

1 **The genome of an apodid holothuroid (*Chiridota heheva*) provides insights**
2 **into its adaptation to deep-sea reducing environment**

3 Running title: Genome assembly of *Chiridota heheva*

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24

25 **Abstract**

26 Cold seeps and hydrothermal vents are deep-sea reducing environments that are characterized
27 by a lack of oxygen, photosynthesis-derived nutrients and a high concentration of reducing
28 chemicals. Apodida is an order of deep-sea echinoderms lacking tube feet and complex
29 respiratory trees, which are commonly found in holothurians. *Chiridota heheva* Pawson &
30 Vance, 2004 (Apodida: Chiridotidae) is one of the few echinoderms that resides in deep-sea
31 reducing environments. Unlike most cold seep and hydrothermal vent-dwelling animals, *C.*
32 *heheva* does not survive by maintaining an epi- or endosymbiotic relationship with
33 chemosynthetic microorganisms. The species acquires nutrients by extracting organic
34 components from sediment detritus and suspended material. Here, we report a high-quality
35 genome of *C. heheva* as a genomic reference for echinoderm adaptation to reducing
36 environments. *Chiridota heheva* likely colonized its current habitats in the early Miocene.
37 The expansion of the aerolysin-like protein family in *C. heheva* compared with other
38 echinoderms might be involved in the disintegration of microbes during digestion, which in
39 turn facilitates the species' adaptation to cold seep environments. Moreover, several hypoxia-
40 related genes were subject to positive selection in the genome of *C. heheva*, which contributes
41 to their adaptation to hypoxic environments.

42

43 **Keywords**

44 Reference genome, *Chiridota heheva*, Echinoderms, Cold seep, Aerolysin, Hypoxia

45

46 **1. Introduction**

47 Echinodermata is a phylum of marine animals comprising 5 extant classes, including
48 Asterozoa (starfish), Ophiurozoa (brittle star), Echinozoa (sea urchin), Crinozoa (feather
49 star), and Holothurozoa (sea cucumber) (Pawson, 2007). Adult echinoderms are
50 characterized by having a body showing pentameral symmetry, a water vascular system with
51 external tube feet (podia), and an endoskeleton consisting of calcareous ossicles (Pechenik,
52 2015). Echinoderms exhibit a high divergence in morphology, from the star-like architecture
53 in Asterozoa to the worm-like architecture in Holothurozoa (Mooi & David, 2008; Smith et
54 al., 2013).

55

56 Compared with other echinoderms, holothurians have a unique body architecture and
57 evolutionary history. The worm-like body of the holothurian preserves the pentameral
58 symmetry structurally along the oral-aboral axis (Li et al., 2020). In addition, holothurians
59 have a soft and stretchable body, in which the ossicles are greatly reduced in size (Pechenik,
60 2015). Variations in body architecture also exist in Holothurozoa. The order Apodida is a
61 group of holothurians that are found in both shallow-water and deep-sea environments
62 (Pawson & Vance, 2004). Phylogenetic analyses showed that Apodida is sister to other orders
63 of Holothurozoa (Lacey et al., 2005; Miller et al., 2017). Apodid holothurians lack tube feet
64 and complex respiratory trees, making them morphologically distinct from other holothurians
65 (Pechenik, 2015). In contrast to other classes of Echinodermata, which experienced a severe
66 evolutionary bottleneck during the Permian-Triassic mass extinction interval, Holothurozoa

67 did not experience family-level extinction through the interval. The deposit-feeding lifestyle
68 of holothurians conferred a selective advantage during the primary productivity collapse of
69 the Permian-Triassic mass extinction (Twitchett & Oji, 2005). As the genome of only one
70 shallow-water holothurian (*Apostichopus japonicus*) has been assembled and analyzed (Li et
71 al., 2018; Zhang et al., 2017), it is critical to study the genomes of more holothurians to
72 dissect their evolutionary history and developmental processes.

73

74 Cold seeps and hydrothermal vents are deep-sea reducing environments that are
75 characterized by high hydrostatic pressure, low temperature, lack of oxygen and
76 photosynthesis-derived nutrients, and high concentrations of reducing chemicals (Levin,
77 2005). However, these harsh environments support a variety of macroinvertebrates, including
78 tubeworms, mussels, clams, and gastropods (Vanreusel et al., 2009). Most of these
79 macrobenthos depend on the epi- or endosymbiotic relationships with chemoautotrophic
80 microorganisms for nutrition (Van Dover et al., 2002). Recent genomic analyses have
81 revealed the genetic basis of adaptation in several seep- and vent-dwelling macrobenthos
82 hosting symbiotic bacteria (Li et al., 2019; Sun et al., 2020; Sun et al., 2017; Y. Sun et al.,
83 2021). However, nonsymbiotic animals residing in deep-sea reducing environments are
84 understudied with only one reported genome (Liu et al., 2020).

85

86 Hydrocarbon fluid seepage from cold seeps is completely devoid of O₂ and comprises
87 high levels of sulfides. After reacting with sulfides contained in the fluid, any free O₂ is

88 removed from the deep-sea water. There are unique mechanisms of hypoxic adaptation in
89 seep-dwelling animals, as their O₂ consumption rates are similar to those of related shallow-
90 water species (Hourdez & Lallier, 2007). However, the genomic basis of hypoxic adaptation
91 in seep-dwelling animals is still lacking.

92

93 Echinoderms are a rare component of deep-sea chemosynthetic ecosystems (Tunnicliffe,
94 1992). *Chiridota heheva* Pawson & Vance, 2004 (Apodida: Chiridotidae) is one of the few
95 echinoderms that occupies all three types of chemosynthetic ecosystems (hydrothermal vent,
96 cold seep, and whale fall) (Thomas et al., 2020). This suggests that the species is well adapted
97 to deep-sea reducing environments. Unlike most seep- and vent-dwelling species, *C. heheva*
98 does not host chemosynthetic bacteria (Pawson & Vance, 2004). *Chiridota heheva* derives
99 nutrients from a variety of sources, extracting organic components from sediment detritus,
100 suspended material, and wood fragments when available (Carney, 2010; Pawson & Vance,
101 2004). A peltate-digitate tentacle structure allows *C. heheva* to exploit various food sources
102 by switching between deposit and suspension feeding (Thomas et al., 2020). The
103 cosmopolitan distribution and special lifestyle of *C. heheva* make it an ideal model to study
104 adaptation to deep-sea reducing environments in nonsymbiotic animals.

105

106 Here, we assembled and annotated a high-quality genome of *C. heheva* collected from
107 the Haima cold seep in the South China Sea. The evolutionary history of *C. heheva* was
108 investigated by inferring the phylogenetic relationship among echinoderms and the

109 demographic history of *C. heheva* and a shallow-water holothurian (*Apostichopus japonicus*).

110 Additionally, comparative genomic analyses were performed to dissect the genomic basis of

111 adaptation to deep-sea reducing environments in *C. heheva*.

112

113 **2 Methods and Materials**

114 **2.1 Sample collection and genome sequencing**

115 The *C. heheva* sample used in this study was collected using manned submersible
116 *Shenhaiyongshi* from the Haima cold seep in the South China Sea (16° 73.228' N, 110°
117 46.143' E, 1,385 m deep) on August 2, 2019. The *C. heheva* individuals were kept in an
118 enclosed sample chamber placed in the sample basket of the submersible. Once the samples
119 were brought to the upper deck of the mothership, the muscle of the individuals was dissected,
120 cut into small pieces, and immediately stored at -80°C. The samples were then transported to
121 Sun Yat-sen University on dry-ice and stored at -80°C until use.

122

123 To construct Nanopore sequencing library, high molecular weight genomic DNA was
124 prepared by the CTAB method. The quality and quantity of the DNA were measured via
125 standard agarose gel electrophoresis and with a Qubit[®]4.0 Fluorometer (Invitrogen).
126 Sequencing library was constructed and sequenced by Nanopore PromethION platform
127 (Oxford Nanopore Technologies). Additionally, DNA was extracted to construct Illumina
128 sequencing library. The quality and quantity of the DNA were measured via standard agarose
129 gel electrophoresis and with a Qubit[®]2.0 Fluorometer (Invitrogen). Sequencing library was
130 constructed and sequenced by Illumina Novaseq platform (Illumina).

131

132 **2.2 Genome assembly**

133 **2.2.1 Mitochondrial genome assembly**

134 Low quality (reads with $\geq 10\%$ unidentified nucleotide and/or $\geq 50\%$ nucleotides having
135 phred score < 5) and sequencing-adaptor-contaminated Illumina reads were filtered with
136 custom C script. The filtered Illumina reads were then trimmed with Fastp (v0.21.0) (Chen et
137 al., 2018) to obtain high-quality Illumina reads, which were used in the following analyses.
138 Mitochondrial genome of *C. heheva* was assembled using the two-step mode of mitoZ (v2.4)
139 (Meng et al., 2019) with the high-quality Illumina reads. And the assembled genome was
140 annotated using mitoZ (v2.4) with parameter "--clade Echinodermata".

141

142 2.2.2 Nuclear genome assembly

143 The size and heterozygosity of *C. heheva* genome were estimated using high-quality
144 Illumina reads by *k*-mer frequency distribution method. The number of *k*-mers and the peak
145 depth of *k*-mer sizes at 17 was obtained using Jellyfish (v2.3.0) (Marcais & Kingsford, 2011)
146 with the -C setting. Genome size was estimated based on the *k*-mer analysis as described
147 previously (Star et al., 2011).

148

149 Low quality Nanopore reads were filtered using custom Python script. Two draft
150 genome assemblies were generated using filtered Nanopore reads with Shasta (v0.4.0) (Shafin
151 et al., 2020) and WTDBG2 (v2.5) (Ruan & Li, 2020), respectively. The contigs of the two
152 draft assemblies were subject to error correction using filtered Nanopore reads with Racon
153 (v1.4.16) (Vaser et al., 2017) three times. The corrected contigs were then polished with high-
154 quality Illumina reads with Pilon (v1.23) (Walker et al., 2014) three times. The error-
155 corrected contigs of Shasta assembly and WTDBG2 assembly were assembled into longer

156 sequences using quickmerge (v0.3) (Chakraborty et al., 2016). The merged contigs were
157 subject to error correction using filtered Nanopore reads with Racon three times, and then
158 using high-quality Illumina reads with Pilon three times. As the heterozygosity of *C. heheva*
159 genome is high, haplotypic duplications in the assembled genome were identified and
160 removed using purge_dups (v1.2.3) (Guan et al., 2020). The completeness and quality of the
161 assembly was evaluated using BUSCO (v4.0.5) (Simao et al., 2015) against the conserved
162 Metazoa dataset (obd10), and SQUAT with high-quality Illumina reads (Yang et al., 2019).

163

164 **2.3 Genome annotation**

165 2.3.1 Repetitive element annotation

166 Repetitive elements in the assembly were identified by *de novo* predictions using
167 RepeatMasker (v4.1.0) (<https://www.repeatmasker.org/>). A *de novo* repeat library for *C.*
168 *heheva* was built using RepeatModeler (v2.0.1) (Flynn et al., 2020). To identify repetitive
169 elements, sequences from the *C. heheva* assembly were aligned to the *de novo* repeat library
170 using RepeatMasker (v4.1.0). Additionally, repetitive elements in *C. heheva* genome
171 assembly were identified by homology searches against known repeat databases using
172 RepeatMasker (v4.1.0). A repeat landscape of *C. heheva* genome was obtained using an R
173 script that was modified from <https://github.com/ValentinaBoP/TransposableElements>.

174

175 2.3.2 Protein-coding gene annotation

176 We applied a combination of homology-based and *de novo* predication methods to build
177 consensus gene models for the *C. heheva* genome assembly. For homology-based gene
178 prediction, protein sequences of *Helobdella robusta*, *Lytechinus variegatus*,
179 *Strongylocentrotus purpuratus*, *Dimorphilus gyrociliatus*, *Apostichopus japonicus* and
180 *Acanthaster planci* were aligned to the *C. heheva* genome assembly using tblastn. The exon-
181 intron structures then were determined according to the alignment results using
182 GenomeThreader (v1.7.0) (Gremme et al., 2005). In addition, *de novo* gene prediction was
183 performed using Augustus (v3.3.2) (Stanke et al., 2006), with the parameters obtained by
184 training the software with protein sequences of *Drosophila melanogaster* and *Parasteatoda*
185 *tepidariorum*. Two sets of gene models were integrated into a non-redundant consensus gene
186 set using EvidenceModeler (v1.1.1) (Haas et al., 2008). To identify functions of the predicted
187 proteins, we aligned the *C. heheva* protein models against NCBI NR, trEMBL, and SwissProt
188 database using blastp (E-value threshold: 10^{-5}), and against eggNOR database (Huerta-Cepas
189 et al., 2019) using eggNOR-Mapper (Huerta-Cepas et al., 2017). In addition, KEGG
190 annotation of the protein models was performed using GhostKOALA (Kanehisa et al., 2016).

191

192 **2.4 Phylogenomic analysis**

193 Protein sequences of 15 metazoan species (*A. planci*, *S. purpuratus*, *Lytechinus*
194 *variegatus*, *A. japonicus*, *Anneissia japonica*, *Saccoglossus kawalevskii*, *Branchiostoma*
195 *floridae*, *Ciona intestinalis*, *Danio rerio*, *Gallus gallus*, *H. robusta*, *Mus musculus*, *Pelodiscus*
196 *sinensis*, *Petromyzon marinus*, and *Xenopus laevis* proteins) were downloaded from NCBI.

197 And protein sequences of *Parastichopus parvimensis* were downloaded from Echinobase
198 (Kudtarkar & Cameron, 2017). OrthoMCL (v2.0.9) (Li et al., 2003) was applied to determine
199 and cluster gene families among these 16 metazoan species and *C. heheva*. Gene clusters
200 with >100 gene copies in one or more species were removed. Single-copy orthologs in each
201 gene cluster were aligned using MAFFT (v7.310) (Kato et al., 2002). And the alignments
202 were trimmed using ClipKit (v1.1.3) (Steenwyk et al., 2020) with “gappy” mode. The
203 phylogenetic tree was reconstructed with the trimmed alignments using a maximum-
204 likelihood method implemented in IQ-TREE2 (v2.1.2) (Minh et al., 2020) with *H. robusta* as
205 outgroup. The best-fit substitution model was selected by using ModelFinder algorithm
206 (Kalyaanamoorthy et al., 2017). Branch supports were assessed using the ultrafast bootstrap
207 (UFBoot) approach with 1,000 replicates (Hoang et al., 2018).

208

209 To estimate the divergent time among echinoderms, single-copy orthologs were
210 identified among *A. japonica*, *A. planci*, *A. japonicus*, *P. parvimensis*, *C. heheva*, *L.*
211 *variegatus* and *S. purpuratus* after running OrthoMCL pipeline as mentioned above. Single-
212 copy orthologs were aligned using MAFFT (v7.310), trimmed using ClipKit (v1.1.3) with
213 ‘gappy’ mode, and concatenated using PhyloSuite (v1.2.2) (Zhang et al., 2020). Divergent
214 time among 7 echinoderms were estimated using the concatenated alignment with MCMCtree
215 module of the PAML package (v4.9) (Tessmar-Raible & Arendt, 2003). MCMCtree analysis
216 was performed using the maximum-likelihood tree that was reconstructed by IQ-TREE2 as a
217 guide tree and calibrated with the divergent time obtained from TimeTree database (minimum

218 = 193 million years and soft maximum = 350 million years between *L. variegatus* and *S.*
219 *purpuratus*).

220

221 **2.5 Demographic inference of *C. heheva* and *A. japonicus***

222 Paired-end Illumina reads of *A. japonicus* (Li et al., 2018) were downloaded from NCBI
223 SRA database. The reads of *A. japonicus* were filtered with custom C script and trimmed with
224 fastp (v0.21.0). The Illumina clean reads of *C. heheva* and *A. japonicus* were aligned to the
225 respective reference genome assembly using BWA (v0.7.17) (Li & Durbin, 2009) with “mem”
226 function. Genetic variants were identified using Samtools (v1.9) (Li et al., 2009). Whole
227 genome consensus sequence was generated with the genetic variants using Samtools (v 1.9).
228 PSMC (v0.6.5) (Li & Durbin, 2011) was used to infer the demographic history of *C. heheva*
229 and *A. japonicus* using the whole genome consensus sequences. The substitution mutations
230 rate and generation time of *C. heheva* and *A. japonicus* was set to 1.0×10^{-8} and 3 years
231 according to the previous study of *A. planci* (Hall et al., 2017).

232

233 **2.6 Homeobox gene analysis**

234 Homeobox genes in *C. heheva* genome were identified by following the procedure
235 described previously (Marletaz et al., 2019). Homeodomain sequences, which were retrieved
236 from HomeoDB database (<http://homeodb.zoo.ox.ac.uk>) (Zhong et al., 2008), were aligned to
237 *C. heheva* genome assembly using tbalstn. Sequences of the candidate homeobox genes were

238 extracted based on the alignment results. The extracted sequences were aligned against NCBI
239 NR and HomeoDB database to classify the homeobox genes.

240

241 **2.7 Gene family evolution**

242 2.7.1 Gene family expansion and contraction analysis

243 r8s (v1.7) (Sanderson, 2003) was applied to obtain the ultrametric tree of 7 echinoderm
244 species, which is calibrated with the divergent time between *A. planci* and *S. purpuratus* (541
245 mya). CAFÉ (v5) (De Bie et al., 2006) was applied to determine the significance of gene
246 family expansion and contraction among 7 echinoderm species based on the ultrametric tree
247 and the gene clusters determined by OrthoMCL (v2.0.9).

248

249 2.7.2 Evolutionary analysis of *C. heheva* NOD-like receptors (NLRs) and other representative 250 metazoan NLRs

251 We used HMMER (v3.1) to search against the proteome of *C. heheva* with the HMM
252 profile of NACHT domain (PF05729) retrieved from Pfam 34.0 as the query and an *e* cut-off
253 value of 0.01. Proteins identified by the HMM search were retrieved from the proteome and
254 aligned with 964 representative proteins from eukaryotes and prokaryotes (Urbach &
255 Ausubel, 2017), and other representative metazoan NLRs (Yuen et al., 2014) using hmalign
256 method implemented in HMMER (v3.1) based on the STAND NTPase domain. The
257 alignment was refined by manual editing. The large-scale phylogenetic analysis was
258 performed using an approximate maximum likelihood method implemented in FastTree

259 (Price et al., 2010). Representative SWACOS and MaIT NTPases were used as outgroups
260 (Urbach & Ausubel, 2017). Significant hits clustering with metazoan NLRs were regarded as
261 NLRs, and protein domain organizations were annotated through hmmscan method
262 implemented in HMMER (v3.1).

263

264 To explore the evolutionary relationships among *C. heheva* NLRs and other
265 representative metazoan NLRs, we reconstructed the phylogenetic tree of NLRs. The NACHT
266 domains of *C. heheva* NLRs and representative metazoan NLRs were aligned using MAFFT
267 (v7.310), and then refined by manual editing. The representative metazoan NLRs were chosen
268 from literature (Yuen et al., 2014). The phylogenetic tree was reconstructed using a
269 maximum-likelihood method implemented in IQ-TREE2 (v2.1.2). The best-fit substitution
270 model selected by using ModelFinder algorithm. Branch supports were assessed using the
271 UFBoot approach with 1,000 replicates.

272

273 **2.8 Identification and analysis of positively selected genes**

274 Branch-site models implemented in the codeml module of the PAML package is widely
275 used to identify positively selected genes (PSGs). Thus, we identified PSGs in the *C. heheva*
276 genome within the single-copy orthologs among 7 echinoderm species, based on the branch-
277 site models using GWideCodeML (v1.1) (Macias et al., 2020). *C. heheva* was set as the
278 ‘foreground’ phylogeny, and the other species were set as the ‘background’ phylogeny. An
279 alternative branch site model (Model = 2, NSsites = 2, and fix_omega = 0) and a neutral

280 branch site model (Model = 2, NSsites = 2, fix_omega = 1, and omega = 1) were tested.
281 Genes with Bayesian Empirical Bayes (BEB) sites > 90 % and a corrected *P*-value < 0.1 were
282 identified to have been subject to positive selection.

283

284 To investigate *LHPP* gene evolution, sequences of *LHPP* from 8 mammals (*Odobenus*
285 *rosmarus*, *Orcinus orca*, *Lipotes vexillifer*, *Tursiops truncatus*, *Physeter catodon*,
286 *Balaenoptera acutorostrata*, *Mus musculus*, and *Homo sapiens*) and 7 echinoderms (*A.*
287 *japonica*, *A. planci*, *A. japonicus*, *P. parvimensis*, *C. heheva*, *L. variegatus* and *S. purpuratus*)
288 were aligned using MAFFT (v7.310). To reconstruct the phylogenetic tree, OrthoMCL
289 (v2.0.9) (Li et al., 2003) was applied to determine and cluster gene families among these 15
290 species. Gene clusters with >100 gene copies in one or more species were removed. Single-
291 copy orthologs in each gene cluster were aligned using MAFFT (v7.310) (Kato et al., 2002).
292 And the alignments were trimmed using ClipKit (v1.1.3) (Steenwyk et al., 2020) with “gappy”
293 mode. The phylogenetic tree was reconstructed with the trimmed alignments using a
294 maximum-likelihood method implemented in IQ-TREE2 (v2.1.2) (Minh et al., 2020). *H.*
295 *robusta* was used as outgroup. The best-fit substitution model was selected by using
296 ModelFinder algorithm (Kalyaanamoorthy et al., 2017).

297 **3. Results**

298 **3.1 Characterization and genome assembly of *C. heheva***

299 The sequenced sample was collected at a depth of 1,385 meters using manned
300 submersible *Shenhaiyongshi* from the Haima cold seep in the South China Sea (16° 73.228' N,
301 110° 46.143' E) (**Figure 1**). We sequenced the sample genome on the Nanopore and Illumina
302 sequencing platforms. In total, 42.43 Gb of Nanopore reads and 49.19 Gb of Illumina reads
303 were obtained (**Table S1 and S2**). Species identity of the sequenced individual was first
304 determined according to its morphological characteristics. In addition, we assembled the
305 mitochondrial genome of the individual using Illumina reads. The sequence identity between
306 the published *C. heheva* mitochondrial genome and our assembled genome was 99.74%,
307 which confirmed the species identity of the sequenced individual (S. Sun et al., 2021). Based
308 on the *k*-mer distribution of Illumina reads, the size of the *C. heheva* genome was estimated to
309 be 1.23 Gb with a high heterozygosity of 2% (**Figure S1 and Table S3**). The *C. heheva*
310 genome was assembled into 4,609 contigs, with a total size of 1.107 Gb and contig N50 of
311 1.22 Mb (**Table 1**). We determined the completeness of the assembled genome by running
312 benchmarking universal single-copy orthologs (BUSCO) and sequencing quality assessment
313 tool (SQUAT) software. BUSCO analysis with metazoan (obd10) gene set showed that the
314 assembled *C. heheva* genome contained 89.6% complete single-copy orthologs (**Table S4**).
315 Additionally, 91.1% of Illumina reads could be aligned to the assembled genome with high
316 confidence in SQUAT analysis (**Table S5**). These results indicate the high integrity of our
317 assembled genome.

318 **3.2 Genome annotation**

319 Repetitive elements represented 624.38 Mb (56.40%) in the *C. heheva* genome
320 assembly (**Table S6**). Long interspersed nuclear elements (LINEs) were the largest class of
321 annotated transposable elements (TEs), making up 9.72% of the genome. DNA transposons,
322 which were the second largest class of TEs, represented 33.59 Mb (3.03%) of the genome.
323 Additionally, the *C. heheva* genome comprised a large proportion (38.39%) of unclassified
324 interspersed repeats. Comparative genomic analysis among *C. heheva* and other echinoderms
325 revealed that the *C. heheva* genome consisted of the largest number of TEs (**Figure 2a and**
326 **2b; Table S7**). Repetitive elements constituted 624.38 Mb of the *C. heheva* genome, and they
327 accounted for 253.98 Mb and 218.2 Mb of the genomes of *A. japonicus* and *P. parvimensis*,
328 respectively. The differences in the repeat content were almost consistent with the size
329 differences between the genomes of *C. heheva* and the other two holothurians. This suggests
330 that repeats contributed to the size differences among the genomes of these three holothurians.
331 Notably, the proportion of LINEs in the *C. heheva* genome was substantially higher than that
332 in the genomes of other echinoderms (**Figure 2b**). Kimura distance-based copy divergence
333 analysis identified a recent expansion of LINEs in the *C. heheva* genome (**Figure 2c**).
334 Protein-coding genes were identified in the genome of *C. heheva* through a combination of *ab*
335 *initio* and homology-based protein prediction approaches. In total, we derived 36,527 gene
336 models in the *C. heheva* genome. The structure of predicted genes in *C. heheva* is slightly
337 different to that of other previously sequenced echinoderm genomes. With longer exon and

338 intron as well as more exons per gene, genes in *C. heheva* are longer than the ones in *A.*
339 *japonicus* (**Table S8**).

340

341 **3.3 Phylogenomic analysis and demographic inference**

342 To investigate the evolutionary history of *C. heheva*, a maximum-likelihood (ML)
343 phylogenetic tree was reconstructed using single-copy orthologs of *C. heheva* and 16 other
344 deuterostomes (**Figure S2**). Consistent with the results of previous analyses, the tree showed
345 that Echinodermata and Hemichordata were sister groups to Chordata. *Chiridota heheva*
346 appeared sister to two other holothurians, which supports the view that Apodida is the sister
347 taxon to the remaining holothuroids (Miller et al., 2017). In addition, divergence times were
348 determined among 7 echinoderms that had whole genome sequences (**Figure 3a**). The
349 divergence time of *A. japonica* and other echinoderms was estimated to be approximately 569
350 million years (Ma), which is generally consistent with the fossil records (Smith, 1988a;
351 Zamora et al., 2013). *Chiridota heheva* and two other holothurians were estimated to have
352 diverged approximately 429 Ma. As Apodid is the basal taxon in Holothuroidea, these results
353 indicate that holothurians started to diverge in the Early Ordovician (Benton & Twitchett,
354 2003).

355

356 We studied the demographic history of the deep-sea (*C. heheva*) and shallow-water (*A.*
357 *japonicus*) holothurians by inferring the histories of ancestral population size using the
358 pairwise sequential Markovian coalescent (PSMC) method (**Figure 3b**). *Chiridota heheva*

359 experienced a decline in population size approximately 21 million years ago, which suggests
360 that this species started to colonize the current habitat at the turn of the Miocene. The decline
361 in population size in *A. japonicus* started in the late Miocene (approximately 8 Ma). *Chiridota*
362 *heheva* also experienced a moderate decline in ancestral population size in the early Pliocene.
363

364 **3.4 *Hox/ParaHox* gene clusters**

365 It has been demonstrated that *Hox* genes play a critical role in embryonic development
366 (Pearson et al., 2005). In addition, previous studies proposed that the presence/absence and
367 expression pattern of *Hox* genes might contribute to morphological patterning and embryonic
368 development in echinoderms (Li et al., 2018; Zhang et al., 2017). Therefore, to determine
369 whether *Hox* genes contribute to morphological divergence in Holothuroidea, we identified
370 *Hox* gene clusters and their evolutionary sister complex, the *ParaHox* gene cluster, in the
371 genomes of *C. heheva* and 6 other echinoderms (**Figure 4**). A *Hox* cluster and a *ParaHox*
372 cluster could be identified in the genomes of all 7 species. The gene composition and
373 arrangement of both *Hox* and *ParaHox* clusters were highly consistent between the genomes
374 of *C. heheva* and *A. japonicus*, suggesting that *Hox/ParaHox* genes do not control the
375 development of tube feet and respiratory trees in Apodida. *Hox4* was missing in Echinodeans
376 and holothurians, and *Hox6* was missing in asteroideans and holothurians. These results
377 support the view that the absence of *Hox* genes might have contributed to the morphological
378 divergence of echinoderms.

379

380 **3.5 NLR repertoire in *C. heheva***

381 NACHT and leucine-rich, repeat-containing proteins (NLRs) are important components
382 of pathogen recognition receptors (PRRs) involved in animal innate immune systems, which
383 can perceive pathogen-associated molecular patterns (PAMPs) of viruses and bacteria (Lange
384 et al., 2011). The bona fide NLRs contain a NACHT (NAIP, CIITA, HET-E, and TP1)
385 domain, which belongs to the signal transduction ATPases with numerous domains (STAND)
386 superfamily, and a series of C-terminal leucine-rich repeats (LRRs) (Ausubel, 2005; Leipe et
387 al., 2004). The Pfam hidden Markov model (HMM) search combined with phylogenetic
388 analysis approach identified only 53 NLRs in *C. heheva* (**Table S9**), compared with a largely
389 expanded set of 203 NLRs in purple sea urchin, a member of the phylum Echinodermata
390 (Hibino et al., 2006). *Chiridota heheva* contained 24 NLRs with one or more N-terminal
391 Death/DED domain, 22 NACHT-only NLRs, 6 NLRs with other domains, including the
392 immunoglobulin V-set domain, which was not identified in sea urchin NLRs, and only one
393 NLR with LRRs (**Table S9**). Taken together, these results indicate that the *C. heheva* NLR
394 repertoire shows different abundances and structural complexities than the sea urchin.

395

396 We performed phylogenetic analysis of *C. heheva* NLRs and other representative NLRs
397 of metazoans, including humans, *Amphimedon queenslandica*, *S. purpuratus*, *Acropora*
398 *digitifera*, *Nematostella vectensis*, *Pinctada fucata*, *Capitella teleta*, mollusks, and arthropods
399 (Yuen et al., 2014). We found that the majority of *C. heheva* NLRs form a monophyletic
400 lineage with sea urchin NLRs (**Figure 5**), supporting the lineage-specific evolution of NLRs

401 in Echinodermata (Zhang et al., 2010). Given that human IPAF (ice protease-activating
402 factor) and NAIP (neuronal apoptosis inhibitory protein) proteins were reported to have
403 originated before the evolution of vertebrates (Zhang et al., 2010), one *C. heheva* NLR
404 clustering with these two proteins indicates that this NLR may have an ancient independent
405 origin (**Figure 5**).

406

407 **3.6 Gene family evolution**

408 We performed gene-family analysis based on the phylogenetic tree of 7 echinoderms
409 (**Figure 3a**). Compared with other echinoderms, 66 gene families were expanded, and 25
410 gene families were contracted in *C. heheva* ($P < 0.05$) (**Tables S10 and S11**). Several
411 significantly expanded gene families are involved in the processes of cell cycle progression,
412 protein folding, and ribosome assembly. As high hydrostatic pressure causes cell cycle delay
413 and affects protein folding (George et al., 2007; Yancey & Siebenaller, 2015), expansion of
414 these families may have contributed to the adaptation of *C. heheva* to cold seep environments.

415

416 Aerolysins, which are pore-forming toxins (PFTs), were first characterized as virulence
417 factors in the pathogenic bacterium *Aeromonas hydrophyla* (Abrami et al., 2000; Dal Peraro
418 & van der Goot, 2016). The homologs of aerolysin in eukaryotes (aerolysin-like proteins,
419 ALPs) originated from recurrent horizontal gene transfer (HGT) (Moran et al., 2012). ALPs
420 of different origins possess diverse functions, including immune defense and predation
421 (Galinier et al., 2013; Szczesny et al., 2011; Xiang et al., 2014). The ALPs were significantly

422 expanded in the genome of *C. heheva* (7 copies) compared with other echinoderms (0 or 1
423 copy) ($P < 0.05$) (**Table S10**). To investigate the possible origin and function of *C. heheva*
424 ALPs, we reconstructed the phylogenetic tree of ALPs in echinoderms and diverse species.
425 Interestingly, *C. heheva* ALPs did not cluster with ALPs from other echinoderms, suggesting
426 that ALPs from *C. heheva* and other echinoderms have different origins (**Figure 6**). *Chiridota*
427 *heheva* ALPs form a clade with ALPs from sea anemones (*Nematostella vectensis* and
428 *Ecaiptasia diaphana*). This indicates that ALPs from *C. heheva* and sea anemones might have
429 similar biological functions. It was shown that ALPs secreted by *N. vectensis* are involved in
430 prey digestion (Moran et al., 2012). Therefore, the expansion of the ALP family in *C. heheva*
431 might contribute to the disintegration of microbes during digestion.

432

433 **3.7 Positively selected genes**

434 To better understand the genetic basis of its adaptation to a deep-sea reducing
435 environment, we identified genes undergoing positive selection (PSGs) in *C. heheva*.
436 Compared with 6 other echinoderms, 27 PSGs were identified in the *C. heheva* genome
437 (**Table S12**). Several hypoxia-related genes (*PKM*, *PAN2*, *LHPP*, and *RRP9*) (Benita et al.,
438 2009; Bett et al., 2013; Chen et al., 2021; Luo et al., 2011), were subject to positive selection
439 in *C. heheva*. Cold seeps and hydrothermal vents are characterized by low oxygen
440 concentrations, which are challenging for endemic species (Hourdez & Lallier, 2007).
441 Therefore, the adaptation of *C. heheva* to a deep-sea reducing environment may be attributed
442 to selection against these hypoxia-related genes. Interestingly, the *LHPP* gene is also

443 positively selected in the genomes of cetaceans, which are hypoxia-tolerant mammals (Tian et
444 al., 2017). In addition, comparative genetic analysis showed that cetaceans and *C. heheva*
445 have the same amino acid substitution at position 118 of the LHPP protein (**Figures S3 and**
446 **S4**), which indicates a possible convergent evolution in the *LHPP* during the adaptation of
447 cetaceans and *C. heheva* to hypoxic environments.

448

449 **4. Discussion**

450 With more than 1,400 extant species, Holothuroidea is one of the largest classes in the
451 phylum Echinodermata (Pawson, 2007). In addition, holothurians are well adapted to diverse
452 marine environments, with habitats ranging from shallow intertidal areas to hadal trenches
453 (Jamieson, 2015; Smirnov et al., 2000). However, due to the lack of body fossils,
454 evolutionary study of Holothuroidea is more difficult than other classes of Echinodermata.
455 The high-quality genome of *C. heheva* presented in this report facilitates the investigation of
456 its evolutionary history. Our phylogenomic analysis revealed that the divergence of
457 echinoderms started in the early Cambrian (~539 Ma), which is consistent with the fossil
458 record. (Bottjer et al., 2006; Smith, 1988b) (**Fig. 3a**). The ancestor of *Chiridota heheva*
459 diverged from the ancestors of two shallow-water holothurians (*A. japonicus* and *P.*
460 *parvimensis*) approximately 429 Ma. As Apodida is the basal taxon in Holothuroidea, these
461 results support the view that holothurians had evolved by the Early Ordovician (Reich, 2010).
462 To better investigate the evolution of holothurians, we inferred the histories of ancestral
463 population sizes of *C. heheva* and *A. japonicus* using PSMC (**Fig. 3b**). *Chiridota heheva*

464 experienced a decline in population size approximately 21 Ma. Ocean temperature increased
465 slowly between the late Oligocene and early Miocene (21-27 Ma) after long-term cooling
466 from the end of the Eocene (Zachos et al., 1997; Zachos et al., 2001). Furthermore, species
467 diversity within Echinodermata started to increase in the early Miocene (Kroh, 2007; Oyen &
468 Portell, 2001). These results indicate that *C. heheva* might have colonized the current habitat
469 in the early Miocene when the climate transition improved adaptations in echinoderms. The
470 oceans experienced a decrease in temperature during the late Miocene (7 to 5.4 Ma) (Herbert
471 et al., 2016). A decline in ancestral population size in *A. japonicus* started approximately 7
472 Ma. *Chiridota heheva* also experienced a moderate decline in population size in the early
473 Pliocene. These results suggest that global cooling and environmental changes in the late
474 Miocene were an important driver of demographic changes in both shallow-water and deep-
475 sea holothurians.

476

477 Apodida do not have tube feet or complex respiratory trees, which are commonly found
478 in other holothurians (Barnes, 1982). It was proposed that *Hox* genes might have contributed
479 to the body development of echinoderms (Li et al., 2018). The gene composition and
480 arrangement of the *Hox/ParaHox* gene cluster were consistent between *A. japonicus* and *C.*
481 *heheva*, indicating that *Hox* genes were unlikely to have been involved in the morphological
482 divergence between Apodids and other holothurians. There are some inconsistent results
483 regarding the gene composition of *Hox* gene clusters in different echinoderm genomes.
484 Previous studies found that *Hox4* and *Hox6* were missing in the genomes of holothurians (Li

485 et al., 2018; Zhang et al., 2017), and *Hox4* was missing in the genomes of echinoids
486 (Cameron et al., 2006). Li *et al.* (2018) proposed that *Hox6* was lost before the split of
487 Echinozoa and Asterozoa (Li et al., 2018), while Li *et al.* (2020) suggested that the loss of
488 *Hox4* or *Hox6* was a lineage-specific event (Li et al., 2020). We found that *Hox4* and *Hox 6*
489 were missing in the genomes of both *C. heheva* and *A. japonicus*. In addition, *Hox6* was
490 missing in the genome of *A. planci*, and *Hox4* was missing in the genomes of both *L.*
491 *variegatus* and *S. purpuratus* (**Fig. 4a**). This suggests that *Hox4* was lost before the split of
492 Echinoidea and Holothuroidea, and *Hox6* was lost in Holothuroidea and Asteroidea. This
493 scenario is not parsimonious, as Holothuroidea is paraphyletic with Asteroidea. As *S.*
494 *purpuratus Hox6* clusters with *A. planci Hox4* in phylogenetic analysis, Baughman et al.
495 (2014) proposed reclassifying *S. purpuratus Hox6* as *Hox4* (Baughman et al., 2014).
496 Following this argument, *Hox6* was missing in Holothuroidea, Echinoidea, and Asteroidea,
497 and *Hox4* was missing in Holothuroidea (**Fig. 4b**). This supports the view that the loss of
498 *Hox6* occurred before the split of Echinozoa and Asterozoa.

499

500 Comparative genomic analysis showed that the ALP gene family was significantly
501 expanded in *C. heheva* compared with other echinoderms. The expansion of the ALP family
502 in *C. heheva* might have contributed to its adaptation to cold seep environments. Cold seeps
503 are areas where methane, hydrogen sulfide, and other hydrocarbons seep or emanate as gas
504 from deep geologic sources (Suess, 2014). Chemosynthetic microbes oxidize the reduced
505 chemicals contained in the fluids to produce energy and fix carbon into organic matter, which

506 supports large benthic communities around the gas source (Levin, 2005). Most seep-dwelling
507 animals survive by hosting chemosynthetic microbes (Petersen & Dubilier, 2009). *Chiridota*
508 *heheva* has a unique feeding habit of acquiring nutrients from sediment detritus, suspended
509 material, and wood fragments when available. The microbial communities of cold seeps are
510 very different from those of other seafloor ecosystems (Ruff et al., 2015). Moreover, some of
511 these microbes have unique cellular structures that might be difficult to disintegrate
512 (Katayama et al., 2020), which impedes nutrient acquisition of *C. heheva* from free-living
513 microbes of cold seeps. As typical pore-forming proteins, aerolysin and related proteins are
514 found in a large variety of species and possess diverse functions (Szczesny et al., 2011). It
515 was proposed that ALPs were derived from recurrent horizontal gene transfer. ALPs of the
516 same origin might have similar functions (Moran et al., 2012). *Chiridota heheva* ALPs and
517 ALPs from other echinoderms are likely to have different origins, as they were clustered with
518 aerolysins from distinct groups of bacteria (**Figure 6**). *Chiridota heheva* ALPs formed a clade
519 with sea anemone ALPs. Furthermore, ALPs from hydra and sea anemones are involved in
520 prey disintegration after predation by lysing cells through pore formation on membranes
521 (Moran et al., 2012; Sher et al., 2008). This suggests that the expansion of the ALP family
522 might have contributed to microbe digestion in *C. heheva*, which in turn facilitated its
523 adaptation to cold seep environments.

524

525 Several genes that are involved in hypoxic responses (*PKM*, *PAN2*, and *LHPP*) and one
526 of the HIF-1 target genes (*PPR9*) were subjected to positive selection in *C. heheva*. The

527 transcription of the *PKM2* gene is activated by HIF-1. PKM2 promotes transactivation of
528 HIF-1 target genes by directly interacting with the HIF-1 α subunit. PKM2 is involved in a
529 feedback loop that reprograms glucose metabolism under hypoxic conditions (Luo et al.,
530 2011). LHPP induces ubiquitin-mediated degradation of PKM2, which results in the
531 inhibition of glycolysis under hypoxia (Chen et al., 2021). Interestingly, the LHPP was also
532 subject to positive selection in cetaceans (Tian et al., 2017). Furthermore, both *C. heheva* and
533 cetaceans have the same amino acid substitution at position 118 of the LHPP protein (**Figs.**
534 **S3 and S4**). These results suggest that the two interacting genes (*PKM2* and *LHPP*) play a
535 key role in the hypoxic adaptation of these hypoxia-tolerant marine animals.

536

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1031 **Data Accessibility**

1032 Raw reads and genome assembly are accessible in NCBI under BioProject number
1033 PRJNA752986. Assembled genome sequences are accessible under Whole Genome Shotgun
1034 project number JAIGNY000000000. Raw reads and genome assembly are also available at
1035 the CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb)
1036 with accession number CNP0002134. The genome assembly and related annotation files are
1037 available at Figshare (<https://doi.org/10.6084/m9.figshare.15302229>).

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1039 **Author Contributions**

1040 M.W and J.G.H. conceived of the project and designed research; J.H. collected the sample;
1041 P.T., L.Z, Y.M., Q.C., Q.Z., L.Z. assembled and annotated the genome; L.Z., Z.G., J.H.,
1042 M.W., S.Q., Y.W. performed the evolutionary analyses; M.W., G.H. wrote the paper the
1043 manuscript with the contribution from all authors.

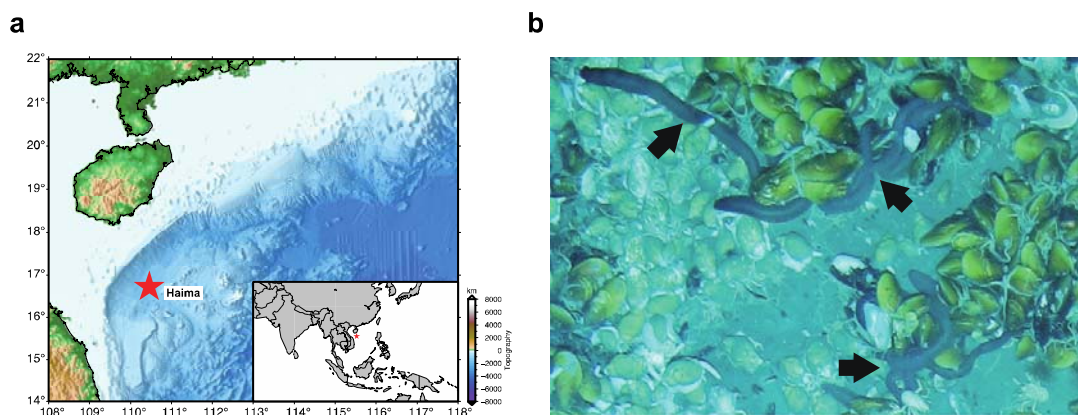
1044

1045 **Table 1 Genome assembly statistics of *C. heheva* and *A. japonicus***

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	<i>C. heheva</i>	<i>A. japonicus</i> (Li et al., 2018)	<i>A. japonicus</i> (Zhang et al., 2017)
Estimated genome size (Gb)	~1.23	~1.0	~1.0
Assembled genome size (bp)	1,106,937,276	952,279,490	804,993,085
Number of contigs	4,609	21,303	7,058
Contig N50 (bp)	1,221,604	45,411	190,269
Scaffold N50 (bp)	-	195,518	486,650

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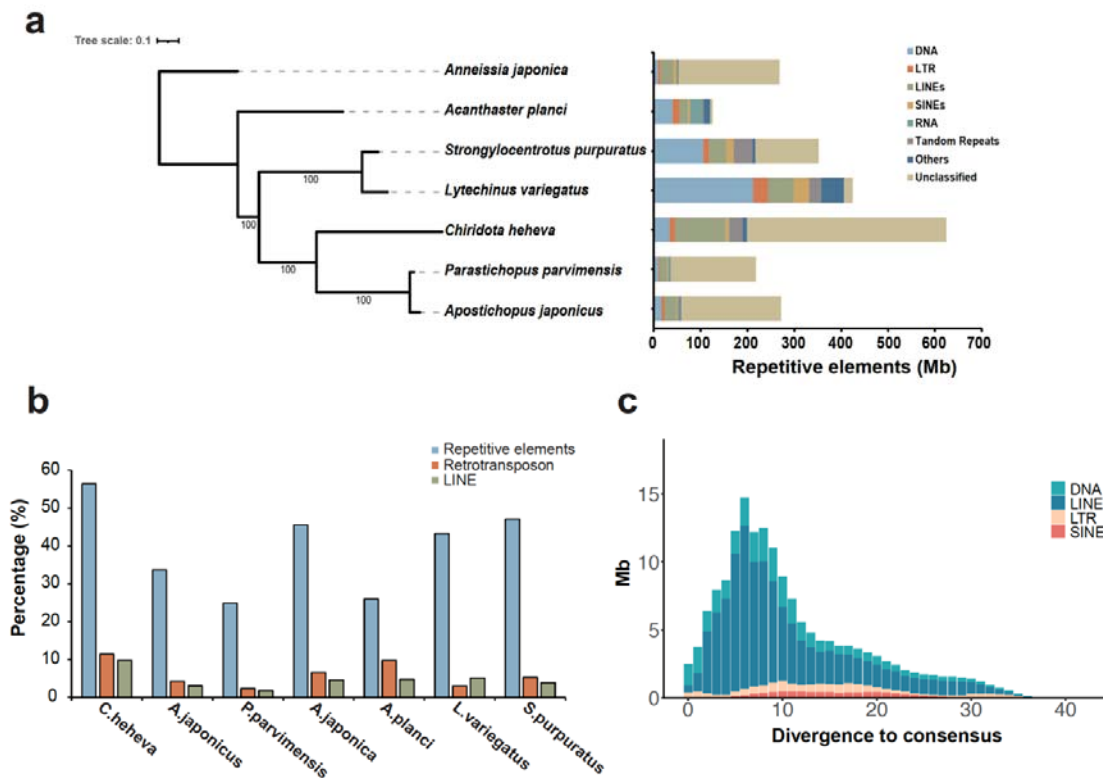


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1049 **Figure 1.** Collection of *C. heheva*. (a) Map showing the sampling site at the Haima cold seep
1050 of northern South China Sea (16° 73.228' N, 110° 46.143' E). (b) *C. heheva* at the sampling
1051 site (depth: 1,385 m), where they cohabit with deep-sea mussels. *C. heheva* individuals are
1052 indicated by black arrows.

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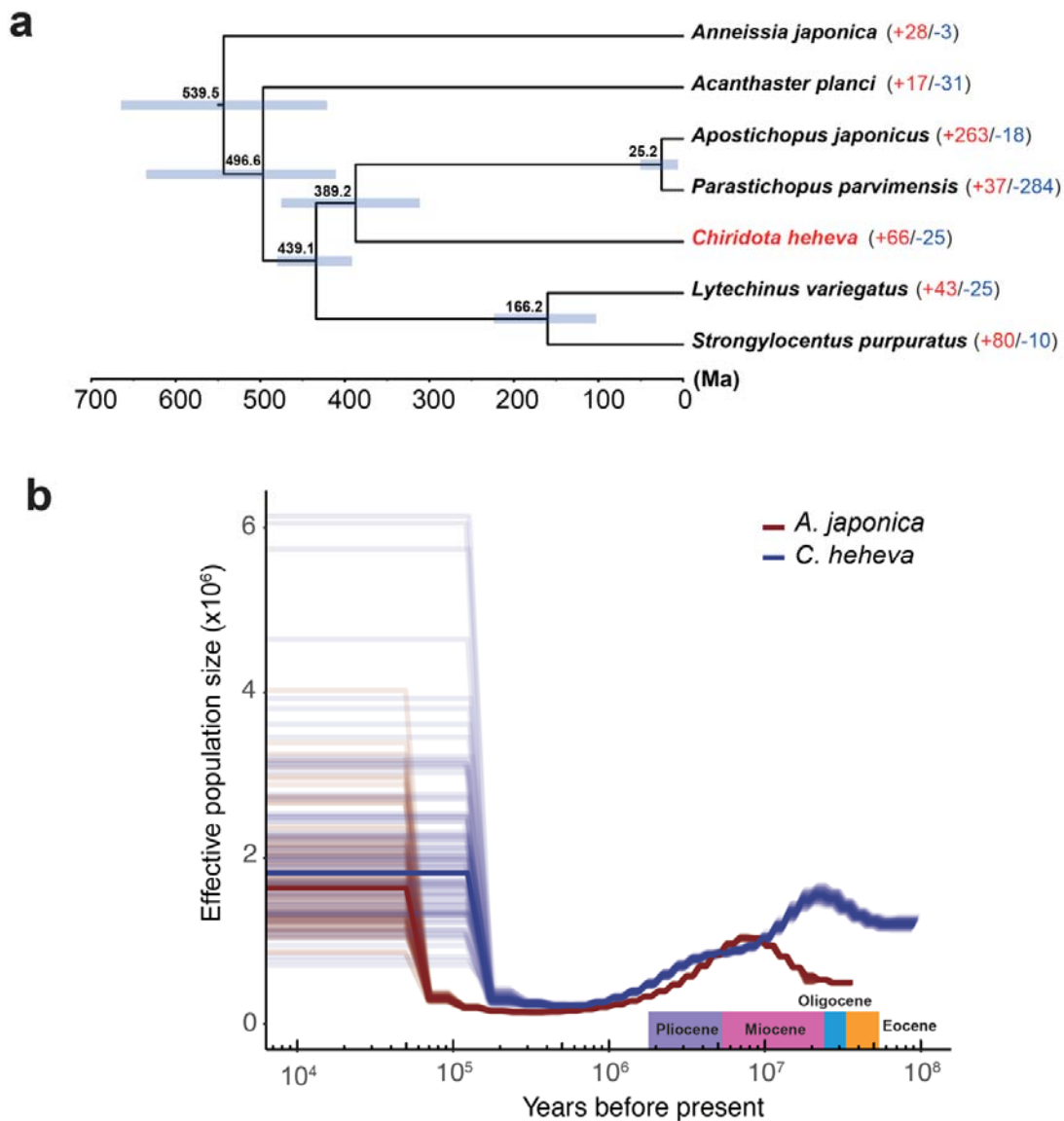
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1056 **Figure 2. Landscape of transposable elements in echinoderm genomes.** (a) Comparison of
 1057 the occurrence and composition of repetitive elements in the genomes of 7 echinoderms. (b)
 1058 Comparison of the proportion of repetitive elements, retrotransposon, and long interspersed
 1059 nuclear elements (LINEs) in the genomes of 7 echinoderms. The proportions of repetitive
 1060 elements and LINEs are higher in the genome of *C. heheva* than that in other echinoderms. (c)
 1061 Transposable element accumulation profile in *C. heheva* genome. A recent burst of LINEs
 1062 was observed in *C. heheva*.

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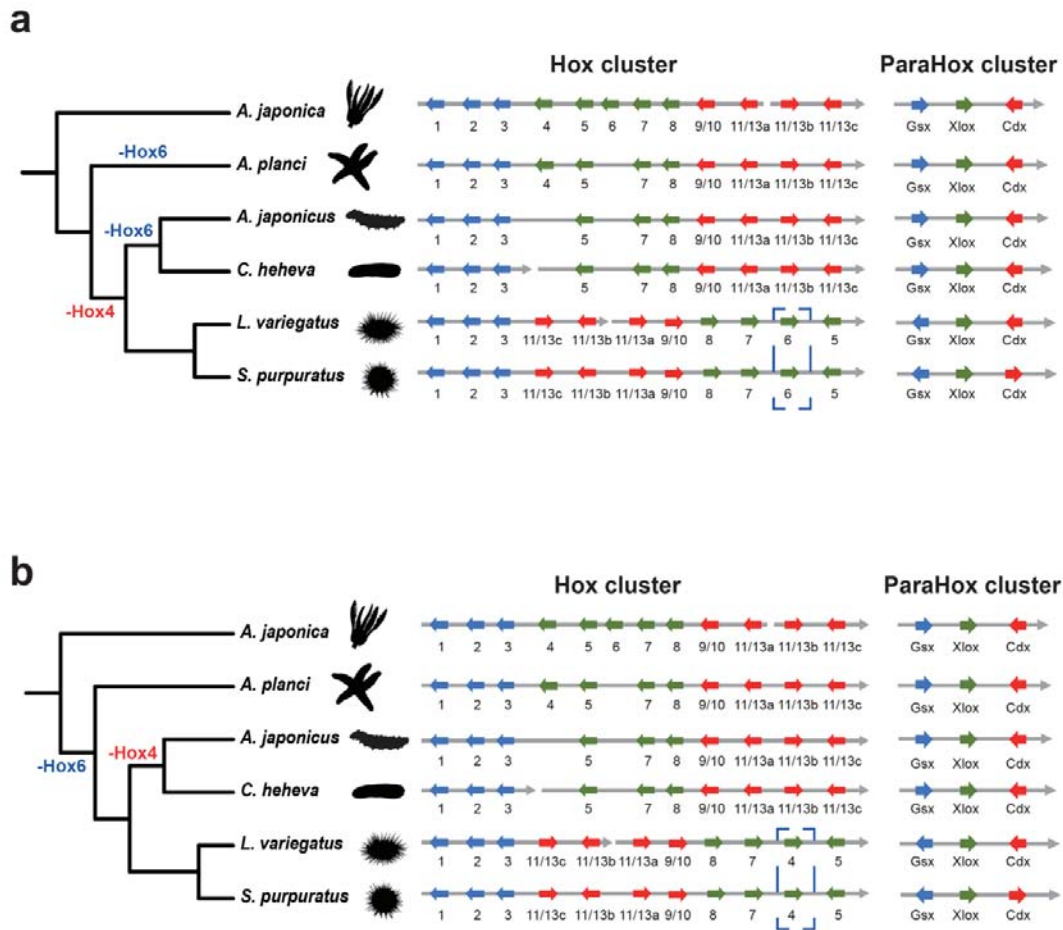


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1066 **Figure 3. Evolutionary history of *C. heheva*.** (a) A species tree of 7 echinoderm species. In
1067 total, 988 single-copy orthologs were used to reconstruct the phylogenetic tree. The
1068 divergence time between species pairs was listed above each node, and 95% confidence
1069 internal of the estimated divergence time was denoted as blue bar. The numbers of protein
1070 families that were significantly expanded (red) or contracted (blue) ($P < 0.05$) in each species
1071 are denoted beside the species names. (b) Demographic history of *C. heheva* (blue) and *A.*
1072 *japonicus* (red). The changes of ancestral population size of *C. heheva* and *A. japonicus*
1073 were inferred using the PSMC method. Time in history was estimated by assuming a generation
1074 time of 3 years and a mutation rate of 1.0×10^{-8} .

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1078 **Figure 4. Genomic organization of *Hox* and *ParaHox* gene clusters in echinoderms. *Hox***

1079 **and *ParaHox* genes are indicated by arrows. The gene composition and orientation of *Hox***

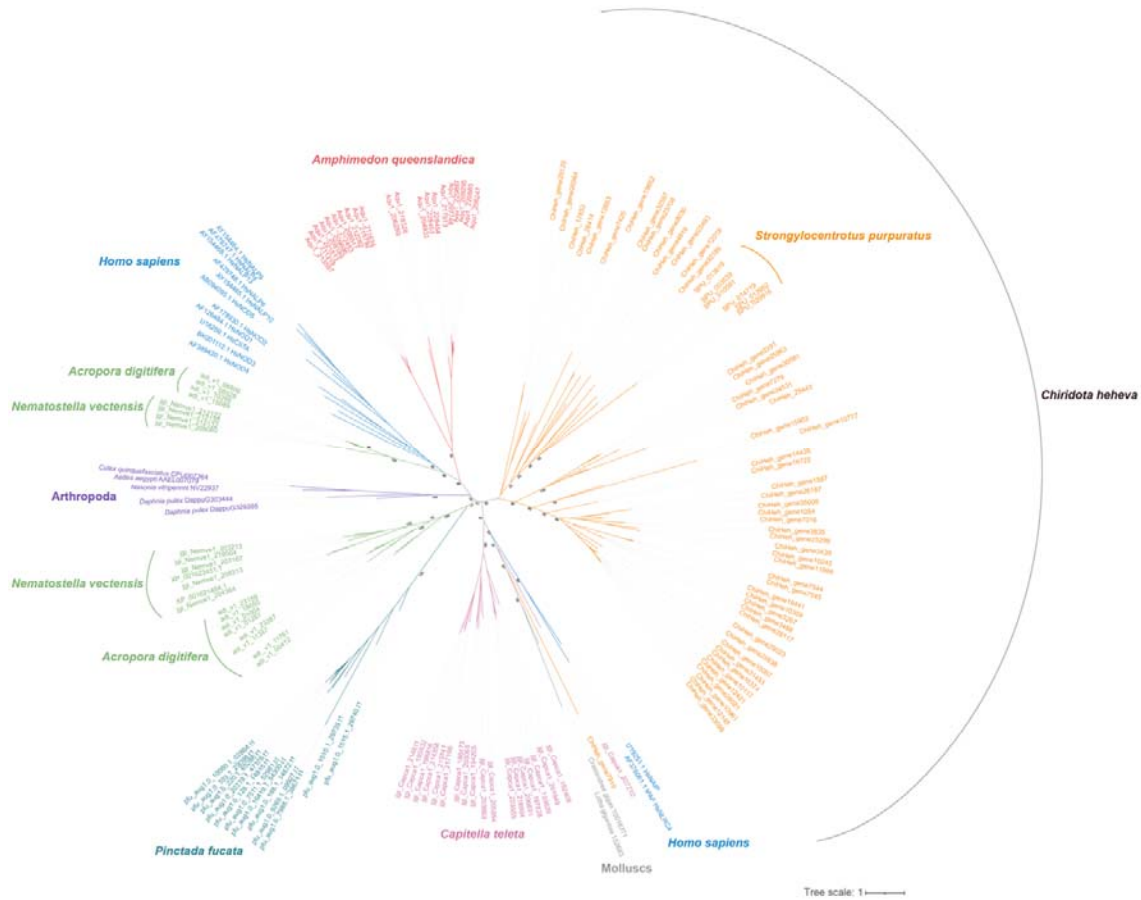
1080 **and *ParaHox* clusters are consistent between two holothurians (*C. heheva*, *A. japonicus*). (a)**

1081 **The organization of *Hox* genes in echinoderms if *Hox4* is lost in echinoids (*L. variegatus*, *S.***

1082 ***purpuratus*). (b) The organization of *Hox* genes in echinoderms if *Hox6* is lost in echinoids.**

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1086 **Figure 5. Evolutionary relationships among *C.heheva* NLRs and other representative**
1087 **metazoan NLRs.** The unrooted phylogenetic tree was reconstructed based on the NACHT
1088 domain sequences using a maximum likelihood method. The values near the nodes are
1089 ultrafast bootstrap (UFBoot) values. NLRs from different types of species are highlighted by
1090 branches of different colors. The species name is shown near the corresponding lineage.

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Figure 6. Evolutionary relationship with aerolysin-like proteins (ALPs) from *C. heheva* and other species. The unrooted phylogenetic tree was reconstructed using a maximum likelihood method. The values near the nodes are ultrafast bootstrap (UFBoot) values. ALPs from different types of species are highlighted by branches of different colors. The species name is shown near the corresponding lineage. ALPs from *C. heheva* do not cluster with ALPs from other echinoderms (*A. japonicus*, *P. parvimensis*), but with the ones from sea anemones (*N. vectensis*, *E. diaphana*).