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Identifikasi Molekuler Ikan Pari Kekeh (Rynchobatus sp.) yang Didaratkan di PPI Rigaih Kabupaten Aceh Jaya Menggunakan Gen COI

Molecular Identification of Wedgefishes Stingrays (Rynchobatus sp.*) at PPI Rigaih, Aceh Jaya Regency by using COI Gene*

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Abstract

Indonesia belongs to one of the world largest shark and ray producing countries. Based on FAO (Food and Agriculture Organization) data, Indonesia is the top country which produce plenty of sharks and rays every year. One of the stingray sharks caught by the fishermen called Kekeh Stingray. Data from the IUCN (International Union for Conservation of Nature and Natural Resources) noted that this kind of ray is included in the Red List and categorized in Appendix II CITES (Convention on international trade of endangered species) due to excessive fishing. In most cases, the identification of Kekeh Stingray is not easy because the information and conservation data are very limited. This study was conducted to identify the species of Kekeh Stingray landed at Rigaih Fish Landing Base, Aceh Jaya Regency using the COI gene (Cytochrome c oxidase subunit I) molecular analysis. Sampling was carried out in June 2021 and then analysed at Indonesian Biodiversity Foundation (BIONESIA) laboratory. The analysis results were adjusted to GenBank data via BLAST (Basic Local Alignment Search Tools). The results of the analysis found per value. The ident of 10 samples ranged around 99-100% each and the query quaver value each sample was 100%. The farthest genetic distance value was 0.34. The phylogenetic tree managed to classify species from each sample, and further form a cluster together with the data obtained from the NCBI GenBank.

INTRODUCTION

Indonesia is one of the largest shark and ray (Elasmobranchii) producing countries in the world, this based on FAO (Food and Agriculture Organization) data report that Indonesia is in the top position which produces plenty of sharks and rays every year (Dharmadi and Fahmi 2014). Seasonal catches up to 106,034 tons or about 13% of the total production of sharks and rays globally. One of the fishermen catches is kekeh stingray (Rynchobatus Sp.) (Azidha *et al*., 2021). The habitat of kekeh stingray is spread throughout almost all Indonesia waters (Yuwandana et al. 2020). In Indonesia, this type of stingray is used for local consumption and as an export commodity (Tindi et al. 2017). All the parts of the body of R. Australiae have a high selling value where the flesh is used as processed food, bones and teeth are used as cosmetic and medical materials, the skin is processed into fashion materials including shoes, bags, and the most demanded of all parts is the fins. (Kyne et al. 2019), besides, this type of ray has the largest fin size compared to other types of shark ray fins (Vista, Nurastri, and Marasabessy 2021). In accord to (Azidha et al. 2021) parts of the body such as teeth, bile, stomach, bones, gills and etc. can be processed for various purposes such as glue, ornaments, animal feed and medical ingredient. The high activity of this fin trading has affected sharks and rays populations which has an impact on decreasing of marine ecosystem quality (Dharmadi and Fahmi 2014).

Kekeh stingray is one of the fisheries natural resources which has a high economic value and is distributed among trade (Azidha et al. 2021). Due to the guaranteed value of selling that benefits fishermen, it possibly becomes vulnerable for overfishing activity (Field et al. 2009). Therefore, it affects the reproducing cycle of the stingray as well as the period of it which takes longer than it had to, moreover the low ability of reproducing also cause the overexploitation threatened to extinction. According to (Wehantouw, Like Ginting, and Wullur 2017), global database that shows the magnificent decreasing of shark stingray stated that this issue is caused by three basic aspects, such as; a) Massive and uncontrolled catch; b) Slow biological reproduction character; and c) Low fecundity, Parmanto (2019). Azidha dkk., (2021) explains that this is also affected by the low fecundity of kekeh stingray and the increasing number of its catching activity

The data of IUCN (International Union for Conservation of Nature and Natural Resources) revealed a certain R. Australiae species is included in the red list or also categorized as CR (Critically Endangered), as the result from over catching so as to extinction. R. Australiae is listed on the second stage of Appendix CITES (Convention on international trade of endangered species) in August 2019, it concludes that the cross country trade has to be managed so it would not endanger the existence (AISHAH et al. 2018).

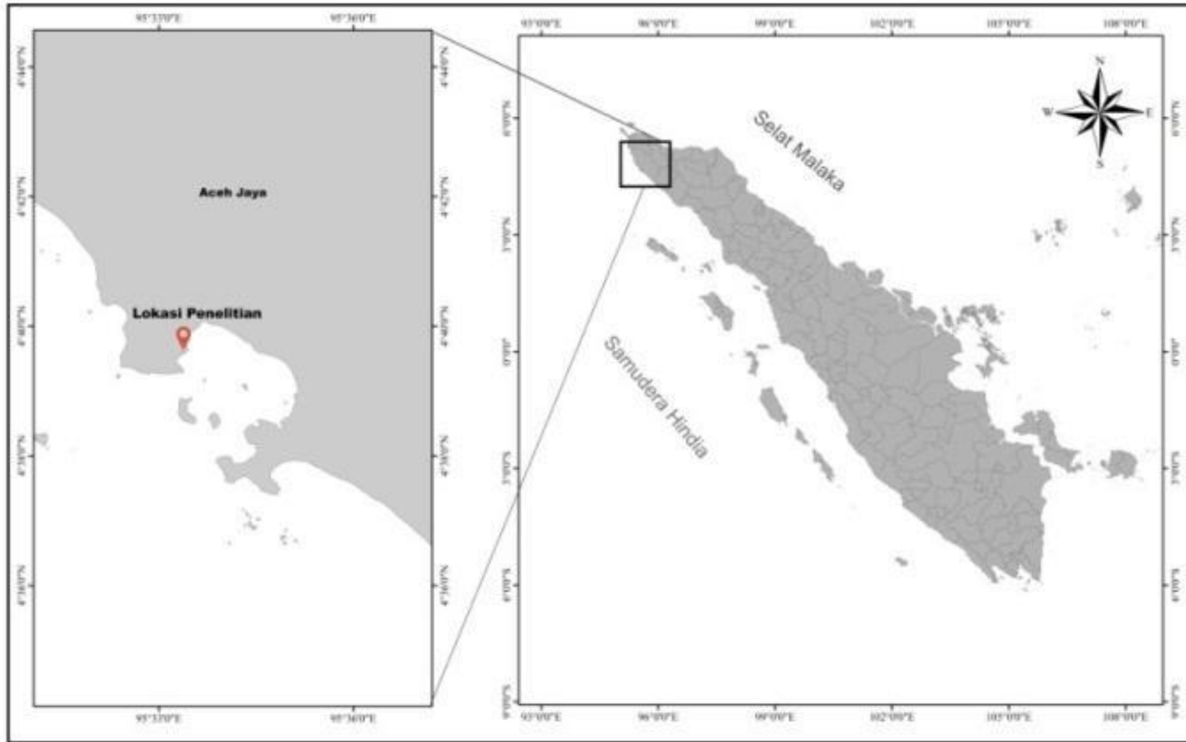
Generally, kekeh stingray information spreading is not easy since the sources and conservations are limited, if only with the species identification morphology is not enough and yet not effective (Larasati, Sabdono, and Sibero 2021). It is hard to be done for the trade distributed through pieces of body and fins (Ward and Holmes 2007). Along with the developed technology in molecular, COI (Cytochrome c oxidase subunit I) is a short sequence gene chosen among various genes are used for the special standard identification based on DNA basroce (AISHAH et al. 2018), could possibly help to identify by taking a little piece of kekeh stingray's body, so the molecular identification method organism is faster and more accurate primacy (Setiati, Peniati, and Maharani 2018), than the other mitochondrial genes, for instance Cyt-b (Rahayu and Jannah 2019).

Aceh Jaya is one out of cities that is located at the coast of southwest Aceh (BARSELA), which is directly connected to the Indian ocean where some kekeh stingray species genuinely habitat (Rhynchobatus. Sp) (Lesmana, et al., 2018). The information source of conservation status of this kekeh stingray that apply "DNA Barcode" in Aceh Jaya is not provided. By using molecular identification of DNA barcoding will help the identification process because it only costs a small amount of the stingray's body cell (Hebert and Gregory 2005). Therefore it is interesting to analyse with the DNA barcode identification from kekeh stingray landed in PPI Aceh Jaya regency.

METHOD

Time and Location

This research is done in June, 2021. The kekeh stingray sample (R.australiae) is directly taken from the fishermen catch landed (PPI) located in Rigaih district, Aceh Jaya regency. The sample amount is 10 pieces of kekeh stingray's fins, each taken from various individuals and differentiated by different kinds of bottles that the fins are put into. Next, those were brought to Yayasan Biodiversitas Indonesia (BIONESIA) laboratory to face the molecular analysing process.



Picture 1. Map of research site

The Sampling

Conducted in PPI Rigaih Aceh Jaya region, Aceh province, which is located in BARSELA watered area in Indian ocean. By cutting small pieces of kekeh stingray's body each from 10 different individuals. And being cut by the sample knives.

Picture 2. *Rynchobatus australiae* sample

Sample Preservation

The aim is to maintain stability, freshness, and to prevent samples from being contaminated by external components that will destroy it. The ingredients and tools used for the preservation are; gloves, tweezers, label paper, petri dish, sterilizer, ethanol, tube, and the sample. Steps; First, use the glove then sterilize the tools using sterilizer, next insert the sample to the petri dish and pour the ethanol into to cleanse it. Right after, insert the sample to another tube using the tweezers and pour the ethanol until it is soaked. Name or code each sample with the label paper so it will not be switched during extraction. This process needs to occur frequently so that the well-being is controlled, according to (Bahri et al. 2017). The liquid is in need of change once its colour changes. Finally, re-sterilize all the tools used along with the process to avoid contamination by other samples.

DNA Extraction

The DNA separation process from a cell tissue and other particles is called extraction. Extraction is an important part of molecular research (Reid 1991). Based on Hajibabaei et al., (2005) there are three extraction and purification methods to produce genomic DNA quickly, such as an extraction with chelex (Chelating Ion Exchange) 10%. A homogeneous 10% Ion Exchange Resin, a styrene divinylbenzene copolymer contains a paired iminodiacetate that can bind polyvalent ions (Cho et al. 2007). Chelex 10% functions to bind DNA and protect against DNAse enzymes by binding the magnesium ions (Mg^{2+}) which are cofactors for DNAse enzymes (Singh et al. 2021).

The extraction using chelex with a concentration of 10% is (Walsh, Metzger, and Higuchi 2013), is relatively simple and fast in obtaining genomic DNA, the chelex method uses few tubes transfers and does not involve toxic organic solvent (Sweet et al. 1996). Before carrying out the extraction, prepare the tools and ingredients will be used, which are; petri dish, scissors, tweezers, infrared sterilizer, 96% ethanol, aquades, tissue, 10% chelex , and the samples to be extracted. The next step is the sterilization process of needed tools then put them afterwards into a pot filled by aquades. Then lift and dry with a tissue, put them back into the infrared sterilizer until it's hot and lift it up again. While waiting for the process, label or give an id sample on the 10% chelex tube. Next, take the fish sample using the tweezers and insert it to the petri dish then cut the sample using scissors on the size of 2 mm. Put the rest of the sample back to the sample bottle for supply. Then, cut the piece smoothly and put it into the bottom of a 10% chelex tube with tweezers. Later, the sample is vortexed for 20 seconds and the sample is centrifuged at 8- rpm for one minute, then is incubated for 45 minutes at 95 C. During incubation, every 20 minute the sample will be vortexed for 20 seconds and centrifuged with 80 rpm for one minute, the aim is to accelerate lysis, then the sample is incubated again until the time is up and it is removed, the sample continuously vortexed and centrifuged.

DNA Amplification

The amplification is processed with PCR (Polymerase Chain Reaction) technique. PCR is an enzymatic method to exponentially a nucleotide sequence, DNA is amplified in vitro using the polymerase chain reaction (Larasati et al. 2021). Based on (Siti and Muhammad 2021) Polymerase Chain Reaction is a technique of enzymatically augmenting (replicating) DNA.

The PCR method follows the protocol in the Bali Biodiversity Foundation (BIONESIA) laboratory, which uses the fish F1 primer and fishR1primer, with the following sequence code:

Table. f1 and r1 sequence code

Primer's name	Skuen Code	Source
Fish F1	TCAACCAACCACAAAGACATTGGCAC	(Ward et al. 2005)
Fish R1	TAGACTTCTGGGTGGCCAAAGAATCA	(Ward et al. 2005)

Before performing PCR it is necessary to prepare strip tubes and 2 1.5 µL tubes consisting of (Master Mix) MM1 and MM2 as reagent containers to be used. The reagents were put into the MMI tube using a micro pipette; ddH2O 9 µL, 10 x PCR Buffer (PE-II) 3 µL, dNTPs 5 µL, MgCl2 4 µL, primer Fish F1 2.5 µL, primer Fish R1 2.5 µL, then vortex for 15 seconds and centrifuged for 30 seconds to homogenize the reagent the. Then fill the MM2 tubes with reagents using a micro pipette; ddH2O 18 µL, 10 x PCR Buffer (PE-II) 2 µL, PE amplitaq 0.25 µL, vortexed for 15 seconds and centrifuged for 30 seconds to homogenize. Next, the MM1 reagent is inserted into 2 strip tubes that have been numbered 1 and 2 of the same size, then strip tube 1 is inserted with 2 µL of ray kekeh DNA, the next stage is inserted into the PCR machine which has been set, then the MM2 reagent component is inserted into the strip tube using a micropipette.

The PCR stage uses 38 cycles then followed by the first denaturation phase (separation of double-stranded DNA) at 80°C for 15 seconds, followed by denaturation at 90°C for 30 seconds, then the annealing stage (primer attachment) at room temperature 53°C takes 30 seconds, then the extension stage (DNA elongation) is carried out at 72°C for 45 seconds, the last extension is at 24°C for 1 minute. This PCR step was repeated for 38 cycles which took place on the Thermocycler.

Electrophoresis

The results of the nucleotide replicas from the PCR method were process an electrophoresis process which aimed to see the quality of the nucleotides using agarose gel. But first carry out the steps for making agarose gel including agarose gel powder required 1% or as much as 0.75 grams which is weighed using a digital scale, 75 ml of Buffer is measured using a beaker, then the material is loaded into a beaker glass container, stirred until smooth, the next step The material is heated using a microwave takes 4 minutes. Furthermore, the liquid agarose gel is poured into a mould that has been paired with a comb to make holes or wells in the gel and wait for up to 30 minutes.

The ready-to-use agarose gel is then loaded into the electrophoresis machine containing TAE Buffer liquid, until the agarose gel is submerged. The next step is to enter the 3 µL ladder and 2 µL biotium which has been mixed into the gel well, then take 3 µL DNA sample, mix it with 1 µL loading dye and 2 µL biotium, put it into the gel well, then add the 3 µL reagent which has no DNA sample. as a negative control. The next step is to close the machine and turn on the setting electrophoresis machine at a voltage of 100 Volts, using 30 minutes with a current of 200 Amperes. The next step is when the running process is complete, the agarose gel is lifted into the UV transilluminator to see the results of the DNA bands.

DNA Sequencing

DNA sequencing is the translation of DNA strands into nucleotides in the form of Adenine (A), Cytosine (C), Guanine (G), and Thymine (T) from the DNA samples that have been electrophoresed to have good DNA bands, then sequenced to obtain the nucleotide sequence (Sianturi et al., 2021).

Data Analysis

Data analysis is an attempt to obtain phylogenetic information. Analysis of the results of the sequencing in the form of F1 and R1 files

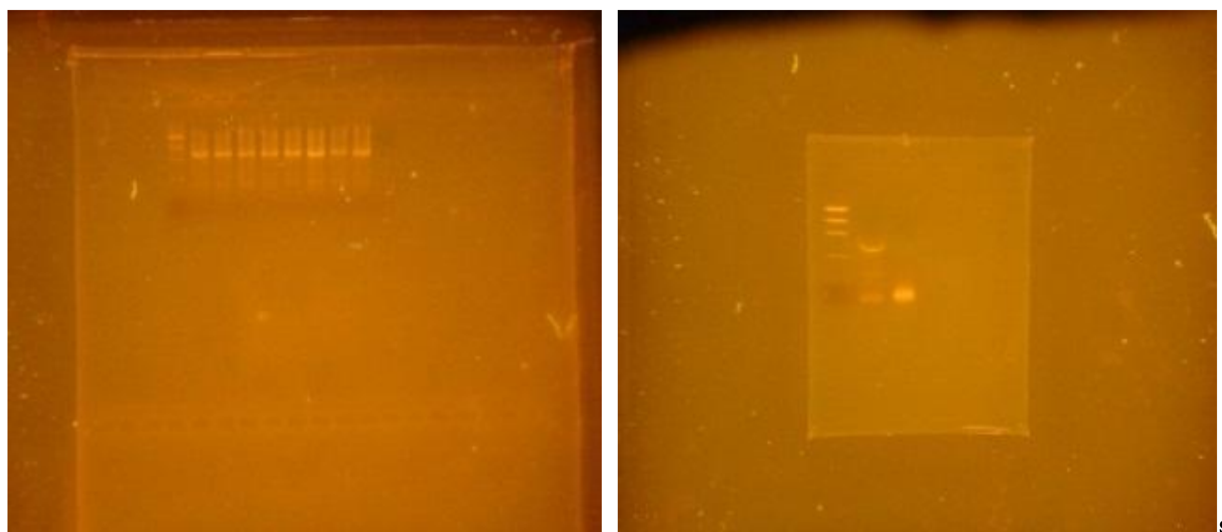
were aligned (edited) using MEGA X (Molecular Evolutionary Genetic Analysis), evolution analysis was performed using MEGA X (Kumar et al. 2018). The next step is the sequence results that have been aligned in BLAST (Basic Local Alignment Search Tool) is to provide comparative data on species similarity that can be accessed through NCBI GenBank data (National Center Biotechnology Information) http://www.ncbi.nih.gov. Furthermore, the preparation of phylogenetic trees using the Neighbor- Joining method (Saitou and Nei 1987), which comes from the phylogeny menu in MEGA X. The shape used is two- parameter kimura with 1000 times of bootstraps (Kumar et al. 2018).

Phylogenetic is a grouping based on the kinship relationship between species and ancestors and the evolutionary relationships between living things and others,. According to Saleky et al., (2020) Phylogenetic is one of the methods used to analyse the relationship of a living thing (phylogenetic relation). The diversity an kinship of living things is based on phylogenetic trees. Phylogenetic tree is a logical approach to showing the evolutionary relationships between organisms. (Schadt, Mullen, and Schmidt 2001). The evolution of living creatures that have the same characteristics and physical are categorized as having the same ancestor, but this is determined by different genetic distances. Phylogenetic studies' purpose is to reconstruct the relationship between organisms (Trianto and Purwanto, 2020), The usage of phylogenetic tree analysis provides information that can determine the branches and relationships between species of the phylogenetic tree. Base on Twindiko et al., (2013) Phylogenetic is a method often used in systematics to understand the diversity of living creatures through the reconstruction of kinship relationships. The study of genetic diversity aims to determine the level of genetic variation that becomes an indication of genetic exchange (gene flow) between populations (Hellberg et al., 2019). Commonly, the trigger of this genetic diversity of a population is caused by mutation, recombination, or gene migration from a place to another (Suryanto, 2003).

DISCUSSION

Identification

Obtaining PCR results multiplied DNA from 10 samples of Rhynchobatus sp. Then electrophoresis was carried out using 1% agarose gel. The electrophoretic DNA bands can be observed in the following figure



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Picture 3. DNA strand discovered from sample

Gel electrophoresis succeeded in visualizing the DNA strand of the sample better than the PCR result which had been performed, denoted by the presence of DNA strands on the

gel using UV transilluminator, the good quality DNA strands appeared bright and no smears (Perwitasari et al 2020) The higher DNA concentration produced, the brighter and sharper the DNA strands will appear (Hartawan et al. 2015). Some of the gel electrophoresis results contained smears that were also visualized according to (Maliza et al. 2021). Smears that appeared on the agarose gel indicated that there is other material which isolated apart from DNA, perhaps the form of protein.

The result of DNA sample sequence of Rynchobatus sp. had been edited then compared to the nucleotide sequence through BLAST (Basic Local Alignment Search Tools) with equivalent data in the GenBank NCBI (National Center Biotechnology Information). Species identification using GenBank NCBI data which can be accessed from this page http://www.ncbi.nih.gov with BLAST method or using the available program in MEGA X software. The following are the results of the selection program shown by megablast.

Table 2. BLAST sampling result

Sampling code	Species	Query cover	Per. Ident	Accession
BIO03.09.012	*R. laevis*	100%	99.02%	KF899689.1
BIO03.09.013	*R. laevis*	100%	99.84%	KF899689.1
BIO03.09.014	*R. australiae*	100%	100.00%	JN022595.1
BIO03.09.015	*R. australiae*	100%	99.67%	MF508696.1
BIO03.09.016	*R. laevis*	100%	99.67%	KF899689.1
BIO03.09.017	*R. australiae*	100%	99.84%	JN022595.1
BIO03.09.018	*R. laevis*	100%	99.67%	KF899689.1
BIO03.09.019	*R. laevis*	100%	99.67%	JN022595.1
BIO03.09.020	*R. laevis*	100%	99.67%	KF899689.1
BI003.09.021	*R. australiae*	100%	100%	JN022595.1

Through BLAST (Basic Local Alignment Search Tools) by matching the data on GenBank NCBI, the query cover value obtained from each sample of Rynchobatus sp. is 100%. From each sample, the good query cover value is obtained as it said from (Nugraha dkk., 2022). The higher the query cover percentage, the higher homology level. The value per. ident from the table above provides information on similarity percentage of identity obtained through alignment of the nucleotide sequences from the sequencing results with the GenBank database, with a percentage value of per. ident of each sample BIO03.09.012 = 99.02%, BIO03.09.013 = 99.84%, BIO03.09.016 = 99.67%, BIO03.09.018 = 99.67%, BIO03.09.019 = 99.67% and BIO03.09.020 = 99.67% hence the sample is a species of Rhynchobatus laevis. While samples of BIO03.09.014 = 100.00%, BIO03.09.015 99.84%, BIO03.09.017 = 99.84%, and BI003.09.021 = 100%, are Rynchobatus australiae species. According to (Bhattacharjee et al. 2012) tells that the percentage of sequence similarity with the GenBank data called significant if the percentage of the similarity reaches 97% -100%. Whereas the average query cover value of each sample from data in GenBank is 100% then it can be called significant.

Genetic Range

Genetic range is the value that is used as a comparison of genes between individuals and between populations (Saitou and Nei 1987). Genetic range is used to discover the genetic relation of each species from ten samples Rynchobatus australiae analyzed. Analysis of interspecies relation can be discovered from genetic range between everyone (Wehantouw et al. 2017). The genetic range from each sample is written into the following table

Table 3. Genetic range each sample

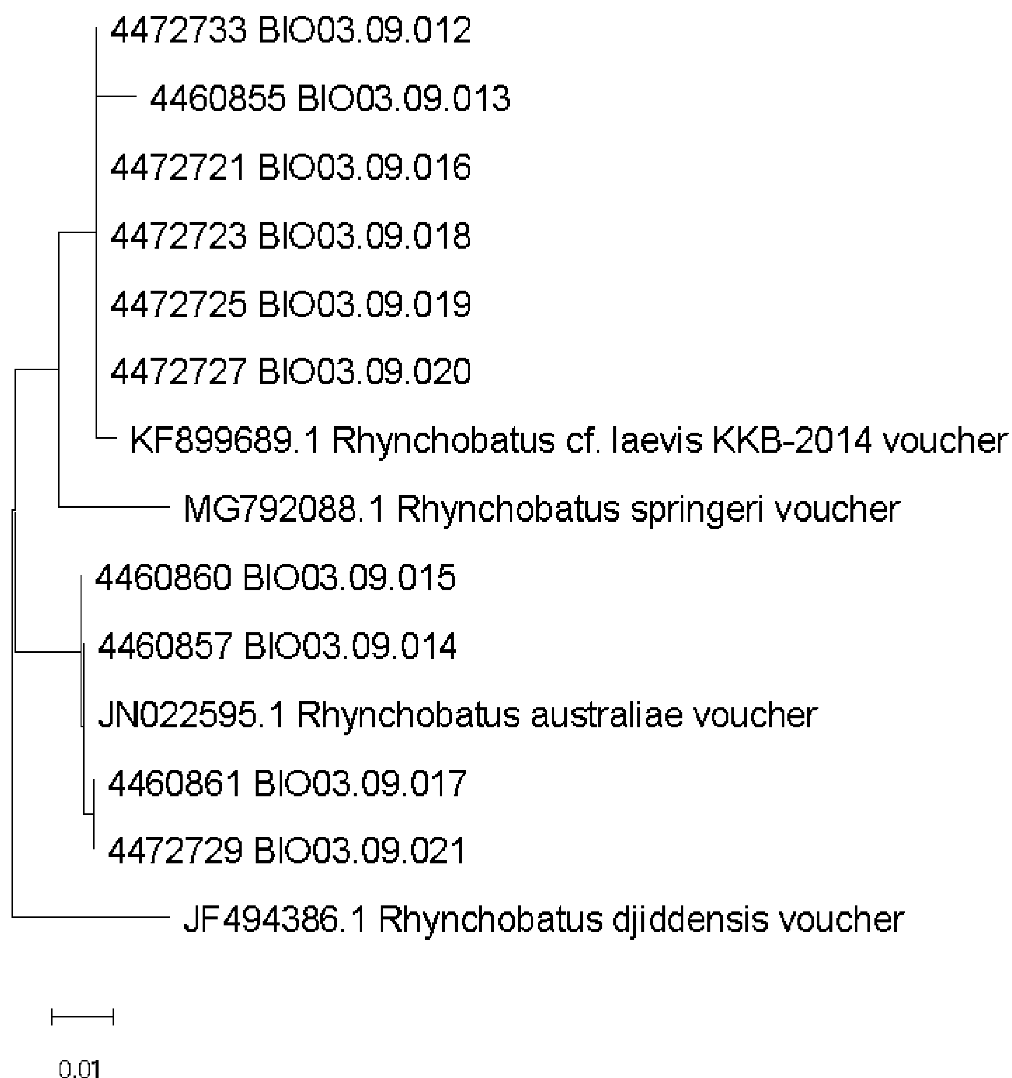
No	Sample code	1	2	3	4	5	6	7	8	9	10
1	BIO03.09.012										
2	BIO03.09.013	0.007									
3	BIO03.09.014	0.025	0.033								
4	BIO03.09.015	0.025	0.033	0.000							
5	BIO03.09.016	0.000	0.007	0.025	0.025						
6	BIO03.09.017	0.027	0.034	0.001	0.001	0.027					
7	BIO03.09.018	0.000	0.007	0.025	0.025	0.000	0.027				
8	BIO03.09.019	0.000	0.007	0.025	0.025	0.000	0.027	0.000			
9	BIO03.09.020	0.000	0.007	0.025	0.025	0.000	0.027	0.000	0.000		
10	BI003.09.021	0.027	0.034	0.001	0.001	0.027	0.000	0.027	0.027	0.027	

The genetic range from the table provides information to see the farthest to the closest range between species, while the farthest range from Rhynchobatus sp. with of 0.034 and the closest range with 0,000. According to (Irawan et al. 2016) explained when two organisms had closer genetic range, the closer genetic relationship will be and vice versa. The genetic range of 0,034 discovered in sample BIO03.09.013 to BIO03.09.017, BI003.09.021. The genetic range of 0,033 was discovered in samples BIO03.09.014, BIO03.09.013, and BIO03.09.015. The genetic range of 0,027 was discovered in samples BI003.09.012, BI003.09.017, BIO03.09.021. BIO03.09.016 to BIO03.09.017 and BI003.09.021. BIO03.09.017 to BIO03.09.018, BIO03.09.019 and BIO03.09.020. BI003.09.021 to BI003.09.018, BI003.09.019 to BI003.09.020. The genetic range ofi 0,25 was discovered in samples BIO03.09.014 and BIO03.09.015 to BIO03.09.012. BIO03.09.016, BIO03.09.018, BIO03.09.019, BIO03.09.020 to BIO03.09.015. The genetic range of 0,007 was discovered in samples BIO03.09.013 to BIO03.09.012. BIO03.09.016, BIO03.09.018, BIO03.09.019, and BIO03.09.020 to BIO03.09.013. The genetic range of 0,001 was discover in samples BIO03.09.017 and BIO03.09.021 to BIO03.09.014. BIO03.09.017 and BIO03.09.021 to BIO03.09.015. As for the closest range with value 0,000 was discovered in samples BIO03.09.016, BIO03.09.018, BIO03.09.019, and BIO03.09.020 to BIO03.09.012. BIO03.09.015 to BIO03.09.014. In accord with Drancourt et al., (2000) the homology level with 99% indicates compatibility interspecies, the homology level with 97% indicates compatibility between genera and the homology level around 89-93% indicates compatibility between families.

Phylogenetic Tree Analysis

Classification inter species can be seen by analyzing phylogenetic trees. Phylogenetic studies aim to reconstruct relationship between organisms (Trianto and Purwanto 2020). Leatemia dkk., (2018) also imply that phylogenetic is one of the analyzing methods to see the phylogenetic relationship of a living creature. Phylogenetic tree analysis using the Nightbor Joining method, The evolutionary relationships of taxa were analyzed using the Neighbor-joining method (Saitou and Nei 1987), By using bootstrap 1000 repetitions, the higher the bootstrap value, the better the confidence level of phylogenetic tree construction (Kumar et al. 2018). According to Xia and Lemey (2012) in the analysis of NJT (Neighbor-joining Tree) to obtain relatively stable data requires bootstrap with a value greater than 70%, the higher the construction. Sahaba et al., 2022) also stated that the higher the bootstrap value of the branch, the stronger the branching in the phylogenetic tree of the species. Making a phylogenetic tree using MEGA X software on the phylogeny menu, with the Nightbor Joining approach, Kimura Model 2 parameters, bootstrap value 1000x (Kumar et al.

2018). The higher the bootstrap value, the better the grouping of the phylogenetic tree arrangement (Saleky et al. 2020). The following is the result of a phylogenetic tree using bootstrap with 1000 repetitions:



Picture 4. The results of phylogenetic tree construction from sample of *Rhynchobatus* sp

Evolutionary tree construction apart from the ten samples which were analysed by including Rynchobatus Springeri and Rynchobatus Djiddensis from the database with the aim of comparing outgroup data. The purpose of the outgroup is to obtain more valid information and analysis in the reconstruction of evolutionary trees (Dharmayanti, 2018). Outgroup Rynchobatus Springeri and Rynchobatus Djiddensis taken from NCBI data, these species still have a very close relationship with the Rhinidae family (Rynchobatus Sp) including Rhynchobatus australiae and Rynchobatus laevis (AISHAH et al. 2018).

The results of the phylogenetic tree reconstruction show four groups (clades), where each clade is arranged because it has the same nucleotide sequence per individual from the sample, the bootstrap value of the phylogenetic tree is 93-99%, it can be said that these groupings have a high degree of similarity (Rahayu and Jannah, 2019). The first clade is occupied by the species Rynchobatus Laevis, followed by

samples BIO03.09.013, BIO03.09.019, BIO03.09.012, BIO03.09.018 and BIO03.09.020. However, the sample code BIO03.09.013 forms a short branch, there may be differences in nucleotide sequences caused by migration of species. The second clade is occupied by Rynchobatus Springeri and as an outgroup is marked by forming a different branch. The third clade is occupied by the species Rynchobatus australiae and followed by samples BIO03.09.014, BIO03.09.015, BIO03.09.017 and BIO03.09.021. The fourth clade is occupied by the species Rynchobatus Djiddensis as an outgroup which also has the most distant branch from all samples.

Conclusion

The results of molecular identification of 10 samples obtained showed that the samples consisted of four individuals from the type of R. australiae and six individuals from R. laevis. Both types of rays show close genetic distances, indicating that these two types have the same gene patterns and characters due to environmental factors that are identical to each other. The results of the phylogenetic tree reconstruction show four groups (clades), where each clade is arranged because it has the same nucleotide sequence per individual from the sample, the bootstrap value of the phylogenetic tree is 93-99%, it can be said that the grouping has a high degree of similarity.

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