Exploited but Unevaluated: DNA Barcoding Reveals Skates and Stingrays (Chordata, Chondrichthyes) Species Landed in the Indonesian Fish Market

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Abstract

Reliable and precise species identification is important to fisheries management and conservation. However, many rays and skates in Indonesia are currently being exploited and landed into traditional fish market without a proper identification. Therefore, this study was conducted to identify species of skates and stingrays that were landed and traded in three fish markets in Indonesia (Palabuhanratu, Muara Saban, and Lampung) using molecular techniques and to determine the conservation status of the identified species based on IUCN (International Union for Conservation of Nature and Natural Resources) as well as defined by CITES (Convention on International Trade in Endangered Species). The mitochondrial cytochrome oxidase I (COI) gene was amplified by polymerase chain reaction (PCR) using a pair of primer, fish-BCL and fish-BCH. Of 29 tissue samples collected from the study sites, a total of five species were successfully identified: Dipturus chilensis (4), Himantura walga (1), Neotrygon kuhlii (11), Taeniura lymma (9) and Rhinoptera javanica (4). The Neighbor Joining phylogeny of mitochondrial lineages, based on partial COI gene sequences, the ingroup haplotypes were clustered into five main clades representing each species. The identified stingrays were being listed as vulnerable (D. chilensis and R. javanica), near threatened (H. walga and T. lymma), and data deficient (N. kuhlii) by IUCN, with two species (D. chilensis and H. walga) population were indicated decreased. Unfortunately, all of identified species have not been evaluated by CITES regarding their trade status. As a consequences, a valuable effort should be placed to create a scientific network for monitoring programmes not only on a local scale, and to make pressure on governments for adopting molecular techniques as tools for controlling and avoiding misidentification.

***Keywords**: Mitochondrial DNA, Phylogeny, Coral Triangle, Taxonomy, Fisheries*

Introduction

Morphology-based identification of skates and stingrays is rather difficult due to their similar characteristics. However, reliable and precise species identification is important to fisheries management and conservation. In Southeast Asia stingrays are exploited intensively, oftentimes without proper enforcement of clear fisheries regulations (White and Kyne, 2010), which results in a particularly high risk of extinction since they mature slowly and have a low fecundity (Stevens *et al*., 2000). Moreover, their habitat and bottom dwelling adaptation increase their exposure to overfishing. Stingray reproduction is ovovivipar with a female stingray species being able to bear children up four baby rays in one mating period, only (Bester, 2011).

Indonesian marine water are the most catched of sharks and rays with high value of they product exports (FAO, 1999). Stingrays are typically traded on many fish markets in Indonesia. The fisherman use all parts of the rays for consumption, skin tanned, and made crafts, heart use to take fish oil, and also bone for glue materials (Rahardjo, 2007). They caught by large amount. In fact, the baby stingrays were often found in fisheries port Palabuhanratu. This may indicates a stingray population began to decline. The conservation status is more problematic, leading them being listed as vulnerable or endangered by IUCN. Of several other species the status is poorly known or unknown, being listed as Data Deficient in the IUCN list (IUCN 2013). In fact, of 77 ray species listed in the IUCN database, six species are categorized as threatened, one species as critically endangered,

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eleven species as vulnerable, eight species as "of little concern". However, at least 39 species - as a the major part – are listed as "data deficient" (IUCN, 2013).This indicates that a systematic and reliable species inventory is still urgently needed to allow for proper monitoring and conservation actions.

In recent years molecular techniques (incl. barcoding) have been developed to solve the problem described above (e.g. Prehadi *et al*. 2015, Sembiring *et al*. 2015, Akbar *et al*. 2014). DNA barcoding is a molecular taxonomic method that uses short genetic markers in an organism's DNA for species identification (Hebert *et al.* 2003, Costa and Carvalo 2007, Jefri *et al.* 2015). The mitochondrial DNA (mtDNA) is widely used in identifying a species (Kyle and Wilson, 2007, Saleky *et al.* 2016). The CO1 mitochondrial DNA loci are able to accurately discriminate species based on the structure and components of the DNA as well as providing information through phylogenetic proximity species (Brooks and McLennan, 1991).

The present study was conducted (1) to identify species of stingrays that were landed and traded in three fish markets in Indonesia (Palabuhanratu, MuaraSaban, and Lampung) with DNA barcoding techniques, and (2) to determine the conservation status of these speciesas defined by IUCN (International Union for Conservation of Nature and Natural Resources) as well as their trade status as defined by CITES (Convention on International Trade in Endangered Species).

Materials and Methods

Tissue sample collection and extraction

A total of 29 tissue samples were collected from the study sites, consisting of 13 samples from Lampung, 12 samples from Palabuhanratu and 14 samples from Muara Saban (Fig. 1). The samples consisted of 0.01-0.5 cm^3 of skin, flesh or fin tissue, dissected on-site with surgical scissors and preserved in 96% ethanol. DNA was extracted using the DNEasy® Tissue Kit following the protocol of the manufacturer (Qiagen GmbH, Hilden, Germany). The DNA extracts were stored at -30 ^oC until they were required for laboratory analyses.

DNA amplification and sequencing

The mitochondrial cytochrome oxidase I (COI) gene was PCR-amplified using the forward primer fish-BCL (5'TCAACYAATCAYAAAGATATYGGCAC') and the reverse primer fish-BCH (5'ACTTCYGGGTGRCC-RAARAATCA') (Weight *et al*., 2011). PCR amplifications were performed in 25 µL reaction mixture containing 2 µL 25 mM $MgCl_2$, 2.5 µL 8µM dNTPs ; 1.25 µL each primer pair 10 mM; 0,125 µL Taq DNA polymerase, 2.5 µL 10xPCR denaturation at 95 ^oC for 2 min followed by 35 cycles of denaturation (94 ^oC for 30 s), annealing (50 ^oC for 45 s), and extension (72 ^oC for 1 min) with the final extension step at 72 ^oC for 2 min.PCR-amplified DNAs were visualized on 1% agarose gels.

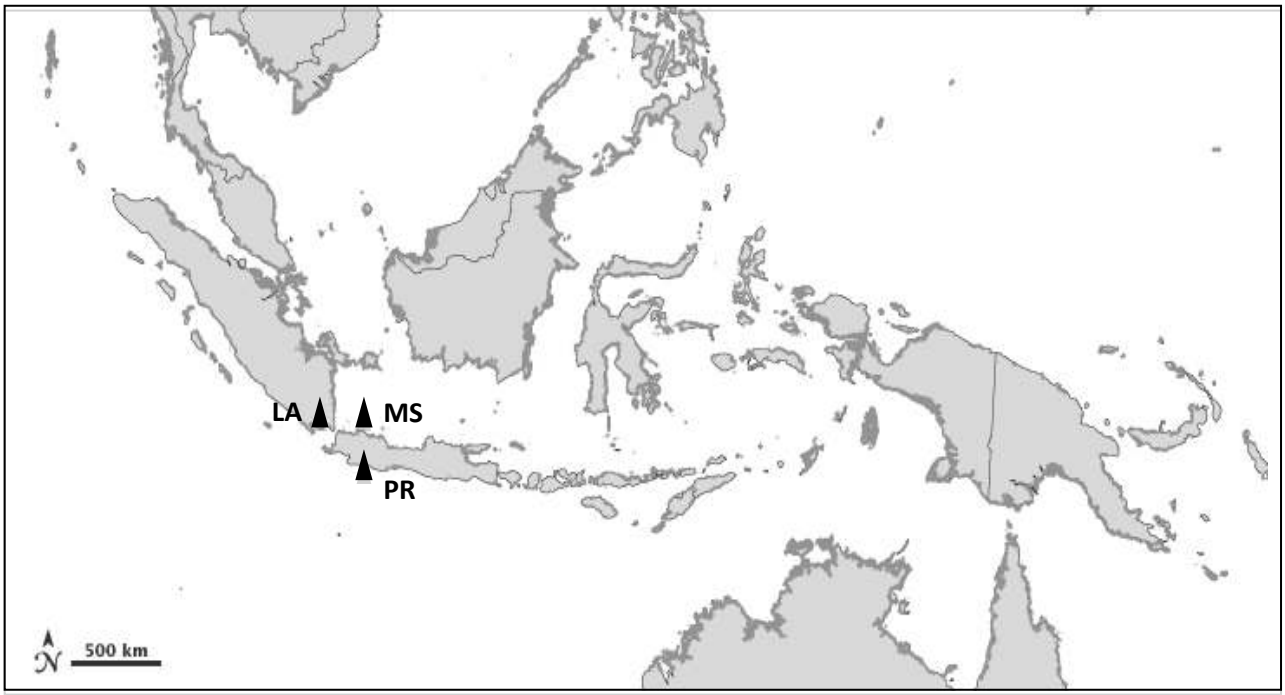

Figure 1. Tissue sampling location in three Indonesian fish markets: Palabuhanratu (PR), Lampung (LA), and Muara Saban (MS)

Prior to sequencing, excess dNTPs and Buffer,2 µL DNA template, 13.38 µL deionize water (ddH_2O). Cycling parameters were an initial oligonucleotides were eliminated from the PCR product using shrimp alkaline phosphatase and exonuclease I (Exo-SAP-IT kit; Affymetrix, Santa Clara CA, U.S.A.) following the manufacturer's protocol. Sequence reactions were performed in both directions using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems), and were loaded into an ABI 3130xl automated sequencer (Applied Biosystems) at the Berkeley Sequencing Facility located in the United States (Sanger *et al*., 1977).

Data analysis

Sequences were edited and aligned in MEGA 5 (Tamura *et al*. 2011), and exploratory identification of samples was performed using the Basic Local Assignment Search Tool (BLAST; Altschul *et al*. 1990). One or more reference sequences with the highest maximum identity to each amplicon sequence were downloaded from the GenBank sequence database for phylogenetic analysis. A neighbor joining phylogenetic trees was reconstructed using Kimura-2 parameter models (Kimura, 1980) with 1000 of bootstrap value.

A review of the conservation status of each species was determined using IUCN (International Union for Conservation of Nature and Natural Resources) online at http://www.iucnredlist.org/. The trade status of identified species was determined using CITES (Convention on International Trade in Endangered Species) online at http://www.cites.org/.

Result and Discussion

Species identification

Of 29 tissue samples collected from study sites, a total of five species were successfully identified, with level of homology is accurate worth more than 99%, with base strands between 300-700 bp (Table 1). The most common species was *Neotrygon kuhlii* with a total of eleven individuals, followed by *Taeniura lymma* (9), *Rhinoptera javanica*(4), *Dipturus chilensis* (4), and *Himantura walga* (1). The five species represented three different families, which were Myliobatidae (Eagle rays), Rajidae (Skates), and Dasyatidae (Stingrays).

The Neighbor Joining phylogeny of mitochondrial lineages, based on partial COI gene sequences (Fig. 2) supported the identification of cartilaginous species. The ingroup haplotypes were clustered into five main clades (I-IV) (Fig. 2). Clade species *H. walga* close with the clade *N. kuhlii*. This means *H. walga* have similarities in genetic and *N. kuhlii* compared with other species. Both of these species have descended from the same family, namely Dasyatidae. Species *T. lymma*have clade close to *N. kuhlii*. Both species have similar body characteristics or morphology and derived from the same family. *D. chilensis* clade close to and *R. javanica*. *D. chilensis* morphologically very different from *H. walga* and *R. javanica*, but in fact they are similar in genetic structure. Clade farthest occurred between *R. javanica* and *N. kuhlii*. This means that the two species have a small similarity in genetic structure. In morphological, both species have snout shape and different spot their body. *R. javanica* has a snout with a slit up in the middle to form two lobes, long tail and does not have blue spots such as *N. kuhlii* (*White et al*., 1997).

The smallest genetic distances between in group species were species *N. kuhlii* and *T. lymma* with distance value 0.15, meaning that they were 15 different bases in 100 nucleotide sequences. This is consistent with the results of phylogenetic analysis that showed the in group clade.

N. kuhlii closer near the clade *T. lymma*, where they both species are in the same family (Dasyatidae). The longest distance between the in group is *R. javanica* and *D. chilensis* with a value of 0.29. This means that in both species here are29 bases in100 different nucleotide sequences.

Table 1. The results of molecular identification of stingray landed in fish market in Palabuhanratu (PR), Lampung (LA), and Muara Saban (MS)

Species	Common name	Genbank max. identification (%)	PR	LA	MS
Dasyatidae:					
Neotrygon kuhlii	Blue-spot stingray	100	-	10	1
Himantura walga	Dwarf whipray	100	-	1	-
Taeniura lymma	Ribbontail stingray	100	-	-	9
Rajidae:					
Dipturus chilensis	Yellownose skate	99	4	-	-
Myliobtidae:					
Rhinoptera javanica	Flapnose ray	100	4	-	-

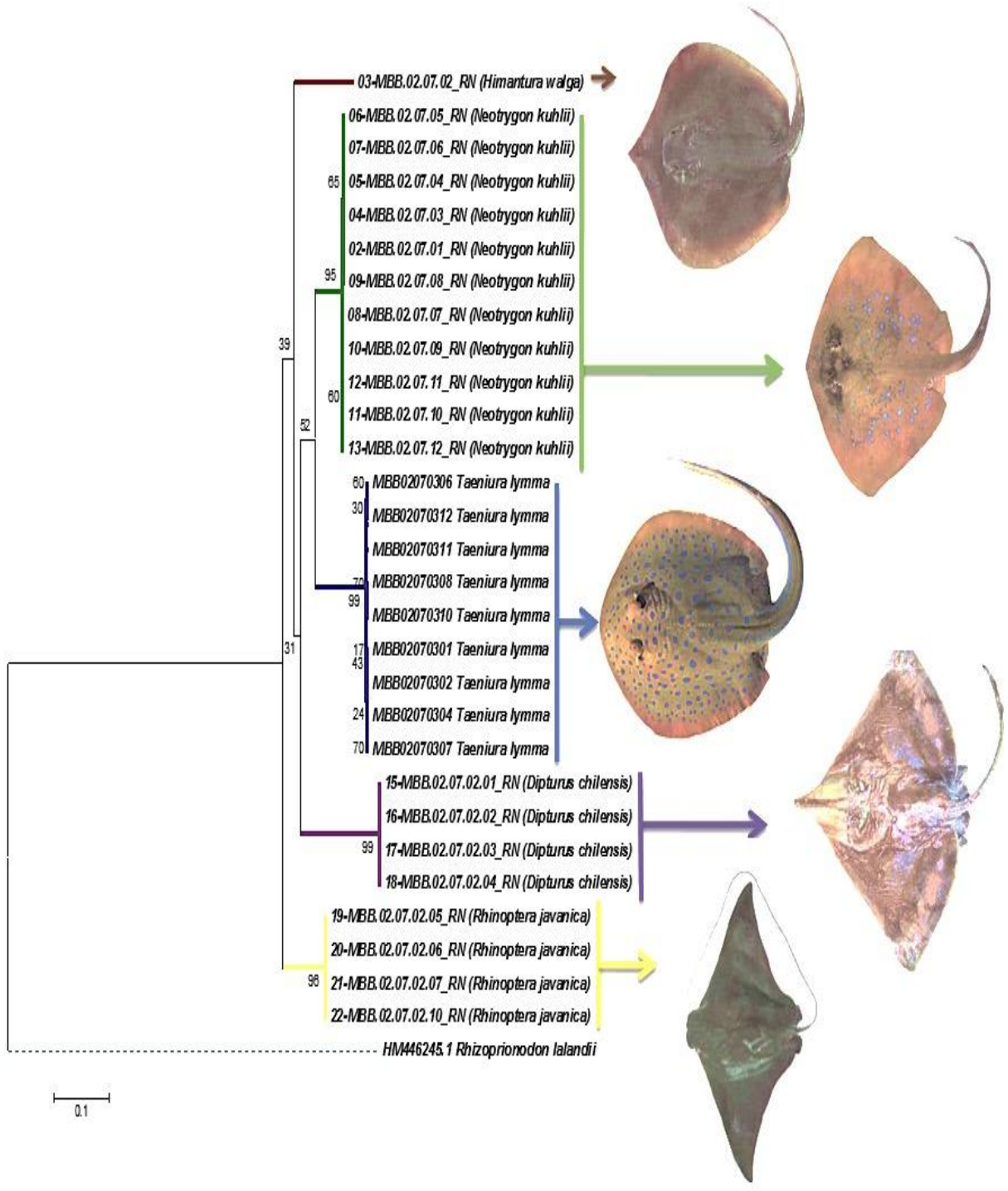

Figure 2. The Neighbour-joining tree based on 29 COI sequence data using Kimura-two-parameter substitution model with 1000 bootstrap

Conservation and trade status

Based on IUCN categories, two species were categorized in vulnerable, two categorized in near threatened, and one species in data deficient (Table 3). Trade status of *N. kuhlii, D. chilensis, R. javanica*, *T. lymma* and *H. walga* had not been evaluated by CITES. The population trend on *Himantura walga* and *Dipturus chilensis* is decreasing based on IUCN (2013). The molecular techniques used in this study using the target locus COI mitochondrial markers could be applied to identify targeted samples.

Table 2. Matrix of genetic distance for stingray species

	1	2	3	4	5	6
1						
2	0.20					
3	0.23	0.26				
4	0.20	0.22	0.29			
5	0.15	0.23	0.25	0.21		
6	1.22	1.29	1.33	1.25	1.41	

Note :
1 *Neotrygon kuhlii* Lampung
2 *Himantura walga* Lampung
3 *Dipturus chilensis* Palabuhanratu
4 *Rhinoptera javanica* Palabuhanratu
5 *Taeniura lymma* Muara Saban
6 *Rhizoprionodon lalandii*

Relationship performed by phylogenetic analysis was able to identify and demonstrate a close relationship between species. Five species were successfully identified including *Neotrygon kuhlii*, *Rhinoptera javanica, Himantura walga, Taeniura lymma* and *Dipturus chilensis*. These species were closelyrelated based on their distance matrix between species in the phylogenetic tree. These species are commonly observed in Indonesia (Arlyza and Adrim, 2007; Last *et al*., 2010).

The genetic analyses have resulted in a better understanding of intra-species diversity across study sites that will ultimately be useful for species identification, fisheries management and conservation of tropical skates and stingrays. Furthermore, the continued updating of sequences lodged on GenBank and BOLD is a vital, but a rarely considered issue in the practical application of barcoding. In addition, there is a potential to discover new species from different part of Indonesia, as described some new species found from different part of Indonesia (Last and White, 2013; Mojica *et al.,* 2013).

Data production of fishing in southern sea of West Java Palabuhanratu only recorded five most common types of rays, i.e. *Taeniura lymma*, *Gymnura micrura*, *Rhinoptera javanica*, *Rhinaan cylostoma*, and *Glaucostegus typus* (KKP, 2013). Only two (*Taeniura lymma* and *Rhinoptera javanica*) of five species from the production data were identified in this study. The annual catch data stingray in Lampung gave information on five stingray species commonly landed in Lampung between 2008-2012 are the same species landed in Palabuhanratu (KKP, 2013).

The most common rays captured between 2008–2012 in the southern seas of West Java, Lampung and Banten are including stingrays (*Dasyatis* spp.) or local name called “Pari Kembang” and Eagle rays (*Myliobatus* spp.) or locally called “Pari Burung” (KKP, 2013). The statistical data by the Ministry of Maritime Affairs and Fisheries (KKP) at southern seas of West Java is based on physical characteristics of captured stingrays, and is mainly recorded as local name (KKP, 2013). Based on their report, a total of five species of rays are commonly found. *T. lymma* recorded only in 2010 with production of 46 tons per year. *Rhinoptera javanica* most captured and production is increasing every year with the highest production in 2011 with 148 tons. Production data is different in each year.

The white spotted wedge fishes (*Rhynchobatus djiddensis*) or called “Pari Kekeh” are not recorded throughout the year 2008-2012. Production of *Taeniura lymma* in 2011 were probably over recorded in amount of 715 tons in Muara Saban, and in the same year in Lampung was

Table 3. Determination of identified species to their conservation status by IUCN (International Union for Conservation of Nature and Natural Resources) and trade status by CITES (Convention on International Trade in Endangered Species)

Species	IUCN (2014)	CITES (2014)	Population Trend (IUCN 2014)
Neotrygonkuhlii	Data deficient	Not evaluated	Unknown
Taeniuralymma	Near threatened	Not evaluated	Unknown
Himanturawalga	Near threatened	Not evaluated	Decreasing
Dipturuschilensis	Vulnerable	Not evaluated	Decreasing
Rhinopterajavanica	Vulnerable	Not evaluated	Unknown

only recorded in amount of one ton of stingrays (*Dasyatis* spp.). There are several possibilities related decline in catches some types of stingrays at the fish auction recorded by KKP local officials. One of them is a very drastic decline may indicate a declining population of stingrays in the waters.

Four of five identified species were categorized vulnerable and near threatened, and two of them (*Himantura walga* and *Dipturus chilensis*) indicate a decrease of the population in their habitat. Based on their production data capture might decrease in the wild population. Species identification is useful in ensuring honest trading exchanges for correct consumer information and for fisheries management and conservation as well. In order to avoid misidentification, which in turn bias fish data catch per species, the use of DNA could contribute on precise identification.

Some studies have revealed that molecular markers have contributed to species authentication by flagging mislabeling and the misidentification of commercial landings (Carvalho *et al*., 2011). This study recommends the establishment of a valid list of commercial and Latin names for the fishes commercialized in Indonesia, starting at the local fish market, as a starting point of the supply chain. Such a reference list would make possible for Ministry of Marine Affairs and Fisheries at all level especially at the local government and local market level to be able to evaluate, regulate and detect fraud, substitution, and the commercialization of threatened species.

Conclusion

The current study has successfully identified 29 tissue samples collected from the study sites using mitochondrial DNA, based on partial COI gene sequences. The identified stingrays were being listed as vulnerable (D. chilensis and R. javanica), near threatened (H. walga and T. lymma), and data deficient (N. kuhlii) by IUCN, with two species (D. chilensis and H. walga) population were indicated decreased. Unfortunately, all of identified species have not been evaluated by CITES regarding their trade status. As a consequences, a valuable effort should be placed to create a scientific network for monitoring programmes not only on a local scale, and to make pressure on governments for adopting molecular techniques as tools for controlling and avoiding misidentification.

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