Column again. Continue to be centrifuged at 12000 rpm for 2 minutes until the pellets dry completely. The Collection Tube is removed from Spin Column and placed in a 1.5 ml micro tube. Added 50 µl TE buffer. Incubated for 2-5 minutes at room temperature and centrifuged at 12000 rpm for 2 minutes.

The result of DNA isolation was measured its concentration and purity using Nano spectrophotometer. 1 µl of DNA isolation was dripped on Nano spectrophotometer lens that had been cleaned using Kim Tech tissue. Then read the absorbance at 260 nm and 280 nm. The quality of DNA isolation was tested by electrophoresis on 0.8% agarose gel with GelRedgel dye and visualization was seen on UV Trans illuminator Gel Documentation.

DNA amplification is done through PCR technique. The primer pair used in this study were FishF1-FishR1 and FishF2-FishR2 with the sequences of each primer presented in Table 1 below:

Table 1. Primer used in research

Primer	Sequences	T_m
FishF1	5'-TCA ACCAACCACAAA GAC ATT GGC AC-3'	66.3
FishR1	5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3'	66.3
FishF2	5'-TCG ACT AAT CAT AAA GAT ATC GGC AC-3'	63.2
FishR2	5'-ACT TCA GGG TGA CCG AAG AAT CAG AA-3'	66.3

Cocktail composition were used in PCR reaction showed in Table 2.

Table 2. PCR *cocktail* composition

No	Material	Volume
1.	Dream Taq Green PCR Master Mix	6.25 µl
2.	Primer Forward	1 µl
3.	Primer Reverse	1 µl
4.	ddH_2O	3.25 µl
5.	DNA sample	1 µl
	Total	12.5 µl

The composition of cocktail PCR that has been mixed is homogeneous using vortex for 15 seconds and spin downed. Then it is entered into Thermal cycle engine that has been programmed for 35 cycles on conditions which presented in Table 3 below.

Table 3. PCR Program

No	Cycles	Temperature (ºC)	Time
1.	Pre-denature	95	3 minutes
2.	Denature	95	30 seconds
3.	Annealing	51	30 seconds
4.	Extension	72	1 minute
5.	Final extension	72	5 minutes

The PCR product was visualized using 2% agarose gel. Making 2% agarose gel by weighing 1 gram of agarose powder and adding TAE 1x in erlenmeyer to 50 ml volume. Heated in microwave until the solution becomes clear. The solution is cooled at room temperature until warm. Added 5 µp GelRed dye in 50 ml of agarose solution and mixed until homogeneous. Agarose solution is poured on a gel mold with a comb and let it freeze. Agarose gel was soaked using TAE 1x buffer solution. 3 µl ladder DNA and 3 µl PCR products were put into gel pit. Running is done at 50 volts for 1 hour. After that, the gel is seen above UV Transilluminator Gel Documentation.

3. Results and Discussion

Five stingray samples taken from fish auction Tasik Agung Rembang were isolated using TIANamp Marine Animals DNA Kit method. The isolated DNA was measured using nano spectrophotometer 260 and 280 nm wavelength. DNA can absorb ultraviolet (UV) light due to the presence of purine and pyrimidine bases. DNA propaganda can absorb UV light at 260 nm, while protein or phenol

contaminants absorb light at 280 nm, so DNA purity can be measured by calculating the absorbance value of 260 nm which divided by the absorbance value of 280 nm. DNA purity values range from 1.8 to 2.0. DNA purity values above 2.0 indicate contaminants in form of RNA. DNA purity values under 1.8 indicate contaminants in form of proteins [11]. The results of measurements on concentration and purity of isolated DNA are presented in Table 4 below.

Table 4. Concentration and Purity of DNA Isolation Result

Sample	Consentration (ng/μL)	Purity (absorbance λ 260/ λ 280)
a	46.5	1.65
b	63.8	1.65
c	27.5	1.52
d	99.3	1.73
e	225.5	1.81

Based on the results of DNA purity measurements showed that from 5 samples, there were 1 sample that produced pure DNA and 4 samples that still contained contaminants with an average purity of all samples is 1.672. Samples that produce pure DNA are sample e with purity is 1.81, while samples contain contaminants are samples a, b, c, and d with the purity of each sample 1.65, 1.65, 1.52, and 1.73. Samples with DNA purity under 1.8 are caused by contaminants in the form of proteins.

Beside measured the purity, DNA from isolation also measured its concentration in order to find out the amount of DNA which contained in solution. [12] explain that the effective concentration range for quantification is between 20 to 100 ng/μL. The concentration of DNA from isolation affects the quality of amplification results. Too low concentrations cause primer attachment to low printed DNA, while too high concentrations may also contain high contaminants so that it can interfere with primer attachment to the printed DNA. DNA concentrations obtained in this study can be said good, with ranges from 27.5-222.5 ng/μL with average 91.92 ng/μL.

The thing that affects the purity and concentration of DNA is technical aspects of implementation of stages carried out, such as in process of destroying imperfect samples, causing DNA in cells not lysis perfectly during the isolation process so that the DNA result is less optimal. When the temperature at incubation stage is not appropriate, it can cause cells not completely lysed and can produce low DNA and contaminants in DNA. DNA in supernatant is in uppermost layer, while proteins form in middle layer, and organic component located at the bottom because it has a large specific gravity. Careless in uptake supernatant can cause unexpected material other than DNA to be taken.

Analysis of DNA purity and concentration isolated qualitatively can be seen based on the characteristics of DNA bands in 0.8% agarose gel. The electrophoresis results can be seen in Figure 1.

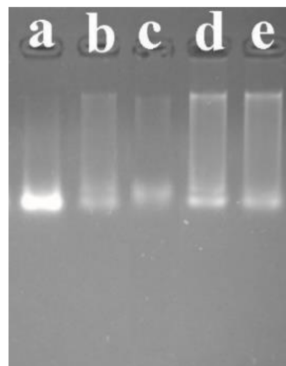

Figure 1. DNA Electrophoresis Results Using 0.8% Agarose Gel. Note: (a) sample a, (b) sample b, (c) sample c, (d) sample d, and (e) sample e.

Analysis of purity and concentration of isolated DNA can be seen qualitatively based on the characteristics of DNA bands in 0.8% agarose gel. Based on the results of electrophoresis carried out 5 samples showed that overall DNA from each sample was isolated, it can be seen from the DNA bands that appear on the gel even though the DNA bands are thick and some are thin and visible smears. Samples a and c showed the thinnest DNA band among the other bands and there was a smear. Sample b shows the resul of DNA band is thin and there is a smear. Samples d and e produced DNA bands that are slightly thicker but still have smears. The difference in results in each sample depends on the amount of DNA concentration isolated. The thicker the DNA band the higher the concentration and vice versa, the thinner the DNA band the lower the concentration. DNA bands contained in smears indicate the level of DNA purity that is not good. Smear on electrophoresis results that appear below near DNA band shows that the isolated DNA is not intact so DNA fragments are formed due to physical treatment during the isolation process such as storing samples in freezers and repeated sample thawing, while the appearance of smears located at the bottom each row indicates contamination in the form of RNA. According to [12] smears at the bottom show the presence of RNA contamination. Based on the appearance of DNA tape isolation results in this study still used for amplification process through PCR technique.

Amplification of CO1 gene for stingray samples in this study use primer pair FishF1-FishR1 and FishF2-FishR2 with annealing temperature according to [13] which is 51°C. This primer pair was success in amplifying various stingray species by several researchers. [6] Ward et al. (2005) success used FishF1, FishR1, FishF2, and Fish R2 primers for stingray identification in Australia with PCR products 655 bp, and in 2008, these primers by Ward et al. success used for identification of stingrays in North Atlantic and Australasia with PCR products between 652 to 655 bp. Likewise in research of [13], the primer pair succeeded in amplifying stingrays in Australian water with 655 bp PCR product. [8] also success used FishF2 primers and FishR2 primers for stingray identification in western Australia with average PCR product lengths at 550 bp.

The results of amplification using 2% agarose gel electrophoresis are presented in Figure 2. Based on the result of gel electrophoresis from CO1 gene amplification of 5 stingray samples in this study showed that FishF1-FishR1 primer pair succeeded in amplifying samples a, b, d, and e, while primer pair FishF2-FishR2 succeeded in amplifying samples b, c, d, and e. with PCR product length around 655 bp. This shows that there is a DNA sequence in primer that complementary or homologous to the sequence in CO1 gene sample that has been successfully amplified. Samples were not successfully amplified could be caused by the poor quality of DNA template. Band that not appear could be occured when primer that used are incompatible with printed DNA. Inappropriate primer cause primer attachment not occur so the polymerase enzyme cannot catalyze the installation of complementary nitrogen basal into DNA template which ultimately does not form new DNA marked by the absence of bands on the gel produced by electrophoresis.

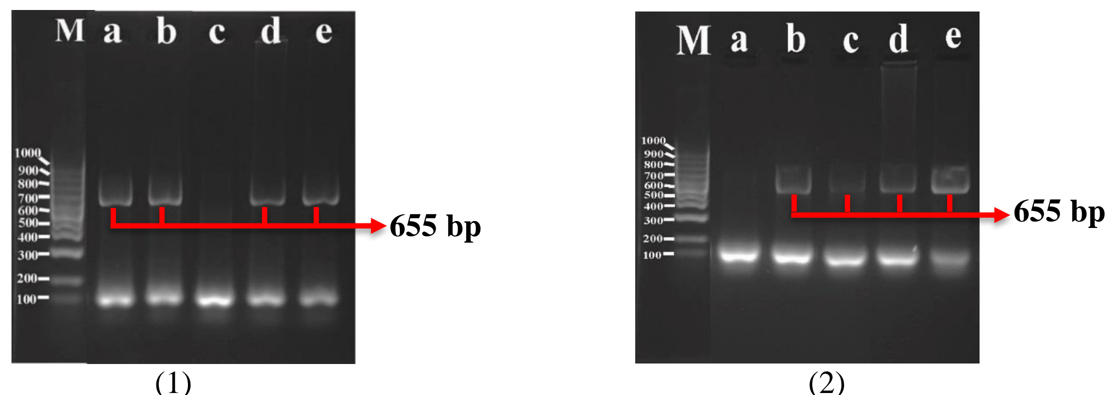

Figure 2. Visualization of PCR Product Results of COI Gene Amplification from 5 Samples Using Primer Pairs (1) FishF1-FishR1 and (2) FishF2-FishR2.

The position of primer attachment in CO1 gene for FishF1-FishR1 primers from basal 6475 to basal 7126 and FishF2-FishR2 primers from basal 6474 to basal 7127 [14]. The number of amplified basal for each primer around 655 bp [6, 13, 15, 16]. The position of primer attachment in CO1 gene can be seen in Figure 3.

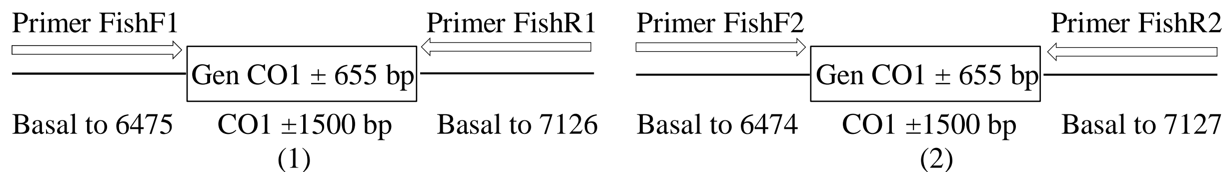

Figure 3. Position of primer attachment (1) FishF1-FishR1 and (2) FishF2-FishR2 in CO1 gene

DNA with success amplifying in this study was demonstrated by the bands appearance on agarose gel electrophoresis, although the thickness of the bands in each sample was different. Thin bands are possible due to the condition of low DNA quality or the template that has few or no homogeneity of DNA taken. In this study, samples that were success in amplified by FishF1-FishR1 and FishF2-FishR2 primers could be used for further identification of stingray studies.

4. Conclusion

FishF1-FishR1 primer pair success in amplifiying COI gene on traded stingray at fish auction Tasik Agung Rembang on sample a, b, d, and e, while FishF2-FishR2 primer success in amplifying samples b, c, d, and e, which marked by the band appearance on long base position around 655 bp.

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