High genetic diversity and mixing of coastal horseshoe crab (*Tachypelus gigas*) across major habitats in Sundaland, Indonesia

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Species with limited dispersal capabilities are often composed of highly genetically structured populations across small geographic ranges. This study aimed to investigate the haplotype diversity and genetic connectivity of the coastal horseshoe crab (*Tachypleus gigas*) in Indonesia. To achieve this, we collected a total of 91 samples from six main *T. gigas* habitats: Bintan, Balikpapan, Demak, Madura, Subang, and Ujung Kulon. The samples were amplified using primers for mitochondrial (mt) AT-rich region DNA sequences. The results showed 34 haplotypes, including six shared and 22 unique haplotypes, ~~from~~ across all localities. The pairwise genetic differentiation (F_{ST}) values were low (0 to 0.13) and not significantly different ($p>0.05$), except ~~in~~ among samples from Ujung Kulon-Madura and Kulon-Subang ($p<0.05$). Additionally, the analysis of molecular variance (AMOVA) showed the most variation within populations (95.23%) compared to less among populations (4.77%). The haplotype network showed evidence of shared haplotypes between populations. Tajima's D and Fu's F_S test values indicated a population expansion. Our results showed a low level of differentiation, suggesting a single stock and high connectivity. Therefore, a regionally-based conservation strategy is recommended for the coastal horseshoe crab in Indonesia.

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Abstract

Species with limited dispersal capabilities are often composed of highly genetically structured populations across small geographic ranges. This study aimed to investigate the haplotype diversity and genetic connectivity of the coastal horseshoe crab (*Tachypleus gigas*) in Indonesia. To achieve this, we collected a total of 91 samples from six main *T. gigas* habitats: Bintan, Balikpapan, Demak, Madura, Subang, and Ujung Kulon. The samples were amplified using primers for mitochondrial (mt) AT-rich region DNA sequences. The results showed 34 haplotypes, including six shared and 22 unique haplotypes, from all localities. The pairwise genetic differentiation (F_{ST}) values were low (0 to 0.13) and not significantly different ($p>0.05$), except in samples from Ujung Kulon-Madura and Kulon-Subang ($p<0.05$). Additionally, the analysis of molecular variance (AMOVA) showed the most variation within populations (95.23%) compared to among populations (4.77%). The haplotype network showed evidence of shared haplotypes between populations. Tajima's D and Fu's F_S test values indicated a population expansion. Our results showed a low level of differentiation, suggesting a single stock and high connectivity. Therefore, a regionally-based conservation strategy is recommended for the coastal horseshoe crab in Indonesia.

Introduction

High rates of gene flow are common in marine organisms that are spread across large geographic ranges (Palumbi, 1994; Crandall et al., 2019). Several marine organisms also exhibit low levels of genetic differentiation across large geographic scales (Avise, 2000). Population structures are ~~reportedly~~ affected by genetic drift, strong post-settlement selection (Hedgecock, 1986), and spatial-landscape patterns (Johnson & Black, 1998; Watts & Johnson, 2004). Species

with limited dispersal capabilities are often composed of highly genetically structured populations with small geographic ranges (Collin, 2001). This creates opportunities to compare the depths and positions of intraspecific genetic differentiation when using location as an extrinsic factor (Bernardi & Talley, 2000).

Horseshoe crabs, an interesting group of marine organisms considered "living fossils" (Eldredge & Stanley 1984), have been extant for almost 500 million years. There are four extant species of horseshoe crabs: the American horseshoe crab (*Limulus polyphemus*) found along the eastern coast of North America from Maine to Mexico (Walls et al., 2002; Rutecki et al., 2004), and three Asian horseshoe crabs species (the mangrove horseshoe crab [*Carcinoscorpius rotundicauda*], the coastal horseshoe crab [*Tachypleus gigas*], and the tri-spined horseshoe crab [*Tachypleus tridentatus*]) (John et al., 2018; Vestbo, 2018) that are sporadically distributed across Southeast Asia and Japan. They are ancient marine arthropods that exhibit life-histories and habitat preferences that suggest a restricted dispersal capability (Sekiguchi, 1988). The Asian 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, horseshoe crabs are highly dependent on environmental conditions in coastal habitats. Most research suggests that they are declining both locally and regionally due to over-harvesting for food, biomedicine, and coastal development (Itow, 1993; Botton, 2001; Chen et al., 2004) and the loss of suitable spawning grounds. *T. gigas* was once relatively common along the northern Java sea. However, coastal and mangrove horseshoe crab populations have an undetermined conservation status due to insufficient data (World Conservation Monitoring Centre, 1996). Furthermore, most population genetic studies on horseshoe crabs have focused on the American horseshoe crab, with little attention paid to the Asian horseshoe crab (Pierce et al., 2000; King et al., 2004; King et al., 2005; Yang et al., 2007; Rozihan & Ismail 2011; King et al., 2015). Therefore, this study examined the genetic diversity, connectivity, and population structure of coastal horseshoe crabs by screening an AT-rich region of mitochondrial DNA, an established genetic marker for arthropods (Brehm et al., 2001). Our aim was to use genetic evidence to facilitate horseshoe crab conservation efforts in Indonesia.

Materials & Methods

Study area and sample collection

With the help of a local fisherman, adult and juvenile *T. gigas* specimens were collected from shallow waters in six locations around Indonesia: Bintan, Balikpapan, Demak, Madura, Subang, and Ujung Kulon (Fig. 1). We collected the hemolymph from a total of 91 *T. gigas* specimens between April 2019 and August 2020. There were eight, 14, 16, 13, 20, and 20 samples from Bintan Island (BT), Balikpapan (BP), Demak (DK), Madura (MD), Subang (SB), and Ujung Kulon (UK), respectively. The hemolymph was collected from each individual and immediately preserved in absolute ethanol. Field experiments were approved by the Research Council of the Study Program from IPB University (letter number 1426/IT3.F3.2/KP.03.03.2019).

Genomic DNA extraction, amplification, and DNA sequencing

Genomic DNA was isolated from the hemolymph using the GeneAiD extraction kit following the manufacturer's instructions. A fragment of the AT-rich region was amplified using a pair of primers, Hb-12S (5'-GTCTAACCGCGGTAGCTGGCAC-3') and Hb-trna (5'GAGCCCAATAGCTTAAATTAGCTTA-3'), designed from the mitochondrial genome of

the ~~Atlantic~~ horseshoe crab (Lavrov et al., 2000). A 25-µL PCR reaction was carried out with 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 reaction mixture was amplified using a polymerase chain reaction (PCR) thermocycler, following Yang et al.'s (2007) amplification steps. The mixture underwent pre-denaturation at 95°C for 3 mins, 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 using electrophoresis on 1% agarose gel in TAE buffer with ethidium bromide at 100 V for 30 min. After electrophoresis, the gel was placed under UV light for band detection to determine the presence of a DNA fragment. The DNA sequencing was performed by 1st BASE DNA Sequencing Services, Selangor, Malaysia.

Data analysis

A total of 91 AT-rich region sequences were obtained, and MEGA X (Kumar et al., 2018) was used to generate multiple alignments of the edited sequences. Genetic diversity was measured using the number of haplotypes (Hn), haplotype diversity (Hd), nucleotide diversity (π), ~~and~~ DNASp v6 (Rozas et al., 2017). The population structure was ~~indicated~~ using Wright's fixation index (F_{ST}) and analysis of molecular variance (AMOVA). The significance level threshold (α), used to determine the pattern of differentiation between locations, was 0.05. The pairwise F-statistic (F_{ST}) was calculated as the genetic distance based on the population differences using DNASp v6 (Rozas et al., 2017). The haplotype network across 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 across all study areas was illustrated in a map to show ~~the~~ distribution and genetic connectivity patterns across the populations. Tajima's D (1989) and Fu's F_S (1997) statistical tests were used to assess ~~the~~ population equilibrium. ~~The analysis was conducted~~ using the Arlequin v.3.5 program (Excoffier & Lischer, 2010).

Results

Genetic diversity

We obtained a total of 91 AT-rich sequences ~~and~~ approximately 670 bp across all sampling locations including Java (UK, SB, DK, and MD), Sumatra ~~Island, and mostly in~~ Bintan and Borneo (Balikpapan). In total, 43 variable nucleotide sites and 34 haplotypes were observed. The haplotypes consisted of both unique (only found in certain locations) and common haplotypes (Table 1). The genetic diversity of the coastal horseshoe crab varied across sampling sites (Table 2). The percentage of A+T composition in each location ~~was slightly different, which~~ was approximately 81%.

At a glance, the obtained haplotype diversity was high, ranging from $h = 0.783$ to 0.945, with a mean gene diversity per population $h = 0.935$. Conversely, the nucleotide diversity was relatively low in all locations, ranging from $\pi = 0.004$ to 0.009. The overall diversity was similar across populations, ~~and~~ DK had the lowest haplotype and nucleotide diversity ($h = 0.783$, $\pi = 0.004$). BP had the highest haplotype and nucleotide diversity ($h = 0.945$ $\pi = 0.009$), followed by UK ($h = 0.942$, $\pi = 0.005$), SB (h=0.926, $\pi = 0.005$), MD (h=0.910, $\pi = 0.006$), and BT ($h = 0.892$, $\pi = 0.006$) (Table 2).

Population structure

Pairwise F_{ST} values ranged from 0 to 0.13 across the populations (Table 3). Generally, the F_{ST} value among locations was not significantly different ($p>0.05$) with the exception of UK-MD and UK-SB, indicating the restricted gene flow in these populations. Populations with higher pairwise F_{ST} values included BT-MD ($p>0.05$), BT-SB ($p>0.05$), UK-MD ($p<0.05$), and UK-SB ($p<0.05$). The pairwise F_{ST} values of UK-BT, DB-DK, and SB-MD were effectively zero. Our AMOVA results showed that the majority of variations were found within (95.23%) rather than among (4.77%) populations (Table 4).

Population connectivity

The relationship of the 34 haplotypes was illustrated using a median-joining network (Fig. 2). The haplotype network showed that there were shared haplotypes (H1, H3, H5, H8, H9, and H18) across the geographic sites. H3 was the most common, and was identified in about 15 individuals. H5 was found in 12 individuals from the BT, BP, DK, SB, and UK populations. However, specific haplotypes were only found in certain locations. The UK population had the highest number of specific haplotypes (seven). Meanwhile, BT had the lowest number of haplotypes (two) (Fig. 3).

We assessed historical demography based on mtDNA AT-rich region haplotype frequencies. There were shared haplotypes in all locations (Fig. 2). Furthermore, the Tajima's D test values (Table 5) were negative across all populations, with the exception of DK, MD, and SB. They showed no significant p-values, indicating that there was no evidence of selection. Similarly, the Fu's Fs test results (Table 5) were negative (except in DK), with no significant p-values across all six populations. This indicated an excess number of haplotypes, as expected due to a recent population expansion.

Discussion

In this study, there was high haplotype diversity in six coastal horseshoe crab populations in the northern Java Sea, Bintan, and Balikpapan waters of Indonesia. There was also a high number of polymorphic sites (43, with 34 defined haplotypes) in Indonesian coastal horseshoe crab populations. The mean haplotype diversity ($h = 0.935$) was quite high, while nucleotide diversity ($\pi = 0.006$) was low across all populations. Similarly high haplotype diversity values were reported in *T. gigas* ($h = 0.797\pm0.129$ and $\pi = 0.058\pm0.001$; Rozihan & Ismail, 2011) in Malaysia and tri-spined horseshoe crab (*T. tridentatus*) in Taiwan ($h = 0.626\pm0.075$ and $\pi = 0.003\pm0.005$; Yang et al., 2007).

Previous studies reported generally high genetic diversity in coastal horseshoe crab. In this study, our results showed high genetic diversity, but low nucleotide diversity. The high number of haplotypes indicates that these populations were large enough to maintain a high level of genetic diversity. These small differences are the signature of rapid demographic expansion from a small and effective population size (Avise, 2000). Nucleotide diversity is a sensitive index when analyzing population genetic diversity (Nei & Li, 1979), which is influenced by life-history characteristics, environmental heterogeneity, large population size (Nei, 1987; Avise, 2000), fishing pressure (Madduppa et al., 2018), reduced larval transport, and limited exchange with other populations (Timm et al., 2017). The rate of mitochondrial evolution and historical factors play an important role in determining genetic variability patterns (Grant et al., 2006; Xiao et al., 2009; Yamaguchi et al., 2010).

We detected very low extensive differentiation across populations (not significant F_{ST} values between 0 and 0.13), with exceptions between UK-MD and UK-SB. This indicated that

there was little subdivision across populations. ~~Conversely,~~ However, the horseshoe crab life-history characteristics and habitat preferences suggested restricted dispersal capabilities (Sekiguchi, 1988). The crab showed limited movement capabilities only in their home range area. Individual distances up to 30 km have been observed in Malaysian crabs (Mohamad et al., 2019), while the movement capabilities of tri-spined horseshoe crab did not exceed 150 km (Yang et al., 2007). Similarly, the American horseshoe crab in the Great Bay Estuary (USA) has a maximum mean annual linear distance ranging between 4.5 km and 9.2 km (Schaller et al., 2010). Studies by Swan (2005) over multiple years found that *Limulus* moved from 104 to 265 km from their release sites. Ecological observations showed that 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, larvae have a strong tendency to concentrate in inshore rather than offshore waters (100-200 km) (Botton & Loveland, 2003), suggesting a limited capability for long-range dispersal between estuaries. Additionally, ~~the~~ low F_{ST} levels reflect inter-population movement over mutigenerational intervals that short-term tagging studies ~~cannot document.~~ Long-term tagging studies have found that horseshoe crabs can move ~~up to~~ 5-500 km ~~dominated in~~ 5-30 km (Beekey & Mattei, 2015), ~~while around~~ 767 km over their long lifetimes (E. Hallerman, 2020, personal communication). A similar study by Rozihan & Ismail (2011) reported that the crab's F_{ST} value along the west coast of peninsular Malaysia ranges from 0.111 – 0.557, indicating moderate to high genetic differentiation (Wright, 1978; Hartl & Clark, 1977). Other reports in the area used microsatellite markers to find a F_{ST} value between 0.144 and 0.846.

There were only ~~six~~ shared haplotypes among the 34 total haplotypes ~~discovered from~~ all 91 samples. The median-joining network analysis ~~showed~~ past population expansions with shared haplotypes among localities. Overall, relationship patterns at the mtDNA level ~~reduced~~ geographical structure. The haplotype network revealed recent demographic processes, but the small sample sizes also limited the possibility of observing the intermediate haplotypes inferred to exist in the network. Moreover, Tajima's D and Fu's Fs test indicated the occurrence of population expansion. Common haplotypes shared between localities can also be explained by the history of biogeography in this Southeast Asian region known as the Sunda Shelves, which includes Java, Sumatera, and Borneo. Historically, Sundaland experienced both dewatering and inundation during the Pleistocene period. Haplotype sharing in this study is attributed to breeding migration and pelagic larvae, as well as the sharing of common ancestors (Frankham, 1996). The occurrence of many specific haplotypes can be explained by the small sample size and perhaps isolation during the Last Glacial Maximum. Many species became isolated in refugia, but genetic differentiation and divergence occurred due to the retreat and dispersal of glacial ice sheets (Hewitt, 2000).

A proactive management approach regarding the Asian coastal horseshoe crab (*T. gigas*) in Indonesia should consider population genetics. High haplotype diversity that occurs with low nucleotide diversity has been associated with population growth or expansion after a period of low effective population growth (Grant & Bowen, 1998). ~~Moreover~~, our findings indicate that *T. gigas* in Indonesia have low genetic differentiation but high population connectivity and expansion. Therefore, ~~all the~~ results suggest that there is a single stock of Indonesia coastal horseshoe crab. ~~However~~, the best conservation strategy is one that combines both local and regional management. ~~Additionally~~, an advanced population genetic analysis based on male and female horseshoe crabs and the nuclear genome (e.g., microsatellites or SNPs) should be

explored in the future. This would also require expanding the scope of geographic sampling around Indonesia.

Conclusion

High genetic diversity and low levels of differentiation across coastal horseshoe crab (*T. gigas*) populations in Indonesia indicated a single species stock with high connectivity. A locally-based conservation management method is suggested as one precautionary approach to conserving the Indonesian coastal horseshoe crab.

Acknowledgements

The authors are grateful to everyone who helped in the field work: local fishermen in all sampling areas, Dr. Qian Tang, Heri Saputro, Agus Alim Hakim, Rani Nuraisah, Siti Mira Rahayu, Ahmad Fauzi Ridwan, and Yunita Multi Cahya Ningrum.

References

Avise JC. 2000. *Phylogeography: The History and Formation of Species*. Cambridge: Harvard University Press 447.

Bandelt H-J, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37-48. PMID: 10331250 DOI: 10.1093/oxfordjournals.molbev.a026036.

Beekey MA, Mattei JH. 2015. The mismanagement of *Limulus Polyphemus* in Long Island Sound, U.S.A.: What are the characteristics of a population in decline? In: Carmichael RH, Botton ML, Shin PKS, Cheung SG, ed. *Changing Global Perspectives on Horseshoe Crab Biology, Conservation and Management*. Berlin: Springer, 433-461.

Bernardi G, Talley D. 2000. Genetic evidence for limited dispersal in the coastal California killifish, *Fundulus parvipinnis*. *Journal of Experimental Marine Biology and Ecology* 255: 187–199. DOI: 10.1016/S0022-0981(00)00298-7.

Brehm A, Harris DJ, Hernandez M, Cabrera V, Larruga J, Pinto F, Gonzalez AM. 2001. Structure and evolution of the mitochondrial DNA complete control region in the *Drosophila subobscura* subgroup. *Insect Molecular Biology* 10: 573–578. DOI: 10.1046/j.0962-1075.2001.00295.x.

Botton ML. 2001. The conservation of horseshoe crab: what can we learn from the Japanese experience ? In: Tanacredi TJ, ed. *Limulus in the Limelight*. New York: Kluwer Academic/Plenum, 41–51.

Botton ML, Loveland RE. 2003. Abundance and dispersal potential of horseshoe crab (*Limulus polyphemus*) larvae in the Delaware estuary. *Estuaries* 26(6): 1472–1479. DOI: 10.1007/BF02803655.

Chen CP, Yeh HY, Lin PF. 2004. Conservation of horseshoe crabs in Kinmen, Taiwan: strategies and practices. *Biodiversity and Conservation* 13: 1889–1904. DOI: 10.1023/B:BIOC.0000035868.11083.84.

Collin R. 2001. The effects of mode of development on phylogeography and population structure of North Atlantic *Crepidula* (Gastropoda: Calyptraeidae). *Molecular Ecology* 10: 2249–2262. PMID: 11555267 DOI: 10.1046/j.1365-294x.2001.01372.x.

Crandall E, Riginos C, Bird C, Liggins L, Treml E, Beger M, Barber PH, Connolly SR, Cowman PF, DiBattista JD, Eble JA, Magnuson SF, Horne JB, Kochzius M, Lessios HA, Liu SYV, Ludt WB, Madduppa H, Pandolfi JM, Toonen RJ, Contributing Members of the Diversity

274 of the Indo-Pacific Network, Gaither MR. 2019. The molecular biogeography of the Indo-
275 Pacific: testing hypotheses with multispecies genetic patterns. *Global Ecology and*
276 *Biogeography* 28(5): 943–96. DOI:10.1111/geb.12905.
277 Eldredge N, Stanley SM. 1984. *Living Fossils*. Berlin: Springer Publishing.
278 Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: A new series of programs to perform
279 population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10:
280 564–567. DOI: 10. 1111/j.1755-0998.2010.02847.x PMID: 21565059.
281 Frankham R. 1996. Relationship of genetic variation to population size in wildlife. *Conservation*
282 *Biology* 10(6): 1500–1508. DOI: 10.1046/j.1523-1739.1996.10061500.x.
283 Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking
284 and background selection. *Genetics* 147(2): 915-925. PMCID: PMC1208208.
285 Grant WS, Bowen BW. 1998. Shallow population histories in deep evolutionary lineages of
286 marine fishes: insight from sardines and anchovies and lessons for conservation. *Genetics*
287 89: 415-426.
288 Grant WS, Spies IB, Canino MF. 2006. Biogeographic evidence for selection on mitochondrial
289 DNA in north Pacific walleye Pollock *Theragra chalcogramma*. *Journal of Heredity* 97(6):
290 571–580. DOI: 10.1093/jhered/esl033.
291 Hartl DL, Clark AG. 1997. *Principles of Population Genetics*, 3nd edn. Sunderland MA: Sinauer
292 Associates, Inc.
293 Hedgecock R. 1986. Is gene flow from pelagic larval dispersal important in the adaptation and
294 evolution of marine invertebrates ? *Bulltin of Marine Science* 39(2): 550–565.
295 Hewitt G. 2000. The genetic legacy of the Quaternary ice ages. *Nature* 405: 907–913. DOI:
296 10.1038/35016000.
297 Itow T. 1993. Crisis in the Seto Inland Sea: the decimation of the horseshoe crab. *EMECS*
298 *Newslett.* 3: 10–11.
299 John BA, Nelson BR, Sheikh HI, Cheung SG, Wardiatno Y, Dash BP, Tsuchiya K, Iwasaki Y,
300 Pati S. 2018. A review on fisheries and conservation status of Asian horseshoe crabs.
301 *Biodiversity and Conservation* 27: 3573-3598. DOI: 10.1007/s10531-018-1650-7.
302 Johnson MS, Black R. 1998. Increased genetic divergence and reduced genetic variation in
303 populations of snail *Bembicium vittatum* in isolated tidal ponds. *Heredity* 80: 163–172.
304 DOI: 10.1046/j.1365-2540.1998.00257.x.
305 King TL, Eackles MS. 2004. Microsatellite DNA markers for the study of horseshoe crab
306 (*Limulus polyphemus*) population structure. *Molecular Ecology Notes* 4: 394–396. DOI:
307 10.1111/j.1471-8286.2004.00663.x.
308 King TL, Eackles MS, Spidle AP, Brockmann HJ. 2005. Regional differentiation and sex-biased
309 dispersal among populations of the horseshoe crab (*Limulus polyphemus*). *Transactions of*
310 *the American Fisheries Society* 134 (2): 441–465. DOI:10.1577/T04-023.1.
311 King TL, Eackles MS, Aunins AW, Brockmann HJ, Hallerman E, Brown BL. 2015.
312 Conservation genetics of the American horseshoe crab (*Limulus polyphemus*): Allelic
313 diversity, zones of genetic discontinuity, and regional differentiation. In: Carmichael RH,
314 Botton ML, Shin PKS, Cheung SG, ed. *Changing Global Perspectives on Horseshoe Crab*
315 *Biology, Conservation and Management*. Berlin: Springer, 65-96.
316 Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular evolutionary
317 genetics analysis across computing platforms. *Molecular Biology and Evolution* 35: 1547–
318 1549. DOI: 10.1093/molbev/msy096.

Lavrov DV, Boore JL, and Brown WM. 2000. The complete mitochondrial DNA sequence of the horseshoe crab *Limulus polyphemus*. *Molecular Biology and Evolution* 17: 813–824. DOI: 10.1093/oxfordjournals.molbev.a026360.

Madduppa HH, Timm J and Kochzius M. 2018. Reduced genetic diversity in the clown anemonefish *Amphiprion ocellaris* in exploited reefs of Spermonde Archipelago, Indonesia. *Frontiers in Marine Science* 5(80):1-8. DOI: 10.3389/fmars.2018.00080.

Mashar A, Butet NA, Juliandi B, Qonita Y, Hakim AA, Wardiatno Y. 2017. Biodiversity and distribution of horseshoe crabs in northern coast of java and southern coast of Madura. *IOP Conference Series: Earth and Environmental Sciences* 54:1-8. DOI: 10.1088/1755-1315/54/1/012076.

Meilana L, Wardiatno Y, Butet NA, Krisanti M. 2016. Morphological character and molecular identification with COI gene marker of horseshoe crabs (*Tachypleus gigas*) at coastal waters of northern Java Island. *Jurnal Ilmu dan Teknologi Kelautan Tropis* 8(1): 145-158. DOI: 10.28930/jitkt.v8i1.12651.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences of the United States of America* 76(10): 5269–5273. DOI: 10.1073/pnas.76.10.5269.

Nei M. 1987. *Molecular Evolutionary Genetics*. New York: Columbia University Press.

Pierce JC, Tan G, Gaffney PM. 2000. Delaware Bay and Chesapeake Bay populations of the horseshoe crab *Limulus polyphemus* are genetically distinct. *Estuaries* 23: 690–698. DOI: 10.2307/1352895.

Rozihan M, Ismail E. 2011. Genetic structure and haplotype diversity of *Tachypleus gigas* population along the west coast of Peninsular Malaysia inferred through mtDNA AT-rich region sequence analysis. *Biotechnology* 10(3): 298-302.

Rozas J, Ferrer-Mata A, Sanchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE. 2017. DnaSP v6: DNA Sequence polymorphism analysis of large datasets. *Molecular Biology and Evolution* 34: 3299–3302. DOI: 10.1093/molbev/msx248 PMID: 29029172.

Rubiyanto E. 2012. Study population of horseshoe crabs (Xiphosura) in peninsular Kuala Tungkal, the district of Tanjung Jabung Barat, Jambi. Master Thesis, University of Indonesia, Indonesia.

Rutecki D, Carmichael RH, Valiela I. 2004. Magnitude of harvest of Atlantic horseshoe crabs, *Limulus polyphemus*, in Pleasant Bay, Massachusetts. *Estuaries* 27: 179–187. DOI: 10.1007/BF02803374.

Schaller SY, Chabot CC, Watson WH. 2010. Seasonal movements of American horseshoe crabs *Limulus polyphemus* in the Great Bay Estuary, New Hampshire (USA). *Current Zoology* 56: 587–598. DOI: 10.1093/czoolo/56.5.587.

Sekiguchi K. 1988. *Biology of Horseshoe Crabs*. Tokyo: Science House.

Shuster CN Jr. A pictorial review of the natural history and ecology of the horseshoe crab *Limulus polyphemus*, with reference to other Limulidae. *Progress in Clinical and Biological Research* 81:1-52. PMID: 6750622.

Swan BL. 2005. Migrations of adult horseshoe crabs, *Limulus polyphemus*, in the middle Atlantic Bight: A 17-year tagging study. *Estuaries* 28: 28–40. DOI: 10.1007/BF02732751.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123:585-595. PMID: 2513255; PMCID: PMC1203831.

Timm J, Kochzius M, Madduppa HH, Neuhaus AI, Dohna T. 2017. Small scale genetic population structure of coral reef organisms in Spermonde Archipelago, Indonesia. *Frontiers in Marine Science* 4: 294. DOI: 10.3389/fmars.2017.00294.

Vestbo S, Obst M, Quevedo Fernandez FJ, Intanai I, Funch P. 2018. Present and potential future distributions of Asian horseshoe crabs determine areas for conservation. *Frontier Marine Science* 5:164. DOI: 10.3389/fmars.2018.00164.

Walls EL, Berkson J, Smith SA. 2002. The horseshoe crab, *Limulus polyphemus*: 200 million years of existence, 100 years of study. *Review Fisheries Sciences* 10: 39-73. DOI: 10.1080/20026491051677.

Watts RJ, Johnson MS. 2004. Estuaries, lagoons and enclosed embayments: habitats that enhance population subdivision of inshore fishes. *Marine and Freshwater Research* 55: 641–651. DOI: 10.1071/MF04051.

Wright S. 1978. Evolution and the Genetics of Population, Variability Within and Among Natural Populations. Chicago: The University of Chicago Press.

World Conservation Monitoring Centre. 1996. *Tachypleus gigas*, The IUCN Red List of Threatened Species 1996: e. T21308A9266907. DOI:10.2305/IUCN.UK.1996.RLTS.T21308A9266907.en.

Xiao YS, Zhang Y, Gao TX, Yanagimoto T, Yabe M, Sakurai Y. 2009. Genetic diversity in the mitochondrial DNA control region and population structure in the small yellow croaker *Larimichthys polyactis*. *Environmental Biology of Fishes* 85: 303–314. DOI: 10.1007/s10641-009-9497-0.

Yamaguchi K, Nakajima M, Taniguchi N. 2010. Loss of genetic variation and increased population differentiation in geographically peripheral populations of Japanese char *Salvelinus leucomaenis*. *Aquaculture* 308: S20–S27. DOI: 10.1016/j.aquaculture.2010.07.032.

Yang MC, Chen CA, Hsieh HL, Chen CP. 2007. Population subdivision of the tri-spine horseshoe crab, *Tachypleus tridentatus*, in Taiwan Strait. *Zoological Science* 24: 219-224. DOI: 10.2108/zsj.24.219.

Table 1:

Variable sites found in a fragment of the AT-rich region of *Tachypleus gigas* in each populations. ~~Fourty~~ 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
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	.
H2	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.

REMOVE THESE LINES

the number of observations of each haplotype can be given as a column.

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

Population	Code	A+T%	n	N*h*	*h*	π
Bintan	BT	81.597	8	6	0.892	0.006
Balikpapan	BP	81.473	14	10	0.945	0.009
Demak	DK	81.568	16	6	0.783	0.004
Madura	MD	81.412	13	8	0.910	0.006
Subang	SB	81.548	20	11	0.926	0.005
Ujung Kulon	UK	81.434	20	12	0.942	0.005
Total			91		0.935	0.0064

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

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	0.00	0.00	-	
UK	0.00	0.08	0.09	0.10*	0.10*	-

Notes : F_{ST} value significantly different from zero (p<0.05)* ; BT= Bintan; BP=Balikpapan; DK= Demak; MD= Madura; SB= Subang; UK= Ujung Kulon

Tabel 4
The 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	0.006
Within populations	85	95.23		
Total	90			

Table 5:
Results of Tajima's D and Fu's F_S tests including associated p-values in all locations

Population	Tajima's D	Fu's F_S
Bintan	-0.646 ns	-0.608 ns
Balikpapan	0.601 ns	0.847 ns
Demak	0.325 ns	-2.941 ns
Madura	-0.875 ns	-1.532 ns
Subang	0.166 ns	-0.891 ns
Ujung Kulon	-0.318 ns	-3.865 ns

No values were

Notes: ~~ns = not~~ significant

Figure 1

Sampling locations of *Tachypleus gigas* ; There were eight, 14, 16, 13, 20, and 20 samples from Bintan Island (BT) = 8, Balikpapan (BP) = 14, Demak (DK)= 16, Madura (MD) = 13, Subang (SB) = 20 and Ujung Kulon (UK) = 20

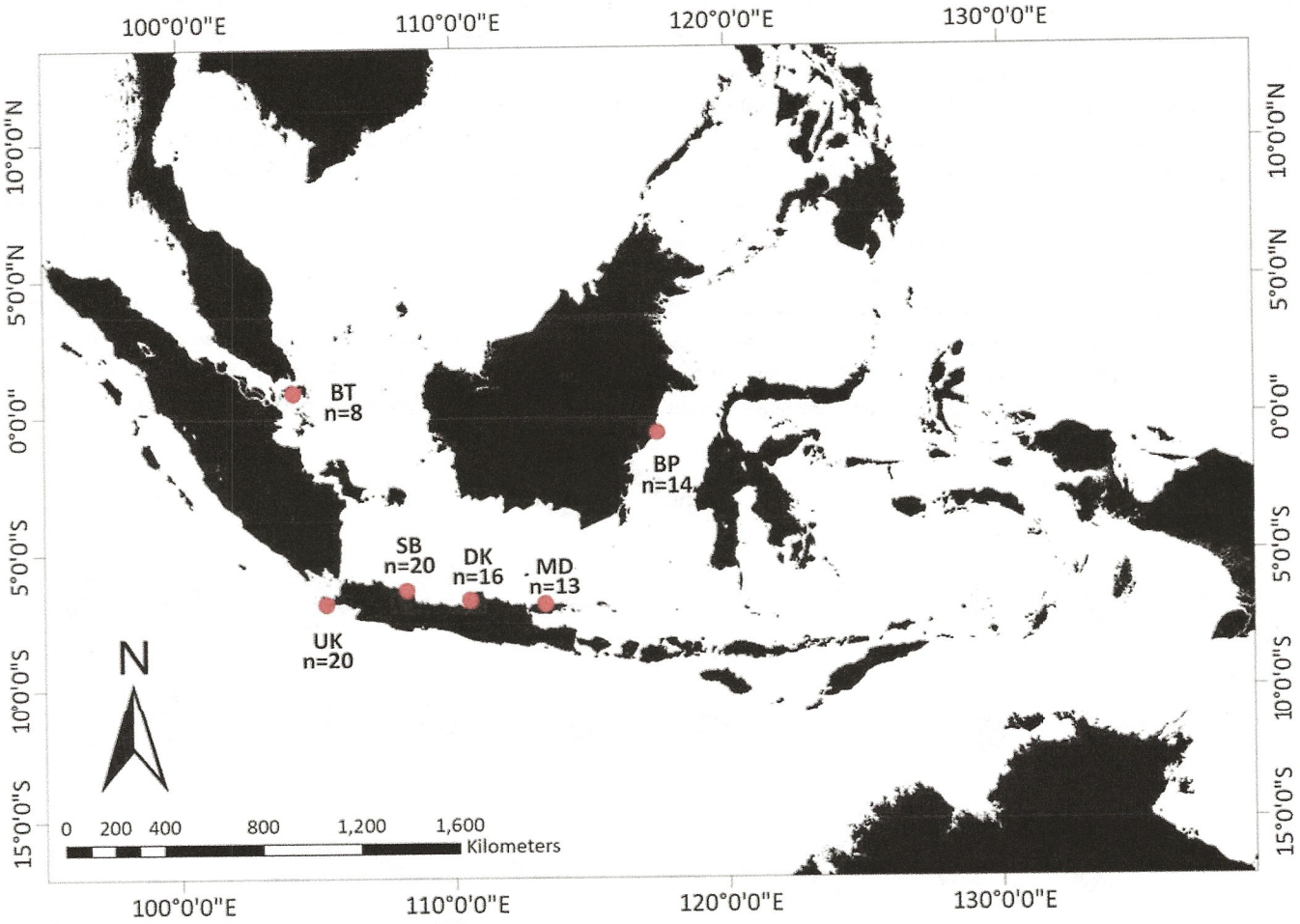

Figure 2

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

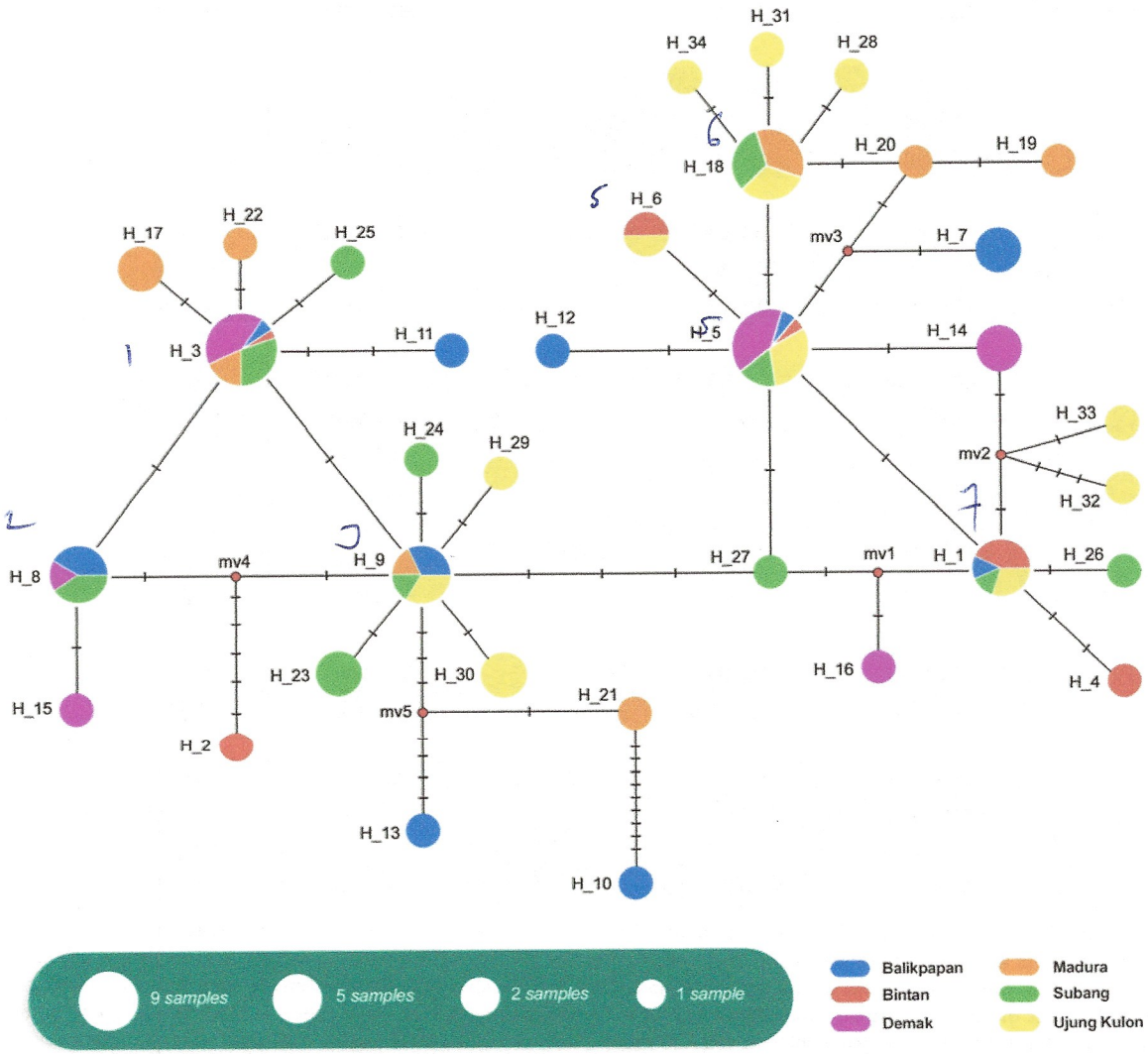

Figure 3

Distribution of 34 haplotypes of *Tachypleus gigas* population ~~from~~ 2C/03J six locations in ~~around~~ Indonesia