

1 **Genomic signature of accelerated evolution in a saline-alkaline lake-dwelling**

2 **Schizothoracine fish**

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14

## 15 Abstract

16 Tibetan Plateau imposes extremely inhospitable environment on most wildlife. Besides the harsh aquatic  
17 environment including hypoxia and chronic cold, high salinity and alkalinity is an increasing threat to  
18 Tibetan endemic fishes. Previous genome-wide studies identified key genes contributed to highland fish  
19 adaptation to hypoxia and long-term cold, while our understanding of saline and alkaline adaptation in  
20 Tibetan fish remains limited. In this study, we performed a comparative genomics analysis in a saline  
21 lake-dwelling highland fish *Gymnocypris przewalskii*, aimed to identify candidate genes that contributed  
22 to saline and alkaline adaptation. We found elevated genome-wide rate of molecular evolution in *G.*  
23 *przewalskii* relative to lowland teleost fish species. In addition, we found nine genes encoding biological  
24 macromolecules associated with ion transport functions underwent accelerated evolution in *G.*  
25 *przewalskii*, which broadly expressed across kidney, gill, liver, spleen, brain and muscle tissues.  
26 Moreover, we found putative evidence of ion transport under selection were interacted by co-expression  
27 in *G. przewalskii* adaptation to high salinity and alkalinity environment of Lake Qinghai. Taken together,  
28 our comparative genomics study identified a set of rapidly evolving ion transport genes and  
29 transcriptomic signatures in Schizothoracine fish adaptation to saline and alkaline environment on the  
30 Tibetan Plateau.

31

32 **Keywords:** Comparative genomics, Schizothoracinae, Accelerated evolution, Saline and alkaline  
33 adaptation

34

## 35 1. Introduction

36 Environments may shape the genetic landscape of wildlife that inhabit them [1]. The world's largest and  
37 highest highland Tibetan Plateau had undergone continuous uplift during the India-Asia collision since  
38 about 45 million years ago, which triggered numerous environmental changes [2,3]. As elevation above  
39 sea level increases, a decrease in barometric pressure results in fewer oxygen molecules in the air, which  
40 causes hypoxia. Besides, other challenging environments high-altitude dwelling wildlife have  
41 encountered are the long-term low temperature and high ultraviolet radiation [4,5]. Understanding how  
42 organism adapt to their dwelling environment is central to answering many ecological and evolutionary  
43 questions, but it remains a formidable task to fully uncover the mechanisms of adaptive process [6].  
44 Adaptation at the molecular level can occur by adaptive mutation in key genes over prolonged  
45 evolutionary time scales [7]. Recent genome-wide studies have identified key genes associated with  
46 hypoxia response and energy metabolism in Tibetan terrestrial animals adaptation to high altitude [8–10].  
47 Nevertheless, the adaptive mechanism of Tibetan aquatic organisms to highland water environment is yet  
48 well-studied [11].

49  
50 Unlike Tibetan terrestrial animal, the draft genomes of very few Tibetan aquatic organisms had been  
51 sequenced [12,13], the genomic basis of aquatic animals adaptation to water environments at high altitude  
52 still remain largely unknown. The Schizothoracine fishes are the predominant aquatic fauna on the  
53 Tibetan Plateau, which had evolved specific phenotypic characteristics to adapt to the harsh aquatic  
54 environments, such as hypoxia, chronic cold and high salinity and alkalinity. Comparative genomics  
55 approaches have the power to facilitate investigation of the genomic basis of evolution and adaptation  
56 [14]. Recent comparative genomics studies based on transcriptomic data of several Schizothoracine  
57 species have identified a number of candidate genes that underwent positive selection during the long-  
58 term adaptive processes to harsh environments on the Tibetan Plateau, such as BYSL and HSF1  
59 associated with hypoxia response [15] and ND1, ATAD2 and ARL3 that involved into cold response  
60 [16,17]. Notably, an increasing number of lakes are existing or towards saline and alkaline due to the  
61 geological evolution and global climate changes on the Tibetan Plateau [3,18]. For instance, Lake  
62 Qinghai, the largest salt lake in China, is highly saline (up to 13‰) and alkaline (up to pH 9.4) water  
63 environment. It is also a typical salt lake with unusually high sodium, potassium and magnesium  
64 concentration [18,19]. Intriguingly, Lake Qinghai used to be freshwater and connected to the Yellow  
65 River, while was late separated with the upper reaches of the Yellow River during the geological event  
66 “Gonghe Movement” (approximately 15 mya) [19,20]. Moreover, the increasing of water salinization is a

67 growing threat to freshwater fish species [21,22]. Tibetan freshwater endemic fishes are long suffering  
68 these harsh conditions challenges [11,18]. The main focus of the genetic mechanism of highland  
69 adaptation in Tibetan fish are still on hypoxia and chronic cold response [15,23–25]. However, the  
70 genomic signature of high salinity and alkalinity adaptation in Schizothoracine fish have yet to be  
71 comprehensively determined.

72

73 Unlike other broadly distributed Schizothoracinae fish species, *Gymnocypris przewalskii* is only endemic  
74 to Lake Qinghai [19,20,26]. Past studies suggested that *G. przewalskii* has gradually evolved from  
75 freshwater fish to tolerate high salinity and alkalinity of Lake Qinghai during the early to late Holocene  
76 [26]. Because of the unique evolutionary history in Lake Qinghai at high altitude, *G. przewalskii* provides  
77 an exceptional model to investigate the genetic mechanisms underlying adaptation to high salinity and  
78 alkalinity environment on the Tibetan Plateau. In this study, we performed a comparative genomics  
79 analysis and identified a set of ion transport genes that showing strong signals of rapidly evolving in *G.*  
80 *przewalskii*. Specifically, we used the *de novo* transcriptome assemblies from multiple tissue RNA-seq  
81 data and five well-annotated teleost fish genomes for comparison. In addition, we estimated the genome-  
82 wide nucleotide substitution rate of each fish species. Moreover, using the tissue-transcriptomics, we  
83 characterized the expression patterns of rapidly evolving ion transport genes in kidney, gill, liver, spleen,  
84 brain and muscle of highland fish, *G. przewalskii*.

85

## 86 2. Materials and methods

### 87 2.1. Data collection and transcriptome assembly

88 We downloaded the transcriptome sequencing data of Schizothoracine fish *G. przewalskii* from NCBI  
89 SRA database (<https://www.ncbi.nlm.nih.gov/sra>). Specifically, we collected six tissues transcriptomics  
90 including kidney, gill, liver, spleen, brain and muscle of *G. przewalskii* (supplementary table S1). At first,  
91 we checked the quality of the raw sequencing reads using FastQC v 0.11.8  
92 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Sequencing adapters and reads with a  
93 quality score < 20 were trimmed with Trimmomatic v.0.36 [27], resulting in clean sequencing reads.  
94 Then, we performed *de novo* transcriptome assembly using Trinity v2.8.5 [28] with default parameters.  
95 After assembly, the redundant transcripts were removed using CD-HIT v4.8.1 [29] with the threshold of  
96 0.90, and only the longest transcript under each cluster was extracted as unigene (unique gene). Next, we  
97 predicted the open reading frame (ORF) of each unigene using TransDecoder

98 (<https://github.com/TransDecoder/TransDecoder>). Finally, we translated the nucleotide sequences of  
99 protein-coding genes from the assemblies of *G. przewalskii* into amino acid sequences using an in-house-  
100 developed perl script.

101

## 102 2.2. Orthologs identification and sequence alignment

103 We included five well-annotated teleost fish genomes for comparative genomics analysis and downloaded  
104 from Ensembl database (<http://useast.ensembl.org/index.html>), including zebrafish (*Danio rerio*), tilapia  
105 (*Oreochromis niloticus*), medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*) and cod (*Gadus morhua*).  
106 Then, we built a local protein database including the sequences from above five fish genomes and *G.*  
107 *przewalskii* transcriptome assemblies. Next, we downloaded the curated orthology map of Actinopterygii  
108 (ray-finned fish) from OrthoDB database (release 8) [30] which contains 21,952 orthologous gene groups  
109 information. Of these seed orthologous groups in HaMStR v13.2.6 [31], we identified the orthologs in  
110 each fish species with E-values of less than  $10^{-20}$ . Moreover, we aligned and trimmed the protein  
111 sequences of the orthologous groups using PRANK [32] and MATFF v 7.450 [33], and trimmed using  
112 trimAl [34] with the parameter “-automated1”. Among the identified orthologs, we identified one-to-one,  
113 one-to-many and many-to-many orthologs in each fish species. For each 1:1 orthologous pair (i.e. genes  
114 for which only one gene from each species matches the given OrthoDB orthologous gene group), we only  
115 selected the longest transcript associated with the gene for each pair of species as putative single-copy  
116 ortholog. Finally, we identified the core single-copy orthologs that were shared by above six fish species.

117

## 118 2.3. Genome-scale concatenation and coalescent based dataset construction

119 We performed the alignment of core shared single-copy orthologs of six fish species using MUSCLE [35]  
120 with default parameters and trimmed using trimAl v1.2 [34] with parameter “-automated1”. In addition,  
121 we filtered the core shared single-copy orthologs with strict constraints, including length (minimum 200  
122 aa) and sequence alignment (maximum missing data 50% in alignments). Next, we prepared two types of  
123 datasets after filtration. Firstly, we concatenated all core shared single-copy genes from each species into  
124 one-line sequence as a supergene using a custom python script (genome-scale concatenation-based  
125 dataset), respectively. Secondly, we conducted a genome-scale coalescent-based dataset including core  
126 shared single-copy genes from each species.

127

## 128 2.4. Molecular evolution analysis

129 We used the clipped species tree (Figure 1A) including above six fish species from a previous study [16].  
130 To estimate lineage-specific evolutionary rates for each fish species, we aligned core shared single-copy  
131 orthologs using MUSCLE [35], derived nucleotide alignments from protein alignments using PAL2NAL  
132 v14 [36], and estimated pairwise non-synonymous to synonymous substitutions (dN/dS) of nucleotide  
133 alignments using the CodeML package in PAML 4.7a [37]. Specifically, we used the free-ratio model  
134 (“several  $\omega$  ratio”) to calculate the ratio of dN to dS nucleotide changes separately for each ortholog and a  
135 concatenation of all alignments of single-copy orthologs from above six fish species. Parameters,  
136 including dN, dS, dN/dS,  $N*dN$ , and  $S*dS$  values, were estimated for each branch, and genes were  
137 discarded if  $N*dN$  or  $S*dS < 1$ , or  $dS > 2$ , following previous studies [11,16,17].

138

139 We sought to identify a set of genes with elevated dN/dS in *G. przewalskii* relative to other five fish  
140 species. At first, we ran two branch models using CodeML package in PAML 4.7a [37] to identify rapidly  
141 evolving genes (REGs) in *G. przewalskii* lineage with corresponding nucleotide alignments, specifically  
142 with the null model assuming that all branches have been evolving at the same rate and the alternative  
143 model allowing the focal foreground branch (*G. przewalskii*) to evolve under a different evolutionary rate.  
144 Next, we used a likelihood ratio test (LRT) in R software, package MASS with  $df = 1$  to discriminate  
145 between the alternative model and the null model for each single-copy orthologs in the genesets. We only  
146 considered the genes as rapidly evolving with a significantly faster rate in the foreground branch if the  
147 adjusted  $P$  value  $< 0.05$  and higher dN/dS in the focal foreground branch than focal background  
148 branches (other four fish species). Finally, we annotated the rapidly evolving genes with gene ontology  
149 (GO) function category using R software, package topGO [38].

150

### 151 2.5. Gene expression analysis

152 After preparation of clean reads from six tissue-transcriptomics (kidney, gill, liver, spleen, brain and  
153 muscle) of *G. przewalskii*, we performed the transcript quantification by mapping all clean reads to the  
154 transcriptome assemblies using RSEM v1.3.1 [39] to obtain expected counts and fragments per kilobase  
155 million (FPKM). In addition, we primarily focused on the expression pattern of rapidly evolving genes  
156 (REGs), and calculated the FPKM value of each REG in each tissue. At last, we annotated the  
157 differentially expressed REGs by gene ontology using R software, package TopGO [38].

158

## 159 3. Results

160 *3.1. Transcriptome assemblies and orthologs*

161 By pooling six tissue-transcriptome sequencing data, the de novo transcriptome assembly of *G.*  
162 *przewalskii* yielded 409,685 transcripts, with an N50 of 1,796 bp and an average length of 986 bp. After  
163 removing redundant isoforms and extraction of longest isoform among alternative transcripts, a total of  
164 357,601 unigenes were obtained, with an N50 of 3,079 bp and a mean length of 1,992 bp. After protein-  
165 coding gene prediction with TransDecoder, we totally obtained 137,539 unigenes with full or partial  
166 length of gene coding regions (CDS) in *G. przewalskii*.

167

168 After identification of orthologs according to the curated orthologous gene groups of Actinopterygii in  
169 each fish species, we obtained a total of 74,107 putative orthologs in 16,379 orthologous gene groups  
170 (Table 1). After strict 1:1 ortholog selection, we identified 16,379 longest orthologs that represent their  
171 gene groups as unique ortholog (Table 1). In addition, we eventually obtained core 10,260 orthologs that  
172 shared by all six fish species, making them suitable for comparative genomics analysis.

173

174 *3.2. Genome-wide nucleotide substitution rate*

175 After estimation of the nucleotide substitution rates of each branch that represented each fish species  
176 based on 6,742 core shared single-copy orthologs, we found that Schizothoracine fish *G. przewalskii* had  
177 elevated terminal genome-wide concatenation-based dN/dS compared to other five fish species (Figure  
178 1B). Furthermore, we also found similar elevated pattern of genome-wide coalescent-based dN/dS in *G.*  
179 *przewalskii* relative to other species (Figure 1C).

180

181 *3.3. Rapidly evolving genes*

182 A set of genes with the signature of an increase rate of non-synonymous changes and underwent  
183 accelerated evolution, namely rapidly evolving genes. We identified 466 putative rapidly evolving single-  
184 copy orthologs (REGs) in *G. przewalskii* (supplementary table S2). Among this set of genes, the most  
185 interesting finding was REGs included genes associated with ion transport functions. This group included  
186 sodium channel subunit beta-3 (SCN3B), solute carrier family 13 member 3 (SLC13A3), transmembrane  
187 protein 175 (TMEM175) and H<sup>(+)</sup>/Cl<sup>(-)</sup> exchange transporter 7 (CLCN7) (Table 2). Moreover, we found  
188 a number of REGs associated with mitochondrial function and also involved ion transport process, such  
189 as sodium/potassium-transporting ATPase subunit beta-2 (ATP1B2), calcium uniporter protein (MCU)

190 and calcium uptake protein 2 (MICU2) (Table 2). Besides the ion transport genes, we found a large  
191 number of genes involved energy metabolism function, such as ATP5c1 and ATP5b associated with ATP  
192 binding and oxidative phosphorylation process (supplementary table S2). Although previous comparative  
193 genomics studies with highland fish identified several candidate genes with the signals of positive  
194 selection [15,23,24], here, we failed to identify any gene that potentially associated with hypoxia  
195 response.

196

### 197 3.4. Tissue expression patterns of rapidly evolving ion transport genes

198 After mapping the clean reads from six tissue-transcriptome sequencing data, we obtained the expression  
199 level of each unigenes based on FPKM value (supplementary table S3). We focused on the expression  
200 pattern of ion transport genes with the signature of accelerated evolution. Notably, we found eight rapidly  
201 evolving ion transport genes were broadly expressed in six tissues, except *transient receptor potential*  
202 *cation channel subfamily V member 6 (TRPV6)* that only expressed in liver (Figure 2A). In addition, the  
203 hierarchical clustering which illustrated by heatmap indicated that four genes (*ATP1B2*, *MCU*, *CLCN7*  
204 and *NALCN*) and another five genes (*MICU2*, *SCN3B*, *TMEM175*, *TRPV6* and *SLC13A3*) showed similar  
205 tissues expression patterns, respectively (Figure 2B). Moreover, gene ontology (GO) enrichment analysis  
206 showed that this set of differentially expressed REGs was significantly enriched multiple functions, such  
207 as ion transport (GO:0006811,  $P = 0.00031$ ), sodium ion transport (GO:0006814,  $P = 0.00047$ ), calcium  
208 ion transport (GO:0006816,  $P = 0.00056$ ), chloride transport (GO:0006821,  $P = 0.00067$ ), response to pH  
209 (GO:0009268,  $P = 0.00069$ ) and response to calcium ion (GO:0051592,  $P = 0.00078$ ) (Figure 2C).

210

## 211 4. Discussion

212 Over the past few years, transcriptome-based assembly approach enables comparative genomics studies  
213 widely employed in many Tibetan endemic organisms to provide insights of highland adaptation [15–  
214 17,23,40,41]. Unlike whole genome data, although transcriptome sequencing is an effective and  
215 accessible approach to initiate comparative genomic analyses on non-model organisms [28], it still can  
216 not cover the whole protein coding gene repertoire of one species. Previous transcriptome studies on  
217 Tibetan fishes mainly included one or two tissues [17,40,41], our present study included six tissues  
218 (kidney, gill, liver, spleen, brain and muscle) RNA-seq data of *G. przewalskii* and generated much more  
219 transcripts than previously assemblies [16,40]. In addition, we used curated orthology mapping approach  
220 [42] and identified more than 15,000 pairwise orthologous genes in each fish species and over 10,000



221 core single-copy orthologs shared by six species, which included much more orthologs than our early  
222 studies [16,17]. These putative single-copy orthologs are the important bases for comparative genomic  
223 analysis. Notably, most high-altitude dwelling Schizothoracine fish species are polyploidy with high  
224 complexity and large size of genomes, the whole genome data is long lacking [11]. Therefore,  
225 comparative genomic analysis based on transcriptome assemblies of Schizothoracine fish will still be the  
226 tendency in recent years.

227

228 Our present study pinpointed that highland fish, *G. przewalskii* has elevated rate of molecular evolution  
229 (dN/dS) on both concatenation and coalescent genomic-scales compared with lowland fish species,  
230 indicating that *G. przewalskii* may be under rapidly evolving. Not surprisingly, this result was consistent  
231 with previous studies in other Tibetan endemic fish species [15–17,23,41]. In addition, this finding  
232 highlighted animals endemic to the Tibetan Plateau underwent accelerated evolution (high dN/dS) relative  
233 to low-altitude dwelling organisms [9,10]. Furthermore, species inhabit similar ecological niches may be  
234 shaped by convergent evolution to form physiological or morphological similarities [43]. Like other  
235 Tibetan terrestrial wildlife, our finding implied that the elevation of genome-wide nucleotide substitution  
236 rate is one of adaptive process of *G. przewalskii* to harsh environment in Lake Qinghai, including the  
237 increasing of water salinization.

238

239 Accelerated evolution at molecular level may be reflected by an increased rate of non-synonymous  
240 changes within genes involved in adaptation [44]. Our present comparative study identifies a set of  
241 rapidly evolving genes associated with ion transport function in *G. przewalskii*. These genes encoded  
242 biological macromolecules which mainly functioning in sodium ion transport, calcium ion transport,  
243 chloride transport and response to pH processes. This result is consistent with findings in an extremely  
244 alkaline environment dwelling fish, *Leuciscus waleckii* [45], indicating that the alkaline environment of  
245 both Lake Qinghai and Lake Dali Nur spurred accelerated evolution of ion transport genes in both fish  
246 species. Notably, the rapidly evolving gene repertoire of *G. przewalskii* included SLC13A3, TMEM175  
247 and CLCN7. Solute carrier (SLC) is a family that encoded transmembrane transporters for inorganic ions,  
248 amino acids, neurotransmitters, sugars, purines and fatty acids, and other solute substrates [46]. Past  
249 evidence suggested that the adaptive evolution of solute carrier genes contribute to high salinity and  
250 alkalinity adaptation in fishes [45–47]. Specifically, SLC13 is a subfamily of sodium sulphate/carboxylate  
251 cotransporters [48]. Moreover, CLC gene is a family of H<sup>+</sup>/Cl<sup>-</sup> exchange transporter that mediate  
252 transmembrane Cl<sup>-</sup> transport [49]. In addition, previous study suggested that TMEM175 is involved in

253 potassium channel activity [50]. Therefore, we suggested that ion transport genes underwent rapidly  
254 evolving is another adaptation strategy for *G. przewalskii* to cope with the severe saline and alkaline  
255 stress in Lake Qinghai.

256

257 Previous studies identified a number of genes under accelerated evolution that mainly involved energy  
258 metabolism pathways [11,15-17,23-25]. Compared with a few rapidly evolving ion transport genes that  
259 were found in *G. przewalskii*, this present study identified a number of candidate genes that related to  
260 energy metabolism and contributed to long-term cold adaptation. Gene associated with energy  
261 metabolism showing signs of adaptive evolution is one of the genomic signatures that had been identified  
262 in Tibetan Plateau dwelling animals [16,17]. Our finding is consistent with previous comparative  
263 genomics studies in highland fishes as well [15-17]. A set of genes functioning in energy supply and  
264 metabolism were under accelerated evolution in *G. przewalskii*, such as ATP5b and ATP5c, ATP  
265 synthase subunit beta. In addition, although hypoxia adaptation is one of the significant adaptive  
266 processes contributed to highland adaptation in endemic animals that dwelt at high altitude [10,16,17], we  
267 still were not able to identify any rapidly evolving genes associated with hypoxia response function in  
268 present study. Indeed, there is a long controversial issue about hypoxic environment and hypoxia response  
269 for Tibetan fish species. Obviously, more physiological, ecological and genomic analysis were required to  
270 reveal the mechanism of highland fish adaptation to hypoxia.

271

272 A set of previous studies indicated that fish gills, kidney, liver and spleen are key tissues that contributed  
273 to saline and alkaline tolerance [51,52]. Using tissue-transcriptomic data, we characterized the expression  
274 profiles of six tissue types. Most of rapidly evolving ion transport genes have broad expression patterns  
275 across all tissues. In addition, these broadly expressed ion transport genes were mainly associated with  
276 sodium ion transport, chloride transport and response to pH function by gene ontology annotation. This  
277 finding indicates that ion transport genes in *G. przewalskii* experiencing accelerated evolution may have  
278 general functions and involve into multiple biological processes. Furthermore, we found a set of rapidly  
279 evolving ion transport genes that involved distinct pathways showed the similar tissue expression  
280 patterns. That is said, these ion transport genes under selection were putatively interacted by cooperation  
281 in *G. przewalskii* adaptation to high salinity and alkalinity environment of Lake Qinghai. Therefore, this  
282 finding indicated that future Schizothoracine fish comparative genomics study, including increasing  
283 sequencing and function assay, can further clarify the molecular basis of saline and alkaline adaptation of  
284 high-altitude dwelling fishes.

285

## 286 5. Conclusion

287 We used comparative genomics based on the *de novo* assemblies from pooled six tissues transcriptomes  
288 to identify the genomic signature of saline and alkaline adaptation in a highland fish, *G. przewalskii*.  
289 These putative genomic signatures included: (1) Schizothoracine fish, *G. przewalskii* had an elevated  
290 genome-wide nucleotide substitution rate than lowland teleost fish species; (2) a number of genes  
291 associated with ion transport and energy metabolism functions were found in *G. przewalskii* with elevated  
292 molecular evolutionary rate (dN/dS) showing the signature of rapidly evolving; (3) most of rapidly  
293 evolving ion transport genes associated with sodium ion transport, calcium ion transport and chloride  
294 transport were broadly expressed in kidney, gill, liver, spleen, brain and muscle of *G. przewalskii*; (4) A  
295 set of rapidly evolving ion transport genes exhibited similar tissue expression patterns and were interacted  
296 by co-expression in *G. przewalskii*. Altogether, our present study will provide the genomic signatures of  
297 rapidly evolving ion transport genes, and gain the insights into the saline and alkaline adaptation of high-  
298 altitude dwelling fishes.

299

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444



445 **Table**

446

447 **Table 1.** Summary of orthologous genes in five fish genomes and *G. przewalskii* transcriptomic  
448 assemblies.

Species	Genes	Genes in orthologous groups	Unique orthologs
<i>Danio rerio</i>	52,089	29,232	17,001
<i>Gadus morhua</i>	22,100	16,884	16,390
<i>Takifugu rubripes</i>	47,841	25,137	16,071
<i>Oryzias latipes</i>	24,674	17,857	15,877
<i>Oreochromis niloticus</i>	26,763	19,432	17,433
<i>Gymnocypris przewalskii</i>	137,539	74,107	16,379

449

450

451 **Table 2.** List of rapidly evolving ion transport genes in *Gymnocypris przewalskii*.

Gene name	Description	Adjusted P-value
SCN3B	Sodium channel subunit beta-3	0.000076
ATP1B2	Sodium/potassium-transporting ATPase subunit beta-2	0.020563
NALCN	Sodium leak channel non-selective protein	0.029246
SLC13A3	Solute carrier family 13 member 3	0.002234
TMEM175	Transmembrane protein 175	0.002100
CLCN7	H(+)/Cl(-) exchange transporter 7	0.000019
TRPV6	Transient receptor potential cation channel subfamily V member 6	0.022243
MCU	Calcium uniporter protein, mitochondrial	0.003946
MICU2	Calcium uptake protein 2, mitochondrial	0.002823

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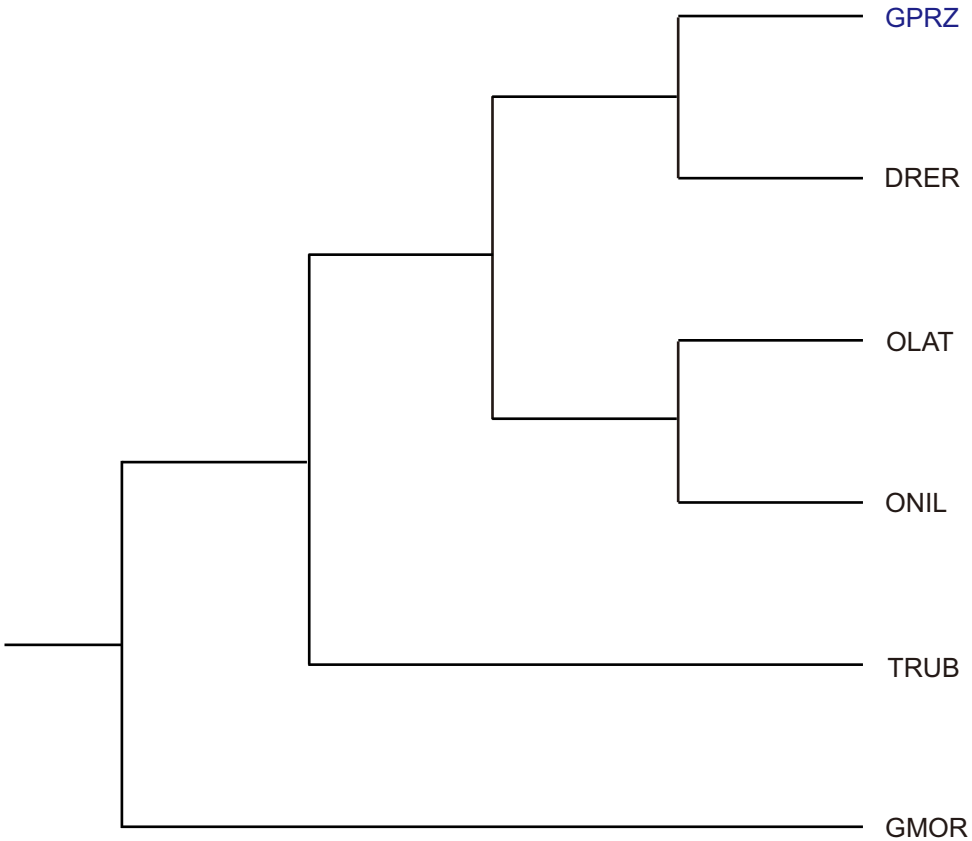
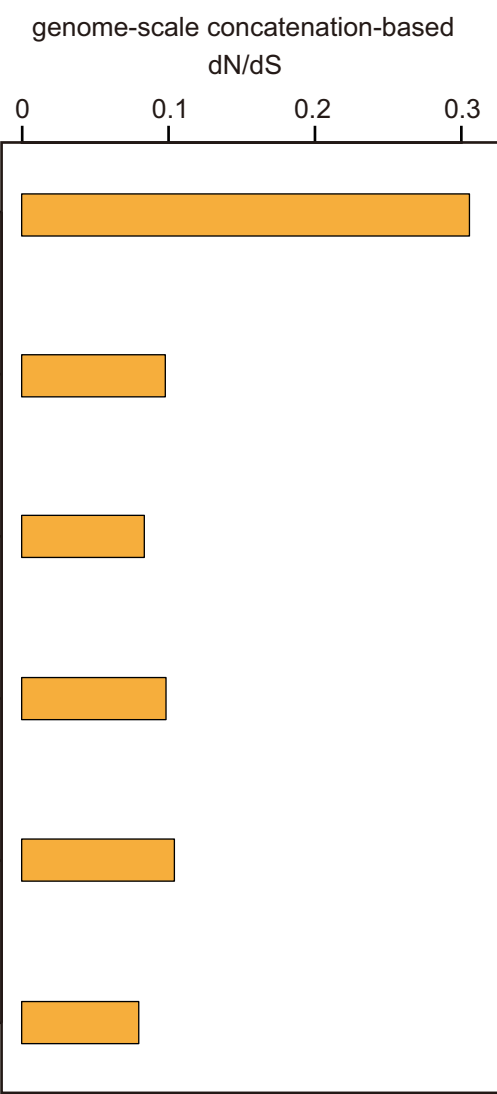
## 454 **Figure legends**

455

456 **Figure 1.** Genome-wide molecular evolutionary feature of six fish species. (A) Clipped species tree used  
457 in this study. GPRZ, *Gymnocypris przewalskii*, DRER, *Danio rerio*, OLAT, *Oryzias latipes*, ONIL,  
458 *Oreochromis niloticus*, TRUB, *Takifugu rubripes*, GMOR, *Gadus morhua*. (B) Barplot depicting the  
459 dN/dS of concatenated supergenes in each fish species. (C) Violin plot depicting the dN/dS of each  
460 coalescent orthologs in each species estimated by free-ratio model.

461

462 **Figure 2.** Expression feature of rapidly evolving ion transport genes (REITGs) in six tissues of *G.*  
463 *przewalskii*. (A) Barplot depicting the expression level of nine REITGs in kidney, gill, liver, spleen, brain  
464 and muscle tissues based on  $\text{Log}_{10}(\text{FPKM} + 1)$  value which estimated from RNA-seq data. (B) Heatmap  
465 depicting the expression level comparison of each REITG based on  $\text{Log}_{10}(\text{FPKM} + 1)$  values. Tissue  
466 type and gene name are shown on the y-axis and x-axis, respectively. Plot colors reflect the expression  
467 level, ranging from low (blue) to high (red). (C) Barplot depicting the significantly enriched gene  
468 ontology for differentially expressed REITGs.

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