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Short Communication: The mitochondrial cytochrome c oxidase subunit I (COI) for identification of batoids collected from landed sites in Medan, Indonesia

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Abstract. *Lubis K, Sudibyo M, Farajallah A, Hanim N. 2020. Short Communication: The mitochondrial cytochrome c oxidase subunit I (COI) for identification of batoids collected from landing sites in Medan, Indonesia. Biodiversitas 21: 5414-5421.* Batoids are member of Elsamoranch subclass which consist of many species. Most of batoids species are overexploited, especially in Medan Indonesia. Up to presents, the information about diversity of rays on the east coast of North Sumatra, Indonesia was very limited. Therefore, this research aimed to investigate the diversity of rays on the east coast of North Sumatra. We examined the morphological trait of 82 individuals of batoid from three landing sites on the east coast of North Sumatra, namely: Tanjung Balai, Belawan, and Percut, then identify its species based on determination key. After that, we collected pectoral muscle tissue from an individual in each species which successfully identified to extract its genomic DNA. Molecular based identification was carried out by using DNA fragment form *COI* gene. The successfully amplificated *COI* gene DNA fragment then was sequenced and analyzed. Based on morphological trait, we successfully identifying nine species of batoid, which is *Maculabatis gerrardi, Gymnura poecilura, Dasyatis zugei, Brevitrygon heterura, Neotrygon kuhlii, Hemitrygon bennettii, Rhinobatos jimbaranensis, Rhinoptera javanica,* and *Taeniura lymma.* The result of identification based on *COI* gene DNA fragment was in congruent with morphological-based identification based on data BLAST-N and genetic distance value within same species. The nucleotide diversity within same species ranged from 0-15 nucleotide variants.

Keywords: *COI* gene, rays, Medan, Indonesia

INTRODUCTION

Indonesian marine waters is known as having the richest diversity of Elasmobranch fishes and also as the center of diversity in the world. Several studies on Elasmobranch diversity had been conducted. A total of 207 species from 44 families of Chondrichthyans were found in Indonesian marine waters, which consist of 109 sharks, 96 batoids, and two ghost sharks (chimeras) (Fahmi 2010). Another study reported that at least 221 species of sharks and batoids found in Indonesian marine waters (Sadili et al. 2015). Recently, a study of Elasmobranch in the North Sumatra reported that eight species of sharks and batoids were caught and landed in Belawan and Batubara (Fadhilah et al. 2019).

Batoid is one of member of Elasmobrach subclass and closely related to sharks. This group consists of fishes that had moderately to greatly flattened body (dorsoventrally), ventral gill slit, enlarged pectoral fin, lack of anal fin, and its enlarged pectoral fin were fused to the side of the head and snout (Compagno 1999; McEachran and de Calvarho 2002). The body and conjoined of head enlarged pectoral fin and snout forming a disc, that delimited from its tail (Compagno 1999; McEachran and de Calvarho 2002). Batoid disk's shape was varied there are wedge-shape, circular, oval, and rhomboidal (Compagno 1999). Besides its shape, the size (length) of this group were varied, from 100 mm to more than 7000 mm (Compagno 1999). As of 7 November 2015, the number of batoid species in the world which has been reported was 630 species (Wiegmann 2016). Several species of batoid had been going through taxonomic review, due to new feature (morphology or molecular) were found, i.e. *Himantura gerrardi, Dasyatis bennetti, Himantura walga* had been under toxonomic review and now were called *Maculabatis gerrardi, Brevitrygon walga* and *Hemitrygon bennettii,* respectively (Last et al 2016; Froese and Pauly 2020).

Morphology-based identification methods were used to identifying species of certain organisms based on morphological features. This identification process was done by comparing the morphological features of sample with morphological features description in the determination key. While this method were of the fastest and cheapest approach, there is certain circumtances that we need to collect or exploit more biological data to successfully identify species of sample, such as specimen species that has similar morphological trait with other species from the same genus, cryptic species, immature specimen, incomplete specimen and hybrid species (Dudgeon et al. 2012). In this circumtances DNA-based identification were solution to collect or exploit more biological data from specimen. DNA-based identification

method or DNA barcoding is a method that used standardized DNA fragment sequence to identify species of an organism interest (Hebert *et al.* 2003a). The mitochondrial genome is accumulating high percentage of neutral mutations that can be helpful in species identification (Siddappa et al. 2013). Up to present, there is several DNA marker from mitochondrial DNA that was known can be used in DNA barcoding of fish, one of them is DNA fragmen from *COI* gene (Dudgeon et al. 2012). Due to commonly used as DNA marker in DNA barcoding (Dudgeon et al. 2012), the amount of data about *COI* gene in GenBank was huge (11680 data for shark and batoids) so we used DNA fragment from *COI* gene for this study.

Batoid fishes was known have high economic value such as it skin used for basic materials of shoes, begs and belts and also it oil, teeth and bones can also be used for medicinal ingredients and glue (Subrata et al. 2017). Based on fisherman explanation, batoid were popular good for expor (personal communication). Due to that, nowadays, batoid were overexploited (Sadili et al. 2015). While the situation is like that, up to present the information about batoid in Indonesia were limited especially molecular information. In North Sumatra the information about batoid that available is about diversity of batoid that inhabit marine water in there (Puteri et al. 2017; Fadhilah et al. 2019). Herein, the present study is devoted to species identification and diversity of batoids in the east coast of North Sumatra, Indonesia based on mitochondrial cytochrome c oxidase subunit I (COI) gene.

MATERIALS AND METHODS

Flesh sample collection and morphological identification

The present study was conducted from September 2019 to October 2019. A total of 82 individuals of batoid fishes, which caught by local fishermens in Malaka Strait area, at 3 landing site in the east coast of North Sumatra, namely Belawan, Tanjung Balai and Percut (Figure 1) were examined for its morphological features and were identified for its species based on White et al (2006). Small part of pectoral fin muscle tissue under the skin from one individual from each species, which has successfully identified, were collected. The muscle tissue sample, then, were placed in 15 mL tube, after that alcohol 96% were added until muscle tissue submerged.

Table 1. Reference DNA sequence of *COI* gene of batoids

Species	Accession number
Brevitrygon heterura	MG792099
Dasyatis zugei	EU398759
Dasyatis bennettii	KF604910
Himantura gerrardi	MH230945
Neotrygon kuhlii	KU497951
Taeniura lymma	KM881715
Gymnura poecilura	EU398804
Rhinoptera javanica	JF494383
Rhinobatos jimbaranensis	EU398994

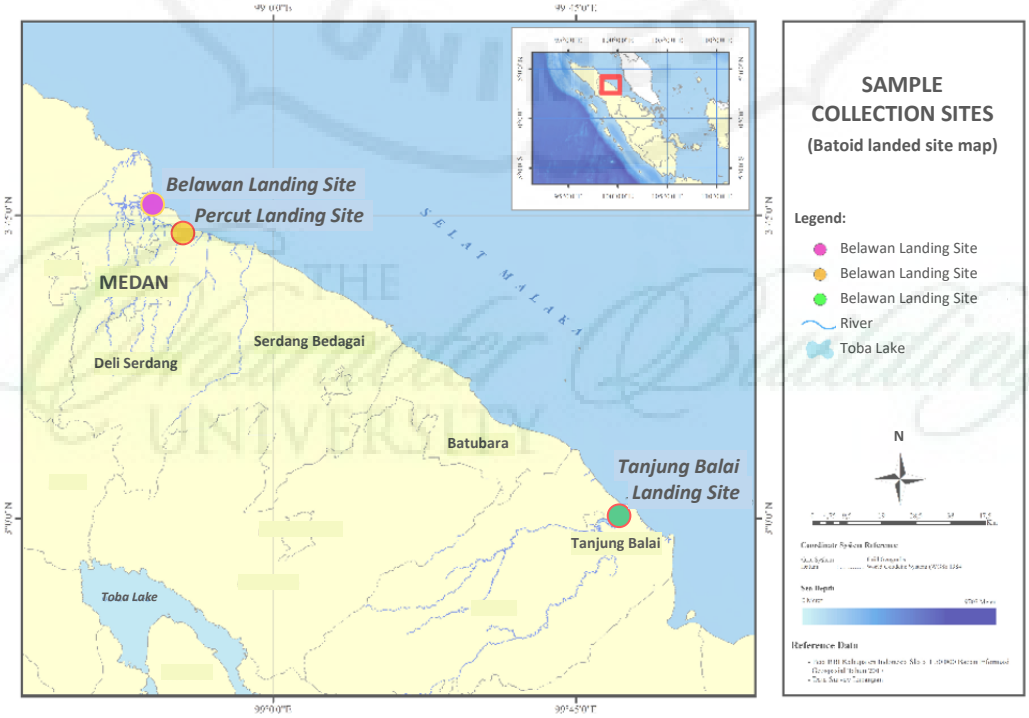

Figure 1. Map of three landed sites of batoids in east Sumatra coast of North Sumatra Province, Indonesia; purple circle is Belawan landing site, orange circle is Percut landing site and green circle is Tanjung Balai landing site

Genomic DNA extraction

Small parts from preserved samples were cut with a sterile surgical scissor, put in the 1.5 mL tube, then soaked in sterile water to remove the preserving liquid for 20-30 minutes. After that, the tubes containing small parts of sample were centrifuged 5000 rpm for 1 minute then the sterile water was removed. The tissue samples were cut to fine pieces in the tubes with a sterile surgical scissor. Then, genomic DNA extraction was carried out using DNA GENEAID Genomic DNA Mini Kit (Tissue) (Geneaid, Canada) and were done according to the protocol of the manufacturer.

Polymerase Chain Reaction (PCR) and Sequencing

The mitochondrial *COI* gene was amplified by polymerase chain reaction (PCR) method using the forward primer AF585 5'-ACCAACCACAAAGACATTGGCAC and the reverse primer AF586 5'-ACTTCTGGGTGGCCAAAG AATCA, which was modified from primer C_FishF1t1 5'-TGTAAAACGACGGCCAGTCGACTAATCATAAAGA TATCGGCAC and C_FishR1t1 5'-CAGGAAACAGCTAT GACACCTCAGGGTGTCCGAARAAYCARAA Ward et al. 2005). Amplification of *COI* gene fragment was conducted by using GoTaq Green Mastermix® DNA Polymerase (Promega) with the following steps: pre-denaturation for 3 minutes at 95°C, followed by denaturation, 30 cycles at 95 °C for 1 minute, annealing for 15 seconds at 52°C, and an extension for 10 minutes at 72°C. All products of PCR were separated using polyacrylamide gel electrophoresis and stained using silver staining method (Byun et al. 2009). These products of PCR were sent to 1st Base for DNA sequencing.

Molecular identification and species diversity analysis

The DNA sequencing products were displayed and edited using BioEdit 7.0.9.0 software (Hall 1999) and followed by aligning sequence from primer forward and reverse from the same sample using ClustalW which embedded in MEGA7 software (Kumar *et al.* 2016). After that, we combined/contig the two sequences to produce the *COI* gene DNA sequence that was ready to be analyzed further. To identify its species, firstly similarity analysis was conducted, using the GenBank BLAST-N program (Altschul *et al.* 1990) to compare *COI* gene DNA fragments from each sample with other mitochondrial DNA fragments in database. After we get the BLAST-N result, the top one sequence was downloaded and used as reference sequence (Table 1), then we aligned all *COI* gene DNA sequence from all sample with it using ClustalW which embedded in MEGA7 software using then we conduct genetic distance analysis with p-distance method to further identifying species of each sequence. Besides that, we also conducting nucleotide variation and construct phylogenetic tree. The phylogenetic tree was constructed using Neighbour-Joining for statistical methods (Saitou and Nei 1987)with Kimura 2-Parameter for subtitution model (Kimura 1980) and bootstrap method for test of phylogeny (Felsenstein 1985) with number of bootstraps was 1000. Neighbor-Joining method with nucleotide substitution model Kimura 2-parameter was used due to this option was the most used option to analyze the diversity pattern of *COI* gene (Hajibabei et al. 2006; Puillandre 2009)

RESULTS AND DISCUSSION

Results

Eighty-two batoids, which were examined its morphological features directly in the three landing sites, were successfully identified belonged to 2 ordo, 4 family, and 9 species (Table 2). Morphological features of the 9 species of batoid were presented in Table 3. Species diversity that we found in this study were different from the previous study of Fadhilah et al. (2019) which also took place in North Sumatra, around Malaka Strait. Fadhilah et al. (2019) only found 6 species of batoid while we found nine (3 species more). Only two species of batoid that were found both in this study and Fadhilah et al. (2019) that is *T. lymna* and *N. kuhlii*, while the other seven species were only found in this study. *Brevitrygon heterura* was batoid species with the most numbered individual which we found in these three landing sites (Belawan, Percut, and Tanjung Balai) and this species had different size of disc width (DW) and tail length (CL) with *B. walga* (*B.heterura* was species of *B. walga that* inhabit in Indonesian marine water) that was described by Simpfendorfer et al. (2017). The size of disc width (DW) and tail length (CL) of this species in our study are 17.14 ± 1.92 cm and 21.36 ± 4.87 respectively, while in Simpfendorfer et al. (2017) is 23-32 cm and 23-50.6 cm (1-2.2 times DW) respectively. We also found 1 individual of *Rhinobatos* jimbaranensis, which were previously reported by Last et al. (2006) as endemic species in Jimbaran Bay (Southern Bali).

Table 2. List of successfully identified batoid fishes and individual frequency of each species which found in three landed site

Order Family	Species	Number of individuals
Myliobatiformes		
Dasyatidae	*Brevitrygon heterura*	28
	Dasyatis zugei	6
	Hemitrygon bennettii	8
	Maculabatis gerrardi	4
	Neotrygon kuhlii	16
	Taeniura lymma	6
Gymnuridae	*Gymnura poecilura*	2
Myliobatidae	*Rhinoptera javanica*	11
Rajiformes		
Rhinobatidae	*Rhinobatos jimbaranensis*	1

Table 3. Morphological features of nine batoid species

Species	Qualitative morphological features	Quantitative morphological features				
		W (g)	TL (cm)	CL (cm)	DL (cm)	DW (cm)
Brevitrygon heterura	No skin fold on ventral surface of tail Profile of disc somewhat oval Tail short, not whip-like (end bulbous in adult females) Tail thorns very elongate, bases nearly half eye diameter in length Mid-disc thorns absent or rudimentary	187.86 ± 83.64	36.61 ± 5.72	21.36 ± 4.87	18.55 ± 2.29	17.14 ± 1.92
Dasyatis zugei	Low skin fold present on ventral surface of tail Tail not banded posterior to sting Snout extremely elongate Anterior margin of disc distinctly concave No oral papillae	255.00 ± 220.43	46.33 ± 12.74	27.17 ± 7.39	21.83 ± 5.67	19.25 ± 4.12
Hemitrygon bennettii	Skinfold present on ventral surface of tail The hind of tail to sting is not striped Very long tail Very small eyes Enlarged thorns were found on the middle of disk and along the tail Whip-like tail There are small denticles skin in the middle of dorsal Tail is covered by small thorn	460.00 ± 278.23	50.48 ± 8.49	31.16 ± 5.46	20.31 ± 4.63	25.06 ± 4.20
Maculabatis gerrardi	No skin fold on ventral surface of tail Profile of disc quadrangular Tail whip-like, with alternating light and dark bands Upper surface greyish brown with numerous white spots, variably distributed over disc (from almost plain to fully spotted)	375 ± 150	79 ± 6.78	62 ± 7.79	20.3 ± 1.71	21.4 ± 2.18
Neotrygon kuhlii	Low skin fold present on ventral surface of tail Low skin fold on dorsal tail beyond sting Tail with broad black and white bands, not whip-like Snout short with black bar through eyes Large, bright, blue spots on upper disc Usually no thorns on tail before sting	460.00 ± 278.23	50.48 ± 8.49	31.16 ± 5.46	20.31 ± 4.63	25.06 ± 4.20
Taeniura lymma	Ventral skin fold on tail moderately deep, extending to tail tip Profile of disc oval Upper surface with bright blue spots Stings present (usually 2), located near end of tail Tail with a long blue stripe extending alongside before sting	655.0 ± 68.63	61.2 ± 3.25	37.7 ± 2.34	27.0 ± 2.28	43.09 ± 3.14
Gymnura poecilura	Dorsal fin absent Tail lacking a sting, and with ~9 dark bands Dorsal surface usually plain, sometimes with faint pale spots	720	39	1845	24	45
Rhinoptera javanica	The snout strongly notched medially to form two lobes The tail is short The head is rather wide	1.315.45 ± 314.69	66.18 ± 6.52	40.45 ± 5.37	30.27 ± 2.83	43.09 ± 3.14
Rhinobatos jimbaranensis	Have dark spots on dorsal area, not white spot Lower lobe of the tail is short Denticles along the midline of dorsal area are not clear Slightly large nostril	1300	77	43	40	24

Note: W: weight; TL: Total Lenght; CL: Tail Lenght; DL: Disc Length; DW: Disc Width; g: gram; cm: centimeter.

Table 5. Genetic distance of *COI* gene fragment between nine batoid species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1																		
2	0.201																	
3	0.182	0.134																
4	0.146	0.191	0.174															
5	0.168	0.166	0.157	0.177														
6	0.190	0.163	0.149	0.180	0.128													
7	0.199	0.199	0.207	0.187	0.188	0.184												
8	0.179	0.191	0.184	0.161	0.165	0.172	0.212											
9	0.199	0.203	0.207	0.176	0.203	0.204	0.223	0.204										
10	0.000*	0.201	0.182	0.146	0.168	0.190	0.199	0.179	0.199									
11	0.204	0.003*	0.134	0.195	0.169	0.166	0.203	0.191	0.206	0.204								
12	0.184	0.136	0.002*	0.176	0.158	0.149	0.209	0.185	0.209	0.184	0.136							
13	0.142	0.193	0.176	0.003*	0.176	0.180	0.188	0.161	0.177	0.142	0.196	0.177						
14	0.166	0.168	0.158	0.176	0.002*	0.127	0.190	0.163	0.201	0.166	0.171	0.160	0.174					
15	0.185	0.163	0.152	0.176	0.128	0.006*	0.179	0.171	0.206	0.185	0.166	0.152	0.176	0.127				
16	0.199	0.199	0.207	0.187	0.188	0.184	0.000*	0.212	0.223	0.199	0.203	0.209	0.188	0.190	0.179			
17	0.185	0.191	0.180	0.168	0.168	0.166	0.210	0.006*	0.207	0.185	0.191	0.182	0.168	0.166	0.165	0.210		
18	0.207	0.204	0.198	0.182	0.206	0.201	0.222	0.195	0.024*	0.207	0.207	0.199	0.184	0.207	0.203	0.222	0.198	

Note: *: genetic distance between same species; 1. *B. heterura;* 2. *D. zugei;* 3. *Hemitrygon bennettii;* 4. *M. gerrardi;* 5. *N. kuhlii;* 6. *T. lymma;* 7. *G. poecilura;* 8. *Rhinoptera javanica;* 9. *Rhinobatos jimbaranensis;* 10. MG792099 *B. heterura*; 11. EU398759 *D. zugei*; 12. KF604910 *D. bennetti*; 13. MH230945 *Himantura gerrardi*; 14. KU497951 *N. kuhlii*; 15. KM881715 *T. lymma;* 16. EU398804 *G. poecilura*; 17. MG792069 *Rhinoptera javanica*; 18. EU398994 *Rhinobatos jimbaranensis*

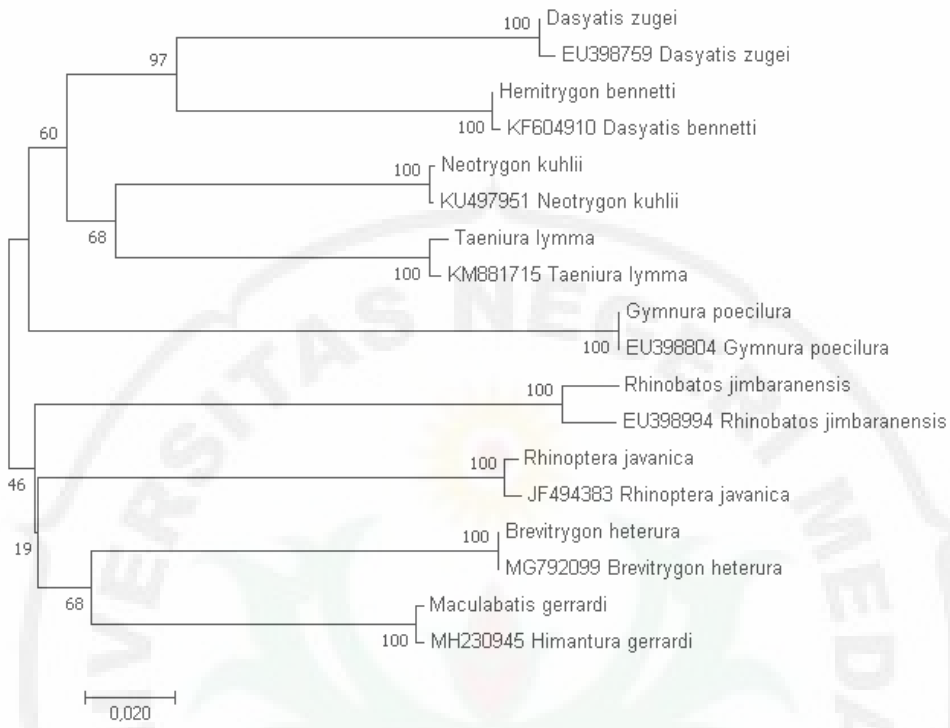

Figure 2. Phylogenetic tree of nine batoid species which found in three landed site based on *COI* gene fragment constructed using Neighbour-joining method with Kimura 2-Parameter model and 1000 bootstrap

Table 4. BLAST-N result of nine batoid species which found in three landed site

Organism species of sample sequence	Organism species of reference sequence	Accession Number	Query Cover	E-Value	Identity percentage
B.heterura	*Brevitrygon heterura*	MG792099	99%	0.0	100.00%
D. zugei	*Dasyatis zugei*	EU398759	96%	0.0	99.68%
Hemitrygon bennettii	*Dasyatis bennettii*	KF604910	99%	0.0	99.85%
M. gerrardi	*Himantura gerrardi*	MH230945	100%	0.0	99.69%
N. kuhlii	*Neotrygon kuhlii*	KU497951	100%	0.0	99.85%
T. lymma	*Taeniura lymma*	KM881715	100%	0.0	99.39%
G. poecilura	*Gymnura poecilura*	EU398804	99%	0.0	100.00%
Rhinoptera javanica	*Rhinoptera javanica*	JF494383	99%	0.0	99.39%
Rhinobatos jimbaranensis	*Rhinobatos jimbaranensis*	EU398994	99%	0.0	97.55%

Table 6. Nucleotide variant of COI gene fragment between same species

Sample >< Reference Sample	Total number of nucleotide variant
B. heterura >< MG792099 *B. heterura*	0
D. zugei >< EU398759 *D. zugei*	2
Hemitrygon bennettii >< KF604910 *D. bennetti*	1
M. gerrardi >< MH230945 *Himantura gerrardi*	2
N. kuhlii >< KU497951 *N. kuhlii*	1
T. lymma >< KM881715 *T. lymma*	4
G. poecilura >< EU398804 *G. poecilura*	0
Rhinoptera javanica >< MG792069 *Rhinoptera javanica*	4
Rhinobatos jimbaranensis>< EU398994 *Rhinobatos jimbaranensis*	15

Table 7. Batoidsconservation status by International Union for Conservation of Nature (IUCN) and trade status by Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 2019.

Species	IUCN	CITES
Rhinobatos jimbaranensis	Vulnerable	Not evaluated
G. poecilura	Near threatened	Not evaluated
Rhinoptera javanica	Vulnerable	Not evaluated
M. gerardi	Vulnerable	Not evaluated
T. lymma	Near threatened	Not evaluated
B. hetura	Not Evaluated	Not evaluated
D. zugei	Near threatened	Not evaluated
N. kuhlii	Data deficient (DD)	Not evaluated
Hemitrygon bennetti	Not Evaluated	Not evaluated

We successfully get *COI* gene fragment sequence from nine species of batoid which had been identified based on morphological features. The length of *COI* gene fragment after we conduct editing and combining primer forward and reverse sequence was 655 bp. By using this 655 bp *COI* gene fragment we conduct similarity analysis using BLAST-N, the result of this analysis can be seen in Table. 4. The result of BLAST-N in all sample sequences above 97 % in "Identity Percentage" value, the lowest was *Rhinobatos jimbaranensis* (97.55%) and the highest was *B. heterura* and *G. poecilura* (100.00%). After we aligning sequence sample and reference sequence, the length of *COI* fragment sequence which can be used in further analysis was 632 bp. Within same species, the range of genetic distance was 0.000-0.024, while between different species was more than 0.1. The lowest value of genetic distance (0.000) was found in *G. heterura* and *G. poecilura*, while the highest was in *Rhinobatos jimbaranensis*.

Our result in nucleotide diversity analysis showed that between same species, nine batoids species which we found, differ in 0-15 nucleotide (Table 6). The lowest number of variant nucleotides found in *B. heterura* and *G. poecilura* (0 nucleotide variant), while the highest (15 nucleotide variant) were found in *Rhinobatos jimbaranensis.* The phylogenetic tree showed that Dasyatidae was divided into two clades, one clade was consist of *B. heterura, M. gerrardi, Rhinoptera javanica*, and *Rhinobatos jimbaranensis*, and the other clade consist of *D. zugei, Hemitrygon bennettii, N. kuhli, T, Lymma* and *G. poecilura* (Figure 2). Even though Dyastidae divided into 2 clades, in each clade, every species of Dyastidae were forming smaller clade, separated from the other family. *Maculabatis gerrardi* were forming a small clade with *B. heterura* and *D. zugei* were forming a small clade with *Hemitrygon bennettii, N. kuhli*, and *T. lymma.*

Based on these results, we conclude that from 82 individuals we found in three landed sites (Tanjung Balai, Belawan, and Percut) were successfully identified belonged to nine species of batoid, both using morphological features identification and DNA identification using *COI* gene fragment. The mitochondrial cytochrome c oxidase subunit I (COI) gene identification could be used to evaluate batoid diversity, monitoring its conservation and fisheries management**.** This study about the use of DNA fragment to identifying batoid species needs further investigation by using another mitochondrial gene fragment to strengthening this finding. Furthermore, almost all batoid species which we found in these three landing sites its population is already.

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