Comparison of genetic diversity and growth traits among Fangzheng silver crucian carp (*Carassius auratus gibelio*) gynogenetic clones

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Abstract

The silver crucian carp (*Carassius auratus gibelio*), a gynogenetic teleost, is a promising model for the study of evolutionary genetics in vertebrates. We identified ten gynogenetic clones (FZ-I~FZ-X) from triploid silver crucian carp, collected from Fangzheng County in Heilongjiang Province, China, using microsatellite markers. The genetic diversity of these gynogenetic clones was analyzed using 52 microsatellite markers. A total of 413 alleles were detected and the length of fragments ranged from 96 to 340 bp. The number of alleles per locus varied from 2~19 (mean=7.9423). The observed heterozygosity at polymorphic loci ranged from 0.10~1.00 (mean=0.80). The average allele count per gynogenetic clone ranged from 1.9423~2.1923. The ratio of the polymorphic locus was from 71.15% (VII) to 84.61% (IX) per clone. The number of genotypes ranged from 2~10 per locus. Ten genotypes were observed by analyzing each of 14 microsatellites. As a result, each gynogenetic clone could be accurately identified. In addition, the growth traits, including body weight, length, and height, among five gynogenetic clones were compared. There was a significant difference among gynogenetic clones. Clone FZ-V exhibited the best growth traits, with the largest body weight (53.17±5.24 g), length (11.38±0.37 cm) and height (4.69±0.18 cm). Our results provide basic data for the identification of silver crucian carp gynogenetic clones and can be used as a guide genetic breeding programs.

Keywords: Silver crucian carp, Gynogenetic clone, Polymorphism, Growth traits, Genetic diversity

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Introduction

The silver crucian carp (*Carassius auratus gibelio*), a naturally occurring gynogenetic triploid, is primarily distributed in the north of China, Korea, Japan, and Europe. Its high level genetic heterogeneity was first documented in the 1980s (Zhu and Jiang, 1987). Subsequent researches identified four gynogenetic clones based on serum protein markers (Zhu and Jiang, 1987) and tissue transplantation (Zhu, 1990). These clones exhibit significant differences in biological characteristics such as length, growth, fecundity, and chromosome number (Zhu and Jiang, 1993). The use of molecular markers such as mitochondrial DNA (mtDNA) (Fan *et al*., 2000), random amplified polymorphic DNA (RAPD) (Zhou *et al*., 2000), sequence characterized amplified regions (SCAR)(Zhou *et al*., 2001a), transferrin (Yang *et al*., 2001), and microsatellites (Zhou *et al*., 2001b) has revealed that individuals of one gynogenetic clone exhibit a high degree of genetic homogeneity, whereas individuals of different gynogenetic clones exhibit a high level of genetic heterogeneity. In addition, Gui and Zhou (2010) documented differences in growth performance and breeding potential among clones, a finding that has played an important role in both genetic breeding researches and the artificial culture of silver crucian carp.

Undoubtedly, however, there remain a number of natural Fangzheng silver crucian carp gynogenetic clones that have not been identified. Comparative studies of triploid and diploid carp found in the same area suggest that there is little difference in genetic diversity between two types (Lu *et al*., 2006; Jia *et al*., 2008; Sun *et al*., 2010). Li and Gui (2007) identified ten clones in the Fangzheng gibel carp using transferring protein markers. These observations highlight that there are a number of gynogenetic clones in wild populations at Fangzheng that have not been adequately studied. Furthermore, there is little information on the growth traits of the clones that have been identified because of the absence of a standard method for their identification. Thus, there is a clear need for the establishment of a standard genotype to identify among different clones for genetic researches and selective breeding of the silver crucian carp.

In recent years, microsatellite markers have been used to identify gynogenetic silver crucian carp because of their high polymorphism, stability, and co-dominant characteristics. In previous studies, four gynogenetic clones of silver crucian carp were identified with different genotypes using eight microsatellites from common carp (*Cyprinus carpio* L.) and nineteen microsatellites from silver crucian carp, respectively (Zhou *et al*., 2001b; Guo and Gui, 2008). However, the available information is limited due to small markers and clones in these earlier studies. Furthermore, the markers were generally analyzed by traditional slab electrophoresis, a technique that is too inaccurate to establish a standard genotype for each clone. In contrast, capillary electrophoresis allows the

accurate and reproducible detection of a base pair of microsatellite alleles. This in turn facilitates the comparison of results obtained by different researchers.

In this study, we transformed the silver crucian carp microsatellite primers (Zheng *et al*., 2010; Cao *et al*., 2011) to universal fluorescent primers. Following this, a capillary genetic analyzer was used to rapidly detect the genotypes of silver crucian carp. We established genotype files of the ten silver crucian carp gynogenetic clones held in our laboratory based on above method. In addition, we conducted a preliminary analysis of the growth traits of some gynogenetic clones. Our results provide a basis for the identification of silver crucian carp gynogenetic clones and can be used as a guide in genetic breeding programs.

Materials and methods

Source of samples

Nearly five hundred silver crucian carps were collected from Shuangfeng and Shuanglong Reservoirs, Fangzheng County, Heilongjiang Province in 2010. One hundred and eighty six triploid silver crucian carp were identified using flow cytometry. Briefly, heparinized whole blood (10 μL) was sampled from each fish and mixed into nuclear isolation and staining solution-10 (490 μL) (NPE systems, Inc. Florida, USA. Lot: 3556). The sufficient mixing solution was placed at 4℃ for 15 min in the dark. Then, it was filtered through a 100 μm nylon mesh to prevent obstruction of the flow chamber by conglutinated cells. Nuclear DNA contents for each sample were automatically measured by the flow cytometer with chicken as a reference standard. The genotypes of 186 triploid silver crucian carp were then detected using polymorphic microsatellite markers. In total, ten gynogenetic clones were identified, FZ-I to FZ-X. In addition, we crossed silver crucian carp from different gynogenic clones with male triploid silver crucian carp to breed homologous gynogenetic clones. Finally, five clones (FZ-I, FZ-IV, FZ-V, FZ-VI, and FZ-VII) were obtained enough fry to further culture. Five hundred 1.5~2.0 cm fry per clone were placed into a single 667 m^2 pond for mixed farming to control for the effect of rearing environment and the stocking density was 3.75 individuals /m^2. The water conditions were suitable for fish growth, i. e. water temperature was 20℃~30℃, the value of pH was 6.5～8.0, the value of dissolved oxygen was 3.5～5.0 mg/L. At the end of September, three hundred 4-month old silver crucian carp were randomly collected and measured for body weight, length, and height. Genomic DNA was also extracted from their fins for further analysis.

Genomic DNA extraction

Approximately, 0.1 g of fin tissue was placed into 200 μL lysate (10 mM EDTA, pH 8.0; 200 μg mL^{-1} proteinase K; 0.5% sodium dodecyl sarcosinate) and digested for 3 h at 55°C, extracted three times using a mixture of phenol,

chloroform, and isoamyl alcohol (Volume ratio of 25:24:1), and then precipitated in anhydrous ethanol. After drying, the precipitate was dissolved in 1/10 TE and stored at 4 °C until further use.

PCR amplification and detection

Deep genetic polymorphism analyses were performed on three samples of each gynogenetic clone. The samples were amplified using a combination of universal (Schuelke, 2000) and specific (Zheng *et al*., 1995; Guo and Gui, 2008;Zheng *et al*., 2010; Cao *et al*., 2011) primers and analyzed by capillary electrophoresis. The universal primer sequence (M13F) was 5'-CACGACGTTGTAAAACGAC-3' (Schuelke, 2000). Universal primer sequences labelled with PET (red), VIC (green), NED (yellow), and 6FAM (blue) were added to the 5' end of the specific forward primer. Of the 52 specific primers, 47 tri nucleotide and tetra nucleotide markers were developed using the biotin capture method and radioactive-labelling hybridization, marked with "HLJYJ" (Zheng *et al*., 2010; Cao *et al.*, 2011); three were from silver crucian carp primers discovered by Guo and Gui (2008), marked as "YJ", and two were derived from carp primers developed by Zheng *et al*. (1995), and denoted as "GF". The total reaction volume was 15 μL consisting of 100 ng DNA, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.2 mM primer, and 1 U *Taq* DNA polymerase. PCR was conducted using the GeneAmp PCR system 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following profile: an initial step at 94°C for 3 min; followed by 25 cycles of 94°C for 30 s, 52°C~60°C for 30 s, 72°C for 30 s; and an extension at 72°C for 5 min. After the reaction, an electrophoresis sample was fabricated by mixing together 0.7 μL of each of the four-color fluorescent PCR products (2.8 μL total), 5.9 μL of Hi-DiTMformamide and 0.1 μL LIZ-500. The mixed sample was subject to PCR at 95°C for 5 min, then immediately placed on ice for 5 min. Capillary electrophoresis was conducted using a ABI-3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Peak Scanner version 1.0 (Applied Biosystems, Foster City, CA, USA) was used to calculate the intensity and size of each peak. Then the genotypes of different gynogenetic clones were manually scored.

Based on this analysis, fragments that differed markedly in size were chosen as markers. Using the markers, 300 four-month old individuals were identified using 8% non-denaturing polyacrylamide gel electrophoresis and the PCR amplifications were made using the corresponding specific primers. The PCR system was the same as described above. The PCR products were visualized by staining the bands with 0.1% $AgNO_3$ solution.

Data analysis

Being codominant markers, microsatellites can be used to directly interpret the genotype of the

gynogenetic clones. Because silver crucian carp is naturally triploid, the proportion of monoallelic, diallelic, and triallelic genotypes were directly calculated for each locus. The number of alleles (N_a) and the observed heterozygosity (H_o) of microsatellite loci, and the proportion of heterozygous loci of each gynogenetic clone were also derived.

The 300 offspring individuals were divided into different gynogenetic clones based on the genotypes determined by microsatellite markers. The traits of each gynogenetic clone were described by a normal distribution. The variance for each trait was then analyzed by one-way analysis of variance (ANOVA). The mean trait values for the different gynogenetic clones were compared using Duncan's test.

Results

Characterization of 52 microsatellite markers

We identified ten gynogenetic clones (FZ-I~FZ-X) from triploid silver crucian carp, collected from Fangzheng County in Heilongjiang Province, China, using microsatellite markers. For each genogenetic clone, three samples were used to analyze the genetic polymorphism of 52 microsatellite loci by capillary electrophoresis. The number of alleles at each microsatellite locus ranged from 2 to 19, the fragment size ranged from 96 to 340 bp; the allele count was 413, and the average number of alleles per locus (N_a) was 7.9423. The observed heterozygosity (H_o) ranged from 0.10 to 1.00 (mean=0.80). Ten clones were heterozygous (diallelic and triallelic) at 20 microsatellite loci, including HLJYJ007, HLJYJ018, and HLJYJ038. The majority of clones exhibited diallelicheterozygosity at 29 microsatellite loci, including HLJYJ004, HLJYJ007, and HLJYJ015 ($H_o \geq 0.50$), and triallelicheterozygosity at 16 microsatellite loci, including HLJYJ018, HLJYJ038, and HLJYJ041 ($H_o \geq 0.50$). In addition, ten clones were primarily homozygous at five microsatellite loci, including HLJYJ011, HLJYJ066, and HLJYJ100 ($H_o < 0.50$). The electrophoresis pattern for HLJYJ119 and HLJYJ205 is illustrated in Fig. 1, and the specific statistics are given in Table 1.

Genetic diversity of silver crucian carp gynogenetic clones

The number of alleles per locus by amplification ranged from one to three for each gynogenetic clone. The number of alleles ranged from 101 to 114 (range in means: 1.9423 to 2.1923), among which the highest were the gynogenetic FZ-I and FZ-IX and the lowest was FZ-VII. Across all 52 microsatellite loci, the FZ-IX clone exhibited the highest (84.61%) proportion of heterozygous loci and the FZ-VII had the lowest (71.15%), which was consistent with the allele count.

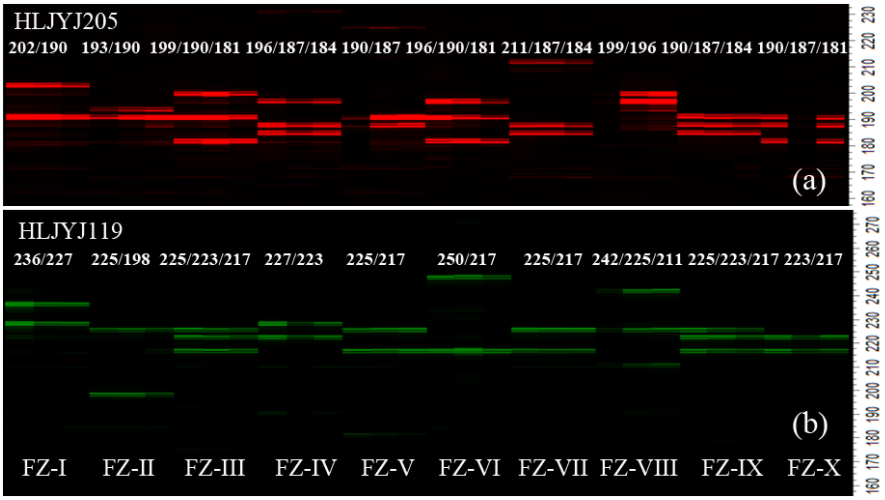

Figure 1: The results of amplification of 10 gynogeneticclones from Fangzheng silver crucian carp at HLJYJ205 (a) and HLJYJ119 (b).

In addition, the heterozygous loci FZ-II, FZ-V, and FZ-IX were primarily diallelic (>50%) (Table 2).

For the 52 microsatellite loci, the number of genotypes amplified at each microsatellite loci ranged from two to ten. All ten genotypes were amplified by each of the 14 microsatellite loci, including HLJYJ015, HLJYJ205, resulting in accurate identification of the ten gynogenetic clones (Fig. 1a). Amplification of 31 microsatellite loci, including HLJYJ004 and HLJYJ119, generated five or more genotypes which can be used to identify the majority of gynogenetic clones (Fig. 1b). The minimum gap size of alleles for 23 microsatellite loci, including HLJYJ011 and HLJYJ015 was ≥3 bp. Thus, it is feasible to distinguish the majority of gynogenetic clones using ordinary tablet electrophoresis. Detailed data are given in Table 3.

Growth traits of silver crucian carp gynogenetic clones

Two thousand and five hundred fry obtained from five clones (FZ-I, FZ-IV, FZ-V, FZ-VI, and FZ-VII) were cultured in a same pond for four months. Three hundred individuals were randomly sampled from the pond. Their mean weight was 45.26±8.73 g, mean body length was 10.76±0.73 cm, and mean body height was 4.50±0.31 cm. Based on the genotypes of their female parents, the 300 individuals were divided into five gynogenetic clones using markers HLJYJ051 and HLJYJ055 (Table 4).We used a Shapiro-Wilk test (SPSS 19.0) to test whether the growth traits were normally distributed. The individuals that deviated significantly from a normal distribution were excluded. The growth traits of the remaining 288 individuals used for further statistical analysis are given in Table 4.

Table 1:Amplification and observed heterozygosity of fifty-two microsatellite markers.

Locus	GenBank No.	Repeat motif	T_a/°C	Size range/ bp	N_a	Homozygosity	H_o	
							Diallelic	Triallelic
HLJYJ004	FJ827508	$(cag)_{12}$	60	167-177	4	0.20	0.70	0.10
HLJYJ007	/	$(agc)_3(agc)_3$	60	96-122	2	0.00	0.90	0.10
HLJYJ011	FJ827510	$(tatc)_3tttt(tatc)_5$	60	172-212	5	0.80	0.20	0.00
HLJYJ015	FJ827511	$(acag)_5(atag)_8$	60	183-206	7	0.10	0.60	0.30
HLJYJ018	FJ827513	$(agac)_9(agat)_{24}$	58	202-262	12	0.00	0.30	0.70
HLJYJ031	FJ827519	$(ctat)_{16}$	60	250-296	10	0.20	0.50	0.30
HLJYJ034	FJ827522	$(tcta)_{13}$	60	143-225	14	0.30	0.40	0.30
HLJYJ038	FJ827523	$(atct)_{12}$	60	183-298	17	0.00	0.30	0.70
HLJYJ041	FJ827526	$(atct)_{15}(tctg)_{21}$	60	189-321	18	0.00	0.20	0.80
HLJYJ048	FJ827529	$(atag)_{11}aa(atag)_{15}$	60	219-283	15	0.20	0.30	0.50
HLJYJ051	FJ827532	$(agat)_{20}$	60	186-254	14	0.00	0.50	0.50
HLJYJ055	FJ827533	$(gata)_{13}(agac)_6$	60	197-326	15	0.30	0.30	0.40
HLJYJ056	FJ827534	$(atct)_{27}$	60	213-288	14	0.20	0.70	0.10
HLJYJ066	/	$(ctg)_7$	60	218-220	2	0.60	0.40	0.00
HLJYJ078	FJ827541	$(cag)_9$	60	213-253	8	0.20	0.60	0.20
HLJYJ080	/	$(cag)_{19}$	60	125-167	5	0.30	0.50	0.20
HLJYJ084	FJ827543	$(gtt)_7$	60	156-172	4	0.00	0.40	0.60
HLJYJ089	FJ827545	$(gct)_9$	60	199-249	9	0.00	0.70	0.30
HLJYJ090	FJ827546	$(ctg)_{10}$	60	192-204	5	0.10	0.70	0.20
HLJYJ091	FJ827547	$(tgc)_{16}$	59	203-209	4	0.10	0.80	0.10
HLJYJ094	FJ827548	$(agc)_7$	60	202-225	9	0.00	0.20	0.80
HLJYJ096	FJ827549	$(cag)_8$	59	240-260	5	0.20	0.50	0.30
HLJYJ100	FJ827550	$(aac)_6(agc)_3$	60	197-205	4	0.50	0.50	0.00
HLJYJ101	FJ827551	$(tgc)_{10}$	60	213-246	7	0.00	0.50	0.50
HLJYJ102	FJ827552	$(cag)_6$	60	253-262	4	0.30	0.40	0.30
HLJYJ115	FJ827554	$(cag)_{12}$	60	200-209	4	0.10	0.80	0.10
HLJYJ119	FJ827557	$(gct)_{10}$	59	198-250	9	0.00	0.70	0.30
HLJYJ122	FJ827560	$(tga)_6$	59	146-304	19	0.00	0.40	0.60
HLJYJ134	FJ827567	$(cag)_9$	60	199-218	6	0.30	0.60	0.10
HLJYJ139	HM449129	$(gat)_{11}$	56	210-225	6	0.00	0.40	0.60
HLJYJ141	HM449130	$(gat)_6gac(gat)_6$	56	152-174	6	0.20	0.80	0.00
HLJYJ143	HM449131	$(tga)_9cga(tga)_6$	58	197-221	9	0.00	0.70	0.30
HLJYJ147	HM449133	$(tga)_8$	52	175-226	9	0.50	0.40	0.10
HLJYJ150	HM449136	$(gat)_{11}$	54	180-198	4	0.60	0.40	0.00
HLJYJ151	HM449137	$(atg)_7gtg(atg)_9$	54	154-179	8	0.00	0.70	0.30
HLJYJ160	HM449140	$(cat)_8$	56	204-216	4	0.40	0.40	0.20
HLJYJ163	HM449141	$(gat)_7$	60	219-240	8	0.20	0.40	0.40
HLJYJ167	HM449143	$(attg)_{11}$	60	189-273	10	0.00	0.60	0.40
HLJYJ168	HM449144	$(gat)_6gaa(gat)_2$	60	159-172	7	0.20	0.50	0.30
HLJYJ177	HM449147	$(gat)_9ggt(gat)_6$	60	158-194	8	0.30	0.50	0.20
HLJYJ179	HM449148	$(tca)_7tcg(tca)_3$	60	162-186	6	0.00	0.50	0.50
HLJYJ188	HM449153	$(ct)_{16}$	60	188-218	9	0.30	0.20	0.50
HLJYJ192	HM449155	$(ac)_{13}(ag)_5$	60	153-177	7	0.00	0.80	0.20
HLJYJ193	HM449156	$(gat)_{11}$	60	160-172	5	0.40	0.50	0.10
HLJYJ204	HM449158	$(tca)_7$	60	259-268	4	0.80	0.10	0.10
HLJYJ205	HM449159	$(tga)_7$	60	181-211	9	0.00	0.40	0.60
HLJYJ207	HM449160	$(attg)_7$	60	207-215	3	0.90	0.10	0.00
YJ0010	/	$(tg)_{11}$	60	150-168	7	0.40	0.40	0.20
YJ0020	/	$(gt)_{10}$	60	152-169	8	0.10	0.20	0.70
YJ0025	/	$(aaac)_5$	60	214-230	9	0.00	0.50	0.50
GF1	U35614	$(gt)_8$	60	302-340	7	0.10	0.80	0.10
GF17	U35616	$(gt)_{13}$	60	184-212	9	0.00	0.50	0.50

T_a: annealing temperature; N_a: allele number; H_o: observed heterozygosity.

Table 2: The number of alleles and proportion of mono-, di-, and triallelic loci for ten gynogenetic clones.

	FZ-I	FZ-II	FZ-III	FZ-IV	FZ-V	FZ-VI	FZ-VII	FZ-VIII	FZ-IX	FZ-X
Number of alleles	114	109	110	107	104	109	101	113	114	107
Number of average alleles	2.19	2.10	2.12	2.06	2.00	2.10	1.94	2.17	2.19	2.06
Proportion of monoallelic loci (%)	19.23	19.23	21.15	25.00	21.15	26.92	28.85	17.31	15.39	23.07
Proportion of diallelic loci (%)	42.31	51.92	46.16	44.23	57.70	36.54	48.08	48.08	50.00	48.08
Proportion of triallelic loci (%)	38.46	28.85	32.69	30.77	21.15	36.54	23.07	34.61	34.61	28.85

Body weight, body length, body height, and the ratio between body length and body height differed significantly among the gynogenetic clones (ANOVA; $p<0.01$). Body weight and body length of clone FZ-V were significantly greater than those of the remaining clones ($p<0.01$). Similarly, body height was highest for FZ-V, but was not different from that of FZ-VI. The body weight and length of clones FZ-IV and FZ-VI were not different and were similar to the mean for all clones. However, the body height of FZ-VI was significantly greater than that of FZ-IV and the ratio of body length to body height was significantly lower than for FZ-IV, suggesting that the body height of FZ-VI was much greater. Clone FZ-VII did not exhibit significantly improved growth traits relative to the other clones. However, the ratio of body length to body height was higher than for the remaining clones, suggesting that FZ-VII was longer.

Discussion

Applied value of different gynogenetic clones of Fangzheng silver crucian carp

Triploid Fangzheng silver crucian carps (*C. autatus gibelio*) exhibit a faster growth rate and are more adaptable relative to diploid crucian carp (*C. auratus auratus* L.) in same conditions (Gui, 1996). Being naturally gynogenetic, the improved growth traits are highly heritable, a characteristic that is of considerable value to breeders. Zhu and Jiang (1993) identified four gynogenetic clones of the Fangzheng silver crucian carp, which have been bred selectively for culture (Gui and Zhou, 2010). Given their potential value, the discovery and identification of new gynogenetic clones have important implications for genetic and breeding research of silver crucian carp. In recent years, Li and Gui (2007) and Sun *et al*. (2010) identified ten (nine novel) and 14 gynogenetic clones from wild Fangzheng silver crucian carp populations, respectively.

Table 3: Identification of 10 silver crucian carp gynogenetic clones at 45 microsatellite loci.

Locus	Number of genotype	Allele size (bp)									
		FZ-I	FZ-II	FZ-III	FZ-IV	FZ-V	FZ-VI	FZ-VII	FZ-VIII	FZ-IX	FZ-X
HLJYJ004	6	170	170	170/168	173/168	173/170	170/168	177/170/168	170/168	170/168	177/170
HLJYJ011	6	192	196/180	212	192/172	180	172	172	180	172	172
HLJYJ015	10	195/191/187	187	187/183	191/183	206/198/195	206/187	198/183	198/187	198/195/191	202/191
HLJYJ018	10	250/223/206	246/223/218	250/238	250/230/226	223/218	262/214/206	234/226	246/206/202	238/218/214	262/226/214
HLJYJ031	9	280/276	288/272/260	288/276	288/272/264	284	292/264/250	296	264/250	288/272	288/272
HLJYJ034	10	209/197/164	206/143	197	168/164	209/177	225/185/143	212/209/164	201	161/156	193
HLJYJ038	10	271/215/211	247/239	298/259	231/227	215/207/199	211/203/183	239/227/219	227/215/207	235/231/223	203/199/195
HLJYJ041	10	313/288/265	274/251	272/265/240	302/267/255	310/267/255	285/255/246	321/310	310/251/246	272/211/189	293/267/211
HLJYJ048	10	251/231/219	275/239	279/267/227	259	259/255	283/248/243	227	267/248/223	283/275/243	271/243
HLJYJ051	10	230/202	222/214/202	238/210/199	246/234/186	238/214	254/246/217	226/214	202/186	226/214/206	234/226
HLJYJ055	10	326/268/220	272/252	313	321/280/228	284/280/197	252	248	260/224	326/248	272/248/216
HLJYJ056	10	229	288/213	262/251/242	262/246	246/242	226	231/226	251/221	274/236	270/266
HLJYJ078	9	250/242	250/247/233	247	253/247	253/242	247	247/242/233	247/242	218/214	226/214
HLJYJ080	6	134/131	134/128/125	134/131	134	134/131	167/131	134	167/134/131	134/125	134
HLJYJ089	8	237/230	227/199	227/224/218	230/224	227/218	249/218	227/218	243/227/212	227/224/218	224/218
HLJYJ090	6	198/195	195/192	195/192	201/198/192	198	198/195	204/198/195	195/192	198/195	198/192
HLJYJ091	6	207/205	207/203	207/205	205/203	207/203	209/207/205	207/205	207	209/207	207/205
HLJYJ094	9	222/213/211	219/205	225/222/213	211/205/202	225/222	225/210/205	219/216/213	219/216/213	225/219/211	219/216/211
HLJYJ096	6	255/252	252/240	260/252/240	240	252/240	255/252/240	252/240	255/252/240	257/240	240
HLJYJ101	8	246/238/231	246/231	246/228/213	238/231	246/228	246/228	234/231	240/234/228	246/238/231	234/231/228
HLJYJ115	6	206/203	206/200	206/203	203/201	206/201	209/206/203	206/203	206	209/206	206/203
HLJYJ119	8	236/227	225/198	225/223/217	227/223	225/217	250/217	225/217	242/225/211	225/223/217	223/217
HLJYJ122	10	292/207/183	304/171	298/216	304/231/216	258/171/146	237/213	207/201/168	288/285/270	292/177	285/258/204
HLJYJ134	9	215/207	215/199	213	218/213	218/207	213	207/199	213/207	207	207/201/199
HLJYJ139	9	222/219/213	222/216/213	225/219/210	216/213	219/213	225/219/210	219/216/213	219/216	222/216/213	222/219
HLJYJ141	8	171/165	168/165	174/168	168/165	168	171	168/162	171/168	171/152	168/165
HLJYJ143	10	212/200	215/206	209/203/200	212/202	209/203	221/203/200	203/200	221/218/200	206/200	206/197
HLJYJ147	8	184	184	193/175	190	181	184	226/217	193/190	181/178	188/181/175
HLJYJ150	6	180	192/180	192	186	186/180	198/180	192	186	186/180	186
HLJYJ151	9	179/164/157	167/154	157/154	173/154	173/167/164	173/164	173/164	167/157	176/173	173/160/154
HLJYJ160	5	216/213/210	213/210	210	210	213/210	216	213/210	216	216/213/204	213/210
HLJYJ163	9	231/229/225	238/231/223	238/229/225	229	238/231/222	238/219	231	240/222	238/231	238/231

Continued Table 3.

Locus	Number of genotype	Allele size (bp)									
		FZ-I	FZ-II	FZ-III	FZ-IV	FZ-V	FZ-VI	FZ-VII	FZ-VIII	FZ-IX	FZ-X
HLJYJ167	10	242/229	259/254/242	254/242/189	242/197	254/225	259/234/225	259/254	273/262/242	259/242	234/225
HLJYJ168	8	172/160	166	172/160	180/172/163	172/163	182/172	172	180/172/160	172/160	172/168/166
HLJYJ177	7	164/158	194/162/158	191/164/158	172/164	158	166/158	166/158	160/158	158	158
HLJYJ179	8	180/171/162	168/162	168/162	180/171/168	186/171/162	183/180	171/168	180/168/162	186/171/168	171/168
HLJYJ188	9	212/198/188	218/206/192	198/188	206	194	206/196/192	200/198	212/206/198	206	206/198/192
HLJYJ192	7	161/159	159/153	170/155/153	177/159/155	171/159	171/159	171/161	171/159	171/159	171/165
HLJYJ193	7	168	166/162	160	171/165	171/165	171/165/162	165	171/165	168/165	165
HLJYJ205	10	202/190	193/190	199/190/181	196/187/184	190/187	196/190/181	211/187/184	199/196	190/187/184	190/187/181
YJ0010	8	164/160	150	152/150	152	168/164	152	166/152/150	162/160/152	168/166	152
YJ0020	10	161/154/152	169/161/154	169/161/158	161/154	164/158/154	158/152	154	161/158/152	169/164/158	164/161/158
YJ0025	9	230/224	226/223/214	223/217	223/217/214	223/219	230/223/221	228/219/217	228/219	226/223/214	223/221
GF1	8	302	308/302	312/304/302	317/308/302	340/308	308/304	308/302	317/302	308/302	329/308
GF17	9	200/194/192	200/196/192	204/192	208/204/192	204/196	212/196/192	192/190	204/192	212/194/184	200/184

Table 4:Mean values and multiple comparisons of body weight, length, body depth of five ynogeneticclones

Lineage	Amount	Weight/g	Length/cm	Height/cm	Length/Height
FZ-I	20	31.74±6.86^{b}	9.37±0.80^{b}	4.05±0.33^{a}	2.32±0.10^{ab}
FZ-IV	85	42.61±4.64^{a}	10.52±0.37^{a}	4.47±0.15^{c}	2.35±0.05^{b}
FZ-V	105	53.17±5.24^{c}	11.38±0.37^{c}	4.69±0.18^{b}	2.43±0.05^{c}
FZ-VI	39	44.93±4.22^{a}	10.69±0.35^{a}	4.69±0.17^{b}	2.28±0.04^{a}
FZ-VII	39	36.59±5.60^{d}	10.49±0.60^{a}	4.11±0.23^{a}	2.55±0.09^{d}

Note: The different values marked by a~d in each column are significantly different (p<0.01).

However, the authors did not explore the genetic polymorphism and production performance of these gynogenetic clones, making it impossible to use these new gynogenetic clones in genetic research and selective breeding. We obtained ten gynogenetic clones (FZ-I~FZ-X) of silver crucian carp over a period of several years. We used 52 microsatellite markers to analyze the genetic characteristics of each of these groups. The proportion of heterozygous loci of the ten gynogenetic clones ranged from 71.15 to 84.61%. Thus, a high degree of genetic diversity was maintained. Comparing the genotypes revealed by 3 microsatellite markers, YJ0010, YJ0020, and YJ0025 with clones A, D, L, and F analyzed by Guo and Gui (2008), only the genotype for YJ0020 in FZ-V (164/158/154) was consistent with that of clone D. In contrast, the genotypes at YJ0010 (168/164) and YJ0025 (223/219)

differed from those of clone D, suggesting that FZ-V was indeed a novel clone. However, the relation was unknown between these clones and other clones reported by Li and Gui (2007) because of different methods. Similarly, the genotypes of the 10 gynogenetic clones, revealed by microsatellites GF1 and GF17, differed from those of the Japanese silver crucian carp clones identified by Ohara *et al*. (2003). This is consistent with results that obtained by Bai *et al*. (2011), and confirms that gynogenetic silver crucian carp and Japan silver crucian carp may have evolved from different origins. Our preliminary assessment of the production performance of five of the gynogenetic clones suggests they have differing growth traits. Growth performance was highest in FZ-V, while the body height of FZ-VI was the highest and the body length of FZ-VII was the longest. Identification of these 10 gynogenetic clones provides additional silver crucian genetic breeding material as well as some basic data describing performance differences that can be used to guide further research and breeding practices.

Effectiveness of identification of gynogenetic clones of Fangzheng silver crucian carp by microsatellite

Microsatellite markers may be the most sensitive and effective tool for identifying gynogenetic clones due to their high polymorphism, stability, and co-dominant characteristics (Ohara *et al*., 1998). The microsatellite markers formed by repetition of trinucleotide and tetranucleotide are more suited for fluorescent labelling and large-scale automated analysis (Edwards *et al*., 1991; Lindqvist *et al*., 1996). The capillary electrophoresis was used to identify 10 silver crucian carp gynogenetic clones based on 52 microsatellite markers with tri nucleotide and tetra nucleotide repeats. Stable and clear bands were obtained for all 52 markers, and no "stutter band" was observed. Of these, 45 markers could be used to identify more than 5 gynogenetic clones, and 14 microsatellites could be used to identify 10 gynogenetic clones, suggesting the microsatellite markers were highly efficient at identifying silver crucian carp clones. Such efficiency is consistent with previous reports in Fangzheng silver crucian carp (*C.autatus gibelio*)(Zhu and Jiang, 1987; Li and Gui, 2007), Japanese Kanto silver crucian carp (*C. langsdorfii*)(Ohara *et al*., 1998; Ohara *et al*., 2003), and Amazon Molly (*Poecilia formosa*)(Lampert *et al*., 2006). As an example, Guo and Gui (2008) used 19 microsatellite markers from silver crucian carp to analyze four gynogenetic clones, 12 of which were able to distinguish completely between clones A, D, L, and F. Sun *et al*. (2010) documented between 1 and 14 genotypes in wild populations of Fangzheng silver crucian carp using microsatellite markers. Similarly, Ohara *et al*.(2003)identified 61 clones in samples collected from six distinct areas

in Japan using only 3 microsatellite markers, of which 39 clones were novel. Interestingly, the number of observed clones ranged from 6 to 28 in a single water body. Furthermore, the authors concluded that more clones may have been distinguished using additional markers. Bai *et al*.(2011) analyzed the gynogenetic clones of Chinese and Japanese silver carp using 10 microsatellite markers. However, only four gynogenetic clones were identified in Chinese farmed and wild populations because of a limited sample size.

Researchers have used a variety of detection methods and microsatellite markers to identify gynogenetic clones. Furthermore, the detected alleles differ in size. Given this, it is not possible to accurately identify a given gynogenetic clone, resulting in difficulties in subsequent studies. To address this, there is a need to establish standard genotypes for each gynogenetic clone so that results from different laboratories are comparable. Capillary gel electrophoresis offers several advantages in this regard, including high accuracy and good reproducibility, compared with ordinary flat electrophoresis (Vemireddy *et al*., 2007). We used capillary gel electrophoresis to develop standard genotypes for 10 gynogenetic clones using 52 microsatellite markers. Our results provide a tool for further genetic and breeding research of gynogenetic clones. Furthermore, the smallest allele gap between 23 markers was ≥3 bp, a size that can be accurately identified by ordinary electrophoresis. Thus, our approach can be used by a variety of researchers and under different experimental conditions, according to the aims of experiments, to accurately distinguish among gynogenetic clones.

Mechanism of gynogenetic clone diversity

Fangzheng silver crucian carp (*C. autatus gibelio*) differs from Japanese silver crucian carp (*Carassius langsdorfii*) because of the existence of 5-25% fertile males within its populations. Studies suggest that the female individuals of Fangzheng silver crucian carp exhibit different responses to heterologous sperm and homologous sperm (Ge *et al*., 1992), leading to two different reproductive modes, i.e., gynogenetic reproduction and sexual reproduction (Lu *et al*., 2007; Gui and Zhou, 2010). Theoretically, the existence of two reproductive modes would enrich the diversity of silver crucian carp gynogenetic clones.

However, several studies have shown that the number of Fangzheng silver crucian carp gynogenetic clones is much lower than for Japanese silver crucian carp (Ohara *et al*., 2003; Li and Gui, 2007), which may be related to their different origins (Murakami and Fujitani, 1997; Shen *et al*., 1997). In addition, the basis of the generated genetic heterogeneity within gynogenetic fish species may derive from the exchange of genetic material. Researches suggest that the line of gynogenetic Amazon molly and

Fangzheng silver crucian carp can infiltrate and exchange genetic material via mini-chromosome between clones or between a clone and a related species (Schartl *et al*., 1995; Zhou and Gui, 2002; Yi *et al*., 2003). Taken together, these observations suggest the mechanism for evolution of gynogenetic clones within the silver crucian carp species is very complex.

In conclusion, this study identified ten gynogenetic clones (FZ-I~FZ-X) from triploid silver crucian carp using microsatellite markers. Deep genetic diversity of these clones was analyzed using 52 microsatellite markers. The average allele and the ratio of the polymorphic locus per clone ranged from 1.9423 to 2.1923 and from 71.15% (VII) to 84.61% (IX), respectively. Each of 14 microsatellites could accurately identify each clone due to different genotypes. In addition, there was a significant difference in growth traits among gynogenetic clones. Clone FZ-V exhibited the best growth traits. Although the 10 gynogenetic clones of Fangzheng silver crucian carp identified in the present study are only a small fraction of the total number of gynogenetic clones, our results provide material for future study and selective breeding. The specific microsatellite markers we have developed also provide a useful tool for the identification and application of these gynogenetic clones.

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