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Phylomitogenomics unravels evolution of symbiosis in Thoracotremata (Decapoda: Cryptochiridae, Pinnotheridae, Varunidae)

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Background: Thoracotreme crabs belong to the large group of “true” crabs (infraorder Brachyura), and they exhibit a wide range of physiological and morphological adaptations to living in terrestrial, freshwater and marine habitats. Moreover, the group comprises various obligately symbiotic species (gall crabs, pea crabs, various varunid crabs) that are specialised to living with an invertebrate host, however, the evolutionary history of these symbiotic crabs is still unresolved.

Methods: Here, we assembled and characterised the complete mitochondrial genomes of three gall crab species (Cryptochiridae): *Troglocarcinus corallicola*, *Kroppcarcinus siderastreicola*, and *Opecarcinus hypostegus*. A phylogenetic tree of the Thoracotremata was reconstructed using 13 protein coding genes and two ribosomal RNA genes of the three new gall crab mitogenomes and a further 70 available thoracotreme mitogenomes. Furthermore, we applied a comparative analysis to characterise mitochondrial gene order arrangement, and performed a selection analysis to test for selective pressure of the mitochondrial protein coding genes in obligately symbiotic Cryptochiridae, Pinnotheridae and one varunid crab.

Results: The results of the phylogenetic reconstruction confirm the monophyly of Cryptochiroidea, which clustered separately from the Pinnotheroidea. The latter clustered at the base of the tree with robust branch values. The symbiotic crab *Asthenognathus inaequipes* clustered with all other Varunidae and the macropthalmid species *Tritodynamia horvathi*, highlighting that obligate symbiosis in the Thoracotremata evolved at least three times. Different gene orders were detected in obligate symbionts and free-living species when compared with the ancestral brachyuran gene order. Lastly, the selective pressure analysis detected one positively selected site in the *atp8* gene of obligate symbionts. This highlights the crucial role of adaptive evolution of mitochondrial protein coding genes, perhaps related to higher energetic demands of a symbiotic lifestyle.

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Abstract

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evolution of mitochondrial protein coding genes, perhaps related to higher energetic demands of a symbiotic lifestyle.

Introduction

Brachyuran crabs, one of the most diverse groups of crustaceans, were divided into three (sub)sections based on the position of the gonopore by Guinot (1978, 1979): Podotremata, Heterotremata, and Thoracotremata. The Podotremata have since been shown to be paraphyletic, whereas the reciprocal monophyly of the Heterotremata and Thoracotremata is supported (Tsang et al., 2014). The Thoracotemata consist of four superfamilies (Grapsoidea, Ocypodoidea, Pinnotheroidea and Cryptochiroidea) together comprising 21 families (Tsang and Naruse, 2023), however, Tsang et al. (2022) proposed a new division into seven superfamilies. Thoracotreme crabs inhabit widely different habitats in terrestrial, freshwater and marine environments across the world, resulting in evolutionary adaptations to these different lifestyles. For example, the majority of ~~Grapsoidea~~ and ~~Ocypodoidea~~ crabs are free-living and can be found in almost all reported habitats for brachyuran crabs (Tan et al., 2016; Wang et al., 2020), while Pinnotheroidea (pea crabs) and Cryptochiroidea (gall crabs) live in obligate symbiotic relationships with a host organism. Pea crabs associate with bivalves, gastropods, echinoids, holothurians, and ascidians (Castro, 2015; Hultgren et al., 2022), whereas gall crabs inhabit dwellings in scleractinian corals (Fize and Serène, 1957; Kropp, 1990). Moreover, some varunids - currently classified in the Grapsoidea - are obligately symbiotic with a host (Castro, 2015).

The monophyly of the Thoracotremata has been confirmed by various studies (Von Sternberg and Cumberlidge, 2001; Tsang et al., 2014; Wang et al., 2020), but the monophyly of the superfamilies within the Thoracotremata has long been debated (Schubart et al., 2006; Tsang

et al., 2014, 2018, 2022; Van der Meij and Schubart, 2014; Chen et al., 2018; Ma et al., 2019, Sun et al., 2022). The superfamily Pinnotheroidea is currently composed of two monophyletic families (Pinnotheridae and Aphanodactylidae), however, Tsang et al. (2018, 2022) suggested that these two families are distantly related. Both families possess the same trait of obligate symbiosis with invertebrate hosts and have similar morphology, which further confuses the interpretation of their evolutionary relationships. Tsang et al. (2022) suggested moving Aphanodactylidae into a new superfamily together with Heloeciidae, Macrophthalmidae, Mictyridae and Varunidae. Whilst the Cryptochiridae is currently classified in its own superfamily - the Cryptochiroidea - Wetzer et al. (2009) questioned this and suggested Cryptochiridae to be considered a family in the Grapsoidea. A later study by Van der Meij and Schubart (2014), using the same gene (16s rRNA) as Wetzer et al. (2009) but with 10 species of cryptochirids instead of one, retrieved the Cryptochiridae as monophyletic and independent from Grapsoidea. Subsequent thoracotreme classification schemes have retained the superfamily status of the Cryptochiroidea (see overview in Tsang et al. 2022), and indicated the need for additional gall crab sequences to elucidate the position of Cryptochiridae in the Thoracotremata, due to weak branch support and uncertainty of tree topology (Sun et al., 2022; Tsang et al., 2022).

Although Pinnotheridae and Cryptochiridae are considered monophyletic, their phylogenetic position within the Thoracotremata - and thus the origin and evolution of thoracotreme symbiosis - is still unresolved (Sun et al. 2022, Tsang et al., 2022). Sun et al. (2022) retrieved pea crabs at the basis of a phylogenetic tree of the Thoracotremata, whilst gall crabs clustered, with equivocal branch support, with an Ocypodoidea lineage (Camptandriidae/Xenophthalmidae/Dotillidae). This is in disagreement with the results of Tsang et al. (2022) whose phylogenetic arrangement showed both families of symbiotic crabs clustering

together – albeit also with equivocal results – far away from the basal branches in the phylogenetic tree. Furthermore, the ~~holothurian symbiotic~~ varunid *Asthenognathus inaequipes* Stimpson, 1858 showed a distant phylogenetic position from gall crabs and pea crabs (Sun et al., 2022). Hence, the question whether species with symbiotic lifestyles in Thoracotremata – as observed in the Pinnotheridae, Cryptochiridae and several Varunidae – have a single origin or evolved independently remains open (Tsang et al., 2022).

The mitogenome of most metazoans has relatively high nucleotide substitution rates, lack of extensive recombination and conserved gene content, thus making it an informative molecular signal for phylogenetic reconstruction and adaptive evolution analysis (Gissi et al., 2008). Moreover, mitochondrial gene order can provide an extra source of phylogenetic information (Basso et al., 2017). In the evolutionary process of a species, the life-strategy of the species in response to different environmental pressures may affect the function of mitochondrial genes and exert selective pressure on them. Numerous examples have shown that taxa adapted to inhabiting a specific niche, ~~undergo~~ positive selection (Li et al., 2018; Chen et al., 2022). The range of life strategies, including symbiosis, in the Thoracotremata allow us to study whether there ~~is~~ positive selection in the mitogenomes of these crabs.

So far, only one mitogenome is available for gall crabs, that of the Indo-Pacific species *Hapalocarcinus marsupialis* Stimpson, 1859 s.l. (Sun et al., 2022; see Bähr et al. (2021) for a discussion of the species complex). Cryptochiridae are a peculiar group of diminutive crabs, obligately associated with scleractinian corals. There are currently 53 described species across 21 genera (WoRMS, 2023); however, recent studies have uncovered large numbers of undescribed cryptochirid species awaiting formal description (e.g., Bähr et al., 2021; Xu et al., 2022). Here we reconstructed a phylogeny of the Thoracotremata using a phylomitogenomic approach based

on 73 species (from 14 out of 21 recognised families), including three newly sequenced Cryptochiridae mitogenomes. Based on the inferred phylogeny reconstruction and comparative analysis, we aim to elucidate: 1) the monophyly and phylogenetic position of Cryptochiroidea; 2) the evolution of symbiosis (single or multiple origin) in Thoracotremata; and 3) by means of a test for selective pressure, whether a symbiotic lifestyle ~~results in~~ positive selection for certain Protein Coding Genes (PCGs) in the mitogenome.

Materials & Methods

Sample collection and mitochondrial genome sequencing

The three gall crab species used in this study were collected from two sites in the Caribbean. *Troglocarcinus corallicola* Verrill, 1908, was sampled from *Orbicella faveolata* (Ellis & Solander, 1786) in Anse à Jacques, Guadeloupe (16°12'29.4"N, 61°25'22.1"W) on the 27th of April 2021. *Kroppcarcinus siderastreicola* Badaro, Neves, Castro & Johnsson, 2012, and *Opecarcinus hypostegus* (Shaw & Hopkins, 1977) were collected from Piscadera Bay, Curaçao (12°7'18.17"N, 68°58'10.66"W). *Kroppcarcinus siderastreicola* was collected from *Siderastrea siderea* (Ellis & Solander, 1786) on the 24th of February 2022 and *O. hypostegus* was collected from *Agaricia humilis* Verrill, 1901, on the 14th of March 2022. The crabs were stored in 70% ethanol and transported to the University of Groningen, and from there the entire specimens were sent to the Beijing Genomics Institute (BGI) in Hong Kong for DNA extraction, and paired-end sequencing using the DNBSEQ-G400 platform.

Sampling in Guadeloupe was authorised by the Direction de la Mer de Guadeloupe under Autorisation N°09/2021. Sampling in Curacao was under collecting permits of the Curaçaoan Government provided to CARMABI (Government reference: 2012/48584).

Mitochondrial genome assembly and annotation

The raw data were filtered by removing adaptors sequences, contamination and low-quality from raw reads using fastp v.0.23.2 (Chen et al., 2018) with default parameters. The clean data were *de novo* ~~assembled~~ with GetOrganelle v.1.7.6.1 (Jin et al., 2020) on the Peregrine high performance cluster of the University of Groningen. The assembled mitogenomes were subsequently imported into MITOS1 on the MITOS Web Server (http://mitos.bioinf.uni-leipzig.de/index.py) (Bernt et al., 2013) for annotation, then we confirmed start and stop codons manually using Geneious v8.1.3. The GCview online service~~s~~ (https://proksee.ca/) was used ~~to~~ complete visualisation of the mitogenome maps. The assembled and annotated ~~mitogenomes~~ with gene features were uploaded to GenBank under accession numbers OQ308778 (*K. siderastreicola*), OQ308779 (*O. hypostegus*), OQ308780 (*T. corallicola*).

Mitochondrial genome characterisation

After complete annotation of the mitogenome, the nucleotide composition for each species was ~~were~~ calculated in MEGA X (Kumar et al., 2018) and the formulas of AT skew = (A − T) / (A + T) and GC skew = (G − C) / (G + C) ~~were used to calculate the composition skew~~. The Relative Synonymous Code Usage (RSCU) for concatenated PCGs was estimated using the ~~in~~ Sequence Manipulation Suite: Codon Usage (https://www.bioinformatics.org/sms2/codon_usage.html) with the invertebrate mitochondrial genetic code (Stothard, 2000) and visualised with the web tool EZcodon (http://ezmito.unisi.it/ezcodon; Cucini et al., 2021) combined with the package *ggplot2* (Wickham, 2016) in R version 4.2.2 (R Core Team 2013). The mitogenome of *H. marsupialis* s.l. (Sun et al., 2022) was included in this analysis because details of the mitogenome were lacking in the original paper. Using the cytochrome c oxidase subunit I (*cox1*)

barcode from the mitogenome by Sun et al. (2022) we identified the specimen as *H. marsupialis* HM.08 (Van der Meij et al. in prep.).

The transfer RNA (tRNA) genes were identified with MiTFi, implemented in the MITOS web server, using the default settings. The secondary structures of tRNAs were visualised with ViennaRNA Web Services (Kerpedjiev et al., 2015).

Phylogenetic analysis

Seventy thoracotreme and five heterotreme crabs (as outgroups) for which whole mitogenomes are available were retrieved from GenBank (Table S1). With the addition of the three new gall crab mitogenomes this resulted in a dataset of 78 mitogenomes for phylogenetic inference. The 13 PCGs and two ribosomal RNA genes (rRNAs; rrnS: 12S ribosomal RNA and rrnL: 16S ribosomal RNA) were aligned separately using MAFFT v.7.407 (Katoh and Standley, 2013) and subsequently Gblocks v.0.91b (Talavera and Castresana, 2007) was applied to remove ambiguously aligned regions using default settings. All PCGs and rRNAs were combined in a concatenated dataset containing 11,193 nucleotides. PartitionFinder 2 (Lanfear et al. 2017) was used to detect the best partition scheme, as well as the best-fit nucleotide substitution models for the respective partitions, based on the corrected Akaike Information Criterion (AICc; Hurvich and Tsai, 1989).

Maximum Likelihood (ML) and Bayesian Inference (BI) approaches were used for the phylogenetic analysis. The selected partition schemes and best-fit substitution models are available in Table S5. ML was inferred in IQ-TREE v1.6.8 (Nguyen et al. 2015) with 20,000 ultrafast bootstraps (Minh et al. 2013), and MrBayes v3.2.7 (Ronquist et al., 2012) was used for the BI analysis; we ran two parallel runs of four chains (one cold and three heated chains) each performing for 10 x 10^6 generations, sampling every 1000 iterations. Consensus trees were

196 constructed in MrBayes with a burnin of 25%, however, the average standard deviation of split
197 frequencies was 0.018, surpassing the recommended threshold (<0.01) proposed by the software
198 authors as a measure of convergence. The trees were visualised in FigTree v1.4.2
199 (http://tree.bio.ed.ac.uk/ software/figtree/).

200 Comparative analysis of mitochondrial gene order

201 The mitogenomes of the rest 70 thoracotreme crabs were annotated by MITOS1
202 (http://mitos.bioinf.uni-leipzig.de/index.py). The Mitochondrial Gene Order (MGO) of
203 *Xenograpsus testudinatus* Ng, Huang & Ho, 2000, is based on Ki et al. (2009) because MITOS 1
204 generated only 20 tRNAs, while it contains 22 tRNAs ~~in~~, the primary paper.
205 The comparative gene order analysis was conducted using CREx (Bernt et al., 2007). In
206 this analysis we compared the MGO of all taxa in our dataset with the ancestral gene order of the
207 Brachyura (Wang et al., 2018). The gene rearrangement scenarios in CREx are based on
208 common intervals, which considers reversals, transpositions, reverse transpositions, and tandem-
209 duplication-random-loss (TDRL) as possible gene rearrangement events, while the control region
210 is excluded from the analysis.

211 Analysis of selective pressure

212 To test for selective pressure on each of the PCGs, the ratio of nonsynonymous (dN) to
213 synonymous (dS) substitutions rates (ω = dN/dS) was calculated using the codon-based
214 maximum likelihood (CodeML) application in PAML 4.7 (Yang, 2007). Codon alignments were
215 implemented in the PAL2NAL web server (http://www.bork.embl.de/pal2nal/; Suyama et al.,
216 2006). Here we used two models to test for selective pressure: (branch model and branch-site
217 model), and a total of 13 PCG genes was computed separately for each of these models. The

topology of the ML tree resulting from the phylogenetic analyses - without outgroups and branch lengths - was used for the selective pressure analysis. The downstream analysis excluded strange ω ratios, which occurred when the dN or dS values were equal to zero due to limited substitution information from the sequences.

The branch model (i.e., one-ratio vs free-ratio model) was used to test if the ω ratios are variable among each branch in the phylogeny. One-ratio model as a null model assumes that ω ratios are constant over terminal branches, while a free-ratio model allows an independent ω ratio for each branch on the phylogenetic tree. Furthermore, the results of ω ratios in the free-ratio model were classified into two groups: obligate symbionts (Pinnotheridae, Cryptochiridae and *A. inaequipes*) and free-living crabs (all other taxa). The branch-site model (i.e., Model A null vs Model A) was applied in this study, which assumes that ω ratios vary among sites and across the branches of the phylogeny. The branches of symbiotic lineages were labelled as foreground branches while the rest were set as background branches in the branch-site model.

The Likelihood-Ratio Test (LRT) was used to compare each pair of models determining the best fitting model to our data. The parameters setting for LRT followed Zhang et al. (2005). Then, the Bayes Empirical Bayes (BEB) was used to calculate the posterior probability that each site acted with positive selection under the alternative model in the site models and branch-site models (Yang et al., 2005). In addition, if the results of LRT indicated that the free-ratio model fit our data better (i.e., one-ratio vs free-ratio model) then the Wilcoxon rank sum test would be applied to determine whether the ω ratios differed significantly between grouped lineages (i.e., symbiotic crabs vs free-living crabs). The visualisation was executed by *ggplot2* v3.4.0 (Wickham, 2016) in R v4.2.2 (R Core Team, 2020).

Results

Mitochondrial genome

The complete mitogenomes of the cryptochirids *T. corallicola*, *K. siderastreicola*, *O. hypostegus* and *H. marsupialis* s.l. were 15,637 bp, 15,629 bp, 15,702 bp and 15,422 bp in size, respectively. They all contained the usual 13 PCGs, two rRNA genes and 22 tRNA genes and non-coding regions (Fig. 1, Table S2) typically observed in decapod mitogenomes. Twenty-two out of the 37 genes were located on the majority strand (J-strand) and the remaining 15 were located on the minority strand (N-strand).

The nucleotide composition and AT-content and GC-content of the mitogenome in 73 thoracotreme crabs are summarised in Table S4. Of the four gall crabs, *K. siderastreicola* had the highest AT-content (74.57%) and *H. marsupialis* s.l. had the lowest value (69.54%).

PCGs comprised 11,144 (*T. corallicola*), 11,149 (*K. siderastreicola*), 11,104 (*O. hypostegus*) and 11,068 (*H. marsupialis* s.l.) codons. Nine (*cox1*, *cox2*, *atp8*, *atp6*, *cox3*, *nad3*, *nad6*, *cob* and *nad2*) PCGs were located on J-strand and four (*nad1*, *nad4*, *nad4l* and *nad5*) on the N-strand. The start and stop codons of the four gall crab species are summarised in Tables S2.

RSCU and amino acid composition of 13 PCGs for four gall crabs are shown in Figure S1. TTA (Leu), ATT (Ile), TTT (Phe) and ATA (Met) were the dominant codons (amino acids) in four gall crabs, and CGC (Arg), CCG (Pro), TCG(Ser) and ACG (Thr) were less commonly used (Table S3).

The majority of tRNAs encoded in the mitogenomes of the four gall crabs showed a clover-leaf secondary structure (Fig. S2). The thymine pseudouracil cytosine (TΨC) loop was

absent in tRNA-Ser1 in *T. corallicola*, while *O. hypostegus* and *H. marsupialis* s.l. only had the TΨC loop but without the TΨC arm. tRNA-Thr only had the dihydroxyuridine (DHU) arm without DHU loop in both *T. corallicola* and *K. siderastreicola*, while the same scenario was found for tRNA-Asn and tRNA-Phe in *T. corallicola*, tRNA-Asp in *O. hypostegus* and tRNA-Gly in *H. marsupialis* s.l. (Fig. S2).

Phylogenetic analysis

ML and BI generated almost identical phylogenetic topologies (Fig. 2, S3); the only difference was that *Varuna litterata* (Fabricius, 1798), *V. yui* Hwang & Takeda, 1986 and *Metaplax longipes* Stimpson, 1858 formed a well-supported clade in BI (Fig. S3), whereas *M. longipes* did not cluster with both *Varuna* species in the ML tree (Fig. 2). The superfamilies Ocypodoidea and Grapsoidea were polyphyletic and the two symbiotic superfamilies were retrieved as separate monophyletic clades with strong nodal support (ML=100, BI=1). The Pinnotheroidea clustered at the base of the phylogenetic tree, while the Cryptochiroidea lineage was located at a distant phylogenetic position compared to the position of pea crabs. The symbiotic varunid crab *A. inaequipes* clustered with *Tritodynamia horvathi* Nobili, 1905 (Macrophthalmidae) in the Grapsoidea.

Comparative analysis of mitochondrial gene order

The comparative analysis of MGO by pairwise comparison with the ancestral gene order of Brachyura detected nine rearrangement patterns in 73 thoracotreme crabs. Within Cryptochiridae, three Atlantic species *T. corallicola*, *K. siderastreicola* and *O. hypostegus* had the same gene order (pattern eight), which differs from Indo-Pacific species *H. marsupialis* s.l. (pattern seven) where *tRNA-His* changed location and was transposed in between *tRNA-Ser2* and

tRNA-Phe (Fig. 3). For Pinnotheridae, pattern one and nine were found. *Amusiotheres obtusidentatus* (Dai in Dai, Feng, Song & Chen, 1980), *Pinnotheres pholadis* De Haan, 1835 and *Pinnaxodes major* Ortmann, 1894 shared pattern one, while *Pinnotheres excussus* Dai in Dai, Feng, Song & Chen, 1980, *Arcotheres sinensis* (Shen, 1932) and *Arcotheres purpureus* (Alcock, 1900) shared pattern nine. Pattern three was only detected in *A. inaequipes*. As for the free-living crabs, five patterns including ancestor gene order of Brachyura were found across the different families. Species with the same pattern always belonged to the same clade (Fig. 2, 3).

Three out of four MGO rearrangement events, including reversals, transpositions and tandem-duplication-random-loss (TDRL), have been detected in 73 thoracotreme crabs. MGO rearrangement ~~of~~ four gall crabs was found to be caused by only one TDRL event and *A. inaequipes* exhibited this event twice, while pea crabs were detected ~~with~~ multiple transposition and reversal events. Furthermore, reversal events only occurred in three pea crab species (Fig. 3).

Selection analysis

We used one-ratio and free-ratio models to calculate selective pressure for terminal branches of the phylogenetic tree. The result of the LRT indicated that the free-ratio model fit our data significantly better than the one-ratio model for 13 PCGs ($p<0.01$; Table S7), indicating that the ω ratio of each gene for the terminal branch was different (Table S6). In particular for the free-ratio model, the average value of ω ratios for 11 PCGs was higher for the ~~obligate~~ symbionts than free-living crabs with exceptions of the other two genes *apt8* and *nad4l* (Table S8). The ω ratio of *atp6*, *cox2*, *nad3*, *nad5* and *nad6* were significantly higher in the symbiotic crabs than the free-living crabs (Fig. 4, Table S8; Wilcoxon rank sum test, $p=0.01$, $p=0.02$, $p=0.02$, $p=0.03$ and $p=0.03$, respectively). Using the branch-site model, we detected evidence of positive selection in 11 symbiotic branches. One amino acid site in the symbiotic group was found in

atp8 by BEB (posterior probability ≥ 95%), as well as fourteen amino acid sites with potential weak positive selection in *cox3*, *nad4*, *nad5* and *nad6* (Table S9).

Discussion

Origin and phylogenetic position of obligate symbiotic crabs

Phylomitogenomics confirmed Cryptochiroidea as an independent monophyletic clade - with robust branch support (ML=100, BI=1) - within the Thoracotremata (Fig. 2, S3), deserving of (super-)family rank. Earlier studies used a single Cryptochiroidea representative, hence no conclusions about the status of the superfamily could be drawn based on those trees (Wetzer et al. 2009; Sun et al. 2022; Tsang et al., 2014, 2018).

A phylomitogenomic study of the Thoracotremata by Sun et al. (2022), based on a similar dataset, retrieved the cryptochirid *H. marsupialis* s.l. as closely related to an Ocypodoidea lineage (Camptandriidae/Xenophthalmidae/Dotillidae), albeit with equivocal branch support. Our phylogenetic reconstruction - with three additional cryptochirid mitogenomes - retrieved Cryptochiroidea as a separate lineage with full support. Recently, Tsang et al. (2022) provided suggestions for taxonomic revisions at superfamilial level, and proposed three new superfamilies in addition to the currently recognised four superfamilies. Our results (Fig. 2) are largely in agreement with their multi-marker analysis, however, the lack of mitogenomic data for various families (e.g., Glyptograpsidae, Leptograpsodidae, Aphanodactylidae and Heloeciidae) and the insufficiency of representative species for several families (e.g., Plagusiidae, Mictyridae, Camptandriidae and Xenophthalmidae) do not allow us to fully compare our results with their proposed new classification. In addition, insufficient representative species within individual families was also a problem in their analysis such as a clade consisting of Leptograpsodidae,

Xenograpsidae and Glyptograpsidae. Future studies working on the classification within Thoracotremata need to contain more species given the large number of undescribed species in some families (Ma et al., 2019).

The ~~obligate~~ symbionts of gall crabs, pea crabs and Varunidae (*A. inaequipes*) were inferred to have evolved three times. The phylogenetic trees ~~of~~ ML and BI (Fig. 2, S3) reveal Cryptochiroidea and Pinnotheroidea ~~in~~ distant, independent clades, eliminating the possibility of a single origin of these obligate symbiotic crabs; a question left unanswered in the study by Tsang et al. (2022). Our result of multiple independent origins of symbiosis in the Thoracotremata is in line with Van der Meij and Schubart (2014) and Wolfe et al. (2022). Moreover, a molecular clock approach employed by Tsang et al. (2014) revealed that pea crabs evolved a symbiotic lifestyle before gall crabs. Monophyly of the Pinnotheridae as currently defined was rejected by Tsang et al. (2018, 2022). Recently Tsang and Naruse (2023) added two new pea crab families in the Pinnotheroidea; the Parapinnixidae and Tetriasidae. Mitogenomic data from these families, as well as the Aphanodactylidae ~~is~~ currently lacking, but given the distant relationships between the Aphanodactylidae and the Pinnotheroidea, symbiosis might have evolved more than three times in the Thoracotremata with one gall crab lineage, two independent pea crab lineages and a varunid lineage (Tsang et al. 2018, 2022; Tsang and Naruse, 2023). Parallel evolution of symbiosis is not unique for the Thoracotremata. It has also been observed in other taxonomic groups (e.g., Gastropoda (Goto et al. 2021); palaemonid shrimps (Horkà et al. 2016); Copepoda (Bernot et al. 2021); etc), and the switch from a free-living to a symbiotic lifestyle appears to be relatively common in marine invertebrates (Horká et al., 2016).

Sun et al. (2022) included *A. inaequipes* in their dataset, but did not recognise the species as an obligate symbiont of Holothuria (see e.g., Lee et al., 2010). The species clusters with *T.*

horvathi (Macrophthalmidae), however, Anker and Ng (2014) discussed that *T. horvathi* has more varunid than macrophthalmid affinities. A transfer of *T. horvathi* to the Varunidae would be in line with our phylogenetic reconstruction (Fig. 2, S3). Tsang et al. (2022) considered *T. horvathi* as free-living in their analysis (a decision we followed in our analyses), however, there is at least anecdotal evidence that the species is symbiotic (Otani et al., 1996). Given the close affinity between *A. inaequipes* and *T. horvathi*, a possible symbiotic status of the latter will not change the conclusion of this study that symbiosis independently evolved at least three times in the Thoracotremata.

Gene arrangement

Gene rearrangements occurred frequently in thoracotreme crabs (Fig. 3). While metazoan mitogenomes are generally conserved, exceptions exist in Mollusca (Serb and Lydeard, 2003), Echinodermata (Perseke et al., 2008), Cnidaria (Kilpert et al., 2012), and Decapoda (Tan et al., 2018; Wang et al., 2021). Interestingly, Tan et al. (2019) reported that the occurrence of MGO rearrangements, with pairwise comparisons to the ancestral arthropod ground pattern, is unevenly distributed across decapod infraorders. They reported on four MGO patterns within 37 brachyuran species, and 13 MGO patterns among 22 anomuran species. However, the diversity of the MGO patterns in Brachyura appears to be ~~an~~ underestimat~~ion~~. Here we report nine MGO patterns occurring within the Thoracotremata alone. Our pairwise comparisons were based on the ancestral brachyuran gene order (Wang et al., 2018) and not on the ancestral arthropod ground pattern, however this comparison does not affect the MGO diversity patterns. Comparative analysis confirmed that all obligate symbionts and 63% of the free-living species have undergone variable MGO rearrangement events (Fig. 2). Thoracotreme crabs could potentially exhibit a greater diversity of MGO patterns if more mitogenomes become available.

Different MGO patterns were detected in gall crabs, pea crabs and a varunid crab, despite similarities in lifestyle. TDRL took place in gall crabs, resulting in two patterns: the Indo-Pacific species *H. marsupialis* s.l. has a unique pattern (pattern seven), and the Atlantic species *K. siderastreicola, O. hypostegus* and *T. corallicola* share the same MGO pattern (pattern eight; Fig. 2). The latter three species are not closely related, hence it seems unlikely that their shared distribution explains their shared MGO (van der Meij and Klaus, 2015; van der Meij and Nieman, 2016). Transposition and reversal events were found in pea crabs, which contributes to them having two MGO patterns (patterns one and nine). Moreover, a unique pattern (three) was found in *A. inaequipes*. The different mitochondrial gene rearrangement scenarios might be related to the hosts they associate with. Gall crabs are obligate symbionts of scleractinian corals, whereas pea crabs associate with a range of invertebrate hosts but not scleractinian corals (Fize and Serène, 1957; Castro, 2015; de Gier and Becker, 2020) and *A. inqequipes* is associated with holothurians (Lee et al., 2010). Sun et al. (2022) proposed that mitochondrial gene rearrangements may correlate with the specialised lifestyles within the Thoracotremata, however, this does not agree with our results of the MGOs being mostly linked to the various thoracotreme clades (Fig. 2). In addition, the pea crab *Pinnotheres excussus*, has a different MGO pattern than *Pinnotheres pholadis* De Haan, 1835. *Pinnotheres excussus* ~~inhabit~~ *Gafrarium* sp. (Dai et al., 1980) and *P. pholadis* live in *Mytilus galloprovincialis* Lamarck, 1819 (Yoo and Takeshi, 1985). Both pea crab species are thus associated with bivalve molluscs, which questions a possible correlation between not only specialised lifestyles but also between host association and MGO rearrangements (Chow et al., 2023). The reasons contributing to variable MGO patterns observed in different lineages within the Thoracotremata are still unclear.

Identical gene orders shared by relatively distinct organisms were observed in this study. Sharing of the same MGO is extremely rare for distinct taxa with a probability of two mitochondrial genomes sharing identical derived genome organisation being of one in 2664 (Dowton et al., 2002; Babbucci et al., 2014). However, Babbucci et al. (2014) observed the ant *Formica fusca* Linnaeus, 1758 (Hymenoptera) sharing the same MGO with Ditrysia (e.g., *Parnassius bremeri* Bremer, 1864; *Aporia crataegi* (Linnaeus, 1758); *Ochrogaster lunifer* Herrich-Schäffer, 1855) which is the largest clade of Lepidoptera. Here we detected MGO pattern four in 12 grapsoids (e.g., *Parasesarma tripectinis* (Shen, 1940), *Orisarma sinense* (H. Milne Edwards, 1853), *Cristarma eulimene* (De Man in Weber, 1897); Fig. 2) and one ocypodoid crab (*Minuca minax* (Le Conte, 1855)). Pattern seven of *H. marsupialis* s.l. is shared by *S. intermedia*, a free-living crab in the Dotillidae (Fig. 2). This result is not in agreement with Sun et al. (2022), who erroneously reported the ancestor MGO pattern of Brachyura for *S. intermedia*. The occurrence of distant lineages sharing the same MGO illustrates convergent evolution in these lineages. In general, however, species sharing a same MGO always belonged to the same clade/group; this scenario has been detected in crabs (Wang et al., 2021; this study), birds (Mindell et al., 1998), insects (Babbucci et al., 2014), and other taxa. Gene order information can be used as an informative character when defining groups at various taxonomic levels (Basso et al., 2017).

MGO has been shown to effectively supplement phylogenetic analysis to investigate evolutionary systematics in invertebrates (Boore and Brown 1998; Dowton et al., 2002). Gall crabs have two distinct gene order patterns (seven and eight), just like pea crabs (one and nine; Fig. 2, 3). The symbiotic varunid *A. inaequipes* is the only crab in our dataset with pattern three. The MGOs, in addition to the phylogenetic analysis, suggests that the obligate symbionts in the

Thoracotremata are only distantly related. Combined with their different host usage this is further evidence that these lineages evolved independently. Adaptive evolution (e.g., by transitioning from a free-living to a symbiotic lifestyle) could promote MGO rearrangements as mitogenomes are more likely to be influenced by evolutionary processes compared to nuclear genes (Shen et al., 2019; Lau et al., 2021). This trend of high MGO diversity has also been found in many marine species such as: crabs (Basso et al., 2017), fishes (Poulsen et al., 2013), bivalves (Yang et al., 2019), echinoderms (Mu et al., 2018; Galaska et al., 2019) or worms (Gonzalez et al., 2021).

Selection analysis

Positive selection drives mitochondrial genes to better adapt to a symbiotic lifestyle. Previous studies have assigned the positive selection signals detected on animal mitochondrial PCGs to oxygen usage and energy metabolism as all 13 PCGs in a mitogenome are involved in aerobic metabolism (Sun et al., 2018; Shen et al., 2019; Lü et al., 2023). The branch-site model identified one amino acid site in *atp8* that had experienced positive selection in symbiotic species, which suggests that adaptive evolution of mitochondrial genes has played a key role in the increased energy cost of symbionts. In addition, the free-ratio model detected that the ω ratios for 11 PCGs were higher in symbiotic species than in free-living species. Notably, *atp6*, *cox2*, *nad3*, *nad5* and *nad6* exhibited significantly higher ratios in symbiotic species than in free-living ones. This indicates that symbiotic species have accumulated more non-synonymous mutations, resulting in advantageous amino acid changes that facilitate adaptation to their symbiotic lifestyle. Similar results have been observed in diverse taxa, including fish, birds, shrimps, insects, and others (Mitterboeck et al., 2017; Wang et al., 2017; Li et al., 2018; Shen et al., 2019). The specimens used in these investigations were based on either animal locomotion or habitat (Shen et al., 2019; Li et al., 2018; Sun et al., 2018). Our study is the first one to compare the evolutionary lifestyle

patterns of ~~the~~ symbiotic and free-living species in ~~Decapoda~~, and ~~highlights~~ the importance of mitochondrial genes in shaping the evolution of symbiotic associations.

Detected positive selection scenarios in symbiotic crabs may be caused by differences in reproduction, body size, mobility (or multiple factors) compared with free-living species. Several factors could influence the oxygen or energy usage. Cryptochiridae and Pinnotheridae have very high reproductive investment~~; symbiotic brachyurans invest a lot more energy in reproduction than~~ their free-living counterparts (Hines, 1992; Hartnoll, 2006; Bähr et al. 2021). Symbiotic crabs live associated with a host, resulting in little movement during the adult stage. The process of settlement as a megalopa~~e~~, however, involves swimming or crawling to reach a suitable location on the host organism which requires high energy expenditure. At the same time, the symbiotic crabs need to expend energy to overcome the host defences, such as the physical barrier on the surface of the host (e.g., Simon-Blecher et al., 1999). The positive selection observed in the mitogenome of the symbiotic crabs could be attributed to these unique ecological and physiological characteristics compared to their free-living counterparts.

Conclusions

The symbiotic lifestyle of Cryptochiroidea, Pinnotheroidea and the varunid *A. inaequipes* evolved independently, thus symbiosis evolved at least three times in the Thoracotremata. The recent inclusion of two additional pea crab families (Tsang and Naruse, 2023), highlights the need for more genetic data on pea crabs (ideally mitogenomes) to study the monophyly of the Pinnotheroidea and the position of the Aphanodactylidae (see Tsang et al., 2022; Tsang and Naruse, 2023). Furthermore, our gene arrangement analysis (Fig. 2, 3, S1) shows how, in general, the MGO pattern is stable between clades, highlighting the potential usefulness of this

analysis in phylogenetic studies. Lastly, our selection analysis indicates a positive selection for several genes in obligately symbiotic crabs in the Thoracotremata. Currently little is known about the exact role and function of these genes, although they are likely linked to aerobic metabolism. Further research is needed to explore the possible link between mobility, reproduction, body size or other factors that are at play in these positive selection scenarios.

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753

Figure 1

The mitochondrial genome maps.

The mitochondrial genome maps of: A) *Kroppcarcinus siderastreicola*; B) *Opecarcinus hypostegus*; C) *Troglocarcinus corallicola;* and D) *Hapalocarcinus marsupialis* s.l. The abbreviated names of mitochondrial transfer RNA genes can be found in Table S2.

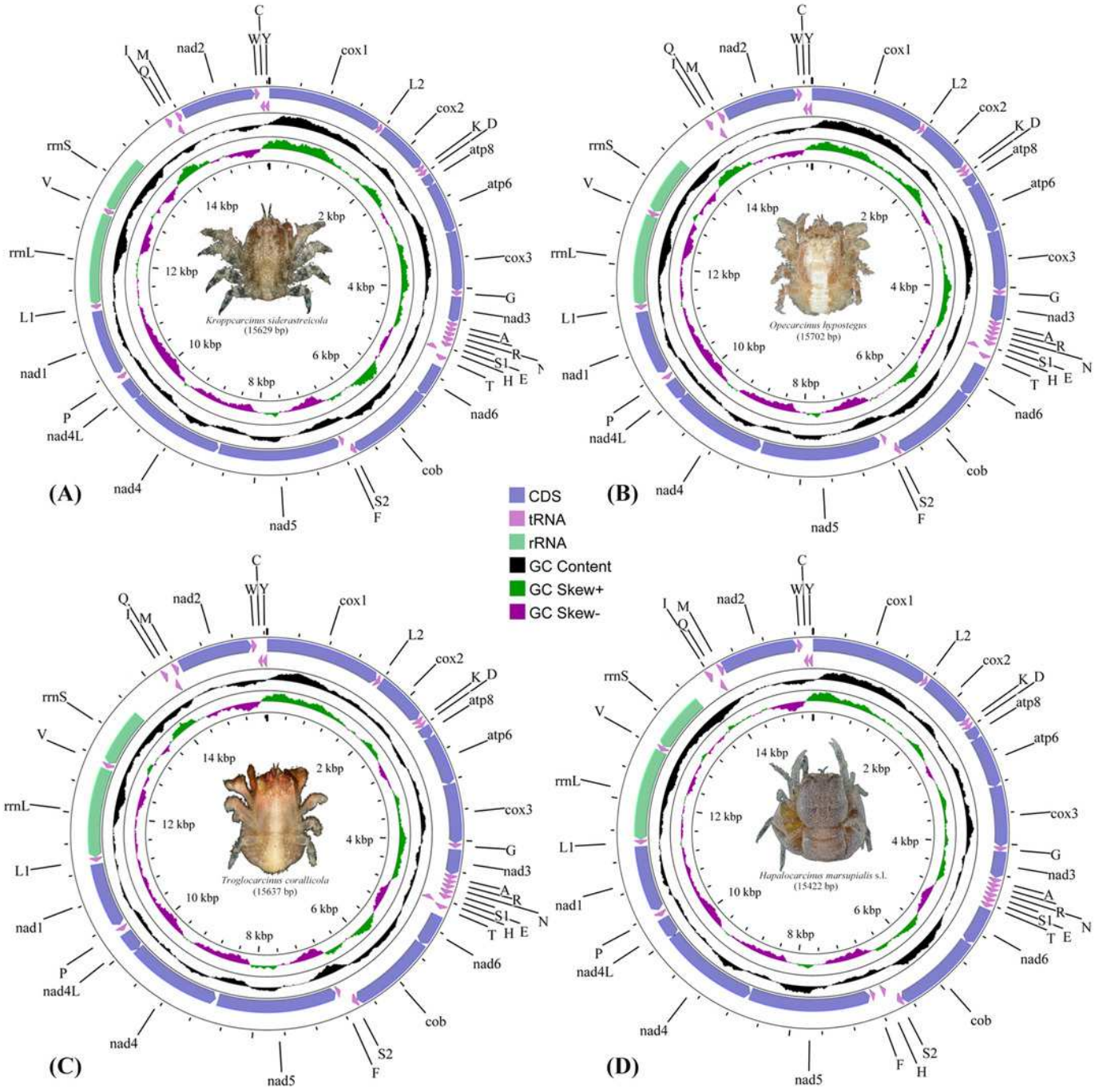
(A)
Kroppcarcinus siderastreicola
(15629 bp)
(B)
Opecarcinus hypostegus
(15702 bp)
(C)
Troglocarcinus corallicola
(15637 bp)
(D)
Hapalocarcinus marsupialis s.l.
(15422 bp)
CDS
tRNA
rRNA
GC Content
GC Skew+
GC Skew-
14 kbp
2 kbp
12 kbp
4 kbp
10 kbp
6 kbp
8 kbp
C
WY
cox1
L2
cox2
K
D
atp8
atp6
cox3
G
nad3
A
R
S1
T
H
E
N
nad6
cob
S2
F
nad5
nad4
nad4L
P
nad1
L1
rrnL
V
rrnS
Q
I
M

Figure 2

Phylogenetic tree with mitochondrial gene order information and lifestyle of each species.

Maximum likelihood (ML) tree based on concatenated genes including 13 PCGs and two rRNA genes. The branch support values are displayed for the major nodes. Values at the branches refer to Bootstrap (BP) values of ML and Posterior Probability (PP) of BI. The stars on either side of the slash indicate that branch support values are under 80 (one star represents BP and two stars represent PP). Terminal tips link mitochondrial gene rearrangement patterns (detailed patterns can be found in Figure 3) detected by CREx. The leftmost column of the heatmap refers to ancestor mitochondrial gene order of Brachyura, and the remaining columns refer to detailed rearrangement events for each terminal species. TDRL is the abbreviation for tandem-duplication-random-loss.

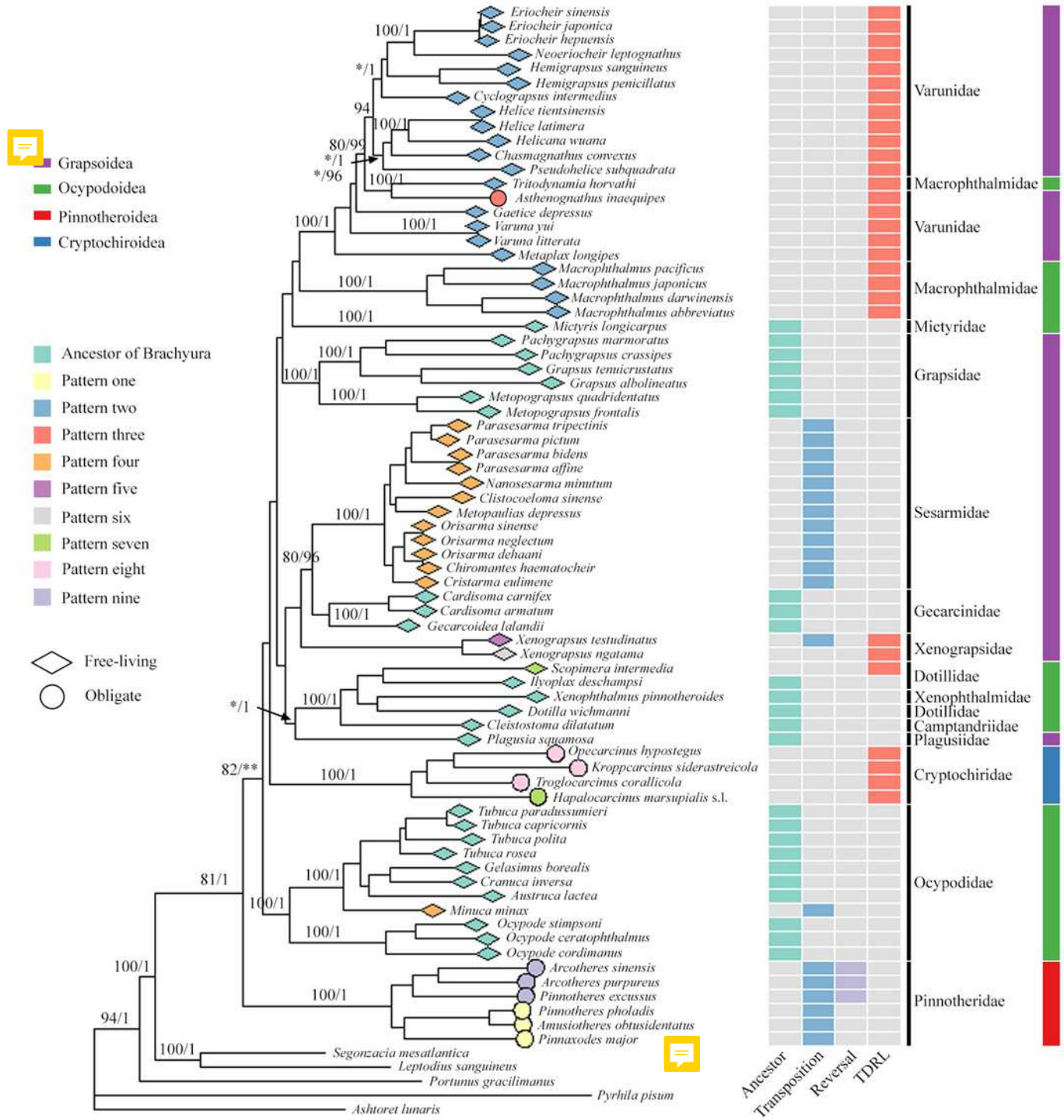
Grapsoidea
Ocypodoidea
Pinnotheroidea
Cryptochiroidea
Ancestor of Brachyura
Pattern one
Pattern two
Pattern three
Pattern four
Pattern five
Pattern six
Pattern seven
Pattern eight
Pattern nine
Free-living
Obligate
Eriocheir sinensis
Eriocheir japonica
Eriocheir hepuensis
Neoeriocheir leptognathus
Hemigrapsus sanguineus
Hemigrapsus penicillatus
Cyclograpsus intermedius
Helice tientsinensis
Helice latimera
Helicana wuana
Chasmagnathus convexus
Pseudohelice subquadrata
Tritodynamia horvathi
Asthenognathus inaequipes
Gaetice depressus
Varuna yui
Varuna litterata
Metaplax longipes
Macrophthalmus pacificus
Macrophthalmus japonicus
Macrophthalmus darwinensis
Macrophthalmus abbreviatus
Mictyris longicarpus
Pachygrapsus marmoratus
Pachygrapsus crassipes
Grapsus tenuicrustatus
Grapsus albolineatus
Metopograpsus quadridentatus
Metopograpsus frontalis
Parasesarma tripectinis
Parasesarma pictum
Parasesarma bidens
Parasesarma affine
Nanosesarma minutum
Clistocoeloma sinense
Metopaulias depressus
Orisarma sinense
Orisarma neglectum
Orisarma dehaani
Chiromantes haematocheir
Cristarma eulimene
Cardisoma carnifex
Cardisoma armatum
Gecarcoidea lalandii
Xenograpsus testudinatus
Xenograpsus ngatama
Scopimera intermedia
Ilyoplax deschampsi
Xenophthalmus pinnotheroides
Dotilla wichmanni
Cleistostoma dilatatum
Plagusia squamosa
Opecarcinus hypostegus
Kroppcarcinus siderastreicola
Troglocarcinus corallicola
Hapalocarcinus marsupialis s.l.
Tubuca paradussumieri
Tubuca capricornis
Tubuca polita
Tubuca rosea
Gelasimus borealis
Cranuca inversa
Austruca lactea
Minuca minax
Ocypode stimpsoni
Ocypode ceratophthalmus
Ocypode cordimanus
Arcotheres sinensis
Arcotheres purpureus
Pinnotheres excussus
Pinnotheres pholadis
Amusiotheres obtusidentatus
Pinnaxodes major
Segonzacia mesatlantica
Leptodius sanguineus
Portunus gracilimanus
Pyrhila pisum
Ashtoret lunaris
Varunidae
Macrophthalmidae
Varunidae
Macrophthalmidae
Mictyridae
Grapsidae
Sesarmidae
Gecarcinidae
Xenograpsidae
Dotillidae
Xenophthalmidae
Dotillidae
Camptandriidae
Plagusiidae
Cryptochiridae
Ocypodidae
Pinnotheridae
Ancestor
Transposition
Reversal
TDRL

Figure 3

Mitochondrial gene order patterns detected by CREx for four gall crabs, six pea crabs, one varunid and 62 free-living thoracotreme crabs.

The solid, coloured circles refer to the number and type of gene rearrangement events. The abbreviated names of mitochondrial transfer RNA genes can be found in Table S2.

Figure 4

Comparison of average ω ratios for 13 individual protein coding genes generated by free-ratio model using CodeML application in PAML between symbiotic and free-living crabs within Thoracotremata.

Red stars refer to the ω ratio in symbiotic species is significantly higher than the one in free-living species ($p<0.05$). The outliers are hidden for better visualisation.