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ORIGINAL ARTICLE

The complete mitochondrial genome of an endangered minnow *Aphyocypris lini* (Cypriniformes: Xenocyprididae): genome characterization and phylogenetic consideration

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Abstract
The mitochondrial genome can provide useful information for analyzing phylogeny and molecular evolution. In this study, the mitochondrial genome of *Aphyocypris lini*, an endangered and endemic minnow from southeast China, was sequenced and compared with other closely related species. The mitogenome of *A. lini* is 16,613 base pairs in length and consists of 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes and one displacement loop region. All of PCGs initiate with the standard start codon ATG except cox1 with GTG and most PCGs terminate with TAA or TAG. The genome composition is highly A + T biased (57.3%), and exhibits a positive AT-skew (0.0614) and a negative GC-skew (−0.2205). Based on PCGs, phylogenetic analysis showed that former Ex-Danioninae subfamily was reclassified as Xenocyprididae and divided into two main clades: Opsariichthyinae and Xenocypridinae. Within Xenocypridinae, genus *Aphyocypris* is not monophyletic but closely related to *Yaoshanicus*, *Nicholsicypris*, and *Pararasbora* genera, suggesting taxonomy should be reconsidered. The present study contributes to understanding the comparative evolution and taxonomy of genus *Aphyocypris*.

Keywords Mitogenome · *Aphyocypris lini* · Garnet minnow · Phylogenetic relationship · Xenocypridinae

Introduction

Mitochondrial genome is a single extrachromosomal circular DNA molecular with 15–18 kb in length and it consistently contains 13 protein coding genes (PCGs), two ribosomal RNA (rRNA), 22 transfer RNA (tRNA) genes, and a noncoding adenine(A) + thymine(T)-rich region (displacement loop region, D-loop) for most vertebrates (Satoh et al. 2016). Compared with nuclear genome, mitogenome is characterized by its small size, simple genomic organization, maternal inheritance, high rate of evolution, and almost unambiguous orthology. Hence, mitochondrial DNA (mtDNA) is typically considered to be an informative molecular marker widely applied in species identification, evolutionary biology, population genetics, and conservation biology (Duchene et al. 2012; Wang et al. 2016; Ko et al. 2018).

The Danioninae is one of the most species-rich subfamilies of the Cyprinidae, comprising approximate 300 species belonging to 50 genera (Tang et al. 2010; Liao et al. 2011a). However, the subfamily Danioninae and its members have had a long and somewhat convoluted taxonomic history (Tang et al. 2010), which had been described as a large assemblage containing most taxa not accommodated by the other subfamilies of the Cyprinidae. In previous phylogenetic studies, the Danioninae is not monophyletic, with putative members scattered throughout Cyprinidae (Tang et al. 2010). Within the Danioninae sensu stricto, it has been distinguished into three major lineages, including tribes *Rasborini*, *Danionini*, and *Chedrini* (Tang et al. 2010; Liao et al. 2011b). Besides, the remaining danionines, corresponding to *Aphyocypris, Opsariichthys*, *Zacco* and others, are still non-monophyletic (Wang et al. 2007; He et al. 2008; Mayden et al. 2008, 2009; Tao et al. 2013) and chaotic within the subfamily. Hence, many studies described these "displaced" danionines as Ex-Danioninae subfamily (Fang 2003; Liao et al. 2011a,

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2011b; Liao and Kullander 2013; Huang et al. 2017), which has also been confirmed in the Fishbase (Froese and Pauly 2019). With the increasing data of molecular markers and genome sequences, recent studies have reclassified these "displaced" danionines into family Xenocyprididae (Tan and Armbruster 2018), which has been confirmed by phylogenetic analyses by mtDNA and nuclear DNA genes (Stout et al. 2016; Schönhuth et al. 2018). In these phylogenetic trees, family Xenocyprididae, including *Aphyocypris, Opsariichthys*, *Zacco* and other genera, is sister to family Danionidae. However, phylogenetic studies based on the mitogenomes are still few.

The cyprinid genus *Aphyocypris* was established in 1868, diagnosed by having a subsuperior mouth, no barbel, no knob on the anterior medium of the low jaw fitting into a notch in the upper jaw, a keel between the base of the pelvic fin and the anus, and an incomplete lateral line or its absence (Chen 1998). Most of the species of genus *Aphyocypris* were narrowly distributed in Southern China, expect for *A. chinensis*, a species widely distributed in East Asia. Therefore, most of the species of genus *Aphyocypris* were reported at the risk of extinction, to date, resulted from low population diversity, narrow distribution and habitat destruction (Hu et al. 2009; Liao et al. 2011c; Zhu et al. 2015; Jiang et al. 2016).

The Garnet minnow *Aphyocypris lini* (Weitzman and Chan, 1966) is an endangered cyprinid endemic to southern China. *Aphyocypris lini*, a subtropical freshwater benthopelagic fish, lives in clear shallow water with thick growth of weeds in river ditches and rivulets (Yue and Chen 1998; Zhu et al. 2013). The distribution of *A. lini* was only recorded in Hong Kong and it has been declared extinct in the wild (Hu et al. 2009). But beyond that, there were no references of this endangered fish. In this study, we firstly collected *A. lini* specimens in Fujian Province, China. It would be the first field report of this endangered species in nearly four decades, as well as a record of new distribution area to be found. Meanwhile, we amplified the first complete mitogenome sequence of *A. lini* and comparatively analyzed the mitogenome among related species endemically distributed in East Asia, regarding genome organization, composition, and its codon usage. Finally, we reconstructed the phylogenetic trees of genus *Aphyocypris* based on the PCGs. Our aim is to understand the features of *A. lini* mitochondrial genome and provide insight into the evolutionary relationships within genus *Aphyocypris*.

Materials and methods

Sampling and DNA extraction

Four specimens were collected from the Minjiang River, Fujian Province, China. For protection of this endangered species, the coordinates are not shown, but can be obtained by contacting the authors if needed. Specimens (Fig. 1) were identified following the diagnostic characteristics described by Chen (1998). Muscle tissues were preserved in 95% ethanol and deposited in the Institute of Oceanology, Minjiang University. Total genomic DNA was extracted by the salt-extraction method (Aljanabi and Martinez 1997).

Primer design, PCR amplification and sequencing

The complete mitogenome of *A. lini* was amplified based on six universal primers and six specific primers described in Table 1. Firstly, six partial genetic fragments were amplified and sequenced using the universal primers. Subsequently, specific primers were designed to amplify long fragments based on the sequenced segments using online tool primer-BLAST (Ye et al. 2012). Polymerase chain reaction (PCR) amplification was carried out in a 20 μL reaction containing 50 ng template DNA, 10 μL SanTaq Plus PCR Mix (Sangon, Shanghai), 0.5 μL each primer (10 μmol/L), and supplemented with sterilized ddH_2O. The PCR conditions were: 94 °C for 3 min, then 35 cycles of 30 s at 94 °C, 45 s at annealing temperature (Table 1) and 1 min at 72 °C, with a final elongation at 72 °C for 5 min. PCR products were examined by electrophoresis using 1.0% TAE agarose gel, and sequenced by Sangon Biotech (Shanghai, China).

Sequences assembly, annotation and analysis

The overlapped fragments were assembled into a linear mitochondrial DNA sequence using SeqMan (DNASTAR), then assembled sequences were manually checked. The mitogenome was annotated using MitoAnnotator web server (http://mitofish.aori.u-tokyo.ac.jp/annotation/input.html) (Iwasaki et al. 2013). Transfer RNA genes and their secondary structures were verified using tRNA-Scan SE web server (http://lowelab.ucsc.edu/tRNAscan-SE/) (Lowe and Chan

Fig. 1 *Aphyocypris lini*, in an aquarium (photographed by J.Z)

Table 1 Information of primers in this study

Amplification	Primer name	Primer sequences (5′→3′)	Annealing temperature (°C)	References
Partial PCRs (Universal primers)	FishF1-COI FishR1-COI	TCAACCAACCACAAAGACATTGGCAC TAGACTTCTGGGTGGCCAAAGAATCA	55	Ward et al. (2005)
	L14504-ND6 H15149-CYB	GCCAAWGCTGCWGAATAMGCAAA GGTGGCKCCTCAGAAGGACAT TTGKCCTCA	52	Inoue et al. (2001)
	L709-12S H1552-12S	TACACATGCAAGTCTCCGCA ACTTACCGTGTTACGACTTGCCTC	57	Inoue et al. (2001)
	16sar 16sbr	CGCCTGTTTATCAAAAACAT CCGGTCTGAACTCAGATCACGT	53	Ivanova et al. (2007)
	L4633-ND2 H5334-ND2	CACCACCCWCGAGCAGTTGA CGKAGRTAGAAGTAHAGGCT	52	Inoue et al. (2001)
	L9514-CO3 H10019-Gly	TTCTGAGCCTTCTAYCA CAAGACKGKGTGATTGGAAG	52	Miya et al. (1999)
Long PCRs (specific primers)	Gap1F Gap1R	CTAGTACCATCAAGCAAAGAGACCT GGAAGGGGGAGACAGTTAAGC	56	In this study
	Gap2F Gap2R	ATATCGACGAGGGGGTTTACG ATAGTGCGGGGTCTACTGTGTG	52	In this study
	Gap3F Gap3R	ACCACCCCTGACAGGATTTATG AGGCGCCCCAATTATAAGAG	57	In this study
	Gap4F Gap4R	ACAGCCGTACTTCTTCTACTTTCC GTTTTCGTTCCCCCTCTATAATG	49	In this study
	Gap5F Gap5R	ACCATTTTACATCCGAACACCAC ACAACGGTGGTTCTTCAAGTCAT	52	In this study
	Gap6F Gap6R	GCAGATAAAATTTCCTTTCACCC GGCATCTGATTAATGATATGAGTAC	55	In this study

2016). A circular display of the mitogenome was drawn using CGView online server (http://stothard.afns.ualberta.ca/cgview_server/) (Grant and Stothard 2008). The composition of amino acids, nucleotide base and relative synonymous codon usage (RSCU) were calculated using MEGA X software (Kumar et al. 2018). The RSCU value for each codon was the observed frequency of this codon divided by its expected frequency under equal usage among the amino acid. Nucleotide composition skewness was carried out using the following formulas: AT-skew = (A - T) / (A + T) and GC-skew = (G – C) / (G + C) (Perna and Kocher 1995).

Phylogenetic analysis

A total of 22 sets of 13 PCG sequences were used to perform phylogenetic analysis. Those from other taxa were downloaded from GenBank (information of species and accession numbers were shown in Table 4), with *Misgurnus anguillicaudatus* (Accession number: MK093946) and *Leptobotia elongate* (Accession number: NC018764) sequences used as outgroups. Phylogenetic analyses were reconstructed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. The best-fit partition model of nucleotide evolution of PCGs was identified by PartitionFinder v2 (Lanfear et al. 2012) in PhyloSuite platform (Zhang et al. 2020) and was GTR + I + G according to the Akaike Information Criterion (Bozdogan 1987). ML and BI analysis were performed with MrBayes v3.2.7 and RaxML v8.2.12 programs following the manuals, separately (Ronquist et al. 2012; Stamatakis 2014). The bootstrap ML analysis was implemented under the GTRGAMMAI model and 1000 replications were used to evaluate the bootstrap support values and search for the best ML tree. BI analysis ran as two simultaneous Markov Chain Monte Carlo (MCMC) chains for 10 million generations, sampled every 1000 generations, and using a burn-in rate of 25%. Phylogenetic trees were visualized through online tool interactive tree of life (iTOL, https://itol.embl.de/) (Letunic and Bork 2019).

Results and discussion

Genome organization and composition

The complete mitogenome sequence of *A. lini* is 16,613 bp in length and contains 13 PCGs, 22 tRNA genes, two rRNA genes, and one CR. The complete closed-circular mitogenome of *A. lini* has been deposited in GenBank under accession number MW338757. All PCGs were encoded in the heavy strand (H) except NADH dehydrogenase subunit (*ND6*) in

the Light strand (L). Eight tRNA ($tRNA^{Gln}$, ^{Ala}, ^{Asn}, ^{Cys}, ^{Tyr}, ^{Ser}, ^{Glu}, ^{Pro}) were located in the L-strand and the remaining 14 tRNAs were in H-strand (Fig. 2). This coding pattern in two strands was consistent with most vertebrates including fishes (Billington and Hebert 1991). There are 14 gene overlaps (from −1 to −31 bp in size) and 7 intergenic spacers (from 1 to 7 bp in length) in the mitogenome. The most overlapped fragment took place between $tRNA^{Asn}$ and $tRNA^{Cys}$ and the two longest spacer presents between ATPase8 and ATPase6, and between ND4 and ND4L (Table 2).

The nucleotide composition of the complete mitogenome of *A. lini* is as follows: A = 5055 (30.4%), T = 4471 (26.9%), G = 2762 (16.6%), C = 4325 (26.0%) (Table 3). It showed a slight A + T bias (57.3%) which was same as most Xenocyprididae species (53.92 ~ 60.07) (Table 4) (Sitoh et al. 2006; Tang et al. 2010; Liaw et al. 2013; Luo et al. 2019). The AT-skew and GC-skew of the complete mitogenomes were also calculated that the composition is skewed away from A in favour of T (AT-skew is 0.0614) but is exceeded of C over G (the GC-skew is −0.2205) (Table 3).

Protein-coding genes and codon usage

The total length of PCGs in *A. lini* was 11,417 bp. The composition of A + T content, AT-skew and GC-skew was significantly biased at different codon positions with the highest A + T content and the lowest value of AT- and GC-skew observed in the first codon position (58.8%, −0.0850 and −0.3155, respectively). It suggests that a relaxed negative selection at neutral sites might affect the base composition of the complete mitogenome (Bachtrog 2007). Additionally, AT-skew in PCGs of *A. lini* is negative which is unusual to most species in Xenocyprididae, except for *Zacco sieboldii* (Temminck & Schlegel, 1846) (Table 4). The result indicated that PCGs of *A. lini* displayed an excess of T over A, whereas PCGs of most teleost fishes were biased towards using A not T (Yu et al. 2019). The reason of the unusual AT-skew of *A. lini* might be attributed to the unique selective pressures or processes which has resulted in the decreasing A in PCGs. The same results were also found in bitterling *Sinorhodeus microlepis* (Li, Liao, Arai & Zhao, 2017) and goby *Rhinogobius leavelli* (Herre, 1935) (Yu et al. 2019; Zhang and Shen 2019).

Of the 13 PCGs, twelve use canonical initiation codon ATG, and cox1 utilizes GTG which codes valine. All PCGs terminate with canonical stop codon TAA or TAG except for four genes (*ND2*, *cox2*, *ND3*, and Cyt*b*) which had incomplete stop codons of T++. Moreover, gene overlapping regions were detected in *ATP8-ATP6* (shared 7 nucleotides), *ND4L-ND4* (shared 7 nucleotides) and *ND5-ND6* (shared 4 nucleotides).

The amino acid codon usages of *A. lini* mitogenome are assessed by relative synonymous codon usage (RSCU) values (Fig. 3). Threonine (*Thr*), proline (*Pro*) and Leucine 1 (*Leu1*) are the most frequently translated amino acids, while Cysteine (*Cys*) is the least used amino acid. The AAA (*Lys*), CCC (*Pro*), AUU (*Ile*), UUA (*Leu*), and ACA (*Thr*), which are commonly used codons in *A. lini* mitogenome, are mostly composed of over-usage of A and T at the third codon position. That indicates the possibility of genome bias, optimal selection of tRNA, or the DNA repair efficiency, referred to other teleost fishes (Fischer et al. 2013).

Transfer and ribosomal RNA genes, non-coding region

The sizes of 22 tRNA genes of *A. lini* range from 68 bp to 76 bp which comprise 9.43% (1556 bp) of the whole mitogenome. Except for $tRNA^{Ser}$, the remaining 21 tRNA could be folded into a typical cloverleaf secondary structure (Online resource 1: Fig. S1), which has been commonly witnessed in many other teleost mitogenomes (Garey and Wolstenholme 1989). However, $tRNA^{Ser}$ showed a D-arm-lacking structure, which was consist with many metazoan mitochondrial tRNAs (Frazer-Abel and Hagerman 2008; Watanabe et al. 2014). The two rRNA genes separated by $tRNA^{Val}$ had a length of 962 bp and 1687 bp, which is close to most danionines (Tang et al. 2010). Similarly, the strong AT-bias was also found in rRNAs. However, the AT content in rRNAs is lower than it in total mtDNA, tRNAs, PCGs, and control region. The strongly positive AT-skew was found in rRNA as well (Table 3), leading to a positive AT-skew in complete mitogenomes.

Finally, we annotated two common non-coding regions (light strand replication origin, O_L and D-loop). O_L, whose length is 31 bp, is located among five continual tRNA. D-loop is 936 bp in length, which is longer than most species within Xenocyprididae (the former Ex-Danioninae). But the short tandem repeats that might resulting to difference of length in D-loop were not found in *A. lini*. Even so, the difference of the length in D-loop suggests to be an important marker in phylogenetic studies (Tang et al. 2017).

Phylogenetic relationships

For better understanding the relationship within the *Aphyocypris* and other related species, the phylogenetic trees were established using ML and BI methods, based on concatenated nucleotide sequences of 13 PCGs from 22 minnows belonging to Danionidae or Xenocyprididae families and 2 outgroup taxa. The results of the phylogenetic trees exhibited a congruent topology by both methods. As Fig. 4 showed, on the overall scale, Danioninae sensu stricto and Ex-Danioninae (now mainly belonging to Xenocyprididae) were

Table 2 Features of the mitochondrial genome of *A. lini*

Feature	position number		Size	Codon		Intergenic nucleotide	Strand
	start	stop		start	stop		
tRNAPhe	1	69	69				H
12SrRNA	70	1031	962				H
tRNAVal	1032	1103	72				H
16SrRNA	1104	2790	1687				H
tRNALeu	2791	2866	76				H
ND1	2868	3842	975	ATG	TAA	-1	H
tRNAIle	3847	3918	72			−4	H
tRNAGln	3917	3987	71			2	L
tRNAMet	3989	4057	69			−1	H
ND2	4058	5102	1045	ATG	T++		H
tRNATrp	5103	5173	71				H
tRNAAla	5175	5243	69			−1	L
tRNAAsn	5245	5317	73			−1	L
tRNACys	5349	5416	68			−31	L
tRNATyr	5418	5488	71			−1	L
CO1	5490	7040	1551	GTG	TAA	−1	H
tRNASer	7041	7111	71				L
tRNAAsp	7115	7188	74			−3	H
CO2	7202	7892	691	ATG	T++	−13	H
tRNALys	7893	7968	76				H
ATPase8	7970	8134	165	ATG	TAG	−1	H
ATPase6	8128	8811	684	ATG	TAA	7	H
CO3	8811	9596	786	ATG	TAA	1	H
tRNAGly	9596	9667	72			1	H
ND3	9668	10,016	349	ATG	T++		H
tRNAArg	10,017	10,086	70				H
ND4L	10,087	10,383	297	ATG	TAA		H
ND4	10,377	11,759	1383	ATG	TAG	7	H
tRNAHis	11,759	11,827	69			1	H
tRNASer	11,828	11,896	69				H
tRNALeu	11,898	11,970	73			−1	H
ND5	11,971	13,806	1836	ATG	TAA		H
ND6	13,803	14,324	522	TTA	CAT	4	L
tRNAGlu	14,325	14,393	69				L
Cytb	14,396	15,536	1141	ATG	T++	−2	H
tRNAThr	15,537	15,608	72				H
tRNAPro	15,608	15,677	70			1	L
D-loop	15,678	16,613	936			−1	H

independently separate which is consistent with the phylogenies resolved from both morphological and molecular analysis (Tang et al. 2010; Liao et al. 2011b, 2011c). However, *Danio albolineatus* (Blyth, 1860) was included in Xenocyprididae clade with a relatively high bootstrap support value, showing an opposite result against the conclusion of a large assemblage of danionines. Although *D. albolineatus* was found at the base of the clade Xenocyprididae, the relevance between Danionidae and Xenocyprididae need to be discussed through more molecular and morphological evidences.

Furthermore, within Xenocyprididae, there are two major tribes. The tribe Xenocypridinae (including genus *Aphyocypris*, *Yaoshanicus*, *Nicholsicypris*, and *Pararasbora*) is sister to the tribe Opsariichthyinae (*Opsariichthys*, *Zacco*, and other genera like *Parazacco*, not mentioned in this study). In addition, some species, such as

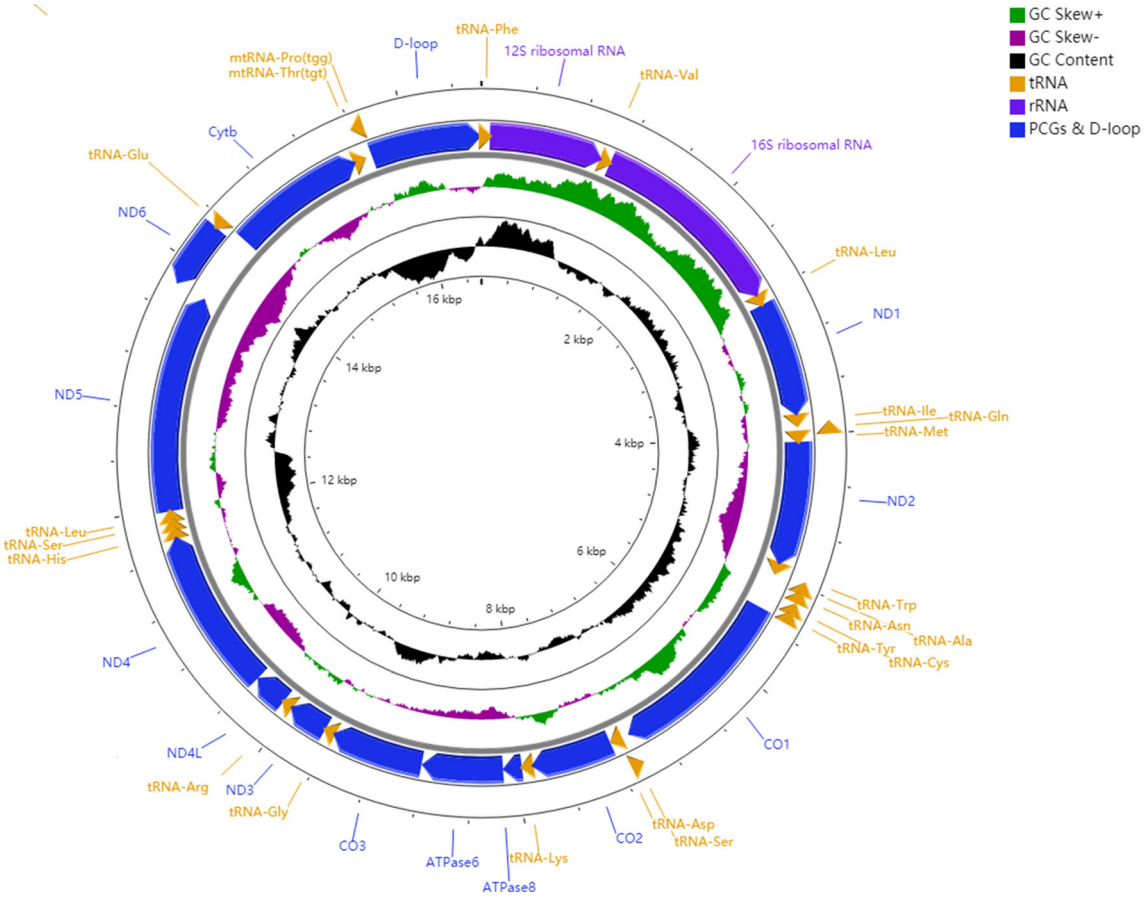

Fig. 2 The schematic illustration for mitochondrial genome of *A. lini*
Note: Gene encoded on H- and L- strands with inverse arrow directions are shown inside and outside the circle. The purple-green ring indicates the GC-skew that the purple is positive, while the green is negative. The black ring shows the GC content that outward and inward peaks demonstrate above and below average GC content, respectively.

Tanichthys albonubes and *Gobiocypris rarus*, were found to be basal of clade Xenocyprididae of the phylogenetic tree. The results are also consistent with Tang et al. (2010), providing strong evidence of taxonomic validity on Xenocyprididae (or Ex-Danioninae in Fishbase) (Tang et al. 2010; Liao et al. 2011b, 2011c; Huang et al. 2017).

More complicatedly, the phylogenetic relationship clearly illustrated that genus *Aphyocypris* is not monophyletic. *Aphyocypris kikuchii* and *A. chinensis* were found to be closely related to *Yaoshanicus arcus*, *Nicholsicypris normalis*, and *Pararasbora moltrechti*. The taxonomy within phylogenetic tribe Aphyocypris is ambiguous, as well as the constant changes of names and classification (Du et al. 2003). Here,

Table 3 The nucleotide composition and AT/GC-skew of the mitochondrial genome of *A. lini*

	Total	A%	A	T%	T	G%	G	C%	C	A+T%	G+C%	AT-skew	GC-skew
mtDNA	16,613	30	5055	27	4471	17	2762	26	4325	57.3	42.7	0.0614	−0.2205
tRNAs	1584	31	487	26	410	20	315	24	372	56.6	43.4	0.0858	−0.083
rRNAs	2649	34	912	21	550	21	557	24	630	55.2	44.8	0.2476	−0.0615
PCGs	11,417	29	3337	28	3203	15	1759	27	3118	57.3	42.7	0.0205	−0.2787
1st codon sites	3809	28	1081	22	846	24	895	26	987	50.6	49.4	0.122	−0.0489
2nd codon sites	3805	22	820	38	1451	13	483	28	1051	59.7	40.3	−0.2779	−0.3703
3rd codon sites	3803	38	1436	24	906	10	381	28	1080	61.6	38.4	0.2263	−0.4784
D-loop	936	34	314	32	301	14	127	21	194	56.6	43.4	0.0245	−0.1649

Note: Numbers correspond to the nucleotides separating different genes. Negative numbers indicate overlapping nucleotides between adjacent genes. H and L denote heavy and light strands, respectively

Table 4 Comparison of characteristics within the mitochondrial genome of members of the *Aphyocypris* genus and related species

species	complete mitogenome							PCGs							Accession number
	size	A	T	G	C	AT-skew	GC-skew	size	A	T	G	C	AT-skew	GC-skew	
Aphyocypris lini Weitzman and Chan, 1966	16,612	30.4	26.9	16.6	26.0	0.0611	−0.2207	11,417	29.2	28.1	15.4	27.3	0.0192	−0.2787	in this study
Aphyocypris chinensis Günther, 1868	16,606	30.7	27.7	16.5	25.1	0.0514	−0.2067	11,419	28.6	30.4	15.9	25.1	−0.0301	−0.2242	NC008650
Aphyocypris kikuchii Oshima, 1919	16,601	30.8	27.7	16.5	25.0	0.0530	−0.2048	11,423	28.8	30.5	15.7	25.0	−0.0295	−0.2272	NC019620
Aphyocypris pulchrilineata Zhu et al., 2013	16,610	30.5	26.6	16.6	26.2	0.0683	−0.2243	11,422	28.2	28.8	16.3	26.6	−0.0101	−0.2405	MK387702
Gobiocypris rarus Ye & Fu, 1983	16,601	29.5	27.6	17.2	25.7	0.0333	−0.1981	11,423	27.3	29.9	16.7	26.1	−0.0444	−0.2199	NC018099
Nicholsicypris normalis Nichols and Pope, 1927	16,619	31.2	27.0	15.9	25.8	0.0722	−0.2374	11,422	29.3	29.6	15.3	25.8	−0.0046	−0.2559	NC015538
Nipponocypris sieboldii Temminck & Schlegel, 1846	16,616	30.1	25.8	16.9	27.2	0.0769	−0.2336	11,429	27.8	27.6	16.6	28.0	0.0036	−0.2541	NC008653
Opsariichthys acutipinnis Bleeker, 1871	16,615	28.2	26.6	18.0	27.2	0.0292	−0.2035	11,425	25.5	28.8	17.9	27.8	−0.0603	−0.2167	NC028595
Opsariichthys bidens Günther, 1873	16,611	27.2	26.7	19.1	27.1	0.0093	−0.1732	11,424	23.9	29.0	19.3	27.7	−0.0958	−0.1783	NC008744
Opsariichthys evolans Jordan & Evermann, 1902	16,656	28.3	26.5	18.1	27.2	0.0328	−0.2009	11,428	25.3	28.8	18.1	27.9	−0.0637	−0.2135	MG650170
Opsariichthys pachycephalus Günther, 1868	16,612	27.8	26.6	18.3	27.3	0.0221	−0.1974	11,425	25.0	28.6	18.3	28.1	−0.0670	−0.2116	MG650171
Opsariichthys uncirostris Temminck & Schlegel, 1846	16,613	27.2	26.7	18.9	27.2	0.0093	−0.1800	11,424	24.2	28.8	19.0	28.0	−0.0881	−0.1905	NC008652
Pararasbora moltrechti Regan, 1908	16,617	31.2	27.4	16.0	25.5	0.0648	−0.2289	11,423	29.3	29.9	15.4	25.4	−0.0114	−0.2465	NC019621
Tanichthys albonubes Lin, 1932	16,547	31.1	29.2	15.8	23.9	0.0315	−0.2040	11,420	29.0	32.0	15.1	23.9	−0.0485	−0.2277	NC015539
Yaoshanicus arcus Lin, 1931	16,617	31.4	27.1	15.7	25.7	0.0735	−0.2415	11,422	29.5	29.7	15.1	25.7	−0.0022	−0.2610	AP011398
Zacco acanthogenys Bleeker, 1871	16,611	29.1	27.2	17.6	26.0	0.0337	−0.1927	11,424	26.6	29.6	17.3	26.4	−0.0532	−0.2077	NC028546
Zacco platypus Temminck & Schlegel, 1846	16,611	29.0	27.2	17.8	26.1	0.0320	−0.1891	11,425	26.6	29.5	17.4	26.5	−0.0505	−0.2073	NC023105

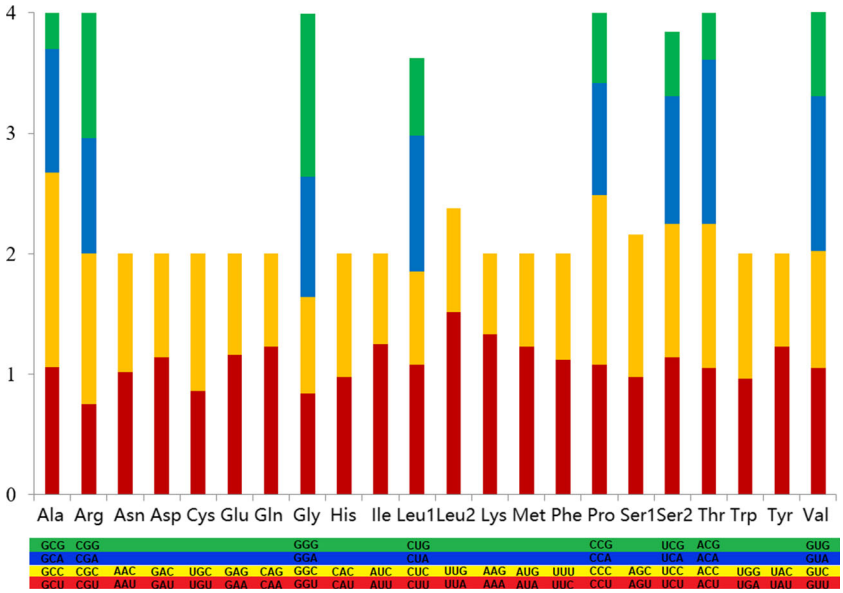

Fig. 3 Codon usage and relative synonymous codon usage (RSCU) in all protein coding genes of *A. lini* mitochondrial genome. Note: The codons are on the X-axis and RSCU values are shown on the Y-axis

we suggest to reconsider the taxonomic status of genus *Aphyocypris* and other close genera. Taxonomists have found differences of key morphological traits between the genera in the past hundred years (Nichols and Pope 1927; Nichols 1943). With the increase in the number of specimens examined, however, intra-species diversity has been also increasing so that the taxonomic status remains to be further discussed. In the present study, the evidence of phylogenetic analysis based on mitogenome supported to integrate genus *Aphyocypris*, *Yaoshanicus*, *Nicholsicypris* and *Pararasbora* into one genus, which was proposed in reclassification by Tan and Armbruster (2018).

Current status and conservation

As mentioned above, *A. lini* was listed as extinct in the wild in Chinese Red List in the 1990s (Yue and Chen 1998). And there have been no studies or reports on this threatened species since then. With the deepening of field sampling and supporting of molecular methods, this endangered minnow

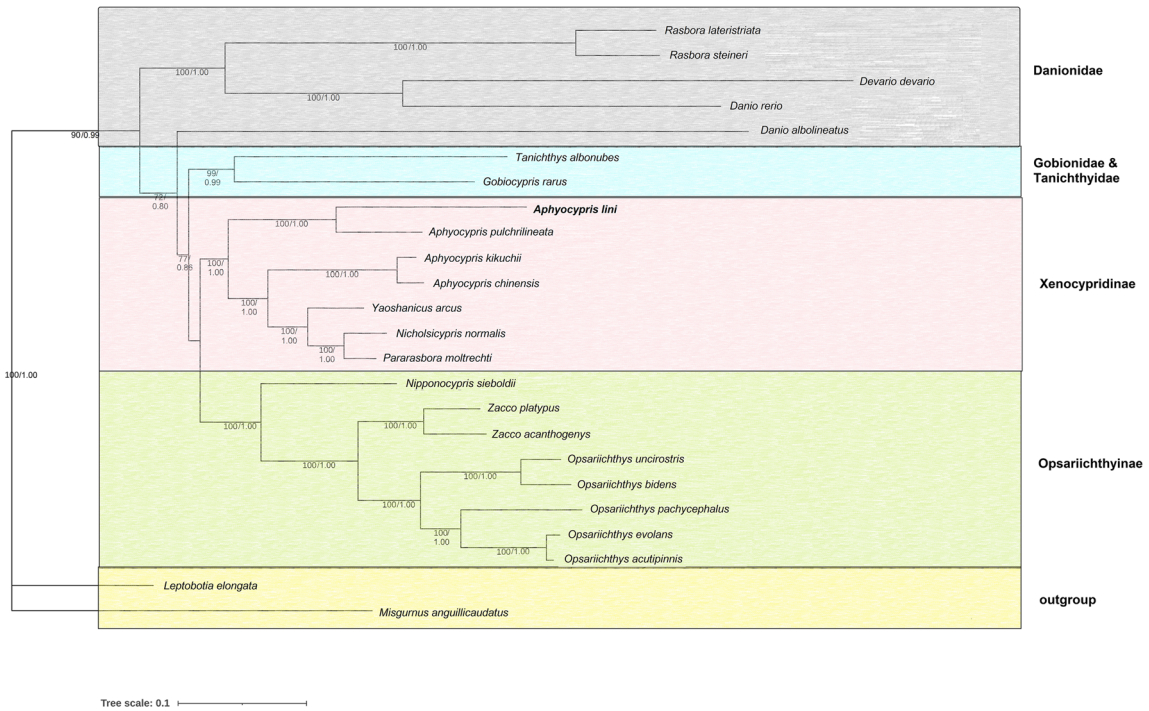

Fig. 4 Phylogenetic relationships of *Aphyocypris* genus and related species inferred from RaxML and Bayesian inference methods. Note: Numbers at the nodes show bootstrap value of 100/100 in ML tree and posterior probability of 1.00/1.00 in BI tree

and its new distribution record was confirmed in more than 30 years. However, it is not optimistic that the detected population in the wild is still small and their habitat requirement is extremely high, which issues high challenges to the protection. In addition to the usual anthropogenic influences such as dam construction, sand mining, and commercial fishing (Maitland 1995; Cooke et al. 2012), we should continue to pay attention to their native habitat (George et al. 2009). Furthermore, from the perspective of population genetics and conservation biology, we need to integrate multiple data to make more comprehensive protection recommendations for these minnows natively habited in the stream or rivulet (Vrijenhoek 1998; Alves et al. 2001).

Conclusion In the present study, we first collected an endangered minnow *A. lini* in the field from a new distribution area. Then we determined the complete mitochondrial genome of *A. lini*, which contains 37 genes and one CR, as is typical of teleost mitogenomes. Comparative analysis of mitogenome structure, base composition, codon usage, and gene order revealed an unusual AT-skew of PCGs of *A. lini*. Further reconstructed phylogeny of Xenocyprididae and other related species suggested non-monophyly within genus *Aphyocypris*, and indicated to reconsider the taxonomy of genus *Aphyocypris* and its phylogenetically closest genera *Yaoshanicus*, *Nicholsicypris* and *Pararasbora* into one integrated genus. Finally, our study provided the genetic basis for the conservation of this endangered minnow.

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Declarations

Conflict of interest The authors declare no conflict of interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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