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Development of Species-specific Molecular Marker as a Tool for Discrimination between Crucian Carp Gengorobuna (*Carassius cuvieri*) Introduced from Japan and Korean Native One (*C. auratus*)

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국내 자연산 붕어와 일본에서 도입된 떡붕어를 구분하기 위한 종특이적 분자마커 개발.

송교홍, 정종우, 구혜영[1], 김 원*(서울대학교 생명과학부, [1]상지대학교 생명과학과)

외래 도입종은 국내 생태계에 빈번히 심각한 문제점들을 일으켜왔고, 이러한 종 중의 하나가 일본에서 도입된 담수어류인 떡붕어(*Carassius cuvieri*)이다. 국내 담수생태계에서 떡붕어의 해로운 영향들을 평가하기 위해 우선적으로 선행되어야 할 연구는 형태적으로 가장 유사한 종(*Carassius auratus*)으로부터 이들을 구분해야 하는 것이다. 전통적인 형태적 동정은 이들 종의 매우 유사한 표현형으로 인해 신뢰할 수 없는 결과들을 자주 보여주기 때문에 더욱 효과적인 방법이 필요하다. 이를 위해 본 연구에서는 3종류의 프라이머(DDF, DDR 그리고 DDR1)를 이용한 효율적인 one-step PCR 분자마커를 개발·적용하였다. 이 분자마커는 국내 담수 생태계의 어류 군집 모니터링에 중요한 역할을 할 것으로 기대된다.

Key words : introduced species, molecular marker, one-step PCR method, crucian carp

INTRODUCTION

The native ecosystem has often been disturbed by the introduced exotic species. They can impact on the ecosystems through habitat alteration, introduction of diseases or parasites, hybridization with the native species, and trophic alteration (Ross, 1991). Such environmental disturbances may result in devastation of environments and biodiversity of the introduced region. Therefore, influences of introduced species must be assessed promptly and precisely to minimize their harmful effects. Then the first step for this purpose is to discriminate correctly the introduced species from the very similar native one. Recently molecular markers have played an important role in this aspect.

The fresh water crucian carp gengorobuna (*Carassius cuvieri*) was introduced to Korea from Japan in 1970s and intentionally released to rivers and reservoirs as food resources. Surpassing other fish species in fertility and adaptation against environmental change, this exotic species has threatened the survival of indigenous fish species. The fish genus *Carassius* (Pisces: Cyprinidae) occurs throughout Eurasian continent and Japanese Islands. Taxonomic status of species belonging to this genus is still under debates. Meng *et al.* (1995) suggested that there are two species-*C. cuvieri* and *C. auratus*-in this genus. *C. auratus* is further divided into several sub-

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species according to habitat divergence and geographic isolation (Ueda and Ojima, 1978; Chen and Leibenguth, 1995). In the recent revision of Japanese *Carassius*, Hosoya (2000) separated *C. cuvieri* as a different species from other subspecies belonging to *C. auratus*. Though Korean native crucian carp was assumed to be *C. a. langsdorfi* based on the analyses of morphological characters and allozyme data for crucian carp populations of Korea, the taxonomic status of this species still needs further study (Yang, 1985; Nam *et al*., 1989).

The gengorobuna *C. cuvieri* and the Korean native *C. auratus* are morphologically quite similar. In the case of juveniles, the identification is even more difficult. This makes researchers very hard to assess harmful effects of gengorobuna on the native environment. Here we designed and tested molecular markers for correct identification of two crucian carp species-introduced species (*C. cuvieri*) and native one (*C. auratus*) inhabiting Korean freshwater based on the species-specific nucleotide sequences of mitochondrial control regions.

MATERIALS AND METHODS

1. Sampling and DNA sequencing

The crucian carp specimens in this study were collected from Woopo Wet Land (N 35° 33′23″, E 128° 24′17″) and Joan (Hangang River) (N 37° 32′18″, E 127° 18′11″) in Korea. Two individuals of each species were used for the nucleotide sequence determination of mitochondrial control region.

Total DNA were extracted from the dorsal tissue of individual specimen using a DNeasy tissue kit (Qiagen). Mitochondrial control region was amplified by PCR using primers-Cara-CR-F and Cara-CR-R (Table 1) which were designed from the flanking regions of control regions, transfer RNA (Pro) and 12S ribosomal RNA, respectively. PCR mixtures were composed of 1X *Taq* polymerase buffer, 1 mM dNTP mix (asymmetric composition of dNTP was used for amplifying AT-rich region), 2.5 mM $MgCl_2$, 0.5 μM of each primer, 2.5～250 pg of total DNA and 0.04 unit μL^{-1} of *Taq* polymerase (Promega Co.). PCR was performed using a GeneAmp PCR system 9700 (Applied Biosys-tems) with the following cycle conditions: 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, 49°C for 50 s and 72°C for 1 min 30 s and followed by a final elongation at 72°C for 7 min. Amplified products were separated on 1% TBE agarose gels, excised under long -wavelength UV light and extracted using a QIAEX II Gel Extraction Kit (Qiagen). After purification, the PCR products were sequenced from both directions with the Big-Dye Terminator Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The reaction products were analyzed on an ABI Prism Genetic Analyzer 3100 (Applied Biosystems). Bidirectional sequences were aligned and visually checked with the Sequence Navigator v1.0.1 (Perkin-Elmer).

Table 1. Primer sequences used in this study.

Primer	Primer sequence (5′-3′)
Cara-CR-F	TTY-TAA-CTC-WCA-CCC-CTG-RCT-MCC
Cara-CR-R	GTK-GCT-GKC-ACG-AGT-TTT-ACC-GGC
DDF	AGT-ACA-TAT-ATG-TAT-TAT-CAC-C
DDR	GGA-TGA-AAT-TTT-ACT-TGA-T
DDR1	ATT-TTT-AGG-TGA-TCA-CTG

2. Design of PCR primers and performing PCRs for species identification

Sequences of the 876 bp-sized mitochondrial control regions of four individuals of two species were aligned with the CLUSTAL option of Sequence Navigator 1.0.1 (Perkin-Elmer) and optimized manually (Fig. 1). Species-specific reverse primers were designed to discriminate two species by different length of amplified PCR fragment of each species. The primer set composed of DDF-DDR1 and DDF-DDR (Table 1), which amplifies 700 bp- and 150 bp-sized fragments from *C. auratus* and *C. cuvieri*, respectively, was selected from various primer combinations. In order to test the primer set, PCR was performed with morphologically identified 25 adult individuals of *C. auratus* and 10 of *C. cuvieri*. Ten unidentified juveniles (9 of *C. auratus* and 1 of *C. cuvieri*) were also tested with the primer set.

The reaction mixture (25 μL) was composed of 3 μL of the templates, 2 μL of $MgCl_2$ (25 mM), 2.5 μL of PCR buffer (10×), 2.5 μL of dNTP mixture (10 mM), 1.25 μL of primer DDF (10 μM), DDR (10 μM), DDR1 (10 μM), 0.2 μL *Taq* polymerase, and 12.3 μL of DW. The best setting was obtained

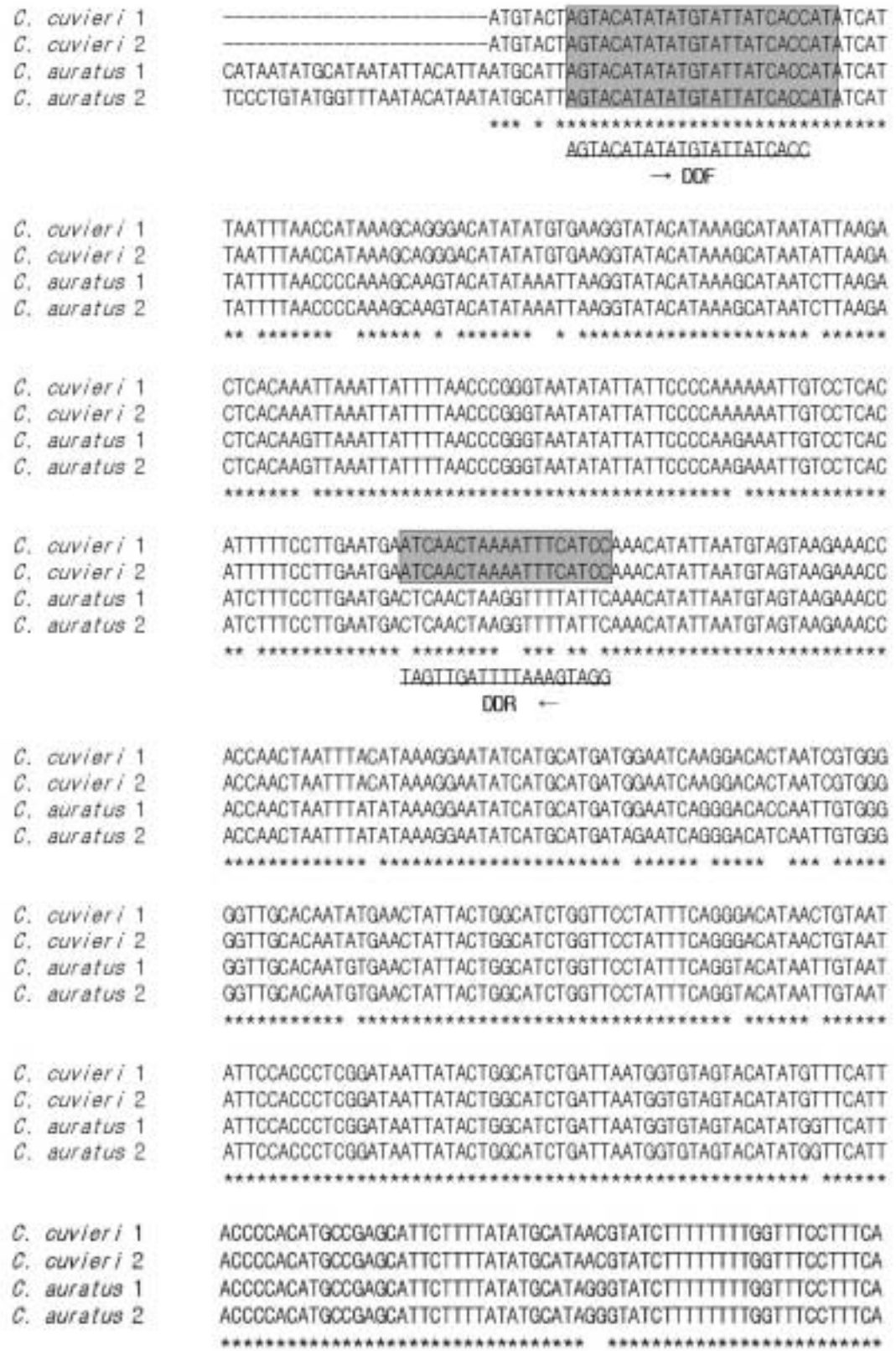

Fig. 1. An alignment set of mitochondrial control region sequences of *Carassius auratus* and *C. cuvieri* from Korea. Locations of PCR primers (DDF, DDR and DDR1) for identification of two species were indicated.

Fig. 1. Continued.

from several different combinations in magnesium concentration and annealing condition. The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 49 °C for 1 min, elongation at 72°C for 1.5 min, and a final extension at 72°C for 2 min.

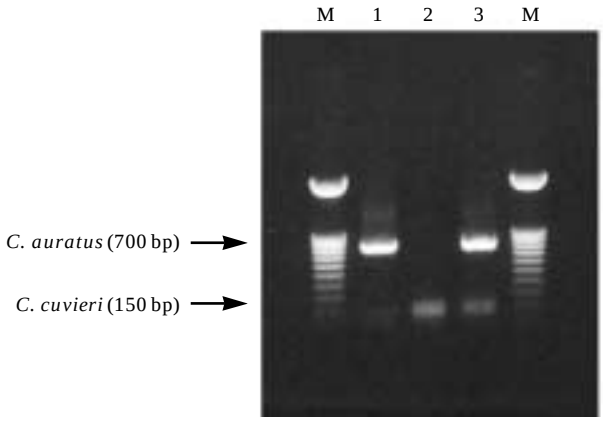

Fig. 2. Gel electrophoresis of PCR products using combined primer set (DDF, DDR and DDR1) for identification of two crucian carp species. M, 100-bp ladder size marker (WelGENE, Daegu, South Korea); lane 1, *C. auratus*, native species of Korea; lane 2, *C. cuvieri*, introduced species; lane 3, *C. auratus*+ *C. cuvieri*; lane 4.

RESULTS AND DISCUSSION

The most suitable primer set resulted in different lengths of PCR products of two species. DDF-DDR1 amplified 700 bp-sized fragments from 25 adult individuals of *C. auratus* and DDF-DDR did 150 bp-sized fragments from 10 adult individuals of *C. cuvieri*. Two species were clearly separated by these molecular markers (Fig. 2). These markers also identified 10 unidentified juveniles as 9 *C. auratus* and 1 *C. cuvieri*. The results proved that two species of *Carassius* inhabiting in Korea can be easily distinguished with just single PCR step method. Without help of molecular markers, it is difficult to identify these species because of considerable morphological interspecies similarities and intra-specific variations according to environmental conditions. Furthermore, two species can hardly be distinguished in their juvenile stages. Compared with most other molecular identification methods composed of two steps including PCR and subsequent nucleotide digestion with restriction enzyme (Lindstrom, 1999), the present one-step PCR method would be simpler and quicker with equal reliability for species identification.

Considerable numbers of fish species have been introduced to Korea. Fifteen species of exotic fishes have been introduced as food resources and about 120 species for aquarium since the 1950s (Jang *et al.*, 2002). Some of these fishes have spread widely in the country and established stable populations in natural environments. However, the distribution and ecology of these introduced fishes has rarely been studied. Correct discrimination of introduced species from the most similar native species may be the starting point of conservation of our environment and native species. The molecular markers produced here by one-step PCR method, developed and tested successfully for identification of two crucian carp species, would be valuable progress for rapid assessment of freshwater fish community structure of Korea.

ABSTRACT

The introduced exotic species has often caused severe problems to the native ecosystem. One of such species is the freshwater fish gengorobuna (*Carassius cuvieri*) introduced from Japan. The first step to assess harmful effects of this species on the Korean freshwater ecosystem is to discriminate it from the most similar native crucian carp (*Carassius auratus*). Because traditional morphological identification often gives unreliable results due to their highly similar phenotype, a new more efficient method is needed. For this purpose, molecular markers produced by the efficient one-step PCR method using three primers (DDF, DDR and DDR1) were developed and tested in the present study. This molecular marker will play an important role in monitoring fish community of Korean freshwater ecosystem.

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