

# Characterisation of microsatellite loci in silver carp and cross amplification in other cyprinid species



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PRIMER NOTE

# Characterization of microsatellite loci in silver carp (*Hypophthalmichthys molitrix*), and cross-amplification in other cyprinid species

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**Abstract**

**Captive populations of silver carp (*Hypophthalmichthys molitrix*), a major aquaculture species in Asia, would undoubtedly benefit from genetic monitoring and improvement programs. We report the isolation and preliminary characterization of 16 microsatellite loci derived from both conventional and microsatellite-enriched libraries. Inheritance studies confirmed the allelic nature of observed polymorphisms at all loci, while identifying null alleles at two loci. These loci, having varying degrees of polymorphism, should provide useful markers for applied genetic studies. A high degree of cross-amplification among 10 other cyprinid species suggests that these loci may have more widespread utility.**

*Keywords*: cross-species amplification, genetic markers, inheritance, microsatellite enrichment, silver carp

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Silver carp, a species native to China, is now widely cultured in Asia, contributing *c*. 22% (> 3 million tonnes) of world carp aquaculture production (FAO 2005). Recently, concerns about deleterious consequences of inbreeding, hybridization, etc. in aquaculture populations have highlighted the urgent need for improved genetic management of silver carp (and other cyprinid) broodstock in Asia (Penman *et al*. 2005). Microsatellite markers could provide practical tools for this purpose, but as yet only a few polymorphic loci (cross-amplified using common carp primers) have been described (Tong *et al*. 2002). We report here the isolation and characterization of 16 polymorphic microsatellite loci from silver carp DNA.

Silver carp genomic DNA libraries (conventional and microsatellite enriched) were constructed using dephosphorylated pBlueScriptII (KS-) phagemid vector (*Eco*RV digested), Epicurian Coli XL2-Blue ultracompetent host cells (both Stratagene) and size-selected (≈ 200–500 bp) restriction-digested silver carp genomic DNA. The DNA (phenol–chloroform extracted from heart tissue pooled from four individuals from the Northwest Fisheries Resource Development and Management Project stock, Parbatipur, Bangladesh) was fragmented by separate *Alu*I, *Hae*III and *Rsa*I digests, subsequently pooled and then size selected by electrophoresis and excision from agarose gels. Conventional libraries were simultaneously screened for di- and trinucleotide repeats following standard colony lift protocols. Microsatellite enrichment employed biotinylated microsatellite motif sequences bound to streptavidin-coated magnetic particles (described in full by Kijas *et al*. 1994). Colonies were picked into 96-well microtitre plates for ordered array screening (Armour *et al*. 1994). For both library types, colonies were transferred onto Hybond-N membrane (Amersham) for hybridization. Screening of both library types used 10 pmol of appropriate radioisotopically ($^{32}$P γ-ATP) end-labelled target oligonucleotide mix (i.e. 2 pmol each $(GT)_{10}$ $(GA)_{10}$ $(ATT)_8$ $(AGC)_5$, & $(GGA)_5$ for conventional library; 3.3 pmol each $(GACA)_4$ $(GATA)_4$, & $(GGAT)_4$ for enriched library). Hybridization was performed overnight, using a rotisserie-style hybridization oven, in 6× SSC; 0.1% SDS; 42 °C with subsequent washes to a stringency of 5× SSC; 0.1% SDS; 42 °C for 30 min. Following autoradiography, those positive clones

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**Table 1** Repeat motif, PCR primer sequences, optimal annealing temperature ($T_a$), $MgCl_2$ concentration for 16 silver carp microsatellite loci and preliminary population characteristics (based on at least 40 broodstock from the Northwest Fisheries Resource Development and Management Project, Parbatipur, Bangladesh)

| Locus name Accession no. | Repeat motif of original clone | Primer sequence (5′–3′) | Fluor | PCR conditions | | Allele size range (bp) | No. of Alleles | Heterozygosity* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $T_a$ (°C) | $MgCl_2$ (mM) | | | $H_O$ | $H_E$ | H/W† |
| Hmo 01 | $(GATA)_6$ | F: TGTTTGCCACACTGTCCAGAGT | HEX | 62 | 1.5 | 161–165 | 2 | 0.40 | 0.32 | NS |
| AM086448 | | R: GCAGTGTAGTTTCCCCAAAAGACT | | | | | | | | |
| Hmo 02 | $(GGAT)_6$ | F: CATCTGTTCTGAGGGGCTGAG | TET | 60 | 1.5 | 160–180 | 5 | 0.40 | 0.62 | < 0.001 |
| AM086449 | | R: CCCCACTTTACCACCAATTATTAT | | | | | | | | |
| Hmo 03 | $(GACA)_4$ | F: GTTACCGAAAGCGAAACCACAC | FAM | 62 | 1.5 | 166–174 | 3 | 0.50 | 0.54 | NS |
| AM086450 | | R: GAGCATGAAGTATTTCCCACTACA | | | | | | | | |
| Hmo 11 | $(GACA)_2$ | F: CTGCTTGATCACAGGGTTTG | FAM | 60 | 1.5 | 142–166 | 6 | 0.63 | 0.60 | NS |
| AM086451 | $(GATA)_8$ | R: CCTTACAGATAGACAGATATTCAG | | | | | | | | |
| Hmo 13 | $(GACA)_6$ | F: AAACCTGGAAGATGTTCACTGAAT | TET | 60 | 1.5 | 136–168 | 6 | 0.54 | 0.74 | NS |
| AM086452 | | R: GCGCGAGTGTTTGAAGTCTG | | | | | | | | |
| Hmo 15 | $(GATA)_7$ | F: TCCTGGAACAGAACCCACTGA | FAM | 60 | 1.5 | 141–149 | 3 | 0.17 | 0.16 | NS |
| AM086453 | | R: ATTTCGTGCACCATCGCTAAAG | | | | | | | | |
| Hmo 25 | $(GT)_{13}$ | F: TGTGCTGCATTTTCACTTCA | FAM | 60 | 1.5 | 137–146 | 5 | 0.72 | 0.65 | NS |
| AM086454 | | R: TTCTTACTATCCACATTTGTTGTATG | | | | | | | | |
| Hmo 26 | $(GT)_{40}$ | F: GATTTCAGGCACATTGCTTATCT | HEX | 60 | 1.5 | 145–238 | 12 | 0.90 | 0.80 | NS |
| AM086455 | (imperfect) | R: GAGCGTTTCTCATTTGTACTTATTTT | | | | | | | | |
| Hmo 27 | $(GT)_{25}$ | F: CTGTAATTCCGTTTTATCTGTGT | TET | 60 | 1.5 | 101–160 | 13 | 0.38 | 0.82 | < 0.001 |
| AM086456 | | R: ATTGCTGTAAACCATAAAATGTAA | | | | | | | | |
| Hmo 31 | $(GT)_5TTGA$ | F: TCCACAGAAGAAAGAAAGTCT | FAM | 57 | 1.5 | 107–160 | 10 | 0.92 | 0.86 | < 0.01 |
| AM086457 | $(GT)_5TT(GT)_8$ | R: CTCAGAGGAAGGAGATGCT | | | | | | | | |
| Hmo 33 | $(GT)_{12}$ | F: GTGCAGCAGTATGTGAATCAGGACAC | FAM | 59 | 1.2 | 86–126 | 10 | 0.82 | 0.77 | NS |
| AM086458 | | R: GTGCTTCGGGATACCACACTCTTG | | | | | | | | |
| Hmo 34 | $(GT)_{19}$ | F: GTTCCCTGAGGCTTTACAA | HEX | 59 | 1.2 | 114–132 | 8 | 0.67 | 0.63 | NS |
| AM086459 | | R: GGGTCATTATCCTCTCACTTT | | | | | | | | |
| Hmo 36 | $(GT)_9$ | F: ATCGGAGGAGTGCTGTTCAGTCTGGA | FAM | 63 | 1.5 | 208–221 | 6 | 0.46 | 0.51 | NS |
| AM086460 | | R: ACGATTGTTGCCGAACGGGTTGAT | | | | | | | | |
| Hmo 37 | $(GTGTGA)_3$ | F: CACAGCGGAGGGGCAAAGGTC | TET | 65 | 1.5 | 148–192 | 16 | 0.90 | 0.86 | NS |
| AM086461 | $(GT)_3(GC)_2(GT)_{20}$ | R: GGACGCCGTGTGACTGGAGATTTT | | | | | | | | |
| Hmo 39 | $(GT)_{12}GA(GT)_5$ | R: ACAGTTATGAGCTAGCAGCAGTTTCT | TET | 59 | 1.2 | 119–143 | 7 | 0.76 | 0.75 | NS |
| AM086462 | | R: TACGTCGTAATACCAGTGTAATACCC | | | | | | | | |
| Hmo 40 | $(GT)_5(N)_6$ | F: CAGGCAGGCATCCACATAGAGAATC | TET | 63 | 1.5 | 208–240 | 5 | 0.58 | 0.58 | NS |
| AM086463 | $(GA)_7AA(GA)_4$ | R: AGAAGAAATCTGATCGTCACCTATGA | | | | | | | | |

*$H_O$, observed heterozygosity; $H_E$, expected heterozygosity (unbiased; Nei 1978); Fluor, fluorescence dyes used.
†probability test for conformance with Hardy–Weinberg expectations using GENEPOP version 3.4 (Raymond & Rousset 1995).
NS, not significant, i.e. $P > 0.05$.

exhibiting particularly strong signal for presumed microsatellite repeats were sequenced (polymerase chain reaction (PCR) cycle sequencing; ABI 377 mediated detection). Primers for PCR amplification were designed for those clones with sufficient unique flanking sequence at both ends of identified repeat sequences (assisted by commercial software; PRIMER SELECT, DNAStar).

Initial characterization of primer sets involved radioisotopic screening of six individuals. Assays comprised (10 μL final volume): *c.* 100 ng template DNA; 1× PCR buffer (ABgene); 0.9–1.5 mM $MgCl_2$; 150 μM of each dNTP; 0.1 μM of each primer (*c.* 10% of one primer end-labelled with $^{32}P$ γ-ATP 4500 Ci/mmol); and 0.5 U *Taq* polymerase (ABgene). The PCR cycling conditions used were: initial denaturation step at 96 °C for 3 min; 28 cycles at 94 °C for 50 s; xx °C annealing for 50 s; 72 °C for 50 s, where xx is annealing temperature for each primer set (see Table 1). Products were separated on 50-cm long 6% denaturing polyacrylamide gels (SequaGel XR; National Diagnostics) followed by autoradiography. Based on initial screening (both primers for each microsatellite locus being alternatively labelled and evaluated), promising primer sets (i.e. those exhibiting clear resolution and apparent polymorphism) were screened on a larger panel of silver carp from

**Table 2** Cross-amplification of 14 silver carp-derived primer sets in 10 other carp species (common names, where known, in parentheses), as determined by agarose gel electrophoresis of amplimers from two individuals

| Locus | *Labeo rohita* (Rohu) | *Catla catla* (Catla) | *Cirrhinus cirrhosus* (Mrigal) | *Labeo fimbriatus* | *Aristichthys nobilis* (Bighead carp) | *Mylopharyngodon piceus* (Black carp) | *Ctenopharyngodon idella* (Grass carp) | *Barbonymus gonionotus* (Silver barb) | *Cyprinus carpio* (Common carp) | *Tor khudree* (Mahseer) |
|---|---|---|---|---|---|---|---|---|---|---|
| Hmo 01 | – | – | 57, 2.0* | 51, 2.0 | + | 60, 1.5 | 57, 1.5 | 53, 1.5 | 59, 2.0 | 53, 2.0 |
| Hmo 02 | 46, 2.0* | 46, 2.0 | 53, 2.0 | 53, 2.0 | + | + | + | + | + | + |
| Hmo 03 | 46, 2.0 | 46, 2.0 | 46, 2.0 | – | + | – | – | – | – | 49, 2.5* |
| Hmo 11 | + | + | + | + | + | + | + | + | + | + |
| Hmo 13 | + | + | + | + | + | + | + | + | + | + |
| Hmo 15 | + | + | + | 60, 2.0 | + | + | + | + | + | + |
| Hmo 25 | 60, 2.5 | 60, 2.5 | 60, 2.5 | 60, 2.5 | + | + | 57, 1.5 | 60, 2.5 | 60, 2.5 | 60, 2.5 |
| Hmo 26 | + | + | + | + | 57, 2.5 | + | + | 60, 2.5 | 53, 1.5 | 57, 1.5 |
| Hmo 33 | 50, 2.0 | 50, 2.0 | 50, 2.0 | 59, 2.5 | + | + | + | + | 44, 2.0* | 56, 2.5 |
| Hmo 34 | + | + | + | + | + | + | + | + | + | + |
| Hmo 36 | + | + | + | + | + | + | + | 59, 1.5* | + | 59, 1.5* |
| Hmo 37 | + | + | + | + | + | + | + | + | + | + |
| Hmo 39 | – | 46, 2.0 | – | – | 49, 2.0* | 50, 2.5 | 50, 2.5* | – | – | – |
| Hmo 40 | – | – | – | – | + | + | + | – | – | – |

+ indicates detection of a discrete fragment of appropriate size amplified using same PCR conditions as of silver carp. For others, the variables altered [$T_a$ (°C), $MgCl_2$ (mM)] to successfully amplify DNA fragments are shown. – indicates no amplification or smeared product; * indicates more than one band.

the farm stock ($n = 48$) and up to 10 informative family panels (sire, dam, 10–48 progeny). Fluorescent detection genotyping was performed on an ABI PRISM 377 DNA sequencer, chromatograms being analysed with ABI proprietary software (GENESCAN version 3.1.2 and GENOTYPER version 2.5).

Approximately 1–2% of clones from the conventional libraries elicited positive hybridization to the di- and trinucleotide motif mixture. Seventy-two colonies with the strongest signal were selected for sequencing, of which 64 gave readable sequence. Sequences with $(GT)_n$ repeats predominated ($n = 51$), the remainder being $(GA)_n$ repeats. Approximately 50% of both motif classes were perfect repeats. No trinucleotide repeat motifs were found. PCR primer pairs could be designed for 24 loci. Over 8% of clones from the enriched library gave positive hybridization signal. From 121 clones successfully sequenced, 110 different inserts were identified, of which 105 contained repeat motifs. Nearly half of the inserts (46%) contained two or more different repeats. $(GGAT)_n$ repeats predominated, present in 44% of clones. PCR primer pairs could be designed for 40 loci.

Of 40 primer sets screened, 16 sets gave reproducible polymorphic patterns (Table 1). Inheritance studies have confirmed disomic segregation at each locus. In some pedigrees, segregation patterns at two loci (Hmo 02 and Hmo 27) indicated the presence of null alleles. Relatively high frequencies for null alleles were also reflected by highly significant excesses of homozygotes at these two loci among the screened farm sample (Table 1). A third locus (Hmo 33) also showed significant departure from Hardy–Weinberg expectations — but this time with an excess of scored heterozygotes. The reason for this departure is unclear but may simply reflect the nonrandom nature of the farm sample. No significant genotypic disequilibrium among loci was detected, as tested by GENEPOP version 3.4 (Raymond & Rousset 1995). Many of the primer sets were potentially informative among 10 other carp species (Table 2). Bighead carp samples amplified at all loci tested. In farmed conditions, this species readily hybridizes with silver carp, producing fertile hybrids. With two to 16 alleles segregating within one farm stock, this set of genetic markers with a range of discriminatory powers should assist future genetic monitoring/improvement of silver carp.

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