High genetic diversity and a mixing 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. This study aims to investigate the haplotype diversity and genetic connectivity of coastal horseshoe crab (*Tachypleus gigas*) in Indonesia. To achieve this, a total of 91 samples were collected from six main habitats of coastal horseshoe crab, 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 to 0.13) and not significantly different ($p>0.05$) except ~~in~~ for Ujung Kulon-Madura and Kulon-Subang ($p<0.05$). Furthermore, the analysis of molecular variance (AMOVA) ~~confirmed a major difference~~ within ~~population~~ (95.23%) rather than among population (4.77%). ~~Also,~~ the ~~construction of~~ haplotype network exhibited evidence of gene flow and sharing haplotype between populations. ~~Based on the~~ Tajima's D and Fu F_S test values indicates a population expansion. In this study, the coastal horseshoe crab around Indonesia suggested ~~a~~ population connectivity that require a ~~local~~ based conservation. ~~Conclusively,~~ The study ~~shed light on the~~ evidence of ~~the~~ gene flow which contradict previous ~~facts~~ that horseshoe crab has ~~visually observed a~~ limited movement ~~area~~ among locations.

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).

Horseshoe crabs, an interesting group of marine organisms and also known as living fossils 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 American 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. 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 Indonesian coastal waters especially in the northern Java sea. However, it is believed that the population of coastal and mangrove horseshoe crab are unidentified based on their conservation status, for which insufficient data are available (World Conservation Monitoring Centre, 1996). Furthermore, the population genetic studies of horseshoe crabs generally focused on American horseshoe crab and a little for Asian horseshoe crab (Pierce et al., 2000; King et al., 2004; King et al., 2005; Yang et al., 2007; Roihan & Ismail 2011; King et al., 2015). 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, six 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. Moreover, the hemolymph of all samples was collected from each individual and immediately preserved with absolute ethanol.

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 analyzed 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).

The Population Structure

The population structure was indicated by Wright's fixation index (F_{ST}), gene flow (Nm), and analysis of molecular variance (AMOVA). Moreover, the significance level threshold (α) to determine the pattern of differentiation between locations is 0.05. 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 Tajima's D test uses the frequency of nucleotide site separation, whereas Fu's Fs uses the haplotype distribution (Fu, 1997). The analysis was conducted using the Arlequin v.3.5 program. 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 Fu's F_S values indicate a recent population expansion (Fu, 1997) and Positive Fu's F_S values suggest that it is steady.

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 analyzed 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 haplotype (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 0.9451 with a mean gene diversity per population of $h = 0.9358$. Conversely, nucleotide diversity was relatively low in all locations, ranging from $\pi = 0.0049$ to 0.0095. The overall diversity was similar among populations, but the lowest haplotype and nucleotide diversity were in DK (h=0.7833 π =0.0049). However, 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) (Table 2).

The Population Structure

The results from pairwise F_{ST} value showed that the entire population was ranging from 0 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 values of pairwise F_{ST} in the comparison between UK-BT, DB-DK and SB-MD were effectively zero value. The low level of F_{ST} means that there is no genetic subdivision among populations also indicates gene flow phenomena. Furthermore, results from AMOVA also confirmed that the majority of variation was found within (95.23%) rather than among populations (4.77%) (Table 4).

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 common haplotypes (H1, H3, H5, H8, H9 and H18) which is an indication of gene flow phenomena among geographic sites. H3 was the most common, being identified in all populations except in UK and consist of 15 individuals. Similarly, 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 test (Table 5) 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. Similarly, the result of Fu's Fs test (Table 5) 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.

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. Furthermore, the mean haplotype diversity ($h = 0.9353$) was quite high while that of nucleotide diversity ($\pi = 0.0064$) was low in all the population. Similarly, high haplotype diversity value has been reported in order *T. gigas* in Malaysia which was around $h = 0.797 \pm 0.129$ and $\pi = 0.058 \pm 0.001$ (Roihan & Ismail, 2011). Other related observations were also reported by Yang et al., (2007) on tri-spined 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, in this study the genetic diversity for coastal horseshoe crab was high but the nucleotide was low. The high number of haplotypes indicates that the populations studied are adequately large to maintain a high level of genetic diversity. 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). The crab has limited movement capabilities only in their home range area. The finding of movement distance of the coastal horseshoe crab (*T. gigas*) in Malaysia was up to 30 km (Mohamad et al., 2019), while the movement capabilities of tri-spined horseshoe crab did not exceed 150 km (Yang et al., 2007). Similarly reported on American horseshoe crab in the Great Bay Estuary (USA) has a mean annual linear range as the maximum distance moved 4.5 km and 9.2 km (Schaller et al., 2010). Over multiple-year studies by Swan, (2005) were documented that *Limulus* moved at 104 until 265 km from their release sites. According to ecological observations, their hatched larvae swim freely for approximately six 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 for the contradictory results is due to the Wahlund effect associated with variable levels of polymorphism and reduced sample size (Dharmarajan et al., 2013). Additionally, the effectively zero value of F_{ST} indicates the low level of differentiation which reflects the higher gene flow around localities. Gene flow phenomena often ensues in marine organisms that are dispersed across wide geographic ranges (Palumbi, 1994). Moreover, the male horseshoe crab moved until 767 km over their long lifetimes regarding to long-term years tagging studies (E Hallerman, 2020, personal communication). As shown in

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 which indicate moderate until high genetic differentiation (Wright, 1978; Hartl & Clark, 1977). Other findings in the area reported that the F_{ST} value, which was analyzed 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 six kinds of sharing haplotypes from the 34 that was discovered from all samples (91 samples). The analysis of the median-joining network shows the population expansions in the past regarding the sharing haplotypes among localities. Overall, patterns of relationship at the mtDNA level have reduced geographical structure. The haplotype network reveals the recent demographic processes but the small number of sample sizes also limiting the possibility of observing the intermediate haplotypes inferred to exist on the network. Moreover, Tajima's D and Fu Fs test indicate the occurrence of the population expansion. The fact that males of horseshoe crabs move several hundred kilometers is both evidence and an answer for the existence of a shared haplotype (E Hallerman, 2020, personal communication). Moreover, the common haplotypes between localities is also explained by the history of biogeography in Southeast Asia region known as Sunda shelves including Java, Sumatera, and Borneo. Historically, Sundaland experienced simultaneous dewatered and then inundated 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). Haplotype sharing in this study and the resulting gene flow is attributed to breeding migration, mutation, pelagic larvae, also sharing of the common ancestors (Frankham, 1996). Whereas the occurrence of the many unique haplotypes is explained by the sample size that was collected also the time during the last glacial maximum phenomena. Many species became isolated in refugia, however, genetic differentiation and divergence occurred due to the retreat and dispersal of glacial ice sheets (Hewitt, 2000).

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. High haplotype diversity that occurs with low nucleotide diversity has been associated with population growth or expansion after period of low effective population size (Grant & Bowen, 1998). 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. 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. Additionally, an advanced analysis based on male and female horseshoe crab needs to be elucidated from the nuclear genome (e.g., microsatellites or SNPs). This also requires the expansion of the number in geographic range around Indonesia.

Conclusions

Conclusively, an overall high genetic diversity within populations of coastal horseshoe crab (*T. gigas*) was observed and the result showed a low level of differentiation, which indicates a single stock population and connectivity. Therefore, the local-based conservation is the preferred management method, which is one of the precautionary approaches to conserving the Indonesian coastal horseshoe crab.

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