



## RESEARCH ARTICLE

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# Mangrove horseshoe crab (*Carcinoscorpius rotundicauda* Latreille, 1802) populations reveal genetic break in Strait of Malacca, with connectivity along southern coasts of Peninsular Malaysia

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## Abstract

1. The mangrove horseshoe crab, *Carcinoscorpius rotundicauda*, has divergent populations between the east and west coasts of Peninsular Malaysia, with the southern coast acting as a land barrier. The actual position of such a genetic break along Peninsular Malaysia as well as the connectivity status of the southernmost *C. rotundicauda* populations with east and west coast populations remain unexplored, however.
2. The aim was to investigate the genetic diversity and structure of *C. rotundicauda* populations from the west (Kuala Sepetang in State Perak), east (Balok in State Pahang), and southern (Pendas in State Johor) coasts of Peninsular Malaysia. Haemolymph samples from adult *C. rotundicauda* specimens ( $n = 152$ ) and eggs from their freshly deposited nests ( $n = 190$ ) were collected monthly (from January 2016 to January 2017) for the sequencing of mitochondrial cytochrome c oxidase subunit I (COI).
3. Gene isolates of *C. rotundicauda* from the present study were compared with National Center for Biotechnology Information (NCBI) GenBank sequences to cover most of the range of the species in Asia. A neighbour-joining tree strongly supported two clades, separating the west-coast populations from the south- and east-coast populations, with further substructure patterns.
4. Both haplotype network and barrier analyses revealed a genetic break within the Strait of Malacca instead of the southern tip of Peninsular Malaysia. The southernmost samples from the Strait of Johor formed a haplotypic diverse gene pool that appeared only as a subclade of the eastern populations. In a detailed

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haplotype network of 347 individuals, individuals with similar *COI* sequences indicate connectivity between *C. rotundicauda* on the east and *C. rotundicauda* on south and south west of Peninsular Malaysia.

5. Overall, the genetic break between *C. rotundicauda* populations is better explained by the convergent ocean currents and available mangrove habitats on the west coast (i.e. Strait of Malacca), rather than the point of the Malay Peninsula acting as a land barrier alone.

#### KEYWORDS

*COI* sequencing, genetic diversity, haplotype network, horseshoe crab, living fossil, Malaysia, phylogeny

## 1 | INTRODUCTION

Among the three extant Asian horseshoe crab species in the world, only *Carcinoscorpius rotundicauda* (Latreille, 1802) is known as the mangrove horseshoe crab, for its preference to live and nest close to mangrove habitat (Cartwright-Taylor, Lee & Hsu, 2009; Yennawar, 2015). In contrast, *Tachypleus tridentatus* (Leach, 1819) and *Tachypleus gigas* (Müller, 1785) are found along sandy beaches (Wada, Itaya & Shuuno, 2010; Obst et al., 2012; Faridah et al., 2016; Kwan et al., 2016). With mangrove habitat extending over 4.4 million ha in Asia (Richards & Friess, 2016), *C. rotundicauda* is widely distributed throughout the continent (Vestbo et al., 2018). In Southeast Asia, the mangrove horseshoe crab is found in Indonesia, Malaysia, Cambodia, Brunei, the Philippines, Singapore, Thailand, and Vietnam (Adibah et al., 2012; Robert, Muhammad Ali & Amelia-Ng, 2014; Cartwright-Taylor, 2015; IUCN, 2019; Meilana & Fang, 2020).

In Malaysia, all three Asian horseshoe crab species are abundant. Whereas *C. rotundicauda* and *T. gigas* are found in Peninsular Malaysia, the distribution of *T. tridentatus* is restricted to Sabah and Sarawak in East Malaysia (Akbar John et al., 2018). The horseshoe crabs of Malaysia have received serious scientific attention only recently, however (Zaleha et al., 2010; Srijaya et al., 2014; Manca et al., 2017). Therefore, the current population size and spawning counts of these horseshoe crabs are uncertain and good baseline data are lacking from several locations in Peninsular Malaysia.

*Carcinoscorpius rotundicauda* populations are believed to have a common ancestry (Rudkin & Young, 2009), yet there have been very few studies on this subject, with most research focused on other horseshoe crab species (Saunders, Kessler & Avise, 1986; Pierce, Tan & Gaffney, 2000; Rozihan & Ismail, 2011; Periasamy, Ingole & Meena, 2017). For Peninsular Malaysia, Adibah, Ng & Tan (2015) investigated the nuclear microsatellite genetic variation of *C. rotundicauda* and demonstrated the low relatedness of the populations between the east and west coasts. An east–west division across Peninsular Malaysia was therefore evident, but the precise geographic position of this genetic break is unknown. Moreover, the identity of populations at potential contact zones along the southern coast of Peninsular Malaysia, as well as their genetic connectivity with

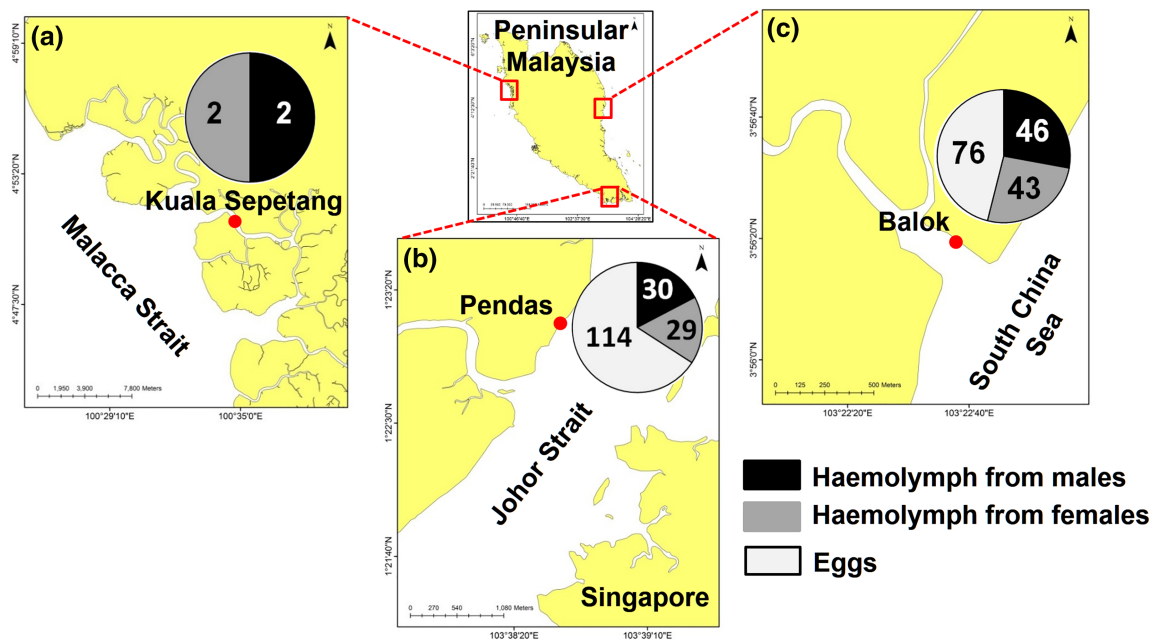
populations from the east or west coasts remain unexplored. The number of horseshoe crabs at the nesting grounds varies seasonally (Brockmann & Johnson, 2011; Akbar John et al., 2012; Nelson et al., 2015; Noor Jawahir et al., 2017) and supports the need for year-round sampling to determine the potential temporal connectivity of migrants. DNA sequencing through mitochondrial cytochrome c oxidase subunit I (*COI*) is considered to be a basic platform for identifying invertebrate species (Mikkelsen, Schander & Willassen, 2007), and is also known for possessing a large range of phylogenetic signals to help discriminate closely allied taxa and distinct haplotype groups within a species (Hebert et al., 2003). Likewise, *COI* markers have demonstrated sufficient polymorphism for the phylogenetic analysis of horseshoe crab species (Liew, Ng & Tan, 2015).

The main objective of the present study was to assess the genetic diversity and structure of *C. rotundicauda* populations, using a maternal marker sequence (*COI*), based on the year-round sampling of adults and eggs from the east, west, and especially the southern coasts of Peninsular Malaysia. Our specific objectives were to: (i) detect a genetic barrier through a comparison of DNA sequences with known horseshoe crab data from Asia; and (ii) estimate the identity and diversity of *C. rotundicauda* populations on the southern coast, and their connectivity with the east and west coasts in Peninsular Malaysia. The approach of temporal and spatial sampling allows a detailed interpretation of the potential connectivity issues and also helped coastal management stakeholders to understand the distribution of horseshoe crabs along coastlines, in particular when dealing with human-assisted introductions.

## 2 | MATERIALS AND METHODS

### 2.1 | Study areas and sample collection

Live samples of *C. rotundicauda* were collected from State Perak on the west coast, State Pahang on the east coast, and State Johor on the southern coast of Peninsular Malaysia (Figure 1). Based on a pilot study (conducted in December 2015) and feedback from local fishermen three sampling areas, namely Kuala Sepetang in Perak, Pendas in



**FIGURE 1** Sampling sites chosen for observing the genetic variation of *Carcinoscopus rotundicauda* in Peninsular Malaysia: (a) Kuala Sepetang in State Perak on the west coast; (b) Pendas in State Johor on the southern coast; and (c) Balok in State Pahang on the east coast. The red circle at each site indicates the place of sample collection and the pie chart therein shows the number of haemolymph and egg samples collected (image source: ARCMAP v10)

Johor, and Balok in Pahang, were chosen. All these areas consist of luxuriant mangrove cover and are known for hosting *C. rotundicauda* populations (Akbar John et al., 2018; Fairuz-Fozi et al., 2018; Zauki et al., 2019a).

The sampling (from January 2016 to January 2017) was conducted every month during the full moon and new moon periods, searching for horseshoe crabs as well as their nests/eggs. In order to find adult *C. rotundicauda* at each site, gill nets (200 m in length and 3 m in height, with a mesh size of 12.7 cm) were deployed during the high tide and retrieved when tidal waters were receding. All live horseshoe crabs trapped in the net were gently removed by hand and 0.5 ml of haemolymph was collected (using a sterilized hypodermic syringe fitted with a fine 22-gauge needle) from the hinge of single females and from amplexus (i.e. male and female in pairs). Surgical cotton swabs were used to prevent bleeding at the point of haemolymph collection and the site was then disinfected with 40% methanol. All specimens were immediately released back into the sea. The search for *C. rotundicauda* nests and eggs continued during low tide. At each site, surface sediment in the intertidal areas was gently excavated (using a hand shovel) to collect between three and five freshly deposited eggs from the nest. The new eggs were differentiated from old eggs on the basis of their morphological characteristics, such as size, shape, and colour, following Fairuz-Fozi et al. (2018). Although nesting activity of *C. rotundicauda* was found throughout the year at Balok and Pendas, the number of adults and the number of nests/eggs varied seasonally in the order of south west > north east > inter-monsoon (for details, see Fairuz-Fozi et al., 2018; Zauki et al., 2019a; Zauki et al., 2019b). The search for *C. rotundicauda* nests/eggs at Kuala Sepetang was unsuccessful, and hence only haemolymph

samples from the specimens collected by local fishers as bycatch were analysed. Immediately after collection, haemolymph samples were transferred into 3-ml sterilized blood collection tubes (sprayed inside with 5.4 mg of ethylenediaminetetraacetic acid, EDTA) and eggs were placed into 1.5-ml disposable microcentrifuge tubes. All samples were transferred to the laboratory in an ice box and preserved in a top-loading freezer (at  $-20^{\circ}\text{C}$ ) for further analyses. In total, 152 haemolymph samples (four from Kuala Sepetang; 89 from Balok; 59 from Pendas) and 190 egg samples (76 from Balok; 114 from Pendas) were analysed.

## 2.2 | Genomic DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from both egg (from one egg per nest, with extra eggs collected during fieldwork to support re-analysis if there were noise peaks, short reads, etc.) and haemolymph (70  $\mu\text{l}$  per individual) samples by using QIAamp DNA Investigator kit (#69504; Qiagen, Hilden, Germany). The targeted regions (partial) of COI were amplified using (forward) LCO1490 (5'-GGTCAACAAATCA-TAAAGATATTGG-3') and (reverse) HCO2198 (5'-TAACTTCAGGGT-GACCAAAAAATCA-3') primers. Polymerase chain reaction (PCR) was performed with 25  $\mu\text{l}$  of master mix containing 1  $\times$  PCR green buffer, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTP mix, 0.4  $\mu\text{M}$  forward primer, 0.4  $\mu\text{M}$  reverse primer, and 0.5 U Taq polymerase (GoTaq<sup>®</sup> Flexi DNA Polymerase, 500 U, 5 U/ $\mu\text{l}$ ; M8295, Promega, Madison, WI, USA). The PCR amplification was started with an initial denaturation at  $95^{\circ}\text{C}$  for 3 min, followed by 40 cycles of denaturation at  $95^{\circ}\text{C}$  for 1 min,

annealing at 40°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 7 min. The amplified PCR products were then checked on 1.2% agarose gel in 1 × TAE (Tris-acetate-EDTA) buffer and stained with ethidium bromide (1 µl EtBr/40 ml gel). These amplified PCR gel products were purified using Wizard® SV Gel and PCR clean-up System (Promega) and then sent to First Base Laboratories Sdn. Bhd. (Selangor, Malaysia) for DNA sequencing. The haemolymph samples sent for DNA sequencing were marked with 'b' (for blood) accompanied by the collection number, and the eggs were marked with 'e' and the collection number. Step-wise methodological details for this study are provided in Appendix S1.

## 2.3 | Data analyses

The DNA sequences containing 617 base pairs (bp) in the present study ( $n = 342$ ) were first checked for mismatches, noise peaks, and short reads (using CHROMAS 2.6), and then compared with 12 sequences of *C. rotundicauda* from other Asian regions (Table 1) using the Basic

Local Alignment Search Tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). All 354 samples from 11 regions in Asia were considered for original alignment with CLUSTALW in MEGA 7.026. In brief, the data contained 74 variable sites (38 with parsimony and 36 singletons) from which horseshoe crab identification measures were attempted. A neighbour-joining (NJ) tree of 12 representative samples showing parsimony (Table 1) was constructed to identify the similarity between the original (present study) and the published DNA sequences of *C. rotundicauda*. Several published sequences were found to be shorter in length (545 bp), however, and hence only 28 variable sites (17 with parsimony) were chosen using the Tamura three-parameter method (Tamura et al., 2013). For phylogenetic interpretation, a maximum-likelihood (ML) tree with partial deletion was performed. ML is a widely used method that allows the full capture of the phylogeny under a given model with high efficiency (Paul & Sahoo, 2014; Godini & Fallahi, 2019). The best model (general time reversible) was used with a Bayesian information criterion (BIC) of 1,517.12, a corrected Akaike information criterion (AICc) of 1,324.86, and an ML value of  $-\ln L = -632.23$  in MEGA, with 1,000 bootstrap replicates (Nei

**TABLE 1** Genetic sequence information of *Carcinoscorpius rotundicauda* (with accession numbers) available from the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov>)

Code	Region	Country	Location	Date of publication	GenBank® accession number
1. INDEC1	Bay of Bengal	Odisha, India	N: 21°07'00.00" E: 087°04'00.00"	30 Sep 2015	KM350551
2. ZMUD:C001/1	Dublar Char	Sundarbans, Bangladesh	N: 21°45'28.40" E: 89°33'48.60"	31 Jul 2017	MF362623
3. CR_T5_3F	Andaman Sea	Phuket, Thailand	N: 07°56'53.74" E: 98°29'43.31"	25 Jul 2016	HQ588754
4. Perak	Strait of Malacca	Perak, Malaysia	N: 04°50'38.36" E: 100°38'29.86"	09 Jun 2018	MF469061 <sup>a</sup>
5. Selangor_d1	Pantai Klang	Selangor, Malaysia	N: 02°48'47.7" E: 101°24'51.2"	16 Mar 2019	MH588641
6. Melaka_c18	Merlimau	Malacca, Malaysia	N: 02°07'42.1" E: 102°24'50.8"	16 Mar 2019	MH588640
7. Melaka_c2	Merlimau-	Malacca, Malaysia	N: 02°07'42.1" E: 102°24'50.8"	16 Mar 2019	MH588639
8. Johor	Strait of Johor	Johor, Malaysia	N: 01°23'11.9" E: 103°38'42.2"	09 Jun 2018	MF469062 <sup>a</sup>
9. AKB124	Sungai Pahang Tua	Pahang, Malaysia	N: 03°36'09.40" E: 103°23'57.10"	25 Jul 2016	JF896106
10. Pahang	South China Sea	Pahang, Malaysia	N: 03°56'24.0" E: 103°22'12.0"	09 Jun 2018	MF469060 <sup>a</sup>
11. Terengganu_e26	Setiu	Terengganu, Malaysia	N: 05°40'47.1" E: 102°42'42.6"	16 Mar 2019	MH588643
12. Terengganu_e11	Setiu	Terengganu, Malaysia	N: 05°40'47.1" E: 102°42'42.6"	16 Mar 2019	MH588645
13. Terengganu_e04	Setiu	Terengganu, Malaysia	N: 05°40'47.1" E: 102°42'42.6"	16 Mar 2019	MH588648
14. CR_T3_2F	Gulf of Thailand	Bang Pu, Thailand	N: 06°54'43.28" E: 101°16'32.49"	25 Jul 2016	HQ588750
15. CR_V1_	South China Sea	Bac Lieu, Vietnam	N: 09°22'37.30" E: 106°13'43.70"	25 Jul 2016	HQ588746

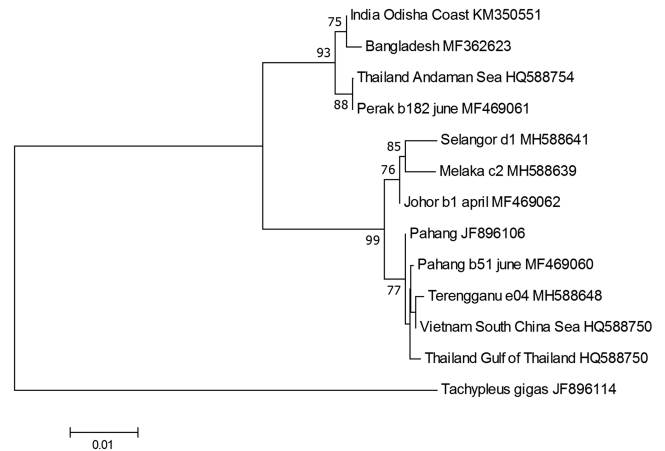
<sup>a</sup>Present investigation.

& Kumar, 2000). The information for *T. gigas* (NCBI GenBank accession number JF896114) was used as an out-group (Liew, Ng & Tan, 2015).

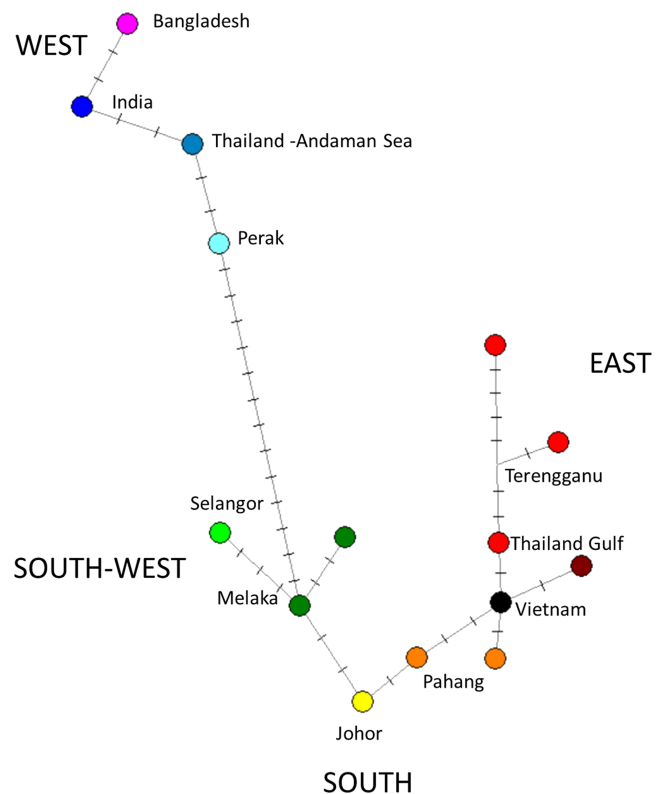
A minimum spanning network conducted in NETWORK 5 (Fluxus Engineering, Colchester, UK) provided haplotype definition for the 11 Asian regions. In this context, 15 gene sequences (12 previously published and three from the present investigation; Table 1) based on 35 variable nucleotide positions, including singletons with comparable (545 bp) alignment, were considered. For detecting mutational steps and shared haplotypes to identify *C. rotundicauda* connectivity, a detailed minimum spanning network of all individual haplotype variants and their frequencies of occurrence was constructed for the south-west, south, and east coastal populations ( $n = 347$  and 44 mutated positions). To determine zones of sharp genetic changes across Peninsular Malaysia, the geographical coordinates of sampling sites and nine nucleotide distance matrices (of all models obtainable from MEGA 7.026) were analysed with Monmonier's algorithm (in BARRIER 2.2) (Manni, Guérard & Heyer, 2004) for a maximum number of two barriers between the neighbouring locations.

### 3 | RESULTS

A positive amplicon of around 710 bp was successfully obtained from both haemolymph and egg samples with high-quality DNA (OD260/280 range 1.7–2.0). The COI sequences of *C. rotundicauda* showed >90% similarity with the GenBank sequences from India, Bangladesh, Thailand, Vietnam, and Malaysia (Table 1). Overall, a mean nucleotide distance (Tamura three-parameter) of  $d = 0.0219$  was observed for all *C. rotundicauda* populations representing the eastern, western, and southern coasts of Peninsular Malaysia; however, the nucleotide distances from east to west ( $d = 0.0382$ – $0.0438$ ) were greater than the within-region distances in the west ( $d = 0$ – $0.0053$ ) and in the east ( $d = 0$ – $0.0133$ ). The NJ tree of 12 representative samples strongly supported two clades (bootstrap value 99) separating the western populations from the south and east populations in Peninsular Malaysia (Figure 2). The southern populations clustered closer to the east than to the west. Furthermore, a minor substructure was found within the western cluster of India and Bangladesh (bootstrap value 75) against the Andaman Sea and the Strait of Malacca (bootstrap value 88). A more pronounced substructure was evident for individuals from the south-west (bootstrap value 85), south (bootstrap value 76), and east coasts (bootstrap value 77). The obtained clusters thus indicate an additional divergence within east and within west populations. The ML phylogenetic tree of the same 12 samples of *C. rotundicauda* also showed two major clades (bootstrap value 100) separating the western populations of India (Odisha, Bay of Bengal), Bangladesh (Bay of Bengal), Thailand (Phuket, Andaman Sea), and Perak (Malaysia, Strait of Malacca) from all other regions (Figure S1). The eastern Malay Peninsula populations of the South China Sea clustered (bootstrap value 87) with Pahang (Malaysia), Terengganu (Malaysia), Bang Pu (Gulf of Thailand), and Bac Lieu (Vietnam).



**FIGURE 2** Neighbour-joining tree of the available GenBank COI sequences of *Carcinoscorpius rotundicauda* from 11 regions in Asia



**FIGURE 3** Haplotype network of 15 GenBank samples from 11 sites in Asia, showing a strong genetic break among *Carcinoscorpius rotundicauda* populations

The haplotype network of 15 GenBank samples from 11 sites (Figure 3; Table 1) showed that the number of mutational steps from the south-western (Selangor and Melaka) and the southern coast (Strait of Johor) populations was much smaller towards the eastern populations (Vietnam, eastern Thailand, and eastern Peninsular Malaysia) than towards the western populations (India, Bangladesh, western Thailand, and western Peninsular Malaysia). A genetic break

of 14 mutational steps in *COI* was found between Perak and Melaka (over a 400-km distance), compared with only one or two steps between most other populations at similar or much larger geographical distances, e.g. between Pahang (Malaysia) and Vietnam (Figure 3).

The detailed haplotype network revealed 15 haplotypes in Johor and nine haplotypes in Pahang (Figure 4). A star-like network in both places indicates radiation from a common haplotype for just one or two mutational steps (seldom more), and confirms the accumulation and maintenance of diversity in *C. rotundicauda* maternal lines locally. The most important observation was that a few haplotypes are shared between distant populations. One individual (an adult male) from Johor contained the main haplotype of Pahang and one individual from Melaka contained the main haplotype of Johor. This shows an occasional stepping-stone dispersal of *C. rotundicauda* from Pahang towards Johor and Melaka (i.e. from east to south and south west). Even more interesting is that several individuals of distant locations, namely Johor (an adult female), Pahang, Terengganu (eastern Peninsular Malaysia), and Bang Pu (Thailand), have a shared haplotype, indicating historical connectivity of a maternal lineage over distances greater than 800 km.

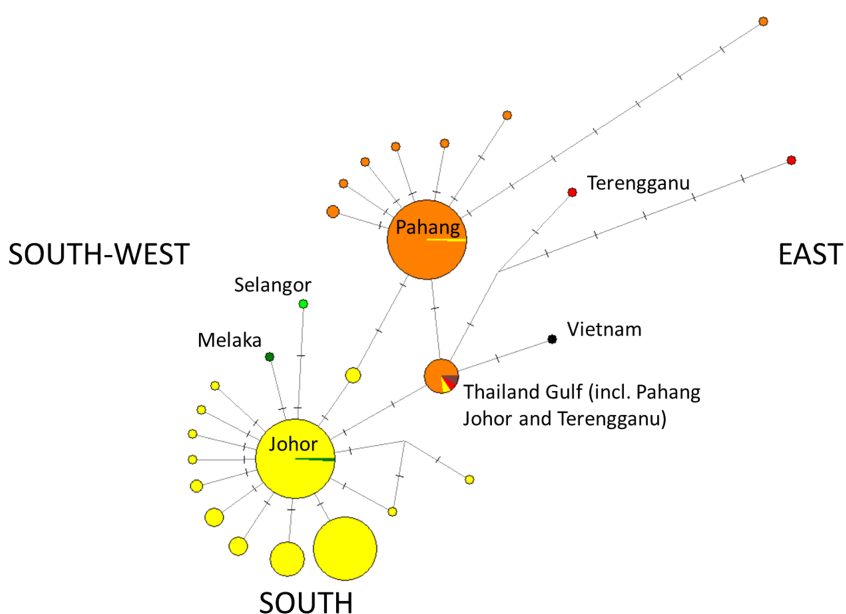
The most common haplotypes in Johor (represented by haemolymph samples b1, female, caught on 21 April 2016, and b12, male, caught on 5 June 2016) were observed year-round, whereas the unique haplotypes were scattered across sites, nests, and various months. No temporal trend was observed, except that the shared haplotypes (two putative migrants in Johor with a haplotype identical to Pahang) were both sampled in August 2016 during the south-west monsoon. First-order barrier analysis revealed the strongest genetic boundary between the east and west coasts: more precisely, between Perak and Selangor on the west coast for neighbouring *C. rotundicauda* populations (Figure 5). Individuals from Selangor also showed a second-order barrier with Melaka. However, when south and south-west populations of *C. rotundicauda* in Peninsular Malaysia

are included, then an oceanic barrier in the Strait of Malacca becomes apparent (Figure 5).

## 4 | DISCUSSION

### 4.1 | Genetic break in Strait of Malacca

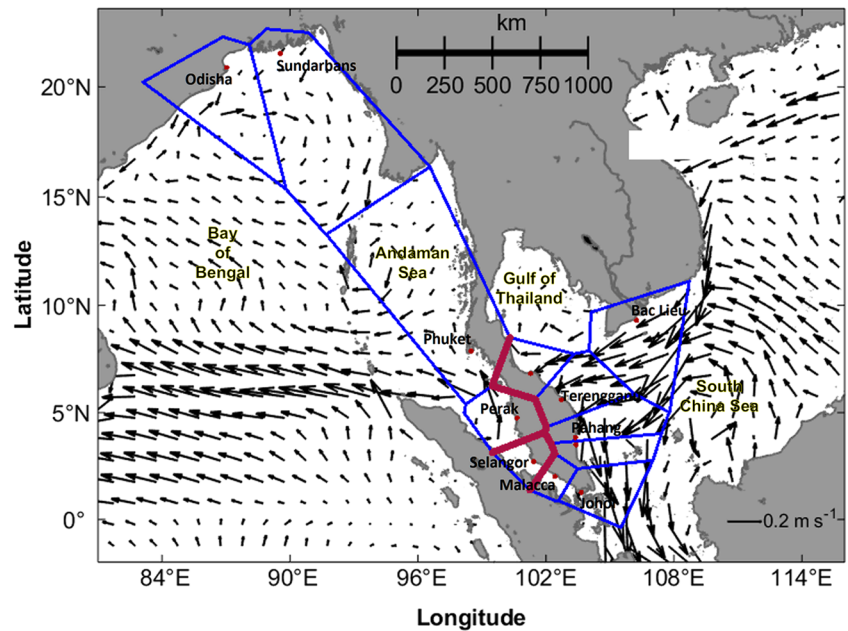
The mitochondrial marker sequences of *COI* from egg and haemolymph samples in the present study confirms a clear separation of *C. rotundicauda* populations on the east and south coasts from those on the west coast in Peninsular Malaysia. This also shows two genetically different *C. rotundicauda* maternal gene pools from seas on the east (South China Sea and Gulf of Thailand) and the west (Andaman Sea and Bay of Bengal) for which Peninsular Malaysia is acting as a land barrier, as reported previously by Adibah, Ng & Tan (2015) for this species, by Ismail et al. (2011) and Liew, Ng & Tan (2015) for *T. gigas*, and by Yang et al. (2009) for *T. tridentatus*. This major land-barrier effect is thought to have existed since the late Pleistocene, when a massive lowland formed and connected the continents, such as the Sunda Shelf (i.e. Sumatra, Java, and Borneo), with multiple glaciation episodes (Wurster et al., 2010; Obst et al., 2012; Pongparadon et al., 2015; Karin et al., 2017; Suppapan et al., 2017; Domingues et al., 2018). Peninsular Malaysia has also been reported as a geographic blockade for other faunal species, such as *Barbonymus schwanenfeldii* and *Varuna litterata*, etc. (Kamarudin & Esa, 2009; Suppapan et al., 2017). In addition, the short-range dispersal ability of *C. rotundicauda* (Cartwright-Taylor & Ng, 2012; Adibah, Ng & Tan, 2015) must be facilitating the sustained ancient separation of populations between east and west coasts. The contemporary enclosed topography of the Strait of Johor is hypothesized to act further as a land barrier and be responsible for the persistence of two distinct major groups of populations (Lessios et al., 1999).



**FIGURE 4** Haplotype network with frequencies showing the connectivity between southern and eastern populations of *Carcinoscorpius rotundicauda*



**FIGURE 5** Barrier analysis showing the main land barriers in the Strait of Malacca on top of the east–west division of Peninsular Malaysia for *Carcinoscorpius rotundicauda*. The background image represents general seawater circulation during the north-east monsoon



The present study also sheds light on the possible existence of an important oceanic barrier within the Strait of Malacca, as shown by both haplotype network and barrier analysis. This suggests that the genetic barrier to connectivity is not situated exactly at the southern tip of Peninsular Malaysia but is instead located further northwards along the west coast. This is a novel observation and also represents a historical or recent movement of *C. rotundicauda* towards the Strait of Malacca, where they encounter a physical barrier most likely resulting from opposing water currents. The population divergence of marine taxa is indeed influenced by seawater current and circulation patterns (Saunders, Kessler & Avise, 1986; Pongparadon et al., 2015), but further evidence is needed in support of this being the case for *C. rotundicauda*. For instance, converging ocean currents (Ngeve et al., 2016) did not mix mangrove propagules but instead kept the gene pools separate. Such a phenomenon of converging ocean currents is a very plausible explanation for the genetic break observed for *C. rotundicauda* populations in the Strait of Malacca. The small sample size ( $n = 4$  for Perak) and limited GenBank data ( $n = 1$  for Selangor and Melaka) did not allow detailed frequency-based haplotype networks for this region, however, and hence it is not possible to confirm this abrupt break within the Strait of Malacca. An important intrinsic trait that may contribute to a persistent barrier is the short dispersal distance of *C. rotundicauda* and the assumption that it follows a stepping-stone migration model. A further very likely extrinsic factor for the establishment of *C. rotundicauda* populations is the availability of their preferred estuarine mangrove habitats.

On the other hand, there was no evidence of historical or recent migration of *C. rotundicauda* from the west coast towards the south west (Selangor, Melaka) or south (Johor) of Peninsular Malaysia, with extreme differences found in their haplotypes (at least 17 mutational steps between Perak and Johor, and 14 mutation steps between Perak and Melaka). Nevertheless, the lack of any haplotype of the west coast population in the extensive sampling at Johor suggests

that the migration of *C. rotundicauda* from the west to the south is most unlikely. Although the observed 14 mutational steps between Perak and Melaka indicates an ancient divergence between these evolutionary units, it still represents a likely underestimation because of data deficiency (e.g. six additional potential mutations from India and Bangladesh were excluded for shorter GenBank sequences, and therefore there was no chance of a detailed comparison). Therefore, the possibility of a more recent mixing and occasional migration of *C. rotundicauda* from the south towards the west coast still needs to be investigated using a larger sample size of the west-coast populations, especially including the potential stepping-stone populations between Johor and Perak.

#### 4.2 | Connectivity of the southern population with eastern Peninsular Malaysia

The southernmost *C. rotundicauda* population at Johor formed a sub-clade or separate haplotype network next to closely related mangrove horseshoe crabs from the eastern Malay Peninsula. They seemed to have a historical connectivity with the South China Sea, but subsequently were separated, however, with only very few mutational steps. This is also a new insight, and future studies focusing on these southern populations (Pendas in Johor) and adjacent *C. rotundicauda* locations along the east coast would be able to provide more clues to the migratory limits as well as the potential for mixing of populations between the Strait of Johor and the South China Sea. In contrast to the NJ tree, the ML tree was not well resolved for samples from the south west (Selangor, Melaka) and south (Johor) because of the lack of nucleotide substitutions with parsimony.

Two (out of 15) of the most commonly found *C. rotundicauda* haplotypes (99/173 and 39/173 individuals) are found at Johor, whereas only one (out of nine) is found at Pahang. This could be

explained in two ways: either the Johor population underwent a mutation, which accumulated, or these populations have different origins, namely one common haplotype from the Gulf of Thailand and another from the Java Sea. Both these options are not supported by the present findings, however. Large sample sizes from both Johor ( $n = 173$ ) and Pahang ( $n = 165$ ) improves the accuracy of identifying genetic relationships. The population of *C. rotundicauda* at Johor showed more diversity and higher frequencies of several haplotypes that occurred more than once ( $n = 15$ ; 4; 4; 3; 2), whereas in Pahang there are mostly singleton mutations. The observation is that in Johor there are particular haplotypes shared by *C. rotundicauda* adults and eggs, meaning that they are less frequent haplotypes established themselves from previous generations. We currently hypothesize that the overall higher diversity and higher frequencies at Johor could be the result of locally accumulated microevolutionary events (the star-like network in Figure 4 supports the idea that most mutations originated from the same common haplotype).

In the case of Pahang, there is one common group ( $n = 147$  out of 165) that includes a male adult from Johor and a group of mixed origin. This Pahang group of four adults and six nests (eggs) is shared with a female adult from Johor and another female from the Gulf of Thailand. Although shared haplotypes between Pahang and Thailand are reflecting historical or recent connectivity, a fraction of the *C. rotundicauda* population at Pahang allowed further dispersal to Johor. Out of the two adults that reached Johor from Pahang (or at least from the same gene pool), the male *C. rotundicauda* indicates a very recent migration, during the north-east monsoon, with the sea-water current moving in a south-west direction (Figure 5). Although the human-assisted introduction of horseshoe crabs from one location to another location may lead to shared haplotypes, this is not the case for the present study where shared haplotypes were found in various regions across the countries (Gulf of Thailand, Terengganu, Pahang, and Johor).

### 4.3 | Implications for horseshoe crab conservation and future perspectives

With the unsuccessful efforts in finding nests/eggs of *C. rotundicauda* at Kuala Sepetang, even the collection of horseshoe crabs was difficult. Gill net hauls at different locations suggested by local fishers (who used to find them as bycatch) did not trap *C. rotundicauda* specimens and therefore the number of haemolymph samples secured for this study remained low. However, the distinct population of *C. rotundicauda* on the west coast of Peninsular Malaysia, India (Odisha, Bay of Bengal), Bangladesh (Sundarbans, Bay of Bengal), and Thailand (Andaman Sea) clearly supported the phylogenetic clustering and relevant haplotype and barrier analyses. Additional samples from Perak and other states on the west coast would be able to reveal the precise location of the genetic barrier within the Strait of Malacca.

Meanwhile, the remains of *C. rotundicauda* carapaces on the mangrove sediment (as moults and leftovers after being eaten by

monkeys; pers. observ., NF-F, NAMZ, BRN, BS) suggests that Kuala Sepetang is perhaps acting as a feeding ground rather than a breeding ground. This could be explained by the fact that *C. rotundicauda* lays eggs in the sediment with a high clay-sand composition (Fairuz-Fozi et al., 2018; Zauki et al., 2019a; Zauki et al., 2019b). The abundance of sand could help the substratum not only to retain moisture and supply oxygen for the development of the eggs (Avisar, 2006), but also make it easier for *C. rotundicauda* to dig holes and place their eggs at suitable depths. As Kuala Sepetang falls under the riverine mangrove category (Goessens et al., 2014), more than 80% of the sediment is dominated by silt and clay (Ariffin & Mustafa, 2013) that is unfavourable for the nesting activity of *C. rotundicauda*. Moreover, the intertidal zones close to mangrove and seagrass beds were found to be important for foraging and the development of juvenile horseshoe crabs (Kwan, Chan & Cheung, 2017). Further scientific evidence, also through considering water current and circulation patterns in the Strait of Malacca, is therefore necessary to identify the local or regional movement of horseshoe crabs from their breeding ground(s) adjacent to Kuala Sepetang, and to confirm its status as a feeding ground.

Considering that the southernmost *C. rotundicauda* population in Peninsular Malaysia has historical connectivity with the South China Sea, future investigations adjacent to Pendas on the east coast would provide more scientific insights into its migration limits and potential mixing of populations between the Strait of Johor and the South China Sea. Also, the studies focusing on nuclear microsatellite DNA of all *C. rotundicauda* individuals (without limitation to females and amplexus) can be recommended. In this context, a priori knowledge on the introduction of horseshoe crabs from one location to another location, if at all, is compulsory to avoid uncertainties in genetic data analysis and interpretation.

Policies for horseshoe crab conservation in Malaysia are not yet in place (Nelson et al., 2016; Akbar John et al., 2018; Fairuz-Fozi et al., 2018). In the light of their selective nesting behaviour, both sandy beaches and mangrove habitats should be protected. All physical infrastructure developments adjacent to the coastline must go through proper environmental impact assessments. On the other hand, the overexploitation of horseshoe crabs for consumption has proven detrimental to the populations (Faridah et al., 2015; Nelson et al., 2019). Although *C. rotundicauda* has no edible value to local communities because of its toxicity (Ngy et al., 2007; Fu et al., 2019; Zheng et al., 2019), they are used for fishmeal and biomedical research purposes (Smith & Brockmann, 2014; Yennawar, 2015; Zauki et al., 2019a). Overall, there is a strong need to raise public awareness and community-based management for the protection as well as genetic conservation of horseshoe crabs in Malaysia.

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## AUTHOR CONTRIBUTIONS

NF-F: methodology, investigation, validation, formal analyses, data curation, visualization, writing of original draft, reviewing, and editing. LT: methodology, software, validation, formal analyses, data curation, visualization, supervision, writing of original draft, reviewing, and editing. NAMZ: methodology, investigation, validation, formal analyses, and data curation. AMK: methodology, investigation, validation, formal analyses, and data curation. BRN: conceptualization, methodology, investigation, resources, visualization, supervision, project administration, fund acquisition, writing, reviewing, and editing. AC: methodology, visualization, writing, reviewing, and editing. MFA: resources, visualization, writing, reviewing, and editing. BS: conceptualization, methodology, investigation, resources, visualization, supervision, project administration, fund acquisition, writing, reviewing, and editing. FD-G: validation, visualization, supervision, writing, reviewing, and editing.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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