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Molecular marker-assisted sex control in half-smooth tongue sole (*Cynoglossus semilaevis*)

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ABSTRACT

Half-smooth tongue sole females grow larger and faster than males. An all-female population would be of significant benefit for tongue sole aquaculture. In the present study, a female-specific AFLP marker (CseF305) was isolated from female genomic DNA of the tongue sole and sequenced. One pair of SCAR primers was designed based on the sequences of the female-specific marker. A PCR method for identifying genetic sex of the sole was developed. PCR amplification of genomic DNA from tongue sole adults using the SCAR primers resulted in a specific fragment in 30 female individuals, but not in 30 males. Secondly, effects of methyltestosterone treatment on the female:male sex ratio of tongue sole fry were examined. Methyltestosterone at a concentration of 20–100 μg/L·H_2O can induce genetic females to reverse to phenotypic males in juvenile tongue sole and produce a high proportion of males (97–100% males). Phenotypic males with female genotype, that is, neo-males, were detected by using female-specific SCAR primers. The neo-males were cultured and matured, and used to mate with normal females to produce progeny. 130,000 fry were produced by using sperm from neo-males. Genetic sex identification demonstrated that 73% of the neo-male progeny fry contained female-specific DNA markers. Three combinations of sex chromosomes (ZZ, ZW and WW) were observed in the neo-male progeny. WW super-female individuals containing 2 huge heteromorphic chromosomes were found in some fry.

1. Introduction

Sex control has important application potential in teleost fish. For many teleosts, one sex grows much faster than the other or has maturation features that are less desirable for production purposes. For example, in certain fish species such as Atlantic halibut, barfin flounder and half-smooth tongue sole, females grow much faster and bigger than males, while in tilapia species, the males grow faster, and therefore are of greater value. Thus, monosex stocks have been developed in various fish species including chinook salmon (Hunter et al., 1983), Nile tilapia (Mair et al., 1997) and halibut (Tvedt et al., 2006). Such monosex stocks are typically generated by combining sex reversal and family selection. Mixtures of genetic males and females are treated with androgens in order to masculinize the genetic females into functional males. The identification of the two types of males is commonly carried out by means of a test crossing of each individual with regular females. The generation and maintenance of monosex stocks require that genetic and phenotypic sexes are independently discernible. The inability to identify the genetic sex of neo-males has hampered the development of monosex stocks in some important cultured fish species.

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). Few reports on sex-linked molecular markers in teleosts are available, although sex-specific molecular markers have been cloned in many mammals. Various molecular markers have been attempted for the assessment of the genetic sex of fish. For example, male-specific DNA markers were isolated and used for genetic sex identification in salmonids (Devlin et al., 2001; Devlin and Nagahama, 2002; Felip et al., 2005). Also, male-specific RAPD markers were isolated from African catfish (*Clarias gariepinus*) (Kovacs et al., 2000). Two male-specific AFLP markers were also identified in the three-spined stickleback, *Gasterosteus aculeatus* (Griffiths et al., 2000), but were found to be not applicable in the nine-spined *Pungitus pungitus* and 15-spined stickleback *Spinachia spinachia* (Griffiths et al., 2000). A sex-determining gene, *DMY* gene, was isolated from medaka (*Oryzias latipes*) (Matsuda et al., 2002); however, this gene, although sex-specific in medaka and *O. curvinotus*, is not the sex-determining gene in any other fish tested (Volff et al., 2003; Kondo et al., 2003). Recently, sex-linked AFLP and microsatellite markers were identified in Nile tilapia (*Oreochromis niloticus*) (Lee et al., 2003; Ezaz

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et al., 2004). So far, marker-assisted sex control was reported only in salmonids (Devlin and Nagahama, 2002). However, no report on marker-assisted sex control was available in marine fish species.

The half-smooth tongue sole (*Cynoglossus semilaevis*) is a newly exploited, commercially important cultured marine flatfish in China. Females grow 2–3 times faster than males. Thus, the development of all-female stock would be of significant benefit for aquaculture. Recently, a female-specific AFLP marker (CseF305) was identified in the sole (Chen et al., 2007), but there is no report on the sequence of the AFLP marker CseF305 or molecular marker-assisted sex control in the half-smooth tongue sole.

In the present study, we isolated and sequenced one female-specific AFLP marker, and developed a PCR method for identifying the genetic sex of tongue sole. Secondly, we obtained neo-males by masculinizing normal female fry by methyltestosterone treatment and confirmed this by genetic sex identification. Thirdly, the neo-males were used for crossing with normal females to produce female-biased fry. Finally, the chromosomal and genetic sex of the neo-male progeny fry were examined.

2. Materials and methods

2.1. Fish and sampling

The experiments were carried out at Laizhou Mingbo Aquatic Ltd. (Shandong Laizhou) and Huanghai Aquaculture Ltd. (Shandong Haiyang). Culture and reproductive manipulation of tongue sole were performed according to routine methods (Jiang and Wan, 2005). Embryos and fry were collected from spawning tanks for sex reversal. Half-smooth tongue sole weighing 100~500 g were obtained from commercial hatcheries. Liver tissues were collected from the above tongue sole and frozen in liquid nitrogen and stored at −80 °C until use. Gonads were collected from the individuals and fixed in Bouin's fixative for assessing phenotypic sex of the individuals by microscopic inspection of histological section of the gonads.

2.2. DNA extraction from tissue

For DNA extraction, a piece of liver or fin tissue of 10–20 mg was homogenized in 500 μl lysis buffer (10 mM/L Tris–HCl, pH 8.0; 100 mM /l EDTA, pH 8.0; 100 mM/l NaCl; 0.5% SDS, and freshly added proteinase K 100 μg/ml). Then the homogenate was lysed at 50 °C for 60–90 min. DNA was extracted with the phenol:chloroform method. After one phenol, one phenol–chloroform and one chloroform extraction, DNA was precipitated with two volumes of ethanol. The DNA was pelleted, washed first in 70% and then in absolute ethanol, dried and dissolved in 100 μl of TE buffer (10 mM Tris–HCl, pH 8.0; 10 mM EDTA, pH 8.0). The quality and concentration of DNA were assessed by agarose gel electrophoresis and measured with a GENEQUANT Pro (Pharmacia Biotech Ltd.) RNA/DNA spectrophotometer. Finally, DNA was adjusted to 100 ng/μl and stored at −20 °C for future use.

2.3. Phenotypic sex identification by histological sectioning

Phenotypic sex of half-smooth tongue sole was determined by histological sectioning and staining as described (Chen et al., 2007). In brief, gonads were removed from sole individuals, fixed in Bouin's fixative and embedded in paraffin. The paraffin containing the tissues was sectioned in 6 μm, and stained with hematoxylin and eosin. The phenotypic sex was determined by microscopical observation.

2.4. Cloning of the female-specific AFLP marker CseF305 and sequence analysis

To clone the female-specific AFLP marker CseF305, AFLP reactions were performed with female-specific AFLP primers (E-ACC/M-CTA), and the PCR products were separated on 6% denaturing polyacrylamide gels. After electrophoresis, gels were silver-stained and air dried at room temperature. The female-specific AFLP bands were excised and reamplified. The target DNA fragments were extracted from the gel using a QIAEXII gel extraction kit and cloned into pMD-18t vectors as described (Chen et al., 2004). PCR was used for screening the clones containing the female-specific fragments. Then female-specific fragments were sequenced on an ABI Prism 377 automated DNA sequencer (PE Corp.).The sequences of several clones were assembled and compared using DNAMAN Version 4.0. Homology of the female-specific fragments to known sequences was assessed (by) using BLASTn in GenBank (Altschul et al., 1990).

2.5. Genetic sex identification using female-specific SCAR markers

Based on the sequences of the female-specific AFLP fragments, a pair of specific SCAR primers (CseF305N1: CTCCCCTGACCTTCCTTT and CseF305C1: 5'-CGG CAGCACAATTATTACA-3') were designed for genetic sex identification. The PCR reaction system (25 μl) consisted of 1 μl of 10 pM of each primer, 1 μl of 2.5 mM of each dNTP, 1 μl of Taq polymerase (Promega, USA), 1.5 μl of 25 mM Mg^{2+}, and 1–2.0 μl of sole DNA as template. PCR was run as follows: initial incubation at 94 °C for 4 min, followed by 30 cycles of 94 °C, 50 s; 56 °C, 50 s; and 72 °C, 50 s, with a final extension of 6 min at 72 °C. DNA was extracted from the livers of 30 female and 30 male soles as described above. The amplification products of 12 μl were resolved on 1.5% agarose gel with a DL2000 DNA marker. A female-specific fragment of 160 bp was amplified from the female genome.

2.6. Induction of sex reversal of fry by androgen

On Days 23–25 after hatching, fry were subjected to 17α-methyltesterone (MT) treatment. In brief, MT dry powder was dissolved in 95% ethanol and then diluted in sea water. Fry were immersed daily for 8–10 h in static sea water containing MT at a concentration of 20 μg/l·H_2O, 30 μg/l·H_2O, 80 μg/l·H_2O or 100 μg/l·H_2O, respectively. MT treatment lasted 60 days. MT-treated and control groups were cultured in aquaria (50 cm×50 cm×50 cm). All groups were kept at 22–24 °C during the experimental period, then cultured until adult under natural water temperature. There were two replicates of each MT-treated group, and each aquarium contained 100 tongue soles.

2.7. Genetic sex identification of MT-treated tongue sole and neo-male progeny

Genetic sex identification of MT-treated tongue sole fry and neo-male progeny was performed as described above.

2.8. Culturing and reproduction manipulation of neo-males

Culturing, reproductive manipulation and artificial induction ovulation to obtain sexually mature neo-male adults were performed according to routine methods (Jiang and Wan, 2005).

2.9. Chromosome analysis of neo-males' progeny fry

Chromosomes were prepared as described (Sha et al., 2003) with some modifications. In brief, fry of 3–6 cm were incubated in sea water containing 0.02% colchicine at room temperature for 6 h. The fin of fry was sheared, transferred into 0.0375 M KCl solution and incubated for 30 min. The fin was fixed in fresh Carnoy's fluid (methanol: glacial acetic acid = 3:1) three times, and each fixation lasted for 20 min. The fin was taken out and put into 50% glacial acetic acid, then lacerated with cuspidal forceps to dissociate the cells. The cells were dropped onto slides on a heated plate, then stained in 10% Giemsa for 20–