Identification of Batoid Fishes from North Sumatra waters, Indonesia: Comparing between 12S and 16S rRNA gene as DNA marker

Khairiza Lubis[1]*, Mufti Sudibyo[1], Achmad Farajallah[2], Nisfa Hanim[2], Person Pesona Renta[3,4]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan*
Jl. Willem Iskandar Pasar V, Medan 20221, North Sumatra, Indonesia
[2]*Departement of Biology, Faculty of Mathematics and Natural Sciences, IPB University*
Jl. Meranti, Kampus IPB Darmaga, Bogor 16680, West Java, Indonesia
[3]*Department of Biotechnology and Bioindustry Sciences, National Cheng Kung University*
No. 1, Daxue Road, Tainan City, Taiwan
Department of Marine Science, Faculty of Agriculture, Universitas Bengkulu
Jl. W.R.Supratman, Kandang Limun, Bengkulu, 38371 Indonesia
Email: khairizalubis@unimed.ac.id

Abstract

Indonesia is one of the biodiversity hotspot areas for chondrichthyan fishes in the world, including batoid fishes. Nevertheless, some of the biological information of batoid fishes in Indonesia are still limited, such as batoid's gene sequences for molecular identification. Two of those DNA marker for identifying batoid species, that it's information still lacking in Indonesia is 12S and 16S gene. Therefore, this study aimed to identify batoid fishes species from North Sumatra using 12S and 16S rRNA gene fragments and comparing the identification result of those two markers. The genomic DNA was extracted from the pectoral fin muscle of eight batoid samples from eight different species. Amplification of both gene fragments was done by using two pairs of primers which were manually designed. The amplicon from both genes was sequenced and then were analyzed. In the present study successfully obtained eight good sequences of each 12S and 16S rRNA gene fragment. Five batoid samples were successfully identified to species level using both of gene fragments due to these identification results were matched with our previous morphological identification results. The identification result of Rhinoptera javanica sample based on 12S rRNA gene fragment was inaccurate due to several reasons. One of the possible reasons was due to the gene region that this work used did not contain sufficient information to differentiate Rhinoptera javanica until species level. The 3 remaining samples were unsuccessfully identified to species level using both genes due to the lack of conspecific targeted gene reference sequences in the database.

Keywords: *12S rRNA gene, 16S rRNA gene, batoid, Medan, Indonesia*

Introduction

The broad area of the sea allows Indonesia to have a high diversity of marine organisms. One of the groups of marine organisms which have a high diversity in Indonesia is chondrichthyan fishes. As per 7 November 2015, the total number of chondrichthyan fishes in the world is 1188, with 221 of them are found in Indonesia and therefore Indonesia was recorded as one of biodiversity hotspots of chondrichthyan fishes in the world (Sadili *et al.*, 2015; Weigmann, 2016).

Morphological-based identification is the most common method that was used due to it is the easiest, fastest, and not time-consuming, but there are certain circumstances species identification of an organism (specimen) could not be done by only using its morphological features, such as specimen species that have similar morphological features with other species from the same genus, cryptic species, immature specimen, incomplete specimen and hybrid species (Dudgeon *et al*., 2012). Due to this reason, an identification method by comparing a certain DNA sequence (called as DNA barcoding) is needed to collect more biological data to get a robust identification result. The use of DNA barcoding to identify certain organism's species depends on the availability of reference sequences in the database (Dudgeon *et al*., 2012) and the strength of DNA fragments in differentiating one species from another (Kress and Erickson, 2008). So, to successfully identifying an organism's species, there is a need in collecting morphological features data, DNA sequences data, and comparative studies of the strength of DNA sequences data in differentiating one species from another.

Batoid is a group of chondrichthyan fishes that have various morphological features. Species

*) Corresponding author

https://ejournal.undip.ac.id/index.php/ijms
DOI: 10.14710/ik.ijms.26.4.298-310
Received : 19-06-2021
Accepted : 06-12-2021

members of this group can be identified by its flattened body (dorsoventrally), has ventral gill slits, enlarged pectoral fin, and lack of anal fin. Its enlarged pectoral fin was fused to the side of the head and snout and this group has disc-like body shape (Compagno, 1999; McEachran and de Calvarho, 2002). The identification process of batoid using DNA barcode has been done by either using certain DNA sequence from *COI, ND2, 12S, 16S* or *cytb* gene (Hoelzel, 2001; Tinti *et al*., 2003; Ward *et al*., 2005; Spies *et al*., 2006; Blaco *et al*., 2008; Ward *et al*., 2008; Holmes *et al*., 2009; Rodrigues-Filho *et al*., 2009). In its taxonomical history, several species of batoid had been going through taxonomic review, due to new features (morphology or molecular) were founded such as *Maculabatis gerrardi and Brevitrygon walga* (Last *et al.,* 2016). The taxonomic review event showed that there is a need in collecting more biological data in batoid fishes to get robust identification result. In North Sumatra, molecular studies of batoids from North Sumatra is limited, with the majority of the information was about the species diversity (morphological-based identification) (Puteri *et al*., 2017; Fadhilah *et al*., 2019). Up to present, there is only one informationof the use of molecular data to identify batoid fishes in North Sumatra by using *COI* gene, and several species of batoid fishes were successfully identified (Sudibyo *et al*., 2020). Even though *12S* and *16S* had been reported could be used in identifying batoid species, *12S* and *16S* data from Indonesian batoid species was lacking. Therefore, this study aimed to identify batoid species from North Sumatra using *12S* and *16S* gene fragments and compare the identification result of these two genes fragment.

Materials and Methods

Batoid samples were collected from three landing sites (Belawan, Tanjung Balai, and Percut) along the east coast of North Sumatra (Figure 1.). These batoid fishes were caught by local fishermen in the Malaka strait area (Figure 1.). Part of pectoral fin muscle tissue under the skin from one individual of each species was taken and immediately put in a 15 mL tube then 96% alcohol was added to preserve it. The batoid fish samples used in this study were the same sample with that used in our previous study. In the previous study, nine batoid species were successfully identified from our samples, both based on morphological characteristics and molecular data (*COI* gene), namely *Maculabatis gerrardi, Gymnura poecilura, Dasyatis zugei, Brevitrygon heterura, Neotrygon kuhlii, Hemitrygon bennettii, Rhinobatos jimbaranensis, Rhinoptera javanica,* and *Taeniura lymma* (Sudibyo *et al*., 2020).

Genomic DNA extraction

Small parts of the pectoral fin muscle were taken using a sterile surgical scissor, then put in the 1.5 mL tube. After that, soaked 30 mg of the muscle tissue sample in sterile water for 20-30 mins to remove the preserving liquid (96% alcohol). Afterward, the tubes were centrifuged, 5000 rpm for 1 min, in next the sterile water was removed. The muscle tissue samples were cut to fine pieces in the tubes with a sterile surgical scissor. Then genomic DNA was extracted using DNA GENEAID Genomic DNA Mini Kit (Tissue) (Geneaid, Canada) following the protocol of the manufacturer.

DNA amplification and sequencing

Both *12S* and *16S rRNA* gene fragments were amplified by polymerase chain reaction (PCR) method using a newly design primer, namely AF587 5'-GAGTTGGTHAATCTCGTGCCAG and AF588 5'-ATTCCAAGTRCACTTTCCAGTA for *12S rRNA* gene and primer AF589 5'-AGGCAAGTCGTAACATGGTAAG and AF590 5'-TGTTTTTGGTAAACAGGCGGAGT for *16S rRNA* gene. Amplification of both gene fragments was carried out by using GoTaq Green Mastermix® DNA Polymerase (Promega). The PCR cycle for both genes comprised of 3 min pre-denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing for 15 secs at 52°C, and an extension for 10 mins at 72°C. All products of PCR (amplicon) were migrated in polyacrylamide gel and stained using silver staining method (Byun *et al*., 2009), then gel which contained migrated amplicon was directly onserved using naked eyes. Later on, amplicons that showed good DNA bands were sent to sequencing service company, 1st Base, to be purified and sequenced.

Molecular identification and species diversity

The DNA sequencing products, chromatogram, were examined and edited, then aligned and combined using BioEdit 7.0.9.0 software (Hall, 1999) and ClustalW which were embedded in MEGA7 software (Kumar *et al.,* 2016), respectively. Several steps were taken to identify the specimens,. Firstly, similarity analysis was carried out, using the GenBank BLAST-N program (Altschul *et al.,* 1990) to compare *12S and 16S rRNA* gene DNA fragment from each sample with all DNA fragment in the database. After the BLAST-N result is obtained, two sequences of *12S* and *16S rRNA* gene within the top five of BLAST result with Query cover value was 100% or nearly 100% were downloaded to be used as reference sequences (Table 1 and 2.), then aligned all *12S* and *16S rRNA* genes of DNA sequence from all samples using ClustalW option which embedded in MEGA7

software, then conducted genetic distance analysis with Kimura 2-Parameter method to further identified the species of each sequence (Kimura, 1980). Kimura-2-parameter method was used to calculate the genetic distance due to it was recommended by the Consortium for the Barcode of Life (CBOL) (Kimura, 1980; Shen *et al*., 2016). This work also conducted nucleotide base composition analysis and constructed a phylogenetic tree using a Maximum Likelihood for statistical method (Nei and Kumar, 2000) with General Time Reversible for substitution model (Tavaré, 1986), Gamma distribution for modeling evolutionary rates differences among sites (5 categories) and bootstrap method for test of phylogeny (Felsenstein, 1985) with the number of bootstraps was 1000.

General Time Reversible for substitution model and Gamma distribution for modeling evolutionary rates (5 categories) were used due to General Time Reversible model with Gamma distribution had the lowest BIC score, 8041.812 for *12S* and 11814.678 for *16S*, after the "Find Best Model" menu in MEGA7 was conducted.

Table 1. Reference DNA sequence of *12S rRNA* gene of batoids

Reference species	Accession Number
Brevitrygon imbricata	MH248229
Gymnura altavela	MH377803
Gymnura poecilura	KJ617038
Hemitrygon akeji	KC526959
Hemitrygon bennetti	KC196067
Himantura uarnak	KR019776
Maculabatis gerrardi	KP091437
Neotrygon kuhlii	KR019777
Rhinobatos formosensis	AF448014
Rhinobatos schlegelii	KJ140136
Rhinoptera brasiliensis	MN883188
Rhinoptera javanica	AF448019
Taeniura lymma	KM881715

Table 2. Reference DNA sequence of *16S rRNA* gene of batoids

Reference species	Accession Number
Gymnura poecilura	KJ617038
Hemitrygon akeji	KC526959
Hemitrygon bennetti	KC633222
Himantura uarnak	KR019776
Maculabatis gerrardi	KP091437
Neotrygon kuhlii	KR019777
Pateobatis hortlei	KP727646
Pateobatis jenkinsii	KU873081
Pteroplatytrygon violacea	KJ641617
Rhinobatos hynnicephalus	KF534708
Rhinobatos schlegelii	KJ140136
Rhinoptera bonasus	KX151652
Taeniura lymma	KM881715

Results and Discussion

Similarity and nucleotide base compotition analysis

After all sequences of *12S* and *16S rRNA* gene fragments that were obtained from sequencing service company, were manually edited and combined, in order that got eight good sequences of *12S rRNA* gene fragment with length of 687-693 bp and eight good sequences of *16S rRNA* gene fragment with 898-911 bp. From the top 5 BLAST-N results, there were 2-3 species samples that showed the absence of *12S* or *16S RNA* sequence data from the same species (Table 3 and 4) such as *Brevitrygon heterura* and *Rhinobatos jimbarensis*. Query Cover value in the top 5 BLAST-N results were greater than 98% in *12S rRNA* sequence fragment and greater that 92% in *16S rRNA* sequence fragment and all E-value were 0 in both gene fragments. The Identity value within the same species in top 5 BLAST-N result ranged 96-100% in *12S rRNA* sequence fragment and 97-99,89 % in *16S rRNA* sequence.

After the alignment and trimming process was conducted between the sample and reference sequences (Table 1 and 2.), in order to got 691 bp of *12S rRNA* dan 944 bp *16S rRNA* gene fragments that were ready for nucleotide base composition, genetic distance analysis and for constructing phylogenetic tree. The overall mean of nucleotide base frequencies for sample and reference *12S rRNA* sequences were T (24,1 %), C (24,3%), A (34,3%) and G (17,4%) (Table 5.). As for the overall mean of nucleotide base frequencies for sample and reference *16S rRNA* sequences were T (23,3 %), C (21,6%), A (39,7%) and G (15,5%) (Table 6.). The nucleotide base composition analysis for the *12S* and *16S rRNA* gene showed both the sample and reference have similar patterns with "A" content was the highest and "G" as the lowest. The overall mean of AT content (58,4% for *12S rRNA;* 63% for *16S rRNA*) of these two gene was higher than GC content (41,7% for *12S rRNA; 37*% for *16S rRNA*).

Genetic distance and phylogenetic tree

The genetic distance value of *12S rRNA* gene sequence within the same species (in red rectangle) was ranged between 0,000-0,035 (Table 7.) with a mean of 0,00983, the smallest one was within *Hemitrygon bennetti* species (0,000) while the largest was within *Rhinoptera javanica* species (0,035). All the genetic distance value within the same species (intraspecific difference) in this gene was much lower than between species (interspecific difference) (most of it was more than 0,1) except genetic distance in *Rhinoptera javanica* sample

Table 3. Top 5 BLAST-N result for *12S rRNA* gene fragment of batoid species from North Sumatra

Sample species	Reference species	Accession number	Query cover	E-value	Identity percentage
Brevitrygon heterura	*Brevitrygon imbricata*	MH248229	98%	0	96,45
	Maculabatis gerrardi	KP091437	100%	0	94,66
	Maculabatis gerrardi	AF447996	100%	0	94,66
	Pateobatis jenkinsii	KU873081	100%	0	94,19
	Pateobatis hortlei	KP727646	100%	0	93,78
Gymnura poecilura	*Gymnura poecilura*	KJ617038	100%	0	99,85
	Gymnura cf. poecilura	MH248236	98%	0	94,07
	Gymnura altavela	MH377803	100%	0	92,01
	Hypanus say	MN883186	100%	0	89,58
	Hypanus say	MN883185	100%	0	89,58
Hemitrygon bennetti	*Hemitrygon bennetti*	KC196067	100%	0	100
	Dasyatis sp.	AF447992	100%	0	100
	Hemitrygon bennetti	KC633222	100%	0	99,86
	Hemitrygon akeji	KC526959	100%	0	98,12
	Dasyatis centroura	MH377784	100%	0	94,22
Maculabatis gerrardi	*Maculabatis gerrardi*	KP091437	100%	0	98,41
	Maculabatis gerrardi	AF447996	100%	0	98,41
	Himantura uarnak	KR019776	100%	0	93,95
	Himantura uarnak	AF447997	100%	0	93,95
	Pateobatis hortlei	KP727646	100%	0	93,94
Neotrygon kuhlii	*Neotrygon kuhlii*	KR019777	100%	0	99,13
	Neotrygon kuhlii	KC992792	100%	0	99,13
	Neotrygon kuhlii	AF447991	100%	0	99,13
	Taeniura lymma	KM881715	100%	0	94,64
	Taeniura lymma	AF448024	100%	0	94,65
Rhinobatos jimbarensis	*Rhinobatos formosensis*	AF448014	100%	0	94,51
	Rhinobatos schlegelii	KJ140136	100%	0	94,22
	Rhinobatos schlegelii	AF448016	100%	0	94,22
	Rhinobatos hynnicephalus	KF534708	100%	0	94,08
	Rhinobatos hynnicephalus	AF448015	100%	0	94,08
Rhinoptera javanica	*Rhinoptera brasiliensis*	MN883188	100%	0	99,27
	Rhinoptera brasiliensis	MN883189	100%	0	99,13
	Rhinoptera bonasus	KX151652	100%	0	96,95
	Rhinoptera steindachneri	KM364982	100%	0	96,66
	Rhinoptera javanica	AF448019	100%	0	96,66
Taeniura lymma	*Taeniura lymma*	KM881715	100%	0	99,56
	Taeniura lymma	AF448024	100%	0	99,13
	Neotrygon kuhlii	KR019777	100%	0	94,8
	Neotrygon kuhlii	KC992792	100%	0	94,78
	Neotrygon kuhlii	AF447991	100%	0	94,78

with *Rhinoptera javanica* (AF448019) (0,035) and with *Rhinoptera brasiliensis* (MN883188) (0,007). The genetic distance value of *16S rRNA* gene sequence between the same species (in red rectangle) ranged 0,002-0,031 (Table 8.) with a mean of 0,0124, the smallest was within *Hemitrygon bennetti* species (0,002) and the largest was within *Maculabatis gerrardi* species. Similar to the genetic distance value of *12S rRNA,* in *16S rRNA* genetic distance value within the same species (intraspecific difference) was much lower than between species (interspecific difference) (most of it was more than 0,1).

Species delimitation based on Hebert *et al*., (2004) by using *COI* gene fragment had an

Table 4. Top 5 BLAST-N result for *16S rRNA* gene fragment of batoid species from North Sumatra

Sample species	Reference species	Accession number	Query cover	E-value	Identity percentage
Brevitrygon heterura	*Maculabatis gerrardi*	KP091437	100%	0	90,23
	Pateobatis jenkinsii	KU873081	99%	0	89,83
	Himantura uarnak	KR019776	99%	0	89,65
	Pateobatis hortlei	KP727646	99%	0	89,68
	Urogymnus granulatus	KF751650	99%	0	88,61
Gymnura poecilura	*Gymnura poecilura*	KJ617038	100%	0	98,78
	Mobula tarapacana	NC_040922	92%	0	85,48
	Mobula japanica	KM364988	92%	0	85,41
	Mobula japanica	KM364984	92%	0	85,41
	Mobula mobular	KM364983	92%	0	85,41
Hemitrygon bennetti	*Hemitrygon bennetti*	KC196067	100%	0	99,89
	Hemitrygon bennetti	KC633222	100%	0	99,78
	Hemitrygon akeji	KC526959	99%	0	97,37
	Bathytoshia brevicaudata	NC_052720	99%	0	92,55
	Himantura microphthalma	KF840390	99%	0	92,34
Maculabatis gerrardi	*Maculabatis gerrardi*	KP091437	99%	0	97,01
	Himantura uarnak	KR019776	100%	0	92,5
	Pateobatis jenkinsii	KU873081	99%	0	91,53
	Pateobatis hortlei	KP727646	99%	0	91,45
	Urogymnus dalyensis	KM244769	99%	0	90,51
Neotrygon kuhlii	*Neotrygon kuhlii*	KR019777	100%	0	98,46
	Neotrygon kuhlii	KC992792	100%	0	97,91
	Taeniura lymma	KM881715	100%	0	89,99
	Mobula japanica	KM364988	100%	0	87,83
	Mobula japanica	KM364984	100%	0	87,83
Rhinobatos jimbarensis	*Rhinobatos schlegelii*	KJ140136	100%	0	94,67
	Rhinobatos hynnicephalus	KF534708	100%	0	93,89
	Anoxypristis cuspidata	KP233202	100%	0	89,28
	Rhinobatos productus	HM140461	100%	0	88,88
	Rhinobatos lentiginosus	AY830717	96%	0	88,82
Rhinoptera javanica	*Rhinoptera bonasus*	KX151652	100%	0	94,96
	Rhinoptera steindachneri	KM364982	100%	0	93,22
	Mobula munkiana	KX151645	100%	0	92
	Mobula tarapacana	NC_040922	100%	0	92
	Mobula hypostoma	KX151646	100%	0	91,89
Taeniura lymma	*Taeniura lymma*	KM881715	100%	0	99,23
	Neotrygon kuhlii	KC992792	100%	0	89,67
	Neotrygon kuhlii	KR019777	100%	0	89,58
	Pteroplatytrygon violacea	KJ641617	99%	0	88,29
	Himantura microphthalma	KF840390	92%	0	89,56

underlying idea that intraspecific difference was lower than interspecific difference. In this study, the term of "success" and "unsuccess" in identifying species level of samples was done by comparing the result of molecular identification based on *12S rRNA* and *16S rRNA* gene fragment (genetic distance data) with morphological identification result of our previous study in Sudibyo *et al*. (2020). If the molecular identification result of a sample based on either *12S rRNA* or *16S rRNA* was matched with morphological identification result, it was "successful", otherwise it was "unsuccessful". Based on *12S rRNA* (from middle to near 3'end region) and *16S rRNA* (from 5'end to middle region) gene fragment, successfully identified the species level of 5 batoid samples, while 3 remaining batoid samples were unsuccessfully identified the species level. The successfully identified samples were *Gymnura poecilura, Hemitrygon bennetti, Maculabatis*

Table 5. Nucleotide compotition of *12S* gene fragment of bataod species from North Sumatra

Species	Origin	Accession number	Percentage of nucleotide bases				Sequence lenght (bp)
			T/U	C	A	G	
Maculabatis gerrardi	North Sumatera	*	24,2	23,9	34,1	17,8	681
Rhinoptera javanica	North Sumatera	*	25,0	24,3	33,6	17,2	676
Rhinobatos jimbarensis	North Sumatera	*	20,6	26,6	35,7	17,2	676
Taeniura lymma	North Sumatera	*	23,5	24,4	35,1	17,0	676
Gymnura poecilura	North Sumatera	*	23,4	24,9	33,6	18,1	675
Hemitrygon bennetti	North Sumatera	*	24,7	24,3	33,6	17,4	679
Neotrygon kuhli	North Sumatera	*	24,8	23,5	34,9	16,8	677
Brevitrygon heterura	North Sumatera	*	24,6	23,2	34,3	17,9	676
Brevitrygon imbricata	Kuwait	MH248229	24,9	22,6	35,2	17,4	674
Gymnura altavela	USA, New Jersey	MH377803	24,3	24,7	34,3	16,7	676
Gymnura poecilura	China	KJ617038	23,6	24,7	33,6	18,1	675
Hemitrygon akeji	China	KC526959	25,3	23,7	33,7	17,4	680
Hemitrygon bennetti	*	KC196067	24,7	24,3	33,6	17,4	679
Himantura uarnak	Taiwan	KR019776	24,8	23,6	34,4	17,2	681
Maculabatis gerrardi	China	KP091437	24,4	23,8	34,4	17,5	681
Neotrygon kuhlii	Philippines: Chebu	KR019777	24,9	23,7	34,6	16,8	679
Rhinobatos formosensis	*	AF448014	22,4	25,5	34,8	17,3	675
Rhinobatos schlegelii	China	KJ140136	22,4	25,5	34,8	17,3	675
Rhinoptera brasiliensis	USA: Mississippi	MN883188	24,6	24,6	33,4	17,5	676
Rhinoptera javanica	*	AF448019	25,0	24,0	33,3	17,8	676
Taeniura lymma	Australia: Ningaloo Reef	KM881715	23,7	24,0	35,4	17,0	676
Average			24,1	24,3	34,3	17,4	677,1

*: this research

Table 6. Nucleotide composition of *16S* gene fragment of bataod species from North Sumatra

Species	Origin	Accession number	Percentage of nucleotide bases				Sequence lenght (bp)
			T/U	C	A	G	
Maculabatis gerrardi	North Sumatera	*	23,6	20,8	40,0	15,6	905
Rhinoptera javanica	North Sumatera	*	23,6	21,6	40,4	14,3	910
Rhinobatos jimbarensis	North Sumatera	*	22,8	21,6	39,8	15,8	898
Taeniura lymma	North Sumatera	*	22,0	22,6	40,5	14,9	906
Gymnura poecilura	North Sumatera	*	21,4	25,2	36,7	16,7	902
Hemitrygon bennetti	North Sumatera	*	23,8	21,2	39,5	15,5	910
Neotrygon kuhlii	North Sumatera	*	23,4	21,4	40,0	15,3	911
Brevitrygon heterura	North Sumatera	*	23,5	20,4	40,1	15,9	905
Gymnura poecilura	China: Haikou	KJ617038	21,5	24,8	37,0	16,7	894
Hemitrygon akeji	China: Dhongsan	KC526959	23,8	21,9	39,0	15,3	913
Hemitrygon bennetti	*	KC633222	24,0	21,0	39,6	15,4	909
Himantura uarnak	Taiwan	KR019776	23,8	20,8	40,4	15,0	900
Maculabatis gerrardi	China	KP091437	23,7	20,8	39,8	15,7	904
Neotrygon kuhlii	Philippines: Chebu	KR019777	23,8	21,1	39,6	15,5	911
Pateobatis hortlei	Indonesia	KP727646	23,6	20,8	40,1	15,5	895
Pateobatis jenkinsii	Thailand: Rhanong	KU873081	25,5	18,8	41,0	14,6	902
Pteroplatytrygon violacea	China: Sanya	KJ641617	23,2	22,1	39,4	15,2	908
Rhinobatos hynnicephalus	China: Dhongsan Island	KF534708	22,4	21,4	40,2	15,9	897
Rhinobatos schlegelii	Dhongsan Island	KJ140136	22,7	21,3	40,1	15,9	898
Rhinoptera bonasus	*	KX151652	24,7	20,6	39,6	15,0	911
Taeniura lymma	Australia: Ningaloo Reef	KM881715	22,0	22,7	40,6	14,8	905
Average			23,3	21,6	39,7	15,5	904,5

*: this research

gerrardi, and *Taeniura lymma*. The unsuccessfully identified samples *Neotrygon kuhli, Brevitrygon heterura, Rhinobatos jimbarensis* and *Rhinoptera javanica*. *Neotrygon kuhli* in Indonesia currently separated into three different species (Last *et al*., 2016). However, based on the database, 12S and

Table 7. Genetic distance of *12S* gene fragment of batoid species from North Sumatra

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
[1]																					
2]	0,133																				
[3]	0,214	0,191																			
[4]	0,128	0,112	0,195																		
[5]	0,166	0,141	0,203	0,149																	
[6]	0,139	0,101	0,179	0,111	0,140																
[7]	0,135	0,117	0,193	0,051	0,143	0,119															
[8]	0,064	0,128	0,186	0,129	0,160	0,131	0,134														
[9]	0,066	0,125	0,186	),117	0,153	0,135	0,124	0,034													
[10]	0,145	0,106	0,194	0,134	0,085	0,127	0,131	0,132	0,137												
[11]	0,168	0,142	0,201	0,149	0,001	0,138	0,141	0,160	0,153	0,087											
[12]	0,136	0,098	0,177	0,108	0,140	0,018	0,111	0,124	0,128	0,125	0,138										
[13]	0,139	0,101	0,179	0,111	0,140	0,000	0,119	0,131	0,135	0,127	0,138	0,018									
[14]	0,069	0,137	0,206	0,126	0,169	0,130	0,135	0,076	0,068	0,138	0,171	0,123	0,130								
[15]	0,016	0,129	0,204	0,119	0,155	0,127	0,126	0,049	0,054	0,134	0,157	0,123	0,127	0,057							
[16]	0,134	0,115	0,195	0,048	0,142	0,120	0,003	0,133	0,122	0,129	0,140	0,113	0,120	0,132	0,125						
[17]	0,216	0,196	0,051	0,193	0,217	0,195	0,201	0,203	0,204	0,198	0,215	0,196	0,195	0,216	0,208	0,202					
[18]	0,216	0,196	0,054	0,195	0,215	0,199	0,205	0,205	0,202	0,198	0,217	0,200	0,199	0,216	0,208	0,207	0,003				
[19]	0,133	0,007	0,191	0,108	0,144	0,103	0,115	0,128	0,125	0,110	0,146	0,100	0,103	0,133	0,129	0,112	0,196	0,196			
[20]	0,134	0,035	0,181	0,117	0,141	0,109	0,124	0,137	0,131	0,119	0,143	0,103	0,109	0,140	0,129	0,122	0,188	0,188	0,027		
[21]	0,130	0,112	0,191	0,004	0,145	0,109	0,052	0,129	0,117	0,134	0,145	0,106	0,109	0,126	0,121	0,049	0,189	0,191	0,108	0,113	

1: *Maculabatis gerrardi* North Sumatra; 2: *Rhinoptera javanica* North Sumatra; 3: *Rhinobatos jimbarensis* North Sumatra; 4: *Taeniura lymma* North Sumatra; 5: *Gymnura poecilura* North Sumatra; 6: *Hemitrygon bennetti* North Sumatra; 7: *Neotrygon kuhli* North Sumatra; 8: *Brevitrygon heterura* North Sumatra; 9: MH248229 *Brevitrygon imbricata;* 10: MH277803 *Gymnura altavela;* 11: KJ617038 *Gymnura poecilura;* 12: KC526959 *Hemitrygon akeji;* 13: KC196067 *Hemitrygon bennetti;* 14: KR019776 *Himantura uarnak;* 15: KP091437 *Maculabatis gerrardi;* 16: KR019777 *Neotrygon kuhlii*; 17: AF448014 *Rhinobatos formosensis;* 18: KJ140136 *Rhinobatos schlegelii;* 19: MN883188 *Rhinoptera brasiliensis;* 20: AF448019 *Rhinoptera javanica;* 21: *Taeniura lymma*

Table 8. Genetic distance of *16S* gene fragment of batoid species from North Sumatra

	[1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
[1]																					
[2]	0,168																				
[3]	0,260	0,227																			
[4]	0,180	0,141	0,256																		
[5]	0,224	0,193	0,246	0,207																	
[6]	0,193	0,156	0,247	0,157	0,228																
[7]	0,188	0,134	0,237	0,097	0,203	0,151															
[8]	0,106	0,170	0,277	0,210	0,221	0,189	0,191														
[9]	0,213	0,189	0,240	0,200	0,006	0,219	0,199	0,213													
[10]	0,187	0,155	0,244	0,151	0,223	0,025	0,152	0,181	0,214												
[11]	0,193	0,155	0,246	0,157	0,227	0,002	0,151	0,186	0,218	0,024											
[12]	0,076	0,170	0,263	0,197	0,215	0,197	0,193	0,106	0,210	0,185	0,197										
[13]	0,031	0,176	0,257	0,185	0,224	0,195	0,185	0,101	0,214	0,189	0,192	0,084									
[14]	0,190	0,144	0,234	0,098	0,203	0,148	0,016	0,196	0,199	0,148	0,148	0,198	0,187								
[15]	0,089	0,173	0,241	0,186	0,210	0,185	0,168	0,099	0,202	0,178	0,185	0,078	0,088	0,173							
[16]	0,091	0,168	0,248	0,192	0,232	0,184	0,187	0,105	0,221	0,181	0,181	0,072	0,086	0,196	0,080						
[17]	0,190	0,160	0,246	0,131	0,212	0,075	0,136	0,191	0,203	0,067	0,075	0,198	0,195	0,133	0,187	0,189					
[18]	0,255	0,234	0,059	0,255	0,245	0,258	0,249	0,261	0,236	0,260	0,256	0,258	0,249	0,249	0,240	0,243	0,249				
[19]	0,267	0,236	0,051	0,254	0,252	0,252	0,250	0,266	0,247	0,254	0,251	0,259	0,262	0,252	0,251	0,243	0,249	0,044			
[20]	0,170	0,049	0,225	0,151	0,194	0,148	0,138	0,167	0,187	0,145	0,144	0,176	0,170	0,137	0,158	0,155	0,150	0,236	0,239		
[21]	0,180	0,141	0,250	0,007	0,202	0,154	0,096	0,208	0,195	0,149	0,154	0,194	0,185	0,097	0,183	0,189	0,129	0,249	0,248	0,151	

1: *Maculabatis gerrardi* North Sumatra; 2: *Rhinoptera javanica* North Sumatra; 3: *Rhinobatos jimbarensis* North Sumatra; 4: *Taeniura lymma* North Sumatra; 5: *Gymnura poecilura* North Sumatra; 6: *Hemitrygon bennetti* North Sumatra; 7: *Neotrygon kuhli* North Sumatra; 8: *Brevitrygon heterura* North Sumatra; 9: KJ617038 *Gymnura poecilura;* 10: KC526959 *Hemitrygon akeji;* 11: KC633222 *Hemitrygon bennetti;* 12: KR019776 *Himantura uarnak;* 13: KP091437 *Maculabatis gerrardi;* 14: KR019777 *Neotrygon kuhlii;* 15: KP727646 *Pateobatis hortlei;* 16: KU873081 *Pateobatis jenkinsii;* 17: KJ641617 *Pteroplatytrygon violacea;* 18: KF534708 *Rhinobatos hynnicephalus;* 19: KJ140136 *Rhinobatos schlegelii;* 20: KX151652 *Rhinoptera bonasus;* 21: KM881715 *Taeniura lymma*

16S DNA sequences only identified this species as *Neotrygon kuhli.*

The Maximum Likelihood tree constructed under General Time Reversible with 5 categories of Gamma Distribution of all 8 samples of North Sumatra and 13 reference species from NCBI (Table 1 and 2.) is provided in Figure 1 for tree based on *12S rRNA* and Figure 2 for tree based on *16S rRNA*. Most batoid species samples from North Sumatra were clustered together in both trees with the same species taken from NCBI. All of these clusters had a very high bootstrap percentage values (more than 90%). Moreover, all batoid species within the same genera also clustered together in both phylogenetic trees.

DNA barcoding is a method in identifying species of sample/specimen by using short, standardized gene regions as internal species tags (Hebert *et al*., 2003). The short and standardized gene was called DNA barcode. In the identification process, DNA barcode from specimen/sample was matched with the existing DNA barcode in the database or with DNA barcode from material voucher specimen, then continued with conducting genetic distance analysis (Hebert *et al*., 2003). Successful or unsuccessful identification process until species level using DNA barcoding is influenced by the availability of comparable conspecific targeted gene reference sequence data either from those that already saved in database (i.e. GenBank) or that was obtained from material voucher specimen. In this study, several unsuccessful identified samples due to the conspecific targeted gene reference sequence were unavailable. There was no reference sequence of both 12S and 16S rRNA gene for *Brevitrygon heterura* and *Rhinobatos jimbarensis*, and for *Rhinoptera javanica* in the 16S rRNA reference. Sequence data of these three species in the database (GenBank) was very limited, up to now, only 5 *COI* gene fragment sequence data of *Brevitrygon heterura*, 2 *COI* gene fragment sequence data of *Rhinobatos jimbarensis,* and 23 sequence data of *COI, 12S rRNA, ND2, ND4, POMC,* and several tRNA genes.

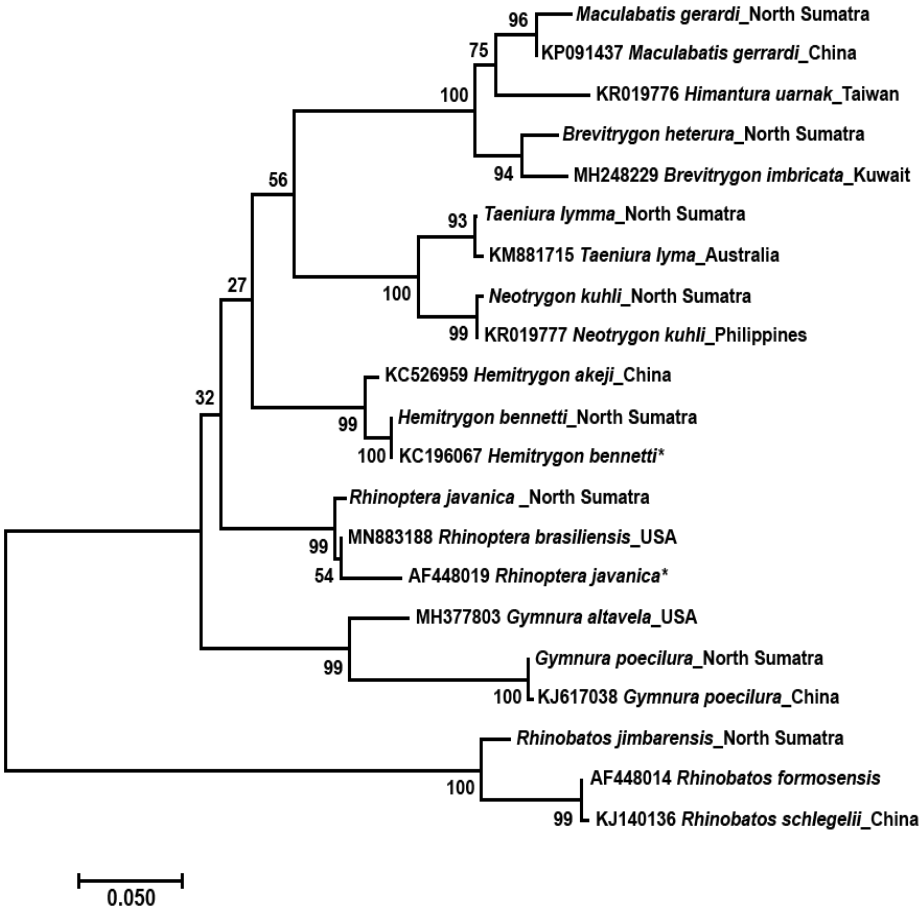

Figure 1. Phylogenetic tree of eight species of North Sumtra batoid fishes based on *12S rRNA* gene fragment, contructed using Maximum Likelihood method with General Time reversible model with Gamma distribution (5 categories) and 1000x bootstrap, *: location data unavailable.

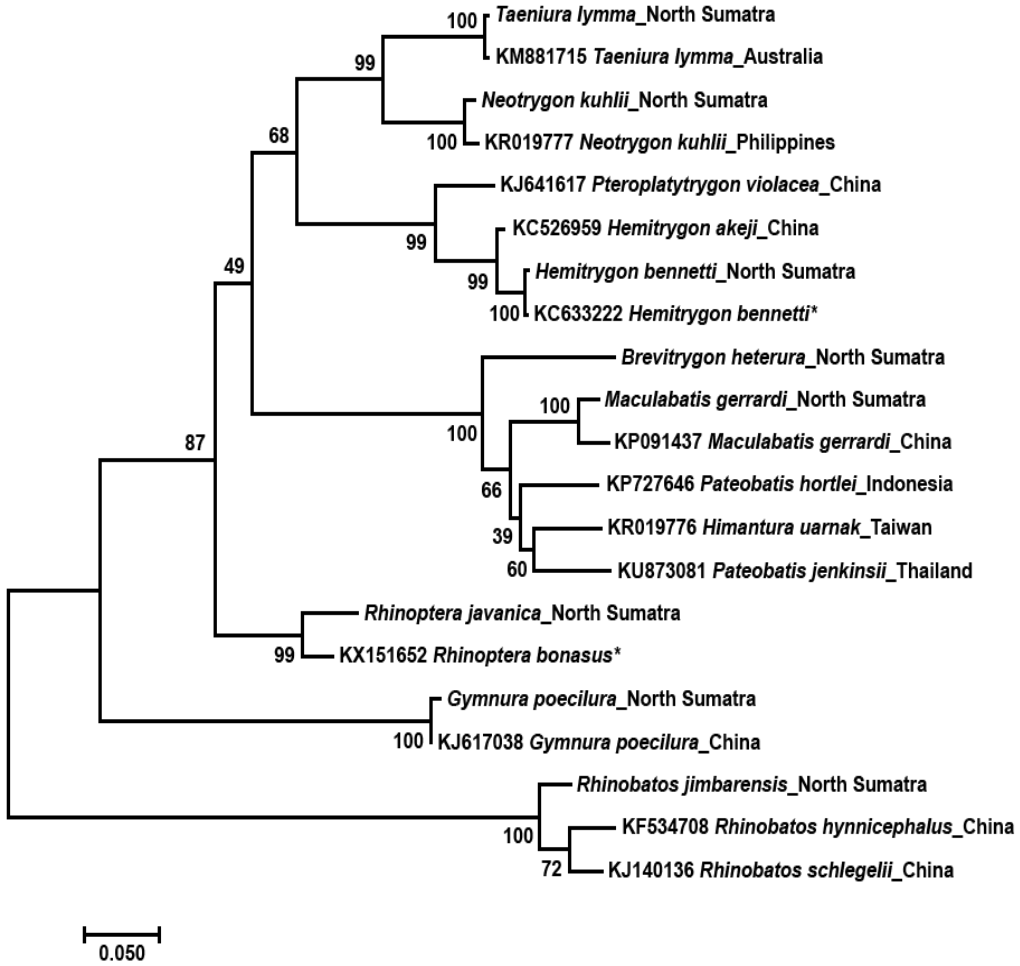

Figure 2. Phylogenetic tree of eight species of North Sumtra batoid fishes based on *16S rRNA* gene fragment, contructed using Maximum Likelihood method with General Time reversible model with Gamma distribution (5 categories) and 1000x bootstrap, *: location data unavailable.

The 12S rRNA gene reference sequence of *Rhinoptera javanica* was available in GenBank but the identification result was inaccurate due to *Rhinoptera javanica* sample was assigned to *Rhinoptera brasiliensis* based on *12S rRNA* gene fragment. This can be seen by observing the interspecific differences (0,035) was larger than its intraspecific difference with *Rhinoptera brasiliensis* (0,007), so it leads to false species assignment. This event might be occurred due the *12S rRNA* gene region that was used as barcode doesn't have sufficient mutation rate. The gene region that was used as "barcode" must have a sufficient mutation rate, which is slow enough to make intraspecific deference is minimised but fairly fast to emphasized interspecific deference, so this region can contain significant species-level genetic variety and divergence data (Hebert *et al*., 2003; Kress and Erickson, 2008)., selecting a certain gene region to be used as "barcode" is a crucial step. If the region that used as "barcode" does not contain significant species-level genetic variety and divergence data, the probability of producing an inaccurate and ambiguous result is higher. According to Nielsen and Matz (2006), false species assignment could occur due to 3 type of errors *i.e.* (1) the true species may not be represented in the database; (2) the random coalescence of lineages in populations and species may not necessarily lead the query sequence to be the most closely related to the true species sequence; (3) the random process at which mutations arise on lineages may cause the sequence representing another species to be more similar to the query.

Conclusion

FIve out of 8 batoid fishes species samples from North Sumatra were successfully identified

using the gene region of 12S and 16S rRNA mtDNA due to the conspecific reference targeted gene sequence are available in GenBank and the molecular identification results were matched with morphological identification results of our previous study. The 3 remaining species samples were unsuccessfully identified due to the conspecific reference targeted gene sequence was unavailable or its molecular identification result was inaccurate (false species assignment). If this study compare the identification result of these two genes, based on the availability of conspecific reference targeted gene sequence in the database, the gene region of 16S rRNA used in this research is more powerful to differentiate species than the 12S rRNA gene region, due to identification result based on 16S rRNA gene fragment was more accurate. Nevertheless, there is a need in exploring another region of both of these genes and or increasing the number of samples to get a more general view of the strength of both genes in differentiating one species from another.

Acknowledgement

This research was funded by Universitas Negeri Medan, Research Expertise Lecturer Group in 2019 budget year, No. 292D/UN33.8/PL/2019.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local aligment search tool. *J. Mol. Biol.,* 215(3): 403-410. doi: 10.1016/S0022-2836(05)80360-2.

Blanco, M., Perez-Martin, R.I. & Sotelo, C.G. 2008. Identification of shark species in seafood products by forensically informative nucleotide sequencing (FINS). *J. Agric. Food Chem.* 56: 9868–9874. doi: 10.1021/jf8015128.

Byun, S.O., Fang, Q., Zhou, H. & Hickford, J.G. 2009. An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Analytical Biochemistry*. 385(1): 174-5. doi: 10.1016/j.ab. 2008.10.024.

Compagnon, L.J.V. 1999. Batoid fishes. In: Carpenter KE (eds) The Living Marine Resourches of the Western Central Pasific, Vol 3: Batoid Fishes, Chimaeras and Bony Fishes Part 1 (Elopidae to Linophrynidae). FAO, Rome. 1397-2068 pp.

Dudgeon, C.L., Blower, D.C., Broderick, D., Giles, J.L., Holmes, B.J., Kashiwagi, T., Krück, N.C., Morgan, J.A.T., Tillett, B.J. & Ovenden, J.R. 2012. A review of the application of molecular genetics for fisheries management and conservation of sharks and rays. *J. Fish Biol.,* 80: 1789–1843. doi: 10.1111/j.1095-8649.2012.0 3265.x.

Fadhilah, A., Susetya, I.E. & Simeon, B.M. 2019. Elasmobranch catch composition of North Sumatera fishers. *IOP Conf. Ser. Earth Environ. Sci.,* 260 (2109): 1-7. doi: 10.1088/1755-1315/260/1/012109.

Felsenstein, J. 1985. Confidence limits on phylogenies: aAn approach using the bootstrap. *Evol.,* 39:783-791. doi: 10.2307/2408678.

Froese, R. & Pauly, D. Editors. 2020. Fishbase Hemitrygon bennettii (Müller &Henle, 1841). http://www.marinespecies.org/aphia.php?p=taxdetails&id=1042846 on 2020-10-21

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.,* 41: 95-98.

Hebert, P.D.N, Ratnasingham, S. & deWaard, J.R. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergence among closely related species. *Proc. R. Soc. Lond. B Biol. Sci.,* 270: S96-S99. doi: 10.1098/rsbl.2003.0025.

Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T.S & Francis, C.M. 2004. Identification of birds through DNA barcodes. *PLoS Biol*., 2(10): 1657-1663. doi: 10.1371/journal.pbio.0020312.

Hoelzel, A. 2001.Shark fishing in fin soup. *Conservation Genetics*. 2: 69-72. doi: 10.1023/A:1011590517389.

Holmes, B.H., Steinke, D. & Ward, R.D. 2009. Identification of shark and ray fins using DNA barcoding. *Fish. Res.,* 95: 280–288. doi: 10.1016/j.fishres.2008.09.036.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.,* 16:111-120. doi: 10.1007/bf0173 1581.

Kress, W.J, & Erickson, D.L. 2008. DNA barcodes: genes genomics, and bioinformatics. *Proc. Nat. Acad. Sci.* 105(8): 2761-2762. doi: 10.1073/pnas. 0800476105.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular evolutionary genetics analysis version

7.0 for bigger datasets. *Mol. Biol. Evol*., 33(7): 1870-4. doi: 10.1007/bf0173 1581.

Last, P.R., Naylor, G.J.P. & Manjaji-Matsumoto, B.M. 2016. A revised classification of the Family Dasyatidae (Chondrichthyes: Myliobatiformes) based on new morphological and molecular insights. *Zootaxa*. 4139(3): 345-368. doi: 10.11 646/zootaxa.4139.3.2.

Last, P.R., White, W.T. & Séret, B. 2016. Taxonomic status of maskrays of the *Neotrygon kuhlii* species complex (Myliobatoidei: Dasyatidae) with the description of three new species from the Indo-West Pacific. *Zootaxa.* 2016 Feb 23; 4083(4):533-61. doi: 10.11646/zootaxa.4083.4.5.

McEachran, J.D & de Calvarho MR. 2002. Batoid fishes. In: Carpenter KE (eds) The Living Marine Resourches of the Western Central Pasific, Vol 1: Introduction, Molluscs, Crustaceans, Hagfishes, Sharks, Batoid Fishes and Chimaeras. FAO, Rome. 507-599 pp.

Nei, M. & Kumar, S. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York.

Nielsen, R. & Matz, M. 2006. Statitistical approach for DNA barcoding. *Soc. Syst. Biol.*, 55(1): 162-169. doi: 10.1080/10635150500431239.

Pratama, O. 2020. Konservasi perairan sebagai upaya menjaga potensi kelautan dan perikanan Indonesia. https://kkp.go.id/djprl/artikel/21045-konservasi-perairan-sebagai-upaya-menjaga-potensi-kelautan-dan-perikanan-indonesia. (Accessed in March 2021)

Pratama, O. 2020. Konservasi perairan sebagai upaya menjaga potensi kelautan dan perikanan Indonesia. https://kkp.go.id/djprl/artikel/21045-konservasi-perairan-sebagai-upaya-menjaga-potensi-kelautan-dan-perikanan-indonesia. (Accessed in March 2021)

Puteri, D., Sitorus, H. & Muhtadi, A. 2017. Fish diversity at mangrove ecosystem of Jaring Halus Village in Langkat Regency, North Sumatera. *J. Ilmu-Ilmu Perairan, Pesisir dan Perikanan*. 6(2): 145-152. doi: 10.13170/depik.6.2.6656 .

Puteri, D., Sitorus, H. & Muhtadi, A. 2017. Fish diversity at mangrove ecosystem of Jaring Halus Village in Langkat Regency, North Sumatera. *J. Ilmu-Ilmu Perairan, Pesisir dan Perikanan*. 6(2): 145-152.

Rodrigues-Filho, L.F.dSD.S., da Rocha, T.C., do Rego, P.S., Schneider, H., Sampaio, I. & Vallinoto, M. 2009. Identification and phylogenetic inferences on stocks of sharks affected by the fishing industry off the Northern coast of Brazil. *Genet. Mol. Biol.,* 32: 405–413. doi: 10.1590/s1415-47572009005000039.

Sadili, D., Dharmadi, Fahmi, Sarmintohadi, Ramli, I. & Sudarsono. 2015. Rencana Aksi Nasional (RAN) Konservasi dan Pengelolaan Hiu dan Pari. Direktorat Konservasi dan Keanekaragaman Hayati Laut, Ditjen Pengelolaan Ruang Laut, Kementrian Kelautan dan Perikanan, Indonesia. www.kkp.go.id

Shen, Y.J., Guan, L.H., Wang, D.Q. & Gan, X.N. 2016. DNA barcoding and evaluation of genetic diversity in Cyprinidae fish in the midstream of the Yangtze River. *Ecol. Evol.,* 6(9): 2702-2713. doi: 10.1002/ece3.2060.

Spies, I.B., Gaichas, S., Stevenson, D.E., Orr, J.W. & Canino, M.F. 2006. DNA-based identification of Alaska skates (Amblyraja, Bathyraja and Raja: Rajidae) using cytochrome c oxidase subunit I (col) variation. *J. Fish Biol.,* 69: 283–292. doi: 10.1111/j.1095-8649.2006.01286.x.

Sudibyo, M., Lubis, K., Farajallah, A. & Hanim, N. 2020. Short communication: the mitochondrial cytochrome c oxidase subunit I (COI) for identification of batoids collected from landed sites in Medan, Indonesia. *Biodiversitas,* 21 (11): 5414-5421. doi: 10.13057/biodiv/d21114910. 13057/biodiv/d211149.

Tavaré, S. & Miura, R.M. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. *Lect. Math. Life Sci.*, 17: 57-86.

Tinti, F., Ungaro, N., Pasolini, P., De Panfilis, M., Garoia, F., Guarniero, I., Sabelli, B., Marano, G. & Piccinetti, C. 2003. Development of molecular and morphological markers to improve species-specific monitoring and systematics of Northeast Atlantic and Mediterranean skates (Rajiformes). *J. Exp. Mar. Biol. Ecol.,* 288: 149–165. doi: 10.1016/s0022-0981(03)00021-2.

Ward, R.D, Zemlak, T.S, Innes, B.H,, Last, P.R. & Hebert PDN. 2005. DNA barcoding Australia's fish species. *Philos. Trans. R. Soc.,* 360: 1847–1857. doi: 10.1098/rstb.2005.1716.

Ward, R.D., Holmes, B.H., White, W.T. & Last, P.R. 2008. DNA barcoding Australasian chondrichthyans: results and potential uses in conservation. *Mar. Freshw. Res.,* 59: 57–71. doi: 10.1071/mf07148.

Weigmann, S. 2016. Annotated checklist of the living sharks, batoids and chimeras (Chondrichthyes) of the world, with a focus on biogeographical diversity. *J. Fish Biol.,* 88: 837-1037. doi: 10.1111/jfb.12874.