DNA Barcoding and CITES-Listed Wedgefish (Rhynchobatidae, Rhinidae) from South Bangka, Indonesia

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Abstract

Overfishing of wedgefish greatly affects its population and the balance of the marine ecosystem. This is exacerbated by their relatively low fecundity, slow growth, and late maturity results in one of the lowest population growth rate within elasmobranch species. However, lacking database information results in insufficient regulations and surveillance of wedgefish fishing. The current situation is feared to the risk of wedgefish's survival, especially in Bangka Belitung Islands, Indonesia. Fundamental to a database is the accurate identification of wedgefish species based on mitochondrial DNA (mtDNA) analysis. This study aimed to use DNA barcodes to identify, determine the conservation status, and the status according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). We collected samples including unidentified fin samples from confiscated illegal fishing catches, the traditional markets and fishing docks, South Bangka. In this research, we used DNA Barcoding (mitocondrial DNA, COI gene) to identify and examine of wedgefish samples. The tissue samples used in this study were identified as species listed in CITES Appendix II, they are Rhynchobatus australiae, Rhynchobatus springeri and Rhina ancylostoma. According to the IUCN Red List, 100% of the wedgefish species found are Critical Endangered at the global level.

Keywords: DNA Barcoding, Wedgefish, endangered species, South Bangka

INTRODUCTION

A major problem in estimating catch rates of sharks and rays is the potential for misidentification. Some shark and ray species are difficult to distinguish morphologically and there are ethnic differences in understanding or interpreting common names (Burgess et al., 2005). The Food and Agriculture Organization (FAO) report described the paucity of good fishery data for elasmobranchs (Castro et al., 1999). The valuable shark fins in South-east Asia has led to an increase in illegal, unreported, and unregulated (IUU) shark and ray fishing. Overfishing of wedgefish, a group of rays also known as Rhynchobatidae in the family Rhinidae, greatly affects its population and the balance of the marine ecosystem. Their relatively low fecundity, slow growth, long lifespan, and late maturity resulted as one of the lowest population growth rate among elasmobranch species, making them more vulnerable for fishing pressure (Dulvy et al., 2014).

Currently, shark and ray populations in Indonesia are going to decline, especially wedgefish species. These fishes are oftenly found in South Bangka in Bangka Belitung Islands, Indonesia. According to the Ministry of Marine Affairs and Fisheries, Indonesia was the world's largest producer of sharks and rays, contributing 12.31 % of the global total catch (Fahmi and Dharmadi, 2013). However, a lack of database information induces insufficient regulation and oversight of the management of wedgefish fishing. The current situation is be alarmed about the risk of wedgefish's survival

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survival of wedgefish, especially in Bangka Belitung Islands. Fundamental to a database is the accurate identification of wedgefish species. Samples from the South Bangka will contribute as a reference for determining the conservation and CITES statuses of wedgefish in the Bangka Belitung Island province.

Species were identified based on mitochondrial DNA (mtDNA) analyses. DNA barcoding is an established powerful tool for fish species identification (Ward et al., 2005; Ward et al., 2009), especially when the entire organism cannot be accessed for morphology. However, there are important limitations concerning its accuracy, which depend on the reference database available and the degree of genetic differentiation among species (Fernanda et al., 2018). This study aimed to use DNA barcodes to identify species of wedgefish from South Bangka, Bangka Belitung Islands, Indonesia, and to determine their conservation status and CITES-listing.

MATERIALS AND METHOD

Samples included unidentified fin samples from confiscated illegal fishing catches and were collected from the traditional markets and fishing docks, South Bangka. They were preserved in a solution of 90 % alcohol or frozen at -4 ℃ (depend on the size of samples).

We extracted genomic DNA from tissue samples around 0,03 to 0,05 g using ZR Tissue and Isect DNA MiniPrep (Zymo Research, D6016), following the manufacturer's protocol. DNA amplification was performed of the targeted locus Cytochrome Oxidase Subunit 1 (COI) gene with a Polymerase Chain Reaction (PCR) using the universal primers (Table 1). We used MyTaq Red Mix (Bioline) for amplification PCR with the following steps: 3 min denaturation at 96 ℃, followed by 35 cycles of denaturation at 94 ℃ for 10 s, 50 ℃ annealing for 30 s, and 72 ℃ extention for 45 s. We examined the PCR products on 1 % agarose gel electrophoresis. The PCR products were sent to sequencing service for DNA sequencing.

Amplicons were sequenced in both forward and reverse directions. The sequencing results of the COI gene nucleotide character were compilating using MEGA X software for trimming, reverse complement, and alignment processes (Tamura et al., 2013). The aligned results were then matched to the nucleotide database in GenBank on the National Center for Biotechnology Information (NCBI) website. Phylogenetic analysis is a taxonomic classification of organisms based on the sample's phylogeny and is an integral part of systematic science and has the aim of determining the phylogeny of organisms based on the sample's characteristics (Mouth, 2001). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Phylogenetic tree was tested with 1000 bootstrap (replicates) to obtain the bootstrap convidence level (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). The analysis allows us to identify close sequences occupying neighboring branches of the tree. When gene families are found in organisms or groups of organisms, phylogenetic relationships between them can predict the possibility of equivalent functions (Mcdonald and Kreitman, 1991; Nielsen and Yang, 1998).

RESULT AND DISCUSSION

We obtained COI barcode sequences for samples included unidentified dried fin samples with sequence lengths ranging around 700 bp. All samples were successfully

Table 1. Primer set in this study

Primer	Sequence	Reference
VF2_t1	5'TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC3'	Ward *et al*. (2005)
FishF2_t1	5' TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC3'	Ward *et al.* (2005)
FishR2_t1	5'CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA3'	Ward *et al.* (2005)
FR1d_t1	5'CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAATCARAA3'	Ivanova *et al.* (2007)

amplified, most barcodes produced clear matches in BLAST allowing for confident assignment of species with > 99 % similarity to database records. These three species had a match with NCBI BLAST sequences, they are *Rhynchobatus australiae* (Whitley, 1939), *Rhynchobatus springeri* (Compagno and Last, 2010), *Rhina ancylostoma* (Bloch and Schneider, 1801).

The tissue samples used in this study were identified as species listed in CITES Appendix II, they have been approved for listing on CITES Appendix II in the 18th CoP meeting in Geneva in August 2019. According to the IUCN Red List, 100% of the wedgefish species found are Critical Endangered at the global level (Kyne, 2019).

The phylogenetic study of wedgefish in this study was based on a ~700 bp sequence of the COI gene. Results from phylogenetic analysis revealed a monophyletic clade for *R. ancylostoma* supported by a 100% bootstrap value. *R. springeri* and *R. australiae* formed a distinct monophyletic clade from each other supported by a 100% and 98% bootstrap value. These findings, together with their closer genetic distance to each other, indicated that wedgefish shared the same common ancestor.

According to CITES Appendix II regulations, any trade of listed shark or ray species requires proof that the nation of origin has methods in place that ensure that the proposed trade is sustainable and not detrimental to any wild populations. Given that sharks and rays are typically characterized by low fecundity, late maturity, and a long gestation period, the finding that 71 % of all sampled fins and gill plates came from species of high-conservational concern suggests that this segment of the global fisheries is anything but sustainable and urgently requires an extensive conservation management response (Steinke *et al*., 2017).

The DNA analysis of our samples indicates *R. springeri*, *R. australiae* and *R. ancylostoma* species are being traded directly exported to other areas. The large amount of samples belonging to this species suggests that they are neither by-catch nor

Table 2. Similarity of species in GenBank

Sample Code	Species	% Query Cover	% Per Ident
MBS_1	*Rhynchobatus australiae*	93%	99.70%
MBS_2	*Rhynchobatus australiae*	94%	99.85%
MBS_3	*Rhynchobatus springeri*	99%	99.41%
MBS_4	*Rhina ancylostoma*	94%	100%

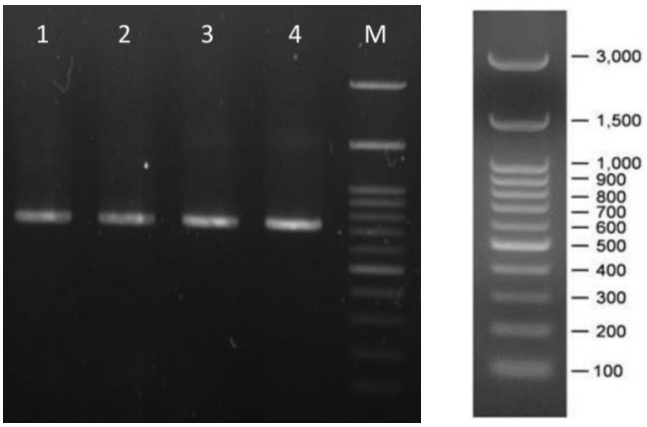

Figure 1. Electrophoresis gel patterns of PCR products shown at sequence lengths between ~700 bp (1= sample code MBS_1; 2= sample code MBS_2; 3 = sample code MBS_3; 4 = sample code MBS_4; M = Marker)

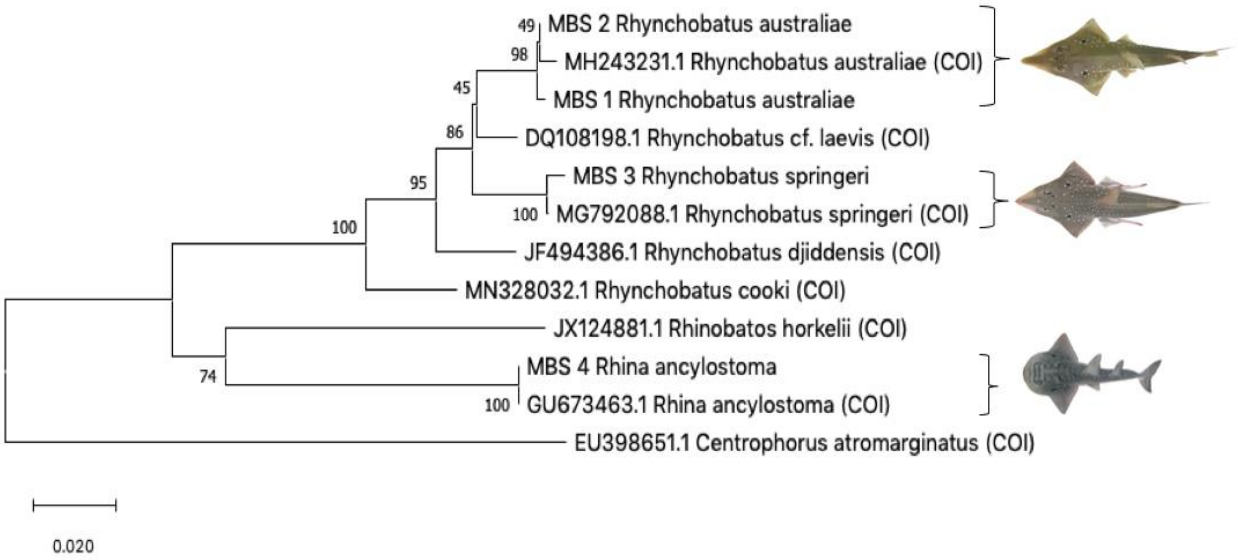

Figure 2. The phylogenetic tree of Cytochrome Oxidase Subunit 1 (COI) gene

imported from small-scale fisheries, but are instead harvested through large-scale fisheries, as has also been documented by other barcoding studies with a focus on shark and ray products (Dent & Clarke, 2015). Our study confirmed that DNA identification of samples using mitochondrial COI barcoding sequencing can act as a powerful market surveillance tool. While shark and ray conservation and management policies are primarily focused on the development of fisheries quotas and marine protected areas, this work demonstrated the importance of market surveillance as a conservation countermeasure that would benefit from large-scale long-term monitoring. The results also raise further concerns about the impacts of trade on the sustainability of these low reproductive species demanding swift and extensive conservation management responses.

CONCLUSION

DNA identification of meat samples and dried ray fins using mitochondrial COI linked them to *R. springeri*, *R. australiae* and *R. ancylostoma* species with a similarity of >99 % with the NCBI sequence. The wedgefish species have been approved for listing on CITES Appendix II in the 18th CoP meeting in Geneva in August 2019. They are also listed in the IUCN Red List categorized as Critically Endangered. Based on exploration in local market and ask to fishermen, the wedgefish species are being traded directly exported to other areas, we urge increased transparency of the trade in this species for monitoring the sustainability and extensive conservation management responses.

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