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PRIMER NOTE

Development of microsatellite DNA markers of silver carp (*Hypophthalmichthys molitrix*) and their cross-species application in bighead carp (*Aristichthys nobilis*)

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Abstract

Forty-four microsatellite DNA markers were developed for silver carp, and used to investigate polymorphisms of 41 wild silver carps and seven wild bighead carps collected from Jingzhou fragment of Yangtze River. In silver carp, 40 markers were polymorphic. A total of 297 alleles were detected at 40 polymorphic loci. The number of alleles per locus varied from two to 16 with an average of 7.4 and the expected heterozygosities of each locus ranged from 0.07 to 0.91 with an average of 0.69. All markers amplified both silver carp and bighead carp DNAs.

Keywords: bighead carp, microsatellite DNA marker, silver carp

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Silver carp and bighead carp are two of the four most important pond-cultured fish species which habitat the major river basins of China. Their cultivation could be dated back more than 1000 years to the Dang Dynasty (Li & Fang 1990). They have been introduced into more than 20 countries and regions in order to improve water quality or harvest for human consumption, although some ecological problems have been associated with their introduction. Silver carp and bighead carp can hybridize with each other artificially (de Almeida-Toledo *et al*. 1995) and may do so in hatchery (Mia *et al*. 2005). These observations imply their close phylogenetic relationship, although the fertility of their filial generation has yet not been assessed. To date, random amplified polymorphic DNA (RAPD) (Zhang *et al*. 2001) and restriction fragment length polymorphism (RFLP) of mtDNA (Song *et al*. 1994; Zhang *et al*. 2002) have been used in studies of their genetic diversity. Microsatellite DNA markers have been used in silver carp and bighead carp, but the number is very limited (Tong *et al*. 2002; Mia *et al*. 2005). In this note, we reported the development of microsatellite DNA markers of silver carp and their cross-species amplifications in bighead carp.

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Genomic DNA of one silver carp individual was extracted from alcohol-preserved muscle tissues using the phenol–chloroform method (Taggart *et al*. 1992). A microsatellite DNA enriched library was constructed using fast isolation by AFLP of sequences containing repeats (FIASCO) method (Zane *et al*. 2002) using a biotinylated $(GT)_{12}$ probe. DNA fragments containing microsatellite DNA were ligated with the pMD18-T vector (TaKaRa), and transferred into *Escherichia coli* JM109 through electroporation. In order to check if simple sequence repeat (SSR) motif locates in the middle of the insert, each recombinant was subjected to three individual polymerase chain reaction (PCR) screenings. In the first reaction universal forward and reverse sequencing primers were used; in the second reaction, the universal forward primer was used in combination with a $(GT)_{12}$ oligonucleotide; and in the third reaction the universal reverse primer was used in combination with a $(GT)_{12}$ oligonucleotide. Recombinant clones that produced products in obviously different lengths with the universal primers as well as with at least one combination of universal primer and $(GT)_{12}$ oligonucleotide were sequenced, trimmed and used to design microsatellite DNA primers using PRIMER PREMIER 5 software (www.premierbiosoft.com/primerdesign/). Primers were used to amplify genomic DNA of 41 silver carp and seven bighead carp individuals

collected from Jingzhou fragment of Yangtze River. PCR amplification reactions were carried out in a total volume of 25 μL containing 1× buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs (each), 200 μM primers (each direction) and about 50 ng DNA as the template. PCR was carried out by denaturing DNA at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at appropriate annealing temperature for each primer (Table 1) for 1 min and extension at 72 °C for 1 min and an extra extension at 72 °C for 10 min. PCR product was separated on a 6% denaturing polyacrylamide gel and visualized by silver staining. Allele size was determined with software QUANTITY VERSION version 4.4 (Bio-Rad) by referring to 20-bp ladder (TaKaRa). POPGEN, version 1.44 (Yeh *et al*. 2000) and GENEPOP web version 3.4 (http://wbiomed.curtin.edu.au/genepop/) were used to calculate the number of alleles and observed and expected heterozygosities, and test Hardy–Weinberg equilibrium and linkage disequilibrium. Significance values of all multiple tests were corrected following sequential Bonferroni procedure (Rice 1989).

Although GT or CA probes were used for screening, some other repeat types, such as those at loci BL8-1, BL82 and BL116 were found. In silver carp, all of the 44 primer pairs tested showed clear band patterns. Forty loci were polymorphic and four were monomorphic (BL23, BL92, BL110 and BL111). A total of 297 alleles were detected at 40 polymorphic loci. The number of alleles per locus ranged from two to 16 with an average of 7.4 and the expected heterozygosities each locus ranged from 0.07 to 0.91 with an average of 0.69. Thirty-one microsatellite DNA loci were in Hardy–Weinberg equilibrium. Two loci showed heterozygote excesses and seven showed heterozygote deficiency. None of the 40 polymorphic loci exhibited genotypic disequilibrium ($P < 0.0125$).

Forty-four markers were tested also for cross-species amplification of seven bighead carp individuals. All the markers worked in bighead carp. A total of 158 alleles were detected at all 44 loci. The number of alleles per locus ranged from one to nine with an average of 4.0. The size range of alleles of bighead carp each locus was similar to that of silver carp. More alleles should be detected in a large collection of big head carp.

The use of these microsatellites DNA markers will certainly facilitate the management and exploitation of the genetic resources of these two fish species and the closely related as well, and assist their genetic improvement to some extent.

Table 1 Microsatellite DNA markers developed for silver carp (*Hypophthalmichthys molitrix*)

Locus	Accession no.	SSR motif	Primers (5′–3′)	Clone size (bp)	T_a (°C)	*Hypophthalmichthys molitrix*					*Aristichthys nobilis*		
						Size range (bp)	Na	H_O	H_E	P	Amplification	Size range (bp)	Na′
BL5	DQ136003	$(TG)_6TA(TG)_{14}TC(TG)_5$	F: CCTGTGCCTTTGAACTCTGA R: CCCTCCACCATACTGACAAG	403	52	393–405	7	0.65	0.82	0.046	+	393–405	7
BL8-1	DQ136005	$(TCCA)_6$	F: TATTGACTGCATCTGGTCTT R: AGGTTATGTTTAGCCCAGTCG	157	59	157–162	3	0.22	0.35	0.000	+	157	1
BL8-2	DQ136005	$(TG)_9$	F: CCCGACTGGGCTAAACATA R: TCATTTGGGAGGCAGACAC	377	50	375–385	5	0.46	0.44	0.489	+	375–377	2
BL11	DQ136006	$(TG)_{11}(AG)_5$	F: GTCATCAAACTAAGCCATCAG R: GCATTTCACCTGTAGCATCTC	200	54	192–206	7	0.68	0.82	0.012	+	192–206	5
BL13	DQ674833	$(AC)_4GC(AC)_4$	F: AATGAGCAATCAGGCACAGAG R: GGGTGTAATGAGGCTATGTTT	278	54	277–231	4	1.00	0.53	0.000	+	278–231	2
BL14	DQ136008	$(GT)_{13}$	F: CGGCACTCAGAAATGATGGGG R: CATGGAGAGCAGGAAGAGTTG	320	54	312–338	9	0.66	0.82	0.001	+	320–332	4
BL15	DQ136009	$(GT)_8$	F: TACTGATACTCCGTCCCCT R: GCACCTGTAATCCCAAAT	191	54	195–199	2	0.46	0.44	1.000	+	191–199	2
BL18	DQ136010	$(AC)_{18}$	F: CGAGACAAATAAGGTTGGATA	229	52	208–229	12	0.68	0.90	0.013	+	208–212	7

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Table 1 *Continued*

						Hypophthalmichthys molitrix					*Aristichthys nobilis*		
Locus	Accession no.	SSR motif	Primers (5′–3′)	Clone size (bp)	T_a (°C)	Size range (bp)	*Na*	H_O	H_E	*P*	Amplification	Size range (bp)	*Na*′
			R: CACAAAGAAACTGGAACAAAGAG										
BL42	DQ136013	$(TG)_{14}$	F: TGCCGATGTTATGTTTGCT R: TGCTTGTGGGTGAGTTTCT	254	52	246–258	7	0.80	0.78	0.245	+	248–256	4
BL46	DQ136014	$(GT)_9$	F: AGTCCTGCTGTTGCTGTATG R: CTCCTGCTCCACCTTCCT	243	52	241–255	7	0.68	0.65	0.552	+	242–247	2
BL52	DQ136015	$(TG)_{12}$	F: CAGAATCCAGAGCCGTCAG R: CACCGAACAGGGAACCAA	220	54	210–220	5	0.51	0.61	0.061	+	216–220	3
BL54	DQ674834	$(CA)_{13}$	F: TGAAACTTAATGAAATACCTCCACG R: TCAAGGTTGTGATGTTTCTGCT	227	52	223–255	9	0.70	0.78	0.020	+	242–247	4
BL55	DQ136016	$(CA)_4CC(CA)_9$	F: AAGGAAAGTTGGCTGCTC R: GGCTCTGAGGGAGATACCAC	220	52	215–256	16	0.78	0.90	0.018	+	215–221	4
BL56	DQ136017	$(AC)_{16}$	F: TTAGGTGAACCCAGCAGC R: AAGAAGCATTAGTGCAGATGAGTAC	310	54	299–308	7	0.82	0.79	0.098	+	278	1
BL58	DQ136018	$(GT)_9$	F: TTCCTGCCTGTGCTCCAT R: TTGCATTGATGCTGTCCC	123	52	119–137	7	0.51	0.68	0.075	+	117–137	4
BL62	DQ136019	$(TG)_{11}$	F: ATATTAACATCTGCCGAAGC R: ACAACCAGCAGTCTGAAGC	232	54	212–244	11	0.66	0.81	0.053	+	214–244	7
BL64	DQ136020	$(TG)_{23}$	F: GCCAGGCTAGAAGAACCACC R: TTGCAGCACAGTTACCAAGACA	147	54	134–158	10	0.63	0.81	0.000	+	132–160	8
BL65	DQ136021	$(AC)_{12}$	F: TTAGAGCCATTAGAGGAAAA R: ACACGGAAGCCATTGTTG	315	54	289–323	12	0.82	0.86	0.061	+	293–302	2
BL66	DQ136022	$(TG)_9$	F: TTTGTTTCCGCCGTGGTG R: GGTTCAGGGTTCAATGTCC	327	54	320–327	5	0.15	0.65	0.000	+	318–322	2
BL67	DQ674835	$(AC)_{17}$	F: GGCAGGCTTCAAAGGACA R: CTCGCCCTCATTGTCGTA	274	54	257–272	11	0.91	0.87	0.160	+	259–263	4
BL69	DQ674836	$(GT)_6(CA)_{12}$	F: CTCGTCTCGTGCTTGTCA R: GGTGGTCATAATCACCATTCC	320	52	320–336	8	0.68	0.82	0.194	+	320–342	6
BL73	DQ674837	$(AC)_6$	F: TGACTTTACACGGCTCCA R: TTACTCTGTTATGGTGGGTCA	242	54	336–341	5	1.00	0.64	0.000	+	336–361	4
BL75	DQ674838	$(TG)_9$	F: GCATACCAGCAGCAAGAAGT R: CAAGTTATAGCCTCTGCCTCAC	313	54	309–315	4	0.65	0.62	0.525	+	311–313	2
BL82	DQ674839	$(GA)_{12}(TG)_4TT(TG)_4$	F: GTTGCTGCTTTATCTTTGGA R: AACCACTTCACATAGGCTTG	260	54	254–272	8	0.73	0.75	0.294	+	246–272	4
BL83	DQ674840	$(AC)_6$	F: CTATCCGCCCTGTTCTGA R: ACCAAACATCCCTCAAGC	297	54	297–307	7	0.61	0.76	0.000	+	297–305	3
BL90	DQ674841	$(CA)_{19}$	F: ATGCGAGGGTGGATGATGGG R: GGAAAGCAAAGCCTGGACTA	326	52	296–330	12	0.66	0.86	0.000	+	298–314	3
BL101	DQ674843	$(AC)_{10}A_7$	F: CCATCAGACAGCCAAAGACAA R: TGAAGGCAAGGTCAAGGTTTT	333	54	327–339	6	0.68	0.71	0.063	+	329–339	4

Table 1 *Continued*

Locus	Accession no.	SSR motif	Primers (5′–3′)	Clone size (bp)	T_a (°C)	*Hypophthalmichthys molitrix*					*Aristichthys nobilis*		
						Size range (bp)	*Na*	H_O	H_E	*P*	Amplification	Size range (bp)	*Na*′
BL106-2	DQ674844	$(AC)_{14}$	F: TTTAATTCTTCTAGCTGGACACG R: CACTCCTCTTCCCTCGTAAAT	232	54	218–246	10	0.93	0.84	0.820	+	226–234	4
BL108	DQ674845	$(GT)_9$	F: GATGAATCGCAGGGCGTGAGG R: GCAGAACACGCACAATGGAGA	383	54	383–389	4	0.63	0.62	0.012	+	383–385	2
BL109	DQ674846	$(TG)_{21}$	F: GTGTCCTGGATTCTAGCCG R: CATGAGAGAAACACCTGAACA	242	54	216–256	15	0.78	0.91	0.273	+	216–256	4
BL116	DQ674849	$(CT)_{15}$	F: GCGGGATGAGTTTGAAGAA R: TATGGACTGGACTGCTGGAT	216	54	212–222	4	0.59	0.63	0.102	+	214–222	2
BL123	DQ674850	$(TG)_9$	F: GCGACAGGAACAGTGAAAAC R: CAAAGAAGGCACAAAGGATT	234	54	227–244	8	0.85	0.69	0.138	+	227–246	6
BL125	DQ674851	$(AC)_4ATAG(AC)_7$	F: AACAGAAAAGCAGTGGAATC R: GGAAAGAGTTTGCTATCAGTG	332	52	229–241	6	0.49	0.46	0.602	+	229–237	3
BL132	DQ674852	$(AC)_{10}$	F: CTTTGACTGCTGGTTGGTTGT R: TTTCTTGTCTTCCTGGCTTCT	155	54	149–161	8	0.68	0.80	0.250	+	151–160	5
BL133	DQ674853	$(AC)_9AT(AC)_2TC(CA)_2$	F: GTTGCTAGTCCATTGGGCTTCA R: GCTGTCCGCTCTGCTGTCCTT	145	54	139–153	7	0.49	0.62	0.003	+	139–145	2
BL138	DQ674854	$(TG)_8$	F: ACTGAAAACATCACTGCCACG R: CTCCTTACATCTGCAAGAACG	154	54	138–162	7	0.61	0.62	0.044	+	156–160	2
BL145	DQ674855	$(TG)_{12}$	F: GTGATTGGACGGGATGAACTA R: TCTTTCTTTTCTGTCCGAGGG	107	52	103–127	8	0.70	0.81	0.084	+	113–129	4
BL151	DQ674856	$TGTT(TG)_7$	F: TCTTCAGACTCACCTGGGAATT R: ACTGGATGTTTGATGGGACG	180	52	178–188	4	0.65	0.55	0.004	+	180–192	4
BL167	DQ674857	$(AC)6$	F: AGTGCGCCTAAAGTAAAAG R: AACCAGGACGAATCAAGTA	248	52	248–258	4	0.05	0.07	0.032	+	248–252	3
BL180	DQ674858	$(TG)_9$	F: ATCGTCAGGTAGGCTATGGT R: ATGTAGCAAGGAAGGGAAAA	190	54	186–200	6	0.46	0.56	0.000	+	186–206	9
BL23	DQ136011	$(GT)_5TT(GT)_4$	F: CCTTCGTTTGACGGACAG R: GATGTGGTGATTTCAGCAGC	305	52	303	1	—	—	—	+	301–307	4
BL92	DQ674842	$(TG)_5CGGT(TG)_3TC(TG)_4$	F: TGGTAACAGATGTGCCCGAC R: AAAGATGACACAGTGGACAGA	292	52	292	1	—	—	—	+	292	1
BL110	DQ674847	$TGAG(TG)_2TA(TG)_5$	F: GTACCGTATGTGGGTGGAC R: GGACTGGAGTGGGAGATGAA	325	52	325	1	—	—	—	+	325	1
BL111	DQ674848	$(TG)_2TT(TG)_5$	F: ATCATCCGTCCGCCCGCACAT R: GGCAAGAAAATGACCGCAAG	162	52	162	1	—	—	—	+	162	1

T_a, temperature of DNA annealing; *Na*, number of alleles each locus in silver carp; H_O, observed heterozyosity; H_E, expected heterozyosity; *Na*′, number of alleles each locus in bighead carp (*Aristichthys nobilis*); *P*, probability of being Hardy–Weinberg equilibrium.

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