RESEARCH ARTICLE

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Screening and identification of a microsatellite marker associated with sex in Wami tilapia, *Oreochromis urolepis hornorum*

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Abstract

In this study, primer pairs of 15 microsatellite markers associated with sex determination of tilapia were selected and amplified in Wami tilapia, *Oreochromis urolepis hornorum*. While one marker, UNH168, on linkage group 3 (LG3) was associated ($P < 0.001$) with the phenotypic sex in the experimental population, nine genotypes were detected in both sexes. Only 99-bp allele was detected in the female samples, while 141, 149 and 157-bp alleles were present in both male and female samples. UNH168 was localized by fluorescence *in situ* hybridization (FISH) on the long arm of the largest tilapia chromosome pair (chromosome 1, equivalent to LG3). This sex-linked microsatellite marker could potentially be used for marker-assisted selection in tilapia breeding programmes to produce monosex male tilapia.

Introduction

The mechanisms of sex determination in fish are highly variable. All sex determination types encountered in higher vertebrates are recorded in fish: dioecious species, hermaphroditic species and sex reversal also exist in fish. The basis of fish sex development is genetic factors mainly as well as endocrine regulation and the influence of environment factors (e.g. temperature). Tilapia are widely distributed in tropical and subtropical regions, and different sex determination mechanisms have been demonstrated in this taxon, namely, XX-female / XY-male (Lee *et al*. 2003; Cnaani *et al*. 2008) and WZ-female / ZZ-male (Cnaani *et al*. 2008; Chen 1969). Owing to its varied sex determination mechanisms, extreme ecological diversity (Greenwood 1991), and importance to tropical and subtropical aquaculture, tilapia has received much scientific interest (Pullin 1991), and is a promising model system for understanding the evolutionary mechanisms of sex determination in vertebrates (Kocher 2004).

Tilapia sex determination are controlled by several genes on different chromosomes and by environmental factors, may

be considered as a quantitative trait (Devlin and Nagahama 2002; Lee *et al*. 2004). Sex-related molecular markers of tilapia have been identified in *O. niloticus*, *O. aureus*, *O. mossambicus*, *O. karongae*, *T. mariae*, *T. zillii* and their hybrids (Campos-Ramos *et al*. 2003; Lee *et al*. 2003, 2004; Ezaz *et al*. 2004; Karayucel *et al*. 2004; Cnaani *et al*. 2004; Shirak *et al*. 2006). In Nile tilapia, 10 microsatellite markers belonging to linkage group 1 (LG1) are linked to dominant male-determining (XY) locus (Lee *et al*. 2003). Another six sex-linked microsatellite markers, including GM354, GM204, UNH168, GM271, GM139 and UNH131 have been detected at one end of LG3 (Lee *et al*. 2005). Five sex-associated markers were found on LG1, LG3, LG19 and LG23 in an F_2 hybrid cross between *O. aureus* and *O. mossambicus* (Shirak *et al*. 2002; Lee *et al*. 2003; Cnaani *et al*. 2004, 2008). Two unlinked microsatellite markers, one located on LG3 and other on LG1 were found to be linked to phenotypic sex in a single family of blue tilapia (Lee *et al*. 2004). In addition, two gene markers (*Amh* and *Dmrta2*) overlapping within two quantitative trait locus (QTL) regions on LG23 were highly associated with sex determination in the hybrids between *O. niloticus* male and *O. aureus* female (Shirak *et al*. 2006). Recently, a novel male-specific duplication of *amh* (denoted as *amhy*) lacking the TGF-β domain was identified and mapped to the QTL region

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on LG23 for sex determination, thus indicating its potential role in sex determination in *O. niloticus* (Eshel *et al.* 2014).

Wami tilapia has been hybridized with *O. mossambicus* to produce F$_1$ hybrids, a new salt-tolerance strain (*Oreochromis mossambicus* female × *O. u. hornorum* male) (Zhu *et al.* 2011). It is tolerant to brackish water and grows well in saline pools, making it suitable particularly for aquaculture by communities living close to the sea in southern China. In this study, we demonstrate that a microsatellite marker on LG3 is associated with sex in Wami tilapia (*O. u. hornorum*) and physically map the marker on metaphase chromosome spreads by fluorescence *in situ* hybridization (FISH).

Materials and methods

Samples

Mature individuals of *O. u. hornorum* ($n=120$) were randomly collected from the Gaoyao Aquaculture Germplasm Conservation Station, Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences. *O. u. hornorum* was introduced from American Aquaculture International in 2001. The body length of these samples ranged from 19.3±2.8 cm. The individuals were temporarily cultured in the laboratory and then sexed by examination of the urogenital papilla (49 males and 71 females). Eleven full-sib families were constructed (with one male mated with one female) for the analysis of segregation pattern of the microsatellite loci. The F$_1$ generation fry were reared until sexual maturation (five months of age) at which stage phenotypic sex could be identified visually.

Genomic DNA extraction

Genomic DNA was extracted from the tailfin tissue according to standard phenol–chloroform procedures (Sambrook and Russell 2001) without sacrificing the animal. The purified total DNA samples were quantified by gel electrophoresis and their quality was verified by spectrophotometry. DNA samples were stored at −20°C until use.

Microsatellite isolation and sequence

Primer pairs for 15 microsatellite markers found to be associated with sex determination in tilapia were selected and designed according to Lee *et al*. (2004, 2005) (table 1). Microsatellite loci were amplified from the genomic DNA of Wami tilapia using the method described by Guo and Gui (2008). Briefly, PCR amplification was performed in 20 μL of a mixture containing 20 ng genomic DNA template, 1× PCR buffer, 100 μmol L^{-1} of each dNTP, 1.5 mmol L^{-1} MgCl$_2$, 0.2 μmol L^{-1} of each primer, and 0.5 unit of *Taq* DNA polymerase. Double-distilled water was added to a final volume of 20 μL. PCR cycling conditions are as follows: an initial denaturation at 94°C for 3 min followed

by 25 cycles of 30 s at 94°C, 30 s at an optimal annealing temperature, 30 s at 72°C, then a final extension step of 10 min at 72°C. Amplified fragments were separated on denaturing 10% polyacrylamide gels and stained with silver nitrate.

The bands that were of interest were cut from the gel with a razor blade and purified using the BioStar Grass milk DNA Purification Kit (BioStar International, Canada) according to the manufacturer's specifications. The DNA fragments were ligated into the plasmid pMD19-T (Promega, Madison, USA), and subsequently transformed into *Escherichia coli* DH5α cells. The recombinant clones that were selected on ampicillin plates were screened by PCR. The inserted DNA fragments in positive clones were sequenced.

Chromosome preparation and FISH

Chromosome were obtained from cephalic kidney tissue by the method of kidney cell-phytohaemagglutinin (PHA) culture *in vivo* as described by Gui (1999) and were subjected to FISH. BAC clones containing the particular microsatellite marker were identified by PCR screening of pooled clones from Nile tilapia libraries as previously described (Peng *et al.* 2009). BAC DNA was extracted from 200 mL overnight cultures of the resulting positive BAC clones using E.Z.N.A.$^{\text{TM}}$ BAC/PAC Maxi Kit (Omega, Norcross, USA) and the purified DNA were used as probes labelled with biotin-11-dUTP by nick translation kit (Roche, Mannhein, Germany), according to the prescribed protocol. FISH was performed according to the method described by Zhu *et al.* (2006). In brief, after treatment with RNase A (100 μg mL^{-1} in 2× SSC), the slides with chromosome spreads were denatured in 70% deionized formamide per 2× SSC for 2 min at 70°C, dehydrated in a 70, 90 and 100% ethanol series for 3 min each and air dried. One-hundred nanograms of labelled probes were denatured for 10 min in boiling water and placed on the slides, covered with a 24 × 50 mm^2 coverslip. Hybridization was carried out overnight at 37°C in a moist chamber. The slides were then washed for 10 min in 2× SSC, with 50% formamide, 0.1× SSC with 0.1% Tween 20, pH 7.0 and then 3 × 5 min at room temperature with 1× PBS. After a series of posthybridization, washes were performed and the spectrum signals were achieved with 50 μL of avidin-FITC (Roche) at 5 μg mL^{-1}. Chromosomes were counterstained with 0.5 μg ml^{-1} (DAPI) in antifade solution which produced adequate bands for chromosome identification.

Images were acquired using a Leica inverted DMIRE2 epifluorescence microscope equipped with a CCD camera and a Leica LCS SP2 confocal image system (Leica, Wetzlar, Germany). DAPI and FITC fluorescence were detected using appropriate filter sets (DAPI filter cube: excitation 340/40 nm, emission 430/50 nm; FITC filter cube: excitation 480/20 nm, emission 510/30 nm). Captured images were coloured and overlapped in Adobe Photoshop 7.0. At least 15 metaphases were analysed for each sample.

Results

Isolation and characterization of microsatellite markers

A total of 15 microsatellite markers were surveyed for analysis of polymorphism (table 1). Of these, UNH168 on LG3 was found produce consistent amplification and to show association with female phenotypic sex in the experimental population. While nine genotypes of the microsatellite loci could be distinguished by the length of the amplicons between phenotypic sex (partial results are shown in figure 1). The 99-bp allele was present only in female samples, while 141, 149 and 157-bp alleles were present in both male and female samples.

Examination of sex-linked microsatellite marker

Chi-square test was conducted to analysis the linkage between phenotypic sex and microsatellite markers segregating within each family. The segregation pattern of this locus among the F_1 generation individuals is shown in table 2. Chi-square test showed that this microsatellite locus was highly associated with the phenotypic sex ($P < 0.001$) based on the result of the alleles segregation in the F_1 generation.

Chromosomal localization of sex-linked microsatellite marker

To localize the sex-linked marker in the tilapia genome, FISH analysis of the microsatellite locus was performed on chromosome spreads of six females and six males of Wami tilapia (*O. u. hornorum*). This mark is located in the middle of the long arm of the largest chromosomes (pair 1, subtelocentric chromosome) (figure 2). Comparing the location of the hybridized signals on the spreads from males and females, no visible differences were observed. In addition, some minor signals were also detected on other chromosomes.

Table 1. List of polymorphic microsatellite primers.

| Primer pair	Primer name	LG no. (Lee *et al.* 2005)	Sequence	Annealing temperature (°C)
1	UNH995	1	CCAGCCCTCTGCATAAAGAC GCAGCACAACCACAGTGCTA	52
2	UNH868	1	TCCTTGTTCAGACCTTGTGG AGCCAGGCTGAAAGGAAATA	54
3	GM201	1	TATTCAGGCTCTTCTTTTGCT CAGAATGAACTCCCTCCAG	52
4	UNH104	1	GCAGTTATTTGTGGTCACTA GTATATGTCTAACTGAAATCC	52
5	UNH115	3	ACCTTCATCTCGGTCAG TCAAGCAGCTGATTTTT	45
6	GM271	3	GCAGCTGGATCAGTCTCTG TGGGAAGTCGTTCATACAAAG	60
7	UNH971	3	GGTGGGCAGTGTGTGTTTTT TTTTCATCCAGGCCTCAGTT	57
8	UNH131	3	CAGAATCAACTTTTGGA GTGATTTTTAAATAGACCTT	48
9	GM633	1	GTGTCCCAAGAAAACCAGGA GACCCAGGACTCATGTGCTT	54
10	UNH168	3	TAAGAAGGTTAGAAAGAAAGTG TATATAATAATTTCCTAAACGGC	58
11	GM354	3	CGGGAGAGCAGGTCAG CACGTTCAGGGTTACTGTGTT	62
12	GM148	1	TAAGGAATAAAGGCAGACACA GGTCGAGGGCTTAAAATG	50
13	GM258	1	CCTTCACCTCCACCACTTTCT AGATCGAACGTCGTCCTCTG	55
14	GM139	3	GTGGGATCTACCAAGAAGAG TTTGAGTAACCACCCTAACAC	53
15	GM180	3	CGGCGGCGACTTGTAGTGT GGACGGTCTGGCGAGGAC	52
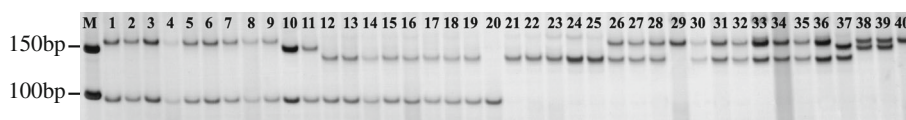
Figure 1. PAGE pattern of PCR products amplified in 40 samples. Lanes 1–20, female samples; lanes 21–40, male samples; lane M, DNA molecular weight marker.

Table 2. Proportions of genotypes for microsatellite marker UNH168 in male and female *O. u. hornorum* of F_1 generation.

Family	Maternal genotype	Paternal genotype	Offspring genotype	Female numbers	Male number	Chi-square test
1	99/157	141/149	99/141	12	1	$\chi^2 = 32.45$
			99/149	11	0	$P < 0.001$
			141/157	1	8	
			149/157	0	7	
2	99/149	141/157	99/141	9	0	$\chi^2 = 36.33$
			99/157	12	0	$P < 0.001$
			141/149	0	8	
			149/157	1	10	
3	99/141	141/149	99/141	10	0	$\chi^2 = 32.73$
			99/149	9	1	$P < 0.001$
			141/141	0	8	
			141/149	1	11	
4	99/149	141/141	99/141	23	1	$\chi^2 = 36.08$
			141/149	0	16	$P < 0.001$
5	99/157	157/157	99/157	21	1	$\chi^2 = 36.17$
			157/157	0	18	$P < 0.001$
6	99/141	149/157	99/149	13	0	$\chi^2 = 30.05$
			99/157	9	0	$P < 0.001$
			141/149	2	5	
			141/157	0	11	
7	99/157	149/157	99/149	8	2	$\chi^2 = 33.58$
			99/157	11	0	$P < 0.001$
			149/157	0	12	
			157/157	0	7	
8	99/149	157/157	99/157	23	0	$\chi^2 = 32.47$
			149/157	2	15	$P < 0.001$
9	99/157	141/141	99/141	16	0	$\chi^2 = 29.47$
			141/157	3	21	$P < 0.001$
10	99/141	141/157	99/141	6	0	$\chi^2 = 36.30$
			99/157	15	0	$P < 0.001$
			141/141	1	11	
			141/157	0	7	
11	99/157	141/157	99/141	9	0	$\chi^2 = 29.61$
			99/157	10	0	$P < 0.001$
			141/157	2	12	
			157/157	1	6	

$P < 0.05$ were considered statistically significant difference.

Discussion

Sex-linked microsatellites (or other sex-linked molecular markers) are useful for sex determination research and genome evolution study (Woram *et al.* 2003; Lee *et al.* 2004; Cnaani *et al.* 2008; Ninwichian *et al.* 2011). Some previous studies have found association of DNA markers with sex in some tilapia species and their hybrids such as UNH995 on LG1 in *O. niloticus* and *O. aureus*, GM201 on LG1 in *O. niloticus, O. aureus* and *O. mossambicus*, UNH148 on LG1 in *O. niloticus* and *O. mossambicus*, GM345 on LG3 in *O. aureus* and *O. karongae* (Lee *et al.* 2005; Cnaani *et al.* 2008). In this study, 15 microsatellite loci were examined in the tilapia species *O. u. hornorum* and one microsatellite marker UNH168 was identified to associate with sex by chi-square test ($P < 0.001$) based on the result of the alleles segregation in the F_1 generation. In this locus of *O. u. hornorum*, four alleles (99, 141, 149 and 157 bp) were detected but the 99-bp allele was found only in females. In *O. tanganicae*, a 123-bp allele of the same locus was observed in only females (Cnaani *et al.* 2008). Cnaani *et al.* (2008) showed that this marker was significantly associated with sex in two of four families analysed from the Israel stock, while for two families of one other stock (Egypt), stronger associations with LG1 were demonstrated. The same marker has also been identified significant association with the sex phenotype in *O. karongae* but was not in *O. tanganicae* (Cnaani and Kocher 2008). These results demonstrated that sex determination in tilapia is complex.
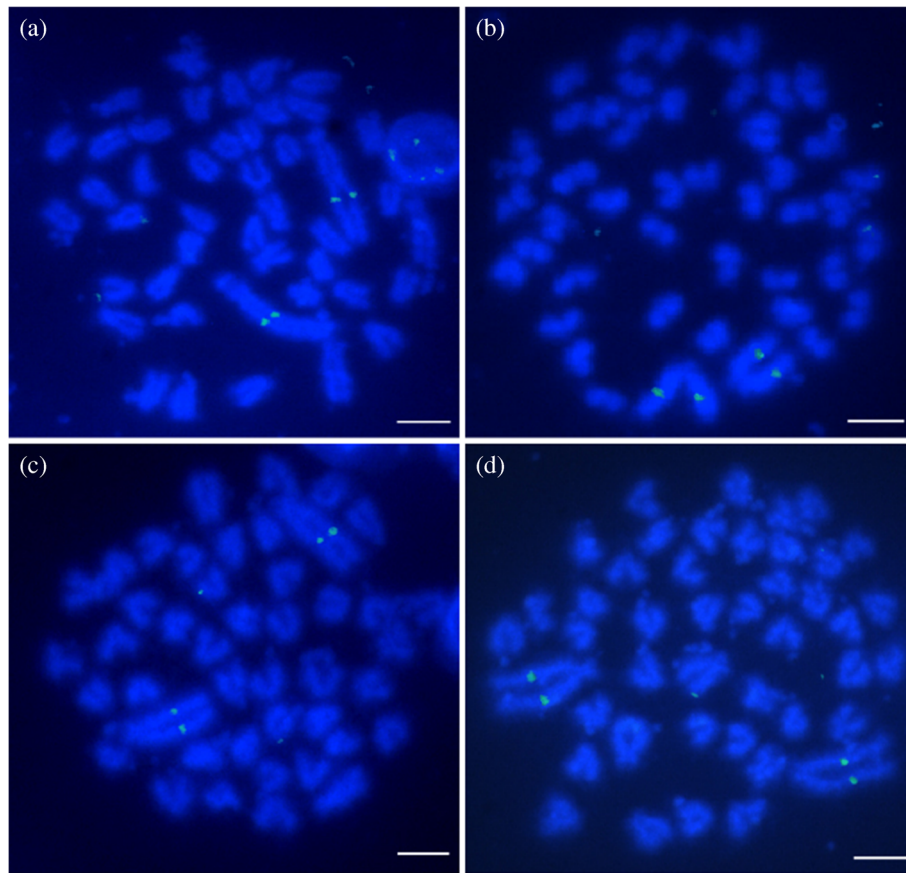
Figure 2. Representative images of metaphase-arrested chromosome spreads of *O. u. hornorum* (a and b, female; c and d, male) hybridized with BAC probes containing marker UNH168 (biotin labeled, green) by FISH. All metaphase chromosomes were counterstained with DAPI and appear blue. The markers are located on the long arm of the largest tilapia chromosome pair. Scale bar represent 10 μm.

Tilapia, one of the most important species groups in worldwide finfish aquaculture, is also an important model species for the analysis of the early events in sex chromosome evolution. Research on the sex determination system in *O. u. hornorum* has provided evidence for genetic sex determination with a ZW female and ZZ male sex determination system (Hickling 1960; Yang *et al.* 2008), but there are no obvious morphological differences between chromosomes in male compared to female. In the present study, we hybridized a BAC probe containing a sex-linked microsatellite to the chromosome spread of *O. u. hornorum* and the hybridization signals were consistently detected on the long arm of the largest chromosome pair (chromosome 1, LG3). Lack of any visible difference of the hybridized signals between the male and female tilapia suggests identities of the microsatellite sequences of both sexes.

It is significant to find more markers associated with phenotypic sex and localize them on chromosomes for sex chromosome evolution study in this tilapia. The largest chromosomes had been considered as sex chromosomes due

to a region of incomplete or delayed chromosome pairing observed in the synaptonemal complex (SC) in meiotic chromosomes of Nile tilapia (Carrasco *et al.* 1999; Campos-Ramos *et al.* 2001; Harvey *et al.* 2002; Ocalewicz *et al.* 2009). Some microsatellite markers associated with phenotypic sex also highly mapped onto chromosome pair 1 in *O. aureus* (WZ–ZZ sex-determining system) (Lee *et al.* 2004; Cnaani *et al.* 2008). Ezaz *et al.* (2004) drew a similar conclusion from the result that sex-linked amplified fragment length polymorphism (AFLP) markers (OniY227 and OniY382) were hybridized to the largest chromosome in mitotic spreads. The OniY227 was located on the boundary of the unpaired region of bivalent one in *O. niloticus* (Ocalewicz *et al.* 2009). However, the microsatellite markers UNH995 and GM201, on the LG1 is strongly associated with sex in *O. niloticus* were mapped on a small pair of chromosomes (Lee *et al.* 2004; Cnaani *et al.* 2008). Three sex-specific AFLP markers (OniY227, OniY382 and OniX420) mapped to LG1 were also hybridized to a small chromosome with no detectable hybridization on the largest chromosome

pair. It is suggested that the XX/XY sex-determining locus is on one of the small chromosomes in *O. niloticus* (Lee *et al.* 2011). These results indicated that the tilapia sex-determining was not clear and suggested that the sex chromosomes in tilapia species are at a very early stage of divergence. Male tilapias were preferred for culture in ponds because of their faster growth and more uniform size than mixed sex culture. The sex-linked microsatellite marker identified in this study could be used to assist breeding for production of monosex tilapia.

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