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Haplotype Diversity and Population Subdivision of the Indian (*Tachypleus gigas*) Horseshoe Crab for monitoring conservation in Indonesia

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INTRODUCTION

Backgound

Gene flow cases often occurs in marine organism that have a dispersal across wide geographic ranges (Palumbi, 1994). Gene flow often precludes genetic subdivision so sampling species with high or intermediate dispersal abilities must be extensive (Lessios et al., 1998). Otherwise, structure of population can be can be separated by reason of genetic drift, strong post-settlement selection (Hedgecock, 1986) and spatial-landscape patterns (Johnson & Black, 1998; Watts & Johnson, 2004) as well as to a limited dispersal capability (Collin, 2001). Species with a limited dispersal capability are often composed of highly genetically structured populations with small geographic ranges, thus providing more opportunities to compare the depths and positions of intraspecific genetic with the locations as extrinsic factors (Bernardi & Talley, 2000).

Horsehoe crabs are one of the interesting groups of marine organism maintaining ther genetic structure virtually unchange over millions of years. These are an exotic aquatic biota and lives almost 500 million years. Horseshoe crab are also known as living fossil animals (Eldredge & Stanley, 1984). Horseshoe crabs are ancient marine arthropods exhibiting life-history and habitat preferences that might indicate a restricted dispersal capability (Sekiguchi, 1988). Generally, horseshoe crab classified as Atlantic horseshoe crab (*Limulus Polyphemus*) the only Atlantic species, inhabits the eastern coast of North America from Maine to Mexico (Rutecki et al., 2004; Walls et al., 2002). Three asian horseshoe crabs including *Carcinoscorpius rotundicauda*, *Tachypleus gigas*, and *Tachypleus tridentatus* (Lee & Morton, 2005; Sekiguchi & Shuster, 2009) are distributed sporadically from Southeast Asia to Japan. These can be found in Indonesian coastal waters, dispersion from Sumatra, Java, Kalimantan and Sulawesi (Rubiyanto, 2012; Mashar et al., 2017).

Throughout its life cycle, the horseshoe crab is highly dependent on environmental conditions in its coastal habitats. Adults spawn on the coarse sand near the high-tide zone and external fertilization during the breeding period. Juveniles inhabit the adjacent intertidal mudflats and gradually migrate to the deeper subtidal zone for maturation then come back to the natal beach for spawning (Sekiguchi, 1988; Chiu & Morton, 1999). The hatched trilobite larvae of *L. polyphemus* swim freely for a short period and settle to the bottom in shallow

waters in intertidal zone near their natal beaches (Shuster, 1982). In contrast, *C. rotundicauda* nests at the high-tide level of mangrove-penetrating tidal creeks just beyond the edges of terrestrial land (Cartwright-Taylor & Hsu, 2012). In addition, *C. rotundicauda* called mangrove horseshoe crab, is known to spend its life within mangrove swamps or sometimes moves to nearby deeper water but does not migrate to the sea (Davidson et al., 2008, Cartwright-Taylor et al., 2012). These life-history characteristics and habitat preferences suggest that the dispersal capability of horseshoe crabs might be restricted (Pierce et al., 2000).

Population genetic studies of horseshoe crabs have generally focused on *Limulus polyphemus* along the eastern coast of North America (Pierce et al., 2000; King et al., 2004). The existence of a genetic division between the Gulf of Mexico and Atlantic populations, a pattern observed for a variety of species (Avise, 2004) and microsatellites analysis (Saunders et al., 1986; King et al., 2004). In contrast, limited gene flow on small scales was reported for sequence variants of cytochrome oxidase I (COI) in the Chesapeake Bay and Delaware Bay (Pierce et al., 2000).

The Indian horseshoe crab was occupied in Southeast Asia to Japan (Sekiguchi, 1988). Most researchers suggest that the Asian horseshoe crabs are declining both locally and regionally. It is due to loss of suitable spawning grounds because of overharvesting for food and biomedical purposes and coastal development (Itow, 1993; Chiu & Morton, 1999; Botton, 2001; Chen et al., 2004). *Tachypleus gigas* was once relatively profuse along the northern of Java sea. It is now thought that the population of Indian horseshoe crab are unidentified based on the conservation status of these, data deficient (IUCN, 2015). Identification spesies (Meilana et al., 2016), dispersal analysis (Mashar et al., 2017), and population structure of *T. tridentatus* (Erwyansyah, 2018) have been done. This study examines population structure and gene flow in Indian horseshoe crab, *T. gigas* using mtDNA AT-rich region, which has proven to be a useful marker in intraspecific studies of some other arthropods (Brehm et al., 2001) in order to facilitate conservation efforts for this species.

In the present study, a nested hierarchical analysis was used to investigate the genetic structure of indian horseshoe crab, *Tachypleus gigas* in the coast of northern Java. A maternally inherited molecular marker, the mitochondrial AT-rich region, was used. Historical and recent perspectives on biogeography and population demography would support this

research. Genetic structure, gene flow, and the potential mechanisms (sea surface currents, demography, and others) creating any population division will be examined, allowing for recommendations of effective marine reserves establishment in the future for horseshoe crab conservation in Indonesia.

Objectives

Research objectives according to the project:

1. Contribute to update IUCN status of *Tachypleus gigas*, now IUCN status of this animal based on the IUCN red list is data deficient
2. Identify the haplotype diversity as the basic data to crosscheck Indonesia horseshoe crab biodiversity with other horseshoe crab from several countries
3. Determine the structure population and gene flow of horseshoe crab in each location
4. Arrange effective management for Indonesian horseshoe crab conservation

Expected output

The expected output of this research according to the project:

1. Contribution to update IUCN status of *Tachypleus gigas* from Indonesia
2. Data about haplotype diversity horseshoe crab (*Tachypleus gigas*) in Ujung Kulon, Bekasi, and Subang
3. Information about population structure and gene flow of horseshoe crab (*Tachypleus gigas*) in Ujung Kulon, Bekasi, and Subang
4. Effective plan management for Indonesian horseshoe crab (*Tachypleus gigas*) conservation
5. This research will be part of my PhD thesis

STATE OF THE ART OF THE RESEARCH

Horseshoe crab is an important component of the macro-benthos community in the coastal waters with fine sand or mud substrate, both in the tropics and in season four worldwide. Actually, horseshoe crabs are more closely related to spiders and scorpions than to

crabs. There are four species of horseshoe crab found around the world. *Limulus polyphemus* (Linnaeus, 1758) occurs along the Atlantic coast of North America, while *Tachypleus tridentatus* (Leach, 1819), *Tachypleus gigas* (Müller, 1785) and *Carcinoscorpius rotundicauda* (Latreille, 1802) found in Asia, from India to Japan and south to Malaysia and Indonesia (Cartwright-Taylor et al., 2011; Ismail et al., 2011; Ismail et al., 2012; Chatterji & Pati., 2014). The life cycle of the horseshoe crab is highly dependent on environmental conditions of the coastal zone (Sekiguchi &Sugita, 1980; Rudloe & Rudloe, 1981; Chen et al., 2004).

Population genetic studies of marine species have shown that, especially along continental margins, high dispersal potential is often associated with only mild genetic differentiation over large scales (Palumbi, 1992). These results suggest high levels of gene flow between populations. There may often be some mechanisms limiting the actual dispersal of marine species with high dispersal potential (Scheltema, 1986), such as isolation by distance, behavioural limits to dispersal, climate change, tectonism, sea-level fluctuations, coastal hydrography and geography, and natural and anthropogenic extirpations, and most of the time these complex mechanisms operated at once (Palumbi, 1994; Charlesworth, 2003; Edwards & Beerli, 2003).

Horseshoe crabs appeared to exhibit marked population subdivisions with mitochondrial COI marker (Pierce et al., 2000). Geographic subdivision of *L. polyphemus* populations along the northwestern Atlantic coast were defined as four to five clades of populations using microsatellite loci in a regional level (King et al., 2005). Another case genetic structure and haplotype diversity of Malaysian horseshoe crab (*Tachypleus gigas*) using mitochondrial DNA (AT rich region = 369 bp) along the west coast of Peninsular Malaysia (Roihan & Ismail 2011). More recently, population subdivisions were found in mitochondrial AT-rich region of *T. tridentatus* in relatively much smaller geographic scale (about 60 km in distance) and showed the sensitivity and potential of AT-rich region for phylogeographic study (Yang et al., 2007). The possible cause of subdivision of horseshoe crabs in above studies could be due to geographic restriction or isolation by distance.

By evaluating species with high dispersal capacities, patterns of genetic homogeneity could be used to establish upper limits of propagule exchange and connectivity on shorter, ecological times scales (i.e seasonal, annual) since genetic homogeneity can be sustained by

exchanging a limited number of individuals (Cowen, 2002). Limited dispersal capacities might reduce gene flow among populations, making them prone to subdivision, even when the distance among populations is small. Single-species models have been important in demonstrating how dispersal distance and the shape of the dispersal curve (Botsford et al., 2001), as well as adult mobility, influence reserve success (Gue´nette & Pitcher, 1999). Direct measurements of mean larval dispersal are needed to understand connectivity in a reserve system, but such measurements are extremely difficult. Genetic patterns of isolation by distance have the potential to add to direct measurement of larval dispersal distance and can help set the appropriate geographic scales on which marine reserve systems will function well (Palumbi, 2004).

The Asian horseshoe crabs are declining both locally and regionally. It is due to loss of suitable spawning grounds because of overharvesting for food and biomedical purposes and coastal development. Furthermore, the biomedical industry utilizes horseshoe crab blood improving the ability of pharmaceutical and medical device manufacturers to assure that their products are free of contaminating endotoxins (Walls, 2002). The population density of horseshoe crab was dramatically decrease from the wild. The serious decline resulted from various factors, including water pollution, loss of suitable spawning and nursing grounds, and overharvesting for food, biomedical, and chitin industry in China due to trade with Japan or the United States. Regarding to the IUCN status, there are two of three species that has data deficient status namely *C. rotundicauda* and *T. gigas.* The IUCN status of another species is Endangered (EN) for *T. tridentatus*. This status has been updated in April 2019.

In addition, horseshoe crab is used as an exclusive food in several countries such as Thailand, Malaysia and Indonesia. In Indonesia, horseshoe crab is also used as an iconic food which is highly sought on certain festival, such as *dugderan* festival in Central Java. The part horseshoe crab that used as food is the eggs from adult female. Based on this, the existence and sustainability of natural horseshoe crab population is very threatened. Another threat that is also a serious problem for the sustainability of yhis biodiversity in Indonesia is the case of smuggling horseshoe crab to several countries in Asia. The case was reported to occur more than once and the number of smuggled was no less than 6000 individuals. Evenly the type of smuggled is *T. gigas* or *T. tridentatus* because their blood component and eeg teste quality. Continuities of these problem will be very detrimental to the Indonesian horseshoe crab

biodiversity and conservasion. Moreover, scientific information for this animal in Indonesia is so limited that it has not been able to crosscheck and compare our Indonesian horseshoe crab with other horseshoe crabs from other countries.

Horseshoe crab's researchs in Indonesia that have been done are about aspect of embrio development (Rahmalia, 1995; Ciptono dan Harjana, 2015), reproductive biology (Eidman, 1997; Mulya, 2004; Muslihah, 2004), morphological character and molecular identification with COI gene marker of horseshoe crabs (*Tachypleus gigas*) at coastal waters of northern Java (Meilani et al., 2016), biodiversity and distribution of horseshoe crabs in northern coast of Java and Southern Coast of Madura (Mashar et al., 2017). The first investigation record of threatened horseshoe crabs in the Banyuasin estuarine, South Sumatra, Indonesia (Fauziyah et al., 2019); The morphometric variability of the mangrove horseshoe crab (*Carcinoscorpius rotundicauda*) from Banyuasin estuarine of South Sumatra, Indonesia (Fauziyah et al., 2019); Population and genetic structure of horseshoe crab (*Tachypleus tridentatus* Leach, 1819) as basic management in the water of Balikpapan coastal (Erwyansyah, 2018). Research about horseshoe crab in Indonesia still limited compared with China, Taiwan, United Stated, Japan, Malaysia and India. There is a lack informations about haplotype diversity of horseshoe crab in Indonesia especially *Tachypleus gigas* because analysis of genetic diversity *Tachypleus tridentatus* have done by Erwyansah (2018).

Research about horseshoe crab based on the haplotypes diversity and population subdivision is very important and highly relevant in conservation genetic for sustainability of horseshoe crab's populations in Indonesia. Success in conservation genetic and preservation existence of horseshoe crabs in Indonesian waters has its own strategic value for Indonesia around the world. This research is quite high complemented by molecular analysis with a high accuracy. The results of this study will have an important contribution in filling the lack of data or information about horseshoe crab according to the IUCN criteria *Tachypleus gigas* data deficient.

METHODS

Study Area and Sampling Technique

T. gigas was surveyed and random collected intertidally from sandy and muddy mudflats at northern of Java sea and east Borneo (Fig. 1). Horseshoe crabs will be collected

from fisherman or directly using hand. All blood samples of each individual were preserved in 100% ethanol immediately after collection. We will put the blood only 1 mL in each individuals then release back to the sea.

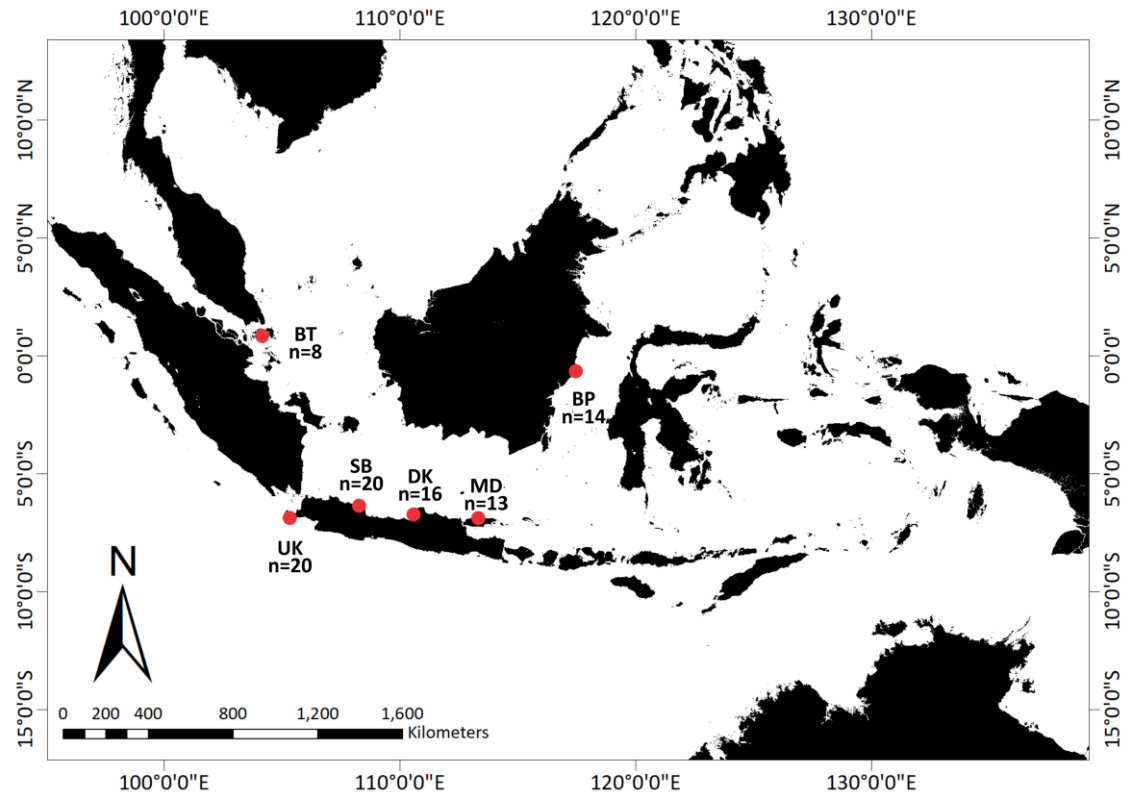

Figure 1 Sampling locations

Genomic DNA extraction, amplification, and DNA sequensing

Total genomic was extracted from hemolymph following the protocol of the GeneAiD extraction kit product. A fragment of AT-rich region was amplified using a pair of primer Hb-12S (5'-GTCTAACCGCGGTAGCTGGCAC-3') and Hb-trna (5'-GAGCCCAATAGCTTAA ATTAGCTTA-3') designed from mt genome of the Atlantic horseshoe crab (Lavrov et al., 2000). PCR reaction was made in total volume 25 µL including 12.5 µL MyTaq HS Red Mix, 9 µL ddH_2O, 1.25 µL forward and reverse primer, and 1 µL DNA template. All of mixture reaction amplified using polymerase chain reaction (PCR) thermocycler and the step following Yang et al. (2007) was pre denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, extension at 72°C for 2 min, and one cycle at 72°C for 2 min and 25°C for 5 min. The PCR product was visualized by gel electrophoresis 1 % agarose in TAE buffer ran with ethidium bromide at 100 V for 30 min. In

addition, to determain the band needs the UV light which indicated the presence of DNA fragment. DNA sequensing was done by 1st BASE, Malaysia.

Data analysis

Genetic diversity

A total of 91 AT-rich region sequences were obtained from the analysed hemolyphs. Alignment of the edited sequences was performed with MEGA X (Kumar et al., 2018) to generate a multiple alignment. The genetic diversity was measured by number of haplotype (Hn), haplotype diversity (Hd), nucleotide diversity (π) using DNASp v6 (Rozas et al., 2017).

Population strucutre

The population structure was indicated by Wright's fixation index (F_{ST}), gene flow (Nm) and analysis of molecular variance (AMOVA) using Arlequin v.3.5 program (Excoffier & Lischer, 2010), with a setting up 1000 permutations, α= 0.05 for significance level threshold, in order to determine the pattern of differentiation between locations. Pairwise F-statistic (F_{ST}) were calculated as genetic distance based on pairwise differences between population using DNASp v6 (Rozas et al., 2017). The value of F_{ST} is ranged from zero to one, F_{ST} close to zero indicated low level of genetic differentiation, while high level of genetic stucture is determined by the F_{ST} value close to one.

Population connectivity

Genetic connectivity among population were estimated using a median joining (MJ) network (Bandelt et al., 1999) calculated using Network v 4.6.1.0 based on haplotype data**.** Haplotype composition was obtained in all study areas then illustrated in an appropriate map to show the pattern of haplotype distribution and genetic connectivity among populations. In this study also performed the Tajima's *D and* Fu's F_S statistical tests were used to test the population equilibrium. Negative values of Tajima's *D* (Tajima, 1989) indicate population expansion and/or purifying selection, whereas positive values indicate a decrease in population size and/or balancing selection. Nevertheless the negative F_S values is indicative of a recent population expansion (Fu, 1997). Positive Fu's F_S values suggest a steady population. The history of effective population size was assessed through the pairwise mismatch distribution in Arlequin. The results will be reflecting the stochastic lineage loss. Unimodal of the result described the expansion population growth and a recent bottleneck effect while the multimodal suggests the equilibrium demografic condition or stationary population.

RESULTS

Genetic diversity

A total of 91 AT-rich sequences each with around 670 bp were obtained in all sampling locations including the sample from Java Island (UK, SB, DK and MD), Sumatera Island especially in Bintan and also Borneo (Balikpapan). Genetic diversity in this study was calculated and analysed based on the initially processes which was sequences alignment. Totally, there were 43 nucleotide sites and 34 haplotypes which were exposed. In general, haplotypes that were obtained consist of the uniqe (only found in certain location) and commond haplotype (Table 1). Genetic diversity of *T. gigas* in each sampling sites was varied in value (Table 2). Percentage of A+T composition in each locations was slightly different which was around 81%.

At a glance, haplotype diversity that obtained in this study was quite high ranging from $h = 0.7833$ to $h = 0.9451$ with a mean gene diversity per population of $h = 0.9353$. In contrast, nucleotide diversity was relatively low in all locations ranging between $\pi = 0.0049$ until $\pi = 0.0095$. Although overall diversity was similar among populations, haplotype diversity and nucleotide diversity were lowest for DK, followed by population in SB and UK. Haplotype diversity and nucleotide siversity was highest for BP followed by UK (h=0.9421 $\pi = 0.0054$), SB (h=0.9263 $\pi = 0.0052$), MD (h=0.9103 $\pi = 0.0066$), and BT (h=0.8929 $\pi = 0.0066$) whereas the lowest haplotype diversity was in DK (h=0.7833 π =0.0049) (Table 2).

Table 1 Variable sites found in a fragment of the AT-rich region of *Tachypleus gigas* in each populations. Fourty three variable sites were found in a fragment of the AT-rich region in 91 horseshoe crabs defining 34 haplotypes (H1–H34)

	Nucleotide positions																																									
	1	1	1	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	6	6	6	6		
	2	3	8	3	7	7	6	6	6	6	7	8	1	4	4	4	5	6	7	7	7	8	0	1	1	1	3	3	4	6	7	7	9	9	0	6	6	6	8	2	4	7	8
	5	2	3	5	4	8	0	1	6	9	4	2	3	2	3	4	9	6	2	3	4	6	1	3	4	5	0	7	6	7	2	7	1	2	2	3	6	7	9	0	7	2	5
H1	T	T	C	C	C	T	G	A	C	A	C	T	T	C	A	A	C	T	T	A	T	A	C	T	T	T	G	A	T	T	A	A	A	C	C	T	A	A	G	C	T	G	C
H2	C	C	.	.	.	C	A	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	C	.	.	.	T	.	.	.	.	.	A	A	.	.
H3	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
H5	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
H7	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	.
H8	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.
H9	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H10	C	C	T	.	T	C	A	G	.	.	.	C	.	T	G	T	.	.	.	.	C	.	T	.	C	C	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H11	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.
H12	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
H13	C	C	T	.	.	C	A	.	T	.	.	C	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	G	.	.	.	G	.	.	.	A	.	.
H14	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H15	C	C	.	.	T	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.
H16	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H17	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.
H18	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H19	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.
H20	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
H21	C	C	T	.	T	C	A	.	.	.	.	C	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H22	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	A	.	.
H23	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.
H24	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.
H25	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.
H26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
H27	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H28	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
H29	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.
H30	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H31	.	C	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.
H33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H34	.	C	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 2 Genetic diversity of *Tachypleus gigas* in each locations

Population	Code	A+T%	n	N*h*	*h*	π
Bintan	BT	81.5973	8	6	0.8929	0.0066
Balikpapan	BP	81.4734	14	10	0.9451	0.0095
Demak	DK	81.5688	16	6	0.7833	0.0049
Madura	DR	81.4125	13	8	0.9103	0.0066
Subang	SB	81.5482	20	11	0.9263	0.0052
Ujung Kulon	UK	81.4344	20	12	0.9421	0.0054
Total			91	34	0.9353	0,0064

Notes: n= number of samples; N*h*= number of haplotype; *h*= haplotype diversity; π= nucleotide diversity

Population structure

Results from pairwise F_{ST} value showed that all populations was low ranging from -0.01 to 0.13 (Table 3). Generally, the F_{ST} value among location was all not significantly different (p>0.05) except for the comparison between UK-MD and UK-SB which indicate the restricted gene flow among population. Amount of four populations that have a higher pairwise F_{ST} value than other populations, BT-MD (p>0.05), BT-SB (p>0.05), UK-MD (p<0.05), UK-SB (p<0.05). The negative value of pairwise F_{ST} were found in the comparison between UK-BT (F_{ST} = -0.01; p>0.05), DB-DK (F_{ST} = -0.02; p>0.05), SB-MD (F_{ST} = -0.01; p>0.05). Low level and negative value of F_{ST} indicates unsubdevided among populations and indication of gene flow phenomena. Additionally, the overall gene flow (Nm) estimated among populations was 6.71. A part from this, the analysis of molecular variation (AMOVA) also confirmed that the majority of variation was found within population (95.23%) rather than among population (4.77%) (Table 4). The mean F_{ST} was calculated as 0.04 (p-value = 0.0069) which indicate the low lavel of genetic differentiation.

Table 3 Pairwise F_{ST} between populations of *Tachypleus gigas* in six sampling locations

	BT	BP	DK	MD	SB	UK
BT	-					
BP	0.05	-				
DK	0.08	0.00	-			
MD	0.13	0.00	0.00	-		
SB	0.11	0.01	-0.02	-0.01	-	
UK	-0.01	0.08	0.09	0.10*	0.10*	-

Notes : F_{ST} value significantly different (p<0.05)* ns: not significant; BT= Bintan; BP=Balikpapan; DK= Demak; MD= Madura; SB= Subang; UK= Ujung Kulon

Table 4The analysis of molecular variation (AMOVA) that conducted based on the haplotype frequencies of *Tachypleus gigas*

Source of variation	d.f	Percentage of variation	F_{ST}	p-values
Among populations	5	4.77	0.04	0.0069
Within populations	85	95.23		
Total	90			

Population connectivity

Thirty four haplotypes were identified using Median-Joining Network of haplotypes created for all samples of *T. gigas* (Fig. 2). Network analysis showed existence of the dominant haplotypes (H1, H3, H5, H8, H9 and H18) which indicates an evolutionary link. Haplotype 3 was the most common haplotype, being identified in all populations except in UK and consist of amount 15 individuals. Similary, haplotype 5 was found in a total of 12 individuals from the BT, BP, DK, SB, and UK populations. On the other hands, there were specific haplotype which were only found in certain location. The highest number of uniqe haplotypes was in UK population whereas the lowest number was in BT. There were two uniqe haplotype (H2 and H4) in samples from BT, three (H14, H15, and H16) in samples from

DK, five (H6, H10, H11, H12 and H13) in samples from BP, five (H17, H19, H20, H21 and H22) in samples from MD, five (H23, H24, H25, H26 and H27) in samples from SB, and seven (H28, H29, H30, H31, H32, H33, and H34) in samples from UK. Regarding to the number of uniqe haplotypes, population of coastal horseshoe crab in UK population has the most number of uniqe haplotypes which were found only in 1 individual for each haplotype (Fig. 3).

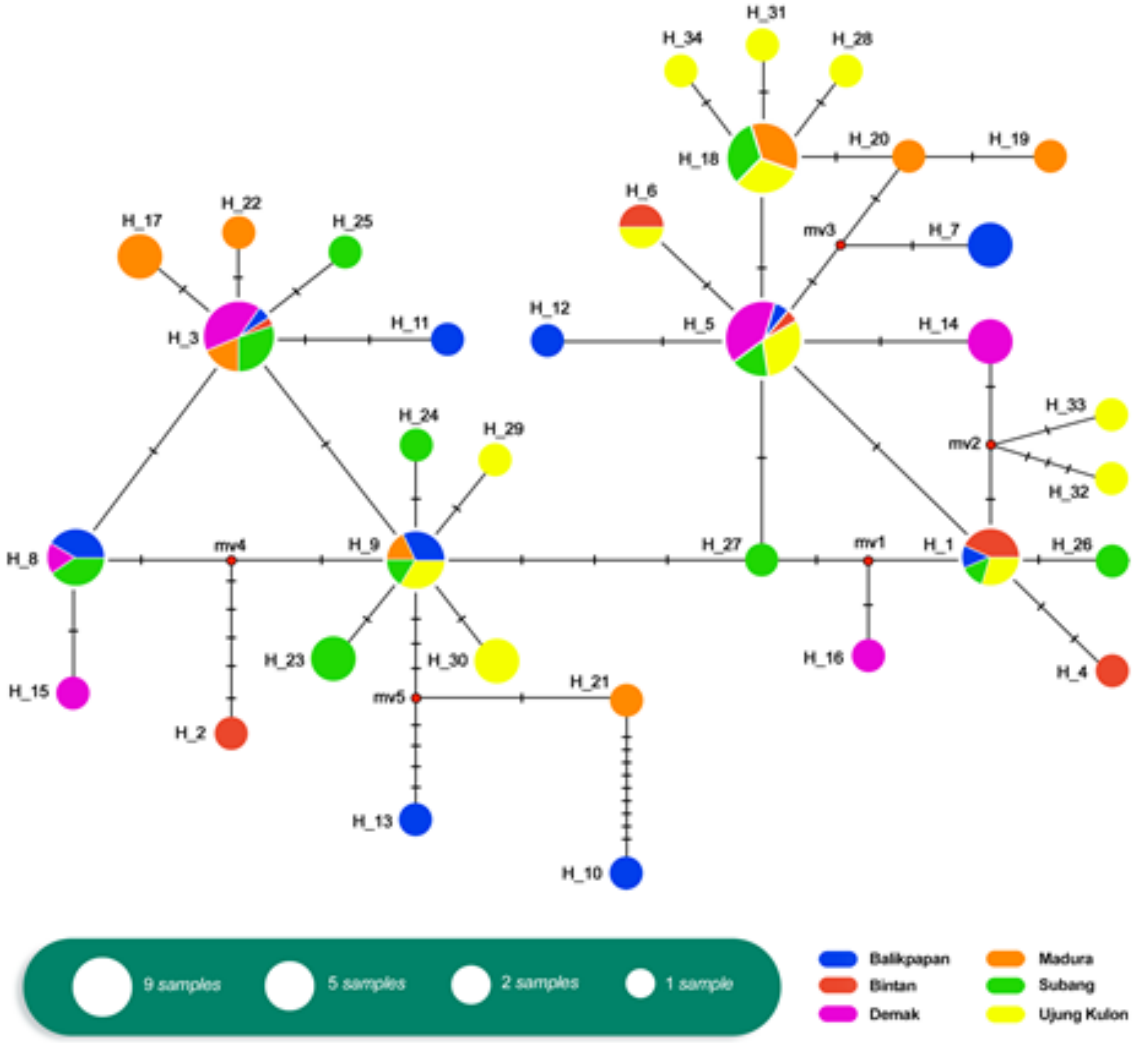

Figure 2 Network analysis based on the haplotype relationship of *Tachypleus gigas*

The historical demography was assased based on the mtDNA AT-rich region haplotype. Resulting of the haplotype network Based on the haplotype network analysis indicated that there was sharing haplotype in all location (Fig. 2). The value of the Tajima's *D* statistics in all

population were negative except in DK, MD and SB with not significant *p*-value, indicating no evidence of selection. Similary, the result of Fu's *F*s test was negative except in DK population with not significant *p*-values for all six populations observed, indicating an excess number of alleles as would be expected from a recent population expantion. However, the results of mismatch distribution expantion were measured to examine the historical of expantion in the past are contradictory. In generally, the mismatch distribution in all population illustrated the multimodal patterns which shown the stationary population or constant in size or no expantion historycal in all study sites. The graphs of the multimodal illustrations are shown in Fig. 4. Hence, based on the value of SSD between the observed and expected mismatch distributions were all statistically insignificant ($p > 0.10$), indicating the presence of non-equilibrium and a population expansion.

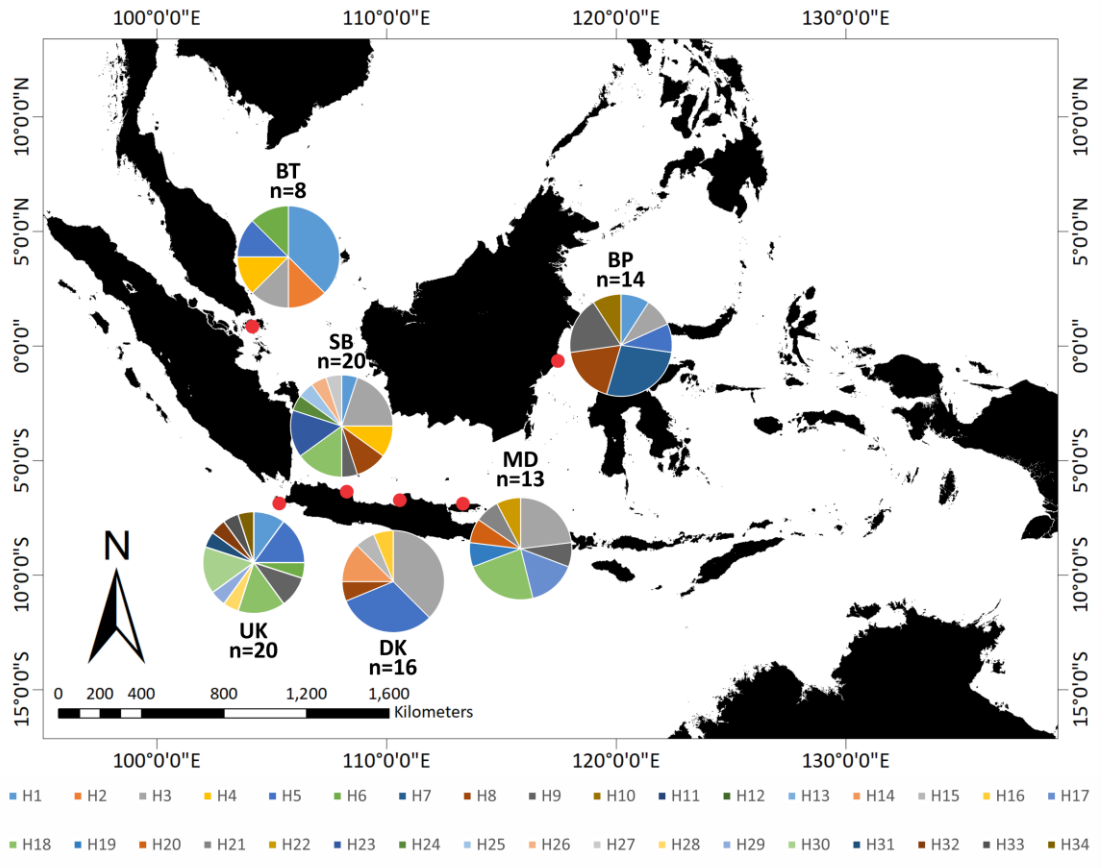

Figure 3 Haplotype composition in each location of *Tachypleus gigas*

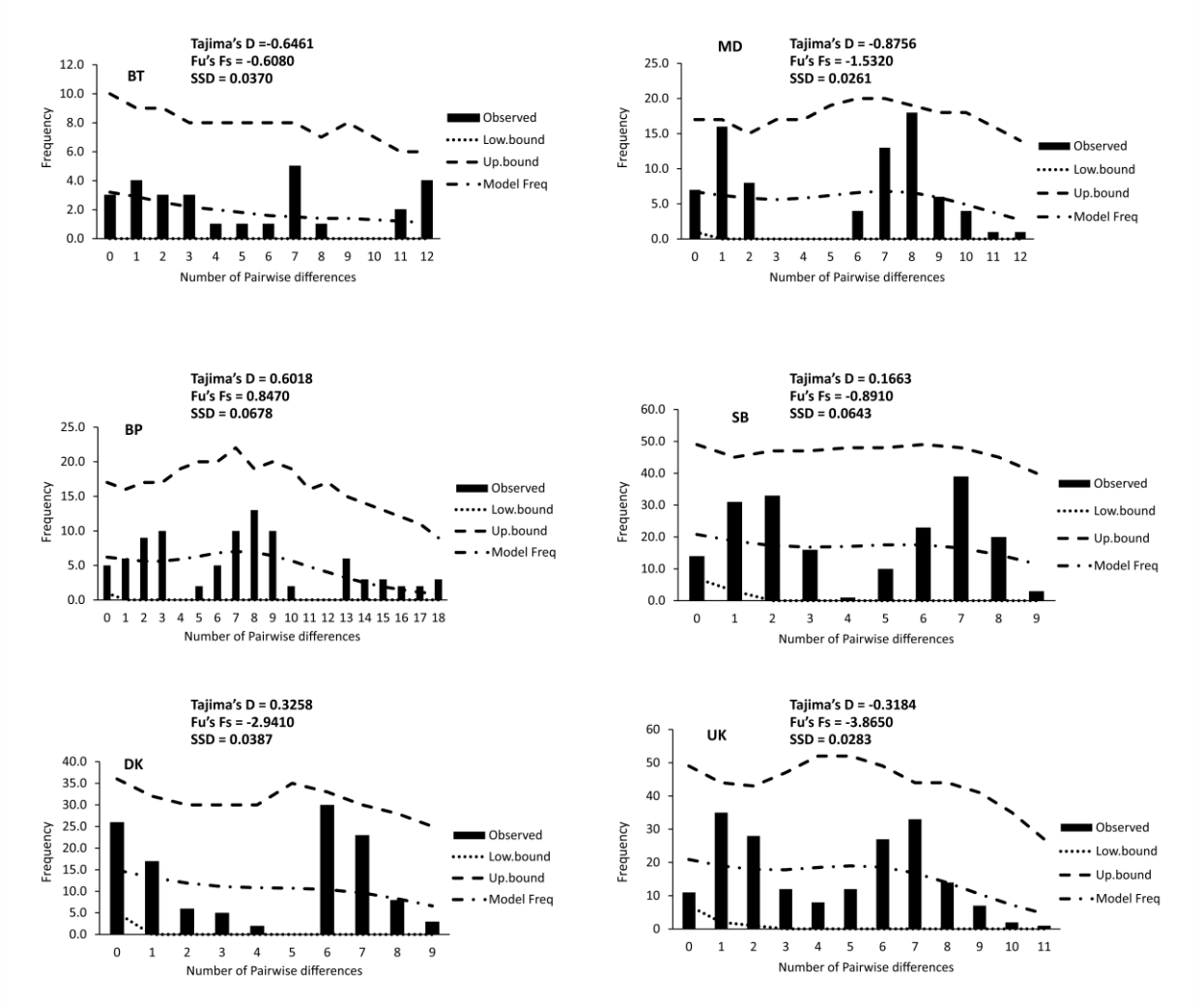

Figure 4 Mismatch Distribution Analysis of *Tachypleus gigas*

DISCUSSION

In this current study, the haplotype diversity in six populations of coastal horseshoe crab in the northern Java Sea, Bintan and Balikpapan were high. There was a high number of polymorphic sites (43 polymorphic sites within 34 haplotypes) in Indonesian coastal horseshoe crab rather than Malaysian horseshoe crab which was only found 13 haplotypes (Roihan & Ismail, 2011). The measured of mean haplotype diversity (h = 0.9353) in this study was high whereas the mean of nucleotide diversity (π = 0.0064) was low in all population. Similar findings showed that the haplotype and nucleotide diversity of Malaysian coastal horseshoe crab (*T. gigas*) was higher h = 0.797±0.129; π = 0.058±0.001 (Roihan & Ismail, 2011). Similar observations were also reported by Yang et al., (2007) in order to tri-spines of

horseshoe crab (*T. tridentatus*) in Taiwan which totally was 0.626±0.075 for genetic variation (*h*) and 0.0039±0.0055 for nucleotide diversity (π). Generally, several results from several studies reported the high genetic diversity but the nucleotide diversity was low for horseshoe crab. The high number of haplotypes in the present study is indication of the high mutation rate of the mtDNA genes. This phenomena is indicate only small differences between haplotypes which can be a signature of a rapid demographic expansion from a small effective population size (Avise, 2000). The nucleotide diversity (π) is a sensitive index for population genetic diversity analysis (Nei & Li, 1979). The genetic diversity may be influenced by the life-history characteristics, environmental heterogeneity, large population size (Nei, 1987; Avise, 2000) also by the fishing pressure (Madduppa et al., 2018), reduced mediated larval transport, limited exchange to other populations (Timm et al., 2017). Furthermore, the rate of mitochondrial evolution and historical factors may play an important role in determining the patterns of genetic variability (Grant et al., 2006; Xiao et al., 2009; Yamaguchi et al., 2010).

Uncommon result, a lack of extensive differentiation among populations (very low F_{ST} 0.02 to 0.1 with not significanly), was found except between population UK-MD and UK-SB. This result indicates there is no subdivion among population. In contrast, the life-history characteristics and habitat preferences of horseshoe crab suggesting that the dispersal capability of this crabs might be restricted (Sekiguchi, 1988). The movement capabilities of the horseshoe crab only in home range area or limited. The finding of Yang et al., (2007) revealed that movement capabilities no more than 150 km. Other findings also reported that the movement analysis on horseshoe crab (*L. polyphemus*) in the Great Bay Estuary, New Hampshire (USA) shows the mean annual linear range for all animals was 4.5 km and the maximum distance moved was 9.2 km (Schaller et al., 2010). Swan, (2005) conducted the largest study to date of *Limulus* migrations and plausible long distance movements were documented for 14 individuals that moved distances ranging from 104–265 km from their release sites, over multiple years. According to ecological observations, the hatched larvae of horseshoe crab swim freely approximately 6 days then settle in the bottom of shallow waters around their natal beaches (Shuster 1982). However, the strong tendency of larvae concentrated in inshore than offshore (100-200 km) (Botton & Loveland, 2003) suggests that their capability for long-range dispersal between estuaries is limited. Hence, one possible elucidation for the contradictory results is the presence of a recent bottleneck effect, which

decreased the overall genetic variation among populations and at the same time increased the chances of inbreeding (Liew et al., 2015). Additionally, the negative value of F_{ST} was found also in this study which indicates no similarities between two random individuals from equal population rather than between two random individuals from separated populations (Arnason & Palsson, 1996). The low level of F_{ST} reflects the higher gene flow around localities. Morover, the results of the mtDNA analysis suggests a moderate level of overall gene flow which was 6.71. Gene flow often ensues in marine organism that have a dispersal across wide geographic ranges (Palumbi, 1994). Similar research conducted by Roihan and Ismail (2011) reported that the F_{ST} value of the coastal horseshoe crab along west coast of peninsular Malaysia was ranging from 0.111 – 0.557. Other findings in Malaysia coastal area reported that the F_{ST} value (analysed using microsatellite marker) was 0.1441-0.8469. Apart from this, AMOVA analysis exposed that most of the genetic variation found was due to variations within populations rather than among populations indicating the extensive gene flow among populations.

There were only six kind of sharing haplotypes from 34 haplotypes that discovered. The median-joining network analysis shows the population expansions in the past regarding to the sharing haplotypes among localities. The evidence of the population expansion also illustrated by a star-like profile whereas the unique haplotypes branched out from the center. Moreover, the Tajima's *D* and Fu *F*s test also indicate the occurance of the population expantion. However, the graphs of mismatch distribution showed the multimodal in all population which elucidate an equiblium population or stationaty population. Contradictory, the movement capabilities of adult horseshoe crab among population was restricted. The presence of sharing haplotypes may give the confused result in this study. Naturally, the crab stays only in the home range area. In addition, the distance among population here was more than 300 km whereas the crab capabilities ranging only from 104-265 km (Swan, 2005). However, the common haplotypes between localities can be explained by the historical of biogeography in Southeast Asia region where known as Sunda shelves including Jawa, Sumatera and Borneo. Historically, Sundaland had experience in simultaneous glaciation and consequent deglaciation during the Pleistocene period. These phenomena were associated with decreased of the sea level which was equally important factors in the dispersal of plants and animals (Voris, 2000). The haplotype sharing and their consequent gene flow may also be attributed to

breeding migration, mutation, pelagic larvae, and sharing of common ancestors (Frankham, 1996). Whereas the occurrence of the many uniqe haplotypes can be explained by the time during the last glacial maximum. There were many species became isolated in refugia, but as the glacial ice sheets retreated and species dispersed, genetic differentiation and divergence ensued (Hewitt, 2000).

IMPLICATION FOR CONSERVATION

The proactive management approach for Asian coastal horseshoe crabs (*T. gigas*) in Indonesia should consider the parameter of population genetic. Generally, the genetic population parameters show that this species in Indonesia indicates a single stock population. The high genetic diversity and low nucleotide diversity elucidate that coastal horseshoe crab has an ability to adapt in the environmental condition and also the nucleotide composition in all population has a low differentiation. Moreover, the findings in this result reveals that *T. gigas* in Indonesia has low genetic differentiation and has indication of population connectivity. Additionally, there is an evidence of the population expansion. Hence, all the results lead to a single stock population of Indonesia coastal horseshoe crab. In contrast, horseshoe crab migrates from its natal beach to the deeper water and returns to the beach again to spawn (Walls et al. 2002). This means that all individuals do not move far away along the coastline. Although the population of Indonesian coastal horseshoe crab reveals a single population stock, the best conservation management strategy that needs in this part is the local based management in conjunction with regional based management.

CONCLUSIONS

This study has observed an overall high genetic diversity within populations of horseshoe crab *T. gigas* and has a low lavel of genetic differentiation which indicates a population connectivity and a single stock population. With regard to the limited movement potential of coastal horseshoe crabs, it should be noted that historical demography was part of the population expansion during the last glacial period. Therefore, local-based conservation is the preferred management method, which can be one of the precautionary approaches to conserving the Indonesian coastal horseshoe crab. Additionally, an advanced analysis based

on male and female horseshoe crab needs to be elucidated with characteristic of population subdivision from the nuclear genome (e.g., microsatellites) and requires the expansion of the number in geographic range around Indonesia.

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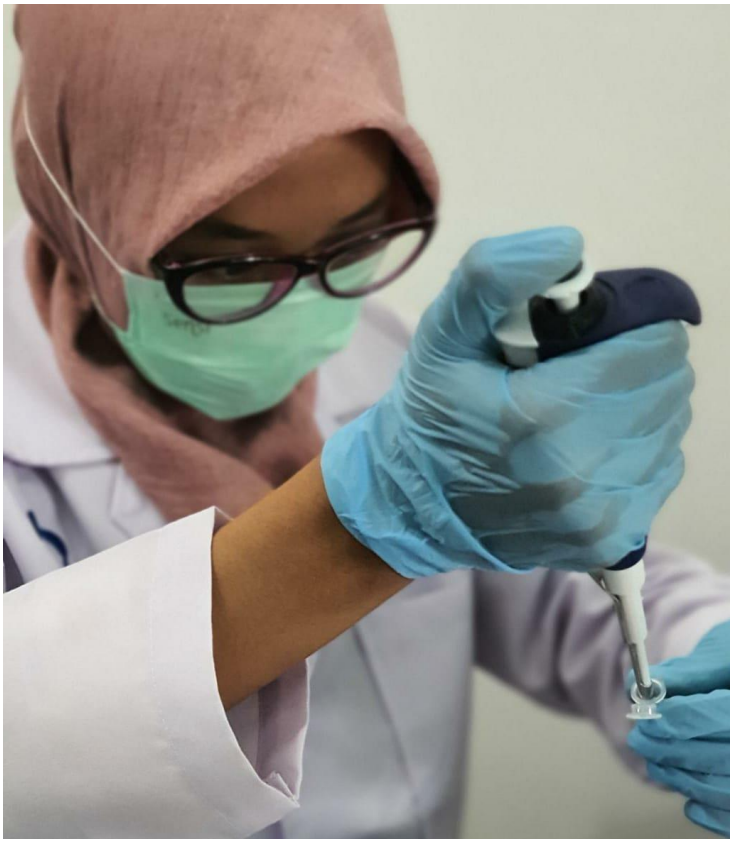
APPENDIX

Appendix 1 Research documentations

Hoeseshoe crab in Balikpapan



Lab work (DNA extraction and PCR)

Blood collection