The implication for conserving high genetic diversity and a panmixing of coastal horseshoe crab (*Tachypelus gigas*) around major habitats in Sundaland, Indonesia

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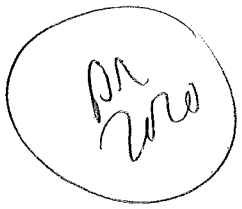

Abstract

The species with limited dispersal capability are often composed of highly genetically structured populations with small geographic ranges. One of the interesting groups of marine organism maintaining their genetic structure, which tends to be unchanged over millions of years is the horseshoe crab. This study aims to investigate the haplotype diversity and genetic connectivity of *Tachypleus gigas* in Indonesia. To achieve this, a total of 91 samples were collected from six main habitats of *Tachypleus gigas,* namely Bintan, Balikpapan, Demak, Madura, Subang, and Ujung Kulon. They were amplified using mitochondrial (mt) AT-rich region DNA sequences. The results showed that 34 haplotypes consist of 6 sharing and 22 unique from all localities. In general, the pairwise genetic differentiation (F_{ST}) value was low (-0.01 to 0.13) and not significantly different ($p>0.05$) except in Ujung Kulon-Madura and Kulon-Subang ($p<0.05$). Furthermore, the overall gene flow was 6.71 and the analysis of molecular variation (AMOVA) confirmed a major difference within (95.23%), rather than among the population (4.77%). Also, the construction of haplotype network exhibited evidence of gene flow and sharing between populations. Based on Tajima's *D* and Fu F_S test, the SSD value indicates expansion whereas the mismatch distribution illustrates the stationary population. In addition, coastal horseshoe crab around Indonesia indicated a population connectivity that require local based conservation. Conclusively, the study shed light on the evidence of the gene flow and the expansion of population which contradict previous facts that adults and juveniles of horseshoe crab has visually observed a limited movement among locations.

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Introduction

The cases of gene flow are common in marine organisms, which are spread over large geographic ranges (Palumbi, 1994; Crandall et al., 2019). Gene flow often precludes genetic subdivision, therefore, an extensive sampling of species with high or intermediate dispersal abilities is required (Lessios et al., 1998). Alternatively, the population structure should be separated by reasons 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). Furthermore, the species with limited dispersal capability are often composed of highly genetically structured populations with small geographic ranges. This provides more opportunities to compare the depths and positions of intraspecific genetic with the locations as extrinsic factors (Bernardi & Talley, 2000).

An One of the interesting groups of marine organism maintaining their genetic structure, which tends to be unchanged over millions of years is the horseshoe crab. This is an aquatic biota, also known as living fossil animals (Eldredge and Stanley 1984), that lives for almost 500 million years. They are ancient marine arthropods exhibiting life-history and habitat preferences that indicate a restricted dispersal capability (Sekiguchi, 1988). Generally, they are classified as Atlantic horseshoe crab (*Limulus Polyphemus*) and are known to be the only Atlantic species that 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 species are found in Indonesian coastal waters, dispersed around Sumatra, Java, Kalimantan, and Sulawesi (Rubiyanto, 2012; Mashar et al., 2017; Meilana et al., 2016).

Throughout their life cycle, they are highly dependent on environmental conditions in the coastal habitats. Furthermore, the population genetic studies of horseshoe crabs generally focused on *L. polyphemus* along the eastern coast of North America (Pierce et al., 2000; King et al., 2004). However, these crabs occupied Southeast Asia to Japan (Sekiguchi, 1988) and most research suggested that they are declining both locally and regionally. This is due to the loss of suitable spawning grounds because of over-harvesting for food, biomedical, and coastal development (Itow, 1993; Botton, 2001; Chen et al., 2004). In addition, *T. gigas* was once relatively profuse along the northern Java sea. However, it is believed that the population of Indian and mangrove horseshoe crab are unidentified based on their conservation status, for which insufficient data are available (World Conservation Monitoring Centre, 1996). Therefore, this study examines the genetic diversity and connectivity, as well as the population structure, of the AT-rich region of mitochondrial DNA from the coastal horseshoe crabs. This has proven to be a useful marker in intraspecific studies of some other arthropods (Brehm et al., 2001) to facilitate conservation efforts for the establishment of horseshoe crab conservation in Indonesia.

Materials & Methods

Study area and sample collection

The adults and juveniles of *T. gigas* were observed and collected from shallow waters with the help of a local fisherman. Also, 6 locations around Indonesia including Bintan, Balikpapan, Demak, Madura, Subang, and Ujung Kulon were observed in this study (Fig. 1). A total of 91 hemolymph of *T. gigas* were collected from April 2019 to August 2020, consisting of 8, 14, 16,

13, 20, and 20 samples from Bintan Island (BT), Balikpapan (BP), Demak (DK), Madura (MD), Subang (SB), and Ujung Kulon (UK), respectively. Also, the hemolymph of all samples was collected from each individual and immediately preserved with absolute ethanol. Field experiments and sampling were approved by the Research Council of Study Program the IPB University (Letter number: 1426/IT3.F3.2/KP.03.03/2019)

Genomic DNA extraction, amplification, and DNA sequencing

All genomic were collected 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). A total volume of 25 µL PCR reaction was carried out including 12.5 µL MyTaq HS Red Mix, 9 µL ddH_2O, 1.25 µL forward and reverse primer, and 1 µL DNA template. The entire mixture reaction was amplified using the polymerase chain reaction (PCR) thermocycler and the step following Yang et al. (2007) was pre-denaturation at 95°C for 3 mins, This was 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, one cycle at 72°C for 2 min, and 25°C for 5 min. The PCR product was visualized by electrophoresis on 1% agarose gel in TAE buffer with ethidium bromide at 100 V for 30 min. In addition, UV light is required to determine the band, which indicates the presence of a DNA fragment. The DNA sequencing was done by 1st BASE, Malaysia.

Data Analysis

Genetic diversity

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

Population Structure

The population structure was indicated by Wright's fixation index (F_{ST}), gene flow (Nm), and the analysis of molecular variance (AMOVA) using Arlequin v.3.5 program (Excoffier & Lischer, 2010), with 1000 permutations. The significance level threshold (α) to determine the pattern of differentiation between locations is 0.05. Furthermore, the Pairwise F-statistic (F_{ST}) were calculated as genetic distance based on the differences between population using DNASp v6 (Rozas et al., 2017). The value of F_{ST} ranges from 0 to 1, F_{ST} close to zero indicates low level of genetic differentiation, while the high level is determined by the value close to one.

Population Connectivity

The genetic connectivity among populations was estimated using a median joining (MJ) network (Bandelt et al., 1999) and was calculated using Network v 4.6.1.0 based on haplotype data. The haplotype composition was obtained in all study areas and then illustrated in an appropriate map to show the pattern of distribution and genetic connectivity among populations. Furthermore, Tajima's *D and* Fu's F_S statistical tests were used to determine the population equilibrium. The 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. However, the negative F_S values indicate a recent population expansion (Fu,

1997) and Positive Eu's F_S values suggest that it is steady. The history of effective population size was assessed through the pairwise mismatch distribution in Arlequin and the results reflect the stochastic lineage loss. Also, the unimodal result described the expansion population growth and a recent bottleneck effect while the multimodal suggests the equilibrium demographic condition or stationary population.

Results

Genetic Diversity

A total of 91 AT-rich sequences each with approximately 670 bp were obtained in all sampling locations including Java (UK, SB, DK, and MD) and Sumatera Island especially in Bintan and also Borneo (Balikpapan). Furthermore, genetic diversity was calculated and analysed based on the initial processes that were sequence alignment. In total, 43 nucleotide sites and 34 haplotypes were exposed. The haplotypes that were obtained consist of the unique (only found in certain locations) and common haplotypes (Table 1). Also, the genetic diversity of *T. gigas* in each sampling sites vary in value (Table 2) and the percentage of A+T composition in each locations was slightly different, which was approximately 81%.

At a glance, the obtained haplotype diversity was quite high ranging from $h = 0.7833$ to 0.9455 with a mean gene diversity per population of $h = 0.9355$. Conversely, nucleotide diversity was relatively low in all locations ranging from $\pi = 0.0049$ to 0.0095. However, the overall diversity was similar among populations. Haplotype and nucleotide were lowest for DK, followed by population in SB and UK. Furthermore, the haplotype and nucleotide diversity was highest for BP, followed by UK (h=0.9421 π = 0.0054), SB (h=0.9263 π = 0.0052), MD (h=0.9103 π = 0.0066), and BT (h=0.8929 π = 0.0066). Meanwhile, the lowest haplotype diversity was in DK (h=0.7833 π =0.0049) (Table 2).

The Population Structure

The results from pairwise F_{ST} value showed that the entire population was ranging from -0.01 to 0.13 (Table 3). Generally, the F_{ST} value among locations was not significantly different (p>0.05), with an exception of the comparison between UK-MD and UK-SB which indicate the restricted gene flow among population. Furthermore, the populations that have a higher pairwise F_{ST} value than others include BT-MD (p>0.05), BT-SB (p>0.05), UK-MD (p<0.05), and UK-SB (p<0.05). Meanwhile, the negative values 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), and SB-MD (F_{ST} = -0.01; p>0.05). The low-level and negative value of F_{ST} indicate a lack of division among populations and also gene flow phenomena. The overall gene flow (Nm) estimated among populations was 6.71 and AMOVA also confirmed that the majority of variation was found within (95.23%) rather than among populations (4.77%) (Table 4). Additionally, the mean F_{ST} was calculated as 0.04 (p-value = 0.0069) which indicates a low level of genetic differentiation.

The Population Connectivity

A total of 34 haplotypes were identified using Median-Joining Network of haplotypes created for all samples of *T. gigas* (Fig. 2). The analysis showed the existence of the dominant haplotypes (H1, H3, H5, H8, H9 and H18) which indicates an evolutionary link. H3 was the most common, being identified in all populations except in UK and consist of 15 individuals. Similary, H5 was found in 12 individuals from the BT, BP, DK, SB, and UK populations. However, there were specific haplotypes, which are only found in certain location. Meanwhile,

the highest number of unique was in UK population whereas the lowest was in BT. There were two unique haplotypes (H2 and H4) in samples from BT, three (H14, H15, and H16) from DK, five (H6, H10, H11, H12 and H13) from BP, five (H17, H19, H20, H21 and H22) from MD, five (H23, H24, H25, H26 and H27) from SB, and seven (H28, H29, H30, H31, H32, H33, and H34) from UK. In terms of the number of unique haplotypes, population of coastal horseshoe crab in UK has the most number, which were found only in 1 individual for each (Fig. 3).

The historical demography was assessed based on the mtDNA AT-rich region haplotype. The result from the analysis of network analysis showed that there was sharing haplotype in all location (Fig. 2). Furthermore, the value of the Tajima's *D* statistics in all population were negative, with an exception in DK, MD, and SB, showing no significant *p*-value, which therefore indicates no evidence of selection. Similary, the result of Fu's and *F*s test was negative except in DK with no significant *p*-values for all six observed, indicating an excess number of alleles as expected from a recent population expansion. However, the results of the mismatch distribution were measured to determine the contradictory expansion in the past. In general, the mismatch distribution illustrated the multimodal patterns, which show the stationary population or no historical expansion in all location (The graphs of the multimodal illustrations are shown in Fig. 4). Furthermore, 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.

Discussion

In this study, the haplotype diversity was high in six populations of coastal horseshoe crab in the northern Java Sea, Bintan, and Balikpapan. There was also a high number of polymorphic sites (43 within 34 haplotypes) in Indonesian coastal horseshoe crab rather than Malaysian, in which only 13 were found (Roihan & Ismail, 2011). Furthermore, the mean haplotype diversity ($h = 0.935$) was high, while that of nucleotide ($\pi = 0.0064$) was low in all the population. Furthermore, the haplotype and nucleotide diversity of Malaysian coastal horseshoe crab (*T. gigas*) was $h = 0.797 \pm 0.129$; $\pi = 0.058 \pm 0.001$ (Roihan & Ismail, 2011). Similar observations were also reported by Yang et al., (2007) on tri-spines horseshoe crab (*T. tridentatus*) in Taiwan, which was 0.626 ± 0.075 for genetic variation (h) and 0.0039 ± 0.0055 for nucleotide diversity (π). Generally, results from several studies reported a high genetic diversity, however, the nucleotide was low for horseshoe crab. Additionally, the high number of haplotypes shows a high mutation rate of the mtDNA genes. These phenomena indicate only small differences, which is the signature of a rapid demographic expansion from a small effective population size (Avise, 2000). The nucleotide (π) is a sensitive index for the analysis of the population genetic diversity (Nei & Li, 1979), which is influenced by the life-history characteristics, environmental heterogeneity, large population size (Nei, 1987; Avise, 2000), fishing pressure (Madduppa et al., 2018), reduced mediated larval transport, and a limited exchange to other populations (Timm et al., 2017). Furthermore, the rate of mitochondrial evolution and historical factors play an important role in determining the patterns of genetic variability (Grant et al., 2006; Xiao et al., 2009; Yamaguchi et al., 2010).

An unusual result, which is a lack of extensive differentiation among populations (very low F_{ST} 0.02 to 0.1 and not significant) was found, with an exception between UK-MD and UK-SB. This indicates that there is no subdivision among populations. Conversely, the life-history characteristics and habitat preferences of horseshoe crabs suggested that their dispersal capability is restricted (Sekiguchi, 1988). Moreover, they have limited movement capabilities only in their

range area. The finding of Yang et al., (2007) revealed that their movement capabilities did not exceed 150 km. Others reported that the movement analysis on horseshoe crab (*L. polyphemus*) in the Great Bay Estuary, New Hampshire (USA) has a mean annual linear range for all animals of 4.5 km and 9.2 km as the maximum distance moved (Schaller et al., 2010). Swan, (2005) conducted the largest study of Limulus migrations to date and plausible long-distance movements were documented for 14 individuals that moved 104–265 km from their release sites, over multiple years. According to ecological observations, their hatched larvae swim freely for approximately 6 days and then settle in the bottom of shallow waters around their natal beaches (Shuster 1982). However, the strong tendency of larvae concentrated in inshore rather than offshore (100-200 km) (Botton & Loveland, 2003) suggests that their capability for long-range dispersal between estuaries is limited. Therefore, a possible cause of the contradictory results is the presence of a recent bottleneck effect, which simultaneously decreased the overall genetic variation among populations and increased their chances of inbreeding (Liew et al., 2015). Additionally, the negative value of F_{ST} indicates no similarities between two random individuals from equal rather than separated populations (Arnason & Palsson, 1996). The low level of F_{ST} reflects the higher gene flow around localities. Moreover, the results of the mtDNA analysis shows a moderate level of overall gene flow which was 6.71. Furthermore, gene flow often ensues in marine organisms that are dispersed across wide geographic ranges (Palumbi, 1994). A similar study by Roihan and Ismail (2011) reported that the F_{ST} value of the crab along the west coast of peninsular Malaysia ranges from 0.111 – 0.557. Other findings in the area reported that the F_{ST} value, which was analysed using microsatellite marker ranges between 0.1441 and 0.8469. In addition, AMOVA analysis exposed that most of the genetic variation found was due to the differences within, rather than among populations, indicating an extensive gene flow.

Furthermore, there was only 6 kinds of sharing haplotypes from the 34 that was discovered. The analysis of the median-joining network shows the population expansions in the past regarding the sharing haplotypes among localities. This evidence was also illustrated by a star-like profile whereas the unique haplotypes branched out from the center. Moreover, Tajima's *D* and Fu *Fs* test indicate the occurrence of the population expansion. However, the graphs of mismatch distribution showed the multimodality in the entire population, which elucidate equilibrium or stationary. In contrast, the movement capabilities of adult horseshoe crab among populations was restricted. The presence of sharing haplotypes may give confusing results in this study, because crab stays only in the home range area. In addition, the distance among populations was more than 300 km whereas the crab capabilities ranging only from 104-265 km (Swan, 2005). The common haplotypes between localities is explained by the history of biogeography in Southeast Asia region known as Sunda shelves including Java, Sumatera, and Borneo. Historically, Sundaland experienced simultaneous glaciation and consequent deglaciation during the Pleistocene period. These phenomena were associated with a decrease in sea level, an equally important factor in the dispersal of plants and animals (Voris, 2000). Furthermore, haplotype sharing and the resulting gene flow is attributed to breeding migration, mutation, pelagic larvae, and sharing of common ancestors (Frankham, 1996). Whereas the occurrence of the many unique haplotypes is explained by the time during the last glacial maximum. Many species became isolated in refugia, however, genetic differentiation and divergence occured due to the retreat and dispersal of glacial ice sheets (Hewitt, 2000).

The Implication for Conservation

The proactive management approach for Asian coastal horseshoe crabs (*T. gigas*) in Indonesia needs to consider the parameter of population genetic. Generally, these parameters show that the species in Indonesia indicates a single stock population. The high genetic and low nucleotide diversity show that the coastal horseshoe crabs are able to adapt to the environmental condition and its composition in all population has low differentiation. Moreover, the findings reveal that *T. gigas* in Indonesia have low genetic differentiation as well as indication of population connectivity and expansion. Therefore, all the results lead to a single stock population of Indonesia coastal horseshoe crab. However, they migrate from their natal beach to the deeper water and return to spawn (Walls et al. 2002). This implies that all individuals do not move far away along the coastline. Although the Indonesian coastal horseshoe crab reveals a single population stock, the best conservation strategy that needs in this part is the local in conjunction with regional-based management.

Conclusions

Conclusively, an overall high genetic diversity within populations of horseshoe crab *T. gigas* was observed and the result showed a low level of differentiation, which indicates a single stock population and connectivity. Furthermore, 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, the local-based conservation is the preferred management method, which is 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). This also requires the expansion of the number in geographic range around Indonesia.

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Table 1

Variable sites found in a fragment of the AT-rich region of *Tachypleus gigas* in each populations. Forty-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	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
H 1	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
H 2	C	C	.	.	.	C	A	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	C	.	.	.	T	.	.	.	.	.	A	A	.	.
H 3	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
H 5	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 6	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
H 7	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	.
H 8	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.
H 9	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H 10	C	C	T	.	T	C	A	G	.	.	.	C	.	T	G	T	.	.	.	.	C	.	T	.	C	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H 11	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.
H 12	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
H 13	C	C	T	.	.	C	A	.	T	.	.	C	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	G	.	.	.	G	.	.	.	A	.	.
H 14	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 15	C	C	.	.	T	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.
H 16	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 17	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H 18	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 19	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.
H 20	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
H 21	C	C	T	.	T	C	A	.	.	.	.	C	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.

H 22	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.
H 23	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.
H 24	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.
H 25	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.
H 26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
H 27	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 28	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
H 29	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.
H 30	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
H 31	.	C	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.
H 33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 34	.	C	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

4 *BT = Bintan; BP= Balikpapan; DK= Demak; DR= Madura; SB= Subang; UK= Ujung Kulon

Tabel 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

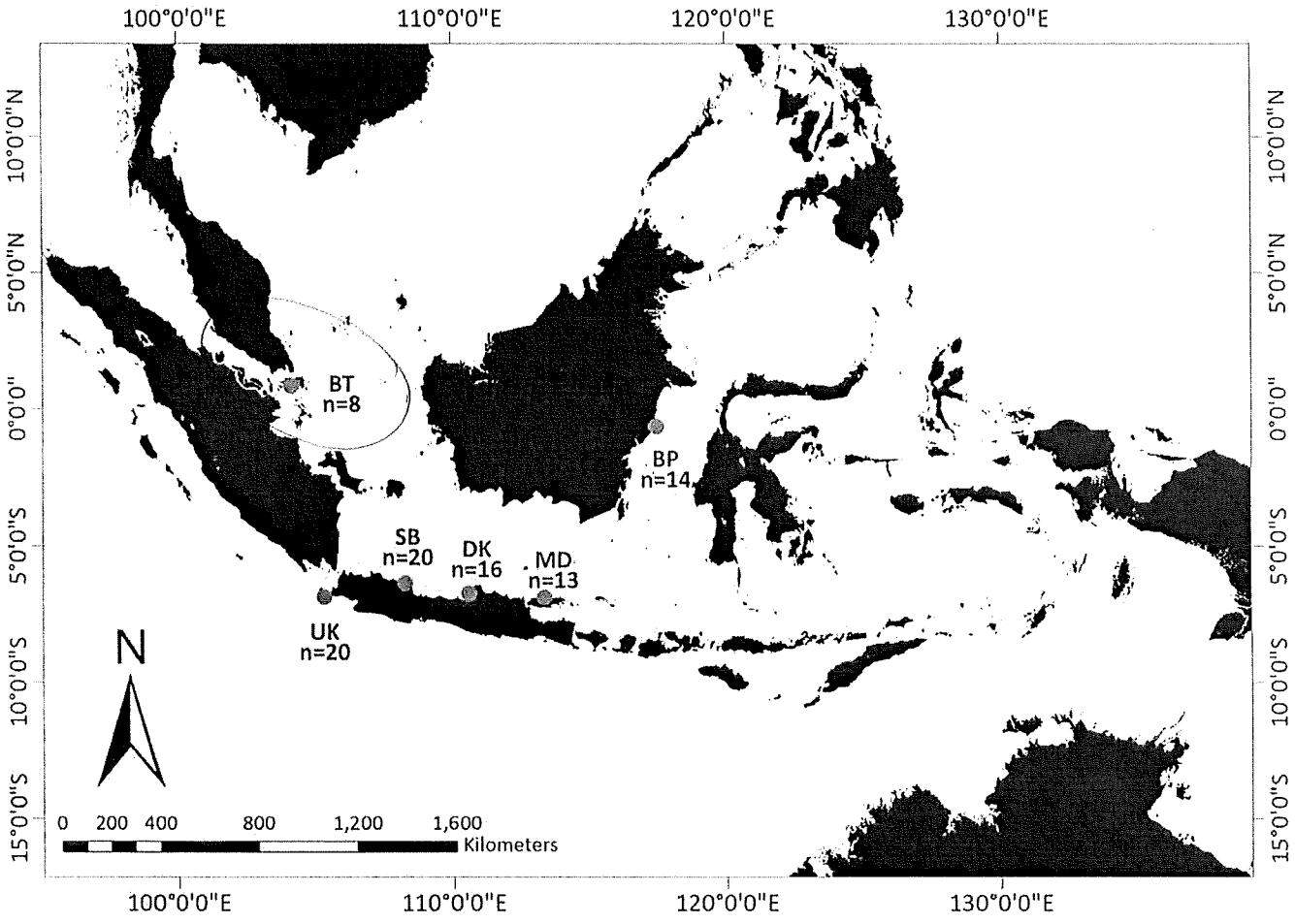
Table 4

The analysis of molecular variance (AMOVA) 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			

Figure 1

Sampling locations of *Tachypleus gigas* (n = number of sample, UK = Ujung Kulon, SB = Subang, DK = Demak, MD = Madura, BP = Balikpapan, BT = Batam)



number small
+ quite small

Figure 2

Haplotype network of *Tachypleus gigas* (n= 91) population in six location around Indonesia, constructed with Median Joining method