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Genetic Differentiation of *Carassius auratus* and *C. cuvieri* by the Cytochrome C Oxidase I Gene Analysis

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Abstract

It is estimated that 27 bony fish species have been introduced into Korean freshwaters for aquaculture and the enhancement of fishery resources. Among them, Carassius cuvieri has completely adapted and is widely distributed throughout Korean freshwater ecosystems. In this study we investigated the genetic relationship between *C. cuvieri* and the closely related native crucian carp species Carassius auratus. Nucleotide sequence variation within the mtDNA cytochrome c oxidase I (COI) gene was used to study the genetic divergence and phylogenetic relationships among the species and to identify their taxonomic status in the family Cyprinidae. We compared partial COI gene sequences (630 base pairs) from three populations of *C. auratus* and three populations of *C. cuvieri*. A total of 46 variable sites were identified from 163 individuals, yielding 23 haplotypes. The two species showed very close genetic relationship with 95% sequence identity in the COI gene, the frequency and distribution of haplotypes showed clear separation, suggesting divergent evolution of these two species. Therefore, despite the morphological similarity and phylogenetic closeness, the populations of these two species should be regarded as separate management units in the ongoing recovery program.

Keywords: Carp; CO1; Genetic differentiation; Hybrid; Triploid

Introduction

Although the introduction of exotic species to aquatic ecosystems can result in many problems, including habitat alternation, the introduction of diseases and parasites, and hybridization with native species, 27 bony fish species have been introduced into Korean freshwaters for aquaculture and the enhancement of fishery resources. Among them, the Japanese crucian carp *Carassius cuvieri*, also known as the white crucian carp, was introduced from Japan in 1972. *C. cuvieri* has since completely adapted and is now widely distributed throughout the domestic freshwater ecosystems of Korea [1].

The classification of species in the genus Carassius is highly controversial due to the morphological similarity of the different species; as a result, crucian carp are classified into either two or five species [2,3]. The species and their distributions in the five species system are as follows: Carassius throughout most of Europe and western Asia; *Carassius langsdorfii* and *C. cuvieri* in Japan; *Carassius auratus* in China; and *Carassius gibelio* in Europe, Siberia, and East Asia [2,4-6]. *C. carassius* is the only species that can be identified easily by its morphological characteristics [7]. The two-species system includes *C. carassius*, and many subspecies of *C. auratus* in different habitats and in geographical isolation: *C. auratus auratus* in China; *C. auratus gibelio* in Europe and China; and another four subspecies, *C. auratus cuvieri*, *C. auratus grandoculis*, *C. auratus buergeri*, and *C. auratus langsdorfii* in Japan [8,9]. In addition, many geographic populations have been reported in China [10-12].

In addition to the morphological similarity and the presence of subspecies, the identification of Carassius species is further complicated by the occurrence of specimens with different ploidy levels. It was long believed that *C. gibelio* and *C. langsdorfii* were triploid, in contrast to the other species, which were believed to be diploid, and ploidy level was used as an important characteristic in species identification [13]. However, diploidy has been observed within *C. gibelio* and *C. langsdorfii*, and triploidy has recently been observed in *C. auratus* [3,14,15]. Also, the existence of hybrids between various Carassius species, such as hybrids between *C. carassius* and *C. auratus*, further complicates species identification [16].

In order to overcome the limitations in the morphological identification of Carassius species, molecular genetic markers, such as random amplified polymorphic DNA *[17]*, microsatellite DNA [18,19], and partial sequences of mitochondrial DNA [20-22] have been used for the identification of species, ploidy, clonal lineages, and phylogenetic relationships. Because of its small size, maternal inheritance, and rapid rate of evolution, mtDNA is often used as a genetic marker in phylogenetic and evolutionary studies in vertebrates, including fish [23]. Within mtDNA, the control region (D-loop), cytochrome b gene, and cytochrome c oxidase subunit I (COI) gene are most frequently used in animal molecular taxonomy and bioidentification studies [24].

Japanese crucian carps into two species, *C. cuvieri* and *C. auratus* were classified [2], and others have confirmed this classification using molecular markers [19,20,21]. Similarly, *C. auratus*, the species endemic to Korea, and C. cuvieri, the exotic species, are two distinct species that live in Korean freshwaters. However, there has been no study of the genetic diversity and phylogenetic relationships among populations of the latter two species. Therefore, we investigated the patterns of sequence variation in the mtDNA COI gene region in the crucian carp population to describe the genetic diversity and genetic structure of the *C. cuvieri* and *C. auratus* populations.

Materials and Methods

Sampling and DNA extraction

C. auratus and *C. cuvieri* samples were collected from the

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Yeongsan, Geum, and Nakdong rivers. Fishes were collected using nets and identified by morphology by a specialist. Among the fishes collected from the Yeongsan, Geum, and Nakdong rivers, the *C. auratus* individuals numbered 45, 20, and 29, and the *C. cuvieri* individuals numbered 28, 16, and 23, respectively. Fin tissues were preserved in 100% ethanol at the sampling site, and then transported to the laboratory for DNA extraction. Tissue (20 mg) was lysed in lysis buffer (MFX-2000, Toyobo, Osaka, Japan) containing 20 mg/ml of proteinase K, and then total DNA was extracted using a Mag Extractor MFX-6100 automated DNA extraction system (Toyobo).

mtDNA COI gene sequence amplification and analysis

The HCO2198 and LCO1490 primers were used for the amplification of a partial sequence of the COI gene [25]. PCR amplification was performed in 30-µl reaction mixtures containing 3 µl template DNA, 1 µl (10 pM/µ1) each of forward and reverse primers, 3 µl of 10× Taq PCR buffer, 2.4 µl of dNTP mixture (2.5 mm each), and 0.6 µl of Taq DNA polymerase (5 U/µl) using a thermo cycler (PTC-220, Bio-Rad, USA). The PCR conditions were as follows: 10 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 51°C, and 1 min at 72°C; and a final extension of 5 min at 72°C. After confirmation of the PCR products on an agarose gel, DNA fragments of approximately 630 base pairs were extracted from the gel using the QIA quick PCR Purification Kit (Qiagen) and sequenced with a 3130XL Genetic Analyzer (Applied Bio systems, Foster City, CA, USA) using the Prism Big Dye Terminator Cycle Sequencing Kit.

Data analysis

DNA sequences were determined using DNA Sequencing Analysis Software (Applied Bio systems) and assembled using SeqMan software (DNASTAR Laser gene 8 package). Haplotype diversity and nucleotide diversity were calculated using Dna SP ver. 4.0 [26]. Population structures were investigated using analysis of molecular variance (AMOVA), and a possible geographical pattern in the distribution of genetic variability was analyzed through Fst estimates using Arlequin 3.0 [27]. Genetic distance matrices were used to construct an unweighted pair group method with arithmetic mean (UPGMA) genetic population relation tree with the POPULATION 1.2.30

Results

Sequence analysis and variation among haplotypes

PCR products of approximately 630 base pairs were obtained by amplification with the primers HCO2198 and LCO1490. The sequence, with the variable sites in bold, is shown in Figure 1. In the 46 polymorphic sites, transitions were observed at 40 sites, whereas transversions were detected at 6 sites. Based on the 46 variable sites, 23 haplotypes were identified among the 163 individuals (Table 1). Haplotype 1 (Hap_1; GenBank number HQ960853) and haplotype 7 (Hap_7; GenBank number HQ536319.1) were the most frequently observed sequences among *C. auratus* (47.3%) and *C. cuvieri* (62.8%), respectively. In contrast, the frequencies of these two haplotypes in opposite species were very low with the haplotype I and haplotype 7 frequency of 4.5% and 4.4% in *C. cuvieri* and *C. auratus*, respectively. The Hap_1 sequences of *C. auratus* and the Hap_7 sequences of *C. cuvieri* share 95% sequence identity. There was a clear difference in the haplotype frequencies for each species. Haplotypes 1, 3, 5, 9, and 16 were dominant in *C. auratus*, each with frequencies above 10%. In contrast, in *C. cuvieri*, haplotypes 7, 11, and 13 had frequencies above 10%. Therefore, there was no overlap of frequent haplotypes between the two species. However, haplotype 3 and 5 which were the main haplotype in *C. auratus* were present as minor haplotype in *C. cuvieri* from Yeongsan River. Similarly, haplotype 7, the other main haplotype in *C. cuvieri*, was observed as a minor haplotype in *C. auratus* from Yeongsan River. Therefore, further investigation on the gene flow between Carassius species in Yeongsan River is necessary.

Population genetic structure and relationships

The genetic relationships among *C. cuvieri* and *C. auratus* populations from the Nakdong, Yeongsan, and Geum rivers were analyzed. As shown in Figure 1, the two species formed clearly separated clusters, each comprising three populations from different locations. Among the *C. auratus* populations, the populations from the Yeongsan and Geum rivers clustered together. Similarly, the *C. cuvieri* populations from the Nakdong and Geum rivers formed a subcluster. Genetic differentiation among populations was assessed using FST pairwise comparisons. The three populations of *C. cuvieri* from the three rivers showed no genetic difference in the COI gene. In contrast, the three populations of *C. auratus* showed significant genetic differences in this gene. Furthermore, the genetic differentiation between the two species was also significant, with high FST values ranging from 0.3347 to 0.7588. AMOVA results showed partitioning of the populations with significant variance (P<0.001). However, only 0.61% of the variation was attributed to the geographical separation of the populations; in contrast, 22.11% and 77.28% of the variations were

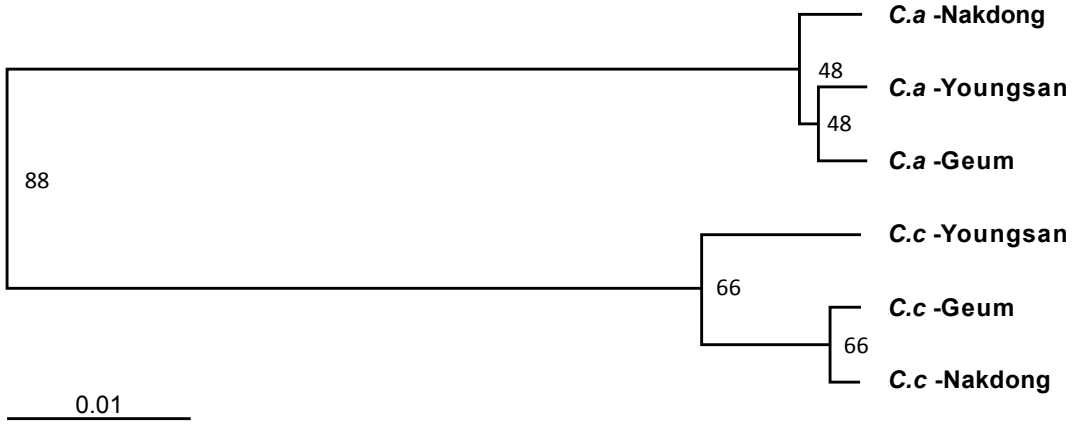

Figure 1: UPGMA tree of Carassiusauratus and *C. cuvieri* from six populations from Korea based on the mtDNA CO1 gene.

Species Carassiusauratus				**Carassiuscutieri**		
Population	**C.a-Youngsan C.a-Nakdong Ca- Geum**			**C.c- Youngsan Cc- Nakdong C.c- Geum**		
	Carassiusauratus			**Carassiuscccuucutiericutieri**		
Haplotwe	Young=	Gem	Nakdong	Young=	Gem	Nakdong
Hap_I	0.444	0.7	0.276	0.071	0.063	
Hap_2	0.022					
Hap_3	0.267	0.15	0.276	0.036		
Hap_4	0.022					
Hap_5	0.156			0.036		
Hap_6	0.022	0.05	0.034			
Hap_7	0.044			0.5	0.688	0.696
Hap_8	0.022					
Hap9		0.05	0.103			
Hap_10		0.05				
Hap_11				0.286		0.217
Hap_12				0.071		
Hap_13					0.125	
Hap_14					0.063	
H_15					0.063	
Hap_16			0.103			
Hap_17			0.034			
Hap_18			0.069			
Hap_19			0.034			
Hap_20			0.034			
Hap_21			0.034			
Hap_22						0.043
Hap_23						0.043
No. of individuals	45	20	29	28	16	23
Nucleotide diversity,(%)	0.0154	0.0024.002	0.0044.002	0.0234.014	0.0234.014	0.020 ± 0.011
No. of Haplotype	8	5	10	6	5	4

Table 1: Summary of the distribution of haplotypes, nucleotide diversity, and number of polymorphic sites among populations of Carassiusauratus and *C. cuvieri.*

Species Population	**Carassi us OR rants**			**Carassiuscusieri**		
	C.a- Youngsan C.a -Nakdong		**C. a-Geuo2**	**C.c - Youngsan Cc-NakdongC.c - Geum**		
Ca-Youngsan	0	0.0177	0.0131	0.1945	0.4744	0.523
Ca-Nalcdong	0.0961*	0	0.0135	0.2394	0.5522	0.6356
Ca-Geum	0.0664*	0.2235*	0	0.2364	0.5442	0.6231
Cc-Youngsan	0.3347*	04710*	0.4427*	0	0.047	0.0731
C.c-Nakdorg	0.5305*	0.7246*	0.7074*	0.066	0	0.0106
C.c-Geum	0.5946*	03588*	0.7421*	0.0941	-0.0243	0

* Significant Fst value (P<0.05)

Table 2: Pairwise genetic distance (above diagonal) and Fst values (below diagonal) of Carassiusauratus and *C. cuvieri.*

attributed to variations between *C. auratus* and *C. cuvieri* (Table 2) and within the populations of each river, respectively.

Discussion

Among the diverse species and subspecies that belong to the genus Carassius, the endemic species C. auratus and the exotic species *C. cuvieri* are present in the freshwater ecosystems of Korea. In this study, the phylogenetic relationships of populations of the two species from three major rivers were analyzed.

Among the 46 polymorphic sites identified from 163 individuals, transitions (40 sites) were more frequent than transversions (6 sites), which is similar to the results of other Carassius mtDNA gene studies [20,28]. Alignment of the 46 polymorphic sites produced a total of 23 haplotypes. In the analysis of the first third of the mtDNA control region (CR; 323 base pairs), 35 haplotypes from 528 individuals were identified [3]. In contrast, only 14 haplotypes in the same region from 47 individuals were identified [29]. In the analysis of the COI gene of 160 individuals of the Carassius genera, 22 haplotypes were identified [28]. Therefore, the number of haplotypes may be related to the number of samples rather than the sequence length of the selected region.

Despite controversy over the classification of Carassius spp., there have been several reports of the genetic separation of *C. cuvieri* and *C. auratus* [20,21,30,31]. In the analysis of the mtDNA cytochrome b (cyt b) and CR, it was demonstrated that *C. cuvieri* and *C. alangsdorfii* diverged later than the continental C. auratus forms (4.0–4.5 million years ago by molecular calibration) [30]. As expected, *C. auratus* and *C. cuvieri* in Korean freshwater systems also showed clear separation based on phylogenetic analysis of the COI gene (Figure 1).

Therefore, our results confirm previous findings [29,32], and support the current taxonomic treatment of *C. cuvieri* as a distinct species [2,33,34]. Although there have been many reports showing the genetic separation between *C. auratus* and *C. cuvieri*, there is no information on the genetic diversity among *C. cuvieri* populations in Japan, from which this species was introduced into Korea. In our analysis, the three populations of *C. cuvieri* from three rivers showed no genetic difference in the COI gene. Considering the geographical separation of the three river systems and the short time since the introduction of *C. cuvieri*, it seems likely that populations from the same origin in Japan were introduced into these rivers. Further study of *C. cuvieri* from Japan could better illustrate the genetic relationships among the *C. cuvieri* populations in Korea and Japan.

In contrast to the *C. cuvieri* populations, the three populations of *C. auratus* showed significant genetic distances. In phylogenetic analyses of a concatenated 4,669-base pair sequence containing the first third of the mtDNA CR, NADH dehydrogenase subunits 4 (ND4) and 5 (ND5), and cyt b gene sequences of the *C. auratus* complex, two old superlineages with high regional specificity, including one population on the Japanese main islands and the other consisting of populations distributed in various regions in and around the Eurasian continent were identified [3]. The molecular clock and fossil record of *C. cuvieri* suggested that these two superlineages separated between 4.01 and 0.39 Mya. Furthermore, seven clades representing the natural populations of each region were estimated by fossil records to have branched 0.2 Mya, and 1.0–1.9 Mya by molecular clock methods. Unfortunately, no sample from the Korean peninsula was included in their analysis. However, considering the origin of samples in their analysis, it is likely that *C. auratus* has branched and evolved over a long period of time as geographically separated populations in each river ecosystem.

Although *C. auratus* is currently one of the more abundant resources in Korean freshwaters, management is still necessary. For example, there have been several reports of recent dramatic decreases of Carassius fishes in Japan, which require special attention and management for recovery [35-37]. The introduction of related Carassius species, such as the case of the introduction of *C. cuvieri* into Korean freshwaters, can affect the ecology of endemic Carassius species. The possibility of hybridization among Carassius species in

natural habitats [38] adds to the importance of resource management of these species. Our results indicate that each population of *C. auratus* in the different rivers of the Korean Peninsula needs to be managed as a separated genetic unit, whereas all *C. cuvieri* populations can be regarded as one genetic unit. However, further information with more samples will be necessary for the efficient management of these species.

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