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Genetic diversity of wedgefishes and guitarfishes at landing sites in east Indonesia using Cytochrome Subunit I (COI)

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Abstract. *Tapilatu ME, Wijayanti DP, Subagiyo, Sembiring A, Yusmalinda NLA, NIngsih EY, Malik MDA, Pertiwi NPD. 2023. Genetic diversity of wedgefishes and guitarfishes at landing sites in east Indonesia using Cytochrome Subunit I (COI). Biodiversitas 24: 3120-3127.* Wedgefish and guitarfish are considered endangered and protected by law in Indonesia due to pressure from overexploitation. They are highly exploited because of their economic value. This condition impacts the decline in the number of populations to the value of genetic diversity. This study used mitochondrial DNA to assess the genetic diversity of wedgefishes and guitarfishes which landed in the eastern part of Indonesia. We found *Rhynchobatus australiae* (Whitley, 1939) to be the most common species (14 out of 26 sequenced samples), with *Glaucostegus typus* (Bennett, 1830) and *Rhinobatos jimbaranensis* (Last, White, & Fahmi, 2006) appearing infrequently. COI sequences were obtained from the NCBI database and utilized in the study to compare population differentiation. Among the *R. australiae* populations, the results showed that the genetic diversity (Hd) values from Papua, Bali, and Lombok were 1.00, 0.67, and 0.75, respectively. In Papua, *G.typus* populations showed genetic diversity values of 0.90. *R. jimbaranensis* from Bali showed a genetic diversity of 0.50. Papua populations indicated higher genetic diversity than Bali and Lombok populations. Furthermore, the analyses of pairwise F_{ST} values and AMOVA indicated moderate genetic divergence across reference populations of *R. australiae* and *G. typus* in this study. Based on this value, a cautious conservation strategy in optimizing fisheries management will be required to limit anthropogenic impacts.

Keywords: COI, genetic diversity, guitarfishes, wedgefishes

INTRODUCTION

Indonesia has been the world's largest producer of sharks and rays since 1998, with an average annual production of 106,034 tons/year, or around 13% of the total production of sharks and rays in the world (Dent and Clarke 2015). According to data from the Directorate General of Fisheries (2016), shark and rays' national production is dominated by rays which reach 60%. Indonesia is one of the world's leading exporters of elasmobranch fins and meats (Dent and Clarke 2015). As a result, rays have become a valuable resource for the national income. Many ray species are vulnerable to fishing pressure due to their relatively low fecundity and slow growth and gonad maturation, such as wedgefishes and guitarfishes. For example, from 1-17 eggs, only 2-4 individuals can survive (Jabado 2019; Jordaan et al. 2021). According to Kyne et al. (2020), these species are currently on the IUCN Red List of Threatened Species. Rays' fisheries resources are now in jeopardy as a consequence.

Wedgefish and guitarfish fishing locations are extensively spread across Indonesia. According to the Sorong Coastal and Marine Resource Management (2018), shark and ray trade in Papua is increasing yearly. The enormous quantities in the market were captured by target and bycatch in trawls, gillnets, and longlines (Alghozali et al. 2019). The Department of Fisheries and Marine Affairs of the Province of West Papua reported in early 2009 that the number of wedgefishes and guitarfishes captured in West Papua weighed 207.50 tons and eventually rose to 270.74 tonnes by 2018 (Bureau of Statistics West Papua Province 2018). Similar events occurred in Bali and Lombok. According to the Indonesian Ministry of Maritime Affairs and Fisheries, the entire output of rays in Bali in 2010 was just 36 tons. However, by 2020, the number of captures had increased to 316 tons (Indonesia Ministry of Marine and Fisheries 2021). Lombok faced a similar problem. The catch in 2010 was 1037 tons, rising to 1418 tons in 2020 (Indonesia Ministry of Marine and Fisheries 2021). In some places, overfishing has directly influenced the decline of population (D'Alberto et al. 2022; Dulvy et al. 2014; Spaet et al. 2015). Domingues et al. (2018) mentioned a link between population number and genetic diversity (mtDNA). Population loss may lead to decreased genetic variety and, consequently, a population's ability to adapt to the environment or disease (Cardeñosa et al. 2014).

Management of wedgefishes and guitarfishes fishing relied on studies of biology, life history, genetics, and population ecology (Domingues et al. 2018). However, the protection still needs to be hampered by the limited scientific data and challenges to balancing conservation efforts (Dulvy et al. 2017; Md-Zain et al. 2018). Wedgefishes and guitarfishes have been challenging research subjects in Papua and other regions of the world. Research on wedgefishes and guitarfishes in Indonesia has covered many aspects of their biology and ecology, but not their genetics. The progress of conservation actions requires information about biodiversity and its conservation status (Turner 2014). Understanding and evaluating the potential for changes in their biodiversity is crucial for ensuring its long-term viability (Hearn et al. 2014). Effective management may be determined by identifying genetic variation among populations, which can be done by employing molecular genetic techniques (Hays et al. 2014). mtDNA, particularly COI is an effective method of determining genetic variation (Zheng et al. 2019). The fast mtDNA evolution rate also helps to study hybrid zones, gene flow, population structure, and other population-related topics (Troast et al. 2016). Despite their economic value, the genetic diversity of wedgefishes and guitarfishes could have been better understood. Previously genetic studies on the population structure of wedgefishes had been conducted in Bangka Belitung. However, an investigation into genetic information from the eastern region of Indonesia has yet to be done or published (Putri et al. 2022). Several studies have also used the COI gene to analyze the genetic diversity of other marine species (Ibáñez et al. 2011; Tapilatu et al. 2022; Toha et al. 2020). Therefore, this research uses COI to assess the genetic diversity of wedgefishes and guitarfishes in the Eastern and Middle parts of Indonesia's landing sites.

MATERIALS AND METHODS

Study area

The sampling areas are located in Sanggeng Fish Auction Place at Manokwari, Hamadi Fish Landing Port at Jayapura, Namatota Landing Port at Kaimana, Tanjung Luar Landing Port at Lombok, and Kedonganan Landing Port at Bali (Figure 1). Specimens are primarily collected from fleets using gillnet and longline. Tissues extracted from each specimen by slicing them into tiny pieces (Villate-Moreno et al. 2021) were then preserved with 96% Merck ethanol. Samples were subsequently transported in a coolbox to the laboratory process.

Molecular examination

Each sample's tissue was dissected using razor blades, scissors, and tweezers autoclaved for DNA extraction (Choo et al. 2021). The sample is placed into a 0.6 ml tube containing 10% Bio-Rad BT Chelex at a ratio of 1: 10 (Walsh et al. 2013). The sample was then vortexed for 10 - 15 seconds until homogenous before being heated at a temperature of 95°C for 45 minutes to break down the specimen's cell wall.

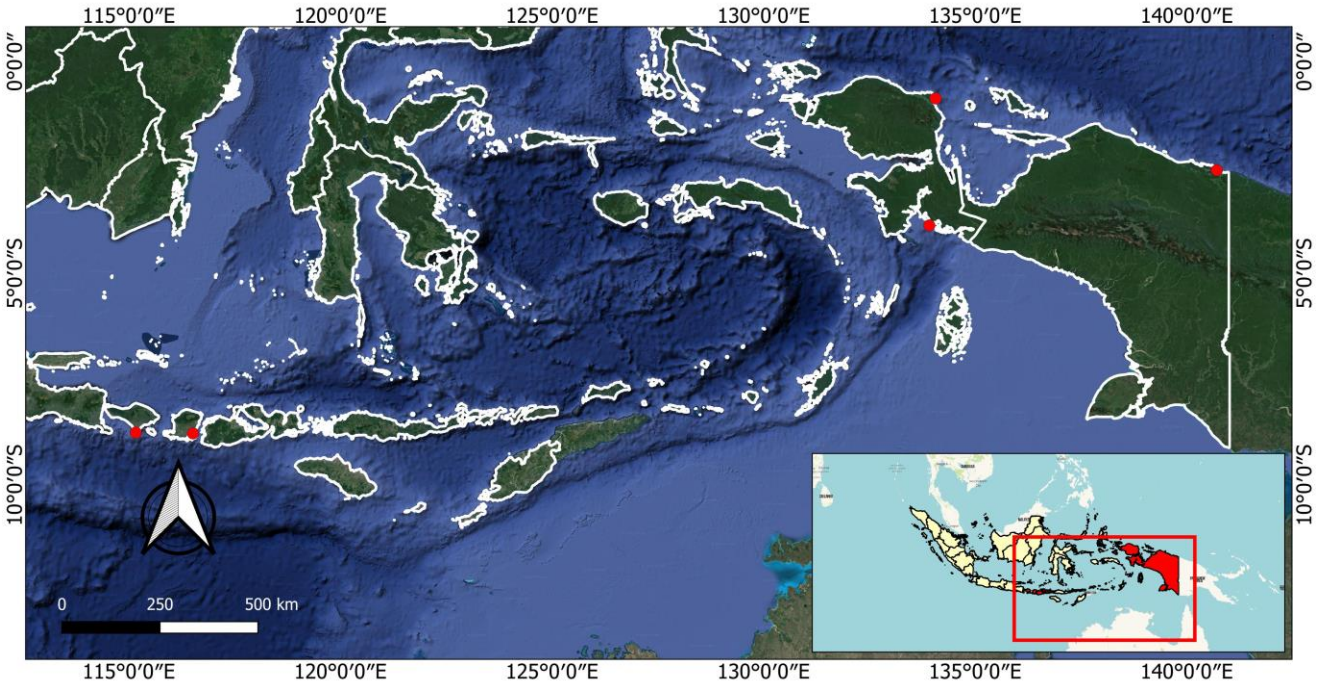

Figure 1. Sampling location in Bali, West Nusa Tenggara, West Papua and Papua provinces, Indonesia

In this study, A part of a fragment of COI locus was amplified using Polymerase Chain Reaction (PCR) methods, using forward primer Fish F1 (5'-TCAACCAA CCACAAAGACATTGGCAC-3') and reverse Fish R1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Bernardo et al. 2020; Bineesh et al. 2014). Qiagen HotStarTaq Plus DNA Polymerase was used as the PCR Master Mix kit, with a 25 L reaction volume containing: 1-2 ng DNA µL-1 and 1.0 µM primer (Abdullah et al. 2020). The thermal cycle profile is 94℃ for 180 seconds, 38 cycles of 94℃ for 30 seconds, 53℃ for 30 seconds, 72℃ for 60 seconds, with a final extension of 72℃ for 120 seconds, and 24℃ for 120 seconds. Each reaction was carried out in a total volume of 25 µL containing 10.0 µL of ddH_20, 1.25 µL of each primer, 12.5 µL of MyTaq Red Mix, and 1 µL of template DNA (Alghozali et al. 2019). Amplification success was determined using 1% agarose gel with Gel Red Biotium. Negative controls without DNA samples were evaluated at each PCR cycle to detect contamination issues. The PCR product was sequenced using forward and reverse primer with Big Dye Chain Termination protocol.

Data analysis

We investigated the sequences of COI mitochondrial DNA genes using MEGA X software (Kumar et al. 2018) and aligned using MUSCLE (Edgar 2004). The highest identity percentage value in the DNA database sequence given in the NCBI BLAST may be used to identify (Basic Local Alignment Search Tool). Using percent identity value and query cover (%), sequences were searched against GenBank (https://www.ncbi.nlm.nih.gov). The best match sequence identity is deemed as an identification.

Reconstruction of the phylogenetic tree studied using the Neighbor-Joining with Kimura-2-parameter model. Kimura's approach for estimating associations between individuals based on genetic distances (Laopichienpong et al. 2016) and utilizing bootstrap 1000 repeats to evaluate the degree of confidence at each node (Bernardo et al. 2020). This approach is suitable for evaluating particular identities based on sequence similarity at a single locus.

All the available COI sequences for *Rhynchobatus australiae* (Whitley, 1939) were obtained from GenBank and were derived from samples collected in Bangka Belitung (Putri et al. 2022). *Rhinobatos jimbaranesis* (Last, White & Fahmi, 2006) compared to species from Lombok. However, no COI sequence reference was discovered for *G. typus* in Indonesia. MEGA X software was used to align them with the samples. DNaSP 5.0 (Librado and Rozas 2009) was used to count the polymorphic sites, genetic diversity, nucleotide diversity, and haplotypes for all species. The haplotype network for COI gene haplotypes was generated with PopART version 1.7 (Leigh and Bryant 2015). Haplotypes were added to the network to identify phylogenetic links between distinct haplotypes and to indicate the frequency of each haplotype in each population. ARLEQUIN 3.5 (Excoffier and Lischer 2010) was used to determine whether there was any population structure at COI in the sampled populations. Analysis of molecular variance (AMOVA) was analyzed using reference groups of COI sequences from various geographic areas. Population differentiation was estimated based on the significance of pairwise genetic differentiation (F_{ST}).

RESULTS AND DISCUSSION

There were a total of 26 samples collected from 5 separate commercial catch landing sites. Species are caught using gillnet boats and long lines. COI mtDNA was successfully amplified with base lengths ranging from 522 to 684 bp. Samples identified as *Rhynchobatus australiae* (Whitley, 1939), *Glaucostegus typus* (Bennett, 1830), *Rhinobatos jimbaranensis* (Last, White & Fahmi, 2006), and *Sphyrna lewini* (Griffith & Smith, 1834). Figure 2 shows a picture of *R. australiae* (Figure 2.B), *G. typus* (Figure 2.A), and *R. jimbaranensis* (Figure 2.C).

A **B** **C**

Figure 2. Photos of: A. *Glaucostegus typus*; B. *Rhynchobatus australiae*; C. *Rhinobatos jimbaranensis* collected at the landing site

Phylogenetic relationship

The phylogenetic tree illustrates the evolutionary connection among samples. Figure 3 shows the results of the Neighbor-Joining (NJ) analysis of the phylogenetic tree. The NJ tree topologies clearly distinguish between the outgroups (*Neotrygon kuhlii* (Müller & Henle, 1841), *Oxynotus paradoxus* Frade, 1929, and *Chiloscyllium griseum* Müller & Henle, 1838), and the ingroup, sharks, and rays.

Genetic diversity between populations

The highest haplotype diversity (Hd) of *R. australiae*, as shown in Table 1, was 1.00 for Manokwari, while the lowest haplotype diversity could be found in Denpasar at 0.67. Table 1 also showed that the guitarfish' genetic diversity (nucleotide) value in Kaimana was relatively high. The lowest haplotype diversity among all the species in the populations showed by *R. jimbaranensis* at Bali.

The results of the haplotype analysis are shown in Table 2. Table 2 shows that out of 4 populations consisting of 34 wedgefishes in Indonesia, there are 11 haplotypes. Haplotypes 1-3 were found to include the specimens studied and also references. Haplotype 1 can be found in 14 individuals from 3 different population groups.

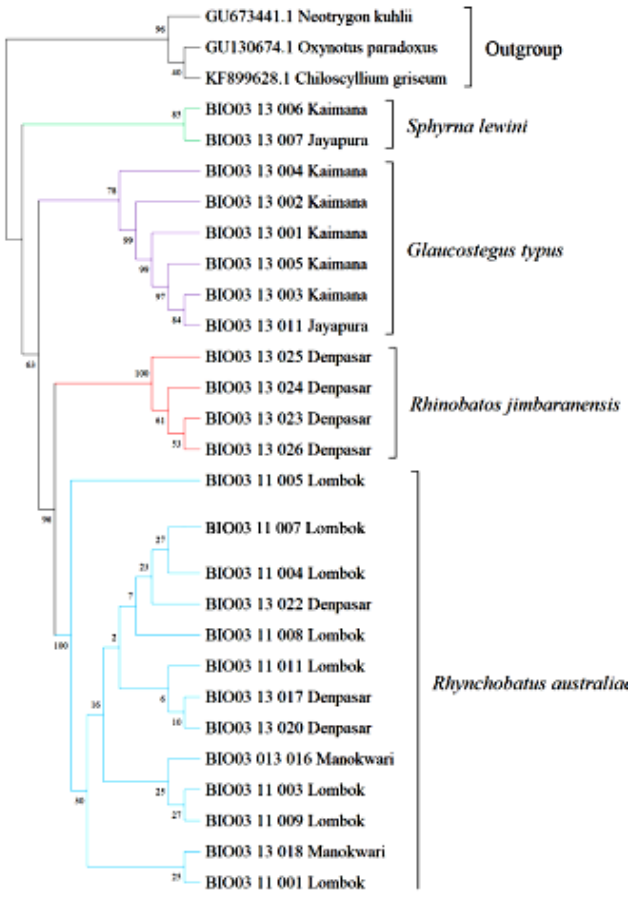

Figure 3. Neighbor-Joining phylogenetic tree

Table 1. Comparison of genetic distances between sample and reference populations

Source	N	Hn	Hd	π
Rhynchobatus australiae				
Denpasar	3	2	0.67	0.00115
Lombok	8	4	0.75	0.00191
Manokwari	2	2	1.00	0.00173
Bangka Belitung (reference)	21	7	0.73	0.00176
Glaucostegus typus				
Kaimana	5	4	0.90	0.04278
Jayapura	1	1	0.00	0.00
Rhinobatos jimbaranensis				
Bali	4	2	0.50	0.00101
Lombok (reference)	2	1	0.00	0.00

Table 2. Haplotype sites

Haplotype	Sample			
Rhynchobatus australiae				
	Bangka Belitung (21)	Bali (3)	Lombok (8)	Manokwari (2)
1	2	-	1	1
2	15	-	4	1
3	-	-	1	-
4	2	2	2	-
5	-	1	-	-
6	1	-	-	-
7	1	-	-	-
Glaucostegus typus				
	Kaimana (5)		Jayapura (1)	
1	3		1	
2	1		-	
3	1		-	
Rhinobatos jimbaranensis				
	Bali (4)		Lombok (2)	
1	3		2	
2	1		-	

Table 2 shows the haplotype sites of the *R. jimbaranensis* sample, as illustrated in Figure 4. From Table 2, it can be seen that *R. jimbaranensis* has two haplotypes, with one specific haplotype consisting of only one individual (Figure 4). Haplotype 1 is the haplotype that has the highest number of individuals, namely five individuals.

Based on Figure 4, there are several haplotype groups of *R. australiae* indicated by haplotype analysis. There are some haplotypes consisting of three populations. Several haplotypes consist of one population, such as Bangka Belitung, Lombok, and Denpasar. These results indicate a different grouping between populations indicated by the absence of haplotype mixing between populations and the possibility of a specific haplotype.

Molecular Variance Analysis (AMOVA) showed in Table 3 indicates whether either population has any significant differences in the population structure (p-value < 0.05).

Table 3. Analysis of molecular variance (AMOVA) for all populations based on mitochondrial COI

Source of variation	d.f.	Sum of Squares	Variance components	Percentage of variation	FST	P-Value
Glaucostegus typus						
Between populations	1	9.933	-24.85 Va	-93.77358	-0.9377	1.0000
Within populations	4	205.400	51.35 Vb	193.77358		
Total	5	170.833	26.50000			
Rhinobatos jimbaranensis						
Between populations	1	11.750	-5.50781 Va	-26.32	-0.2632	1.0000
Within populations	4	105.750	26.43750 Vb	126.32		
Total	5	117.500	6.38281			
Rhynchobatus australiae						
Between populations	3	57.204	1.37701 Va	11.64	0.11638	0.1036
Within populations	30	313.649	10.45496 Vb	88.36		
Total	33	370.853	11.83197			

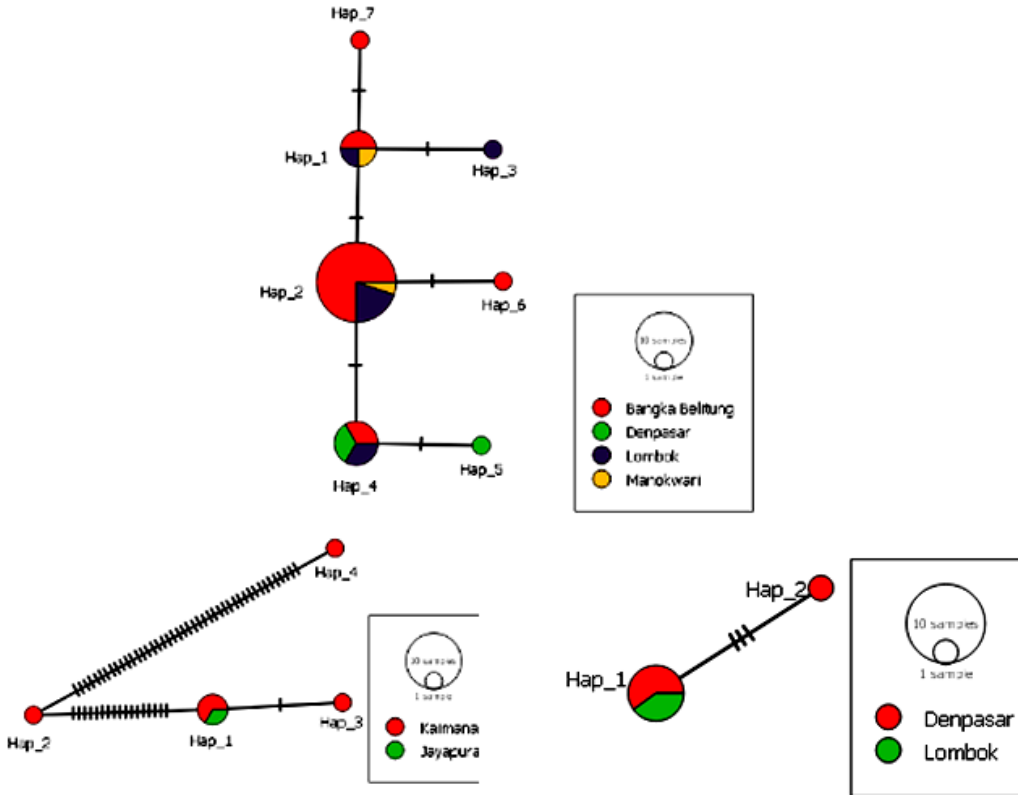

Figure 4. Minimum spanning haplotype network of (a) *Rhynchobatus autraliae*, (b) *Glaucostegus typus*, (c) *Rhinobatos jimbaranensis*

Genetic diversity between species

The highest haplotype diversity (Hd) was observed in *R. australiae*. A similar result was observed for overall genetic diversity in wedgefishes (Table 4). Meanwhile, the lowest haplotype diversity among all of the species in the populations showed by *R. jimbaranensis.*

The results of the haplotype analysis are shown in Table 5. Table 5 shows that out of 3 species, there are eight haplotypes. Haplotypes 1-3 were found to include *G. typus*. Haplotypes 4-6 were included in *R. australiae*. Last, Haplotype 7-8 identified as *R.jimbaranensis.*

Molecular Variance Analysis (AMOVA) showed in Table 6 indicates either population has any significant differences in the population structure.

Table 4. Comparison of genetic distances between species

Species	N	Hn	Hd	π
Rhynchobatus australiae	13	3	0.71	0.00239
Glaucostegus typus	6	3	0.60	0.04036
Rhinobatos jimbaranensis	4	2	0.50	0.00133

Table 5. Analysis of molecular variance (AMOVA) for all species

Source of variation	d.f.	Sum of Squares	Variance components	Percentage of variation	F_{ST}	P-Value
Between populations	2	950.368	68.50816 Va	80.61	0.80612	0.0000
Within populations	20	329.545	16.47724 Vb	19.39		
Total	22	1279.913	84.98541			

Table 6. Haplotype sites between species

Haplotype	Species		
	***Glaucostegus typus* (6)**	***Rhynchobatus australiae* (13)**	***Rhinobatos jimbaranensis* (4)**
1	4	-	-
2	1	-	-
3	1	-	-
4	-	5	-
5	-	5	-
6	-	3	-
7	-	-	3
8	-	-	1

Discussion

Differences in PCR primers and PCR amplicon concentrations can cause differences in variations in the length of gene fragments. Three main clades were detected in the NJ tree: *R. australiae*, *R. jimbaranensis*, and *G. typus*. All of the species form monophyletic clades within each clade.

The high value of genetic diversity found in the *G. typus* population indicates a high population size in Papua. The high value of genetic diversity is also believed to be related to population size (Cardeñosa et al. 2014). This is related to the populations of *R. jimbaranensis* found in Bali. The low value of genetic diversity in this population is assumed to be related to overexploitation and catching sharks using large nets, thus impacting the decline of diversity value. Chiu et al. (2013) stated that population size, reproduction, migration/distribution, gene mutations, and natural selection could affect these. Manokwari has the highest genetic diversity of *R. australiae* than other populations. According to data on the production of sting rays each year, Papua has far less catch than other regions (Bureau of Statistics West Papua Province 2018), which explains the high diversity value in this region. Overexploitation can affect the value of diversity, affecting the population's ability to adapt and survive in their environment (Kardos et al. 2021).

The node sizes of *R.australiae* were larger in Haplotype 1, Haplotype 2, and Haplotype 4 than the others shown in Figure 4A, indicating the number of COI sequence haplotype frequencies. Long lines connecting Lombok and Bangka Belitung haplotypes refer to linkage distances representing one base pair difference per dot. It means more distant nodes are more distant haplotypes (more base pair difference). More considerable distances between populations could increase genetic heterogeneity (Saleky et al. 2016). Respectively, there are three populations closely related in Haplotype 2 (Bangka Belitung, Lombok, and Manokwari). This pattern usually showed an aged population with one or several ancestral haplotypes and many rare descendants separated from it by mutation. Based on the Analysis of Molecular Variance (AMOVA) showed significant differences in the population structure of *G. typus* in the two populations means each population comes from the same population. A little genetic distance value indicated the close diversity of these two populations. Ocean currents and suitable habitat conditions can trigger the genetic similarity of species. Currents in the Papua region are strongly influenced by the Papua New Guinea Coastal Current (NGCC), which connects the subtropical water masses with the equator (and is an extension of the South (SEC). This means that westward currents carry cold water and genetic material from along the north coast of Papua.

The number of haplotypes found in the *R. jimbaranensis* population in Bali and Lombok was two COI haplotypes. Meanwhile, *R. jimbaranensis* found in Bali has a value of genetic diversity (nucleotides) 0.50. Differences in the value of this genetic diversity can occur because this area is very suitable for the life of the rays populations. After all, it is rich in food sources. These differences can result in phylogenetic, anatomical, and morphological differences in a population (Näslund and Johnsson 2016). Bali waters become the Indonesian Through-Flow (ITF) route, where the mass flow of water is carried from the Pacific Ocean to the Indian Ocean. This causes the area of Bali to be rich in nutrients (Wijaya et al. 2020). The low genetic diversity value, which reached a value of 0.00 in the Lombok region, could occur due to the significant demand for sharks and rays. This vast demand may lead to a decrease in the population size of Lombok.

The haplotype diversity (Hd) value of the wedgefishes population landed in Manokwari was 1.00, complemented by nucleotide diversity (π) of 0.00173. The genetic diversity of wedgefishes in Denpasar is 0.67 and 0.00115 nucleotides. Genetic diversity in the Lombok population showed a value of haplotype diversity 0.75 and nucleotide diversity of 0.00191 for nucleotide diversity. This value indicates the presence of genetic diversity within the population and between populations. The high value of haplotype diversity indicates the potential that the number of arrests in the area is still in normal condition so that the condition of the population has not been much disturbed. This genetic diversity can occur due to physical or geographical separation from other populations in different places (Manel et al. 2020).

Close genetic distance was also found in Lombok and Bangka Belitung populations at 0.09399. This is also

similar to the genetic distance value between the Lombok and Bali populations of 0.03067. The low genetic distance value between the Lombok and Bangka Belitung population groups indicates the closeness of the *R. australiae* population groups. It is suspected that this could occur due to fishing activities that can be carried out outside the Lombok area or the delivery of *R. australiae* from Bangka Belitung to TPI Tanjung Luar.

A global review of wedgefishes and guitarfishes fisheries indicates that it is necessary to implement strategic management plans for stocks and address overfishing as one of the critical reasons for the decline and extinction of ray and shark populations. The Ministry of Maritime Affairs and Fisheries has tried to control the rate of the catch of wedgefishes and guitarfishes through Ministry Regulation Number 12 of 2022. This has been done by setting several national catch quotas each region may catch. The Papua region gets a quota of 5000 individuals, West Papua 1000 individuals, West Nusa Tenggara 200 individuals, and Bali 1500 individuals for the *R. australiae*. The quota for *G. typus* obtained by Papua was 4800 individuals, West Papua was 1900 individuals, and Bali was 4950 individuals. The Ministry of Maritime Affairs and Fisheries recommends catching wedgefishes and guitarfishes if it is 1.7 meters long and *G. typus* is 1.8 meters long. Restrictions on arrest quotas have been carried out, but these have not been accompanied by supervision from the authorities (Imanuel et al. 2018). Based on the results of a questionnaire conducted by Imanuel et al. (2018) showed that 86% of fishermen stated that there had been no monitoring activities for catching sharks and rays.

Rays have an essential ecological role in marine ecosystems that maintain functional marine ecosystems (Booth et al. 2018). The results of this study indicate that the genetic diversity of wedgefishes and guitarfishes populations is moderate to very high. Bonde (2012) states that populations with high genetic diversity values have the opportunity to adapt and avoid the risk of extinction. Therefore, populations with high genetic diversity still need to be preserved. This population is essential as a genetic resource supplying genetic flow to other populations. This value can also be used for culture management in selecting quality broodstock for cultivation. Meanwhile, the low value of genetic diversity can be caused by anthropogenic pressures, especially over-exploitation, and poaching. Overexploitation can lead to a decrease in population size, reducing the degree of genetic variability.

Strategies in management and conservation are needed for the management and conservation of wedgefishes and guitarfishes. Therefore a strategy is needed in management and conservation based on local wisdom. Fishermen could run and restore by Sasi, which played a role in protecting its habitat and ecosystem. In addition to controlling and monitoring activities, there is a need for strategic advocacy to fishermen or business actors by taking into account substantial socio-economic problems, specifically for shark and ray fishermen who use this biota as their main catch. Efforts that can be made include limiting the types and sizes of sharks and rays that may be caught, setting the size of the mesh or line, limiting the number of fishing gear and fishing vessels, and limiting fishing efforts.

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