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No sex-specific markers detected in bluegill sunfish *Lepomis macrochirus* by AFLP

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The amplified fragment-length polymorphism (AFLP) technique was used to identify sex-specific markers in bluegill sunfish. A total of 12 835 loci were produced by 256 primer combinations, of which nine (0·73‰) exhibited presumed sex-associated amplifications in the pooled samples; however, none of which revealed sex specificity upon individual evaluation.

Key words: AFLP; *Lepomis macrochirus*; sex-specific marker.

The bluegill sunfish *Lepomis macrochirus* Rafinesque is currently recognized as one of the most valuable North American recreational fish species and an increasing economically important aquaculture species in the U.S.A. The common problems in small impoundments and farm ponds stocked with this species are overcrowding, prolific reproductive nature and early maturation (Mitzner, 1984; Arslan, 2002). An alternative approach to the management of *L. macrochirus* in small impoundments is to stock monosex populations. Monosex populations can eliminate the problem of prolific reproduction, precocious maturity and their consequences (Dunham, 1990). A recent study has shown that the male *L. macrochirus* also appear to hold a greater potential for the food market due to their fast growth relative to females (Hayward & Wang, 2006). These findings suggest that monosex (all-male) culture will hold considerable potential as a method to increase the efficiency and profitability of *L. macrochirus* aquaculture. Such monosex populations are typically produced by sex reversal and selective breeding. A lack of obvious heteromorphic sex chromosomes in this species, however, has resulted in limited knowledge on sex chromosomal system (XY or ZW, *etc*.) (Childers, 1968). In addition, sex determination cascades and sex-specific markers have not been reported in *L. macrochirus*. This has limited the possibility of increasing aquaculture production through a monosex culture approach.

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Teleosts exhibit reduced sexual dimorphism and a variety of sex determination systems (Bull, 1983). Sex chromosomes in fishes are thought to have evolved on parallel pathways and often show signs of high plasticity (Price, 1984; Solari, 1994; Devlin & Nagahama, 2002). The morphologically distinct sex chromosomes were described only in a few species such as lake trout *Salvelinus namaycush* (Walbaum), rainbow trout *Oncorhynchus mykiss* (Walbaum) and sockeye salmon *Oncorhynchus nerka* (Walbaum) (Hartley, 1987). Most teleosts do not exhibit morphological differentiation in sex chromosomes. Thus, alternative techniques based on molecular-level differences are required for sex identification. DNA-based tests can solve this problem, but sex-specific markers should initially be isolated (Devlin *et al*., 2001). Sex-specific markers have been identified in several fish species using different approaches, including rapid amplification of polymorphic DNA (RAPD) as (Kovács *et al*., 2001), AFLPs (Griffiths *et al*., 2000; Woram *et al*., 2003; Felip *et al*., 2005; Chen *et al*., 2008), microsatellite markers (Sakamoto *et al*., 2000; Nichols *et al*., 2003; Palti *et al*., 2004) and a single locus marker (Iturra *et al*., 2001). Nevertheless, the variable in sex chromosome organization in fishes implies that the number and type of loci involved in sex determination in fishes can be fluid among or within species (Devlin & Nagahama, 2002; Volff *et al*., 2003). The first sex-determining gene (dmY/dmrt1Y) has been identified in a non-mammalian vertebrate, the teleost fish medaka *Oryzias latipes* (Temminck & Schlegel) (Matsuda *et al*., 2002, 2003); however, it is not the universal sex-determining gene in fishes (Kondo *et al*., 2003; Volff *et al*., 2003). Thus, it is essential to isolate sex-specific DNA markers for different fish species. Among the methods for identifying sex-specific markers, the amplified fragment-length polymorphism (AFLP) approach has been frequently used due to the simplicity and high efficiency in whole genome scanning (Griffiths & Orr, 1999).

In this study, the AFLP technique was used to identify sex-specific markers in *L. macrochirus* based on pooled DNA samples from known male and female individuals, which were sampled from three different populations.

Lepomis macrochirus were originally collected from two wild populations in southern (Hocking: 39° 22′ N; 82° 16′ W) and northern (Wooster: 40° 48′ N; 81° 56′ W) Ohio, and one domesticated population (Hebron) in the Ohio Hebron State Hatchery in 2005. The fish were maintained at the Ohio State University South Centers Wet Laboratory. In September 2007, finclip samples were collected and stored in 95% ethanol at −20° C. Phenotypic sex of fish was determined by examining external morphology during the reproductive season (McComish, 1968) or gonadal structure by histological analysis (Wang *et al*., 2008).

Total genomic DNA was extracted from 50 mg tissue sample according to Waters *et al*. (2000). A sex-type pool strategy for the identification of sex-linked polymorphisms using an AFLP approach was set up as follows: four pooled DNA samples were prepared using 10 μl (100 ng μl^{-1}) of genomic DNA from each individual. Pools A and C had 10 females and 10 males from the Hocking population, respectively. Pools B and D had 10 females (Dave 5 and Hebron 5) and 10 males (Dave 5 and Hebron 5), respectively. The AFLP analysis was essentially carried out using the methods reported by Vos *et al*. (1995) with minor modification. Genomic DNA (*c*. 500 ng) was digested with *Eco*RI and *Mse*I in a double enzymes digestion buffer system (Promega; www.promega.com). The *Mse*I and *Eco*RI adapters were ligated to the DNA fragments using T4 DNA ligase (Promega). Adapter common primers (Table I) were used for pre-amplification. Selective amplification by

TABLE I. The amplified fragment-length polymorphism (AFLP) adapters, common primers and selective primers used in this study for sex-specific marker screening in *Lepomis macrochirus*

Primer	Primer sequence (from 5′ to 3′ direction)	
	*EcoR*I	*Mse*I
Adapter 1	CTC GTA GAC TGC GTA CC	GAC GAT GAG TCC TGA G
Adapter 2	AAT TGG TAC GCA GTC	TAC TCA GGA CTC AT
Common primer	GAC TGC GTA CCA ATT C (E00)	GAT GAG TCC TGA GTA A (M00)
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
	E9: E00 + ACC	M9: M00 + CAC
	E10: E00 + ACG	M10: M00 + CGT
	E11: E00 + AGT	M11: M00 + CTC
	E12: E00 +AGG	M12: M00 + CTG
	E13: E00 + TGT	M13: M00 + GTT
	E14: E00 + TGA	M14: M00 + GTA
	E15: E00 + TCT	M15: M00 + GCT
	E16: E00 + TCA	M16: M00 + GCA

256 different primer combinations (Table I) was conducted with diluted (50-fold) pre-amplification product. Polymerase chain reaction (PCR) products were separated using 4·5% denatured polyacrylamide gels and visualized using silver staining. The primer combinations yielding AFLP bands that were associated with a single sex of bluegill sunfish were re-analysed in individual DNA samples of each pool.

A total of 12 835 loci were produced using 256 AFLP primer combinations, including 531 (4·14%) polymorphic loci among different pools. Among the 256 primer combinations, only nine (3·52%) primer combinations yielded sex-associated amplifications across the pooled DNA samples (Table II). Four AFLP loci (0·03%) were initially considered as possibly being female specific because they were only amplified in two female DNA pools, and another five AFLP loci (0·04%) were only amplified in two male DNA pools (Table II). When these loci were re-analysed in all samples, including all individual samples composed of DNA pools, however, the sex-specific markers were only observed in a limited number of individuals of putative sex (Table II). These results revealed that for each putative sex-specific marker, the putative sex-specific bands in the pooled DNA samples were virtually caused by the individual polymorphism.

Many studies have demonstrated that the AFLP technique in combination with a sex-typed pool strategy is a robust approach for identification of sex-specific markers in teleost fish (Griffiths *et al*., 2000; Woram *et al*., 2003; Felip *et al*., 2005; Chen *et al*., 2008). As the identity of the sex chromosomes is very labile, sex-linked

TABLE II. Summary of candidate sex-specific amplicons based on bulked samples

Primer combinations that yielded sex-specific bands (loci name)	Sex of DNA pools with sex-specific bands	Per cent of individuals with sex-specific bands
E4/M10 (Lma429)	Female	45
E12/M8 (Lma695)	Female	30
E14/M6 (Lma341)	Female	25
E15/M9 (Lma756)	Female	25
E5/M14 (Lma870)	Male	45
E8/M16 (Lma342)	Male	20
E9/M1 (Lma1092)	Male	25
E9/M3 (Lma566)	Male	25
E14/M2 (Lma237)	Male	25

Loci were named with the abbreviation of the bluegill sunfish scientific name and the size of the band.

genes could differ according to the species, races or even populations. In Nile tilapia *Oreochromis niloticus* L., Ezaz *et al*. (2004) found four family-specific sex-linked markers that did not show sex specificity in the unrelated individuals. The studies on rainbow trout *O. mykiss* (Walbaum) revealed that Y-chromosome-linked AFLP markers were essentially strain specific resulting from the genetic differences on the Y-chromosome and suggested an intraspecific genetic polymorphism of *O. mykiss* Y-chromosome (Felip *et al*., 2005). Sriphairoj *et al*. (2007) found that the presumed sex-associated AFLP fragments in the initial samples of striped catfish *Pangasianodon hypophthalmus* (Sauvage) did not show sex specificity in the another population, which suggested a possibility of being the population sex-specific markers. The male-specific marker identified by Chen *et al*. (2009) in Yellow River common carp *Cyprinus carpio* L. did not show sex specificity in the other three populations. These reports reveal that the presumed sex-linked markers may vary among strains due to the DNA mutation, insertion or deletion events that occur on or near the sex-specific site. In the present study, three different populations were used to avoid the strain-specific or population-specific phenomenon existing in the detection of sex-specific markers. The sex-specific markers, however, were still not identified in any single population.

The success of the identification of sex-specific markers depends mainly on the presence of a sex chromosome, *e.g*. as in African catfish *Clarias gariepinus* (Burchell) (Kovács *et al*., 2001), Chinook salmon *Oncorhynchus tshawytscha* (Walbaum), chum salmon *Oncorhynchus keta* (Walbaum) and coho salmon *Oncorhynchus kisutch* (Walbaum) (Brunelli & Thorgaard, 2004), *O. mykiss* (Felip *et al*., 2005) or non-chromosomal genetic sex-determining mechanisms in the target species, as in the three-spined stickleback *Gasterosteus aculeatus* L. (Griffiths *et al*., 2000). In contrast, failures to identify sex-specific markers have been reported in species without detectable sex chromosomes or genetic sex-determining systems, *e.g*. green-spotted pufferfish *Tetraodon nigroviridis* (Marion de Procé) (Li *et al*., 2002), sturgeons (Wuertz *et al*., 2006) and *P. hypophthalmus* (Sriphairoj *et al*., 2007). The sex-determining system in *L. macrochirus* is still unclear. Haldane's rule (Haldane, 1922) pointed out that 'when in the F_1 offspring of a cross between two animal species or races, one sex is absent, rare, or sterile, that sex is always the heterozygous

sex'. Given that hybrid *L. macrochirus* populations usually exhibit skewness towards maleness (Krumholz, 1950; Crandall & Durocher, 1980) or predominantly male sex ratios (Brunson & Robinette, 1987), female bluegill sunfish are probably heterozygametic (Childers, 1968). Moreover, as >50% intersex and small number of males and females were produced by oral administration of androgens to sexually undifferentiated *L. macrochirus* fry, Al-Ablani (1997) suggested that sex determination in *L. macrochirus* is probably polygenic, where autosomal genes may be involved in the process of sex differentiation and the sex of the fry may be determined as a result of a balance between the sum of sex-determining genes. In the present study, despite 12 835 AFLP loci being tested and a considerable proportion of the *L. macrochirus* genome screened, however, not a single sex-specific marker was found. The failure in search of sex-specific DNA markers could be due to the size of genome, the number of markers screened and the proportion of the genome that is sex specific in the species studied (Keyvanshokooh *et al*., 2007; Penman & Piferrer, 2008).

This study was the first attempt to find sex-specific markers in *L. macrochirus*. Despite the failure to find such markers, these data offer useful information for further studies targeting similar goals. For future investigations, we recommend the use of an alternative approach, possibly focusing on the gene expression patterns during the course of sex determination and differentiation.

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