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The Malay Peninsula as a barrier to gene flow in an Asian horseshoe crab species, *Carcinoscorpius rotundicauda* Latreille

A.B. Adibah, W.L. Ng, S.G. Tan*

Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Selangor, Malaysia

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ABSTRACT

Horseshoe crabs are marine arthropods that are amongst the oldest living creatures that still exist today. Among the four extant species of horseshoe crabs, *Carcinoscorpius rotundicauda* differs from the other species by having poisonous eggs and lays its eggs in sandy-mud areas near river mouths. With the rapid development of coastal areas worldwide, *C. rotundicauda* habitats are decreasing. Until now, however, there has not been any study on the species' genetic variation. Simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) markers were employed to study the genetic variation in five *C. rotundicauda* populations from the east and west coasts of the Malay Peninsula. Both markers showed differing levels of genetic variation, but concurred on the pattern of genetic structuring among populations of the species. This includes showing that little, although significant, genetic differentiation is present among populations, suggesting a low rate of gene flow among populations. The results also suggested that *C. rotundicauda* may be subjected to the land barrier effect of the Malay Peninsula, whereby gene flow is limited between populations occurring on both sides of the peninsula, increasing their genetic differentiation through time.

1. Introduction

Horseshoe crabs are marine arthropods that are amongst the oldest living creatures that still exist today. They consist of four extant species – the American (*Limulus polyphemus*) and the Asian (*Tachypleus tridentatus*, *Tachypleus gigas*, and *Carcinoscorpius rotundicauda*) horseshoe crabs. Populations of the Asian horseshoe crabs are not well known and have never been extensively studied due to their low commercial values. *L. polyphemus* is currently considered as a 'lower risk/near threatened' species according to the IUCN Red List, while all three species of the Asian horseshoe crabs are only listed under the 'data deficiency' category and knowledge of their diversity and distribution patterns are still fragmentary (http://www.iucnredlist.org, accessed November 2014). Nonetheless, populations of the American horseshoe crab and the Asian horseshoe crabs are thought to be decreasing worldwide (Morton, 1999; Botton, 2001; Chen et al., 2004). This decline may have resulted from the combined effects of pollution, degradation of the estuarine spawning habitats, and commercial fishing activities (Berkson and Shuster, 1999; Botton, 2001; Walls et al., 2002; Chen et al., 2004).

* Corresponding author. Tel.: +603 89468098.
E-mail address: wsgtan@gmail.com (S.G. Tan).

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Among the three species of Asian horseshoe crabs, only *T. gigas* and *C. rotundicauda* can be found to inhabit sandy beaches and mangrove areas on the Malay Peninsula. However, unlike the other species of horseshoe crabs, *C. rotundicauda* and its eggs are not eaten as food as they are known to be poisonous. In terms of breeding habitat, it also differs from the other species by laying its eggs in the sandy-mud areas near river mouths (Sekiguchi et al., 1977). Given the rapid development of coastal areas in Asia, it is thus not surprising that suitable breeding habitats are decreasing, affecting the health of *C. rotundicauda* populations. Also, horseshoe crabs are known to exhibit life history and habitat preferences that may indicate a restricted dispersal capability (Sekiguchi, 1988). In this case, a species' dispersal over long distances, or over a physical barrier, may be hindered. The Malay Peninsula is a popular modern-day land barrier that limits the dispersal of marine species across the peninsula, significantly reducing gene flow between populations of the South China Sea and the Indian Ocean (Parnell, 2013). With this in mind, it is important to understand the levels and patterns of genetic variation in the horseshoe crab populations in order to identify areas of conservation priority, and to devise more soundly methods for species management and conservation.

In this study, we analysed genetic variation in *C. rotundicauda* populations collected from around the Malay Peninsula using simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) markers. Comparisons of SSR marker variation levels between species and populations have also proven useful in the assessment of overall genetic variation (Gottelli et al., 1994; Taylor et al., 1994). They can be used to gain insights into the degree of population substructuring and the genetic relationships among the various subpopulations (Bowcock et al., 1994; Forbes et al., 1995; Estoup et al., 1996). ISSR markers are also useful with its ability to generate a high number of loci across the genome in species that lack genomic information, like in *C. rotundicauda*. As far as we are aware, this study represents the first ever study on the population genetics of *C. rotundicauda*. In particular, we show that like in many other marine species, the Malay Peninsula may have played an important role in shaping the population structure of *C. rotundicauda* by limiting its dispersal across the peninsula. This is especially so since the causeway between the Malay Peninsula and Singapore island also blocks the water flow across the Straits of Johore.

2. Material and methods

2.1. Sample collection

A total of 127 *C. rotundicauda* samples were collected from five locations on the Malay Peninsula, namely Kuala Juru (A), Kg. Permatang (B), Kg. Sg. Pulai (C), Kg. Chuah (D), and Kg. Sekokoh (E). Table 1 and Fig. 1 show the details of the sampling sites.

Total genomic DNA was extracted from 100 mg of *C. rotundicauda* muscle tissue using the CTAB method described by Winnepennincx et al. (1993) with minor modifications to the CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl pH8.0), and was subsequently used for SSR and ISSR genotyping.

2.2. PCR amplification and genotyping

Seven SSR primer pairs previously developed by Adibah et al. (2012) were used in this study. Markers that generated null alleles were excluded from this study. PCR reactions were carried out in a total volume of 10 μl consisting of 100 ng of template DNA, 1× PCR buffer, 1.0–1.5 mM of $MgCl_2$, 0.25 mM of dNTP mix, 0.5 μM of each primer, and 0.2 U of *Taq* DNA polymerase (Promega, USA). A touchdown PCR procedure was used. The conditions set for the thermocycler included an initial denaturation step of 95 °C for 3 min, followed by 10 cycles of 30 s at 94 °C, 30 s at [10 °C + annealing temperature (T_a)] with a decrement of 1 °C per cycle, and 45 s at 72 °C, continued with 30 cycles of 30 s at 94 °C, 30 s at T_a, and a final extension step at 72 °C for 20 min. The primer sequences and the corresponding T_a and $MgCl_2$ concentrations are listed in Supplementary Table S1. The amplification products were separated by electrophoresis on 4% MetaPhor™ agarose gel. Gels were subsequently stained with EtBr and viewed under UV illumination.

Twelve ISSR primers from Usmani (2002), Kumar (2003), and Hoh (2005) were used in this study. PCR reactions were carried out in a total volume of 10 μl consisting of 100 ng of template DNA, 1× PCR buffer, 3.75 mM of $MgCl_2$, 0.25 mM of dNTP mix, 0.5 μM of primer, and 0.15 U of *Taq* DNA polymerase (Promega, USA). The conditions set for the thermocycler included an initial denaturation of 3 min at 95 °C, followed by 40 cycles of 30 s denaturation at 95 °C, 30 s at T_a, and 30 s extension at 72 °C.

Table 1
Details on the sampling sites of *C. rotundicauda*.

No.	Site	Location	Coordinates	Relative location to the Malay Peninsula	Sample size
1	A	Kuala Juru, Penang, Malaysia	5°21′ N 100°25′ E	West	25
2	B	Kg. Permatang, Perak, Malaysia	4°11′ N 100°39′ E	West	25
3	C	Kg. Sg. Pulai, Selangor, Malaysia	3°43′ N 100°55′ E	West	27
4	D	Kg. Chuah, Negeri Sembilan, Malaysia	2°36′ N 101°46′ E	West	25
5	E	Kg. Sekokoh, Pahang, Malaysia	3°30′ N 103°28′ E	East	25
	Total				127