

Occurrence of a Natural Intergeneric Hybrid between *Rhodeus pseudosericeus* and *Acheilognathus signifer* (Pisces: Cyprinidae) from the Namhangang (river), Korea

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ABSTRACT A natural intergeneric hybrid between *Rhodeus pseudosericeus* and *Acheilognathus signifer* was collected in their sympatric area, the Jucheongang (stream), the Namhangang (river), Korea. Morphological characters as well as mitochondrial cytochrome *b* (*mt-cyb*) gene were investigated to confirm the natural hybrid origin. In the comparison of morphological characters, the natural hybrid appeared to have intermediate characters between two parental species in several key characters such as the band of dorsal fins, the color pattern of body, and the body spot and stripe. Also, the collected specimen had an incomplete lateral line with a large number of pored scales. In analysis of *mt-cyb* gene, it revealed that the maternal species of the natural hybrid appeared to be *R. pseudosericeus* due to their 100% sequence identity. Thus, such results gave convincing evidence for the occurrence of a natural intergeneric hybrid between *Rhodeus pseudosericeus* and *Acheilognathus signifer*.

Key words : *Rhodeus pseudosericeus*, *Acheilognathus signifer*, a natural hybrid, Jucheongang

INTRODUCTION

Bitterlings (Cyprinidae, Acheilognathinae) are small and deep-bodied freshwater fishes which include approximately 60 species in the world and 14 species in Korea (Kim and Park, 2002; Nelson, 2006). They are usually found in South-Eastern Asia, China mainland, Korea, and Japan, except *Rhodeus sericeus*, *R. amarus* and *R. colchicus* (Damme *et al.*, 2007). Female bitterlings develop a long ovipositor that they use to lay their eggs in the gills of mussels through the mussel's exhalant siphon. Males eject their sperm into the inhalant of the mussel, so that fertilization is taken within the gills of a host. However, there has been much taxonomic confusion due to a large variety of the shapes resulted from frequent hybridizations of Acheilognathinae fish (Okazaki *et al.*, 2001).

Many natural hybrids among the teleost fishes have been often occurred and discovered especially in cyprinid

fishes (Hubbs, 1955). Hybridizations between fish species frequently were discussed for reproductive isolating mechanisms and system relationships through interspecies or intergeneric external fertilizations (Trautman, 1981). In Korea, there have been reports on the occurrence of natural hybrids in the subspecies of *Pungitius sinensis* (Chae and Yang, 1990), and the species of *C. sinensis* and *C. longicarpus* (Kim and Lee, 1990), *Pungtungia herzi* and *Pseudopungtungia nigra* (Kim *et al.*, 1991), *Moroco oxycephalus* and *M. lagowskii* (Min and Yang, 1992), *C. longicarpus* and *Misgurnus anguilicaudatus* (Hwang *et al.*, 1995), and *R. uyekii* and *Acheilognathus signifer* (Kim *et al.*, 2010).

The Korean bitterling *A. signifer* and the Hangang bitterling *R. pseudosericeus* are distributed only in the Hangang (river) of Korea and both designated as endangered wild animals in Korean (NIBR, 2011). During a survey of ichthyofauna at the Jucheongang (stream) of the Namhangang (river) in Korea, we collected a natural hybrid between *R. pseudosericeus* and *A. signifer* in their sympatric area. So the aim of the present study is to report the morphological characters and analysis of mitochon-

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drial cytochrome *b* (*mt-cyb*) genes of the natural hybrid for verifying the natural hybrid origin.

MATERIALS AND METHODS

1. Collection of fish specimen

A natural hybrid specimen (81.2 mm SL) was collected at the Jucheongang (stream) of the Namhangang (river), Anheung-myeon, Hoengseong-gun, Gangwon-do, Korea on April, 25, 2014. Specimens of its supposed parental species, *R. pseudosericeus* (n=5), and *A. signifer* (n=5) were captured at the same area where the natural hybrid was found. For the comparison of body coloration of *R. pseudosericeus*, *A. signifer*, and the natural hybrid, we took pictures in the field shortly after capturing them. The collected specimen was fixed in 10% formalin solution and deposited at the Department of Biological Science, Chonbuk National University, Jeonju (CNUC).

2. Morphological characters

To examine the morphological characters of the collected specimens, counting and measuring were done by the method of Hubbs *et al.* (2004), and fin rays and vertebral counts were observed by a soft-X ray photograph (Nikon SMZ-10, Japan). A total of 18 morphological characters were chosen to calculate hybrid index (Hindex) using the following formula (Nikoljukin, 1972).

$$\text{Hybrid index (Hindex)} = (\text{H} - \text{M1}) / (\text{M2} - \text{M1})$$

where H is the numerical value of a character of the natural hybrid, while M1 and M2 are numerical values of the same character in *R. pseudosericeus* and *A. signifer*, respectively. Approach 50 of Hindex indicates intermediate characters between two parental species. If Hindex was lower than 30, the natural hybrid was considered to resemble *R. pseudosericeus* while Hindex was higher than 70, the natural hybrid was considered to resemble *A. signifer*. In addition if Hindex was between 30 and 70, the natural hybrid was considered to be intermediate (Šorić, 2004).

3. Analysis of mitochondrial cytochrome *b* (*mt-cyb*) gene

Mitochondrial DNA is haploid, located in the cytoplasm, and is maternally inherited without recombination (Lee *et al.*, 2009). To confirm the maternal hybrid origin *mt-cyb* genes were investigated. For DNA analysis a piece of pectoral fin was dissected from each specimen and stored in 100% Ethyl alcohol. Total DNA was purified with the genomic DNA Prep Kit for blood and tissue (QUIAGEN Co., USA). A segment of 1,141 bp containing a complete *mt-cyb* gene was amplified by the poly-

merase chain reaction. A pair of primers (forward primer: TGA CTT GAA GAA CCA CCG TTG, reverse primer: GGA TTA CAA GAC CGA TGC TTT) was used.

PCR consisted of 1 cycle of 5 min. denaturing step at 95°C and 30 cycles of 30 sec. At 94°C, 30 sec. at 56°C, and 1 min. at 72°C. Finally 1 cycle of 7 min. extension step at 72°C. Products were purified by using the Gel extraction kit according to the manufacturer's instructions (Real biotech corporation, Taipei) and sequenced using ABI3730XL automatic sequencer (Applied Biosystems, USA).

To evaluate the phylogeny of *mt-cyb* gene of the natural hybrid and its supposed parental species (*R. pseudosericeus* and *A. signifer*), 8 Acheilognathinae and 2 out group

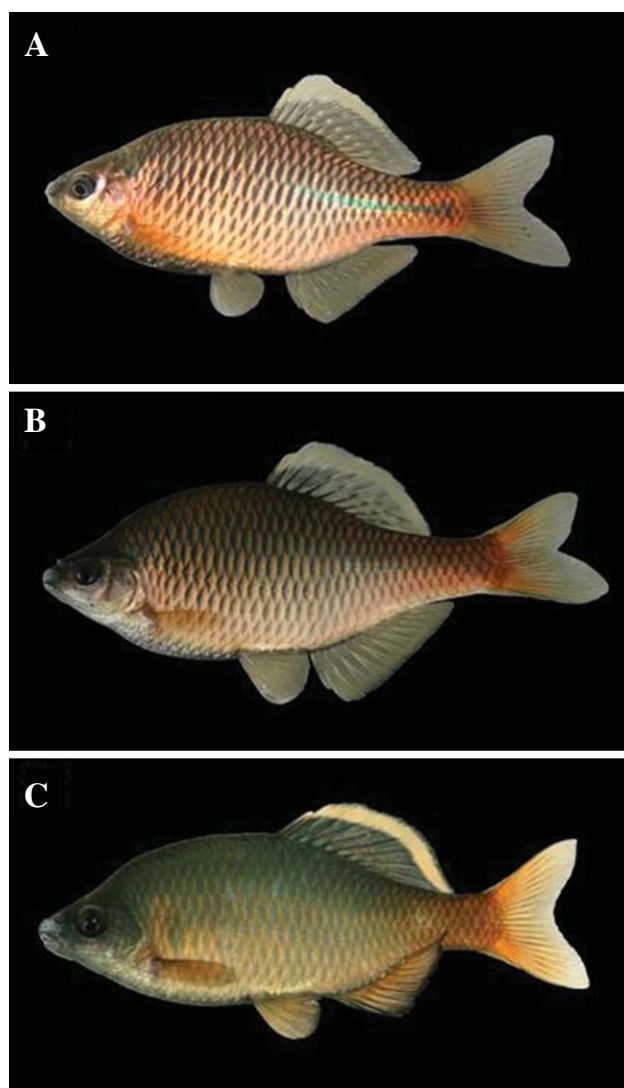


Fig. 1. Photographs of *Rhodeus pseudosericeus* (A: 73.0 mm SL), a natural hybrid (B: 81.2 mm SL) and *Acheilognathus signifer* (C: 79.3 mm SL) at the Jucheongang (stream) of the Namhangang (river), Anheung-myeon, Hoengseong-gun, Gangwon-do, Korea on April, 25, 2014.

sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Sequences were aligned using Clustal Omega. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 6. Phylogenetic tree was established by Maximum Likelihood method and bootstrap phylogeny test with 1,000 replications (Tamura and Nei, 1993).

RESULTS

1. Body coloration and morphological characters

At the present area, a total of three bitterling species amounting to 427 specimens was found: 267 individuals of *R. pseudosericeus* (62.5%), 160 individuals of *A. signifer* (37.4%), and only 1 individual of the natural hybrid (0.2%). For the natural hybrid, the nuptial coloration was intermediate between those of the parental species and the body coloration was totally bluish green but not shown more deep color than those of *A. signifer*. Whereas, the dark spots of the body scales were similar to those of *R. pseudosericeus*. The central body has a stripe thinner than that of *R. pseudosericeus*. The outer membrane occupying about 1/3 ~ 1/2 of the dorsal fin has a horizontal yellow band like *A. signifer* and there were two spotted white lines below the yellow band as those of *R. pseudosericeus* (Fig. 1).

The results of data for 12 morphometric and 6 meristic characters with hybrid index (Hindex) are shown in Table 1. The natural hybrid was intermediated for only 1 character (Hindex=44), the number of lateral line scales (pored scales). Of the morphometric and meristic characters, the hybrid was closer to *R. pseudosericeus* in 3 the characters: the pre-ventral length, the inter-orbital width, and the number of the dorsal fin ray (Hindex=83 ~ 100), but to *A. signifer* in 2 characters including pre-anal length and the number of anal fin rays (Hindex=0 ~ 26). However, some hybrid characters were outside of range.

R. pseudosericeus have an incomplete lateral line scales (2 ~ 4 pored lateral line scales) without barbels while *A. signifer* had a complete lateral line scales (33 ~ 36 pored lateral line scales) with a pair of barbels. Whereas, the natural hybrid possesses an incomplete lateral line scales having 17 pored scales, which show an intermediate character between the two parental species. In addition, they lack barbels.

2. Analysis of mitochondrial cytochrome *b* (*mt-cyb*) gene

The total length of Acheilognathinae fish including the natural hybrid between *R. pseudosericeus* and *A. signifer* *mt-cyb* gene was 1,141 bp positions. Phylogenetic analysis using ML tree showed that *R. pseudosericeus* and *A. signifer* constructed their own lineages supported by high

Table 1. Comparison of 12 morphometric and 6 meristic characters of the natural hybrid with hybrid index (Hindex) and its supposed parental species between *Rhodeus pseudosericeus* and *Acheilognathus signifer* at the Jucheongang (stream) of the Namhangang (river), Anheung-myeon, Hoengseong-gun, Gangwon-do, Korea on April, 25, 2014

Characters	<i>R. pseudosericeus</i> (n=5)		Hybrid (n=1)	<i>A. signifer</i> (n=5)		Hindex
	CNUC 38984-38988		CNUC 38983	CNUC 38973-38977		
	Range	Mean ± SD		Mean ± SD	Range	
Standard length (mm)	48.3 ~ 73.0		81.2	62.2 ~ 79.3		
Proportions of standard length						
Body depth	39.1 ~ 43.1	41.3 ± 1.8	39.8	40.1 ± 2.4	37.9 ~ 43.5	131
Head length	21.6 ~ 24.0	23.0 ± 0.9	22.1	23.5 ± 0.9	22.3 ~ 24.2	-195
Pre-dorsal length	48.8 ~ 51.3	50.3 ± 0.9	54.0	53.0 ± 1.5	51.3 ~ 54.5	134
Pre-ventral length	41.2 ~ 44.9	42.6 ± 1.6	45.0	45.1 ± 0.7	44.1 ~ 45.7	97
Pre-anal length	58.6 ~ 61.8	60.3 ± 1.4	61.1	63.2 ± 1.7	60.9 ~ 64.7	26
Caudal peduncle length	17.6 ~ 20.8	19.3 ± 1.3	21.9	18.7 ± 1.3	17.4 ~ 20.7	-421
Caudal peduncle depth	13.2 ~ 14.9	13.8 ± 0.7	13.2	13.4 ± 0.9	12.4 ~ 14.3	138
Proportions of head length						
Caudal peduncle length	73.1 ~ 96.4	84.3 ± 8.4	99.2	79.7 ± 5.0	73.9 ~ 85.6	-329
Caudal peduncle depth	55.3 ~ 64.2	59.9 ± 3.9	60.0	57.0 ± 3.8	53.1 ~ 62.7	-6
Snout length	25.2 ~ 27.3	26.5 ± 0.9	30.7	28.5 ± 1.0	27.4 ~ 30.2	211
Eye diameter	31.2 ~ 37.6	33.3 ± 2.6	27.8	32.0 ± 1.1	30.4 ~ 33.4	416
Inter-orbital width	40.7 ~ 50.6	45.2 ± 3.6	41.1	40.3 ± 2.6	37.3 ~ 44.3	83
No. of dorsal fin rays	9		8	8		100
No. of anal fin rays	10		10	8 ~ 9		0
No. of lateral scales (pored scales)	30 ~ 33 (2 ~ 4)		35 (17)	(32 ~ 36)		44
No. of lateral line scales				32 ~ 36		
No. of vertebrae	33 ~ 34		36	33 ~ 36		200
Barbels	Absent		Absent	Present		

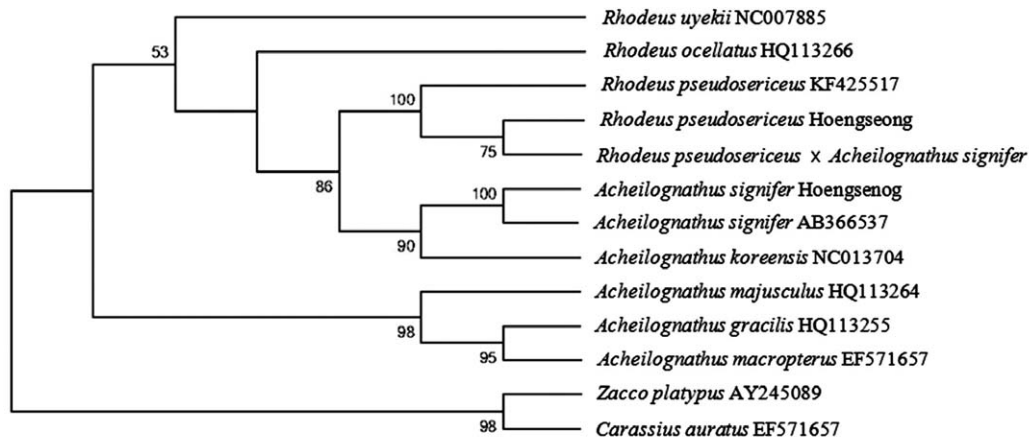


Fig. 2. Molecular phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993).

bootstrap value. Comparison of genetic distance was coincident with the result of the phylogenetic analysis. Nucleotide sequence difference of *mt-cyb* gene was very low at *A. signifer* clades (0.1% ~ 0.2%) and identical at *R. pseudosericeus* clades by Maximum Composite Likelihood genetic distance calculate model. On the other hand, the natural hybrid was identical with *R. pseudosericeus* lineage in *mt-cyb* gene sequence by ML tree and phylogenetic analysis.

DISCUSSION

By the morphological characters, the natural hybrid was identified an intergeneric hybrid between *Rhodeus pseudosericeus* and *Acheilognathus signifer*. And another genetic method such as *mt-cyb* gene indicated that the maternal species of the natural hybrid appeared to be *R. pseudosericeus* due to its 100% sequence identity.

Main factors in the natural hybrid have been usually happened because of its similar spawning season, insufficient spawning site, and sneaker behavior (Jansson *et al.*, 1991). *R. pseudosericeus* and *A. signifer* are sympatric and have almost similar spawning periods from April to July (Personal observation). Not only two bitterlings did not seem to be different habitat preferences but also used to place their eggs in one mussel species, only *Unio douglasiae sinuolatus* living in the collecting site. In addition, although the males defended its territory around the mussels, it was said that sneaking is a common phenomenon under natural conditions (Back *et al.*, 2004; Smith *et al.*, 2004). Furthermore, there was a report that *A. signifer* engage in an intergeneric hybridization with *R. uyekii* (Kim *et al.*, 2010). Therefore, the coexistence of the two parental species may seem to increase the possibility of hybridization.

The existence of a complete lateral line and barbels in the bitterlings, known as key intergeneric characters for Acheilognathinae fish (Kim, 1997), plays an important role in identifying them in nature. The intermediate traits of the body color patterns and two morphological characters such as an incomplete lateral line scales and lacking barbels strongly supported that the collected specimen was a natural intergeneric hybrid. The results of the present study were quite similar to those found in the earlier studies in *R. uyekii* and *A. signifer* (Kim *et al.*, 2010).

Yun (2009) had shown that *A. signifer* had a lower level of inter-population divergence possibility and there was no difference, based on the result of phylogenetic analysis in mitochondrial *COI* sequences and nuclear *RAG1* from the Imjingang (river), the Bukhangang (river), and the Namhangang (river). Also in the present study, *A. signifer* formed a single lineage including GenBank dataset in *mt-cyb* gene. By Lee *et al.* (2009), the natural hybrid between *Cobitis tetralineata* and *Iksookimia longicorpa* was identical with *C. tetralineata* in mitochondrial *COI* analysis and then the natural hybrid was considered with a paternal side of *I. longicorpa* and a maternal of *C. tetralineata*. From the present study, as the natural hybrid between *R. pseudosericeus* and *A. signifer* was identical with *R. pseudosericeus* lineage, it was possible that the paternal side is *A. signifer* and maternal side was *R. pseudosericeus*.

Hybridization was frequently occurred by the anthropogenic disturbances of natural spawning grounds, as well as by the habitat fragmentations including a man-made construction of reservoirs, hydroelectric power plants, and an introduction of non-native species, and other installations causing drastic changes of aquatic eco-systems (Hubbs, 1955; Rhymer and Simberloff, 1996; Scribner *et al.*, 2001; Šorić, 2004). Our present study, however, still remains limited to make the hybrid origin clear from

just only one hybrid specimen collected. Thus, it will need further studies through breeding experiments in the aquarium and seeking of various forms in nature.

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한강납줄개 *Rhodeus pseudosericeus*와 묵납자루 *Acheilognathus signifer* (Pisces: Cyprinidae)의 속간 자연 잡종 출현

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간추림 : 한강납줄개 *Rhodeus pseudosericeus*와 묵납자루 *Acheilognathus signifer*의 속간 잡종으로 추정되는 개체를 한강 수계의 주천강에서 채집하였다. 잡종의 기원을 확인하기 위하여 형태적 특징과 mitochondrial cytochrome *b* (*mt-cyb*) gene을 조사하였다. 형태 형질 분석에서, 조사된 표본의 등지느러미의 반문, 체색, 체측 반점과 종대 무늬 등은 두 부모종의 중간적 형질을 보였다. 또한, 잡종 개체의 측선은 불완전하였고 측선유공린수는 부모종의 중간값을 보였다. *mt-cyb* gene 분석 결과 잡종 개체는 한강납줄개와 염기서열이 100% 일치하여 한강납줄개가 모계임이 밝혀졌다. 따라서 두 자료의 분석 결과 본 연구에서 조사한 개체는 한강납줄개와 묵납자루의 속간 자연 잡종 개체임을 확인하였다.

찾아보기 낱말 : *Rhodeus pseudosericeus*, *Acheilognathus signifer*, 자연 잡종, 주천강