

The complete mitochondrial genome of the *Tanichthys flavianalis* (Cypriniformes: Tanichthyidae) and its phylogeny

Yuzhou Gong^{a,b,*}, Tiezhu Yang^{c,d,*}, Chaoqun Su^{c,d}, Liangjie Zhao^{c,d} and Fan Li^{a,e}

^aBranch of Shanghai Science and Technology Museum, Shanghai Natural History Museum, Shanghai, China; ^bCAS Key Laboratory of Mountain Ecological Restoration and Bioresource Utilization & Ecological Restoration and Biodiversity Conservation Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, China; ^cCollege of Fisheries, Xinyang Agriculture and Forestry University, Xinyang, China; ^dFishery Biological Engineering Technology Research Center of Henan Province, Xinyang, China; ^eInstitute of Hydrobiology, The Key Laboratory of Aquatic Biodiversity and Conservation of Chinese Academy of Sciences, Chinese Academy of Sciences, Wuhan, China

ABSTRACT

In the present study, we have sequenced, annotated and characterized the complete mitogenome of *Tanichthys flavianalis* Li, Liao & Shen, 2022, which is an endemic minnow to Hainan Province, China. The entire mitogenome size is 16,545 bp, containing 37 genes coding for 13 mitochondrial proteins, 22 tRNAs, 2 rRNAs, and one putative control region. It has the typical fish mitochondrial gene arrangement, though with 9 gene overlaps and 17 intergenic spacers scattering throughout the mitogenome, and a high A + T bias (60.8%) comparing to other Cyprinids. ATG is used as the start codon in most mitochondrial protein coding genes (PCGs) except for *cox1* which uses GTG, whereas the canonical TAA/TAG are prevailing stop codons except for *cox2*, *cox3*, *nad4* and *cytb*. Phylogenetic analyses with other reported species from Tanichthyidae were conducted using IQ-TREE and MrBayes, based on the concatenated 13 PCGs. The results clustered *T. flavianalis* with the newly described *T. albiventris* Li Bohlen & Liao, 2022, and proved the reciprocally monophyletic relationship between Chinese and Vietnamese *Tanichthys*.

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Introduction


Tanichthys is a genus of freshwater minnows in the family Tanichthyidae of Cypriniformes (Chen and Mayden 2009; Mayden and Chen 2010). They inhabit streams in southeastern China, and northern and central Vietnam. Despite being famous ornamental fish due to their bright colors (Weitzman and Chan 1966), until recently, the type species, *T. albonubes* Lin 1932, was the only one known. After 2000, eleven new *Tanichthys* species were discovered (Nguyễn and Ngô 2001; Bohlen et al. 2019; Freyhof and Herder 2021; Jin et al. 2022; Li et al. 2022), and so far, six of them are widely recognized (Froese and Pauly 2023). However, there is very little information available on these species, such as population trends and threats, habitat and ecology, and genetic resources. The insufficient information emphasizes the necessity for increased attention to this group within the academic community (Lin 1932; Nguyễn and Ngô 2001; Freyhof and Herder 2011; Bohlen et al. 2019; Li et al. 2022; Yang et al. 2023).

Since 1989, wild populations of *Tanichthys* have held a class II status under state protection in China (Yue and Chen

1998). Recent studies have identified multiple cryptic species within the genus, shedding light on the potentially extensive genetic diversity of *Tanichthys* (Jin et al. 2022; Li et al. 2022). Over the years, *Tanichthys*' taxonomic classification has been a topic of contention, with debates on its placement within various subfamilies, such as Leuciscinae (Chu 1935; Yang and Huang 1964), Danioninae (Chen 1998; Nelson et al. 2016), Tincinae (Tang et al. 2010), or as a sister group to Acheilognathinae within the family Cyprinidae (Liao et al. 2011). However, the prevailing consensus now leans toward establishing a new family, Tanichthyidae, to accommodate *Tanichthys*. This shift in classification aligns with the elevation of the former Cyprinidae to superfamily status, supported by emerging molecular phylogenetic evidence (Chen and Mayden 2009; Mayden and Chen 2010). Within this updated taxonomic framework, our focal species, *T. flavianalis*, takes its place as a recently discovered member of the *Tanichthys* genus, originating from Hainan Province, China. It sets itself apart from its congeners with a higher number of branched dorsal- and anal-fin rays, and distinctive reddish-orange and golden margins on the dorsal- and anal-fins, respectively

CONTACT Liangjie Zhao  a850924t@163.com  College of Fisheries, Xinyang Agriculture and Forestry University, Xinyang 464000, China; Fan Li  lfaqua@gmail.com  Branch of Shanghai Science and Technology Museum, Shanghai Natural History Museum, Shanghai 200041, China.

*These authors contributed equally to this work.

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(Figure 1). Studying the mitochondrial genome of *T. flavianalis* holds significance as it provides further genetic insights into this species, aiding in a deeper understanding of the genetic diversity and evolutionary history of *Tanichthys*, while also serving as valuable scientific groundwork for wildlife conservation and protection efforts.

Materials

A *T. flavianalis* was collected from the upper reach of the Jiuqu River (110.2861°E, 19.0451°N) in Qionghai City, Hainan



Figure 1. The morphological traits of *T. flavianalis*, note the reddish-orange dorsal-fin and golden anal-fin (photographed by Fan Li).

Province, China, 15 June 2020, and euthanized with eugenol, then preserved in 95% ethanol, and stored in the Aquatic Museum of Xinyang Agricultural and Forestry University (<https://www.xyafu.edu.cn/>, Liangjie Zhao, a850924t@163.com, voucher number XYAFU-Mu-1803245).

Methods

Genomic DNA was extracted from caudal-fin using the Ezup Column Animal Genomic DNA Purification Kit (#B518251-0100, Sangon Biotech, Shanghai, China) and verified for quality and quantity using 1.2% agarose gel electrophoresis and NanoDrop2000 Spectrophotometers (Thermo Scientific, USA), respectively. Isolated DNA was stored at -20°C , until we used long and accurate PCR (LA-PCR) with nine pairs of primers designed specifically for *Tanichthys* species (Table S1) to amplify the mitochondrial sequences. The LA-PCR Kit (#B639285-0100, Sangon Biotech) was used for 50 μL volume reactions which contain 1.5 μL template DNA (2 ng/ μL), 5 μL 10 \times Long PCR Buffer, 1 μL dNTP Mix (10 mM), 0.5 μL each primer (10 μM), 0.5 μL Long and Accurate Enzyme, and 41 μL sterilized ddH₂O. PCR conditions were adjusted based on primer TM values and amplicon sizes according to the manufacturer's instruction. PCR products were sequenced using an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The sequencing result was uploaded to GenBank as a fastq file containing base-calling quality information. Amplicons ranged from 850 bp to 3876 bp (see Figure S1, supplementary material),

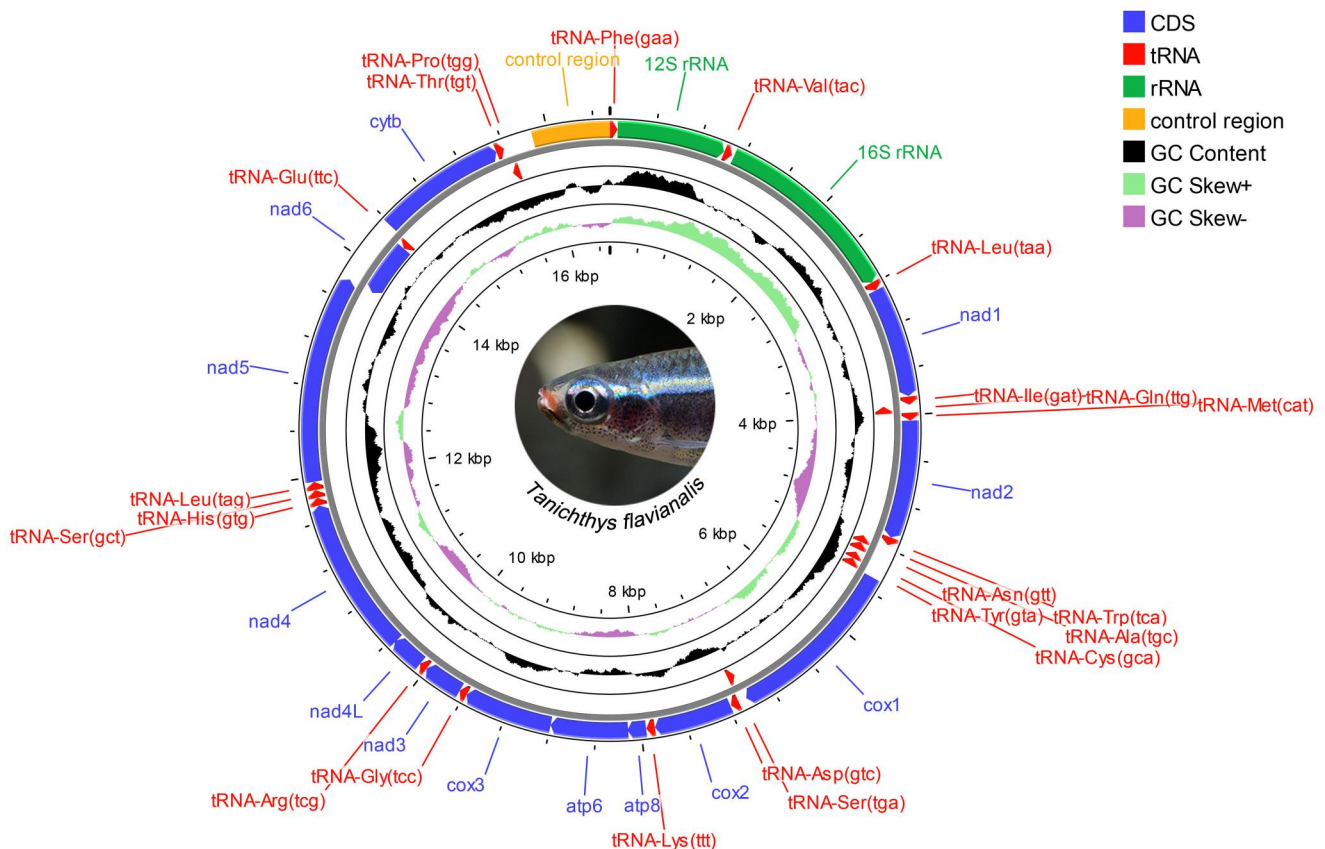


Figure 2. Circular map of the assembled *Tanichthys flavianalis* mitogenome. Gene encoded on H- and L- strands with inverse arrow directions are shown outside and inside the circle, respectively. Plots of GC content and skew used a window size of 500 and reflect GC content/skew on a scale of 0 to 1 using a baseline of 0.5. Positive and negative skew are indicated by values above and below the midpoint respectively. This map was drawn using the Proksee web server.

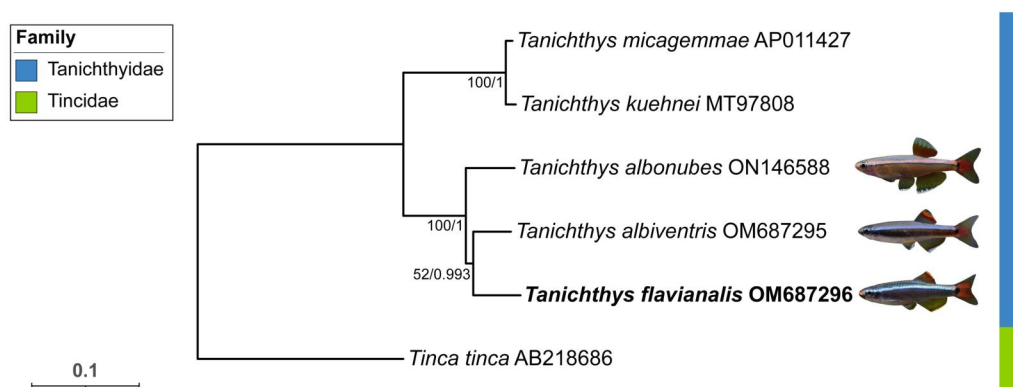


Figure 3. The phylogenetic tree constructed based on 13 protein coding genes of five Tanichthyidae mitogenomes and an outgroup. The support values of the corresponding nodes are shown (bootstrap values for ML/Bayes posterior probabilities for BI). The focal species is in bold, and pictures of the three Chinese *Tanichthys* were provided by Fan Li. The following sequences were used: *T. micagemmae* AP011427 (unpublished), *T. kuehnei* MT97808 (unpublished), *T. albonubes* ON146588 (unpublished), *T. albiventris* OM 687295 (Yang et al. 2023), *T. flavianalis* OM687296 (this study), and *Tinca tinca* AB218686 (Saitoh et al. 2006).

empowering us to assemble the entire mitogenome of the species by using DNAMAN (ver8.0.8, Lynnon Biosoft, Canada, see Figure S2, supplementary material). MITOS2 WebServer (Donath et al. 2019) was used to annotate the assembled mitogenome, and the circular display was made by using CGViewBuilder in Proksee web server (Stothard and Wishart 2005) (Figure 2).

In order to understand the phylogenetic position of *T. flavianalis* within the *Tanichthys* genus, we collected all reported mitochondrial reference genomes of species in Tanichthyidae from GenBank. *Tinca tinca* was introduced as an outgroup. The corresponding sequence accession numbers were listed in the legend of Figure 3. The 13 PCGs of each mitogenome were extracted, and then aligned. The alignment was performed using 'L-INS-I' strategy in MAFFT v7.505 (Katoh and Standley 2013). We concatenated 13 aligned mitochondrial PCGs blocks, each contains six sequences, into a multi-locus dataset as the input for downstream phylogenetic analyses. Maximum-likelihood (ML) phylogenies were inferred using IQ-TREE v2.2.0 (Nguyen et al. 2015) under Edge-linked partition model with 1000 standard bootstrap, and Bayesian inference (BI) using MrBayes v3.2.7a (Ronquist et al. 2012) ran four simultaneous MCMC chains for one million generations with every 1000 generations to sample. The first 25% of samples were discarded as burn-in. ModelFinder v1.6.12 (Kalyaanamoorthy et al. 2017) embedded in PhyloSuite v1.2.3 (Xiang et al. 2023) was employed to determine the optimal partitioning strategies and the corresponding models. Visualization of the resulted trees was achieved by using iTOL (Letunic and Bork 2019).

Results

T. flavianalis has a 16545 bp-long mitogenome, and it consists of 13 PCGs, 22 tRNA genes, 2 rRNA genes, and a noncoding AT-rich control region (Figure 2). There are nine gene overlaps (from 1 to 7 bp in size) and 17 intergenic spacers (from 1 to 199 bp) in the mitogenome. The most overlapped fragment is between *atp8* and *atp6*, while the longest spacer locates between *tRNA-Pro* and *tRNA-Phe*, which contains the control region. Of the 13 PCGs, codon ATG is overwhelmingly used as the start codon with the exception of *cox1*, which

starts with GTG. All PCGs terminate with canonical stop codon TAA or TAG except for four genes that have incomplete stop codons of 'T-' (*cox2* and *cytb*) or 'TA-' (*cox3* and *nad4*). The overall nucleotide composition of the *T. flavianalis* mitogenome is 31.34% A (5185), 29.47% T (4876), 15.50% G (2565), and 23.69% C (3919).

Topological consistency is observed between the ML and BI trees of Tanichthyidae species. Here we display the ML tree and indicate the branch support values with both the standard bootstrap value, and the Bayes posterior probability (Figure 3). The two clades within *Tanichthys*, which could be told apart by their geographical distributions (*T. micagemmae* and *T. kuehnei* from Vietnam, and *T. albonubes*, *T. flavianalis* and *T. albiventris* from China), were in reciprocal monophyly.

Discussion and conclusion

In the mitogenome of *T. flavianalis*, all PCGs are encoded in the heavy strand (H) except *nad6*, fourteen tRNA genes are located in the H-strand and the remaining eight tRNAs are in L-strand, which is consistent with most fish mitogenomes (Billington and Hebert 1991; Satoh et al. 2016). *T. flavianalis* shows a relatively high A + T bias (60.81%) in its mitogenome when compared to other Cyprinidae species (Tang et al. 2010; Liaw et al. 2013; Luo et al. 2019; Zhang et al. 2021), but coincides with the situation in other *Tanichthys* species (Yang et al. 2023).

Our mitogenome-based phylogenetic analyses support the placement of *T. flavianalis* to the genus *Tanichthys*, and show its close relationship to other Chinese *Tanichthys* species, which is consistent with previous morphologically based conclusion (Li et al. 2022). Our observation of phylogenetic connections aligning with geographical regions implies that local environmental factors may have driven speciation in this genus. This situation, in particular, raise intriguing questions about the possible presence of undescribed cryptic species within widely distributed species, such as *T. albonubes*. Further investigation into the genetic diversity of *Tanichthys* has the potential to enhance our understanding of the evolutionary history and conservation considerations for these fascinating fish species.

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Ethical approval

Experiment permission was issued by the Ethics Committee of Xinyang Agriculture and Forestry University (XYNLXY20210911), and all procedures involving animal attendances were strictly followed the Chinese Association for the Laboratory Animal Sciences and the Institutional Animal Care and Use Committee (IACUC) protocols.

Author contributions

LZ and FL conceived and designed the study, and conducted fundraising. FL and CS collected the samples. TY performed the experiments and uploaded the sequence information to GenBank. YG analyzed data and wrote the manuscript. All authors have proofread the manuscript and agreed to publish this version.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The genome sequence data of the study are deposited in GenBank of NCBI (<https://www.ncbi.nlm.nih.gov/>) under the accession no. OM687296. The associated BioProject, Bio-Sample and Run numbers are PRJNA957284, SAMN34248125, and SRR25113585, respectively.

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