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Verification of mitotic gynogenesis in ornamental (koi) carp (*Cyprinus carpio* L.) using microsatellite DNA markers

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Abstract

The Japanese ornamental (koi) carp is a popular decorative fish all over the world. In koi, clones have not yet been obtained, although production of fish with identical colour patterns could be of commercial interest. Mitotic gynogenetic progenies are essential for subsequent production of clones in fish. However, resulting late-shocked progenies may be contaminated with meiotic gynogens from spontaneous suppression of the second meiotic division in eggs. In this study, microsatellite DNA markers were used to confirm mitotic gynogenetic origin of obtained late-shocked progenies. Recombination rate (y) and mapping distance relative to centromere (M-C) of 10 microsatellite loci were determined based on percentage of heterozygotes in meiotic gynogenetic progenies. The range of y varied from 0.01 to 0.96 and the M-C map ranged from 0.5 to 48 cM. The mean value of y over the 10 loci was 0.481. Six loci, which had y 0.47 and higher, were used as markers in two late-shocked gynogenetic progenies. Complete homozygosity was revealed at all six microsatellite loci indicating mitotic gynogenetic origin of analysed progenies.

Keywords: common carp, koi, gynogenesis, DNA markers, microsatellites

Introduction

The Japanese ornamental (koi) carp *Cyprinus carpio* is a popular decorative fish in many countries all over the world. Koi were developed approximately two centuries ago in Japan and are characterized by a wide diversity of colours and colour patterns (Kuroki 1981; Davies 1989).

Clones (genetically identical groups of fish) have been produced in several fish species (review article Komen & Thorgaard 2007) including common carp (Komen, Bongers, Richter, Muiswinkel & Huisman 1991; Ben-Dom, Cherfas, Gomelsky, Avtalion, Moav & Hulata 2001). In koi, clones have not yet been obtained, although production of fish with identical colour patterns could be of commercial interest. Mitotic gynogenetic progenies are essential for subsequent production of clones in fish (Komen & Thorgaard 2007). However, late-shocked, presumably mitotic gynogenetic progenies may be contaminated with meiotic gynogens, which can arise from spontaneous suppression of the second meiotic division in eggs (Gomelsky, Emelyanova & Recoubratsky 1992; Arai 2001; Morishima, Nakayama & Arai 2001). Microsatellite DNA markers can be used to identify the type of gynogenesis. For this purpose, microsatellite loci with high meiotic segregation frequencies should be identified based on the proportion of heterozygotes in meiotic (early-shocked) gynogenetic progenies. Homozygosity at these loci in late-shocked progenies would indicate their mitotic gynogenetic origin.

Previously, Morishima, Nakayama and Arai (2001) and Lahrech, Kishioka, Morishima, Mori, Saito and Arai (2007) have used microsatellites to prove mitotic gynogenesis in the loach *Misgurnus anguillicaudatus* (Cantor) and barfin flounder

Verasper moseri (Jordan & Gilbert) respectively. The current study was designed to use microsatellite DNA markers for the same purpose in koi.

Materials and methods

The study was performed at the Aquaculture Research Center of Kentucky State University, Frankfort, Kentucky, USA. Fish breeders were caught from 0.04 ha earthen ponds and placed for induced spawning into indoor 2.5 m^3 raceways supplied with dechlorinated tap water. A total of 10 fish breeders (five females and five males) were used in crosses; mean weight [± standard error (SE)] of females was 3.6 ± 0.9 kg whereas mean weight of males was 2.4 ± 0.6 kg. To induce ovulation and spermiation fish breeders were injected with carp pituitary extract (CPE) (Sigma Chemical Company, St Louis, Missouri, USA) at 3 mg kg^{-1} of body weight.

Scheme of crosses and techniques for production of different types of progenies

Normal amphimictic, meiotic and mitotic gynogenetic progenies were obtained from five females. The females differed with regard to the types of progenies obtained from them.

For production of amphimictic or gynogenetic progenies, separate batches of approximately 140 000 eggs were artificially inseminated in plastic bowls; 2.5 L of a water-cow milk mixture (with volumetric ratio 8:1) was added into bowls to remove eggs adhesiveness. For gynogenetic progenies, sperm were irradiated using a FisherBiotech UV microprocessor-controlled Crosslinker (FB-UVXL-1000; Fisher Scientific, Chandler, AZ, USA) with a dosage 3000 J m^{-2}. This dosage was determined based on results of preliminary experiment on insemination of koi eggs with common carp sperm irradiated with different doses; dominant alleles controlling melanin synthesis in larvae were used as genetic markers to prove genetic inactivation of paternal chromosomes (see Gomelsky, Cherfas, Ben-Don & Hulata 1996). Before irradiation, milt was diluted with 145 mM saline solution (1 mL of milt per 9 mL of saline solution). For irradiation, 2 mL of diluted milt was placed in a 60 mm glass Petri dish. The uniform irradiation of spermatozoa was achieved by placing Crosslinker on a shaker table to keep the diluted milt in motion during treatment.

Meiotic and mitotic gynogenetic progenies were obtained using heat shocks by suppression of the second meiotic division in eggs and first mitotic division in haploid embryos respectively. Parameters of heat shocks were chosen according to previous studies on induced diploid gynogenesis and polyploidy in common carp (Recoubratsky, Gomelsky, Emelyanova & Pankratyeva 1992; Cherfas, Gomelsky, Peretz, Ben-Dom, Hulata & Moav 1993). The water temperature before application of heat shocks was 20°C. For meiotic gynogenesis, 2-min heat shock (39°C) was initiated 5 or 6 min after insemination; for mitotic gynogenesis, 2-min heat shock (39.5–40°C) was initiated 42–45 min after insemination. Embryos from each progeny were incubated separately in McDonald hatching jars.

Sample collection for microsatellite analysis

Fin clips were collected from all brood fish used for the crosses and individually stored in 95% ethanol. Larvae from gynogenetic and amphimictic progenies were randomly sampled at 2–3 days after hatch and individually stored in 95% ethanol. From amphimictic progeny 40 larvae were collected; whereas 30 and 20 larvae were fixed from meiotic and mitotic gynogenetic progenies respectively.

DNA Extraction

Genomic DNA was extracted from either fin clips (fish parents) or whole larvae (offspring) using the Promega Wizard® Genomic DNA purification Kit (Promega Corporation, Madison, WI) mouse tail extraction protocol. The fin clips were cut into small pieces (~3–5 mm in length) to aid digestion. Following extraction, the total DNA concentration was determined using a GeneQuant™ *pro* RNA/DNA Calculator Spectrophotometer (GE Healthcare-Life Sciences, Piscataway, New Jersey). Following quantification, a working solution was prepared by diluting a portion of the total DNA to 10 ng/μL for use as a template source in Polymerase Chain Reactions (PCR).

Microsatellite Amplification

Variability at 10 microsatellites loci (*MFW4*, *MFW7*, *MFW26*, *Koi29-30*, *Koi105-106*, *Koi115-116*, *Cca02*, *Cca04*, *Cca24* and *Cca-21*) was analysed in the present study. First nine loci listed above were included by Yue, Ho, Orban and Komen (2004) in the list of 21 microsatellite loci recommended for genetic diversity studies of common carp. Primer sequence information was taken from the following sources: for loci *MFW4*, *MFW7* and *MFW26* from Crooijmans, Bierbooms, Komen, Van der Poel and Groenen (1997), for loci *Koi29-30*, *Koi105-106* and *Koi115-116* from David, Rajasekaran, Fang, Hillel and Lavi (2001), for loci *Cca02*, *Cca04* and *Cca24* from Yue *et al.* (2004), and for locus *Cca-21* from Aliah, Takagi, Dong, Teoh and Taniguchi (1999). All primer sets were either forward or reverse labelled with a 6-FAM™ fluorophore (Integrated DNA Technologies, Coralville, IA).

Polymerase chain reactions (PCR) were performed using Techne® TC-215 gradient thermal cycler (Bibby Scientific, UK). Each reaction contained 40 ng of template DNA, 0.25 pmol of forward and reverse primers (Integrated DNA Technologies), 0.25 mM of each dNTP (Promega, Madison, WI), 1.5 mM $MgCl_2$ (Promega), 0.25 U *Taq* DNA polymerase (Promega), 5× PCR reaction buffer (initial concentration) (Promega), and PCR H_2O to a final volume of 10 μL.

The PCR profile was initial denaturation at 94°C for 3 min; then 30–35 cycles at 94°C for 30 s, annealing temperature for 45 s, and 72°C for 1 min; followed by a final extension for 5 min at 68°C.

Scoring of Amplified Products

Amplified products were resolved via capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Fragment sizes were determined using GeneMapper® software version 3.5 (Applied Biosystems) by comparison against a GenScan™ 500 LIZ™ (Applied Biosystems), internal size standard.

Data analysis

Recombination rate (y) was estimated from the frequency of heterozygotes in meiotic gynogenetic progenies for a target locus. The values of y were converted to map distances in centimorgans (cM) based on formula cM = 100 (y/2), assuming complete chiasma interference (Thorgaard, Allendorf & Knudsen 1983). Mitotic gynogens were identified by complete homozygosity at microsatellite loci that demonstrated high recombination rates (y). Chi-square test (Zar 1999) was performed to evaluate significance of difference between observed genotype segregations in amphimictic progeny with Mendelian theoretical ratios. A ratio of 1:1 between two classes of homozygotes for each locus in meiotic gynogenetic progenies was also compared using Chi-square test.

Results

A total of five crosses were produced; descriptions of parents and types of progenies obtained in each cross are given in Table 1. In Cross 1, normal amphimictic (AMPH-1), meiotic (MEI-1) and mitotic (MIT-1) progenies were obtained. In Crosses 2–4, meiotic progenies (MEI-2, MEI-3, MEI-4) were obtained and in Cross 5, meiotic (MEI-5) and mitotic (MIT-5) progenies were obtained (Table 1).

Data on segregation of 10 microsatellite loci in the amphimictic (AMPH-1) progeny are given in Table 2. All loci segregated in agreement with the Mendelian expected ratios of 1:1 or 1:1:1:1 (Table 2).

Data on segregation of 10 microsatellite loci in meiotic gynogenetic progenies obtained from heterozygous females are given in Table 3. All offspring in all progenies obtained from homozygous females, as expected, demonstrated the same genotypes as the respective maternal female (these data are not shown in Table 3). The complete absence

Table 1 Description of fish parents used in crosses and characteristics of obtained progenies

	Fish Parents		Type and designation of progeny		
No. of cross	Female	Male	Amphimictic	Meiotic gynogenetic	Mitotic gynogenetic
Cross 1	Koi x Common Carp hybrid	White-Red Koi	AMPH-1	MEI-1	MIT-1
Cross 2	White Koi	Common Carp	–	MEI-2	–
Cross 3	Koi x Common Carp hybrid	White-Red Koi	–	MEI-3	–
Cross 4	White Koi	Common Carp	–	MEI-4	–
Cross 5	White-Red Koi	Common Carp	–	MEI-5	MIT-5

Table 2 Genotypic segregation of 10 microsatellite loci in amphimictic progeny AMPH-1

Locus	Parental genotypes (bp)		Genotypic segregations in offspring					
	Female	Male	Observed (expected)				*n*	χ^2
MFW4	147/191	155/197	147/155	147/197	155/191	191/197		
			13 (10)	7 (10)	11 (10)	9 (10)	40	2.00
MFW7	179/201	221/235	179/221	179/235	201/221	201/235		
			5 (10)	12 (10)	13 (10)	10 (10)	40	3.80
MFW26	128/154	140/140	128/140	140/154				
			17 (20)	23 (20)			40	0.90
Koi29-30	256/260	252/260	256/260	252/256	260/260	252/260		
			15 (10)	10 (10)	11 (10)	4 (10)	40	6.20
Koi105-106	192/222	198/198	192/198	198/222				
			22 (20)	18 (20)			40	0.40
Koi115-116	252/256	248/248	248/252	248/256				
			19 (20)	21 (20)			40	0.10
Cca02	175/177	175/175	175/175	175/177				
			15 (20)	25 (20)			40	2.50
Cca04	226/234	216/216	216/226	216/234				
			20 (20)	20 (20)			40	0.00
Cca24	221/231	245/260	221/245	221/260	231/245	231/260		
			8 (10)	10 (10)	7 (10)	15 (10)	40	3.80
Cca-21	75/83	79/83	75/83	75/79	83/83	79/83		
			11 (10)	8 (10)	9 (10)	12 (10)	40	1.00

of paternal specific alleles was observed in all progenies indicating their gynogenetic origin (see Table 3). Recombination frequencies for 10 loci in offspring produced from heterozygous females ranged from 0.01 at locus *Cca24* to 0.96 at locus *Cca-21*; the estimated microsatellite-centromere map distance ranged from 0.5 to 48 cM (Table 3). The mean value of recombination frequencies (y) for all analysed loci was 0.481.

Six microsatellite loci with recombination frequencies (y) 0.47 and higher (*MFW4*, *MFW26*, *Koi29-30*, *Koi105-106*, *Koi115-116* and *Cca-21*) were used to evaluate the mitotic gynogenetic origin of two late-shocked progenies MIT-1 and MIT-5. Only homozygous genotypes and no heterozygotes were observed at all six analysed loci in these progenies (Table 4).

Discussion

Analysis of microsatellites has confirmed complete inactivation of paternal inheritance by UV-irradiation of sperm; no individuals with paternally specific alleles at 10 analysed microsatellite loci were found in all analysed meiotic (early-shocked) and mitotic (late-shocked) gynogenetic progenies.

Meiotic (early-shocked) gynogenetic progenies were used to determine recombination rates (y) and the M-C distance maps in relation to the centromere of 10 microsatellite loci. Until now, only one study has been published on determination of recombination rate for microsatellites in common carp or koi. Aliah and Taniguchi (2000) examined segregation of six microsatellite loci in meiotic gynogenetic koi progenies; the mean segregation rate ranged from 0.040 at locus *MFW7* to 0.919 at locus *Cca-21*. In the current study, the mean recombination rate at locus *MFW7* was higher (0.13), while the highest mean recombination value (0.96) was detected at the same locus *Cca-21*.

In this study, two late-shocked, presumably mitotic gynogenetic progenies were produced. If these progenies were of mitotic gynogenetic origin, they had to demonstrate complete homozygosity. Six microsatellite loci, which revealed high recombination rates (y), were used as markers for determination of late-shocked progenies origin. Complete homozygosity was observed for all six analysed loci which prove the mitotic gynogenetic origin of these progenies.

It is known that the common carp genome passed through duplication. The chromosome number of common carp ($2n = 100$) is twice that of most fish in the family *Cyprinidae*, and the DNA content is high (Ohno, Muramoto, Christian &

Table 3 Genotypes of fish parents and segregation of ten microsatellite loci in meiotic gynogenetic progenies

Locus	Gynogenetic progeny	Parental genotypes (bp)		Genotypic segregation in offspring				Recombination frequency (*y*)	M-C distance (cM)	χ^{2*}
		Female (*a/b*)	Male	*aa*	*ab*	*bb*	*n*			
MFW4	MEI-1	147/191	155/197	7	10	13	30	0.33	17	1.80
	MEI-2	187/197	145/151	7	13	10	30	0.43	22	0.53
	MEI-4	147/197	141/147	5	20	5	30	0.67	33	0.00
	MEI-5	147/155	141/141	5	17	8	30	0.57	28	0.69
	Mean							0.50	25	
MFW7	MEI-1	197/201	221/235	12	7	11	30	0.23	12	0.04
	MEI-5	201/221	197/197	17	1	12	30	0.03	2	0.86
	Mean							0.13	7	
MFW26	MEI-1	128/154	140/140	2	26	2	30	0.87	43	0.00
	MEI-3	140/154	128/140	3	20	7	30	0.67	33	1.60
	MEI-4	128/140	144/154	5	21	4	30	0.70	35	0.11
	MEI-5	140/144	132/144	3	20	7	30	0.67	33	1.60
	Mean							0.73	37	
Koi29-30	MEI-1	256/260	252/260	6	15	9	30	0.50	25	0.60
	MEI-3	252/256	252/252	8	13	9	30	0.43	22	0.06
	Mean							0.47	24	
Koi105-106	MEI-1	192/222	198/198	5	24	1	30	0.80	40	2.67
	MEI-2	182/192	220/222	4	24	2	30	0.80	40	0.67
	MEI-3	175/192	180/204	1	29	0	30	0.97	48	1.00
	MEI-4	182/198	164/222	0	29	1	30	0.97	48	1.00
	MEI-5	180/182	222/222	1	28	1	30	0.93	47	0.00
	Mean							0.89	45	
Koi115-116	MEI-1	252/256	248/248	4	25	1	30	0.83	42	1.80
	MEI-2	252/280	256/256	2	26	2	30	0.87	43	0.00
	MEI-3	248/256	248/252	3	20	7	30	0.67	33	1.60
	MEI-4	248/252	256/256	4	22	4	30	0.73	37	0.00
	MEI-5	248/256	256/256	3	20	7	30	0.67	33	1.60
	Mean							0.75	38	
Cca02	MEI-1	175/177	175/175	9	5	16	30	0.17	8	1.96
	MEI-3	157/177	175/175	14	1	15	30	0.03	2	0.03
	MEI-4	157/175	177/177	15	1	14	30	0.03	2	0.03
	MEI-5	167/175	177/177	11	2	17	30	0.07	3	1.29
	Mean							0.08	4	
Cca04	MEI-1	226/234	216/216	9	14	7	30	0.47	23	0.25
	MEI-2	214/216	232/234	12	4	14	30	0.13	7	0.15
	MEI-3	226/242	210/214	14	8	8	30	0.27	13	1.64
	Mean							0.29	15	
Cca24	MEI-1	221/231	245/261	14	0	16	30	0.00	0.00	0.13
	MEI-3	212/231	244/246	12	1	17	30	0.03	2	0.86
	MEI-4	212/260	236/238	11	0	19	30	0.00	0.00	2.13
	MEI-5	212/244	236/260	11	0	19	30	0.00	0.00	2.13
	Mean							0.01	0.5	
Cca-21	MEI-1	75/83	79/83	1	29	0	30	0.97	48	1.00
	MEI-2	79/83	75/75	3	27	0	30	0.90	45	3.00
	MEI-3	75/83	79/83	1	29	0	30	0.97	48	1.00
	MEI-5	75/83	75/75	0	30	0	30	1.00	50	-
	Mean							0.96	48	

*Chi-square test for equal numbers of homozygotes (d.f. = 1).

Atkin 1967; David, Blum, Feldman, Lavi & Hillel 2003). Based on segregation patterns in amphimictic progenies, David *et al.* (2003) have described that many microsatellite loci in common carp are duplicated and each of these duplicated loci has disomic inheritance. The most demonstrative indication of locus duplication was the presence of more than two amplified fragments per

Table 4 Segregation of six microsatellite loci in two late-shocked gynogenetic progenies

Locus	Mean *y**	Gynogenetic progeny	Maternal genotype (bp) *a/b*	Genotypic segregation in offspring *aa*	*ab*	*bb*	*n*
MFW4	0.50	MIT-1	147/191	10	0	10	20
		MIT-5	147/155	14	0	6	20
MFW26	0.73	MIT-1	128/154	10	0	10	20
		MIT-5	140/144	14	0	6	20
Koi29-30	0.47	MIT-1	256/260	10	0	10	20
Koi105-106	0.89	MIT-1	192/222	12	0	8	20
		MIT-5	180/182	7	0	13	20
Koi115-116	0.75	MIT-1	252/256	10	0	10	20
		MIT-5	248/256	14	0	6	20
Cca-21	0.96	MIT-1	75/83	8	0	12	20
		MIT-5	75/83	8	0	12	20

*Determined based on proportion of heterozygotes in meiotic gynogenetic progenies.

individual (David *et al.* 2003). In the present study, no signs of microsatellite locus duplication were observed and no individuals with more than two fragments were detected among parents or amphimictic offspring. Theoretically, duplication of microsatellite loci could result in appearance of individuals with two amplified fragments in mitotic gynogenetic progenies; in this case the appearance of two fragments would indicate not to heterozygosity but on presence of different alleles at duplicated loci. Earlier, Spruell, Pilgrim, Greene, Habicht, Knudsen, Lindner, Olsen, Sage, Seeb and Allendorf (1999) observed two amplified fragments at duplicated microsatellite loci in haploid gynogens of pink salmon *Oncorhynchus gorbuscha* (Walbaum).

The present study demonstrates the effectiveness of using microsatellite markers to confirm the mitotic gynogenetic origin of koi progenies obtained by application of late shock. Application of microsatellite markers has also confirmed the exclusion of paternal chromosomes in gynogenetic progenies. In addition, this study has provided useful information on inheritance of microsatellite loci in amphimictic, meiotic and mitotic gynogenetic progenies.

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