Mitochondrial COI Haplotype Diversity of Rhynchobatus australiae Collected from Ketapang Fish Port, Bangka Belitung Islands

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Mitochondrial COI Haplotype Diversity of *Rhynchobatus australiae* Collected from Ketapang Fish Port, Bangka Belitung Islands

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Abstract

Rhynchobatus australiae is a member of the Rhinidae family and can be found in the Indo Pacific. This species is categorized as Critically Endangered according to The International Union for Conservation of Nature and Natural Resources (IUCN) Red List and listed as Appendix II Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), due to population declines. Sustainable fisheries management is urgently needed. Information related to genetic diversity is one of the most important aspects of information for appropriate sustainable fisheries management. Therefore, this research aims to investigate the genetic diversity of Rhynchobatus australiae collected from Ketapang Fish Port Bangka Belitung Islands. Total 21 samples were collected at Ketapang Fish Port. DNA extraction was carried out using the 10% chelex method and amplification was done through PCR method on the mitochondrial DNA using Fish BCL and Fish BCH primer. Sequences in size of 653 base pairs were successfully obtained from all fish samples showing the existence of 2 clades from the construction of the phylogeny tree with close genetic distance. Results showed high haplotype diversity (Hd: 0.733 ± 0.082) and low nucleotide diversity (π: 0.00176± 0.00036) with 6 polymorphic sites (S) from 7 unique haplotypes (h). This research provides basic information of genetic diversity of Rhynchobatus australiae collected from Ketapang Fish Port and complements other information to better understand the status of the threatened Rhynchobatus australiae population.

Keywords: *Genetic Diversity, Wedgefish, Rhynchobatus australiae, Bangka Belitung Islands*

Introduction

Sharks and rays are vulnerable to extinction due to their slow growth and sex maturity level. Despite the long cycle of reproduction exhibited by sharks and rays, only a few offspring are successfully born (Stevens *et al*., 2000). Fahmi and Darmadi (2005), stated that from the beginning of 1988 the demand for shark and rays' fin has grown and it leads to a significant increase in sharks and rays' fins trading activities. Three Chondrichtyans in the Rhinidae Family are considered the most vulnerable, this includes the Rhynchobatus (Dulvy *et al*., 2014).

Rhynchobatus australiae is endemic to the Indo-West Pacific and can be found in Southeast Asia and Australia including Thailand, Taiwan and Indonesia to the Australian sub-tropics (Last *et al*., 2013; Giles *et al*., 2016). *R. australiae* population-level in certain areas are declining significantly for almost 80% (Jabado *et al*., 2019). This species is commonly caught as a target catch that are sought primarily or else they are caught unintentionally in fishing gears such as gillnets, trawl and longlines (Kurniawan *et al*., 2017). The largest fishing ground for *R. australiae* are located in WPP 711, 712 and 713, among them are The Java Sea, Flores Sea, The Natuna Sea, Makassar Strait, Malacca Strait and Karimata Strait (Yuwandana *et al.*, 2020). *Rhynchobatus* spp. more mobile than the predominantly sedentary batoid species but spends part of their time motionless at the bottom and thus has a medium-sized activity space (White *et al.*, 2014).

Ketapang Fish Port is a fish landing site under the region of WPP 711. The condition of natural fisheries resources is at an alarming state, it is shown by the result of density measurement toward rays that

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were caught in the WPP 711 in November 2017. From the measurement, only 68 kg.km^{-2} of average density of rays caught by trawl was collected. The occurrence was caused by high fishing activities in the area (Yusuf *et al*., 2018).

Genetic diversity of species in a certain population is advantageous because it enables some individuals to adapt and survive from many conditions of the environment while maintaining the survival of the population (Booy *et al*., 2000). Genetic diversity helps the population maintain its health as it allows every gen a specific yet varies response to environmental change (Yusron, 2005). However, biological data and information availaible on the genetic diversity of *R. australiae* is still limited. This research aims to study the genetic diversity of *Rhynchobatus australiae* collected from Ketapang Fish Port.

Materials and Methods

Study site and sample collection

Total of 21 samples of *R. australiae* were collected from Ketapang Fish Port, Bangka Belitung Islands, Indonesia (Figure 1.). The specimens obtained came from the waters around Pulau Tiga, Pulau Tujuh to the border of Riau Archipelago. The samples were dissected and preserved using 96% ethanol. Sample of each individual identified based on the description by Giles (2016).

DNA extraction, PCR amplification and electrophoresis

The DNA isolation was conducted using a modified Chelex 100 method. Sample extraction was carried out by placing a 0.5 cm tissue sample comprising 500 µL of Chelex resin (10%) mixed with 7 µL proteinase K (10 mg.mL^{-1}) in an *eppendorf* tube. The mixtures were then incubated using a heat block at 55°C for 90 min to degrade protein. To inactivate proteinase K, incubation continued at 100°C for 20 min (Walsh *et al*., 1991; Galal-Khallaf *et al*., 2014).

The extracted DNA was used as a template for amplification at the cytochrome oxidase I (COI) locus in mitochondrial DNA (mtDNA). The amplification of the DNA product was performed using the Polymerase Chain Reaction technique with the forward primer fish-BCL (5'–TCA ACY AAT CAY AAA GAT ATY GGC AC–3') and the reverse primer fish-BCH (5'–ACT TCY GGG TGR CCR AAR AAT CA–3') (Baldwin *et al*., 2009). PCR was carried out in a 26 µL reaction mixture containing 12.5 µL of MyTaq Red Mix, 1.25 µL 10 mM of forward primer, 1.25 µL 10 mM of reverse primer, 1 µL DNA template and 10 µL ddH2O (Alghozali *et al*., 2019).

PCR cycling was adjusted according to the standard protocol of MyTaq Red Mix under the following conditions: initial denaturation for 1 min at 95°C, 34 cycles of denaturation for 15 s at 95°C, annealing for 15 s at 50°C, elongation for 10 s at 72°C, and final elongation for 5 min at 72°C. The amplicons were loaded into 1% agarose gel with

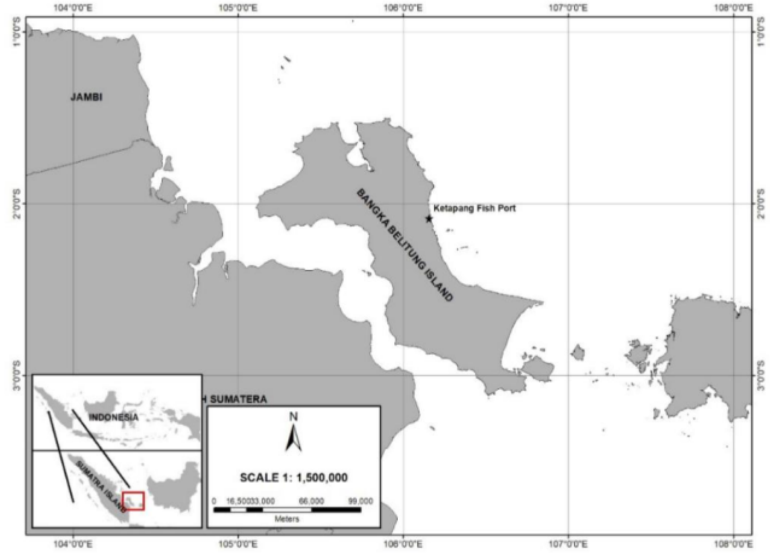

Figure 1. Maps of Ketapang Fish Port, Bangka Belitung Islands, Indonesia

Florosafe DNA Stain, electrophoresed (100 V for 30 min) for band visualization. Amplicons were sent for purification and sequencing to Genetika Science Indonesia.

Data analysis

The mtDNA COI gene sequences from 21 individuals of *R. australiae* were edited using BioEdit software (Hall, 1999). Samples identification were done by using DNA barcoding. Sequences of samples were compared to the highest identity percentage value on the DNA database sequence contained in the BLAST (Basic Local Alignment Search Tool) NCBI (National Center for Biotechnology Information). Each sample was then submitted to Genbank under the accession number of MW509711-30. Phylogenetic tree reconstruction was analyzed using several sequences from GenBank as their ingroup and outgroup. The method used was Neighbor-Joining with the Kimura-2-Parameter model (Saitou and Nei, 1987) and using bootstrap of 1000 replications to test the confidence level at each node (Felsenstein, 1985). Pairwise distance tools with the Kimura-2-Parameter model were used to determine the genetic distance. Polymorphic sites, genetic diversity and nucleotide diversity were calculated and unique haplotypes were quantified using DNASP version 4.0 (Librado and Rozas, 2009).

Results and Discussion

Phylogenetic analysis

A segment length of sequenced cytochrome C oxidase I (COI) were visualized with a UV transilluminator and showed DNA bands of 600-700 base pairs (bp). This is consistent with the variation in the target length of the COI gene (Cytochrome Oxidase subunit I), which is between 675 - 820 bp (Lv *et al.*, 2014). The percentage identity of all sequences was identified as *R. australiae* with values of 99.40-100% (Table 1.).

The phylogenetic tree resulted in two lineages; clade 1 which consisted most of the collected samples except for two samples (*R. australiae* 17 and *R. australiae* 18) that clustered into clade 2. The pairwises genetic distance were ranging from 0.000 – 0.007 and the furthest was observed between *R. australiae* 17 and *R. australiae* 18.

R. australiae from clade 1 were observed to be closely related to those from Australia, India, Malaysia, Thailand and Papua New Guinea. This results indicated this subtype is widely distributed across the eastern part of the Indo-Pacific region. The low genetic distance between samples originating from WPP 711 and from other regions could be due to population migration in habitat use which affect the phylogeography of this species (Fetzner Jr and Crandall 2001; Ferreira *et al.*, 2017).

In addition, the samples in clade 2 are likely to be firstly recorded in the current study. However, whether these variations are endemic to this specific locality, requires further study with more sample numbers from various localities.

Despite the limited studies on seasonal migratory patterns of *R. australiae* across the wider range of ocean regions, the previous studies revealed its possible pattern between Indonesia and Australia which applied an episodical patterns (Giles *et al.,* 2016). Moreover, Jabado *et al.* (2018) added that the seasonal migratory of Batoids species around the Arabian Sea were related to the life-history stages and events including the birth that occurred around the coastal or shallower waters area. The study by White *et al.* (2013) in northern Australia, examined the residence and spatial ecology of *R. australiae,* provide evidence of certain individuals to leave the area for periods varying from days to weeks.

Genetic diversity

Table 3 and 4 showed that among 21 samples, total 7 haplotypes were discovered with 6 polymorphic sites from 653 sites of COI. The haplotype diversity (Hd) were at moderate level which consistent with the Hd of *R. australiae* two populations in Indonesia, Jakarta and Kalimantan based on control region (Giles *et al*., 2016). Meanwhile, unlike the Hd value, the nucleotide diversity (π) were observed to be relatively low (Table 2) which partially represent a low polymorphism level (Qin *et al*., 2021).

According to the Hd, a relatively high genetic diversity could be observed in this population. Previous study reported that a species with high migration rates like hammerhead shark (*Sphyrna lewini)* tend to have a large population size with high nucleotide substitution and high gene flow which eventually lead to high genetic diversity (Hadi *et al.,* 2020). Thus, it could be assumed that the high genetic diversity of *R. australiae* in Bangka-Belitung Islands was resulted by migratory event.

Moreover, Camacho *et al.* (2017) and Avise (2000) mentioned, the high diversity of haplotypes and the low diversity of nucleotides usually corresponds to a relatively bottleneck event followed by rapid demographic expansion from a small effective population size, assuming this population had sufficient time to form high diversity of haplotypes but insufficient to increase the diversity

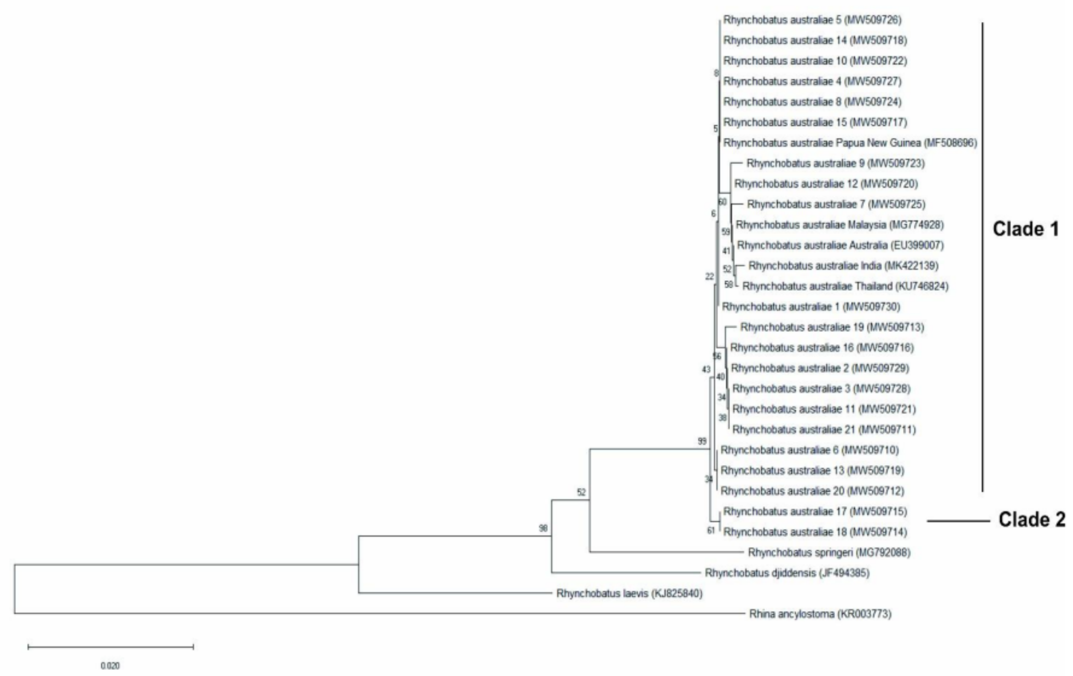

Figure 2 Phylogenetic tree of *Rhynchobatus australiae* collected from Ketapang Fish Port Bangka Belitung Islands constructed by Neighbor-Joining method with Kimura 2-parameter model and 1000 bootstrap replications.

Table 1. Results of nucleotide bases BLAST in GenBank

Sample Code	BLAST Results	Identify (%)	Accesion Code
Rhynchobatus australiae 1	*Rhynchobatus australiae*	99.85%	MW509730
Rhynchobatus australiae 2	*Rhynchobatus australiae*	99.70%	MW509729
Rhynchobatus australiae 3	*Rhynchobatus australiae*	99.70%	MW509728
Rhynchobatus australiae 4	*Rhynchobatus australiae*	99.70%	MW509727
Rhynchobatus australiae 5	*Rhynchobatus australiae*	99.70%	MW509726
Rhynchobatus australiae 6	*Rhynchobatus australiae*	99.55%	MW509710
Rhynchobatus australiae 7	*Rhynchobatus australiae*	99.85%	MW509725
Rhynchobatus australiae 8	*Rhynchobatus australiae*	99.70%	MW509724
Rhynchobatus australiae 9	*Rhynchobatus australiae*	99.85%	MW509723
Rhynchobatus australiae 10	*Rhynchobatus australiae*	99.70%	MW509722
Rhynchobatus australiae 11	*Rhynchobatus australiae*	99.55%	MW509721
Rhynchobatus australiae 12	*Rhynchobatus australiae*	100.00%	MW509720
Rhynchobatus australiae 13	*Rhynchobatus australiae*	99.55%	MW509719
Rhynchobatus australiae 14	*Rhynchobatus australiae*	99.70%	MW509718
Rhynchobatus australiae 15	*Rhynchobatus australiae*	99.85%	MW509717
Rhynchobatus australiae 16	*Rhynchobatus australiae*	99.70%	MW509716
Rhynchobatus australiae 17	*Rhynchobatus australiae*	99.41%	MW509715
Rhynchobatus australiae 18	*Rhynchobatus australiae*	99.40%	MW509714
Rhynchobatus australiae 19	*Rhynchobatus australiae*	99.55%	MW509713
Rhynchobatus australiae 20	*Rhynchobatus australiae*	99.55%	MW509712
Rhynchobatus australiae 21	*Rhynchobatus australiae*	99.55%	MW509711

Table 2. Genetic diversity of *Rhynchobatus australiae* collected from Ketapang Fish Port Bangka Belitung Islands based on number of samples (n), polymorphic sites (S), number of haplotype (h), haplotype diversity (Hd) and nucleotide diversity (π).

n	S	h	Hd ± SD	(π) ± SD
21	6	7	0,733 ± 0.082	0,00176 ± 0,00036

Table 3. Haplotype and Polymorphic Sites

	Polymorphic Sites					
	2	3	3	5	6	6
Haplotype	7	4	8	4	3	3
	0	8	4	4	3	9
Haplotype 1	C	G	A	T	A	A
Haplotype 2	.	.	.	.	G	.
Haplotype 3	A	.	G	.	.	.
Haplotype 4	.	.	G	.	.	G
Haplotype 5	.	.	G	.	.	.
Haplotype 6	.	.	.	C	.	.
Haplotype 7	.	A	.	.	G	.

Table 4. The sample code for each haplotype

Haplotype	Total Sample	Sample Code
Haplotype 1	10	*Rhynchobatus australiae* 1 (MW509730) *Rhynchobatus australiae* 4 (MW509727) *Rhynchobatus australiae* 5 (MW509726) *Rhynchobatus australiae* 6 (MW509710) *Rhynchobatus australiae* 8 (MW509724) *Rhynchobatus australiae* 10 (MW509722) *Rhynchobatus australiae* 13 (MW509719) *Rhynchobatus australiae* 14 (MW509718) *Rhynchobatus australiae* 15 (MW509717) *Rhynchoatus australiae* 20 (MW509712)
Haplotype 2	5	*Rhynchobatus australiae* 2 (MW509729) *Rhynchobatus australiae* 3 (MW509728) *Rhynchobatus australiae* 11 (MW509721) *Rhynchobatus australiae* 16 (MW509716) *Rhynchobatus australiae* 21 (MW509711))
Haplotype 3	1	*Rhynchobatus australiae* 7 (MW509725)
Haplotype 4	1	*Rhynchobatus australiae* 9 (MW509723)
Haplotype 5	1	*Rhynchobatus australiae* 12 (MW509720)
Haplotype 6	2	*Rhynchobatus australiae* 17 (MW509715) *Rhynchobatus australiae* 18 (MW509714)
Haplotype 7	1	*Rhynchobatus australiae* 19 (MW509713)

of nucleotides. Population bottleneck is an event that reduces the size of a population, limiting the genetic diversity of a species that can be caused by various events (Ali and Roossinck, 2008). In regards to confirm this hypothesis and for a deeper understanding of the *Rhynchobatus australiae* population further study is necessary.

The conservation status of *Rhynchobatus australiae* population according to the catch data is currently at the state of overexploitation and severe population depletion. This fish is included in 80% of the target gillnet fisheries in Indonesia. This high fishing pressure may lead to the decrease of effective population size and subsequently genetic variability lost due to genetic drift (Marty *et al.,* 2015; Jabado, 2019; Kyne *et al.,* 2020).

The genetic diversity surveyed in the present study may provide a baseline data for the following *Rhynchobatus australiae*. Further studies can be conducted using larger sample size and area to fully understand the genetic diversity of this species.

Conclusion

The genetic diversity of *Rhynchobatus australiae* collected from Ketapang Fish Port, Bangka Belitung Islands, showed high haplotypes diversity and low nucleotides diversity. Phylogeny tree construction and genetic distance values showed the close relationship between the 2 clades.

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