

Molecular Systematics of Korean Cobitids Based on Mitochondrial Cytochrome *b* Sequence

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Key Words:

Cobitis
Mitochondrial cytochrome
b gene
Phylogenetic relationship
Secondary sexual
dimorphism

We compared the complete mitochondrial cytochrome *b* gene sequences of Korean and European cobitids to provide independent evidence for assessment of systematic and biogeographic relationships of species in the genus *Cobitis*. The data suggested monophyly of the genus *Cobitis* and the inclusion of Korean *Cobitis* species within the group having one lamina circularis, a primitive condition. Also, all the phylogenetic analyses using maximum parsimony, maximum likelihood, and neighbor joining methods showed a monophyletic relationship among *Cobitis*. The basal position of the Caspian *C. cf. sibirica* reported here reflects the eastern Asiatic origin of the European *Cobitis* and establishes *C. cf. sibirica* as an independent lineage. The Korean *C. pacifica* diverged next to *C. cf. sibirica* in basal group from the genus *Cobitis*. This result is in agreement with the hypothesized Asiatic origin of some European freshwater fish lineages. The phylogenetic relationships in this study showed a close affinity between *C. zanadreae* and *C. sinensis*. Two new species, *C. tetralineata* and *C. pacifica* in Korea also are closely related to monophyletic group clustering the type species of the *Acanestrinia* subgenus (*C. elongata*) with all the endemic Italian species (*C. bilineata* and *C. zanadreae*). This may suggest that the affinity between the Korean and Danubian-Italian imply genetic convergence or genetic plesiomorphic state between allopatric species that are separated for the Miocene. The mtDNA-based phylogeny for the species of the genus *Cobitis* from Korea and Europe permits phylogenetic assessment of the morphological transitions of lamina circularis.

The family Cobitidae (Cypriniformes) comprises 16 genera and about 200 species of freshwater fishes, distributed in Eurasia and northern Africa (Nalbant, 1994). Nalbant (1999) identified 11 valid species in the genus *Cobitis* from Eastern Asia (China, Viet-Nam, Laos, Russia-the basin of the river Amur, and Korea), along with two new species, *C. tetralineata* and *C. pacifica* in Korea. Kim et al. (1999) confirmed the identity of *C. tetralineata* and *C. pacifica* which were reported as *C. striata* and *C. melanoleuca* by Kim (1997).

Traditional phylogenetic inference regarding the biogeography and evolution of the family Cobitidae was mainly based on correct interpretation of transitions between morphological states of secondary sexual characters (e.g., lamina circularis). The phylogenetic utility of the secondary sexual characters is one of the most recurrent topics in evolutionary biology (Ryan et

al., 1990). The systematics and phylogeny of the family Cobitidae present a good example of the use of secondary sexual characters for phylogenetic purposes. Males of the genus *Cobitis* are characterized by thickening of the second pectoral fin ray, referred to as the lamina circularis (Vladykov, 1928). All species of the genus *Cobitis* have the peculiar lamina circularis at the base of their pectoral fin in the male as a secondary sexual characteristic, microstructures of which are important in identification of the cobitid species (Kim and Park, 1997). Presence, duplication and loss of the lamina circularis are the principal character states defining the *Cobitis* subgenera, *Acanestrinia*, *Bicane-strinia*, *Cobitis s. str.*, and *Iberocobitis* (Bacescu, 1962). Thus, the evolutionary transitions represented by the character states of lamina circularis have played a predominant role in interpretation of the biogeography and systematic relationships of the species in the genus *Cobitis*. Polymorphism in the number of lamina circularis within species (Economidis and Nalbant, 1996) provided a key to the evolutionary lability of this character. However, some cobitid fishes, *C. elongata*

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and *C. calderoni*, which have no lamina circularis, are not closely related in phylogeny and this suggests that the absence of the lamina circularis is not a good character state to define phylogenetic relationships (Perdices & Doadrio, 2001).

Our molecular systematic investigation of *Cobitis* is based on DNA sequence from complete mitochondrial cytochrome *b* gene. These molecular data provide an independent set of characters required for testing of the phylogenetic utility of lamina circularis for identification of natural groups within the genus *Cobitis*, for revealing evolutionary history of these sexual characters and for examination of the phylogenetic relationship between the Korean and European *Cobitis*.

Materials and Methods

Specimens Examined

Four Korean cobitids fishes, one species of *Orthrias* (Nemacheilinae) and 15 species from Europe representing the geographic and nominal taxonomic range of *Cobitis* subgenera (Table 1) were used for their phylogenetic relationships based on the complete sequence of cytochrome *b* gene. The classification of the family

Cobitidae followed those of Kim (1997) and Kim *et al.* (1999). The specimens were collected from either rivers or small streams in Korea (Table 1) and deposited in the Laboratory of Ichthyology, Faculty of Biological Sciences, Chonbuk National University (CNU). Our molecular systematic analysis included three additional outgroup genera, *Misgurnus* and *Orthrias* and the Cyprinidae genus *Barbus*.

Caudal and anal fins were taken from frozen or living specimens from each species for sequence analysis. All tissues were stored at -70°C under laboratory conditions. The details of species samples used in this study and downloaded sequences from GenBank are presented in Table 1.

DNA extraction

Total genomic DNA was extracted from fin tissues with Proteinase K in extraction buffer (50 mM Tris, 100 mM EDTA, and 1% SDS, 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), vacuum-dried and resuspended in TE buffer (Maniatis *et al.*, 1982). DNA was quantified by

Table 1. Taxonomic sampling, localities and GenBank accession numbers for cobitid and cyprinid mitochondrial cytochrome *b* gene sequences examined in this study

Taxon ¹	Locality	Accession No.
Outgroups		
Family Cyprinidae		
<i>Barbus Barbus</i>	R.Axios/Axiopolis/Greece	AY004753
<i>B. bocagei</i>	R.Deuro/Carrascal del Rio/Spain	AY004728
Ingroups		
Family Cobitidae		
Subfamily Balitoridae		
<i>Orthrias nudus</i>	Samcheok/Korea	—
<i>O. barbatulus</i>	R.Esca/Burgui/Spain	AF263098
Subfamily Cobitinae		
<i>Misgurnus fossilis</i>	R.Dyje/Kosarske'Louky/Czech Rep.	AF263097
Subgeneric group		
Acanestrinia	<i>Cobitis calderoni</i>	R.Arlanzon/Arlanzon/Spain
Acanestrinia	<i>C. elongata</i>	R.Nara/Sasca/Rumania
Bicanestrinia	<i>C. arachthosensis</i>	R.Arachthos/Atra/Greece
Bicanestrinia	<i>C. sp</i>	R.Thiamis/Parapothamus/Greece
Bicanestrinia	<i>C. trichonica</i>	LakeTrichonis/Panetolis/Greece
Bicanestrinia	<i>C. meridionalis</i>	Lake Prespa/Psarades/Greece
Cobitis s. str.	<i>C. vardarensis</i>	R.Agiaki/Kastanies/Greece
Cobitis s. str.	<i>C. elongatoides</i>	R.Timis/Albina/Rumania
Cobitis s. str.	<i>C. cf sibirica</i>	R.Sinukha/Krasnodarskij Kraj/Russia
Cobitis s. str.	<i>C. taenia</i>	Haaren Creek/Oldenburger/Germany
Cobitis s. str.	<i>C. bilineata</i>	R.Reno/Vergato/Italy
Cobitis s. str.	<i>C. Zanandreai</i>	Lago di Foundi/San Rafeale/Italy
Iberocobitis	<i>C. maroccana</i>	R.Kherrouba/Kherrouba/Morocco
Iberocobitis	<i>C. paludica</i>	Fuente del Roble/Talayuela/Spain
Iberocobitis	<i>C. vettonica</i>	R.Arrago/Cadalso de Gata/Spain
	<i>C. tetralineata</i>	Hwasun/Korea
	<i>C. lutheri</i>	Yeongsan River/Korea
	<i>C. pacifica</i>	Samcheok/Korea
	<i>C. sinensis</i>	Hyeongsan River/Korea

¹ Classification of subgeneric group follows Bacescu (1962). Dashes denote the present study

spectrophotometry and quantifications were confirmed by electrophoresis on a 1.2% agarose gel.

PCR amplification, molecular cloning and sequencing

Total genomic DNA of fish was used as a template for PCR amplification. PCR amplications were performed following Kocher et al. (1989) using 50 ng of genomic DNA and 1 unit of Ex-Taq DNA polymerase (Takara, Japan) per 50 µg of reaction volume. Thermal cycling was: initial hot start 4 min at 95°C; followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, 90 s at 72°C, and an additional 7 min interval at 72°C. The complete cytochrome *b* gene were amplified using the primers cbup1 and cblo2 (Table 2). The PCR products were prepared for cloning by removing the primers using the GENE CLEAN kit (BIO 101). The fragment was inserted into pGME-T easy vector (Promega) and used to transform JM 109 cell line. Automated DNA sequencing was performed using a ABI PRISM 377 DNA sequencer, according to the manufacture's protocol. Sequencing and PCR primers used were listed in Table 2.

Sequence analysis

Sequences were aligned using the Clustal X 1.81 (Thompson et al., 1997). All codon positions were included in the phylogenetic analysis. All phylogenetic analyses were executed using PAUP 4.0b4a (Swofford, 2001). The aligned data sets were analyzed by maximum-parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) methods. The NJ analysis was done using the HKY85 substitution model to calculate divergence distances, allowing for different rates of Ti and Tv and unequal base frequencies. In the MP analysis, only minimal trees were retained and zero-length branches were collapsed. In all cases, the heuristic search with TBR branch swapping and 10 replicates of random addition of taxa were used. Bootstrap analysis with 1000 replicates was used to assess the relative robustness of inferred monophyletic groups. For ML analysis, we used the HKY model of evolution (Hasegawa et al., 1985) with gamma parameter and proportion of invariable positions. ML analyses with empirical base frequencies and Ti/Tv ratio of 6.91 were performed using PAUP. To test constancy of rates of divergence behavior of cytochrome *b*, a log-likelihood ratio test was performed in PAUP with a χ^2 test to statistically estimate the validity of the constancy of rates hypothesis (Page & Holmes, 1998).

Results

Nucleotide variation

The cytochrome *b* genes were fully sequenced from all 5 species examined and other sequences have been downloaded in GenBank under Accession Nos.

Table 2. List of primers used in this study

Name	Position	Sequence	Usage*
cbup1	tRNA ^{Glu}	5'-GGATTACAAGACCGATGCTTT-3'	P
cblo2	tRNA ^{Thr}	5'-TGACTTGAAGAACCACCGTTG-3'	P
cytup3	cyt b	5'-ACTTATCCGCAACATTCATGC-3'	C
cytup4	cyt b	5'-CCCACATCTGCCGAGATGTA-3'	C
cytup5	cyt b	5'-TGACTAATTCGAAGCATGCAC-3'	C
cytup6	cyt b	5'-CTGATATCTCCACCGCTTCT-3'	C
cytup7	cyt b	5'-CTGATATCTCCACCGCTTCT-3'	C
cytup8	cyt b	5'-GACGCAGACAAAGTATCATTCA-3'	C
cytup9	cyt b	5'-CTCGGACGCAGATAAAATTTTC-3'	C
cytup10	cyt b	5'-TTTGCATTCCACTTCCTTCT-3'	C
cytup11	cyt b	5'-ACTTATCCGCAACATTCATGC-3'	C
cytup12	cyt b	5'-CCCACATCTGCCGAGATGTA-3'	C
cytup13	cyt b	5'-TGACTAATTCGAAGCATGCAC-3'	C

* P; PCR amplification, C; DNA sequencing.

AF263037-AF263098 (Table 1). *Barbus barbuis* (AY-004753) and *Barbus bocagei* (AY004728) were designated as outgroup taxa in all analyses. The nucleotide composition of the cytochrome *b* gene was homogeneous among congeners and did not differ significantly between cobitid genera ($\chi^2 = 87.029$, $df = 69$, $P = 0.070$). The sequences showed a noticeable anti-G bias pattern described for numerous fishes (Cantatore et al., 1994): a global deficit of guanine (G: 15.4%) and approximately equal frequencies of the other three nucleotides (A: 27.47%, C: 26.0%, T: 31.2%).

Phylogenetic analysis

In MP analysis with heuristic search, 1140 nucleotide positions were used (86 variable and 417 informative sites). The estimated Ti:Tv ratio among all taxa for the complete data set was 6.91. Maximum parsimony analysis recovered a most parsimonious tree (consistency index = 0.4331). Results are as follows (Fig. 1a): Cobitidae is monophyletic with respect to the other noncobitidae species in the analysis. The genus *Orthrias* diverged first and the genus *Misgurnus* differentiated next. *C. cf. sibirica* from Russia in the third clade is the sister group of the other *Cobitis* species. The *C. pacifica* from Korea was diverged early in this clade. The Korean *Cobitis* species was closely related with the subgenus *Acanestrinia* (*C. elongata*) plus all the Italian endemic species (*C. bilineata* and *C. zanandreai*). The MP and NJ analyses of the sequence data produced congruent topologies which supported the monophyly of the genus *Cobitis* (96% and 91% bootstrap values, respectively). This monophyly of *Cobitis* was not corroborated in the ML analysis, where this relationship received low support. All analyses unambiguously placed *Misgurnus fossilis* basal to the genus *Cobitis* (Fig. 1). However, the sister relationship between *Cobitis* and *Misgurnus* supported in the MP and NJ analyses (100%) was not corroborated in the ML analysis, where this relationship received low support (73%) (Fig. 1b). *C. cf. sibirica* from the Caspian area and *C. pacifica* from Korea

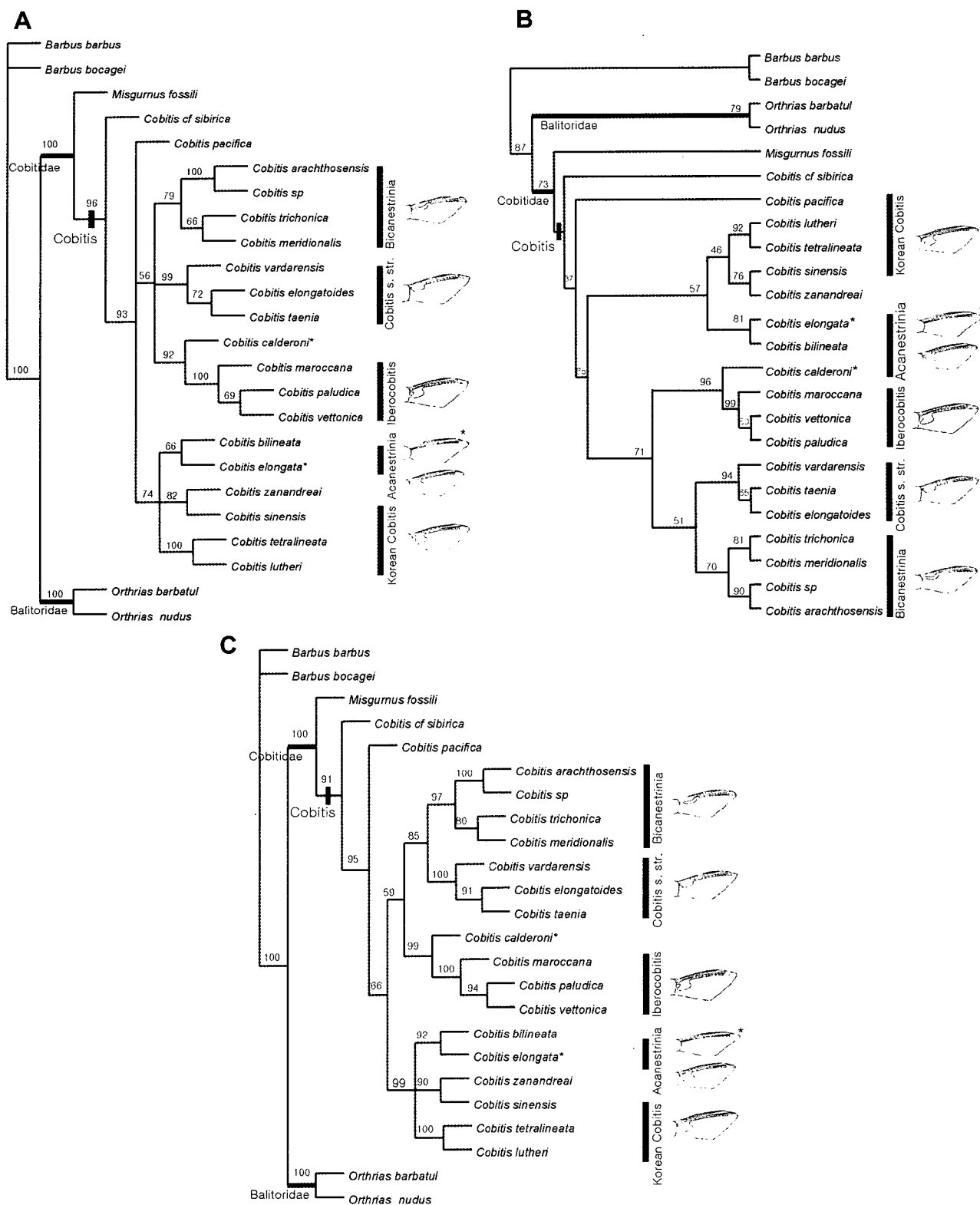


Fig. 1. Phylogenetic relationships of complete sequences of cytochrome *b* gene for Korean and European species of the family Cobitidae. A, Single MP tree (TL=1704; CI=0.4331). B, ML tree obtained with HKY85 distance. C, NJ tree obtained with K2P distance. Scale below NJ tree indicates 10 substitution per nucleotide. Values above branches are the bootstrap supports of 1000 replication and quartet puzzling values in ML, respectively. Subgeneric monophyletic clades of the genus *Cobitis* recovered are listed to the right (Bacescu, 1962). The two species classified to subgenus *Acanestrinia* which defined by Bacescu (1962) are marked with asterics.

consistently occupied the basal position of the ingroup species of the genus *Cobitis* in all analyses.

The monophyly of the *Bicanestrinia* and *Iberocobitis* subgenera *sensu* Bacescu (1962) was supported in all analyses with high bootstrap values (70-100%). However, this heuristic approach for the monophyly of the subgenera *Acanestrinia* and *Cobitis s. str. sensu* Bacescu (1962) did not support the monophyly of both subgenera (Fig. 1, Table 1).

All analyses revealed paraphyletic relationship between the type species of the *Acanestrinia* subgenus (*C. elongata*) and all the endemic Italian species (*C. bilineata* and *C. zanandrea*). The members of the subgenus *Iberocobitis* formed a strongly supported clade (>99%) and *C. calderoni* from the Iberian Peninsula was its sister group. The subgenus *Bicanestrinia* (70-97%) included all four *Bicanestrinia* species analyzed.

The phylogenetic analyses showed paraphyletic relationship between the Korean *Cobitis* group (*C. sinensis*, *C. tetrakineata*, and *C. lutheri*) and *Acanestrinia* (*C. elongata*) plus all the endemic Italian species (*C. bilineata* and *C. zanandrea*). MP and NJ analyses revealed strongly supported (74-99%) monophyletic relationship between the Korean *Cobitis* species and the type species of the *Acanestrinia* subgenus (*C. elongata*) plus all the endemic Italian species (*C. bilineata* and *C. zanandrea*). But this monophyly was supported with a weak and puzzling value (57%) in the ML analysis. In all analyses this monophyletic group formed the basal lineage of *Cobitis*.

The data showed a sister relationship between the *Misgurnus* and the *Orthrias* with strong bootstrap support (>73%). The genus *Orthrias* was a well supported (>79% bootstrap) monophyletic group. Sequence divergence values (26.1-32.2%) between *Orthrias* and *Cobitis* corresponded to interfamilial sequence divergences (24.4-26.8%) between the Cobitidae and Cyprinidae.

Discussion

Most molecular phylogenetic studies of vertebrates have been based on DNA sequences of 13 mitochondrial-encoded genes (Briolay et al., 1998; Miguel et al., 1997). Mitochondrial DNA evolves rapidly and is thus particularly useful for resolving relationships among recently evolved groups (Briolay et al., 1998). Cytochrome *b* was selected for the present study because it contains discrete character classes (i.e., the three codon positions) which exhibit rates of mutation ranging from rapid to conservative (Irwin et al., 1991).

In this study, phylogenetic relationships between the Korean and European *Cobitis* (Cypriniformes : Cobitidae) were investigated by comparing the DNA sequences of cytochrome *b* gene from 23 species, among which 5 Korean *Cobitis* species were newly sequenced. Secondary sexual characters have traditionally been used in

cobitid systematics and their derived biogeographical inferences (Vladykov, 1928; Bacescu, 1962; Banarescu & Nalbant, 1998). Results of the phylogenetic analyses partially supported the morphological lineages formerly defined (Bacescu, 1962). The presence of the scale of lamina circularis in males is the synapomorphy that relates all species of the genus *Cobitis* (Vladykov, 1928). A very simple scale was suggested as the primitive state and has always been considered a nonreversible condition (Banarescu & Nalbant, 1998). Our phylogeny supports the presence of one scale of lamina circularis as the ancestral condition in the genus *Cobitis*, as well as the monophyly of the group previously suggested on the basis of morphological and sexual characters (Banarescu et al., 1972).

MtDNA phylogeny revealed six unambiguous *Cobitis* lineages that partially corresponded to the morphological groups defined by Bacescu (1962). The subgenera *Cobitis s. str.* and *Iberocobitis* exhibit one scale of lamina circularis. The *Bicanestrinia* lineage has duplicated lamina circularis, while scaleless taxa *C. elongata* and *C. calderoni* were grouped in the subgenus *Acanestrinia* (Bacescu, 1962).

Traditional hypotheses suggest that the duplication or loss of the laminar circularis are derived states. Consequently, all members sharing these derived states are included in the same evolutionary lineage. However, the phylogenetic hypothesis derived from cytochrome *b* suggests that the absence of the laminar circularis has been derived more than once (Perdices and Doadrio, 2001). Thus, morphologically defined lineages might not correspond to natural groups. All phylogenies obtained indicate frequent reversions in the primitive cypriniform state represented by the absence of the laminar circularis. Our data do not support the monophyly of the subgenus *Acanestrinia* as traditionally conceived (Bacescu, 1962). The monophyly of the *C. sinensis* from Korea and the *C. zanandrea* from Italy was strongly supported by a high bootstrap value. Also the monophyly between *Acanestrinia* (*C. elongata*) plus two Italian endemic species (*C. bilineata* and *C. zanandrea*) and three Korean *Cobitis* species was strongly supported with a high bootstrap value. Members of this lineage showed a loss of laminar circularis or one laminar circularis. Our results indicated that species having laminar circularis were not phylogenetic closely related and suggested that the absence of laminar circularis was not a good character state to define phylogenetic relationships. Despite the fact that the laminar circularis seems a good character to identify all members of the genus *Cobitis*, the above evidence suggests that the use of the secondary sexual character states is inappropriate to define the monophyletic lineages included in this genus.

In all analyses *M. fossilis* tends to group as a sister to species of the genus *Cobitis*. This result remains tentative because neither of the *M. anguillicaudatus* or

M. mizolepis, the other congenitors, was included in this study. Although some of our phylogenetic relationships are not congruent with traditional systematics views, our results are congruent with the previous biogeographical hypotheses for freshwater fishes (Banareescu, 1990). Zoogeographical studies on the European freshwater fish fauna have suggested the eastern Asiatic origin of the European cobitids based on the high cobitid diversification in these areas (Banareescu, 1992). Recent molecular phylogenies based on complete cytochrome *b* sequences have also found evidence of the Asiatic origin of other European cyprinid groups (Zardoya and Doadrio, 1999; Durand et al., 1999). The basal position of the Caspian *C. cf. sibirica* reported here reflects the eastern Asiatic origin of the European *Cobitis* and establishes *C. cf. sibirica* as an independent lineage. The Korean *C. pacifica* is the next basal group diverged early from the genus *Cobitis*. This result is in agreement with the hypothesized Asiatic origin of some European freshwater fish lineages and shows the unique origin of all European *Cobitis* lineages. The phylogenetic relationships in this study showed close affinities between *C. zanadreae* and *C. sinensis*. Also, Nalbant (1999) identified 2 valid species in the genus *Cobitis* from Korea along with two new species, *C. tetralineata* and *C. pacifica* in Korea. Kim et al. (1999) confirmed the identity of *C. tetralineata* and *C. pacifica* which were discriminated as *C. striata* and *C. melanoleuca* by Kim (1997) from Korea. These new species are also closely related to monophyletic group clustering the type species of the *Acanestrinia* subgenus (*C. elongata*) with all the endemic Italian species (*C. bilineata* and *C. zanadreae*). It may suggest that the affinity between Korean and Danubian-Italian imply genetic convergence or genetic plesiomorphic state between the allopatric species that are separated for the Miocene.

Also, new interesting relationships were found in the present study. The family Cobitidae of Korea comprise two subfamilies, Cobitinae including *Cobitis* and Nemacheilinae including *Orthrias* (Nalbant, 1993). Sequence divergences between the Cobitinae and the Nemacheilinae ranged from 26.1% to 32.2%. This range of values corresponds to that of interfamilial sequence divergence between the Cobitidae and the Cyprinidae (24.4-26.8%). Also, *Orthrias* appeared as an early divergence in the Cobitidae. This suggest a clue for supporting the hypothesis that *Orthrias* should be classified into the family Balitoridae (Nelson, 1994).

According to the estimated dates of 0.76%/Mya for teleost (Zardoya and Doadrio, 1999), extant species of *Cobitis* from Korea diverged from *C. cf. sibirica* mostly during the early Miocene (21-23.4 Mya). This supports that the cobitids diverged earlier from ancestor populations which founded from southern China to Korea through Paleo Hwangho River or through Paleo Amur River (Siberia) than the Pliocene or Pleistocene (Sawada, 1982; Kim, 1997). However, for more con-

clusive decision on possible explanations, further studies on the molecular phylogeny of *Cobitis* are required. In conclusion, estimates of genetic distinction and relationships afforded by analysis of mitochondrial DNA sequences provide an excellent source of information that, when combined with an analysis of morphological characters, should yield predictive classification.

Acknowledgement

This work was supported by Korea Research Council of Fundamental Science and Technology Grant KBM4111 to H.-Y. P.

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[Received January 25, 2002; accepted February 16, 2002]