A THESIS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

MOLECULAR PHYLOGENY AND NATURAL HYBRIDIZATION OF COBITID FISHES IN KOREA

REZA MALEKZADEH VIAYEH

DEPARTMENT OF MARINE BIOTECHNOLOGY
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

2005.12

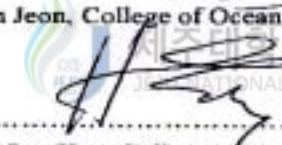
MOLECULAR PHYLOGENY AND NATURAL HYBRIDIZATION OF COBITID FISHES IN KOREA

REZA MALEKZADEH VIAYEH

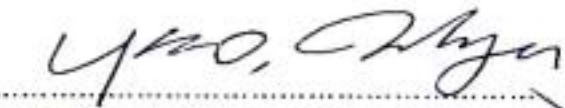
(SUPERVISED BY PROFESSOR CHOON BOK SONG)
A thesis submitted in partial fulfillment of the requirements for
The degree of Doctor of Philosophy
2005. 12
This thesis has been examined and approved by

Chairperson of the supervising committee
Professor Ki-Wan Lee, College of Ocean Science, Cheju National University

Professor Yu-Jin Jeon, College of Ocean Science, Cheju National University



Professor Moon-Soo Heo, College of Ocean Science, Cheju National University



Professor In-Kyu Yeo, College of Ocean Science, Cheju National University

Professor Choon Bok Song, College of Ocean Science, Cheju National University

DEPARTMENT OF MARINE BIOTECHNOLOGY
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

DEDICATION

I WOULD LIKE TO DEDICATE THIS WORK IN GRATITUDE TO MY FATHER **MR. HASAN MALEKZADEH** AND MY MOTHER **MRS. SEYYEDEH BANOO MOOSAVI** FOR THEIR AND PATIENCE.

TABlE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY

The family Cobitidae (Cypriniformes) comprises 16 genera and about 200 species of freshwater fishes, distributed in Eurasia and northern Africa (Kim et al., 2002). In Korea, 16 species belonging to six genera of *Misgurnus*, *Cobitis*, *Iksookimia*, *Koreocobitis*, *Niwaella*, and *Kichulchoia* have been ascribed to this family (Kim et al., 2003). To date, the systematic ranking of cobitid fishes have mainly been based on their morphological and phenotypic features. In a few molecular studies on Cobitid lineages in Korea, only a limited number of taxa have been examined (Kim et al., 2002; Kitagawa et al., 2005). In this study, the phylogenetic relationships among Korean cobitid fishes have been investigated by comparison of their DNA sequences of mitochondrial cytochrome *b* gene. The genomic DNAs of all 16 identified cobitid species of Korea were extracted and their entire cytochrome b gene was PCR amplified using Glu-F3 and Pro-R as forward and reverse primers, respectively. The purified PCR products were then cloned and sequenced. A total of 78 complete 1140bp long cytochrome b sequences, including 10 GeneBank deposited data were used for the analyses. Three outgroups were chosen from two families of Balitoridae and Cyprinidae. Hybrids of two species of *Cobitis hankugensis* and *Iksookimia longicorpa* were also included in our specimens. DNAssist, MEGA 3.0 and PAUP 4.0d54 were used for sequencing and the phylogenetic analyses. Phylogenetic trees were constructed according to the neighbor-joining (NJ) and maximum parsimony (MP) methods in 1000 replications. All codon positions and both transition and transversion were considered in the analyses.

In the total sequence data, G and T had the lowest and the highest contents respectively, while C and A had almost equal rates. The phylogenetic trees had a general congruency and in some cases conflict with the current systematic scheme of the family Cobitidae. The polyphyly of the genus *Iksookimia* was notable. The positional proximity and the low sequence divergence between *I. longicorpa* and *I. hugowolfeldi*, suggested that they may not in fact be two distinct taxa at the species level. The association of *I. longicorpa* with two groups of *C. hankugensis* and the hybrid samples in the molecular phylogeny can be explained by a possible gene flow among these three taxa through the process of natural hybridization. *I. pumila* was the sister group of *I. koreensis* with a high bootstrap support (100%). The monophyly of two taxa of *C. lutheri* and *C. tetralineata* observed in this study was obtained in previous reseraches. However, the paraphyly of *C. lutheri* has raised a question over its taxonomy. One considerable outcome was that the examined species of Genus *Niwaella* from Korea (*N. multifasciata*) had a distant position and high genetic difference to its congener from Japan (*N. delicata*). Some questionable relationships within the genus *Misgurnus* were also uncovered. *M. mizolepis* did not fall into the branch composed of its congeners and had a relatively high sequence divergence to *M. anguillicatatus* and *M. fossilis.* The distant position of *C. sinensis* from *C. hankugensis* was considerable. Within the ingroup taxa, *Koreocobitis* occupied the most basal position in almost all the generated trees.

In conclusion, the obtained phylogeny provides a new insight into the relationships among cobitid fishes. Further researches are needed to answer the question of incongruencies between traditional and

molecular approaches. Especially the relationships among the taxa belonging to Genus *Iksookimia*, the systematics and nomenclature of *Niwaella*, the relationships within *Misgurnus* group and also the unusual positioning of *Cobitis* lineages. Using more molecular and cellular markers can lead to the construction of a more realistic phylogeny for this taxonomically complicated family.

요약

미꾸리과(Cobitidae)는 잉어목에 포함되며 세계적으로 16 속, 약 200 종이 담수역에 분포한다(Kim et al., 2002). 한국산 미꾸리과 어류의 계통분류학적 연구는 70 년대부터 시작되어서 다수의 연구가 진행되 었으 며(Kim et al, 2003) 현재 우리나라의 미꾸리과 어류는 6 속(*Misgurnus*, *Cobitis, Iksookimia, Koreocobitis, Niwaella,* 그리고 *Kichulchoia*), 16 종으로 보고되고 있다. 미꾸리과 어류의 계통분류학적 연구는 주로 형태 적 특징과 표현형 특징을 대상으로하여 수행되어 왔으며, 이들에 관한 분자적 연구의 경우, 한국산 미꾸리과 어류 중 일부를 대상으로 한 연구(e.g. Kim et al., 2002; Kitagawa et al., 2005)가 수행된 바었으나 우리나라 서식하는 미꾸리과 전체 어종을 대상으로 한 연구는 없는 실정이다. 따라서 본 연구는 미토콘드리아 cytochrome *b* 유전자 서열을 이용하여 한국산 미꾸리과 어류의 분자계통분류학적 관계성를 조사하였다.

본 연구에서는, 한국산 미꾸리과 어류, 16 종로부터 genomic DNA 를 분리한 후 Glu-F3 와 Pro-R 를 이용한 PCR 을 통해서 전체 cytochrome *b* 유전자 서열을 증폭하였다. 증폭된 PCR 산물은 정제를 한 후 cloning 하였다.

GenBank 에 등록된 10 개의 어류를 포함하여 총 78 개체의 전체 cytochrome *b* 유전자인 1141 bp nucleotide 를 이용하여 분자계통분류학적 관계성를 조사하였으며 참조분류군으로 종개과와 잉어아과 어류를 이용하였다. 또한 잡종(hybrid)인 2 개의 종 *Cobitis hankugensis* 와 *Iksookimia longicorpa* 를 이 연구에 포함하였다. 분자계통분류학적 분석은 MEGA 3.0 과 PAUP 4.0d54 프로그램을 이용하여 수행하였다. 계통수는 neighbor-joining (NJ)와 maximum parsimony (MP) 방법으로 통해서 작성하였으며 계통수 분지의 통계학적 검정을 위하여 1000 회 boostrap-ing 실시하였다. 그리고 각 염기조성을 계산하였고 transition 과 transver- sion 의 포화수준을 조산한 후 계통수 작성을 하였다.

미꾸리과 어류의 염기조성에 있어서, G 함량(14.9%)은 낮은 값을 나타냈고 T 함량(31.3%)은 높은 값을 나타났으며 C 함량(26.3%)과 A 함량(27.4%)은

유사한 값을 보였다. 작성된 계통수는 현재 사용하고있는 형태적인 분류체계와 일반적으로는 유사하게 나타났지만 몇몇의 분류군에 있어서는 차이를 보였다. *Iksookimia* 속은 polyphyletic group 를 형성하여 흥미로운 결과가 나타났으며 *I. longicorpa* 와 *I. hugowolfeldi* 사이에 낮은 sequence divergence (0-2%)가 나타나 종 수준에서의 구별된 으로 보기에는 어렵다고 생각되었다. 계통분석에서 *I. longicorpa* 는 두개의 *C. hankugensis* 의 잡종 그룹과 같이 묶이는 현상이 나타났고 이들 어종 간 자연적 잡종을 통해서 일어나는 3 개의 집단간 유전자 흐름을 확인할 수 있었다. 두개의 분류군인 *I. pumila* 와 *I. koreensis* 은 구분되며 단진화군을 형성하였다. *C. lutheri* 와 *C. tetralineata* 는 같은 그룹을 형성하여 대부분 단진화군 임을 관찰할 수 있었지만 탐진강에서 채집된 *C. lutheri* 는 paraphyletic group 를 형성하여 한강과 함평에서 채집된 개체와 유전적 차이를 관찰 할 수 있었다. 한국산 *Niwaella* 속(*N. multifasciata*)을 일본산 (*N. delicata*)과 비교한 결과 계통학적 위치로나 유전적으로 분명한 차이를 나타냈다. 또한 *Misgurnus* 속내의 계통분류학적 관계성는 불분명하였는데, 특히 *M. mizolepis* 는 *Misgurnus* 속 그룹과 묶이지 않았으며 *M.anguillicaudatus* 와 *M. fossilis* 와 비교한 결과 높은 sequence divergence (15.6-16.5%)를 나타내였다. *C. hankugensis* 는 *C. sinensis* 와 계통수에 있어서 분자계통유전학적으로 분명한 차이를 나타내었으나 *Koreocobitis* 속은 단화군을 형성하였다.

이 연구는 한국산 미꾸리과 어류 전체 종과 약간의 중국, 그리고 일부 일본산 미꾸리과 이들를 대상으로 이들 어류 사이의 분자계통분류헉인 관계를 조사하였다. 이 연구의 결과, *Iksookimia*, *Niwaella* 속 분류와 명명법 및 *Misgurnus* 그룹 내의 관계에 대하여 기존분류체계의 문제점을 제시하였고 이를 통해서 미꾸리과 어류의 분류체계의 재정립을 시도하였다.

1. INTRODUCTION

1.1. Cobitid Fishes; Ichthyological Aspects and Occurrence In Asiatic Waters

The spined loaches, Family Cobitidae, (Order Cypriniformes) comprise 16 genera and about 200 species of freshwater fishes, distributed in Eurasia and northern Africa (Kim et al., 2002). Nelson (1994) introduced the members of Family Cobitidae as having wormlike to fusiform bodies with subterminal mouth, 3-6 pairs of barbels, eractile spine below their eye, one row of pharyngeal teeth and a maximum body length of 40cm. Similar to the other members of the order Cyprinifomes, the Weberian apparatus, typical of Ostariophysi (Jamieson, 1991), is present in this family.

Liu et al. (2002) studied the phylogenetic relationships of Cobitidae with the other families of the order Cypriniformes by using their sequences of the mitochondrial control region gene. Two proposed intra-ordinal relative positions of the families of Cypriniformes is illustrated in the cladogram of Fig 1.

Several morphological traits which have generally been used in identification of genera and species within the family Cobitidae include body coloration, the appearance and arrangement of Gambetta zone on the body sides, presence and structure of suborbital spine, number, position and length of barbels, dark- color spots on the base of caudal fin, predorsal swelling, the arrangement of subdorsal scales, the structure of mouth, metric and meristic measures, shape and size of pectoral fin, the elongate second pectoral fin ray in some males, the number of branched rays in the caudal fin, and most specifically, the number and shape of an ossified plate-like extension on the base of the

second pectoral fin ray, referred to as the scale of Canestrini or lamina circularis, in the males (Sezaki et al., 1994; Kim and Park, 1997; Erkakan et al., 1998; Erkakan et al., 1999; Kotusz, 2000; Perdices and Doadrio, 2001; Ludwig et al., 2001; Kim et al., 2002; Kim et al., 2003). In addition, it has been noted that sexual dimorphism in cobitid fishes can cause considerable morphological differences between sexes (Kim et al., 1976; Kotusz, 2000; Vladykov, 2005).

Nelson (1994) divided Cobitidae into two subfamilies of Cobitinae, comprised of 15 genera with one pair of rostral barbels and a rounded caudal fin, and Botiinae including three genera which posses two pairs of rostral barbels and a deeply forked caudal fin. Recently, Kim et al. (2003) described the subfamily Cobitinae as having 16 genera and about 100 species, which are widely distributed in the Old World. Kim and Lee (2000) introduced this subfamily as small freshwater fishes with an elongate body, minute scales, and a small suborbital spine, occurring through Europe and Asia. *Cobitis* is the most specious genus in the family Cobitidae, with about 50 valid species known at present (Kim et al., 2003). All species of the genus *Cobitis* have the peculiar lamina circularis at the base of their pectoral fin in the males as a secondary sexual characteristic, microstructures which are important in the identification of the cobitid species (Kim et al., 2002).

Cobitids are bottom dwelling species (Nelson, 1994), and they live at down, middle or up stream of the rivers with mostly sandy or somewhat muddy bottoms (Kim et al., 2005). Besides the biological roles of Cobitidae in freshwater ecosystems, some members of the family are considered as nutritious food items (e.g. mud loach, *Misgurnus mizolepis*; Kim et al., 1997; Nam et al., 2001). Some cobitid

fishes are highly demanded by the ornamental-fish traders (e.g. eel loaches Genus *Pangio*; Ruzainah et al., 2003). Also, cobitid fishes have been found to be useful organisms for biological control of pests in the agriculture fields (Lee, 2000). Some of them live in extreme and peculiar environmental conditions such as caves, resulting in acquisition of special morphological and physiological features, mainly losing optical sense (ascribed to 'eyeless' fish) (Greenwood, 1976; Romero and Green, 2005). Further more, some of the species of this family are facing the high risk of extinction and are included in the list of endangered fish fauna (Baillie et al., 2004), being considered for intensive conservation programs. Many scientists are interested to employ cobitid fishes as model organisms in biological and genetic researches, because of their several attractive characteristics such as small body size, transparent embryo, fast embryonic development, short generation time, superior tolerance to low oxygen tension and diseases, year-round spawning under control conditions, and high fecundity (Noh et al, 2003; Kim et al., 2004). The earliest fossil records for cobitid fishes are found in the Miocene and are dated to an age of about 15 million years (Ludwig et al., 2001). Perdices and Doadrio (2001), following the molecular clock estimation, concluded that the major lineages of the genus *Cobitis* originated mainly during a period of the Miocene between 8 and 9.3 million year ago (Mya), where as the within-lineage speciation occurred during Pliocene (5.8-3.9 Mya).

Eastern as well as southern Asian freshwater fish fauna represent the richest ichthyofauna in the Sino-Indian region. There are more than 25 primary freshwater fish families described with at least 12 families endemic to the Asiatic waters (Perdices et al., 2004). The fishes of

order Cypriniformes, that are the largest component of the Ostariophysans (Liu et al., 2002), have their greatest diversity in Southeast Asia (Jamieson, 1991). The species richness of Cyprinidae, the largest fish family, has been found to be the highest in East Asia (He et al., 2004). A number of 13 valid species in the genus *Cobitis* was described in Eastern Asia, which inhabit China, Korea, Laos, Russia and Vietnam (Kim et al., 2002). Erkakan et al. (1998) marked this area as having undergone large transformational events and consequently faster species evolution. Zoogeographic studies on the European freshwater fish fauna have suggested the eastern Asiatic origin of the European Cobitidae based on the high cobitid diversification in this area (Kim et al., 2002).

The Korean peninsula bears a pronounce part of fish diversity and endemism of East Asia, with having its own exclusive ichthyofauna. The number of identified freshwater fishes in Korea is about 212 species, by which some 50 species are distinguished to be endemic to the fluvio-lacustrine systems of the peninsula (Kim and Park, 2002). Lee (2004), based on analyzing the fossil records of some cyprinid fishes, concluded that this group of fish might have existed on the Korean peninsula since the early Pliocene (More than 5 million years ago). This region is also considered as a pathway for biogeographic convergence, speciation, and evolutionary processes. It has been presumed that most of the Japanese species have originated from the Korean Peninsula (Kitagawa et al., 2005). Like wise, Kitagawa et al. (2003) proposed that the Japanese *Cobitis* species were introduced to Japan from the Asian continent through the Korean peninsula when the country and the Asian mainland were joined by a land bridge. Kim et al.

(2003) suggested that endemism richness in Korea might have resulted from long-term isolation in this area.

In Korea, the scientific name of the Cobitid fishes has been confused for a long time owing to their misidentification. The history of records on systematic studies of Family Cobitidae in Korea exceeds 70 years (Kim et al., 2003). In one of the earliest reports on cobitid fishes of Korea, they were all categorized as a single species of *Cobitis taenia* Linnes which having different colour patterns (Kim, 1975). Later, several discriminative characteristics were discovered in this group of fishes, and new species were erected by detail morphological analysis of the specimens (i.e. Kim, 1975; Kim et al., 1976; Kim and Son, 1984; Kim and Lee, 1995; Kim and Park, 1997).

The genus *Niwaella* was introduced as a new taxon that differed from the other cobitids by having a small head, sucker-like mouth with small barbels and lack of a lamina circularis in the pectoral fin of the males (Kim and Lee, 1995). By the year 1993, the number of genera in Korean Cobitidae had been raised to three genera of *Cobitis*, *Misgurnus* and *Niwaella*. In this year, *Iksookimia* was erected as a new genus based on its characteristics including an elongated first pectoral fin ray and the absence of four Gambetta zone on the body sides (Kim and Park, 1997). *Koreocobitis* is the latest emerged genus of Korean Cobitidae, which has been introduced as a distinct taxon, according to its diagnostic characteristics of the body colour patterns and its moveable suborbital spine (Kim et al., 2000). Currently, the family Cobitidae in Korea includes 16 species in six genera of *Misgurnus*, *Cobitis*, *Iksookimia*, *Koreocobitis*, *Niwaella*, and *Kichulchoia*, of which, three genera and 12 species are endemic to Korea (Kim et al., 2003).

Kim et al. (2002) suggested Korean Cobitidae belonged to two subfamilies of Cobitinae including *Cobitis* and Nemacheilonae including *Orthrias*. As for Genus *Cobitis*, nine species and subspecies are native to Korea, and of them five are endemic (Sezaki et al., 1994; Kim and Lee, 1995). During the past decades, several cobitid species have been transferred to other genera from the genus *Cobitis*, and now, four species are presented in the genus *Cobitis* of Korea (Kim et al., 2003). An illustrative literature of Korean ichthyofauna, including cobitid fishes, has been provided by Kim et al. (2005).

Several hypotheses have been presented considering the biogeographical distribution of freshwater fishes in the Korean peninsula (Jeon, 1986). In the case of cobitid fishes of Korea, an obvious disjunct occurrence of different species has been discovered in the peninsular waters (Kim and Park, 1997; Kim et al., 2003). Figure 2 demonstrates the distribution of species of Family cobitidae in different localities of Korea. As can be seen in the figure some of the species such as *Misgurnus anguillicaudatus* and *M. mizolepis* have large ranges of distribution, while some others like *Ikssokimia pumila* and *I. choii* occur in limited geographic areas.

To date, the location and nomenclature of genera and species in the systematic scheme of the family Cobitidae have been mainly determined based on their morphological and phenotypic structures. Park and Lee (1991) studied the differences in populations of *Nemacheilus toni*, a putative cobitid fish, by using genetic variations in their mitochondrial DNA (mtDNA). Kim et al. (1999) carried out a chromosomal study on Korean cobitid *Iksookimia yondokensis*. Molecular systematics of some Korean cobitid fishes was studied based

on their mitochondrial cytochrome *b* sequences by Kim et al. (2002). Kim et al. (2003) published a review on the species of Genus C*obitis* of Korea. The karyotype of *I. hugowolfeldi* and a bibliography of the cytological studies on the genus *Iksookimia* during the years 1986-1999 were presented by Kim et al. (2003). In a study by Saitoh et al. (2004), the mitochondrial gene introgression was observed in a hybrid loach and its parents sampled from Korea.

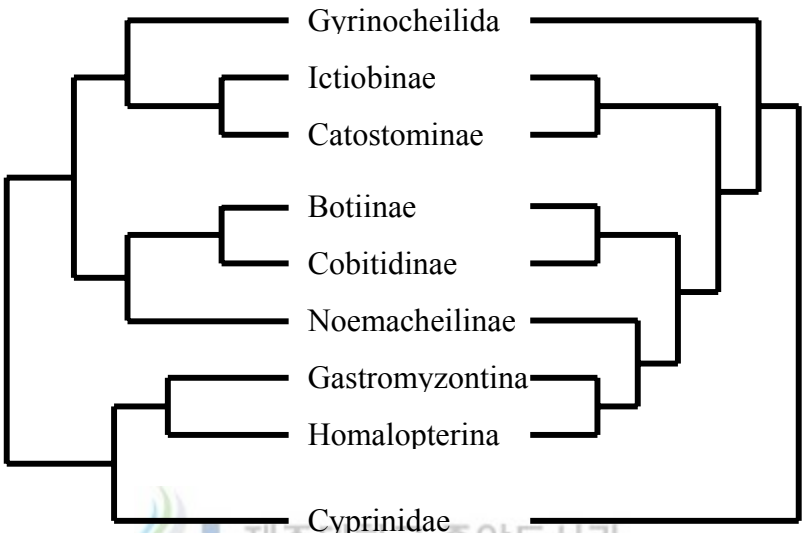


Fig.1. Two hypotheses concerning the phylogeny of Cypriniformes (Adapted from Liu et al., 2002). Note that Cobitididae Is an alternative incorrect spelling of Cobitidae (Nelson, 1994).

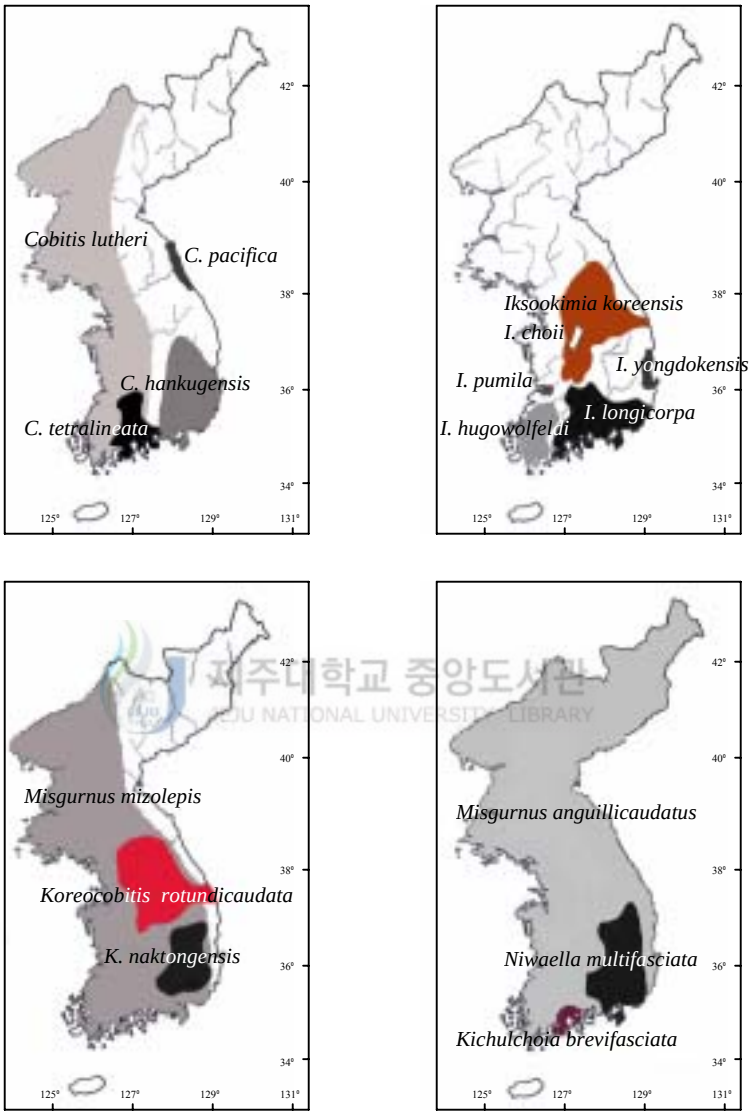


Fig. 2. Distribution of cobitid fishes In Korean peninsula. The distinct areas of distribution for different species are shown by different colours.

1.2. Hybridization, Ploidy and Speciation In Cobitidae

Natural hybridization is now increasingly seen as an important evolutionary factor to initiate speciation and adaptive radiations (Hanfling et al., 2005). The presence of hybrids in a population is usually inferred when sympatric specimens are found that are morphologically intermediate between two species (Kim and Lee, 1990). Berrebi (1995) proposed that hybrids form a buffer population between the two species, so that, this 'hybrid zone' is stable in time and the space.

Hybrids are deemed to occur in 56 families of fishes, and are most common within the Salmonidae (Salmoniformes), Esocidae (Esociformes), Cyprinidae and Catostomidae (Cypriniformes), Centrarchidae and Percidae (Perciformes) (Le Comber and Smith, 2004). The fishes of Family Cobitidae demonstrate some rates of the intergeneric and interspecific hybridization. This hybridization has been stated as being associated with the generation of all-female triploid or some times diploid clones of one species which reproduce by gynogenesis (Sezaki et al., 1994).

Kim and Lee (1990), in their study on the cobitid fishes of the Nakdong River system in Korea, observed many individuals having intermediate forms and found them to be the hybrids between two species of *Cobitis sinensis* and *C. longicorpus*. These hybrids were almost always females, outnumbering the parent species at some localities, and occurring as both diploids and triploids. Biological aspects, ploidy complexes, and artificial hybridization of this hybrid were investigated by Kim and Lee (1995) and Kim and Lee (2000). In Korea, one other natural hybrid between two species of *Cobitis*

longicorpus and *Misgurnus anguilocaudatus* was introduced by Hwang et al. (1995). On the other hand, Bohlen and Ritterbusch (2000) proposed that mating of the females of each of the spined loaches Genus *Cobitis* with the male of any other fish species is most unlikely, because of the late spawning season and the specific spawning habits in this group of fish. Sezaki et al. (1994), by using biochemical genetic evidences, proved the hybrid origin of the Japanese cobitid, *C. taenia taenia*. Hubbs (1955) proposed that occurrence of natural hybrids is correlated with evolutionary expectation, in that, they were most likely to be found in (1) disturbed environments; (2) localities where one parental species had been introduced; (3) localities where one parental species was rare and the other abundant; and (4) areas of stress where heterosis would enhance abundance. He also noted that hybridization was inversely associated with fish diversity. So that in freshwater fish it occurs more than in marine fish. Tsigenopoulos et al. (2002) and Toth et al. (2005) suggested that the natural process of hybridization is facilitated by the changes of environmental condition and human activities. Briolay et al. (1998), as an evolutionary interpretation of this phenomenon, concluded that true hybrids occur between individuals whose last common ancestor is as old as 10-15 million year ago or more. More over, hybrids have been called as being rather the by-products of an accidental secondary contact of different evolutionary lineages, which are not fully isolated reproductively (Kotusz, 2005; pers.com).

In fish, some families such as Cyprinidae or Cobitidae are known for containing polyploid or polyploid-origin species or populations (Kitagawa et al., 2003). Many ichthyologists and herpetologists have

concentrated on the evolutionary biology of polyploids. This field of study is attractive, not only because of the particular mode of speciation, but also it provides insights into the evolution of higher vertebrates by gene duplication (Saitoh et al., 2000). Polyploidy has been linked to carcinogenesis in mammals, but occurs widely in some groups of fishes (Lingenfelser et al., 1997). In some instances, polyploidy in fish involves entire taxa above the level of species. These include the tetraploid Salmondae and Catostomidae and the tetraploid and hexaploid *Barbus* (Berrebi, 1995).

In the Moscow River drainage of Russia, the diploid-polyploid complex of Genus C*obitis* was described as a gynogenetic form or hybridization (Kim and Lee, 2000). In a similar report, a triploid-tetraploid gynogenetic form of the genus *Cobitis* including two bisexual species, *Cobitis taenia* and *C. granoei* was introduced from the Moscow River (Kim and Lee, 1990). Slechtova et al. (2000) explored the occurrence of two ploid complexes of cobitid fish in Czech. Like wise, a combination of different ploidy in Genus *Cobitis* has been observed in the natural waters of Poland (Kotusz, 2000). Hybridization between *C. taenia* and other cobitid fishes resulted in polyploid offspring (Sezaki et al., 1994). The aforementioned Korean hybrid species of *C. sinensis-longicorpus* found to be of the two levels of ploidy (diploid-triploid) (Kim and Lee, 1990; Kim and Lee, 1995). In an artificial hybridization between *Misgurnus mizolepis* and *M. anguillicaudatus* some triploid individuals were produced together with diploid ones (Park and Kim, 2000). Among the known hybrid vertebrates, only for *M. anguillicaudatus* simultaneous production of haploid and triploid eggs had been reported (Alves et al., 2004)

Nelson (1994) suggested that a diploid chromosome number of 48 might be primitive for the all fish of order Cypriniformes. In *C. sinensis-longicorpus*, the parental species have 2n chromosome numbers of 48 and 50, while the chromosome number of diploid and triploid forms of this hybrid estimated to be 49 and 73 or 74, respectively (Kim and Lee, 2000). A chromosome number of 100 was obtained from *Iksookimia yongdokensis*, suggesting that at least part of the populations of this species in Korea may exist as tetraploid (Kim et al., 1999). Karyotypic studies revealed the existence of at least two different types of mud loach, tetraploid (in China) and diploid (in Japan) (Alam and Rahman Khan, 2001). *Cobitis biwae* also includes the tetraploid form, which has 96 chromosomes, as well as the diploid form with 48 chromosomes. In the latter species, two ploidy forms have been represented as a single nominal species (diploid-tetraploid complex), because of a lack of critical morphological differentiations between them (Kitagawa et al., 2003).

The occurrence of unisexual (all-female) populations of hybrid origin in some of the loaches, which contain diploid-polyploid complexes, is emphasized to be a source of establishment of gonochoric tetraploid population (Saitoh et al., 2004). Triploid offspring resulting from the hybridization between *C. sinensis* and *C. longicorpus* accounted for approximately 40% of the cobitid fishes in Nakdong River and related streams (Sezaki et al., 1994). Polyploid females were estimated as 87% of the spined loach, *C. taenia,* population from the middle Dnieper basin, Ukraine (Mezhzherin and Chudakorova, 2002). A high rate (6.9%) of triploid individual of loach *Misgurnus anguillicaudatus* has been reported to be among the samples

from a fish farm in Japan (Arai et al., 2000). Bohlen and Ritterbusch (2000) hypothesized that the numerical dominance of polyploids in nature is likely because of their higher reproductive rates or an advantage in competition.

At present, alloploidy is the only way of speciation that has been experimentally confirmed. This phenomenon is rare in vertebrates, and among these, is restricted to poikilotherms. One of these rare examples is a species complex that is conventionally termed spined loach, *Cobitis taenia*, and actually represents a polyploidy series of diploids, triploids, tetraploids, and possibly, pentaploid. Two to three percent of all individuals of a population of this loach species are identified as pentaploids (Mezhzherin and Chudakorova, 2002). Mable (2004) suggested that polyploidy should be more common in temperate than in tropical breeders because environmental fluctuations can promote unreduced gamete formation.

Polyploid forms usually display characteristics that are intermediate between the diploid parental species, which confirms their hybrid origin (Kotusz, 2000). Recent studies have shown that several polyploid species of the genus *Cobitis* are of hybrid origin (Kitagawa et al., 2003). Polyploidy in fish has been associated with traits including large body size, fast growth rate, long life, sterility, higher survival rate and ecological adaptability (Cogswell et al., 2002). In some of triploid fishes, suppression in gonadal development and reproductive processes has been reported (Krisfalusi et al., 2000; Felip et al., 2001; Le Comber and Smith, 2004). Fish having different ploidy also showed differences in some blood factors (i.e. triploid fish had larger erythrocytes, increased hemoglobin content, and less erythrocyte count than diploid

fish; Cogswell et al., 2002). A review on the physiological and behavioral impacts of ploidy in fish has been issued by Benfey (1999).

Le Comber and Smith (2004) claimed that polyploidy effects all levels of the genome, from the chromosome to DNA sequence, and can have important consequences for both the arrangement of sequences within the genome and for the sequences themselves. The presence of three different genomes was discovered in the hybrid complex of *C. taenia*, *C. melanoleuca* and *C.* sp. (Slechtova et al., 2000). Lingenfelser et al. (1997) identified fish of the different ploidy by comparison of the genomic DNA contents in their blood cells. Kotusz (2000) found significant differences in some hematological factors and metric measures among the fish of different ploidy levels. Mezhzherin and Chudakorova (2002) suggested that using morphological analysis of hybrids makes it more likely to find out the origin of polyploid loaches, because polyploids are similar in appearance to the nominative species if its genome is prevalent in the polyploid genome. In biochemical study of particular enzyme systems, polyploid fish showed asymmetrical intensity distribution of individual isozyme having more than two separate alleles in the respective locus. Such individuals were then assumed to be triploids or tetraploids (three or four different alleles, respectively) (Slechtova et al., 2000). However, no differences were observed between diploid and tetraploid specimens of *C. biwae* in the starch gel electrophoretic and isoelectric focusing patterns of sarchoplasmic proteins (Sezaki et al., 1994). Guo et al. (2004) proposed that mitochondrial genes are very useful in the analysis of the genetic relationship in the polyploid fish. In contrast, Berrebi (1995) reached different conclusions by using both allozymic and molecular markers

for studying on polyidy in fish and concluded that mitochondrial markers were of no use in this respect. Saitoh et al. (2000) following the use of mtDNA as a marker of divergence among fish having different levels of ploidy, suggested that the depths of mitochondrial branches between tetraploids and their closest diploid relatives were usually much smaller than branches among diploids, indicating a recent establishment of tetraploids. Guo et al. (2004) found that even after eleven generations the examined F11 allotetraploid fish and its female parent had the identical amino acid sequences of the mitochondrial ATPase8 and ATPase6 genes. Measurement of DNA content of blood or body cells by flow cytometry (FCM) has been found as a confident approach to distinguish fish with different levels of ploidy (Lingenfelser et al., 1997; Lamatsch et al., 2000; Iguchi et al., 2003; Alves et al., 2004). The other conventional methods for ploidy determination are known to be estimating the size of erythrocytes nucleus area (Felip et al., 2001; Toth et al., 2005) and analysis of hemoglobin content by spectrophotometry (Cogswell et al., 2002). However, the most reliable way of ploidy estimation seems yet to be karyotyping for chromosome counts as Lamatsch et al. (2000) did on *Poecilia Formosa*, and Saitoh et al. (2000) and Kim and Lee (1990) applied on cobitid fishes of Japan and Korea, respectively.

1.3. Outline of Systematic Studies In Cyprinformes and Cobitid Fishes

Knowledge of the correct phylogenetic relationships among animals is crucial for the valid interpretation of evolutionary trends in biology (Chen et al., 2004). Freshwater fishes are particularly informative for the study of historical events, because the island-like property of freshwater habitats, the relative instability of these habitats, the impacts of climatic changes on rivers and lakes and the availability of geological data for aquatic systems (Salzburger et al., 2003). Not surprisingly, and in accordance with the prominent numerical (inclusion nearly 80% of all species of the order Cypriniformes; Howes, 1991) and commercial superiority of the family Cyprinidae, most taxonomic, phylogenetic and biogeographic studies on the Cypriniformes have been concentrated on this family and its subfamilies. In this regard, external and phenotypic parameters of fish have been the preliminary applied character states. Phylogenetic relationships of Cyprinid fish were investigated by using morphological and osteological aspects (Nam and Yang, 1998; Dimmick and Burr, 1999; Chen and Chen, 2001). Similarly, morphological and osteological traits such as body colour patterns, shape and size of the pectoral fin, structure of the suborbital spine, structure of the mouth and the shape of lamina circularis have so far been the main criteria in species identification of Family Cobitidae (Sezaki et al., 1994; Erkakan et al., 1998; Erkakan et al., 1999; Kotusz, 2000; Perdices and Doadrio, 2001; Kim et al., 2002; Kim et al., 2003). It has also been realized that special host-parasite interactions can lead to an understanding of phylogenetic relationships in some cyprinid fish (Xiao et al., 2001).

Jamieson (1991) and Kim et al. (1998), following their ultrasructural studies on some variable means of spermatozoa such as tail length, the relative position of the centrioles, and the number of mitochondria, proposed the existence of a correlation between phylogeny and sperm structure, especially its number of mitochondria.

Among the vertebrates, fish have perhaps been the most intensely studied group for local and regional genetic structure, largely due to the sustained long-term interest in the geographical structure of intraspecific diversity for fishery management (Bernatchez and Wilson, 1998). Cytogenetic surveys on phylogeny of ciprinid fishes are limited primarily to visualizations of the chromosomal nucleolus organizer regions (NORs). In most extensive cases, NORs variations have been examined within and among sixty-nine North American cyprinids, which the obtained results highlighted the applicability of these regions as taxonomic or systematic characteristics in the analyzed fish (Buth et al., 1991).

An increasingly sophisticated realm of techniques has been developed since the mid-1970s to study the molecular similarities of organisms (Kocher and Stepien, 1997). Molecular studies of fish populations commenced some thirty-five years ago (Imsiridou et al., 1998). The advances in molecular biology during the past decade led to the accumulation of a considerable amount of sequence data of ray-finned fish in the GeneBank (Chen et al., 2004). Further more, the improvements in molecular biology (e.g. DNA cloning) have opened frontiers in evolutionary biology (Buth et al., 1991). Also, it has been taken into consideration that since molecular characteristics are less likely related to adaptive evolution than morphologic traits (Briolay et

al., 1998), they could be useful to resolve the evolutionary dependence among organisms.

At the protein level, allozymes have been used in phylogenetic studies of some cyprinid fishes (Dimmick and Burr, 1999; Laroche et al., 1999; Hanfling and Brandl, 2000; Tsigenopoulos et al., 2002). However, the utility and power of allozyme sequence data for deep splits within studied fish found to be doubtful (Hanfling and Brandl, 2000). During the past decade, geneticists and taxonomists have used restriction endonuclease, rather than sequencing to examine variation within and between species in specific segments of DNA (Kocher et al., 1989). In the study of Hwang et al. (2002), beta-actin gene was employed to resolve the phylogeny of cyprinid fishes.

Most evolutionary or systematic studies, which have focused upon differences at the sequence level, have employed mitochondrial DNA (mtDNA). Several unique properties of mtDNA make it particularly useful for molecular investigations: (1) it is relatively small, circular molecule ranging in size from 16.2 to 19.5 kilobases (kb) in fishes; (2) it is highly compact with few non-coding regions, and its gene content and order appear to be conserved in all vertebrates; (3) it is maternally and clonally inherited without recombination, and usually exists in only one genotype per individual, although there are exceptions; and (4) in vertebrates, it evolves five to ten times as rapidly as scnDNA (single copy nuclear DNA). Because of its rapid evolution, mtDNA usually varies among individuals within a species. This variability, coupled with haploid inheritance, makes mtDNA a sensitive marker for analyses of population genetic structure and estimates of gene flow between populations (Buth et al., 1991).

Several mitochondrial genes have been used to explore the phylogenetic and systematic relationship among members of order Cypriniformes and to construct their phylogenetic trees. For instance, 12S (Harris and Mayden, 2001) and 16S (Gilles et al., 1998; Dimmick and Burr, 1999; Gilles et al., 2001; Harris and Mayden, 2001) ribosomal DNA mitochondrial genes, and mitochondrial ATPases 6 and 8 genes (Machordom and Doadrio, 2001). Some other mitochondrial genes, which their sequences have been used in phylogenetic analyses, are ND4L, ND4, ND2 and tRNA genes (Dimmick and Burr, 1999; Xiao et al., 2001). The comparative sequences of some other component of mitochondria such as the control region (Gilles et al., 2001; Liu et al., 2002; Salzburger et al., 2003; Zhou et al., 2004) and the D-loop region (Imsiridou et al., 1998) were applied to find out the extent and sort of convergence in different cyprinid lineages.

Among the 37 protein-coding genes in mtDND, cytochrome *b* gene codes for one of the several factors responsible for electron transport in electron-chain phosphorylation (Fuchs et al., 2000). The cytochrome *b* gene has been widely used in systematic studies to resolve divergences at many taxonomic levels and to answer many systematic questions, from ‘deep’ phylogeny to the population and recent divergence levels. It has been considered as one of the most useful genes for phylogenetic researches, and is probably the best-known mitochondrial gene with respect to structure and function of its protein product (Farias et al., 2001). It has also been found to provide phylogenetic resolution among a variety of fish groups (Zardoya et al., 1999). A growing number of scientists have recruited partial sequences (Gilles et al., 1998; Durand

et al., 1999; Fuchs et al., 2000; Gilles et al., 2001; Tsigenopoulos et al., 2002) as well as complete sequences (Zardoya and Doadrio, 1998; Briolay et al., 1998; Zardoya et al., 1999; Tsigenopoulos and Berrebi, 2000; Xiao et al., 2001; Machordom and Doadrio, 2001; Durand et al., 2002; Kim et al., 2002; Berendzen et al., 2003; Zhou et al., 2004; Perdices et al., 2004; He et al., 2004) of the cytochrome *b* gene to address the systematic and phylogenetic relationships among members of the order Cypriniformes at different taxonomic levels.

In the case of molecular works in cobitid fish, Perdices and Doadrio (2001) studied the molecular systematics and biogeography of European cobitids based on their mtDNA sequences. Ludwig et al. (2001) studied the phylogenetic relationships and historical biogeography of the members of two cobitid genera of *Cobitis* and *Sabanejewia* by analyzing their 12S rRNA gene sequences. Alam and Rahman Khan (2001) examined the intraspecific genetic variation in the Japanese loach (*Misgurnus anguillicaudatus*) by the aid of RAPD analysis. The phylogeny of the fishes order Cypriniformes (including some species of Cobitidae) was studied by Liu et al. (2002) through the comparison of the sequence variation of their mitochondrial DNA control region. Arai et al. (2000) certified the clonal nature of the gynogens produced from *M. anguillicaudatus* by analyzing their DNA fragments. Saitoh et al. (2000) searched for the relationships among the populations of different ploidy complexes of Japanese loach. Ruzainah et al. (2003) applied random amplified polymorphic DNA (RAPD) in taxonomy of two eel loch species. Kitagawa et al. (2003) studied the phylogeography and maternal origin of a tetraploid *Cobitis biwae* from Japan by means of mtDNA analysis. Most recently, the relationships

among the spined loaches sampled from Japan and Korea were analyzed by Kitagawa et al. (2005), by comparisons of some segments of their mitochondrial DNA.

Regarding molecular systematics of Cobitidae, and biogeography and phylogenetic classification of the whole family based on the genetic parameters, the existing literature attests an apparent scarcity of information. Meanwhile, no former study subjected the phylogenetic relationship among all cobitid fishes of Korean peninsula in molecular level.

In this study we aimed to examine the phylogenetic relationships in the fishes of Family Cobitidae in Korea by comparison of their complete sequences of mitochondrial cytochrome *b* gene with a review on previous works on taxonomy and nomenclature of this fishes.

2. MATERIALS AND METHODS

2.1. Sample Collection

The list of fish species used in this study and their sampling localities are presented in Table 1. In total, all 16 identified cobitid species, in Korean waters, were sampled and their genomic DNAs were extracted. For some localities more than one specimen of each species were examined. Besides, the sequences of some Asiatic cobitid and cyprinid fishes were taken from the GeneBank, under their corresponding accession numbers (Table 1) to extend our tree accuracy and to delineate inter and intra-familial relationships. Three species of *Lefua costata* from the family Balitoridae, and *Cyprinus carpio* and *Carassius auratus* from the family Cyprinidae, were used as outgroups. These outgroup species were selected because of their relative closeness to the family Cobitidae in conventional taxonomic ordering. Further more, a number of 4 fish were sampled as suspected to be hybrids between two species of *Cobitis hankugensis* (revised *C. sinensis*) and *Iksookimia longicorpa* as reported by Kim and Lee (1990). The distribution of sampling localities is indicated in Figure 3.

2.2. Total DNA Extraction

Total cellular DNA was extracted from the muscle of deceased samples or part of the fin of the live fishes, using an AccuPrep Genomic DNA Extraction Kit (BIONEER corporation) according to the kit manual instruction. Briefly, a section of 25 to 50 mg tissue was disrupted and placed in a 1.5 ml tube, suspended in 200µl tissue lysis buffer (TL) plus 20µl proteinase K and incubated at 60ºC for 3 hours or more for protein digestion. The sample in the tube was resuspended in 200µl biding

buffer (GC) and incubated once more at 60ºC for 10 min. The procedure was followed by adding 100µl Isopropanol, transferring the sample to a spin column, washing the extra residual, and eluting the bound DNA from the glass fiber column of the tube by 100µl elution buffer (EL). The concentration of the final extracted DNA was determined by electrophoresis of products loaded on a 0.8% agarose gel.

2.3. Amplification of Cytochrome *b* Gene

The entire cytochrome *b* gene of each sample including parts of tRNA regions (the 3' end of the glutamine transfer RNA, the complete threonine transfer RNA, and the 5' end of proline transfer RNA), was amplified with the flanking tRNA primers including the forward primer of Glu-F3 and the reverse primer of Pro-R. (Fig.4). The sequence structures of these primers are displayed in Table 2. The PCR reaction was performed in a final volume of 50µl in 0.5 ml PCR tubes containing 1-4µl of genomic DNA depended on its concentration, 2.5µl each of forward and reverse primers, 5µl 10x reaction buffer, 5µl PCR nucleotide mixture (containing the sodium salts of dATP, dCTP, dGTP, and dTTP), and 1µl (1 unit/µl) Ex *Taq* polymerase (Takara Bio Inc.). The remaining volume up to 50µl was completed with ddH2O. Approximately 2 drops of mineral oil from a 200µl micropipette tip was added prior to the initiation of cycling, to serve as an evaporation barrier. The amplification was performed in a Programmable Thermo Controller (PTC-100, MJ Research Inc.). Typical PCR protocol used in this study to amplify cytochrome *b* gene consisted of an initial denaturating step at a temperature of 94ºC for 2 min, followed by 29 or 35 cycles of DNA denaturation at 94ºC for 45 sec, primer annealing at

43ºC or 45ºC for 1 min, primer extension at 72ºC for 1.5 min, and a final extension at 72ºC for 7min. The size of PCR product was checked against a 1kb DNA ladder on a 0.8% agarose gel (Agarose LE, Promega Co.), stained with a 0.5µl ethidium bromide in 1x TAE buffer. The initial PCR product was purified by electrophoresis on a low-melt stained agarose gel. The gel slice that contained the desired band was cut out as small as possible and purified using AccuPrep Gel Purification Kit (Bioneer Corp.), according to the manual instruction.

2.4. Cloning of the PCR Product

For cloning of the PCR product pBluescript II SK (Stratagene) was used as host or vector**.** To prepare the vector for hosting, 2.5 µl of it was digested with Hinc II restriction enzyme at 37ºC for 2 hour. The digested vector was purified after running on an agarose gel, by using a gel purification kit (Bioneer Corp.), according to the manufacturer recommendation. Consequently, the concentration of the restricted vector was determined and compared with that of the PCR product of each sample by electrophoresis. Ligation was carried out with 12µl reaction mixtures containing 1µl of the restricted vector, 1.5 to 9µl of gel purified DNA (according to the relative concentration or band brightness in electrophoresis), 1µl of 10x T4 ligation buffer and 1 unit of T4 ligase (Takara Bio Inc.). The mixture was incubated at 16ºC for 18 hours for a complete insertion of DNA into vector.

In the next step, five microliter of each ligated product was mixed with 50µl of *E. coli* XL1-blue competent cells and the mixture was left for 40 min in ice, with latter heat-shocks by putting at 42ºC and then in ice for 1.5 and 2 min, respectively. This procedure was followed by

adding a volume of 95µl of LB broth to the tube components and mixing them by pipette. The final mixture was incubated at 37ºC for 30 min.

The tube content was spread on a Luria-Bertani (LB) agar plate containing ampicillin, X-gal, and IPTG, and the medium was incubated overnight at 37ºC. The produced colonies, having white colour, were scratched and inoculated into a 4 ml LB broth solution containing ampicillin, and were left to grow for 16-18 hours at 37ºC in a shaking incubator. Finally, the plasmid DNAs of potential clones were isolated by using a Plasmid Miniprep Kit (Dyne Bio Inc. Korea), according to the kit manual, and prepared for sequencing after checking their size by a further electrophoresis on the agarose gel.

2.5. Sequencing

The sequencing of purified selected DNA fragments was accomplished by handing over a volume of 15µl genomic DNA of each individual to a commercial sequencing company (Macrogen, Korea). The sequencing has been done based up on our recommended protocol by using a Pbluescript II SK vector in a concentration of 0.10 µg/µl, and two specific cytochrome *b* forward and reverse primers of T3 and T7, respectively. The structural sequences of these primers are displayed in Table 2.

2.6. Sequence Analysis

A total of sixty-eight complete 1140 bp long cytochrome *b* from 16 species of Family Cobitidae and one species of Balitoridae *sensu* Kim et al. (2005), plus 10 GeneBank-deposited sequences with their

corresponding accession numbers (Table 1), were aligned manually using a DNAssist alignment editor (shareware, version 2.2; Patterton and Graves, 2000). The base composition bias, pattern of substitution for pairwise comparisons and codon arrays were computed by applying MEGA 3.0 software (Kumar et al, 2004). Phylogenic trees were constructed according to the maximum parsimony (MP; Fitch, 1971) and Neighbor-joining (NJ; Saitou and Nei, 1987) methods, using MEGA 3.0 and PAUP 4.0d54 (Swofford, 1998) programs. To take into account the ratio of transition to transversion (TS/TV) bias and intrasequence base composition bias, pairwise distances between nucleotide sequences were assessed according to Kimura's (1980) two-parameter model and Tamura and Nei's (1993) distance model. A combination of several models and methods were tested using first, second and third codon positions separately or in conjugation to evaluate the possible effect of the application of each data set and also to eliminate the noise resulted from saturated data. The statistical significance of branching orders was calculated by the bootstrap resampling process (Felsenstein, 1985), in a replication number of 1000. In order to increase the level of accuracy in our phylogeny, and have a better comparison between two different tree-making methods, consensus trees were recovered using both NJ and MP methods. It has been achieved by giving a priority to the topologies supported with replication units higher than 50%.

Table 1. Species names and their abbreviate forms (Abr.), sampling localities and GeneBank accession numbers of the Cobitid, Balitorid and Cyprinid fishes examined in this study

Species	Common name	Abr.	Localities	Accession No.
Cobitis hankugensis	Korean spine loach	C. han	Youngcheon (3) Namwon (14) Geochang (7) Namwon, Korea	AB120176
Cobitis lutheri	Sand spine loach	C. lut	Tamjin (2) Hampyung Daedong (6)	
Cobitis pacifica	Northern loach	C. pac	Gangreung (1)	
Cobitis tetralineata	Striped spine loach	C. tet	Imsil (1) Namwon (1)	
Iksookimia choii	Miho spine loach	I. cho	Geum River (1)	
Iksookimia hugowolfeldi	Southern king spine loach	I. hug	Hampyung Daedong dam (2)	
Iksookimia koreensis	Korean spine loach	I. kor	Puhang (1) Youngcheon (1)	
Iksookimia longicorpa	King spine loach	I. lon	Imsil (2)	
Iksookimia pumila	Buan spine loach	I. pum	Buan (1)	

Numbers in parentheses indicate the number of specimen from each locality.
* Outgroup species.

Table 1. Continued

Species	Common name	Abr.	Localities	Accession No.
Iksookimia yongdokensis	Eastern spine loach	I. yon	Angang (2)	
Kichulchoia brevifasciata	Little loach	K. bre	Geogeum-do (2)	
Koreocobitis naktongensis	Spotted white nose loach	K. nak	Suncheong (1)	
Koreocobitis rotundicaudata	White nose loach	K. rot	Han River (1)	
Misgurnus anguillicaudatus	Muddy loach	M. ang	Nakdong River (1)	
			China	AF051868
			Aomori, Iwaki, Japan	AB080185
Misgurnus mizolepis	Chinese muddy loach	M. miz	Imsil (1)	
Misgurnus fossilis	Weather fish	M. fos	Japan	E28664
Niwaella multifasciata	Su su Miguri (Korean name)	N. mul	Geochang (1)	
			Suncheong (1)	
Niwaella delicata	Ajime loach	N. del	Japan	AB039352
Cobitis sinensis	Siberian spiny loach	C. sin	China	AY625699
			Taiwan	AY526868
			Taiwan	NC007229
**Lefua costata*	Eight barbell loach	L. cos	Imsil (1)	
**Cyprinus carpio*	Common carp	C. car	Taiwan	NC001606
**Carassius auratus*	Red crucian carp	C. aur	Jeju-Korea	
c.f. Hybrid		Hyb	Suncheong (4)	
			Namwon, Korea	AB120177

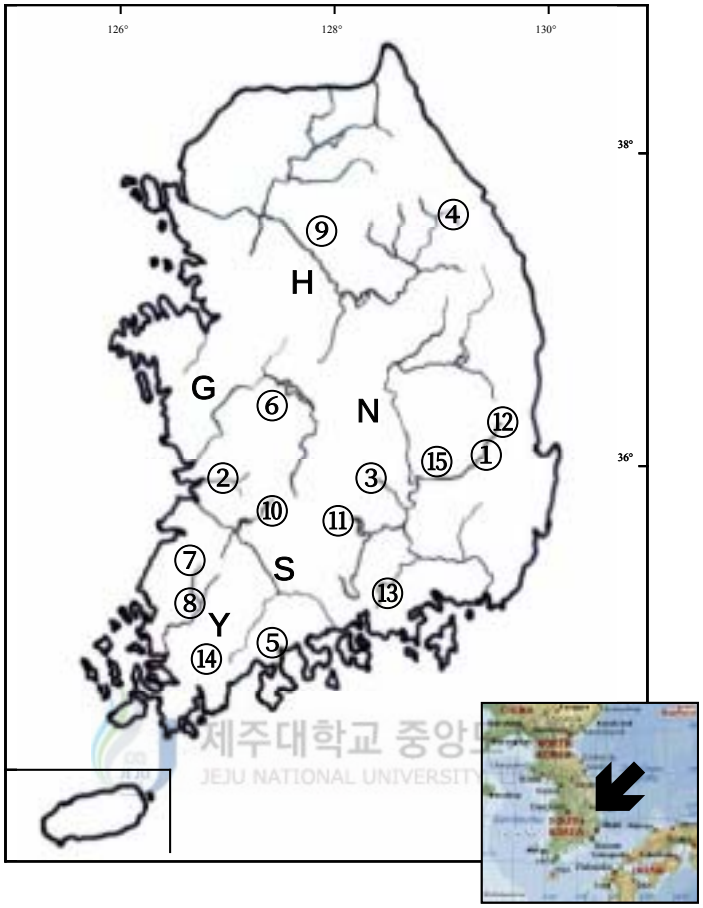


Fig. 3. Map of five main river systems in the southern part of Korean peninsula and the distribution of sampling localities of cobitid fishes used in this study. River systems are shown by letters: H: Han River; N: Nakdong River; G: Geum River; S: Samjin River; Y: Youngsun River. Sampling localities: 1. Angang; 2. Buan; 3. Geochang; 4. Gangreung; 5. Geogeumdo; 6. Geum River; 7. Hampyungdaedong; 8. Hampyungdaedong dam; 9. HanRiver; 10. Imsil; 11. Namwon; 12. Puhang; 13. Sancheong; 14. Tamjin; 15. Youngcheong.

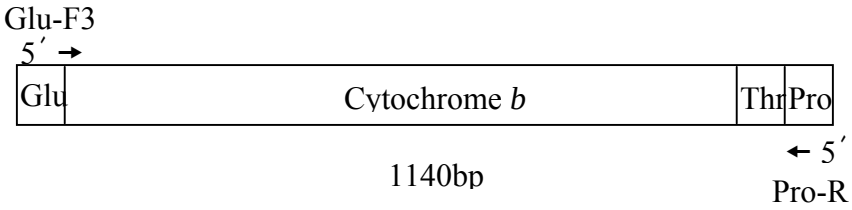


Fig. 4. Strategy for PCR amplification and sequencing of the cytochrome *b* gene and its flanking tRNA genes (Glu, Pro and Thr). Arrows denote the primers. Glu: glutamine; Pro: proline; Thr: threonine.

Table 2. The sequences of the primers used in PCR and sequencing of the cytochrome *b* gene

Primer	Gene Position	Sequence
Glu-F3	$tRNA^{Glu}$	5'-ACCACCGTTGTHNTTCAACTA-3'
Pro-R	$tRNA^{Pro}$	5'-TAGAATYYTRGCTTTGGGAG-3'
T7		5'-TAATACGACTCACTATAGGG-3'
T3		5'-TAACCCTCACTAAAGGGA-3'

3. RESULTS

3.1. Base Composition Bias

In total, the complete cytochrome *b* (1140bp) of 22 species, besides the sequences of a group of putative hybrids (having intermediate phenotype of two species of *C. hankugensis* and *I. longicorpus*), were used for phylogenetic analyses. Base compositions biases were analyzed to interpret the total and positional frequencies of each of four bases in individual DNA sequences regarding their sampling locality (Table 3). The results of base composition in all studied fish revealed low average G content (14.9%), high T content (31.3%) and almost equal rates of C and A (26.3% and 27.4%, respectively). Mean contents of G at first, second and third codon positions were 26.0%, 13.2% and 5.6%, respectively. The average rates of T at first, second and third codon positions estimated to be 25.1%, 41.5% and 27.5 %, respectively. The average contents of C at the first, second and third codon positions were 25.0%, 25.5% and 28.3%, respectively. The average rates of A at the first, second and third codon positions were 23.8%, 19.8% and 38.6%, respectively. According to these results, the low G content could be due to its low rate at the third and second codon position, while at the second codon position T has the highest rate compare to the other three bases. As is revealed in the table, the contents of all four bases at the first codon position were almost similar, whereas the rate of A was largest at the third codon position. There was a relative homogeneity in the content of each of four bases among the sequences. So that, T contents ranged from 26.2 to 33.2%, C and A had rates between 25.0 and 30.0% and the content of G varied between 14.1 and 15.8%. In the fishes examined, the second codon position showed the

least interspecific variability (standard deviation-SD- ranged from 0.07 to 0.28), while in the third codon position the rates of base composition had the highest variations (SD valued between 0.99 and 2.41). Results showed a relative homology in base composition within each family. So that, for instance, the content of T in cobitid fishes varied from 30.9 to 32.8%, in balitorid fishes ranged from 28.0 to 31.5% and in examined cyprinid fishes fluctuated between 26.2 and 29.0%. Similarly, the content of G in Cobitidae ranged from 14.5 to 15.8%, in Balitoridae ranged from 15.2 to 18.5% and in Cyprinidae varied between 14.1 and 14.5%.

3.2. Sequence Evolution

Except for two Genebank-deposited species of *M. anguillicaudatus* (Accession No. AB080185) and *N. delicata* (Accession No. AB039352) with partial cytochrom *b* sequences, no size differences were observed among the 22 species examined. Of the 1140 nucleotide site, 479 were variable and 403 were phylogenetically informative for parsimony analysis. Evidently, the remaining number out of variable sites have been considered as conserved positions in all specimens (661 nucleotide sites). As was predictable, the third codon position was the most variable site in the aligned data. The average numbers of identical, trasitional and transversional base pair substitutions at each of the three codon positions of the sequence data are indicated in Table 4. According to the table, the total numbers of identical base pair substitution in all codon positions were much higher than those of trasnsitional and transversional nucleotide substitutions. However, the number of identical base substitution at the third codon position was

lower than those at two other positions. The estimates of transition to transversion ratios ranged from 2.1 in the second codon position to 8.3 in the first codon position (Table 4).

Aligned cytochrome *b* gene sequences showed a starting codon of ATG (encoding methionine amino acid) and a termination codon of CTT (encoding leucine) in all studied fish groups. Table 5 illustrates the average frequencies of different triplet amino acid mediating-codons arrays in cytochrome *b* sequence data of examined fishes. As is evident in table 4, although only two base arrays encoding each of the amino acids of 'isoleucine' and 'phenylalanine' are included in the sequences, these codons have the highest frequencies among all other arrangements of codons, while in contrast, 'arginine' and 'serine' with several encoding arrangements occurred with low rates in the sequences. The average number of codons in all examined sequence data was 378.

Table 3. Total base composition and estimated base composition bias at each codon position in the sequences of cytochrome *b* gene of the studies fish species considering their sampling localities

Species	Location	Codon Position															
		All				First				Second				Third			
		T	C	A	G	T1	C1	A1	G1	T2	C2	A2	G2	T3	C3	A3	G3
C. han	Namwon 1M	31.1	26.7	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.6	29.7	38.9	4.7
	Namwon 3	31.1	26.7	27.4	14.7	25.5	24.7	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 4F	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 5M	31.0	26.8	27.3	14.8	25.2	24.9	23.4	26.5	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 6M	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 7F	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 8M	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 9F	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon10M	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 11	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 12	30.9	26.9	27.4	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 13	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Namwon 14	31.1	26.6	27.7	14.6	24.9	25.2	23.9	26.0	41.3	25.8	19.7	13.2	27.1	28.7	39.5	4.7
	Namwon 15	30.9	26.8	27.5	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	26.3	29.7	39.2	4.7
	Namwon AB120176	31.2	26.5	27.6	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7

Refer to Table 1 for species abbreviations. F: female; M: male

Table 3. Continued

Species	Location	Codon Position															
		All				First				Second				Third			
		T	C	A	G	T1	C1	A1	G1	T2	C2	A2	G2	T3	C3	A3	G3
C. han	Geochang 1M	30.9	26.9	27.3	14.8	25.2	24.9	23.4	26.5	41.6	25.5	19.7	13.2	26.1	30.3	38.9	4.7
	Geochang 2	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Geochang 3	31.2	26.5	27.6	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7
	Geochang 4	31.2	26.5	27.6	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7
	Geochang 5	31.2	26.5	27.6	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7
	Geochang 6	31.2	26.4	27.5	14.8	24.7	25.3	23.7	26.3	41.6	25.5	19.7	13.2	27.4	28.4	39.2	5.0
	Geochang 7	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Youngcheon 1M	31.2	26.5	27.6	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7
	Youngcheon 2M	31.9	25.8	27.1	15.2	24.7	25.5	23.9	26.0	41.6	25.5	19.7	13.2	29.5	26.3	37.6	6.6
	Youngcheon 1F	32.0	25.7	27.1	15.2	24.7	25.5	24.1	25.7	41.6	25.5	19.7	13.2	29.7	26.1	37.4	6.8
	Youngcheon 2F	32.0	25.7	27.1	15.2	24.7	25.5	23.9	26.0	41.6	25.5	19.7	13.2	29.7	26.1	37.6	6.6
	Sancheon 1 F	31.9	25.8	27.1	15.2	24.7	25.5	23.9	26.0	41.6	25.5	19.7	13.2	29.5	26.3	37.6	6.6
	Sancheon 1 M	31.9	25.8	27.1	15.2	24.7	25.5	23.9	26.0	41.6	25.5	19.7	13.2	29.5	26.3	37.6	6.6
	Sancheon 2 F	31.9	25.8	27.1	15.2	24.7	25.5	23.9	26.0	41.6	25.5	19.7	13.2	29.5	26.3	37.6	6.6
	Sancheon 2 M	32.0	25.7	26.9	15.4	24.7	25.5	23.4	26.5	41.6	25.5	19.7	13.2	29.7	26.1	37.6	6.6
	Sancheon 3 F	31.9	25.8	27.0	15.3	24.7	25.5	23.6	26.2	41.6	25.5	19.7	13.2	29.5	26.3	37.6	6.6

Table 3. Continued

Species	Location	Codon Position															
		All				First				Second				Third			
		T	C	A	G	T1	C1	A1	G1	T2	C2	A2	G2	T3	C3	A3	G3
C. han	Sancheon 3 M	32.0	25.7	27.1	15.2	24.7	25.5	23.9	26.0	41.6	25.5	19.7	13.2	29.7	26.1	37.6	6.6
	Sancheon 4F	32.0	25.7	27.1	15.2	24.7	25.5	23.9	26.0	41.6	25.5	19.7	13.2	29.7	26.1	37.6	6.6
C. lut	Hampyung daedong 4	32.7	25.0	27.0	15.3	25.2	24.9	24.1	25.7	41.6	25.5	19.5	13.4	31.3	24.5	37.4	6.8
	Hampyung daedong 5	32.8	25.0	26.7	15.5	25.2	24.9	23.9	26.0	41.6	25.5	19.7	13.2	31.6	24.5	36.6	7.4
	Hampyung daedong 6	32.6	25.1	26.9	15.4	25.2	24.9	23.9	26.0	41.6	25.5	19.7	13.2	31.1	24.7	37.1	7.1
	Tamjin 1	31.5	26.3	27.5	14.7	24.9	25.2	24.4	25.5	41.6	25.5	19.7	13.2	27.9	28.2	38.4	5.5
	Tamjin 2	31.6	26.1	27.5	14.7	24.9	25.2	24.4	25.5	41.6	25.5	19.7	13.2	28.4	27.6	38.4	5.5
	Hampyung daedong 1	32.2	25.4	27.1	15.3	25.2	24.9	24.1	25.7	41.6	25.5	19.5	13.4	29.7	25.8	37.6	6.8
	Hampyung daedong 2	32.6	25.0	27.3	15.2	25.2	24.9	24.1	25.7	41.6	25.5	19.7	13.2	31.1	24.5	37.9	6.6
	Hampyung daedong 3	32.5	25.1	27.2	15.2	25.2	24.9	24.1	25.7	41.6	25.5	19.7	13.2	30.8	24.7	37.6	6.8
C. tet	Namwon	32.1	25.5	27.3	15.2	24.9	25.2	24.1	25.7	41.8	25.3	19.7	13.2	29.5	26.1	37.9	6.6
	Imsil	32.2	25.4	27.3	15.1	24.9	25.2	24.1	25.7	41.6	25.5	19.7	13.2	30.0	25.5	38.2	6.3

Table 3. Continued

Species	Location	Codon Position															
		All				First				Second				Third			
		T	C	A	G	T1	C1	A1	G1	T2	C2	A2	G2	T3	C3	A3	G3
C. pac	Gangreung	30.9	26.0	28.1	15.0	24.4	25.7	23.9	26.0	41.3	25.5	20.0	13.2	26.8	26.8	40.5	5.8
C. sin	China AY625699	31.7	25.9	27.0	15.4	26.2	23.9	23.6	26.2	40.8	25.5	20.3	13.4	28.2	28.2	37.1	6.6
	Taiwan AY526868	31.7	26.2	27.0	15.1	25.5	24.7	23.6	26.2	41.3	25.8	19.7	13.2	28.2	28.2	37.7	5.8
	Taiwan NC007229	31.7	26.2	27.0	15.1	25.5	24.7	23.6	26.2	41.3	25.8	19.7	13.2	28.2	28.2	37.7	5.8
I. lon	Namwon	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
	Imsil 1	31.2	26.5	27.7	14.6	24.9	25.2	23.9	26.0	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7
	Imsil 2	31.2	26.5	27.6	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7
I. hug	Hampyung Daedongdam 1	31.0	26.7	27.6	14.6	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	29.7	39.5	4.5
	Hampyung Daedongdam 2	30.9	26.8	27.6	14.6	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	26.3	29.7	39.5	4.5
I. yon	Angang 1	31.4	26.3	27.3	15.0	24.9	25.2	23.9	26.0	41.1	25.5	20.3	13.2	28.2	28.2	37.9	5.8
	Angang 2	31.3	26.3	27.5	14.9	24.9	25.2	23.9	26.0	41.1	25.5	20.0	13.4	27.9	28.2	38.7	5.3
I. kor	Youngcheon	31.6	25.4	27.4	15.6	24.9	25.2	23.6	26.2	41.3	25.5	20.0	13.2	28.4	25.5	38.7	7.4
	Puhang	31.2	25.8	27.3	15.8	24.4	25.7	23.4	26.5	41.3	25.5	20.0	13.2	27.9	26.1	38.4	7.6

Table 3. Continued

Species	Location	Codon Position															
		All				First				Second				Third			
		T	C	A	G	T1	C1	A1	G1	T2	C2	A2	G2	T3	C3	A3	G3
I. pum	Buan	31.5	25.6	27.7	15.2	25.2	24.9	23.6	26.2	41.3	25.5	20.0	13.2	27.9	26.3	39.5	6.3
I. cho	GeumRiver	31.3	25.9	27.7	15.2	25.7	24.4	24.1	25.7	41.3	25.5	20.0	13.2	26.8	27.6	38.9	6.6
K. bre	Geogeumdo 1	31.3	26.4	27.2	15.2	24.9	25.2	24.1	25.7	41.3	25.8	19.7	13.2	27.6	28.2	37.6	6.6
	Geogeumdo 2	31.3	26.4	27.1	15.2	24.9	25.2	24.1	25.7	41.3	25.8	19.7	13.2	27.6	28.2	37.4	6.4
K. rot	HanRiver	31.1	26.4	27.6	14.9	25.7	24.4	24.1	25.7	41.3	25.3	20.0	13.4	26.3	29.5	38.7	5.5
K. nak	Suncheong	30.9	26.1	28.2	14.7	25.5	24.7	24.4	25.5	41.3	25.3	20.0	13.4	26.1	28.4	40.3	5.3
M. mis	Imsil	30.9	26.5	27.5	15.2	24.4	25.7	24.1	25.7	41.3	25.5	20.0	13.2	26.8	28.2	38.4	6.6
M. ang	Geochang	30.9	26.6	27.3	15.2	25.5	24.7	24.7	25.2	41.6	25.3	20.0	13.2	25.8	30.0	37.1	7.1
	ChinaAF051868	31.3	26.1	27.5	15.1	25.7	24.7	23.6	26.0	40.8	26.1	20.0	13.2	27.4	27.6	38.9	6.1
	JapanAB080185	31.8	26.2	26.9	15.1	26.8	23.9	23.9	25.4	42.1	25.1	19.9	12.9	26.6	29.5	36.8	7.0
	Japan E28664	31.8	25.8	27.0	15.4	26.0	24.4	23.9	25.7	41.1	25.8	20.0	13.2	28.4	27.1	37.1	7.4
N. mul	Suncheong	31.0	26.7	27.6	14.6	24.4	25.7	24.7	25.2	41.3	25.8	19.7	13.2	27.4	28.7	38.4	5.5
	Geochang	31.3	26.6	27.6	14.5	24.4	25.7	24.7	25.2	41.3	25.8	19.7	13.2	28.2	28.2	38.4	5.3
N. del	JapanAB039352	33.2	25.1	26.8	14.9	28.1	21.9	23.1	26.9	39.8	25.7	21.2	13.3	31.8	27.7	36.0	4.5
L. cos	Imsil	29.5	26.6	28.7	15.2	26.0	23.1	24.1	26.8	41.1	25.8	19.7	13.4	21.6	30.8	42.4	5.3

Table 3. Continued

Species	Location	Codon Position															
		All				First				Second				Third			
		T	C	A	G	T1	C1	A1	G1	T2	C2	A2	G2	T3	C3	A3	G3
Hyb	Sancheong 1F	31.2	26.6	27.5	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.9	39.2	4.7
	Sancheong 2F	31.2	26.5	27.6	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7
	Sancheong13F	31.2	26.5	27.6	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7
	Sancheong12F	31.2	26.5	27.6	14.7	24.9	25.2	23.6	26.2	41.6	25.5	19.7	13.2	27.1	28.7	39.5	4.7
	Namwon * AB120177	31.0	26.8	27.4	14.7	25.2	24.9	23.6	26.2	41.6	25.5	19.7	13.2	26.3	30.0	38.9	4.7
C. car	Taiwan NC001606	26.2	30.0	29.7	14.1	23.1	26.5	25.2	25.2	41.8	25.0	20.0	13.2	13.7	38.4	43.9	3.9
C. aur	Jeju	29.0	28.0	28.6	14.5	23.4	26.5	24.9	25.2	41.8	25.0	20.0	13.2	21.8	32.4	40.8	5.0
Average		31.3	26.3	27.4	14.9	25.1	25.0	23.8	26.0	41.5	25.5	19.8	13.2	27.5	28.3	38.6	5.6
SD		0.87	0.71	0.43	0.31	0.61	0.60	0.37	0.35	0.28	0.16	0.22	0.07	2.41	2.11	1.20	0.99

* Triploid

Table 4. The average numbers of identical, transitional and transversional base substitution at different codon positions of the cytochrome *b* sequences of the studied fish groups

Codon Position	Identical Base Pairs					Transitional (Ts) Pairs			Transversional (Tv) Pairs					Ts/Tv
	TT	CC	AA	GG	Total	TC	AG	Total	TA	TG	CA	CG	Total	
First	89	87	86	95	357	12	6	18	1	0	1	0	2	8.3
Second	155	95	74	49	373	1	0	1	0	0	0	0	0	2.1
Third	68	76	121	10	275	49	21	70	12	3	14	3	32	2.2
*Average	312	258	281	154	1005	62	27	89	13	3	15	3	34	2.6

* The mean value of base pairs over all taxa.

Table 5. The average frequencies of codons and their corresponding encoded amino acids in cytochrome *b* gene sequences

UUU (F)	17.1 (1.16)	UCU (S)	4.2 (1.09)	UAU (Y)	9.1 (1.38)	UGU (C)	0.3 (0.21)
UUC (F)	12.5 (0.84)	UCC (S)	4.4 (1.15)	UAC (Y)	4.1 (0.62)	UGC (C)	2.7 (1.79)
UUA (L)	12.3 (1.20)	UCA (S)	12.9 (3.36)	UAA (*)	0.0 (0.00)	UGA (W)	12.2 (1.89)
UUG (L)	0.9 (0.09)	UCG (S)	0.6 (0.14)	UAG (*)	0.0 (0.00)	UGG (W)	0.7 (0.11)
CUU (L)	11.8 (1.15)	CCU (P)	4.0 (0.77)	CAU (H)	3.7 (0.62)	CGU (R)	1.5 (0.76)
CUC (L)	8.5 (0.83)	CCC (P)	9.0 (1.72)	CAC (H)	8.3 (1.38)	CGC (R)	0.3 (0.18)
CUA (L)	23.5 (2.29)	CCA (P)	7.6 (1.46)	CAA (Q)	5.7 (1.93)	CGA (R)	5.9 (2.97)
CUG (L)	4.5 (0.44)	CCG (P)	0.2 (0.04)	CAG (Q)	0.2 (0.07)	CGG (R)	0.2 (0.09)
AUU (I)	20.4 (1.33)	ACU (T)	5.2 (0.91)	AAU (N)	8.2 (0.92)	AGU (S)	0.0 (0.00)
AUC (I)	10.3 (0.67)	ACC (T)	5.6 (0.98)	AAC (N)	9.7 (1.08)	AGC (S)	1.0 (0.26)
AUA (M)	6.3 (1.38)	ACA (T)	11.3 (1.98)	AAA (K)	7.8 (1.74)	AGA (*)	0.0 (4.00)
AUG (M)	2.8 (0.62)	ACG (T)	0.7 (0.12)	AAG (K)	1.2 (0.26)	AGG (*)	0.0 (0.00)
GUU (V)	6.5 (1.01)	GCU (A)	4.6 (0.60)	GAU (D)	5.5 (1.01)	GGU (G)	1.5 (0.24)
GUC (V)	6.9 (1.06)	GCC (A)	12.2 (1.58)	GAC (D)	5.4 (0.99)	GGC (G)	6.0 (0.97)
GUA (V)	10.6 (1.64)	GCA (A)	12.5 (1.62)	GAA (E)	4.9 (1.65)	GGA (G)	12.5 (2.01)
GUG (V)	1.9 (0.29)	GCG (A)	1.6 (0.21)	GAG (E)	1.0 (0.35)	GGG (G)	4.8 (0.78)

The numbers in parentheses indicate the bias of the frequency data. The amino acids are indicated using single-letter standard symbols (Appendix 2). The termination codons are displayed by asterisks.

3.3. Saturation Analysis

To recognize the possible noise in sequence data and applicability of data in each position for our phylogenetic comparisons, plotted graphs emerged from the relationships between absolute number of transitionlan and tranversional substitutions (Kimura-two parameters, Kimura, 1980) against sequence divergence or number of mutation were constructed for the first, second and third codon positions separately and in conjugation (Figs. 5 and 6. As is displayed in the figures, although at third codon position saturation was occurd in a weak extend, in total, the rate of saturation observed was ignorable, so that the data of all codon positions were included in our phylogenetic analyses. Further more, both transitions and transversions data accumulated with nearly linear arrangements. The distinct segregation of the data in the plots also may show the distances among three fish families have been used in this experiment.

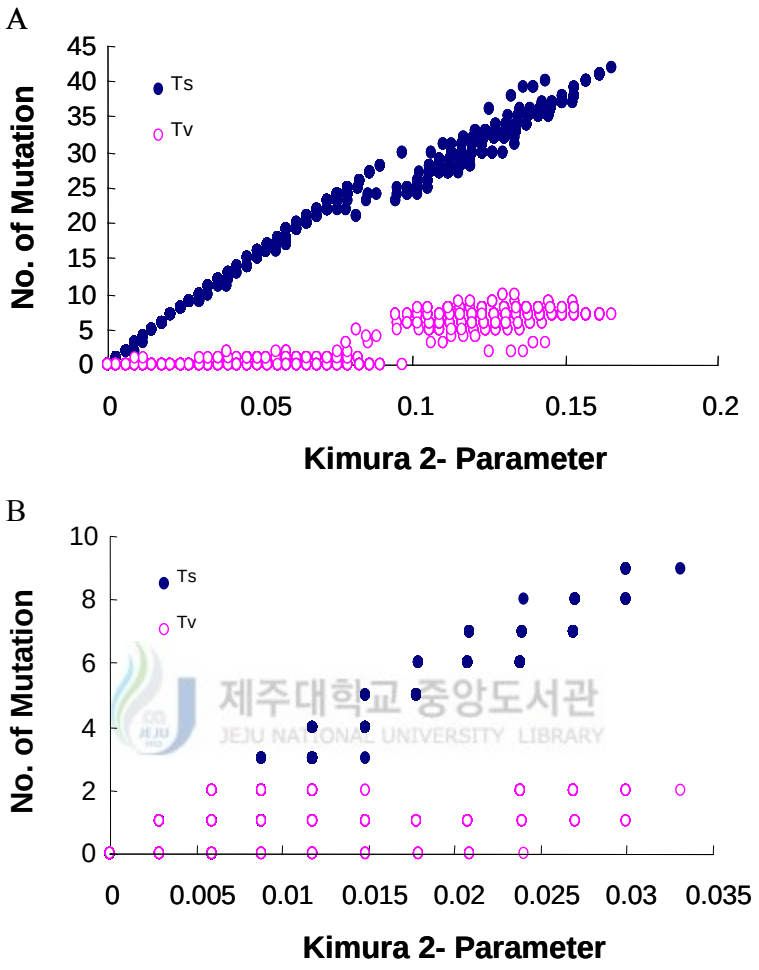


Fig. 5. Pairwise sequence comparison scatterplots indicating absolute number of transitions and transversions against percentage sequence divergence (Kimura-two parameter) for the sequence data of cytochrome *b* gene at first (A) and second (B) codon positions.

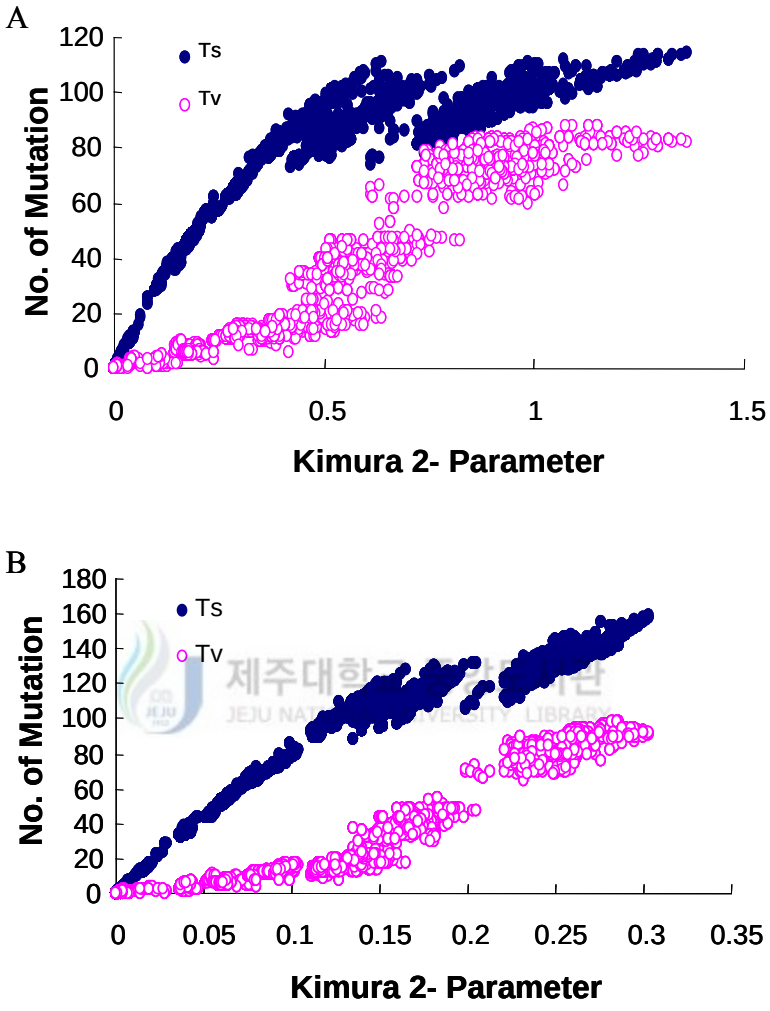


Fig. 6. Pairwise sequence comparison scatterplots indicating absolute number of transitions and transversions against percentage sequence divergence (Kimura-two parameter) for the sequence data of cytochrome *b* gene at third codon position alone (A) and all three codon positions (B).

3.4. Outgroups Comparisons

The sequence divergence among the main examined fishes of Family Cobitidae and two outgroup families is presented in table 6. As is inferred from the table, all three families have a relatively high but similar sequence distance from each other ranging from 17.3 to 21.3%. These results might support the accuracy of our outgroup selection for more reliable comparison of the ingroup fishes.

Table 6. Percentage inter-familial sequence divergence (*p*-distance) ranges among three examined families

Binary Families	*P*- distance
Cobitidae vs Balitorida	17.3 – 21.3
Cobitidae vs Cyprinidae	18.8 – 21.4
Cyprinidae vs Balitoridae	20.0 – 20.8

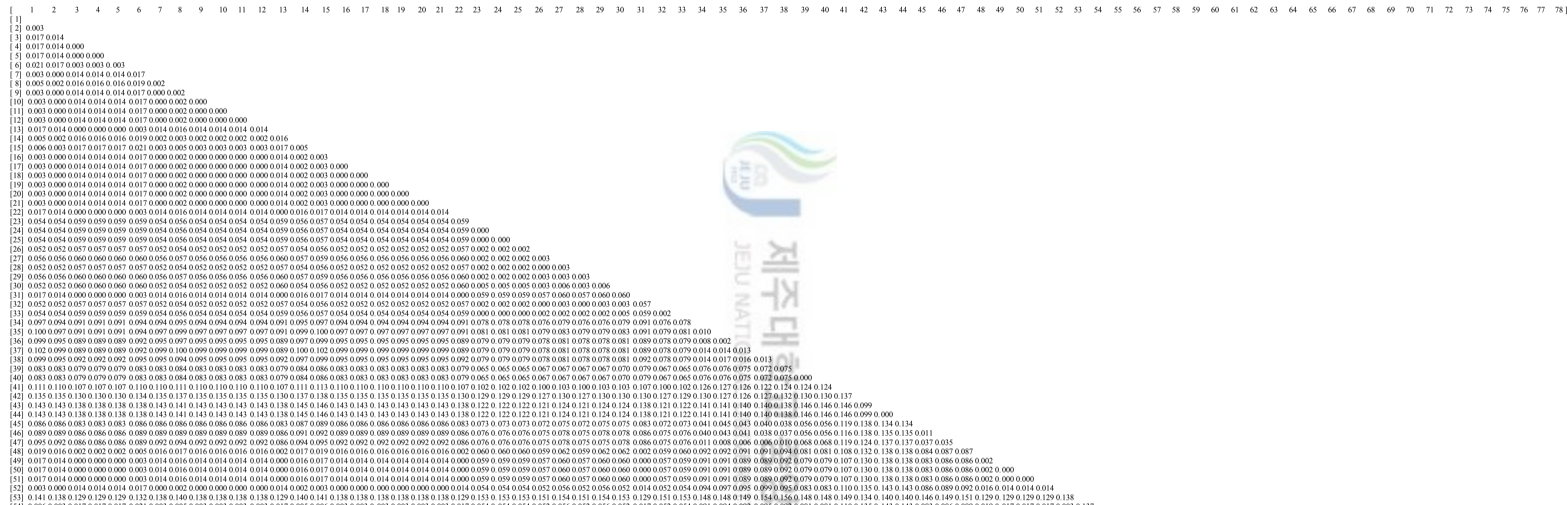
3.5. Ingroup Sequence Comparisons

The results of estimating the sequence divergence (*p*-distance values) among all examined taxa is shown as a cross matrix in Table 7. According to these results, the number of specimens and variations in sampling localities of the members of each species could affect the intraspecific sequence divergence among all cobitid species. Thus, in general, an accurate comparison of variation among different species could not be made because of a different number of samples belonging to each species. Intrageneric divergence rates in the genus *Cobitis* was from 3.5 to 14.7%. These rates in Genus *Iksookimia* ranged from 0.2 to 14.0%, and in *Misgurnus* varied between 2.3 and 16.5%. Two species of Genus *Koreocobitis* had a divergence rate of 7.3%. Intrafamilial–sequence divergence within whole studied cobitid fishes was estimated to be from 0.0 to 17.4%.

Based on the *p*-distance data matrix, the divergence among the taxa in a generic level could be evaluated (Table 8). Accordingly, the *p*-distance between two genera of *Cobitis* and *Iksookimia* had the lowest values and ranged between 0.0 and 6.0%. Distance between *Cobitis* and *Kichulchoia* was from 6.4 to 12.5, between *Cobitis* and *Koreocobitis* was from 12.7 to 16.7%, between *Cobitis* and *Misgurnus* was from 10.1 to 16.7% and between *Cobitis* and *Niwaella* had a rate of 4.9 to 12.2%. *Iksookimia* had a distance to *Kichulchoia* ranged from 6.4 to 15.7%, to *Koreocobitis* ranged from 12.7 to 14.9%, to *Misgurnus* from 11.1 to 16.9% and to *Niwaella* from 4.9 to 15.0%. The *p*-distance between *Kichulchoia* and *Koreocobitis* was estimated to be between 14.5 and 16.4%, while *Kichulchoia* had a distance to *Misgurnus* that ranged

from 11.9 to 17.4%, and to *Niwaella* from 6.2 to 6.4%. *Koreocobitis* had a sequence divergence to *Misgurnus* that ranged between 13.1 and 16.9%, and to *Niwaella* that ranged from 14.1 to 14.4%. Finally, the sequence divergence between *Misgurnus* and *Niwaella* turned out to be from 11.2 to 15.8%.

Table 7. Sequence divergence (*p*-distance) estimated among studied fish goups.



Numbers correspond to the specimens listed in appendix 1.

Table 8. Summarized intergeneric sequence-divergence (*p*-distance)* ranges of cobitid fishes from Korea

	Cobitis	*Iksookimia*	*Kichulchoia*	*Koreocobitis*	*Misgurnus*
Cobitis					
Iksookimia	0.0- 6.0				
Kichulchoia	6.4-12.5	6.4-15.7			
Koreocobitis	12.7-16.7	12.7-14.9	14.5-14.6		
Misgurnus	10.1-16.7	11.1-16.9	11.9-17.4	13.1-19.6	
Niwaella	4.9-12.2	4.9-15.0	6.2-6.4	14.1-14.4	11.2-15.8

* Values in percent

3.6. Phylogenetic Analysis

Phylogenetic trees were constructed according to both distance-based methods and parsimony-based methods. Based on the saturation analysis (Figs. 5 and 6), using the sequence data of all codon positions could lead to the generation of actual phylogenetic trees. However, for detail comparisons, we made all possible trees using several arrays and options. Only the trees constructed by using all of the codon position data according to the Kimura-2 parameter distance model are presented here. As is revealed in the trees of Figures 7 and 8, there is a general congruence in the topologies of the trees made by both applied methods of neighbor- joining (NJ) and maximum parsimony (MP). However, few taxa are differently positioned in the two cladograms. For instance, there are some differences in the position of clade including the specimens of *C. hankugensis* from two localities of Youngcheon and Sancheon and the complex of *C. lutheri* and *C. tetralineata* in the two trees. The tree recovered by NJ method placed the *C. hankugensis* group closer to the two other synonymous group and its hybrids, while in MP the monophyletic group of *C. lutheri* and *C. tetralineata* has a closer affinity to the first large group on the top of the tree. However, this affinity is supported by low bootstrap value (23%). The other incongroency in the two topologies is the position of the clade including two sister taxa of *Iksookimia choii* and *Niwaella delicata*, which has affinity with upper clades in NJ method, but a basal position in MP tree. Three outgroups of the two families of Balitoridae and Cyprinidae have clustered together with a bootstrap support of 82%. Inclusion of additional outgroups (member of family Balitoridae) did

not effect the topology and position of ingroup taxa, but supported the monophyly of the family Cobitidae as well as intrafamilial conjugations with higher bootstrap values (data not shown). *Cobitis hankugensis* with the largest number of specimens, including its hybrid lineages had polyphyletic positions on the trees. Part of this species, from two localities of Namwon and Geochang, made monophyly with *Iksookimia longicorpa* and *I. hugowolfoldi* together with one hybrid individual with bootstrap values of 97 to 98%. The other group of *C. hankugensis* stood with more hybrids and two other *I. longicorpa* supported by 100% replication values. The third group of *C. hankugensis* from two water bodies of Youngchon and Sancheon made a separate monophyly with the highest bootstrap supports. Both samples of *Niwaella multifasciata* from two localities of Sancheon and Geochang made a monophyly with a bootstrap value of 100%, while, the other congener species from Japan, *N. delicata*, had a relatively distant position to them and found to be as the sister taxon of *I. choii.* However, this sistergroup received low replication support (52%). Expected monophylies were observed for different specimens of *I. yongdokensis*, *Kichulchoia brevifasciata* and *Misgurnus anguillicaudatus*. Among the three *Misgurnus* species *M. mizolepis* had a far distance to the other congeners and made monophyly with two species of Genus *Koreocobitis* with bootstrap rates of 68 to 71%. The specimens of *C. lutheri* had polyphyletic positions, some grouped with *C. tetralineata* with a 99% support. However, the monophyly of whole complex groups including *C. lutheri* and *C. tetralineata* recovered with high bootstraps of 98 to 100%. As is revealed from the trees, the genus *Iksookimia* discloses an evident paraphyly. A distinct group of

Iksookimia containing two species of *I. pumila* and *I. koreensis* made monophyly with a high bootstrap support (100%) in both trees, with a further grouping to *C. pacifica* supported by quite high bootstrap values of 68 to 78%. The GeneBank-depositied samples of *C. sinensis* from China and Taiwan represented a basal position with respect to all other species of *Cobitis*, while, they did not display close affinity to their congener species from Korea. *Koreocobitis* had the most basal position of the family Cobitidae in the tree recovered by NJ method. In MP tree, a weakly supported clade including two species of *N. delicata* and *I. choii* occupied the most basal place within ingroup taxa.

For a detail examination of the incongruency in two topologies and as in both applied methods some of the nodes illustrated low bootstrap support, we generated more reliable phylogenies which could be recovered in at least half of the replications (50% consensus trees) (Figs. 9 and 10). In this regard, *I. hugowolfoldi* turned out to be a separated clade, which weakly (bootstrap support of 51%) clustered to the first clade including *C. hakugensis* and *I. longicorpa*. Also, the monophyly of the second and third *C. hankugensis* groups has been disrupted in consensus trees and they have branched inside their main clades, but with slightly low bootstrap support (61-67%). In maximum parsimony consensus topology most of the clustering patterns are supported by relatively high bootstrap proportions. Similar to the regular topologies, there are two main incongruencies in the consensus trees of the two methods. So that, the monophyletic group including *C. lutheri* and *C. tetralineata* has a different position in the MP tree than it has in the NJ tree. Besides, in MP, the clade containing *I. choii* and *N. delicata* has moved to the most basal part of the tree relative to ingroup fishes.

However, the multifurcation of the four most basal groups as well the branching of the outgroups received low replication rates in both consensus trees (68- 57%).

In order to reinforce our findings and recheck the power of inconsistency in the two tree making methods, especially in the case of monophyly of three *C. hankugensis* clusters, we carried out an analysis considering parts of the taxa which were located in closer distance to the questioned clades. In other word, we omitted some distant groups to clear the ingroup relationships with a higher resolution. In this regard, the *Misgurnus* group has been selected to play the role of outgroup for the deeper branches. This strategy led to the topologies (Figs 11, 12) which confirmed our preliminary results showing that all of the three last branched clades including different specimens of *C. hankugensis* located as a large monophyletic complex in both NJ and MP methods and the affinity of the group comprising *C. lutheri* and *C. tetralineata* to part of upper nodes was temporary and unresolved phenomenon.

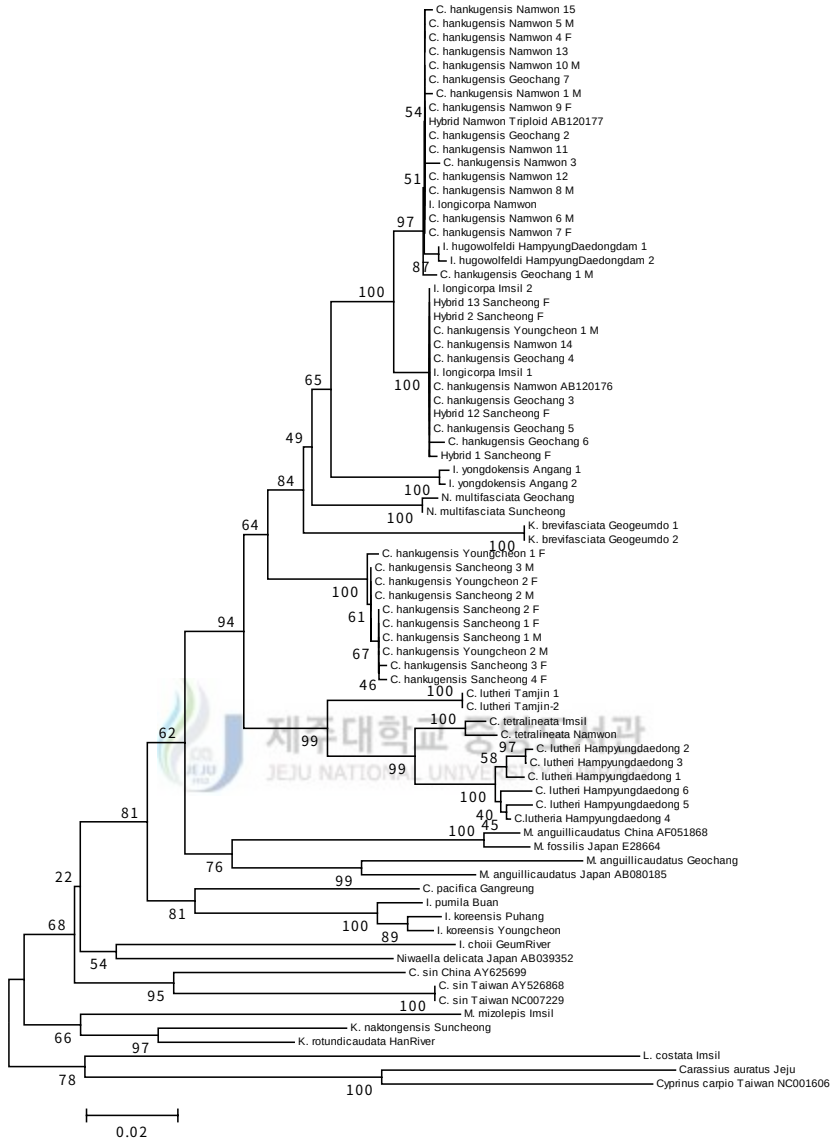


Fig 7. The tree constructed from 78 cytochrome *b* sequence data of 19 species of Family Cobitidae and three outgroups from two families of Balitoridae and Cyprinidae by neighbor-joining (NJ) method, considering both transitional and transversional substitutions and based on Kimura-two parameter distance model. The numbers are bootstrap proportions obtained for the nodes using 1000 replicates.

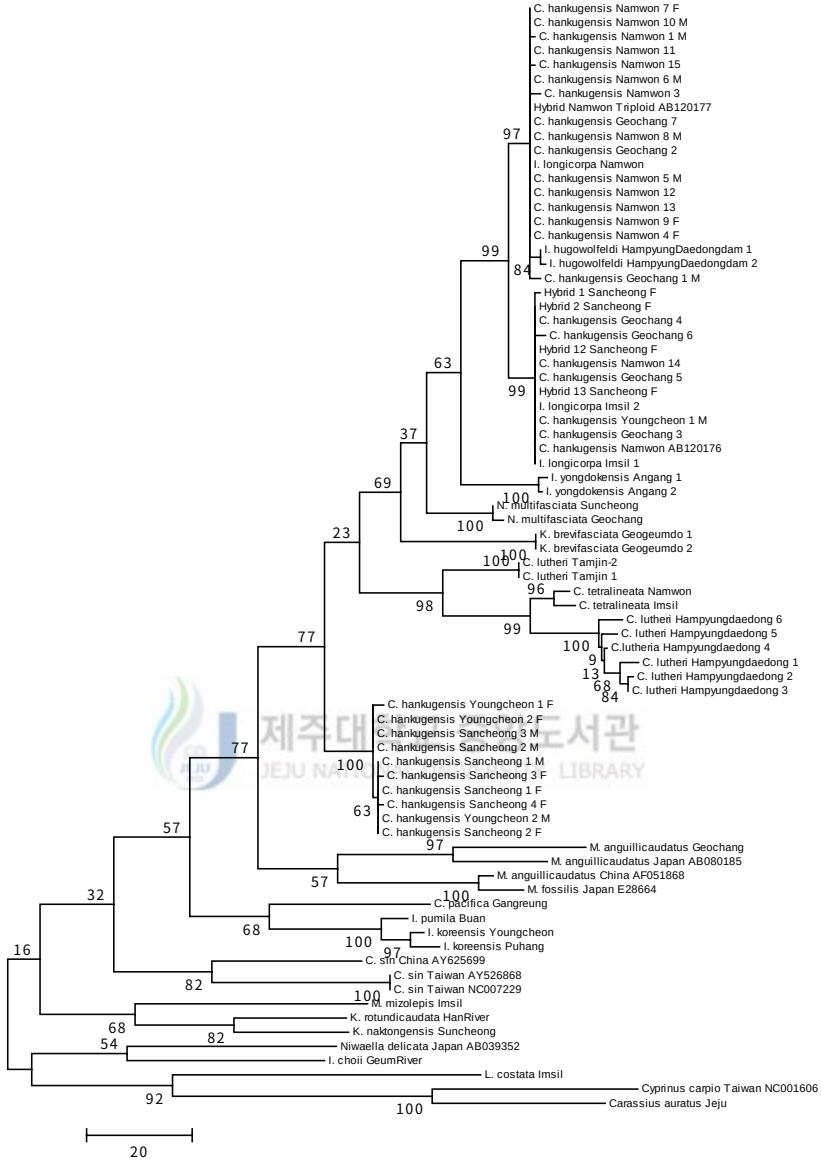


Fig 8. The tree constructed from 78 cytochrome *b* sequence data of 19 species of Family Cobitidae and three outgroups from two families of Balitoridae and Cyprinidae by maximum parsimony (MP) method, considering both transitional and transversional substitutions and based on Kimura-two parameter distance model. The numbers are bootstrap proportions obtained for the nodes using 1000 replicates.

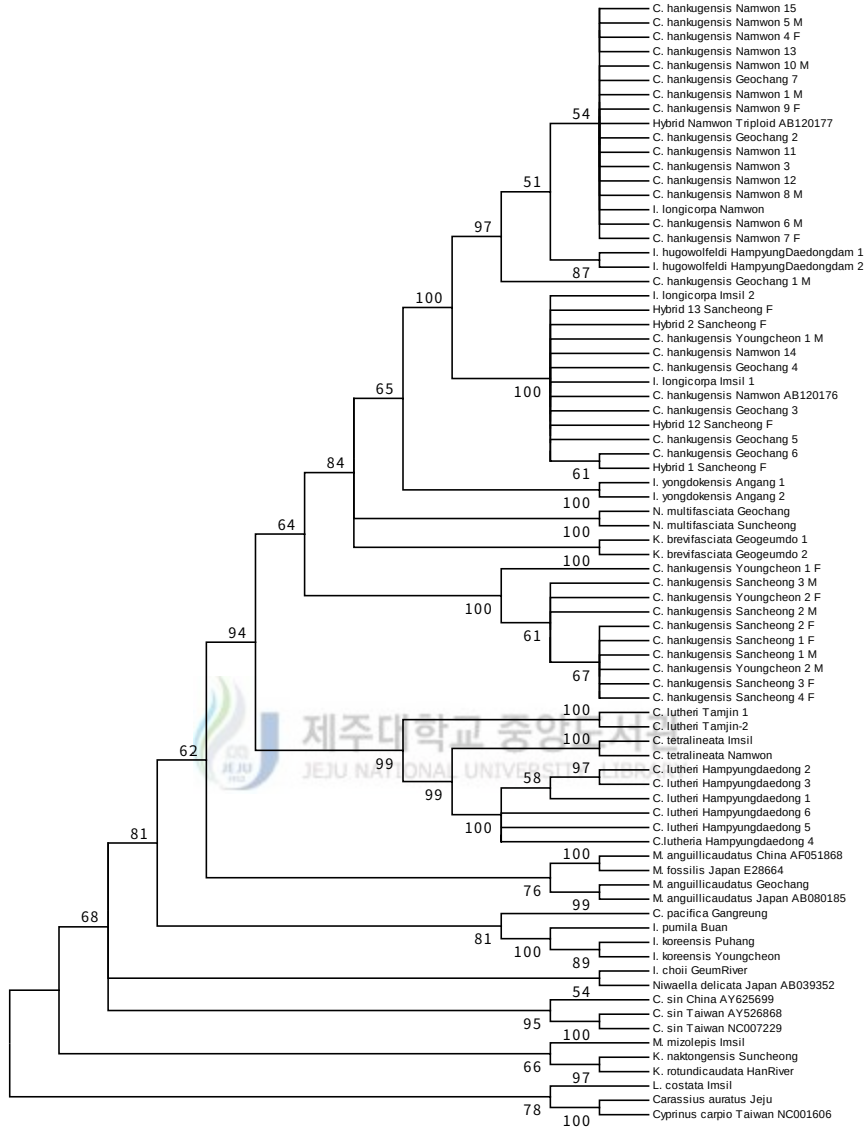


Fig. 9. The 50% majority-rule consensus tree obtained from NJ analysis and base on Kimura-two parameter distance model using 78 cytochrome *b* sequence data of cobitid fishes and the outgroups. The numbers are bootstrap proportions obtained for the nodes using 1000 replicates.

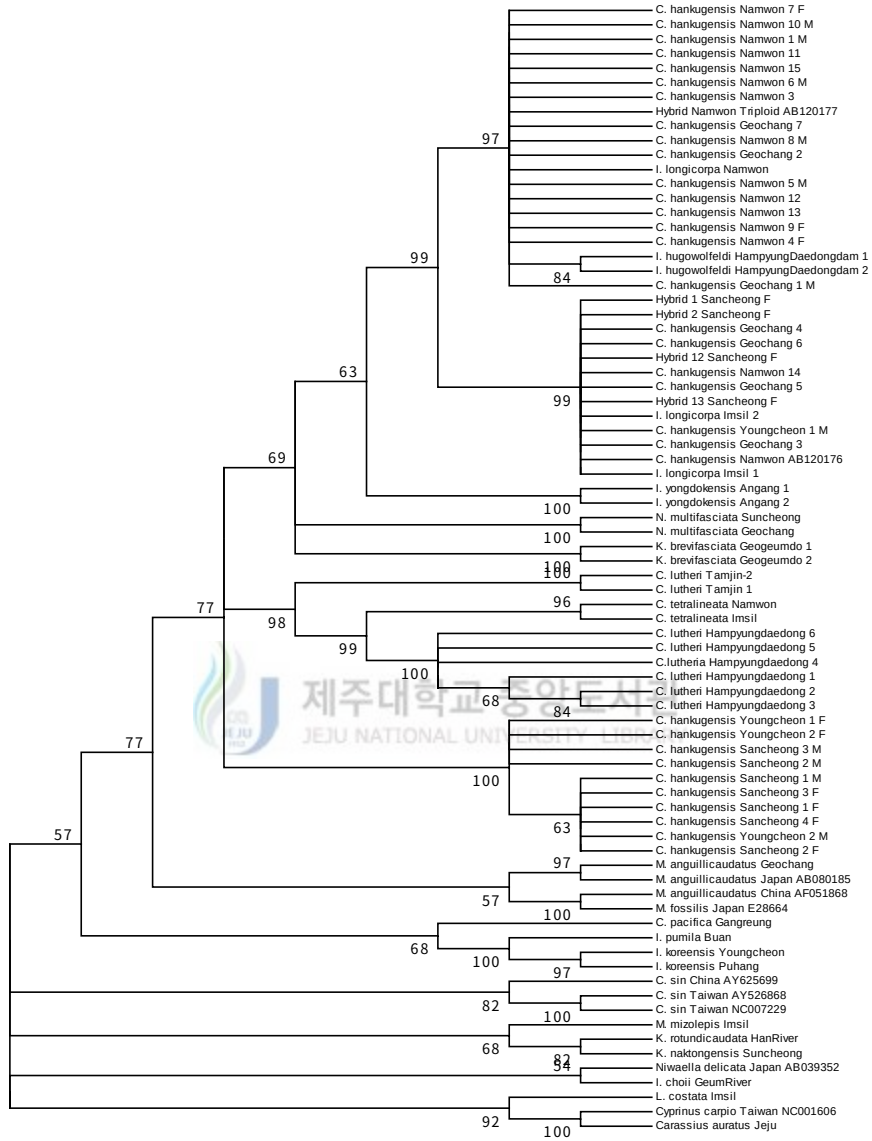


Fig. 10. The 50% majority-rule consensus tree obtained from MP analysis and base on Kimura-two parameter distance model using 78 cytochrome *b* sequence data of cobitid fishes and the outgroups. The numbers are bootstrap proportions obtained for the nodes using 1000 replicates.

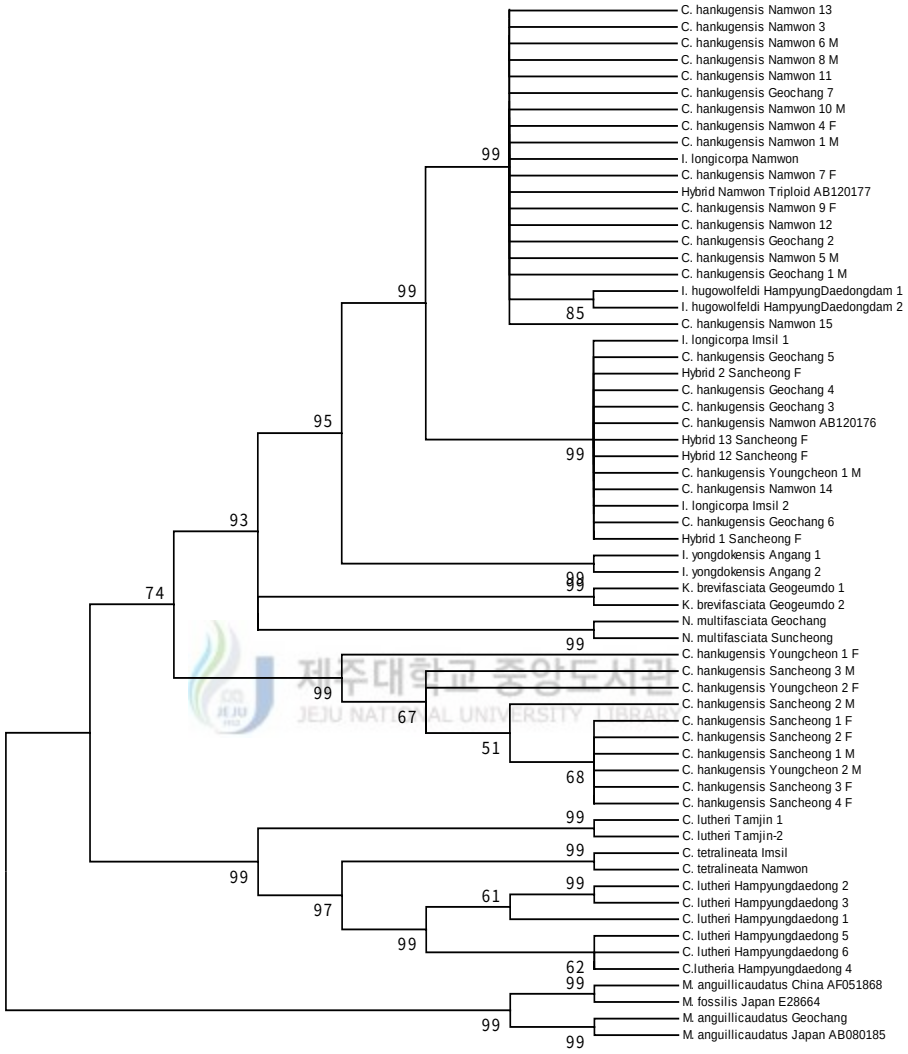


Fig. 11. The 50% consensus tree recovered from NJ analysis for part of closely related ingroup taxa to resolve the accuracy of general topology. Note that in this analysis the monophyletic *Misgurns* group is considered as outgroup for the inner clades.

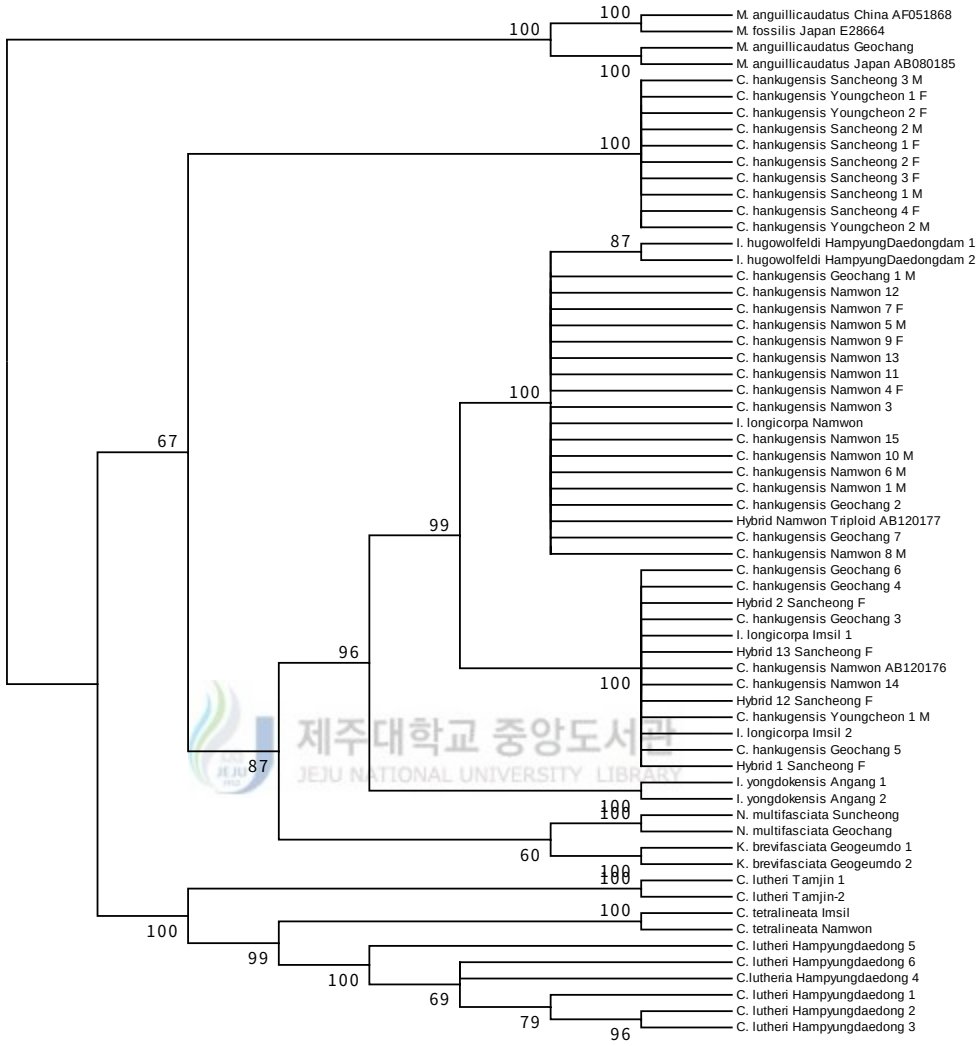


Fig. 12. The 50% consensus tree recovered from MP analysis for part of closely related ingroup taxa to resolve the accuracy of general topology. Note that in this analysis the monophyletic *Misgurns* group is considered as outgroup for the inner clades.

4. DISCUSSION

In the primary systematics of spined loaches (Family Cobitidae), most of the Eurasian records of various species were described to be belonging to a single genus of *Cobitis* (Ludwig et al., 2001). The evolution of cobitid fishes on the whole, from the Miocene to the present, has raised many interesting problems in connection with the evolution of their lineages (Erkakan et al., 1998). These taxonomic problems implied to be because the species are morphologically little differentiated (Ludwig et al., 2001).

Many authors have used mitochondrial DNA (mtDNA) sequences because they are easily accessible, have high rates of evolution, and generally follow a clonal pattern of inheritance well suited to phylogenetic reconstruction (Kocher and Stepien, 1997). Imsiridou et al. (1998) suggested that mtDNA would be particularly useful in providing phylogenetic resolution among closely related taxa, because of its rapid rate of change. Among the mitochondrial genes, sequences of cytochrome *b* gene have been found to provide phylogenetic resolution among a variety of fish groups (Zardoya et al., 1999). It is a powerful source of information that can yield an improved taxonomic classification (Tsigenopoulos and Berrebi, 2000). Further more, nucleotide sequences in the cytochrome *b* gene appear to be highly conserved and easily aligned between even very distantly related species, presumably because of its structural and functional constraints at the protein level (Song, 1994).

In this study, we analyzed the implications of cytochrome *b* for resolving the phylogenetic relationships among cobitid fishes of Korea.

With regard to sequence analysis, the relative frequencies of nucleotides in the sequence data have been so far as a traditional and primary means for comparisons of taxa to be considered. The composition of bases in each codon position is correlated with the structure and function of protein coded by applied gene (Kocher and Stepien, 1997). The observed base composition bias in this study was very similar to the estimated rates for the sequences of European cobitid fishes (Perdices and Doadrio, 2001), was close to the bias rates in some Japanese and Korean cobitid species (Kim et al., 2002), and was slightly different from those of cyprinid fishes (Briolay et al., 1998), with having more difference while comparing with those of Percid fishes (Song, 1994). This may indicate the utility of base composition bias as a criterion to delineate the differences among fishes in a familial or ordinal level. However, the low content of G was a common consequence of all these estimations. In case of substitution rate in sequence level, our data demonstrated low rate of transition and transvertion compare to identical substitution in the examined taxa (Table 4), which may pointing to the relative closeness of the studied fish groups. Kocher et al. (1989) found that among very close relatives such as species within a genus most of the changes in DNA sequences were transitions at synonymous sites, while transversions were evident among more distant taxa such as genera within a family or order. Their study confirmed the general view that transition occurs more often than transversion and that amino acid replacements occur less often than transversions at synonymous positions in cytochrome *b* codons.

Nucleotide substitutions in the third codon position showed a slight saturation for distantly related species (Fig. 6). However, excluding the

third codon position emerged the problem of long-branch effects, and significantly decreased the rates of bootstrap support (data not shown). Several studies have concluded that removal of the third positions diminished the possibility of finding the true tree, especially at low levels of variation (Farias et al., 2001). In the present study, all of the three codon-data were included in the phylogenetic analyses, and the third positions have been realized to carry valuable information that could be used for finding the real topologies.

The trees reconstructed by using both applied methods, (NJ and MP) involving all 78 sequence data sets, displayed high rates of congruency, especially while representing new structure of relationships among the members of Family Cobitidae in Korea. However, few taxa or clades were translocated in the two resulted topologies. (Figs.7 and 8). In both the distance and parsimony methods, the results gained were strongly compatible for the different models. Only the topologies that were generated based upon the Kimura-two parameter are shown here. The results of our molecular systematic study are in some extent in conflict with the currently accepted scheme of nomenclature of genera and species in the Korean cobitid fishes. The most interesting finding is the paraphyletic distribution of the *Iksookimia* group with the taxa which occupied different phylogenetic positions on the trees. *Iksookimia hugowolfoldi* had a close affinity with *I. logicorpa*. In all constructed trees, these two species together with a group of hybrid individuals made a monophyly, which except in the 50% NJ consensus tree was supported with a high bootstrap value (97%). According to the *p*-distance estimation, the rate of pairwise sequence divergence between *I. logicorpa* and *I. hugowolfoldi* was

between 0.00 and 2%, while the conspecific samples of *I. logicorpa* from two different localities of Namwon and Imsil vary in nucleotide composition with a value of 1%. This range of divergence between two species is almost similar to the amounts of cytochrome *b* sequence difference, 0.1 to 1.4%, among the different specimens of a single species estimated in cobitid fish (Perdices and Doadrio, 2001). Relative genetic closeness between the two *Iksookimia* species suggests that they may not actually be two distinct taxa at the species level and may be fused in a single species or at least one of them would be considered as a subspecies of the other. In the study of latter authors, the sequence divergences among the conspesific taxa were estimated to be from 4.6 to 6.9%. Park and Lee (1991) proposed an intraspecific *p*-distance for freshwater fish mtDNA to be less than 2%.

Another notable outcome of our phylogeny is the position of hybrid samples. As described earlier, these hybrids bear the intermediate morphological features of two species of *C. hankugensis* and *I. longicorpa*. The presence of these natural hybrids in Korean waters was first reported by Kim and Lee (1990). Sequence closeness and clustering of these hybrids with *I. longicorpa* and two groups of *C. hankugensis* may be explained by the possible existence of a process of gene flow among the parental species and their putative hybrids. Therefore, sustained long-term hybridization and probable introgression in natural habitats could have caused a genomic interchange across these species. This monophyly more likely shows that the specimens on the two last clades which had similar morphology to that of *C. hankugensis* are in fact the hybrids carrying the maternal genome received from *I. longicorpa*. Thus, their similar genomic

patterns have located them in the same position of the tree. On the other hand, it is worth noticing that according to our survey, the presence of true or original individuals of *I. longicorpa* s*ensu* Nalbant (1993) in Korean freshwaters is doubtful, as the morphology and color pattern of almost all sampled specimens did not completely match to those of the introduced haplotype of this species. A wide range of sequence divergence among the individuals of the species so-called *C. hankugensis* (0-6%) and close distance among some members of this taxon to some samples of putative species of *I. longicorpa* might be because of the influence of the hybridization process. Saitoh et al. (2004), following the observation of similar mitochindrial genome in two species of *C. hankugensis* and *C. longicorpa* (a former name for *I. longocopa*), suggested the occurrence of mitochondrial gene introgression between these two species mediated by the produced hybrid complex. They highlighted the role of this diploid-triploid hybrid complex, as could be a vehicle of mitochondrial genome exchange between the two *Cobitis* lineages. Kitagawa et al. (2005) reached the conclusion that the existence of natural hybridization in cobitid fishes having introduced complications in the results of mtDNA phylogenetic studies as a result of mtDNA introgression and allopolyidy. Gilles et al. (1998) hypothesized that introgression may take place only when two conditions are met: first, the introgressed mitochondrial haplotype must be clustered with haplotypes of different morphology and/or geographic origin with high bootstrap support, and second, the introgressed individual is characterized by a hybrid morphology.

Among the remaining *Iksookimia* group, *I. yongdokensis* had a relatively closer position to the two aforementioned species namely *I. longicorpa and I. hugowolfeldi,* and its conjugation with the two last branches in the trees received quite high support values being between 63 and 66%. In the review of Kim and Park (1997) on the genus *Iksookimia*, these three species were appreciated to have close relationship by their morphological similarities. The distant positioning of two *Niwaella* species, *N. multifasciata* from Korea and *N. delicata* of Japan, is considered as an unexpected result and raise the question about the validity of the criteria used for their enumeration, and may necessitate further detailed review on these two species. Loaches of the genus *Niwaella* are distributed in East Asia and have been found only in Japan and South Korea (Chen and Chen, 2005). *Niwaella* was erected as a distinct genus from the other cobitid genera on the basis of a small head, sucker-like mouth with small barbells, and the absence of lamina circularis (Kim and Lee, 1995). As only the partial cytochrome *b* sequence of *N. delicata* was accessible in the GeneBank, we aligned it with the same sequence length of all other taxa to obtain a more realistic relative phylogeny. Interestingly, the tree that resulted from partial sequences (725bp in length), had an almost identical topology with that of from complete sequence data (not shown). This congruency not only emphasizes the question of the relationship between two *Niwaella* groups, but may also reflect the power of cytochrome *b* for resolving the phylogenetic relationship among the cobitid fishes. The genetic distance (*p* value) between the two *Niwaella* species while aligned with the same sequence length was from 12.3 to 12.6%. A group of *C. hankugensis* from two localities of Youngcheon and

Sancheong, both belonging to the Nakdong River system, but with as nearly as far of a geographic distance, made a highly supported monophyly. This monophyletic group had different positions in the NJ and MP trees and replaced the group including *C. lutheri* and *C. tetralineata* in the MP. However, detail analysis of some closely related taxa by using new outgroups, *Misgurnus* taxa, (Figs. 10 and 11) ascertained the general topology of the NJ method showing convergence of all three clades containing *C. hankugensis*. Nevertheless, the branch composed of *C. lutheri* and *C. tetralineata* also had a highly supported affinity to the last branches in most of the generated trees. The two latter species formed one of the most predictable clades with strong bootstrap support (98-100%). In this group, two local units of *C. lutheri* had paraphyletic positions to *C. tetralineata*. Based on the similar stripped colouration on their body sides, *C. lutheri* and *C. tetralineata* are called "striata complex." Their monophyly as well as the paraphyly of the members of *C. lutheri* have been observed in previous studies (Kim et al., 2002; Kitagawa et al., 2005). Paraphyly of the latter species has generated a debate on its systematics. Kitagawa et al. (2005), putting together the mitochondrial differences among the members of *C. lutheri* with the previous reports on their chromosomal polymorphisms, suggested that the populations of this spined loach in Korea could be divided in interspecific level. In our study the sequence divergence among the samples of *C. lutheri* ranged between 0 and 5.6%. Ludwig et al. (2001), by estimating the sequence divergence among the populations of European cobitid fishes, proposed that many of those populations could be referred to as distinct species. On the other hand, the level of sequence divergence among the

different populations of a single species estimated to be from 0.14 to 9.24 in some cyprinid and salmonid fishes (Imsiridou et al. 1998). In the genus *Barbus* even higher intraspecific sequence variations of up to 9.5% have been recorded (Tsigenopoulos and Berrebi, 2000). Saitoh et al. (2004) concluded that loach populations are prone to diverge even within a single basin.

Among the three species of Genus *Misgurnus*, *M. anguillicaudatus* and *M. fossilis* linked together with bootstrap supports ranged between 57 and 76%. *M. anguillicaudatus*, from Korea, was the sistergroup of Japanese conspecific taxon by high confirmed bootstrap rates (97-100%), while the other sistergroup with the highest replication value was formed by *M. anguillicaudatus* from China and *M. fossilis* of Japan. Molecular results placed *M. mizoleis* in a relatively far distance from the congener taxa at an almost basal position within the cobitid fishes. The latter species had a pronounced genetic distance (*p* value) with the other examined *Misgurnus* lineages in the rates of 15.6 to 16.5%. This difference in positioning and the high rate of nucleotide substitution may call for a further inspection of the phylogenetic relationships within *Misgurnus* groups in Korea. The observed proximity of *M. fossilis* to the monophyletic group of *C. sinensis* in addition to its relative basal position, resembles previous results of the phylogeny of the cobitid genera (Perdices and Doadrio, 2001). The distant location of *C. sinensis* to its morphologically-close species, *C. hankugensis,* is a surprising consequent of their molecular phylogeny. Indeed, until recent examination of the type specimens of *C. sinensis* by Son and Kim, (2002) and the review of Korean *Cobitis* by Kim et al. (2003), these two species were considered as one single taxon namely *C.*

sinensis. Nonetheless, these results may testify the idea that there are no actual *C. sinensis* in Korean waters.

Iksookimia pumila made a monophyly with *I. koreensis* with a high bootstrap support (100%). These two congener taxa associated with *C. pacifica* in all the topologies, where their affinity was supported by pretty high replication rates (68-81%). *I. choii* was the most basal species of the *Iksookimia* group with an isolated position relative to the other congeners, which placed it as a sistergroup of the Japanese *Niwaella.* However, the conjugation of this sistergroup was poorly resolved (52-54%). The genus *Koreocobitis* consistently occupied the basal position of the ingroup phylogeny in both constructed trees and several tree making methods, although in the MP tree it is replaced by the node including *N. delicata* and *I. choii*. This substitution could be ignorable, because it did not receive high replication support.

Our tree making analyses were trialed by using different arrays of outgroups. The applied outgroups found to be suitable for illustrating the relationships among examined fishes of Family Cobitidae. Using more balitorid taxa as outgroups did not influence the structure of the topologies, unless few changes in the bootstrap values of the phylogeny of the ingroup taxa (data not shown). Although the two cyprinid outgroups had some differences in their genetic distance to the species of cobitid fishes, their overall divergence to the ingroup taxa was almost similar (Table 6).

A comparative review on the morphological-based systematics of the cobitid fishes and our molecular phylogeny perhaps can be useful to uncover the rate of consistency between two different methods. The main phenotypic characteristics that have been considered as

discriminative elements in Cobitidae are: body coloration, the size of pectoral fin in the males, presence and shape of lamina circularis in the males, existence and shape of a suborbital spine, and an area of the focal part on the scales (Kim and Park, 1997; Erkakan et al., 1998; Perdices and Doadrio, 2001; Kim et al., 2002; Kim et al., 2005). Two genera of *Niwaella* from Korea and *Kichulchoia,* which were closely placed in our molecular phylogeny, are synapomorphic for the absence of lamina circularis in their males and are similar in body coloration. Four close taxa on the trees namely *C. hankugensis*, *I. longicorpa*, *I. hugowolfoldi* and *I. yongdokensis* share the common character state of having circular lamina circularis, whereas *C. hankugensis* has different colour patterns on its body sides. As was discussed before, the monophyly of latter species with three *Iksookimia* groups could display a gene flow from a morphologically different species. Two genera of *Koreocobitis* and *Misgurnus* also have a circular lamina cicularis. Kim et al (2000) compared these genera as being similar in shape and body colour, while having a movable suborbital spine and a larger scale focal area in the former genus. The structure of lamina circularis is similarly elongated in three species of *I. choii*, *I. pumila* and *I. koreensis*. These three species are also similar by having a large focal area on their scales, but are somewhat different in their body colouration (Kim et al., 2005). Two species of *M. mizolepis* and *M. anguillicaudatus* share the characteristic of having no suborbital spine at all. While the former species is similar to *C. pacifica* for bearing an incomplete lateral line. On the whole, in a comparison between the phylogenetic systematics inferred from cytochrome *b* and that emerged from morphological similarities, some degrees of consistency as well as some contradictions

may be observed between the outgrowths of the two approaches. Meanwhile, having more phenotypic criteria such as morphometric and meristic measures, more plesiomorphic character states might be found among the cobitid lineages, which consequently would led to reaching more inconsistencies between molecular and phenotypic outcomes. However, as noted, in some cases, molecular results support the relationships recovered by morphological analyses. In some of the previous molecular researches, different phylogenetic ordering was gained from those that had acquired from morphological routes (Perdices and Doadrio, 2001; Harris and Mayden, 2001; Kitagawa et al, 2005). On the other hand, a striking agreement between morphologically and molecularly-based phylogenies have been reported for different studied fish (Briolay et al., 1998). With respect to these conflicting results, it has been suggested that cytochrome *b* comparisons are useful because molecular characters are less likely related to adaptive evolution than are morphologic characters (Briolay et al., 1998; Tsigenopoulos and Berrebi, 2000). Whereas, a number of phynotypic and morphologic traits appeared to be instable and unreliable. For instance, it has been found that the body colour in cobitids is often subject to environmental influences (Ruzainah et al., 2003). Perdices and Doadrio (2001) in their phylogenetic study based on both mitochondrial sequence data and the secondary sexual characteristics, concluded that the absence of lamina circularis was not a good character state to define phylogenetic relationships among cobitid fishes. Durand et al. (2002) noted that morphometric characteristics used by some authors to infer the phylogenetic relationships in some cyprinid fishes are sometimes irrelevant.

Notwithstanding, Kocher and Stepien (1997) believed that in general, the overall concordance between morphological and molecular studies has been reasonable. On the other hand, Suk et al. (1996) emphasized that genetic similarity is not necessarily creditable in recognition of species, and the level of reproductive isolation must also be considered in making up the phylogenetic relationships among taxa. Tsigenopoulos and Berrebi (2000) deduced that phylogenetic relations among some *Barbus* species were more strongly correlated with their geographic distribution than with their morphological similarities. One other subject that seems to be worth mentioning is to interfere the results from karyotying examinations with our comparisons. Freyhof et al. (2000) marked the importance of the study of karyotype as a main diagnostic characteristic in the spined loach. Although there is no complete chromosome data of all cobitid fishes, and some of the studied species found to be of the different ploidy levels, chromosome counts may be applicable for systematic comparisons. For example, according to the kartyotyping studies on the genus *Iksookimia,* two molecularly-close taxa of *I. koreensis* and *I. pumila* possess similar karyotypic measures (Kim et al., 1999; Kim et al., 2003).

In conclusion, our data provide a new insight into the relationships among the fishes of Family Cobitidae. This is the first phylogenetic study considering all described species of cobitid fishes of Korea at the molecular level, which offers an actual systematic ranking of the members of this family. The bootstrap values in both tree-making methods were enough robust to support the affinity of the examined taxa and to guarantee of their phylogenetic positions. However, low bootstrap support for some of the deeper branches can be a reminder of

the impotence of cytochrome *b* to resolve the relations within the taxa in this level, as was implied by Briolay et al., (1998). This low bootstrap could also be because of the fast evolution in some of the cobitids. Erkakan et al. (1998) called the fishes occur in East Asia as to be subjected to fast evolution. Our results suggest the need for detail examinations and possible revisions in grouping of some taxa, especially recently introduced genus so-called *Iksookimia*, as the contradiction between current taxonomy and our molecular data is the highest for this group. The other thing could be appreciated from molecular phylogeny is the unusual fractioning of the members of Genus *Cobitis* in the familial trees, as well as equivocal relations in the genus *Niwaella*. Moreover, the present results can also lead to evaluation of the evolutionary history of the studied lineages. In this point of view, the taxa placed at the basal positions might be considered as ones that have evolved faster than the others in a given time scale. A previous mitochondrial phylogentic study revealed that the cobitid fishes had the most recent evolution among the other members of order Cypriniformes (Liu et al., 2002). The long term debates in cobitid systematics, as remarked by Kim et al. (2003) can in part be reasoned by the overlapping morphological similarities and conflicting ranges of molecular differences (i.e. sequence divergence) in species and generic levels. In this regard, it is more surprising that even some of the valid sources for the definition of the family did not give a comprehensive and isolative description of all cobitid taxa. For example, in the definition of Nelson (1994) having suborbital spines is a common morphological characteristic of the whole family, whereas in some of the fishes belonging to the genus *Misgurnus* this characteristic is absent.

The new phylogeny that somewhat rejects the traditional morphological-based systematics, may be of interest for researchers who deal with the investigation in both fields of ichthyology and molecular phylogeny and may encourage them to extend the knowledge of fish systematics and evolution. It may also open a new window for scientists to focus their future surveys on discovering the mechanisms underlying the diversity of the fishes, by using a combination of systematic criteria such as morphological and molecular elements. A firmly established phylogeny of cobitid fishes may additionally need complimentary approaches using more cellular, nuclear and allozymic markers. Kocher and Stepien, (1997) recommended that testing for consistencies among the relationships derived from independent data sets is a particularly robust approach to systematic problems. Another fascinating issue for latter attempts would be the study of phylogeography of cobitid fishes in Korea and East Asia. Finally, a well-supported phylogeny is also required to address the question of hybridization and the role and position of fish having different levels of ploidy within this family.

REFERENCES

Alam M.S. and Rahman Khan M.M., 2001. Intraspecific genetic variation in the Japanese loach (*Misgurnus anguillicaudatus*) revealed by random amplified polymorphism DNA (RAPD) analysis. Pakistan Journal of Biological Science 4 (7), 877- 880.

Alves M.J., Gromicho M., Collares-Pereira M. J., Crespo-Lopez E. and Coelho M. M., 2004. Simultaneous production of triploid and haploid eggs by triploid *Squalius alburnoides* (Teleostei: cyprinidae). Journal of Experimental Zoology. 301A, 552-558.

Arai K., Morishima K., Momotani S., Kudo N. and Zhang Q., 2000. Clonal nature of gynogens induced from spontaneous diploid eggs in the loach, *Misgurnus anguillicaudatus*. Folia Zoologica. 49, 31-36.

Baillie J.E.M., Hilton-Taylor H. and Stuart S.N., 2004. IUCN red list of threatened species. IUCN, Gland, Switzerland.

Benfey T.J., 1999. The physiology and behavior of triploid fishes. Reviews in Fisheries Science. 7 (1), 39-67.

Berendzen P. B., Simons A.M. and Wood R.M., 2003. Phylogeography of the northern hogsucker, *Hypentelium nigricans* (Teleostei: Cypriniformes): genetic evidence for the existence of ancient Teays River. Journal of Biogeography. 30, 1139-1152.

Bernatchez L. and Wilson C.C., 1998. Comparative phylogeography of Nearctic and Palearctic fishes. Molecular Ecology. 7, 431- 452.

Berrebi P., 1995. Speciation of the genus *Barbus* in the North Mediterranean basin: recent advances from biochemical genetics. Biological conservation. 72, 237-249.

Bohlen J. and Ritterbusch D., 2000. Which factors affect sex ratio of spined loach (Genus *Cobitis*) in Lake Muggelsee? Environmental Biology of Fishes. 59, 347-352.

Briolay J., Galtier N., Brito R.M. and Bouvet Y., 1998. Molecular phylogeny of cyprinidae inferred from cytochrome *b* DNA sequences. Molecular Phylogenetics and Evolution. 9 (1), 100-108.

Buth D.G., Dowling T.E. and Gold J.R., 1991. Molecular and cytological investigations. In Cyprinid fishes; Systematics, Biology and Exploitation (Eds.Winfield I. and Nelson J.), New York, Chapman & Hall. PP.83-126

Chen W.J., Orti G. and Mayer A., 2004. Novel evolutionary relationship among four fish model systems. Trends in Genetics. 20 (9) 424-431.

Chen Y. and Chen Y., 2005. Revision of the Genus *Niwaella* in China (Pisces, Cobitida), with description of two new species. Journal of Natural History. 39, 1641-1651.

Chen Z.M. and Chen Y.F., 2001. Phylogeny of the specialized Schizothoracine fishes (Teleostei: Cypriniformes: cyprinidae). Zoological Studies. 40 (2), 147-157.

Cogswell A.T., Benfey T.J. and Sutterlin A.M., 2002. The hematology of diploid and triploid transgenic Atlantic salmon (*Salmo salar*). Fish Physiology and Biochemistry. 24, 271- 277.

Dimmick W.W. and Burr B.M., 1999. Phylogenetic relationships of the suckermouth minnows, Genus *Phenacobius*, inferred from parsimony analyses of nucleotide sequence, allozymic and morphological data (Cyprinidae: Cypriniformes). Biochemical Systematic and Ecology. 27, 469-485.

Durand J.D., Persat H. and Bouvet Y., 1999. Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe. Molecular Ecology. 8, 989-997.

Durand J.D., Tsigenopoulos, Unlu E. and Berrebi P., 2002. Phylogeny and biogeography of the family cyprinidae in the Middle East inferred from cytochrome *b* DNA evolutionary significance of this region. Molecular Phylogenetics and Evolution. 22 (1), 91-100.

Erkakan F., Atalay-Ekmekcl F.G., Nalbant, T.T., 1998. Four new species and one new subspecies of the genus *Cobitis* (pisces: Ostariophysi: Cobitidae) from Turkey. Turkish Journal of Zoology. 22, 9-15.

Erkakan F., Atalay-Ekmekcl F.G., Nalbant, T.T., 1999. A review of the genus *Cobitis* in Turkey (Pisces: Ostariophysi: Cobitidae). Hydrobiologia. 403, 13-26.

Farias I.P., Orti G., Sampaio I., Schneider H. and Mayer A., 2001. The Cytochrome *b* gene as a phylogenetic marker: the limits of resolution for analyzing relationships among cichlid fishes. Journal of Molecular Evolution. 53, 89-103.

Felip A., Piferrer F., Carrillo M. and Zanuy S., 2001. Comparison of the gonadal development and plasma levels of sex steroid hormones in diploid and triploid Sea bass, *Dicentrarchus labrax* L. Journal of Experimental Zoology. 290, 384-395.

Felsenstein J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39(4), 783-791.

Freyhof J., Rab P. and Bohlen J., 2000. The valid names of some European species of the genus *Cobitis* (Teleostei, Cobitidae). Folia Zoologica. 49, 3-7.

Fitch W.M., 1971. Toward defining the course of evolution: Minimum change for a specific tree topology. Systematic Zoology. 20, 406-416.

Fuchs H., Schlee P., Blusch J., Werner T., Stein H. and Rottmann O., 2000. Phylogenetic studies in cyprinid species from central Europe by cytochrome *b* sequences of mitochondrial DNA. Journal of Applied Ichthyology. 16, 79-82.

Gilles A., Lecointre G., Faure E., Chappaz R. and Brun G., 1998. Mitochondrial phylogeny of the European cyprinids: implications for their systematics, reticulate evolution, and colonization time. Molecular Phylogenetics and Evolution. 10 (1), 132-143.

Gilles A., Lecointre G., Miquelis A., Loerstcher M., Chappaz R. and Brun G., 2001. Partial combination applied to phylogeny of European cyprinids using the mitochondrial control region. Molecular phylogenetics and Evolution. 19 (1), 22-33.

Greenwood P.H., 1976. A new and eyeless cobitid fish (Pisces, Cypriniformes) from the Zagros Mountain, Iran. Journal of Zoology (London) 129-137.

Guo X.H., Liu S.J. and Liu Y., 2004. Evidence for maternal inheritance of mitochondrial DNA in polyploid fish of crosses by ATPase8 and ATPase6 genes. Acta Zoologica Sinica. 50(3), 408-413.

Hanfling B., Bolton P., Harley M. and Carvalho R., 2005. A molecular approach to detect hybridization between crucian carp (*Carssius carassius*) and non-indigenous carp species (*Carassius* spp. and *Cyprinus carpio*). Freshwater Biology.50, 403-417.

Hanfling B. and Brandle R., 2000. Phylogenetics of European cyprinids: insights from allozymes. Journal of Fish Biology. 57, 265-276.

Harris P.M. and Mayden R.L., 2001. Phylogenetic relationships of major clades of Catostomidae (Teleostei: cypriniformes) as inferred from mitochondrial SSU and LSU rDNA sequences. Molecular Phylogenetics and Evolution. 20 (2), 225-237.

He S., Liu H., Chen Y., Kuwahara M., Nakajima T. and Zhong Y., 2004. Molecular phylogenetic relationships of Eastern Asian cyprinidae (Pisces: cypriniformes) inferred from cytochrome *b* sequences. Science in China Series C Life Science. 47 (2), 130-138.

Howes, G.J., 1991. Systematics and biogeography: an overview. In Cyprinid fishes; systematics, Biology and Exploitation (Eds. Winfield I. and Nelson J.), New York, Chapman & Hall. PP.1-54.

Hubbs C.L., 1955. Hybridization between fish species in nature. Systematic Zoology. 4, 1-20.

Hwang U.W., Han M.S., Kim I.C., Lee Y.S. Aoki Y. and Lee J.S., 2002. Cloning and sequences of beta-actin genes from *Rhodeus notatus* and the silver carp *Hypophtalmichthys molitrix* (cyprinidae) and the phylogeny of cyprinid fishes inferred from beta-actin genes. DNA sequence. 13 (3), 153-159.

Hwang Y.J., Ra M.S. and Choi C.G., 1995. A natural hybrid between spinous loach, *Cobitis longicorpus* and cyprinid loach *Misgurnus anguilicaudatus* (Pices, Cobitidae). Korean Journal of Ichthyology. 7 (2), 203-207 (in Korean).

Iguchi K., Yamamoto G., Matsubara N. and Nishida M., 2003. Morphological and genetic analysis of fish of a *Carassius* complex

(Cyprinidae) in Lake Kasumigaura with reference to the taxonomic sta tus of two all-female triploid morphs. Biological Journal of the Linnean Society. 79 (2), 351-357.

Imsiridou A., Apostolidis A.P., Durand J.D., Briolay J., Bouvet Y. and Triantaphyllidid C., 1998. Genetic differentiation and phylogenetic relationships among Greek chub *Leuciscus cephalus* L. (Pisces, Cyprinidae) populations as revealed by RFLP analysis of mitochondrial DNA. Biochemical Systematics and Ecology. 26, 415-429.

Jamieson B.G.M., 1991. Fish evolution and systematics: evidence from spermatozoa. Cambridge University Press.

Jeon, S.R., 1986. Studies on the key and distribution of Serranid pheripheral freshwater fishes from Korea. Ph.D. thesis. Sookmyng Women University, Korea. 18, 335-335 (in Korean).

Kim D. S., Kim B. S., Lee S.J., Park I. S. and Nam Y. K., 2004. Comparative analysis of inherited patterns of the transgene in transgenic mud loach *Misgurnus mizolepis* lines carrying the CAT reporter gene. Fisheries Science. 70, 201- 210.

Kim D.S., Nam Y.K. and Jo J.Y., 1997. Effect of oestradiol- 17β inmmersion treatments on sex reversal of mud loach, *Misgurnus mizolepis* (Gunther). Aquaculture Research. 28, 941- 946.

Kim I.S., 1975. A new species of cobitid fish from Korea (Cobitis koreensis). Korean Journal of Lomnology. 8 (3-4), 51-57.

Kim I.S. Choi E.K., Yang H. and Koh M.H., 2003. Karyotype analysis of Southern spined loach, *Iksookimia hugowolfeldi* (Pisces, Cobitidae). Korean Journal of Ichthyology. 15 (2), 127- 129.

Kim I.S., Choi K. C. and Nalbant T.T., 1976. *Cobitis longicorpus*, a new cobitid fish from Korea. Korean Journal of Zoology. 19 (4), 171-178.

Kim I.S., Choi Y., Lee C.L., Lee Y.J., Kim B. J. and Kim J. H., 2005. Illustrated book of Korean fishes. Kyohak Publishing Co., Ltd. Soul, Korea. 615 pp.(in Korean).

Kim I.S. and Lee E.H., 1995. Interspecific hybridization between triploid hybrid fish, *Cobitis sinensis-logicorpus* and two diploid species from Korea. Korean Journal of Ichthyology. 7 (1), 71-78.

Kim I.S. and Lee E.H., 2000. Hybridization experiment of diploid-triploid cobitid fishes, *Cobitis sinensis- longicorpus* complex (Pisces: Cobitidae). Folia Zoologica. 49, 17-22.

Kim I.S. and Lee J.H., 1990. Diploid-triploid hybrid complex of the spined loach *Cobitis sinensis* and *C. longicorpus* (Pices, Cobitidae). Korean Journal of Ichthyology. 2 (2), 203-210.

Kim I.S. and Lee W.O., 1995. *Niwaella brevifasciata*, a new cobitid fish (Cypriniformes: cobitidae) with a revised key to the species of *Niwaella*. Japanese Journal of Ichthyology. 42 (3/4) 285-290.

Kim I.S. and Park J. Y., 1997. *Iksookimia yongdokensis*, a new cobitid fish (Pisces: Cobitidae) from Korea with a key to the species of *Iksookimia*. Ichthyological Research. 44 (3), 249- 256.

Kim I.S. and Park J.Y., 2002. Fresh water fishes of Korea. Kyo-Hak Pub.Co.Ltd. Seoul Korea. 727 pp. (In Korean).

Kim I.S., Park J.Y and Nalbant T.T., 2000. A new species of *Koreocobitis* from Korea with redescription of *K. rotundicaudata*. Korean Journal of Ichthyology. 12 (2), 89-95.

Kim I.S., Park J.Y., Son Y.M. and Nalbant T.T., 2003. A review of the Loaches, Genus *Cobitis* (Teleostomi: cobitida) from Korea, with the description of a new species *Cobitis hankugensis*. Korean Journal of Ichthyology. 15 (1), 1-11.

Kim I.S. and Son Y.M., 1984. *Cobitis choii*, a new cobitid fish from Korea. Korean Journal of Zoology. 27 (1), 49-55.

Kim K.H., Kwan A.S. and Lee Y.H., 1998. Spermatozoal ultrastructure and phylogenetic relationships of the subfamily Gobioninae (cyprinidae)2. Ultrastructue of spermatozoa in the Korean gudgean, *Squalidus chankaensis tsuchigae*. Korean Journal of Limnology. 31(3), 159-164.

Kim S.Y., Kim C.B., Kim I.S., Park J.Y. and Park H.Y., 2002. Molecular systematics of Korean cobitids based on mitochondrial cytochrome *b* sequence. Korean Journal of Biological Science. 6, 45-51.

Kim S.Y., Park J.Y. and Kim I.S., 1999. Chromosome of spined loach, *Iksookimia yongdokensis* (Pisces: Cobitidae) from Korea. Korean Journal of Ichthyology. 11 (2), 172-176.

Kimura M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution. 16, 111-120.

Kitagawa T., Jeon S.R., Kitagawa E., Yoshioka M., Kashiwagi M. and Okazaki T., 2005. Genetic relationships among the Japanese and Korean striated spined loach complex (Cobitidae: *Cobitis*) and their phylogenetic positions. Ichthyological Research. 52, 111-122.

Kitagawa T., Watanabe M., Kitagawa E., Yoshioka M., Kashiwagi M. and Okazaki T., 2003. Phylogeography and the maternal origin of

the tetraploid form of the Japanese spined loach, *Cobitis biwae*, revealed by mitochondrial DNA analysis. Ichthyological Research. 50, 318-325.

Kocher T.D. and Stepien C.A., 1997. Molecular systematics of fishes. Academic Press. San Diego, USA. 314pp.

Kocher T.D., Thomas W.K., Meyer A., Edwards S.V., Paabo S., Villablanca F.X. and Wilson A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. 86, 6196-6200.

Kotusz J., 2000. Intra-and interpopulation morphological variability in diploid and varied-ploidy *Cobitis* from Poland. Folia Zoologica. 49, 219-226.

Krisfalusi M., Wheeler P.A., Thorgaard G. H. and Cloud J. G., 2000. Gonadal morphology of female diploid gynogenetic and triploid Rainbow Trout. Journal of Experimental Zoology. 286, 505-512.

Kumar S., Tanura K. and Nei M., 2004. Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics. 5(2), 150-163.

Lamatsch D.K., Steinlein C., Schmid M. and Schartl M., 2000. Noninvasive determination of genome size and ploidy level in fishes by flow cytometry: detection of triploid *Poecilia Formosa*. Cytometry. 39, 91-95.

Laroche J., Durand J.D., Bouvet Y., Guinand B. and Brohon B., 1999. Genetic structure and differentiation among populations of two cyprinids, *Leuciscus cephalus* and *Rutilus rutilus*, in large European river. Canadian Journal of Fisheries and Aquatic Science. 56, 1659-1667.

Le Comber S.C. and Smith C., 2004. Polyploidy in fishes: pattern and processes. Biological Journal of the Linnean Society. 82, 431-442.

Lee D.K., 2000. Predation efficiency of the fish muddy loach, *Misgurnus mizolepis*, against Aedes and Culex mosquitoes in laboratory and small rice plots. Journal of American Mosquito Control Association. 16 (3), 258- 261.

Lee Y.N., 2004. The first cyprinid fish and small mammal fossils from the Korean peninsula. Journal of Veterinary Paleontology. 24 (2), 489-493.

Lingenfelser S.K., Dallas C.E., Jagoe C.H., Chesser R.K., Smith M.H. and Lomakin M., 1997. Variation in blood cell DNA in *Carassius carassius* from ponds near Chernobyl, Ukraine. Ecotoxicology. 6, 187-203.

Liu H., Tzeng C.S. and Teng H.Y., 2002. Sequence variations in the mitochondrial DNA control region and their implications for the phylogeny of the Cypriniformes. Canadian Journal of Zoology. 80, 569- 581.

Ludwig A., Bohlen J., Wolter C. and Pitra C., 2001. Phylogenetic relationships and historical biogeography of spined loaches (Cobitidae, *Cobitis* and *Sabanejewia*) as indicated by variability of mitochondrial DNA. Zoological Journal of the Linnean Society, 131 (3), 381- 392.

Mable B.K., 2004. Why polyploidy is rare in animals than in plants; myths and mechanisms. Biological Journal of the Linnean Society. 82 (4), 453-466.

Machordom A. and Doadrio I., 2001. Evolutionary history and speciation modes in the cyprinid Genus *Barbus*. Proceeding of the

Royal society of London series B, biological science. 268 (1473), 1297-306.

Mezhzherin S.V. and Chudakorova T. Y., 2002. The genetic structure of the diploid-polyploid complex of the spined loach *Cobitis taenia* (Cypriniformes: Cobitidae) from the Middle Dnieper basin. Russian Journal of Genetics. 38 (1), 70-75.

Nalbant T.T., 1993. Some problems in the systematics of the genus *Cobitis* and its relatives (Piesces, Ostariophysi, Cobitidae). Review Roumain de Biologie Animals. 38 (2), 101-110.

Nam Y.K., Noh J. K., Cho Y.S., Cho H. J., Cho K.N., Kim C. G. and Kim D. S., 2001. Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach *Misgurnus mizolepis*. Transgenic Research 10: 353-362.

Nam M.M. and Yang H.J., 1998. On the circumorbital bone of the cyprinid fishes in Korea. Korean Journal of Ichthyology. 10 (1), 128-138. (in Korean).

Nelson J.S., 1994. Fishes of the world. Third edition. John Wiley and Sons, Inc. USA.

Noh J.K., Cho K.N., Han E.H., Kim A., Lee J.S., Kim D.S. and Kim C. G., 2003. Genomic cloning of mud loach *Misgurnus mizolepis* (Cypriniformes, cobitidae) β- actin gene and usefulness of its promotor region for fish transgenesis. Marine Biotechnology. 5, 244-252.

Park I.S. and Kim D.S., 2000. Comparison of some tissues in diploid and triploid hybrid between mud loach, *Misgurnus mizolepis* and cyprinid loach, *M. anguillicaudatus*. Developmenta Reproduction. 4, 19-28.

Park C.S. and Lee H.Y., 1991. Systematic study on the fishes of Family Cobitidae (Pisces: Cypriniformes): Extensive variation in mitochondrial DNA among geographic populations on *Nemacheilus toni*. Korean Journal of Ichthyology. 3 (2), 140-147.

Patterton H. G. and Graves S., 2000. DNAssist: the integrated editing and analysis of molecular biology sequences in Windows. Bioinformatics. 16 (7), 652-653.

Perdices A., Cunha C. and Coelho M.M., 2004. Phylogenetic structure of *Zacco platypus* (Teleostei, Cyprinidae) populations on the upper and middle Chang Jiang (=Yangtze) drainage inferred from cytochrome *b* sequences. Molecular Phylogenetics and Evolution. 31, 192-203.

Perdices A. and Doadrio I., 2001. The molecular systematics and biogeography of the European cobitids based on mitochondrial DNA sequences. Molecular Phylogenetics and Evolution. 19 (3), 468- 478.

Romero A. and Green S.M., 2005. The end of regressive evolution: examining and interpreting the evidence from cave fishes. Journal of Fish Biology. 67, 3-32.

Ruzainah A., Siti Azizah M.N., Patimah, I. and Amirrudim A., 2003. RAPD fingerprinting of the eel-loaches *Pangio filinaris* and *Pangio piperrata*: preliminary evaluation. Aquaculture Research. 34, 959-965.

Saitoh K., Kim I.S. and Lee E.H., 2004. Mitochondrial gene introgression between spined loaches via hybridogenesis. Zoological Science. 21, 795-798.

Saitoh K., Kobayashi T., Ueshima R. and Numachi K.I., 2000. Analyses of mitochondrial and satellite DNAs on spined loaches of the genus *Cobitis* from Japan have revealed relationships among populations of three diploid-tetraploid complexes. Folia Zoologica. 49, 9-16.

Saitou N. and Nei M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 4(4), 406-425.

Salzburger W., Brandstatter A., Gilles A., Parson W., Hempel M., Sturmbauer C. and Meyer A., 2003. Phylogeography of the vairone (Leuciscus souffia, Risso 1826) in Central Europe. Molecular Ecology. 12, 2371- 2386.

Sezaki K., Watabe S., Ochiai Y. and Hashimoto K., 1994. Biochemical genetic evidence for a hybrid origin of spined loach, *Cobitis taenia taenia*, in Japan. Journal of Fish Biology. 44, 683-691.

Slechtova V., Luskova V., Slechta V., Lusk S., Halacka K. and Bohlen J., 2000. Genetic differentiation of two diploid-polyploid complexs of spined loach Genus *Cobitis* (Cobitidae), in the Czech Republic, involving *C. taenia*, *C. elongatoides*, and *C.* spp.: Allozyme interpopulation and interspecific differences. Folia Zoologica. 49, 67- 78.

Son, Y.M. and Kim I.S., 2002. Study on the specimens of *Cobitis sinensis* of the Museum National d'histoire Naturelle (MNHN), France. Korean Journal of Ichthyology. 14 (3), 240-244.

Song C.B., 1994. Molecular evolution of the cytochrome *b* gene among percid fishes. Ph.D. thesis in biology. University of Illinois. Urbana-Champaign. U.S.A.111pp.

Suk H.Y., Kim J.B., Min M.S. and Yang S.Y., 1996. Genetic differentiation and reproductive isolation among three types of the floating goby (Chaenogobius annularis) in Korea. Korean Journal of Zoology. 39, 147- 158.

Swofford D.L., 1998. PAUP: Phylogenetic analysis using parsimony, version 4. Sinauer Associates, Sunderland, MA.USA.

Tamura K. and Nei M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution. 10 (3), 512-526.

Toth B., Varkonyi E., Hidas A., Meleg E.E. and Varadi L., 2005. Genetic analysis of offspring from intra-and interspecific crosses of *Carassius auratus gibelio* by chromosome and RAPD analysis. Journal of Fish Biology. 66, 784-797.

Tsigenopoulos C.S. and Berrebi P., 2000. Molecular phylogeny of North Mediterranean freshwater barbs (Genus *Barbus*: cyprinidae) inferred from cytochrome *b* sequences: Biogeographic and systematic implications. Molecular Phylogenetics and Evolution. 14 (2), 165-179.

Tsigenopoulos C.S., Kotlik P. and Berrebi P., 2002. Biogeography and pattern of gene flow among *Barbus* species (Teleostei: Cyprinidae) inhabiting the Italian peninsula and neighbouring Adriatic drainages as revealed by allozyme and mitochondrial sequence data. Biological Journal of the Linnean Society. 75, 83-99.

Vladykov V.D., 2005. Secondary sexual dimorphism in some Chinese cobitid fishes. Journal of Morphology. 57, 275-302.

Xiao W., Zhang Y. and Liu H., 2001. Molecular systematics of Xenocyprinae (Teleostei: cyprinidae): taxonomy, biogeography, and coevolution of a special group restricted in East Asia. Molecular Phylogenetics and Evolution. 18(2), 163-173.

Zardoya R. and Doadrio I., 1998. Phylogenetic relationships of Iberian cyprinids: systematic and biogeographical implications. Proceeding of the Royal society of London series B, biological science. 265, 1365-1372.

Zardoya R., Economidis P.S. and Doadrio I., 1999. Phylogenetic relationship of Greek Cyprinidae: molecular evidence for at least two origins of the Greek cyprinid fauna. Molecular Phylogenetics and Evolution. 13 (1), 122-131.

Zhou J., Wu Q., Wang Z. and Ye Y., 2004. Molecular phylogeny of three subspecies of common carp *Cyprinus capio*, based on sequence analysis of cytochrome *b* and control region of mtDNA. Journal of Zoological Systematic and Evolutionary Research. 42, 266-269.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my academic supervisor, Professor Choon Bok Song for giving me the opportunity to study at his laboratory, teaching me, being kind and patient and helping in different ways. I would also like to extend a hearty thanks to my labmates, Beom-Seok Koh, Meang-Jin Kim, Young-Deuk Won, Sang-Gyu Oh, Mi-Ran Jo, Yoon-Chul Yang, He-Young Yang and Song-Hun Han for their help, kindness, friendship and understanding. My especial and immense thanks to the examiners and referees of my thesis, professors Ki-Wan Lee, You-Jin Jeon, Moon-Soo Heo and In-Kyu Yeo for their invaluable advices and suggestions.

I would further like to express herewith my gratitude to the former president of Cheju National University, professor Man Keun Boo and present president of the university professor Chung Seok Goh, their vice presidents, the president and vice president of the College of Ocean Science, as well as the staff and officers of the university and college for their consideration, support and effort.

Many thanks as well to the professors, students and members of the laboratories at the College of Ocean Science.

I present my approbation and respect to my foreign schoolmates and to many other Korean and foreign friends for their kindness and friendship during my study in Korea.

I am grateful to the authorities and staff of the Brain Korea 21 (BK-21) program and the Korean Ministry of Maritime Affairs and Fisheries for their financial support of my study and research.

I am indebted to Dr. Duk-Hyun Yoon who took the first steps towards my studying in Korea.

Finally, I would particularly like to express my heartfelt gratitude to my family, friends and relatives for their encouragement, patience and support.

Appendix 1

No	Taxa	No	Taxa
1	*C. hankugensis* Geochang 1M	26	*C. hankugensis* Sancheong 2 M
2	*C. hankugensis* Geochang 2	27	*C. hankugensis* Sancheong 3 F
3	*C. hankugensis* Geochang 3	28	*C. hankugensis* Sancheong 3 M
4	*C. hankugensis* Geochang 4	29	*C. hankugensis* Sancheong 4 F
5	*C. hankugensis* Geochang 5	30	*C. hankugensis* Youngcheon 1 F
6	*C. hankugensis* Geochang 6	31	*C. hankugensis* Youngcheon 1 M
7	*C. hankugensis* Geochang 7	32	*C. hankugensis* Youngcheon 2 F
8	*C. hankugensis* Namwon 1 M	33	*C. hankugensis* Youngcheon 2 M
9	*C. hankugensis* Namwon 10 M	34	*C. lutheri* Hampyungdaedong 1
10	*C. hankugensis* Namwon 11	35	*C. lutheri* Hampyungdaedong 2
11	*C. hankugensis* Namwon 12	36	*C. lutheri* Hampyungdaedong 3
12	*C. hankugensis* Namwon 13	37	*C. lutheri* Hampyungdaedong 5
13	*C. hankugensis* Namwon 14	38	*C. lutheri* Hampyungdaedong 6
14	*C. hankugensis* Namwon 15	39	*C. lutheri* Tamjin 1
15	*C. hankugensis* Namwon 3	40	*C. lutheri* Tamjin 2
16	*C. hankugensis* Namwon 4 F	41	*C. pacifica* Gangreung
17	*C. hankugensis* Namwon 5 M	42	*C. sinensis* China AY625699
18	*C. hankugensis* Namwon 6 M	43	*C. sinensis* Taiwan AY526868
19	*C. hankugensis* Namwon 7 F	44	*C. sinensis* Taiwan NC007229
20	*C. hankugensis* Namwon 8 M	45	*C. tetralineata* Imsil
21	*C. hankugensis* Namwon 9 F	46	*C. tetralineata* Namwon
22	*C. hankugensis* Namwon AB120176	47	*C. lutheria* Hampyungdaedong 4
23	*C. hankugensis* Sancheong 1 F	48	Hybrid 1 Sancheong F
24	*C. hankugensis* Sancheong 1 M	49	Hybrid 12 Sancheong F
25	*C. hankugensis* Sancheong 2 F	50	Hybrid 13 Sancheong F

Appendix 1. List of the studied fish taxa and their corresponding numbers. The name of each taxon includes its sampling locality and specimen number. F: females; M: male

Appendix 1

No	Taxa	No	Taxa
51	Hybrid 2 Sancheong F	65	*K. brevifasciata* Geogeumdo 2
52	Hybrid Namwon Triploid AB120177	66	*K. naktongensis* Suncheong
53	*I. choii* GeumRiver	67	*K. rotundicaudata* HanRiver
54	*I. hugowolfeldi* HampyungDaedongdam 1	68	*M. anguillicaudatus* China AF051868
55	*I. hugowolfeldi* HampyungDaedongdam 2	69	*M. anguillicaudatus* Geochang
56	*I. koreensis* Puhang	70	*M. anguillicaudatus* Japan AB080185
57	*I. koreensis* Youngcheon	71	*M. fossilis* Japan E28664
58	*I. longicorpa* Imsil 1	72	*M. mizolepis* Imsil
59	*I. longicorpa* Imsil 2	73	*N. multifasciata* Geochang
60	*I. longicorpa* Namwon	74	*N. multifasciata* Suncheong
61	*I. pumila* Buan	75	*Niwaella delicata* Japan AB039352
62	*I. yongdokensis* Angang 1	76	*L. costata* Imsil
63	*I. yongdokensis* Angang 2	77	*Carassius auratus* Jeju
64	*K. brevifasciata* Geogeumdo 1	78	*Cyprinus carpio* Taiwan NC001606

Appendix 2

No.	Symbols	Amino acid
1	A	Alanine
2	C	Cysteine
3	D	Aspartic acid
4	E	Glutamic acid
5	F	Phenylalanine
6	G	Glycine
7	H	Histidine
8	I	Isoleucine
9	K	Lysine
10	L	Leucine
11	M	Methionine
12	N	Asparagines
13	P	Proline
14	Q	Glutamine
15	R	Arginine
16	S	Serine
17	T	Threonine
18	V	Valine
19	W	Tryptophan
20	Y	Tyrosine

Apendix 2. The alphabetic symbols and their corresponding amino acids

Vitae

Reza Malekzadeh Viayeh
Department of Marine Biotechnology,
Cheju National University- Ara-Dong 690-756.
Jeju City, Republic of Korea.

Born September 21, 1969, Roudbar, Iran

Education

1988-1993 Faculty of Natural Resources, University of Tehran, Iran
B.SC. In Fisheries and Environment
1993-1996 Faculty of Natural Resources, University of Tehran, Iran
M.SC. In Fisheries
2002-2006 Graduate School, Cheju National University, Korea
Ph.D. In Marine Biotechnology

Professional Experience

- Research Center for Natural Resources and Livestock- Tabriz Iran (1995-1997).
- "Abzi Gostar" Aquaculture Management Consultant Company. Tehran- Iran (1995-1996)
- "Ahrar" Aquaculture Complex. Ahvaz- Iran (1995).
- International Sturgeon Research Institute. Rasht- Iran (1999-2002).

Honors and Awards

- Highest scored contestant in M.Sc. entrance exam of the country in 1993.
- Research lecturer of the Iranian Ministry of Agriculture (Since 2001).
- Recipient of the scholarship of BK-21 program from Korean government for study in PhD (2002)

Projects and Research Activities

- Identification and study of the mollusks and shellfishes in the tidal zone of Persian Gulf (1992-1993).
- Identification and study of ichthyofauna in the freshwaters of western Iran (1995-1997).
- Study of aquatic fauna of Hamoon Lake in East Iran (1995-1996).
- Histological studies on the Caspian sturgeons (2000).
- Study on osmoregulation in *Acipenser persicus* (2000-2002).
- Reproductive physiology and artificial breeding in *A. stelatus* using hormonal induction (2000-2002).
- Study of steroid hormones in *A. persicus* brood fish (2001-2002).
- Study on sex determination in Persian sturgeon by ELISA of steroid hormones (uncompleted).
- Isolation, cultural condition, and demographic study of the rotifer *Brachionus rotundiformis* (2002-2003).
- Selective breeding in the rotifer *B. rotundiformis* (2003-2004).
- Mollecular phylogeny in the fishes of order Cypriniformes inferred from cytochrome *b* gene (2003-2005).
- Molecular phylogeny and natural hybridization of cobitid fishes in Korea. PhD. thesis (2003-2005).

Workshops

- Advanced course on artemia culture and processing (Urmia-Iran, 1997).
- Workshop on applications of GC and HPLC in Marine Biotechnology (Bandar Abbas-Iran, 2002).
- Training course on Reproduction physiology of fish (Rasht-Iran, 2002).

Seminars and Scientific Meetings

- Kazemin, R., M. Bahmani, A. Hallajian, M. Mohseni and **R. Malekzadeh**, 2001. Morphological comparisons of reared juvenile sturgeons in South Caspian Sea and economical evaluation of artificial breeding. The fourth International Symposium on Sturgeon. Oshkosh-USA.
- Bahmani, M., R. Kazemi, M. Pourdehghani, **R. Malekzadeh**, A. Hallajian, S. Dejandian, M. Mohseni, H. Mostafavi and B. Mojazi Amiri, 2002. A new approach in artificial reproduction of Stellate

sturgeon, *Acipenser stellatus*, by administrating a combination of GnRH and Dopamine antagonist. Second National and Regional Symposium on Sturgeon. Rasht-Iran.

- Hallajian, A., M. Bahmani, R. Kazemi, **R. Malekzadeh**, S. Dejandian, M., Mohseni, M. Alizadeh, H.R. Pourali, 2002. Caviar production from unfertile breeders of Persian sturgeon. Second National and Regional Symposium on Sturgeon. Rasht-Iran.
- **R. Malekzadeh Viayeh** and Choon Bok Song, 2004. Effect of salinity on the demographic characteristics of the rotifer *Brachionus rotundiformis*. Korean Society of Aquaculture Seasonal Meeting. Yosu - Korea
- **R. Malekzadeh Viayeh**, M.A. H. Webb, A. Hallajian and R. Kazemi, 2005. Biochemical and morphometric parameters as indicators of sex and gonadal stage of maturity in Persian sturgeon, *Acipenser persicus*. The fifth International Symposium on Sturgeon. Ramsar- Iran.
- **R. Malekzadeh Viayeh** and Choon Bok Song, 2005. Selective breeding in a Korean strain of the rotifer *Brachionus rotundiformis*. Korean Society of Aquaculture Seasonal Meeting. Busan- Korea.
- **R. Malekzadeh Viayeh,** Meang-Jin Kim and Choon-Bok Song, 2005**.** Molecular phylogeny and natural hybridization of cobitid fishes in Korea. The Ichthyological Society of Korea. Fall Meeting. Gunsan - Korea.

Publications

- **Reza Malekzadeh Viayeh** and Choon Bok Song, 2004. Effect of salinity on the demographic traits of the rotifer *Brachionus rotundiformis*. Journal of Fisheries Science and Technology.
- **R. Malekzadeh Viayeh**, M.A. H. Webb, A. Hallajian and R. Kazemi, 2005. Biochemical and morphometric parameters as indicators of sex and gonadal stage of maturity in Persian sturgeon, *Acipenser persicus*. (Under review by the Journal of Applied Ichthyology)
- **R. Malekzadeh Viayeh**, A. Hallajian and A. Houshmand. A study on the levels of steroid hormones in Persian sturgeon prior to their spawning migration. (Article In press for the Scientific Bulletin of Iranian Fisheries Research Institute)

Unpublished works

- Selective breeding in rotifer
- Maximum thermal tolerance and effect of temperature on the population density and body size of the rotifer *Brachionus rotundiformis*.
- Effect of UV irradiation on survival and body size of the rotifer *B. rotundiformis*.
- Phylogenetic relationship in fish order Cypriniformes by comparisons of the sequences of cytochrome *b* gene.

Contact Address

Postal Code 44619- Roudbar- Gilan- Iran
Tel: 0098 132 6222221
E-mail: rmalekvia@yahoo.com