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Phylogenetic relationship and taxonomic status of *Gymnocypris eckloni* (Schizothoracinae) based on specific locus amplified fragments sequencing

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Accurately delimiting phylogenetic relationships and taxonomic status is important for understanding species diversity and distributions and devising effective strategies for biodiversity conservation. However, species delimitation is controversial in *Gymnocypris eckloni*, a schizothoracine fish endemic to the Qinghai–Tibetan Plateau. The aim of this study is robustly identifying the phylogeny of G. eckloni in the Yellow River (YR) population and Qaidam basin (QB) population. The specific-locus amplified fragments sequencing (SLAF-seq) is employed with comprehensively sampling of schizothoracine fishes. In total, 350,181,802 clean reads and 5,114,096 SNPs are identified from SLAF-seq. Phylogenetic analysis recovers a non-monophyletic population of *G. eckloni* between YR and QB populations, representing an independent phylogenetic relationship between the two populations. Species delimitation analyses by SNAPPER and GMYC methods using the genome-wide SNP data confirm that their taxonomic statuses are separated. This study highlights the importance of further reconsidering clearer taxonomy, which would improve the genetic diversity conservation of Tibetan highland fishes.

KEYWORDS

genetic diversity conservation, non-monophyletic, population phylogeny structure, species delimitation, SLAF-seq, species synonymy

Introduction

The *Gymnocypris eckloni* Herzenstein (Teleostei: Cyprinidae) is a schizothoracine fish endemic to the Qinghai–Tibetan Plateau. This species includes two geographical populations, the Yellow River population (YR) and the Qaidam basin (QB) population. The holotype of *G. eckloni* was described in 1891 by a specimen from the QB population (Herzenstein, 1888).

The phylogenetic relationship between the YR and QB populations was a controversial topic. Some studies reported that (Wu and Wu, 1992; Li et al., 2020) the morphological and phenotypic characteristics were generally the same between the YR and QB populations of *G. eckloni*. Based on a single mitochondrial cytochrome *b* gene, the previous phylogenetic study (He and Chen, 2007) indicated that the two populations formed monophyly (Wu and Wu, 1992; He and Chen, 2007; Li et al., 2020). On the contrary, by increasing species, sample sizes, and the number of informative genetic markers, our previous studies (Zhao et al., 2005, 2009; Zhang et al., 2020) accidentally found that YR and QB populations were clustered into two lineages. The opposite findings might result from the limited information provided by a single mitochondrial cytochrome *b* gene (Cummings et al., 1995; Zou and Ge, 2008).

Specific locus amplified fragment sequencing (SLAF-seq) is a newly developed simplified deep genome sequencing technology for large-scale single nucleotide polymorphism (SNP) discovery and genotyping, with advantages of low cost, high accuracy, specificity, and repeatability (Chen et al., 2013; Jiang et al., 2015; Liu et al., 2018; Jing et al., 2020). To illustrate the taxonomic status of YR and QB populations of *G. eckloni*, we thus adopted this advanced method with a comprehensive sampling of schizothoracine fishes (**Figure 1**), which reached a convincing result based on nuclear markers.

Materials and methods

Sample collection

The *G. eckloni* samples were collected in the Yellow River and Qaidam basin. Samples were net-captured to contain pelvic fin and stored immediately in liquid nitrogen for DNA extraction. The Qinghai Provincial Bureau of Fishery approved and supervised field investigations. All the animal experiments were conducted according to procedures described in "Guidelines for Animal Care and Use" and approved by the Animal Care and Use Committee at the Northwest Institute of Plateau Biology, Chinese Academy of Sciences.

Library construction and data processing

Genomic DNA was isolated from the fin using a standard phenol–chloroform method (Sambrook et al., 1989). DNA quality and concentration were assessed by a NanoDrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA) and gel electrophoresis. The preparations of SLAF sequencing libraries were according to the description by Tang et al. (2019). Briefly, genomic DNA was digested by RsaI and HaeIII enzymes, respectively, and then ligated to Duplex Tag-labeled sequencing adapters with T4 DNA ligase (Life Technologies, Carlsbad, CA, USA) (Kozich et al., 2013). PCR reactions were performed using diluted restriction-ligation samples, dNTP, Q5® High-Fidelity DNA Polymerase (NEB China, Beijing, China), and then purified using Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK). The 300–700 bp fragments were selected and purified with QIAquick Gel Extraction Kit. According to the manufacturer's recommendations, the SLAF library was sequenced in the Illumina HiSeq 2500 system (Illumina, Inc., San Diego, CA, USA).

Raw reads of four samples of *G. eckloni* from two geographic populations, together with raw data from 14 schizothoracine fishes available in the NCBI SRA dataset (**Table 1**), formed the dataset for the current study.

The raw data were filtered to generate high-quality clean reads, increasing the confidence of variant calling. This step was processed using the FASTQ preprocessor (Chen et al., 2018) in terms of three stringent filtering standards: (1) removing reads with $\geq$10% unidentified nucleotides; (2) removing low-quality reads (the percentage of bases with quality Phred-scaled quality score $\leq$ 20); and (3) removing reads aligned to the barcode adapter. Based on the genome size and guanine-cytosine content of *G. eckloni*, the whole genome sequencing of *Gymnocypris przewalskii* (NCBI SRA database Bioproject ID PRJNA664553) was used as the reference genome. The clean reads were aligned against that reference genome using the Burrows–Wheeler Aligner (Abuín et al., 2015; Wanghe et al., 2020) with the setting "mem 4 -k 32 –M", where "k" was the minimum seed length and "-M" was an option used to mark shorter split alignment hits as secondary alignments (Li and Durbin, 2009). Variant calling was performed using the GATK's Unified Genotyper. SNPs were filtered using GATK's Variant Filtration with proper standards (-Window 4, -filter "QD < 2.0 || FS > 60.0 || MQ < 40.0", -G_filter "GQ < 20").

Phylogenetic analysis

The phylogenetic analysis by the maximum likelihood (ML) method was performed. The ML analyses were constructed using tree-building software raxmlGUI v1.3.1

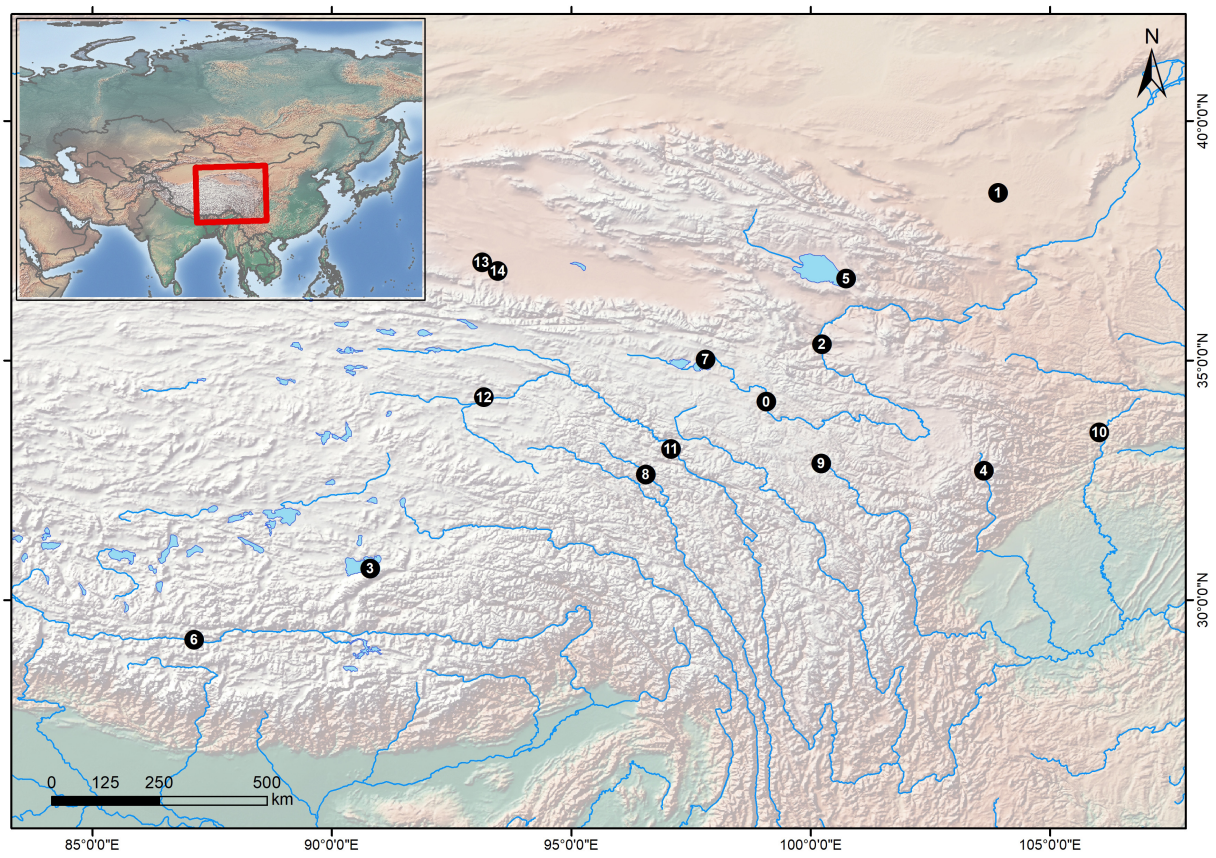

FIGURE 1
Sampling localities of the specimens. The black points are sampling sites, and the numbers in those points refer to the locality numbers in **Table 1**.

(Silvestro and Michalak, 2012) under the substitution GTRGAMMAI model, a graphical interface, and a toolkit for phylogenetic analyses using RAxML (Silvestro and Michalak, 2012; Edler et al., 2021). The node support estimation was assessed by 1,000 bootstrap replicates, and the other parameters were set to default. The option of combining all ML trees into a single file was selected to generate a consensus tree (Edler et al., 2021). *Oxygymnocypris stewartii* was regarded as the out-group for the phylogenetic analysis. The consensus tree was viewed and visualized by FigTree v1.4.4 software (Rambaut, 2009), and the publication-ready figure of the tree was produced by the Adobe Illustrator CC 2020 software (Wang et al., 2019).

Species delimitation

Generalized mixed Yule coalescent model

The Generalized Mixed Yule Coalescent (GMYC) model (Fujisawa and Barraclough, 2013) was implemented to detect shifts in branching rates between intra- and interspecific relationships. The GMYC web server at the multiple-threshold version on https://species.h-its.org/gmyc/ was used to set up this model. We transformed the initial RAxML tree to a time-calibrated phylogenetic tree as the input of the GMYC model by the RelTime method (Tamura et al., 2018). Based on our previous studies (Wanghe et al., 2017; Tang et al., 2019), the most recent common ancestor between *Schizopygopsis microcephalus* and *Schizopygopsis pylzovi* set at 1.10–0.70 MA and between *Gymnocypris przewalskii* and *G. eckloni* (YR population) was set at 0.15 MA.

SNAPPER analyses

SNAPP (SNP and AFLP Package for Phylogenetic analysis) is a method applied to infer species trees and demographics from independent biallelic markers such as well-spaced SNPs in a full coalescent analysis (Bryant et al., 2012). SNAPPER is a computationally more efficient method compared to SNAPP (Stoltz et al., 2021). The SNAPPER v1.0.2 package in BEAST v2.6.7 (Stoltz et al., 2021) was used to implement this analysis. The parameter of path sampling was set as 48 steps, with MCMC length = 100,000 and pre-burnin = 1,000, following

TABLE 1 Taxonomic information, sampling sites, and GenBank accession numbers of all species used in phylogenetic analysis.

No.	Species	Accession no.	Locality (number in Figure 1)
1	*Chuanchia labiosa*	SRR7628160	Gande, Qinghai (0)
2	*Chuanchia labiosa*	SRR7628161	Gande, Qinghai (0)
3	*Gymnocypris chilianensis*	SRR7628111	Wuwei, Gansu (1)
4	*Gymnocypris chilianensis*	SRR7628131	Wuwei, Gansu (1)
5	*Gymnocypris eckloni* (YR)	PRJNA842780	Golmud, Qinghai (13)
6	*Gymnocypris eckloni* (YR)	PRJNA842780	Golmud, Qinghai (13)
7	*Gymnocypris eckloni* (QB)	SRR7628145	Tongde, Qinghai (2)
8	*Gymnocypris eckloni* (QB)	SRR7628148	Tongde, Qinghai (2)
9	*Gymnocypris namensis*	SRR7628119	Damxung, Tibet (3)
10	*Gymnocypris namensis*	SRR7628155	Damxung, Tibet (3)
11	*Gymnocypris potanini*	SRR7628133	Songpan, Sichuan (4)
12	*Gymnocypris potanini*	SRR7628117	Songpan, Sichuan (4)
13	*Gymnocypris przewalskii*	SRR7628112	Haiyan, Qinghai (5)
14	*Gymnocypris przewalskii*	SRR7628113	Haiyan, Qinghai (5)
15	*Oxygymnocypris stewartii*	SRR7628120	Zhongba, Tibet (6)
16	*Oxygymnocypris stewartii*	PRJNA842780	Zhongba, Tibet (6)
17	*Platypharodon extremus*	SRR7628143	Madoi, Qinghai (7)
18	*Schizopygopsis anteroventris*	SRR7628116	Yushu, Qinghai (8)
19	*Schizopygopsis anteroventris*	SRR7628110	Yushu, Qinghai (8)
20	*Schizopygopsis chengi*	SRR7628164	Banma, Qinghai (9)
21	*Schizopygopsis chengi*	SRR7628163	Banma, Qinghai (9)
22	*Schizopygopsis kessleri*	PRJNA842780	Golmud, Qinghai (14)
23	*Schizopygopsis kessleri*	PRJNA842780	Golmud, Qinghai (14)
24	*Schizopygopsis kialingensis*	SRR7628154	Têwo, Gansu (10)
25	*Schizopygopsis kialingensis*	SRR7628153	Têwo, Gansu (10)
26	*Schizopygopsis malacanthus*	SRR7628135	Yushu, Qinghai (11)
27	*Schizopygopsis malacanthus*	SRR7628136	Yushu, Qinghai (11)
28	*Schizopygopsis microcephalus*	SRR7628134	Golmud, Qinghai (12)
29	*Schizopygopsis microcephalus*	SRR7628132	Golmud, Qinghai (12)
30	*Schizopygopsis pylzovi*	SRR7628142	Gande, Qinghai (0)
31	*Schizopygopsis pylzovi*	SRR7628144	Gande, Qinghai (0)

Andrea et al. (Quattrini et al., 2019). Samples were assigned to the following alternative species model (**Figure 2**): (1) run A, lumping by the current taxonomy delimited based on morphological discrimination, (2) run B, lumping by morphological discrimination but splitting the two populations of *G. eckloni*; (3) run C, lumping by genus; (4) run D, lumping by GMYC; (5) run E, lumping by basin/habitat; and (6) run F, lumping by basin but merging the two populations of *G. eckloni*. Marginal likelihood estimates (MLE) were obtained for each different model run in SNAPP analyses. The different species delimitation models were then ranked using the BFD* (Bayes factor delimitation with genomic data) methods (Leaché et al., 2014). Bayes factor (BF) was calculated between each alternative model by subtracting the MLE between two models, then multiplying the difference by two (Eq. 1). A positive BF value indicates support in favor of model 1, and a negative BF value indicates support in favor of model 2 (Kass and Raftery, 1995; Leaché et al., 2014). The strength of support from BF comparisons of competing models can be evaluated using the framework proposed by Kass and Raftery (1995). The BF scale is as follows: $0 < BF < 2$ is not worth more than a bare mention, $2 < BF < 6$ is positive evidence, $6 < BF < 10$ is strong support, and $BF > 10$ is decisive:

$$BF = 2 \times \left(\text{model 1} - \text{model 2}\right) \quad (1)$$

In Eq. 1, model 1 and model 2 are the MLE values obtained by two alternative runs of SNAPPER analyses.

Results

Specific-locus amplified fragment sequencing and single nucleotide polymorphism discovery

In total, 31 individuals (**Figure 1** and **Table 1**) generated 350,181,802 clean reads, with an average quality score of 96.81%. We identified 5,114,096 SNPs with a MAF $\geq$ 0.05 and integrity $\geq$ 80%. The number of insertions and deletions were 261,371 and 312,411, respectively. All SLAF-seq raw data are available on NCBI SRA with accession numbers. The detailed information on the SNP number, index number, raw reads, clean reads, and mapping the ratio (**Supplementary Table 1**).

Phylogenetic analyses

Using 5,114,096 high-quality SNPs, 31 accessions from 15 species delimited based on morphological discrimination were classified into three major clades. A majority of the identified morphospecies formed well-supported monophyletic clades in the clades (Run A in **Figure 2**). While the same genus (Run C in **Figure 2**) and the fish species in the same basin/habitat (Run E in **Figure 2**) were not grouped with a monophyletic clade. Interestingly, we found that the YR and QB populations of *G. eckloni* were not grouped (**Figure 2**). YR population and *G. przewalskii* were clustered into one lineage and formed a paraphyletic relationship with the QB populations.

Species delimitation

Results of generalized mixed Yule coalescent

The multi-species coalescent thresholds of the GMYC model were 0.70 and 0.95 MA (**Supplementary Figure 1A**), indicating that the time before all nodes reflected speciation events and after which all nodes reflected coalescent events (Milan et al., 2020). The GMYC model delimited six primary lineages (Run D

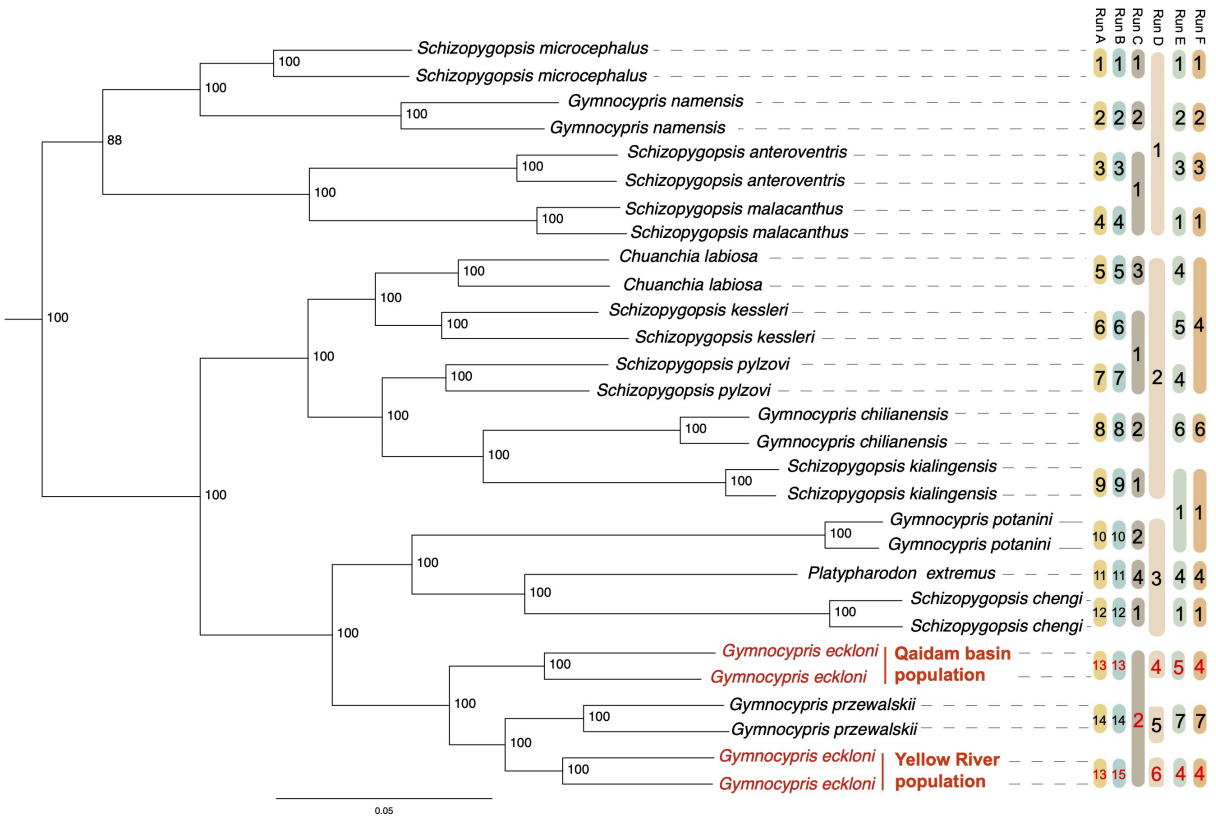

FIGURE 2
ML tree constructed by SALF-seq, plus a bar named Run D of GMYC results. Bootstrap support values were labeled in each node, and YR and QB populations of *Gymnocypris eckloni* were highlighted in red. Outgroup *Oxygymnocypris stewartii* was excluded from this tree. Remarks: The bars from Run A to Run F are the alternative species model for SNAPPER analyses. The same code in a bar refers to the same group for an alternative model. (1) Run A, lumping by the current taxonomy delimited based on morphological discrimination. (2) Run B, lumping by morphological discrimination but splitting the two populations of *G. eckloni*. (3) Run C, lumping by genus. (4) Run D, lumping by GMYC. (5) Run E, lumping by basin/habitat. (6) Run F, lumping by basin/habitat but merging the two populations of *G. eckloni*.

in Figure 2 and Supplementary Figure 1B) and recovered YR and QB populations of *G. eckloni* as separate individual groups.

Results of SNAPPER

The results of species delimitation by SNAPPER (Table 2) also decisively supported two separate individual groups between YR and QB populations of *G. eckloni*. The higher MLE value indicates a more likely alternative species model. Run A was the currently defined morphospecies with the second maximum MLE. The MLE of Run B was the maximum. Compared with Run A, Run B assumed that the YR and QB populations of *G. eckloni* were split into two independent species. The BF value between Run A and Run B was −644, which decisively supported in favor of Run B. Compared with Run E, Run F merged the two populations of *G. eckloni*, but the BF [value = (−2,743 − −2759) × 2 = 32] significantly supported that Run E (i.e., the two populations were separated) was the more likely alternative scenario.

Discussion

Gymnocypris eckloni is an essential freshwater germplasm species in the Tibetan Plateau (Li et al., 2020). Clarifying the phylogeny of this species would play a significant role in understanding the evolution of highland fishes and their biodiversity conservation. The previous studies (Zhao et al., 2005, 2009; He and Chen, 2007) did not agree on the phylogenetic relationship and taxonomic status of *G. eckloni*. In this study, we empirically confirmed that the phylogenetic relationship between the two populations was independent and that their taxonomic statuses were separated by applying the SLAF-seq of available schizothoracine fish. Those results accorded with our previous studies (Zhao et al., 2005, 2009), inferring that the YR population of *G. eckloni* was a substantially older divergence compared with the lineage of the QB population. Some related studies on fish species reported that (Cui et al., 2013; Tang et al., 2019) the convergent evolution, caused by

TABLE 2 SNAPPER results for different species delimitation models.

Model	Run A	Run B	Run C	Run D	Run E	Run F
MLE[1]	−2,088	−1,766	−2,783	−2,299	−2,743	−2,759
BF[2]	0	−644	1,390	423	1,310	1,343
Rank	2	1	6	3	4	5

[1]MLE, the marginal likelihood estimate obtained by the SNAPPER analyses.
[2]BF, the Bayes factor calculated by equation 1 compared with the MLE value of Run A = model 1.

dwelling in the same ecological environment, would produce an extensive reticulate evolution process, resulting in morphological similarity within some genetically close species. The above-discussed research would help explain the misled taxonomic definition of *G. eckloni*. Therefore, we suggested that a more definite taxonomy of *G. eckloni* in the Qaidam basin population should be reconsidered to improve the conservation of genetic diversity for this endemic fish.

In future studies, genome-wide SNP data would probably produce some direct evidence (Leaché et al., 2014; Kim and Roe, 2021). Additionally, the hidden morphological divergence between the two populations of *G. eckloni* needs to be examined by advanced approaches (Li et al., 2020), such as modern geometric morphometrics (Wang et al., 2017) and micro-computed tomography (Li et al., 2020), to test the unknown species taxon in the Qaidam basin population (Tang et al., 2016).

Conclusion

This study using SLAF-seq, a newly developed simplified deep genome sequencing technology for large-scale SNP discovery and genotyping, provide information for further understanding of the phylogenetics, adaptation, and evolution of *G. eckloni*. Our results emphasized that the phylogenesis between the YR and the QB population of *G. eckloni* was genetically independent. In summary, the current study underlines the great significance of *G. eckloni* in the Qaidam basin in protecting Tibetan highland fishes, laying the foundation for reconsidering a more straightforward taxonomy of Tibetan highland fishes and providing new insights for further taxonomic study.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the **Table 1**.

Ethics statement

All animal experiments were conducted according to procedures described in "Guidelines for Animal Care and Use" and approved by the Animal Care and Use Committee in Northwest Institute of Plateau Biology, Chinese Academy of Sciences.

Author contributions

KW, CF, and YT: conception, sampling, and writing – original draft. DQ and SA: analysis. GN, XL, GW, LJ, and SL: critical review. KZ and FT: funding acquisition and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.933632/full#supplementary-material

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