

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

2 **Schizothoracine fish**

3

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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 ω 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⁽⁺⁾/Cl⁽⁻⁾ 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⁺/Cl⁻ exchange transporter that mediate
252 transmembrane Cl⁻ 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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302

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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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453

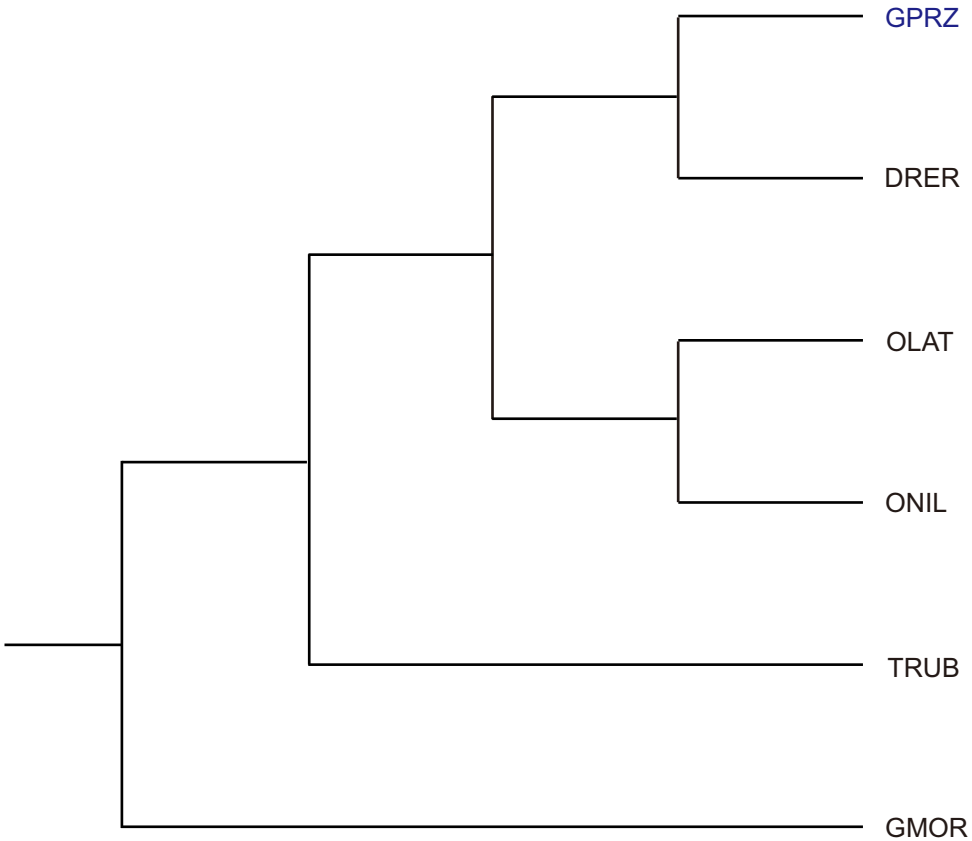
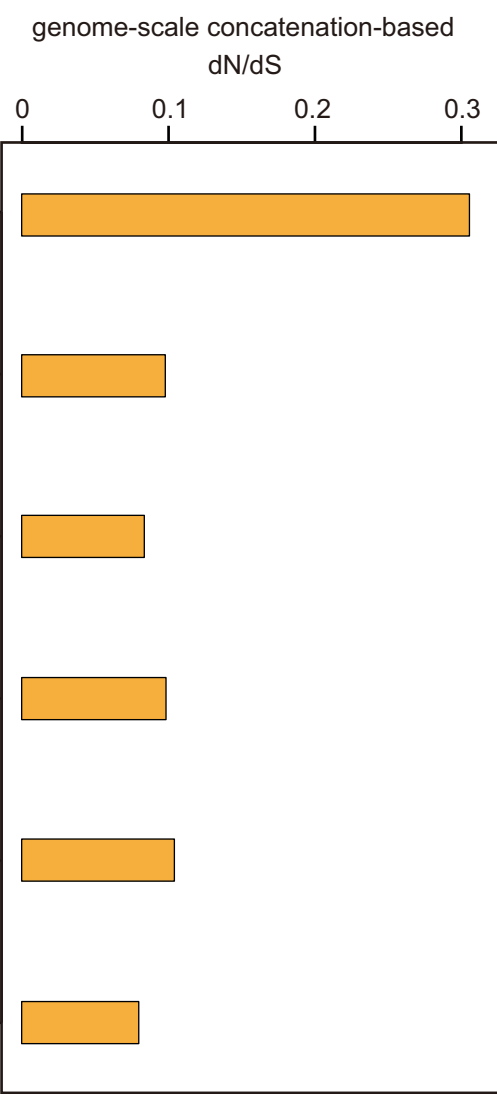
454 **Figure legends**

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**