Development of DNA Microsatellite Loci in Chinese Carps and Application to Detection of Hybridization in Broodstock

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Abstract
Microsatellite DNA loci have been developed from silver carp (*Hypophthalmichthys molitrix*) DNA for use in broodstock management and research. Here we describe how three of these loci (*Hmo*1, *Hmo*3 and *Hmo*11) can be used to distinguish between silver carp and bighead carp (*Aristichthys nobilis*). These loci are being used in the analysis of samples collected from hatcheries in different regions of Bangladesh, in parallel with a survey of broodstock management practices. Analysis of samples from two of the hatchery areas revealed a fairly low proportion of hybrid genotypes among broodstock: this included fish which were identified as silver and bighead carp by the hatcheries. The genetic analysis suggested that while some of these fish might be F1 hybrids, others had more complex genotypes suggesting further generations of hybridization or introgression between the species. The study

is ongoing and will attempt to identify factors associated with hybridization in hatchery broodstock. The methodology could also be applied to detect hybrids in nursery or ongrowing stocks.

Introduction

Aquaculture now accounts for nearly 40% of total fish production in Bangladesh (Hussain and Mazid, 2001), with carps, produced in polyculture systems, dominating. The vast majority of the seed for aquaculture in Bangladesh is produced in private hatcheries, of which it is estimated that there are in excess of 600. There is much anecdotal evidence of genetic deterioration of the stocks of carps in Bangladesh, through inbreeding, negative selection and hybridization (Hussain and Mazid, 2001). Stocks of exotic (i.e. non-indigenous) species of carps are particularly vulnerable to such degradation, given that the opportunities to go back to wild populations for broodstock replenishment are very limited. Furthermore, anecdotal evidence suggests that hybridization between the silver carp and bighead carp is common, at least partly due to a shortage of mature bighead carp males towards the end of the breeding season. The silver carp accounts for 25% (126000 t) of reported major carp production in Bangladesh, while there was no reported bighead carp production in the same set of statistics (FAO, 2002), suggesting that the actual levels are low.

Several types of genetic markers have been developed which have potential application to fisheries and aquaculture. Used appropriately, these have the potential to differentiate between species, populations and individuals. Microsatellite DNA loci (Estoup and Angers, 1998) have a core of short, repeated units (generally 2-5 base pairs), flanked by unique sequence DNA. Primers for polymerase chain amplification (PCR) can be designed from the flanking DNA, ensuring specific amplification of single loci, with variation between alleles coming largely from variation in the number of repeat units in the central core region. Some microsatellite loci have very high numbers of alleles per locus (>20), making them very useful for applications such as parent-offspring identification in mixed populations, while others have lower numbers of alleles and may be more suited for population genetics and phylogeny (O'Connell and Wright, 1997; Estoup and Angers, 1998). Primers developed for one species will often cross-amplify microsatellite loci in closely related species (Estoup and Angers, 1998).

As part of the project which is the focus of this workshop, microsatellite loci were developed from a DNA library produced from the silver carp (unpublished data). Preliminary screening showed that some of these loci could be used as species-specific markers to distinguish between silver and bighead carp. Although allozymes can also be used to distinguish between these species, the simplicity of sample

collection (biopsy – e.g. fin tissue or scales), preparation and transportation (fixation in 95% ethanol and transportation at room temperature) for PCR-based DNA techniques make sampling under field conditions much easier.

A survey was designed in which fin samples and information about hatchery management techniques were collected from several hatcheries in each of five major hatchery areas in Bangladesh. This paper reports initial analysis of samples from two of these regions.

Materials and methods

The microsatellite loci were developed using a slight modification of the library enrichment methodology of Kijas *et al.* (1994), from size-fractionated silver carp DNA (unpublished results). The names assigned to the microsatellite loci were derived from the Latin name of the species (*Hypophthalmichthys molitrix*) and a serial number. Four loci were initially identified as being potentially diagnostic for the silver and bighead carp, but one of these (*Hmo*5) was discarded as it gave an inconsistent multiple banding pattern in the bighead carp. The remaining three loci (*Hmo*1, *Hmo*3 and *Hmo*11) were used for this study.

Fin samples were collected from Chinese carps from the reference populations at NFEP, Parbatipur, from six private hatcheries in the Mymensingh region and five private hatcheries in the Jessore region. Each sample was removed from the edge of a fin using scissors, dried and placed into a microcentrifuge tube containg approximately one millilitre of 95% ethanol. The ethanol solution was discarded and replaced later the same day. The samples were then stored at ambient temperature during transportation and at 4-6°C in the laboratory until DNA extraction and analysis.

DNA was extracted using protocols based on either phenol-chloroform or chelex. The three loci were amplified by the polymerase chain reaction (PCR) in separate reactions, and then run in 1.2% standard agarose gels for routine analysis, or 2% Metaphor agarose (Flowgen) to allow higher resolution and closer examination of the allele sizes.

Results

Table 1 summarises the samples analysed from the reference population and the two hatchery regions, with the data from the hatcheries within each region pooled. This shows that five fish with hybrid genotypes were found in the Mymensingh region, and none in the Jessore region. Two of these had been identified as silver carp at the

time of sampling on morphological, while three were identified as bighead carp. Table 1 shows the genotypes of these five fish for each of the three microsatellite loci. Figures 1 and 2 show the appearance of some of these genotypes on agarose gels (in high resolution Metaphor and standard agarose respectively).

Table 1. Genotypes of broodstock from reference and hatchery stocks of silver and bighead carp in Bangladesh. The stocks at NFEP Parbatipur were imported from China in 1994 and served as reference samples for this study; the others were collected from hatcheries in the Mymensingh and Jessore areas (data pooled among hatcheries within each region). The identification of the hatchery stocks to species at the time of sampling was made on morphological grounds and for the purposes of this study was considered to be preliminary until the genotype was confirmed.

Region	Species	Genotype			Total
		Silver	Hybrid	Bighead	
Parbatipur (wild stocks)	Silver	30	0	0	30
	Bighead	0	0	30	30
Mymensingh	"Silver"	74	2 (2.6%)	0	76
	"Bighead"	0	3 (17.6%)	14	17
Jessore	"Silver"	122	0	0	122
	"Bighead"	0	0	12	12

Table 2. Genotypes of the five individuals considered to be hybrids on the basis of analysis using three microsatellite loci *Hmo*1, *Hmo* 3 and *Hmo* 11. S/S = homozygote for the allele found in the silver carp reference population; B/B = homozygous for the allele found in the bighead carp reference population; S/B = heterozygous, having one copy of each of these two alleles.

Hybrid		Microsatellite locus genotype		
		***Hmo*1**	***Hmo*3**	***Hmo*11**
"Silver"	1	S/B	S/S	S/B
	2	S/B	S/B	S/B
"Bighead"	1	S/B	S/B	S/B
	2	S/B	S/B	S/B
	3	S/B	B/B	B/B

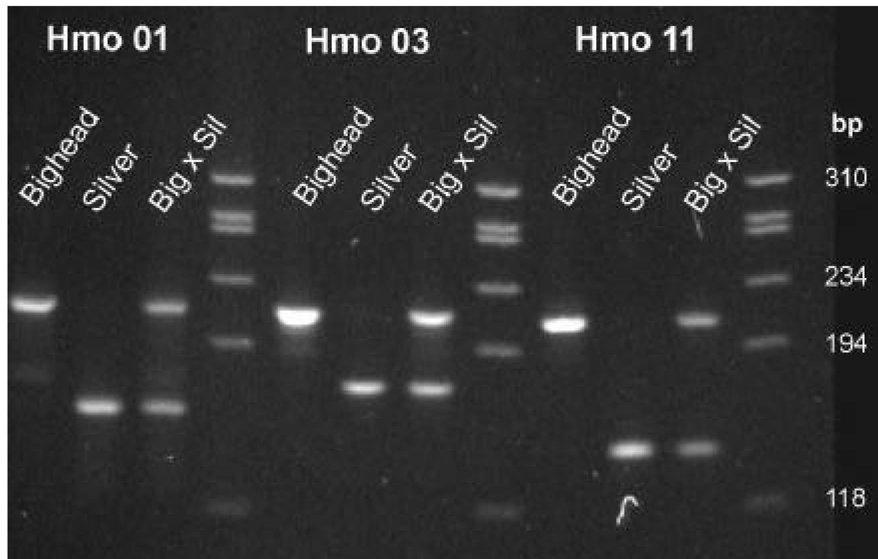

Figure 1. Electrophoresis of PCR-amplified microsatellite loci Hmo1, Hmo3 and Hmo11, showing an example of pure silver carp, pure bighead carp and hybrid (Big x Sil) genotypes for each locus. The right hand lane of each set contains molecular weight standards, with the sizes in base pairs (bp) given at the extreme right of the figure.

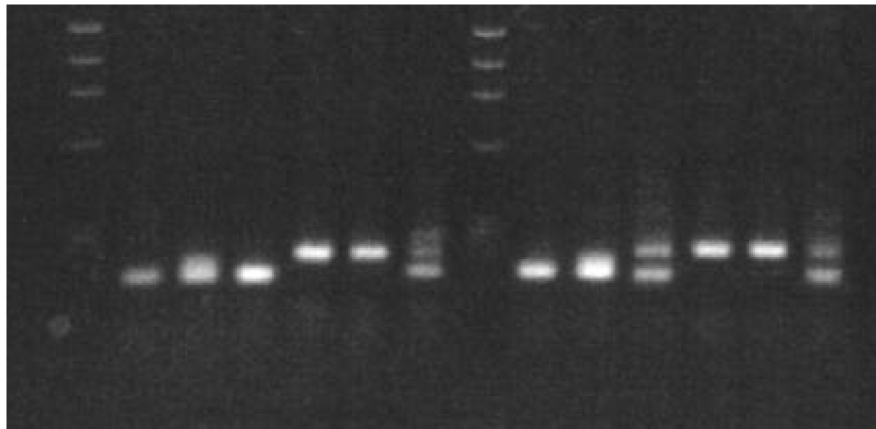

Figure 2. Electrophoresis of PCR-amplified microsatellite loci Hmo3 (left side of gel) and Hmo1 (right side of gel). For each locus, the order of the lanes is molecular weight standards, two reference silver carp samples, "silver" carp hybrid #1 (see Table 2), two reference bighead carp samples and "bighead" carp hybrid #1 (see Table 2).

Discussion

The methodology described in this paper appears to offer a rapid and simple procedure for the detection of hybridization and introgression between silver and bighead carps. Sample collection is quick and easy, and does not require that broodstock are killed. This would also be true for fish of only a few grams in weight, as the reliance on PCR allows even a tiny piece of fin to be used as the source of DNA. Sample storage is also easy, which is important during field work with limited facilities. The use of the chelex-based extraction method reduces the time taken for DNA extraction, and the analysis could be made even more efficient (although more expensive) if all three loci were amplified and analysed together in a "multiplex", where one of each pair of primers for each locus is labelled with a fluorescent dye, allowing separate detection of the alleles of each locus in a single sample in an automatic sequencer (e.g. Fishback *et al*., 1999).

Two of the five hybrid fish detected during this preliminary analysis can be concluded not to be F1 hybrids ("silver" #1 and "bighead" #3 in Table 2), since they displayed a mixture of both heterozygous and homozygous genotypes for the different loci. This would only be expected from backcrosses to one of the parent species, F2 hybrids or further generations of hybridization or introgression. The other three hybrids may have been F1s (heterozygous at all three loci) but this cannot be stated with any degree of certainty from analysis of only three loci. More detailed analysis would require larger numbers of loci.

The proportion of hybrids detected in this preliminary analysis was low (2.6% in silver carp and 17.6% in bighead carp in one of the two hatchery regions studied). It is intended to extend this survey to cover five major hatchery regions of Bangladesh and to attempt to correlate the presence of hybrids with factors such as geographical region, broodstock replacement strategy, presence of one or both parental species in the hatchery, or experience of hatchery manager. We also intend to survey nurseries for the presence of hybrid fingerlings. Anecdotal evidence suggests that F1 hybrids are produced towards the end of the bighead carp spawning season, when mature males are no longer available but mature silver carp males are.

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