Isolation and Inheritance of Microsatellite Markers in the Common Carp *Cyprinus carpio*

Ratu Siti Aliah,^{*1} Motohiro Takagi,^{*2} Shi Dong,^{*1}
Chin Tick Teoh,^{*1} and Nobuhiko Taniguchi^{*1,†}

^{*1}Department of Aquaculture, Faculty of Agriculture, Kochi University, Monobe,
Nankoku, Kochi 783-8502, Japan

^{*2}Limnological Station, National Fisheries University, Ooaza Ono, Ube,
Yamaguchi 754-1311, Japan

(Received August 3, 1998)

Three microsatellite loci, *Cca-17**, *Cca-21**, and *Cca-30**, were isolated from a partial genome library of common carp *Cyprinus carpio*. Primers for PCR amplification of template DNA were designed based on the unique sequences flanking each motif. The total number of alleles detected from *nishikigoi* and wild type common carp population were five, six and nine at loci *Cca-17**, *Cca-21**, and *Cca-30**, respectively. Inheritance was shown to be Mendelian by analysis of genotype ratio in F1 offspring of six families of *nishikigoi*. Allele *69 at locus *Cca-21** and allele *266 at locus *Cca-30** were detected only in wild type common carp with frequencies of 0.304 and 0.458, respectively, while allele *288 was observed only in the *nishikigoi* with the frequencies ranged from 0.600–0.610.

Key words: Common carp, *Cyprinus carpio*, microsatellite DNA, Mendelian inheritance

Isozyme polymorphism has been used effectively as a genetic marker to characterize the population structure of various fish species. However, highly polymorphic markers are needed to detect the inbreeding in aquacultural stocks, in pedigree analysis and in the study of breeding success in wild populations. At the levels of individual, sub population and population, the investigation of variable number tandem repeat loci (VNTRs) using multi-locus and single locus DNA fingerprinting showed a high level of polymorphism. However, these methods have limited application due to technical difficulties in interpreting the results.¹⁾

More recently microsatellites or simple sequence repeats, which consist of short tandemly arrayed di-, tri- or tetranucleotide sequences, flanked on either side by regions of unique DNA,²⁾ have been shown to be highly polymorphic in fish.³⁾ Primers synthesized complementary to these flanking sequences may amplify alleles at microsatellite loci by the PCR. Size of alleles can be accurately determined by electrophoresis on polyacrylamide gel. Due to the relatively high polymorphism, microsatellite loci may be used as markers in studies of population genetics, pedigree analysis and phylogeny.⁴⁾ To date, microsatellite markers in fish have been effectively used in population study,^{3,5–8)} parentage and kinship analysis.^{9–11)}

In this study we report the isolation of microsatellite loci from the genome of common carp *Cyprinus carpio*. Primers were designed for three loci and their amplification conditions were optimized. After analysing the Mendelian inheritance in *nishikigoi*, the Japanese ornamental (*koi*) carp, the level of polymorphism of the three markers was examined in *nishikigoi* and the wild type of common carp.

Materials and Methods

Fish Samples and DNA Extraction

Six families of *nishikigoi* were produced in our laboratory and used for analysis of the Mendelian inheritance. According to Japanese classification,¹²⁾ they were consisted of 1 family of *kouhaku* (white fish with red patches), 2 families of hybrid *kouhaku* and *showa sanke* (black fish with red and white patches) and a family of either *tancho* (white fish with one red circled patch on the head), *taisho sanke* (white fish with red and black patches) or *showa sanke* type (Table 1). These parent fish and their offspring (20–22 individuals/family), together with the wild type of common carp population collected from Shimanto River, Nakamura City, Kochi Prefecture, were analyzed for microsatellite loci polymorphism and their genetic variability.

DNA samples were extracted from a fin clip of each individual by standard SDS-phenol/chloroform procedures¹³⁾ and stored at 4°C prior to PCR amplification.

Table 1. Description of the six families of *nishikigoi* studied

Family	Parental	
	Female	Male
1	<i>Kouhaku</i>	<i>Kouhaku</i>
2	<i>Tancho</i>	<i>Tancho</i>
3	<i>Taisho sanke</i>	<i>Taisho sanke</i>
4	<i>Showa sanke</i>	<i>Showa sanke*</i>
5	<i>Kouhaku</i>	<i>Showa sanke*</i>
6	<i>Kouhaku</i>	<i>Showa sanke*</i>

* same individual.

† Corresponding author.

Cloning and Sequencing of Microsatellite

For cloning purposes, genomic DNA was extracted from pooled blood sample of five mixed sex common carp and *nishikigoi* individuals belonging to either *matsubaogon* (golden metallic with a reticulated net-like pattern), *kouhaku* or *sanke* type. Positive clones were screened with (GT)₁₅ synthetic oligonucleotide and sequenced, and primers were designed for the unique flanking sequences following the protocols described by Takagi et al.¹⁴

Microsatellite Polymorphism

The reverse primer was 5' end labeled with [γ -³²P]ATP. PCR was carried out using an MJ Thermal cycler. The 5 μ l of reaction contained 15 ng of template DNA, 0.20 μ M each unlabelled primer, 0.02 μ M labeled reverse primer, 10 mM of Tris-HCL (pH 8.3), 1 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 0.20 mM each dNTPs and 0.12 unit of *Taq* polymerase (TaKaRa). The PCR was programmed for 7 cycles consisting of denaturation for 1 min at 94°C, annealing for 30 s at the appropriate temperature (Table 2), and extension for 30 s at 72°C, followed by 30 cycles consisting of denaturation for 30 s at 90°C, annealing for 30 s at annealing temperature, extension for 30 s at 72°C. Following amplification the samples were mixed with an equal volume of denaturing stop dye (Pharmacia), heated for 15 min at 95°C, electrophoresed on 8% polyacrylamide gel (7 M urea, 8% acrylamide), dried and finally exposed to autoradiography film (Fuji RX-U). Microsatellite alleles were sized relative to a M13 sequence ladder.

Mendelian Inheritance of Microsatellite Loci

The microsatellite loci previously identified were scored in parents and offspring. Genotype ratios of the offspring were tested for conformance with Mendelian expectations using χ^2 test. The symbols of locus, allele, phenotypes and genotypes were written based on Shaklee et al.¹⁵ The loci were designated by the number of the colony isolated, while the alleles were designated by molecular size of the amplified fragments relative to a M13 sequence ladder.

Results

Detection of Microsatellite Loci and Their Polymorphism

Among a total of 30 microsatellites isolated from a partial genomic library of common carp, clear amplification and scorable variation were observed in 3 microsatellites—*Cca-17**, *Cca-21**, and *Cca-30**. Two of them were perfect

motifs [(T)₂₅ and (GT)₁₁], and one was a compound motif [(GT)₁₈ + (GA)₂₀]. The primer sequences, annealing temperature, number of alleles, amplified product size range, and heterozygosities for the three loci are shown in Table 2. Based on data obtained from 34 samples, five alleles were observed at the locus *Cca-17**, six alleles at *Cca-21**, and nine alleles at *Cca-30**. The length of alleles ranged from 179 to 199 bp for *Cca-17**, from 69 to 81 bp for *Cca-21** and from 260 to 318 bp for *Cca-30**.

Mendelian Inheritance of Microsatellite Loci

Genotypic frequencies of parents and offspring are shown in Table 3 and Fig. 1. At locus *Cca-17**, all parent fish were homozygote and gave all homozygote offspring. At *Cca-21**, 5 out of 6 families had a heterozygous parent, and the proportion of homozygote and heterozygote offspring was not significantly different from the 1:1 Mendelian ratio. Male and female parents of family 4 were homozygotes at alternative alleles and gave all heterozygous offspring as expected. At the locus *Cca-30**, both parents of family 1 were heterozygous (*288/300 and *288/318) and shared only one alleles, therefore giving an expected ratio of 1:1:1:1 of homo- and heterozygotes, and this result did agree with the expectation. Four families had both parents possessing the same heterozygous genotype and gave genotype not statistically different with a ratio of 1:2:1 in the offspring. Family 4 had a parent possessing heterozygous genotype thus gave an offspring genotype ratio not significantly different from 1:1.

Genetic Variation

At the *Cca-17** locus, only one allele (*185) was observed in parent *nishikigoi*, while a total of 5 alleles including allele *185 were found in the wild type (Table 4). Allele *185 was a common allele among wild type, with a frequency of 0.562. At the locus *Cca-21**, 3 alleles found in *nishikigoi* (*75, *77, and *79) were similar to those found in the wild type, in which a total of 6 alleles were observed. However, the two groups were dominated with different alleles, allele *79 (frequency=0.550) in *nishikigoi* and allele *69 (frequency=0.301) in the wild type. At the locus *Cca-30**, only one allele was shared between the two groups (allele *300) with low gene frequency (0.050 in *nishikigoi* and 0.043 in the wild type). Three alleles (*288, *300, and *318) were found in *nishikigoi*, while 7 alleles (*260, *262, *266, *268, *292, *296, and *300) were observed in the wild type. The mean observed heterozygosity of three loci

Table 2. Nucleotide sequence of common carp microsatellites and primers for amplification of loci by PCR

Locus		Sequence 5' → 3'	Annealing temperature (°C)	No. of alleles	Size (bp)
<i>Cca-17*</i>	F* ¹	(T) ₂₅ CAACACATAACCGCATTTCT	47	5	179–199
	R* ²	CTTTCTGTGATTGACCTCC			
<i>Cca-21*</i>	F	(GT) ₁₁ AAGGTGAGTGTGACGACGG	53	6	69–81
	R	AGCACAACACACGGTCAG			
<i>Cca-30*</i>	F	(GT) ₁₈ + (GA) ₂₀ CTGCCTTCTTCTACTCTACAC	50	9	260–318
	R	TTGCCTCTATGCTTGATTTT			

*¹ Forward primer.

*² Reverse primer.

Table 3. Genotypes of parents and offspring of *nishikigoi* at 3 microsatellite loci

Locus	Family	Parental Genotype		F ₁ offspring genotype				n
		Female	Male	Observed number* ¹ (expected in each class)				
Cca-17*	1	*185/185	*185/185	*185/185 20(20)				20
	2	*185/185	*185/185	*185/185 21(21)				21
	3	*185/185	*185/185	*185/185 21(21)				21
	4	*185/185	*185/185	*185/185 20(20)				20
	5	*185/185	*185/185	*185/185 21(21)				21
	6	*185/185	*185/185	*185/185 22(22)				22
Cca-21*	1	*75/79	*75/75	*75/75 11(10)	*79/75 9(10)			20
	2	*77/79	*79/79	*77/79 9(11)	*79/79 13(11)			22
	3	*79/79	*75/79	*79/75 8(11)	*79/79 14(11)			22
	4	*79/79	*77/77	*79/77 22(22)				22
	5	*75/79	*77/77	*75/77 10(11)	*79/77 12(11)			22
	6	*75/79	*77/77	*75/77 13(11)	*79/77 9(11)			22
Cca-30*	1	*288/300	*288/318	*288/288 6(5.5)	*288/318 5(5.5)	*300/288 5(5.5)	*300/318 6(5.5)	22
	2	*288/318	*288/318	*288/288 3(5.5)	*288/318 14(11)	*318/318 3(5.5)		22
	3	*288/318	*288/318	*288/288 7(5)	*288/318 9(10)	*318/318 4(5)		20
	4	*288/288	*288/318	*288/288 15(11)	*288/318 7(11)			22
	5	*288/318	*288/318	*288/288 8(5.5)	*288/318 7(11)	*318/318 7(5.5)		22
	6	*288/288	*288/318	*288/288 9(10.5)	*288/318 12(10.5)			21

*1 Non significant at $P < 0.05$.

Fig. 1. Microsatellite alleles of *nishikigoi* and its F₁ offspring at locus *Cca-21** (lane 1: female parent, lane 2–11: F₁ offspring, lane 12: male parent). The size standard is a sequencing ladder of M13 mp18.

Table 4. Allele frequency of three microsatellite loci in parent stock of *nishikigoi*, F₁ offspring of *nishikigoi* and the wild type of common carp

Allele	<i>Cca-17*</i>			Allele	<i>Cca-21*</i>			Allele	<i>Cca-30*</i>		
	Parent	F ₁ offspring	Wild type		Parent	F ₁ offspring	Wild type		Parent	F ₁ offspring	Wild type
No. of fish	10	130	24		10	130	24		10	129	24
*179	—	—	0.188	*69	—	—	0.304	*260	—	—	0.250
*181	—	—	0.062	*71	—	—	0.196	*262	—	—	0.062
*185	1.000	1.000	0.562	*75	0.300	0.238	0.218	*266	—	—	0.458
*197	—	—	0.021	*77	0.150	0.288	0.043	*268	—	—	0.021
*199	—	—	0.167	*79	0.550	0.474	0.043	*272	—	—	0.125
				*81	—	—	0.196	*276	—	—	0.042
								*288	0.600	0.610	—
								*300	0.050	0.044	0.042
								*318	0.350	0.346	—

Table 5. Genetic variability of three microsatellite loci of *nishikigoi* and the wild type of common carp

			<i>Cca-17*</i>	<i>Cca-21*</i>	<i>Cca-30*</i>	Mean (within population)
Crossbreeding experiment of <i>nishikigoi</i>	Parent stock	No. of sample	10	10	10	
		No. of allele	1	3	3	2.3
		Heterozygosity (Ho)	0	0.556	0.889	0.482
		(He)	0	0.650	0.572	0.407
	Offspring	(Ho/He)	—	0.855	1.554	1.184
		No. of sample	125	130	127	
		No. of allele	1	3	3	2.3
		Heterozygosity (Ho)	0	0.906	0.472	0.459
		(He)	0	0.707	0.562	0.423
		(Ho/He)	—	1.282	0.840	1.085
Wild type of common carp		No. of sample	24	23	24	
		No. of allele	5	6	7	6.0
		Heterozygosity (Ho)	0.694	1.014	1.064	0.924
		(He)	0.684	0.867	0.782	0.778
		(Ho/He)	1.015	1.170	1.361	1.188

in wild type was 0.924, twice as high as in the *nishikigoi* parent (0.482) and offspring (0.459) (Table 5).

Discussion

Two loci, *Cca-21** and *Cca-30**, have been shown to follow the Mendelian inheritance in 6 families of *nishikigoi*. Two to three alleles were detected in each pair of parental genotype. A microsatellite marker has also been applied for pedigree analysis in rainbow trout⁹⁾ and sea bass.¹¹⁾ The results also showed that the observed frequencies of the offspring genotype did not differ significantly from the Mendelian expectation. Seven microsatellites have been used for pedigree analysis in 1 family of sea bass, and 3 to 4 alleles were detected in each pair of parental genotypes. While in rainbow trout, 3 microsatellites were used for pedigree analysis in 3 full-sib families and showed 2 to 3 alleles were detected in each pair of parental genotype. Therefore, since a microsatellite marker has detected two or more alleles in one pair of parental genotype, it can be used as a marker for pedigree analysis.

Genetic study on qualitative and quantitative traits of the European common carp is well established.¹⁶⁾ Furthermore, biochemical marker studies also have been carried out in Chinese, European, Japanese and Indonesian common carp.¹⁷⁻¹⁹⁾ This study of microsatellite DNA markers

shows that the variability observed in microsatellite loci was higher than that of isozymes in the wild type of common carp and *nishikigoi*. Sumantadinata and Taniguchi¹⁹⁾ has identified 23 gene loci encoding 11 enzymes and protein systems from 167 fish samples (4 lots) of both *nishikigoi* and the wild type of common carp stock and found an average of 1.261 alleles per locus in *nishikigoi* and 1.171 alleles per locus in wild type. Using microsatellite DNA, a larger number of alleles per locus were observed (2.3 in *nishikigoi* and 6.0 in wild type) than through isozymes analysis, even though we used only a small number of fish samples (10 individuals in the parent stock and 24 individuals in the wild type). Based on the isozyme loci, the observed heterozygosities of the wild type of common carp and *nishikigoi* were almost the same, 0.042 and 0.047 respectively,¹⁹⁾ while in this study, the observed heterozygosity of wild type common carp at microsatellite DNA loci was almost twice as high as in *nishikigoi*, 0.924 and 0.482 respectively. Genetic variability was also observed in the natural and hatchery populations of red sea bream using isozymes and microsatellite DNA markers.²⁰⁾ No distinct difference was found in the average heterozygosity between the natural population and hatchery population of red sea bream based on isozyme data, while using microsatellite DNA the apparent reduction of the average heterozygosity of hatchery population was observed.

These results were similar to those found in the wild type population of common carp and *nishikigoi*. However, the observed heterozygosity in the wild type of common carp (0.924) was higher than that of the natural population of red sea bream (0.810)¹⁴ although the smaller number of alleles per locus was detected in common carp compared to those of red sea bream, 6.0 and 22.4 alleles per locus respectively.

This study demonstrated the usefulness of microsatellite DNA marker for pedigree analysis in *nishikigoi* and detection of genetic variability in both the wild type of common carp and *nishikigoi*. Only 2 loci were successfully used for pedigree analysis; thus more polymorphic loci must be isolated.

References

- 1) M. Lynch: Analysis of population genetic structure by DNA fingerprinting, in "DNA Fingerprinting: Approaches and Applications" (ed. by T. Burke, G. Dolt, A. J. Jeffrey, and R. Wolff), Birkhauser Verlag, Basel, Switzerland, 1991, pp. 113-126.
- 2) D. Tautz: Hyperiability of simple sequence as a general source of polymorphic DNA markers. *Nucleic. Acid Res.*, **17**, 6463-6471 (1989).
- 3) A. L. Brooker, D. Cook, P. Bentzen, J. M. Wright, and R. W. Doyle: Organization of microsatellites differs between mammals and cold-water teleost fishes. *Can. J. Fish. Aquat. Sci.*, **51**, 1959-1966 (1994).
- 4) L. K. Park and P. Moran: Developments in molecular genetic techniques in fisheries, in "Molecular Genetics in Fisheries" (ed. by G. R. Carvalho, and T. J. Pitcher), Chapman and Hall, London, 1995, pp. 1-28.
- 5) B. Angers, L. Bernatchez, A. Angers, and L. Desgroseillers: Specific microsatellite loci for brook charr reveal strong population subdivision on a microgeographic scale. *J. Fish Biol.*, **47** (Suppl. A), 177-185 (1995).
- 6) S. McConnell, L. Hamilton, D. Morris, D. Cook, D. Paquet, P. Bentzen, and J. Wright: Isolation of salmonid microsatellite loci and their application to the population genetics of Canadian east coast stocks of Atlantic salmon. *Aquaculture*, **137**, 19-30 (1995).
- 7) S. K. McConnell, P. O'Reilly, L. Hamilton, J. M. Wright, and P. Bentzen: Polymorphic microsatellite loci from Atlantic salmon (*Salmo salar*): genetic differentiation of North American and European populations. *Can. J. Fish. Aquat. Sci.*, **52**, 1863-1872 (1995).
- 8) D. B. Morris, K.R. Richard, and J. M. Wright: Microsatellites from rainbow trout (*Oncorhynchus mykiss*) and their use for genetic study of salmonids. *Can. J. Fish. Aquat. Sci.*, **53**, 120-126 (1996).
- 9) A. Estoup, P. Presa, F. Kreig, D. Vaiman, and R. Guyomard: (CT)n and (GT)n microsatellites: a new class of genetic markers for *Salmo trutta* L. (brown trout). *Heredity*, **71**, 488-496 (1993).
- 10) H. I. Kincaid: An evaluation of inbreeding and effective population size in salmonid broodstocks in federal and state hatcheries. *American Fisheries Society Symposium*, **15**, 193-204 (1995).
- 11) F. J. Garcia de Leon, B. Chatain, M. Canonne, J. J. Versini, and F. Bonhomme: Development and use of microsatellite markers in sea bass, *Dicentrarchus labrax* (Linnaeus, 1758) (Perciformes: Seranidae). *Mol. Mar. Biol. and Biotech.*, **4**, 62-68 (1995).
- 12) M. Tamadachi: The cult of the koi. Second edition, Expanded, T. F. H. Publication, Inc. Neptune City, 1994. 320 p.
- 13) A. S. Harris, S. Bieger, R. W. Doyle, and J. M. Wright: DNA fingerprinting of tilapia, *Oreochromis niloticus*, and its application to aquaculture genetics. *Aquaculture*, **92**, 157-163 (1991).
- 14) M. Takagi, N. Taniguchi, D. Cook, and R. W. Doyle: Isolation and characterization of microsatellite loci from red sea bream *Pagrus major* and detection in closely related species. *Fisheries Sci.*, **63**, 199-204 (1997).
- 15) J. B. Shackle, F. W. Allendorf, D. C. Morizot, and G. S. Whitt: Gene nomenclature for protein-coding loci in fish. *Transactions of the American Fisheries Society*, **119**, 2-15 (1990).
- 16) V. S. Kirpichnikov: Genetic Bases of Fish Selection, Springer-Verlag, Berlin, 1981, 410 p.
- 17) T. Brody, D. Kirsht, G. Parag, G. Wolfarth, G. Hulata, and R. Moav: Biochemical genetic comparison of the Chinese and European races of the common carp. *Anim. Blood Group Biochem. Genet.*, **10**, 141-149 (1979).
- 18) Y. Kosaka, Y. Fujio, and R. Suzuki: Genetic characteristics of races and colonies in *Cyprinus carpio*. *Suisanikusu*, **5**, 22-26 (1980) (in Japanese).
- 19) K. Sumantadinata and N. Taniguchi: Comparison of electrophoretic allele frequencies and genetic variability of common carp stocks from Indonesia and Japan. *Aquaculture*, **88**, 263-271. (1990).
- 20) N. Taniguchi, M. Takagi, and S. Matsumoto: Genetic evaluation of quantitative and qualitative traits of hatchery stocks for aquaculture in red sea bream. *Bull. Natl. Res. Inst. Aquacult., Suppl.* **3**, 35-41 (1997).