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Short Communication

An AFLP-based approach for the identification of sex-linked markers in blunt snout bream, *Megalobrama amblycephala* (Cyprinidae)

H.O. Rao, J.C. Deng, W.M. Wang and Z.-X. Gao

College of Fishery, Key Lab of Agricultural Animal Genetics,
Breeding and Reproduction of Ministry of Education/
Key Lab of Freshwater Animal Breeding, Ministry of Agriculture,
Huazhong Agricultural University, Wuhan, Hubei, China

Corresponding author: Z.-X. Gao
E-mail: gaozx@mail.hzau.edu.cn

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ABSTRACT. Sex-specific DNA markers are useful for studying sex-determination mechanisms and establishment of monosex populations. Three widely spaced geographical populations (Liangzi, Poyang and Yuni Lakes in China) of blunt snout bream (*Megalobrama amblycephala*) were screened with AFLPs to search for sex-linked markers. Female and male pools (10 individuals in each pool) from each population were screened using 64 different primer combinations. A total of 4789 genomic fragments were produced, with a mean frequency of 75 bands per primer pair. Three different primer combinations produced putative sex-associated amplifications and were selected for individual screening in the three populations. However, none showed sex specificity when we converted these three markers into sequence characterized amplified region markers and evaluated all the individuals from the three populations.

Key words: AFLP; Sex-specific marker; SCAR;
Megalobrama amblycephala

INTRODUCTION

Blunt snout bream (*Megalobrama amblycephala* Yih, 1955), commonly known as Wuchang fish, is an endemic species in China. Its natural distribution is restricted to the middle and lower reaches of Yangtze River, such as Liangzi (LZ), Poyang (PY) and Yuni (YN) Lakes (Li et al., 1991). *M. amblycephala* is a species of ray-finned fish in the genus *Megalobrama*, which includes other three species *M. skolkoii*, *M. hoffmanni* and *M. pellegrini*. Among these four species, *M. amblycephala* has been widely favored for its delicacy and recognized as a main aquaculture species in the freshwater polyculture system since 1960s in China. As the success of artificial propagation and its high economic value, *M. amblycephala* aquaculture industry has been greatly developed in the past decades. As early as 2001, the total output reached 541,115 tons (CAFS, 2001). However, growth depressions have been reported in the culture stock of *M. amblycephala* due to inbreeding during artificial propagation. Moreover, as its widespread through the transplant and artificial breeding, germplasm resources of the bream were under threat of recession and mixture (Li and Yang, 1996). Nowadays, the cultured *M. amblycephala* is gradually exhibiting early sexual maturity, low growth rate and susceptible to disease. By comparison, early sexual maturity of female *M. amblycephala* can result in reduced feeding and growth as reported in other fish species (Simpson et al., 1996). Monosex male populations can eliminate the problem of precocious female maturity and their consequences (Dunham, 1990). Therefore, sex control has important application potential in *M. amblycephala* aquaculture. Such monosex populations are typically produced by sex reversal and selective breeding. The generation and maintenance of monosex stocks require that genetic and phenotypic sex can be independently discernible.

The molecular marker technique has been demonstrated to be an effective tool for both the identification of sex-specific genetic markers and sex control (Liu and Cordes, 2004). Amplified fragment length polymorphism (AFLP) analysis in combination with bulked segregant analysis (BSA) approach has been shown to be a fast and efficient way to identify markers linked to target genes responsible for desirable traits (Michelmore et al., 1991). AFLP screening with BSA approach was used successfully in the isolation of sex-specific markers of aquatic animals including the Nile tilapia *Oreochromis niloticus* (Ezaz et al., 2004), the rainbow trout *Oncorhynchus mykiss* (Felip et al., 2005), and the spotted halibut *Verasper variegatus* (Ma et al., 2010).

In the present study, the AFLP technique was used to identify sex-specific markers in *M. amblycephala* based on pooled DNA samples from known male and female individuals, which were sampled from its three natural populations.

MATERIAL AND METHODS

M. amblycephala were collected from three wild populations in Liangzi, Yuni and Poyang Lakes (Table 1). Caudal fin samples were taken from 20 mature individuals (10 females and 10 males) of each population. The phenotypic sex of fish was determined by distinguishing external morphology during the reproductive season or gonadal structure of the histological section. Total genomic DNA was extracted from 50 mg tissue sample according to Waters et al. (2000). The quality and concentration of DNA was measured by NanoDrop 2000 and then adjusted to 100 ng/µL.

Table 1. Samples and geographic information of sampling sites.

Name	Locality	Area (km^2)	Latitude	Longitude	No. of samples
Liangzi Lake	Hubei Province	227.15	30.34	114.51	20
Yuni Lake	Hubei Province	152.1	29.85	112.12	20
Poyang Lake	Jiangxi Province	2262	29.24	115.95	20

AFLP technique in combination with BSA approach was performed as previously described (Michelmore et al., 1991; Vos et al., 1995; Ezaz et al., 2004) with minor modification. Genomic DNAs of female and male from each population were pooled to generate female (N = 10 fish) and male (N = 10 fish) pools. Six pooled DNA samples were prepared using 10 µL (100 ng/µL) genomic DNA from each individual. Each pool (500 ng) was digested with *Eco*RI/*Mse*I prior to ligation with restriction site-specific adaptors (Table 2). Pre-amplication was carried out utilizing adaptor common primers, and selective amplification by 64 different primer combinations (Table 2) was conducted with diluted (50-fold) pre-amplification product. AFLP-PCR products were separated on 6% denaturing polyacrylamide gels and visualized by silver staining.

Table 2. The AFLP adapters, common primers and selective primer used in this study for sex-specific marker screening in *Megalobrama amblycephala*.

Primer	Primer sequence (from 5' to 3' direction)	
	*Eco*RI	*Mse*I
Adapter	CTC GTA GAC TGC GTA CC	GAC GAT GAG TCC TGA G
Common primer	E00: GAC TGC GTA CCA ATT C	M00: GAT GAG TCC TGA GTA A
Selective primer	E1: E00+AAC	M1: M00+CAT
	E2: E00+AAG	M2: M00+CAA
	E3: E00+ACA	M3: M00+CCA
	E4: E00+ACT	M4: M00+CCT
	E5: E00+AGA	M5: M00+CTA
	E6: E00+AGC	M6: M00+CTT
	E7: E00+ATC	M7: M00+CGA
	E8: E00+ATG	M8: M00+CGC

Sex-specific bands were directly excised from the 6% denaturing polyacrylamide gel and eluted from the gel by incubation in 30 µL sterilizing water at 4°C overnight. The re-amplification PCR was performed with the corresponding primer pairs under the same conditions as before. PCR products were recovered from agarose gels using gel extraction kit and cloned into PMD-18 T vector as described (Ma et al., 2010) and then selected for sequencing. The sequences of different clones of the same marker and the sequences of the different markers were assembled and compared using the DNAMAN version 4.0 software. Based on the sequence information, the primer pairs were designed with the Primer Premier 5.0 software and synthesized commercially (Sangon, Shanghai, China). SCAR primers were employed to amplify the genomic DNA of the female and male individuals from three populations.

RESULTS AND DISCUSSION

A total of 4789 legible genomic fragments were identified with an average of 75 bands per primer combination. Based on the primary screens across the pooled DNA samples, 30, 28 and 21 different primer combinations, which gave putative sex-associated amplifications, were selected for individual screening in different geographical populations. Based on the analysis of the individual DNA samples, three primer combinations showed sex-associated bands in different populations (Table 3). However, when we converted the three markers into SCAR markers, none showed a different band pattern between female and male individuals from three populations.

Table 3. Summary of putative sex-linked markers and their percent in females and males *Megalobrama amblycephala*, respectively.

Primer combinations	Populations	Size	Sex of DNA pools with sex-specific bands	Percent of individuals with sex-specific bands
M7/E7	Poyang Lake	846 bp	Male	70%
M2/E1	Yuni Lake	830 bp	Male	90%
	Poyang Lake		Male	50%
M5/E5	Yuni Lake	886 bp	Female	80%
	Poyang Lake		Female	40%

Recently, AFLP screening and BSA were used successfully in the isolation of sex-specific markers of aquatic animals. However, many studies have demonstrated that sex-specific markers could vary among species, strains or even geographical populations. The female-specific marker identified by Xia et al. (2011) in loach *Paramisgurnus dabryanus* from the ancient Yellow River Wetland did not show sex specificity in the closely related species *Misgurnus anguillicaudatus* and other two geographical populations. Chen et al. (2009) demonstrated that the male-specific markers observed in the common carp *Cyprinus carpio* from the Yellow River did not show sex specificity in three other common carp populations. Other sex-linked markers found in the Antarctic icefish *Chionodraco hamatus* (Capriglione et al., 1994) and the piapara semen *Leporinus elongatus* (Nakayama et al., 1994) both show variation among strains. These studies suggest that sex-specific markers may vary among strains and geographical populations owing to the DNA mutation, insertion or deletion events that occur on or near the sex-specific sites (Gao et al., 2010). In the present study, the AFLP fragments that produced by M2/E1 and M5/E5 showed a significant difference between females and males when evaluated in the individual females and males from related populations. But, none showed sex specificity when we converted the AFLP markers into SCAR markers.

The study to identify sex-specific markers in fish species seems laborious owing to the variability and plasticity in sex chromosome organization. The success of the identification of sex-specific markers in fish species is relevant with the presence of a sex chromosome or non-chromosomal genetic sex-determining mechanisms in the target species (Sriphairoj et al., 2007). In *M. amblycephala*, sex chromosomes have not been identified by classical karyotype (Lu et al., 1984). In addition, sex determination mechanism and sex-linked markers have not been studied in *M. amblycephala* in the published literature. The failure in search of sex-specific DNA markers could be due to the size of the genome, the number of markers screened and the proportion of the genome that is sex specific in the species studied (Penman and Piferrer, 2008). Our failure to identify sex-specific markers seems to strengthen the possibility that sex chromosomes are either not present or they weakly differentiated in the genomes of *M. amblycephala*.

Our study was the first attempt, to our knowledge, to find sex-specific markers in *M. amblycephala*. Despite the failure to find such markers, our data provide some useful information for further studies targeting similar goals.

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