The mitogenome of freshwater loach *Homatula laxiclathra* (Teleostei: Nemacheilidae) with phylogenetic analysis of Nemacheilidae

Mengfei Cao¹ | Ling Tang¹ | Juan Chen¹ | Xiaoyu Zhang¹ | Russell H. Easy² | Ping You¹

¹School of Life Sciences, Shaanxi Normal University, Xi’an, China
²Department of Biology, Acadia University, Wolfville, NS, Canada

Correspondence
Ping You, School of Life Sciences, Shaanxi Normal University, No. 199, South Chang’an Road, Yanta District, Xi’an 710062, China. Email: youping@snnu.edu.cn

Funding information
This study was supported by grants from the National Natural Science Foundation of China (31872203) and the Natural Science Foundation of Shaanxi Province (2017JM3014).

Abstract
The complete mitogenome can provide valuable genetic information to reconstruct relationships between species. In this study, we sequenced a stone loach, *Homatula laxiclathra* (Teleostei: Nemacheilidae), which is found in the northern region of the Qinling Mountains in China. The size of the *H. laxiclathra* mitogenome is 16,570 bp, which contains 37 typical mitochondrial genes including 13 protein-coding genes, 22 transfer RNAs, two ribosomal RNAs, and a control region (D-loop) with a total AT content of 55.8%. This is similar to other Nemacheilidae sequences published in GenBank. Furthermore, a mito-phylogenomic analysis of 46 Nemacheilidae species places *H. laxiclathra* in a robust monophyletic *Homatula* cluster with other *Homatula* species. Our results contribute toward a better understanding of a true phylogeny of these species based on large-scale taxonomic samplings as well as to help grasp the evolution of fish mitogenomes.

KEYWORDS
*Homatula laxiclathra*, mitogenome, Nemacheilidae, phylogenetic analysis

1 | INTRODUCTION

Mitochondrial DNA can provide valuable taxon information to reconstruct evolutionary relationships between species. The fish mitogenome is circular, 15–19 k bp in size, and comprises 13 protein-coding genes (PCGs), two ribosomal RNA genes (125 rRNA and 165 rRNA), 22 transfer RNA genes (tRNAs) and two noncoding control regions (O₁ and CR) (Miya, Kawaguchi, & Nishida, 2001). Mitogenomes are widely used for molecular systematics, phylogeography and taxa identification due to their small and simple structure, rapid evolution, maternal inheritance, and high gene conservation (Boore, 1999). In addition, molecular data for mtDNA, such as secondary structure of tRNAs and rRNAs, amino acid sequence, and codon usage can provide additional data for phylogenetic analyses (Boore, 1999; Zhu, Yan, Song, & You, 2018).

Loaches are small-bodied freshwater fishes, which are widely distributed across Eurasia, Africa, and North America. They are popular in China due to their distinctive flavor and diverse body color. From a commercial fisheries and ornamental trade value, it is crucial to identify mtDNA mutations to avoid genetic diseases in these fish (Kipp et al., 2010). Partial mtDNA genes from the Nemacheilidae have been used for species identification and systematics (Liu et al., 2012). Unfortunately, partial mitochondrial genes do not contain complete phylogenetic information to accurately define a phylogeny (Cunha, Grande, & Zardoya, 2009; Lee, Conroy, Howel, & Kocher, 1995; Parhi, Tripathy, Priyadarshi, Mandal, & Pandey, 2019).
An effective solution is to conduct comparisons of whole mtDNA from representative species of each genus (Betancur et al., 2017; Shi, Xing, Chen, Yang, & You, 2014). So far, 207 complete mitogenomes of teleostean species have been published in the GenBank database, but only 56 species from Nemacheilidae are available.

In this study, we sequenced the complete mitogenome of *Homatula laxiclathra* Gu & Zhang, 2012, which is only distributed in the northern region in Qinling Mountains. The genome structure and gene characterization of *H. laxiclathra* are compared with those reported for other *Homatula* species. To assess the deeper phylogenetic relationships of Nemacheilidae, we reconstructed the tree using Maximum Likelihood (ML) and Bayesian inference (BI) methods. The investigation of the *H. laxiclathra* mitogenome may provide valuable evidence about teleost evolution as well as aid in species identification.

2 | MATERIALS AND METHODS

2.1 | Sample collection and DNA extraction

Adult specimens of *Homatula laxiclathra* Gu & Zhang, 2012 were collected from Xinguansi (33.98°N, 109.11°E), Chang’an County, the Dayu River located on the north slope of the Qinling Mountains of Shaanxi Province, Central China (Figure 1). Specimens were preserved in 95% ethanol. Animal processing was approved by the Animal Care and Use Committee of Shaanxi Normal University. Total genomic DNA was extracted from muscle tissues using a TIANamp Animal DNA Kit (Tiangen Biotech), according to the manufacturer’s protocol. Voucher specimens deposited in the Fish Disease Laboratory, Shaanxi Normal University (Accession number: HL20160124).

2.2 | PCR amplification and sequencing

Using a primer-walking strategy, thirty conserved fish primers were designed to amplify the mitogenome (Miya & Nishida, 1999). PCR amplifications were performed with FastPfu Fly DNA polymerase (TransGen Biotech), following published PCR reaction conditions (Zhu et al., 2018).

2.3 | Genome annotation and sequence analysis

Raw sequences were assembled using the Staden Package v1.7.0 (Staden, Beal, & Bonfield, 2000). Gene predictions were compared with published mitogenomes of *Homatula* fishes. PCGs and rRNAs were

FIGURE 1 Map of sampling localities of *Homatula laxiclathra*. The map was downloaded from the Wikimedia Commons with slight modification (https://commons.wikimedia.org/wiki/File:East_Asia_topographic_map.png)
| Family      | Species                  | Size (bp) | Whole genome composition | Accession number |
|-------------|--------------------------|-----------|--------------------------|-----------------|
|             |                          | A%        | C%           | G%        | T%        |                     |
| Nemacheilidae| Acanthocobitis botia     | 16,660    | 30.5 26.5 15.9 27.1  | AP012138        |
|             | Acanthocobitis zonalternans | 16,642  | 30.1 27.1 16.5 26.4  | AP012140        |
|             | Barbatula barbatula       | 16,630    | 28.5 27.1 18.2 26.2  | KP715096        |
|             | Barbatula nuda            | 16,619    | 28.4 27.2 17.9 26.5  | KF574248        |
|             | Barbatula toni            | 16,617    | 28.5 27.3 17.8 26.4  | AB242162        |
|             | Homatula potanini         | 16,569    | 30.1 26.9 16.7 26.3  | KM017732        |
|             | Homatula variagata        | 16,571    | 29.5 27.1 17.3 26.1  | JX144893        |
|             | Homatula laxiclaathra     | 16,570    | 29.6 27.0 17.2 26.1  | MK279351        |
|             | Lefua costata             | 16,579    | 29.9 26.5 16.8 26.9  | KT943751        |
|             | Lefua echigonia           | 16,559    | 30.7 24.8 16.1 28.4  | AB054126        |
|             | Lefua nikkonis            | 16,589    | 29.9 26.3 16.7 27.1  | AP011300        |
|             | Oreonectes furcudalis     | 16,569    | 31.1 29.5 12.9 26.5  | KX778472        |
|             | Oreonectes platycephaulus | 16,580    | 30.2 26.9 16.1 26.8  | AP011296        |
|             | Schistura balteata        | 16,564    | 31.7 27.0 15.3 26.0  | AB242172        |
|             | Schistura corica          | 16,572    | 29.7 26.6 17.3 26.4  | AP011445        |
|             | Schistura fasciolata      | 16,560    | 30.9 26.9 16.2 26.1  | KY404236        |
|             | Schistura geisleri        | 16,819    | 30.0 28.2 17.0 24.9  | AP013295        |
|             | Schistura jarutanini      | 16,594    | 30.3 28.3 16.9 24.4  | AP011307        |
|             | Schistura kaysonei        | 16,575    | 30.6 28.2 16.5 24.8  | AP011297        |
|             | Schistura notostigma      | 16,568    | 29.8 27.9 17.1 25.2  | AP011308        |
|             | Schistura pridii          | 16,576    | 30.8 28.4 16.3 24.5  | AP011443        |
|             | Schistura reticulofasciata| 16,603    | 30.8 27.7 16.5 25.0  | KY379150        |
|             | Schistura scaturigina     | 16,585    | 30.8 27.0 16.4 25.8  | KU380330        |
|             | Schistura sikmaiensis     | 16,581    | 33.8 21.1 13.5 31.6  | KY379151        |
|             | Triplphysa anterodorsalis | 16,567    | 27.4 25.7 18.4 28.6  | KJ739868        |
|             | Triplphysa bleekeri       | 16,568    | 27.1 25.8 18.5 28.6  | JX135578        |
|             | Triplphysa dorsalis       | 16,572    | 26.9 26.1 16.1 30.9  | KT241024        |
|             | Triplphysa lixinensis     | 16,570    | 27.8 25.4 18.4 28.5  | KT966735        |
|             | Triplphysa orientalis     | 16,562    | 27.4 25.5 18.7 28.5  | KJ631323        |
|             | Triplphysa pappenheimi    | 16,572    | 28.2 25.4 18.1 28.3  | KY419201        |
|             | Triplphysa robusta        | 16,570    | 28.2 25.3 18.0 28.4  | KM406486        |
|             | Triplphysa rosa           | 16,585    | 31.8 25.3 15.6 27.3  | JF268621        |
|             | Triplphysa siluroides     | 16,574    | 28.8 25.0 17.5 28.7  | KJ781206        |
|             | Triplphysa stenura        | 16,569    | 27.8 25.4 18.4 28.4  | KX354975        |
|             | Triplphysa stewarti       | 16,567    | 27.8 25.4 18.4 28.4  | KJ631324        |
|             | Triplphysa stoliczkan     | 16,571    | 28.1 25.2 17.9 28.8  | JQ663847        |
|             | Triplphysa strauchii      | 16,590    | 28.3 25.4 17.8 28.5  | KP297875        |
|             | Triplphysa tenuis         | 16,571    | 27.5 25.7 18.6 28.2  | KT224363        |
|             | Triplphysa tibetana       | 16,574    | 26.9 25.6 19.1 28.3  | KT224364        |
|             | Triplphysa venusta        | 16,574    | 27.8 26.9 18.4 26.9  | KT008666        |
|             | Triplphysa wuweiensis     | 16,681    | 28.0 25.7 18.1 28.2  | KT224365        |
|             | Triplphysa xiangxiensis   | 16,598    | 30.8 26.3 16.0 26.8  | KT751089        |
|             | Triplphysa xichangensis   | 16,570    | 28.6 25.3 17.6 28.6  | KT224366        |
|             | Triplphysa yarkandensis   | 16,574    | 31.9 30.4 17.4 20.3  | KP050360        |
| Cyprinidae  | Hemibarbus labo           | 16,612    | 29.7 27.1 17.2 26.0  | DQ347953        |
|             | Hemibarbus longirostris   | 16,608    | 27.7 27.2 18.7 26.3  | DQ347952        |
identified through DOGMA using default settings (Wyman, Jansen, & Boore, 2004). All tRNA genes and their secondary structures were verified with tRNA-scan SE (Lowe & Eddy, 1997). The secondary structure of tRNA genes and O L was drawn by RNAstructure 6.1 and modified by StructureEditor (Mathews, 2014). MEGA 7 was used to calculate the relative synonymous codon usage (RSCU) and base composition of each gene (Kumar, Stecher, & Tamura, 2016). Nucleotide composition skew values of 13 PCGs were counted by the formulas: (AT-skew = \(\frac{A - T}{A + T}\), GC-skew = \(\frac{G - C}{G + C}\)) (Perna & Kocher, 1995). The complete sequence and annotation were constructed using MitoFish, including a graphic circular map (Iwasaki et al., 2013).

2.4 Phyllogenetic analysis

A total of 43 GenBank-retrieved mitogenomes of species from the Nemacheilidae was used to reconstruct phylogenetic relationships (Table 1). Two species from the Cyprinidae (Hemibarbus labeo GenBank: DQ347953, Hemibarbus longirostris GenBank: DQ347952) were selected as outgroups. Nucleotide sequences of 12 PCGs were aligned separately by MEGA 7 using the default setting. The ND6 gene was excluded for phylogenetic analysis due to a high degree of heterogeneity (Miya et al., 2003). The 12 PCGs were concatenated to a combination sequence without termination codon due to a high degree of degeneracy. Maximum likelihood (ML) and Bayesian inference (BI) analyses were used to define phylogenetic relationships among the Nemacheilidae (Kumar et al., 2016; Ronquist et al., 2012). The phylogenetic trees were modified by FigTree v1.4.3 (Vlad, Balaji, Vikas, Ramani, & Larry, 2008).

3 RESULTS AND DISCUSSION

3.1 Mitochondrial genomic structure and composition

The complete mitogenome of H. laxiclathra is a circular molecule of 16,570 bp (Figure 2) and is deposited in the GenBank database under accession numbers MK279351. It consists of 37 typical genes, including 13 protein-coding genes (PCGs), 22 transfer RNA genes, two rRNA genes, and a noncoding region (Table 2). Nearly, all the genes are transcribed on the heavy strand, whereas ND6 and eight tRNA genes are located on the light strand. The structure and composition of H. laxiclathra is identical to other mitogenomes of nemacheilids to date (Vlad et al., 2008). The nucleotide composition of the H. laxiclathra mitogenome has a gently biased A + T content for 55.7%. The overall base composition of H. laxiclathra is the following: A,
29.6%; T, 26.1%; C, 27.0%; G, 17.2%. The overall AT- and GC-skew of *H. laxiclathra* mitogenome are −0.013 and −0.233S. The nucleotide frequency of each protein-coding gene is A + T > C + G, respectively, showing a strong AT bias (Table 3). For analyses within the genus, the same information from *H. potanini* and *H. variegata* was calculated. *H. potanini* showed the highest A + T frequency at 56.4% with *H. variegata* and *H. laxiclathra* having the most robust AT-skew. The whole mitogenome base composition of *H. variegata* is highly similar to *H. laxiclathra* with A for 29.5%, T for 26.1%, C for 27.1%, and G for 17.3%, suggesting they share a deep homology.

### 3.2 Protein-coding genes

The 13 PCGs of *H. laxiclathra* are similar in component and length to other familial fishes, ranging from 168 bp for ATP8 to 1839 bp for

| Gene | Position From-to | Length (bp) | Intergenic length | Strand | Start codon | Stop codon |
|------|------------------|-------------|-------------------|--------|-------------|------------|
| tRNAPhe | 1–69 | 69 | H |
| tRNAVal | 1,021–1,092 | 72 | 0 | H |
| 16SrRNA | 1,093–2,764 | 1672 | 0 | H |
| tRNAAsn(UUR) | 2.765–2.839 | 75 | 0 | H |
| ND1 | 2,840–3,814 | 975 | 0 | H | ATG | TAA |
| tRNAAs | 3,822–3,893 | 72 | 7 | H |
| tRNAGlu | 5,388–5,456 | 69 | −1 | L |
| tRNAArg | 9,984–10,053 | 70 | 0 | H |
| tRNATrp | 3,964–4,032 | 69 | 1 | H |
| COI | 5,458–7,008 | 1,551 | 1 | H | GTG | TAA |
| tRNAPhe(UCN) | 7,009–7,079 | 71 | 0 | L |
| COII | 7,169–7,859 | 691 | 13 | H | ATG | T |
| tRNATrp | 5,220–5,292 | 73 | 1 | L |
| tRNAAsn | 5,323–5,388 | 66 | 30 | L |
| tRNAIle | 3,822–3,893 | 72 | 7 | H |
| tRNAGln | 3,892–3,962 | 71 | −2 | L |
| tRNATyr | 3,964–4,032 | 69 | 1 | H |
| COI | 5,458–7,008 | 1,551 | 1 | H | GTG | TAA |
| tRNAAsp | 7,083–7,155 | 73 | 3 | H |
| ND1 | 2,840–3,814 | 975 | 0 | H | ATG | TAA |
| ATP8 | 7,009–7,079 | 71 | 0 | L |
| ATP6 | 2,840–3,814 | 975 | 0 | H | ATG | TAA |
| ND3 | 9,635–9,983 | 349 | 0 | H | ATG | T |
| ND4 | 10,344–11,725 | 1,382 | −7 | H | ATG | TAA |
| COIII | 7,169–7,859 | 691 | 13 | H | ATG | T |
| tRNAAsn | 7,860–7,935 | 76 | 0 | H |
| COIII | 7,169–7,859 | 691 | 13 | H | ATG | T |
| tRNAThr | 15,508–15,579 | 72 | 0 | H |
| tRNATyr | 11,807–11,862 | 56 | 11 | H |
| D-loop | 15,648–15,670 | 923 | 0 | H |
| ND2 | 4,033–5,077 | 1,045 | 0 | H | ATG | T |
| tRNAAsp | 5,078–5,147 | 70 | 0 | H |
| tRNAGlu | 9,562–9,634 | 73 | 0 | H |
| tRNAAsp | 7,083–7,155 | 73 | 3 | H |
| tRNALeu(UUR) | 7,169–7,859 | 691 | 13 | H | ATG | T |
| tRNAIle | 7,083–7,155 | 73 | 3 | H |
| tRNAGln | 7,083–7,155 | 73 | 3 | H |
| tRNAAsp | 7,083–7,155 | 73 | 3 | H |
| tRNALeu(UUR) | 7,169–7,859 | 691 | 13 | H | ATG | T |
| tRNAIle | 7,083–7,155 | 73 | 3 | H |
| tRNAGln | 7,083–7,155 | 73 | 3 | H |
| tRNAAsp | 7,083–7,155 | 73 | 3 | H |
| tRNALeu(UUR) | 7,169–7,859 | 691 | 13 | H | ATG | T |
| tRNAIle | 7,083–7,155 | 73 | 3 | H |
| tRNAGln | 7,083–7,155 | 73 | 3 | H |
| tRNAAsp | 7,083–7,155 | 73 | 3 | H |
| tRNALeu(UUR) | 7,169–7,859 | 691 | 13 | H | ATG | T |
| tRNAIle | 7,083–7,155 | 73 | 3 | H |
| tRNAGln | 7,083–7,155 | 73 | 3 | H |
| tRNAAsp | 7,083–7,155 | 73 | 3 | H |

**Table 2** Annotation of mitochondrial genome of *Homatula laxiclathra*
ND5. All these PCGs are coded by the heavy strand except ND6 which is coded by the light strand (Miya & Nishida, 2000). Similar to other loaches, the COI gene has a GTG start codon, whereas other twelve PCGs start with ATG. Five PCGs end with complete termination codon TAA and others with T- or TA-. The total length of 13 PCGs is 11,441 bp, which contain 12 intergenic spacers, the smallest spacer is only 1 bp in size, whereas the longest spacer can be up to 30 bp located between tRNA (Asn) and tRNA (Cys). There are six overlaps ranging from 1 to 10 bp, and the longest region is located between ATP8 and ATP6. Among the 13 protein-coding genes, ATP6 showed the highest A + T content with 58.9% and COIII at the lowest A + T content with 52.8%.

Codon usage and relative synonymous codon usage (RSCU) of the *H. laxiclathra* mitogenome is summarized (Table 4). Almost all codons are present in *H. laxiclathra* except for the specific mammals stop codons AGA and AGG. The most common amino acids in protein-coding genes are leucine (463), alanine (331), and threonine (298). Leucine was coded by CUA

| Gene | Nucleotide frequency (%) | A + T (%) | AT-skew | CG-skew |
|------|--------------------------|-----------|---------|---------|
| ATP6 | 28.7 30.2 27.1 14.1 | 58.9 | -0.025 | -0.316 |
| ATP8 | 31.0 26.8 26.2 16.1 | 57.8 | 0.073 | -0.239 |
| COI  | 24.6 29.4 25.9 20.2 | 54.0 | -0.089 | -0.124 |
| COII | 29.2 25.8 28.4 16.6 | 55.0 | 0.062 | -0.262 |
| COIII| 26.8 26.0 28.8 18.4 | 52.8 | 0.015 | -0.220 |
| ND1  | 27.9 27.7 29.6 14.8 | 55.6 | 0.004 | -0.333 |
| ND2  | 31.3 24.0 31.0 13.7 | 55.3 | 0.132 | -0.387 |
| ND3  | 25.2 30.1 27.2 17.5 | 55.3 | -0.089 | -0.217 |
| ND4  | 27.8 26.9 29.2 16.1 | 54.7 | 0.016 | -0.289 |
| ND4L | 22.6 29.0 31.0 16.6 | 55.3 | -0.012 | -0.027 |
| ND5  | 30.1 27.4 28.2 14.4 | 57.5 | 0.047 | -0.324 |
| ND6  | 40.2 16.1 30.8 12.8 | 56.3 | 0.428 | -0.413 |
| Cytb | 28.0 28.4 27.4 16.1 | 56.4 | -0.007 | -0.260 |
| 12SrRNA | 29.0 19.9 27.3 22.9 | 49.8 | 0.201 | -0.088 |
| 16SrRNA | 35.8 20.5 23.2 20.5 | 56.3 | 0.272 | -0.062 |
| D-Loop | 32.5 33.6 19.5 14.4 | 66.1 | -0.017 | -0.150 |
| Total | 27.3 28.1 26.9 16.8 | 55.8 | -0.013 | -0.233 |

**TABLE 4** Relative synonymous codon usage (RSCU) in all proteins of *Homatula laxiclathra*

| Codon | n (RSCU) | Codon | n (RSCU) | Codon | n (RSCU) | Codon | n (RSCU) |
|-------|----------|-------|----------|-------|----------|-------|----------|
| UUU(F) | 108 (1) | UCU(S) | 32 (0.81) | UAU(Y) | 41 (0.78) | UGU(C) | 8 (0.64) |
| UUC(F) | 108 (1) | UCC(S) | 61 (1.55) | UAC(Y) | 64 (1.22) | UGC(C) | 17 (1.26) |
| UUA(L) | 108 (1.1) | UCA(S) | 85 (2.16) | UAA(*) | 0 (0) | UGA(W) | 89 (1.53) |
| UUG(L) | 16 (0.16) | UCG(S) | 7 (0.18) | UAG(*) | 0 (0) | UGG(W) | 27 (0.47) |
| CUA(L) | 94 (0.96) | CCA(P) | 38 (0.73) | CAU(H) | 25 (0.48) | CGU(R) | 8 (0.45) |
| CUG(L) | 94 (0.96) | CCC(P) | 67 (1.28) | CAC(H) | 79 (1.52) | CGC(R) | 10 (0.56) |
| CUA(L) | 213 (2.18) | CCA(P) | 88 (1.68) | CAA(Q) | 78 (1.58) | CGA(R) | 47 (2.65) |
| CUG(L) | 62 (0.63) | CGG(P) | 16 (0.31) | CAG(Q) | 21 (0.42) | CGG(G) | 6 (0.34) |
| AUU(I) | 175 (1.24) | ACU(T) | 35 (0.47) | AAU(N) | 41 (0.74) | AGU(S) | 8 (0.2) |
| AUC(I) | 107 (0.76) | ACC(T) | 120 (1.61) | AAC(N) | 70 (1.26) | AGC(S) | 43 (1.09) |
| AUA(M) | 118 (1.4) | ACA(T) | 129 (1.73) | AAA(K) | 61 (1.61) | AGA(*) | 0 (0) |
| AUG(M) | 50 (0.6) | AGA(T) | 14 (0.19) | AAG(K) | 15 (0.39) | AGG(*) | 0 (0) |
| GUU(V) | 53 (1.03) | GCU(A) | 43 (0.52) | GAU(D) | 21 (0.58) | GGU(G) | 34 (0.61) |
| GUC(V) | 37 (0.72) | GCC(A) | 147 (1.78) | GAC(D) | 51 (1.42) | GGC(G) | 47 (0.85) |
| GUA(V) | 92 (1.8) | GCA(A) | 129 (1.56) | GAA(E) | 69 (1.41) | GGA(G) | 85 (1.53) |
| GUG(V) | 23 (0.45) | GCG(A) | 12 (0.15) | GAG(E) | 29 (0.59) | GGG(G) | 56 (1.01) |
in *H. laxiclathra* PCGs, the same as in *H. variegata* and *H. potanini*. GCC (147) and GCA (129) are shared equally, coding for alanine, and the same trend is shown by threonine: ACC (120) and ACA (129).

### 3.3 | Transfer RNA genes and ribosomal RNA genes

All 22 tRNA genes are found in the mitogenome of *H. laxiclathra*. Comparative analysis on potential secondary structures of *H. laxiclathra* tRNAs is shown (Figure 3). Fourteen tRNAs were located on the heavy strand whereas the other tRNAs were on the light strand. The length of all tRNAs was similar, ranging from 56 bp to 75 bp. Nearly, all tRNA genes were predicted to have typical cloverleaf structures, with the exception of tRNA-Ser (AGN) which lacked a stable DHU stem (Figure 2). This missing stem occurs in most teleost mitogenomes as previously reported (Lee & Kocher, 1995). In addition, some tRNAs showed mismatched pairs in stems (e.g. U-G and A-C in the acceptor arm.

**FIGURE 3**  Secondary structures of transfer RNA genes in *Homatula laxiclathra*, generated from RNAstructure 6.1 and SturctureEditor (Mathews, 2014)
of tRNA-Arg for three *Homatula* species). These conserved mismatched pairs may be similar to the molecular synapomorphy for the genus. The length of 12S rRNA and 16S rRNA of *H. laxiclathra* were 951 bp and 1672 bp, respectively. The values were similar with *H. potanini* and *H. variegata*, falling well into the size range in fishes. The A + T contents of the 12S rRNA and 16S rRNA of *H. laxiclathra* were 49.8% and 56.3%, respectively, thus indicating some diversity in nucleotide distribution. Both 12S rRNA and 16S rRNA had a positive AT-skew (0.201 and 0.272), and a negative GC-skew (−0.088 and −0.062) at the same locations on the heavy strand. Similar to other Nemacheilidae species, 12S rRNA was located between tRNA-Phe and tRNA-Val and the 16S rRNA was located between tRNA-Val and tRNA-Leu.

### 3.4 Noncoding regions

The mitogenome of *H. laxiclathra* has two noncoding regions, the D-loop and O$_\gamma$. The 923 bp D-loop is located between tRNA-Pro and tRNA-Phe with 66.1% A + T content (Figure 4). The O$_\gamma$ is 30 bp in length, located in the WANCY region between tRNA-Asn and tRNA-Cys with a putative hairpin structure (Figure 5). The D-loop region is complex and highly variable and can determine the replication pattern of the mitogenome (Liu, Zhang, Tang, Yu, & Zhou, 2010).

By comparing with other Nemacheilidae, the D-loop can be divided into three functional segments, the termination associated sequence (TAS-1 and TAS-2), the central conserved sequence block (CSB-D, CSB-E, and CSB-F) and the conserved sequence block (CSB-1, CSB-2, and CSB-3). The termination associated sequence varies markedly among different lineages, although it can play vital
roles in determining the fate of the heavy strand. The core conserved sequence TACAT and complementary sequence ATGTA were detected in TAS, folded into a stable hairpin structure. Two poly-T stretches and a conserved motif (TA)₅ were found by comparing against other fishes. Significant tandem repeats were not recognized in the H. laxiclathra D-loop.

3.5 | Phylogenetic analysis

Phylogenetic relationships of the Nemacheilidae were reconstructed using two methods, Bayesian inference (BI) and maximum likelihood (ML) (Figure 6). Twelve PCGs from 41 nemacheilid species were concatenated to a matrix and used for phylogenetic analyses; two Cyprinididae species were selected as the outgroups. The phylogenetic trees generated a similar topology that confirmed the findings from a previous study for loach classification (Sgouros, Page, Orlofske, & Jadin, 2019). Both phylogenetic trees consistently showed three major clades, including (I) Acanthocobitis and Schistura, (II) Oreonectes and Lefua, (III) Homatula, Barbatula, and Triplophysa. All the congeneric species represented a single cluster for each genus (Acanthocobitis, Homatula, Barbatula, Lefua, Oreonectes, Schistura, and Triplophysa), and, the relationship of the Nemacheilidae was consistent with other phylogenetic and morphological studies on these species (Prokofiev, 2010; Stout, Tan, Lemmon, Lemmon, & Armbruster, 2016). Thus, Homatula was shown to be valid as an inherent Asian fish group according to where the genus falls out on both trees. Further, Homatula shares a close ancestor with Oreonectes and Lefua making it a sister group.

The topology also demonstrated monophyly of three Lefua species (Miyazaki et al., 2011). This molecular information provides a more robust data set to support fish classification and identification. In addition, several related articles adapt various standards to classify species, such as phylogeny based on single mitogenome genes or nuclear genes (Liu et al., 2012; Powell, Barker, & Lanyon, 2013; Tang, Liu, Mayden, & Xiong, 2006). Our results are based on the highest coverage of Nemacheilidae mitogenomic data to date and provide an updated view of Nemacheilidae phylogeny.
4 | CONCLUSIONS

In this study, we present the complete mitogenome of Homatula laxiclathra and provide a comparison of this sequence against other Homatula species to examine the architecture of mitogenome structure, the location of coding genes, and codon usage. The results integrate updated mitogenomic information of the Nemacheilidae and generate a new phylogeny and relationship among different genera of these fishes. However, many genus-level taxonomy studies lack robust molecular data and thus the true phylogeny of the loach remains unresolved.

ACKNOWLEDGMENTS

The authors would like to thank Fe Ye, Jun Yan, and Tao Chen (College of Life Sciences, Shaanxi Normal University) for collecting the specimens and data, and Dr. Fei Ye for editorial comments on the draft of the manuscript. Dr. David K. Cone (Department of Biology, Saint Mary’s University, Canada) for editorial comments and revision of the final version.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

Mengfei Cao: Data curation (equal); Formal analysis (equal); Investigation (equal); Resources (equal); Software (equal); Writing-original draft (equal); Writing-review & editing (equal). Ling Tang: Data curation (equal); Investigation (equal); Resources (equal). Juan Chen: Data curation (equal); Formal analysis (equal); Investigation (equal); Resources (equal). Xiaoou Zhang: Data curation (equal); Resources (equal). Russell H. Easy: Writing-review & editing (equal). Ping You: Conceptualization (equal); Funding acquisition (equal); Investigation (equal); Project administration (equal); Supervision (equal); Writing-original draft (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

DNA sequences: The complete mitogenome sequence of Homatula laxiclathra was deposited in the GenBank database under accession numbers MK279351. The data have been uploaded to Dryad and available on https://doi.org/10.5061/dryad.nvx0k6dnz.

ORCID

Ping You https://orcid.org/0000-0002-2913-1544

REFERENCES

Betancur, R. R., Wiley, E. O., Arratia, G., Acero, A., Bailly, N., Miya, M., ... Ortí, G. (2017). Phylogenetic classification of bony fishes. BMC Evolutionary Biology, 17. 162. https://doi.org/10.1186/s12862-017-0958-3

Boore, J. L. (1999). Animal mitochondrial genomes. Nucleic Acids Research, 27(8), 1767-1780. https://doi.org/10.1093/nar/27.8.1767

Cunha, R. L., Grande, C., & Zardoya, R. (2009). Neogastropod phylogenetic relationships based on entire mitochondrial genomes. BMC Evolutionary Biology, 9, 210. https://doi.org/10.1186/1471-2148-9-210

Gu, J. H., & Zhang, E. (2012). Homatula laxiclathra (Teleostei: Balitoridae), a new species of nemacheiline loach from the Yellow River drainage in Shaanxi Province, northern china. Environmental Biology of Fishes, 94(4), 591–599. https://doi.org/10.1007/s10641-011-9965-1

Iwasaki, W., Fukunaga, T., Isagozawa, R., Yamada, K., Maeda, Y., Satoh, T. P., ... Nishida, M. (2013). MitoFish and MitoAnnotator: A mitochondrial genome database of fish with an accurate and automatic annotation pipeline. Molecular Biology and Evolution, 30(11), 2531–2540. https://doi.org/10.1093/molbev/ms3141

Kipp, B. R., Barr Fritcher, E. G., Clayton, A. C., Gores, G. J., Roberts, L. R., Zhang, J., ... Halling, K. C. (2010). Comparison of KRAS mutation analysis and FISH for detecting pancreatobiliary tract cancer in cytology specimens collected during endoscopic retrograde cholangiopancreatography. Journal of Molecular Diagnostics, 12(6), 780–786. https://doi.org/10.2353/jmoldx.2010.100016

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution, 33(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Lee, W. J., Conroy, J., Howel, W. H., & Kocher, T. D. (1995). Structure and evolution of teleost mitochondrial control regions. Journal of Molecular Evolution, 41(1), 54–66. https://doi.org/10.1007/BF00174041

Lee, W. J., & Kocher, T. D. (1995). Complete sequence of a sea lamprey (Petromyzon marinus) mitochondrial genome: Early establishment of the vertebrate genome organization. Genetics, 139(2), 873–887.

Liu, H. L., Zhang, Q., Tang, Y. L., Yu, F. Y., & Zhou, J. Y. (2010). Structure and genetic diversity of mtDNA D-Loop sequences among Trachidermus fasciatus stocks in Yellow sea and Bohai sea of china. Marine Science Bulletin, 29(3), 283–288.

Liu, S. Q., Mayden, R. L., Zhang, J. B., Yu, D., Tang, Q. Y., Deng, X., & Liu, H. Z. (2012). Phylogenetic relationships of the Cobitoidea (Teleostei: Cypriniformes) inferred from mitochondrial and nuclear genes with analyses of gene evolution. Gene, 508(1), 60–72. https://doi.org/10.1016/j.gene.2012.07.040

Lowe, T. M., & Eddy, S. R. (1997). tRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Research, 25(5), 955–964. https://doi.org/10.1093/nar/25.5.955

Mathews, D. H. (2014). Using the RNA structure software package to predict conserved RNA structures. Current Protocols in Bioinformatics, 46, 12.4.1-22. https://doi.org/10.1002/0471250953.b1204s46

Miya, M., Kawaguchi, A., & Nishida, M. (2001). Mitogenomic exploration of higher teleostean phylogenies—a case study for moderate-scale evolutionary genomics with 38 newly determined complete mitochondrial DNA sequences. Molecular Biology Evolution, 18(11), 1993–2009. https://doi.org/10.1093/oxfordjournals.molbev.a003741

Miya, M., & Nishida, M. (1999). Organization of the mitochondrial genome of a deep-sea fish, Gonostoma gracile (Teleostei: Stomiiformes): First example of transfer RNA gene rearrangements in bony fishes. Marine Biotechnology, 1, 416–426. https://doi.org/10.1007/PL00011798

Miya, M., & Nishida, M. (2000). Use of mitogenomic information in teleostean molecular phylogenetics: A tree-based exploration under the maximum-parsimony optimality criterion. Molecular Phylogenetics and Evolution, 17(3), 437–455. https://doi.org/10.1006/mpev.2000.0839

Miya, M., Takeshima, H., Endo, H., Ishiguro, N. B., Inoue, J. G., Mukai, T., ... Nishidoi, M. (2003). Major patterns of higher teleostean phylogenies—a new perspective based on 100 complete mitochondrial DNA sequences. Molecular Phylogenetics and Evolution, 26(1), 121–138. https://doi.org/10.1016/S1055-7903(02)00332-9

Miyaizaki, J., Dobashi, M., Tamura, T., Beppu, S., Sakai, T., Mihara, M., & Hosoya, K. (2011). Parallel evolution in eight-barbel loaches of the genus Lefua (Balitoridae, Cypriniformes) revealed by mitochondrial and nuclear DNA phylogenies. Molecular Phylogenetics and Evolution, 60(3), 416–427. https://doi.org/10.1016/j.ympev.2011.05.005
Parhi, J., Tripathy, P. S., Priyadarshi, H., Mandal, S. C., & Pandey, P. K. (2019). Diagnosis of mitogenome for robust phylogeny: A case of Cypriniformes fish group. *Gene*, 713, 143967. https://doi.org/10.1016/j.gene.2019.143967

Perna, N. T., & Kocher, T. D. (1995). Patterns of nucleotide composition at fourfold degenerate sites of animal mitochondrial genomes. *Journal of Molecular Evolution*, 41(3), 353–358. https://doi.org/10.1007/BF00121582

Powell, A. F., Barker, F. K., & Lanyon, S. M. (2013). Empirical evaluation of partitioning schemes for phylogenetic analyses of mitogenomic data: An avian case study. *Molecular Phylogenetics and Evolution*, 66(1), 69–79. https://doi.org/10.1016/j.ympev.2012.09.006

Prokofiev, A. (2010). Morphological classification of loaches (Nemacheilinae). *Journal of Ichthyology*, 50(10), 827–913. https://doi.org/10.1134/S0032945210100012

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., ... Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61(3), 539–542. https://doi.org/10.1093/sysbio/sys029

Sgouros, K., Page, L. M., Orlofske, S. A., & Jadin, R. C. (2019). A revised molecular phylogeny reveals polyphyly in Schistura (Teleostei: Cypriniformes: Nemacheilidae). *Zootaxa*, 4559(2), 349–362. https://doi.org/10.11646/zootaxa.4559.2.8

Shi, Y. R., Xing, L. X., Chen, T., Yang, M., & You, P. (2014). Sequencing and analysis of the complete mitochondrial genome of *Paracobitis variagatus*. *Journal of Shannxi Normal University (Natural Science Edition)*, 42(4), 50–56.

Staden, R., Beal, K. F., & Bonfield, J. K. (2000). The Staden package, 1998. *Methods in Molecular Biology*, 132, 115–130.

Stout, C. C., Tan, M., Lemmon, A. R., Lemmon, E. M., & Armbruster, J. W. (2016). Resolving Cypriniformes relationships using an anchored enrichment approach. *BMC Evolutionary Biology*, 16, 244. https://doi.org/10.1186/s12862-016-0819-5

Tang, Q., Liu, H., Mayden, R., & Xiong, B. (2006). Comparison of evolutionary rates in the mitochondrial DNA cytochrome b gene and control region and their implications for phylogeny of the Cobitoidea (Teleostei: Cypriniformes). *Molecular Phylogenetics and Evolution*, 39(2), 347–357. https://doi.org/10.1016/j.ympev.2005.08.007

Vlad, I. M., Balaji, V. S., Vikas, C. R., Ramani, D., & Larry, S. D. (2008). Automatic online tuning for fast Gaussian summation. *Advances in Neural Information Processing Systems (NIPS)*, 2008, 1113–1120.

Wyman, S. K., Jansen, R. K., & Boore, J. L. (2004). Automatic annotation of organellar genomes with DOGMA. *Bioinformatics*, 20(17), 3252–3255. https://doi.org/10.1093/bioinformatics/bth352

Zhu, W. B., Yan, J., Song, J. R., & You, P. (2018). The first mitochondrial genomes for Pyralinae (Pyralidae) and Glaphyriinae (Crambidae), with phylogenetic implications of Pyraloidea. *PLoS ONE*, 13(3), e0194672. https://doi.org/10.1371/journal.pone.0194672

How to cite this article: Cao M, Tang L, Chen J, Zhang X, Easy RH, You P. The mitogenome of freshwater loach *Homatula laxiclaithra* (Teleostei: Nemacheilidae) with phylogenetic analysis of Nemacheilidae. *Ecol Evol*. 2020;10:5990–6000. https://doi.org/10.1002/ece3.6338