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Molecular Identification of Stingrays (Dasyatidae) from Gresik, East Java

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ABSTRACT

Stingrays (Dasyatidae) are the Elasmobranchii or cartilaginous fish. In Indonesia, stingrays have long been one of the fish resources with high economic value with a fairly high export value. High exploitation and lack of conservation efforts can lead to reduced stingray stocks in some waters in Indonesia. Identification of stingray species is necessary to determine their conservation status as a conservation effort by maintaining genetic resources, species and ecosystems of stingrays. The method used for fish identification is very fast and accurate. DNA barcoding is a new identification technique with a molecular approach that provides an alternative for the fast, accurate, and unambiguous identification of an organism. The purpose of this study was to identify stingray species that landed on Delegan Gresik Beach, East Java to determine the relationship between species through phylogenetic trees. The analysis and reconstruction of the phylogenetic tree were carried out by MEGA X software with the neighbour-joining algorithm and the Kimura-2 parameter model with a bootstrap value of 1000. The results obtained 2 species of stingrays, Telatrygon zugei and Neotrygon kuhlii. Phylogenetic analysis showed a close relationship between the two species with a genetic distance of 0.185. The conservation status of the Telatrygon zugei species is Near Threatened (NT) category and the Neotrygon kuhlii species is in the Data Deficient (DD) category.

INTRODUCTION

The Delegan Beach is a tourist attraction located on the northern coast of Java, which is in Delegan Village, Panceng District, Gresik Regency, East Java. This coastal area has a variety of natural resources so apart from being a tourist spot, the coastal area is also an area of high fishing activity, especially for the family stingray (Dasyatidae) **(Bahar, 2016)**. In addition to having high potential, the area located adjacent to the fish auction site has the opportunity for excessive fishing activities that can affect the existing conditions of the coastal area and the existence of the ecosystem of several marine species. One of the most exploited capture fisheries commodities is stingray.

Stingrays (Dasyatidae) are Elasmobranchii or cartilaginous fish **(Wijayanti *et al.*, 2018)**. Stingrays have an important ecological role as benthic predators in marine waters. As top-level predators, stingrays are considered to have an important role in maintaining the health of marine ecosystems **(Abubakar *et al.*, 2016)**. Based on data from the **KKP**

(2018), the total production of stingray capture fisheries in 2018 reached 22.4 tons. This increasing production is feared to cause availability in nature to be increasingly limited. Information on stingray species is very important in relation to information on stingray species diversity, management activities, and at the same time stingray conservation in Indonesia The technique that can be used to identify stingray species accurately and quickly is the molecular identification technique **(Wehantouw *et al.*, 2017)**.

Molecular identification is a method to identify an organism species. DNA (Deoxyribose Nucleic Acid) barcoding is one of the molecular techniques used to speed up and simplify identifying organisms using certain gene pieces **(Bangola *et al.*, 2014)**. The DNA barcoding technique has the advantage of identifying a species in various taxa that may be difficult to distinguish morphologically or by traditional identification methods. Diagnosis at the molecular scale through DNA barcoding provides an alternative to organism identification that is fast, accurate, and unambiguous (biased) **(Virgilio *et al.*, 2012)**. The molecular identification approach is also able to identify stingrays which are morphologically difficult to distinguish **(Zein and Prawiradilaga, 2013)**.

MATERIALS AND METHODS

Materials

Sample Collection

Sampling was conducted at Delegan Beach, Gresik, East Java, Indonesia. The samples obtained two samples of stingray tissue specimens were taken at the caudal part of the base of the tail and then inserted into a microtube filled with 1 ml of 96% ethanol and labelled according to each sample. The sample tube and ethanol were stored in the Laboratory of Microbiology and Fish Diseases, Faculty of Fisheries and Marine Affairs, Airlangga University, Surabaya.

DNA Extraction and Amplification

DNA extraction aims to destroy cells and take tissue in the sample. The extraction method was carried out in several stages from the lysis stage, binding stage, washing stage to the elution stage with extraction materials consisting of Aquabides, DNA Template, Proteinase K, Buffer GT 1 and 2, Buffer W1 and W2, and Elution Buffer. The DNA amplification process using the Polymerase Chain Reaction (PCR) method is a technique or method of enzymatic DNA propagation (replication). The main components in PCR are DNA templates, PCR mixes, and primers. The primer used is universal Primer Fish LCO-HCO. The PCR process was carried out using a PCR machine (Thermocycler) which consisted of several processes, namely the separation of double-stranded DNA (Pre-denaturation) at 94^{o}C for 3 minutes followed by 40 cycles (denaturation) at 94^{o}C for 30 seconds, the attachment process primer (annealing) at 55^{o}C for 30 seconds and DNA segment lengthening (Extention) at 72^{o}C for 45 seconds for 30 cycles and post-extension at 72^{o}C for 5 minutes **(Andriyono and Suciyono, 2020)**.

Electrophoresis

The electrophoresis stage is an advanced stage to see positive or negative DNA from the resulting PCR product. The initial step is to make 10% Agarose Gel by mixing 1 gram of agarose powder with 100 mL of TAE 1x in an Erlenmeyer tube. Heat in the

microwave for 1 minute until the agarose looks dissolved. Then pour it into an agarose mould and attach a comb then wait for 15-25 minutes until a gel is formed **(Zain and Prawiradilaga, 2013)**. Then enter the PCR sample using a pipette by mixing it with gel-rad as a dye. The mixing stage is carried out using a pipette. After that put it into the gel mould. Run the electrophoresis machine at 200 V and a current of 400 mA. Wait up to 30 minutes, then see the results using a UV lamp at a wavelength of 254 nm then the results are photographed. Then the electrophoresis results were sent to PT Genetics Science 1st Base for DNA sequencing to determine the nucleotide sequence contained in the DNA of the stingray species.

Data analysis

Data analysis was carried out using the first, namely the trimming of the nucleotide and part of sequence quality control by Chromas software version 2.6.6 (http://technelysium.com.au/wp/chromas/). Besides, forward and reverse sequences were alignment by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic tree and nucleotide analysis have been generated by MEGA10 (Molecular Evolutionary Genetic Analysis) including the diversity of nucleotides **(Tamura *et al*., 2007)**. The nucleotide alignment data obtained were then matched with the data available on the GeneBank at NCBI (National Center for Biotechnology Information). The method used is BLASTN (Basic Local Alignment Search Tool Nucleotides) through the online system (http://blast.ncbi.nlm.nih.gov). Reconstruction of a phylogenetic tree using MegaX software **(Kimura *et al*., 2018)** with the neighbour-joining method and Kimura-2 parameter model with a bootstrap value of 1000 **(Kimura, 1980)**.

RESULTS

Identification of Stingray Morphology

The morphology identification showed two specimens of stingrays obtained from Delegan Beach, Gresik successfully conducted based on a previous publication on Dasyatidae **(Last *et al*., 2016a; Last *et al*., 2016b)**. It identified as the sample code for stingray GRPI01 has similarities with the pale-eyed stingray (*Telatrygon zugei*). The body shape and colour of the stingray have a fairly close resemblance. The particularity of the species *Telatrygon zugei* is the presence of pale-eyed, the front of the body plate tends to be concave, the snout is long and tapered, and the back of the body is brown to dark, with a whitish belly. The size of T. zugei is range from 30 cm to 2 meter **(Moazzam and Osmany 2021)**. The results are also supported by morphometric measurement data. The total length measurement for sample code GRPI01 is 40 cm, the standard length is 17 cm, and the body width is 12 cm. The sample code GRPI02 has similarities to the (*Neotrygon kuhlii*) which is commonly known as a spotted stingray. The body shape and colour of these fish have a fairly close resemblance. The distinctive feature of this species is its spotted body with blue spots at the back of the dorsal **(Sudibyo *et al*., 2020)**. The morphometric measurement data for sample code GRPI02 with a total length of 38 cm, a standard length of 12 cm and a body width of 9 cm. A distinctive feature of both species is the presence in (Figure 1).

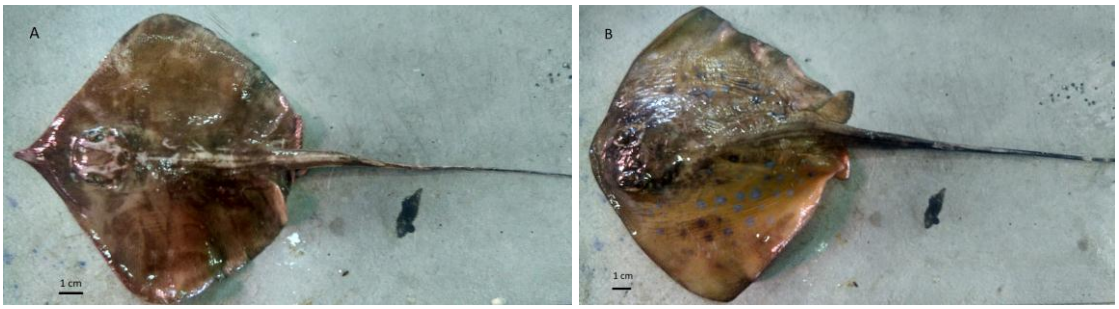

Figure 1. Morphological characteristics of stingrays landed on Delegan Beach, Gresik, East Java. A. *Telatrygon zugei* and B. *Neotrygon kuhlii*.

Molecular Identification of Stingrays

Molecular identification in stingrays was carried out through several stages, DNA extraction, PCR, and electrophoresis to the sequencing process to obtain sequencing results in the form of nucleotide base sequences. The results of the BLASTN analysis showed that the specimen code GRPI01 identified the type of *Telatrygon zugei* (Pale-edged Stingray) with the accession number MH085752 with a score of 1129 against the GRPI01 sample, with a sequence similarity level of 100%. While the specimen code GRPI02 *Neotrygon kuhlii* (Spotted Stingray) with access number MH085753 has a score of 1112 against the GRPI02 sample, with a sequence similarity level of 100%. The high percentage value indicates that the sample sequences are identical to the species sequences in the database, which is the highest percentage analysis results in each sample. Based on the results of BLASTN it can be concluded that the specimen GRPI01 is a species of *Telatrygon zugei* and GRPI02 is a species of *Neotrygon kuhlii*. The results of the BLASTN analysis of stingray samples from Delegan Beach, Gresik can be seen in (Table 1).

Table 1. The results of the BLASTN analysis based on the homology match of the Stingray sample with the Gene Bank database

Sample Code	**Species**	**Common Name**	***% Identity***	***GenBank Accession No.***
GRPI01	*Telatrygon zugei*	Pale Edged Stingray	100%	MH085752
GRPI02	*Neotrygon kuhlii*	Spotted Stingray	100%	MH085753

The BLAST data obtained are then aligned to create a phylogenetic tree by editing using the alignment tools in the MEGAX Software. Then aligned with the ClustalW method to see the diversity of nucleotide bases. The results of the alignment were then used to construct a phylogenetic tree to see the level of relationship between species and to determine the genetic distance of each species. The phylogenetic tree can be made using the neighbour-joining Tree method, the Kimura 2-parameter evolution model and 1000x bootstraps replication (Kimura, 1980). The results of the phylogenetic reconstruction of stingrays from Delegan Beach, Gresik can be seen in (**Figure 2**).

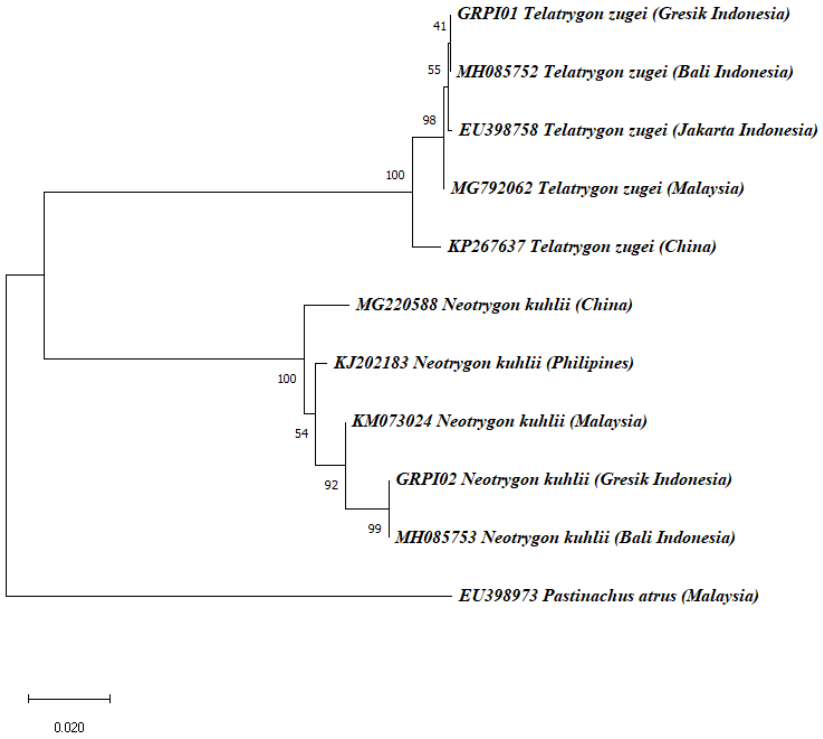

Figure 2. Stingray phylogenetic tree was generated by MEGAX.

Genetic distance analysis was carried out using MEGA X software. Based on the results of genetic distance analysis, it was shown that the genetic distance in the stingray sample code GRPI01 *Telatrygon zugei* obtained from Delegan Beach, Gresik, had no difference with the sequence from the Genbank database from Bali (MH085752) with zero genetic distance. Meanwhile, the sequence from Jakarta (EU398758) and Malaysia (MG792062) had a genetic distance of 0.001, and the sequence from China (KP267637) had a genetic distance of 0.017. Then in the sample code (GRPI02) *Neotrygon kuhlii* obtained from Delegan Beach, Gresik, there is no difference with the database sequence from Bali (MH085753) with zero genetic distance. Meanwhile, the sequence from the Philippines (KJ202183) has a genetic distance of 0.018, China (MG220588) has a genetic distance of 0.025 and Malaysia (KM073024) has a genetic distance of 0.010. And the genetic distance between the species *Telatrygon zugei* and *Neotrygon kuhlii* is 0.185. The results of genetic distance can be seen in **Table 2**.

The results of stingray identification that have been obtained are then analyzed for their conservation status by referring to the IUCN (International Union for Conservation of Nature and Natural Resources) site. IUCN also recommends limiting trade in endangered species by issuing an international agreement, namely the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The conservation status of stingray species can be seen in **Table 3**.

Table 2. The genetic distance of stingray with sequences from Genbank.

No	Species	1	2	3	4	5	6	7	8	9	10	11
1	GRPI01 *Telatrygon zugei* (Gresik Indonesia)											
2	MG792062 *Telatrygon zugei* (Malaysia)	0,001										
3	EU398758 *Telatrygon zugei* (Jakarta Indonesia)	0,001	0,003									
4	MH085752 *Telatrygon zugei* (Bali Indonesia)	0,000	0,001	0,001								
5	KP267637 *Telatrygon zugei* (China)	0,017	0,015	0,015	0,017							
6	GRPI02 *Neotrygon kuhlii* (Gresik Indonesia)	0,185	0,183	0,187	0,185	0,185						
7	MH085753 *Neotrygon kuhlii* (Bali Indonesia)	0,185	0,183	0,187	0,185	0,185	0,000					
8	KJ202183 *Neotrygon kuhlii* (Philipines)	0,165	0,163	0,168	0,165	0,165	0,018	0,018				
9	MG220588 *Neotrygon kuhlii* (China)	0,175	0,172	0,177	0,175	0,175	0,025	0,025	0,020			
10	KM073024 *Neotrygon kuhlii* (Malaysia)	0,172	0,170	0,174	0,172	0,172	0,010	0,010	0,012	0,018		
11	EU398973 *Pastinachus atrus* (Malaysia)	0,219	0,217	0,217	0,219	0,214	0,202	0,202	0,198	0,189	0,191	

Description :

= Interspecies genetic distance from the samples obtained.

Table 3. Stingray conservation status based on IUCN and CITES.

No	Specimen Code	Species	IUCN Status	CITES	Threat To Humans
1	GRPI01	*Telatrygon zugei*	NT	Not Evaluated	Harmless
2	GRPI02	*Neotrygon kuhlii*	DD	Not Evaluated	Venomous

Note: IUCN Status ; (NT) *Near Threatened* and (DD) *Data Deficient;* CITES Status; *Not Evaluated*.

DISCUSSION

Molecular identification of stingrays is carried out to determine the accuracy of the species of an organism by going through several stages, DNA extraction, sample PCR, and electrophoresis which then results obtained. Based on the sequencing results of the two samples of stingrays with the specimen codes GRPI01 identified as *Telatrygon zugei* (Pale-edged Stingray) with a sequence similarity level of 100%. While the specimen code GRPI02 *Neotrygon kuhlii* (Spotted Stingray) with a sequence similarity level of 100%. Regarding the habitat of *N. kuhlii*, population from Java and Bali not significat different due to the sama in Java Sea **(Fahmi *et al*., 2017)**. The high percentage value indicates that the sample sequences are identical to the species sequences in the database, which is the highest percentage of BLASTN analysis results in each sample. The higher the score, the higher the similarity between the sample sequence and the database sequence. Scores <50 are declared to have no similarity **(Claverie and Notredame, 2003)**. In addition, the similarity between sample sequences and database sequences is also characterized by the similarity of percentage values **(Narita *et al*., 2012)**. The percentage level of sequence similarity which is at a value of 100% indicates that the species being compared are the same **(Drancourt *et al*., 2000**). So based on the results of BLASTN it can be concluded that the specimen GRPI01 is a species of *Telatrygon zugei* and GRPI02 is a species of *Neotrygon kuhlii*. The results of the sequences were then arranged in the phylogenetic tree.

The arrangement of the phylogenetic tree in this study was formed from 2 sequences with the addition of the sequence results contained in GenBank. There are 4 species of *Telatrygon zugei* and 4 species of *Neotrygon kuhliii*. In compiling the phylogenetic tree, it is necessary to outgroup species obtained from GenBank with taxa that are not too far apart. The outgroup species functioned as a comparison in determining the species in the ingroup. The phylogenetic formed was clear and strong to classify a relationship between individuals and species **(Mount, 2001)**. The outgroup species used is a sequence of the stingray *Pastinachus atrus* (Broad-cowtail ray) which is used as an outgroup of the Family Dasyatidae and Genus *Pastinachus* **(Froese and Pauly, 2021)**. The phylogenetic tree reconstruction method used the Neighbor-Joining method and the Kimura-2 parameter model with a bootstrap value of 1000. Based on the phylogenetic tree

construction, the stingray samples obtained from Delegan Beach Gresik showed that there were 2 clades formed in the Dasyatidae family. Clade I consists of the genus *Telatrygon* and clade II consists of *Neotrygon*.

In the construction of the phylogenetic tree formed, in clade I there is a code added from the *Telatrygon zugei* sequence on GenBank which consists of the sequence code MH085752 from Bali, MG792062 from Malaysia, EU398758 from Jakarta and KP267637 from China. While in clade II, the *Neotrygon kuhlii* sequence consists of sequences MH085753 from Bali, KM073024 from Malaysia, KJ202183 from the Philippines, and MG220588 from China. The phylogenetic tree in (Figure 2) shows a scale of 0.020 which indicates that from 100 nucleotide sequences there are 2 different bases in each branch.

Reconstruction of the stingray phylogenetic tree can be identified by species through phylogenetic branching that forms groups. There is a bootstrap value on a scale of 1-100% of the repetition value of 1000 times to determine the level of accuracy of branching phylogenetic trees. The higher the bootstrap value (maximum value of 100%), the higher the level of accuracy and positional determination of the branching of the phylogenetic tree formed **(Ubaidilah and Sutrisno, 2009)**. The bootstrap value from the reconstruction of stingray species at each branch has a value of 100, which means that the branching of the two clades is accurate.

Based on its conservation status, the species *Telatrygon zugei* is in the Near Threatened (NT) category **(White, 2016)**. The conservation status is determined by the international body IUCN (International Union for Conservation of Nature and Natural Resources) which focuses on the conservation of sharks and rays (Elasmobranchii). Near Threatened (NT) is a conservation status given to species that may be in a state of threat or near threatened with extinction even though they are not included in the threatened status. While the *Neotrygon kuhlii* species is included in the Data Deficient (DD) category or lacking data (**Kyne and Finucci, 2018)**. This status is given to species that have been evaluated but still lack data to be included in one of the categories. Then based on their trading status according to CITES, these two species of stingray are included in the Not Evaluated category, which indicates that no evaluation was carried out because they did not meet the criteria for evaluating their trade status, so they are still classified as safe for international trade.

CONCLUSION

In this study, two specimens of stingrays were obtained from Delegan Gresik Beach, East Java. The stingray in specimen code GRPI01 was identified as *Telatrygon zugei*, and specimen code GRPI02 was identified as *Neotrygon Kuhlii*. Based on the results of the compilation of the phylogenetic tree, shows that the two species of stingrays have a genetic distance of 0.185. Based on its conservation status, the species *Telatrygon zugei* is in the Near Threatened (NT) category and the *Neotrygon kuhlii* species is in the

Data Deficient (DD) category. Meanwhile, the trade status of these two species is in the Not Evaluated category.

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