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PRIMER NOTE

Isolation and characterization of polymorphic trinucleotide microsatellites of the polyploid crucian carp (*Carassius auratus*)

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Abstract

In this study, we isolated and characterized nine polymorphic trinucleotide microsatellites (CAG and CCT) from the crucian carp (*Carassius auratus*). The number of alleles per locus ranged from two to six. Five loci showed a significantly excess homozygosity, and a genetic linkage between CAL0102 and CAL0495 was strongly suggested. Our results confirmed the triploidy of Korean individuals, and the microsatellites were found to be useful for analysing the allelic state of the polyploid crucian carp.

Keywords: *Carassius auratus*, cross-specific amplification, Korea, trinucleotide microsatellite, triploid

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In addition to its popularity in the fishery and aquarium industries, the crucian carp (*Carassius auratus*) is well known as the first fish species that was established worldwide via anthropogenic introduction. This species causes several ecological problems in introduced regions; these problems may be enhanced by the fact that *C. auratus* has high pollution tolerance and can easily hybridize with indigenous fish species (Page & Burr 1991). In addition, the genetic background of this species is noteworthy because some subspecies comprise gynogenetic polyploid individuals that may exist as clonal lineages as well as diploid sexual individuals (Fan & Liu 1990). In order to understand the species evolution and pathways by which this species was introduced, it is necessary to determine the hybridization state and identify the clonal lines. From the results of previous studies (Ohara *et al*. 2000, 2003), it is considered that microsatellites may be adequate for this purpose. In this study, we isolated trinucleotide microsatellites and characterized their genetic polymorphism by using Korean triploid individuals. We also tested the cross-specific amplification of these loci.

Total DNA was isolated from the muscle tissue present below the scales of the crucian carp by following the protocol of Sambrook & Russel (2001). A genomic library containing trinucleotide microsatellites (CAG and CCT repeats) was constructed by the enrichment method (Hammond *et al*. 1998). The polymerase chain reaction (PCR) conditions used during the enrichment process were as follows. The PCR mixture (total volume 25 μL) comprised 2 μL of linker-ligated DNA, 6.25 μL of primer SAULA (10 μM), 1.5 μL of dNTP solution (10 mM), 5 μL of $MgCl_2$ (25 mM), 2.5 μL 10× PCR buffer, 0.5 μL of *Taq* DNA polymerase (Promega), and 7.25 μL of distilled water (DW). Amplifications were performed using the GeneAmp 9700 system (ABI) under the following conditions: initial denaturation at 72 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s, elongation at 72 °C for 2 min, and final extension at 72 °C for 7 min. The enrichment process was repeated three times.

Transformed clones from the genomic library were selected and replated onto new agar plates. These clones were then used for colony hybridization assays in order to confirm the presence of microsatellites by using DIG-labelled probes and a DIG detection kit (Roche). Strongly positive clones were selected and incubated in liquid Luria-Bertani (LB) medium, and the plasmids from these clones were recovered using the QIAprep Spin Miniprep kit (QIAGEN). The inserted fragments were sequenced by using the BigDye Terminator Ready Reaction kit (ABI) and M13 primers. The sequences were determined by using the ABI PRISM 3770 Genetic Analyzer system, and they were finally confirmed visually by using SEQUENCE NAVIGATOR 1.1 (PE). The PCR primers that were used to amplify the microsatellite regions were designed from each clone sequence by using the PRIMER 3 program (Rozen & Skaletsky

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Table 1 Trinucleotide microsatellites in triploid crucian carp (*Carassius auratus*)

Locus (GenBank No.)	Repeat motif	Primer (5′–3′)	T_a (°C)†	No. of alleles	Size range (bp)	Observed frequency of the allelic state (expected frequency)‡			*P* value
						D	M	T	
CAL0102 (DQ532361)	$(CAT)_4CAA(CAG)_5$	F: FAM-GCTTCAGCATCACCAGCAACAG R: TAGGTGGCTGGAGATGCGTT	59	6	159–186	0.65 (0.55)	0.13 (0.11)	0.22 (0.34)	0.47
CAL0428 (DQ532362)	$(CAG)_6$	F: FAM-TTTTTGGTCACCTTTAGCAATG R: GTGGATGTTCTGGGCTTTGT	55	2	164–167	0.11 (0.14)	0.89 (0.86)		0.42
CAL04100 (DQ532363)	$(CAG)_5N_{12}(CAG)_3N_{21}(CAG)_5$	F: HEX-TTGGAGGTTTGGATGTTGGT R: ATCTGCTGTTGCATGTGTTG	55	6	193–226	0.44 (0.49)	0.08 (0.12)	0.48 (0.39)	0.61
CAL0410 (DQ532364)	$(TGT)_4TGA(TGC)_4A(G(C/T)T)_{10}$	F: FAM-CGTGTGGTCAGCTACTGAGG R: AGCAAGGCACCTTTGCAG	57	2	232–247	0.88 (0.63)	0.12 (0.37)		< 0.0001
CAL0461 (DQ532365)	$(TGC)_4TGT(TGC)_2$	F: HEX-TAGACACCGATGCCACGTT R: CTGTACTGAAGACCCAGCAG	57	6	86–107	0.69 (0.57)	0.06 (0.15)	0.25 (0.28)	0.05
CAL0174 (DQ532366)	$(GAG)_5N_{12}(CAG)_3N_{21}(CAG)_5$	F: FAM-GCCACAGTGCTTCACATCAC R: TCAATGTGTTGTGCTTTAATGTAAT	55	4	117–126	0.69 (0.65)	0.25 (0.15)	0.06 (0.2)	< 0.05
CAL0192 (DQ532367)	$(GCT)_6$	F: FAM-AGTGAGGAAGGATGGCAC R: CTTCCTCTTCTTCTTCTCACTC	55	5	254–266	0.38 (0.6)	0.56 (0.26)	0.06 (0.14)	< 0.001
CAL0156 (DQ532368)	$(GCT)_6$	F: FAM-CGTAAAAATGGCATGTCGAA R: GCGTGAGTTTGAGGGATTGT	54	2	144–147	0.25 (0.64)	0.75 (0.36)		< 0.0001
CAL0495 (DQ532369)	$(TGC)_5TGT(TGA)_4$	F: NED-TAGGTGGCTGGAGATGCGTT R: TCCAGCATCTTCAGCAGC	56	5	194–218	0.57 (0.58)	0.26 (0.11)	0.17 (0.31)	< 0.05

†, T_a, annealing temperature; ‡, D (diallelic), M (monoallelic) and T (triallelic) state.

Table 2 Number of linkage disequilibrium states between the loci tested by using randomly selected diploid genotypes from triploid ones with 100 repeats

Locus	CAL 0102	CAL 0428	CAL 04100	CAL 0410	CAL 0461	CAL 0174	CAL 0192	CAL 0156	CAL 0495
CAL0102	—								
CAL0428		—							
CAL04100	1		—						
CAL0410	3		1	—					
CAL0461	5		2		—				
CAL0174	1			3	5	—			
CAL0192	1			4	1	49	—		
CAL0156	24	3	9	2		2	2	—	
CAL0495	97		7	2	3	3	2	11	—

2000). One of the two primers that were used to amplify each locus was labelled with a fluorescent dye such as HEX, NED and FAM.

Fifteen primer pairs were designed from 68 clone sequences. Each microsatellite locus was tested by PCR amplification of sequences from 26 individuals that were collected from 13 locations in Korea. The genomic DNAs of these samples were extracted according to the standard protocol of Sambrook & Russel (2001). The microsatellites were amplified under the following conditions. The reaction mixture (total volume 25 μL) comprised 1.0 μL of template DNA, 0.7 μL of each primer (10 μM), 1.0 μL of dNTP solution (10 mM), 2.0 μL of $MgCl_2$ (25 mM), 2.5 μL of 10× PCR buffer, 0.2 μL of *Taq* DNA polymerase (Promega), and 16.9 μL of DW. The PCR were performed using the GeneAmp 9700 system (Applied Biosystems) and included the following steps: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at a set temperature depending on the locus (Table 1) for 30 s, elongation at 72 °C for 30 s, and final extension at 72 °C for 30 min. The PCR products were determined using the ABI 3730 Genetic Analyser system and analysed using GENESCAN 3.7 and GENOTYPER 3.7 (ABI).

From the results, nine polymorphic loci were observed. The allelic states were expressed as diallelic (AAB), triallelic (ABC) and monoallelic (AAA) genotypes (Table 1). Heterozygosity, i.e. the frequency of diallelic or triallelic genotypes, ranged from 0.11 to 0.94; the number of alleles at each locus ranged from two to six. Triallelic heterozygotes were not observed in CAL0410, CAL0428 and CAL0156 loci. The expected frequencies of the allelic states, which were calculated using MATHEMATICA 4.1 (Wolfram Research), were compared with the observed frequencies, and the significance of their differences was evaluated by the *G*-test (Dytham 2003) using Excel 2003 (Microsoft). From the results, a significantly lesser heterozygosity was observed at five loci, including CAL0410. For linkage disequilibrium (LD) analysis, diploid genotypes were randomly selected from triploid ones using Excel 2003 (Microsoft) with 100 repeats. One hundred data were successively tested by GENEPOP 3.4 (Raymond & Rousset 1995). The pairs in significant LD ($P < 0.05$) were counted (Table 2). A linkage between CAL0102 and CAL0495 was strongly suggested by our results. We examined the cross-specific amplification of each locus in four cyprinid species—diploid *C. auratus*, *Rhodeus notatus*, *Rhodeus notatus ocellatus* and *Squalidus gracilis*. In all species, we were able to amplify the following five loci: CAL0102, CAL04100, CAL0461, CAL0174 and CAL0495.

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