

1 **Phylogenomic analyses shed light on the relationships of chiton superfamilies and**  
2 **shell-eye evolution**

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14

15 **Abstract**

16 Mollusca is the second-largest animal phylum with over 100,000 species among eight distinct  
17 taxonomic classes. Across 1000 living species in the class Polyplacophora, chitons have a  
18 relatively constrained morphology but with some notable deviations. Several genera possess  
19 “shell eyes”, true eyes with a lens and retina that are embedded within the dorsal shells,  
20 which represent the most recent evolution of animal eyes. The phylogeny of major chiton  
21 clades is mostly well established, in a set of superfamily and higher-level taxa supported by  
22 various approaches including multiple gene markers, mitogenome-phylogeny and  
23 phylotranscriptomic approaches as well as morphological studies. However, one critical  
24 lineage has remained unclear: *Schizochiton* was controversially suggested as a potential  
25 independent origin of chiton shell eyes. Here, with the draft genome sequencing of  
26 *Schizochiton incisus* (superfamily Schizochitonoidea) plus assembly of transcriptome data  
27 from other polyplacophorans, we present phylogenetic reconstructions using both  
28 mitochondrial genomes and phylogenomic approaches with multiple methods. Phylogenetic  
29 trees from mitogenomic data are inconsistent, reflecting larger scale confounding factors in  
30 molluscan mitogenomes. A consistent robust topology was generated with protein coding  
31 genes using different models and methods. Our results support Schizochitonoidea is a sister  
32 group to other Chitonoidea in Chitonina, in agreement with established classification. This  
33 suggests that the earliest origin of shell eyes is in Schizochitonoidea, which were also gained  
34 secondarily in other genera in Chitonoidea. Our results have generated a holistic review of  
35 the internal relationship within Polyplacophora, and a better understanding on the evolution  
36 of Polyplacophora.

37

38 **Keywords:** Polyplacophora, Chiton, Phylogenomics, Mollusca, shell eyes

## 39 **Introduction**

40 Molluscs represent the second most species rich animal phylum with the broadest  
41 morphological disparity of body plans. The class Polyplacophora, also known as chitons,  
42 includes around 1000 living species and over 400 fossil species (Stebbins et al. 2009).  
43 Chitons are exclusively marine, and their most distinctive feature is eight separate aragonitic  
44 valves or plates on their dorsal side (Ladd 1966; Stebbins et al. 2009; Irisarri et al. 2020).  
45 They attach to the substratum with a muscular ventral foot and feed with an iron-mineralised  
46 radula (Joester et al. 2016). They have no head or cephalised senses, and therefore lack  
47 conventional eyes. However, the dorsal valves are densely innervated with a complex array of  
48 sensory pores called aesthetes which can have densities of over 1000 mm<sup>-2</sup>.

49  
50 Aesthete pores are present in all chitons, with substantial differences in morphology, size,  
51 arrangement, densities, and presumably also functions, and aesthete morphology is often used  
52 to discriminate species in taxonomic descriptions (Sirenko 2006). A number of chiton species  
53 are demonstrably photosensitive, and some have pigmented aesthetes that apparently function  
54 as photoreceptors. In the most elaborate variation, in a few genera, some of the larger  
55 “megalaesthete” pores have further developed into shell eyes. These are true eyes, embedded  
56 in the shell matrix, with a crystalline lens and a pigmented photoreceptive retina (Sigwart et  
57 al. 2021).

58  
59 The evolution of chiton shell eyes occurred much more recently than any other animal eyes.  
60 The oldest fossil shell eyes are known from the fossil genus *Incissiochiton* from the lower  
61 Palaeocene (61-66 Mya), which is a member of the family Schizochitonidae, the only family  
62 in a superfamily Schizochitonoidea (Sirenko 2006; Sirenko 2013). Members of  
63 Schizochitonidae (*Incissiochiton* and the Recent genus *Schizochiton*), as well as species in the  
64 two subfamilies Acanthopleurinae and Toniciinae, possess shell eyes. The only previous  
65 molecular phylogenetic study that included *Schizochiton* dates back to 2003 with five gene  
66 fragments (Okusu et al. 2003), and those authors suggested that the phylogenetic position of *S.*  
67 *incisus* in those analyses was “unstable” and deserved further discussion. Most importantly,  
68 the unresolved phylogenetic position of *Schizochiton* raised the possibility that shell eye  
69 structures evolved not only relatively recently, but in two separate events. However, in the  
70 last 20 years this hypothesis has not been tested further, due to a lack of appropriate specimen  
71 material for molecular data from this important lineage *Schizochiton*.

72  
73 Phylogenetic systematics of Polyplacophora has been developed using both morphological  
74 and molecular characters (Albano 2021). Extant chitons are divided into three well-resolved  
75 orders: Lepidopleurida, Callochitonida, and Chitonida (Giribet et al. 2020). Lepidopleurida  
76 consists of mainly deep-sea species with distinctive morphological synapomorphies including

77 aesthete arrangement, gills, and a specialized sense organ called the Schwabe Organ (Sigwart  
78 et al. 2014). The position of *Callochiton* was equivocal in earlier studies but usually resolved  
79 as sister to Chitonida (Koch et al. 1990; Sigwart et al. 2013) and the single family  
80 Callochitonidae is now recognized as comprising a separate order-ranked clade  
81 Callochitonida (Sigwart et al. 2013; Giribet et al. 2020; Moles et al. 2021). Most living  
82 chitons are in the order Chitonida, which is further divided into two suborders, Chitonina  
83 (including two superfamilies, Chitonoidea and Schizochitonoidea) and Acanthochitonina  
84 (including two superfamilies, Mopaliaoidea and Cryptoplacoidea). The backbone phylogeny  
85 of chitons is well understood especially at the level of superfamilies, for all clades except for  
86 Schizochitonoidea.

87

88 Various genomic and transcriptomic data in Polyplacophora are now available on NCBI but  
89 were generated independently for several different research purposes (Table 1). There are  
90 only two chiton genomes available, *Acanthopleura granulata* (Varney et al. 2021) and  
91 *Hanleya hanleyi* (Varney et al. 2022). Meanwhile, two independent phylogenomic studies  
92 based on transcriptome sequencing, generated data for species and genera that cover all  
93 Recent superfamilies: *Callochiton*, *Tonicia schrammi*, *Chiton tuberculatus*, *Chiton*  
94 *marmoratus*, *Chaetopleura apiculata*, *Lepidozonia mertensii*, *Mopalia muscosa*, *Katharina*  
95 *tunicata*, *Tonicella lineata*, *Nutallochiton* sp., *Cryptoplax japonica* and *Cryptoplax*  
96 *larvaeformis* (Varney et al. 2021) and *Lepidopleurus cajetanus* (SRX5063921), *Callochiton*  
97 *septemvalvis*, *Stenoplax bahamensis*, *Cryptoplax japonica* and *Choneplax lata* (Moles et al.  
98 2021). There are also some other studies examining the gene expression profiles, which  
99 includes *Leptochiton cascadiensis* (Halanych et al. 2014), *Acanthopleura lochooana* (Liu et  
100 al. 2022), *Rhyssoplax olivacea* (Riesgo et al. 2012), *Cryptochiton stelleri* (Nemoto et al.  
101 2019), *Acanthochitona crinita* (De Oliveira et al. 2016), *Acanthochitona rubrolineata*  
102 (SRP179406) and *Acanthochitona fascicularis* (SRR13862580). These data collection can  
103 support a phylogenomic construction with larger taxon coverage. And due to the important  
104 position of *Schizochiton* for us to better understand chiton evolution, we newly sequenced and  
105 assembled the genome and mitogenome of *Schizochiton incisus*. Combining this with other  
106 available chiton data from NCBI and previous studies, we aimed to reconstruct a phylogeny  
107 of Polyplacophora at the superfamily level with different phylogenomics inferences and tree  
108 reconstruction methods, specifically to test the position of *S. incisus* and Schizochitonoidea.

109

## 110 **Material and Methods**

### 111 **1. Sample collection**

112 All genomes and transcriptomes used in this study are listed in Table 1. To increase taxon  
113 sampling, we newly sequenced an individual of *Schizochiton incisus* and also *Leptochiton*  
114 *asellus*. *Schizochiton incisus* was collected from a rock on a coral reef at the depth of 80 m of

115 Livock Reef (10°10'N, 115°19'E) by fishing net in the South China Sea on July 11, 2020 (Fig.  
116 1). The whole animals *S. incisus* was preserved in 95% EtOH, which was later stored at room  
117 temperature, and a small piece of girdle tissue was removed for DNA extraction. The *S.*  
118 *incisus* sample was deposited in the malacology collections at the Senckenberg Museum,  
119 Frankfurt with catalogue number SMF 386201. *Leptochiton asellus* was collected on the  
120 rocky shore in September 2019, at Ballyhenry Island, Strangford Lough, at Portaferry, N.  
121 Ireland. For *L. asellus*, five tissues, including foot, perinotum, aesthetes, viscera, and shell  
122 edge were dissected and fixed in RNAlater (ThermoFisher) at 4 degree and transferred to -80  
123 deep freezer for storage.

124

## 125 **2. Genome and RNA sequencing**

126 Total genomic DNA of *S. incisus* was extracted with a DNeasy Blood & Tissue Kit (QIAGEN,  
127 Germantown, Maryland), which was further sequenced for 150bp paired-end Illumina  
128 sequencing to generate approximately 40Gb of raw data on NovaSeq 6000 platform at  
129 Novogene (Beijing).

130

131 RNA of *Leptochiton asellus* was extracted using Trizol (ThermoFisher) and sent to Novogene  
132 (Beijing) for Eukaryotic type transcriptome library preparation and further sequenced on  
133 NovaSeq 6000 platform. Approximately 6Gb of raw reads were generated for each tissue.

134

## 135 **3. Mitogenome analysis**

### 136 **3.1 Mitogenome assemble and annotation**

137 The raw data were trimmed using Trimmomatic v.0.39 (Bolger et al. 2014) with strict  
138 filtering settings (ILLUMINACLIP: adapters.fa:2:30:10 LEADING:20 TRAILING:20  
139 SLIDINGWINDOW:4:20 MINLEN:140) to remove low-quality reads and adapters  
140 contaminated reads. The resultant clean reads were initially assembled by SPAdes v.3.15.3  
141 (Prjibelski et al. 2020) with default settings, and then the partial COI sequence of *S. incisus*  
142 was extracted from the assembled contigs, which was later used as the “seed input” in  
143 NOVOplasty v.4.2 (Dierckxsens et al. 2016) to obtain the complete mitogenome of *S. incisus*.  
144 The mitogenome was then annotated using the MITOS web server (Donath et al. 2019) with  
145 the invertebrate genetic code and the rest default settings, followed by a manual mitogenome  
146 annotation confirmation by comparing with other chiton mitogenomes (Irisarri et al. 2020).

147

### 148 **3.2 Matrix construction**

149 All .gb files of chiton mitogenomes available on NCBI were downloaded and imported into  
150 Phylosuite v.1.2.2 (Zhang et al. 2020), which is an application that allows users to perform  
151 phylogenetic analyses on relatively small datasets. All procedures of mitogenome  
152 phylogenetic analyses, except for tree constructing and visualization, were carried out

153 through Phylosuite built-in plugins. In brief, 13 protein-coding genes and 2 rRNA genes were  
154 extracted from the chiton mitogenomes. Afterwards, MAFFT v. 7.471 was used to align  
155 sequences, followed by trimAL v. 1.2rev57 with the “automated1” option to remove spurious  
156 sequences and misaligned regions. After that, trimmed sequences were concatenated,  
157 generating 3 different matrices. Amino acid sequences of 13 protein-coding genes (PCGs)  
158 were extracted and concatenated into a Matrix1. As for Matrix2, all nucleotides of 13 PCGs  
159 and 2 rRNA were concatenated. To avoid the phylogenetic signal saturation on the third  
160 codon, the third codons of 13 PCGs were replaced by degenerate bases (A, G replaced by R  
161 and C, T replaced by Y), then these modified sequences were concatenated, named Matrix3.  
162 Generated gene matrix and the corresponding partition file were later used for maximum  
163 likelihood (ML) and Bayesian inference (BI) tree construction.

164

### 165 **3.3 Mitogenome phylogeny**

166 For the ML framework, IQ-Tree v.2.1.3 (Minh et al. 2020) was implemented using -MFP to  
167 select the best-fit model for each partition. Besides, an additional empirical profile mixture  
168 model, C60, was also carried out on the AA matrix (Matrix1). All ML analysis were  
169 performed with 1000 replicates of ultrafast bootstrapping (-bb 1000).

170

171 BI was carried out using PhyloBayes MPI v.1.8c (Lartillot et al. 2013) with CAT-GTR+Γ4  
172 models. For each matrix, four independent Monte Carlo Markov chains (MCMC) were run  
173 simultaneously and convergence was checked with the bpcomp program. Then a consensus  
174 tree was obtained after discarding the first 10% cycles as a burn-in.

175 All trees obtained were then visualized with Figtree  
176 (<http://tree.bio.ed.ac.uk/software/figtree/>).

177

### 178 **4. Genome assembly and annotation**

179 The Illumina raw data was filtered with Trimmomatic v.0.39 (Bolger et al. 2014) with  
180 settings of “PE ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:10 TRAILING:10  
181 SLIDINGWINDOW:4:15 MINLEN:40”. Afterwards, genome features were calculated by  
182 using jellyfish v.2.3.0 (Marçais et al. 2011) (19mer) and GenomeScope2 (Vurture et al. 2017).  
183 A benchmark of commonly used assemblers for Illumina data, including Platanus v.1.2.4  
184 (Kajitani et al. 2014) and MaSuRCA v.4.0.3 (Zimin et al. 2013), was performed based on  
185 BUSCO v.5.1.2 score by searching against metazoan odb10 database. Afterwards, purge-dups  
186 v.1.2.5 (Guan et al. 2020) was used to remove redundant contigs, and the resultant contigs  
187 were further scaffolded by using PEP-scaffolder (Zhu et al. 2016) with the help of protein  
188 sequences from the concatenation of the genome of *Acanthopleura granulata* (Varney et al.  
189 2021).

190

191 A custom repeat library of *S. incisus* was *de novo* generated by RepeatModeler v.2.0.2a  
192 (Flynn et al. 2020). RepeatMasker v.4.1.0 (Tarailo-Graovac et al. 2009) was performed with  
193 the species-specific repeat library mentioned above, followed by a second round of  
194 RepeatMasker but with Repbase library 2018 (<https://www.girinst.org/replib/>). Afterwards,  
195 BRAKER v.2.1.6 (Hoff et al. 2019) was run to train an *ab initio* gene predictor Augustus  
196 v.3.4.0 (Stanke et al. 2006) with ODB10 v.1 database downloaded from OrthoDB  
197 (Kriventseva et al. 2018), generating a config file of *S. incisus*, which was used as one piece  
198 of evidence while running the genome annotator MAKER v.3.01.04 (Holt et al. 2011).  
199 Because there was no transcript evidence available, all Mollusca proteins on NCBI were  
200 downloaded (Date: Jan 20 2022), and redundancy was removed with CD-HIT v.4.8.1 with the  
201 setting of “-c 0.9”. These protein sequences were regarded as the protein homology evidence  
202 in MAKER. And the proteins generated from MAKER was used for further phylogenetic  
203 analyses.

204

## 205 **5. Transcriptome assembly and filtration**

206 The protein coding genes of *Acanthopleura granulata*, *A. loochooana* (Liu et al. 2022) and  
207 all other available transcriptomes were downloaded from NCBI SRA database. For  
208 transcriptome SRA datasets as well as the transcriptome sequencing of *L. asellus*, the raw  
209 reads were *de novo* assembled in Trinity v.2.13.2 or v.2.14.0 (Haas et al. 2013), using the  
210 “--trimmomatic” setting, followed by one round of CD-HIT v.4.8.1 (Fu et al. 2012) with the  
211 strictest threshold (-c 0.8) to remove redundant sequences. CD-HIT was run multiple times  
212 which was continuously monitored by BUSCO5 aiming to get a best score with highest “S”  
213 score and lowest “D” (duplicated BUSCO) score. Afterwards, Transdecoder v.5.5.0 (Douglas  
214 2018) was performed to search for open reading frames with the “--single\_best\_only” option.  
215 And the generated peptide files were filtered using CD-HIT with the “-c 0.8” option again to  
216 make sure the “D” score wouldn’t drop any more. This step aimed to remove as many  
217 heterozygous and transcript isoforms as possible so that they would not mislead orthology  
218 inference.

219

## 220 **6. Orthology inference and matrix construction**

221 Orthology inference was accomplished with a pipeline that was generated from former  
222 studies (Kocot et al. 2017; Sun et al. 2021) with slight modifications. We ran Orthofinder  
223 v.2.5.4 (Emms et al. 2019) to search for orthologues within selected taxa. Then in the  
224 “Orthogroup\_Sequences” directory of the Orthofinder output, OG heads were fixed with a  
225 custom shell script to make sure that the orthology inference pipeline could be error less.  
226 After the preparation, PREQUAL v.1.02 (Whelan et al. 2018) was used to detect and mask  
227 non-homologous characters. Then sequences shorter than 100 amino acids were deleted.  
228 Occupancy was set to 50%, and redundant sequences were then removed with another custom

229 shell script named `uniqHaplo.pl`. The leftover `.fasta` files were aligned using MAFFT v.7.490  
230 (Kato et al. 2013) with default settings. Afterwards, HmmCleaner (Di Franco et al. 2019)  
231 was used to remove misaligned regions, followed by trimming alignment with BMGE v.1.12  
232 (Crisuolo et al. 2010). Then FastTree2 (Price et al. 2010) was used to construct fast-ML  
233 trees for each remaining OGs. Last but not least, PhyloPyPruner v.1.2.4  
234 (<https://pypi.org/project/phylo-pypruner>) was performed to identify putative orthology  
235 sequences based on the former FastTree2 result, resulting in an initial matrix containing 3593  
236 OGs.

237

238 We performed genesortR (Mongiardino Koch 2021) to sort and select “best” OGs based on  
239 seven commonly used phylogenetic gene properties, thus genes with best phylogenetic  
240 signals can be used for down streaming analysis. An ML tree for the initial matrix was  
241 constructed with the IQ-Tree “-MFP” model as input. Also, ML trees for each gene were  
242 constructed in IQ-Tree with the same settings. At last, four matrices, including an initial  
243 matrix (Matrix1), best 800 genes matrix (Matrix2), best 1300 genes matrix (Matrix3), and  
244 best 2700 genes matrix (Matrix4) generated by genesortR, were prepared for phylogenetic  
245 analysis.

246

## 247 **7. Phylogenomics**

248 ML phylogenetic analysis was performed using IQ-Tree 2 (Minh et al. 2020) on the four  
249 matrices generated above. The ML approach was carried out using the best-fitting model for  
250 each partition (-m MFP). Regarding the `.contree` file generated by the MFP model as the  
251 guide tree, PMSF model was then performed in IQ-Tree 2 with site-specific frequency  
252 models (C20, C40 and C60). All ML analyses were carried out with 1000 ultrafast bootstrap.  
253 As for BI analysis, all matrices mentioned above were too large to run in PhyloBayes MPI  
254 v.1.8c, thus the fifth matrix, produced by random 300 genes from Matrix1, was brought out.  
255 Four independent chains were run simultaneously until convergent with CAT-GTR+Γ4  
256 model.

257 A coalescent approach, in contrast to concatenated-based phylogenetic analysis, was also  
258 performed to evaluate evolutionary relationships in polyplacophora with ASTRAL v.5.7.1  
259 (Sayyari et al. 2016). An AU-test was performed with IQ-tree 2 on two topologies, which  
260 were ((Chitonoidea, Schizochitonoidea), Acanthochitonina) and ((Acanthochitonina,  
261 Schizochitonoidea), Chitonoidea), respectively.

262

## 263 **Results**

### 264 **Mitochondrial genome**

265 We assembled the complete mitochondrial genome of *S. incisus*, which was 15,491 bp in  
266 length circularized with 13 PCGs, 2 rRNA, and 22 tRNA, a typical mitogenome architecture

267 of bilaterians. Protein-coding genes are coded with normal invertebrate mitochondrial codons  
268 including the start and stop codons. The mitogenome of *S. incisus* follows the proposed  
269 hypothetical ancestral gene order for Polyplacophora (Irisarri et al. 2020), except for an  
270 inversion of trnG-trnE (Fig. 2b). The mitogenome gene order seems to be relatively  
271 conserved in Polyplacophora compared to those in gastropods or bivalves (Irisarri et al.  
272 2020).

273

### 274 **Mitochondrial phylogeny**

275 The phylogenetic trees reconstructed with mitogenome data showed significant discordance  
276 among different methods and matrices. There were 3 distinct topologies for the position of *S.*  
277 *incisus*, which were ((Chitonoidea, Schizochitonoidea), Acanthochitonina)(13PCGs with  
278 MFP, PB based on modified 3rd codon), ((Acanthochitonina, Schizochitonoidea),  
279 Chitonoidea) (13PCGs with C60, PB, PCGs + rRNA with MFP, PCGs + rRNA with PB) and  
280 ((Chitonoidea, Acanthochitonina), Schizochitonoidea) (modified 3rd codon), respectively.  
281 The statistical support of the *S. incisus* node was lower than 95% in all methods, except for  
282 BI, indicating these nodes were not well supported with mitogenomic data. We note that in  
283 addition to *Schizochiton*, the position of *Plaxiphora albida* also varied from one clade to  
284 another (Fig. 2a). And in the presentative tree, *Tonicina zschau*i was sister to the rest  
285 Chitonoidea.

286

### 287 **Genome and transcriptome assembly**

288 Genome features of *S. incisus* were estimated with Illumina sequencing reads, which resulted  
289 in an estimated genome size of 1.1 GB and genome heterozygosity of 0.93%. Draft genome  
290 assembly from MaSuRCA generated a better result (C:73.8% [S:68.1%, D:5.7%]) than the  
291 Platanus version [C:17.9% (S:13.7%, D:4.2%)], which was used for down-stream analyses.  
292 After further scaffolding with protein sequences from other chitons with available genomes  
293 and removing heterozygous contigs, the final assembly has a BUSCO score of C:73.8%, N50  
294 of 13.2Kb and the assembled size of 971 Mb.

295

296 By collecting the evidence from the *ab initial* method and protein evidence, a total of 23,444  
297 protein coding genes were predicted in *S. incisus* with a BUSCO score of C: 40.8% (S: 37.0%,  
298 D: 3.8%) and F: 19.8%. Though the score is lower than the *Acanthochitona rubrolineata*  
299 genome (Varney et al. 2021), 12,419 of them (52%) can find their reciprocal best hits BLAST  
300 in *A. rubrolineata*, suggesting that a good coverage of protein coding genes for the  
301 phylogenomic analyses.

302

303 The transcriptome of *Leptochiton asellus* generated from five tissues was assembled into  
304 390,724 contigs with an N50 value of 1.68Kb, and the BUSCO score is C:94.5%



305 (S:83.1%,D:11.4%). For the rest transcriptome assembly of the publicly available data, the  
306 BUSCO completeness ranges from 12.9% (*Lepidopleurus cajetanus*) to 95.8% (*Callochiton*  
307 *septemvalvis*) (for the species list and their corresponding BUSCO score, see Table 1).

308

### 309 **Phylogenomics**

310 The phylogenomic analysis was based on the combination of transcriptome and genome data,  
311 covering all the extant superfamilies in Polyplacophora (Table 1). There were four matrices  
312 generated by genesortR forming seven distinct phylogenetic signals. Minimum occupancy for  
313 all matrices was set to 50%. The sites contained in the four matrices are 696,897 (3593 genes,  
314 all genes, Matrix 1), 194,356 (best 800 genes, Matrix 2), 299,710 (best 1300 genes, Matrix 3),  
315 554,857 (best 2700 genes, Matrix 4), respectively (Fig. 3).

316

317 The phylogenetic trees reconstructed from nuclear data, including coalescent approach results,  
318 showed a high degree of consistency about the position of *S. incisus*, as sister to Chitonoidea  
319 (Fig. 4). Support for this Schizochitonoidea + Chitonoidea clade retrieved node support of  
320 100% in all analyses except for PMSF-C20 of Matrix1 (which is 59), showing a relatively  
321 stable topology. The support for all superfamily level groups and their arrangement was  
322 consistently high. However, the positions of some tips are unsettled: *Chaetopleura apiculata*,  
323 *Lepidozonia mertensii* and *Stenoplax bahamensis* resolved in variable positions within the  
324 superfamilies. The relationship of *Choneplax* relative to the members of genus  
325 *Acanthochitona* is also changeable.

326

### 327 **Topology test**

328 We performed AU-test on two topologies based on Matrix1 to determine the better supported  
329 tree topology. Given results with  $P$ -value  $< 0.05$  will be rejected. The results showed that the  
330 first tree topology ((Chitonoidea, Schizochitonoidea), Acanthochitonina) was accept with a  
331  $P$ -value of 0.952, and the second topology ((Acanthochitonina, Schizochitonoidea),  
332 Chitonoidea) was rejected with a  $P$ -value of 0.0476.

333

### 334 **Discussions**

335 The phylogenetic relationships of chiton at the order and superfamily levels are relatively  
336 stable and well resolved. Based on a consensus of phylogenetic analyses, Polyplacophora is  
337 divided into three orders, Lepidopleurida, Callochitonida and Chitonida (Irisarri et al. 2020;  
338 Moles et al. 2021), which is also recovered in the present analyses. At the superfamily level,  
339 former molecular studies lacked data to test the position of Schizochitonoidea, and our results  
340 support the sister relationship of Chitonoidea + Schizochitonoidea in a monophyletic  
341 suborder Chitonina, as proposed from integrated morphological and anatomical evidence  
342 (Sirenko 2006).

343

344 The mitogenome data were much less informative than nuclear transcriptome and genomic  
345 data. We used mitogenome data of available chitons to reconstruct phylogenetic trees with  
346 different approaches, including ML and Bayesian inference, but the results below superfamily  
347 level are unstable. For example, in the representative tree selected for mitochondrial analyses,  
348 *Tonicina zschau* formed a sister group to other remaining Chitonoidea, whereas current  
349 systematics would predict a placement for *Tonicina* within the small clade formed by the  
350 genera *Lepidozona*, *Ischnochiton* and *Chaetopleura*. The topology we illustrated is not  
351 supported by 4 of 7 trees reconstructed by corresponding methods, so this placement should  
352 be taken as unresolved. As already suggested in the previous mitogenome phylogeny of  
353 chitons (Irisarri et al. 2020), this could be a result by poor taxon sampling, but it was not  
354 improved by adding a few additional taxa here. Indeed, this issue of low phylogenetic signal  
355 in mitogenome phylogeny has also been raised in data from another molluscan class,  
356 Monoplacophora (Stoger et al. 2016), and confounding features occur in many molluscan  
357 mitogenomes (Ghiselli et al. 2021).

358

359 Interestingly, *Schizochiton* possesses a unique mitogenome gene order, differing from any  
360 other chitons with available mitogenomes, which might imply relatively fast evolution of the  
361 species. Mitogenome phylogenies are currently not reliable for reconstructing detailed  
362 phylogenies for Polyplacophora and potentially other molluscan clades. This may be  
363 improved with better taxon sampling, or may be a fundamental problem of insufficient  
364 phylogenetic signal. It is clear that currently phylogenomic approaches are needed to  
365 reconstruct phylogeny of chitons at or below superfamily level resolution.

366

367 All the phylogenomic results for the main lineage in this study shared the same topology with  
368 strong node support except for Matrix1-C20. The topology is consistent with what is by now  
369 a well-established backbone phylogeny for Polyplacophora and also concordant at  
370 superfamily and higher level with the mitogenome phylogeny (Sigwart et al. 2013; Irisarri et  
371 al. 2020; Moles et al. 2021). Lepidopleurida is sister to the remaining Polyplacophora.  
372 *Callochiton*, representing the order Callochitonida is sister to Chitonida. This latter order is  
373 divided into two clear clades representing the suborders Chitonina and Acanthochitonina.

374

375 Our phylogeny of polyplacophora based on phylogenomic approach possesses more  
376 advantages than former molecular studies (Okusu et al. 2003; Sigwart et al. 2013; Irisarri et al.  
377 2020; Moles et al. 2021) , including a broader taxon sampling and massive genes, has  
378 resolved the relationships among main lineages of chitons. The genus and family level  
379 arrangement of taxa in this study are largely concordant with established taxonomy or with  
380 other molecular studies from smaller data matrices. Within Lepidopleurida, the family

381 Leptochitonidae s.s. is restricted to the NE Atlantic species, represented here by *Leptochiton*  
382 *asellus* and *Lepidopleurus cajetaus*, with the Pacific *Leptochiton cascadiensis* outside that  
383 clade, as is already established from previous molecular studies using Sanger sequencing  
384 (Sigwart et al. 2011; Sigwart 2016).

385

386 Acanthochitonina is known to be divided into two clades based on egg hulls and hexagon  
387 edges projections; one of the clades, Mopaliioidea, includes *Cryptochiton*, *Mopalia*,  
388 *Katharina* and *Tonicella* (Okusu et al. 2003) which is also well supported by every other  
389 molecular phylogeny including our results and modern phylogenetic systematics (Sigwart et  
390 al. 2013). Family level arrangement is difficult to test with limited taxon sampling, but the  
391 genera in our study group into these four genera that are closely allied to Mopaliidae as  
392 separate from a second clade of *Nuttallina* + *Cyanoplax*, also as found in previous studies  
393 (Irisarri et al. 2020). In the other superfamily Cryptoplacoidea, *Nuttallochiton* is sister to the  
394 rest of Cryptoplacoidea, in accordance with previous molecular studies (Okusu et al. 2003;  
395 Sigwart et al. 2013; Irisarri et al. 2020). However, the position of *Plaxiphora* within  
396 Acanthochitonina is equivocal; this has been a persistent problem in every molecular  
397 phylogeny of chitons, although multiple morphological characters unite *Plaxiphora* with the  
398 family Mopaliidae (Sirenko 2006).

399

400 *Schizochiton* resolved as sister to Chitonoidea, forming a monophyletic suborder Chitonina  
401 with full support except for Matrix1-C20 method, being sister group with the larger order  
402 Chitonida. The only prior molecular analysis to include *Schizochiton* also recovered it as  
403 sister to the remaining Chitonina in one version of their analyses, but concluded that its  
404 position within the phylogeny was effectively unresolved (Okusu et al. 2003: fig 5). The  
405 position of *Schizochiton* was controversial because of an unusual combination of  
406 morphological characters. The balance of evidence placed this group in the suborder  
407 Chitonina (Sirenko 2006). *Schizochiton* possess a caudal sinus in tail valve that is similar to  
408 others in Mopaliioidea as well as egg hulls with cupules that are simpler but comparable to  
409 other Mopaliioidea. Based on the new phylogenetic tree, we can infer these features may be  
410 plesiomorphic for the larger order Chitonida.

411

412 One important morphological feature of *Schizochiton* that differs from almost all other  
413 chitons is their shell eyes. Shell eyes were described in 1884 from specimens of *Schizochiton*  
414 *incissus* (Moseley 1884), and were immediately recognized as modifications of the chiton  
415 aesthete system (Moseley 1885). All chitons possess aesthete pores in their shell plates and  
416 some are photosensitive (Kingston et al. 2018). But shells eyes are restricted to only a few  
417 genera, in the family Schizochitonidae and the family Chitonidae. Those genera in the family  
418 Chitonidae with shell eyes form a monophyletic clade and have a fossil record only dating

419 back to the Miocene (Sirenko 2006). Phylogenetic and fossil evidence suggests that shell eyes  
420 evolved first in Schizochitonidae and again a second time very recently in the history of  
421 Chitonidae.

422

423 Recognizing *Schizochiton* within a superfamily level group Schizochitonoidea, sister to  
424 Chitonoidea, confirms the relationship predicted by morphological systematics. This is now  
425 confirmed from molecular evidence and a more stable phylogeny than earlier preliminary  
426 results. This also reaffirms that the multiple lines of evidence from morphological,  
427 anatomical, and gamete characters already recognized in chitons provide a robust basis for  
428 phylogenetic systematics.

429

#### 430 **Data availability**

431 The raw Illumina sequencing data was deposited on NCBI SRA database with the accession  
432 No. of PRJNA909482, and the assembled mitogenome on NCBI nucleotide database with the  
433 No. of XXXX. The assembled genomic contigs, predicted gene models can be accessed via  
434 FigShare with the URL of [10.6084/m9.figshare.21709742](https://www.figshare.com/10.6084/m9.figshare.21709742).

435

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444

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- 610
- 611

612 **Figure legend:**

613 Figure 1. *Schizochiton incisus* (b) and the position where it was collected (a, marked with a  
614 red spot). C shows the whole genomic pipeline used in this study, including sample  
615 preparation, mitogenome analysis, draft genome assembling and annotation and  
616 phylogenomic approach. Photos courtesy of Prof. Xiaoqi Zheng.

617

618 Figure 2. Mitogenome analyses of *Schizochiton incisus*; (a) mitogenome phylogeny of  
619 Polyplacophora, and (b) *S. incisus* mitochondrial gene order comparing with hypothetical  
620 ancestral mitochondrial gene order of chitons.

621

622 Figure 3. The occupancy of the four matrices generated by genesortR.

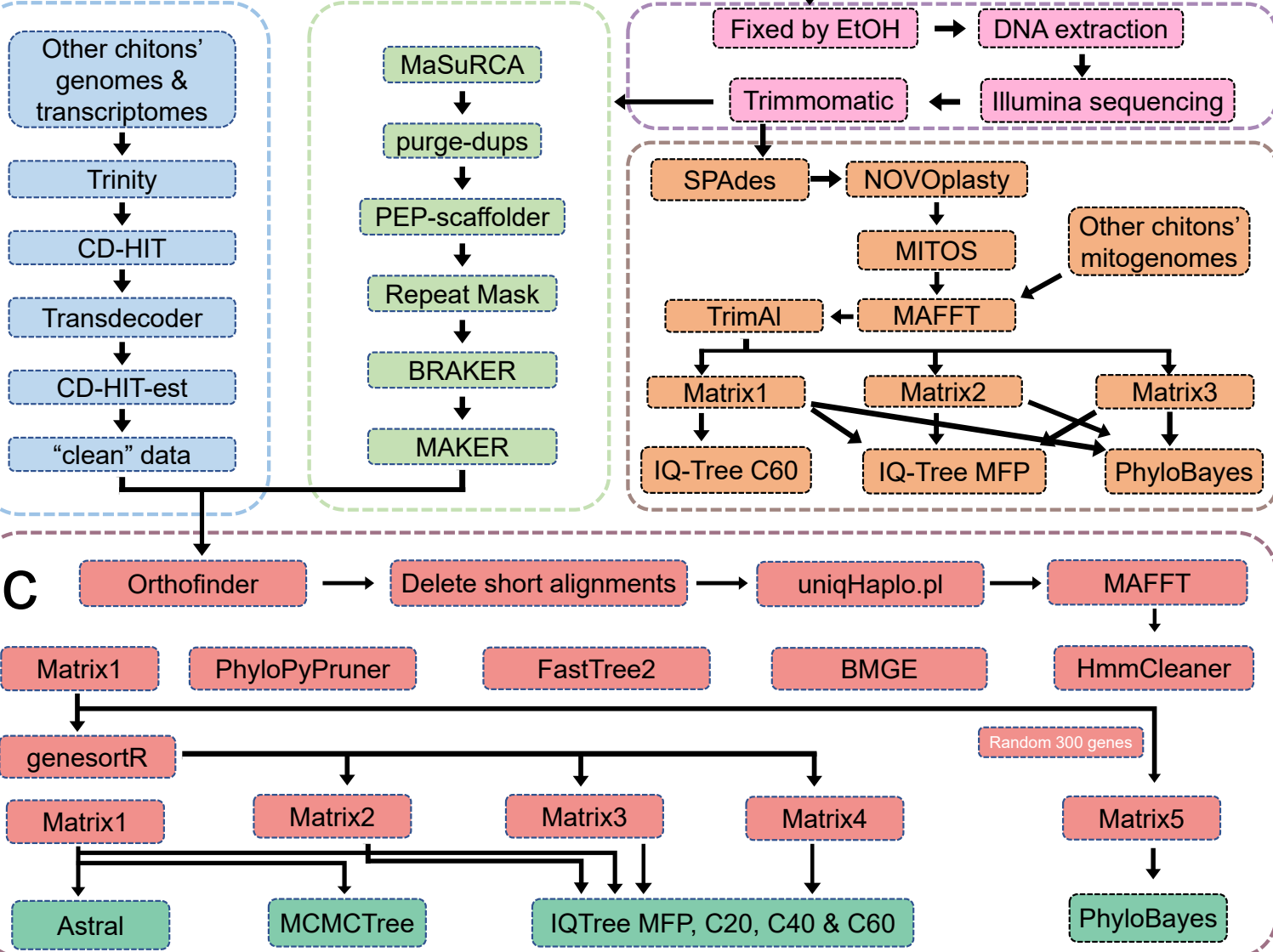
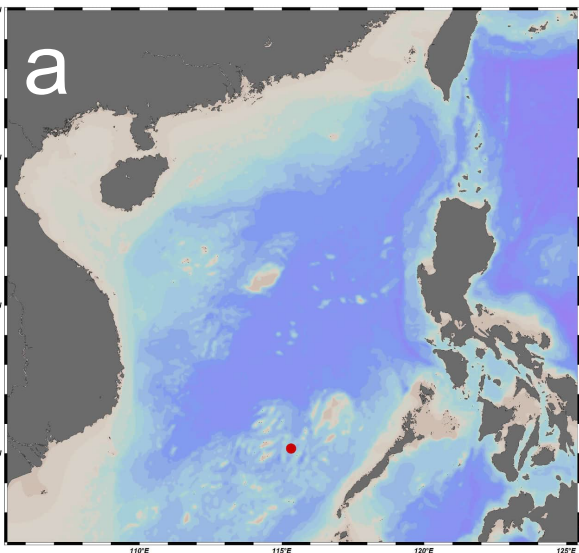
623

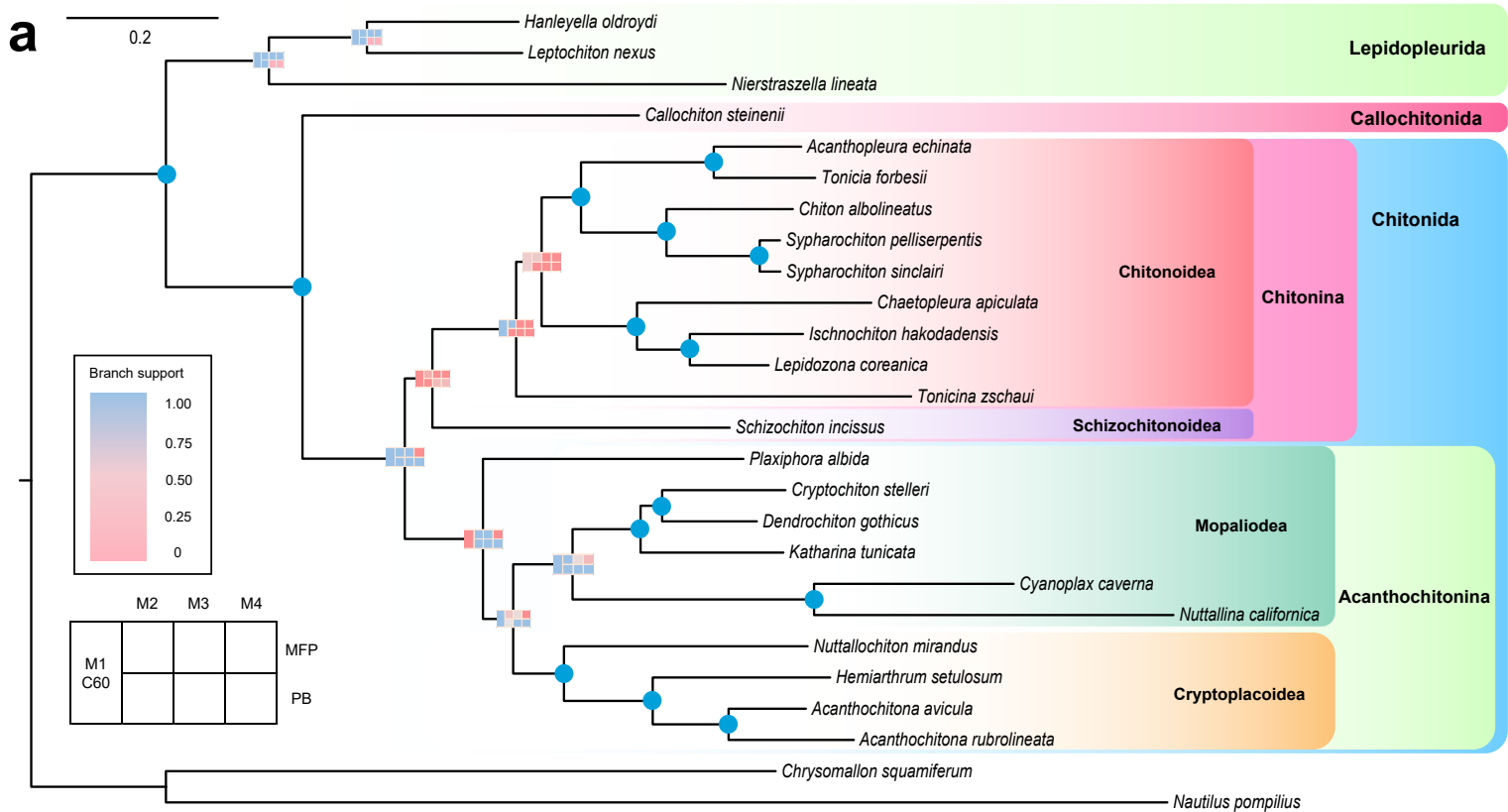
624 Figure 4. Phylogeny of chiton based on phylogenomic approach with different methods.  
625 Node support are transferred into matrices colored with a continuous scale bar ranging from 0  
626 to 1. Blue indicates 100% support and pink indicates the topology is not supported by the  
627 representative tree. And node with blue spot indicates full support in all methods. M1-M5,  
628 matrix 1-5; MFP, IQ-Tree MFP model; C20-C60, profile mixture models C20-C60; M1 Astral,  
629 coalescent analysis based on Matrix1; M5 PB, PhyloBayes analysis based on Matrix5.



Table 1 Statistics of chiton genomes and transcriptomes used in this study, including number of contigs and BUSCO scores after filtering.

| Species                            | SRA No.     | No. of proteins | BUSCO score                    | Source                            |
|------------------------------------|-------------|-----------------|--------------------------------|-----------------------------------|
| <b>Lepidopleurida</b>              |             |                 |                                |                                   |
| <i>Hanleya hanleyi</i>             | SRR11674123 | 47,786          | C:81.7%[S:80.7%,D:1.0%]        | Varney, Yap-Chiongco et al. 2022  |
| <i>Lepidopleurus cajetanus</i>     | SRX5063921  | 10,479          | C:12.9%[S:12.7%,D:0.2%]        | -----                             |
| <b><i>Leptochiton asellus</i></b>  | -----       | <b>81,610</b>   | <b>C:94.7%[S:86.9%,D:7.8%]</b> | <b>this study</b>                 |
| <i>Leptochiton rugatus</i>         | SRR1611558  | 23,030          | C:79.4%[S:77.9%,D:1.5%]        | Halanych and Kocot 2014           |
| <b>Callochitonida</b>              |             |                 |                                |                                   |
| <i>Callochiton septemvalvis</i>    | SRR13010089 | 30,618          | C:95.9%[S:87.8%,D:8.1%]        | Moles, Cunha et al. 2021          |
| <i>Callochiton</i> sp.             | SRR11674125 | 8235            | C:28.2%[S:26.1%,D:2.1%]        | Varney, Speiser et al. 2021       |
| <b>Chitonida</b>                   |             |                 |                                |                                   |
| <i>Acanthopleura granulata</i>     | -----       | 19,621          | C:93.8%[S:93.3%,D:0.5%]        | Varney, Speiser et al. 2021       |
| <i>Acanthopleura loochooana</i>    | -----       | 44,182          | C:90.4%[S:85.3%,D:5.1%]        | Liu, Liu et al. 2022              |
| <i>Tonicia schrammi</i>            | SRR11674132 | 16,274          | C:67.4%[S:67.1%,D:0.3%]        | Varney, Speiser et al. 2021       |
| <i>Chiton tuberculatus</i>         | SRR11674134 | 18,002          | C:83.2%[S:82.8%,D:0.4%]        | Varney, Speiser et al. 2021       |
| <i>Chiton marmoratus</i>           | SRR11674135 | 5848            | C:26.5%[S:26.5%,D:0.0%]        | Varney, Speiser et al. 2021       |
| <i>Rhysoplax olivacea</i>          | SRR618506   | 27,356          | C:67.1%[S:65.3%,D:1.8%]        | Riesgo, Andrade et al. 2012       |
| <i>Chaetopleura apiculata</i>      | SRR11674124 | 18,915          | C:79.3%[S:79.1%,D:0.2%]        | Varney, Speiser et al. 2021       |
| <i>Lepidozona mertensii</i>        | SRR11674130 | 13,531          | C:72.1%[S:71.5%,D:0.6%]        | Varney, Speiser et al. 2021       |
| <i>Stenoplax bahamensis</i>        | SRR13010087 | 24,602          | C:39.7%[S:39.0%,D:0.7%]        | Moles, Cunha et al. 2021          |
| <b><i>Schizochiton incisus</i></b> | -----       | <b>20,902</b>   | <b>C:40.9%[S:37.5%,D:3.4%]</b> | <b>this study</b>                 |
| <i>Cryptochiton stelleri</i>       | DRP005555   | 19,101          | C:82.2%[S:81.7%,D:0.5%]        | Nemoto, Ren et al. 2019           |
| <i>Mopalia muscosa</i>             | SRR11577121 | 13,262          | C:77.0%[S:76.6%,D:0.4%]        | Varney, Speiser et al. 2021       |
| <i>Katharina tunicata</i>          | SRR11674131 | 15,542          | C:89.7%[S:88.4%,D:1.3%]        | Varney, Speiser et al. 2021       |
| <i>Tonicella lineata</i>           | SRR11577222 | 13,780          | C:79.0%[S:77.7%,D:1.3%]        | Varney, Speiser et al. 2021       |
| <i>Nutallochiton</i> sp.           | SRR11674133 | 57,110          | C:74.3%[S:67.4%,D:6.9%]        | Varney, Speiser et al. 2021       |
| <i>Cryptoplax japonica</i>         | SRR13010086 | 14,963          | C:34.6%[S:34.3%,D:0.3%]        | Moles, Cunha et al. 2021          |
| <i>Cryptoplax larvaeformis</i>     | SRR11674126 | 20,128          | C:88.1%[S:87.7%,D:0.4%]        | Varney, Speiser et al. 2021       |
| <i>Choneplax lata</i>              | SRR13010088 | 16,971          | C:14.3%[S:13.4%,D:0.9%]        | Moles, Cunha et al. 2021          |
| <i>Acanthochitona rubrolineata</i> | SRP179406   | 44,221          | C:91.8%[S:71.2%,D:20.6%]       | -----                             |
| <i>Acanthochitona crinita</i>      | SRR5110525  | 22,678          | C:91.4%[S:91.0%,D:0.4%]        | De Oliveira, Wollesen et al. 2016 |
| <i>Acanthochitona fascicularis</i> | SRR13862580 | 17,427          | C:88.9%[S:88.5%,D:0.4%]        | -----                             |





**b**

*Schizochiton incisus* mitochondrial gene order

cox1 cox2 D atp8 atp6 T P E G cox3 K A R N I nad3 S1 nad2  
F nad5 H nad4 nad4L S2 cob nad6 nad1 L2 L1 rrL V rrS M C Y W Q

Hypothetical ancestral mitochondrial gene order of chitons

cox1 cox2 D atp8 atp6 T P cox3 K A R N I nad3 S1 nad2  
F nad5 H nad4 nad4L S2 cob nad6 nad1 L2 L1 rrL V rrS M C Y W Q G E

aligned length: 0-500



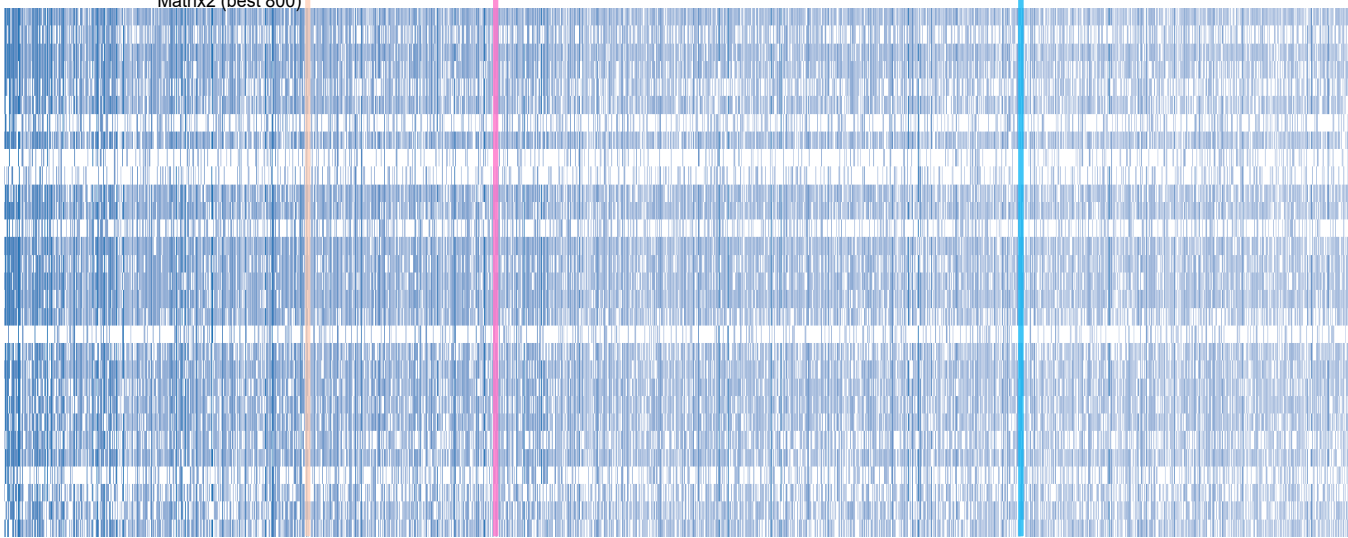
Matrix1 (all 3593)

Matrix4 (best 2700)

Matrix3 (best 1300)

Matrix2 (best 800)

*Acanthochitona crinita*  
*Acanthochitona fascicularis*  
*Acanthochitona rubrolineata*  
*Acanthopleura granulata*  
*Acanthopleura loochooana*  
*Callochiton septemvalvis*  
*Callochiton* sp.  
*Chaetopleura apiculata*  
*Chiton marmoratus*  
*Chiton tuberculatus*  
*Choneplax lata*  
*Chrysomallon squamiferum*  
*Cryptochiton stelleri*  
*Cryptoplax japonica*  
*Cryptoplax larvaeformis*  
*Hanleya hanleyi*  
*Katharina tunicata*  
*Lepidopleurus cajetanus*  
*Lepidozona mertensii*  
*Leptochiton asellus*  
*Leptochiton rugatus*  
*Mizuhopecten yessoensis*  
*Mopalia muscosa*  
*Nautilus pompilius*  
*Nutallochiton* sp.  
*Rhyssoplax olivacea*  
*Schizochiton incissus*  
*Stenoplax bahamensis*  
*Tonicella lineata*  
*Tonicia schrammi*



← OGs sorted by genesrotR score

