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ANIMAL GENETICS

# DNA Barcoding of Fishes in Irtysh River China[1]

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**Abstact**—DNA barcoding was a molecular diagnostic method that provided rapid and accurate species identification. The 650 bp-length cytochrome c oxidase subunit I (COI) gene of 33 species in Irtysh River China was sequenced and analyzed in this study. The average intra-species, -genus, -family, and -order of Kimura two parameter (K2P) distances were 0.003, 0.060, 0.163 and 0.240, respectively. The genetic distance between genus *Barbatula* and *Cobitis* was the largest whereas that between genus *Hypophthalmichthys* and *Aristichthys* was the smallest. The neighbour-joining tree constructed by all 44 haplotypes was divided into two major clusters: Cypriniformes fishes and other fishes. A cryptic species of *Barbatula barbatula* was detected according to 2% genetic threshold.

*Keywords:* Irtysh River, fishes DNA barcoding, species identification, cryptic species

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## INTRODUCTION

Irtysh River that locates in northern Xinjiang is the only international river belonging to Arctic Ocean water system in China. The length is 633 km (4248 km in total) with the basin area of 57.000 square kilometers in China. Kuyierte and Kayierte River, two upstream tributaries of Irtysh River originate from the Qigeertai Daban in southern slope of the Altai Mountains, after flowing through the confluence Temeke it is called Irtysh River. The river runs into the Zaysan Lake in Kazakhstan, flows northward through Ob River in Russia and finally disembogues into the Kara Sea of the Arctic Ocean [1].

The studies of fish in Irtysh River hadn't been carried out until 1960's. The book *China's economic animals: Freshwater Fishes* and *The cyprinid fishes of China* gave briefly descriptions of fishes in Irtysh River [2, 3]; Li et al. investigated the river and discovered twenty-three kinds of fishes, including a new subspecies and five new recorded species in China [4], and the book *Fishes of Xinjiang* published in 1979 further described details of these fishes on this basis [5]. Sporadic surveys were conducted during the next twenty years. Up till the early 2000, a systematic and comprehensive investigation of fish resources in this river was carried out over the course of two years. More than five thousand fish specimens were collected and analyzed, a total of 35 fish (subspecies) species belonging to 31 genera, 12 families and 6 orders, including 23 indigenous fish (subspecies) species were found in Irtysh River system [1].

It was proposed that the sequence of a single gene region could be used as the basis of a global bio-identification system for animals and plants [6, 7]. Extensive researches confirmed that an approximately 650bp-long sequence of mitochondrial cytochrome c oxidase subunit I (COI) gene was more suitable for DNA barcoding than other genes [8–11]. It has advantages in species identification from just a single collection, highlighting cases of range expansion for known species, flagging previously overlooked species and enabling identifications where traditional morphology methods cannot be applied [12]. It is suggested that DNA barcodes could separate about 98 and 93% of already described marine and freshwater fish species, respectively [13]. Genetic researches of fishes in Irtysh River basin have obtained some achievements at present [14–18], but DNA barcoding analysis has not been reported so far. In this study, 18 species were collected and DNA barcodes were analyzed from 83 samples in order to fill up the blank of fish identification in Irtysh River, accumulate DNA barcoding data and provide protection and utilization references for fish resources research.

[1] The article is published in the original.

**Table 1.** Latin name, taxonomic status and GenBank accession number

| Species | Taxonomic status | Source | GenBank accession number |
|---|---|---|---|
| | | Acipenseriformes | |
| *Acipenser baeri* | Acipenseridae | Genbank | NC017603 |
| *Acipenser ruthenus* | | Genbank | NC022453 |
| | | Salmoniformes | |
| *Hucho taimen* | | Genbank | KJ711550 |
| *Brachymystax lenok* | Salmonidae | This study | KT716377 |
| *Stenodus leucichthys nelma* | | Genbank | JX960967 |
| *Thymallus arcticus arcticus* | Thymallidae | This study | KT716357, KT716358 |
| *Hypomesus olidus* | Osmeridae | This study | KT716362, KT716363 |
| *Protosalanx hyalocranius* | Salangidae | Genbank | NC024109 |
| *Esox lucius* | Esocidae | This study | KT716353 |
| | | Cypriniformes | |
| *Phoxinus Phoxinus ujmonensis* | | This study | KT716375 |
| *Tinca tinca* | | This study | KT716360 |
| *Abramis brama orientalis* | | This study | KT716372, KT716373, KT716374 |
| *Rutilus rutilus lacustris* | | This study | KT716365 |
| *Ctenopharyngodon idellus* | | Genbank | JN673561 |
| *Leuciscus leuciscus baicalensis* | | This study | KT716354, KT716355, KT716356 |
| *Leuciscus idus* | | This study | KT716361 |
| *Pseudorasbora parva* | Cyprinidae | Genbank | KJ415113 |
| *Gobio gobio acutipinnafus* | | This study | KT716366, KT716367 |
| *Abbottina rivularis* | | Genbank | KM081703 |
| *Cyprinus carpio* | | Genbank | KT716369 |
| *Carassius carassius* | | Genbank | JQ911695 |
| *Carassius auratus gibelio* | | This study | KT716381, KT716382 |
| *Aristichthys nobilis* | | Genbank | KJ746966 |
| *Hypophthalmichthys molitrix* | | Genbank | NC010156 |
| *Barbatula barbatula nuda* | | This study | KT716378, KT716379, KT716380 |
| *Triplophysa strauchii* | Cobitidae | Genbank | KP297875 |
| *Cobitis granoei* | | Genbank | KF908768 |
| | | Gadiformes | |
| *Lota lota* | Gadidae | This study | KT716368 |
| | | Perciformes | |
| *Perca fluviatilis* | | This study | KT716364 |
| *Lucioperca lucioperca* | Percidae | This study | KT716376 |
| *Acerina cernua* | | This study | KT716370, KT716371 |
| *Hypseleotris swinhonis* | Eleotridae | Genbank | NC021763 |
| | | Scorpaeniformes | |
| *Cottus sibiricus altaicus* | Cottidae | This study | KT716359 |

## MATERIALS AND METHODS

### *Sample Collections*

Eighty-three fish specimens were captured from Irtysh River in China in April 2014 (Table 1). Fresh fins were removed and stored in 95% ethyl alcohol immediately. All samples were frozen at −20°C until performing the experiments. In addition, fifteen COI gene sequences were also downloaded from Genbank for analysis.

### *DNA Extraction and PCR*

Genomic DNA was extracted by Phenol Tris-Chloroform method [19]. Fragments of the 5' region of mitochondrial COI gene were amplified with the primers FishF1 (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and FishR1 (5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3') [20]. The PCR reaction was performed in a volume of 50 μL containing 50 ng template DNA, 5 μL 10 reaction buffer, 4 μL dNTPs (10 mM), 1 μL each primer (20 μM) and 2 U

Taq DNA polymerase (Takara). The parameters of PCR amplifications consisted of an initial denaturation at 94°C for 5 minutes followed by 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 45 s, a final extension at 72°C for 10 minutes and then held at 4°C. Amplification products were detected by 1.0% agarose gel electrophoresis. Meanwhile, negative controls (template-free PCR reactions) were carried out to assure the fidelity of PCR reactions. PCR products were purified with the Gel Midi purification Kit (Tiangen Biotech) and bidirectionally sequenced using Sanger method.

### *Sequence Verification and Submission*

Sequence homology retrieval was preliminary conducted using BLAST (Basic Local Alignment Search Tool), to ensure that the obtained sequence was the target one. Taxonomic status or valid name was verified in BOLD Identification System (IDS) further. Finally sequences were submitted to NCBI and GenBank numbers were obtained (Table 1).

### *Data Analysis*

Sequences were edited and aligned using DNASTAR Larsergene package (Version 7.1.0). Nucleotide composition and average Kimura two parameter (K2P) distances [21] were calculated by MEGA software (Version 6.0) [22]. Neighbor-joining (NJ) tree based on K2P distance was constructed to provide a graphic representation of divergence and phylogenetic relationships between species by bootstrapping with 1000 replications in MEGA.

## RESULTS

### *Sequence Characteristic and K2P Distance Analysis*

All sequences were aligned and no nucleotide insertions or deletions were found. A length of 650 bp COI gene sequence segment was obtained. Forty-four haplotypes belonging to 33 species, 30 genera and 12 families have been detected. Overall nucleotide frequencies were 29.3% T, 28.3% C, 23.8% A and 18.6% G. The average nucleotide compositions of different families were showed in Table 2. G content was the lowest of all except for family Osmeridae, and C content was the highest in Acipenseridae, Salangidae and Cottidae, while T content was the highest in the rest. GC content was lower than AT content in most families, but Acipenseridae and Salangidae were just the opposite. The first-position GC content (GC1) was greater than AT1, while GC2 was lower than AT2. However, there were significant differences in the occurrences of GC contents in the third codon positions among different families. In the present analysis, GC3 content ranged from 32.0 to 61.1%. Interestingly, GC3 was obviously higher than GC1 only in Salangidae (Fig. 1).

**Table 2.** The average nucleotide composition of different families

| Family | Number of species | T | C | A | G | G+C |
|---|---|---|---|---|---|---|
| Acipenseridae | 2 | 26.7 | 30.7 | 23.0 | 19.7 | 50.4 |
| Salmonidae | 3 | 29.6 | 29.2 | 22.7 | 18.5 | 47.7 |
| Thymallidae | 1 | 31.4 | 27.3 | 21.9 | 19.4 | 46.7 |
| Osmeridae | 1 | 30.8 | 28.4 | 20.1 | 20.7 | 49.1 |
| Salangidae | 1 | 25.6 | 34.2 | 20.8 | 19.4 | 53.6 |
| Esocidae | 1 | 31.7 | 27.7 | 23.1 | 17.4 | 45.1 |
| Cyprinidae | 15 | 28.9 | 27.6 | 25.2 | 18.3 | 45.9 |
| Cobitidae | 3 | 30.2 | 28.3 | 23.0 | 18.5 | 46.8 |
| Gadidae | 1 | 29.4 | 28.4 | 23.7 | 18.5 | 46.9 |
| Percidae | 3 | 29.8 | 28.6 | 23.4 | 18.2 | 46.8 |
| Eleotridae | 1 | 32.7 | 25.1 | 23.6 | 18.6 | 43.7 |
| Cottidae | 1 | 28.5 | 30.8 | 22.5 | 18.2 | 49.0 |

**Table 3.** K2P distances within various taxonomic levels

| Comparisons within | Maximum | Minimum | Mean |
|---|---|---|---|
| Species | 0.008 | 0 | 0.003 |
| Genera | 0.065 | 0.053 | 0.060 |
| Families | 0.273 | 0.043 | 0.163 |
| Orders | 0.276 | 0.163 | 0.240 |

Genetic divergences within various taxonomic levels were summarized in Table 3 and Fig. 2. The average K2P distance of individuals within species was 0.003 compared with 0.060 for species within genera, which was about 20 times as large as the former. There was a wide distribution of genetic variation among species within families ranging from 0.043 to 0.273. The mean divergence among species within families was 0.163, and among species within orders it increased to 0.240. Thus, the rate of increase gradually declined with the taxonomic categories becoming higher.

### *Phylogenetic Analysis*

Neighbor-joining phylogenetic tree (Fig. 3) was constructed based on K2P model with 1000 replications of bootstrapping test, and it was obviously divided into two clades: one was the Cypriniformes, and the other one was the rest including Acipenseriformes, Salmoniformes, Gadiformes, Perciformes and Scorpaeniformes. Different species had its specific haplotype, and individuals of the same species clustered together with high support rates.

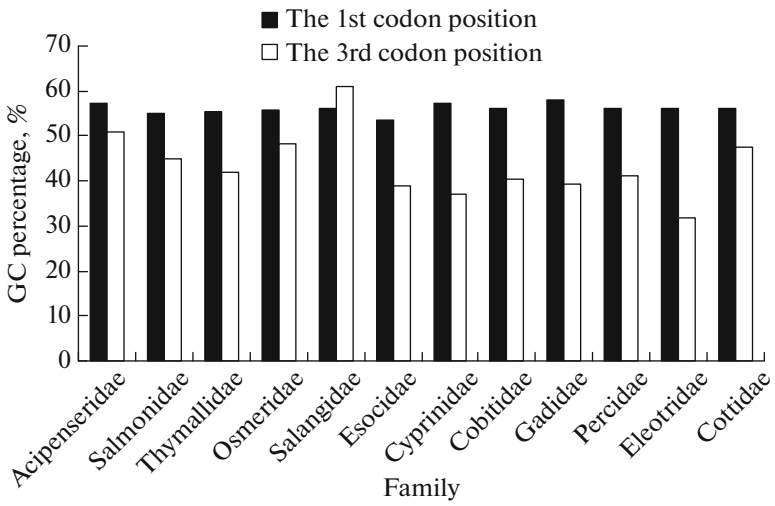


**Fig. 1.** The average GC content of the first and third codon positions for different families.

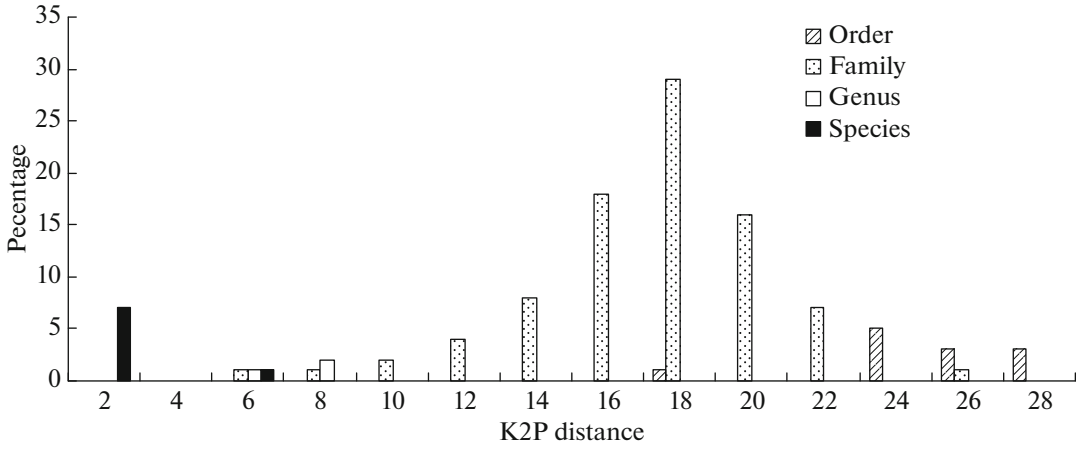


**Fig. 2.** Distribution of K2P distances (percent) for COI gene within different taxonomic categories.

## DISSCUSSION

The concept of DNA barcoding was first proposed by Paul Hebert, the taxonomist of Guelph University in 2003 [6]. Now, it has been an emerging global standard for identifying species using gene sequences and widely used in taxonomic identification and identification of cryptic species. Bickford et al. defined 'cryptic species' like this: two or more species are or have been classified as a single nominal species because they are at least superficially morphologically indistinguishable [23]. Tree-based distance method was considered to be a standard method for the DNA barcoding data analysis. According to this, two percent of genetic threshold was suggested identifying different species [6], and then a standard screening threshold of sequence difference (10 × average intraspecific variations) was proposed to speed the discovery of new animal species [24]. In this article, the mean distances between conspecific and congeneric species reached 0.003 and 0.060. The latter was nearly twenty times larger than the former, and the similar results were obtained in researches on DNA barcoding of other fish faunas. For instance, the congeneric species variation was about 25 times, 31 times and 29 times more than conspecific individuals in Australia, Canada and South Korea fish fauna, respectively [20, 25, 26].

The intraspecific and interspecific genetic difference formed obvious barcoding gap and most species could be identified and distinguished effectively. However, the genetic distance among *Barbatula barbatula* was from 0.002 to 0.087 with the mean genetic distance 0.058, which was much higher than 2% threshold implying the potential existence of a cryptic species in Irtysh River. Beared stone loach was initially recorded as *Nemachilus* (*Barbatula*) *barbatula toni* in Xinjiang [5], and then renamed *B.barbatula nuda* by

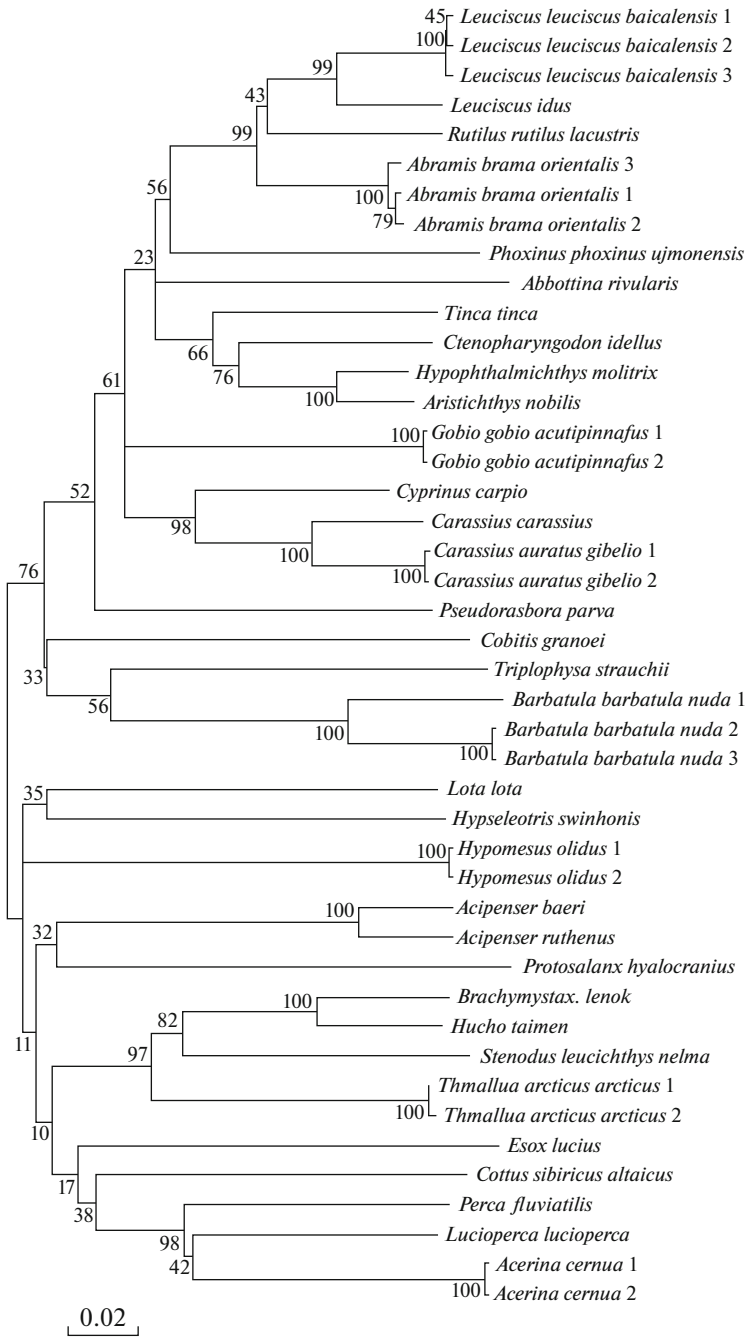


**Fig. 3.** The neighbor-joining tree of forty-four haplotypes based on K2P genetic distances (Bootstrap values were 1000 replications).

Zhu in book *The Loaches of the Subfamily Nemacheilinae in China* [27]. Whether they were synonyms or two different species was still in dispute [28]. According to data of FishBase, the former was mainly distributed in the Ob River, and the latter was found in China, Korea and Japan. Sequences of both two Latin names have been uploaded in Genbank hitherto. The sequence alignment proved that haplotype 2 and 3 had closer relationships with *B. barbatula nuda* (EU670789) from Korea, but distant with *B. barbatula toni* (AB242162) from Russia. It was worth mentioning that haplotype1 clustered with *B. barbatula* (KP715096) from Europe with 100% bootstrap value, which was never reported in fish researches of Irtysh River before. To sum up, we came to a conclusion that there should be two kinds of beard stone loach *B. barbatula* and *B. barbatula nuda* in Irtysh River. In consideration of high genetic variation between them

(above 8%), population genetic analysis should be necessarily adopted for the further study in the future.

K2P genetic distances showed that the rate of increase declined in the higher taxonomic categories due to substitutional saturation. The distribution of genetic divergence within different taxonomic levels varied from 0.002 to 0.276 with some overlaps. The genetic distance between Salmonidae and Thymallidae (0.163) was strikingly lower than average genetic distance among families within order, indicating a close genetic relationship between them. The similar result was also reflected in NJ tree: *Brachymystax lenok, Hucho taimen* and *Stenodus leucichthys nelma* first clustered together and then assembled with *Thymallus arcticus arcticus* with the higher confidence value.

The distribution range of genetic distance among genera within family was the widest. The distance between genus *Barbatula* and *Cobitis* was the largest whereas that between *Hypophthalmichthys* and *Aristichthys* was the smallest. So far, there have long been international controversies in the classification of Cobitidae fishes. Chen and Zhu considered that family Cobitidae contained three monophyletic groups (subfamilies): Cobitinae, Noemacheilinae and Botiinae [29, 30]. Sawada, Nelson and Kottelat suggested transferring subfamily Noemacheilinae from the family Cobitidae to Homalopteridae [31–33]. Nalbant intended to promote the subfamilies Cobitinae, Noemacheilinae and Botiinae to the family level [34]. The relatively large genetic distance between genus *Barbatula* and *Cobitis* belonging to subfamily Noemacheilinae and Cobitinae respectively also proved that they had rather distant phylogenetic relationships.

The taxonomic relationship of Silver carp *Hypophthalmichthys molitrix* and bighead carp *Aristichthys nobilis* at the generic level remained equivocal. Different ichthyologists classified them into either the same genus [35, 36] or two distinct genera [37, 38]. Li et al compared and analyzed the complete mitochondrial genomes of *H. molitrix* and *A. nobilis*, and their results supported that the two species belonged to the genus *Hypophthalmichthys* [39]. In this paper, the K2P distance between them was 0.043, which was lower than average intergeneric genetic distances of cyprinid genera (0.093). From the perspective of molecular systematics, our research trended to agree with the first view, although a set of morphological characters were developed to distinguish *Aristichthys* from *Hypophthalmichthys*.

The distribution of the four nucleotides in the mitochondrial genome was not uniform in the animal mitochondrial genome [40, 41]. Various biases affect such estimates, including GC ratio, codon usage bias, transition-transversion ratio and degree of saturation [42]. In this study, the anti-G bias was observed in the average nucleotide composition of different families. Clayton considered this phenomenon might be due in part to selection against less stable G nucleotides on the light strand, which was exposed as a single strand for a considerable length of time during the asymmetrical replication of mtDNA [43].

In 1980, Grantham et al. proposed "genome hypothesis", which indicated that codon bias was species-specific, namely similar patterns of codon usage were existed within species or among species closely related [44]. The base composition of most freshwater fishes showed a strong AT bias, except for Acipenseridae and Salangidae in this research. The similar results were also found in other researches [45–47], which revealed that these two families perhaps had relatively close genetic relationship. Meanwhile, species of Acipenseridae and Salangidae that clustered together in NJ tree also supported this conclusion.

This study was the first comprehensive molecular assessment and DNA barcoding analysis of fishes in Irtysh River. Our research confirmed that as a DNA barcode, mitochondrial COI gene could be used to effectively identify fishes at the species level in this river. In addition, it also provided a strong evidence for fish larvae and eggs identification. The genetic relationships of Irtysh River fishes among different taxonomic category were clarified. Meanwhile, two species or subspecies of *Barbatula barbatula* might be existed in Irtysh River. It is believed that the research will lay the foundation for the further fish taxonomy and molecular systematics in Irtysh River.

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