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Letter to the editor Open Access

Comparative analysis and phylogenetic and evolutionary implications of mitogenomes of Chinese *Sinocyclocheilus* cavefish (Cypriniformes: Cyprinidae)

DEAR EDITOR,

Cavefish are of considerable interest due to the remarkable morphological changes that occurred during their adaptation to cave or subterranean river habitats. These changes are thought to involve trade-offs in metabolic requirements and energy utilization. Mitochondria play a vital role in oxygen use and energy metabolism; thus, mitochondrial genes are likely to have experienced specific selective pressures during cavefish evolution. In this study, we assembled and annotated the mitogenome of *Sinocyclocheilus sanxiaensis*, a typical cavefish species in China. Using this sequence and other available mitogenomes from the NCBI database, we reconstructed an updated phylogeny of *Sinocyclocheilus* based on 29 species. Five species groups were revealed, with the recently erected *S. microphthalmus* species group supported. To determine whether divergent selective pressures have acted on the protein-coding genes (PCGs) of the 29 *Sinocyclocheilus* species exhibiting different morphological features (including eye shape and body color), the fish were classified into three groups according to their cave-related traits. The branch model test revealed higher nonsynonymous/synonymous substitution ratios (ω) in the cave-dwelling groups, potentially resulting from a balance between adaptive selection and relaxed functional constraints. Sophisticated analysis using site and branch-site models identified 24 highly credible positive selection sites located on nine PCGs, with most sites also showing radical changes in amino acid properties. This study sheds light on the phylogeny and mitogenomic evolution of *Sinocyclocheilus* cavefish, highlighting their successful adaptation and survival in subterranean environments.

Cavefish, also known as hypogean fish, constitute a specialized group of fish that spend much (or all) of their lives restricted to cave or subterranean river habitats (Ma et al., 2019). The study of cavefish evolution has greatly benefited from the traditional model species *Astyanax mexicanus*, including extensive exploration of the biological mechanisms and genetic basis of the gain and loss of traits in cavefish compared to surface morphs (Jeffery, 2020). China harbors the largest number of cavefish species in the world, with approximately 150 species predominantly distributed in southwestern karst areas (Ma et al., 2019). Among these fish, the cyprinid genus *Sinocyclocheilus* represents the most expansive known group (Zhao & Zhang, 2009), currently comprising 77 recognized species. This genus exhibits a spectacular diversity of morphological forms, ranging from typical surface-dwelling fish characterized by well-developed eyes and conspicuous pigmentation to cave-dwelling forms that typically lack eyes and pigmentation (Borowsky, 2018). Given its remarkable species diversity and phenotypes, the *Sinocyclocheilus* genus has emerged as an excellent model for investigating adaptive evolution in cavefish, with the increasing availability of sequenced species helping to unravel the genetic architecture governing the evolution of various traits (e.g., Yang et al., 2016).

Previous studies have reconstructed the phylogenetic relationships of *Sinocyclocheilus* using several mitochondrial DNA markers, including the *CYTB* gene sequence (Jiang et al., 2019; Zhao & Zhang, 2009). Although mitochondrial sequences have been extensively used in phylogenetic studies, relatively little attention has been devoted to the study of molecular adaptation based on mitochondrial PCGs. Mitochondria serve as essential energy factories in metazoans and play critical roles in adenosine triphosphate (ATP) synthesis and heat generation via cellular respiration. Increasing evidence supports the occurrence of adaptive evolution within mitogenomes, particularly in extreme habitats such as the Qinghai-Xizang (Tibet) Plateau, where fish require intense cold and hypoxic adaptation (Wang et al., 2016). Cavefish typically exhibit efficient metabolism, allowing for the accumulation of fat (a kind of gain) even under poor food availability (Borowsky, 2018). However, whether these constructive traits display adaptive selection signals in energy metabolism-related genes, such as mitochondrial PCGs, remains an interesting but unanswered question.

In this study, we assembled and delineated the complete mitogenome of *S. sanxiaensis*, a typical cave-dwelling species (Jiang et al., 2019), and investigated its detailed characteristics (GenBank accession number: OP745534). The mitogenome of *S. sanxiaensis* was a circular molecule 16 589 bp in length, containing typical teleost mitogenomic content (Supplementary Figure S1 and Table S1). All transfer RNAs (tRNAs) were folded into a typical cloverleaf secondary structure, except for tRNA^{Ser1}, which lacked the dihydrouridine

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(DHU) arm (Supplementary Figure S2). The nucleotide compositions, as well as AT- and GC-skew and relative synonymous codon usage, of *S. sanxiaensis* were comparable to those observed in other congeners (Supplementary Figure S3A, B and Table S2).

To facilitate analysis, we classified 29 *Sinocyclocheilus* species with available mitogenomes (including *S. sanxiaensis* from this study) into three groups according to their cave-related traits: (I) surface-dwelling group with normal eyes and regular body color; (II) semi-cave-dwelling group with reduced eyes and partial pigment loss; and (III) cave-restricted group with absent eyes and albinism (Figure 1). The nucleotide diversities showed the highest values for group (III), followed by group (I) and group (II) (Supplementary Figure S3C). Both the *ATP8* and *ND3* genes showed some ratio of non-synonymous (*K*a) and synonymous (*K*s) substitutions (*K*a/*K*s) values > 1 between congeneric species pairs (Supplementary Figure S3D), indicating that positive selection may occur within *Sinocyclocheilus* species.

According to the phylogenetic tree constructed using maximum-likelihood (ML) and Bayesian inference (BI) analyses, *Sinocyclocheilus* could be divided into five major clades (A–E, Figure 1), corresponding to the five known species groups: i.e., *S. jii* (A), *S. angularis* (B), *S. microphthalmos* (C), *S. cyphotergous* (D), and *S. tingi* (E) (Zhao & Zhang, 2009). The *S. jii* species group (A) diverged first, followed by the *S. angularis* species group (B). The *S. microphthalmos* species group (C) is a recently defined clade (Wen et al., 2022), which was established to accommodate species previously classified in the *S. angularis* species group (B) but later presenting as a mutually paraphyletic lineage. This group was supported in our study and consisted of *S. microphthalmos* and *S. anshuiensis*. The *S. cyphotergous* (D) and *S. tingi* (E) species groups were recovered as sister clades, and contained most species within *Sinocyclocheilus.* Interestingly, the four cave-restricted species (*S. furcodorsalis*, *S. anshuiensis*, *S. sanxiaensis*, and *S. anophthalmus*) exhibited parallel evolution distinct from the other four species groups/clades (B, C, D, and E, respectively) after divergence from the *S. jii* species group (A).

We assessed the selective pressures acting on the 13 PCGs in the 29 *Sinocyclocheilus* species based on the three-group classification (I, II, and III). The PAML branch tests revealed that the two-ratio (M_2) and three-ratio models (M_3) were generally better than the one-ratio model (M_0, P=0.000). Furthermore, the groups assigned to the M_3 and $M_{2\text{-}2}$ models ((II & III) vs. I) demonstrated better fit of the data compared to the $M_{2\text{-}1}$ model (III vs. (I & II); P=0.000, Supplementary Table

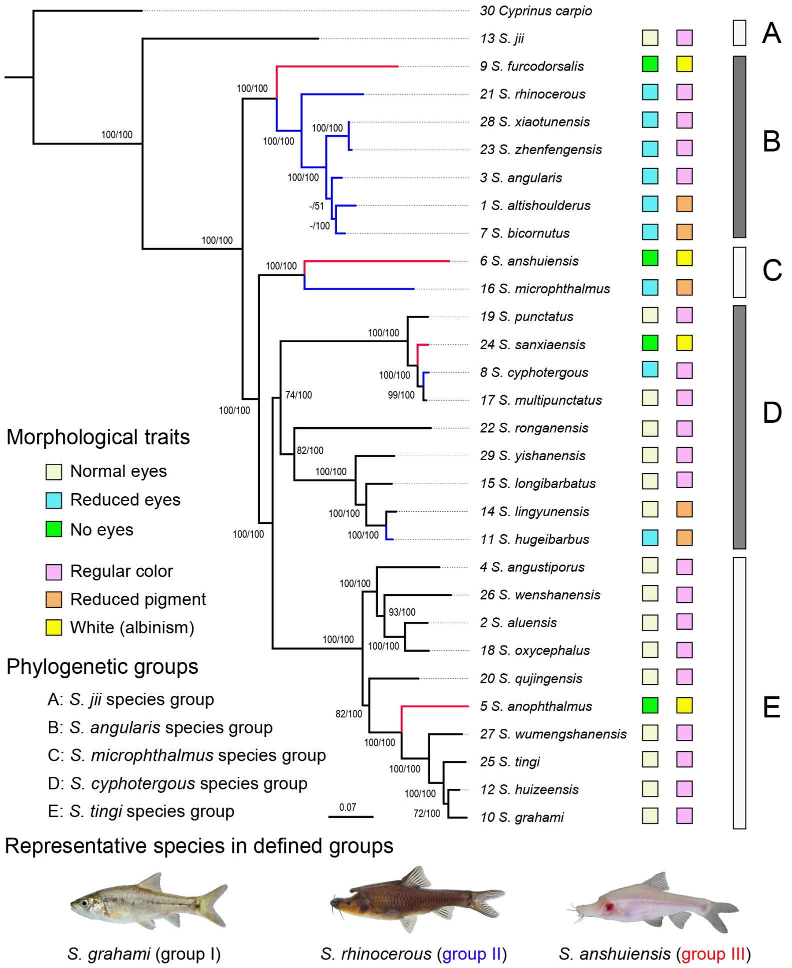

Figure 1 Phylogenetic relationship within *Sinocyclocheilus* based on 29 species

Numbers around nodes are bootstrap values and posterior probabilities from ML and BI methods, respectively. Number before species name is listed according to Supplementary Table S2. Branches marked in black, green, and red indicate species assigned to surface-dwelling fish group (I), semi-cave-dwelling fish group (II), and cave-restricted fish group (III), respectively. Pictures of one representative species in each defined group are provided. Photos by W.S. Jiang.

S3). Under the free-ratio model (M_1), the estimated ω ratios of branches from the cave-dwelling groups (II & III) were significantly larger than that of the surface-dwelling group (I) (mean ω: 0.126 (II & III) vs. 0.057 (I); P=0.003). However, RELAX analysis indicated that the cave-dwelling groups (II & III) were under significant selection relaxation compared to the surface-dwelling group (I; K=0.8 & P=0.000).

A total of 44 (out of 3 794) codon sites were identified as being under positive selection using the PAML site model, while 50 sites were identified using the branch-site model. A total of 26 and 31 codon sites were found to be under positive selection using the fixed-effects likelihood (FEL) and mixed-effects model of evolution (MEME) methods, respectively. Together, 11 sites were found to be under positive selection based on at least two methods, while three sites were identified based on three methods. A total of 24 sites located within nine genes were considered to be highly credible positive selection sites according to their high Bayes empirical Bayes (BEB) values (>0.95) and overlap between the PAML and HyPhy tools (Supplementary Table S4). Among these sites, 20 were also detected by TreeSAAP as being under radical substitution at the physicochemical amino acid level (Supplementary Table S4). Nine sites identified via the branch-site models were further examined in relation to the relevant protein structures, with many being situated within the functional domain of the α-helix, especially near or on the connection sites of the α-helix and loop area (Supplementary Figure S4), which may be responsible for conformational stability.

In conclusion, the cave-dwelling groups (II & III) exhibited significantly higher ω ratios than the surface-dwelling group (I) across the 13 mitochondrial PCGs in *Sinocyclocheilus*, suggesting weaker purifying selection and greater accumulation of nonsynonymous mutations in the cave-dwelling species. A high ω value can be attributed to either positive selection, leading to the fixation of beneficial nonsynonymous mutations, or relaxed functional constraints, which decrease the degree of purifying selection and lead to the fixation of certain deleterious mutations (Li et al., 2017). While it is difficult to distinguish between these two possibilities based exclusively on ω ratios, our RELAX analysis results suggested that relaxed functional constraints may contribute significantly, or at least partially, to the higher ω ratios observed in the cave-dwelling groups (II & III). However, the harsh physiological demands imposed by cave environments can trigger strong selective pressure on energy metabolism-related genes, including mitochondrial PCGs. Hence, positive selection may also play a partial role here, particularly considering the highly credible positive selection sites identified (Supplementary Table S4). In addition, cavefish are often susceptible to population bottlenecks due to restricted migration, resulting in small effective population sizes, which may also significantly increase the ω ratio. In summary, the higher ω ratios in the *Sinocyclocheilus* cave-dwelling groups (II & III) may be attributable to a complex balance between intense ecological and physiological selective pressures and demographic restrictions leading to genetic drift, resulting in partially relaxed selection. Overall, we identified 24 highly credible positive selection sites in nine out of the 13 mitochondrial PCGs, indicating that the extensive parallel evolution of the cave-dwelling *Sinocyclocheilus* species likely entailed parallel but distinct genetic pathways that facilitated their adaptation to the extreme subterranean environments found across the vast karst areas of southwestern China.

SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Permission for field surveys in Hubei Province was granted by the Ministry of Agriculture and Rural Affairs of China (MOA). Project approval (No. 171821301354052150) was issued by the Bureau of Fisheries of MOA.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.S.J., J.X.Y., and J.B.C. designed the study. J.L. collected the fish. C.S. carried out the molecular experiments. W.S.J. and H.M.X. analyzed the data. W.S.J. and J.L. wrote the draft manuscript. J.X.Y. and J.B.C. revised the manuscript. All authors read and approved the final manuscript.

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