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Development of Microsatellite Markers Reveals Moderate Genetic Diversity in Sabah's Tri-Spine Horseshoe Crab, *Tachypleus tridentatus* (Leach, 1819)

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ABSTRACT

The tri-spine horseshoe crab, *Tachypleus tridentatus* (Leach,1819), is a species under conservation pressure. Anecdotal observations suggest a decline in its populations along the coasts of Sabah, northern Borneo, raising concerns over the potential loss of genetic diversity. In this study, a total of 36 individuals were collected from three localities (Sandakan, Kota Belud, and Kudat) representing both the east and west coasts of Sabah. Genomic DNA from these samples was used to develop novel microsatellite markers, enabling the assessment of population-level genetic variation. Here, we report on the characterization of 14 polymorphic microsatellite loci. All loci were observed to be polymorphic with the number of alleles ranging from 2 to 5 per locus. The overall observed heterozygosity for the 14 analyzed loci ranged from 0.03 to 0.4667 while the expected heterozygosity ranged from 0.2466 to 0.7706. The results indicate that genetic variability in the populations is moderate with signs of inbreeding and restricted gene flow. These markers have a potential application in population management where it will be used to determine if there is a loss of genetic diversity of the Asian horseshoe crab species that are present in Sabah.

Keywords: *Microsatellites, horseshoe crab, simple sequence repeat, molecular markers, Sabah*

INTRODUCTION

Horseshoe crabs are often referred to as 'living fossils' because they have existed for more than 450 million years, retaining their distinctive three-part armor-like exoskeleton (Bennett *et al*. 2018; Størmer, 1952). Only four extant species are known today, which are, the American species *Limulus polyphemus*, and three Asian species, *Tachypleus tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda* all of which are distributed across their respective geographical regions (Carmichael and Brush, 2012; Fu *et al*., 2019). Throughout their life cycle, horseshoe crabs occupy diverse habitats, particularly sandy beaches near the high-tide line for nesting, intertidal mudflats for juvenile development, and shallow coastal waters less than 30 meters deep as feeding grounds (Chen *et al*., 2015; Faridah *et al*., 2015; Tanacredi *et al*., 2009).

Horseshoe crabs play an essential role in both marine ecosystems and the biomedical industry, making them commercially significant. For instance, the *Limulus*/*Tachypleus* amebocyte lysate (LAL/TAL), a vital component in sterility testing that guarantees the safety of drugs and medical devices for millions of patients each year, is derived from horseshoe crab hemolymph (Krisfalusi-Gannon et al., 2018; Tinker-Kulberg et al., 2020). However, the commercial harvesting and bleeding of horseshoe crabs for hemolymph extraction have raised serious concerns about population decline and the long-term sustainability of both the species and the LAL/TAL supply chain.

Extensive studies have been conducted on the American horseshoe crab, *L. polyphemus*, covering molecular biology, population dynamics, reproductive ecology, and spawning behavior (Jackson *et al*., 2014; Nelson *et al*., 2015; Smith *et al*., 2017). In contrast, research relevant to the Asian horseshoe crabs remains limited, with ongoing studies mainly concentrated in East, South and Southeast Asian countries (Obst *et al*., 2012). The IUCN Red list of vulnerable species includes Asian horseshoe crabs under the "Data Deficient" category for *T. gigas* and *C. rotundicauda*, while *T. tridentatus* has been listed under the 'Endangered' category since 2019.

The declining status of the tri-spine horseshoe crab, *T. tridentatus*, has been reported globally specifically in China and Japan, where adult populations have become increasingly rare compared to observations from three to four decades ago (Berkson *et al*., 2009; Shin *et al*., 2009; Manca *et al*., 2017). These declines have been largely attributed to coastal development, habitat loss, overharvesting and the degradation of spawning and nursery grounds. In response, conservation efforts, including long-term monitoring of juvenile populations have been implemented in regions including Hong Kong and Taiwan, where field surveys have documented reduced recruitment and localized population fragmentation (Kwan et al., 2016). Genetic studies in these regions have further revealed significant population structuring and variable levels of genetic diversity using mitochondrial and microsatellite DNA markers (Nishida and Koike, 2009), indicating that habitat connectivity and effective population size are critical determinants of genetic resilience.

Within the Malaysian context, research on *T. tridentatus* remains limited compared to that on *T. gigas* and *C. rotundicauda* (Robert *et al*., 2014). This is largely because *T. tridentatus* is geographically limited to northern Borneo, whereas the other two species are found in both Peninsular Malaysia and the island of Borneo. A recent genetic study in Sabah using mitochondrial and microsatellite markers reported polymorphic variation among horseshoe crab populations across multiple districts, underscoring the presence of genetic structure within the region (Kntayya, 2015). Independent studies have also provided the first population size estimates of *T. tridentatus* in Tawau and highlighted morphometric differences among Sabah populations, offering useful ecological baselines for management (Mohamad et al., 2016; Manca et al., 2017). Given the limited genetic data available, there is a need to employ molecular markers to assess the genetic diversity of *T. tridentatus* populations in Malaysia. Understanding their genetic variation is vital for developing effective conservation strategies.

This present study aims to evaluate the genetic diversity of *T. tridentatus* across three spatially distributed populations in northern Borneo i.e., Sandakan, Kota Belud and Kudat, respectively using microsatellite markers. The findings are expected to contribute valuable insights for the conservation and management of this endangered species.

MATERIALS & METHODS

Sample Collection

A total of 36 *T. tridentatus* individuals were sampled from three coastal locations in Sabah, Malaysian Borneo: Sandakan (n = 12), Kota Belud (n = 12), and Kudat (n = 12), representing populations from the east and west coasts of Sabah. The specimens were obtained opportunistically from individuals incidentally caught in fishing nets or available at wet markets or local *tamu*. Tissue samples were obtained from the walking legs of individuals and preserved immediately at 4 ^{o}C for subsequent genetic analysis. All samples used in this study were collected between 2011 and 2014, prior to the implementation of the Sabah Biodiversity Centre (SaBC) permit requirement notified by Pusat Penyelidikan Inovasi (PPI), Universiti Malaysia Sabah.

Microsatellite Isolation and Marker Development

Genomic DNA was isolated from a single *T. tridentatus* individual and subjected to partial shotgun sequencing using the PacBio RS II long-read sequencing platform. The resulting sequence reads were then mined for simple sequence repeat to identify potential microsatellite loci suitable for marker development. The sequencing data are available at GenBank (SRA: SRR22722188, BioProject: PRJNA761241).

In this study, we focused on perfect microsatellites with a combination of di,-tri- and tetra-nucleotides motifs as these loci generally exhibit greater genetic variation than other repeat types (Merritt *et al*., 2015; Temnykh *et al*., 2001). Mononucleotide repeats were excluded due to their higher likelihood of sequencing or assembly errors, which can complicate accurate genotyping (Cai *et al*., 2013; Wattanadilokchatkun *et al*., 2022). The search criteria utilized to find the relevant microsatellite markers were restricted to a minimum of ten repeats for dinucleotides, four for trinucleotides and tetranucleotides respectively.

Search parameters for microsatellite identification were set to a minimum of ten repeat units for dinucleotides and a minimum of four repeat units for trinucleotides and tetranucleotides. Primer pairs were designed using Primer3 software (version 3) (https://primer3.ut.ee), with parameters optimized for an expected product size of 150–350 bp, primer length of 17–30 bp, and melting temperature (Tm) range of 50–65℃. From the identified microsatellite loci, sixteen markers with the longest repeat motifs were selected for downstream population genetic analyses to maximize polymorphic information content (PIC) and enhance discriminatory power among populations (Table 1).

Microsatellite Genotyping and Analysis

Total genomic DNA was isolated from the tissues using Wizard Genomic Purification Kit (Promega, USA) according to manufacturer's instruction with minor modification. The purified DNA was then used as a template for microsatellite genotyping. The PCR amplification was performed in a 10 µL reaction volume containing approximately 20 ng of genomic DNA, 0.1 U GoTaq Flexi DNA Polymerase (Promega, USA), 1x buffer, 1.0 mM $MgCl_2$, 10 pmol of each primer, 0.2 mM of dNTPs in a C1000 thermal cycler (Bio-Rad, USA). The PCR protocol was as follows: initial denaturation at 94℃ for 2 min followed by 40 cycles of denaturation at 94℃ for 25 s; annealing at 47-63℃ (depending on the primer pair) for 25 s; extension at 68℃ for 25 s; and final extension at 68℃ for 2 min. Then, the PCR products were separated using the QIAxcel

Advanced capillary electrophoresis system (Qiagen, Germany). The QIAxcel ScreenGel software was used to generate the output file from the electrophoresis for allele scoring.

Genetic diversity indices, including the number of alleles per locus, observed heterozygosity (H_o), and expected heterozygosity (H_e), were calculated using PopGene version 1.32 (Yeh et al., 1999). The same software was used to test for Hardy–Weinberg equilibrium (HWE) and to estimate the inbreeding coefficient (F_{IS}) for each locus.

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Table 1 Microsatellite primer sequences and amplification information for *T. tridentatus*.

Locus	Accession Number	Repeat motif	Primer Sequences (5′-3′)	Annealing temperature (°C)	Fragment size (bp)
TT02	OK396648	$(AAGT)_4$	F: CGTGTACCGTGTGCAGTATT R: ACTGCTGGGAGTCATTGTCA	55	167-188
TT03	OK396649	$(AGAT)_4$	F: AGACACTCACACACACACAGA R: GATCGGCAAGCTGTGGAAAG	62	196-216
TT04	OK396650	$(AACC)_5$	F: GGCTGCAAAATGTCGGGATA R: GACAACCAAAGCATGTATCAGGA	58	231-274
TT05	OK349170	$(AAG)_5$	F: GGGTGGAGTGCTCGAGAG R: CATCCAGGATTCAATACACAGGA	55	280-309
TT07	OK349171	$(AAC)_5$	F: GCTCAAGCGGCATGTCTG R: TGTCCTTCTTCGTACGTGGT	53	186-217
TT08	OK274271	$(AC)_{36}$	F: ACACACCCTCCACAATCCAT R: TGTAGAATGGACGGCAACTG	57	143-216
TT10	OK396651	$(AAAG)_4$	F: ACGGAGCTGGCATTCCTAAT R: GCTTCGTGACCACCTTAAAGA	55	240-263
TT12	OK349172	$(AGC)_4$	F: TGTTGGAAAGTTTGGCCACA R: CAAGACATCACAAGCCCTGG	55	182-208
TT13	OK349173	$(AAC)_6$	F: TGTGTGTCCCCTTGCAGTAT R: TCACTGCCTCGGTAACTCTC	54	204-225
TT15	OK274274	$(AG)_{11}$	F: AGGGAAAAGTGCACTGGAGA R: CCTGGACTGTACACTTGTGG	58	166-293
TT16	OK247272	$(GT)_{22}$	F: CCTAAGGACTGGGAGATAGCA R: AAGGTACACTGACGCCCTAC	59	150-204
TT17	OK349175	$(ATG)_6$	F: ACGACTTGCTAAAACTCAACAAA R: TCAGTGTCGGAGGTTCTTGA	52	201-213
TT18	OK349176	$(AAG)_5$	F: TGCCGCAGATTTGACACATG R: ACACAATTACCCATACGAACACC	55	203-225
TT20	OK396653	$(AAAC)_6$	F: CCCTTGAGTAGCTTTGCGTG R: ACCAACAAGAGAAGGAGTCCA	53	177-211

RESULTS

A total of 16 microsatellite loci were initially identified from the *Tachypleus tridentatus* genome. Of these, 14 loci were successfully amplified and found to be polymorphic across the 36 individuals analyzed (Table 1). The repeat motifs consisted mainly of di-, tri-, and tetranucleotide repeats, with the number of repeat units ranging from four to thirty-six. The amplified fragment sizes ranged from 143 to 309 bp, and the annealing temperatures for the primers varied between 52°C and 63°C.

Across the pooled dataset of 36 individuals, the number of alleles per locus (N_A) ranged from 2 to 5, with a mean of 3.4 alleles per locus, indicating moderate polymorphism of the developed markers (Table 2). Expected heterozygosity (H_E) values ranged from 0.2003 (TT15) to 0.7524 (TT04), with an average of 0.5659, while observed heterozygosity (H_O) ranged from 0.0278 (TT02, TT03) to 0.7222 (TT16), averaging 0.3367 across all loci (Table 2). Loci TT04, TT12, and TT16, exhibited comparatively higher heterozygosity and were particularly informative for genetic analyses.

Deviations from Hardy–Weinberg equilibrium ($P_{HWE} < 0.05$) were detected at most loci when all samples were pooled, with the exception of TT07. These deviations are likely influenced by population sub-structuring and heterogeneity among sampling locations rather than reflecting locus-specific biases alone. Overall, the pooled analysis confirms that the microsatellite loci developed in this study are sufficiently polymorphic and suitable for population-level genetic analyses.

Table 2 Polymorphism characteristics and heterozygosity estimates of 14 microsatellite loci developed for *Tachypleus tridentatus* across all sampled individuals.

Locus	Accession Number	N_A	H_E	H_O	P_{HWE}
TT02	OK396648	4	0.6960	0.0278	0.00
TT03	OK396649	2	0.5035	0.0278	0.00
TT04	OK396650	5	0.7524	0.5429	0.00
TT05	OK349170	4	0.6076	0.3056	0.00
TT07	OK349171	2	0.3416	0.3143	0.63
TT08	OK274271	4	0.4803	0.3143	0.01
TT10	OK396651	3	0.5904	0.0833	0.00
TT12	OK349172	4	0.7039	0.4571	0.00
TT13	OK349173	4	0.6553	0.3889	0.00
TT15	OK274274	2	0.2003	0.0556	0.00
TT16	OK247272	3	0.6467	0.7222	0.02
TT17	OK349175	2	0.5057	0.2941	0.01
TT18	OK349176	3	0.5622	0.6944	0.05
TT20	OK396653	4	0.6762	0.4857	0.01
	Mean		0.5659	0.3367	

Genetic diversity indices calculated separately for each of the three *T. tridentatus* populations using 14 newly isolated microsatellite markers are presented in Table 3. Mean expected heterozygosity (H_E) ranged from 0.434 in Kudat to 0.490 in Kota Belud, while the mean observed heterozygosity (H_O) ranged from 0.286 in Sandakan to 0.396 in Kota Belud. Here, all three populations show lower observed heterozygosity levels compared to the expected heterozygosity, indicating a general deficit of heterozygotes. Sandakan population was observed to have the lowest observed heterozygosity compared to the Kota Belud and Kudat populations.

Table 3 Population-specific genetic diversity indices for three *Tachypleus tridentatus* populations in Sabah based on 14 microsatellite loci.

Population	N	H_E	H_O	F_{IS}
Kota Belud	12	0.490	0.396	0.187
Kudat	12	0.434	0.328	0.267
Sandakan	12	0.459	0.286	0.393

The inbreeding coefficient (F_{IS}) varied among populations, with the highest value observed in Sandakan (0.393), followed by Kudat (0.267) and Kota Belud (0.187). The relatively higher F_{IS} values in all three populations indicate a general deficit of heterozygotes, which may reflect limited gene flow, small population size, or localized breeding within sampling sites. Collectively, the population-based analysis demonstrate moderate levels of genetic diversity among the *T. tridentatus* populations studied, with Kota Belud showing the highest heterozygosity and lowest inbreeding coefficient, suggesting a comparatively more genetically diverse and stable population.

DISCUSSION

This study represents one of the few molecular investigations on *Tachypleus tridentatus* populations from Malaysian waters, particularly in Sabah. Using newly developed microsatellite markers, we assessed the genetic diversity of three geographical separated populations from Kota Belud, Kudat and Sandakan, and detected moderate levels of genetic variation across loci. Although genetic variation is still present, as reflected by allelic richness and heterozygosity estimates (Tables 2 and 3), the overall pattern suggest it may be declining, likely due to limited gene flow and increasing habitat fragmentation in coastal areas.

A total of 46 alleles were identified across the three populations, with the number of alleles per locus ranging from 2 to 4 in Kota Belud and Sandakan, and from 2 to 5 in Kudat. These values indicate a modest level of genetic variation within Sabah's *T. tridentatus* populations. Our findings are consistent with a previous study on the Tuaran population, located at the west coast of Sabah (Kntayya, 2015), which reported 1 to 3 alleles per locus. In contrast, *T. tridentatus* populations in China exhibited considerably higher allelic richness, averaging 3 to 8 alleles per locus (Cai *et al*., 2015). According to Xu *et al*. (2000), higher numbers of alleles across microsatellite loci are often associated with increased mutation rates, which may in turn shape the genetic structure of populations. These comparisons suggest that *T. tridentatus* populations in China are genetically more diverse than those in Sabah, possibly due to larger effective population sizes and wider habitat connectivity. Given that the distribution of *T. tridentatus* in Malaysia is largely confined to Sabah, the lower allelic variation observed here implies a greater susceptibility to genetic drift and diversity loss.

All loci, except TT07 and TT18, deviated significantly from Hardy–Weinberg equilibrium (HWE). Such deviations may result from a combination of biological and demographic processes, including non-random mating, population sub-structuring (Wahlund effect), limited dispersal among sites, and small effective population sizes. The influence of null alleles and sampling effects associated with modest sample sizes may also contribute to these departures, as reported in other population genetic studies (Wittke-Thompson et al., 2005). Therefore, deviations from equilibrium observed in this study likely reflect both genuine population structure and methodological constraints.

Genetic diversity among the three Sabah populations was further assessed based on heterozygosity indices. In all populations, mean observed heterozygosity (H_o) was consistently lower than mean expected heterozygosity (H_e), indicating a general deficit of heterozygotes. This pattern is commonly observed in fragmented or declining populations and may arise from restricted gene flow, localized breeding, inbreeding, or recent population bottlenecks. This pattern mirrors previous findings in Tuaran (Kntayya, 2015) but contrasts with populations from Japan and China, where higher H_O values have been reported (Li *et al*., 2009; Nishida and Koike, 2009). Despite documented population declines in those regions, their *T. tridentatus* populations appear to retain higher genetic diversity than populations in Sabah, highlighting regional differences in demographic history and habitat connectivity.

The inbreeding coefficient (F_{IS}) provides additional insight into population structure and the degree of homozygosity within populations. Positive F_{IS} values were observed in all three Sabah populations, indicating a deficit of heterozygotes, consistent with the heterozygosity results. The highest F_{IS} value was found in Sandakan, followed by Kudat and Kota Belud, implying varying degrees of inbreeding or genetic isolation. These differences likely reflect varying environmental pressures and habitat connectivity along the Sabah coastline. Similar patterns of local population differentiation have been reported in *T. tridentatus* from Hong Kong, Taiwan, and mainland China, where coastal development and declining spawning grounds have fragmented populations and reduced gene flow (Liao *et al*., 2019; Yang *et al*., 2007). Taken together, the relatively low allelic richness, reduced heterozygosity, and positive F_{IS} values observed in this study suggest limited genetic exchange among Sabah populations of *T. tridentatus*. Such patterns have been linked to habitat degradation and the loss of spawning beaches in other parts of Asia (Liao *et al*., 2019; Chen *et al*., 2015; Nishida and Koike, 2009; Yang *et al*., 2007). Continuous genetic monitoring, along with expanded sampling across Borneo and Peninsular Malaysia, will be crucial to clarify the extent of population connectivity and to inform conservation efforts for this endangered species.

Seasonal movements and environmentally driven population flux may also contribute to the genetic patterns observed in this study. Horseshoe crabs are known to exhibit seasonal migration associated with spawning activity, tidal cycles, and monsoonal changes, with adults moving between offshore feeding grounds and intertidal nesting beaches. Seasonal population flux has also been documented in Sabah for other horseshoe crab species. Robert *et al*. (2014) reported marked seasonal variation in the abundance and mating behaviour of *Carcinoscorpius rotundicauda* in the Kota Kinabalu region, with spawning activity closely linked to tidal cycles and environmental conditions. In the South China Sea region, coastal currents and monsoon-driven circulation patterns have been suggested to influence larval dispersal and juvenile distribution, potentially shaping connectivity among local populations (Yang *et al*., 2007; Liao *et al*., 2019). However, despite this potential for seasonal connectivity, the observed heterozygosity deficits and positive F_{IS} values in Sabah populations suggest that such movements may be insufficient to counteract the effects of habitat fragmentation and localized breeding.

The microsatellite markers developed in this study included a combination of di-, tri-, and tetranucleotide repeat motifs to provide a comprehensive assessment of genetic variation. No consistent pattern of higher allele numbers was observed among tetranucleotide loci compared to other repeat types (Table 2), indicating that allelic richness was more strongly influenced by locus-specific characteristics, such as repeat length and mutation dynamics, rather than repeat motif class alone. Genetic analyses were conducted under the Infinite Alleles Model (IAM), which assumes that each mutation produces a novel allele and is appropriate for evaluating overall heterozygosity and allelic diversity across mixed microsatellite repeat types. The absence of a clear relationship between repeat motif class and allele number further supports the suitability of this model for the present dataset.

The moderate overall genetic diversity observed in this study provides a valuable foundation for future conservation genetics research on *T. tridentatus* in Sabah, Malaysian Borneo. However, the relatively small sample size and the limited availability of specimens posed significant challenges to this work. The scarcity of individuals along the Sabah coastline suggests the species' declining population and the practical difficulties of obtaining adequate samples for population-level analyses. Observations from Kota Kinabalu indicate that even relatively common horseshoe crab species such as *C. rotundicauda* exhibit strong site fidelity to spawning areas and are sensitive to coastal disturbance (Robert *et al*., 2014), emphasizing the need to protect key breeding habitats for T. tridentatus along the Sabah coastline. Consequently, the patterns of genetic diversity reported here should be verified through broader sampling efforts, if possible.

Future studies incorporating larger sample sizes and additional molecular markers will be essential to confirm the genetic structure and connectivity of *T. tridentatus* populations in Sabah waters. Given that the species is listed as "Endangered" by the IUCN, such information is critical for guiding effective conservation and management strategies. Protecting key breeding beaches, minimizing anthropogenic disturbances, and preserving habitat connectivity among populations should remain central priorities to ensure the long-term survival of this ancient and ecologically important species.

CONCLUSION

Overall, the microsatellite loci developed in this study proved to be informative for assessing genetic diversity in *T. tridentatus* populations from Sabah. The moderate levels of heterozygosity observed across loci and populations suggest that some degree of genetic variation is still maintained despite the species' restricted distribution in northern Borneo. However, the relatively high inbreeding coefficients, particularly in the Sandakan population, indicate potential genetic erosion due to population isolation or small effective population sizes. These findings highlight the need for continued genetic monitoring and conservation management to prevent further loss of diversity and to support the long-term viability of *T. tridentatus* populations in Sabah waters.

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