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Genetic Distance and Conservation Status of Stingray Species at TPI Tasik Agung Rembang, Central Java based on the Mitochondrial Cytochrome Oxidase I Gene

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ABSTRACT

Genetic distance and conservation status of stingray species at TPI Tasik Agung Rembang, Central Java utilizes the mitochondrial cytochrome oxidase I (COI) gene. The objective of this research is to examine the genetic distance and conservation status of five stingray species discovered in TPI (Fish Auction) Tasik Agung: *Himantura uarnacoides, Himantura walga, Himantura gerrardi, Rhinobatos penggali*, and *Brevitrygon heterura*. Eventually, the acquired data can be applied as important basic information to preserve genetic diversity in stingray populations. The DNA barcode approach was performed on the COI gene using the polymerase chain reaction (PCR) technique. The sequences obtained were edited in the Basic Local Alignment Search Tool (BLAST) program before being analyzed in MEGA 10 software for phylogenetic tree reconstruction and genetic distance measurement. The genetic distance between the most closely related species was 0.002 (*Dasyatis zugei* and *Telatrygon zugei*), and the most significant genetic distance was 0.231 (*Rhinobatos penggali* and *Brevitrygon heterura* with *Himantura walga*). The IUCN conservation status of stingrays observed during the study revealed four species in the vulnerable category (VU) and one species in the near threatened (NT) category.

Keywords: Conservation; mtDNA COI gene; Phylogenetic; Stingray; TPI Tasik Agung

1. Introduction

As a maritime country that has a massive number of marine resources, Indonesia indeed meets the potential to become a commodity in the fishery sector. Fish production increased by 3.36% over the last five years, according to the Ministry of Maritime Affairs and Fisheries, in conjunction

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with a 7.23% growth in fish consumption, or 50.69 kg/capita per year [1].

Stingrays are considered a fish commodity in Indonesia due to their high economic value. According to data from the Central Java Provincial Statistics Agency for 2020, with 136358.77 tons/year in 2017, Rembang Regency has the highest marine capture fisheries production among Central Java districts [2]. This is related to the number of fish caught at TPI Tasik Agung, one of Rembang Regency's fish auction sites, which increased from August to December 2019 [3].

Previous research by Zain et al. [4] discovered that only a few stingray species were caught, with species including *Dasyatis kuhlii, Gymnura micrura,* and *Rhynchobatus austraiae*. Hence, it is feared that the availability of stingrays in nature will decline and even lead to extinction at some point. Therefore, phylogenetic analysis was used to describe the relationship of each stingray species in TPI Tasik Agung Rembang based on genetic distance for the purpose of genetic conservation efforts [5].

If stingray consumption is not balanced with conservation efforts, it could lead to a rapid decline in population and even species extinction, which would take a long time to recover from [6- 8]. According to data from the International Union for Conservation of Nature and Natural Resources (IUCN), three stingray species in Java are critically endangered, six are endangered, seven are vulnerable, three are near threatened, three are species of least concern, and six are deficient [9]. This fact is aggravated by its biological characteristics, in which stingrays' growth rate and maturity are slow, and their fecundity is relatively low. These aspects of stingray biology underline the necessity of conservation efforts to ensure their long-term viability [10].

Phylogenetic analysis is one of the solutions which examines the relationship between organisms based on evolutionary history [11]. Conservation research on stingrays has been conducted in general, but research on stingray genetics is still limited. To investigate the genetic diversity of stingray species, mitochondrial DNA (mtDNA) at the COI gene locus was studied [12].

The COI gene in mtDNA is widely used for species identification because it is frequently used as a DNA barcode to distinguish between species [14]. COI gene fragments are rarely subjected to amino acid substitution, but they are subjected to silent mutations (DNA mutations that have no effect on amino acid sequence) [15]. As a result, the COI gene can be used to reconstruct phylogenetic diversity at levels lower than species [16].

The COI area's capability to serve as a marker for specific aquatic animals in fish, including both freshwater and saltwater fish, has been widely used [17]. Several studies have used the COI area as a marker, including those on Telaga Sari's typical fish [18] and stingrays in Indonesia's western Pacific region [19].

2. Materials and Methods

2.1 Study area

Molecular analysis, DNA isolation, polymerase chain reaction (PCR), and sequencing were all used in this study. Five different species of stingrays from TPI Tasik Agung Rembang were purchased for preparation [20]. Muscle tissue from stingray fins was used for mtDNA extraction.

2.2 Procedures

2.2.1 DNA extraction and isolation

The TIANamp Marine Animals Kit protocol was used for DNA isolation. A total of 30 mg of stingray fin and 200 µl of Buffer GA were weighed and vortexed for 15 seconds. The mixture was then vortexed and incubated at 56-70ºC for 1 hour with 20 µl of Proteinase K, 200 µl of Buffer GB, and 200 µl of absolute ethanol. The purpose of vortexing and incubating was to create a lysable and homogeneous solution.

The incubated suspension was transferred to the Spin Column CB3 (located in the collection tube) and centrifuged for 30 seconds at 12,000 rpm. In the collection tube, the supernatant was filtered.

Furthermore, 500 µl of Buffer GD was added and centrifuged at the same speed and time, twice. Spin Column CB3 was centrifuged for 2 minutes at 12,000 rpm until the membrane was completely dry. Spin Column CB3 was placed in a new, sterile 1.5 ml microtube, along with 50-200 µl of Buffer TE in membrane center. It was then incubated for 2-5 minutes at room temperature (15-25ºC) before being centrifuged for 2 minutes at 12,000 rpm. The DNA samples were kept in the freezer until they were needed later.

2.2.2 PCR Amplification and Visualization of DNA Fragments

PCR was used to amplify the mitochondrial locus gene. At this stage, DNA templates, Taq DNA polymerase, dNTPs, PCR buffer, MgCl2, and two universal primer pairs were used:

1) FishF1 forward primer (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and FishR1 reverse primer (5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3')
2) FishF2 forward primer (5'-TCG ACT AAT CAT AAA GAT ATC GGC AC-3') and FishR2 reverse primer (5'- ACT TCA GGG TGA CCG AAG AAT CAG AA-3') [21].

The PCR mixture contained 4 µl of DNA samples, 4 µl each of forward and reverse primers, 25 µl of 2xTaq Polymerase Mix Kit, and 18 µl of ddH2O. The total volume was 50 µl. The PCR reaction was performed using a thermal cycler machine at 94ºC for 3 minutes under pre-denatured conditions. The PCR cycle was repeated 35 times, with denaturation at 94ºC for 30 seconds, annealing at 51ºC for 30 seconds, extension at 72ºC for µl minute, and final extension at 72ºC for 5 minutes. The resulting PCR product had a size of 655 bp. On 2% agarose gel with 1X TAE buffer, the PCR product was examined for quality. A UV transilluminator was used to observe the agarose gel after it had been stained with gelRed [22].

2.2.3 PCR Product Sequencing

The COI gene PCR product in this study was a single band measuring 655 bp. The sequencing analysis was performed by Macrogen in South Korea.

2.3 Data analysis (phylogenetic analysis)

The DNA sequences of the COI gene were edited with the DNA Baser Assembler v5 program. The alignment of the nucleotide sequences of the COI gene was analyzed using the ClustalW program within the MEGA software, version 10. The edited nucleotide sequence then underwent BLAST analysis with the nucleotide sequence from GenBank on the NCBI website. The MEGA version 10 program and the bootstrap Neighbor-Joining *(*NJ*)* method with 1000 repetitions were used for phylogenetic analysis. The MEGA program generated a phylogenetic tree and a genetic distance matrix based on nucleotide base similarities and differences [23].

3. Results and Discussion

3.1 Results

DNA fragments from the COI gene were sequenced from samples from 8 stingrays and tested in the NCBI BLAST program (Table 1).

Table 1. List of test sample results from BLAST GenBank.

Test Sample	Sample Code	Species	Sequence length (bp)	Accession Number	Homology Percentage
Stingray	A3	*Himantura gerrardi*	655	EU398840.1	99%
	A3.2	*Maculabatis macrura**	655	MG774914.1	99%
	A4	*Himantura uarnacoides*	655	EU398870.1	98%
	A5	*Himantura walga*	655	EU398873.1	99%
	A5.2	*Brevitrygon heterura**	655	MG774919.1	99%
	A6	*Dasyatis zugei*	655	EU398761.1	98%
	A6.2	*Telatrygon zugei**	655	MH085752.1	98%
	B9	*Dasyatis zugei*	655	KM073022.1	99%
	B13	*Himantura gerrardi*	655	KX219583.1	99%
	B13.2	*Maculabatis macrura**	655	MG792063.1	99%
	B16	*Rhinobatos penggali*	655	EU398996.1	99%
	B18	*Himantura walga*	655	KX219580.1	100%

Note: *is accession number which has similar percentage.
A: primer pair FishF1-FishR1, B: primer pair FishF2-FishR2.

The results of the sequencing were aligned between the forward and reverse primers so that the nucleotide sequence length was 655 bp. The average nucleotide base composition in the samples was 30.9% thymine (T), 25.5% adenine (A), 26.8% cytosine (C), and 16.7% guanine (G) (Table 2).

Table 2. Average nucleotide base composition in samples.

No	Test Sample	T(U)	C	A	G
1	MG774919.1 *Brevitrygon heterura*	31.1	27.1	24.5	17.4
2	KX219580.1 *Himantura walga*	31.1	27.1	24.5	17.4
3	EU398873.1 *Himantura walga*(2)	31.1	27.1	24.5	17.4
4	MG792063.1 *Maculabatis macrura*	29.9	28.3	25.3	16.5
5	MG774914.1 *Maculabatis macrura*(2)	29.9	28.3	25.3	16.5
6	KX219583.1 *Himantura gerrardi*	29.9	28.3	25.3	16.5
7	EU398840.1 *Himantura gerrardi*(2)	29.8	28.2	25.3	16.6
8	EU398761.1 *Dasyatis zugei*	32.8	24.0	27.8	15.5
9	KM073022.1 *Dasyatis zugei*(2)	33.0	23.8	27.6	15.6
10	MH085752.1 *Telatrygon zugei*	32.8	24.0	27.6	15.6
11	EU398870.1 *Himantura uarnacoides*	30.3	27.1	24.7	17.9
12	EU398996.1 *Rhinobatos penggali*	29.5	28.5	24.8	17.2
	Avg.	30.9	26.8	25.5	16.7

Note: (2) the same species from different samples.

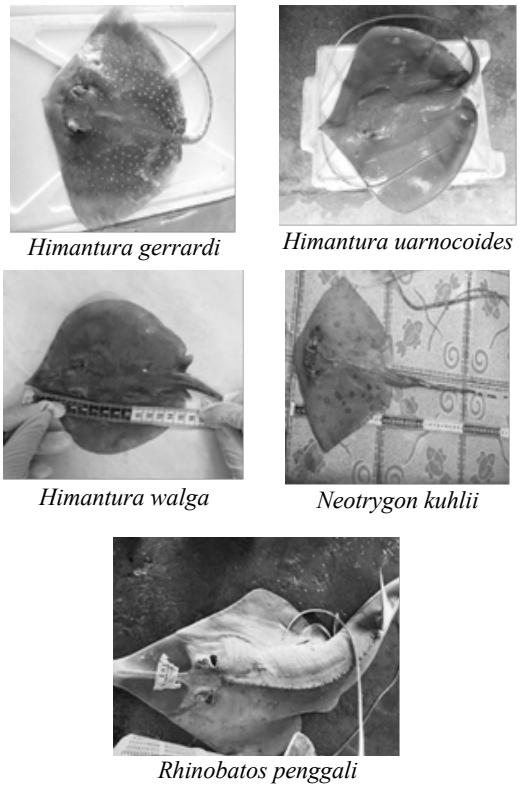

Fig. 1. Stingrays found in TPI Tasik Agung (5 Species).

The phylogenetic construction of stingray samples with GenBank collection was obtained using neighbor-joining (NJ) with Bootstrap 1000x replications. Phylogenetic tree reconstruction in stingray samples is shown in Fig. 1.

Genetic distance results are presented in Table 3.

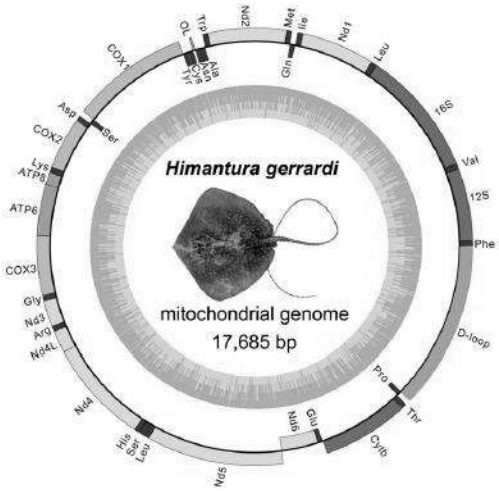

Fig. 2. Mitochondrial DNA genome mapping of *Himantura gerrardi* [12].

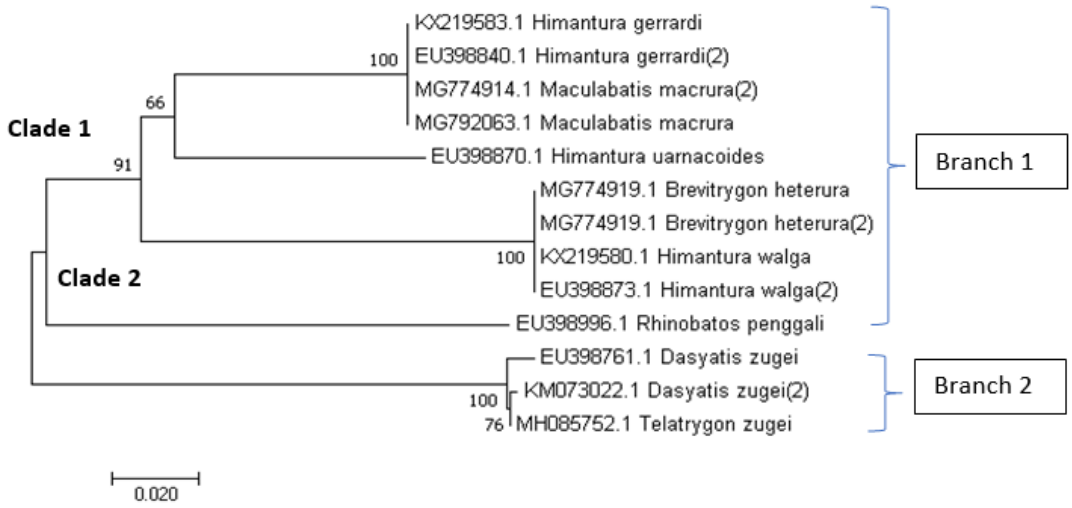

Fig. 3. Reconstruction of the 655 bp stingray phylogenetic tree from the COI gene fragment using the Neighbor-Joining method, bootstrapped 1000 times with the p-distance model.

Table 3. Matrix of genetic distance of COI gene fragments in stingray test samples.

No	1	2	3	4	5	6	7	8	9	10	11	12
1												
2	0,000											
3	0,000	0,000										
4	0,153	0,153	0,153									
5	0,153	0,153	0,153	0,000								
6	0,153	0,153	0,153	0,000	0,000							
7	0,153	0,153	0,153	0,000	0,000	0,000						
8	0,219	0,219	0,219	0,202	0,202	0,202	0,202					
9	0,214	0,214	0,214	0,200	0,200	0,200	0,200	0,009				
10	0,212	0,212	0,212	0,198	0,198	0,198	0,198	0,007	0,002			
11	0,150	0,150	0,150	0,111	0,111	0,111	0,111	0,217	0,212	0,210		
12	0,231	0,231	0,231	0,186	0,186	0,186	0,186	0,226	0,221	0,219	0,195	

Note: *(1) Brevitrygon heterura, (2) Himantura walga, (3) Himantura walga (2), (4) Maculabatis macrura, (5) Maculabatis macrura (2), (6) Himantura gerrardi, (7) Himantura gerrardi (2), (8) Dasyatis zugei, (9) Dasyatis zugei (2), (10) Telatrygon zugei, (11) Himantura uarnacoides, (12) Rhinobatos penggali.*

Table 4. Conservation status of stingrays found in TPI Tasik Agung Rembang.

Family	Latin Name	Local Name	Conservation Status
Dasyatidae	*Brevitrygon heterura*	Pari Kikir	NT
Dasyatidae	*Himantura walga*	Pari bersisik	VU
Dasyatidae	*Himantura gerrardi,*	Pari Bintang	VU
Rhinobatidae	*Rhinobatos penggali*	Pari Penggali	VU
Dasyatidae	*Himantura uarnacoides*	Pari Tanjung	VU

Based on the conservation status in the IUCN catalog, 4 species of stingrays found at Tasik Agung Fish Auction Site are classified VU, 1 species is classified as NT.

3.2 Discussion

According to the PCR results, the COI fragment was amplified well at 655 bp using the two primer pairs, FishF1-FishR1 and FishF2-FishR2. The purity of extracted DNA, reagent composition, and proper PCR

conditions, particularly during the annealing process, all have an impact on the success of PCR amplification (primary attachment) [24]. For the primer to attach to both ends of the DNA template, the annealing process requires a specific temperature *(*melting temperature*)*. The high temperature prevents the primer from attaching to the DNA template, however, if the melting temperature is too low, mispriming (the primer sticking to the incorrect location on the DNA template) will occur [5].

According to Table 1, the COI gene sequencing results showed a sequence length of 655 bp for each primer from all samples. Based on the BLAST analysis of sample A3, the percentage of homology in *Himantura gerrardi* and *Maculabatis macrura* is 99%, sample A4 shows 98% homology in *Himantura uarnacoides*, and sample A5 shows 99% homology in *Himantura walga* and *Brevitrygon heterura*. The percentage of homology is 98% in *Dasyatis zugei* and *Telatrygon zugei* species in sample A6, 99% in *Dasyatis zugei* species in sample B9, 99% in *Himantura gerrardi* and *Maculabatis macrura* in sample B13, 99% in burrowing *Rhinobatos penggali* species in sample B16, and 100% in *Himantura* species in sample B18. Conforming to the findings of this analysis, there were 5 species of stingray sampled from TPI Tasik Agung Rembang.

The A-T base content in stingray COI mtDNA was greater than the G-C base content (Table 2). These findings corroborate those of Le Porth et al., [10]. Clustal W was used in the Mega software 7.0 program to align the percentage of base content results, which had previously been used to reconstruct the stingray phylogenetic tree.

3.2.1 Relationship of Stingrays Using Genetic Distance

Kinship relationships can be analyzed using phylogenetic analysis. This is done to determine the degree of similarity between the discovered stingray species [25]. A phylogenetic analysis tree is a phylogenetic analysis-based branching system. The tree's branches show species that are getting closer together based on phylogenetic analysis [23].

The phylogenetic tree reconstruction (Fig. 1) depicts the relationship between 5 stingray species that were discovered to demonstrate confidence in the phylogenetic topology in branch representatives with bootstrap values. The bootstrap value in Mega 10 software is the number of tree creation repetitions, which is 1000 repetitions in this study [26].

The bootstrapping test results are shown in a cladogram, along with the bootstrap value for each branch. Each branch's level of accuracy is determined by the bootstrap value. The higher the bootstrap value of a branch, the higher the level of branch accuracy. Branching indicates a species' descent from an ancestor. The base difference in each branch is represented by the number 0.020. This number indicates that each branch of 200 nucleotide base sequences contains one different base. Based on the reconstruction of the phylogenetic tree, two large branches were formed, branch 1 consisting of 6 ray species and branch 2 consisting of 2 ray species. Branch 1 is divided into two clades, with clade 1 consisting of 5 species and clade 2 consisting of only 1 species, *Rhinobatos penggali*.

According to Fig. 1, the phylogenetic results of branch 1 species *Himantura gerrardi* and *Maculabatis macrura* have a similar genetic structure, and these 2 species are closely related to *Himantura uarnacoides* due a common ancestor. Morphologically, the 3 species are very similar in skin color and body shape, and they are all members of the same family, *Dasyatidae*. The 3 species also share a common ancestor and similar genetic structure with *Brevitrygon heterura* and *Himantura walga*. However, morphologically, this species has colored spots on its skin.

The 5 species, *Himantura gerrardi, Maculabatis macrura, Himantura uarnacoides, Brevitrygon heterura*, and *Himantura walga*, belong to a single clade that differs from clade 2, which includes burrowing *Rhinobatos* species from a different order,

Rhinopristiformes, and are also morphologically similar to sharks. According to the branching of the phylogenetic tree, the *Dasyatis zugei* species has the same genetic structure as *Telatrygon zugei* species in branch 2. Regarding genetic structure, the two species have little in common with branch 1, but morphologically, they share clade 1 in branch 1. Base or genetic structure differences indicate genetic differences within the species. Setiati *et al.,* stated that this could be caused by a variety of factors, including the process of genetic code deletion, mutation, and transversion [27].

The number of bases that change determines the genetic distance. The more base differences there are, the higher the mutation rate [28]. Genetic distance is used to support phylogenetic data. The closer the species is, the smaller the genetic distance value. According to Table 3, *Dasyatis zugei* and *Telatrygon zugei* have the smallest genetic distance with a distance value of 0.002, implying that there are 2 different bases in 1000 nucleotide base sequences. This is consistent with the findings of phylogenetic analysis, which show that *Dasyatis zugei* is more closely related to *Telatrygon zugei*. These two ray species are members of the same family, *Dasyatidae*. With a distance 0.231, *Rhinobatos penggali* is the furthest away from the species *Brevitrygon heterura* and *Himantura walga*. This means that in 1000 nucleotide base, there are 231 bases that don't match.

The IUCN VU category is given to species that have been proven to meet the criteria in the IUCN, one of which is a reduction in population size that occurs in less than 10 years and can be sure to face extinction in nature. Factors that cause species to fall into the vulnerable category include large-scale exploitation, especially for meat, skin, and fins; therefore, the number of these stingray species in nature has decreased sharply.

Another cause is that almost all of these species are viviparous (giving live birth), hence the number of offspring produced is small and the growth of young animals tends to be slow. Another cause is natural predation. For these reasons, it is necessary to socialize and educate fishermen and consumers of stingrays at Muara Angke Fish Auction Site not to catch and consume the nine types of stingrays. Furthermore, to the relevant institutions, namely the Ministry of Maritime Affairs and Fisheries of the Republic of Indonesia to prohibit the capture of stingray species whose conservation status is vulnerable.

4. Conclusion

The genetic relationships between stingray species can be revealed through phylogenetic analysis. This study has shown that at TPI Tasik Agung Rembang, there are 5 different species of stingray present: *Himantura uarnacoides, Himantura walga, Dasyatis zugei, Himantura gerrardi,* and *Rhinobatos penggali*. These 5 species all have a close genetic relationship. The species *Himantura uarnacoides, Himantura walga,* and *Himantura gerrardi* are closely related to *Rhinobatos penggali*. Meanwhile, because the genetic distance is low, *Dasyatis zugei* is closely related to *Telatrygon zugei*. The greater the genetic distance between species, the greater the genetic diversity; conversely, the greater the genetic distance between species, the less genetic diversity. Conservation is neither urgent nor non-threatened, based on phylogenetic analysis and genetic distance between each of these species.

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