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A Molecular Genetic Variation Among Intra-populations of Korean Shiner, *Coreoleuciscus splendidus* Mori (Cyprinidae)

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We examined the genetic diversity in intra-populations of Korean shiner, *Coreoleuciscus splendidus*, in six major rivers (Bukhan, Namhan, Geum, Osipcheon, Nakdong and Seomjin river) of Korea based on two different mitochondrial genes, the mitochondrial cytochrome *b* and the 16S rRNA. Analysis of sequence variation in a 657-bp segment of the mitochondrial cytochrome *b* gene revealed deep divergence among populations (98.2～99.9%) and high genetic diversity from geographically isolated populations. Intra-specific variation in this 697-bp segment of the 16S rRNA gene sequences was very low and nearly identical. Six isolate populations of *C. splendidus* showed a high similarity (97.7%～99.7%). This result may be indicative of a complex history of connection and isolation of the rivers in the Korea peninsula.

Key words : *Coreoleuciscus splendidus*, Cyprinidae, cytochrome *b*, 16S rRNA, Korea

Introduction

Phylogeography is a study of the geographical distribution of evolutionary lineages and of the geographical processes that influence such distribution with a focus on diversity within and between closely related species (Avise, 2000). The present and historical connections among rivers are one possible basis for the interpretation of the observed genetic relationships among populations (Bernatchez and Wilson, 1998; Aurelle *et al*., 2002). Historical biogeographical analyses of freshwater fishes provide a natural link between the geological and biotic evolution of a region.

The family Cyprinidae, containing about 2000 species, is one of the most extensive freshwater fish families, whose biogeography, phylogeography and systematics are based on morphological characters (Nelson, 1994). Korean shiner, *Coreoleuciscus splendidus* is one of the endemic freshwater cyprinid fishes in Korea (Kim, 1997). Most individuals dwell at the bottom in riffle area of streams and feed on aquatic insects (Song and Kwon, 1993). Most streams of this study area are divided no connection to neighbouring drainage systems. Geographical isolation is one of the primary barriers that produce changes of genetic composition. By integrating past and present biogeographical records and the information provided by molecular data, this conjunction has led to important insights in population genetics, evolutionary biology and ecology (Schluter, 1997; McCusker *et al*., 2000; Bernatchez, 2001). Molecular systematics is now recognized as an integral part of efforts to conserve rare species (Soltis and Gitzendanner, 1999). By delineating boundaries between evolutionary lineages, phylogenetic hypotheses focus attention on genetically distinct populations or groups of

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populations that may require special protection or warrant independent management strategies (Moritz, 1994). In recent years, mitochondrial DNA, because of its fast evolution, has been widely applied in systematics (Bermingham and Martin, 1998). As regards molecular studies, which in other fields have proved to be quite useful, a single specific work has been conducted on this family, during which partial sequences of the mitochondrial 16S rRNA gene and cytochrome *b* gene were obtained in many species (Kotlik and Berrebi, 2001; Machordom and Doadrio, 2001). Further, phylogenetic reconstruction identifies the relationships between these lineages and reveals the extent to which cohesive evolutionary groups have diverged. The objectives of the present study were to investigate for phylogeographic patterns at hierarchical scales of between populations. This study is an investigation of the phylogeny of *C. splendidus*, using two mitochondrial genes, the 16S ribosomal gene and the cytochrome *b* gene for intra-specific divergence.

Materials and Methods

A total of 46 specimens of *C. splendidus* from six populations (Bukhan, Namhan, Geum, Osipcheon, Nakdong and Seomjin river) in Korea were collected (Fig. 1 and Table 1).

Genomic DNA was extracted from muscle tissue by a standard phenol/chloroform protocol as described by Sambrook and Russel (2001). The two gene regions were amplified by the polymerase chain reaction (PCR) from 20~40 ng of DNA. For the 16S rRNA coding gene, the primers used, N984 (5′-CGCCTGTTTACCAAAAACATCG-3′) and 3259 (CCGCTTTGAGCTCAGATCA-3′), were as described by Kocher *et al.* (1989). PCR amplification was conducted over 30 cycles using the following conditions: 1 min at 93°C (one cycle), 15 sec at 93°C, 45 sec at 48°C, and 2 min 30 sec at 72°C (five cycle), 15 sec at 93°C, 45 sec at 55°C, and 2 min 30 sec at 72°C (30 cycles), with a final extension of 7 min at 72°C for 16S rRNA coding gene. PCR amplication of the mitochondrial cytochrome *b* gene was carried out using the primers New-For (5′-AGCCTACGAAAAACCCACCC-3′) and 34 Rew (5′-AAACTGCAGCCCCTCAG-AATGATATTTGTCCTCA-3′) designed by Chang *et al.* (1994) and Cantatore *et al.* (1994). Double-stranded product was amplified using the following cycling profile: initial denaturation for 1 min at 92°C; 15 sec at 92°C, 45s at 48°C, and 2 min 30s at 72°C (5 cycle); 15s at 92°C, 45s at 52°C, and 2 min 30s at 72°C (30 cycle); and a final ex-

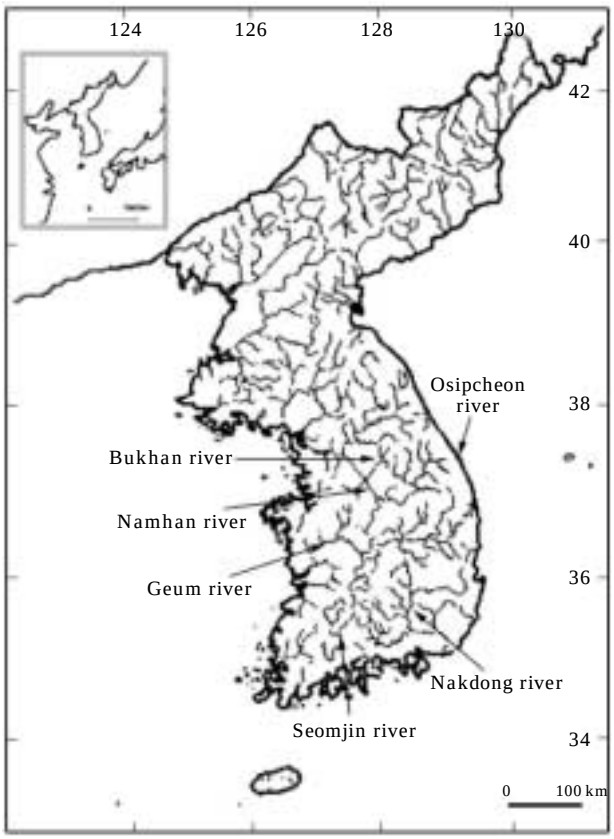

Fig. 1. Map illustrating the location of sampling sites.

Table 1. Collection localities and specimen numbers of *Coreoleuciscus splendidus* examined in this study

Population	Specimen No.	Locality
Bukhan River	7	Wontong-ri, Buk-myeon, Inje-gun, Gangwon-do
Namhan River	8	Najeon-ri, Bukpyeong-myeon, Jeongseon-gun, Gangwon-do
Geum River	7	Cheongnyang-ri, Seolcheon-myeon, Muju-gun, Jeollabuk-do
Osipcheon River	9	Singi-ri, Singi-myeon, Samcheok-si, Gangwon-do
Nakdong River	8	Docheon-ri, Myeongho-myeon, Bonghwa-gun, Gyeongsangbuk-do
Seomjin River	7	Gwanchon-ri, Gwanchon-myeon, Imsil-gun, Jeollabuk-do

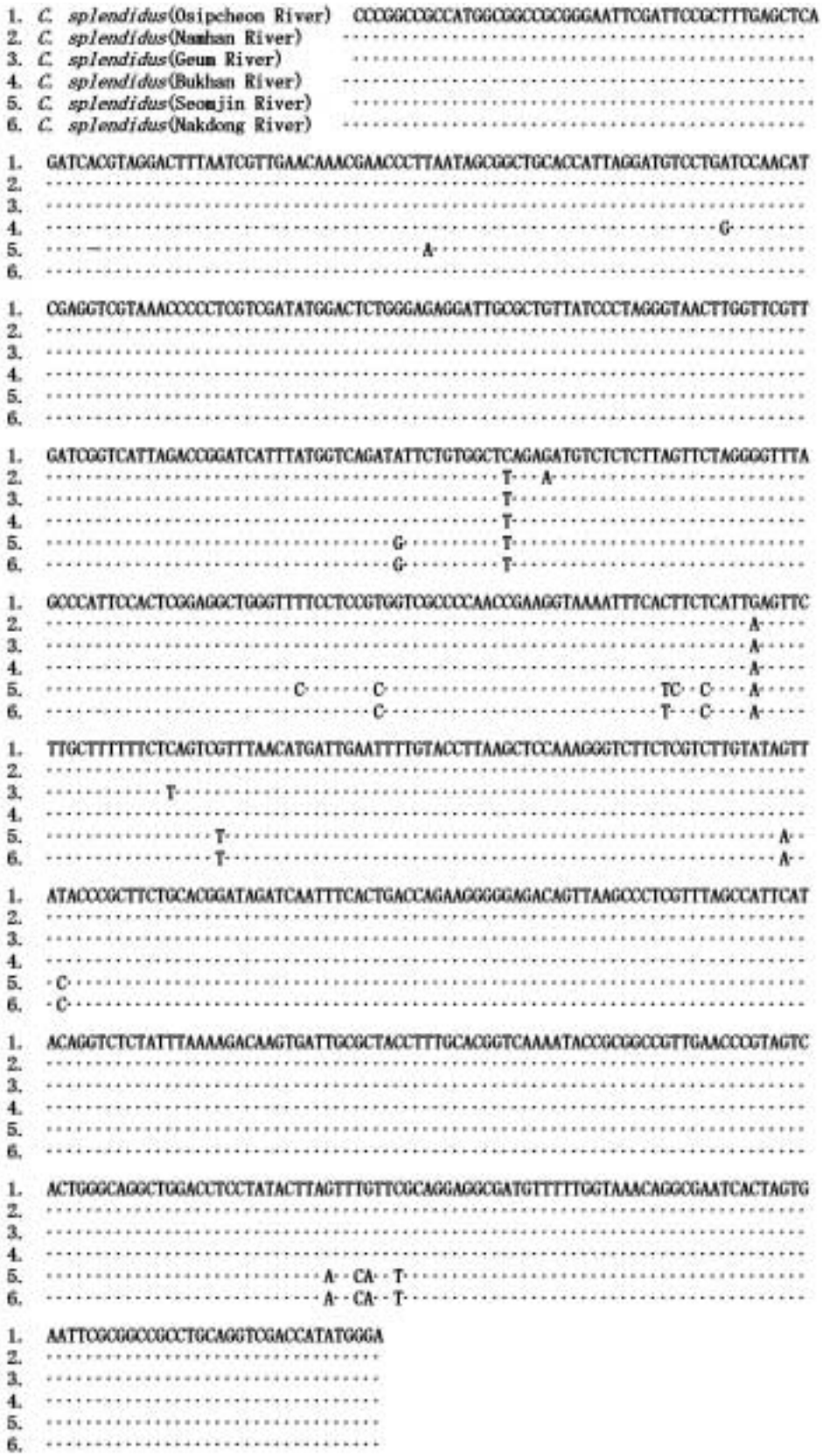

Fig. 2. Sequence alignment of 16S rRNA gene sequences from six geographical isolates for *Coreoleuciscus splendidus* of Korea. Dots (•) denote identical bases; dashes (−), gaps inserted for alignment purposes.

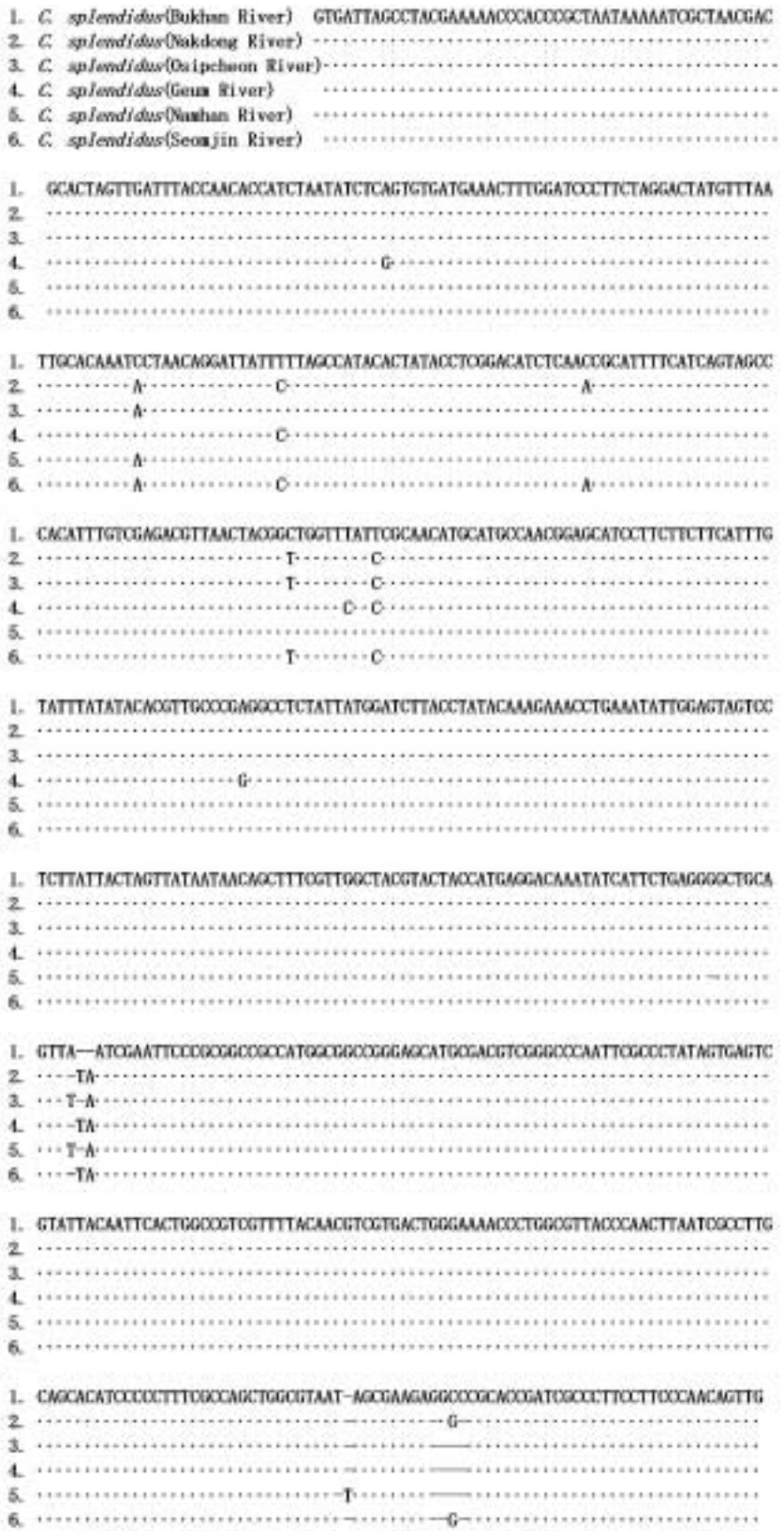

Fig. 3. Sequence alignment of mitochondrial cytochrome *b* gene sequences from six geographical isolates for *Coreoleuciscus splendidus* of Korea. Dots (•) denote identical bases; dashes (−), gaps inserted for alignment purposes.

tension of 7 min at 72°C. The PCR products were purified using an Ultra Clean DNA purification kit (MO BIO Labs), and ligated into a pGEM-T easy vector (Promega). DNA from positive recombinants was purified using the QIAprep spin plasmid kit (Qiagen Co.). DNA sequencing was performed using the dideoxy chain termination method and an automated DNA sequencer. At least two clones were sequenced per isolate, and additional clones were sequenced as necessary to resolve ambiguous sites. Nucleotide sequences were aligned using Clustal X 1.81 (Thompson *et al*., 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar *et al*., 2001). Gaps were considered as an additional character state in pairwise comparisons.

Table 2. Pairwise differences among 16S rRNA gene sequences of populations *Coreoleuciscus splendidus*

	1	2	3	4	5	6
1. Osipcheon river		3 (99.6)	2 (99.7)	2 (99.7)	16 (97.9)	12 (98.4)
2. Namhan river			3 (99.6)	3 (99.6)	17 (97.7)	14 (98.1)
3. Geum river				2 (99.7)	16 (97.9)	13 (98.3)
4. Bukhan river					16 (97.9)	13 (98.3)
5. Seomjin river						3 (99.6)
6. Nakdong river						

Table 3. Pairwise differences among mitochondrial cytochrome *b* gene sequences of populations *Coreoleuciscus splendidus*

	1	2	3	4	5	6
1. Bukhan river		11 (98.3%)	9 (98.6)	12 (98.2)	9 (98.6)	12 (98.2)
2. Nakdong river			6 (99.1)	8 (98.8)	10 (98.5)	1 (99.9)
3. Osipcheon river				8 (98.8)	4 (99.4)	5 (99.2)
4. Geum river					10 (98.5)	7 (98.9)
5. Namhan river						9 (98.6)
6. Seomjin river						

Results

The 16S rRNA and mitochondrial cytochrome *b* gene were sequenced in 7 to 9 individuals from six populations by a 697 bp and 657 bp segment of the 5′ end of the gene. The nucleotide composition of these two gene were homogeneous among congeners. Sequence data of the 16S rRNA and mitochondrial cytochrome *b* gene obtained from this study were aligned and compared (Fig. 2 and Fig. 3). Pairwise differences among 16S and cytochrome *b* of mitochondrial gene sequences of intra-populations are shown in Table 2 and Table 3. The 697 bp 16S rRNA of the six *C. splendidus* isolates was obtained. The six populations of *C. splendidus* of Korea had almost identical 16S sequences and differed at either two or 16 sites (0.3～2.3% divergence) (Table 2). Figure 4 shows the relationships among the intra-species of *C. splendidus* used as inferred from their 16S sequences. The phylogenetic tree shows that *C.*

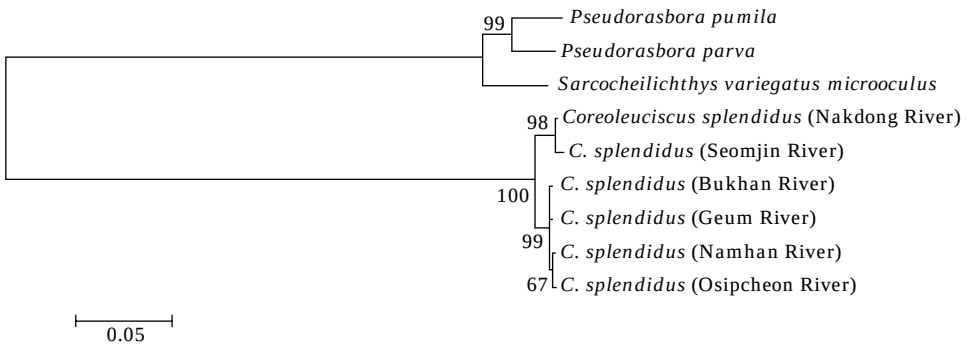

Fig. 4. Phylogenetic relationships among the taxa analyzed, based on 16S rRNA coding gene sequences. Tree depicting relationships between *Coreoleuciscus splendidus* and *P. pumila* (GenBank Accession no., AB025214), *P. parva* (AB025204) and *S. variegatus microoculus* (AB054124) used as outgroup taxa. A distance matrix was calculated using the Kimura-2-parameter model and the tree was constructed using the minimum evolution. Numbers on branches indicate the percentage of 100 bootstraps supporting the branching pattern shown.

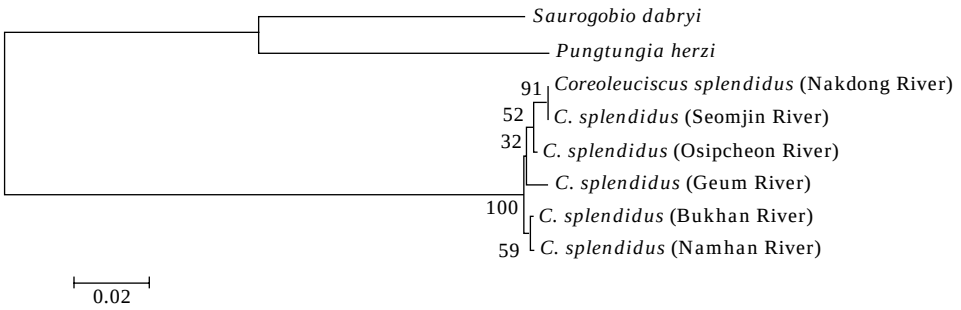

Fig. 5. Phylogenetic relationships among the taxa analyzed, based on mitochondrial cytochrome *b* gene sequences. Tree depicting relationships between *Coreoleuciscus splendidus*, and *S. dabryi* (AF245091) and *P. herzi* (AF375864) as outgroup taxa. A distance matrix was calculated using the Kimura-2-parameter model and the tree was constructed using the minimum evolution. Numbers on branches indicate the percentage of 100 bootstraps supporting the branching pattern shown.

splendidus populations formed two monophyletic clades, which were consistently supported by high bootstrap (98% and 99%). Nakdong and Seomjin river populations clustered together in one clade and Bukhan, Geum, Namhan and Osipcheon river populations in the other (Fig. 4). The 657 bp mitochondrial cytochrome *b* gene of the six *C. splendidus* isolate populations was obtained. Intra-species variations were detected at a low level of 0.1∼1.8% in the six *C. splendidus* isolates. The phylogenetic tree shows the relationships among the intra-species based on the cytochrome *b* sequences when other cyprinid species, *Saurogobio dabryi* and *Pungtungia herzi* were used as the outgroup (Fig. 5). The patterns of relationships among the six major rivers, based on nucleotide diversity, showed a twin grouping of Nakdong and Seomjin river populations in one group, and Namhan and Osipcheon river populations clustered together with Bukhan and Geum river populations in the other.

Discussion

Phylogeography, the combination of phylogenetics and population genetics with biogeography, has existed as a formal discipline for over 15 years (Avise *et al*., 1987; Avise, 1998, 2000; Bernatchez and Wilson, 1998; Machordom and Doadrio, 2001). Freshwater fish are well suited for phylogeographic studies, because they are dependent upon water routes for dispersal and their phylogeographies are therefore likely to reflect historical causes more closely than those of terrestrial species (Bernatchez and Wilson, 1998).

Most molecular phylogenetic studies of vertebrates have been based on DNA sequences of mitochondrial encoded genes. Mitochondrial DNA evolves rapidly and is thus particularly useful for resolving relationships among recently evolved groups (Lydeard and Roe, 1997). The phylogeographical patterns and intra-population genetic structures of *C. splendidus* in Korea were investigated based on the genetic diversity of nucleotide sequences of the mitochondrial cytochrome *b* gene and 16S rRNA of mtDNA. In the present study, the genetic variation of the intra-species of *C. splendidus* was very low; in fact, they were almost identical. Sequences for the six *C. splendidus* isolates differed at 2 to 17 of the 697 base positions of the 16S rRNA gene and at 1 to 11 of the 657 base positions of the mitochondrial cytochrome *b* gene. Genetic variation is the raw material in species populations which enables them to adapt to changes in their environment. New genetic variation arises in a population from either spontaneous mutation of a gene or by immigration from a population of genetically different individuals.

Our phylogenetic analysis of 16S rRNA and cytochrome *b* gene variation within six *C. splendidus* populations provides clear evidence for two lowly differentiated with monophyletic lineages. Phylogenetic relationships within the genus seem to be well established. However, the relationships between species and different populations have barely been investigated due to a lack of biogeographical understanding. Indeed, the evolutions of the species *C. splendidus* and its biogeographical history remain unclear and

the question arises as to which barriers or events were the most important for promoting speciation. The patterns of relationships among the six major rivers in the Korean peninsula, based on nucleotide diversity, showed a twin grouping of Nakdong and Seomjin river populations in one group, and Namhan and Osipcheon river populations clustered together with Bukhan and Geum river populations in the other. The reason for two group was that the rivers in the same drainage basin were isolated by uplift of sea level in western and southern part of the Korean peninsula, and in land, Baekdu mountain range separated in western part (west Koean subdistrict) which contained Han and Geum river and southern part (south Korean subdistrict) which belong to Nakdong and Seomjin river (Lindberg and Krasyrkova, 1975; Kim, 1997). Interestingly, Osipcheon river populations which another drainage basin with Han river are phylogenetically closer to Namhan river populations. This reason suggested that Namhan river populations were introduced to Osipcheon river by stream capture (Choi, 1973). Compared analysis of predicted assemblage from the 16S gene does appear to resolve the relationships within the group containing six isolates (Fig. 4). On the other hand, the result of cytochrome *b* gene sequences does appeare for different assemblage as compared with 16S gene sequences (Fig. 5). Additional sequences data from other conserved protein-encoding genes will be required to further test the proposed relationships.

Genetic variation in mtDNA is not representative of evolutionary forces acting on nuclear DNA (nDNA) because individuals are haploid and mtDNA genotypes are transmitted through maternal lines. The effective population size for mtDNA is considerably smaller than for a nuclear gene because each individual has only one copy and because of uniparental inheritance (Birky *et al*., 1989). In this study, the *C. splendidus* populations showed high levels of pairwise sequence divergence ranging from 0.3 to 2.3% of 16S and 0.1 to 2.8% of cytochrome *b* gene. The most striking result of this study is the pattern of deep divergences among the major rivers. Populations in major rivers form reciprocally monophyletic clades with high levels of sequence divergence. The phylogeographic structure and Quaternary history of the Korean peninsula freshwater fishes have been only recently addressed, and they still remain largely unknown (Kim, 1997). Application of a molecular clock indicated that the divergence time among rivers reflects the vicariant events that occurred during the late Pliocene to present. This result is suggestive of a complex history of connection and isolation of rivers in the Korean peninsula. Further studies in other fish groups with similar patterns of geographic distribution may provide additional insights, not only into the history and evolutionary relationships among fish species, but also into the events that may have caused speciation and dispersion in the Korean peninsula.

Acknowledgements

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한국산 쉬리, *Coreoleuciscus splendidus* (잉어과)의 종내 집단간 분자 유전 변이

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한국산 쉬리, *Coreuleuciscus splendidus*의 종내 집단간 유전자 다양성을 알기 위해 6개 주요 강 (북한강, 남한강, 금강, 오십천, 낙동강, 섬진강)으로부터 채집된 개체를 대상으로 16S rRNA 유전자와 미트콘드리아 cytochrome *b* 유전자에 근거하여 비교 · 분석하였다. 미트콘드리아 cytochrome *b* 유전자의 657 bp 길이의 염기서열 분석결과, 6개 집단간에 차이는 98.2~99.9%로 나타났으며 지리적으로 격리된 집단간에 높은 유전적 다양성을 보였다. 16S rRNA 유전자는 697 bp의 염기서열을 얻었으며, 종내 변이는 큰 차이가 없이 거의 동일하였다. 16S rRNA 유전자의 6개 집단간에는 97.7%에서 99.7%의 높은 유사성을 보였다.