Gene Arrangement and Adaptive Evolution in the Mitochondrial Genomes of Terrestrial Sesarmid Crabs Geosesarma faustum and Geosesarma penangensis

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INTRODUCTION

Decapoda order (Crustacea and Malacostraca) is the most diverse and species-rich taxonomy group comprising many of the well-known invertebrates such as shrimps, prawns, crayfishes, lobsters, and true crabs (Shen et al., 2013). The true crabs belong to Brachyura, the largest infraorder within Decapoda with approximately 7,200 described species and some of which are economically important (De Grave et al., 2009; Ahyong et al., 2011). Brachyura crabs have a diverse range of forms and adaptations, making them one of the most important groups to study in terms of evolution (Castro et al., 2015). Brachyura encompasses Podotremata, Heterotremata, and Thoracotremata, the latter two of which are collectively referred to as Eubrachyura (de Saint Laurent, 1980; Ahyong et al., 2007). Within Eubrachyura, the family Sesarmidae includes terrestrial, semiterrestrial, or tree-climbing species occurring mainly in the mangroves (Cumberlidge et al., 2005; Serrano-Sánchez et al., 2016). Sesarmid crabs, due to their role in nutrient cycling, have been considered keystone species in the mangrove ecosystem (Smith et al., 1991). Multiple transition routes have been proposed for this family, which comprises members that have colonized land via both marine and freshwater environments (Watson-Zink, 2021).

The genus Geosesarma De Man, 1892, belonging to the Sesarmidae family, is represented by 67 species at present. The genus is distributed in Malaysia, Indonesia, Philippines, Thailand, Papua New Guinea, and the Andamans (Ng et al., 2008; Ng and Wowor, 2019; Shy and Ng, 2019; Naruse and Ng, 2020). Geosesarma species are commonly known as vampire crabs due to the bright yellow eyes of some species (Ng et al., 2015). Geosesarma species are amphipodous or terrestrial, and they sometimes live far from water (Hartnoll, 1988). As many of the species display abbreviated or no larval development and low fecundity, Geosesarma species often have restricted distributions and a high degree of endemism (Schubart and Ng, 2014). Geosesarma faustum and Geosesarma penangensis are found on Penang Hill, Malaysia, with G. faustum associated with phytotelms and living at altitudes greater than 700 m, whereas G. penangensis at lower altitudes (Ng, 2017). Geosesarma species are small crabs with carapace widths that rarely exceed 10 mm (Hartnoll, 1988). Their small size, high level of endemism, and cryptic nature account for the scarcity of information on the genus.
The animal mitogenomes are circular double-stranded molecules of 15–18 kb in size and generally contain 37 genes such as 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (12S rRNA and 16S rRNA) genes, and an A + T-rich control region (Boore, 1999). Crustacean mitogenomes exhibit exceptions to this organization with instances of duplications of tRNA gene and rearrangements of the gene order (Segawa and Aotsuka, 2005; Basso et al., 2017). Due to its smaller effective population size and lack of recombination, mitochondrial DNA (mtDNA) is more strongly influenced by evolutionary processes than nuclear DNA and has thus been used to analyze genetic diversity and adaptive evolution (Shen et al., 2019; Peng et al., 2021). The high level of endemism exhibited by Geosesarma crabs makes them an index for the study of adaptation to the local environment. Nevertheless, genomic and genetic resources are lacking for the genus Geosesarma. Previous studies have assigned Geosesarma to Sesarmidae based on morphology (Ng et al., 2008; Ng, 2017; Ng and Wowor, 2019; Shy and Ng, 2019); nevertheless, no genetic analysis has been performed to show the position of Geosesarma within Sesarmidae. In this study, the complete mitogenomes of G. faustum and G. penangensis were analyzed and compared with other Brachyura mitogenomes. The results illustrate the taxonomic placement of Geosesarma within Brachyura, gene arrangement of Geosesarma, and adaptive evolution in mitochondrial genes related to adaptation to hill habitat.

MATERIALS AND METHODS

Sample Collection and Mitogenome Sequencing

Adult specimens of G. faustum and G. penangensis were collected from Penang Hill, Malaysia (5.4085° N, 100.2773° E) in April 2021. Voucher specimens of G. faustum and G. penangensis were deposited at the Zoological Reference Museum Collection, Universiti Sains Malaysia. Total DNA was extracted from muscle tissues of three individuals in total for each species using the DNeasy Blood and Tissue Kit (QIAGEN, Germany) according to the instructions of the manufacturer and pooled together. DNA quality was assessed using gel electrophoresis, and DNA concentration was quantified using the dsDNA HS Assay Kit on the Qubit Fluorometer (Thermo Fisher, United States). Sequencing libraries were constructed using the TruSeq PCR-Free DNA Library Kit (Illumina, United States) and sequenced on an Illumina NovaSeq 6000 platform. Paired-end 150-bp sequencing yielded approximately 5.8 Gb data for each species.

Mitogenome Assembly, Annotation, and Comparative Analyses

Raw reads were assembled by baiting and iterative mapping to the reference mitogenome sequence of Sesarmops sinensis (NC_030196) using MITObim (v1.9.1) (Hahn et al., 2013) and MIRA (v4.0.2). Annotations were performed with the MITOS pipeline (Bernt et al., 2013) using the invertebrate mitochondrial genetic code. The tRNA genes were annotated, and their secondary structures were inferred using the MiTFi module in the MITOS. Secondary structures of the predicted tRNAs were visualized on the Forna web server. Tandem Repeats Finder was used to detect repeat sequences in the control region, and their secondary structures were predicted with Mfold software. The base composition and the relative synonymous codon usage were determined using MEGA X. The skew in the nucleotide composition was calculated according to the formulas as follows: AT-skew = (A–T)/(A + T) and GC-skew = (G–C)/(G + C). Circular mitogenomes representations were drawn using CGView online server. The ratio of non-synonymous to synonymous substitutions (o = dN/dS) was estimated using the codon-based maximum likelihood (ML) method in PAML (v4.9) (Yang and Nielsen, 2000). The branch-site test was applied with G. faustum or G. penangensis as the foreground branch and the rest of the Sesarmidae as the background branch. A likelihood ratio test was used to compare model A against the null model A. The posterior probability of the positively selected sites was computed by the Bayes Empirical Bayes method.

Phylogenetic Analyses

The phylogenetic analyses were performed using 13 PCG sequences from 76 species covering representative Brachyura families, and Clibanarius infraspinatus was used as the outgroup (Supplementary Table 1). The nucleotide sequences of the PCGs were aligned using MAFFT (v7.313) in auto mode and then concatenated. The ambiguous sequences or poorly aligned regions in the alignment were removed using Gblocks (v0.91b) with the default setting. Potential saturation in the nucleic acid sequences was assessed using DAMBE (v7.3.5). None of the tests yielded the index of substitution saturation (Iss) greater than the critical one (Iss > Iss.cAsym for the third codon position in asymmetrical topology (Iss > Iss.cAsym) except for the third codon position in asymmetrical topology (Iss > Iss.cAsym) (Supplementary Table 2). The best-fitting evolutionary model was determined using ModelFinder (v2.1.1) based on the Akaike Information Criterion. The ML tree was built in IQ-TREE (v1.6.8) (Nguyen et al., 2015) with 1,000 bootstrap replicates and the GTR + F + I + G4 model. The Bayesian inference (BI) was performed with MrBayes (v3.2.7) (Ronquist et al., 2012) using the GTR + I + G model. Markov Chain Monte Carlo was run for 10 million generations with four independent chains. Sampling was performed every 1,000 generations, and the initial 25% of the generations were discarded as burn-in. The convergence of the runs was assessed using Tracer (v1.7.2). The phylogenetic trees were visualized using FigTree (v1.4.4) software1.

RESULTS AND DISCUSSION

Genome Structure, Organization, and Composition

In this study, the complete mitochondrial genome sequences of the vampire crabs G. faustum and G. penangensis were determined. The mitogenomes of G. faustum and G. penangensis

1http://tree.bio.ed.ac.uk/software/figtree/
are 15,880- and 15,955-bp long (Figure 1A), respectively, which fall within the range of other Sesarmidae mitogenomes from 15,611 bp (Parasesarma pictum) to 15,920 bp (Chiromantes neglectum) (Supplementary Table 3). The mitogenomes follow a typical metazoan mtDNA structure comprising 37 genes (13 PCGs, 22 tRNAs, and 2 RNAs) and a major non-coding region known as the control region (Supplementary Table 4). As a result of the compactness of mitogenomes, there are a total of 45- to 71-bp overlap between genes, and the longest overlap occurs between trnL1 and large rRNA (rrnL) (25–55 bp). The mitogenomes also contain 331- to 372-bp intergenic sequences distributed in 20 regions ranging from 1 to 187 bp in size. The whole mitogenomes of Geosesarma are biased toward AT nucleotides (78.44–78.49%) (Supplementary Figure 1), showing significant strand asymmetry in a similar manner to other Sesarmidae mitogenomes. The overall AT- and GC-skews for the mitogenomes were calculated and compared (Supplementary Table 3). The GC-skews for all Sesarmidae are negative, ranging from –0.194 (P. pictum) to –0.232 (Metapopaulias depressus), and the AT-skews are weakly negative, ranging from –0.010 (Chiromantes dehaani and C. neglectum) to –0.032 (P. pictum).

The mitogenomes of G. faustum and G. penangensis have 13 typical PCGs found in brachyuran species consisting of seven NADH dehydrogenases (nad1-6 and nad4L), two ATPases (atp6 and atp8), three cytochrome c oxidases (cox1–3), and one cytochrome b (cob), which account for 11,178–11,181 bp in length. Nine PCGs (i.e., cox1, cox2, atp8, atp6, cox3, nad3, nad6, cob, and nad2) are encoded on the majority strand and the remaining (nad5, nad4, nad4L, and nad1) on the minority strand. While cox1, cox2, atp8, cox3, nad4, and nad4L initiated by the standard ATG codon, atp6, nad3, nad5, nad6, cob, and nad1 (G. penangensis) used ATA, and nad6 (G. faustum) used GTG. Most PCGs, i.e., atp8, atp6, cox3, nad3, nad5, nad4, nad6, and cox1 (G. penangensis), were terminated by TAA, whereas nad2 used TAG as stop codon, and cox2, cob, and cox1 (G. faustum) terminated by a single T. These incomplete stop codons have been observed in many brachyuran mitogenomes (Wang et al., 2019; Zhang et al., 2020) and are thought to be completed by posttranscriptional polyadenylation (Ojala et al., 1981).

The relative synonymous codon usage for 13 PCGs was analyzed (Supplementary Figure 1). Both the mitogenomes of G. faustum and G. penangensis consist of 3,716 codons (Supplementary Figure 2). In the 13 PCGs, the most frequently utilized amino acids were Leu (12.94%), Ile (10.09–10.15%), and Phe (9.58–9.85%). The usages of both the twofold and fourfold degenerate codons are biased toward codons abundant in A or T. This amino acid bias trend is consistent with the higher representation of nucleotides A and T in the whole mitogenome.

Similar to other Brachyura mtDNAs, G. faustum and G. penangensis mitogenomes contain a complete set of 22 tRNA genes ranging in size from 63 bp (trnF) to 73 bp (trnV). The majority of tRNAs fold into a typical cloverleaf secondary structure, although trnD, trnH, and trnR (G. faustum) have truncated dihydrouridine (DHU) loop compared with normal tRNA (Supplementary Figure 3). The trnS also lacks the thymidine-pseudouridine-cytidine (TPC) stem and loop, whereas trnC (G. penangensis) has reduced the DHU stem. Aberrant tRNA structures in mitogenomes appear to be a common phenomenon in many species (Li et al., 2020; Wang et al., 2020); previous research on nematodes and metazoans suggests that such features have no effect on tRNA function (Watanabe et al., 2014; Foroudrilis et al., 2018).

The rrnL and small rRNA (rrnS) genes of G. faustum and G. penangensis are 1,364–1,394 bp and 825–844 bp in length, respectively, which both are located between trnL1 and control region, and separated by trnV. The control region located between rrnS and trnQ spans 712–785 bp and has functional importance related to the initiation of replication and transcription of the mitogenome (Clayton, 1992). This region exhibits high A + T content (82.56–83.69%) and contains features such as tandem repeat, microsatellite sequence (TA)n, and stem-loop structure (Supplementary Figure 4).

Selection Analyses
To analyze the selective pressure on G. faustum and G. penangensis compared with other Sesarmidae species, the non-synonymous/synonymous substitution ratios of the mitochondrial PCGs were estimated under the branch-site test (Figure 1B). The analysis detected signals of positive selection in cob of G. faustum, as well as nad5 and atp6 of G. penangensis at seven codon sites. The different positively selected sites detected in the two Geosesarma species could be attributed to differences in the mechanisms used by crabs endemic to different locations. Previous studies have found evidence for positive selection in mitochondrial genes that may play a role in adaptive evolution in organisms inhabiting extreme environments or with requirements for extreme metabolic demands (Li et al., 2018; Sun et al., 2018; Shen et al., 2019). In Chinese snub-nosed monkeys and galliform birds living in high-altitude environments, the selections on mitochondrial genes are driven by the requirements for higher energy demand and oxygen metabolism (Yu et al., 2011; Zhou et al., 2014). In brachyurans, a positive selection of mitochondrial genes has only been reported in a deep-sea representative (Zhang et al., 2020). We showed here, for the first time, the occurrence of positive selection detected in a land crab, which suggests a relationship between altered metabolic requirements and high-altitude colonization. Terrestrial brachyurans display morphological, behavioral, and physiological adaptations to sustain prolonged periods from the water source (Lim and Goh, 2021). Among these, osmotic and ionic regulation, waste excretion, aerial respiration, and thermoregulation are pivotal changes (Watson-Zink, 2021). The positive selection of PCGs in the two Geosesarma species showed the importance of adaptation in respiratory physiology for survival at higher altitudes. The differences in positive selection sites and genes between G. faustum and G. penangensis, which occupy different altitude niches, warrant further investigation.

Gene Rearrangement
Compared to the hypothetical ancestral pancrustacean gene order (Boore et al., 1998), it is evident that the Geosesarma experienced mitochondrial gene rearrangement (Figure 1C).
FIGURE 1  |  The complete mitochondrial genomes of Geosesarma faustum and Geosesarma penangensis. (A) Circular map of the G. faustum and G. penangensis mitogenomes. (B) Non-synonymous/synonymous substitution (ω) ratios for G. faustum and G. penangensis mitochondrial protein-coding genes (PCGs) and possible sites under positive selection (p-value < 0.05). The ω values for the cob of G. faustum, nad5, and atp6 of G. penangensis not plotted in the chart are shown in the table. Only posterior probability > 0.95 sites is shown. (C) The linear representation of the mitochondrial gene arrangement in Brachyura. Cox1 has been designated the start point for all mitogenomes.
FIGURE 2 | Phylogenetic tree inferred from the nucleotide sequences of 13 PCGs of the mitogenomes using the maximum-likelihood analysis. Bootstrap values greater than 50 are indicated at nodes, and sequences generated in this study are indicated in bold.
Typically, \textit{trnH} located between \textit{nad4} and \textit{nad5} in the pancrustacean ground pattern had been shifted into the \textit{trnE} and \textit{trnF} junction in the \textit{Geosesarma} mitogenomes. This translocation is a relatively common event in the brachyuran mitogenomes (Chen et al., 2019; Wang Q. et al., 2020). In the most common brachyuran gene order, the tRNA gene arrangement between the control region and \textit{nad2} is \textit{trnL}--\textit{trnQ}--\textit{trnM}. Nevertheless, \textit{G. faustum} and all Sesarmidae mitogenomes (except \textit{G. penangensis}) shared a transposition of \textit{trnQ} that resulted in the \textit{trnL}--\textit{trnQ}--\textit{trnM} order. The tandem duplication-random loss model is one of the most widely used mechanisms to explain mitochondrial gene arrangement (Moritz and Brown, 1987; Macey et al., 1997). In this case, tandem duplication in the \textit{trnF}--\textit{nad5}--\textit{trnH} region, followed by loss of supernumerary genes, is the most likely process for forming \textit{trnH}--\textit{trnF}--\textit{nad5} gene order. Meanwhile, the \textit{trnQ}--\textit{trnL}--\textit{trnM} arrangement could also be caused by slipped-strand mispairing followed by gene deletion. Despite belonging to the \textit{Geosesarma} genus, \textit{G. penangensis} differs from \textit{G. faustum} and the rest of the Sesarmidae by showing \textit{trnL}--\textit{trnQ}--\textit{trnM}. This feature raises the possibility of a secondary independent reversal to the plesiomorphic brachyuran gene arrangement. Such reversion is extremely rare; endemism in \textit{Geosesarma} species may have promoted the evolutionary transition. Brachyura families such as Varunidae, Macrophthalmidae, Xenograpsidae, Majidae, Potamidae, and Parathelphusidae also showed signatures of gene arrangement with respect to the ancestral pancrustacean pattern.

**Phylogenetic Analyses**

The phylogenetic relationships were analyzed based on the nucleotide sequences of 13 PCGs from 76 Brachyura species and one out-group. ML and BI analyses resulted in tree topologies that were largely congruent (Figure 2 and Supplementary Figure 5). In this study, \textit{G. faustum} and \textit{G. penangensis} clustered in one branch with high support values, indicating that the two have a sister group relationship. This is the first phylogenomic analysis that placed \textit{Geosesarma} as the sister group to the \textit{[(C. dehaani + C. neglectum) + (Chiromantes haematocheir + S. sinensis)]} clade. This result confirmed that \textit{Geosesarma} belongs to Grapsoida, Sesarmidae, congruent with the morphological assignments (Ng et al., 2008; Ng, 2017; Ng and Wowor, 2019; Shy and Ng, 2019). All Sesarmidae species cluster into a clade consisting of two sister groups, which was consistent with previous findings (Wang et al., 2019; Li et al., 2020; Lu et al., 2020; Wang Z. et al., 2020). Together, \textit{[(C. dehaani + C. neglectum) + (C. haematocheir + S. sinensis)]} + \textit{[(C. faustum + G. penangensis) + (Chiromantes eulime)]} and \textit{[(Parasesarma affine + Perisesarma bidens) + Nanosesarma minutum] + (P. pictum + Parasesarma tripectinis) + (Clistocoleoma sinense + M. depressus)]} groups form the polyphyletic Sesarmidae clade.

Sesarmidae species are most closely related to Gecarcinidae, constituting part of the Grapsidea group, which is in accordance with the results of the studies by Chen et al. (2019), Wang et al. (2019), Lu et al. (2020), and Zhang Y. et al. (2020). The mitochondrial phylogenomic analyses including our dataset have shown the divergent phylogenetic status of Grapsidae and Dotilidae (Wang et al., 2019; Li et al., 2020; Wang Z. et al., 2020; Zhang Y. et al., 2020). In the ML tree, Grapsidae and Dotilidae form a sister group and constitute the base of the \textit{[(Grapsidae) + Gecarcinidae] + Xenograpsidae} + \textit{Ocyopodidae} + \textit{[Varunidae + (Macrophthalmidae + Mictyridae)]} clade. Nevertheless, the BI tree placed Grapsidae and Dotilidae group at the basal to the \textit{[(Grapsidae) + Gecarcinidae] + Xenograpsidae} + \textit{Ocyopodidae} clade. While ML tree recovered Dynomenidae + \textit{[Homolidae + (Raninidae)]} relationship that was similar to those reported in the studies by Lu et al. (2020) and Wang Z. et al. (2020), BI tree exhibited Homolidae + \textit{[Dynomenidae + (Raninidae)]} topology. The presence of only one or a small number of representatives for certain families could have resulted in unstable phylogenetic relationships.

In this study, the complete mitogenomes of \textit{G. faustum} and \textit{G. penangensis} were characterized and compared with that of other Brachyura. The phylogenetic analyses confirmed that \textit{Geosesarma} belongs to Grapsoida, Sesarmidae. Evidence of positive selection was detected in \textit{Geosesarma} mitochondrial genes, highlighting adaptive evolution in crabs for the colonization of terrestrial hill habitat. Unlike \textit{G. faustum} and all other Sesarmidae mitogenomes, \textit{G. penangensis} has a \textit{trnL}--\textit{trnQ}--\textit{trnM} gene arrangement, suggesting a reversal to the plesiomorphic brachyuran pattern. Importantly, our study provides genomic resources that are pivotal to understand the biology of endemic \textit{Geosesarma} crabs.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: MZ725940, MZ725941, and SRR15514280-1).

**AUTHOR CONTRIBUTIONS**

ACS-C and N-SL conceived and designed the experiments. N-SL analyzed the data and wrote the manuscript. K-KS performed the experiments. AA, K-AS, and AWAZ collected and identified the samples. All authors reviewed the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.778570/full#supplementary-material
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