

## COI Gene Analysis of Asian Horseshoe Crab in Banyuasin Estuarine Waters, Sumatra, Indonesia

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### ARTICLE INFO

#### Article history:

Received April 20, 2022

Received in revised form July 29, 2022

Accepted December 1, 2022

#### KEYWORDS:

Bottleneck,  
DNA barcoding,  
Haplotype diversity,  
Nucleotide diversity

### ABSTRACT

There is limited study on DNA barcoding of horseshoe crabs in South Sumatra, Indonesia. The present study was the first to record horseshoe crabs' COI DNA barcoding in three localities of Banyuasin estuarine waters. The nucleotide composition was in a strong adenine thymine bias (AT = 63.90%) with high haplotypic diversity ( $Hd = 0.858$ ) relative to low nucleotide diversity ( $\pi = 0.0534$ ). A total of 16 haplotypes were recorded, of which two were in Carat Cape, 4 in Makati Jaya, and 12 in the Banyuasin river estuary (BRE), and Hap 1 was a dominant type (62.5%). The population differentiation ( $F_{ST}$ ) value was varied, and only in the comparison of BRE-Carat Cape was significant ( $F_{ST} = 0.637$ ), while the gene flow ( $Nm$ ) value in Makati Jaya-BRE was high (6.563). The neutrality test, Tajima's  $D$  (0.4142), and Fu's  $F_s$  values were negative (-0.492), suggesting these populations have experienced a current bottleneck or multiplication. Analysis of molecular variation (AMOVA) suggested that 68% was distributed within populations. The haplotype network, PCoA, and phylogenetic of COI DNA sequences clustered in three groups correspond to *Carcinoscorpius rotundicauda*, *Tachypleus gigas*, and *Tachypleus tridentatus*. This finding is a matter of concern for managing and conserving horseshoe crab species in Banyuasin estuarine waters.

## 1. Introduction

Until now, there are four species of horseshoe crabs in the world. *Tachypleus tridentatus* (Leach), *Carcinoscorpius rotundicauda* (Latreille), and *Tachypleus gigas* (Muller), known as Asian horseshoe crabs (Lee and Morton 2005), and *Limulus polyphemus* (Linnaeus) that can be found in the Atlantic coast of North America (Walls *et al.* 2002). The existence of *C. rotundicauda* and *T. gigas* as two Asian horseshoe crabs is declared Data Deficient by the IUCN Red List (Center for World Conservation Monitoring 1996a, 1996b), while *T. tridentatus* is threatened with extinction (Laurie *et al.* 2019).

In Indonesia, the distribution of horseshoe crabs can be found on Sumatera Island (Anggraini *et al.* 2017; Fauziyah *et al.* 2019a), Java Island (Meilana *et*

*al.* 2016; Meilana and Fang 2020; Silver *et al.* 2017), Kalimantan Island (Erwyansyah *et al.* 2018), and Sulawesi Island (Meilana and Fang 2020). Recently, it was reported that the populations of horseshoe crabs in 62 districts from Java, Sumatra, Kalimantan, and Sulawesi Island are declining (Meilana and Fang 2020).

Studies related to horseshoe crabs have been carried out in various countries. However, in Indonesia, there are limited studies of horseshoe crabs based on COI DNA barcoding. The horseshoe crab research in Indonesia include food preferences (Nuraisah *et al.* 2020), biomaterial compounds, and bioactivity (Asih *et al.* 2018; Romadhon *et al.* 2018), morphometrics (Ahmad *et al.* 2017; Anggraini *et al.* 2017; Fauziyah *et al.* 2019b), environmental variability (Sari *et al.* 2020), biodiversity and distribution (Mashar *et al.* 2017; Rubiyanto 2012). Limited studies about COI DNA barcoding, such as the COI gene of *T. gigas* from five locations in the

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northern coastal waters of Java (Meilana *et al.* 2016), and the phylogenetic tree of COI gene of *T. tridentatus* from Balikpapan, East Kalimantan revealed a cluster of *T. tridentatus* from Balikpapan in the same group with *T. tridentatus* from Malaysia.

In the Banyuasin estuarine waters of South Sumatera, the horseshoe crabs were found as discarded catch (Fauziyah *et al.* 2018), and the first investigation of these horseshoe crabs was identified using their morphometric measurement (Fauziyah *et al.* 2019b). However, there needs to be more information on horseshoe crab species regarding their DNA barcoding and haplotype diversity in Banyuasin estuarine waters. This study was the first record of DNA barcoding of horseshoe crabs in Banyuasin estuarine waters, South Sumatera, Indonesia. This study aims to investigate the variations of COI DNA sequences of horseshoe crabs and elucidate their population and genetic diversity along the Banyuasin estuarine waters and provide published COI DNA sequences retrieved from GenBank for phylogenetic comparison. The knowledge of the population genetic diversity of these species is essential to develop new insight into the management and conservation of endangered marine animals.

## 2. Materials and Methods

### 2.1. Materials

A total of 26 Asian horseshoe crabs (17 of *C. rotundicauda*, 6 of *T. gigas*, and 3 of *T. tridentatus*) were provided from the survey location (Figure 1). It was collected from 11 stations in Makarti Jaya, Banyuasin river estuary (BRE), and Carat Cape along the Banyuasin estuarine of South Sumatera Province (N 104.89'28.9" to 105.00'27.7" and E 02.26'09.5" to 02.29'87.7") which were conducted in July 2019.

### 2.2. DNA Sequencing

DNA was extracted from the blood of each specimen using DNeasy blood and tissue kit following manufacture protocol (Qiagen), and the barcode region of the mitochondrial COI gene was amplified using primer LCO1490F: GGCAACAATCATAAAGATATTGG and HCO2198R: TAACTTCAGGGTGACCAAAAAATCA (Folmer *et al.* 1994). COI PCR reactions with a total volume of 50  $\mu$ l were prepared using five  $\mu$ l of DNA template, 19  $\mu$ l ddH<sub>2</sub>O, 25  $\mu$ l of Mix (MyTaq DNA Polymerase), and one  $\mu$ l of combined primer (10  $\mu$ M). The PCR amplification was generated using the Biorad

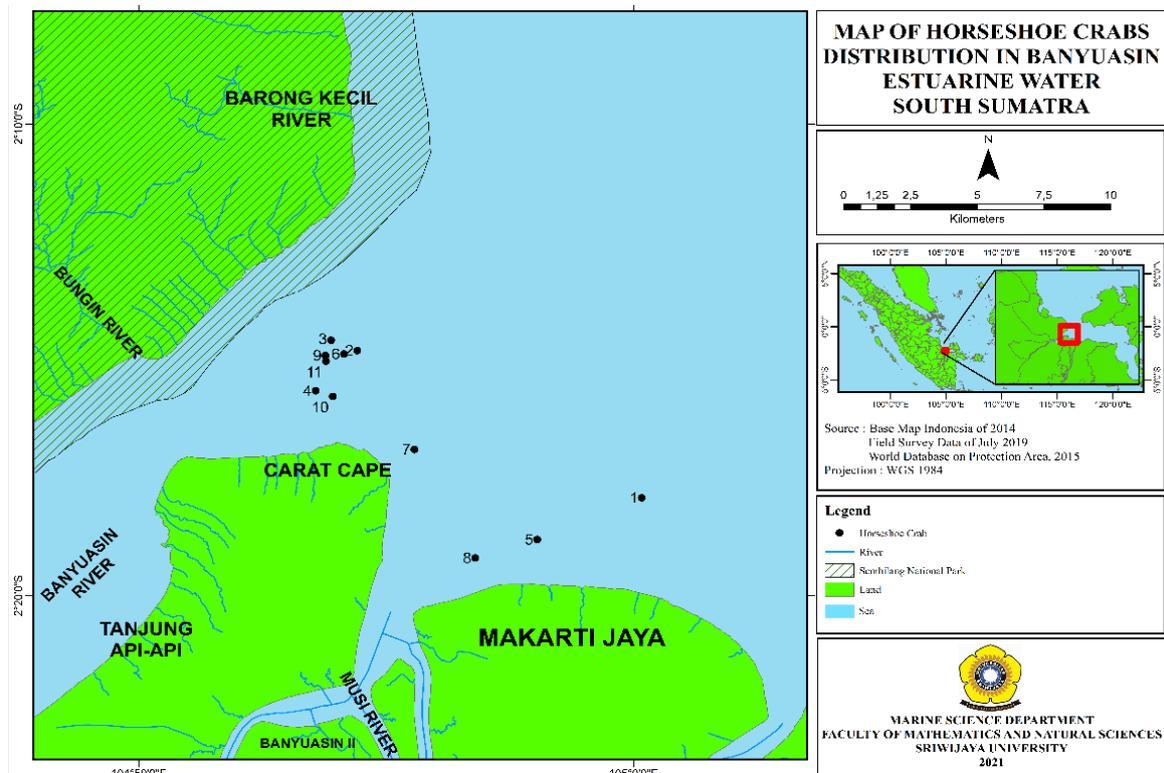


Figure 1. Sampling location of horseshoe crab in 11 stations in Banyuasin estuarine waters, South Sumatera

Thermal Cycler PCR machine by following PCR conditions: 95°C denaturations for 5 minutes at one cycle, 94°C denaturations for 30 seconds at 35 cycles, 55°C for 30 seconds for annealing temperature, and 72°C for 30 seconds for extension, with 72°C for 10 minutes for a final extension and the PCR products were maintained at 4°C. The PCR products were assessed on 2% agarose gel electrophoresis and visualized using gel documentation to ensure the size. The PCR products were sent to 1st Base (Singapore Science Park II, Singapore) for purification and DNA sequencing in both directions.

### 2.3. Data Analysis

The COI sequences were constructed into a contig sequence and then submitted to NCBI BLAST to determine the homology level (<https://blast.ncbi.nlm.nih.gov/>). The COI sequences were aligned by ClustalW and the phylogenetic tree was created using maximum likelihood (ML) with a Kimura two-parameter model using (Kumar *et al.* 2018). The nucleotide diversity ( $\pi$ ) and haplotype diversity ( $h$ ) were measured as a reflection of gene diversity for each population. Neutrality tests (Tajima's  $D$  test and Fu's  $F_s$ ) were calculated using MEGA software (Librado and Rozas 2009). The relationships among the COI haplotypes were examined by Median-Joining Network (MJN) analysis. The analysis of molecular variances (AMOVA) was used to examine the differences in genetic structure among or within

populations. Principal Coordinate Analysis (PCoA) and pairwise genetic divergence between populations of  $F_{st}$  values and gene flow ( $N_m$ ) were calculated using GenAlex 6.41. (Peakall and Smouse 2006).

## 3. Results

### 3.1. Haplotype Diversity and Distribution

Amplification of horseshoe crab DNA generated around 600-700 bp of PCR product of COI gene sequence from 26 horseshoe crab at three locations acrossed Banyuasin estuarine waters was used for investigation. The nucleotide arrangement of COI DNA sequence in horseshoe crab was A for 30.0%, T for 33.9%, C for 20.1%, and G for 16%. The nucleotide arrangement of the COI DNA sequences revealed that the content of AT (63.90%) was higher than the content of GC (36.2%).

Totally, it was obtained 43 variable sites which engaged 0.934%, containing 9 singletons and 34 parsimoniously informative sites, of which 31 transitions (Ts's) and 12 transversions (Tv's) without inversions/deletions. Sixteen haplotypes were assigned from all 26 fragments of COI gene sequences of 3 localities in Banyuasin estuarine waters (2, 4, and 12 haplotypes found in Carat Cape, Makarti Jaya, and BRE, respectively). Nonetheless, fifteen out of sixteen haplotypes (93.75%) were obtained only one time (Table 1). The haplotype distribution of Hap 1 was the prominent type

Table 1. The variation of nucleotide position from sixteen haplotypes of the horseshoe crab of COI DNA sequence in Banyuasin estuarine waters

Nucleotide position	Haplotypes (Hap)																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
63	1	A	T	.	.	T	.	T	T	.	T	T	.	.	T	.	T
66	2	G	.	.	.	T	.	T	T	.	T	.	.	.	.	.	T
69	3	T	.	.	.	C	.	C	C	.	C	.	.	.	.	.	C
72	4	C	.	.	.	T	.	T	T	.	T	.	.	.	.	.	T
81	5	T	.	.	.	C	.	C	C	.	C	C	.	.	.	.	C
82	6	C	.	.	.	T	T	T	T	.	T	T	.	.	.	.	T
103	7	A	C	.	.	C	.	C	C	.	C	T	.	.	C	.	C
112	8	A	.	.	.	C	.	C	C	.	C	.	.	.	.	.	C
117	9	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
119	10	A	.	.	.	G	.	G	G	.	.	.	.	.	.	.	G
122	11	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
125	12	G	A	.	.	.	.	.	.	.	.	A	.	A	A	.	.
140	13	T	.	.	.	C	.	C	C	.	C	.	.	.	.	.	C
149	14	C	T	.	.	T	.	T	T	.	T	T	.	.	T	.	T
152	15	A	.	.	.	T	.	T	T	.	T	.	.	.	.	.	T
161	16	C	.	.	.	T	.	T	T	.	T	.	.	.	.	.	T
162	17	G	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
167	18	T	.	.	.	C	.	C	C	.	C	.	.	.	.	.	C

Table 1. Continued

Nucleotide position	Haplotypes (Hap)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
170	19	A	T	.	.	.	.	.	.	.	C	.	.	C	.	.
180	20	C	.	.	.	T	.	T	T	.	T	.	.	T	.	T
185	21	T	C	.	C	.	.	.	.	C	.	.	.	C	.	.
195	22	A	.	.	.	T	.	T	T	.	T	.	.	.	.	T
198	23	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.
199	24	A	.	.	.	G	.	G	.	.	.	.	.	.	.	G
211	25	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.
218	26	A	.	.	.	T	.	T	T	.	T	.	.	.	.	T
222	27	C	.	.	.	T	.	T	T	.	T	.	.	.	.	T
231	28	T	.	.	.	C	.	C	C	.	C	.	.	.	.	C
234	29	C	A	.	.	A	.	A	A	.	A	T	T	.	A	T
241	30	T	.	.	.	C	.	C	C	.	C	.	.	.	.	C
255	31	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.
258	32	A	.	.	.	G	.	G	G	.	G	.	.	.	.	G
261	33	A	T	.	.	T	.	T	T	.	T	.	.	T	.	T
264	34	T	C	.	.	C	.	C	C	.	C	.	.	C	.	C
267	35	C	.	.	.	.	.	.	.	.	.	.	.	T	.	.
269	36	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.
278	37	A	.	.	.	.	.	.	.	.	.	.	.	C	.	C
280	38	C	T	T	.	T	T	T	T	.	T	T	T	.	.	T
282	39	T	.	.	C	.	.	.	.	.	.	.	.	C	.	.
289	40	T	.	.	.	.	.	.	.	.	.	.	.	.	.	G
298	41	C	T	T	.	.	.	.	.	.	.	.	.	T	.	.
307	42	T	.	.	.	C	.	C	C	.	C	.	.	.	.	C
310	43	C	.	.	.	T	.	T	T	.	T	.	.	.	.	T

remarked in Makarti Jaya and BRE, and its frequency was 62.5% from a total of 14 females and 12 males horseshoe crabs used in this study. Based on the species, the number of haplotypes found in *C. rotundicauda* was 8 haplotypes found in BRE, and Makarti Jaya, *T. gigas* was 5 haplotypes found in BRE, Makarti Jaya, and Carat Cape while *T. tridentatus* was 3 haplotypes found in BRE (Table 2).

The haplotypic diversity ( $h$ ) value was high in all three localities, ranging from 0.836 to 1.00. Otherwise, the nucleotide diversity ( $\pi$ ) value was low, ranging from 0.00153 to 0.07669. The mean of nucleotide diversity ( $\pi$ ) and haplotype diversity ( $h$ ) were 0.0534 and 0.858, respectively. The results of horseshoe crab populations showed that along with the BRE the value of Tajima's  $D$  (-0.7866) and Fu's  $F_s$  (-0.217) were negative. In contrast, Tajima's  $D$  (1.3552) and Fu's  $F_s$  (2.805) were positive, along with the Makarti Jaya and Carat Cape. The results for the whole data set of horseshoe crab populations along the Banyuasin estuarine waters showed that Tajima's  $D$  was 0.4142 while Fu's  $F_s$  was negative (-0.492) (Table 3). The results of horseshoe crab species based on haplotype network cluster revealed that the Fu's  $F_s$  (-5.569) values of the genetic group

2 of *C. rotundicauda* and Fu's  $F_s$  (-1.121) values of the genetic group 3 of *T. gigas* were negative. In contrast, Fu's  $F_s$  (1.272) of the genetic group 1 of *T. tridentatus* was positive (Table 4).

### 3.2. Population Genetic Structure

The value of population differentiation ( $F_{ST}$ ) varied from 0.037 to 0.637 and only the  $F_{ST}$  value in the comparison Banyuasin River estuary-Carat Cape (BRE-CC) was statistically significant ( $F_{ST} = 0.637$ ;  $P < 0.05$ ) while the gene flow ( $N_m$ ) value in Makarti Jaya-Banyuasin river estuary (MJ-BRE) was high (6.563) (Table 5). The AMOVA analysis revealed that 68% variance was shared among individuals or within populations yielding the  $F_{ST}$  value of 0.317 ( $P < 0.01$ ) (Table 6).

### 3.3. Population Genetic Analysis

The correlation between haplotypes was described on a median-joining network (MJN). The MJN examined 16 haplotypes network of the COI DNA sequences of horseshoe crab in Banyuasin estuarine waters. The MJN (Figure 2A) showed obvious haplotype clustering for *C. rotundicauda* (Hap1, Hap3, Hap4, Hap6, Hap9, Hap12, Hap13, Hap15), *T. gigas*

Table 2. The distribution of sixteen haplotypes in the fragment of 26 COI gene sequence at three localities of three species of horseshoe crab in Banyuasin estuarine waters

Haplotype (Hap)	Distribution of haplotypes						Total	Overall frequency
	Locations			Species				
	MJ	CC	BRE	Cr	Tg	Tt		
1	2		8	10			10	0.625
2			1			1	1	0.0625
3			1	1			1	0.0625
4	1			1			1	0.0625
5	1				1		1	0.0625
6			1	1			1	0.0625
7					1		1	0.0625
8		1	1		2		2	0.125
9		1	1	1			1	0.0625
10	1				1		1	0.0625
11			1			1	1	0.0625
12			1	1			1	0.0625
13			1	1			1	0.0625
14			1			1	1	0.0625
15			1	1			1	0.0625
16			1		1		1	0.0625
Total	5		19	17	6	3	26	
Female	2	2	10	7	5	2		
Male	3	2	9	10	1	1		

MJ = Makarti Jaya, CC = Carat Cape, BRE = Banyuasin River Estuary, Cr = *Carcinoscorpius rotundicauda*, Tg = *Tachypleus gigas*, Tt = *Tachypleus tridentatus*

Table 3. Genetic Diversity Statistics for Horseshoe crab in three localities in Banyuasin estuarine waters

Locations	n	S	Eta	h	Varh	$\pi$	ThetaNuc	k	ThetaG	Tajima D	Fu Fs
MJ	5	36	36	0.900	0.02592	0.07669	0.06496	20.400	17.2800	1.3552 <sup>ns</sup>	2.805
CC	2	1	1	1.000	0.25000	0.00153	0.00153	1.0000	1.0000	n.d.	0.000
BRE	19	42	46	0.836	0.00754	0.04210	0.05223	10.6082	13.1613	-0.7866 <sup>ns</sup>	-0.217
Overall	26	43	46	0.858	0.00453	0.05346	0.04822	13.3662	12.0546	0.4142 <sup>ns</sup>	-0.492

MJ = Makarti Jaya, CC = Carat Cape, BRE = Banyuasin River Estuary, S = number of segregating sites, n = sample size, Eta = number of mutations, h = haplotype diversity, Varh = haplotype variance,  $\pi$  = nucleotide diversity, ThetaNuc = estimated population mutation rate per site, k = average number of differences of nucleotide, ThetaG = estimated population mutation rate per sequence and Tajima D value and Fu Fs value for neutrality assessment

Table 4. Genetic diversity statistics for horseshoe crab species based on haplotype network cluster in Banyuasin estuarine waters

Population	n	S	Eta	h	Varh	$\pi$	ThetaNuc	k	ThetaG	Tajima D	Fu Fs
Cr	17	8	8	0.735	0.01374	0.00525	0.00880	1.4118	2.3664	-1.4267 <sup>ns</sup>	-5.569
Tg	6	9	9	0.933	0.01481	0.00753	0.00908	3.2667	3.9416	-1.0199 <sup>ns</sup>	-1.121
Tt	3	17	17	1.000	0.07407	0.03803	0.03803	11.3333	11.3333	n.d.	1.272

Cr = *Carcinoscorpius rotundicauda*, Tg = *Tachypleus gigas*, Tt = *Tachypleus tridentatus*

Table 5. FST and gene flow (Nm) values among three populations of horseshoe crab for a fragment of the COI gene

	Carat cape (CC)	Makarti jaya (MJ)	Banyuasin river estuary (BRE)
Carat Cape (CC)	-	0.710	0.143
Makarti Jaya (MJ)	0.260	-	6.563
Banyuasin river estuary (BRE)	0.637*	0.037	-

Nm values are above the diagonal, and the values of FST are under the diagonal (\*significant P<0.01)

Table 6. AMOVA analysis for horseshoe crab from three populations

Source	df	SS	MS	Est. Var.	%	$F_{ST}$	P
Among Populations	2	78.943	39.472	2.58	32	0.317	0.01
Among Individuals	23	255.211	11.096	5.548	68		
Within Individuals	26	0	0	0	0		
Total	51	334.154		8.128	100		

df = degree of freedom, SS = sum of square, MS = mean squared deviation, Est.Var = estimated of variance component, % = percentage of total variance contributed by each component,  $F_{ST}$  = population pairwise, P = probability

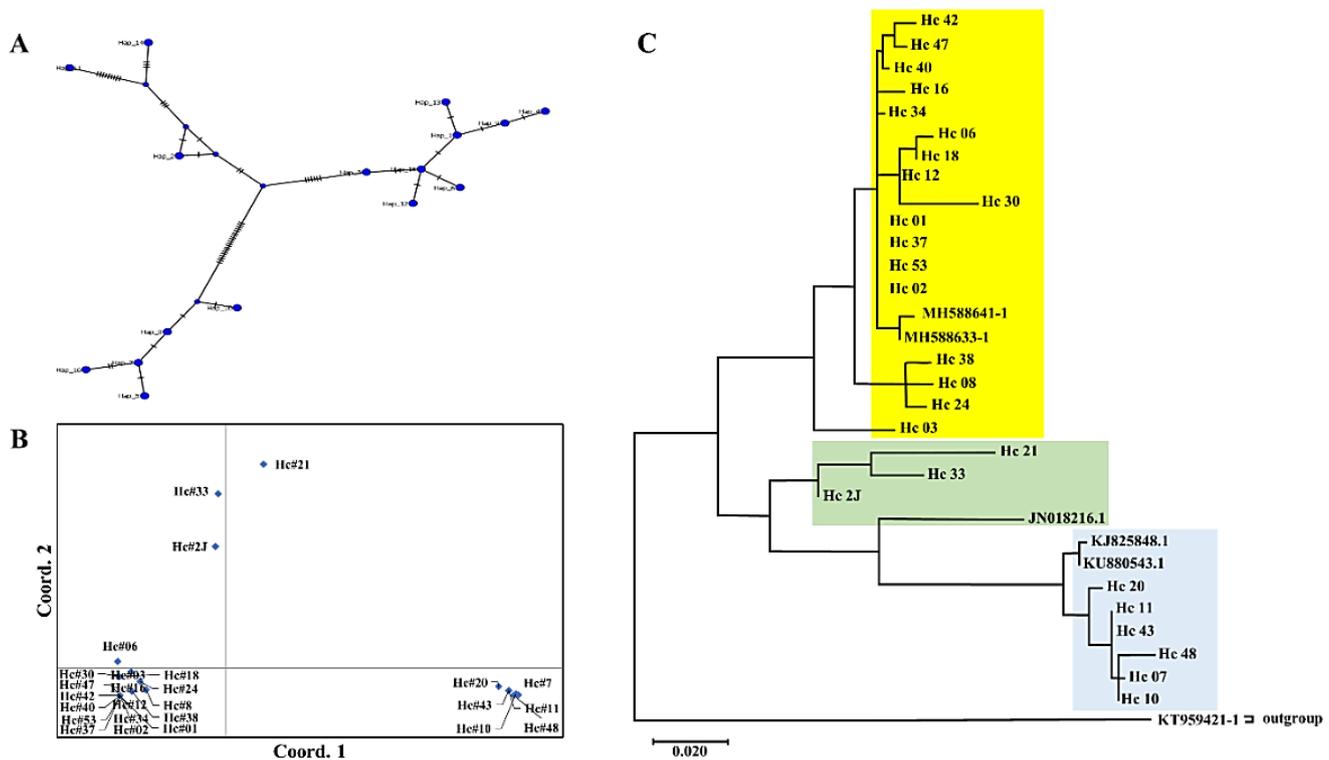


Figure 2. Haplotype network and clustering analysis depicting similarity of horseshoe crab in Banyuasir estuarine waters: (A) Haplotype network using median-joining network (MJN) of 16 haplotypes, (B) the PCoA chart of the genetic distances defined the variation percentage by Coord.1 (73.49%) and Coord.2 (16.10%), (C) phylogenetic tree constructed for horseshoe crab in Banyuasir estuarine waters and reference sequences according to the COI gene

(Hap5, Hap7, Hap8, Hap10, Hap16), and *T. tridentatus* (Hap2, Hap11, Hap14). The PCoA clustering displayed three groups of the horseshoe crab (Figure 2B). The upper points (group 1) represent the samples collected were *T. tridentatus* while the bottom left points (group 2) represent *C. rotundicauda* and the bottom right points (group 3) represent *T. gigas*. It was considered that the horseshoe crab populations were grouped by their species. The PCoA revealed that the genetics of group 2 (*C. rotundicauda*) and group 3 (*T. gigas*) turned out to be a self-contained groups. The COI phylogenies tree constructed using Maximum likelihood produced identical topologies (Figure 2C). The first group consisted of seventeen horseshoe crabs that correspond to *C.*

*rotundicauda* (MH588633, MH588641), the second group consisted of three horseshoe crabs that corresponded to *T. tridentatus* (JN018216) and the third group consisted of six horseshoe crabs that corresponded to *T. gigas* (KU880543, KJ825848), and one outgroup was *Limulus polyphemus* (KT959421).

#### 4. Discussion

The nucleotide arrangement of the COI DNA sequences had a strong adenine thymine bias, of which the content of AT was higher than the GC content. It was in agreement with Meilana *et al.* (2016) that 61% of AT content of *T. gigas* originated from five locations in Java. In this study, forty-three

variable sites were detected, 31 Ts's and 12 Tv's without inversions/deletions. Transversions were expected to interfere with Transcription Factor binding, generating more significant alteration in gene expression, and local divergence in the Ts: Tv ratios were representative of the evolutionary screening of genes (Guo *et al.* 2017).

Sixteen COI haplotypes were revealed from 26 individuals of 3 localities in Banyuasin estuarine waters designating that the new haplotypes have lacked numerous in these populations. This study's number of haplotypes was higher than the seven haplotypes obtained from three species of horseshoe crab from 10 locations in the northern coastal waters of Java (Meilana *et al.* 2016). In this study, Hap 1 was the dominant type of haplotype observed in BRE and Makarti Jaya. Interestingly, this study identified additional COI haplotypes in Carat Cape (Hap 7) despite the small sample size studied beside the common one (Hap 2), which was also detected in BRE. An additional compelling concomitant from this analysis was the lack of shared haplotypes in three-neighboring locations (BRE, Makarti Jaya, and Carat Cape) for *T. tridentatus*. This haplotype highlighted that BRE had its haplotype cluster corresponding to *T. tridentatus*.

The haplotype diversities of horseshoe crabs from the Carat Cape and Makarti Jaya are much higher than that of the BRE populations as the native regions suggesting that these two localities had been engaged several times by horseshoe crabs from BRE. The high mean haplotypic diversity concerning the low mean nucleotide diversity of horseshoe crabs in three localities of Banyuasin estuarine waters suggested that the populations had experienced a current bottleneck or multiplication by rapid population expansion and heap of mutations (Avice 2000). The horseshoe crab across the China shore revealed high haplotypic diversity and low nucleotide diversity, suggesting an intense assessment should be taken to preserve the scarce marine organisms (Weng *et al.* 2012).

The recapitulation statistics for examining drift-mutation equilibrium are based on the divergence from neutrality Fu' Fs and Tajima's D test. It designated that the populations that fell under genetic group 2 and group 3 could had been under the multiplication stage. In contrast, the value of Fu's Fs in genetic group 1 was positive, indicating that this population was under a population bottleneck.

Besides, the imbalance in abundance between males and females used in this study was thought to had existed when unpaired male horseshoe crabs congregated on the beach and acted as satellites to wait for their turn to spawn.

The significant population genetic differentiation (FST) in the BRE-CC populations that existed proposed that there was no intercrossing or that the populations were fixed. If populations were referred to as fixed, it means that they did not share any alleles. They were completely isolated from one another. Presumably, the strait's geographical constraint was apparently an essential point in restricting the gene flow among *T. tridentatus* populations. It was also realizable that BRE near TNS (Taman Nasional Sembilang) or Sembilang National Park (Desmiwati and Surati 2017; Pratama *et al.* 2017) had put a harsh quarantine investigation on the horseshoe crab to hinder human-mediated shipment with ongoing intensive traffic business. As a consequence, these *T. tridentatus* populations could have been thriving separately. *T. tridentatus* lived in the sea ground and traveled around to flat spots for propagation in the adult stage (Sekiguchi *et al.* 1988). After hatching, the larvae would settle, and the juvenile would feed near the beach (Chen *et al.* 2004; Sekiguchi *et al.* 1988). These features exhibited finite movement in larvae and juveniles but swam along with the sea flow in the adult stage. Besides that, the continuous travel across the boundary of traffic and expedition between BRE and neighboring localities (Makarti Jaya and Carat Cape) has been widely extensive. The geographic distance from BRE to Makarti Jaya was longer compared with Makarti Jaya to Carat Cape and BRE to Carat Cape. However, the gene flow (Nm) value in BRE-MJ existed on a large scale compared to MJ-CC and BRE-CC. The AMOVA analysis suggested that there was a superior level of the genetic framework among these three groups. It showed highly significant differences among individuals or within populations. This result indicated that considerable variability tends to retain in outbreeding species (Hamrick and Godt 1996).

The haplotype network revealed three groups of haplotype clusters. The first group of haplotype clusters in Makarti Jaya had genetic relatedness to BRE populations that corresponded to the same species, *T. tridentatus*, which means that it was the most common species found in both locations.

Meilana et al. (2016) mentioned that *T. tridentatus* was the most common species found in 10 locations in the northern coastal waters of Java, except in the Banten area. However, Banten waters had a depth of no more than 13 meters. They were muddy mixed with sand where *T. tridentatus* lived in brackish water with muddy substrates, usually in mangrove areas, and the distribution pattern of *T. tridentatus* was random (Rubiyanto 2012). *T. tridentatus* frequently occurs in high density in estuarine waters within close range of mangroves (Meilana and Fang 2020). This result indicated the ability of an individual to tolerate the availability of environmental resources (Cartwright-Taylor et al. 2009).

However, in this study the stations of BRE location were near the offshore waters, not in the muddy area, so, another possibility was that the *T. tridentatus* travel such a long distance. Thus, results in horseshoe crab with the lowest indices of body condition occurred in BRE reflecting an environmental condition closely offshore (Sari et al. 2020). Another possibility was that it was raised by artificial transport for frequently smuggled to other places for economic interests. The unlawful exportation and large-scale rearing of horseshoe crabs had become headlines in the newspaper of Banyuasin, Asahan, Langkat, and North Sumatra (Meilana and Fang 2020). Consequently, we assumed that the *T. tridentatus* populations in Makarti Jaya perhaps have derived from BRE.

Furthermore, the second group of haplotype clusters revealed that the horseshoe crab in Carat Cape and Makarti Jaya have genetic relatedness to BRE populations corresponding to the same species *T. gigas*. Therefore, it could be hypothesized that the *T. gigas* populations in Makarti Jaya and Carat Cape also presumably had their origin from BRE. While the third group of haplotype clusters corresponds to *T. tridentatus* found only in BRE as the native region.

The species phylogeny tree generated by 26 individual horseshoe crabs of COI DNA sequence from Banyuasin estuarine waters demonstrates that *T. tridentatus* and *T. gigas* were closely related but independent species differed from *T. tridentatus* and *L. polyphemus*. This was in line with a study in the coastal waters of Kuala Kemaman (Malaysia) which reported that the phylogenetic analysis of the mitochondrial genome showed that *T. tridentatus* was more closely related to *T. gigas* than to *T. tridentatus* (Sarmiento et al. 2021).

In conclusion, the variations in COI DNA sequences of the horseshoe crabs in Banyuasin estuarine waters revealed 16 unique haplotypes. The gene flow in Makarti Jaya-BRE was high indicating these populations had experienced a current bottleneck or multiplication. The horseshoe crab populations in BRE and the neighboring regions, such as Makarti Jaya was genetically close but well separated from the Carat Cape population. The haplotype network, PCoA, and phylogenetic of COI DNA sequences compared to published COI DNA sequences clustered in three groups correspond to *T. tridentatus*, *T. tridentatus*, and *T. gigas*.

### Acknowledgements

The authors thank the Indonesian Ministry of Research, Technology and Higher Education for providing the Applied Research Fund (Grant SP DIPA-042.06.1.401516/2019, 023.17.2.677515/2022 and No. 299/SP2H/LT/ DRPM/2021). The authors also would like to thank Annisa and Mushlihatun Baroya for their technical support.

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