

1 **High-quality reference genome of *Fasciola gigantica*: Insights into  
2 the genomic signatures of transposon-mediated evolution and  
3 specific parasitic adaption in tropical regions**

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27 **Abstract**

28 *Fasciola gigantica* and *Fasciola hepatica* are causative pathogens of *fascioliasis*,  
29 with the widest latitudinal, longitudinal, and altitudinal distribution; however, among  
30 parasites, they have the largest sequenced genomes, hindering genomic research. In the  
31 present study, we used various sequencing and assembly technologies to generate a new  
32 high-quality *Fasciola gigantica* reference genome. We improved the integration of gene  
33 structure prediction, and identified two independent transposable element expansion  
34 events contributing to (1) the speciation between *Fasciola* and *Fasciolopsis* during the  
35 Cretaceous-Paleogene boundary mass extinction, and (2) the habitat switch to the liver  
36 during the Paleocene-Eocene Thermal Maximum, accompanied by gene length  
37 increment. Long interspersed element (LINE) duplication contributed to the second  
38 transposon-mediated alteration, showing an obvious trend of insertion into gene regions,  
39 regardless of strong purifying selection. Gene ontology analysis of genes with long  
40 LINE insertions identified membrane-associated and vesicle secretion process proteins,  
41 further implicating the functional alteration of the gene network. We identified 852  
42 excretory/secretory proteins and 3300 protein-protein interactions between *Fasciola*  
43 *gigantica* and its host. Among them, copper/zinc superoxide dismutase genes, with  
44 specific gene copy number variations, might play a central role in the phase I  
45 detoxification process. Analysis of 559 single-copy orthologs suggested that *Fasciola*  
46 *gigantica* and *Fasciola hepatica* diverged at 11.8 Ma near the Middle and Late Miocene  
47 Epoch boundary. We identified 98 rapidly evolving gene families, including actin and  
48 aquaporin, which might explain the large body size and the parasitic adaptive character  
49 resulting in these liver flukes becoming epidemic in tropical and subtropical regions.

50 **Introduction**

51 *Fasciola gigantica* and *Fasciola hepatica*, known as liver flukes, are two species  
52 in the genus *Fasciola*, which cause *fascioliasis* commonly in domestic and wild  
53 ruminants, but also are causal agents of *fascioliasis* in humans. *Fascioliasis* reduces the  
54 productivity of animal industries, imposes an economic burden of at least 3.2 billion  
55 dollars annually worldwide [1], and is a neglected zoonotic tropical disease of humans,  
56 according the World Health Organization's list [2]. *F. gigantica*, the major fluke  
57 infecting ruminants in Asia and Africa, has been a serious threat to the farming of  
58 domesticated animals, such as cows and buffaloes, and dramatically reduces their feed  
59 conversion efficiency and reproduction [3]. The prevalence *F. gigantica* infection has  
60 greatly affected subsistence farmers, who have limited resources to treat their herds,  
61 and has hindered economic development and health levels, especially in developing  
62 countries.

63 The various omics technologies provide powerful tools to advance our  
64 understanding of the molecules that act at the host-parasite interface, and allow the  
65 identification of new therapeutic targets against *fascioliasis* [4]. To date, four  
66 assemblies for *F. hepatica* and two assemblies for *F. gigantica* have been deposited at  
67 the NCBI [5-8]. These assemblies reveal a large genome with a high percentage of  
68 repeat regions in *Fasciola* species, and provided valuable insights into features of  
69 adaptation and evolution. However, these assemblies are based on the short read  
70 Illumina sequencing or hybrid sequencing methods, with limited ability to span large

71 families of repeats. Various limitations have led to the current assemblies in the genus  
72 *Fasciola* being fragmented (8 kb to 33 kb and 128 kb to 1.9 Mb for contig and scaffold  
73 N50s, respectively). Subsequent gene annotation analysis using current assemblies  
74 were also challenging, with abundant transposition events occurring over evolutionary  
75 history, which significantly increased the repeat components in intron regions, resulting  
76 in considerable fragmentation in gene annotation.

77 Infection by *Fasciola* causes extensive damage to the liver, and excretory/secretory  
78 (E/S) proteins play an important role in host-parasite interactions. Parasite-derived  
79 molecules interact with proteins from the host cell to generate a protein interaction  
80 network, and these proteins partly contribute to *Fasciola*'s striking ability to avoid and  
81 modulate the host's immune response [9]. Previous proteomics of E/S proteins have  
82 highlighted the importance of secreted extracellular vesicles (EVs) and detoxification  
83 enzymes to modulate host immunity by internalizing with host immune cells [10, 11].  
84 The anthelmintic drug, triclabendazole (TCBZ), is currently the major drug available  
85 to treat *fascioliasis* at the early and adult stages, which acts by disrupting  $\beta$ -tubulin  
86 polymerization [1]; however, over-reliance on TCBZ to treat domesticated ruminants  
87 has resulted in selection for resistance to liver flukes [12]. Drug and vaccine targets for  
88 molecules associated with reactive oxygen species (ROS)-mediated apoptosis have  
89 recently been validated as an effective tools in multiple helminth parasites [13].  
90 Increased understanding of host-parasite and drug-parasite interactions would facilitate  
91 the development of novel strategies to control *fascioliasis*.

92 In recent years, there have been increasing numbers of human cases of *fascioliasis*,  
93 becoming a major public health concern in many regions [14, 15]. However, high  
94 quality genome assemblies for liver flukes are still insufficient. In the present study, we  
95 combined multiple sequencing technologies to assemble a chromosome-level genome  
96 for *F. gigantica* and provided integrated gene annotation. Protein-protein interactions  
97 were analyzed between the predicted *F. gigantica* secretome and host proteins  
98 expressed in the small intestine and liver. In addition, gene family analysis identified a  
99 series of genes expansions in *F. gigantica*. Interestingly, the distribution of repeat  
100 sequences in the genome exhibit an excess of long interspersed element (LINE)  
101 duplications inserted into intronic regions, potentially helping to explain the  
102 duplications of transposable element (TE) plasticizing gene structures and possibly  
103 acting as long-term agents in the speciation of *Fasciola*.

## 104 Results

### 105 Pacbio long reads-based *de novo* assembly and gene annotation

106 The *F. gigantica* genome contains abundant repeat sequences that are difficult to  
107 span using short read assembly methods, and the complex regions also hinder integrated  
108 gene annotation of the genome. Therefore, in the present study, multiple sequencing  
109 technologies, have been applied: (1) Single-molecule sequencing long reads (~91 $\times$   
110 depth) using the Pacbio Sequel II platform; (2) paired-end reads (~66 $\times$  depth) using the  
111 Illumina platform; and (3) chromosome conformation capture sequencing (Hi-C) data  
112 (~100 $\times$  depth) (Supplementary Table 1). The initial assembly was performed using the  
113 Pacbio long reads, followed by mapping using single-molecule sequencing and  
114 Illumina sequencing reads to polish assembly errors and sequencing mistakes, resulting

115 in a contig N50 size of 4.89 Mb (**Fig. 1A**). The Hi-C data were used to build final  
116 super-scaffolds, resulting in a total length of 1.35 Gb with a scaffold N50 size of 133  
117 Mb (**Fig. 1B, Table 1, Supplementary Table S2-3, Supplementary Fig. S1**). The final  
118 assembly consists of 10 pseudo-chromosomes covering more than 99.9% of the *F.*  
119 *gigantica* genome, and the length distribution was approximate equal to the estimation  
120 by karyotype in previous research (**Supplementary Fig. S2, Supplementary Table S4**)  
121 [16]. The assessment of nucleotide accuracy shows that the error rate was  $5.7 \times 10^{-6}$  in  
122 the genome. QUAST analysis [17] showed a high mapping and coverage rate using  
123 both Illumina short reads and Pacbio long reads, in which 99.73% of reads mapped to  
124 99.85% of the genome with more than 10 $\times$  depth (**Supplementary Table S5**).

125 Combing *de novo*/homolog/RNA-seq prediction, a total of 12,503 protein coding  
126 genes were annotated in the *F. gigantica* genome. BUSCO assessment [18] indicated  
127 that the genome is 90.4% complete and 5.6% fragmented, underscoring the significant  
128 improvement of the genome continuity and gene-structure predictions compared with  
129 previous assemblies (**Supplementary Table S6**). Specifically, the average gene length  
130 in the annotated data is 28.8 kb, nearly twice the length of that in other digenetic species,  
131 but contrasted with the similar average length of the coding sequences (CDSs). Through  
132 functional annotation, we found that 8569 of the genes could be characterized in the  
133 InterPro database [19, 20], 7892 of them were mapped to the gene ontology (GO) terms,  
134 and 5353 of them were identified by the Kyoto Encyclopedia of Genes and Genomes  
135 (KEGG) pathways database (**Supplementary Fig. S3-4, Supplementary Table S7**).

### 136 The unique repeat duplications in *Fasciola*

137 TEs are insertional mutagens and major drivers of genome evolution in eukaryotes,  
138 and replication of these sequences, resulting in variation of gene structure and  
139 expression, have been extensively documented [21, 22]. Besides, TEs are molecular  
140 fossils, being remnants of past mobilization waves that occurred millions of years ago  
141 [23]. In the present study, we identified repeat sequences combined the analysis from  
142 RepeatModeler [24] and RepeatMasker [25], and detected a significant proportion of  
143 them neglected by previous studies. In the *F. gigantica* genome, we identified 945 Mb  
144 of repeat sequences, which was approximate 20% more than that identified in other  
145 assemblies in *Fasciola* species, while the lengths of non-repeat sequences were nearly  
146 identical. The most convincing explanation for the additional assembled repeat  
147 sequences was that the contigs constructed from Pacbio long reads spanned longer  
148 repeat regions, which were compressed in previous assemblies. Among these repeat  
149 sequences, there were 408 Mb of LINEs (corresponding to 30.3% of the assembled  
150 genome), 285 Mb of long terminal repeats (LTRs, corresponding to 21.2% of the  
151 assembled genome), and 162 Mb of unclassified interspersed repeats (corresponding to  
152 12.0% of the assembled genome) (**Supplementary Fig. S5, Supplementary Table S8**).  
153 According to the repeat landscapes, we found that there were two shared expansion  
154 events for LINEs and LTRs that occurred approximately 12 million years ago (Ma) and  
155 65 Ma, and an additional expansion event at 33 Ma for LTRs (**Supplementary Fig. S6-7**).  
156 The abundant repeat sequences in the *Fasciola* genomes aroused our interest  
157 concerning the role of repeats in evolution (**Fig. 2A**), and inspired us to hypothesize  
158 that the expansion of TEs enlarged the genome size of an ancestor of *Fasciola* to gain a

159 new advantage by rewiring gene networks. To test this hypothesis, we focused on the  
160 genome-wide repeats distribution and test signatures of selection.

161 For new TE insertions to persist through vertical inheritance, transposition events  
162 must be under strong purifying selection among gene loci to avoid disturbing their  
163 biological function. However, we observed many intronic repeat elements in *Fasciola*,  
164 resulting in a larger intron size per gene. If there are equal selection effects on newly  
165 inserted TEs in intronic and intergenic regions, there would a high correlation between  
166 the distribution of insertion time and retained TE lengths between these two regions.  
167 By contrast, there would be fewer accumulated repeat sequences existing under  
168 purifying selection. In this study, we use the relative proportion of TEs between intronic  
169 and intergenic regions as a simple indicator, and use the inferred size of intronic and  
170 intergenic regions over evolutionary history as a control to estimate the signatures of  
171 selection. The results showed that TE insertions into intronic regions are under  
172 persistent intense purifying selection, except for LINEs. There was an excess of  
173 persistent LINE insertions into intronic regions between 41 Ma and 62 Ma, indicating  
174 different modes of accumulating LINEs into intronic regions compared with that in  
175 other periods (Fig. 2B). Specifically, the time of the ancient intronic LINE expansion  
176 (~51.5 Ma) was different to the genome-wide LINE expansion time (~68.0 Ma),  
177 whereas the time was coincident with two important environmental change events, the  
178 Cretaceous-Paleogene boundary (KPB) mass extinction (~66.0 Ma) and the Paleocene-  
179 Eocene Thermal Maximum (PETM) (~55.8 Ma). Both the PETM and KPB events  
180 recorded extreme and rapid warming climate changes; however, rapid evolutionary  
181 diversification followed the PETM event, as opposed to near total mass extinction at  
182 the KPB [26]. Therefore, we selected genes with different LINE lengths, derived  
183 between 41 Ma and 62 Ma, and expected to identify a transposon-mediated alterative  
184 gene network contributing to the host switch and the shift from intestinal to hepatic  
185 habitats.

#### 186 **LINE-mediated alterative gene network**

187 We identified a substantial proportion of genes with LINE insertions, derived  
188 between 41 Ma and 62 Ma, indicating a universal effect of the gene network. We  
189 selected 1288 genes with the LINE insertions of more than 10 kb, representing more  
190 than one third of the average gene length, and annotated the genes using Gene Ontology  
191 (GO) terms and processes and Kyoto encyclopedia of genes and genomes (KEGG)  
192 pathways (Fig. 2C, Supplementary Table S9-11). These genes involve molecules  
193 internalizing substances from their external environment, including  
194 membrane-associated and vesicle secretion process proteins. Meanwhile, the gene  
195 network was likely adapted to the evolution of protein biosynthesis and modification  
196 of histones.

197 Enrichment analysis of GO terms showed that membrane and  
198 membrane-associated proteins are over-represented, involving “synaptic membrane” ( $P$   
199 = 3.52E-04), “clathrin-coated vesicle membrane” ( $P$  = 1.08E-03), and “synaptic vesicle”  
200 ( $P$  = 3.02E-03), as well as vesicles secretion processes, such as “endocytosis” ( $P$  =  
201 7.06E-06), “Golgi organization” ( $P$  = 7.45E-05), “COPII vesicle coating” ( $P$  = 2.72E-  
202 04), “intracellular signal transduction” ( $P$  = 5.16E-04), and “endosomal transport” ( $P$  =

203 2.47E-03). The over-representation of genes involved in membrane transport was  
204 particularly interesting because helminth parasites interfere with the host immune  
205 system by secreting molecules from surface tegument or gut. The *TMED10* gene in *F.*  
206 *gigantica* (encoding transmembrane P24 trafficking protein 10) was used as an example.  
207 *TMED10* is a cargo receptor involved in protein vesicular trafficking along the secretory  
208 pathway [27, 28], and the genes has an 11.1 kb LINE insertion in the third intron,  
209 resulting in an over three-fold increment in the gene length (**Fig. 2D**). The enrichment  
210 suggests that the gene network related to secretion could have experienced adaptive  
211 evolution during LINE transposition events. We further compared our dataset with the  
212 proteome result from *F. hepatica* extracellular vesicles (EVs) [29], and found 21  
213 proteins that were also identified as surface molecules associated with EV biogenesis  
214 and vesicle trafficking (*IST1*, *VPS4B*, *TSG101*, *MYOF*, *ATG2B*, *STXBP5L*, and 15 Rho  
215 GTPase-activating related proteins). Specifically, *IST1*, *VPS4B*, and *TSG101* are  
216 members of the endosomal sorting complex required for transport (ESCRT) pathway,  
217 which promotes the budding and release of EVs. *TSG101*, a crucial member of the  
218 ESCRT-I complex, has an important role in mediating the biogenesis of multi-vesicular  
219 bodies, cargo degradation, and recycling of membrane receptors. Besides, the ESCRT  
220 pathway promotes the formation of both exosomal carriers for immune communication.  
221 During the formation of the immunological synapse between T-cells and antigen-  
222 presenting B cells, *TSG101* ensures the ubiquitin-dependent sorting of T-Cell Receptor  
223 (*TCR*) molecules to exosomes that undergo *VPS4*-dependent release into the synaptic  
224 cleft[30].

225 The most significant KEGG pathway was aminoacyl-tRNA biosynthesis ( $P =$   
226 7.16E-04), containing 15 out of 38 annotated aminoacyl tRNA synthetases (AAASs).  
227 AAASs are the enzymes that catalyze the aminoacylation reaction by covalently linking  
228 an amino acid to its cognate tRNA in the first step of protein translation. The large-scale  
229 insertion of LINEs reside in AAAS genes suggested that the ancestor of *Fasciola* may  
230 have profited from the effect of transposition, with changes to protein biosynthesis and  
231 several metabolic pathways for cell viability. In addition, a significant number of genes  
232 are strongly associated with histone modulation, including “histone deacetylase  
233 complex” ( $P = 1.89E-03$ ), “histone methyltransferase activity (H3-K36 specific)” ( $P =$   
234 1.08E-03), and “methylated histone binding” ( $P = 2.37E-03$ ). Histone modifications  
235 play fundamental roles in the manipulation and expression of DNA. We found nine  
236 histone deacetylases and Histone methyltransferases in the gene set (*HDAC4*, *HDAC8*,  
237 *HDAC10*, *KMT2E*, *KMT2H*, *KMT3A*, *KDM8*, *NSD1*, and *NSD3*). Histone  
238 modifications can exert their effects by influencing the overall structure of chromatin  
239 and modifying and regulating the binding of effector molecules [31, 32]; therefore, the  
240 variation of these genes might bring about evolution from a disturbed gene structure to  
241 a mechanism of genome stabilization to tackle a continuous genome amplification  
242 process in evolutionary history.

#### 243 **Genome-wide host-parasite interaction analysis**

244 In the *Fasciola* genome, we predicted genes encoding 268 proteases, 36 protease  
245 inhibitors (PIs), and 852 excretory/secretory (E/S) proteins that are commonly involved  
246 in interacting with hosts and modulating host immune responses. The largest class of

247 proteases was cysteine peptidases ( $n = 113$ ), which was also identified in the *F. hepatica*  
248 genome (**Fig. 3A, Supplementary Table S12**). The largest ( $n = 19$ , 52.8% of PIs) PI  
249 family was the I02 family of Kunitz-BPTI serine protease inhibitors, which bind to  
250 Cathepsin L with a possible immunoregulatory function [33] (**Supplementary Table**  
251 **S13**). GO enrichment analysis of E/S proteins showed that proteins related to  
252 “activation of cysteine-type endopeptidase activity” ( $P = 6.14\text{E-}19$ ), “peroxidase  
253 activity” ( $P = 3.79\text{E-}07$ ) and “protein disulfide isomerase activity” ( $P = 3.75\text{E-}06$ ) are  
254 over-represented (**Fig. 3B, Supplementary Table S14-15**). Indeed, there were 38  
255 cysteine peptidases identified as E/S proteins, including cathepsin L-like, cathepsin B-  
256 like, and legumain proteins, which participate in excystment, migration through gut  
257 wall, and immune evasion [34].

258 In parasites, as in mammalian cells, ROS are produced as a by-product of cell  
259 metabolism and from the metabolism of certain pharmacological agents. The ability of  
260 a parasite to survive in its host has been directly related to its antioxidant enzyme  
261 content [35]. To further analyze host-parasite interactions, we identified the  
262 protein-protein interactions (PPIs) between the *F. gigantica* secretome and human  
263 proteins expressed in the small intestine and liver. In total, we identified 3300 PPIs,  
264 including rich interactions that directly or indirectly participated in the two phases of  
265 detoxification pathways (**Fig. 3C**). Superoxide dismutase [Cu-Zn] (*SOD*, PPIs = 49)  
266 was first highlighted because of its important role on phase I detoxification against ROS,  
267 in which it catalyzes the dismutation of the superoxide radical to molecular oxygen and  
268 hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) [36]. Gene family analysis identified six *SOD* paralogs in *F.*  
269 *gigantica*, and two of them contained a signal peptide (**Fig. 4D**). Previous enzyme  
270 activity assays also confirmed a significant difference between *SOD* activities and  
271 concentration in E/S proteins of two *Fasciola* species [37], suggesting an intense ability  
272 to resist superoxide radical toxicity. Meanwhile, the metabolite of phase I,  $\text{H}_2\text{O}_2$ , can  
273 also damage parasites, which requires detoxification enzymes, including  
274 glutathione-dependent enzymes *GPx*, glutathione reductase, and other peroxidases.  
275 Protein disulfide-isomerase (*P4HB*, PPIs = 132) and phospholipid hydroperoxide  
276 glutathione peroxidase (*GPX4*, PPIs=28) were as functioning in phase II detoxification.  
277 *GPx* catalyzes the reduction of hydroperoxides (ROOH) to water, using glutathione  
278 (*GSH*) as the reductant. *P4HB* also participates in the process by mediating homeostasis  
279 of the antioxidant glutathione [38]. However, we did not identify E/S proteins in the  
280 Cytochrome P450 (*CYP450*) family in phase III detoxification. Therefore, we  
281 speculated that successful parasite defense against *F. gigantica* is mainly depends on  
282 the strong superoxide activity and efficient hydrogen peroxide detoxification.

### 283 **Gene family analysis**

284 Gene family analysis was performed using eight taxa (*F. gigantica*, *F. hepatica*,  
285 *Fasciolopsis buski*[39], *Clonorchis sinensis* [40], *Schistosoma mansoni*)[41], *Taenia*  
286 *multiceps* [42], swamp buffalo [43], and human [44], which identified 17,992 gene  
287 families (**Fig. 4A**). Phylogeny analysis of 559 single-copy orthologs showed that *F.*  
288 *gigantica* and *F. hepatica* shared a common ancestor approximately 11.8 million years  
289 ago (2.2-22.5 Ma, 95% highest posterior density [HPD]) near the Middle and Late  
290 Miocene Epoch boundary. The Miocene warming began 21 million years ago and

291 continued until 14 million years ago, when global temperatures took a sharp drop at the  
292 Middle Miocene Climate Transition (MMCT). The divergence of the two *Fasciola*  
293 species may have resulted from the consequences of rapid climate changes, such as  
294 migration of the host causing geographic isolation. Our estimation is between the  
295 previously suggested date of 5.3 Ma based on 30 nuclear protein-coding genes [45],  
296 and 19 Ma based on cathepsin L-like cysteine proteases [46]. Although we used a more  
297 integrative gene dataset, the wide HPD interval could not be neglected, raising possible  
298 uncertainty from the complex process of speciation or inappropriate protein sequence  
299 alignment between members of the genus *Fasciola*.

300 The distribution of gene family size among different species is used to estimate  
301 which lineages underwent significant contractions or expansions. Compared with *F.*  
302 *hepatica*, *F. gigantica* shows more gene family expansion events (643 compared to 449)  
303 and a similar number of gene family contractions (713 compared to 672). The result  
304 emphasize the general trend that, relative to the common ancestor of *Fasciola*, the  
305 ancestor of *F. gigantica* apparently underwent a higher extent of gene-expansion than  
306 did the ancestor of *F. hepatica*. Gene duplication is one of the primary contributors to  
307 the acquisition of new functions and physiology [47]. We identified 98 gene families,  
308 including 629 genes, as rapidly evolving families specific to *F. gigantica*. Family  
309 analysis showed a fascinating trend of gene duplication, with substantial enrichment for  
310 the “structural constituent of cytoskeleton” ( $P = 3.52\text{E-}24$ ), “sarcomere organization”  
311 ( $P = 2.29\text{E-}14$ ), “actin filament capping” ( $P = 6.19\text{E-}13$ ), and “spectrin” ( $P = 3.03\text{E-}11$ )  
312 in *F. gigantica* (**Supplementary Table S16**). There were 24 actin paralogs in *F.*  
313 *gigantica*, in contrast to 8 actin paralogs in *F. hepatica*. Actin is one of the most  
314 abundant proteins in most cells, and actin filaments, one of the three major cytoskeletal  
315 polymers, provide structure and support internal movements of organisms [48]. They  
316 are also highly conserved, varying by only a few amino acids between algae, amoeba,  
317 fungi, and animals [49]. We observed three types of actin proteins in flukes, according  
318 to their identity from human actin family. Seventeen of the 24 actin proteins in *F.*  
319 *gigantica* are highly conserved (Identity > 95%) (**Fig. 4B**). Consistent with the accepted  
320 role of the epidermal actin cytoskeleton in embryonic elongation [50, 51], we  
321 speculated that the significant expansion of actin and spectrin genes increased the body  
322 size of *F. gigantica* via cell elongation or proliferation during morphogenesis. Another  
323 rapidly evolving family is the aquaglyceroporin subfamily in the membrane water  
324 channel family. We found six aquaglyceroporin paralogs in *F. gigantica*, which were  
325 over-represented in the GO term “water transport” ( $P = 2.10\text{E-}06$ ) (**Fig. 4C**).  
326 Aquaglyceroporins are highly permeated by glycerol and other solutes, and variably  
327 permeated by water, as functionally validated by several studies [52, 53]. The  
328 mammalian aquaglyceroporins regulate glycerol content in epidermal, fat, and other  
329 tissues, and appear to be involved in skin hydration, cell proliferation, carcinogenesis,  
330 and fat metabolism. A previous study showed that *F. gigantica* could withstand a wider  
331 range of osmotic pressures compared with *F. hepatica* [54], and we speculated that a  
332 higher aquaglyceroporin gene copy number might help explain this observation.

333 It is worth mentioning that 57.6% of rapidly evolving expansion genes specific to  
334 the *F. gigantica* genome were driven by tandem duplication, such that the newly formed

335 duplicates preserved nearly identical sequences to the original genes. The newly formed  
336 genes would accumulate non-functionalizing mutations, or develop new functions over  
337 time. We found only few tandem duplicated genes that had non-functionalizing  
338 mutations, suggesting that adaptive evolution could have an important role in the  
339 consequences of these genes via a dosage effect or neo-functionalization.

### 340 Discussion

341 The genome of *Fasciola* species contains a large percentage of repeat sequences,  
342 making them the largest parasite genomes sequenced to date. Since the first assembly  
343 of *F. hepatica* was submitted in 2015 [6], several studies have aimed to improve the  
344 quality of assembly and gene annotation [5, 7, 8]. With advances in long read  
345 sequencing assembly and Hi-C scaffolding technologies, it is now viable to resolve the  
346 genomic “dark matter” of repetitive sequences, and other complex structural regions at  
347 relatively low cost [55]. Therefore, we present the highest quality genome and gene  
348 annotation for *F. gigantica* to date, and provide long-awaited integrated genome  
349 annotation for *fascioliasis* research.

350 Our research determined the TE sequences among intronic and intergenic regions.  
351 TE sequences of *F. gigantica* experienced massive expansion through the genome via  
352 a ‘copy-and-paste’ model of transposition [56]. Especially, the speciation between  
353 *Fasciola* and *Fasciolopsis* was most likely caused by a *Fasciola*-specific whole genome  
354 repeat expansion event during the KPB mass extinction, and similarly, the speciation  
355 between the *Fasciola* and *Fascioloides*—a habitat switch from the small intestine to the  
356 liver in the host—occurred during the PETM, accompanied by LINE expansion biased  
357 toward intronic regions (Fig. 5). These synchronous events informed a new hypothesis  
358 of adaptive evolution driven by transposition events and will prompt investigations of  
359 how such differences contribute mechanistically to the morphological phenotypes of  
360 liver flukes and related species. This hypothesis could be tested by targeted genome  
361 assembly of *Fascioloides* species and estimating whether they had a different pattern of  
362 LINE duplication among intronic regions. There are also many studies in other species  
363 supporting the hypothesis that TE invasions endured by organisms have catalyzed the  
364 evolution of gene-regulatory network [57]. For example, Eutherian-specific TEs have  
365 the epigenetic signatures of enhancers, insulators, and repressors, and bind directly to  
366 transcription factors that are essential for pregnancy and coordinately regulate gene  
367 expression [58]. Similarly, genes with large-scale insertion of TEs in *Fasciola* species  
368 identified here, represent a signature of *Fasciola*-specific evolutionary gene network to  
369 distinguish other flukes of the family *Fasciolidae*. These genes overlap significantly  
370 with host-parasite interaction genes, including proteases and E/S proteins, and are  
371 enriched in the pathways of EV biogenesis and vesicle trafficking.

372 The data from genomic, transcriptomic, and proteomic studies can form a good  
373 complementary relationship to further our understanding of helminth parasites and their  
374 interaction with their hosts. Previous studies have identified a rich source of  
375 stage-specific molecules of interest using transcriptomic and proteomic analysis [59,  
376 60]. Here, we provided a comprehensive list of predicted E/S proteins in *F. gigantica*  
377 and predicted 3300 PPIs at the host-parasite interface, extending our understanding of  
378 how the phase I and phase II detoxification enzymes counteract the effect of ROS. The

ability of *Fasciola* species to infect and survive in different tissue environments is underpinned by several key E/S protein gene duplications. Both *Fasciola* species have a common expansion in the secretion of papain-like cysteine peptidase family (Clan A, family C1) [6]. Besides, *F. gigantica* has a specific variation in the *SOD* gene copy number, allowing it to regulate the catalytic activity of the superoxide radical released by the host. The effect of specific gene duplications can also be reflected in the increased body size of *F. gigantica*, which is an important morphometric character to distinguish *Fasciola* species and has a decisive influence on the final host species [61], although a gene level study of this phenotype is barely reported.

Overall, our study demonstrated that the combination of long-read sequencing with Hi-C scaffolding produced a very high-quality liver fluke genome assembly and gene annotation. Additionally, identification of the repeat distribution among the gene regions extended our understanding of the evolutionary process in *Fasciola* species. Further detailed functional studies of secretion might be of great scientific significance to explore their potential application in *fascioliasis* treatment.

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395

## 396 Materials and Methods

### 397 Sample collection and *de novo* sequencing.

398 All animal work was approved by the Guangxi University Institutional Animal  
399 Care and Use Committee. For the reference genome sequencing, *F. gigantica* was  
400 derived from infected buffalo in the Guangxi Zhuang Autonomous Region. Nucleic  
401 acids were extracted using a QIAGEN DNeasy (DNA) kit (Qiagen Hilden, Germany).  
402 Three *de novo* genome sequencing methods were performed on the liver fluke: We  
403 generated (1) 122.4 Gb (~88× depth) PacBio Sequel II single-molecule long reads, with  
404 an average read length of 15.8 kb (PacBio, Menlo Park, CA, USA); (2) 89.5 Gb (~66×  
405 depth) Illumina HiSeq PE150 pair-end sequencing to correct errors (Illumina, San  
406 Diego, CA, USA); and (3) 134 Gb (~100× depth) chromosome conformation capture  
407 sequencing (Hi-C) data (sequenced by Illumina platform).

### 408 *De novo* assembly and assessment of the genome quality.

409 A PacBio-only assembly was performed using Canu v2.0 [62, 63] using new  
410 overlapping and assembly algorithms, including an adaptive overlapping strategy based  
411 on *tf-idf* weighted MinHash and a sparse assembly graph construction that avoids  
412 collapsing diverged repeats and haplotypes. To remove haplotigs and contig overlaps  
413 in the assembly, we used Purge\_Dups based on the read depth [64]. Arrow  
414 (<https://github.com/PacificBiosciences/GenomicConsensus>) was initially used to  
415 reduce the assembly error in the draft assembly, with an improved consensus model  
416 based on a more straightforward hidden Markov model approach. Pilon [65] was used  
417 to improve the local base accuracy of the contigs via analysis of the read alignment  
418 information based on paired-end bam files (thrice). As a result, the initial assembly  
419 resulted had an N50 size of 4.89 Mb for the *F. gigantica* reference genome. ALLHiC  
420 was capable of building chromosomal-scale scaffolds for the initial genome using Hi-C  
421 paired-end reads containing putative restriction enzyme site information [66].

422 Three methods were used to evaluate the quality of the genomes. First, we used

423 QQuality ASsessment Tool (QUAST) [67] to align the Illumina and PacBio raw reads to  
424 the *F. gigantica* reference genome to estimate the coverage and mapping rate. Second,  
425 all the Illumina paired-end reads were mapped to the final genome using BWA [68],  
426 and single nucleotide polymorphisms (SNPs) were called using Samtools and Bcftools  
427 [69]. The predicted error rate was calculated by the homozygous substitutions divided  
428 by length of the whole genome, which included the discrepancy between assembly and  
429 sequencing data. Thirdly, we assessed the completeness of the genome assemblies and  
430 annotated the genes using BUSCO [18].

### 431 **Genome annotation**

432 Three gene prediction methods, based on *de novo* prediction, homologous genes,  
433 and transcriptomes, were integrated to annotate protein-coding genes. RNA-seq data of  
434 *F. gigantica* were obtained from the NCBI Sequence Read Archive, SRR4449208 [70].  
435 RNA-seq reads were aligned to the genome assembly using HISAT2 (v2.2.0) [71] and  
436 subsequently assembled using StringTie (v2.1.3) [72]. PASA (v2.4) [73] was another  
437 tool used to assemble RNA-seq reads and further generated gene models to train *de*  
438 *novo* programs. Two *de novo* programs, including Augustus (v3.0.2) [74] and SNAP  
439 (v2006-07-28) [75], were used to predict genes in the repeat-masked genome sequences.  
440 For homology-based prediction, protein sequences from UniRef100 [76]  
441 (plagiorchiida-specific, n = 75,612) were aligned on the genome sequence using  
442 TBLASTN [77] (e-value < 10<sup>-4</sup>), and GeneWise (version 2.4.1) [78] was used to identify  
443 accurate gene structures. All predicted genes from the three approaches were combined  
444 using MAKER (v3.1.2) [79] to generate high-confidence gene sets. To obtain gene  
445 function annotations, Interproscan (v5.45) [80] was used to identify annotated genes  
446 features, including protein families, domains, functional sites, and GO terms from the  
447 InterPro database. SwissProt and TrEMBL protein databases were also searched using  
448 BLASTp [81] (e-value < 10<sup>-4</sup>). The best BLASTp hits were used to assign homology-  
449 based gene functions. BlastKOALA [82] was used to search the KEGG ORTHOLOGY  
450 (KO) database. The subsequent enrichment analysis was performed using  
451 clusterProfiler using total annotated genes as the background with the “enricher”  
452 function [83].

### 453 **Repeat annotation and analysis**

454 We combined *de novo* and homology approaches to identify repetitive sequences  
455 in our assembly and previous published assemblies, including *F. gigantica*, *F. hepatica*,  
456 and *Fasciolopsis buski*. RepeatModeler (v2.0.1) [24] was first used to construct the *de*  
457 *novo* identification and accurate compilation of sequence models representing all of the  
458 unique TE families dispersed in the genome. Then, RepeatMasker (v4.1.0) [25] was run  
459 on the genome using the combination of *de novo* libraries and a library of known repeats  
460 (Repbase-20181026). The relative position between a repeat and a gene was identified  
461 using bedtools [84], and the type of repeat was further divided to intronic and intergenic  
462 origin. The repeat landscape was constructed using sequence alignments and the  
463 complete annotations output from RepeatMasker, depicting the Kimura divergence  
464 (Kimura genetic distances between identified repeat sequences and their consensus)  
465 distribution of all repeats types. The most notable peak in the repeat landscapes was  
466 considered as the most convincing time of repeat duplication in that period. The

467 transition between the Kimura divergence and age was performed by dividing the  
468 divergence by the two-fold mutation rate per year ( $T = d/2\mu$ ). The mutation rate ( $\mu$   
469 =  $1.73 \times 10^{-9}$ ) was calculated using MCMCTree [85] based on the CDS sequence  
470 alignment of single-copy gene families.

#### 471 **Genome-wide host-parasite protein interaction analysis**

472 In addition to the genome data that we generated for *F. gigantica*, we downloaded  
473 genome annotation information for human (GCA\_000001405.28), swamp buffalo  
474 (GWHAJZ00000000), *F. hepatica* (GCA\_002763495.2), *Fasciolopsis buski*  
475 (GCA\_008360955.1), *Clonorchis sinensis* (GCA\_003604175.1), *Schistosoma mansoni*  
476 (GCA\_000237925.2), and *Taenia multiceps* (GCA\_001923025.3) from the NCBI  
477 database and BIG Sub (China National Center for Bioinformation, Beijing, China).  
478 Proteases and protease inhibitors were identified and classified into families using  
479 BLASTp ( $e$ -value <  $10^{-4}$ ) against the MEROPS peptidase database (merops\_scan.lib;  
480 (European Bioinformatics Institute (EMBL-EBI), Cambridge, UK)), with amino acids  
481 at least 80% coverage matched for database proteins. These proteases were divided into  
482 five major classes (aspartic, cysteine, metallo, serine, and threonine proteases). E/S  
483 proteins (i.e., the secretome) were predicted by the programs SignalP 5.0 [86], TargetP  
484 [87], and TMHMM [88]. Proteins with a signal peptide sequence but without a  
485 transmembrane region were identified as secretome proteins, excluding the  
486 mitochondrial sequences. Genome-wide host-parasite protein interaction analysis was  
487 performed by constructing the PPIs between the *F. gigantica* secretome and human  
488 proteins expressed in the tissues related to the liver fluke life cycle. For the hosts, we  
489 selected human proteins expressed in the small intestine and liver, and located in the  
490 plasma membrane and extracellular region. The gene expression and subcellular  
491 location information were obtained from the TISSUES [89] and Uniprot (EMBL-EBI)  
492 databases, respectively. For *F. gigantica*, secretome molecules were mapped to the  
493 human proteome as the reference, using the reciprocal best-hit BLAST method. These  
494 two gene datasets were used to construct host-parasite PPI networks. We downloaded  
495 the interaction files (protein.links.v11.0) in the STRING database [90], and only highly  
496 credible PPIs were retained by excluding PPIs with confidence scores below 0.7. The  
497 final STRING network was plotted using Cytoscape [91].

#### 498 **Gene family analysis**

499 We chose the longest transcript in the downloaded annotation dataset to represent  
500 each gene, and removed genes with open reading frames shorter than 150 bp. Gene  
501 family clustering was then performed using OrthoFinder (v 2.3.12) [92], based on the  
502 predicted gene set for eight genomes. This analysis yielded 17,992 gene families. To  
503 identify gene families that had undergone expansion or contraction, we applied the  
504 CAFE (v5.0.0) program [93], which inferred the rate and direction of changes in gene  
505 family size over a given phylogeny. Among the eight species, 559 single-copy orthologs  
506 were aligned using MUSCLE (v3.8.1551) [94], and we eliminated poorly aligned  
507 positions and divergent regions of the alignment using Gblock 0.91b [95]. RAxML (v  
508 8.2.12) was then used with the PROTGAMMALGF model to estimate a maximum  
509 likelihood tree. Divergence times were estimated using PAML MCMCTREE [85]. A  
510 Markov chain Monte Carlo (MCMC) process was run for 2,000,000 iterations, with a

511 sample frequency of 100 after a burn-in of 1,000 iterations under an independent rates  
512 model. Two independent runs were performed to check the convergence. The  
513 fossil-calibrated eukaryote phylogeny was used to set the root height for the species  
514 tree, taken from the age of Animals (602–661 Ma) estimated in a previous  
515 fossil-calibrated eukaryotic phylogeny [96] and the divergence time between the  
516 euarchontoglires and laurasiatheria: (95.3–113 Ma) [97].

517

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## 523 **DATA AND MATERIALS AVAILABILITY**

524 The whole genome assembly (contig version) and gene annotation reported in this  
525 paper have been deposited in the Genome Warehouse in BIG Data Center [98], Beijing  
526 Institute of Genomics (China National Center for Bioinformation), Chinese Academy  
527 of Sciences, under accession number GWHAZTT00000000 that is publicly accessible  
528 at <http://bigd.big.ac.cn/gwh>. The AGP file for Hi-C was uploaded as supplement file.  
529 The Pacbio sequencing reads has been deposited into the genome sequence archive  
530 (GSA) in BIG under accession code CRA003783. The whole genome assembly also  
531 can be obtained in the National Center for Biotechnology Information (NCBI) under  
532 Bioproject PRJNA691688.

## 533 **AUTHOR CONTRIBUTIONS**

534 Q.L., J.R. and Y.W. conceived and designed the project. Q.L., K.Q., Z.Q., Z.J., Z.P.,  
535 W.K., W.D. and D. W. collected the samples and performed experiments. J.R., Q.L.,  
536 X.L., Z.Q., X.W., and T.F. analyzed the data. X.L., Q.L., K.Q. and Z.Q. drafted the  
537 manuscript. J.R., Y.W., W.Y., X.Q., and J.Y revised the manuscript.

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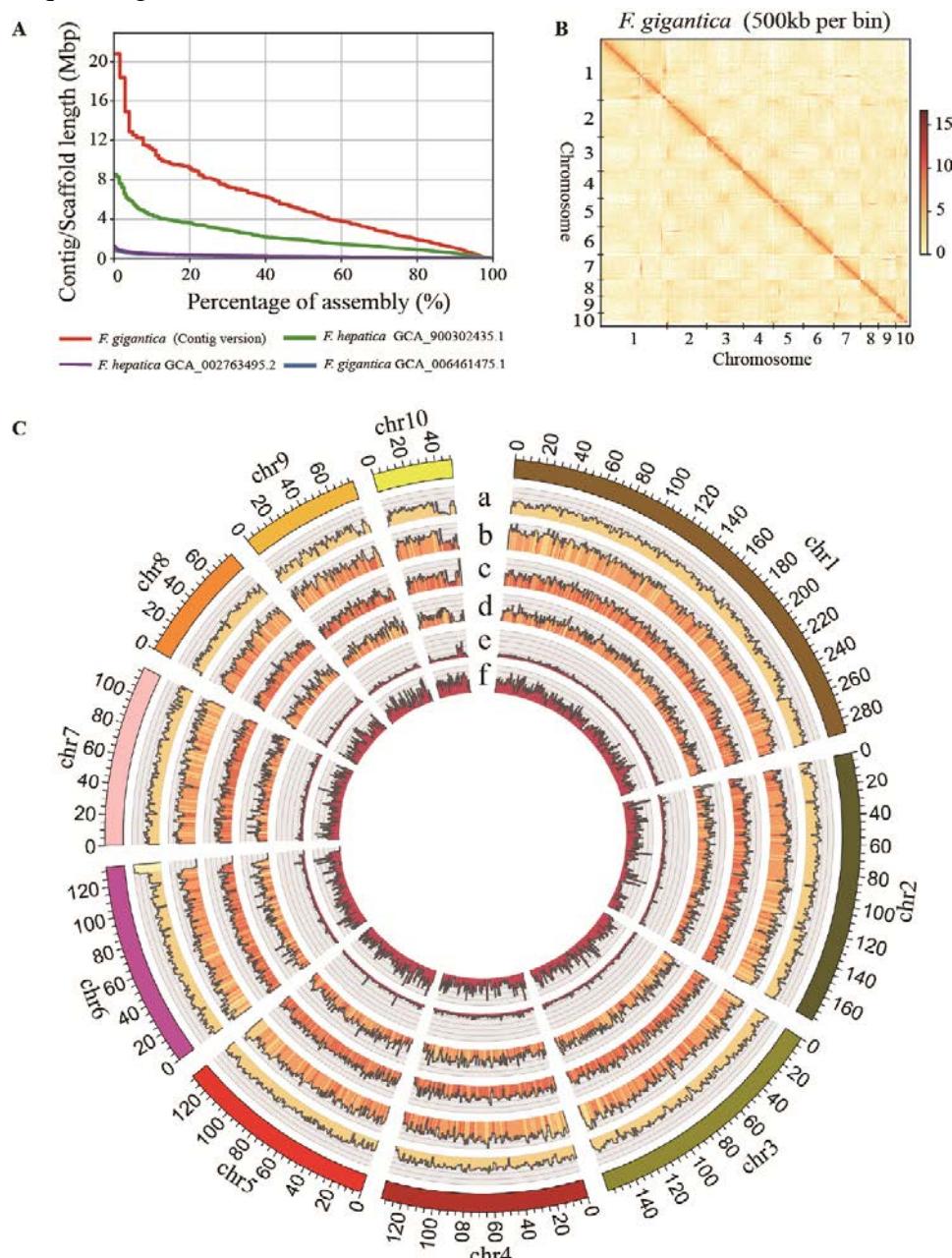
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851 **Fig. 1 Landscape of the *Fasciola gigantica* genome.**

852 (A) Comparisons of the assembled contigs and scaffold lengths (y-axis) and tallies  
853 (x-axis) in *Fasciola* species. (B) Hi-C interactive heatmap of the genome-wide  
854 organization. The effective mapping read pairs between two bins were used as a signal  
855 of the strength of the interaction between the two bins. (C) Integration of genomic and  
856 annotation data using 1 Mb bins in 10 Hi-C assembled chromosomes. (a) Distribution  
857 of the GC content (GC content > 39% and < 52%); (b) distribution of the long  
858 interspersed element (LINE) percentage > 0% and < 50%; (c) distribution of the long  
859 terminal repeat (LTR) percentage > 0% and < 50%; (d) distribution of the gene  
860 percentage > 0% and < 70%; (e) distribution of the heterozygosity density of our sample  
861 (percentage > 0% and < 1%); (f) distribution of the heterozygosity density of  
862 SAMN03459319 in the NCBI database. Hi-C, chromosome conformation capture

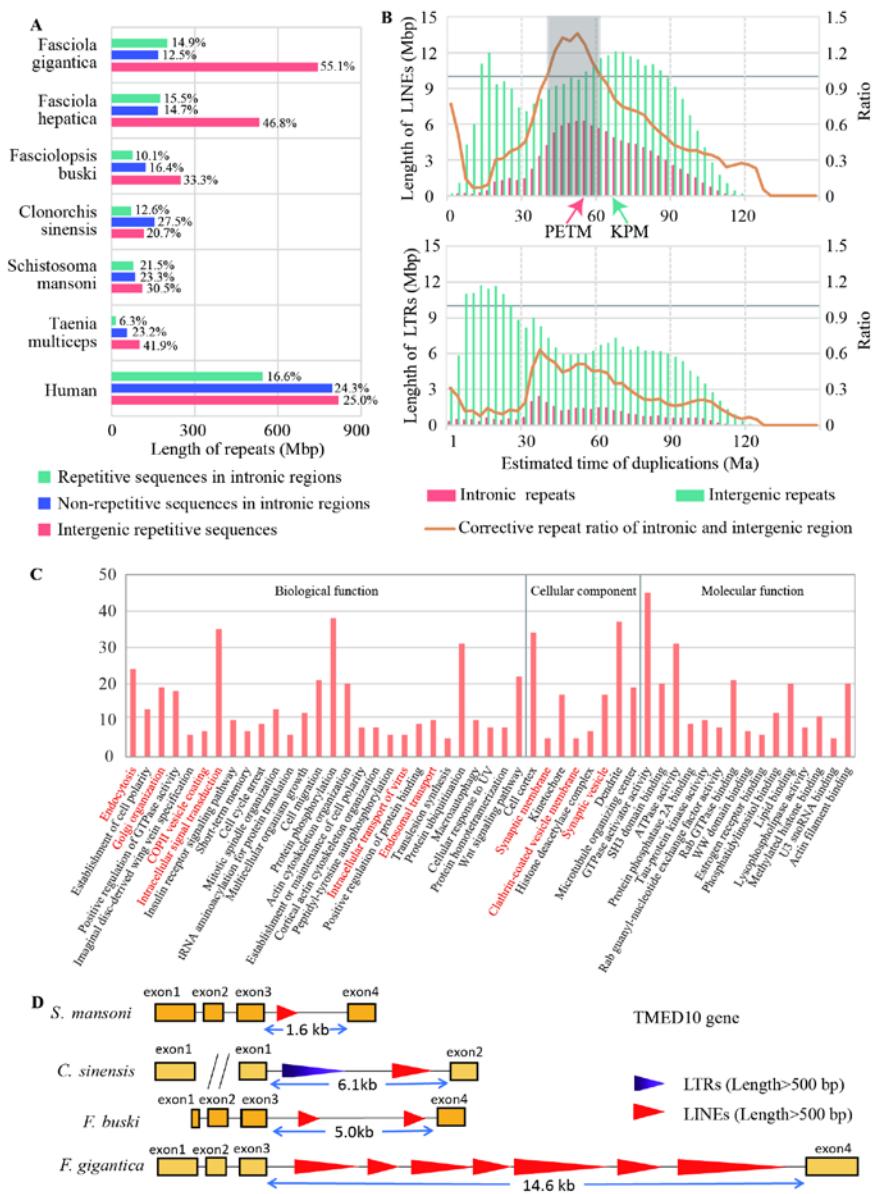
863 sequencing;



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865 **Fig. 2 Identification of repeat expansion and alternative gene networks in the**  
866 ***Fasciola gigantica* genome.**

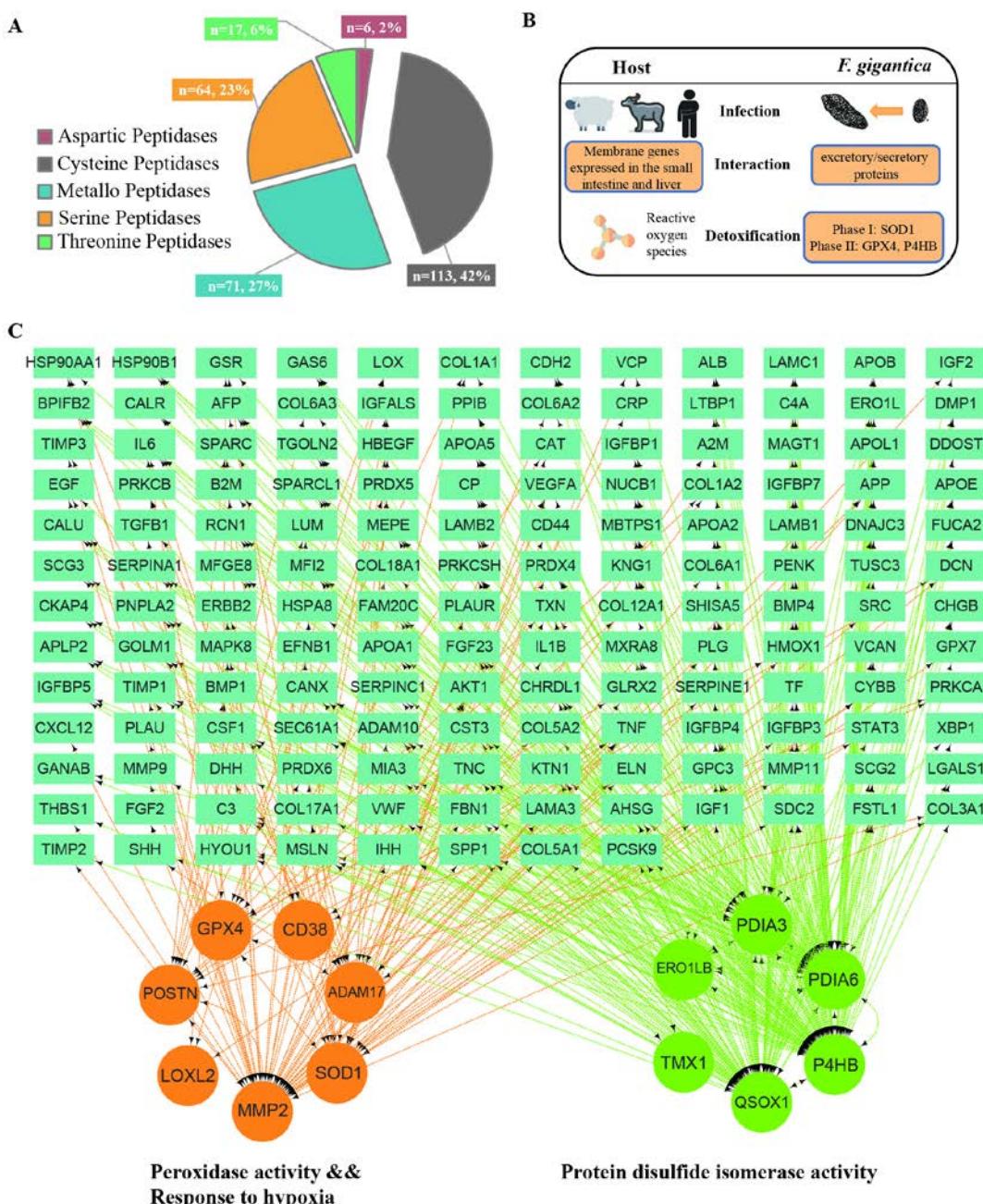
(A) The distribution of repetitive sequence length among the genomes of six flatworms and the human genome. (B) Landscape of LINEs and LTRs distribution in the *Fasciola gigantica* genome. The x-axis shows the expansion time of TEs calculated by the divergence between repeat sequences. The mutation rate was set as  $1.73 \times 10^{-9}$  per year. The orange line represents the repeat length ratio, used to estimate the signatures of selection, which was corrected by the total length of intronic and intergenic regions in history. (C) The functional enrichment of genes with more than 10 kb LINE insertions between 41 Ma and 62 Ma by Gene Ontology (GO) classification. The GO terms related to vesicle secretion are marked in red. (D) *TMED10* gene structure map. LINEs original between 41 Ma and 62 Ma and longer than 500 bp identified by RepeatMasker were plotted. LTRs longer than 500 bp were plotted. Long interspersed element, LINE; long terminal repeat, LTR; TE, transposable element; TMED10, transmembrane P24 trafficking protein 10.



882 **Fig. 3 Genome-wide host-parasite interaction analysis.**

883 (A) Pie chart for proteases identified in *Fasciola gigantica*. (B) The interaction mode  
 884 between the adult *Fasciola gigantica* and the host. (C) The protein-protein interaction  
 885 (PPI) network of redox-related pathways in *Fasciola gigantica* with host proteins. The  
 886 genes indicated in the three gene ontology (GO) terms were significantly enriched and  
 887 have their encoded proteins have PPIs with excretory/secretory (E/S) proteins.

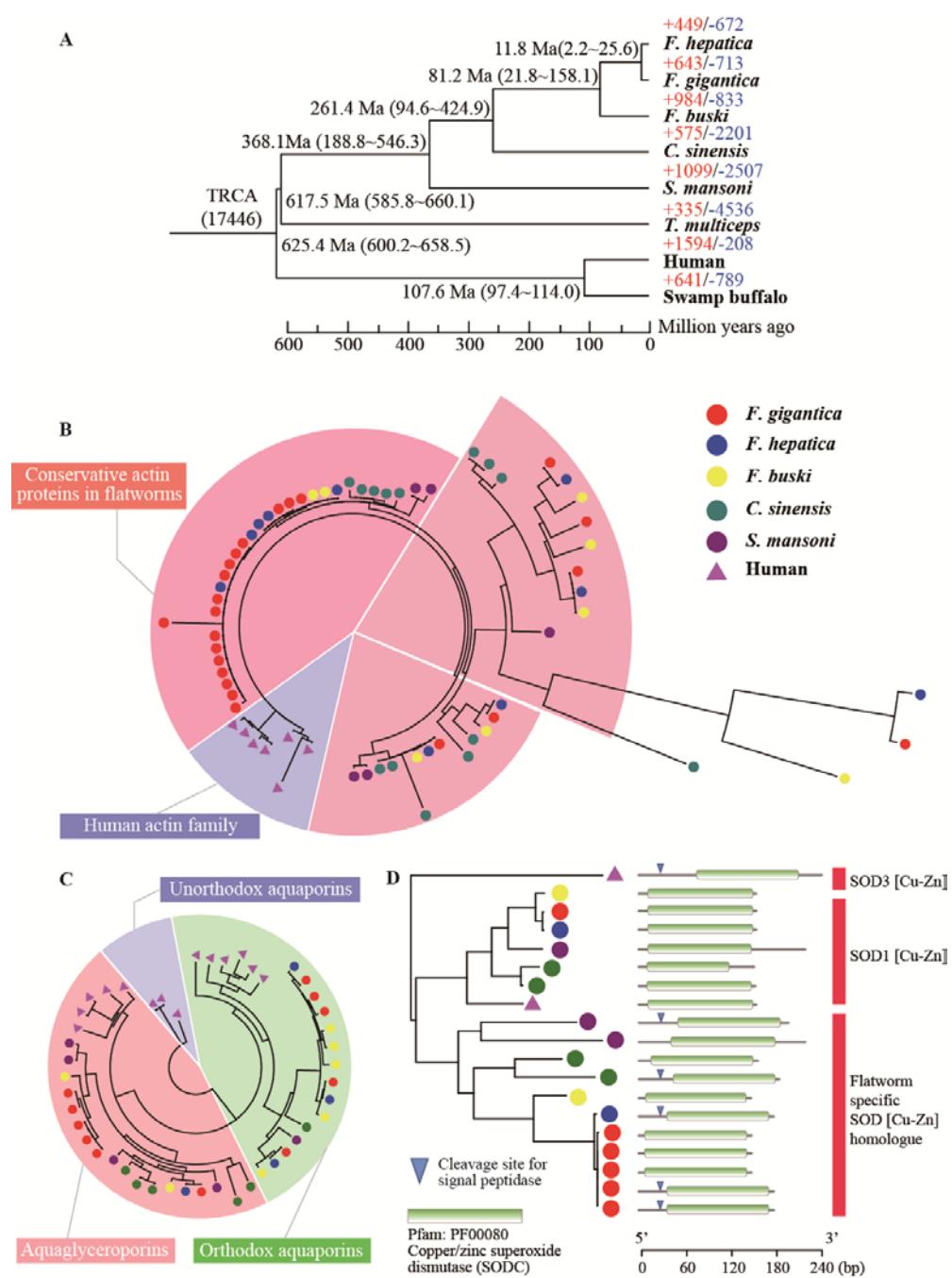
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891 **Fig. 4 Phylogenetic tree and gene family analysis.**

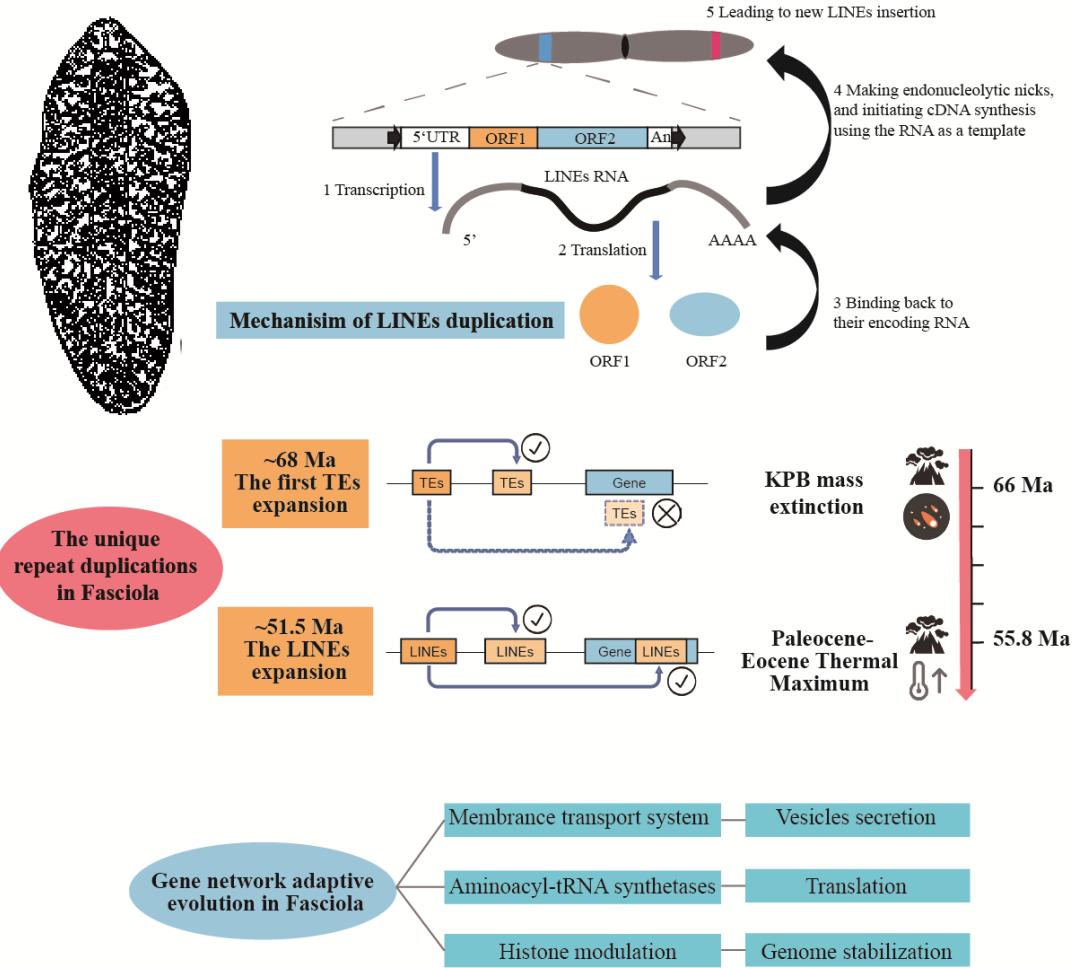
892 (A) A phylogenetic tree generated using 559 single-copy orthologous genes. The  
 893 numbers on the species names are the expanded (+) and contracted (-) gene families.  
 894 The numbers on the nodes are the divergence time between species. (B) A phylogenetic  
 895 tree of actin genes in flatworms and humans. All human homologue genes are selected  
 896 as outgroup. (C) Phylogenetic tree of aquaglyceroporin (AQP) family genes in  
 897 flatworms and humans. The human homolog genes (*AQP11*, *AQP12A*, and *AQP12B*)  
 898 were selected as the outgroup. (D) A phylogenetic tree of copper/zinc superoxide  
 899 dismutase (*SOD*) genes in flatworms and humans. The midpoint was selected as the  
 900 root node.



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903 **Fig. 5 Schematic diagram of the process of *Fasciola*-specific repeat expansion**  
904 **during evolution.**



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908 **Table 1. Summary statistics for the genome sequences and annotation.**

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<i>F. gigantica</i>		
Genome	Total Genome Size (Mb)	1,348
	Chromosome Number	10
	Scaffold Number <sup>a</sup>	10+24
	Scaffold N50 (Mb)	133
	Scaffold L50	4
	Contig Number	1,022
	Contig N50 (Mb)	4.89
Heterozygosity Rate (%)		
Annotation	Total Gene Number	12,503
	Average CDS Length (bp)	1552.7
	Average Gene Length (kb)	28.8
	Percentage of Genome Covered by CDSs (%)	1.5%
	BUSCO Assessment	90.4%
	Repeat Content	70.0%

<sup>a</sup> number of chromosome level scaffolds and unplaced scaffolds.

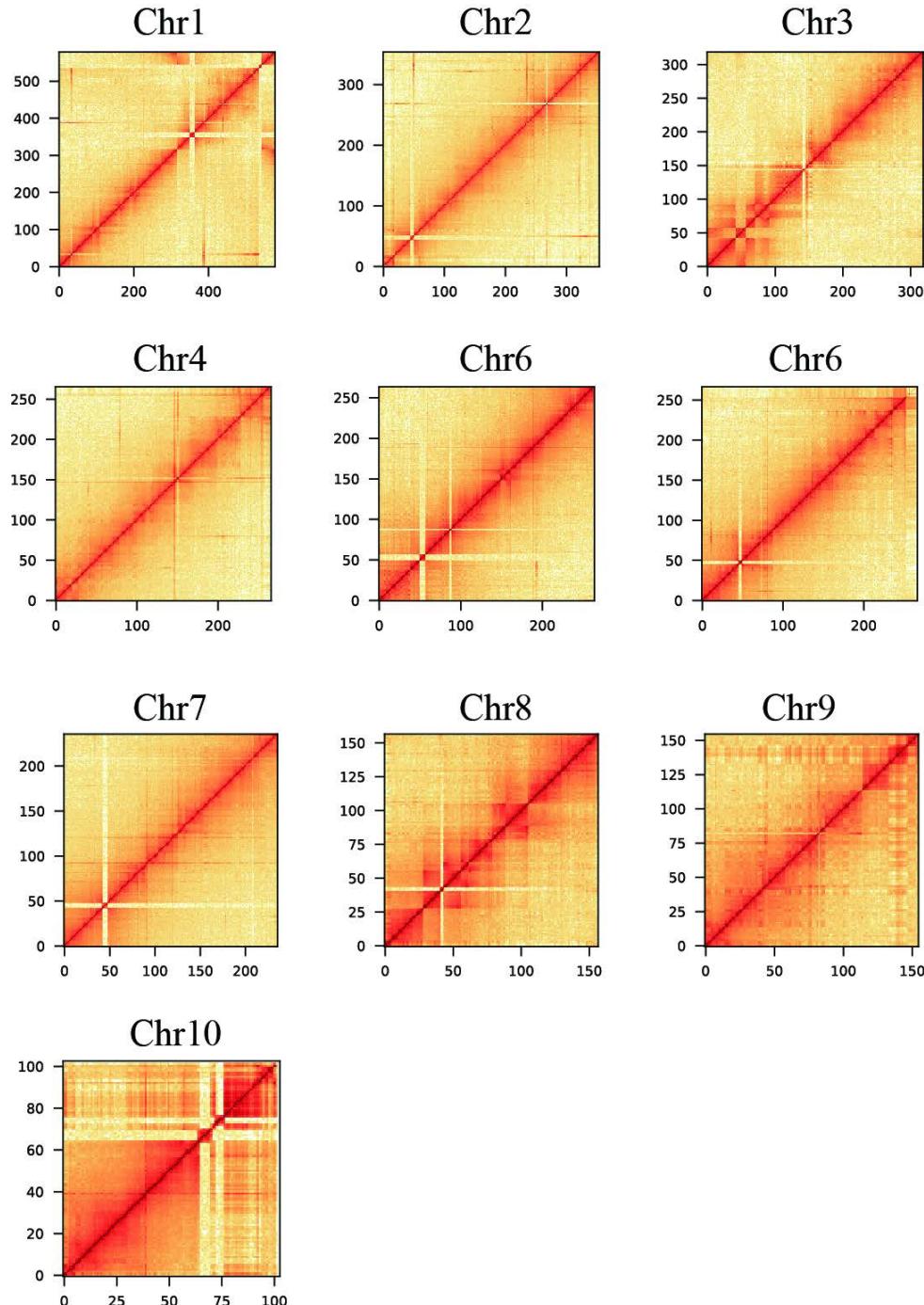
CDS, coding sequence.

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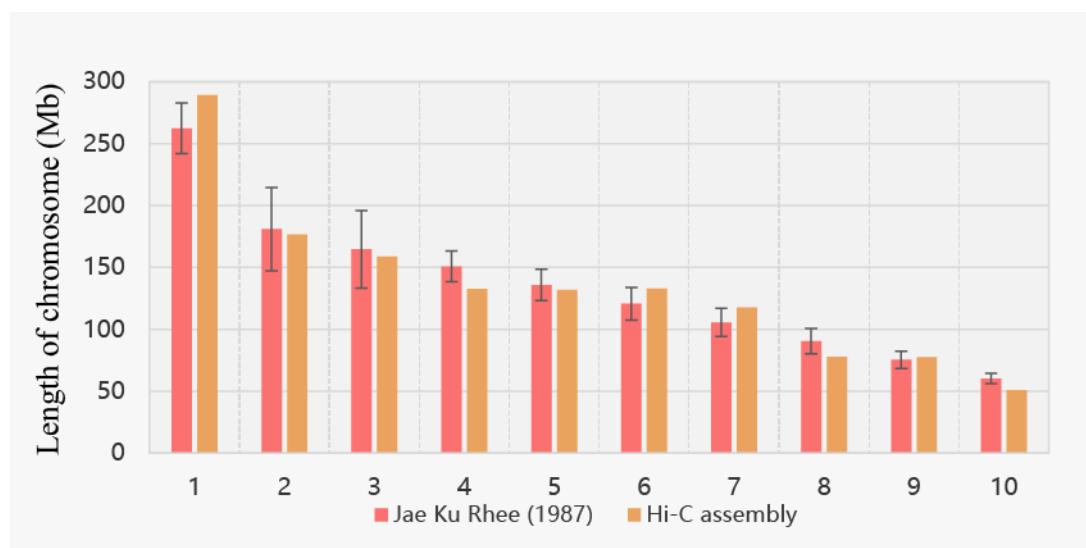
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913 Fig. S1. Genome-wide all-by-all chromosome conformation capture sequencing (Hi-C)  
914 interaction in *F. gigantica* (Bins = 500 K).



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918 Fig. S2. Comparison of chromosome length between the chromosome conformation  
919 capture sequencing (Hi-C) assembly and estimates from published karyotype data by  
920 Jae Ku Rhee.



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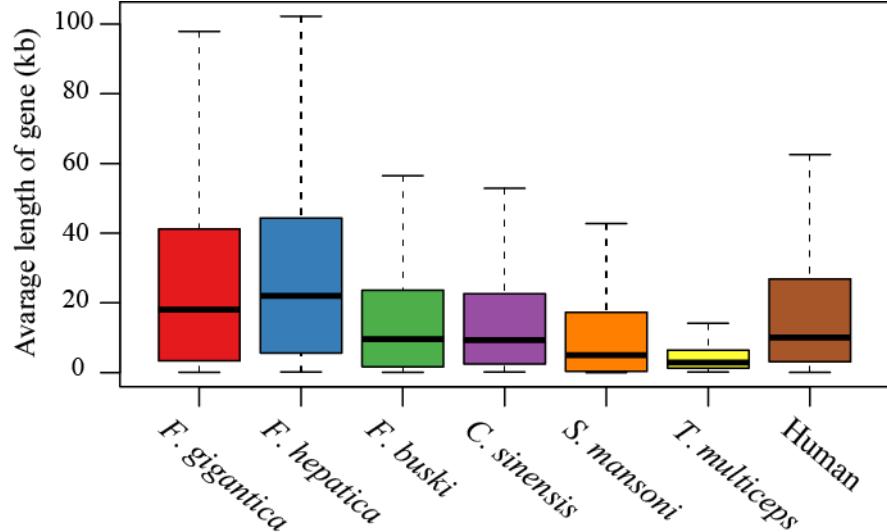
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928 Fig. S3. Boxplot of average gene length.



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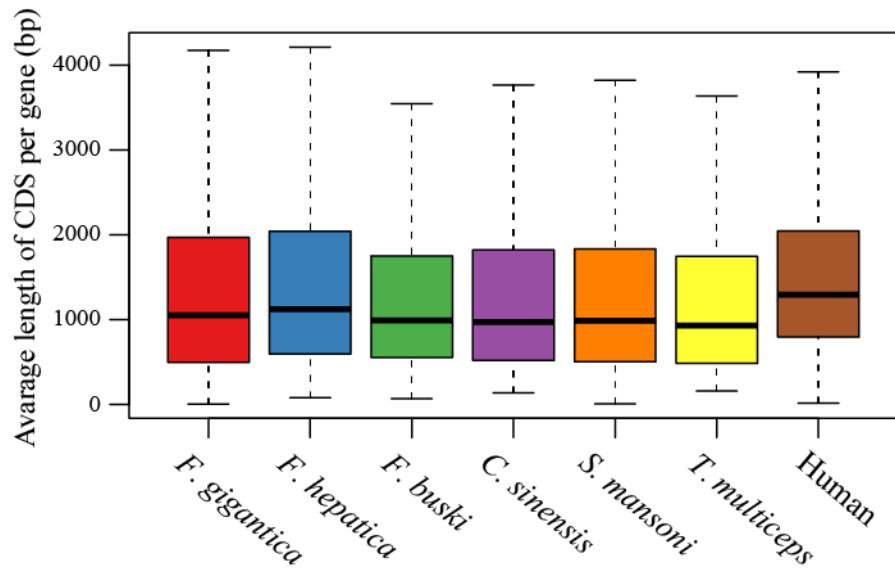
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935 Fig. S4. Boxplot of average coding sequence (CDS) length per gene.



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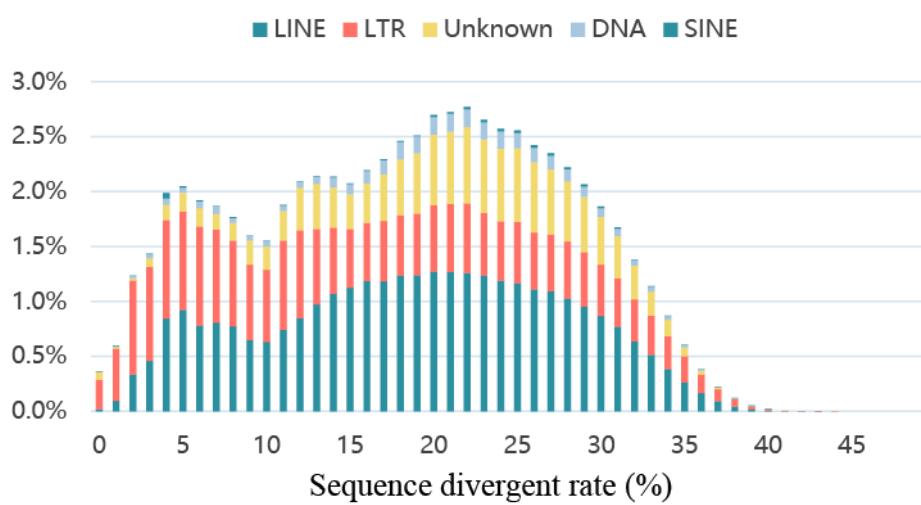
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943 Fig. S5. Divergence distribution of classified families of transposable elements. The  
944 classified transposon families in *F. gigantica*.



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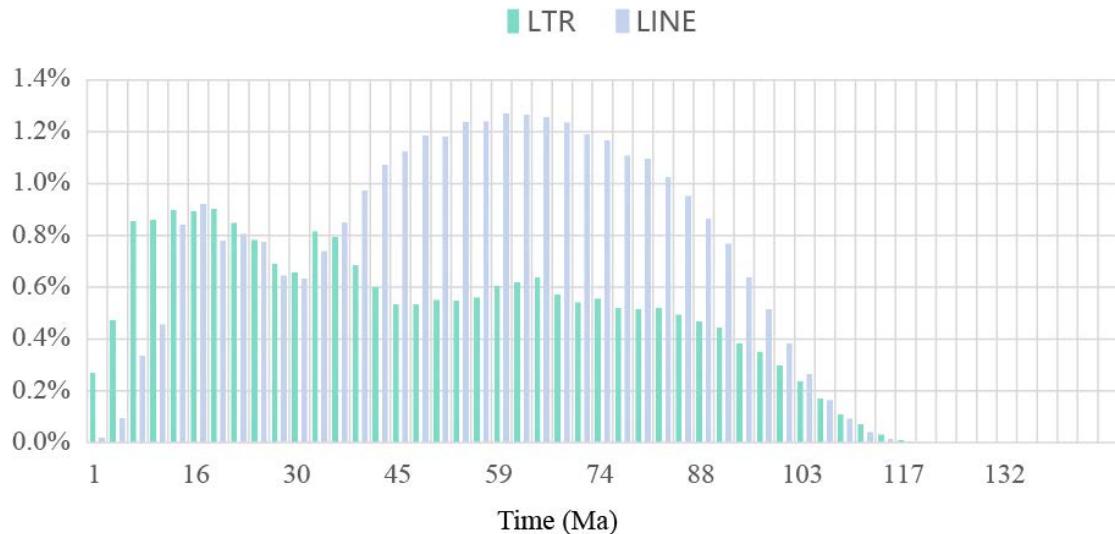
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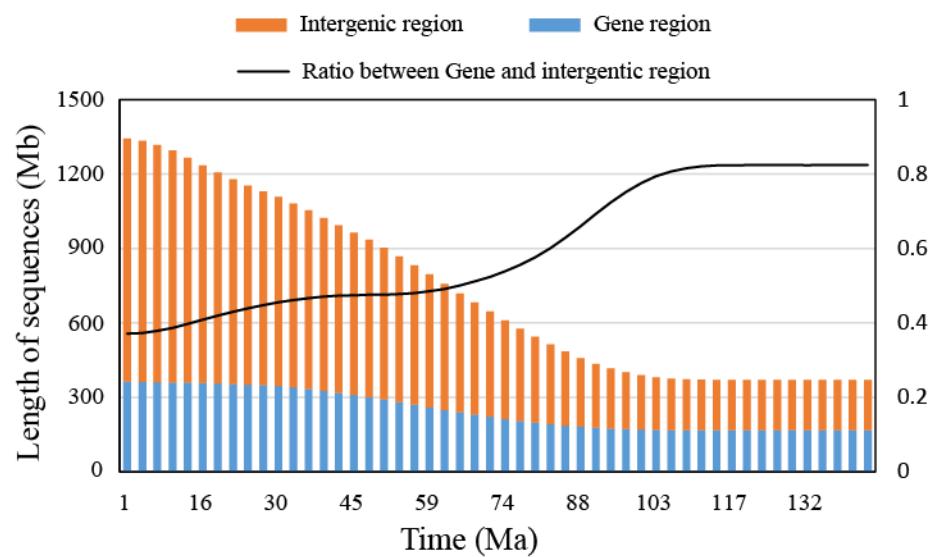
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950 Fig. S6. Expansion time of long terminal repeats (LTRs) and long interspersed elements  
951 (LINEs). The mutation rate was  $1.73 \times 10^{-9}$ .



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961 Fig. S7. Estimation of *F. gigantica* genome size based on the expansion time of repeat  
962 sequences during evolution. The mutation rate was  $1.73 \times 10^{-9}$ .



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968 Supplementary Table 1. Genome sequencing strategy for buffaloes  
969 Supplementary Table 2. Summary of the *Fasciola gigantica* genome assembly  
970 Supplementary Table 3. Summary of different assemblies in *Fasciola* species  
971 Supplementary Table 4. Summary of chromosome conformation capture sequencing  
972 (Hi-C) assembly of the chromosome length in *Fasciola gigantica*  
973 Supplementary Table 5. Assessment of the completeness and accuracy of the genome  
974 Supplementary Table 6. BUSCO assessment of the genome  
975 Supplementary Table 7. Number of genes with functional classification gained using  
976 various methods  
977 Supplementary Table 8. Transposable element content of *Fasciola gigantica* genome  
978 Supplementary Table 9. The list of genes with more than 10 kb of long interspersed  
979 element (LINE) insertion between 41 Ma and 62 Ma  
980 Supplementary Table 10. Gene ontology (GO) term category enrichment for genes with  
981 more than 10 kb of long interspersed element (LINE) insertion between 41 Ma and 62  
982 Ma  
983 Supplementary Table 11. Kyoto Encyclopedia of Genes and Genomes (KEGG pathway  
984 enrichment for genes with more than 10 kb of long interspersed element (LINE)  
985 insertion between 41 Ma and 62 Ma  
986 Supplementary Table 12. Kyoto Encyclopedia of Genes and Genomes (KEGG)  
987 pathway enrichment for genes with more than 10 kb of long interspersed element (LINE)  
988 insertion between 41 Ma and 62 Ma  
989 Supplementary Table 13. Protein inhibitors in the *Fasciola gigantica* genome  
990 Supplementary Table 14. Excretory/secretory (E/S) proteins in the *Fasciola gigantica*  
991 genome  
992 Supplementary Table 15. Gene ontology (GO) term category enrichment for  
993 excretory/secretory (E/S) proteins  
994 Supplementary Table 16. Gene ontology (GO) term category enrichment for rapidly  
995 evolving families specific to *F. gigantica*.

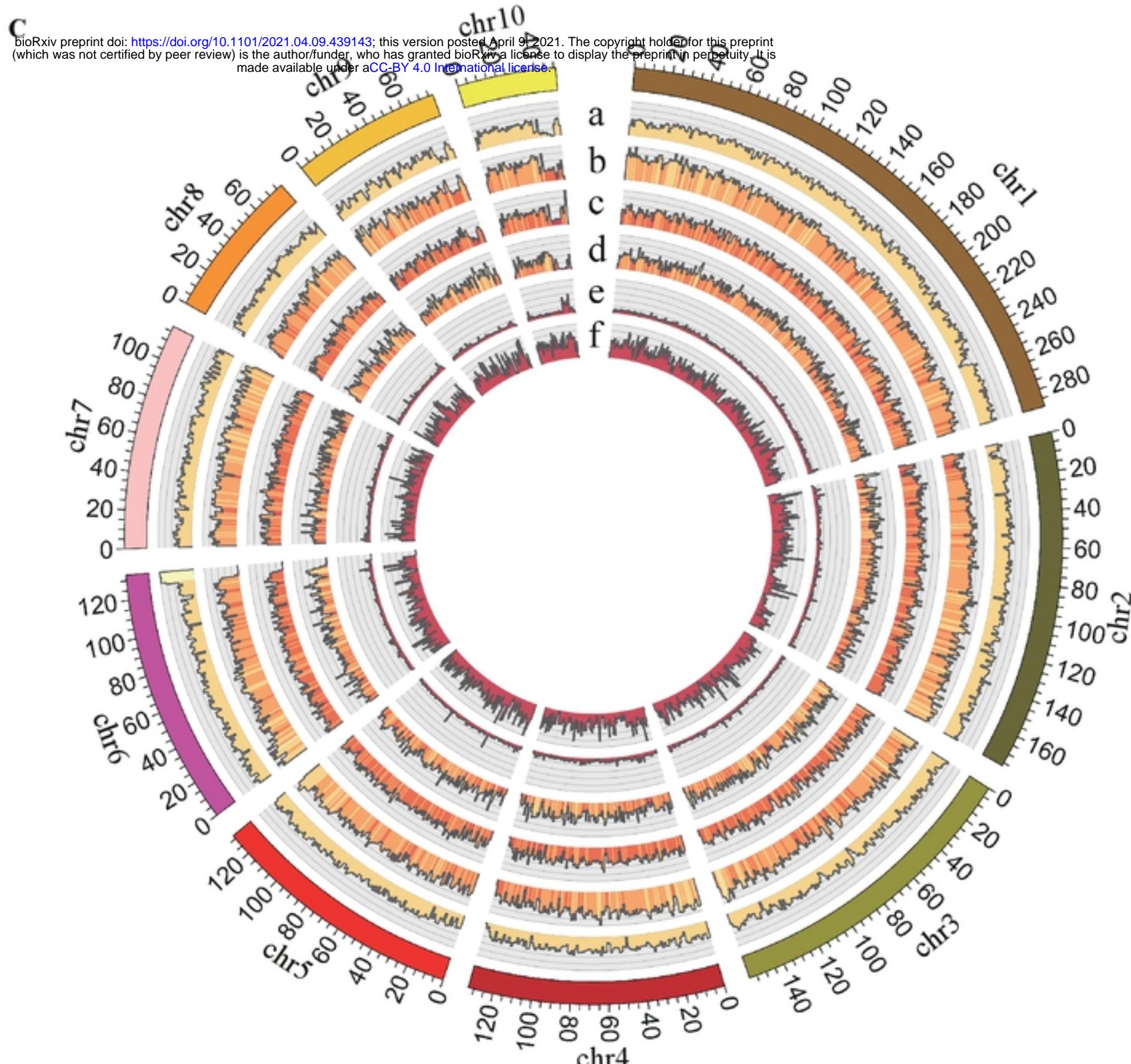
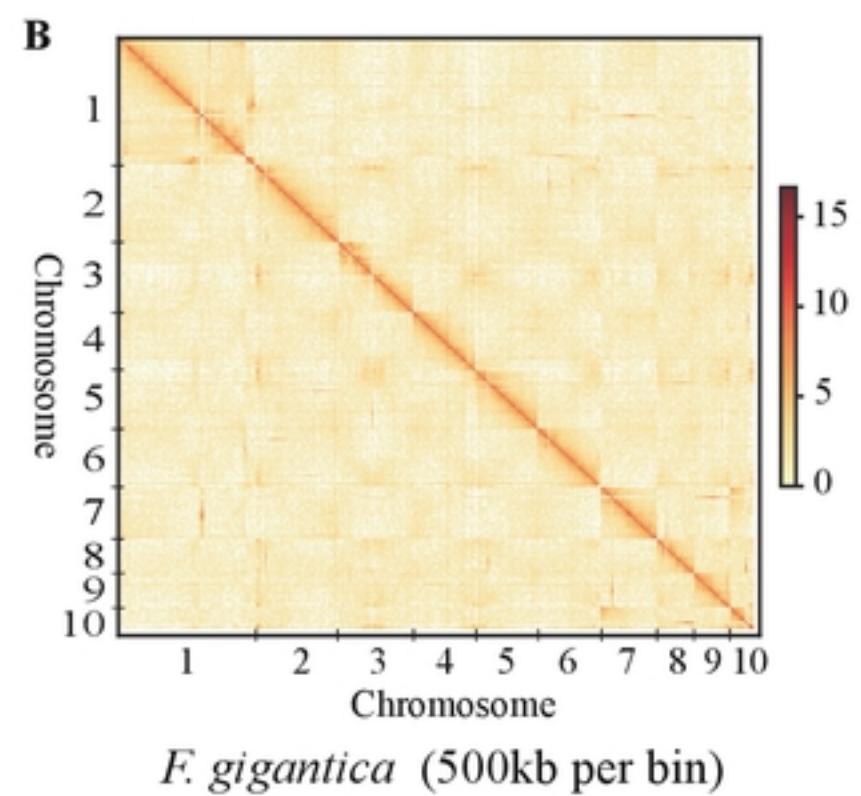
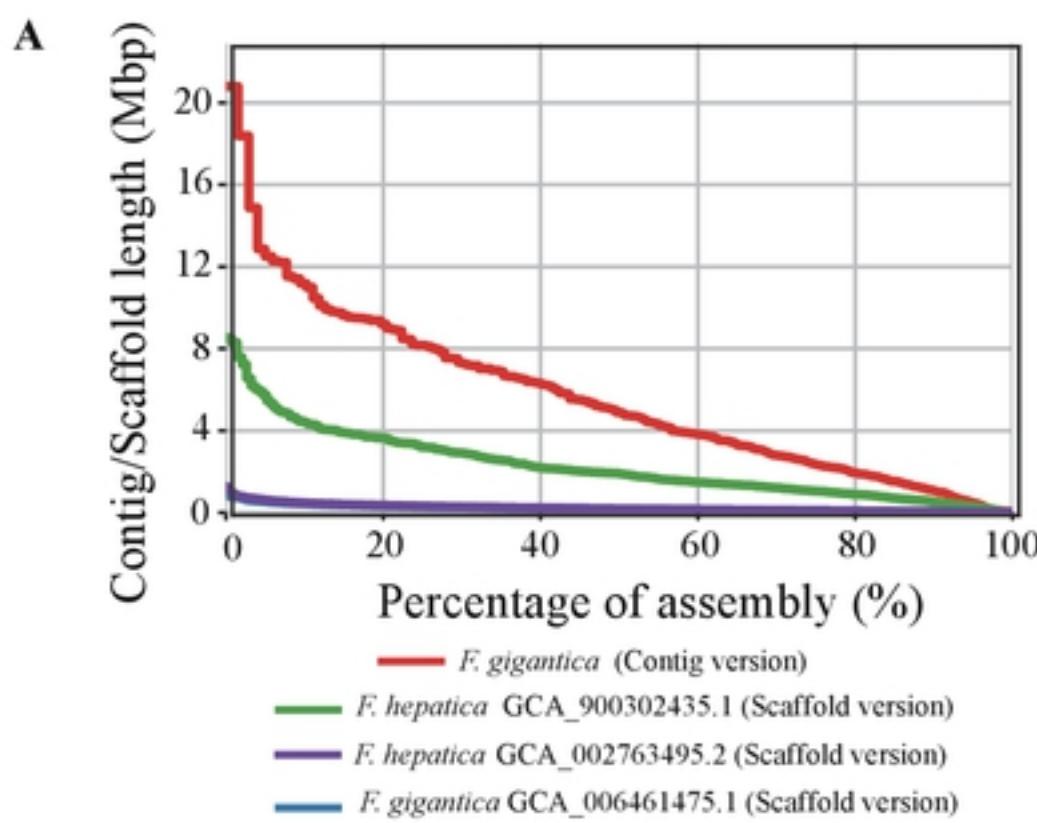


Figure 1

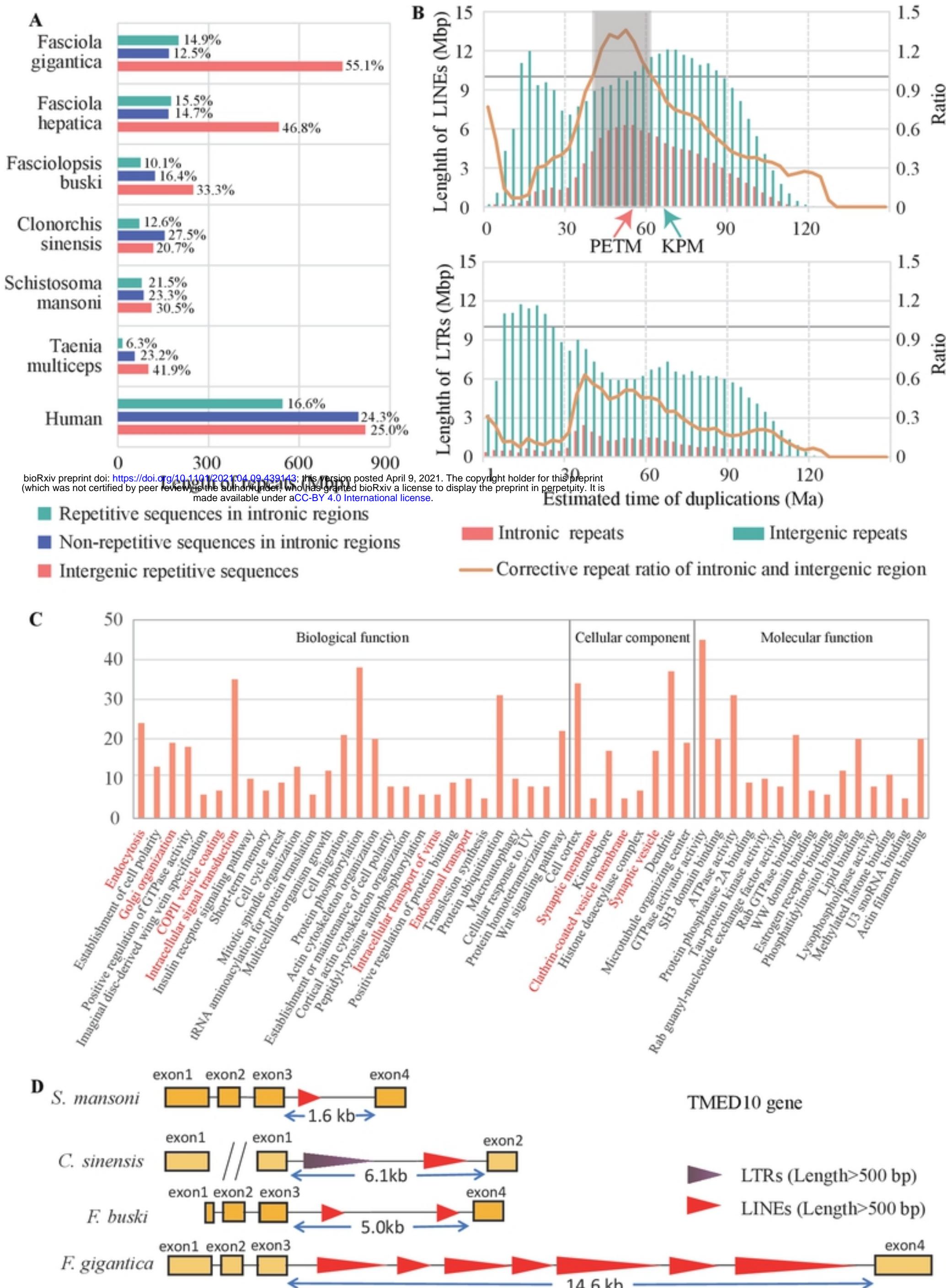
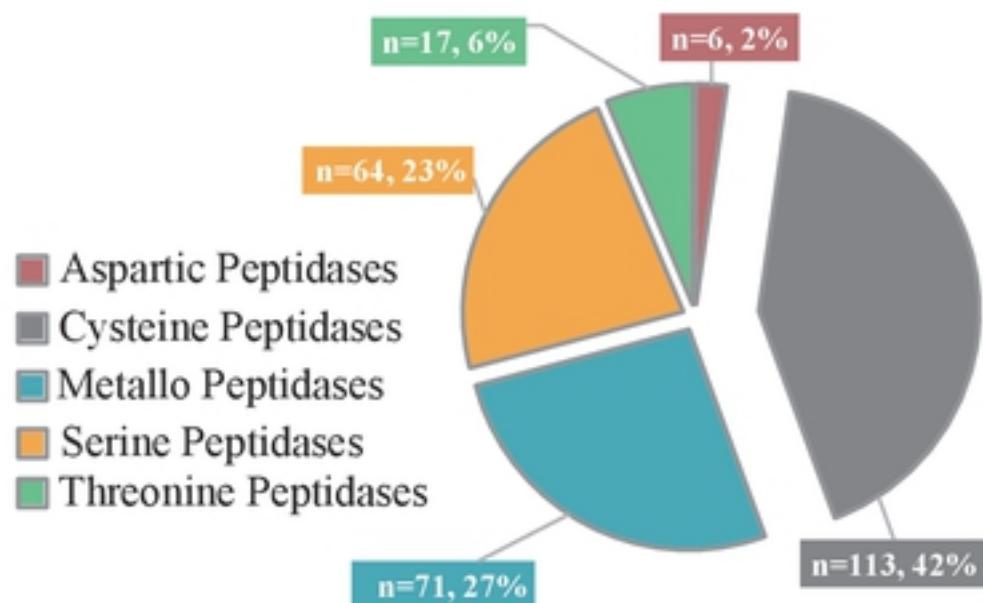
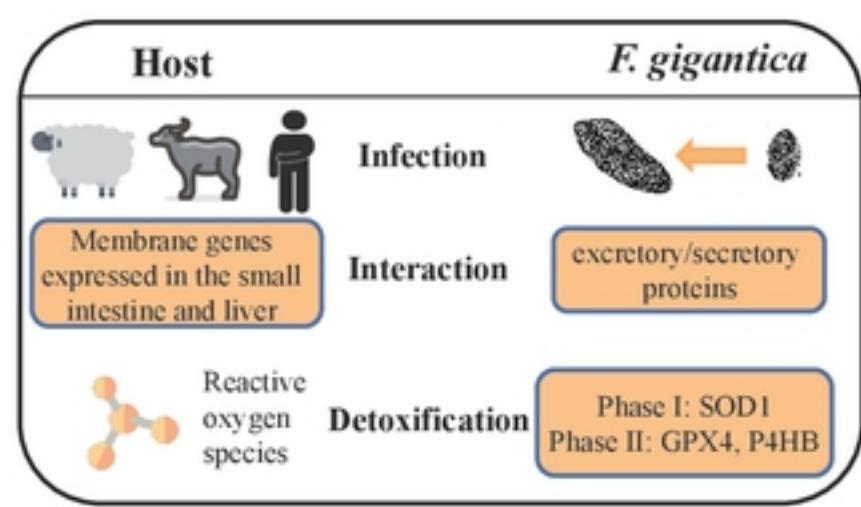
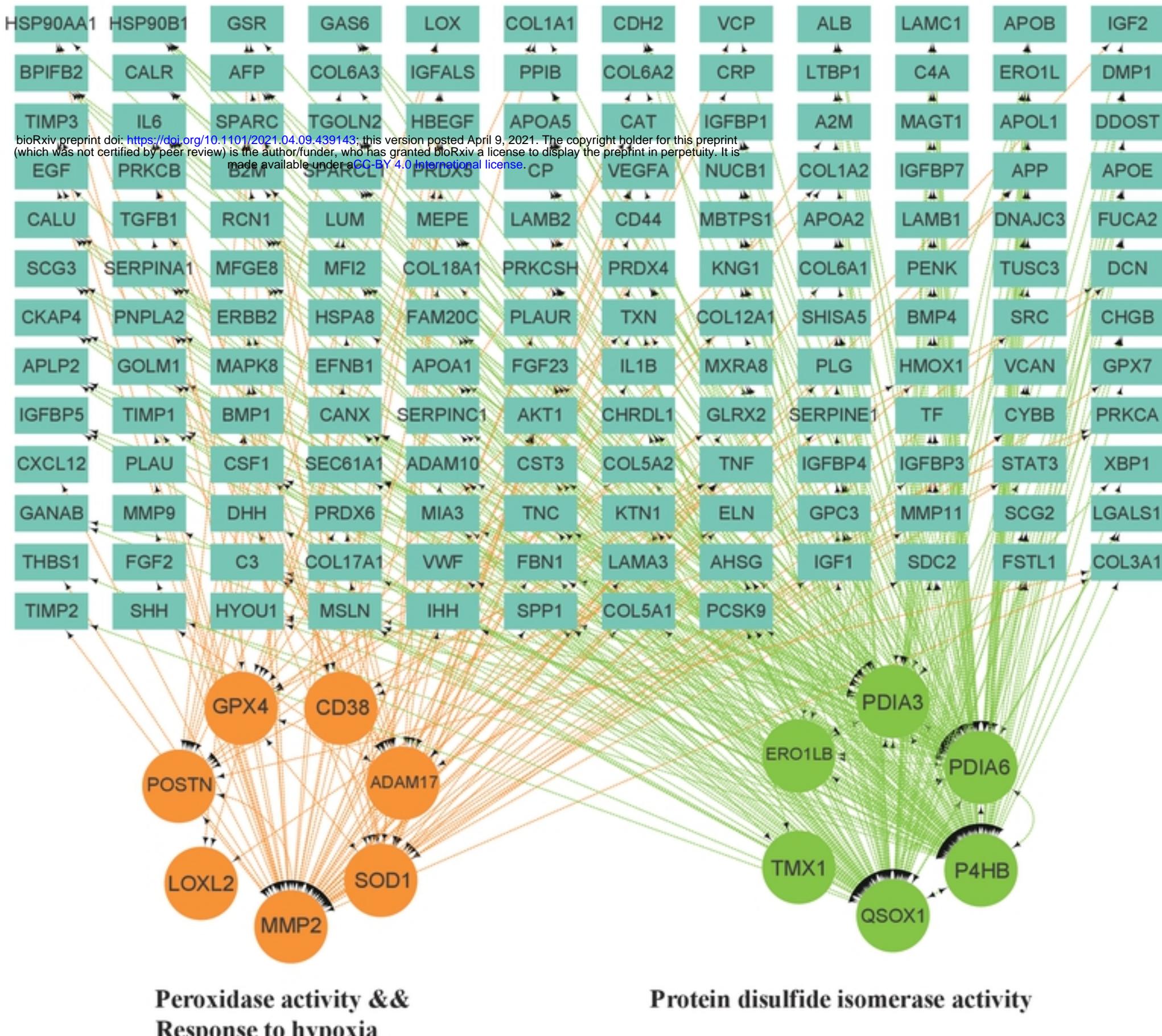


Figure2

**A****B****C****Figure3**

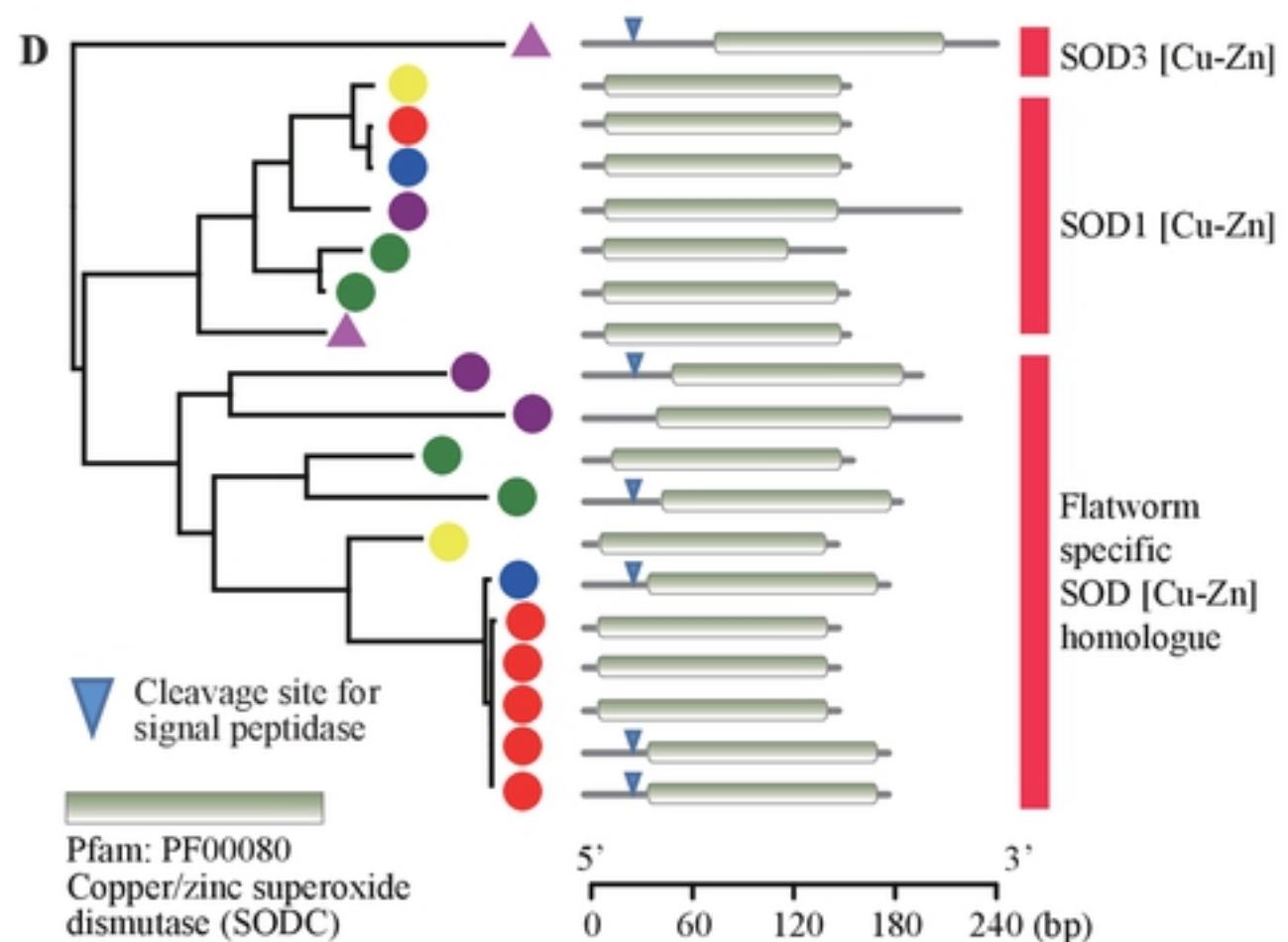
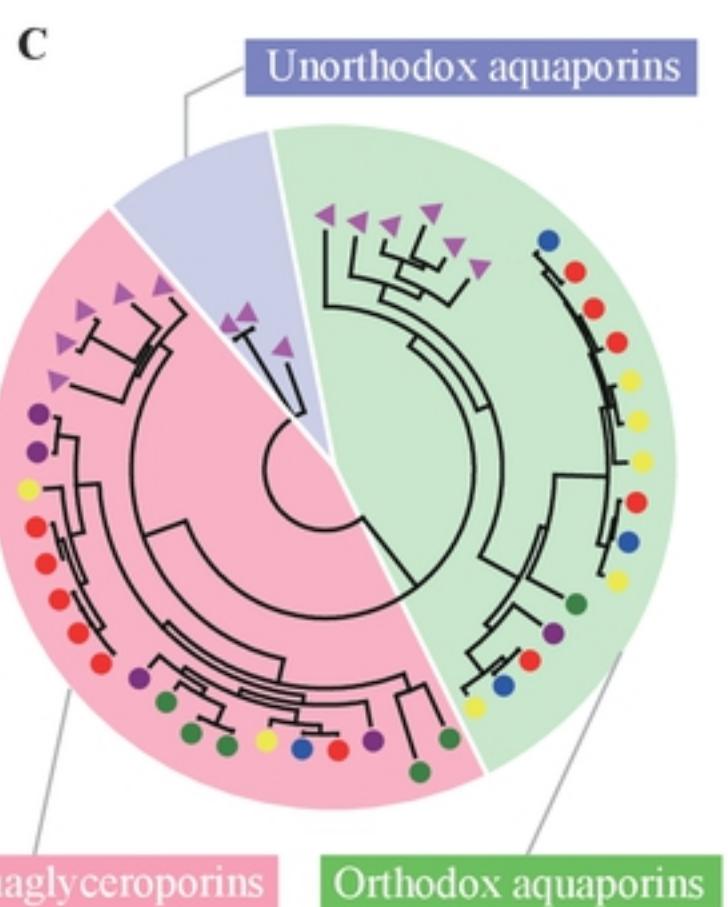
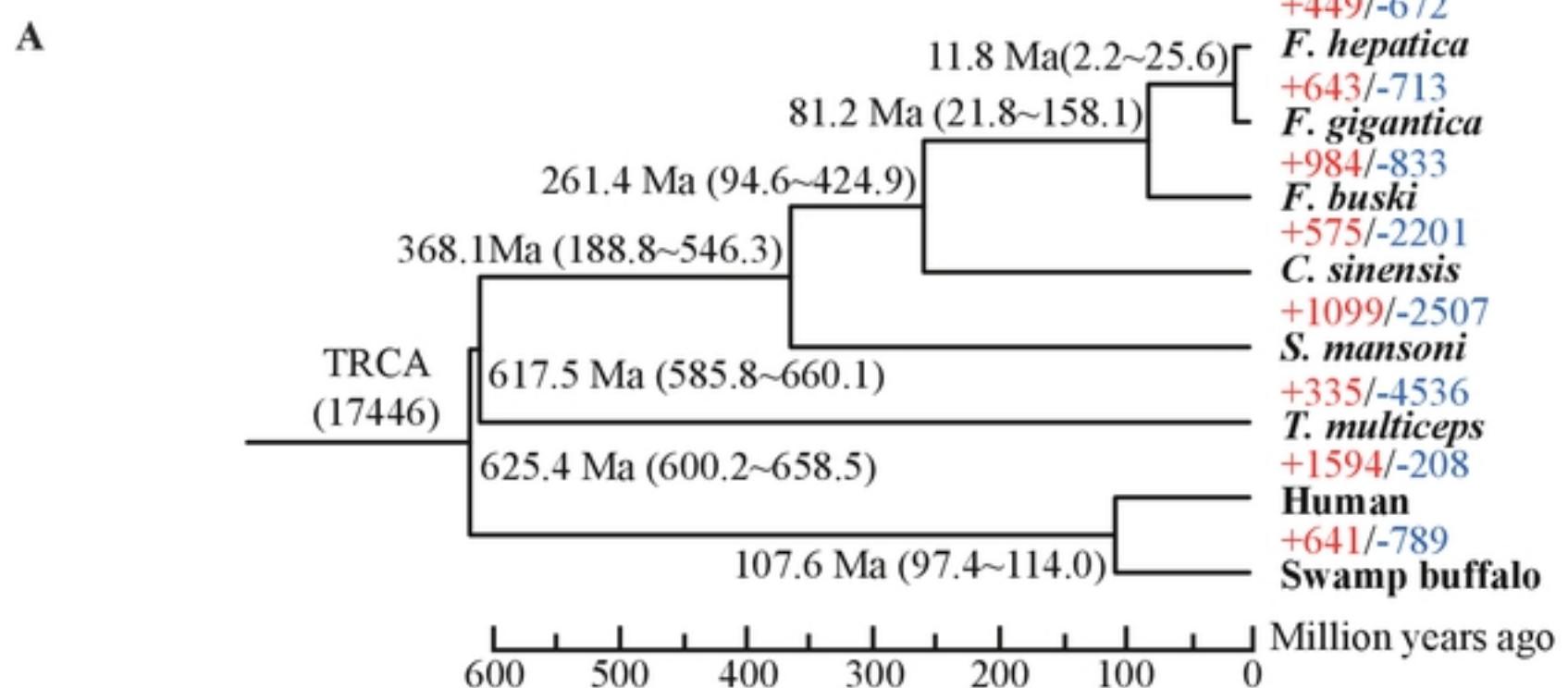
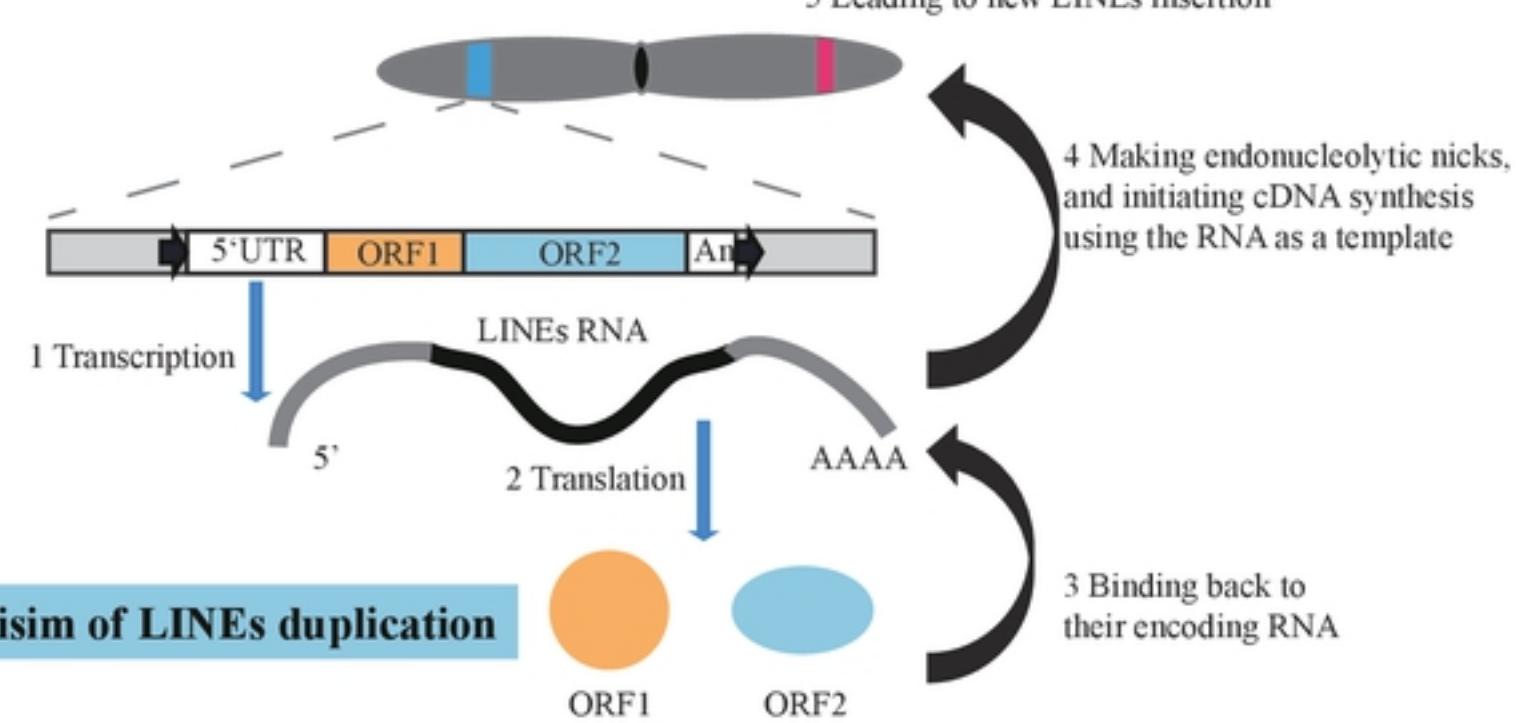


Figure4



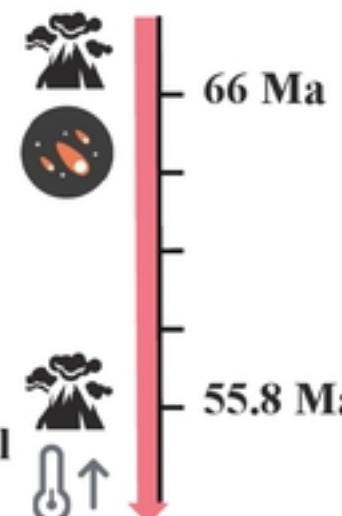
### Mechanism of LINEs duplication

The unique repeat duplications in *Fasciola*

~68 Ma  
The first TEs expansion

~51.5 Ma  
The LINEs expansion

KPB mass extinction



Gene network adaptive evolution in *Fasciola*

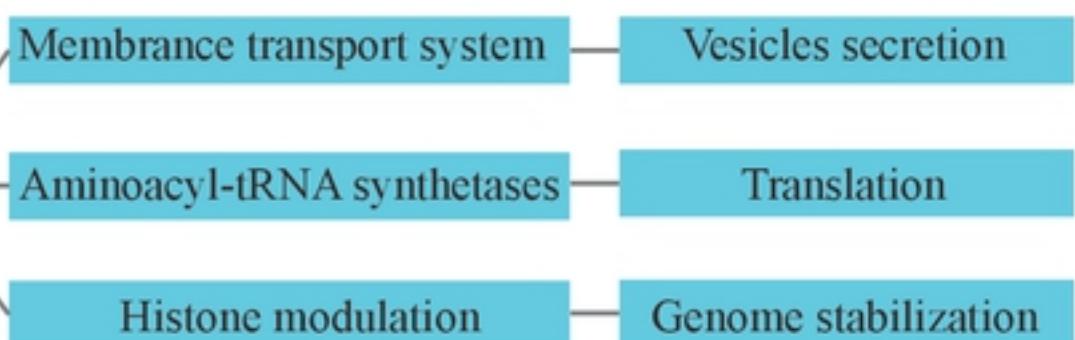


Figure5