



Screening and characterization of sex-linked DNA markers and marker-assisted selection in the Nile tilapia (*Oreochromis niloticus*)



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ABSTRACT

The Nile tilapia is a cultured teleost fish in which males grow faster and larger than females, and mono-sex culturing could avoid unwanted reproduction during grow-out. Sex control in tilapia is an important issue in aquaculture. In the present study, randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) fingerprinting were used to screen pooled and individual DNA samples from XX, XY, and YY fish for sex-linked markers. Four sex-linked markers (Marker-1 from RAPD and Markers-2, -3, and -4 from AFLP) were obtained and mapped to LG23, a small chromosome, by sequence analysis and fluorescence in situ hybridization (FISH). In addition, a total of 32 pairs of primers were designed based on the sequences of scaffolds 7, 29, and 101 on LG23, and these were used to screen genetic differences between X and Y by PCR. One of these (Marker-5) produced a detectable difference between XX, XY, and YY individuals. Eight pairs of primers based on the sequence characterized amplified region (SCAR) were designed and successfully converted to SCAR markers, which were used to sex progeny from different crosses of known genotypes for validation. Subsequently, Markers-4 and -5 were used to sex eight populations of Nile tilapia collected from different fish farms in China, which gave concordance rates that ranged from 76 to 100%. Based on the recombination rate derived from the progeny of XX (♀) × XY (♂), Markers-1, -2, -3, -4, and -5 were estimated to be around 5, 3, 5, 2 and 0 cM away from the sex-determining locus (SD), respectively. Marker-5, which mapped close to the SD reported previously (Eshel et al., 2012), was used for selective breeding of genetic male tilapia (GMT).

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1. Introduction

Tilapias are currently the second most cultured fish in the world (behind carps), with annual production exceeding 3.7 million tons in 2011 (FAO, 2013). The farming of tilapia is presently in a period of dynamic expansion to satisfy both domestic and international markets, and tilapias are now cultured in 85 countries. Although many varieties of tilapia are cultured, the most widely farmed is the Nile tilapia (*Oreochromis niloticus*). Manipulation of tilapia sex has important applications in aquaculture as males grow faster and reach a larger size than females, and mono-sex culturing could avoid unwanted reproduction during grow-out (Cnaani and Levavi-sivan, 2009; Mair et al., 1995, 1997). Culturing all-male Nile tilapia populations is the best way to rapidly increase yields of this important food fish. Traditionally, production

of all-male tilapia was achieved by manual separation of males from females by visual observation of the genital papilla (the male genital papilla is elongated while the female papilla is rounded) (Popma et al., 1984). Some interspecific crosses such as Mozambique tilapia (*Oreochromis mossambicus*) females with *Oreochromis urolepis hornorum* males and Nile tilapia females with blue tilapia (*Oreochromis aureus*) males (Fishelson, 1966; Hickling, 1960; Hulata et al., 1983) also produced all male progeny. Currently the most widely used method is hormonal sex reversal (Beardmore et al., 2001; Hopkins et al., 1979; Mair et al., 1995, 1997; Rosenstein and Hulata, 1994). However, all of these manipulations can be troublesome, time consuming, and unreliable. Another effective method for producing genetically male tilapia (GMT) on a large scale is to cross normal females XX with supermale YY broodstock (Mair et al., 1997). However, the difficulty of rapidly identifying the genotypes of the neomale XX (♂), male XY (♂), and supermale YY (♂) progeny has hampered the development of monosex stocks (Mair et al., 1997; Yang et al., 1980). Molecular marker-assisted selection (MAS) has been demonstrated to be an effective method for both sex identification and sex control (Gui and Zhu, 2012; Liu and Cordes, 2004; Liu et al., 2013b), but first sex-linked markers need to be identified. In aquaculture, molecular markers for sex have been

Abbreviations: RAPD, randomly amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; SCAR, sequence characterized amplified region; SD, sex-determination locus; MAS, marker-assisted selection; GMT, genetically male tilapia; E2, 17β-estradiol; F, Fadrozole; LG, linkage group.

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identified in many species including chinook salmon (Devlin et al., 1994, 2001), African catfish (*Clarias gariepinus*) (Kovacs et al., 2000), three-spined stickleback (*Gasterosteus aculeatus*) (Griffiths et al., 2000), rainbow trout (*Oncorhynchus mykiss*) (Felip et al., 2005), half-smooth tongue sole (*Cynoglossus semilaevis*) (Chen et al., 2007), yellow catfish (*Pelteobagrus fulvidraco*) (Dan et al., 2013; Wang et al., 2009), turbot (*Scophthalmus maximus*) (Martínez et al., 2009), Brycon (*Brycon amazonicus*) (Da Silva et al., 2012), and rock bream (*Oplegnathus fasciatus*) (Xu et al., 2013). In addition, several sex-determining genes that can be used as sex-specific markers have been isolated in fishes in recent years, including *Dmy* in medaka (*Oryzias latipes*) (Matsuda et al., 2002), *Amhy* in Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori et al., 2012), *Gsdf^β* in medaka (*Oryzias luzonensis*) (Myosho et al., 2012), and *Irf9* in rainbow trout (Yano et al., 2012). In the last two decades, MAS has been used in chinook salmon (Devlin et al., 1994), half-smooth tongue sole (Chen et al., 2008), and yellow catfish (Wang et al., 2009). However, there are often only limited genetic differences between the sexes, and in many cases markers obtained in one species proved not to be sex-linked in other, even closely related species (Devlin et al., 2001; Griffiths et al., 2000; Kondo et al., 2003). Even different strains and families of the same tilapia species can segregate different sex-determining genes (Cnaani et al., 2008; Eshel et al., 2011, 2012; Ezaz et al., 2004; Lee et al., 2003, 2004, 2011; Liu et al., 2013a; Shirak et al., 2006).

The sex of Nile tilapia is proved to be an XX/X Y male heterogametic system determined by a major gene, even though it was also influenced by autosomal genes and environmental factors (especially temperature) (Abucay et al., 1999; Baroiller et al., 1995, 2009; Eshel et al., 2011, 2012; Harvey et al., 2003; Mair et al., 1991). However, there are no gross morphological differences between any of the chromosome pairs that identify putative X and Y chromosomes in mitotic chromosome spreads (Crosetti et al., 1988; Kornfield, 1984; Majumdar and McAndrew, 1986). Several studies revealed that the longest chromosome corresponding to the linkage group 3 (LG3) shows features (e.g. delayed pairing in meiosis in XY but not XX or YY) expected in sex chromosomes (Carrasco et al., 1999; Foresti et al., 1993; Harvey et al., 2002, 2003; Ocalewicz et al., 2009). Other studies, using linkage mapping and FISH, have mapped sex chromosome to a small chromosome corresponding to LG1 (Cnaani et al., 2008; Ezaz et al., 2004; Lee et al., 2003, 2011). On the other hand, two studies using SSR markers demonstrated that another small chromosome corresponding to LG23 is the sex chromosome in another strain of Nile tilapia (Eshel et al., 2011, 2012).

To determine the sex chromosome in our Nile tilapia strain and to produce mono-sex progeny for laboratory use and aquaculture, we isolated and sequenced five sex-linked markers. All of them mapped to a small chromosome corresponding to LG23. Using most closely linked marker (Marker-5), we developed a simple PCR method for identifying the genetic sex of our Nile tilapia strain as well as several populations from fish farms in China. This method was subsequently used for production of mono-sex progeny for basic research as well as for establishment of MAS-GMT in our strain.

2. Materials and methods

2.1. Fish and sampling

The founder strain of the Nile tilapia, which was first introduced from Egypt in Africa, was obtained from Prof. Nagahama (Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki, Japan) and reared in indoor tanks with a circulating aerated freshwater system. Fish were maintained at ambient temperature (26 °C) under natural photoperiod. Δ XX neomales, Δ XY and Δ YY neofemales were obtained by hormonal masculinization of XX fry and feminization of XY and YY fry, respectively (Mair et al., 1987) or by drug treatment (see Section 2.2). The YY genomic DNA used in this experiment was

obtained from the laboratory of Prof. Nagahama, or from progeny of Δ XY neofemales crossed with YY “supermales” in our laboratory. Progeny were obtained by artificial fertilization of eggs from normal females (XX) or neofemales (Δ XY[♀]) with spermatozoa from sex-reversed neomales (Δ XX), normal males (XY), or “supermales” (YY). The artificially fertilized eggs were then incubated in re-circulating water at 26 °C in an artificial incubation system to obtain fry. To test the reliability of the selected markers, double-blind checks of the phenotypic (identified by histological examination, Section 2.4) and genetic (identified by sex-linked markers) sex of progeny from crosses, 160 progenies from 6 XX (♀) × Δ XX (♂) crosses, 160 progenies from 6 XX (♀) × XY (♂) crosses, 170 progenies from 6 XX (♀) × YY (♂) crosses, 75 progenies from 2 Δ XY (♀) × XY (♂) crosses, and 81 progenies from 2 Δ XY (♀) × YY (♂) crosses were performed independently by two individuals, before the data were analyzed by a third person. In addition, 215 Nile tilapia from eight different populations (HN, HN1, HN2, T1, T2, THS, NG, and TH) were collected from different farms in Guangdong, Guangxi, Hainan, Fujian Provinces, and Shanghai City. The phenotypic sexes of these fish were then determined using the same methods as described below (Section 2.4). The genotypic sexes of these fish were determined by Markers-4 and -5. Finally, the concordance rates between the identified genotypes and phenotypes were calculated. All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University, China.

2.2. Sex reversal

Δ XX neomales, Δ XY and Δ YY neofemales were generated by treating fry with the aromatase inhibitor Fadrozole (F) (Novartis, Switzerland), or 17 β -estradiol (E2) (Sigma, USA), respectively. Drug treatment was applied to the fry from 3 to 30 days after hatching (dah), the critical period for Nile tilapia sex differentiation. First, the fry were immersed in either Fadrozole (200 μ g/l) or E2 (300 μ g/l) from 3 to 7 dah. Then fish were treated with feed sprayed with 100% ethanol containing Fadrozole (250 μ g/g) or E2 (200 μ g/g) until 30 dah. Control fish were fed with 100% ethanol-sprayed feed. Histological examination of the gonads from 80 fish (20 for each group) at 90 dah confirmed that F (XX) and E2 (XY) treatment resulted in 100% and 70% sex reversal, respectively; while the control groups remained as 100% (XX) female and (XY) male.

2.3. DNA extraction

Liver tissues or fin clips were collected and frozen in liquid nitrogen and stored at −80 °C until use. Genomic DNA was isolated by proteinase K digestion followed by phenol/chloroform extraction, as previously described (Sambrook et al., 1989). The quality and concentration of DNA were then assessed by agarose gel electrophoresis, and measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Finally, the DNA was diluted to a concentration of 20 ng/ μ l, and stored at −20 °C for future use.

2.4. Phenotypic sex identification by histological sectioning and staining

Phenotypic sex of tilapia was determined by histological sectioning and staining at 90 dah. In brief, gonads were removed from the fish, fixed in Bouin's solution and embedded in paraffin. The paraffin embedded tissues were cut into 5–6 μ m sections, and stained with hematoxylin and eosin. The phenotypic sex was then determined by microscopic inspection.

2.5. RAPD-PCR fingerprinting

Bulked segregant analysis (Michelmore et al., 1991) was used for identification of sex-specific markers. Samples from 10 XX, 10 XY, and

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