Molecular Phylogeny of Korean Loaches Inferred from Mitochondrial DNA Cytochrome b Sequences

So-Young Kim, Ik-Soo Kim, Kwang-Yeop Jahng and Mi-Hee Chang
Division of Biological Sciences, Chonbuk National University, Chonju 561-756, Korea

Phylogenetic relationships between 8 species Korean loaches (Cobitidae) were investigated by comparing mitochondrial cytochrome b gene sequences. However our results are in accordance with finding observed using other morphological studies, new interesting interspecific variation in Korean loaches were found. Orthrias and Lefua appeared to be paraphyletic in Cobitidae observed. Their sequence divergence value was agreed with interfamilic sequence divergences between Cobitidae and Cyprinidae ranged from 0.184 to 0.272. Otherwise, the present results support that two species of Iksookimia and Cobitis melanoleuca were early diverged respectively. And another remarkable result was sequence divergence between Misgurnus anguillicaudatus from China and M. anguillicaudatus from Yongdok, Korea. That was 0.099, which was interspecific value. Also the phylogenetic location of some Iksookimia species was suggested as the cobitid intergeneric hybrid origin.

Key words: Cobitidae, mitochondrial cytochrome b gene, phylogenetic relationship

Introduction

The family Cobitidae is composed of 16 genera and about 100 species of freshwater fish which are confined to Eurasia and northern Africa (Nalbant, 1994). Despite the intensive study of cobitid taxonomy, hypotheses on the relationship among the cobitid species based on morphology, cytotagentic, reproduction, histology and behavior have led to conflicting classifications (Kobayasi, 1976; Kim, 1980; Sawada, 1982; Kim and Lee, 1986; Lee and Lee, 1989; Kim and Park, 1997).

Kim (1997) and Nalbant (1993, 1994) recognized the following 8 genera in Korea: Orthrias, Lefua, Misgurnus, Iksookimia, Koreocobitis, Cobitis, Niwaella and Choia. The hypothesis of relationships presented by Kim and Nalbant (1993) was based on the condition of the first pectoral fin ray in males and the colour pattern of the body sides. All species of the two genera Cobitis and Iksookimia have the peculiar lamina circularis at the base of their pectoral fin in the male as a secondary sexual characteristic, which is also an important characteristic to identify the cobitid species (Kim and Park, 1997). All loaches of Iksookimia are endemic to Korea, which were erected from the genus Cobitis by Nalbant(1993).

Recently, geographical variations of mitochondrial DNA were investigated through the genetic differences between the western and eastern Cobitis koreensis groups (Yang and Kim, 1995). In addition, extensive variations in mitochondrial DNA were studied among geographic populations of Nemacheilus toni (Park and Lee, 1991). At present, no study has provided a widely accepted formulation of the evolutionary relationships within the family Cobitidae using genetic characteristic. In this paper we describe for the first time sequences of the mitochondrial cytochrome b gene of 8 cobitids and use them to evaluate the monophyly of the Cobitis and the paraphyly of the Orthrias and Lefua. We specifically address the phylogenetic relationships among the species of Cobitidae using one species of Cyprinidae (Carassius auratus) as an outgroup.
Materials and Methods

1. Sample

Details of the samples and species used in this study are presented in Table 1. The specimens were collected in the field and identified to species level using the method by Kim (1997). Tissue samples (fin) were dissected, avoiding cross-contamination, and stored at -80°C.

2. DNA extraction, amplification, and sequencing

Tissues were digested with proteinase K in the presence of extraction buffer (10-14 h at 55°C). DNA was purified with standard phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) extractions. Ethanol-precipitated DNA pellets were rinsed (70% ethanol), air dried and resuspended in TE buffer (Maniatis et al., 1982).

PCR amplifications were performed following Kocher et al. (1989) using 50 ng of genomic DNA and 1 unit of Taq DNA polymerase (Ex-Taq Taqara) per 50 µl of reaction volume. Thermal cycling was: initial hot start 4 min at 95°C; followed by 30 cycles of 30s at 94°C, 30s at 58°C, and 1 min 30s at 72°C, and an additional 7 min interval at 72°C. The 1,140 bps of the cytochrome b were amplified using primer cbup 1 and cblo 2 (Table 2). PCR products were prepared for cloning by removing the primers using the GENE CLEAN kit (BIO 101). The fragment was inserted into pGEM-T vector (Promega) and used to transform JM 109 cell line. Automated DNA sequencing was performed using ABI PRISM 377 DNA sequencers, according to manufacturer protocols. Sequencing primers were custom made (Table 2).

3. Phylogenetic analysis

Sequencing base composition, patterns of substitution for pairwise comparisons along the mitochondrial DNA cytochrome b sequences were aligned using the Clustal × 1.81 (Thompson et al., 1997). All phylogenetic analyses were executed using PAUP* test vision 4.0b4a (written by D. L. Swofford, 2000).

Different approaches to phylogenetic reconstruction were used to analyze the data set. They included maximum parsimony (MP), maximum likelihood (ML) and neighbor joining (NJ) trees. MP analyses were performed by the bootstrap.

Table 1. List of Cobitidae species for analysis in the present study

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Accession number</th>
<th>Locality/area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthrias toni</td>
<td>Cobitidae</td>
<td>–</td>
<td>Samchok/Korea</td>
</tr>
<tr>
<td>Lefua costata</td>
<td>Cobitidae</td>
<td>–</td>
<td>Samchok/Korea</td>
</tr>
<tr>
<td>Misgurnus anguillicaudatus</td>
<td>Cobitidae</td>
<td>AF051868</td>
<td>China</td>
</tr>
<tr>
<td>Misgurnus anguillicaudatus</td>
<td>Cobitidae</td>
<td>–</td>
<td>Yongdok/Korea</td>
</tr>
<tr>
<td>Ihsookimia koreensis</td>
<td>Cobitidae</td>
<td>–</td>
<td>Gosan/Korea</td>
</tr>
<tr>
<td>Ihsookimia pumila</td>
<td>Cobitidae</td>
<td>–</td>
<td>Buan/Korea</td>
</tr>
<tr>
<td>Cobitis sinensis</td>
<td>Cobitidae</td>
<td>–</td>
<td>Hyongsan R./Korea</td>
</tr>
<tr>
<td>Cobitis melanoleuca</td>
<td>Cobitidae</td>
<td>–</td>
<td>Samchok/Korea</td>
</tr>
<tr>
<td>Niwaella multifasciata</td>
<td>Cobitidae</td>
<td>–</td>
<td>Nam R./Korea</td>
</tr>
<tr>
<td>Carassius auratus</td>
<td>Cyprinidae</td>
<td>AB006953</td>
<td>Japan</td>
</tr>
</tbody>
</table>

-, present work

Table 2. List of primers used in this study, Usage (PCR amplification – P, Cycle sequence – C)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene</th>
<th>Sequence</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>cbup 1</td>
<td>Cyt b</td>
<td>GGATTACAAGACCCGATGCTTT</td>
<td>P</td>
</tr>
<tr>
<td>cblo 2</td>
<td>Cyt b</td>
<td>TGACTTGAAGAACCCACCGTGT</td>
<td>P</td>
</tr>
<tr>
<td>cytop 3</td>
<td>Cyt b</td>
<td>ACTTAATCGACATTCGATGC</td>
<td>C</td>
</tr>
<tr>
<td>cytop 4</td>
<td>Cyt b</td>
<td>CCCACATCTGCAGATGTA</td>
<td>C</td>
</tr>
<tr>
<td>cytop 5</td>
<td>Cyt b</td>
<td>TGACTAATCGACATTCGAC</td>
<td>C</td>
</tr>
<tr>
<td>cytop 6</td>
<td>Cyt b</td>
<td>CTGATATCTCCACCGCTCTT</td>
<td>C</td>
</tr>
<tr>
<td>cytop 7</td>
<td>Cyt b</td>
<td>CCCATAGGATTAACCTCGACG</td>
<td>C</td>
</tr>
<tr>
<td>cytop 8</td>
<td>Cyt b</td>
<td>GACGCGACACAGATACATTC</td>
<td>C</td>
</tr>
<tr>
<td>cytop 9</td>
<td>Cyt b</td>
<td>CTGACGCGACATGATTTTTC</td>
<td>C</td>
</tr>
<tr>
<td>cytop 10</td>
<td>Cyt b</td>
<td>TTTGCATTCCTCCTCCTTC</td>
<td>C</td>
</tr>
</tbody>
</table>
method with heuristic searches with following search parameters: ignore uninformative characters, retain minimal trees, collapse zero length branches and branch swapping algorithm (tree-bisection-reconnection: TBR). ML analyses were performed using quartet puzzling with the parameter model of evolution of Hasegawa–Kishino–Yano (HKY85; Hasegawa et al., 1985). HKY model has been requested transitions/transversions ratio = 2 (kappa = 4.00347) and used empirical base frequencies. All sites were assumed to have evolved at the same rate.

A search for the NJ (Saito and Nei, 1987) was performed using the Kimura two-parameter distance (Kimura, 1980), which allows for unequal rates of transitions and transversions. The NJ tree was estimated to 1,000 bootstrap replicates to assess branch support.

Result

1. Nucleotide variation

The cytochrome b genes were sequenced in all 8 sampled specimens by a 650 bp segment of the 5' end of the gene. Completed cytochrome b sequences from 1 non-cobitid species, Carassius auratus (from Japan) and Misgurnus anguillicaudatus (from China) were downloaded from Genbank. Carassius auratus was designated as outgroup taxa in all analyses. The nucleotide composition of the cytochrome b gene was homogeneous among conegers. The nucleotide composition of the cytochrome b sequences followed the pattern described for numerous fish (Cantatore et al., 1994): a global deficit of guanosine (G: 15.9%) and approximately equal frequencies of the other three nucleotides (A: 27.8%, C: 25.9%, T: 30.3%). Base composition was quite constant among species: G+C content ranged from 39.4% to 42.8% (31.6% to 47.1% for variable sites only).

2. Phylogenetic analysis

Phylogenetic signal was detected in the aligned cytochrome b data set. In maximum parsimony analysis with heuristic search, over the 650 bp segment for 10 cobitid species, 415 nucleotide positions were constant, 84 variable characters were uninformative and 151 were informative for parsimony analysis. Maximum parsimony analysis recovered a single most parsimonious tree, with a length of 453 steps and a consistency index (excluding uninformative characters) of 0.684 (Fig. 1). Cobitidae was monophyletic with respect to the other non-cobitid species in the analysis, and this clade is supported with high bootstrap (100%). Misgurnus is recovered as the sister taxon of a monophyletic Cobitis-Iksoo kimia-Niwaella clade. The monophyly of the Cobitis-Iksoo kimia-Niwaella clade is supported with high bootstrap (81%) and decay (5) values (Fig. 1). Orthrias and Lefua are recovered as monophyletic. This clade receives high support in bootstrap (98%) and has a branch length of 33 steps (Fig. 1). But, it was found that this clade was paraphyletic from other Cobitidae.

The two species, Cobitis sinensis and Niwaella multijasciata were strongly monophyletic and continued to be placed outside the rest of cobitids. This clade was supported high bootstrap (88%) and decay (5) value (Fig. 1). Cobitis melanoleuca clade was recovered as the sister taxon of a monophyletic clade consisting of Iksoo kimia-koreanis and L. pumila and this Iksoo kimia clade supported with high bootstrap (99%) and decay (9) values, and the length of the branch leading to this clade is 17 steps. Also, Misgurnus anguillicaudatus from China and M. anguillicaudatus from Yongdok were paraphyletic, but this clade was weak.

Maximum likelihood tree analyzed by quartet puzzling (Fig. 1). Number of puzzling step was 1,000. This tree was similar to the maximum parsimony tree with heuristic search but some difference resided in the placement between Misgurnus anguillicaudatus from China and M. anguillicaudatus from Youngdok, Korea. Their monophyly was supported high puzzle value (97%).

The neighbor joining (NJ) tree was constructed using Kimura’s (Kimura, 1980) two-parameter distance (Table 3). This tree was very similar to the maximum likelihood tree with quartet puzzling (Fig. 1). The hypothesis of Kim (1997) and Nalbant (1994) was not significantly different from the topology using maximum parsimony, maximum likelihood and neighbor joining criteria. Some surprising results from this analysis were that a paraphyletic C. melanoleuca from C. sinensis clade is strongly supported in bootstrap analysis (89%) and Orthrias-Lefua clade is early diverged from other cobitids and this clade high bootstrap value (98%).
Fig. 1. Phylogenetic relationships of 10 species recovered from cytochrome \( b \) sequences (650 bp) according to the maximum parsimony method (a), the maximum likelihood method with quartet puzzling (b) and the neighbor joining methods (c). Bootstrap values (1,000 replication) are indicated below branches. Decay values (in box) are given above branches.

**Discussion**

Most molecular phylogenetic studies of vertebrates have been based on DNA sequences of mitochondrial-encoded genes. Mitochondrial DNA evolves rapidly and is thus particularly useful for resolving relationships among recently evolved groups. Cytochrome \( b \) was selected because it contains discrete character classes (i.e., the three codon positions), which exhibit rates of muta-
Table 3. Pairwise distance matrix of Kimura 2-parameter

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. N. multifasciata</td>
<td>-</td>
<td>0.05478</td>
<td>-</td>
<td>0.10860</td>
<td>0.09736</td>
<td>-</td>
<td>0.10671</td>
<td>0.09551</td>
<td>0.01089</td>
<td>-</td>
</tr>
<tr>
<td>2. C. sinensis</td>
<td>0.05478</td>
<td>-</td>
<td>0.09736</td>
<td>0.10860</td>
<td>0.09736</td>
<td>-</td>
<td>0.11572</td>
<td>0.11191</td>
<td>0.07416</td>
<td>0.07952</td>
</tr>
<tr>
<td>3. I. koreensis</td>
<td>0.10671</td>
<td>0.09736</td>
<td>-</td>
<td>0.10860</td>
<td>0.09736</td>
<td>-</td>
<td>0.11572</td>
<td>0.11191</td>
<td>0.07416</td>
<td>0.07952</td>
</tr>
<tr>
<td>4. I. pumila</td>
<td>0.09551</td>
<td>0.10736</td>
<td>0.09736</td>
<td>-</td>
<td>0.10860</td>
<td>0.09736</td>
<td>-</td>
<td>0.11572</td>
<td>0.11191</td>
<td>0.07416</td>
</tr>
<tr>
<td>5. C. melanolouca</td>
<td>0.01089</td>
<td>0.07416</td>
<td>0.07952</td>
<td>0.07416</td>
<td>-</td>
<td>0.12185</td>
<td>0.12379</td>
<td>0.13106</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6. M. anguillicaudatus (from Yongdok)</td>
<td>-</td>
<td>0.10671</td>
<td>0.09551</td>
<td>0.10736</td>
<td>0.09736</td>
<td>-</td>
<td>0.11572</td>
<td>0.11191</td>
<td>0.07416</td>
<td>0.07952</td>
</tr>
<tr>
<td>7. M. anguillicaudatus (from China)</td>
<td>0.11715</td>
<td>0.12326</td>
<td>0.12948</td>
<td>0.12751</td>
<td>0.13087</td>
<td>0.09907</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. C. auratus</td>
<td>0.20437</td>
<td>0.18396</td>
<td>0.19021</td>
<td>0.18812</td>
<td>0.20924</td>
<td>0.18828</td>
<td>0.18960</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. L. costata</td>
<td>0.20734</td>
<td>0.19876</td>
<td>0.20956</td>
<td>0.20742</td>
<td>0.21811</td>
<td>0.21811</td>
<td>0.21209</td>
<td>0.20904</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10. O. toni</td>
<td>0.28013</td>
<td>0.26914</td>
<td>0.26195</td>
<td>0.25957</td>
<td>0.28352</td>
<td>0.26702</td>
<td>0.28202</td>
<td>0.27186</td>
<td>0.22776</td>
<td>-</td>
</tr>
</tbody>
</table>

Phylogenetic relationships between Korean loaches (Cypriniformes: Cobitidae) were investigated by comparing cytochrome b gene sequences from 8 species, which were newly sequenced. As a result, four groups of Korean cobitids were found. Specimen composition in these groups was almost unchanged in all the reconstructed trees. The first identified clade included specimens from the Nakdong river basin, namely *Cobitis sinensis* and *Niwaella multifasciata*. The second clade included *Iksookimia koreensis* from the Mankyong river and *I. pumila* from the Buan Baechon river. The third clade included *Misgurnus anguillicaudatus* from China and Youngdok, Korea. The final clade included *Orthrias toni* and *Lefua costata*.

Most phylogenies agree to a certain extent with previous phylogenetic hypotheses based on morphological studies. Disagreement between the previous hypothesis about *Cobitis* suggests that *C. melanolouca* was early diverged from *C. sinensis*. Also, new interesting relationships were found: *Orthrias–Lefua* appeared to be paraphyletic in Cobitidae observed and *Iksookimia* clade was a sister to *Cobitis melanolouca*. It was agreed that *Orthrias* and *Lefua* were classified by the other family Balitoridae (Nelson, 1994). Interfamilic sequence divergences between Cobitidae and Cyprinidae ranged from 0.184 to 0.272. This value coincided with sequence divergence value between *Orthrias–Lefua* and *Cobitis–Iksookimia*. We might conclude from these statement that *Orthrias–Lefua* had been reclassified as the family Balitoridae. The intergeneric sequence divergences of Cobitidae ranged from 0.097 to 0.282 by pairwise distance of Kimura 2-parameter (Table 3). Intragenic sequence divergence between *C. sinensis* and *C. melanolouca* was 0.112. The value was higher than genus *Sebastes* (Teleostei, Scorpaenidae) which was low levels (<10%) of intragenic sequence divergence (Rocha–Olivares et al., 1999). Also Cobitidae was monophyletic with respect to the other noncobitidae species in the analysis. But intragenus *Cobitis* appeared to be paraphyletic.

The phylogenetic location of some *Iksookimia* species was pervaded to *Cobitis*. The problem of intergeneric cyprinid hybrids was addressed (Briolay et al., 1998). The present study also was addressed the question of cobitid intergeneric hybrids. Intergeneric hybrids by interbreeding seems to be the consequence of a high genetic flexibility in cobitid fishes. The placement of *C. sinensis* and *C. melanolouca* confused the recovery of a monophyletic *Cobitis*. The phylogenetic placement of these two species within Cobitidae cannot be resolved with the cytochrome b data set. The aberrant placement of *Cobitis sinensis* was critically examined by including a complete cytochrome b sequence of *C. lutheri*, *C. striata* and *N. brevifasciata* (unpublished data).

Presently, a 2% mtDNA divergence rate pr MY has been assumed for bony fishes, however, divergence rates as low as 0.5% have been suggested (Cantatore et al., 1994). Given the relatively slow rate of nucleotide substitution in fish mitochondrial DNA relative to "conventional" rate estimates for vertebrates (Martin et al., 1992; Bermingham et al., 1997), these levels of specific sequence differentiation are remarkable.
and suggest that many of these populations have been isolated for millions of years. Long term isolation may explain the unique color patterns of cobitid fishes. Specific sequence divergences between I. koreensis and I. pumila detected 1.1%. Thus, we concluded that geographic isolation leads to different values of sequence divergence, and on the other hand, genes which are under strong environmental selection (e. g. for pigmentation) would still reflect the special adaptations of the different stocks on their habitat. This leads to a great variability of species. However, for a final conclusion among the possible explanations, further studies on the molecular phylogeny of loaches are required.

Overall, we observed considerable genetic divergence among species, and the data seems to confirm the growing necessity for taxonomic revision of Cobitidae. Estimates of genetic distinction and relationships afforded by analysis of mitochondrial DNA sequences provide an excellent source of information that, when combined with analysis of morphological characters, should yield a predictive classification. Mitochondrial DNA evolves rapidly and is thus particularly useful for resolving relationships among recently evolved groups. However, it has the disadvantage that all of the mitochondrial genes are inherited as a single linkage group so that only one independent gene tree can be inferred, regardless of the number of sequenced genes. Introns of nuclear genes are attractive candidates for independent sources of rapidly evolving DNA. As a consequence, compared with cytochrome b, introns were expected to enter more distinct character states over the course of evolution and had fewer multiple substitutions and lower levels of homoplasy (Prychitko and Moore, 2000).

The phylogenetic results we reported from molecular data were mostly congruent with previous morphology based studies, although some new points were made. This suggests that both approaches are reliable for the study of fish phylogeny. Therefore, in this study, the cytochrome b sequence data of mtDNA was also useful in elucidating the phylogenetic relationships among Cobitidae which show great ecological adaptability. These analyses have provided further evidence for the utility of cytochrome b sequence in addressing phylogenetic relationships among Cobitidae.

References


Molecular Phylogeny of Cobitidae

The rate of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature, 357 : 153 ~ 155.

Swofford, D.L. 2000. PAUP* Ver. 4.0b4a, Sinauer, Sunderland, MA.

Received : November 1, 2000
Accepted : December 8, 2000

Mitochondrial Cytochrome b gene의 분석에 의한 한국산 미꾸리과 어류(Cobitidae)의 계통

김소영 · 김익수 · 장광열 · 장미희

전북대학교 생물학부

한국산 미꾸리과 어류의 제통유전학적 관계를 고찰하고자 8종의 mitochondrial cytochrome b의 유전자의 서열을 비교한 결과 대부분 이전의 형태학적 연구의 결과와 일치하였다. 그러나 종계 속 *Orthrias*와 말미꾸리속 *Lefua*의 분류학적 위치는 미꾸리과 Cobitidae와 paraphyletic group으로 나타났으며 이 두 속의 sequence divergence는 0.184 ~ 0.272으로 나타나 미꾸리과와 어류와 사이의 divergence와 유사하였다. 한편 참조계속 *Iksookimia* 2종과 북방참조 *Cobitis melanoleuca*는 각각 다르게 분화한 결과를 보여 주었으며 또한 한국산 미꾸리과 한국산 영덕 미꾸리의 sequence divergence는 0.099로 종간의 divergence를 보여 주어 주목되었다. 미꾸리과 어류 가운데 참조계속의 일부 어류는 분류학적 위치로 보아 이들의 기원이 미꾸리과의 속간 참조계속으로 생중된.