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S. F. Li
USGS Columbia Environmental Research Center, sfli@shou.edu.cn

J. W. Xu
Shanghai Ocean University

Q. L. Yang
Shanghai Ocean University

C. H. Wang
Shanghai Ocean University

Q. Chen
Shanghai Ocean University, wangch@shou.edu.cn

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S. F. Li, J. W. Xu, Q. L. Yang, C. H. Wang, Q. Chen, D. C. Chapman, and Guoqing Lu

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A comparison of complete mitochondrial genomes of silver carp *Hypophthalmichthys molitrix* and bighead carp *Hypophthalmichthys nobilis*: implications for their taxonomic relationship and phylogeny

S. F. LI||†, J. W. XU*, Q. L. YANG*, C. H. WANG*, Q. CHEN*, D. C. CHAPMAN† AND G. LU‡

∗Key Laboratory of Aquatic Genetic Resources and Utilization, Ministry of Agriculture, Shanghai Ocean University, Shanghai, 200090, China, †USGS Columbia Environmental Research Center, Columbia, MO 65201, U.S.A. and ‡Department of Biology, University of Nebraska at Omaha, Omaha, NE 68182, U.S.A.

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Based upon morphological characters, Silver carp *Hypophthalmichthys molitrix* and bighead carp *Hypophthalmichthys nobilis* (or *Aristichthys nobilis*) have been classified into either the same genus or two distinct genera. Consequently, the taxonomic relationship of the two species at the generic level remains equivocal. This issue is addressed by sequencing complete mitochondrial genomes of *H. molitrix* and *H. nobilis*, comparing their mitogenome organization, structure and sequence similarity, and conducting a comprehensive phylogenetic analysis of cyprinid species. As with other cyprinid fishes, the mitogenomes of the two species were structurally conserved, containing 37 genes including 13 protein-coding genes, two ribosomal RNA genes, 22 transfer RNA (tRNAs) genes and a putative control region (D-loop). Sequence similarity between the two mitogenomes varied in different genes or regions, being highest in the tRNA genes (98·8%), lowest in the control region (89·4%) and intermediate in the protein-coding genes (94·2%). Analyses of the sequence comparison and phylogeny using concatenated protein sequences support the view that the two species belong to the genus *Hypophthalmichthys*. Further studies using nuclear markers and involving more closely related species, and the systematic combination of traditional biology and molecular biology are needed in order to confirm this conclusion.
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Key words: bighead carp; generic taxonomy; mitochondrial genome; molecular phylogeny; sequence comparison; silver carp.

INTRODUCTION

According to the Linnaean taxonomic system, a genus is a low-level rank used to categorize a group of closely related species that descend from a common ancestor. Traditionally, the classification of species relies mainly on the characterization of

||Author to whom correspondence should be addressed. Tel.: +86 21 6190 0450; fax: +86 21 6190 0450; email: sfli@shou.edu.cn

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homologous morphological characters. Such characters, however, are lacking or not easy to identify in certain closely related groups. With the advent and application of molecular phylogeny, significant progress has been achieved in resolving some taxonomic questions, particularly when data from molecular biology are combined with data from traditional biology (Hedges & Poling, 1999; Giribet *et al*., 2001; Saitoh *et al*., 2006). Successful examples in fishes have been reported, based on the analysis of complete mitochondrial genome sequences (Miya *et al*., 2003; Peng *et al*., 2006; Saitoh *et al*., 2006).

Silver carp *Hypophthalmichthys molitrix* (Valenciennes) and bighead carp *Hypophthalmichthys nobilis* (Richardson) belong to the family Cyprinidae. They were originally described as species of the genus *Leuciscus* and subsequently placed in the genus *Hypophthalmichthys* until Oshima (1919) reclassified *H. nobilis* to the genus *Aristichthys*. A set of morphological characters were developed to distinguish *Aristichthys* from *Hypophthalmichthys*, including gill raker morphology, position of the abdominal keel and pharyngeal dentition as well as pectoral-fin length. Consequently, some ichthyologists classified silver carp in the genus *Hypophthalmichthys* and bighead carp in *Aristichthys* (Cheng & Zheng, 1987; Wu, 1964), whereas other ichthyologists placed both species in the genus *Hypophthalmichthys* (Howes, 1981; Kolar *et al.,* 2007).

Hypophthalmichthys molitrix and *H. nobilis* are native to eastern Asia and have been broadly introduced to southern Asia, Europe and North America (Kolar *et al*., 2007). The two carps adapt to various environments very well and are widely distributed in the world. Their production in 2005 reached 3·52 and $2{\cdot}18 \times 10^6$ t in China and 4·15 and $2{\cdot}21 \times 10^6$ t in the world, respectively (FAO, 2005). Because of their importance in aquaculture and in biological control of water quality, both species have been extensively studied in a variety of areas, including traditional biology (Li *et al*., 1997, 1998; Kolar *et al*., 2007), cytogenetics (Li, 1998), isoenzymes (Xia *et al*., 1996; Zhao & Li, 1996; Jiang *et al*., 1998) and molecular genetics Fan *et al*., 1994; Lu *et al*., 1997, 2005; Jian & Xia, 1999; Zhang *et al*., 1999*a*, *b*, 2001, 2002; Zhang, 2002; Geng *et al*., 2006; Shan *et al*., 2006; Zhu *et al*., 2007. To date, however, the complete mitochondrial genome, consisting of important evolutionary information, has not been sequenced in *H. molitrix* and *H. nobilis*.

In this study, the complete mitochondrial genomes of *H. molitrix* and *H. nobilis* are reported, the results of sequence comparison at the genome and gene levels are described, and the phylogeny of 53 cyprinid fishes using concatenated protein sequences is presented. In addition, issues pertaining to the taxonomic and phylogenetic relationships between the two species at the generic level are discussed.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DNA EXTRACTION

Three samples of each species with body mass of 400–500 g were collected in the lower Yangtze River (32° 15′ N; 119° 25′ W) in 2005. A small piece of the caudal fin from each sample was taken and stored in 95% ethanol. Whole genomic DNA, including mitochondrial DNA (mtDNA), was extracted using a proteinase K and phenol-chloroform procedure (Sambrock & Russell, 2001). The quantity and quality of the extracted DNA were estimated on 1% agarose gels stained with ethidium bromide (EB).

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PRIMER DESIGN, PCR AMPLIFICATION AND SEQUENCING

Eighteen pairs of primers (Table I) were designed to amplify the complete mtDNA sequence of these two species, according to the alignment of reported complete mitogenome sequences of common carp *Cyprinus carpio* L., crucian carp *Carrasius auratus* (L.) and tench *Tinca tinca* (L.) (Chang *et al*., 1994; Murakami *et al*., 1998; Mabuchi *et al*., 2006; Saitoh *et al*., 2006). Polymerase chain reaction (PCR) was performed using an Eppendorf Thermal Cycler (www.eppendorf.com) within a reaction mixture of 50 μl containing 2 units of Taq DNA polymerase (Tiangen Inc.; www.tiangen.com/eng), 5 μl 10× PCR buffer (Tiangen Inc.), 50 ng/μl template DNA, 0·4 mM each dNTP and 0·2 μM each primer, in distilled water. The reaction was denatured at 94° C for 5 min, followed by 30 cycles at 94° C for 30 s, 54° C for 30 s and 72° C for 1 min; a final extension was done at 72° C for 10 min. All amplified products were purified using a 3S Spin PCR Product Purification Kit (Biocolor Inc.; www.biocolor-online.com) following the supplier's instructions. The purified products were sequenced on an Applied Biosystems ABI 3730 capillary sequencer (www.appliedbiosystems.com).

SEQUENCE ASSEMBLY AND GENE IDENTIFICATION

DNA sequences were examined using the basic local alignment search tool (BLAST) search tool available at the NCBI website (www.ncbi.nlm.nih.gov) to make sure that the correct DNA targets were amplified. The BioEdit 7.0 package was then used to edit and assemble mtDNA sequences for the complete genomes of these two species (Hall, 1998). tRNA genes were identified using tRNAscan-SE 1.21 (Lowe & Eddy, 1997) with the following settings: (1) default search mode, (2) mitochondrial–chloroplast DNA as the source and (3) vertebrate mitochondrial genetic code for tRNA structure prediction. Protein and ribosomal RNA genes were determined based on sequence similarity as compared with *C. carpio*, *C. auratus* and *T. tinca*. The 5′-end of the protein-coding genes was inferred using start codons ATG, GTG, TTG and GTT whereas the 3′-end termini were inferred using the stop codons TAA, TAG, AGA and AGG.

Protein-coding genes are commonly more informative in inferring species phylogeny (Peng *et al*., 2006). Mitochondrial protein sequences of 53 cyprinid species were thus retrieved from the NCBI Genome database for the phylogenetic analysis. The concatenated protein sequences of 13 genes were aligned using MUSCLE (Edgar, 2004). Excluding *H. molitrix* and *H. nobilis*, the remaining 51 cyprinid species were divided into two groups, *i.e*. intrageneric and intergeneric, to evaluate their taxonomic relationships at the generic level. The *JTT* model, based on a recounting of the number of observed changes in amino acids (Jones *et al*., 1992), was used to calculate genetic distances of pairwise sequences under MEGA 4.0 (Tamura *et al*., 2007). Phylogenetic analyses were performed using neighbour-joining (NJ) in MEGA, maximum likelihood (ML) in Multiphyl (Keane *et al*., 2007), and Bayesian Markov-Chain-Monte-Carlo (MCMC) method in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Based on tests of 88 amino acid substitution models, MtMam + I + G + F (-lnL = 49935.9755, Gamma distribution parameter $a = 0{\cdot}59$, invariable sites Pinv = 0·58) were selected for the ML analyses of concatenated mitochondrial protein sequences. Two million generations with four chains were run for Bayesian analysis and the trees sampled prior to reaching convergence were discarded before computing the consensus tree and posterior probabilities. Two independent runs were used to provide additional confirmation of the convergence of the posterior probability distribution. Bootstrap values and posterior probabilities were used to evaluate the above phylogenetic trees.

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TABLE I. Polymerase chain reaction (PCR) and sequencing primers designed for amplifying mitochondrial genomes of *Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis**

Forward	Sequences (5′ to 3′)	Reverse	Sequences (5′ to 3′)
SA0	CATGCCGAGCATTCTTTT	SB0	GAGACTTGCATGTGTAAG
SA1	CAAAGCATAGCACTGAAGATGC	SB1	TTTTGACAGGGGAGAGTGA
SA2	CCAGCCTATATACCGCCGT	SB2	CTATCACCAGGTTCGGTAGG
SA3	TTAGCCAGTACACCCAAGCA	SB3	AAAGACAAGTGATTGCGCT
SA4	AAGGAACTCGGCAAACACAA	SB4	TGGTGCTCATAAGGTTATGG
SA5	CCATCCACATCATCCCCA	SB5	ATTGGCGGAGGAGGGACTTT
SA6	CCCTACCAATTGCACTAGCA	SB6	GTTTGTAGGATCGAGGCCTT
SA7	AGACCAAAAGCCTTCAAAGC	SB7	TGTGGCTAATCAGCTAAA
	ACAACTCACCCTTCTCGCT		*CGTGAAGGACAATGTCAAGTG*
SA8	TCCGCAACAATAATTATCGC	SB8	TGGGACTGCGTCCATTTTTA
SA9	CACCCAACGCAACTAGGT(A)TT	SB9	GGCTTGCAAATTGGTCGAA
SA10	CAAATGAACCAACCCCAGTA	SB10	TGCAATTGTGAAGGGTGCTT
	CACCCACAACACAACTATCA		*TGAGCCTCATCAATAGATGG*
SA11	CTTCACAATTGCAGAT(C)GGG(A)G	SB11	AGCTG(A)AAATGTACGGGTGTC
SA12	AGAATGAGCAGAATAGGGA	SB12	TTCGTTCATAGGCTGTGTT
SA13	ATCAATTTGCCTCCGACA	SB13	GATTTGTTGAATTTCTCAGG
	GGTGGATACGGAATAATACGA		*GGCACAGGTGGCTGTAAATA*
SA14	ATGATGACAT(C)GGACGA(G)GCAG	SB14	GCGGCTGATTGT(A)CCTAGA(G)GT
SA15	AGCAGCCCTCA(C)TA(C)GTAACAA	SB15	TTGAATAACAACGGTGGTTC
	GACTTGCCTGAGGAAGCATTA		*GAACAGCCCGGTTAGGATTT*
SA16	CTTGCTCAGACTTTAACCGA	SB16	GTTTAGAATTCTGGCTTTGG
SA17	GGTCTTGTAATCCGAAGATC	SB17	GGGGTTTGACAAGGATAA

*Primer sequences are identical for both species, except for the pairs 7, 10, 13 and 15, where primers for *H. nobilis* are shown in italic; the single bases in parentheses are degenerate positions.

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RESULTS

MITOCHONDRIAL GENOME ORGANIZATION AND COMPOSITION

The mitochondrial genomes of *H. molitrix* and *H. nobilis* are similar to those of other cyprinids (Fig. 1). The size of the complete genome is 16 620 bp for *H. molitrix* and 16 621 bp for *H. nobilis*. Both species contain 13 protein-coding genes (Cytb, ATP6, ATP8, COI to III, ND1 to 6, ND4L), 22 transfer RNA genes, two ribosomal RNA genes (12S rRNA and 16S rRNA) and a putative control region (Table II). Overlaps between adjacent genes were found in both species, as revealed in other cyprinids. The overall base composition of the mitochondrial genomes is highly similar between these two species: A = 31·8%, C = 26·9%, G = 15·7%, T = 25·6%, A + T = 57·4% in *H. molitrix*; A = 31·6%, C = 27·1%, G = 16·0%, T = 25·3%, A + T = 56·9% in *H. nobilis* (Fig. 2). Overall nucleotide sequence similarity between the two mitochondrial genomes is 95·1%.

PROTEIN-CODING GENES

As with other cyprinids, protein-coding genes of the mitochondrial genome in *H. molitrix* and *H. nobilis* contain the strand start codon ATG except the gene COI, which contains GTG instead. The open reading frames (ORF) for the protein-coding genes are terminated using TAA (ND1, COI, ATPase8, ATPase6, COIII, ND4L, ND5 and ND6), TAG (ND2 in *H. molitrix*, ND2 to 4 in *H. nobilis*) and incomplete stop codon T- (COII and Cytb in *H. molitrix*; ND1, COII and Cytb in *H. nobilis*). Incomplete stop codons were observed commonly in the protein-coding genes of teleost mitochondria (Peng *et al*., 2006; Kartavtsev *et al*., 2007). Of the 13 protein-coding genes, 12 are encoded on the H-strand of the mtDNA whereas only the ND6 gene is encoded on the L-strand (Table II).

Three reading-frame overlaps were observed in mitochondrial genomes of the two species. In *H. molitrix* one nucleotide overlaps between ATP6 and COIII whereas 10 nucleotides overlap between ATP8ase and ATP6ase, and seven between ND4 and ND4L. In *H. nobilis* only seven nucleotides overlap between ATPase8 and ATPase6, and the other two overlaps are the same as in *H. molitrix*. The total length of the 13 protein-coding genes is 11 431 bp for *H. molitrix*, accounting for 68·78% of the whole mitogenome and 11 429 bp for *H. nobilis*, which accounts for 68·76% of the whole mitogenome. Both species contain nucleotide G least frequently in the third codon position. Nucleotide sequence similarity between mitochondrial protein-coding genes of both species is 94·2%.

NON-CODING SEQUENCE

The length of the major non-coding (D-loop) region, located between $tRNA^{Pro}$ and $tRNA^{Phe}$, is 936 bp in *H. molitrix* and 938 bp in *H. nobilis*, respectively. As with other fish species (Kartavtsev *et al*., 2007), the D-loop region can be divided into three domains. The first domain is hypervariable and consists of a termination-associated sequence (TAS: TACATATGTA in *H. molitrix*; TAS: TACAT AAT GTA CTA ATA CCT ATA TATGTATTAT in *H. nobilis*). The second domain is the central

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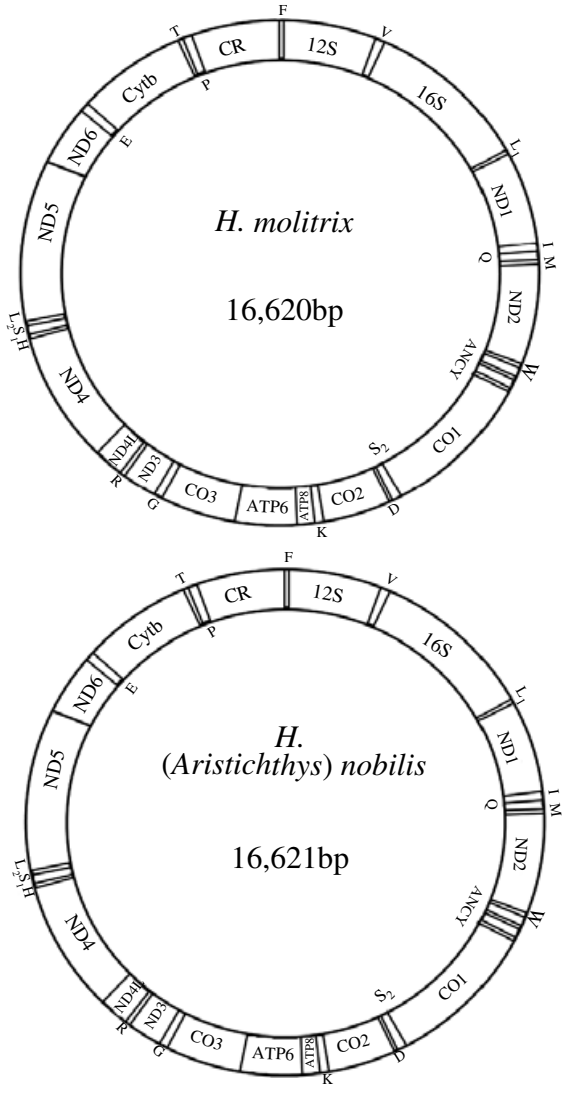

FIG. 1. The mitochondrial genomes of *Hypophthalmichthys molitrix* and *Hypophthalmichthys (Aristichthys) nobilis*. L-strand is designated on the inside and H-strand on the outside of the molecule. 12S and 16S, genes of the 12S and 16S ribosomal RNA; ND1-6 and 4 L, nicotinamide adenine dinucleotidede hydrogenase subunits 1-6 and 4 L; COI-III, cytochrome *c* oxidase subunits I-III; ATP6 and ATP8, ATPase subunits 6 and 8; cyt*b*, cytochrome *b*; CR, control region; tRNAs are designated by single-letter amino acid codes except leucine and serine, which are labelled as L_1(tRNA$^{Leu(UUR)}$), L_2(tRNA$^{Leu(CUN)}$), S_1(tRNA$^{Ser(AGN)}$) and S_2(tRNA$^{Ser(UCN)}$).

conserved region with 375–575 bp. The third domain consists of three conserved blocks (CSB-1, CSB-2, and CSB-3) and contains a TA-dinucleotide microsatellite repeat. Sequence similarity between D-loop regions of both species is 89·4%.

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TABLE II. Organization and profile of the mitochondrial genomes for *Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis**

	Position number			Codon			
	Start	Stop	Size (bp)	Start	Stop	Intergenic nucleotide	Strand
$tRNA^{Phe}$	1	69	69				H
12SrRNA	70/71	1031/1029	962/959			0/1	H
$tRNA^{Val}$	1032/1030	1103/1101	72			0	H
16S rRNA	1104/1102	2794/2792	1691			0	H
$tRNA^{Leu(UUR)}$	2795/2793	2870/2868	76			1/0	H
ND1	2872/2870	3846/3844	975	ATG	TAA / T++	4	H
$tRNA^{Ile}$	3851/3849	3922/3920	72			−2	H
$tRNA^{Gln}$	3921/3919	3991/3989	71			1	L
$tRNA^{Met}$	3993/3991	4061/4059	69			0	H
ND2	4062/4060	5108/5106	1047	ATG	TAG	−2	H
$tRNA^{Trp}$	5107/5105	5177/5175	71			1	H
$tRNA^{Ala}$	5179/5177	5247/5245	69			1	L
$tRNA^{Asn}$	5249/5247	5321/5319	73			32	L
$tRNA^{Cys}$	5354/5352	5421/5419	68			−/1	L
$tRNA^{Tyr}$	5423/5421	5493/5491	71			1	L
CO I	5495/5493	7045/7043	1551	GTG	TAA	0	H
$tRNA^{Ser(UCN)}$	7046/7044	7116/7114	71			3	L
$tRNA^{Asp}$	7120/7118	7193/7191	74			13	H

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TABLE II. Continued

	Position number			Codon			
	Start	Stop	Size (bp)	Start	Stop	Intergenic nucleotide	Strand
CO II	7207/7205	7897/7895	691	ATG	T++	1/0	H
$tRNA^{Lys}$	7898/7896	7973/7971	76			1	H
ATPase8	7975/7973	8142/8137	168/165	ATG	TAA	−10/−7	H
ATPase6	8133/8131	8816/8814	684	ATG	TAA	−1	H
CO III	8816/8814	9601/9599	786	ATG	TAA	−1	H
$tRNA^{Gly}$	9601/9599	9673/9671	73			0	H
ND3	9674/9672	10024/10022	351	ATG	TAG	−2	H
$tRNA^{Arg}$	10023/10021	10091/10090	69/70			−2/0	H
ND4L	10092/10091	10388/10387	297	ATG	TAA	−8/−7	H
ND4	10382/10381	11764/11763	1383	ATG	TAA	−1	H
$tRNA^{His}$	11764/11763	11832/11831	69			0	H
$tRNA^{Ser(AGN)}$	11833/11832	11901/11900	69			1	H
$tRNA^{Leu(CUN)}$	11903/11902	11975/11974	73			0	H
ND5	11976/11975	13811/13810	1836	ATG	TAA	−4	H
ND6	13808/13807	14329/14328	522	ATG	TAA	0	L
$tRNA^{Glu}$	14330/14329	14398/14397	69			4	L
Cytb	14403/14402	15542/15542	1141	ATG	T++	1/4	H
$tRNA^{Thr}$	15544/15543	15615/15614	72			−1/0	H
$tRNA^{Pro}$	15615/15614	15684/15683	70			0/ −1	L
D-loop	15685/15684	16620/16621	936/938			0	H

*Forward slashes (/) denote values of *H. molitrix*/*H. nobilis*; otherwise, both are identical.

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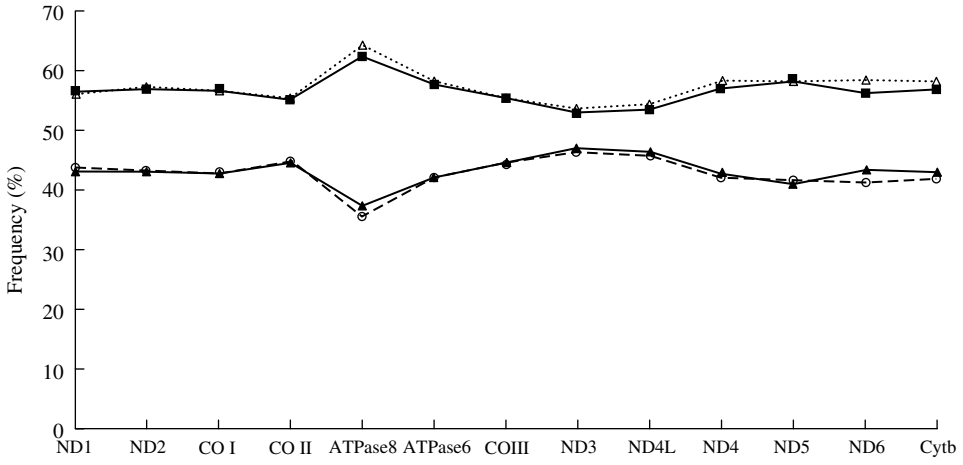

FIG. 2. Comparison of base composition in the mitochondrial protein-coding genes between *Hypophthalmichthys molitrix* [A + T (···△···) and C + G (– –○– –)] and *Hypophthalmichthys nobilis* [A + T (—■—) and C + G (—▲—)].

In both *H. molitrix* and *H. nobilis*, a small non-coding region of 42 bp, the putative origin of light strand replication (O_L), is located in a cluster of $tRNA^{Trp}$–$tRNA^{Ala}$–$tRNA^{Asn}$–$tRNA^{Cys}$–$tRNA^{Tyr}$ region (*ie*. the WANCY region, a hot spot for gene order rearrangements). This region has the potential to fold into a stable stem–loop secondary structure with 19 bp in the stem and 11 bp in the loop. The conserved motif 5′-GCCGG–3′, located at the bottom of the $tRNA^{Cys}$ stem is regarded to be associated with the transition from RNA synthesis to DNA synthesis, (Hixson *et al*., 1986).

RIBOSOMAL AND TRANSFER RNA GENES

The mitochondrial genomes of *H. molitrix* and *H. nobilis* contain two subunits of ribosomal RNA, a small one (12S) and a large one (16S). These two subunits are separated by $tRNA^{Val}$. The length of the 12S rRNA gene is 962 bp for *H. molitrix* and 949 bp for *H. nobilis*. This length is similar in other fish species (Chang *et al*., 1994; Murakami *et al*., 1998; Guo *et al*., 2006; Mabuchi *et al*., 2006). The 16S rRNA gene has the same length (1691 bp) in both *H. molitrix* and *H. nobilis*. Length variation has been, however, found in other species such as *T. tinca*, *C. carpio* and *C. auratus* (Chang *et al*., 1994; Murakami *et al*., 1998; Guo *et al*., 2006; Mabuchi *et al*., 2006). The average sequence similarity of mitochondrial rRNA genes between the two species is 98·6%.

Twenty-two tRNA genes are interspersed by the rRNA and protein-encoding genes and their lengths range from 68 to 76 bp. There are overlaps between adjacent tRNA genes, *e.g*. one nucleotide overlapping between $tRNA^{Thr}$ and $tRNA^{Pro}$, two between $tRNA^{Ile}$ and $tRNA^{Gln}$ and three between $tRNA^{Cys}$ and $tRNA^{Tyr}$. The average A+T content of tRNAs is 56·1% for *H. molitrix* and 55·8% for *H. nobilis*, respectively. These values are higher than those for rRNA genes (54·9% in *H. molitrix* and 54·7% in *H. nobilis*), but lower than those for the D-loop region (68·5% in *H. molitrix* and

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67·4% in *H. nobilis*) (Table III). Sequence similarity between tRNA genes for the two species was 98·8% on average.

TAXONOMIC AND PHYLOGENETIC ANALYSIS OF *H. MOLITRIX* AND *H. NOBILIS*

The JTT distance estimated based on mitochondrial protein sequences between *H. molitrix* and *H. nobilis* is 0.009. The intrageneric JTT distance for 19 species that belong to eight genera (*Barbus, Carassius, Cyprinella, Hemibarbus, Labeo, Opsariichthys, Puntius* and *Rhodeus*) was estimated to be 0.063 on average, ranging from 0.003 to 0.193. In terms of intergeneric analysis, the distance for 32 species that belong to distinct cyprinid genera was estimated to be 0.093 on average, ranging from 0.01 to 0.288. The genetic distances among 51 species suggest that *H. molitrix* and *H. nobilis* be placed in the same genus. The ML phylogenetic tree of 53 cyprinids reveals that the number of amino acid substitutions, as denoted by branch length between *H. molitrix* and *H. nobilis*, is relatively small compared with species that belong to different cyprinid genera [Fig. 3(a)]. In addition, the monophyletic relationship between these two carps is highly supported by the bootstrap values as well as the Bayesian posterior probabilities [Fig. 3(b)].

DISCUSSION

In this study, the mitochondrial genomes of *H. molitrix* and *H. nobilis* were revealed to comprise the same number of genes and have an identical structure. This is consistent with the observations that most animal mitochondrial genomes contain the same 37 genes and that the gene order is highly conserved among vertebrates (Broughton *et al*., 2001). There was only 1 bp difference found between the two mitogenomes, with the whole length close to those of other fish species (Chang *et al*., 1994; Murakami *et al*., 1998; Broughton *et al*., 2001; Wang *et al*., 2008). Both mitogenomes contain a number of overlaps between adjacent genes,

TABLE III. Base composition (%) of mitochondrial genes (or regions) for *Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis*

	Gene/fragment*				
Genes(regions)	A	C	G	T	A+T
Protein coding	31.1/30.8	28.5/28.6	14.3/14.5	26.1/26.0	57.2/56.8
First	29.1/28.7	26.8/26.8	23.6/23.9	20.5/20.6	49.6/49.3
Second	20.1/20.1	27.5/27.5	13.7/13.7	38.7/38.8	58.8/58.9
Third	44.1/43.6	31.2/31.6	5.5/6.1	19.2/18.7	63.3/62.3
tRNA	30.5/30.3	24.2/24.3	19.7/19.9	25.6/25.5	56.1/55.8
srRNA	34.8/34.8	24.3/24.2	20.8/21.1	20.1/19.9	54.9/54.7
D-loop	34.2/35.4	18.6/20.6	12.9/12.0	34.3/32.0	68.5/67.4
Overall	31.8/31.6	26.9/27.1	15.7/16.0	25.6/25.3	57.4/56.9

*Values of *H. molitrix*/*H. nobilis*.

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indicating the high efficiency in RNA transcription and protein translation (Anderson *et al*., 1981). In protein-coding genes, a strong bias (*c*. 6 %) against G has been observed in both carps as well as other vertebrates (Broughton *et al*., 2001). The control region is involved in the regulation of replication and transcription (Clayton, 1982, 1991; Shadel & Clayton, 1997). In *H. molitrix* and *H. nobilis*, extensive variability was found in the control region, but several regulatory elements were conserved, demonstrating that the secondary or the tertiary structures rather than the primary structure (*i.e*. sequence) are important in regulating RNA transcription and DNA replication.

One of the salient features in this study is to explore the boundary of genetic divergence at the genus level. Concatenated protein sequences of the two carps as well as 19 intrageneric and 32 intergeneric cyprinid species were analysed. The genetic distance between *H. molitrix* and *H. nobilis* (0.009) is below the smallest distance estimated among the intergeneric species (0.01 between *Chanodichthys mongolicus* (Basilewsky) and *Megalobrama amblycephala* Yih, but within the genetic distances among intrageneric species (0.003–0.193). This provides the first molecular evidence in support of classifying silver and bighead carps in the same genus, *i.e*. *Hypophthalmichthys*. From the viewpoint of reproductive isolation, *H. molitrix* and *H. nobilis* are genetically closely related to each other. In portions of the introduced ranges of *H. molitrix* and *H. nobilis*, it has been observed that the two species can hybridize and produce fertile offspring (Verigin *et al*., 1979; Kolar *et al.,* 2007; Chapman *et al*., in press). This would partially support placement of *H. molitrix* and *H. nobilis* in the same genus. There are a great number of cases, however, where hybridization occurs between species from different genera (Scribner *et al*., 2001).

In addition, there is a great deal of information that seems to support the viewpoint of placing *H. molitrix* and *H. nobilis* into two different genera. Besides the work by *Oshima* (1919), a series of previous studies revealed significant differences in ecological, biological and biochemical, as well as molecular characteristics between *H. molitrix* and *H. nobilis* (Li *et al*., 1997, 1998). For example, the native range of *H. molitrix* extends approximately from 20 to 54° N, covering the Red River (northern Vietnam), Zhujiang (Pearl) River (southern China) and north to the Heilongjiang (Amur) River (the China–Russia border); the native range of *H. nobilis* is more narrow, approximately from 21 to 40° N, covering the Zhujiang River north to the Huanghe (Yellow) River (northern China), but not to the Heilongjiang River (Li, 1996). The karyotypes also differ: *H. molitrix* has the karyotype 10m + 9sm + 5st, while *H. nobilis* has the karyotype 12m + 9m + 3st. They also differ in isozyme electrophoresis patterns. For instance, the relative activity of A1, A2B1, A2B2, A1B3 and B4 of LDH in muscle is 45·0, 32·3, 18·6, 2·8, and 1·4% in *H. molitrix*, but 54·1, 26·0, 15·9, 2·0, and 1·9% in *H. nobilis* (National Inspection Bureau for Quality and Technology, People's Republic China, 1999*a*, *b*). Chapman *et al*. (in press) recently identified 22 different morphometric ratios that were significantly different between *H. molitrix* and *H. nobilis*. For five of the ratios, the range of observed values did not overlap between the two species in a comparison of >100 fish of each species.

One major concern pertaining to the use of mtDNA in resolving phylogeny is that the entire mitogenome is essentially a single locus; linkage of all mitochondrial genes might increase systematic errors, *e.g*. compositional biases (Gadagkar *et al*.,

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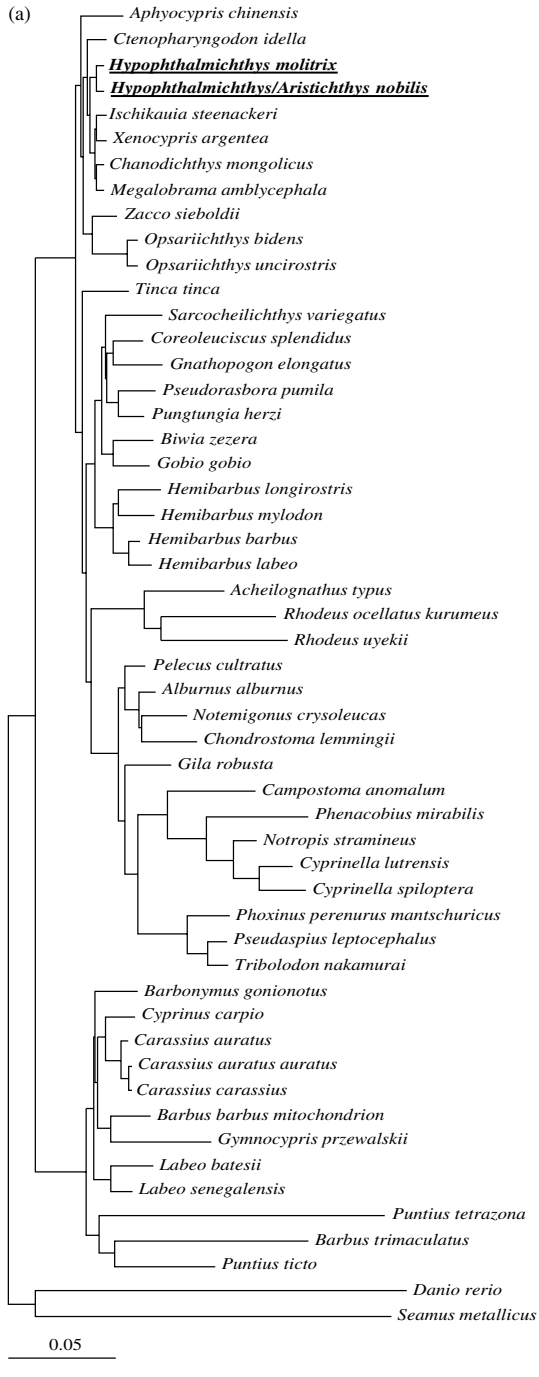

FIG. 3. Maximum likelihood (ML) phylogenetic trees of 53 cyprinid fishes; including *Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis* inferred from concatenated mitochondrial protein sequences. (a) Neighbour-joining (NJ) tree with branches scaled by 0·05 substitution per site and (b) majority rule tree (50%) with the numbers representing bootstrap values from the NJ and ML analyses and posterior probabilities from the Bayesian analysis, respectively.

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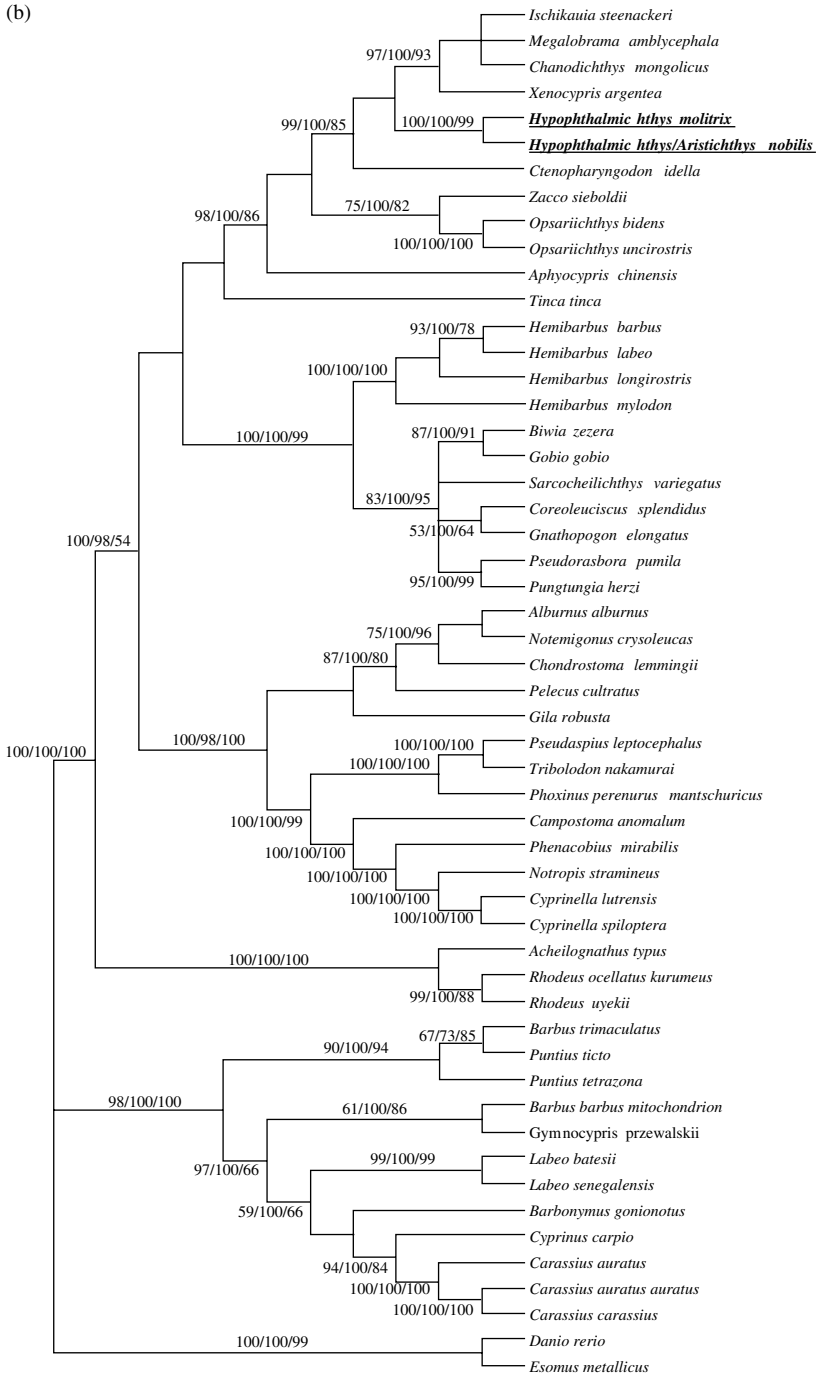

FIG. 3. Continued.

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2005). A more robust strategy is therefore to use combined datasets of complete mitogenomes and nuclear genes for the study of species phylogeny (Miya *et al*., 2007). In addition, another species, large-scale silver carp *Hypophthalmicthys harmandi Sauvage* is native to the Red River of northern Vietnam and the Hainan Island of southern China and is closely related to *H. molitrix*. Therefore, in order to attain a unified taxonomy for *H. molitrix* and *H. nobilis* at the generic level, more molecular markers such as nuclear genes, more closely related species such as *H. harmandi*, and systematic combination between traditional biological and molecular biological studies are required for further investigations in the future.

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References

Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijin, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, C. D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R. & Young, I. G. (1981). Sequence and organization of the human mitochondrial genome. *Nature* **290,** 457–65.

Broughton, R. E., Milam, J. E. & Roe, B. A. (2001). The complete sequence of the zebrafish (*Danio rerio*) mitochondrial genome evolutionary patterns in vertebrate mitochondrial DNA. *Genome Research* **11,** 1958–1967.

Chang, Y. S., Huang, F. L. & Lo, T. B. (1994). The complete nucleotide sequence and gene organization of carp (*Cyprinus carpio*) mitochondrial genome. *Journal of Molecular Evolution* **38,** 138–155.

Chapman, D. C., Deters, J. E. & King, T. (in press). Occurrence of bighead carp X silver carp hybrids in the Missouri River, and an examination of morphological methods for determination of hybridization and sex. In *Proceedings of the Asian Carp Symposium* (Chapman, D. C. & Hoff, M., eds). Bethesda, MD: American Fisheries Society.

Cheng, Q. T. & Zheng, B. S. (1987). *Systematic Synopsis of Chinese Fishes* Beijing, China: Science Press (in Chinese).

Clayton, D. A. (1982). Replication of animal mitochondrial DNA. *Cell* **28,** 693–705.

Clayton, D. A. (1991). Nuclear gadgets in mitochondrial DNA replication and transcription. *Trends in Biochemical Sciences* **16,** 107–111.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32,** 1792–1797.

FAO (2005). *Yearbook of Fishery Statistics 2005–Aquaculture Production*. Rome, Italy: Food and Agriculture Organization.

Fan L. C., Cui, J. X. & Yu, Q. X. (1994). Restriction endonuclease map of mtDNA of *Aristichthys nobilis*. *Journal of Wuhan University* (*Natural Science Edition*) **40,** 121–125 (in Chinese).

Gadagkar, S. R., Rosenberg, M. S. & Kumar S. (2005). Inferring species phylogenies from multiple genes: concatenated sequence tree versus consensus gene tree. *Journal of Experimental Zoology B* **304,** 64–74.

Geng, B., Sun, X. W., Lian, L. Q., Ouyang, H. S. & Tong, J. G. (2006). Microsatellite analysis of genetic diversity of *Aristichthys nobilis* in China. *Hereditas* (Beijing) **28,** 683–688 (in Chinese).

Giribet, G., Edgecombe, G. D. & Wheeler W. C. (2001). Arthropod phylogeny based on eight molecular loci and morphology. *Nature* **413,** 157–161.

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Journal compilation © 2009 The Fisheries Society of the British Isles,

Guo, X., Liu, S. & Liu, Y. (2006). Evidence for recombination of mitochondrial DNA in triploid crucian carp. *Genetics* **172,** 1745–1749.

Hall, T. A. (1998). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acid Symposium Series* **41,** 95–98.

Hedges, S. B. & Poling, L. L. (1999). A molecular phylogeny of reptiles. *Science* **283,** 998–1001.

Hixson, J. E., Wong, T. W. & Clayton, D. A. (1986). Both the conserved and divergent 5'-flanking sequences are required for initiation at human mitochondrial origin of light strand replication. *Journal of Biological Chemistry* **261,** 2384–2390.

Howes, G. (1981). Anatomy and phylogeny of the Chinese major carps *Ctenopharyngodon* Steind., 1866 and *Hypophthalmichthys* Blkr., 1980. *Bulletin of the British Museum of Natural History and Zoology* **41,** 1–52.

Jian, J. C. & Xia, D. Q. (1999). Cloning of minisatellite DNA from bighead carp. *Journal of Fishery Science of China* **6,** 18–20 (in Chinese).

Jiang, J. G., Xiong, Q. W. & Yao, R. H. (1998). Comparative studies on isoensymes of black carp, grass carp, silver carp and bighead carp. *Hereditas* (Beijing) **20,** 19–22 (in Chinese).

Jones, D. T., Taylor, W. R. & Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *CABIOS* **8,** 275–282.

Kartavtsev, Y. P., Jung, S. O., Lee, Y. M., Byeon, H. K. & Lee, J. S. (2007). Complete mitochondrial genome of the bullhead torrent catfish, *Liobagrus obesus* (Siluriformes, Amblycipididae): Genome description and phylogenetic considerations inferred from the Cytb and 16S rRNA genes. *Gene* **396,** 13–27.

Keane, T. M., Naughton, T. J. & McInerney, J. O. (2007). MultiPhyl: a high-throughput phylogenomics webserver using distributed computing. *Nucleic Acids Research* **35,** 33–37.

Kolar, C. S., Chapman, D. C., Courtenay, W. R., Housel, C. M., Williams, J. D. & Jennings, D. P. (2007). Bigheaded carps, a biological synopsis and environmental risk assessment. *American Fisheries Society Special Publication* **33**.

Li, S. F. (1996). *Germplasm Resources and Conservation of Freshwater Fishes in China*. Beijing, China: China Agriculture Press (in Chinese).

Li, S. F., Zhou, B. Y., Lu, G. Q., Zhao, J. L, Yao, D. X. & Shen, W. K. (1997). A study on the criteria and inspection of brooders of silver carp, bighead carp, grass carp and black carp originated from the Yangtze River. *Journal of Fisheries of China* **21,** 143–151 (in Chinese).

Li, S. F. (Ed.) (1998). *Genetic Characterization of Major Freshwater Culture Fishes in China*. Shanghai, China: Shanghai Scientific & Technical Publishers (in Chinese).

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,** 955–964.

Lu, G. Q., Li, S. F. & Bernatchez, L. (1997). Mitochondrial DNA diversity, population structure and conservation genetics of four native carps within the Yangtze River, China. *Canadian Journal of Fisheries and Aquatic Sciences* **54,** 47–58.

Lu, C. Y., Sun, X. W. & Lian, L. Q. (2005). Isolation of microsatellite markers in bighead carp *Aristichthys nobilis*. *Journal of Fishery Science of China* **12,** 192–196 (in Chinese).

Mabuchi, K., Miya, M., Senou, H., Suzuki, T. & Nishida, M. (2006). Complete mitochondrial DNA sequence of the Lake Biwa wild strain of common carp (*Cyprinus carpio* L.): further evidence for an ancient origin. *Aquaculture* **257,** 68–77.

Miya, M., Takeshima, H., Endo, H., Ishiguro, N. B., Inoue, J. G., Mukai, T., Satoh, T. P., Yamaguchi, M., Kawaguchi, A., Mabuchi, K., Shirai, S.M. & Nishida, M. (2003). Major patterns of higher teleostean phylogenies: a new perspective based on 100 complete mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* **26,** 121–138.

Miya, M., Holcroft, N., Satoh, T. P., Yamaguchi, M. & Nishida, M. (2007). Mitochondrial genome and a nuclear gene indicate a novel phylogenetic position of deep-sea tube-eye fish (Stylephoridae). *Ichthyological Research* **54,** 323–332.

© 2009 The Authors

Murakami, M., Yamashita, Y. & Fujitani, H. (1998). The complete sequence of mitochondrial genome from a gynogenetic triploid 'ginbuna' (*Carassius auratus langsdorfi*). *Zoological Science* **15,** 335–337.

National Inspection Bureau for Quality and Technology, People's Republic of China (1999*a*). *National Standard for Silver Carp*. GB 17717: 1999 (in Chinese).

National Inspection Bureau for Quality and Technology, People's Republic of China (1999*b*). *National Standard for Bighead Carp*. GB 17718:1999 (in Chinese).

Oshima M. (1919). Contributions to the study of freshwater fishes of the island of Formosa. *Annals of the Carnegie Museum* **12,** 169–328.

Peng Z., Wang, J. & He X. (2006). The complete mitochondrial genome of the helmet catfish *Cranoglanis bouderius* (Siluriformes: Cranoglanididae) and the phylogeny of otophysan fishes. *Gene* **376,** 290–297.

Ronquist, F. & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19,** 1572–1574.

Saitoh, K., Sado, T., Mayden, R. L., Hanzawa, N., Nakamura, K., Nishida, M. & Miya, M. (2006). Mitogenomic evolution and interrelationships of the Cypriniformes (*Actinopterygii: Ostariophysi*): the first evidence toward resolution of higher-level relationships of the world's largest freshwater fish clade based on 59 whole mitogenome sequences. *Journal of Molecular Evolution* **63,** 826–841.

Sambrock, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual* 3rd edn. New York, NY: Cold Spring Harbor Laboratory Press.

Scribner, K. T., Page, K. S. & Bartron, M. L. (2001). Hybridization in freshwater fishes: a review of case studies and cytonuclear methods of biological inference. *Reviews in Fish Biology and Fisheries* **10,** 293–323.

Shadel, G. S. & Clayton, D. A. (1997). Mitochondrial DNA maintenance in vertebrates. *Annual Review of Biochemistry* **66,** 409–435.

Shan, Q., Dong, S., Wu, H. F. & Taniguchi, N. (2006). Diversity analysis on mtDNA D-loop region of three populations of *Aristichthys nobilis*. *Journal of Fishery Science of China* **13,** 174–180 (in Chinese).

Tamura K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24,** 1596–1599.

Verigin, B. V., Makeeva, A. P. & Shubnikova, N. G. (1979). A case of natural hybridization between *Hypophthalmichthys molitrix* and *Aristichthys nobilis* (Pisces, Cyprinidae). *Zoologicheskii Zhurnal* **58,** 190–196.

Wang, C. H., Chen, Q., Lu, G., Xu, J. W., Yang, Q. L. & Li, Si. F. (2008). Complete mitochondrial genome of the grass carp (*Ctenopharyngodon idella*, Teleostei): insight into its phylogenic position within Cyprinidae. *Gene* **424,** 96–101.

Wu, X. W. (1964). *Fishes of Cyprinidae in China*, Vol. 1. Shanghai, China: Shanghai Scientific Press (in Chinese).

Xia, D. Q., Yan, H., Wu, T. T., Dong, Z. J., Jian, J. C., Cao, Y. & Zhang, Y. S. (1996). Study on the population genetic structure of black carp, grass carp, silver carp and bighead carp in Tian-E-Zhou open old course of the Yangtze River. *Journal of Fishery Science of China* **3,** 11–18 (in Chinese).

Zhang, D. C. (2002). Study on the genetic diversity of cultivated population of bighead carp (*Aristichthys nobilis*). *Journal of China Three Gorges University (Natural Sciences)* **24,** 379–381 (in Chinese).

Zhang, S. M., Deng, H. & Wang, D.Q. (2001). Population structure and genetic diversity of silver carp and grass carp from populations of the Yangtze River system revealed by RAPD. *Acta Hydrobiologica Sinica* **25,** 324–330 (in Chinese).

Zhang, S. M., Wang, D. Q., Deng, H. & Yu, L. N. (2002). Mitochondrial DNA variation of silver carp and grass carp of middle reaches of the Yangtze River revealed by using PCR-AFLP. *Acta Hydrobiologica Sinica* **26,** 142–147 (in Chinese).

Zhang, X. Y., Yang, J. Q., Zhang, D. C., Deng, F. J., Yu, L. N. & Fang, Y. L. (1999a). RAPD analysis on *Hypophthalmichthys molitrix* and *Anistchthys noblils*. *Progress in Biochemistry and Biophysics* **26,** 469–472 (in Chinese).

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Zhang, X. Y., Zhang, X. Y., Yang, D. S., Yu, L. N., Fang, Y. L., Deng, F. J. & Liu, S. Y. (1999b). Studies on genetic diversity of bighead carp (*Aristichthys nobilis*) in the Yangtze River. *Journal of Wuhan University (Natural Science Edition)* **45,** 857–860 (in Chinese).

Zhao, J. L. & Li, S. F. (1996). Isoenzyme analysis of population diversity of silver carp, bighead carp, grass carp and black carp in the middle and low stream of Changjiang River. *Journal of Fisheries of China* **20,** 104–110 (in Chinese).

Zhu, X. D., Geng, B., Li, J. & Sun, X. W. (2007). Analysis of genetic diversity among silver carp populations in the middle and lower Yangtze River using thirty microsatellite makers. *Hereditas* **29,** 705–713 (in Chinese).

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