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AFLP reveals no sex-specific markers in Persian sturgeon (*Acipenser persicus)* or beluga sturgeon (*Huso huso*) from the southern Caspian Sea, Iran

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The late sexual maturity in sturgeon and the absence of morphological differences between males and females makes sex discrimination difficult. Identification of sex at an early life stage is of high interest in caviar production because it allows efficient selection of females. In this study, the genome of 10 mature male and 10 mature female specimens of Persian sturgeon *(Acipenser persicus)* and beluga sturgeon (*Huso huso*) were screened using AFLP and 100 primer combinations. Results showed a total of 3771 and 3779 scoreable bands in *A. persicus* and *H. huso*, respectively. Approximately 30% of markers in *A. persicus* and 29.6% *H. huso* were polymorphic. No sex specific makers were identified. The results of the present study suggest that the sex chromosomes are not extensively differentiated in sturgeon species, or possibly the methods utilized were not sufficiently sensitive to recognize them. © 2011 Progress in Biological Sciences, Vol 1, No.1, 55-60.

Key words: Persian sturgeon, beluga, sex determination, AFLP, *Acipenser persicus*, *Huso huso*

INTRODUCTION

Sturgeon, the source of caviar, inhabit the northern hemisphere exclusively (Billard and Lecointre, 2001). Overfishing, deterioration of natural spawning grounds and pollution have seriously threatened sturgeon species to the verge of extinction (Pourkazemi, 2006). The decline in natural populations has increased interest in aquaculture caviar production, and the number of aquaculture farms is rapidly increasing. However, fishes can be sexed only after several years of rearing (Logan et al., 1995, Bahmani and Kazemi, 1998), and the rearing of males for the first years significantly increases production costs, up to 30% of the total cost (Wuertz et al., 2006). Even after maturity, sexing by ultrasound, endoscopy, or by measuring plasma levels of hormone is time consuming and often stressful for the animals (Hett and Ludwig, 2005). The identification of a molecular marker and the consequent development of a PCR-based method that allows sex identification at early life stages would be a valuable innovation, significantly benefiting aquaculture and reducing depletion of natural populations. Heteromorphic sex chromosomes have not been revealed in any sturgeon species (Fontana and Colombo, 1974; Holcik, 1986; Van Eenennaam, 1997). However, studies of gynogenetic females indicated a heterogamety (WZ/ZZ) mechanism in white sturgeon *A. transmontanus* (Van Eenennaam et al., 1999), bester sturgeon (*H. huso* female × *A. ruthenus* male) (Omoto *et al*., 2005) and shortnose sturgeon *Acipenser brevirostrum* (Flynn et al., 2006). Identification of sex-linked genomic polymorphisms has been pursued through the comparative screening of previously sexed animals using Amplified Fragment Length Polymorphism (AFLP) (Wuertz et al., 2006) and randomly amplified polymorphic DNA (RAPD)

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(Keyvanshokooh et al., 2007) approaches without satisfactory results. This lack of evidence suggests that sex determination may be environmental (in Lake Sturgeon (*Acipenser fulvescens*) McCormick et al., 2008). However, the hypothesis of genetically based sex determination in sturgeon is supported by the balanced sex ratio consistently observed with artificial reproduction (Van Eenennaam, 1997).

Among marker systems currently available, AFLP is dominant, providing a high level of polymorphism detection and multilocus and genome-wide marker profiles (Vos et al., 1995). Amplified Fragment Length Polymorphism has been used to assess sex-specific markers in the three-spined stickleback (*Gasterosteus aculeatus)* (Griffiths et al., 2000) and rainbow trout *(Onchorhynchus mykiss)* (Sato et al., 2001). It also widely used for studying sex specific markers in avian species (Griffith and Orr, 1999).

Persian sturgeon *A. persicus* Borodin 1897 and the beluga sturgeon *Huso. huso* L. 1758 are the most important species in the Caspian Sea and are considered candidate species for aquaculture. Persian sturgeon is chiefly distributed in the southern Caspian Sea (Pourkazemi, 2006). Considering the endangered status of these species, artificial reproduction and stocking is necessary for their survival.

Male and female genomes of *A. persicus* and *H. huso* have been randomly screened by AFLP (Vos et al., 1995). The present study presents the first comparative genome analysis of male and female Persian and beluga sturgeons from the Caspian Sea using AFLP technique. The search for possible sex-linked markers was performed despite previous negative results with other sturgeon species, as sex determination mechanisms may be different even among closely related fish species.

MATERIAL AND METHODS

Fish sampling and DNA extraction

Fin tissue samples were prepared from 20 adult beluga and 20 adult Persian sturgeons of both sexes (10 of each sex) and preserved in ethanol. Spawners were caught in March 2007 from the Caspian Sea coastline and transferred to the Shahid Beheshti Sturgeon Propagation Complex, located in Rasht, Iran. Sex of spawners was identified by examination of testes and ovaries.

Total genomic DNA was extracted from preserved fin tissue using the phenol–chloroform procedure (Pourkazemi et al., 1999). The quantity and quality of extracted DNA was assayed using Nanodrop (ND1000, USA) and 1% agarose electrophoresis.

Polymerase chain reaction

The AFLP procedure was conducted as described by Vos et al. (1995). An aliquot of total DNA (250 ng) was digested using 10U *MseI* and *EcoRI* in Tango buffer (Fermentas, France) in a 20 µl reaction at 37^{o}C for 90 min. Ligation was immediately initiated with a 5 µl mix of *Mse*I (50 mM) and *Eco*RI (5 mM) adapters, T_4 DNA Ligase, and 1X ligation buffer and carried out at 37^{o}C for 3 h. Preamplification PCR was performed on 15 µl volumes, with 5 µl diluted restricted/ligated DNA, 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM of each dNTPs, 10 pmol *Mse*I+C and *Eco*RI+A primers (Table 1).

All PCR amplifications were carried out with an Eppendorf thermocycler (Mastercycler ep gradient, 96 plus, Eppendorf, Germany) with 30 sec denaturation at 94^{o}C, 30 sec annealing at 56^{o}C, and 1 min extension at 72^{o}C, for 22 cycles. For selective amplifications, preliminary trials were conducted using 100 primer combinations. Selective PCR amplifications were performed on 15 µl volumes, with 3 µl diluted (1:10) preamplification products as template, 1X PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs (CinnaGene, Tehran, Iran), and 10 pmol *M*+4 and *E*+3 primers (Table 1). PCR consisted of 1 min denaturation at 94^{o}C, 10 cycles of 30 sec at 94^{o}C, 30 sec at 63^{o}C, and 2 min at 72^{o}C with the annealing temperature decreasing from 63^{o}C in 1^{o}C increments in cycles 2-10. This was followed by 26 cycles of 30 sec at 94^{o}C, 30 sec at 54^{o}C, and 2 min at 72^{o}C.

Electrophoresis

After PCR, an equal volume of loading buffer (98% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added. The reaction mixture was then heated for 10 min at 95^{o}C and immediately cooled on ice. The gel

Table 1. Sequences of primers and adaptors for DNA-AFLP analysis.

Oligonucleotide	Sequence
*Eco*RI adapter	5′- CTC GTA GAC TGC GTA CC-3′ 5′- AAT TGG TAC GCA GTC TAC-3′
*Mse*I adapter	5′-GAC GAT GAG TCC TGA G-3′ 5′- TAC TCA GGA CTC AT-3′
*Eco*RI+1 primer	5′- GAC TGC GTA CCA ATT C A-3′
*Mse*I+1 primer	5′-GAT GAG TCC TGA GTA A C-3′
*Eco*RI+3 primers*	5′- GAC TGC GTA CCA ATT C ANN-3′
*Mse*I+4 primers**	5′-GAT GAG TCC TGA GTA A CNNN-3′

**Eco*RI+3 primers are: *E*-AAT, ACA, ATT, ACC, AAC, AAA, ACG, ATA, ATC, and AAG
***Mse*I+4 primers are: *M*- CACA, CGAA, CGAT, CCTT, CATA, CATT, CGTC, CTGC, CCGT, and CATT

Table 2. The estimated number of markers screened by AFLP.

Species	Number of primer pairs	Length of Markers (bp)	Number of counted markers	Number (%) of polymorphic markers
A. persicus	100	50-600	3771	1132 (30%)
H. huso	100	50-600	3779	1120 (29.6%)

was pre-electrophoresed at 55°C, 95 W for 20 min, then 5 µl of the amplified DNA was loaded and run on a 6% polyacrylamide gel (19:1 acrylamidae/bisacrylamidae; 7.5 M Urea; 1X TBE buffer) with 1X TBE buffer on a vertical gel electrophoresis system (BIO-RAD sequi Gen, GT, 38 ×30 cm/ PowerPAC 5000, USA) at 85 W and 50°C for 90 min. After electrophoresis, the bands were visualized using silver staining. Clear and unambiguous bands of lengths ranging from 40 to 1000 bp were considered for further analysis.

Data analysis

One hundred combinations of primer sets were tested and only the recognizable AFLP markers were scored. The scored AFLP bands were dark, consistent, and repeatable in individuals across polymerase chain reactions and gels. The obtained results from male and female individuals were scored as presence (1) and absence (0) of band in each polymorphic locus. The number of polymorphic, monomorphic, and total alleles per each primer combination for each species was manually scored.

RESULTS

Figures 1 and 2 show the denatured acrylamide gel of male and female Persian and beluga sturgeons using *E*+3/*M*+4 primer combination of AFLP technique. Amplified Fragment Length Polymorphism band patterns in *A. persicus* and *H. huso* showed high levels of variation among individuals, unrelated to sex.

A set of 100 (*E+3* and *M+4*) primer combinations in *A. persicus* and *H. huso* yielded a total of 3771 and 3779 scoreable bands, respectively, of which 30% in *A. persicus* and 29.6% in *H. huso* were polymorphic. The fragments ranged from 50 to 600 bp without revealing any sex-specific markers (Table 2).

DISCUSSION

The 100 primer combinations used and the markers analyzed, ranging from 50 to 600 bp, revealed no sex markers in the sturgeon species studied. Previously, Wuertz et al. (2006) used AFLP to search for sex-specific DNA markers in four sturgeon species, *A. naccarii*, *A. baerii*, *A. gueldenstaedtii*, and *A. ruthenus*. Although

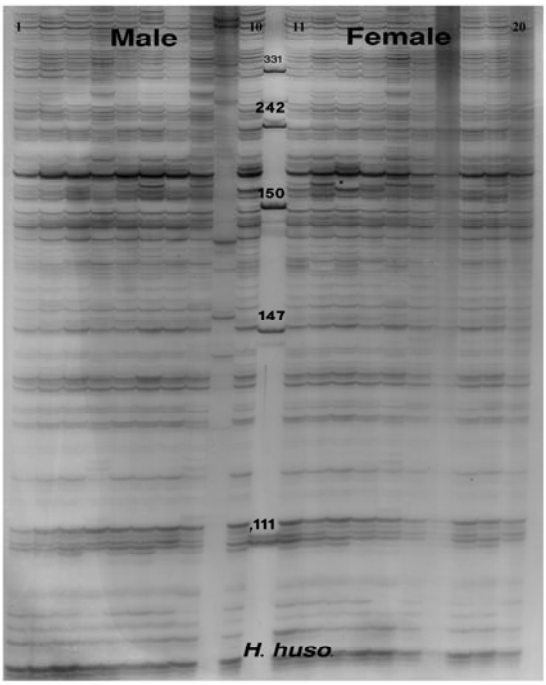

Fig. 1. AFLP generated from DNA samples of 20 *Huso huso* using a set of *Eco*+3 / *Mse*+4 primers. 1-10 male and 11-20 female, (M) ladder, pUC Mix Marker, 8 (Ferments, France).

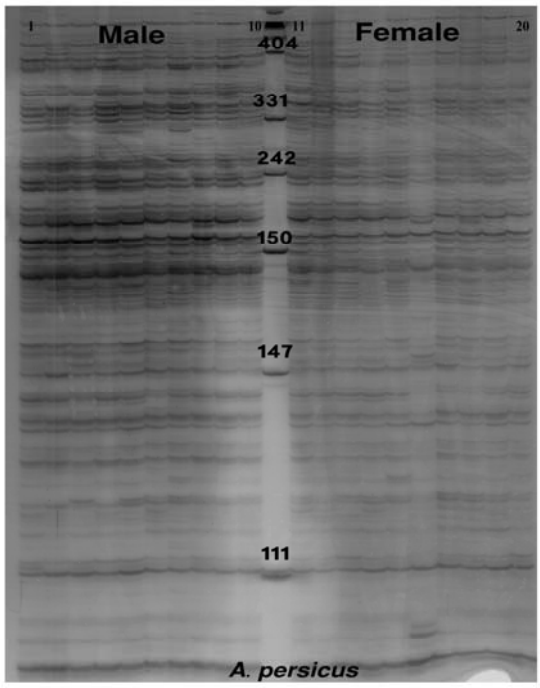

Fig. 2. AFLP generated from DNA samples of 20 *Acipenser persicus* using a set of *Eco*+3/*Mse*+4 primers. 1-10 male and 11-20 female, (M) ladder, pUC Mix Marker, 8 (Ferments, France).

1100-9230 bands were screened per species, sex-specific markers were not detected.

Several molecular techniques have been used for the determination of sex in fish, including sex-specific markers in rainbow trout using RAPD and *FISH* (Fluorescence *In Situ* Hybridization) techniques (Ittura et al., 1998) and the three-spined stickleback *Gasterosteus aculeatus* using AFLP (Griffith et al., 2000). DNA markers from a gynogenetic triploid ginbuna genome were studied using representational differences analysis (RDA), and sex-specific markers in *Takifugo rubripes* were characterized by a cDNA-AFLP technique (Cui et al., 2006).

However, all techniques tested have failed to find sex-specific markers in sturgeon, including RAPD (Wuertz et al., 2006; Keyvanshokooh et al., 2007, 2009), single-strand conformation polymorphisms (SSCP), AFLP, inter-simple sequence repeats (ISSR) (Wuertz et al., 2006), RDA, subtractive hybridization (McCormick et al., 2008), and a candidate gene approach (Hett and Ludwig, 2005; Hett et al., 2005). In addition to screening at the genomic level, studies of expression patterns such as proteomics have also failed to find sex-specific markers (Keyvanshokooh et al., 2009).

McCormick et al. (2008) suggested that an environmental sex determination system may exist in sturgeon. However, environmental sex determination leads to variations in sex ratios (Bull, 1983), and the sex ratio in adult populations of sturgeon is 1:1, both in natural and aquaculture populations (Chapman et al., 1996).

Some authors have also argued that sturgeon contain no sex chromosomes (Wuertz et al., 2006; Keyvanshokooh et al., 2007; McCormick and DeWoody, 2008) which is congruent with the results of the present study. However, genetic evidence for female heterogametic sex determination (WZ female, ZZ male), for example, in white sturgeon (Van Eenennam et al., 1999), bester sturgeon (Omoto et al., 2005), and shortnose sturgeon (Flynn et al., 2006) would suggest that a genetic based sex determination exists. The absence of DNA markers in sturgeon could be due to several factors, e.g., genome size, the proportion of genome analyzed, and the number

of markers in the studied species (Keyvanshokooh and Gharaei, 2010).

Alternatively, it may be that homologous chromosomes carrying the sex-determining factors are not extensively differentiated in sturgeon species, or that the methods utilized did not have the required sensitivity (Wuertz et al., 2006). Due to these findings, we believe that alternative approaches based on gene expression at different stages of gonadal maturation should be used.

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