The different fates of two Asian horseshoe crab species with different dispersal abilities

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Abstract
Impending anthropogenic climate change will severely impact coastal organisms at unprecedented speed. Knowledge on organisms’ evolutionary responses to past sea-level fluctuations and estimation of their evolutionary potential is therefore indispensable in efforts to mitigate the effects of future climate change. We sampled tens of thousands of genomic markers from ~300 individuals in two of the four extant horseshoe crab species across the complex archipelagic Singapore Straits. Carcinocorpus rotundicauda Latreille, a less mobile mangrove species, has finer population structure and lower genetic diversity compared with the dispersive deep-sea Tachypleus gigas Müller. Even though the source populations of both species during the last glacial maximum exhibited comparable effective population sizes, the less dispersive C. rotundicauda seems to lose genetic diversity much more quickly because of population fragmentation. Contra previous studies’ results, we predict that the more commonly sighted C. rotundicauda faces a more uncertain conservation plight, with a continuing loss in evolutionary potential and higher vulnerability to future climate change. Our study provides important genomic baseline data for the redirection of conservation measures in the face of climate change and can be used as a blueprint for assessment and mitigation of the adverse effects of impending sea-level rise in other systems.

Keywords
benthic dispersal, climate change, conservation genomics, demographic reconstruction, seascape genomics, Sunda shelf
1 | INTRODUCTION

Global sea-level change is one of the most prominent factors shaping coastal and marine natural communities through constant range shifts and changes in connectivity (Bird et al., 2007; Sheaves, 2009). Depending on life-history parameters such as habitat preference and dispersal ability, coastal marine species differ in their evolutionary responses to environmental fluctuations in the wake of sea-level change (Leprieur et al., 2016; Ludt & Rocha, 2015; Ni et al., 2014). An expanding genomic infrastructure and the recent development of powerful analytical approaches provide an opportunity to investigate the interplay between life-history parameters and evolutionary responses (Gagnaire, 2020; Gagnaire et al., 2015). Understanding an organism’s evolutionary responses to such range shifts is a critical prerequisite for the conservation of coastal marine biodiversity in this era of anthropogenic climate change (Chuang & Peterson, 2016; Miller et al., 2020; Nadeau & Urban, 2019). To evaluate species viability during environmental changes, most previous studies have estimated the evolutionary potential (e.g., genetic variation) of expanding populations by comparing them with populations in the core range (Berthouly-Salazar et al., 2013; Braasch et al., 2019; Chen et al., 2018; Pierce et al., 2000; Robalo et al., 2020; Yang et al., 2016).

However, many environmental fluctuations result in original habitats becoming uninhabitable, rendering core habitats difficult to identify, or individuals in core habitats undergo major demographic events (e.g., bottlenecks) which hamper a meaningful estimation of the evolutionary potential of expanding and receding populations. Two horseshoe crab species across the Singapore Straits in South-East Asia, one of the world’s most dynamic coastal landscapes, provide us with a unique opportunity to estimate the evolutionary potential of expanding coastal marine species whose source habitats during the last glacial maximum (LGM) remain unknown.

Horseshoe crabs are long-lived marine arthropods, often referred to as living fossils because of their nearly unchanged physical appearance over 455 million years (Rudkin et al., 2008). They occur across some of world’s seas in four extant species. Two of them, Carcinus maenas and Tachypleus gigas Müller, are widespread across coastal South-East Asia, with highly overlapping ranges of distribution (John et al., 2018, 2021). Even though the two horseshoe crab species under various threat categories, for example ‘vulnerable’ for C. rotundicauda and ‘endangered’ for T. gigas in Singapore (Davison et al., 2018). In this study, we screened hundreds of thousands of genome-wide single nucleotide polymorphisms (SNPs) over 300 individuals of the two horseshoe crab species with a comprehensive geographic coverage across the Singapore Straits, a complex archipelagic nexus on the Sunda Shelf that has undergone dramatic habitat transformation during the Holocene (i.e., roughly the past ~10,000 years).

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2 | MATERIAL AND METHODS

Our sampling covered the Singapore Straits at a radius of 100 km around Singapore island (1°22′N, 103°48′E). A total of 188 C. rotundicauda and 116 T. gigas were collected across 46 locations (Figure 1; Table S1). All samples were captured alive and released after muscle tissue had been collected from one of their ambulatory legs, or
blood had been drawn from the hinge, to ensure minimal intrusive- ness. Tissue and blood samples were preserved in absolute etha- nol and stored at −20°C for subsequent processing. We extracted genomic DNA using the DNeasy Blood & Tissue Kit (Qiagen). We followed a double- digest RAD (ddRAD) library preparation protocol (Peterson et al., 2012) with few modifications. In brief, we used the restriction enzymes EcoRI and MSeI to digest the extracted genomic DNA and ligated the digested fragments with adapters. We pooled and selected ligated fragments of ~350 bp size using Pippin Prep (Sage Science) and performed eight PCR cycles on the size- selected fragments for final library preparation before submission for next- generation sequencing (Illumina HiSeq 4000, paired-end 150 bp read length).

We checked sequence quality using FastQC (Babraham Bioinformatics) and demultiplexed all sequences using process_rad- tag as implemented in Stacks v2.4 (Rochette & Catchen, 2017). We aligned raw reads to whole-genome sequences of C. rotundicauda (Shingate, Ravi, Prasad, Tay, Garg et al., 2020) (GenBank accession number VWRL00000000) and T. gigas (Shingate, Ravi, Prasad, Tay, & Venkatesh, 2020) (GenBank accession number JAALXS0000000000), respectively, using BWA-MEM as implemented in BWA v0.7.15 (Li, 2013). Reads with MAPQ scores lower than 20 were discarded. Using ref_map.pl, as implemented in Stacks v2.4, we called 2,720,972 SNPs for C. rotundicauda (average stack depth 22.8x) and 2,779,046 SNPs for T. gigas (average stack depth 21.7x) without prior popu- lation assignment (per sample coverage weighted by sample size is 8.98– 54.89 for C. rotundicauda and 7.85– 31.33 for T. gigas). We did not assign individuals to populations based on their sampling locality because our sampling focused on maximizing spatial cov- erage to guarantee as much differentiation as possible in pairwise spatial distances for increased robustness of the IBD-related analy- ses (see below), leaving uneven numbers of individuals at each site. Therefore, we excluded some subsequent analyses that require popu- lation assignment, for example, the calculation of private alleles per population. Moreover, as the area of study is relatively small compared with the mobility of horseshoe crabs, analyses based on sampling locality determined population assignment may not be in- formative. We used PLINK v1.9 (Purcell et al., 2007) to filter missing
data. PLINK was also run to remove physically linked loci for some analyses but not for others (see below) (indep-pairwise algorithm with a 25-SNP window sliding 10 SNPs during each step; unphased-hardcall $r^2$ threshold = 0.95).

To accommodate different requirements of downstream analytical programs, we generated six SNP datasets, three for each species: (C0) C. rotundicauda with 0% missing data and no linked loci (104,252 SNPs); (COL) C. rotundicauda with 0% missing data while retaining linked loci (116,670 SNPs); (C1) C. rotundicauda with ~10% missing data and no linked loci (785,339 SNPs); (TO) T. gigas with 0% missing data and no linked loci (94,013 SNPs); (TOL) T. gigas with 0% missing data while retaining linked loci (108,728 SNPs); and (T1) T. gigas with ~10% missing data and no linked loci (432,544 SNPs). Detailed scripts outlining data quality control are appended in the supplement.

To examine genetic variation within individuals, within sampling localities, and among sampling localities for C. rotundicauda and T. gigas in our study area, we performed AMOVA, as implemented in the R package poppr (Kamvar et al., 2014), on datasets C0, C1, T0, and T1. We calculated observed and expected homozygosity as well as method-of-moments $F$ coefficients for datasets C0, C1, T0, and T1 using the $-het$ function available in PLINK v1.9. We averaged $F$ for individuals collected at identical sampling localities to check spatial patterns in the distribution of genetic diversity. To obtain an alternative proxy of genetic diversity, we calculated effective population size based on genome-wide linkage disequilibrium (LDNe), which is considered one of the superior ways of computing resistance to dispersal using genome-wide differentiation or the ‘outlier’ loci under selection, we carried out two analyses based on AMOVA results for PCA and ADMIXTURE respectively. For the results of PCA, we calculated the correlations between the dominant principal component, PC1, and the LDNe genotypes using function snpgdsPCACorr, as implemented in SNPRele. For the results of ADMIXTURE, we ran a 20,000 bp sliding window moving in steps 5000 bp, performing $F_{st}$ calculation for the nonadmixed individuals (with over 80% ancestry fraction) between probable ancestral populations using VCFtools v0.1.16 (Danecek et al., 2011).

To examine spatial-genetic patterns, we calculated genetic distance matrices for datasets C0 and T0 using a relative dissimilarity approach (diss.dist) as implemented in the R package poppr (Kamvar et al., 2014) and geographic distance matrices using the least-cost approach implemented in the R package gdistance (Etten, 2017) to account for landmasses between paired individuals. Subsequently, using GenAlEx v6.51 (Peakall & Smouse, 2006), we modeled linear isolation by distance (IBD) and performed Mantel tests with 999 permutations to check for a spatial-genetic correlation and correspondence. We also ran spatial autocorrelation analysis (Smouse & Peakall, 1999), as implemented in GenAlEx v6.51, for 999 permutations and 999 bootstraps, to examine dispersal patterns at different geographic distance classes. We mapped the spatial distribution of resistance to dispersal using DReSd (Keis et al., 2013). DReSd intakes the distance matrices of geographic and genetic distances to model nonlinear IBD, calculates IBD residuals of individual pairs, and maps the weighted means of IBD residuals to visualize the distribution of resistance to dispersal. We overlaid bathymetric data (SRTM15+) (Tozer et al., 2019) to verify whether sea depth is a barrier to the dispersal of the two horseshoe crab species.

### RESULTS

Overall, genetic variation is mostly contributed by within-sample variation (~90%) for both horseshoe crab species across the Singapore Straits, while variation in individuals between sampling localities contributes ~2% for C. rotundicauda and 0% for T. gigas (Table S2). C. rotundicauda and T. gigas across the Singapore Straits are characterized by slight inbreeding, indicated by positive $F$ coefficients values calculated for all four SNP datasets: (C0) 0.0529, (C1) 0.1003, (T0) 0.0324, and (T1) 0.0988. When applying equal regimes of missing data filtering (datasets C0 vs. T0, C1 vs. T1), C. rotundicauda has a lower genetic diversity, as indicated by higher $F$. 

To visualize genomic diversification among individuals, we performed principal component analysis (PCA) based on the SNP genotype data, as implemented in SNPRele, for datasets C0, C1, T0, and T1. In addition, we ran ADMIXTURE (Alexander et al., 2009), which uses maximum-likelihood ancestry estimation, across datasets C0, C1, T0, and T1 to detect whether there are any population subdivisions within our study area. We performed discriminant analysis of principal components (DAPC, Jombart et al., 2010) on datasets C0 and T0 to provide a model-free alternative for potential population subgrouping using the R package poppr (Kamvar et al., 2014). To verify whether the population genetic structure is contributed by genome-wide differentiation or the ‘outlier’ loci under selection, we carried out two analyses based on the results of PCA and ADMIXTURE respectively. For the results of PCA, we calculated the correlations between the dominant principal component, PC1, and the SNP genotypes using function snpgdsPCACorr, as implemented in SNPRele. For the results of ADMIXTURE, we ran a 20,000 bp sliding window moving in steps 5000 bp, performing $F_{st}$ calculation for the nonadmixed individuals (with over 80% ancestry fraction) between probable ancestral populations using VCFtools v0.1.16 (Danecek et al., 2011).
coefficients (Figure 1), than T. gigas. In both species, populations at
the southwest end of the Singapore Straits exhibit the highest levels
of genetic diversity (Figure 1), as shown by relatively low F coef-
ficients when considering loci shared among all individuals (datasets
C0 and T0). A relatively low genetic diversity in C. rotundicauda as
compared to T. gigas is also indicated by pronounced differences in
estimates of contemporary LDNe (age = 0), 395.8 in C. rotundicauda
and 4237.6 in T. gigas (Figure 2), even though both species are esti-
mated to have had a comparable LDNe at the end of the last glacial
maximum (LGM) around 14,000 years ago (Figure 2). The LDNe of
C. rotundicauda has continuously dropped throughout the Holocene
whereas the effective population size of T. gigas has undergone a
delayed but steeper drop over the last 5000 years, which roughly
coincided with the recent rapid mangrove establishment across the
Singapore Straits, following a period of more general stability in the
early Holocene (Figure 2). We detected 45 pairs of related individu-
als (r < 0.0325, sharing an ancestor ≤5 generations ago) in C. rotundi-
cauda but none in T. gigas. A total of 39 out of these 45 pairs were
sampled at the southeast end of the Singapore Straits; 24 of them
were collected at overlapping sites; and 19 of them at one particular
pair of sites ~10 km from Bintan Island (Table S3).

Principal component analysis (PCA) failed to reveal major
subdivisions within the two horseshoe crab species (Figure 1). Populations of C. rotundicauda are geographically arranged along a
northwest–southeast genomic cline (Figure 1). In contrast, T. gigas
emerges as virtually panmictic across the study area. Based on the
cross-validation error calculated for all tested K values (1-15) in
the ADMIXTURE analyses (Table S4), the most likely number of
ancestral populations is one for both species (i.e., K = 1). The second
likely number of ancestral populations is two, with the second low-
est cross-validation error across all four datasets, for both species.

When enforcing K = 2 (Figure S1), individuals of T. gigas display no
geographic structure while individuals of C. rotundicauda are divided
into two subgroups by the Singapore Straits, with intermediate indi-
cividuals on Pulau Semakau (Figure 3), a small island in the middle
of the Singapore Straits ~7 km south of Singapore. Based on the
Bayesian information criterion, DAPC detected a single population
for C. rotundicauda as well as for T. gigas (Figure S2). Even though
C. rotundicauda displayed a spatially structured regional population
across the Singapore Straits, the genetic divergence is shallow and
does not associate with specific genotypes or regions across the
genome (Figures S3 and S4). The PCA and ADMIXTURE results also
suggest the population genetic pattern of spatial divergence in C. ro-
tundicauda is most likely the result of isolation by distance rather
than admixture of two distinct populations or natural selection.
Otherwise, we would have observed genomic regions or genotypes
that are highly associated with the spatial divergence.

Both horseshoe crab species display positive linear isolation
by distance (IBD) in the study area, C. rotundicauda significantly
so (p = 0.001, R² = 0.217) but T. gigas not (p = 0.32, R² = 0.0006).
Results from spatial autocorrelation analyses (Figure 4) indicate that
C. rotundicauda has a genetic patch size of ~35 km. In T. gigas, auto-
correlation coefficients at initial distance classes do not significantly
deviate from the null model, indicating that this species routinely
disperses beyond the range of our study area (~200 km). In C. ro-
tundicauda, DReS-D analysis detects significantly high resistance to
dispersal around the Singapore Deeps (Bird et al., 2006) between
Singapore and Batam Island around the deepest areas of sea of the
entire study area (Figure 3). In T. gigas, relatively high resistance val-
ues emerge in a plain of shallow sea southwest of Singapore but with
a lack of statistical power to support the robustness of these results
(Figure S5).

![Effective population size across the Holocene of two horseshoe crab species.](image)

**FIGURE 2** Effective population size across the Holocene of two horseshoe crab species. Effective population size is displayed with 95% confidence intervals (shaded area). Sea-level fluctuations and carbon isotope composition in the Singapore Straits during the Holocene are based on Bird et al. (2010).
4 | DISCUSSION

We detected a relatively lower genetic diversity and finer population structure in the less dispersive, depth-limited, mangrove-adapted *C. rotundicauda* as compared to the more dispersive, open sea-adapted *T. gigas*. Based on our study, we predict further steep declines in the genetic diversity of local *C. rotundicauda* with the impending human-caused sea-level rise. Further significant losses of genetic diversity directly translate into losses in evolutionary potential (Frankham et al., 2014; Palstra & Ruzzante, 2008), which will contribute to an unpredictable fate of *C. rotundicauda* populations in the Singapore Straits during the periods of intense environmental change expected for the coming decades and centuries. In contrast, the genetic diversity of *T. gigas* may be maintained or even be favored by a rapid sea-level rise of up to 0.7 m (Stocker et al., 2013), but precise responses will critically depend on habitat availability and human disturbance.

Our population genetic analyses with a comprehensive geographic coverage yield the first firm evidence on differences in dispersal ability between the two horseshoe crab species (Figure 4), particularly as regards the depth-limited dispersal of *C. rotundicauda* (Figure 3). Our approach provides an analytical framework for the study of movement ecology and life history where conventional marking and tracking methods are not as cost-effective, especially in long-lived species such as horseshoe crabs whose benthic life remains cryptic (Rudloe, 1979; Sekiguchi, 1988). Given size is important for the dispersal distance of active dispersers (Jenkins et al., 2007), our findings on the more limited dispersal in the smaller *C. rotundicauda* as compared to the larger *T. gigas* suggest that active benthic dispersal of adults, rather than passive phytoplanktonic dispersal of larvae, better explains the differences in dispersal distances of the two species. This is consistent with observations of limited larval dispersal in the other two horseshoe crab species from the Atlantic and

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**FIGURE 3** Map of resistance to *C. rotundicauda* dispersal across the Singapore Straits, generated using DResD. Red indicates areas of high resistance whereas green indicates areas of low resistance; sampling localities are indicated with yellow dots, bathymetry is illustrated with blue contour lines. *ADMixTure* bars (*K* = 2, dataset C0) are placed adjacent to correspondent sampling localities.
East Asia (Botton & Loveland, 2003; King et al., 2005; Pierce et al., 2000; Yang et al., 2009).

Our sampling of horseshoe crabs from randomly mixed cohorts may underestimate LDNe (Waples et al., 2014). Given that the two horseshoe crab species have a similar lifespan and generation time, we expect the same level of bias in LDNe for both species, ruling out systematic biases in our results. Our results indicate that the more dispersive _T. gigas_ has lost almost half of the genetic diversity during the expansion from the source population whereas the less dispersive _C. rotundicauda_ has lost more than 95% (Figure 2). Loss of genetic diversity, as reflected in reduced effective population size, may be the consequence of sequential founder effects (Clegg et al., 2002), which both species would have needed to undergo in order to re-expand across the vast shelf. However, differences in the degree of diversity loss between the two species may correspond to their different dispersal abilities. _T. gigas_ was able to maintain genetic exchange across fairly distant populations, keeping diversity loss in check (Berthouly-Salazar et al., 2013), while _C. rotundicauda_ branched out into increasingly fragmented and isolated populations across the Sunda Shelf. Besides differences in dispersal ability, differences in their ability to adapt to novel coastal habitats created by rapid sea-level rise may also have contributed to the difference in genetic diversity. However, this can be only tested with exclusive sampling beyond our study area to verify whether populations of _C. rotundicauda_ from across the Sunda Shelf display the same level of low genetic diversity.

Our results provide important baseline data for the conservation of horseshoe crabs in the Singapore Straits and for mitigation against the future effects of climate change. These baseline data, which allow insights into both genetic diversity and dispersal ability, illustrate how dispersal ability may have contributed to present-day patterns of genetic diversity and therefore may offer superior conservation recommendations as compared to the conventional baseline data of census population size or effective population size alone. The gradual and continuous drop of effective population size in _C. rotundicauda_ over the last 10,000 years stands in stark contrast to the massive expansion and coastal domination of its preferred mangrove habitat in the region (Bird et al., 2010). This seeming contradiction underscores the importance of including a species’ dispersal ability and evolutionary trajectory in conservation planning (Hoban et al., 2013; Laikre, 2010). Our dataset affords surprising small-scale resolution by capturing ongoing sequential founder effects of _C. rotundicauda_ populations on Batam and Bintan islands to the south of the Singapore Straits. Individuals sampled around the latter two islands exhibit the region’s lowest genetic diversity, emerging as isolated from all other regional populations across the deep-water barrier of the Singapore Straits. Individuals sampled around the latter two islands exhibit the region’s lowest genetic diversity, emerging as isolated from all other regional populations across the deep-water barrier of the Singapore Straits. Individuals sampled around the latter two islands exhibit the region’s lowest genetic diversity, emerging as isolated from all other regional populations across the deep-water barrier of the Singapore Straits. Habitat restoration should primarily focus on creating corridors to provide increased connectivity between isolated populations characterized by low diversity. In the immediate future, further loss of genetic diversity in the local _C. rotundicauda_ population unfortunately appears inevitable—intense conservation efforts notwithstanding—so long as sea levels continue to rise or persist at the current level. As for _T. gigas_, rapid future sea-level rise may facilitate a slight increment in the level of genetic diversity. However, the precise fate of local populations also depends on small-scale habitat dynamics and human disturbance and development, all of which need to be taken into consideration by conservation planners (Di Nitto et al., 2014; Jiang et al., 2016).

Our results identified the southeastern end of the Straits of Malacca as a stronghold featuring the highest genetic diversity for
both horseshoe crab species (Figure 1), so this area should be considered of especially high conservation value. High genetic diversity may reflect a high abundance of individuals (Frankham, 1996), and this—in turn—is possibly linked to relatively high levels of productivity as indicated by the regionally highest annual levels of chlorophyll a concentration in this area (Siswanto & Tanaka, 2014). Systematic in-depth population surveys would provide invaluable additional data to evaluate each species’ conservation status for long-term conservation planning.

Recent conservation concern for horseshoe crabs in the Singapore Straits has revolved around both species at equal measure, with suggestions that T. gigas may even be the more endangered species because of fewer detections (Cartwright-Taylor, Davison et al., 2008). Our results demonstrate that T. gigas, with its panmictic population structure and strong recolonization potential, is of much lesser concern than the poorly dispersive C. rotundicauda, which suffers from a long-term trend of decreasing effective population size throughout the Holocene. This dichotomy highlights the importance of studies such as ours in identifying the right conservation targets in our efforts to mitigate the effects of future climate change. While limited to horseshoe crabs, our study provides a blueprint for impending research to help safeguard the Earth’s coastal and marine organismic communities.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available in the Dryad digital repository at http://doi.org/10.5061/dryad.5mkkwh75h.

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