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Two unlinked loci controlling the sex of blue tilapia (*Oreochromis aureus*)

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Sex determination in the blue tilapia (*Oreochromis aureus*) is thought to be a WZ-ZZ (female heterogametic) system controlled by a major gene. We searched for DNA markers linked to this major gene using the technique of bulked segregant analysis. We identified 11 microsatellite markers on linkage group 3 which were linked to phenotypic sex. The putative W chromosome haplotype correctly predicts the sex of 97% of male and 85% of female individuals. Our results suggest the W locus lies within a few centimorgans of markers GM354, UNH168, GM271 and UNH131. Markers on LG1 also showed a strong association with sex, and indicate the segregation of a male-determining allele in this region. Analysis of epistatic interactions among the loci suggests the action of a dominant male repressor (the W haplotype on LG 3) and a dominant male determiner (the Y haplotype on LG1). These markers have immediate utility for studying the strength of different sex chromosome alleles, and for identifying broodstock carrying copies of the W haplotype.
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Introduction

The mechanisms of animal sex determination are diverse and highly labile (Bull, 1983). Even among species where sex is genetically (rather than environmentally) determined, different genes have been implicated as the primary regulators of sexual differentiation. For example, in evolutionary terms, the Y chromosome of mammals is recent, having first appeared between 170 and 300 MY ago. *Sry*, the gene that initiates the male differentiation cascade in mammals, does not control the sex of monotremes, and so must have taken on a role in sex determination within the last 170 MY (Graves, 2002). A few species of mole have recently lost *Sry*, and accomplish sex determination by a still unknown mechanism (Just *et al*, 2002). Birds have a female heterogametic (WZ) system which arose from a different pair of autosomes than the XY chromosomes of mammals some 350 MY ago (Nanda *et al*, 1999). Birds apparently lack *Sry*, and the genetic mechanism for sex determination in these species remains a mystery.

The diversity of sex-determining mechanisms has slowed the development of a unifying theory for the evolution of sex-determining pathways. It now appears that a variety of primary signals regulate one or a few ancient proximate pathways of differentiation (Zarkower, 2001). Wilkins (2002) proposed a hypothesis for the elaboration of sex-determining pathways by retrograde addition of upstream regulators. In his model, the downstream steps in the genetic pathway are conserved, but new regulators, especially inhibitory factors, are frequently recruited at upstream steps. Wilkins' model is consistent with what is known about the structure of sex-determining pathways in insects, nematodes and mammals (Gilbert, 1997).

Teleosts display a wonderful variety of mechanisms for sex determination and sex differentiation (reviewed in Devlin and Nagahama, 2002). Sex differentiation of fishes is remarkably plastic, and sex is determined by environmental factors in many species (Baroiller *et al*, 1999). A few species even undergo sex change in response to behavioral cues (Devlin and Nagahama, 2002).

Fishes provide interesting material for studying the evolution of sex chromosomes. Although genetic factors probably regulate sex determination in most fishes, relatively few teleosts have karyotypically distinct sex chromosomes (Arkhipchuk, 1995). In most species, the sex chromosomes are still in early stages of differentiation, and do not show distinct differences in length or gene content. Both XY and WZ gonosomal systems have evolved repeatedly in various groups of fishes (Devlin and Nagahama, 2002). Additional autosomal loci also contribute to sex determination in many species (Kosswig, 1964). In only one fish species (medaka) has the primary sex-determining gene been identified (Matsuda *et al*, 2002; Nanda *et al*, 2002). DMY is a new doublesex/mab-3 gene most closely related to DMRT1 (Kondo *et al*, 2003). It is expressed in developing male (but not female) gonads and appears to be necessary for differentiation of testes. Surprisingly, no sex-linked markers have been identified in the well-studied zebrafish, or the pufferfish (Li *et al*, 2002).

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Tilapia reach sexual maturity in just a few months and often begin reproducing in grow-out ponds before they reach a marketable size, reducing the yield and value at harvest. Therefore, commercial production of tilapia often relies on monosex culture of males. Beginning with the work of Hickling (1960), a variety of methods have been used to produce unisex fingerlings, including interspecific hybridization (Wohlfarth and Hulata, 1983), hormone treatment (Phelps and Popma, 2000) and YY supermales (Mair *et al*, 1997). These methods are not entirely reliable, in part because of their technical complexity (eg Wohlfarth, 1994), but also because the sex of tilapia is affected by environmental factors such as temperature (Baroiller *et al*, 1995; Desprez and Mélard, 1998; Abucay *et al*, 1999), and may also be influenced by additional genes (Mair *et al*, 1991; Hussain *et al*, 1994; Sarder *et al*, 1999).

*Oreochromis aureu*s has been described as having a predominantly female-heterogametic (WZ) system of sex determination (Mair *et al*, 1991). Crosses of hormonally sex-reversed ZZ phenotypic females with normal ZZ males usually produce 100% male offspring, but slight deviations have been observed (Hopkins *et al*, 1979; Mair *et al*, 1987; Lahav, 1993; Rosenstein and Hulata, 1994). Gynogenesis has also been used to study sex determination. If *O. aureus* females are WZ, then a ratio of one female (WW): one male (ZZ) is expected in their gynogenetic offspring. Yet, these fish produced a predominance of females in the F_1 generation. Penman *et al* (1987) explained this by hypothesizing a recombination of sex-determining genes in prophase of the first meiotic division. Thus a single crossover would yield an all-female (WZ) population, while double crossovers would produce equal numbers of males and females. The large number of crossovers suggested a distance of about 25 cM between the centromere and the sex-determining genes (Penman *et al*, 1987). This hypothesis was further investigated by Avtalion and Don (1990), who found that WZ females can produce, in all descending gynogenetic generations, offspring expressing a male genotype (ZZ) and two different female genotypes (WW and WZ), thus leading to a greater fraction of female progeny. Further studies by Mair *et al* (1991) confirmed female heterogamety, but also suggested the involvement of an autosomal recessive modifier. This study, as well as those of Hopkins (1979) and Mélard (1995), demonstrated male homogamety for this species. A monofactorial sex determination system with two sex chromosomes (WZ) in *O. aureus* was also supported by results from the analysis of progeny sex ratios from pseudofemales (Desprez *et al*, 2003), who showed that it is possible to obtain high proportions of male progeny from successive generations of pseudofemales by hormonal sex reversal and progeny testing.

The sex chromosomes of tilapia are relatively undifferentiated. There are no gross morphological differences in any chromosome pair that would identify the sex chromosomes (Kornfield, 1984; Majumdar and McAndrew, 1986). Campos-Ramos *et al* (2001) visualized the synaptonemal complex of *O. aureus* and observed incompletely paired segments in the longest bivalent and a smaller bivalent, which they suggested could be the sex-determining regions. Association between loci with deleterious alleles and distorted sex ratios has recently been reported in an inbred line of *O. aureus* (Shirak *et al*, 2002), but to this point, no DNA sequence markers for the major sex-determining locus in *O. aureus* have been described. We have recently constructed a linkage map for tilapia which contains more than 550 microsatellite markers (Lee *et al*, in prep.). Here we use markers selected from this map to rapidly scan the genome for sex-linked markers in bulked segregants. We then study genotypes of individual fish to localize the sex-determining regions and study epistatic interactions among loci.

Materials and methods

Fish source and DNA extraction

A single family of tilapia (*O. aureus*), produced at the Agricultural Research Organization, Israel, was used for this study. The history of cultured stocks of tilapia is typically uncertain, but this stock is to the best of our knowledge free of introgression from other species. Crossing males of this stock with *O. niloticus* females results in 100% male offspring, which further supports it purity. Offspring were sexed at the age of $\sim$4 months (at a mean size of $\sim$25 g) by macroscopic inspection of gonads, or by microscopic examination using the technique of Guerrero and Shelton (1974). Fin-clips from each fish were then dried and sent to the University of New Hampshire for genotyping. DNA was extracted from the fin-clips using the standard phenol/chloroform method (Kocher *et al*, 1989).

Marker selection

We selected 119 microsatellite markers, at intervals of approximately 20 cM, based on a linkage map produced from an F_2 population from the interspecies cross of *O. aureus* with *O. niloticus* (Lee *et al* in prep; see map at http://hcgs.unh.edu/comp). Those markers consisted of 92 UNH markers, 24 GM markers and three genes (*CLCN5*, *RAS-GRF2*, *UV-Opsin*), all of which are deposited in GenBank. Linkage group numbers in this manuscript are harmonized, to the extent possible, with the previous linkage map of tilapia (Kocher *et al*, 1998). Note in particular that the markers on the *O. niloticus* sex chromosome described by Lee *et al* (2003) are to be found on LG1 of the new map.

Bulked segregant analysis

To accelerate the identification of sex-linked markers, we used the technique of bulked segregant analysis (Michelmore *et al*, 1991). We made separate pools of 24 male and 24 female DNAs. Before pooling, the concentration of the DNAs was quantified using a DyNA Quant2000 spectrofluorometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and each DNA was diluted to a final concentration of $\sim$10 ng/μl. This allowed us to equalize the contribution of each individual to the pool. PCR was performed in a total volume of 20 μl for 2 min at 94°C followed by 28 cycles of 30 s at 94°C, 30 s at 55–60°C, 60 s at 72°C, with a final elongation step of 5 min at 72°C. One primer in each pair was labeled with a HEX, TET or FAM fluorescent dye (Operon Technologies, Alameda, CA, USA). PCR products were separated on an ABI377 DNA sequencer and fragment sizes were analyzed using ABI GeneScan 3.1.2 software (Applied Biosystems, Foster City, CA, USA).

Genotyping and statistics

For those markers that showed a qualitative difference in allelic composition in the pooled DNA, we repeated the genotyping on individual DNA samples from 48 females and 45 males using the same PCR conditions described above. We genotyped these individuals for all available markers on LG1 (nine markers) and LG3 (11 markers; Table 1). Goodness-of-fit (G-tests) were used to assess whether there were significant differences in genotypic distributions between males and females (Sokal and Rohlf, 1981). Significance thresholds were Bonferroni corrected for the number of chromosome arms (27), as reported by Majumdar and McAndrew (1986).

Mapping of sex-linked markers

Linkage maps for the sex-linked chromosomes were constructed from the segregation in this family. Linkage

Table 1 Genotypic proportions for sex-linked markers in male and female *O. aureus*. Asterisks indicate the Bonferroni-corrected *P*-values for each test

LG3					*LG1*				
Marker	*Genotypes*	*Female*	*Male*	*G-test*	*Marker*	*Genotypes*	*Female*	*Male*	*G-test*
GM139	193/193	6	1	35.58***	GM041	232/232	8	18	8.03
	193/223	14	16			232/238	26	20	
	193/218	13	0			238/238	13	5	
	218/223	3	21						
					UNH213	170/170	18	32	10.6*
GM354	129/137	4	24	78.69***		170/226	30	13	
	129/169	4	19						
	137/137	23	0		UNH148	161/148	28	12	9.2
	137/169	17	0			161/157	19	31	
GM271	121/125	5	28	54.4***	GM201	165/179	13	10	20.89**
	125/125	34	1			165/204	16	1	
						179/190	9	19	
UNH168	158/170	3	22	84.11***		190/204	9	15	
	158/174	4	23						
	170/174	24	0		UNH104	137/185	16	1	20.94**
	170/170	16	0			137/189	14	16	
						181/185	11	11	
UNH131	193/193	5	35	75.69***		181/189	6	17	
	187/193	38	0						
					UNH995	174/223	16	2	14.54
UNH115	168/182	8	24	66.04***		174/228	10	14	
	168/184	21	0			219/223	9	7	
	170/182	2	18			219/228	9	16	
	170/184	16	1						
					UNH868	220/216	20	33	9.28
CLC5	191/256	1	18	57.89***		220/224	26	11	
	191/281	16	0						
	191/191	13	24		UNH846	179/203	10	8	11.55
	256/281	12	0			179/213	15	3	
						203/213	8	15	
GM024	117/142	16	1	44.65***		213/213	13	18	
	117/154	13	1						
	125/142	9	22		GM258	130/130	15	2	11.83
	124/154	2	16			130/174	14	17	
						174/174	19	25	
UNH971	214/230	18	1	44.4***					
	214/234	15	2						
	216/230	10	23						
	216/234	3	17						
GM150	132/132	32	14	16.6**					
	132/186	13	34						
GM635	226/226	15	22	31.18***					
	226/228	18	0						
	228/228	7	19						

*$P<0.05$; **$P<0.01$;*** <0.001.

analysis was performed by Crimap (Green *et al*, 1990) using the TWO-POINT command with a LOD of 3.0. Map orders were decided by the ALL routine and confirmed by FLIPS. The sex-specific and sex-averaged maps were made using the BUILD command.

Results

Identification of sex-linked markers in pooled DNA

Amplification was successful for 102 of the 119 markers. Nine of these showed differential allelic segregation between male and female DNA pools. Five of these nine markers (CLC5, GM271, GM354, UNH131 and UNH971) belong to LG3. The 256 and 281 bp alleles of CLC5 were more frequent in the female pool, while the 191 bp allele was present in both the male and female pools. Both sexes had a 193 bp allele at UNH131, but females also carried a 187 bp allele. Alleles unique to the male pool were found at GM271 (121 bp) and GM354 (129 bp). UNH971 had 230 and 234 bp alleles in both pools but the female pool had a unique 213 bp allele and the male pool had a unique 215 bp allele.

Two markers on LG1 (UNH213 and UNH868) also showed a difference between the male and female pools. UNH213 showed a 170 bp allele in both sexes and an extra 226 bp allele in the female pool. UNH868 showed 220 bp in both sexes, an additional 224 bp in the female pool and a 216 bp allele in the male pool.

The other two markers (GM210 and UNH129) appear to be false positives. We tested another marker (UNH424) located only 5 cM from GM210, but it showed no difference between the male and female pools. The extra band in the female pool for UNH129 was determined to be extraneous signal bleeding from an adjacent lane of the gel.

Analysis of individual genotypes

These preliminary results encouraged us to individually genotype animals for these and other markers on LG1 and LG3. The genotypic proportions in males and females, and the associated G-tests, are shown in Table 1. The strongest associations were with a female-determining haplotype on LG3. All individuals with the 187 bp allele at UNH131 were females. Figure 1 plots the proportion of individuals whose phenotypic sex was consistent with the hypothesized female chromosome. The graph shows a broad peak around 30 cM in females, which corresponds to markers GM354, UNH168, GM271 and UNH131. Flanking markers show a decreasing correspondence with phenotypic sex, as recombination breaks up the association with the putative female haplotype. All males are homozygous for a haplotype marked by a 193 bp allele at UNH131.

This family is also segregating for a male-determining factor on LG1, which is epistatic to the locus on LG3. Individuals homozygous for the 193 bp allele at UNH131 can be either male or female, depending on their genotype for the locus on LG1. All 193/193 (UNH131) individuals with an 189 bp allele at UNH104 were males (Table 2). In all, 10 of the 193/193 (UNH131) individuals not having the 189 bp allele at UNH104 were male, but five were phenotypic females.

Although our family sizes are small, there are some clear differences in the pattern of recombination in the male and female parents (Figure 2). The male map shows reduced recombination in the vicinity of the sex-determining locus on both LG1 and LG3, and an expansion relative to the female map in distal regions. These large variations along the chromosome make it difficult to tell which sex has greater recombination on average, and we cannot yet relate the pattern to the location of centromeres.

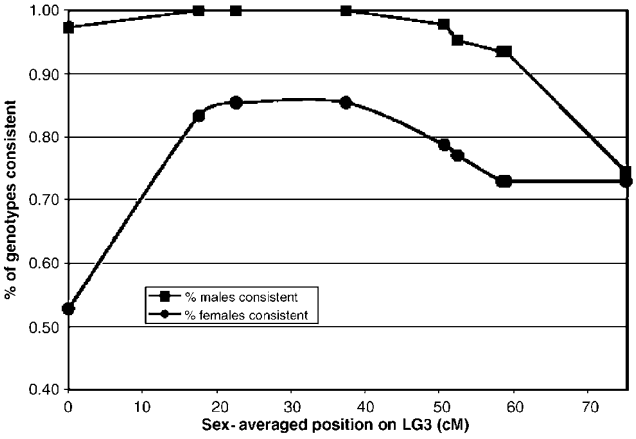

Figure 1 Proportion of individuals whose phenotypic sex is consistent with the hypothesized female determining haplotype on LG3. The map spans markers GM139 to GM150; markers UNH168 and GM271 both map to 22.6 cM. The parents were not informative for marker GM635 (88 cM).

Discussion

We detected two unlinked loci which interact to determine sex in this family of *O. aureus*. Our interpretation of the data, and our use of the WXYZ notation for sex-determining loci (or chromosomes), is as follows:

The first locus is located on LG3. Significant differences in genotypic proportions, suggestive of a W haplotype, are detected for 11 microsatellite markers on this linkage group. The sex-determining locus appears to lie near markers GM354, UNH168, GM271 and UNH131. The 193 bp allele at UNH131 is a marker for the Z allele, and the 187 bp allele is a marker for the W. Hence, the ZZ genotype corresponds to the homozygote 193/193, while the WZ genotype is represented by the 187/193 heterozygote. Essentially, 100% of the individuals with the W haplotype are females.

Among ZZ individuals, sex is primarily determined by the genotype of markers on LG1. This second locus is closely associated with marker UNH104. An 'XY' sex-determining locus at this position was also detected in two *O. niloticus* families described in Lee *et al* (2003). The 189 bp allele at UNH104 is a marker for the Y chromosome and any alternative allele is a marker for the X allele. Hence, the XY genotype corresponds to heterozygotes carrying the 189 bp allele, and all other allelic combinations at UNH104 represent the XX genotype. ZZ individuals carrying a putative Y haplotype are 100% male.

Our results suggest that the XY and WZ systems are not allelic. This is in contrast to the assumptions of many previous theoretical treatments of the system. Most tilapia geneticists publishing in the late 1970s assumed

that maleness in the WZ system would be determined by the action of a male-determining Z, rather than the absence of a dominant female determiner (W). According to a four gonosome model, *O. niloticus* would consist of XX females and XY males, while *O. aureus* would consist of WZ females and ZZ males, where Z = Y. Hybrid crosses of presumptive XX *O. niloticus* females with presumptive ZZ *O. aureus* males would be expected to produce all-male (XZ) offspring.

In our terminology, we must consider a dilocus genotype. We do not know the allelic state for the LG3 WZ locus in *O. niloticus*, or the allelic state for the LG1 XY locus in *O. aureus*. So, we postulate the *O. niloticus* female as (??XX) and the *O. aureus* male as (ZZ??). The hybrids are then (Z?X?) and expected to be largely male, but may show some proportion of females depending on their genotype for additional sex-modifying loci. Pruginin *et al* (1975) observed anywhere from 52 to 100% males in such pair crosses. This may, however, have been the result of using impure/contaminated stocks of one or both species. Later studies carried out in Israel have shown that 100% males can be obtained when 'good' stocks are being used (eg Lahav and Lahav, 1990; Hulata *et al*, 1995). These 'good' stocks have presumably been purged of any sex-modifying variation. The presence of both males and females in the putative ZZXX genotypic class of our family is consistent with the influence of additional 'autosomal' loci affecting sex ratio.

Table 2 Epistatic interactions of the sex-determining loci on LG1 and LG3 in a family of *O. aureus*

UNH131	*UNH104*	
	A/A	*A/189*
187/193	20 females 0 males	18 females 0 males
193/193	5 females 10 males	0 females 25 males

'A' stands for alleles other than 189 bp at locus UNH104 (eg 137, 181 or 185 bp).

It is now commonly accepted (eg Wohlfarth and Wedekind (1991) and Trombka and Avtalion (1993)) that sex determination in tilapias is based on major (sex chromosome) genes and minor (autosomal) modifiers. The LG1 locus identified in the present work may well be the 'autosomal locus' suggested by Hammerman and Avtalion (1979), affecting sex ratios through epistatic interactions with the major WZ locus located on LG3 in *O. aureus*. This same locus, probably inherited by both *O. aureus* and *O. niloticus* from a common ancestor, could in turn be the major sex-determining gene operating in *O. niloticus*. It should be noted, however, that our notation does not fit exactly the model of Hammerman and Avtalion (1979). They based their model on an assumption that each species (both males and females) is homozygous for a different allele at the modifying autosomal locus, which becomes heterozygous in the hybrids. If our LG1 locus is that autosomal modifying locus, then it appears to have different allelic combinations in males and females of *O. aureus*.

Our results are largely consistent with the model of Mair *et al* (1991). They postulated a WZ sex chromosome system with an autosomal recessive allele inducing female sex. Since the homozygous ZZXX animals in our family are of mixed sex (67% male), we suggest that

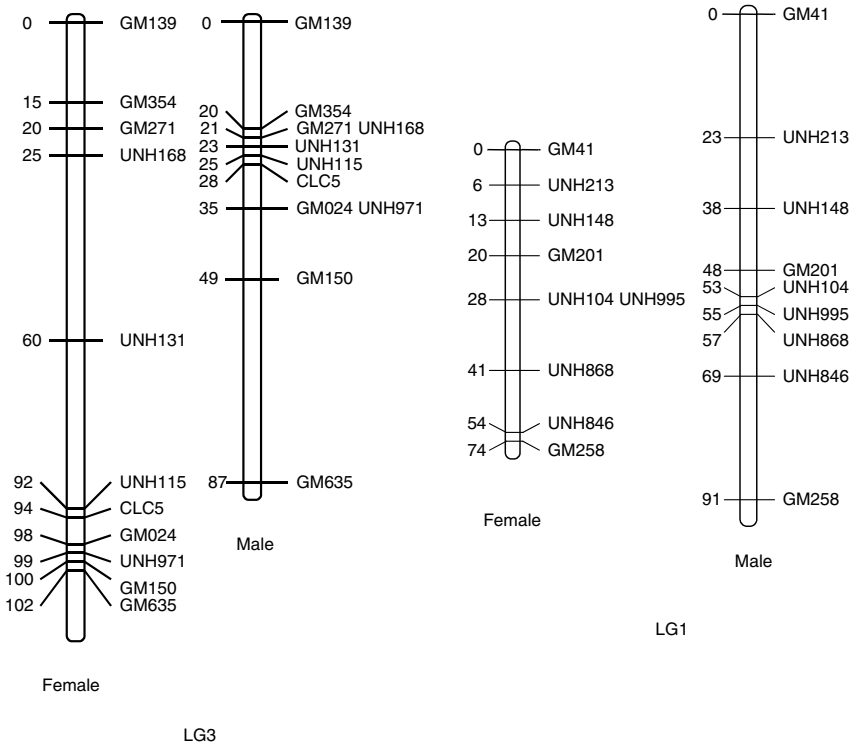

Figure 2 Sex-specific linkage maps for LG3 and LG1 in *Oreochromis aureus*.

additional genetic factors may be affecting determining the sex of these homozygotes. It is worth noting that we observed no effect of the putative WZ locus in three families of *O. niloticus* (Lee *et al*, 2003). This species may be fixed for a Z-like allele at the LG3 locus.

Sex-specific spatial variation in recombination rate has been observed in several fish species. Sakamoto *et al* (2000) observed much higher rates of recombination near the centromere in female rainbow trout. Conversely, male recombination rates were higher in the telomeric regions. We observed a similar sex-specific pattern of recombination, and predict that a centromere will be found near UNH131. In medaka, male recombination is suppressed in the region around the sex-determining gene and female recombination is suppressed in the telomeric regions (Kondo *et al*, 2001). Our results are also consistent with these patterns, suggesting they may be general for teleost fishes.

Our results begin to explain the variety of sex ratios that have been observed in pure and hybrid crosses of tilapia species. These DNA markers have immediate utility for tracking sex-linked haplotypes in breeding programs aimed at controlling the sex of fingerlings for commercial production. They also can be used in experiments aimed at quantifying the strength of different W- and Y-chromosome alleles from different individuals, strains and species. Marker-assisted selection could then be used to select genotypes that give a higher percentage of males for commercial production.

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