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Original Article

Genetic differentiation and heavy metal pollution influence on horseshoe crab *Tachypleus gigas* populations in Peninsular Malaysia: A comprehensive allozyme analysis

Chee Kong Yap[1] • **Khalid Awadh Al-Mutairi**[2]

[1] *Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[2] *Department of Biology, Faculty of Science, University of Tabuk, Tabuk, P.O. Box 741, Saudi Arabia*

Correspondence
Chee Kong Yap; Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
yapchee@upm.edu.my

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Abstract
This study aimed study the genetic diversity and differentiation of Asian horseshoe crab *Tachypleus gigas* (Müller, 1785) populations across three distinct locations in Peninsular Malaysia: Kampung Pasir Puteh (KPPuteh), Kuala Sungai Ayam (KSAyam), and Kampung Pulau Sayak (KPSayak). A total of 28 individuals were collected, and their genetic structure was analyzed using starch gel electrophoresis. The study employed multiple buffer systems optimized for separating 20 different enzymes, each contributing to understanding the genetic variability within and between populations. The findings of this study, which revealed significant genetic differentiation, particularly between populations in polluted and less impacted environments, as reflected by high FST values and distinct clustering in the UPGMA dendrogram, are of paramount importance. The results indicate that environmental factors, especially metal-polluted sites at KPPuteh and KSAyam, have played a critical role in shaping the genetic structure of these populations. Populations from the two polluted sites, exhibited higher genetic diversity and greater deviations from Hardy–Weinberg equilibrium, suggesting localized selection pressures. Conversely, from a less polluted site, the unpolluted site at KPSayak population showed lower genetic variability and was genetically distinct from the others. These findings underscore the importance of considering environmental influences in conservation strategies for *T. gigas*, highlighting the need for site-specific management to preserve genetic diversity and ensure long-term survival of the species.

Keywords: environmental pollution; genetic differentiation; population genetics; starch gel electrophoresis; *Tachypleus gigas*

1 | INTRODUCTION

The Asian horseshoe crab, *Tachypleus gigas* (Müller, 1785), is an ancient and ecologically important species in coastal ecosystems across Asia (Liew *et al.* 2015). These organisms play crucial roles in marine environments, serving as indicators of ecosystem health and contributing to the dynamics of coastal and estuarine habitats, which are increasingly threatened by natural events and human activities (Xu *et al.* 2021). Given the importance of *T. gigas* in maintaining ecological balance, understanding the genetic diversity within its populations is vital for effective management and conservation strategies as anthropogenic pressures continue to escalate in their habitats (Rozihan and Ismail 2012). It is important to elucidate the genetic variability within *T. gigas* populations across selected sites, providing critical insights for addressing the

declining population trends due to environmental stresses and habitat degradation (Xu *et al.* 2021).

Allozyme analysis has been widely employed as a robust tool for assessing genetic diversity in natural populations, as it can reveal subtle genetic differences that may not be detected using other molecular techniques (Bert *et al.* 2002; Johnson *et al.* 2006). The findings from this comparative analysis are expected to yield valuable data on the levels of genetic variation present in *T. gigas* populations, as well as potential correlations between genetic diversity and environmental stressors, informing management practices and conservation initiatives for this ecologically significant species (Linh and Huyền 2020; Manel *et al.* 2020; Aini *et al.* 2021; Zee *et al.* 2022).

The genetic diversity and population structure of *T. gigas* in Peninsular Malaysia have not been extensively studied despite the species' ecological importance and increasing habitat threats (Aini *et al.* 2021). Understanding the genetic diversity within this species is imperative, as it may provide insights into its adaptive potential and long-term survival in changing environments. Genetic diversity can be a key factor influencing resilience to habitat loss and pollution (Manel *et al.* 2020). Additionally, the erosion of genetic diversity can lead to the loss of locally adapted populations, negatively impacting the species' overall resilience and adaptive capacity. Therefore, identifying and documenting genetic variability among different populations is critical to understanding how these populations might cope with environmental changes and to ensuring the long-term viability of *T. gigas*, especially in light of the on-going pressures from human activities in its coastal habitats (Donaldson 2002; Weiss 2005; Manel *et al.* 2020).

The current research addresses several significant gaps in understanding the genetic diversity of *T. gigas*, a species crucial to coastal ecosystems across Asia. Despite its ecological importance, limited studies have explored the genetic structure and variability of *T. gigas* populations, particularly in Peninsular Malaysia, where increasing anthropogenic pressures and habitat degradation pose serious threats (Aini *et al.* 2021). Previous studies have focused primarily on the ecological roles of the species but have not comprehensively examined the genetic differentiation across various populations or how environmental factors, such as pollution and habitat changes, impact genetic diversity. This presents a clear research gap that this study seeks to fill by using allozyme analysis, a proven method for detecting subtle genetic variations often overlooked by other molecular techniques (Bert *et al.* 2002; Johnson *et al.* 2006).

The significance of this study lies in its potential to contribute to both the scientific understanding of *T. gigas* and broader conservation efforts. As coastal ecosystems face mounting pressures, maintaining genetic diversity is a key to ensuring species resilience and long-term survival. This study's contribution is twofold: it provides empirical data on the genetic structure of *T. gigas* populations, which is currently lacking, and it underscores the importance of site-specific management practices that account for environmental influences on genetic differentiation. Ultimately, the study's findings will enhance conservation strategies, helping to preserve the genetic diversity and ecological functions of *T. gigas*, while addressing the broader implications of environmental change for vulnerable coastal species.

Therefore, the objectives of this study are twofold. First, it aims to assess the genetic variability within *T. gigas* populations across selected coastal sites in Peninsular Malaysia. Second, it seeks to investigate the potential correlations between genetic diversity and environmental stressors, such as pollution, habitat loss, and other anthropogenic pressures. By focusing on these objectives, the study provides valuable insights into the genetic health and adaptability of *T. gigas* populations, information critical for developing effective management and conservation strategies. The findings are expected to inform conservation initiatives by identifying areas where genetic diversity is threatened and where management efforts can be focused to preserve population resilience and adaptive capacity in response to environmental changes.

2 | METHODOLOGY

2.1 Study area and sample collection

The study was conducted across three distinct locations in Peninsular Malaysia (Table 1). Kampung Pasir Puteh (KPPuteh), located in the Pasir Gudang area of Johor, Malaysia, is a significant industrial hub dominated by heavy industries. Key activities include petrochemical production, oil and gas operations, shipping, and logistics through Pasir Gudang Port. The area is also home to manufacturing plants specializing in rubber, plastics, and automotive components, alongside palm oil refining and processing facilities. Additionally, the region supports heavy engineering, metal processing, and the production of various industrial chemicals, making it a vital contributor to Malaysia's industrial and economic landscape.

Kampung Sungai Ayam (KSAyam) in Batu Pahat, Johor, is primarily focused on agriculture and small-scale industrial activities. Key sectors include traditional farming of crops like oil palm, fisheries, and poultry farming. Small and medium-sized enterprises (SMEs) contribute through food processing, particularly in palm oil extraction and fish processing, as well as furniture manufacturing, which is a notable industry in the region. Additionally, some small-scale industries support the construction sector by producing building materials. These activities collectively sustain the local economy and provide employment opportunities in the area.

Kampung Pulau Sayak (KPSayak) in Kedah is primari-

ly a coastal village where traditional livelihoods dominate. The local economy revolves around fishing, aquaculture, and small-scale agriculture, with residents relying on the sea and land for food production. Additionally, cottage industries, particularly the production of seafood-based snacks and traditional delicacies, support the community. Tourism and eco-tourism are also growing, as visitors are drawn to the village's scenic coastal environment and nearby natural attractions. These activities collectively sustain the local economy, with a focus on traditional and small-scale enterprises.

Previously, KKPuteh and KSAyam were reportedly polluted by heavy metals by Yap *et al.* (2002a) and Yap and Noorhaidah (2011), respectively. This selection allows for a comparative analysis of genetic diversity that could help understand the resilience of *T. gigas* populations in the face of varying anthropogenic impacts, which is essential for formulating targeted conservation actions (Rozihan and Ismail 2012; Nelson *et al.* 2016).

These locations were selected to capture a range of environmental conditions that could potentially influence the genetic structure of *T. gigas* populations. The sampling was conducted on 3 and 11 May 2008, ensuring consistent temporal conditions across sites. The use of older sample collection data, such as from May 2008, in scientific research can be justified by several factors. One key reason is the need for long-term studies or trend analysis, where historical data are critical for understanding changes over time. In environmental monitoring, biomonitoring, or ecological studies, comparing older data with current findings allows researchers to identify trends and assess the progression of environmental or biological conditions. Additionally, data from 2008 may serve as baseline or reference data, providing a comparative framework to evaluate shifts in metal concentrations, ecological health, or contamination levels. In some cases, the rarity of specific environmental events, conditions, or species may necessitate using older data, especially if more recent samples are unavailable. Furthermore, the consistent application of methodologies across time periods enhances the reliability and comparability of the findings. Thus, including older data can offer valuable insights, particularly in cases where significant environmental changes have not occurred, ensuring the relevance of data to current research objectives.

A total of 28 *T. gigas* individuals were collected, 12 individuals sampled from both KPPuteh and KSAyam, and 4 individuals from KPSayak. The specimens were carefully handled and transported to the laboratory for subsequent genetic analysis.

TABLE 1 Sampling locations of horseshoe crab *Tachypleus gigas* in Peninsular Malaysia.

Location	GPS coordinates	Sampling date	Major activities	Industry type
Kampung Pasir Puteh (KKPuteh), Johor	01°26′7.5″N 103°56′7.68″E	3 May 2008	Heavy industries: petrochemical; oil & gas; shipping & logistics; manufacturing (rubber, plastics); palm oil refining; heavy engineering; metal processing; chemical production	Heavy industrial
Kuala Sungai Ayam (KSAyam), Johore	01°45′12.5″N 102°55′ 45.4″E	3 May 2008	Agriculture, fisheries, poultry & livestock farming; food processing (palm oil, fish); furniture manufacturing; construction-related manufacturing	Agricultural and small-scale manufacturing
Kampung Pulau Sayak (KPSayak), Kedah	05°40′0″N 100°20′0″E	11 May 2008	Fishing; aquaculture; small-scale agriculture; cottage industries (seafood snacks, dried fish); tourism & eco-tourism	Traditional and small-scale

2.2 Starch gel electrophoresis

The genetic analysis was performed using starch gel electrophoresis, a method chosen for its effectiveness in separating allozymes (enzyme variants) based on their electrophoretic mobility (Shaw and Prasad 1970). The electrophoresis was conducted using an 11% starch gel, prepared by dissolving 44.0 g of Sigma starch in 400 ml of buffer solution (Table 2). The buffer for the gel was chosen based on its ability to maintain a stable pH and ionic strength during the electrophoretic process. The gel was thoroughly mixed and poured into casting trays, where it was allowed to set at room temperature (Harris and Hopkinson 1976).

Horizontal Starch Gel Electrophoresis (STAGE) was carried out at a temperature range of 3 – 5°C, applying 230 V per gel slab. The starch gels were prepared using a 12% starch solution (Sigma, St. Louis, MO). After an initial 30-minute run, the inserts were removed, and electrophoresis was resumed until the tracking dye, bromophenol blue, reached the anodal end of the gel (Shaw and Prasad1970; Harris and Hopkinson 1976).

TABLE 2 Chemical ingredients for 11% starch gel used in the present study.

Chemical ingredients	Amounts (weight or volume)
Sigma starch	44.0 g
Buffer for gel	400.0 ml
Total in ml	400.0 ml

Once the gel was prepared, it was loaded with tissue extracts from the collected *T. gigas* individuals. The tissue samples were homogenized in an appropriate extraction buffer, and the extracts were applied to the gel using filter paper wicks. The loaded gel was then placed in an electrophoresis apparatus, and an electric current was applied to separate the proteins based on their charge and size. The electrophoresis was conducted at a constant voltage, with the duration adjusted to ensure adequate separation of the enzymes. Following electrophoresis, the gels were sliced and subjected to enzyme staining using recipes adapted from Shaw and Prasad (1970) and Harris and Hopkinson (1976), with slight modifications.

For the genetic analysis, the most common allele in the control population from Penang was designated as allele 100. All other alleles were annotated based on their electrophoretic mobility in mm relative to the position of the allele 100 band, either more anodally or cathodally.

2.3 Buffer systems

Muscle tissue samples were stored at –70°C until needed for electrophoretic analysis. When required, frozen tissue samples were homogenized manually in a buffer solution containing 0.2 M Tris-HCl and glycerol. The manually homogenized fluid sample was imbibed onto Whatman No. 1 filter-paper inserts. Different buffer systems were used for the electrophoresis to optimize the separation of various enzymes (Table 3). The CA-7, Tris-citric, and Tris-citric-EDTA buffer systems were selected for their ability to maintain enzyme activity and stability during electrophoresis (Shaw and Prasad 1970; Ayala *et al.* 1972; Steiner and Joslyn 1979). The CA-7 buffer system, consisting of Trizma base and citric acid, was adjusted to a pH of 7.10 for the gel and 6.90 for the electrode buffer. This buffer system was used for enzymes such as Lactate Dehydrogenase (LDH) and Glucose-6-phosphate Dehydrogenase (G6PDH) (Ayala *et al.* 1972; Steiner and Joslyn 1979).

The Tris-citric buffer system was adjusted to a pH of 7.00 for both the gel and electrode, providing a stable environment for the separation of enzymes like Alcohol Dehydrogenase (ADH) and Alkaline Phosphatase (ALKP). The Tris-citric-EDTA buffer, also adjusted to pH 7.00, was used for enzymes requiring additional stability provided by EDTA, such as Glutamate Dehydrogenase (GDH) and Glutamic-oxaloacetic Transaminase (GOT). Each buffer was freshly prepared before use to ensure optimal performance during electrophoresis (Shaw and Prasad 1970; Steiner and Joslyn 1979).

TABLE 3 Buffer systems and chemical ingredients for starch gel electrophoresis used in the present study.

System	Buffer type	Adjusted to pH	Chemical ingredients (g L^{-1})
CA-7 (Steiner and Joslyn 1979)	Gel	7.10	Trizma base: 1.10 Citri Acid. H_2O: 0.63
	Electrode	6.90	Trizma base: 16.35 Citri acid. H_2O: 8.40
Tris-citric-EDTA (Ayala *et al.* 1972)	Gel	7.00	Tris: 1.09 Citric acid: 0.63 EDTA(Na_2): 0.47
	Electrode	7.00	Tris: 16.35 Citric acid: 9.45 EDTA(Na_2): 0.47
Tris-citric (Ayala *et al.* 1972)	Gel	7.00	Tris: 1.09 Citric acid: 0.63
	Electrode	7.00	Tris: 16.35 Citric acid: 9.45

2.4 Staining procedures

Following electrophoresis, the separated enzymes were visualized using specific staining procedures (Table 4). The staining involved immersing the gel in a staining solution containing substrates and dyes appropriate for the enzyme of interest. The staining solutions were prepared using various buffer systems. For instance, a 0.2 M Tris-HCl buffer at pH 8.0 was used to stain enzymes such as Acid Phosphatase (ACP) and Aconitase (ACON). The staining reactions were conducted at room temperature and monitored closely to prevent overdevelopment (Shaw and Prasad 1970; Steiner and Joslyn 1979).

Each enzyme required a specific staining protocol, involving different chemical ingredients. For example, Acid Phosphatase was stained using a solution containing sodium α-naphthyl phosphate and Fast blue RR in an acetate buffer at pH 5.0 (Table 5). The staining reaction was allowed to proceed until clear, distinguishable bands appeared on the gel, indicating the presence of specific enzyme variants. The gels were then rinsed with distilled water to stop the staining reaction and were subsequently photographed for documentation and analysis (Shaw

and Prasad 1970; Steiner and Joslyn 1979).

3.5. Enzyme analysis and data collection

The study focused on 20 different enzymes, chosen for their relevance in population genetics and their ability to reveal genetic variability among *T. gigas* populations (Table 5). Each enzyme's electrophoretic profile was analyzed to identify different alleles (allozyme variants) present in the populations. The banding patterns observed on the gels were compared to determine the presence or absence of specific alleles and to assess the genetic diversity within and between the sampled populations (Shaw and Prasad 1970; Steiner and Joslyn 1979).

TABLE 4 The staining buffer systems used in the present study.

Staining buffer systems	Ingredients
0.2 M Tris-HCl buffer system (pH 8.0)	24.22 g Tris (hydroxymethyl) aminomethane; 900 ml DW; Adjusted pH to 8.0 with concentrated HCl and make up to 1 liter with distilled water.
0.5 M Tris-HCl buffer system (pH 8.0)	60 g Tris (hydroxymethyl) aminomethane; 900 ml DW; Adjusted pH to 8.0 with concentrated HCl and make up to 1 liter with distilled water.
0.5 M Tris-HCl buffer system (pH 8.7)	60 g Tris (hydroxymethyl) aminomethane; 900 ml DW; Adjusted pH to 8.7 with concentrated HCl and make up to 1 liter with distilled water.
0.5 M Tris-HCl buffer system (pH 7.1)	60 g Tris (hydroxymethyl) aminomethane; 900ml DW; Adjusted pH to 8.7 with concentrated HCl and make up to 1 liter with distilled water.
0.04 M Tris 0.04 Maleate (pH 6.0)	4.84 g Tris (hydroxymethyl) aminomethane; 4.64g Maleic acid; 0.082g NaOH; 1000 ml DW.
0.5 M phosphate (pH 7.0)	28.55 g $Na_2HPO_4.2H_2O$; 22.00g $Na_2H_2PO_4.2H_2O$; 1000 ml DW
0.05 M acetate (pH 5.0)	6.8 g Sodium acetate.$3H_2O$; 14.8 ml 1 N HCl; 985.2 ml DW

TABLE 5 Enzyme names and abbreviations, enzyme codes (E.C.), enzyme structures, electrophoretic buffer systems, and ingredients used for allozyme study of horseshoe crabs in the present study.

Enzyme	E.C.	ES	BS	Ingredients
Acid Phosphatase (ACP)	3.1.3.2	monomeric / dimeric	CA-7	0.04 g Sodium α-naphthyl phosphate; 0.04 g Fast blue RR; 40 ml 0.5 M acetate (pH 5.0) buffer.
Aconitase (ACON)	4.2.1.3		CA-7	0.1 g cis-Aconitic acid; 0.01 g $NADP^+$; 0.015 g PMS; 0.007 g MTT; 0.15 g $MgCl_2.6H_2O$; 20 ml 0.2 M tris-HCl buffer (pH 8.0); 30 ml DW.
Alcohol Dehydrogenase (ADH)	1.1.1.1	dimeric	Tris-citric	0.04 g NAD^+; 0.001 g PMS; 0.01 g MTT; 1 ml 0.5 M tris-HCl buffer (pH 8.7); 10 ml DW.
Aldolase (ALD)	4.1.2.13	tetrameric	CA-7	0.02 g NAD^+;0.0025 g PMS; 0.0075 g MTT; 0.06 g sodium arsenate; 60 µl fructose 1,6-diphosphate; 12.5 ml 0.5 M tris-HCl buffer (pH 8.0); 29.5 ml DW.
Alkaline Phosphatase (ALKP)	3.1.3.1	monomeric	Tris-citric	0.05 g β-napthyl Na phosphate; 0.05 g Fast blue RR; 0.123 g $MgSO_4.7H_2O$; 100 ml DW.
α–Esterase (α-EST)	3.1.1.1	monomeric / dimeric	Tris-citric-EDTA	0.02 g α- napthyl acetate; 0.04 g Fast blue RR; 60 ml 0.5 M phosphate buffer; 2 ml acetone.
Glucose 6-Phosphate Dehydrogenase (G6PDH)	1.1.1.49	dimeric	CA-7	0.06 g $NADP^+$; 0.004 g NBT; 0.04 g PMS; 10 ml 0.5 M tris-HCl buffer (pH 7.1); 0.4 g Na_2 glucose 6 phosphate H_2O.
Glutamate Dehydrogenase (GDH)	1.4.1.2	dimeric	Tris-citric-EDTA	0.06 g NAD^+;0.002 g PMS; 0.003 g NBT; 5 ml 1 M L-Glutamate; 25 ml 0.5 M phosphate buffer.
Glutamic-oxaloacetic Transaminase (GOT)	2.6.1.1	dimeric	Tris-citric-EDTA	0.4 g L-aspartic acid; 0.18 g 2-Oxoglutaric acid; 0.04 g Fast blue B salt; 100 ml 0.5 M tris-HCl buffer (pH 8.7).
Glycerol-3-phosphate Dehydrogenase (G3PD)	1.1.1.8	Dimeric	CA-7	0.05 g NAD^+;0.002 g PMS; 0.003 g NBT; 10 ml 0.1 M Glycerol-3-phosphate; 43 ml 0.5 M tris-HCl buffer (pH 8.0); 47 ml DW.
β-Hydroxybutyrate Dehydrogenase (HBDH)	1.1.1.30	Dimeric	CA-7	0.3 g gluconic acid; 0.1 g NAD^+; 0.002 g PMS; 0.025 g NBT; 25ml 0.5 M phosphate buffer (pH 7.4); 0.102 g $MgCl_2$; 0.575 g NaCl; 65 ml DW.

TABLE 5 Continued.

Enzyme	E.C.	ES	BS	Ingredients
Lactate Dehydrogenase (LDH)	1.1.1.27	Monomeric / dimeric / tetrameric	CA-7	0.027 g NAD^+;0.007 g PMS; 0.007 g NBT; 60 ml 0.2 M tris-HCl buffer (pH 8.0); 12 ml 0.5M D-lactic acid.
Leucine Aminopeptidase (LAP)	3.4.11.1	monomeric	CA-7	0.03 g L-leucyl β-napthylamide (dissolved in few amount of acetone); 0.03 g Black K; 60 ml 0.4 M tris 0.04 maleate buffer (pH 6.0).
Malate Dehydrogenase (MDH)	1.1.1.37	dimeric	CA-7	0.02 g NAD^+; 0.004 g PMS; 0.06 g NBT ; 0.24 g DL-malic acid; 10 ml 0.5 M tris-HCl buffer (pH 8.7); 34 ml DW.
Malic Enzyme (ME)	1.1.1.40	dimeric	CA-7	0.008 g $NADP^+$; 0.002 g PMS; 0.03 g NBT; 0.12 g DL-malate 2-Na; 5 ml 0.5 M tris-HCl buffer (pH 8.0); 17 ml DW.
NADH Diaphorase (DIA[NADH])	1.8.1.4	dimeric	CA-7	0.01 g NADH; 0.002 g DCIP; 0.007 g MTT; 50 ml 0.2 M tris-HCl buffer (pH 8.0).
Phosphogluco-isomerase (PGI)	5.3.1.9	dimeric	CA-7	0.008 g $NADP^+$; 0.002 g PMS; 0.03 g NBT; 0.06 g fructose 6-phosphate; 15 µl G6PDH; 5 ml 0.5 M tris-HCl buffer (pH 8.0); 1 ml 1 M $MgCl_2$; 17 ml DW.
Phosphoglucomutase (PGM)	2.7.5.1	monomeric	CA-7	0.016 g $NADP^+$; 0.004 g PMS; 0.06 gNBT; 0.16 g Na glucose 1-phosphate; 60µl G6PDH; 10 ml 0.5 M tris-HCl buffer (pH 8.0); 2 ml 1 M $MgCl_2$; 34 ml DW.
Sorbitol Dehydrogenase (SDH)	1.1.1.14	dimeric	CA-7	0.02 g NAD^+; 0.004 g PMS; 0.006 g NBT; 10 ml 0.5 M tris-HCl buffer (pH 8.7); 34 ml DW.
Superoxide Dismutase (SOD)	1.15.1.1	dimeric	CA-7	0.006 g PMS; 0.010 g MTT; 0.015 g $MgCl_2.6H_2O$; 10 ml 0.5 M tris-HCl buffer (pH 8.7); 34 ml DW.

3.6 Data analysis

Swofford and Selander's (1989) BIOSYS-1 computer package was used to calculate allelic frequencies, the proportion of polymorphic loci (P) based on the 0.95 criterion, mean heterozygosity (H), and genetic distance (D), as well as the identity (I) values of Nei (1978) and *F*-statistics (Nei 1977; Wright 1978). The dendrogram based on Nei's I was drawn using the unweighted-pair group method with arithmetic averaging (UPGMA) of Sneath and Sokal (1973).

3 | RESULTS

The genetic analysis of *T. gigas* populations from three distinct locations in Peninsular Malaysia revealed significant genetic variability across nine loci. Allele frequency distributions in Table 6 demonstrate considerable variation among the populations. For instance, at the LDH locus, allele A was completely absent in the KPPuteh and KSAyam populations but present in KPSayak at a frequency of 0.750. Similarly, allele B was absent in KSAyam but present in KPPuteh and KPSayak, albeit at different frequencies. Such disparities in allele presence across loci suggest that these populations are genetically diverse, possibly due to differing environmental pressures in their respective habitats.

TABLE 6 Allele frequencies of 9 loci in *Tachypleus gigas*.

Locus	Population		
	KPPuteh	KPSayak	KSAyam
LDH			
(N)	10	4	2
A	0	0.75	0
B	0.1	0.25	0
C	0.9	0	1
ALD			
(N)	10	4	2
A	0.15	1	0.25
B	0.85	0	0.75
GOT-1			
(N)	10	4	2
A	0.85	0.875	0.5
B	0.15	0.125	0
C	0	0	0.5
GOT-2			
(N)	10	4	2
A	0.65	0	0.75
B	0.15	0	0
C	0.1	1	0.25
D	0.1	0	0
PGI			
(N)	10	4	2
A	0.3	1	0.5
B	0.55	0	0.5
C	0.15	0	0

TABLE 6 Continued.

Locus	Population		
	KPPuteh	KPSayak	KSAyam
MDH			
(N)	10	4	2
A	0	0.75	0
B	1	0.25	1
LAP			
(N)	5	2	1
A	0	1	0
B	1	0	1
PGM-1			
(N)	5	2	1
A	0.8	1	0
B	0.2	0	1
PGM-2			
(N)	5	2	1
A	0.6	1	0
B	0.2	0	1
C	0.2	0	0

Phosphoglucomutase (PGM); Phosphogluco-isomerase (PGI); Glutamic-oxaloacetic Transaminase (GOT); Leucine Aminopeptidase (LAP); Malate Dehydrogenase (MDH); Aldolase (ALD); Lactate Dehydrogenase (LDH).

The observed heterozygosity (H_o) and expected heterozygosity (H_e), along with χ^2 tests for Hardy–Weinberg equilibrium, are detailed in Table 7. Significant deviations from Hardy- Weinberg equilibrium were observed in several loci, particularly in the KPPuteh and KSAyam populations, indicating possible factors like selection, genetic drift, or population structure affecting these populations. For example, the GOT-2 locus in KPPuteh showed a significant χ^2 value (20.205, $p < 0.05$), suggesting a deviation from equilibrium. This locus also displayed a negative Selander's Ds value (–0.112), implying a potential deficit in heterozygosity, possibly due to inbreeding or selection pressures in this metal-polluted environment. Similarly, the PGM-1 and PGM-2 loci in KPPuteh exhibited significant χ^2 values (9.143 and 18.400, respectively, $p < 0.05$) and negative Ds values (–1.000 for both), reinforcing the evidence of non-random mating and genetic structuring within these populations.

Table 8 provides an overview of genetic variability, with KPPuteh exhibiting the highest level of genetic diversity among the three populations. This population had a mean of 2.2 alleles per locus and 77.8% polymorphic loci, compared to only 33.3% in KPSayak and 44.4% in KSAyam. The observed heterozygosity in KPPuteh (0.244 ± 0.102) was slightly lower than the expected heterozygosity (0.320 ± 0.080), which could indicate a slight inbreeding effect or selective pressure in this population. Conversely, KSAyam showed a higher observed heterozygosity (0.333 ± 0.144) compared to the expected (0.259 ± 0.104), which might suggest outbreeding or a recent mixing of different gene pools, potentially as a response to environmental stress in the polluted habitat.

TABLE 7 Observed heterozygosity (H_o), expected heterozygosity (H_e); Selander's D_s and χ^2 tests of goodness of fit to the Hardy–Weinberg model for three populations of *Tachypleus gigas* (*n* = number of individuals scored).

Locus		KPPuteh	KPSayak	KSAyam
ALD	*n*	10	4	2
	H_o	0.300	0.000	0.500
	H_e	0.268	0.000	0.500
	χ^2	0.199 ^{NS}		0 ^{NS}
	D_S	0.118		0
GOT-1	*n*	10	4	2
	H_o	0.300	0.250	1.000
	H_e	0.268	0.250	0.667
	χ^2	0.199 ^{NS}	0 ^{NS}	1.000 ^{NS}
	D_S	0.118	0	0.500
GOT-2	*n*	10	4	2
	H_o	0.500	0.000	0.500
	H_e	0.563	0.000	0.500
	χ^2	20.205		0 ^{NS}
	D_S	-0.112		0
LAP	*n*	5	2	1
	H_o	0.000	0.000	0.000
	H_e	0.000	0.000	0.000
	χ^2			
	D_S			
LDH	*n*	10	4	2
	H_o	0.200	0.500	0.000
	H_e	0.190	0.429	0.000
	χ^2	0.059 ^{NS}	0.200 ^{NS}	
	D_S	0.056	0.167	
MDH	*n*	10	4	2
	H_o	0.000	0	0.000
	H_e	0.000	0.429	0.000
	χ^2		7.200	
	D_S		-1.000	
PGI	*n*	10	4	2
	H_o	0.900	0.000	1.000
	H_e	0.616	0.000	0.667
	χ^2	5.891 ^{NS}		1.000 ^{NS}
	D_S	0.462		0.500
PGM-1	*n*	5	2	1
	H_o	0	0.000	0.000
	H_e	0.356	0.000	0.000
	χ^2	9.143		
	D_S	-1.000		
PGM-2	*n*	5	2	1
	H_o	0	0.000	0.000
	H_e	0.622	0.000	0.000
	χ^2	18.400		
	D_S	-1.000		

NS= not significant; others are significant at $p < 0.05$. Phosphoglucomutase (PGM); Phosphogluco-isomerase (PGI); Glutamic-oxaloacetic Transaminase (GOT); Leucine Aminopeptidase (LAP); Malate Dehydrogenase (MDH); Aldolase (ALD); Lactate Dehydrogenase (LDH).

TABLE 8 Genetic variability (mean ± standard error) at nine loci in the three populations of *Tachypleus gigas*.

Population	sample size per locus	Number of allele per locus	Percentage of polymorphic loci	Heterozygosity	
				Observed	Expected
1. KPPuteh	8.3 ± 0.8	2.2 ± 0.3	77.8	0.244 ± 0.102	0.320 ± 0.080
2. KPSayak	3.3 ± 0.3	1.3 ± 0.2	33.3	0.083 ± 0.059	0.123 ± 0.064
3. KSAyam	1.7 ± 0.2	1.4 ±0.2	44.4	0.333 ± 0.144	0.259 ± 0.104

Note: A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95 (Nei 1978)

The *F*-statistics results, summarized in Table 9, highlight significant genetic differentiation among the populations. The F_{ST} values, which quantify genetic differentiation among populations, were notably high for certain loci, such as LDH (0.646) and PGM-1 (0.778), indicating substantial genetic divergence, likely driven by localized selection pressures or restricted gene flow. The overall positive F_{IT} values suggest that inbreeding or genetic drift could influence these populations' genetic structure. Notably, loci such as MDH, LAP, and PGM-2 showed F_{IT} and F_{ST} values of 1.000, indicating extreme levels of genetic differentiation and possible fixation of alleles in some populations.

Table 10 presents Nei's genetic identity and distance metrics, further elucidating the genetic relationships among the populations. The genetic identity values indicate that KPPuteh and KSAyam, both from metal-polluted sites, share a higher degree of genetic similarity (0.836), compared to KPSayak, which is genetically more distinct with a lower identity value (0.430) when compared to KPPuteh. The genetic distances between these populations underscore the effect of environmental factors, particularly pollution, in driving genetic differentiation.

Finally, the UPGMA dendrogram in Figure 1 visually represents the genetic relationships among the three populations. The dendrogram clusters KPPuteh and KSAyam together, reflecting their shared environmental conditions in metal-polluted sites and their closer genetic similarity. KPSayak, on the other hand, is distinctly separated, suggesting that its relatively unpolluted environment has led to a different genetic trajectory.

TABLE 9 *F*-statistics values for nine loci of three populations of *Tachypleus gigas*.

Locus	F_{IS}	F_{IT}	F_{ST}
LDH	–0.291	0.544	0.646
ALD	–0.27	0.464	0.578
GOT-1	–0.592	–0.249	0.216
GOT-2	–0.099	0.421	0.473
PGI	–0.751	–0.23	0.298
MDH	1	1	0.667
LAP	-	1	1
PGM-1	1	1	0.778
PGM-2	1	1	0.661
Mean	-0.1	0.547	0.588

Overall, the results highlight the substantial genetic differentiation among *T. gigas* populations in Peninsular Malaysia, strongly influenced by local environmental conditions, particularly metal pollution. The observed deviations from Hardy–Weinberg equilibrium, coupled with significant F_{ST} values and distinct genetic clusters, point to the influence of selective pressures and restricted gene flow in shaping the genetic structure of these populations. These findings underscore the importance of considering environmental factors when studying population genetics and the evolutionary dynamics of species in fragmented and polluted habitats.

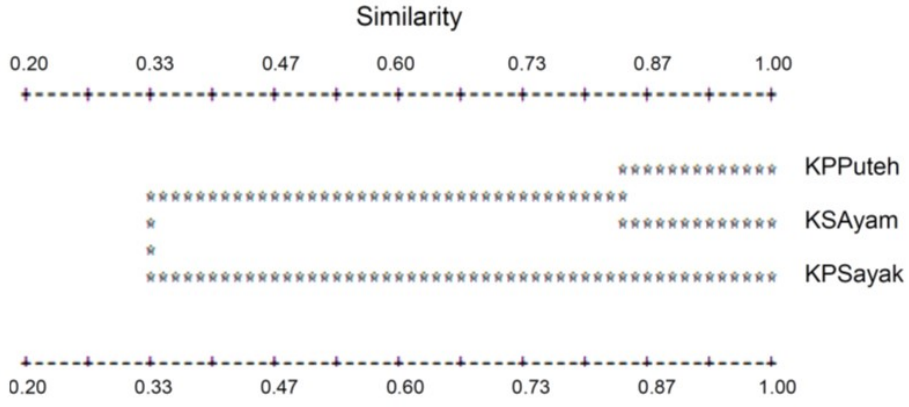

FIGURE 1 An UPGMA dendrogram of genetic relationships among three populations of *Tachypleus gigas* based on Nei's (1978) identity values. Note: KPPteh and KSAyam are metal-polluted sites.

TABLE 10 Nei's (1978) genetic identity (below diagonal) and genetic distance (above) for three populations of Asian horseshoe crab *Tachypleus gigas* from Peninsular Malaysia.

Population	KPPuteh	KPSayak	KSAyam
KPPuteh	-	0.844	0.179
KPSayak	0.430	-	1.458
KSAyam	0.836	0.233	-

4 | DISCUSSION

4.1 Genetic differentiation of *T. gigas* populations in metal-polluted habitats

The analysis of genetic differentiation among *T. gigas* populations from KPPuteh, KPSayak, and KSAyam reveals substantial variability across loci, indicating that environmental factors, particularly metal pollution, play a significant role in shaping these genetic patterns (Aini *et al.* 2021). The high F_{ST} values observed at loci such as LDH and PGM-1 underscore considerable genetic divergence among the populations, consistent with findings highlighting the relationship between environmental stressors and genetic variation in marine species subjected to pollution (Aini *et al.* 2021). This divergence is likely driven by localized selection pressures imposed by the differing environmental conditions at each site, especially the polluted habitats of KPPuteh and KSAyam—a pattern well-documented in studies examining the effects of metal contamination on genetic structure in coastal organisms (Piras *et al.* 2013; Lebrun *et al.* 2015).

The genetic differentiation observed is further supported by the clustering of KPPuteh and KSAyam in the UPGMA dendrogram, indicating a close genetic relationship between these two populations, both inhabiting metal-polluted sites. This clustering suggests that shared environmental stressors have resulted in similar selective pressures, driving the populations toward a convergent genetic structure (Depledge 1998). This phenomenon has been observed in other species facing analogous environmental challenges, reinforcing the link between ecological factors and genetic adaptation. Furthermore, significant deviations from Hardy–Weinberg equilibrium at several loci, particularly in the KPPuteh and KSAyam populations, provide additional evidence of environmental stressors' impact on genetic structure (Yap *et al.* 2002b). For example, the significant χ^2 values at loci such as GOT-2 in KPPuteh indicate a departure from random mating, suggesting adaptive genetic responses to high metal levels in these habitats, reflecting on-going evolutionary pressures in polluted environments (Aini *et al.* 2021).

In contrast, KPSayak, which is relatively less polluted, exhibits greater genetic distinctiveness, as reflected in the lower genetic identity values and its separate clustering in the dendrogram. This distinctiveness suggests that KPSayak is a refuge for genetic diversity, maintaining a unique allele frequency spectrum that may be more resilient to environmental changes (Nevo *et al.* 1986). This could be a pattern observed in other populations subjected to varying degrees of pollution and stress (Mastana and Papiha 1994; Karadede-Akin and Ünlü 2006; Wang *et al.* 2023). The differentiation at KPSayak likely results from the different selective pressures and environmental conditions at this site, which have preserved a distinct genetic pool within the population (van Straalen and Timmermans 2002).

These findings align with the understanding that populations in stressful environments, such as those polluted with metals, often experience higher rates of genetic differentiation due to selective pressures favouring alleles conferring resistance or adaptation to these conditions (Abdul-Aziz and Ali 2009). The genetic structure of *T. gigas* populations in the studied regions appears strongly influenced by heterogeneous environmental conditions, particularly metal pollution, which has led to significant genetic differentiation and adaptive responses in the affected populations (Lopes *et al.* 2004; Timmermans *et al.* 2007). These adaptive responses are exemplified by the increased genetic diversity observed in contaminated sites, which can enhance resilience against further environmental stressors. This pattern echoes findings in other studies where metal-stressed populations displayed higher genetic diversification and the emergence of pollution-related alleles (Nevo *et al.* 1986). Specifically, significant allelic variation at loci such as metallothionein in metal-contaminated populations suggests that these genes may confer a fitness advantage in polluted environments, highlighting the importance of adaptive divergence in response to anthropogenic stressors (Timmermans *et al.* 2007; Janssens *et al.* 2008).

The results of this study contribute to our understanding of the complex interplay between environmental factors, genetic variation, and evolutionary processes in marine organisms facing the challenges of pollution and environmental degradation (Breitwieser *et al.* 2018). The observed patterns of genetic differentiation among *T. gigas* populations underscore the role of allele frequency shifts in response to heavy metal exposure. These shifts affect phenotypic traits and drive species' evolutionary trajectories through the selective pressures exerted on their genetic architecture (Anderson *et al.* 1994). This may lead to an overall increase in population tolerance to lethal and sublethal environmental conditions, as observed in various studies examining the effects of heavy metals on genetic adaptation in diverse species (Posthuma and Straalen 1993; Lopes *et al.* 2004).

These findings highlight the necessity for on-going research to elucidate the molecular mechanisms underlying such adaptations and their evolutionary implications. Integrative approaches that consider both genetic and ecological factors are crucial when assessing the impacts

of environmental pollution on biodiversity and population dynamics in marine ecosystems (Ungherese *et al.* 2010). Understanding these dynamics is essential for conservation efforts to protect biodiversity in affected areas. Adapting to such environmental stressors is not only a matter of genetic resilience but also essential for maintaining ecological balance and population viability in increasingly polluted ecosystems—an issue of growing concern across a wide range of taxa and habitats (Timmermans *et al.* 2007; Bhandari 2016; Houslay *et al.* 2022; Krishna *et al.* 2023).

Conservation strategies that emphasize the role of genetic adaptation in responding to environmental stressors will provide further insights into the strategies employed by populations to withstand anthropogenic pressures, ultimately informing practices aimed at preserving biodiversity in these increasingly challenged ecosystems (Posthuma and Straalen 1993; Timmermans *et al.* 2007).

4.2 Impact of environmental pollution on genetic variability

The results from the genetic analysis underscore the significant impact of environmental pollution on the genetic variability of *T. gigas* populations. This adaptive response to chemical stress highlights the resilience of these populations. It emphasizes the necessity for long-term conservation strategies that consider genetic diversity as a crucial factor for survival in polluted ecosystems (Tenji *et al.* 2020). The ability to cope with environmental stressors like pollution is influenced by genetic factors, making it essential to maintain genetic diversity to enable populations to withstand future environmental challenges and recover from anthropogenic impacts on their habitats (Bhandari 2016; Friedli *et al.* 2020; Rebolledo *et al.* 2021).

Notably, the populations at KPPuteh and KSAyam, located in metal-polluted environments, exhibited higher genetic diversity than KPSayak. This is indicated by the greater number of polymorphic loci and alleles per locus (Lopes *et al.* 2004; Timmermans *et al.* 2007). Such increased genetic diversity in populations exposed to environmental stressors suggests that selection pressures in polluted areas may promote the preservation of alleles associated with detoxification and stress response mechanisms, as observed in other organisms adapting to chemical pollution (Timmermans *et al.* 2007; Janssens *et al.* 2008). This adaptive evolution in response to metal stress may be occurring in *T. gigas*, allowing these populations to better cope with pollution-induced challenges (Yap *et al.* 2002b, 2013).

In contrast, KPSayak, a site less impacted by pollution, exhibited lower genetic variability, with fewer polymorphic loci and alleles per locus. The reduced genetic diversity in this population could be attributed to the lack of strong selective pressures in a relatively stable environment, resulting in a more homogenous genetic structure (Prakoon *et al.* 2010). However, such a lack of variability may hinder the population's ability to adapt to future environmental changes, potentially increasing its vulnerability to extinction under new stressors or habitat alterations, a concern echoed in other studies focused on the consequences of reduced genetic diversity (Vincenzi *et al.* 2017; Kyriazis *et al.* 2021).

The observed heterozygosity values further illuminate the impact of pollution on genetic variability. In KPPuteh and KSAyam, the observed heterozygosity was generally lower than expected, suggesting that inbreeding or selection against certain genotypes may occur (Rajaei *et al.* 2015). This could exacerbate the effects of pollution on these already stressed populations. The pattern of reduced heterozygosity aligns with findings from other studies that indicate environmental stress can lead to allele frequency shifts favouring more tolerant genotypes, potentially limiting the genetic resources available for future adaptation (Janssens *et al.* 2008; Tenji *et al.* 2020). The negative Selander's Ds values at several loci, particularly in KPPuteh, support this interpretation, indicating a deficit in heterozygosity likely due to environmental stress, which compounds the risks associated with reduced genetic diversity (Mussali-Galante *et al.* 2014).

These findings have significant implications for understanding how populations respond to environmental stressors (Cross and Rebordinos 2003). The increased genetic variability in polluted sites suggests that these populations are undergoing adaptive changes in response to the selective pressures imposed by their environment, potentially maintaining higher fitness in compromised habitats (Jordaens *et al.* 2006). However, the lower diversity in less polluted areas, combined with the reduction in heterozygosity in the more polluted sites, highlights the potential vulnerability of these populations to future environmental changes. This underscores the need for conservation efforts that prioritize genetic diversity as a crucial factor in ensuring the long-term resilience of *T. gigas* populations (Depledge 1998).

Conservation strategies should, therefore, prioritize the maintenance and enhancement of genetic diversity, as it plays a pivotal role in the resilience and adaptability of populations to environmental fluctuations and stressors, thereby ensuring their survival in the face of ongoing anthropogenic influences (Lopes *et al.* 2004; Timmermans *et al.* 2007; Janssens *et al.* 2008). These strategies must also consider the potential for deleterious mutations, as evidence suggests that managing genetic variation to minimize these negative effects is essential for enhancing population viability in the face of on-going environmental challenges (Kyriazis *et al.* 2021). Conservation programs must integrate genetic assessment tools to identify both beneficial alleles and potentially harmful mutations, ensuring a balanced approach that fosters adaptive resili-

ence while safeguarding against the risks associated with inbreeding depression and genetic erosion (Hellmair and Kinziger 2014; Kyriazis *et al.* 2021).

4.3 Implications of genetic structure for population management

The genetic structure observed in *T. gigas* populations has significant implications for their management and conservation (Kim *et al.* 2003). The pattern of inbreeding observed within these populations may increase the risk of extinction, especially regarding environmental change and habitat degradation (Larsson *et al.* 2016). Inbreeding compromises each population's adaptive potential due to the accumulation of deleterious alleles, which weakens their resilience to changing conditions (Kyriazis *et al.* 2021). Addressing inbreeding issues through targeted conservation strategies is crucial, as minimizing the presence of strongly deleterious mutations can significantly enhance population resilience against on-going environmental pressures, ultimately supporting their long-term survival (Kyriazis *et al.* 2021).

The high levels of genetic differentiation observed among *T. gigas* populations, particularly between those from polluted and non-polluted sites, suggest that these populations should be managed as distinct units (Weiss 2005; Fenster *et al.* 2018). Implementing active genetic management strategies, such as facilitating gene flow between fragmented populations, could mitigate the effects of inbreeding depression and enhance adaptive capacity to environmental changes. This, in turn, would reduce extinction risks associated with low genetic diversity (Fenster *et al.* 2018). Moreover, fostering genetic connectivity through managed translocations or the establishment of wildlife corridors could help maintain genetic diversity and combat the adverse effects of inbreeding across *T. gigas* populations, thereby improving their overall resilience to ecological changes and anthropogenic threats (Weiss 2005; Fenster *et al.* 2018; Kyriazis *et al.* 2021).

Furthermore, the significant F_{ST} values observed indicate limited gene flow between *T. gigas* populations, potentially due to environmental barriers or behavioural factors restricting movement (Muller *et al.* 2007). This limited connectivity has led to the development of unique genetic identities within each population, as they adapt to specific environmental conditions. Consequently, understanding the genetic dynamics within and among these populations is essential for formulating effective conservation strategies that address current threats and anticipate future ecological challenges (Fenster *et al.* 2018).

This situation underscores the need for conservation programs that monitor genetic variation and implement strategies to reverse the impacts of inbreeding and genetic isolation (Nevo *et al.* 1986). Such strategies are vital for fostering a more resilient population in the face of environmental changes and ensuring the survival of T. gigas in fragmented landscapes (Fenster *et al.* 2018; Manel *et al.* 2020; Aini *et al.* 2021; Kyriazis *et al.* 2021). These goals can be achieved by integrating empirical genetic studies and adaptive management approaches, allowing conservationists to make informed decisions that support both the genetic health and ecological integrity of *T. gigas* populations over the long term (Kyriazis *et al.* 2021).

Additionally, on-going research into the genetic diversity and population structure of *T. gigas* is critical for guiding these management efforts (Depledge 1998). Such research will provide insights into each population's specific genetic vulnerabilities and adaptations, which can inform tailored conservation actions to preserve their genetic and ecological viability. It is also essential to recognize that genetic diversity alone may not ensure population stability (Larsson *et al.* 2016). A comprehensive understanding of the ecological context and the specific threats facing each *T. gigas* population is necessary to formulate effective conservation strategies responsive to their unique circumstances (Manel *et al.* 2020; Aini *et al.* 2021).

4.4 Conservation Implications and Future Research Directions

The *T. gigas* populations hold significant ecological and economic importance. This species is a crucial component of marine ecosystems, playing a vital role in the food web and contributing to the health of coastal environments. These factors underscore the necessity for effective management and conservation efforts to ensure the species' survival and the sustainability of its habitats (Manca *et al.* 2017; Mashar *et al.* 2017; Liew *et al.* 2023). However, the genetic diversity and population dynamics of *T. gigas* remain understudied, limiting our understanding of the factors influencing its population structure and hindering the development of targeted conservation strategies (García-Enríquez *et al.* 2023).

Hence, the present presented the genetic diversity and population differentiation of *T. gigas* across its range, with particular attention to environmental factors, such as pollution, in shaping genetic variation. The findings have important implications for the conservation of *T. gigas*, highlighting the need for further research to better understand the genetic and environmental factors influencing population dynamics. Notably, the significant correlation between genetic diversity and resilience to environmental fluctuations underscores the critical need for conservation strategies that consider both genetic and ecological factors to mitigate the risks posed by habitat degradation and pollution (Hellmair and Kinziger 2014).

Moreover, integrating genetic assessments into conservation planning can enhance our understanding of population resilience and adaptive potential, which is vital for the long-term survival of *T. gigas* amid on-going envi-

ronmental changes and anthropogenic pressures (Nelson *et al.* 2016). In light of these findings, future conservation initiatives must incorporate genetic monitoring and adaptive management practices to respond to emerging environmental challenges and support the sustainable use of this species (Manel *et al.* 2020). Additionally, breeding programs prioritising genetic diversity can proactively bolster population sizes and enhance resilience against environmental stressors, fostering the recovery of *T. gigas* populations in affected regions (Xu *et al.* 2021).

In summary, addressing the genetic and ecological dimensions of *T. gigas* conservation is critical, as these factors are interrelated and directly influence the species' adaptive capacity and long-term viability under varying environmental conditions (Manel *et al.* 2020). The significant genetic differentiation among populations, coupled with the impact of environmental pollution on genetic variability, suggests that conservation strategies should focus on preserving genetic diversity within and among populations (Krisfalusi-Gannon *et al.* 2018). This approach is particularly important as genetically distinct local populations may be at higher risk of extinction, gradually eroding genetic diversity and adaptive potential (Manel *et al.* 2020).

This erosion emphasizes the necessity of understanding the global distribution of intraspecific genetic variation and its key drivers, as current knowledge often does not adequately reflect the on-going threats to genetic diversity in the face of environmental change (Manel *et al.* 2020). Elucidating the mechanisms underlying genetic variation and population structure is essential for designing effective conservation schemes, as diverse populations are generally more resilient to environmental fluctuations. This highlights the importance of biocomplexity at the population level to ensure the long-term persistence of *T. gigas* (Carmichael *et al.* 2015).

Finally, the study underscores the need for a collaborative conservation approach involving multiple stakeholders, including local communities, government agencies, and conservation organizations. By working together, these stakeholders can develop and implement conservation strategies that are informed by the best available science and tailored to the specific needs of *T. gigas* populations. Such an approach would help ensure long-term survival of the species and preserve its unique genetic diversity in the face of on-going environmental change (John *et al.* 2018).

5 | CONCLUSIONS

The populations of *T. gigas* in Peninsular Malaysia show extensive genetic divergence, demonstrating the deep influence of environmental conditions, notably metal contamination, on genetic structure and variability. Populations from contaminated areas like KPPuteh and KSAyam have more genetic diversity and considerable departures from Hardy–Weinberg equilibrium, possibly due to selective pressures favouring pollutant-resistant genes. The less polluted KPSayak site population has lower genetic diversity and more genetic uniqueness, suggesting that differing environmental circumstances have impacted their evolutionary trajectories.

The large F_{ST} values and grouping of populations in the UPGMA dendrogram emphasize the need to manage these populations as discrete entities due to their genetic makeup and environmental concerns. Inbreeding in contaminated populations highlights the need for conservation techniques that encourage gene flow and genetic diversity to strengthen populations. These findings emphasize the need for genetic variation within and between *T. gigas* populations for long-term survival. Future studies should examine genetic and environmental variables affecting population differentiation and ecological and behavioural processes affecting gene flow and population connectedness. By incorporating genetic insights into conservation initiatives, stakeholders may better manage environmental change and help preserve this species in its native settings.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Conceptualization, CKY and KAA-M; methodology and validation, CKY and KAA-M; formal analysis, CKY; investigation, CKY; resources, KAA-M; data curation, CKY; writing—original draft preparation, CKY; writing—review and editing, CKY and KAA-M. Both authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of the study will be made available on a reasonable request from the corresponding author.

REFERENCES

Abdul-Aziz KK, Ali AE (2009) The health status and genetic variations of the bivalve, *Pinctada radiata* affected by environmental pollution. The Egyptian Science Magazine 6(3): 22–30.

Aini N, Wardiatno Y, Effendi H, Mashar A, Madduppa H (2021) High genetic diversity and mixing of coastal horseshoe crabs (*Tachypleus gigas*) across major habitats in Sundaland, Indonesia. PeerJ 9: e11739.

Anderson S, Sadinski W, Shugart L, Brussard P, Depledge M, ... Wogan G (1994) Genetic and molecular ecotoxicology: a research framework. Environmental Health Perspectives 102(Suppl 12): 3–8.

Ayala FJ, Powell JR, Tracey ML, Mourao CA, Perez-salas S (1972) Enzyme variability in the *Drosophila willistoni* group. IV. Genic variation in natural populations of *Drosophila willistoni*. Genetics 70(1): 113–139.

Bert TM, Seyoum S, Tringali MD, McMillen-Jackson AL (2002) Methodologies for conservation assessments of the genetic biodiversity of aquatic macroorganisms. Brazilian Journal of Biology 62(3): 387–408.

Bhandari RK (2016) Medaka as a model for studying environmentally induced epigenetic transgenerational inheritance of phenotypes. Environmental Epigenetics 2(1): dvv010.

Breitwieser M, Becquet V, Thomas-Guyon H, Pillet V, Sauriau PG, ... Viricel A (2018) Population structure and genetic diversity in the variegated scallop, *Mimachlamys varia* (Linnaeus, 1758), a novel bioindicator of chemical pollution on the French coastline. Journal of Molluscan Studies 84(4): 417–425.

Carmichael RH, Botton ML, Shin PK, Cheung SG (Eds) (2015) Changing global perspectives on horseshoe crab biology, conservation and management (No. 11406). Springer International Publishing.

Cross I, Rebordinos L (2003) Effect of marine contamination on the genetic population structure of the bivalve *Crassostrea angulata*. Ciencias Marinas 29(2): 239–250.

Depledge MH (1998) The ecotoxicological significance of genotoxicity in marine invertebrates. Mutation Research – Fundamental and Molecular Mechanisms of Mutagenesis 399(1): 109–122.

Donaldson TJ (2002) Phylogeny, reef fish conservation biology, and the live reef fish trade. Fisheries Science 68(sup1): 143–147.

Fenster CB, Ballou JD, Dudash MR, Eldridge MDB, Frankham R, ... Sunnucks P (2018) Conservation and genetics. Biological Conservation 91(4): 491–501.

Friedli A, Williams GR, Bruckner S, Neumann P, Straub L (2020) The weakest link: haploid honey bees are more susceptible to neonicotinoid insecticides. Chemosphere 242: 125145.

García-Enríquez JM, Machkour-M´ Rabet S, Rosas-Correa CO, Hénaut Y, Carrillo L (2023) Genetic study of the American horseshoe crab throughout its Mexican distribution: conservation and management implications. Biodiversity and Conservation 32(2): 489–507.

Harris H, Hopkinson DA (1976) Handbook of enzyme electrophoresis in human genetics. North Holland Publishing Company, Amsterdam.

Hellmair M, Kinziger AP (2014) Increased extinction potential of insular fish populations with reduced life history variation and low genetic diversity. PLOS ONE 9(11): e113139.

Houslay TM, Earley RL, White SJ, Lammers W, Grimmer AJ, ... Wilson AJ (2022) Genetic integration of behavioural and endocrine components of the stress response. eLife 11. DOI 10.7554/elife.67126

Janssens T, Lopéz RDR, Mariën J, Timmermans MJTN, Montagne-Wajer K, ... Roelofs D (2008) Comparative population analysis of metallothionein promoter alleles suggests stress-induced microevolution in the field. Environmental Science & Technology 42(10): 3873–3878.

John BA, Nelson BR, Sheikh HI, Cheung SG, Wardiatno Y, ... Pati S (2018) A review on fisheries and conservation status of Asian horseshoe crabs. Biodiversity and Conservation 27: 3573–3598.

Johnson RL, Mitchell RM, Harp GL (2006) Genetic variation and genetic structuring of a numerically declining species of darter, *Etheostoma moorei* Raney & Suttkus, endemic to the upper Little Red River, Arkansas. American Midland Naturalist 156(1): 37–44.

Jordaens K, de Wolf H, van Houtte N, Vandecasteele B, Backeljau T (2006) Genetic variation in two land snails, *Cepaea nemoralis* and *Succinea putris* (Gastropoda, Pulmonata), from sites differing in heavy metal content. Genetica 128: 227–239.

Karadede-Akin H, Ünlü E (2006) Heavy metal concentrations in water, sediment, fish and some benthic organisms from Tigris River, Turkey. Environmental Monitoring and Assessment 131(1-3): 323–337.

Kim SJ, Rodriguez-Lanetty M, Suh JH, Song JI (2003) Emergent effects of heavy metal pollution at a population level: *Littorina brevicula* a study case. Marine Pollution Bulletin 46(1): 74–80.

Krisfalusi-Gannon J, Ali W, Dellinger K, Robertson L, Brady TE, ... Dellinger AL (2018) The role of horseshoe crabs in the biomedical industry and recent trends impacting species sustainability. Frontiers in Marine Science 5: 185.

Krishna S, Lemmen C, Örey S, Rehren J, Pane JD, ... Wirtz K (2023) Interactive effects of multiple stressors in coastal ecosystems. arXiv. DOI 10.48550/arxiv.2312.07306

Kyriazis CC, Wayne RK, Lohmueller KE (2021) Strongly deleterious mutations are a primary determinant of extinction risk due to inbreeding depression. Evolution Letters 5(1): 33–47.

Larsson J, Lönn M, Lind EE, Świeżak J, Smolarz K, Grahn M (2016) Sewage treatment plant associated genetic differentiation in the blue mussel from the Baltic Sea and Swedish west coast. PeerJ 4: e2628.

Lebrun JD, Geffard O, Urien N, François A, Uher E, Fechner LC (2015) Seasonal variability and inter-species comparison of metal bioaccumulation in caged gammarids under urban diffuse contamination gra-

dient: implications for biomonitoring investigations. Science of the Total Environment 511: 501–508.

Liew PL, Ng WL, Tan SG (2015) Levels and patterns of genetic variation in an Asian horseshoe crab species, *Tachypleus gigas* Müller, from the Malay Peninsula. Marine Biology Research 11(8): 879–886.

Linh NQ, Huyền KT (2020) Multiple population structure of the giant eel *Anguilla marmorata* in Thua Thien Hue, Vietnam base on COI sequences. Research Square. DOI: 10.21203/rs.3.rs-40345/v1

Lopes I, Baird DJ, Ribeiro R (2004) Genetic determination of tolerance to lethal and sublethal copper concentrations in field populations of *Daphnia longispina*. Archives of Environmental Contamination and Toxicology 46(1): 43-51.

Manca A, Mohamad F, Ahmad A, Sofa MFAM, Ismail N (2017) Tri-spine horseshoe crab, *Tachypleus tridentatus* (L.) in Sabah, Malaysia: the adult body sizes and population estimate. Journal of Asia-Pacific Biodiversity 10(3): 355–361.

Manel S, Guérin P, Mouillot D, Blanchet S, Velez L, Pellissier L (2020) Global determinants of freshwater and marine fish genetic diversity. Nature Communications 11(1): 692.

Mashar A, Butet N, 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 Science 54: 012076.

Mastana S, Papiha SS (1994) Genetic structure and microdifferentiation among four endogamous groups of Maharashtra, Western India. Annals of Human Biology 21(3): 241–262.

Muller LA, Vangronsveld J, Colpaert JV (2007) Genetic structure of *Suillus luteus* populations in heavy metal polluted and nonpolluted habitats. Molecular Ecology 16(22): 4728–4737.

Mussali-Galante P, Tovar-Sánchez E, Valverde M, Rojas E (2014) Genetic structure and diversity of animal populations exposed to metal pollution (pp. 79–106). In: Whitacre D (Ed) Reviews of Environmental Contamination and Toxicology Volume 227. Springer Cham.

Nei M (1977) F-statistics and analysis of gene diversity in subdivided populations. Annals of Human Genetics 41(2): 225–233.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89(3): 583–590.

Nelson BR, Satyanarayana B, Moh JHZ, Ikhwanuddin M, Chatterji A, Shaharom F (2016) The final spawning ground of *Tachypleus gigas* (Müller, 1785) on the east Peninsular Malaysia is at risk: a call for action. PeerJ 4: e2232.

Nevo E, Noy R, Lavie B, Beiles A, Muchtar S (1986) Genetic diversity and resistance to marine pollution. Biological Journal of the Linnean Society 29(2): 139–144.

Piras P, Chessa G, Cossu M, Fiori G, Piras P, Ledda G (2013) Lead and other heavy metals (cadmium and mercury) accumulation in bivalve mollusks (*Mytilus galloprovincialis*, *Ruditapes* spp., and *Crassostrea gigas*) sampled in Sardinia in 2008-2012. Italian Journal of Food Safety 2(3): 49.

Posthuma L, Straalen NMV (1993) Heavy-metal adaptation in terrestrial invertebrates: a review of occurrence, genetics, physiology and ecological consequences. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 106(1): 11–38.

Prakoon W, Tunkijjanukij S, Nguyen TT, Na-Nakorn U (2010) Spatial and temporal genetic variation of green mussel, *Perna viridis* in the Gulf of Thailand and implication for aquaculture. Marine Biotechnology 12(5): 506–515.

Rajaei M, Farahmand H, Poorbagher H, Mortazavi MS, Farhadi A (2015) Sympatric morphological and genetic differentiation of the pearl oyster *Pinctada radiata* (Bivalvia: Pterioida) in the northern Persian Gulf. Journal of the Marine Biological Association of the United Kingdom 95(3): 537–543.

Rebolledo UA, Páez-Osuna F, Fernández R (2021) Single and mixture toxicity of As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, and Zn to the rotifer *Proales similis* under different salinities. Environmental Pollution 271: 116357.

Rozihan M, Ismail ES (2012) Impact of Malaysian Continental Drift on the genetic diversity of horseshoe crab inferred through mtDNA sequence analysis. International Journal of Biology 4(1): 104–110.

Shaw CR, Prasad R (1970) Starch gel electrophoresis of enzymes. Biochemical Genetics 4(2): 297–320.

Sneath PHA, Sokal RR (1973) Numerical taxonomy: the principles and practice of numerical classification. WF Freeman & Co., San Francisco. 573 pp.

Steiner WWM, Joslyn DJ (1979) Electrophoretic techniques for the genetic study of mosquitoes. Mosquito News 39(1): 35–54.

Swofford DL, Selander RB (1989) BIOSYS-1: a computer program for the analysis of allelic variation in population genetics and biochemical systematics (Release 1.7). Illinois Natural History Survey, Illinois, USA.

Tenji D, Mićić B, Sipos S, Miljanović B, Teodorović I, Kaišarević S (2020) Fish biomarkers from a different perspective: Evidence of adaptive strategy of *Abramis brama* (L.) to chemical stress. Environmental Sciences Europe 32(1): 47.

Timmermans MJTN, Ellers J, Straalen NMV (2007) Allelic diversity of metallothionein in *Orchesella cincta* (L.): traces of natural selection by environmental pollution. Heredity 98(5): 311–319.

Ungherese G, Mengoni A, Somigli S, Baroni D, Focardi S,

Ugolini A (2010) Relationship between heavy metals pollution and genetic diversity in Mediterranean populations of the sandhopper *Talitrus saltator* (Montagu) (Crustacea, Amphipoda). Environmental Pollution 158(5): 1638–1643.

van Straalen NM, Timmermans MJ (2002) Genetic variation in toxicant-stressed populations: an evaluation of the "genetic erosion" hypothesis. Human and Ecological Risk Assessment 8(5): 983–1002.

Vincenzi S, Mangel M, Jeseňsek D, Garza JC, Crivellì AJ (2017) Genetic and life-history consequences of extreme climate events. Proceedings of the Royal Society B: Biological Sciences 284(1848): 20162118.

Wang T, Meng H, Wang N, Zheng S, Jiang Y, ... Kozlowski G (2023) Adaptive divergence and genetic vulnerability of relict species under climate change: a case study of *Pterocarya macroptera*. Annals of Botany 132(2): 241–254.

Weiss S (2005) Conservation genetics of freshwater organisms. Knowledge and Management of Aquatic Ecosystems 376–377: 571–583.

Wright S (1978) Evolution and the genetics of populations: variability within and among natural populations (volume 4). University of Chicago Press, Chicago.

Xu P, Bai H, Xie X, Wang C, Huang X, ... Kwan KY (2021) Tri-spine horseshoe crab aquaculture, ranching and stock enhancement: perspectives and challenges. Frontiers in Marine Science 8: 608155.

Yap CK, Cheng WH, Ong CC, Tan SG (2013) Heavy metal contamination and physical barrier are main causal agents for the genetic differentiation of *Perna viridis* populations in Peninsular Malaysia. Sains Malaysiana 42(11): 1557–1564.

Yap CK, Ismail A, Tan SG, Omar H (2002a) Concentrations of Cu and Pb in the offshore and intertidal sediments of the west coast of Peninsular Malaysia. Environment International 28: 467–497.

Yap CK, Noorhaidah A (2011) Gill and digestive caecum of *Telescopium telescopium* are biomonitors of Pb bioavailability and contamination by Pb in the tropical intertidal area. Sains Malaysiana 40(10): 1075–1085.

Yap CK, Tan SG, Ismail A, Omar H (2002b) Genetic variation of the green-lipped mussel *Perna viridis* (L.) (Mytilidae: Mytiloida: Mytilicae) from the west coast of Peninsular Malaysia. Zoological Studies 41(4): 376–387.

Zee MJVD, Whiting JR, Paris JR, Bassar RD, Travis J, ... Fraser BA (2022) Rapid genomic convergent evolution in experimental populations of Trinidadian guppies (*Poecilia reticulata*). Evolution Letters 6(2): 149–161.

CK Yap http://orcid.org/0000-0003-0317-0999
KA Al-Mutairi http://orcid.org/0000-0003-2356-0724